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Phytochemical Inhibition of Aflatoxigenicity in *Aspergillus flavus* by Constituents of Walnut (*Juglans regia*)

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Tulare walnut, a cultivar highly resistant to aflatoxin formation, was investigated for endogenous phytochemical constituents capable of inhibiting aflatoxigenesis in *Aspergillus flavus*. The activity, located entirely in the pellicle (seed coat), was extractable to various degrees with polar solvents, although some activity remained unextractable, indicating that the bioactivity resided in a complex of hydrolyzable tannins. These tannins can be hydrolyzed by a fungal tannase present in *A. flavus*, yielding gallic acid and ellagic acid, testing of which showed that only gallic acid had potent inhibitory activity toward aflatoxin biosynthesis. Comparison of the gallic and ellagic acid content in the pellicle of Tulare and Chico cultivars, over the 2002 and 2003 growing seasons, showed that the gallic acid content increased rapidly with maturation of the nut and was 1.5−2 times higher in Tulare than in Chico. Gallic acid content in the pellicle at maturity of a series of commercial English walnut cultivars, and two black walnut species, was determined as an indicator of potential for inhibition of aflatoxigenesis. Regulation of gallic acid levels in the hydrolyzable tannins of walnuts by conventional breeding or genetic manipulation has the potential to provide new cultivars with high resistance to aflatoxigenesis.

KEYWORDS: Aflatoxin; *Aspergillus* spp.; walnuts; *Juglans regia*; *Juglans hindsii*; *Juglans nigra*; hydrolyzable tannins; gallic acid; ellagic acid

INTRODUCTION

Tree nuts, primarily almonds, pistachios, and walnuts, are extremely important agricultural crops in California, where virtually all of the U.S. commercial production is located, with a large proportion of their value accruing from the export market. In calendar year 2001 their aggregate export value was $974 million (*1*). However, this market is threatened by potential food safety and quality concerns arising from potential contamination by aflatoxins, metabolites produced by various strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, which can infect not only tree nuts but also other major agricultural crops such as corn and peanuts. These mycotoxins are highly regulated, especially in the European Community, which has imposed exceptionally low tolerance levels of 2 ng/g for aflatoxin B_1 and 4 ng/g total aflatoxins (*2*), compared to the U.S. Food and Drug Administration maximum guidance level limit of 20 ng/g (20 ppb) for tree nuts (shells included) intended for human consumption within the United States (*3*). Rejection or destruction of shipments by the European Community due to the presence of aflatoxins in excess of regulatory amounts is therefore a serious economic concern to producers and exporters.

Postharvest processing to prevent growth of microorganisms and destroy aflatoxins is unlikely to ensure that shipments conform to tolerance levels or to be acceptable to regulators or consumers. Together with other approaches (*4*), we have therefore investigated the potential for factors naturally present in the crop to confer resistance to *Aspergillus* colonization and growth or suppress aflatoxin biosynthesis. Such methods should provide a product safe for consumption and in conformance to regulations. If present, such factors could be enhanced through selection of cultivars or crop-breeding programs. We have previously shown that naphthoquinones in walnut hulls can delay germination of *A. flavus* and affect levels of aflatoxin production (*5*) and that natural products in plants and spices can inhibit the biotransformation of aflatoxin B_1 to mutagenic compounds (*6*). More recently, we have demonstrated that walnuts have significantly more resistance to aflatoxigenesis than almonds and pistachios (*7*). Although walnuts are apparently well-protected against fungal infection by the hull, shell, and pellicle (seed coat tissue) surrounding the kernel, these can be breached by insect attack, providing an entry for fungal spores. The source of this resistance is therefore likely to be due to the presence of phytochemical constituents rather than a physical defense.

Walnut production in California, consisting of about 11 major cultivars of *Juglans regia* (family Juglandaceae), amounted to 254,000 metric tons in the 2001 season, with an export value of $179.1 million (*1*). These cultivars display different susceptibilities to aflatoxin formation (*7*), providing a resource by means of which specific phytochemical constituents could be

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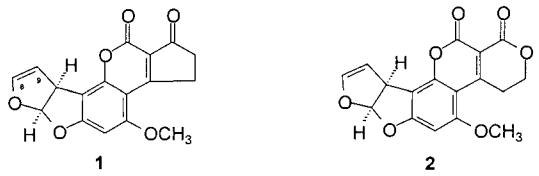

Figure 1. Chemical structures of aflatoxin B_1, **1**, and aflatoxin G_1, **2**; aflatoxins B_2 and G_2 are the 8,9-dihydro derivatives of **1** and **2**, respectively.

identified and their respective amounts compared with aflatoxin levels. More particularly, the walnut cultivar Tulare has been shown to exhibit extraordinary resistance to aflatoxigenesis even when the kernel is directly inoculated with *A. flavus* in vitro (*7*). Furthermore, the source of this resistance was shown to be in the pellicle, increasing with maturity of the walnut, and not in the kernel.

The objectives of this study were therefore to identify the pellicle constituents that confer resistance to aflatoxin contamination, to determine the levels of these components in cv. Tulare walnut throughout the growing season, in comparison with the more susceptible cv. Chico, and to compare the levels at maturity with those of other commercial walnut varieties. The information thus obtained should indicate possible mechanisms whereby aflatoxigenic *Aspergillus* species are rendered atoxigenic and potential sources for introduction of resistance factors into cultivated crops by breeding or genetic manipulation.

MATERIALS AND METHODS

Materials. Cv. Tulare and Chico walnuts were acquired biweekly during the 2002 and 2003 seasons, starting at the "jelly" stage in early June and continuing until full maturity in early September, from the Wolfskill Experimental Orchard, Winters, Solano County, California, which contains two trees of each variety. Other walnut cultivars were collected at maturity during the first week of September. The pellicle of 10 nuts from each tree of every variety examined was separated from the kernel by hand and the yield from the 20 nuts combined. Nut kernels and pellicle were ground to pass through a 1 mm screen with a Retsch model ZM-1 mill (Brinkmann, Westbury, NY).

Gallic acid, ellagic acid, and tannic acid were obtained from Sigma-Aldrich (St. Louis, MO). The gallic and ellagic acids were purified by passage through a column of LH-20 in ethanol; tannic acid was used as received.

Preparation of Media. Vogel's medium N (VMN), a defined medium, was prepared according to literature methods (*8*, *9*). Gallic, ellagic, and tannic acids were added at various levels (w/v) and autoclaved with the media. Petri dishes (60 mm diameter) containing 10 mL of the media were prepared in triplicate. Control samples contained none of the added gallic, ellagic, or tannic acids.

Walnut kernel medium (*7*) consisted of 5% ground Tulare kernel from which the pellicle had been removed manually, in 1.5% agar. Walnut tannin or gallic acid was added at various levels (w/v) and autoclaved, and Petri plates were prepared as for VMN.

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, **1**, with only trace levels of aflatoxin B_2 (8,9-dihydroaflatoxin B_1) and no aflatoxin G_1, **2**, or G_2 (**Figure 1**). 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 VMN and walnut kernel medium (10 mL per 60 mm Petri plate). Plates were prepared in triplicate and incubated for 7 days at 30 °C in the dark. For the time course experiment, triplicate sets of plates incubated under the same conditions were analyzed daily from day 0 (control) to day 11. The effect on fungal growth was evaluated by visual assessment of the extent of mycelium radial expansion at each time point.

Analysis for Aflatoxin. The fungal mats, including spores and media from each of the three replicate Petri plates, were extracted with MeOH (50 mL) and analyzed individually. 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 fluorescence detection, with excitation at 365 nm and detection of emission at 455 nm (*10*, *11*). The lower detection limit was 0.02 μg per Petri dish or 10 mL of medium. Aflatoxin B_2 was detected at levels that were insignificant relative to aflatoxin B_1 (~0.1%) and was therefore not quantitated. Each data point was calculated as the average of three replicates.

Sequential Extraction of Walnut Pellicle. Ground Tulare walnut pellicle (18.8 g) was extracted sequentially by sonication for 1 min with hexane, acetone, methanol, and water (400 mL each). The extracts were evaporated to dryness, and the acetone, methanol, and water extractables, together with the fully extracted pellicle residue, were analyzed for their ability to inhibit aflatoxin production at 0.25% incorporation in VMN medium. The hexane residue was too insoluble for incorporation into the medium, and a portion of the residue after hexane extraction was therefore evaluated for aflatoxin inhibitory activity and found to be the same as that of unextracted pellicle. Yields of extractables as percent of the total unextracted pellicle and aflatoxin inhibitory activity relative to control (percent) were as follows: hexane, 12.1% (nd); acetone, 27.1% (100); methanol, 29.1% (100); water, 8.2% (100); residue, 23.5% (76.8).

Analysis of Gallic Acid and Ellagic Acid in Walnut Pellicle and Extracts. Duplicate analyses were performed for every sample from each collection and time period. Methanolic HCl (Alltech Associates, Inc., Deerfield, IL) was added to ground walnut pellicle (20 mg) and stirred at 100 °C for 1 h. After cooling, aliquots (200 μL) were evaporated to dryness under N_2 at 40 °C, redissolved in methanol (1.0 mL), and filtered through 0.2 μm nylon syringe filters (Gelman). Aliquots (10 μL) were analyzed for methyl gallate and ellagic acid by reversed-phase HPLC on a 250 mm × 4.6 mm i.d. Vydac 201SP104 C18 column, using a gradient from 100% water containing 0.3% TFA to 100% methanol over 25 min at a flow rate of 1.0 mL/min, with detection at 252 and 280 nm using an Agilent 1100 series diode array detector.

The extractables and residue from sequential solvent extraction of the pellicle were analyzed in the same manner, with the following results for gallic and ellagic acids (weight percent), respectively: hexane, 0 and 0.02; acetone, 5.0 and 25.4; methanol, 4.6 and 20.3; water, 2.0 and 15.7; residue, 0.7 and 2.2.

RESULTS AND DISCUSSION

Previous in vitro experiments showed that the Tulare walnut cultivar exhibited exceptional resistance to aflatoxigenesis, whereas Chico was the most susceptible of the nine commercial cultivars tested. Thus, no aflatoxin was detected for Tulare, whereas Chico produced 28 μg/plate when *A. flavus* was grown on media consisting of 5% ground nut kernels added to plain agar (*7*). Furthermore, analogous experiments with Tulare nuts separated into the kernel without pellicle (endosperm) and pellicle alone showed that at levels of 400 mg/plate of endosperm or pellicle, the aflatoxin levels were 400 and 3% of control, respectively. However, when pellicle was added back to endosperm material in a proportion approximating that in the whole kernel, aflatoxin production was reduced to 0.8% of control (*7*). These results demonstrated that the resistance factors were located entirely within the pellicle material, and experiments were therefore designed to isolate specific fractions and assay for activity.

Identification of Aflatoxin Resistance Factors in the Pellicle. Ground pellicle material was sequentially extracted with hexane, acetone, methanol, and water. The hexane extract, comprising only 12% of the total, consisted primarily of lipophilic substances, and the remaining pellicle had essentially

the same activity as a control (unextracted) sample and none of the aflatoxin resistance factor was therefore removed from the pellicle by this solvent. The primary lipid constituent of walnut kernels is known to be linoleic acid, either in its free state or as a triacylglycerol constituent (*12*), but the high level of lipids in the pellicle alone was unexpected. When tested at an incorporation level of 0.25% (w/v) in VMN, a defined medium, the acetone, methanol, and water extracts all reduced the aflatoxin content by 100%, relative to control, indicating that the compounds responsible had a range of polar characteristics. The largest proportions of material were extracted by acetone and methanol, together comprising 56% of the total. However, the residual unextractable material amounted to 23.5%, and this still inhibited aflatoxin production by 77% of control, indicating that not all of the inhibitory compounds were extracted, even by very polar solvents.

The acetone, methanol, and aqueous extracts were analyzed by GC-MS. Nut kernels are known to contain high levels of phenolic compounds, and the aqueous extract was trimethylsilylated prior to analysis to ensure that such compounds would be converted into volatile derivatives. The only compound detected was methyl gallate, present in trace amounts (data not shown), insufficient to account for the potent reduction of aflatoxigenicity produced by the extracts. It therefore appeared to be unlikely that the active constituents were discrete low molecular weight compounds. The fact that the activity was extractable by solvents exhibiting a range of polarities, and that some of the inhibitory activity was not extractable, suggested that a complex of relatively high molecular weight substances was responsible. The most likely class of compounds was hypothesized to be hydrolyzable tannins because walnut pellicle is well established as having high levels of these compounds, which are responsible for the astringency of the nuts.

In 1956, Jurd (*13*) showed that walnut pellicle, obtained from a processing stream (personal communication) and therefore likely to be composed of a mixture of commercial varieties, contained 5−10 wt % of a complex mixture of hydrolyzable tannins. A pure ellagitannin named juglanin was subsequently isolated from this mixture and characterized as a glucose moiety esterified with one molecule of gallic acid and one molecule of hexahydroxydiphenic acid (*14*). Juglanin was structurally similar to corilagin (*15*), a major constituent of divi-divi, the tannin obtained from the pods of *Caesalpinia coriaria* (Leguminoseae), but differed in melting point and optical rotation, and its structure was therefore postulated as an isomer of corilagin. It has been speculated that hydrolyzable tannins serve an antimicrobial protective role in plants, inhibiting the decay of essential tissues. However, in vitro assays to determine the minimum inhibitory concentrations (MIC) to inhibit growth of filamentous fungi generally require levels in excess of 0.5 g/L (*16*). For example, although a series of hydrolyzable tannins were shown to have activity toward opportunistic yeasts, there was no activity against a diverse selection of filamentous fungi at levels up to 1000 μg/mL (*17*). When walnut pellicle tannin, extracted by methanol and purified from low molecular weight compounds by passage through Sephadex LH-20 (*18*), was tested against *A. flavus* grown on walnut endosperm media, it initially delayed radial growth and resulted in sparser mycelium relative to control, although this effect was overcome during the time course of the experiment. The tannin potently suppressed aflatoxin production (**Figure 2**), with the level being reduced to 50% of control at only 0.05% (w/v) tannin incorporation and reduced to 0.6 and 0%, respectively, at 0.25 and 0.5% (w/v) incorporation, indicating that the hydrolyzable tannin was directly or indirectly responsible for inhibition of aflatoxin biosynthesis.

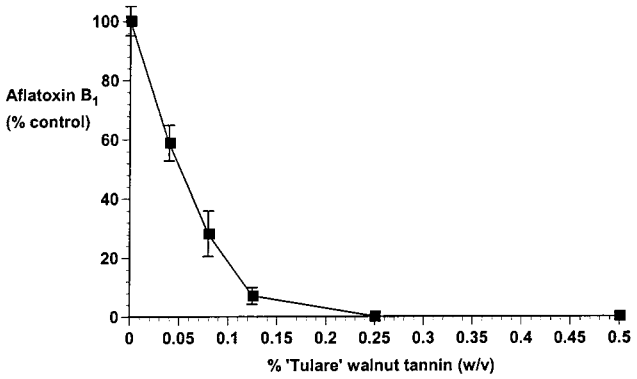

Figure 2. Suppression of aflatoxin formation by *A. flavus* grown on walnut endosperm media containing various levels of cv. Tulare walnut hydrolyzable tannin purified by Sephadex LH-20 chromatography.

A number of fungi, especially *Aspergillus* and *Penicillium* species, are able to grow on media containing tannins as the sole carbon source and are even capable of thriving on the surface of tanning vats containing very high tannin levels (*16*). Among these, a fungal tannase has been purified from *A. flavus* strain IFO 5839 (*19*). This extracellular tannase was shown to hydrolyze the ester linkages in tannic acid, glucose-1-gallate and methyl gallate, respectively, in each case yielding gallic acid. To establish whether the strain of *A. flavus* used in our experiments (NRRL 25347; isolated from pistachio) had similar activity, it was screened using a simple plate assay in which the fungus was grown on tannic acid media. A broad zone of clearing was observed, the extent of which has previously been shown to correlate closely with the extent of tannase production (*20*). Hydrolyzable tannins consist of a carbohydrate, usually glucose, esterified with gallic and/or hexahydroxydiphenic acid moieties; a representative structure of the simplest type, **3**, analogous to that postulated for juglanin by Jurd (*14*), is illustrated in **Figure 3**. Further complexity can result from depside linkages of additional gallic acid moieties with the primary esterifying acids, and oligomers may result from oxidative coupling of monomers (*21*). On hydrolysis (**Figure 3**), gallic acid, **4**, is released as such, but the hexahydroxydiphenic acid, **5**, spontaneously lactonizes to ellagic acid, **6**, and cannot be isolated in the free form. The presence of an extracellular tannase is probably necessary for the fungus to gain access to the glucose moiety of the tannin fraction, which is a nutrient for *A. flavus* growth. Conversely, the ability of the fungus to hydrolyze tannin indicated that it could be one or more of the other hydrolysis products, namely, gallic and ellagic acids, rather than the tannin fraction itself, that suppresses aflatoxin production. Pure gallic and ellagic acids were therefore tested as aflatoxigenesis inhibitors.

An 11-day time course comparison of aflatoxin inhibition by gallic acid and ellagic acid, added at 0.2% to VMN, showed that aflatoxin production peaked at days 5−7. Gallic acid reduced aflatoxin levels to 4% of control on day 6, whereas the ellagic acid had only a slight effect, reducing aflatoxin to 84% relative to control on the same day and by a smaller amount on other days (**Figure 4A**). However, at a lower level of incorporation (0.05%) ellagic acid actually stimulated aflatoxin to 148% of control (data not shown), an effect that we have also observed with walnut naphthoquinones (*5*). Whereas gallic acid had no effect on radial growth of the fungus, ellagic acid showed an effect similar to that seen with walnut tannin, namely, a delay in growth and sparser mycelium, which was eventually over-

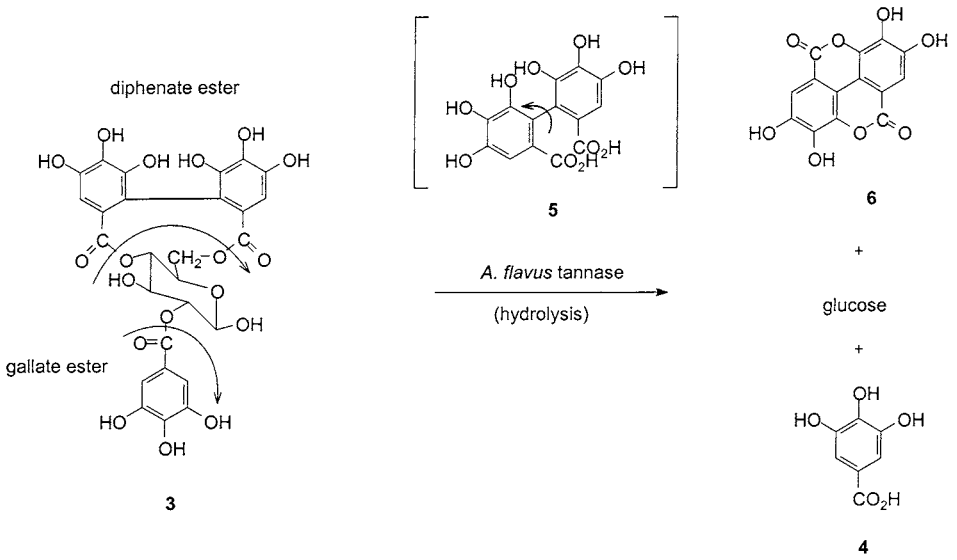

Figure 3. Representative structure of walnut hydrolyzable tannin, **3**, and hydrolysis by fungal tannase to gallic acid, **4**, and hexahydroxydiphenic acid, **5**, which undergoes spontaneous lactonization to ellagic acid, **6**.

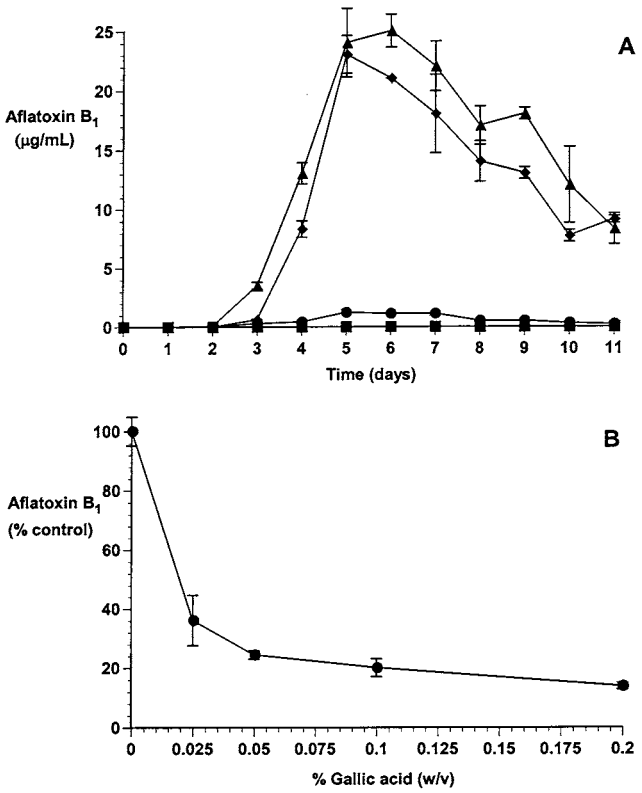

Figure 4. (**A**) Time course comparison over 11 days of aflatoxin production by *A. flavus* in Vogel's medium N (▲) and relative inhibition by gallic acid (●), ellagic acid (◆), and tannic acid (■); (**B**) suppression of aflatoxin formation by *A. flavus* grown on walnut endosperm media containing various levels of gallic acid.

come, with growth appearing normal at later time stages. For comparison purposes, commercial tannic acid, which contains only gallate ester moieties (26.4%) and no hexhydroxydiphenate (ellagic acid precursor) functionalities, was tested at 0.4% incorporation with complete inhibition of aflatoxin production and no effect on growth (**Figure 4A**). It is not possible to evaluate the effect of the ellagic acid precursor, hexahydroxydiphenic acid, **5**, which may be ephemerally present, because this compound cannot be isolated without lactonization. Nevertheless, the potency of gallic acid suggests that this compound alone is substantially responsible for aflatoxin inhibition by walnut tannin. Gallic acid was also incorporated into media consisting of walnut endosperm and was found to be an inhibitor of aflatoxin production at low levels (**Figure 4B**). Aflatoxin was reduced to 50% of control at only 0.02% (w/v) incorporation and to 13.8% at 0.2% (w/v) incorporation.

Correlation of Walnut Pellicle Gallic Acid Content with Aflatoxin Resistance. To correlate the gallic acid content of walnut pellicle with relative inhibition of aflatoxigenesis, samples of Tulare and Chico walnuts were collected throughout the growing season for two consecutive years (2002−2003). The pellicle was removed, and the gallic and ellagic acid contents were determined by HPLC analysis after hydrolysis with anhydrous methanolic HCl, a technique developed for analysis of both acids in wood and food products (*22*). Under these conditions, gallic acid was determined as methyl gallate, and ellagic acid per se, with monitoring at 280 and 252 nm, respectively. Linear calibration curves were generated for each compound in concentration ranges of 0.1−4.0 and 0.1−1.0 μg/mL for methyl gallate and ellagic acid, respectively. The method was modified by using a diode array detector to ensure that each compound was correctly identified, not only by retention time but also by matching of their UV spectra.

The gallic and ellagic acid analyses for Tulare and Chico walnuts are shown in **Figure 5**. The year 2003 results extended over a slightly longer time period than for 2002 because observations from the previous year indicated that sufficient pellicle for separation from endosperm was formed even at the "jelly" stage prior to hardening of the kernel and that the gallic acid content was already high by the beginning of June. Collections were therefore commenced 2 weeks earlier, in mid-May, and collections were also made on a weekly basis for the first 6 weeks, during rapid development of the kernel, to reveal any rapid fluctuations in gallic and ellagic acid contents. Gallic acid levels (**Figure 5A**) were consistently higher by a factor of 1.5−2 times in Tulare than in Chico during both seasons. The year 2003 results showed an initial rapid increase in gallic acid for both cultivars, attaining a maximum 3 weeks after the first collection, which was followed by a noticeable decline. The established biosynthetic pathway (*23*, *24*) to all hydrolyzable tannins from their common precursor pentagalloyl glucose, **7**

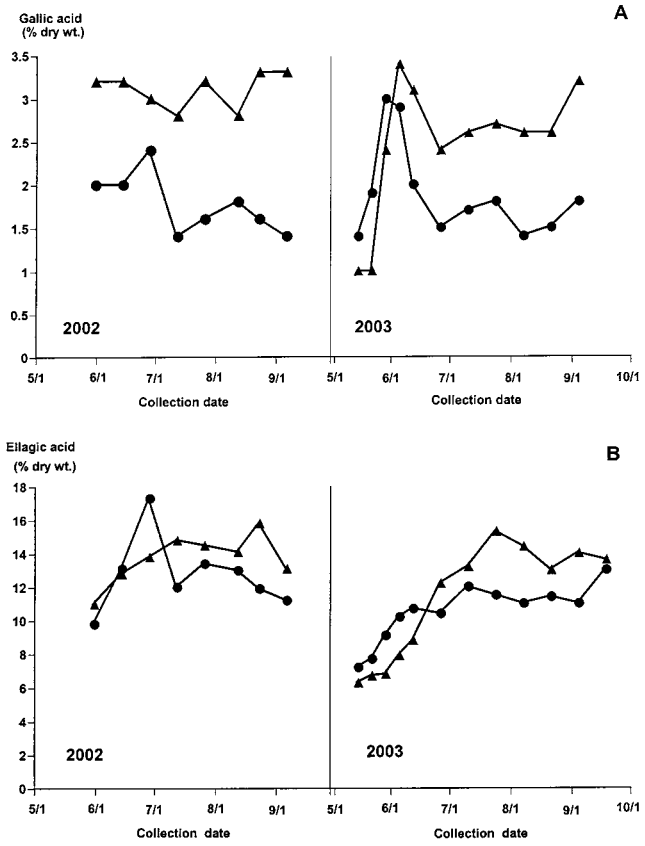

Figure 5. Hydrolyzable tannin constituent content of cv. Tulare (▲) and Chico (●) pellicle as a function of walnut development for the 2002 and 2003 growing seasons: (**A**) gallic acid; (**B**) ellagic acid.

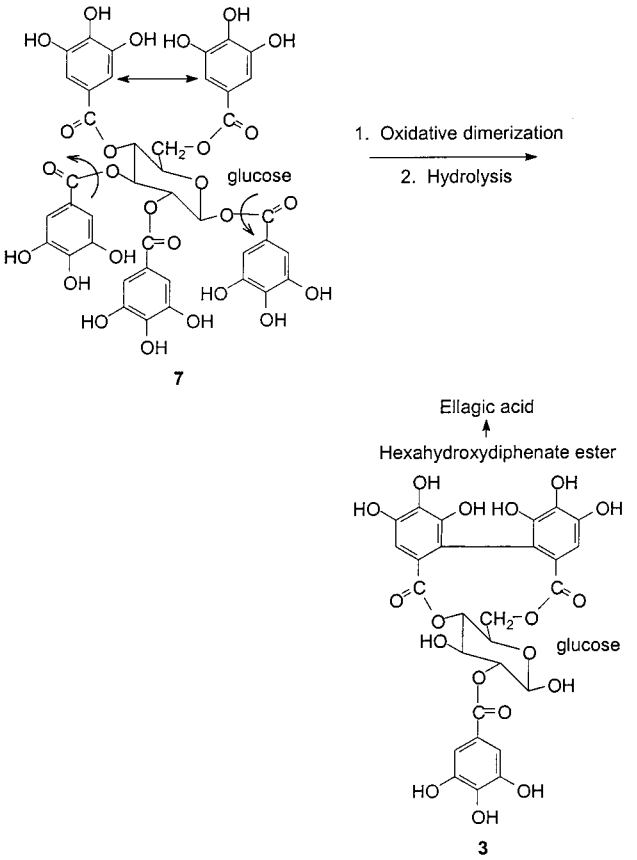

Figure 6. Representation of biosynthesis of walnut hydrolyzable tannin, **3**, from pentagalloylglucose, **7**, through oxidative dimerization of adjacent galloyl ester moieties and partial hydrolysis of galloyl esters.

(**Figure 6**), suggests a probable explanation for this effect. This precursor subsequently undergoes hydrolysis to remove some of the galloyl moieties and oxidative dimerization to generate the hexahydroxydiphenate ester group (**Figure 6**). A combination of these reactions would give a simple hydrolyzable tannin such as **3**, resulting in a net reduction in gallic acid content relative to the peak level corresponding to pentagalloyl glucose. Hydrolyzable tannins increase in complexity as the plant matures due to subsequent addition of gallic acid residues through oxidative couplings or depside linkages to the core structure. The relative amounts of gallic and ellagic acids are therefore likely to vary as these reactions proceed.

The biosynthetic correlation was also consistent with the ellagic acid content for both cultivars, which showed a general tendency to increase from the time of the first collection, followed by a leveling off or slight decline from midseason to maturity; this was particularly noticeable during the 2003 season (**Figure 5B**). It was also apparent that although ellagic acid levels were significantly higher than those of gallic acid, the differences between the two cultivars were much less noticeable than for gallic acid. This observation, considered in concert with its relative lack of antiaflatoxigenic activity (**Figure 4**), indicates that ellagic acid is not the constituent responsible for the potent atoxigenic effect on *A. flavus* of Tulare walnut and that this is essentially attributable to gallic acid.

Comparison of Gallic and Ellagic Acid Contents in Walnut Cultivars. In addition to comparing the gallic and ellagic acid contents of Tulare and Chico walnuts throughout the growing season, a number of other California cultivars from the 2003 season were analyzed at maturity, together with two black walnut species, *Juglans nigra* and *Juglans hindsii*. The results are shown in **Table 1**, arranged in order of increasing gallic acid content for the English walnut and black walnut groups. Tulare and Red Zinger were the only English walnut cultivars with gallic acid levels exceeding 3% dry weight, and the pellicles of both of these varieties had been shown in previous work (*7*) to reduce aflatoxin B_1 levels to 0.2 and 0.02 μg/plate, respectively. Four varieties of English walnut had gallic acid contents of 2.0−2.6%, and two varieties, Chandler and Chico, had levels of 1.4 and 1.8%, respectively. Comparison with our previous study (*7*) for those varieties that were analyzed in both studies suggests that gallic acid levels of 2.2% or below are insufficient to prevent aflatoxin formation, because the aflatoxin B_1 produced ranged from 3.5 to 28 μg/plate. The black

Table 1. Gallic and Ellagic Acid Composition of Pellicle from Walnut (*Juglans*) Species and Varieties Analyzed in 2003

walnut species/variety	gallic acid (% dw)[a]	ellagic acid (% dw)[a]	ellagic/gallic ratio
English walnut (*J. regia*)			
Chandler	1.4 ± 0.05	10.0 ± 0.50	7.1
Chico	1.8 ± 0.05	11.0 ± 0.70	6.1
Serr	2.0 ± 0.05	11.8 ± 0.03	5.9
Payne	2.0 ± 0.05	12.3 ± 0.10	6.2
Hartley	2.2 ± 0.00	13.3 ± 0.10	6.0
Tehama	2.6 ± 0.05	11.0 ± 0.15	4.2
Tulare	3.2 ± 0.05	14.0 ± 0.15	4.4
Red Zinger	3.4 ± 0.15	15.9 ± 0.20	4.7
black walnut (*J. hindsii*)			
Rawlins	1.0 ± 0.05	3.1 ± 0.10	3.1
black walnut (*J. nigra*)			
Thomas	1.1 ± 0.00	2.6 ± 0.05	2.4

[a] Values are averages of two determinations.

walnut species, *J. hindsii* cv. Rawlins and *J. nigra* cv. Thomas, had much lower gallic acid levels of 1.0 and 1.1%, respectively. This low gallic acid value was consistent with a previously determined aflatoxin level of 44 μg/plate for *J. hindsii* (*7*).

For English walnuts, the increase in ellagic acid content paralleled that of gallic acid, with the exception of the Tehama cultivar, which had a lower ellagic acid level than Hartley and Tulare cultivars (**Table 1**). However, when the ellagic acid/gallic acid ratio was calculated, there was a trend for the ratio to decrease with increasing gallic acid content. Thus, Chandler had an ellagic/gallic ratio of 7.1, whereas that of Tulare was 4.4. This is consistent with a biosynthetic process in which the higher gallic acid levels are a consequence of less oxidative dimerization of their ester moieties to hexahydroxydiphenic ester (**Figure 6**), and consequently ellagic acid, on hydrolysis. In contrast, the ratios for black walnut did not fit this pattern, having lower values than any of the English walnuts but with very low gallic acid contents (**Table 1**). Ellagic acid levels were also ~25% of those in English walnuts, suggesting that biosynthesis of hydrolyzable tannins is substantially reduced in black walnuts.

Structure—Activity Considerations. The total gallic acid levels measured by methanolic HCl hydrolysis do not necessarily correlate directly with the effective levels of gallic acid required to induce atoxigenicity in *A. flavus*. Gallic acid can be present in hydrolyzable tannins as gallate esters of the carbohydrate moiety or in depside linkages with these carbohydrate-bound primary gallate or hexahydroxydiphenate esters. Hydrolysis of both gallic acid esters of carbohydrates and depside bonds by fungal tannase has established that there are several isoenzymes (*25*), but fractionation into esterase and depsidase activity has not been achieved. Such differential activity suggests that gallic acid may be generated more readily from some structural classes of hydrolyzable tannins than from others, depending upon whether the gallate residues are bound as carbohydrate esters or as depsides. This was also apparent from the gallic and ellagic acid analyses of the various solvent extracts and the unextractable residue. The hexane extract showed the presence of neither of these acids, consistent with its lack of antiaflatoxigenic activity. In contrast, the acetone, methanol, and water extracts all exhibited potent activity, equivalent to that of the unextracted pellicle, and contained significant amounts of gallic (2.0−5.0%) and ellagic acids (15.7−25.4%); there was a general trend for the amounts to decline as the polarity of the solvent increased. The unextractable residue retained antiaflatoxigenic activity but had lower levels of gallic (0.7%) and ellagic (2.2%) acids. These values probably reflect an increasing tendency for the tannins to consist of complex polymerized structures in the more polar and unextractable fractions.

The earlier work of Jurd (*14*) showed that juglanin, a major constituent of walnut pellicle, was a hydrolyzable tannin consisting of glucose bearing one gallate and one hexahydroxydiphenate ester moiety with the putative structure **1**. However, our analyses of gallic and ellagic acid contents in the pellicle of 10 walnut varieties show that at maturity the ellagic acid content exceeds that of gallic acid by 2.4−7.1-fold (**Table 1**), although gallic acid proportions were established as being greater in Chico and Tulare varieties at earlier stages of growth (**Figure 5**), as predicted from biosynthetic considerations. Recently, 3 new hydrolyzable tannins named glansrins A−C, present in very small amounts, and 13 known tannins have been isolated from walnut kernels consisting of both endosperm and pellicle of an unspecified walnut variety (*26*). The major individual tannins were tellimagrandin, comprising 0.007% of the weight (undried) of the kernel, pedunculagin (0.006%), and strictinin, casuarictin, and casuarinin (0.002% each). Pedunculagin has no galloyl residues, whereas the gallic acid/ellagic acid ratios of the other major tannins ranged from 0.5 to 2.0. Although this study reveals some of the exceptional structural diversity of hydrolyzable tannins in walnuts, it is difficult to correlate these findings with our results because the total kernel was examined, not just the pellicle to which the tannins are confined, and tannins were isolated only from selected chromatographic fractions of the total extract. Many of the more complex tannins with high proportions of hexahydroxydiphenoyl ester moieties, such as a *Juglans* species leaf constituent sanguiin H-6 (*27*), may not have been present in these fractions. Furthermore, the lack of information regarding the specific walnut variety selected (*26*) renders direct comparison with our cultivars problematic.

Mechanism of Antiaflatoxigenic Action. The mechanism by which gallic acid suppresses aflatoxin formation is not yet established. It is noteworthy that no fluorescence was observed from the extracts of the Petri plates during aflatoxin analysis, indicating an absence of expected intermediates accumulating from interruption of the biosynthetic pathway prior to aflatoxin formation. Similarly, no color development was observed due to the presence of norsolorinic acid, an early metabolite in the aflatoxin pathway. This suggests that gallic acid may be affecting genes identified as controlling specific steps of aflatoxin biosynthesis (*28*, *29*), in particular those controlling the fatty acid synthase or polyketide synthase enzymes involved in the synthesis of the polyketide precursor of norsolorinic acid. The aflatoxin biosynthesis genes occur in a 75 kb cluster, and the effect of gallic acid could be upstream of this gene cluster. Alternatively, the regulatory gene, *aflR*, expression of which is essential for the functioning of other genes in the cluster (*30*), encodes for a zinc-containing, DNA-binding protein, and it is conceivable that the gallic acid acts as a chelator of this metal ion through sequestration by catecholic hydroxyl groups. Other metal ions essential for fungal growth, such as copper and iron, could be complexed in a similar manner (*31*). However, this possibility is refuted by the fact that ellagic acid, which has the same metal-ion complexing catechol groups, does not markedly affect aflatoxin production. The effect of gallic acid on specific genes involved in aflatoxin biosynthesis is currently under active investigation (J. W. Cary, personal communication).

Another alternative is that aflatoxigenesis, which is a consequence of increased oxidative stress resulting in the formation of peroxides and epoxides through enhanced lipid peroxidation (*32*), is suppressed by the antioxidant capacity of gallic acid. Gallic acid and its esters are potent antioxidants and free radical scavengers (*26*) and could therefore mitigate or over-ride the stress response. Eugenol (2-methoxy-4-allylphenol) has been shown to reduce aflatoxin production to 30% of control at 0.45 mM without any decrease in fungal growth due to inhibition of lipid peroxidation. However, at lower concentrations, both fungal growth and aflatoxin production were enhanced (*33*), an effect that was also observed in our earlier experiments with naphthoquinones from walnut hulls (*5*). Gallic acid has recently been shown to alleviate oxidative stress in yeast deletion mutants lacking oxidative stress genes of which orthologous genes are known in *A. flavus* (J. Kim, personal communication).

Many phytochemicals and other natural products have fungicidal activity. However, the ability to suppress biosynthesis of fungal metabolites without affecting fungal growth is atypical. For example, it has been reported that 4-acetylbenzoxazolin-2-one isolated from an ear-rot resistant corn hybrid suppresses

aflatoxin B_1 production to 20% of control at 2.26 mM, without significant reduction in fungal growth, but provided no further reduction even at 50 mM. The structurally related compounds benzoxazilinone and 6-methoxybenzoxazilinone eliminate aflatoxin production at 3.0 mM, but this is accompanied by a corresponding termination of mycelial growth (*34*). In contrast, gallic acid reduces aflatoxin B_1 to 4% of control at 12 mM, and tannic acid completely inhibits production at 2 mM (**Figure 4**), with little or no effect on growth. Desjardins et al. (*35*) have demonstrated that the furanocoumarins imperatorin B and bergapten completely inhibit production at 5 mM in vitro of T-2 toxin and its biosynthetic precursor, trichodiene, by *Fusarium sporotrichioides*, with no effect on fungal dry weight. Other compounds belonging to the same structural class inhibited T-2 toxin production but also resulted in the accumulation of trichodiene and had an inhibitory effect on fungal growth. It is noteworthy that many natural products having antifungal effects or reducing mycotoxin production are phytoalexins, inducible metabolites produced de novo after fungal invasion. In contrast, the hydrolyzable tannins are endogenous constituents or phytoanticipins (*36*, *37*), constitutive metabolites present in situ, either in the active form or easily generated from a precursor. Levels of such compounds sufficient to have the desired effect of suppressing mycotoxin biosynthesis are more responsive to manipulation in the plant than phytoalexins, which are produced only at highly localized sites after fungal attack and are therefore unlikely to be effective in completely inhibiting mycotoxin biosynthesis.

The results of our investigation demonstrate the role of hydrolyzable tannins, present in a physical and chemical defensive tissue surrounding the edible portion of a crop plant susceptible to food safety concerns and economic losses, in eliminating formation of aflatoxins. It is apparent that a high gallate ester level in the tannins of pellicle tissue is desirable with respect to suppression or elimination of aflatoxin biosynthesis; it is not necessary for there to be a corresponding increase in the ellagic acid precursor, hexahydroxydiphenate ester. An increase in the gallate ester ratio should be achievable by conventional breeding. Analysis of pellicle tissue in the parents of the Tulare cultivar and other walnut germplasm may provide a useful indicator of selections with high potential for inheritability of this trait. Alternatively, a directed genetic approach could be adopted, focused on the biosynthetic pathway to gallic acid and the hydrolyzable tannins, through the shikimate pathway (*38*). Stimulation of galloyl ester biosynthesis with accompanying down-regulation of subsequent transformations such as oxidative dimerization and gallate hydrolysis should lead to pellicle tannin constituents with the optimum gallic acid content to prevent aflatoxin biosynthesis. The question of whether repeated exposure of the fungus to hydrolyzable tannins results in permanent atoxigenicity remains to be resolved and is currently under investigation in our laboratory.

Manipulation of composition and levels of hydrolyzable tannins will have implications for the organoleptic qualities of walnuts. The astringency of this product is associated with the tannins present in the pellicle. Thus, black walnuts are known to be much less astringent than English walnuts, but the latter have become the commercially desirable crop because of their thinner shell and ease of separation of the kernel after cracking. Our analyses (**Table 1**) show that the gallic acid content of black walnut species (1.0 and 1.1%) is not much lower than that in the English walnut variety Chandler, with the lowest level (1.4%). In contrast, ellagic acid levels in black walnuts are considerably lower than those in English walnuts, suggesting that astringency is more likely to be associated with ellagic acid or its hexahydroxydiphenic ester precursor. A directed increase in gallic acid content to suppress aflatoxin formation therefore would not necessarily result in increased astringency, particularly if ellagic acid production was suppressed at the same time.

The generally undesirable astringent taste properties of hydrolyzable tannins may be counterbalanced by a number of favorable effects. Mild astringency in medicinal plants has been shown to be beneficial for treatment of digestive disorders (*31*). In addition, hydrolyzable tannins have been reported to have significant biological and pharmacological activities, including bacteriocidal, anthelmintic, and antihepatoxic properties, suppression of human immunodeficiency (HIV) and herpes simplex (HSV) viral replication, and inhibition of glucosyl transferases of *Streptococcus mutans*, the organism responsible for dental caries (*31*). With regard to commercial properties of the walnut itself, the antioxidant activity of hydrolyzable tannins (*26*) and the demonstrated activity of sucrose gallate esters as radical scavengers in lipid peroxidation (*39*) suggest that appropriate levels should prevent or delay the development of rancidity. It therefore appears that there should be no major contraindications to attempts to increase gallic acid levels in walnut pellicle in order to suppress aflatoxin formation. A primary objective of our future research will be to extend these findings to other tree nut species, such as almonds and pistachios, and eventually to other crops that are also adversely affected in domestic and export markets by aflatoxin contamination.

SAFETY

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

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