
6 Health Aspects and Antiaflatoxigenic Activity of Phytochemicals in Tree Nuts

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6.1 INTRODUCTION

Tree nuts, while not generally regarded as a staple food, are particularly appreciated by consumers for their flavor and convenience. Snack foods in the United States such as chips, pretzels, and popcorn are often highly processed, but in many other countries, nuts and seeds play a more prevalent role and these undergo little or no postharvest treatment, although occasionally they may be smoked or flavored with spices. Even when incorporated into baked goods or savory dishes, or utilized as nut butters or marzipan, they are subject to minimal processing, consisting primarily of chopping, grinding, or blanching. Because of such factors, most consumers regard nuts as “natural” foods and have little concern for adverse or deleterious effects other than occasional allergenicity in some individuals. Development of off-flavors from rancidity and infection with spoilage microorganisms (generally *Aspergillus niger*, *Penicillium* spp., and *Rhizopus* spp.) are so obvious that such nuts are usually discarded and not eaten.

Nuts contain high levels of protein, fiber, and dietary fats, which in association with their pleasant flavor and convenience, has led to the recommendation that they should be an essential part of a

healthy diet. This was recently endorsed by allowance of a qualified health claim for a relationship between the consumption of nuts and reduced risk of coronary heart disease (CHD) by the Food and Drug Administration (FDA) [1]. Most tree nuts have low levels of saturated fats but high levels of desirable unsaturated fats. Monounsaturated fatty acids (MUFA) such as oleic acid (18:1 ω 9) occur in almonds and hazelnuts and polyunsaturated fatty acids (PUFA) such as linoleic acid (18:2 ω 6) and α -linolenic acid (18:3 ω 3) predominate in walnuts [2]. A consistent decrease in serum cholesterol levels and reduced risk of CHD in humans was established by meta-analysis of five controlled diet clinical intervention trials with walnuts [3] and analogous effects have been found with other nuts [4].

Although they are not a primary nutrient source, and may sometimes be perceived almost as a "condiment" crop, tree nuts are an extremely valuable agricultural commodity in national and international trade. In California, where almost all almonds, pistachios, and walnuts in the United States are produced, they had an aggregate value of \$3.42 billion in 2005, with 40%–60%, valued at \$2 billion, being exported [5,6]. In spite of this, there is one serious constraint on the marketing of tree nuts, namely the potential presence of aflatoxins, which are highly regulated because of food safety concerns. The European Community (EC) in particular applies an extremely low tolerance level of 2 ng/g for aflatoxin B₁ and 4 ng/g total aflatoxins [7]; in contrast, the FDA has a domestic maximum guidance level for tree nuts intended for human consumption of 20 ng/g (e.g., 20 ppb) [8]. In 2005, 94% of the rapid alerts or notifications from the EC Rapid Alert System for Food and Feed (RASFF) for mycotoxins in tree nuts were for aflatoxins, with 28 for almonds and 13 for pistachios from the United States [9]. While this is a small number relative to the total of 827 alerts for all mycotoxins in tree nuts, it represents considerable economic loss to California producers and exporters, due to lost revenue, return, or reprocessing of the shipment and increased sampling of subsequent imports. Additional costs are incurred for preshipment quality control and from the rigorous sampling protocol mandated by the EC [7].

6.2 AFLATOXINS IN TREE NUTS

Aflatoxins are metabolites produced by many strains of the fungi *Aspergillus flavus* and *A. parasiticus*, which commonly infect major agricultural crops such as corn, peanuts, cotton, and tree nuts. Contamination of human foods and animal feeds by these compounds is of great concern because they are classified as carcinogens, particularly in humans infected with hepatitis [10,11]. There are also episodes of acute toxicity, the most recent being caused by contaminated maize in eastern Kenya in 2004 with 317 diagnosed cases and 125 deaths; analysis of maize samples revealed aflatoxin B₁ concentrations as high as 4400 ppb [12].

Structurally, aflatoxins are polyketide derivatives and can be classified into B and G groups, which share a common difurochromanone core but with furanone and δ -lactone moieties appended, respectively (Figure 6.1). As a general rule, *A. flavus* produces the B group aflatoxins whereas *A. parasiticus* produces both B and G groups, although not all strains of either species are capable of producing aflatoxins and these are regarded as atoxigenic. The most common metabolites are aflatoxins B₁ and G₁, accompanied to a lesser extent by aflatoxins B₂ and G₂, which are 8,9-dihydro derivatives of B₁ and G₁, respectively. Aflatoxins B₁ and G₁ are of greatest concern because the presence of the furan double bond permits biotransformation into the 8,9-epoxide through *in vivo*

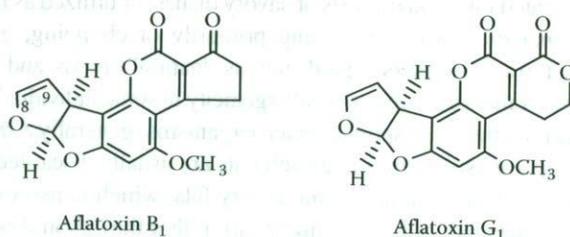


FIGURE 6.1 Structures of aflatoxin B₁ and aflatoxin G₁.

hepatic oxidation by cytochrome P450 [13]. Aflatoxin B₁-*exo*-8,9-epoxide has been shown to be the proximate carcinogen, intercalating into double-stranded DNA [14].

Since tree nuts are subjected to minimal processing, there are few postharvest opportunities to reduce aflatoxin levels and these are primarily influenced by nut species or cultivar and their proper handling during harvesting, drying, and packing. Because of the perception of tree nuts as a "natural" food, treatment with fungicides to reduce fungal infection or processing to destroy aflatoxins is unlikely to be acceptable to consumers or regulators. Such methods would involve the use of toxic antifungal compounds and could change the organoleptic characteristics of the product. Furthermore, the additional expense would add to the cost of an already relatively expensive product, leading to reduced consumption. There is an association of aflatoxins in tree nuts with damage by insect pests such as codling moth and navel orange worm, permitting ingress of fungal spores, and sorting to remove obviously damaged nuts can reduce the overall aflatoxin load. One method of preventing such damage would be to breed new cultivars with increased physical barriers to microorganisms, including the husk or hull, the shell, and the pellicle or seed coat (papery tissue surrounding the kernel). However, this could result in undesirable characteristics such as difficulty in cracking or shattering and even then the toxins can often be found in nuts which show no evidence that the integrity of the shell has been breached. It appears that the fungus may enter through the relatively less impenetrable suture or stem end, or be present in the flowers at the time of fertilization and thus incorporated into the fruit as it matures.

An alternative general strategy to limit aflatoxin formation is therefore to investigate natural factors within each nut species that might confer resistance to *Aspergillus* colonization and growth and/or aflatoxin biosynthesis. Such factors could then be incorporated and enhanced during breeding for new cultivars. The toughness of the shell and its lignin-derived composition suggests that it primarily presents a physical barrier, but the protective abilities of the husk, pellicle, and possibly the kernel itself are more likely to be due to the presence of natural chemical constituents. A bioactivity-directed strategy of determining the most aflatoxin-resistant nut species and varieties and focusing upon the natural product composition of these to isolate and identify specific resistance factors has proved fruitful. Obviously, reduction of aflatoxin biosynthesis is the most desirable outcome because merely eliminating aflatoxigenic fungi may result in their replacement by other toxic or spoilage microorganisms. Circumstantial evidence that aflatoxin biosynthesis can be influenced lies in the fact that mutant strains of *Aspergillus* exist that either do not produce the mycotoxins or their precursors, or that possess a disrupted biosynthetic pathway, suggesting that aflatoxigenesis is not absolutely essential to the fungus. For example, one mutant of *A. parasiticus* produces only the bright orange-colored norsolorinic acid, the initial product resulting from polyketide cyclization. Analogous mutants have been discovered for each intermediate product of aflatoxin biosynthesis [15].

6.3 AFLATOXIN RESISTANCE PHYTOCHEMICALS IN TREE NUTS

Phytochemical metabolites with antifungal activity fall into two distinct classes, phytoalexins and phytoanticipins [16]. Whereas phytoalexins are biosynthesized in significant amounts only in response to fungal attack, phytoanticipins are constituents always present in the plant, either in their active form or as precursors (e.g., glycosides) from which they can be generated. Phytoalexins are usually produced and located only in close proximity to the point of fungal attack and in variable amounts. This latent process means that even if the *Aspergillus* infection is ultimately overcome, there may have been sufficient time for significant amounts of aflatoxin to have been produced. In contrast, phytoanticipins are a characteristic of any particular plant species or variety, and therefore capable of genetic control for optimal effect. Identification of specific anticipins in tree nuts that confer resistance to aflatoxigenesis should be possible by first determining the relative inhibitory activity of different species and cultivars. Individual tissues of the most active with respect to aflatoxin suppression can then be examined for those having the highest potency, and such materials extracted and analyzed for content and concentrations of bioactive compounds. Since such constituents should be highest in

the most active tissues, isolation in sufficient amounts for biological testing should thus be facilitated. The general approach to identifying antiaflatoxigenic compounds in tree nuts is therefore to determine the most resistant nut species, then the varieties within that species, and finally the location of the factors within the individual nuts.

6.3.1 VARIETAL RESISTANCE OF TREE NUT SPECIES

Both anecdotal evidence, and the RASFF notification system for aflatoxins in products entering the EC [9], suggests that there is a species-related propensity for tree nuts to become contaminated. Thus, in 2005, almonds were subject to the greatest number of notifications, followed by pistachios, whereas there were none for walnuts. Furthermore, from experience, walnut producers and exporters are far more concerned with spoilage microorganisms, having little concern for aflatoxin contamination. *In vitro* experiments with selected cultivars and breeding lines of all three species confirmed this assumption. Comparison of the ability of *A. flavus* to produce aflatoxins when grown on agar medium containing 5% by weight of ground kernels (including seed coat) from 23 varieties of almonds (*Prunus dulcis*), 26 varieties of English walnuts (*Juglans regia*), single varieties each of pistachio (*Pistacia vera*, 'Kerman'), and black walnut (*Juglans hindsii*, 'Rawlins') showed that the English walnut was considerably more active in suppressing aflatoxigenesis [17]. Specifically, aflatoxin levels for almond ranged from 34 to 179 $\mu\text{g}/\text{plate}$ (average 91 $\mu\text{g}/\text{plate}$) versus 0 to 28 $\mu\text{g}/\text{plate}$ (average 4.2 $\mu\text{g}/\text{plate}$) for English walnuts, after 7 days of incubation at 30°C. The pistachio and black walnut kernels were intermediate in activity with values of 40 and 44 $\mu\text{g}/\text{plate}$, respectively. Most significantly, as shown in Figure 6.2, the 'Tulare' walnut allowed no aflatoxin formation, indicating that this variety would be the most rewarding to investigate, since it would most probably have the highest level of antiaflatoxigenic compounds.

6.3.2 ANTIAFLATOXIGENIC CONSTITUENTS IN SPECIFIC TISSUES

The potent antiaflatoxigenic activity of 'Tulare' walnuts established that the bioactive constituents were located either throughout the edible portion of the nut, or alternatively specifically within the

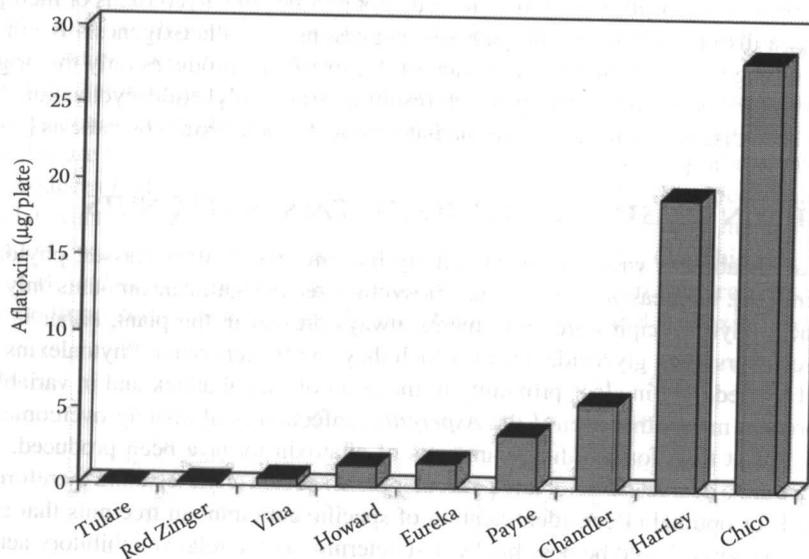


FIGURE 6.2 Differential production of aflatoxin B_1 by selected walnut varieties on agar media containing 5% by weight of ground kernels.

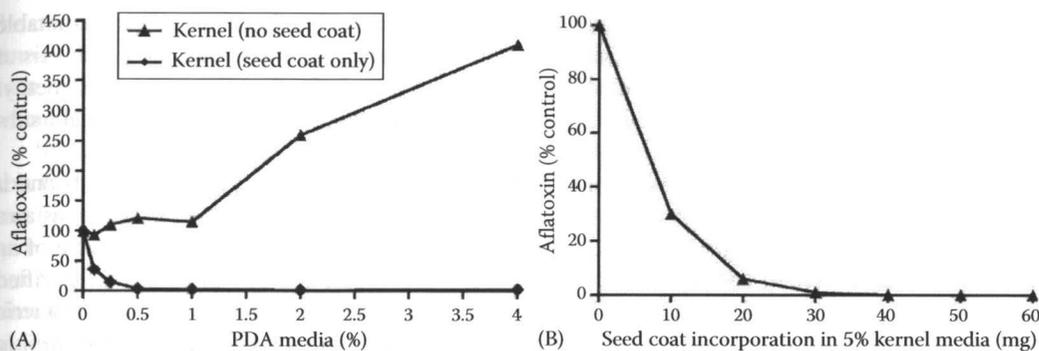


FIGURE 6.3 (A) Aflatoxin B_1 production on potato dextrose agar (PDA) media incorporating various amounts of kernel only or seed coat only of 'Tulare' walnut. (B) Decrease in aflatoxin B_1 production by incorporation of increasing amounts of seed coat material into agar media containing 5% by weight of ground kernels.

kernel itself (endosperm) or the pellicle (seed coat), the latter being the thin tissue surrounding the kernel. Samples of each tissue from 'Tulare' nuts were therefore obtained by peeling the pellicle from the whole kernel and aflatoxin production was measured *in vitro* with endosperm or pellicle incorporated into potato dextrose agar (PDA) medium as previously done for the complete kernel. As shown in Figure 6.3, there was no inhibition of aflatoxin by the endosperm alone, and at incorporation levels greater than 1% in PDA media, the aflatoxin production rapidly increased to approximately 4-fold at 4% incorporation. This effect is probably caused by an increase in levels of nutrients such as sugars and fatty acids. In contrast, incorporation of pellicle alone is completely different, with aflatoxin suppressed to 3% of control at an incorporation level of 1%. At levels as low as 0.1%, aflatoxin production was reduced to about one-third that of control [17].

These results established that antiaflatoxic constituents were located entirely in the pellicle and this was confirmed by adding back pellicle material to 5% endosperm media in agar. Under these conditions, the ability of kernel to enhance aflatoxin production was negated. Aflatoxin production was inversely proportional to the amount of added pellicle, with an incorporation level approximating the weight proportion of seed coat to endosperm in whole kernels reducing aflatoxin to 0.8% of control (Figure 6.3) [17].

6.4 ISOLATION AND IDENTIFICATION OF WALNUT ANTIAFLATOXIGENIC CONSTITUENTS

The identification of 'Tulare' walnut pellicle as the source of antiaflatoxic activity greatly simplified procedures for isolation of the bioactive constituents since it made it unnecessary to extract the whole kernel, which would have introduced large quantities of extraneous material. Attention was therefore focused on the constituents of pellicle alone.

6.4.1 BIOASSAY-DIRECTED FRACTIONATION

The extraction and fractionation of 'Tulare' pellicle adopted conventional bioassay-directed procedures commonly used for isolation of natural products. Sequential Soxhlet extraction of finely ground pellicle with solvents of increasing polarity gave extracted material, after evaporation of the solvent, which was suitable for testing *in vitro* in a similar way to the unextracted tissues. The results were unusual in that acetone, methanol, and water extracts all exhibited activity, as did the extracted pellicle material; only the hexane extract lacked activity [18]. This suggested that hexane-extractable

material such as lipids did not have any effect on aflatoxin formation but that compounds extractable by more polar solvents consisted of a complex of constituents with variable solubilities. Analysis of extracts by gas chromatography/mass spectrometry (GC/MS) showed only trace amounts of methyl gallate in the water extract, indicating that a series of polar, relatively high molecular weight substances were the active compounds.

These results were considered in relation to literature on composition of walnut kernels and it was concluded that they were consistent with the established presence of hydrolysable tannins as a major component of the pellicle. In the 1950s, these compounds were extracted from pellicle of an unidentified walnut variety obtained from a commercial processor, and one of these was purified from the acetone-soluble fraction and named juglanin [19,20]. The latter was shown to be isomeric with, but not identical to, corilagin, a constituent of the seed pods of the legume *Caesalpinia coriaria* [21]. Corilagin possesses one of the simplest representative structures of a hydrolysable tannin and it is assumed that juglanin must have a different configuration with regard to substitution pattern or stereochemistry because of the differences in melting point and optical rotation. Hydrolysable tannins in walnuts consist of a glucose core, esterified with hexahydroxydiphenic acid and gallic acids, and as 1-*O*-galloyl-3,6-*(R)*-hexahydroxydiphenoyl- β -D-glucopyranose, corilagin contains only one of each moiety. A series of 17 more complex tannins have recently been isolated from walnut pellicle of the Chandler variety [22,23].

This information, in concert with the fact that no aflatoxin-suppressing activity is extractable with hexane, but with the demonstrated occurrence of activity in all other solvent extracts and even in exhaustively extracted residue, is consistent with activity being associated with the hydrolysable tannin content or composition of the pellicle.

6.4.2 ANALYSIS OF HYDROLYSABLE TANNIN CONTENT IN WALNUTS

The extraordinary structural complexity and diversity of hydrolysable tannins, especially in walnuts [22,23], would appear to make their correlation with anti-aflatoxic activity exceptionally difficult. However, in most cases, acid hydrolysis of almost all of the tannins gives only three products, namely glucose, gallic acid, and hexahydroxydiphenic acid, although the latter cannot be isolated as such because it spontaneously lactonizes to ellagic acid. This is illustrated in Figure 6.4 for strictinin, one of

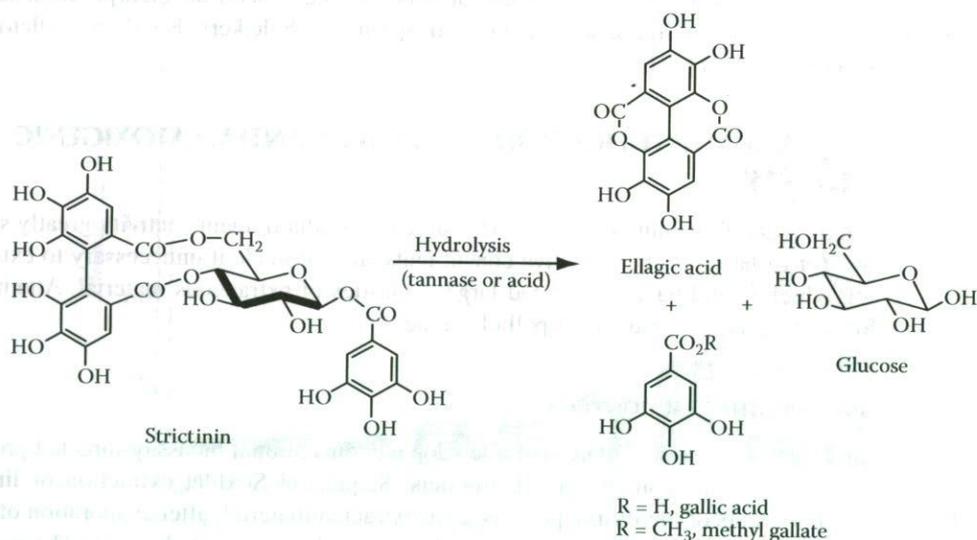


FIGURE 6.4 Structure of strictinin, a simple hydrolysable tannin present in walnuts and its hydrolysis products, ellagic acid, gallic acid, and glucose.

the major simple tannins in walnuts [22]. Interestingly, *A. flavus* is known to possess an extracellular tannase that is capable of hydrolyzing the tannins in an identical manner [24]. Furthermore, the presence of this tannase in any particular fungal strain can be demonstrated by observing a zone of clearing when the particular fungus is grown on media consisting of commercial tannic acid in agar [25].

Since gallic and ellagic acids are the ultimate products of the action of the fungus on hydrolysable tannins, these compounds may be used as surrogates for hydrolysable tannin content. This analysis can be easily achieved by hydrolysis of plant tissue samples with methanolic hydrochloric acid to give methyl gallate and ellagic acid (Figure 6.4). Both of these products can be measured in a single run by high-performance liquid chromatography (HPLC) on a reversed phase C₁₈ column with simultaneous monitoring at 252 and 280 nm, using a diode array detector (DAD), at concentrations as low as 0.1 µg/mL [26].

6.4.3 GALLIC AND ELLAGIC ACID CONTENT IN TREE NUTS

6.4.3.1 Variation of Gallic and Ellagic Acids with Maturity

The rapidity and convenience of the HPLC procedure has permitted analysis of a large number of samples, so that changes in gallic and ellagic acid levels with maturity could be compared for walnuts throughout the growing season. 'Tulare' was chosen as the aflatoxin-resistant cultivar and "Chico" as a more susceptible variety. During the 2002 growing season, samples were taken monthly, but on the basis of these results, this was changed to biweekly and collections started earlier during 2003 [18]. As shown in Figure 6.5, even before the kernel became firm ("jelly" stage), high levels of gallic acid were present, increasing by 3-fold over the first month of development, followed by a rapid decline and then reaching a relatively constant level throughout the rest of growth. Ellagic acid showed a contrasting pattern, increasing fairly steadily throughout kernel development. This is quite consistent with hydrolysable tannin biosynthesis, in which pentagalloyl glucose, the initially product, loses a proportion of gallic acid moieties either via hydrolysis or by dimerization to form hexahydroxydiphenic esters; as a consequence, the ellagic acid content, derived from the latter, would be expected to increase. Further examination of the results showed that the gallic acid content of 'Tulare' was consistently higher than that of 'Chico,' indicating a higher overall level of hydrolysable tannins in the pellicle of the former. Ellagic acid levels were also higher overall in 'Tulare' relative to 'Chico' but the effect was less remarkable.

The ability of hydrolysable tannins to inhibit aflatoxin production, therefore, correlates well with the relative amounts of gallic acid contained within the structures of all of the individual tannins, considered as a whole, but the ellagic acid content appears to have little influence. Since the proportion of ellagic acid to gallic acid generally increases as biosynthesis of the tannins proceeds. It proceeds, it would be expected that antiaflatoxic activity should decline with maturity of the nut, but this may be offset by the fact that kernel is much less vulnerable than in early growth due to the physical barrier to infection provided by hardening of the shell. However, maintenance of gallic acid content above a specific, not yet determined, level should enable inhibitory activity to be preserved.

6.4.3.2 Variation of Gallic and Ellagic Acids with Cultivar

In addition to analyzing gallic and ellagic acid content throughout the growing season, the HPLC method can be used to compare levels of these compounds between cultivars and with other nut species. As shown in Table 6.1 for tree nuts harvested in the 2003 season, English walnut varieties have gallic acid contents ranging from 1.4% to 3.4% of the dry weight (dw) of the pellicle. These values generally correlate with the ability of the varieties to suppress aflatoxin production *in vitro* (Figure 6.2), although it should be recognized that such activity is not solely related to gallic acid but must also depend to some extent on ellagic acid content and on the activity of the intact hydrolysable tannins prior to tannase hydrolysis. Comparison of the ellagic acid/gallic acid ratio for the walnuts

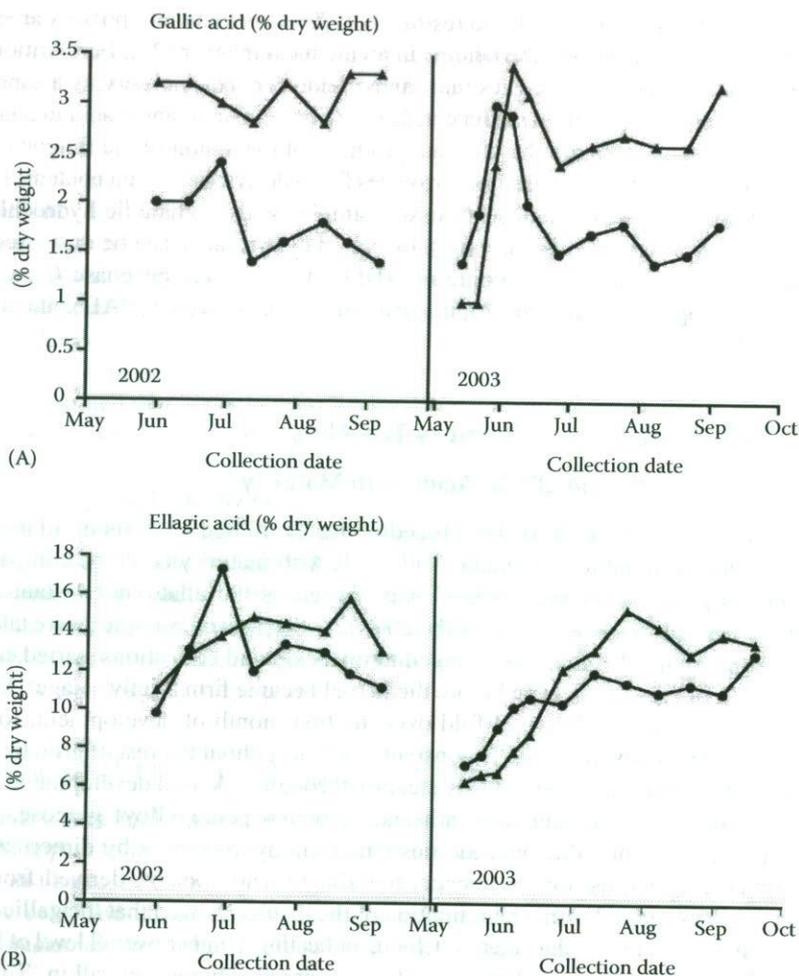


FIGURE 6.5 Variation in gallic acid and ellagic acid content in kernels of walnut varieties 'Tulare' (▲) and 'Chico' (●) during the growing seasons 2002 and 2003.

showed that there was a general trend to lower values for those varieties most resistant to aflatoxigenesis, with 'Tulare' having a ratio of 4.4 and 'Chico' a ratio of 6.1.

Black walnut cultivars 'Rawlins' and 'Thomas' had considerably lower gallic acid levels of ~1% dw, quite close to that of the pistachio cultivar 'Kerman' (0.5% dw), and *in vitro* aflatoxin production for the walnut 'Thomas' and pistachio 'Kerman' were also similar at 45 and 40 $\mu\text{g}/\text{plate}$, respectively [17]. The hydrolysable tannin in pistachios has a core of quinic acid, rather than glucose (unpublished results), and its stereochemistry is such that dimerization of gallic acid moieties to hexahydroxydiphenic esters, and consequent formation of ellagic acid, cannot occur. Any aflatoxin inhibitory activity is, therefore, dependent on either the tannin itself or gallic acid.

In contrast to walnuts and pistachios, almond pellicle contains no hydrolysable tannin and therefore no gallic acid was detectable in the cultivars 'Nonpareil' and 'Mission.' This is consistent with their propensity to accumulate aflatoxins [9] and their ability to support aflatoxin biosynthesis. However, there are considerable differences in aflatoxin production *in vitro* between cultivars and these must be due to compounds other than hydrolysable tannins. Almond pellicle has been shown to contain other phenolic constituents, primarily phenolic acids and flavonoids, which may suppress aflatoxin production although less effectively than the tannins [27–29].

TABLE 6.1
Gallic Acid and Ellagic Acid Content of Seed Coat at Maturity
from Selected Nut Species Harvested in California in 2003

Species/Cultivar	Gallic Acid (% dw)	Ellagic Acid (% dw)	Ellagic/Gallic Ratio
<i>Walnut, English (Juglans regia)</i>			
Red Zinger	3.4	15.9	4.7
Tulare	3.2	14.0	4.4
Tehama	2.6	11.0	4.2
Hartley	2.2	13.3	6.0
Payne	2.0	12.3	6.2
Serr	2.0	11.8	5.9
Chico	1.8	11.0	6.1
Chandler	1.4	10.0	7.1
<i>Walnut, Black</i>			
<i>Juglans nigra</i> 'Thomas'	1.1	2.6	2.4
<i>Juglans hindsii</i> 'Rawlins'	1.0	3.1	3.1
<i>Pistachio (Pistacia vera)</i>			
Kerman	0.5	nd	—
<i>Almond (Prunus dulcis)</i>			
Nonpareil	<0.1	nd	—
Mission	<0.1	nd	—

Note: nd, not detected.

6.4.3.3 Structure–Activity Relationships

Although overall gallic acid content of the pellicle has been shown to be a primary indicator of the ability of any particular cultivar to resist aflatoxin formation, incorporation of this finding into breeding programs will depend on a much more complete analysis of structural relationships to the hydrolysable tannins from which it derives. Furthermore, a fundamental understanding of the mechanism by which this and other compounds suppress aflatoxin biosynthesis should open new directions to control of the contamination problem. Gallic acid in hydrolysable tannins is bound primarily as gallate esters of the carbohydrate core or as depsides of the underlying gallate or hexahydroxydiphenate moieties. Hydrolysis of such ester linkages is not likely to proceed at the same rate and may also be dependent on the substitution position on the carbohydrate and degree of steric hindrance. In addition, the fungal tannase consists of several isozymes with individual esterase and depsidase activities [30], while some strains of *Aspergillus* may not even possess such enzymes; in the latter situations, aflatoxin suppression will be dependent on the activity of the tannins themselves. *In vitro* bioassays therefore cannot be regarded as a comprehensive model for the situation *in vivo*, although they are convenient as a first approximation for evaluating and comparing antiaflatoxic potential. *Aspergillus* growing on the kernel of the nut will be directly exposed to the hydrolysable tannin in the seed coat and gallic acid will be generated where metabolic activity, including tannase production and aflatoxin biosynthesis, is the greatest. As a consequence, the effective gallic acid concentration at this point source may be much higher than in the *in vitro* bioassays where it is distributed throughout the media. It is, therefore, necessary to evaluate different structural types of hydrolysable tannins and other types of pellicle constituents, such as those in almonds, in order to develop a theoretical basis for understanding aflatoxin biosynthesis and control.

6.5 MECHANISM OF ANTIAFLATOXIGENIC ACTIVITY

The discovery of hydrolysable tannins and their derivatives as natural antiaflatoxigenic constituents is significant with regard to practical control of aflatoxin contamination in tree nut crops. Enhancement of levels of these compounds in new cultivars should enable growers to provide a product to wholesalers and exporters that, together with postharvest screening of nuts likely to be infected, will generally pass regulatory inspections. However, the information and compounds can also be used as a scientific tool to investigate the fundamental question of the role of aflatoxins in the life cycle of the fungus itself. Although many natural products are known to be fungicidal, the ability to affect metabolite biosynthesis without inhibiting fungal growth is unusual and raises the question as to how essential aflatoxins are to survival of the fungus. Initial observations suggest that it is not merely the penultimate stages of aflatoxin formation that are disrupted but rather that the whole biosynthetic pathway is affected, including the genes controlling fatty acid or polyketide synthases involved in the earliest stages of aflatoxin biosynthesis. If this is so, a basic physiological and biological response in the fungus must be involved.

6.5.1 EFFECT OF PHENOLIC ANTIOXIDANTS ON FUNGAL OXIDATIVE STRESS RESPONSE

It has been suggested that aflatoxin production in *A. parasiticus* is a response to oxidative stress [31]. This implies that the aflatoxins themselves serve as antioxidants. However, it is noteworthy that although many of their precursors are phenolic compounds and therefore structurally feasible antioxidants, aflatoxins are not phenolic; it may be that the total biosynthetic network needs to be evaluated from this perspective. Defense responses [32] and environmental conditions such as drought [33] generate reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) in the plant that result in oxidative stress induced in the fungus, which may be alleviated by induction of aflatoxin biosynthesis. The presence of hydrolysable tannins and hydrolytic products such as gallic acid, which are potent antioxidants and free radical scavengers, could relieve the fungus of the costs associated with such biosynthetic necessity. This suggests that any phytochemical antioxidant, especially plant phenolics, could to some extent be used to reduce or eliminate aflatoxigenesis.

Comparative tests of phenolic compounds known to occur in walnuts, pistachios, and almonds have shown that these reduce aflatoxin levels *in vitro* (unpublished results). The most effective were pentagalloyl glucose, representative of a walnut-hydrolysable tannin, and 3,4-digalloylquinic acid, representative of a pistachio tannin, together with caffeic acid, all of which reduced aflatoxin production by >98% relative to the control. Gallic acid caused an aflatoxin reduction of 84% and ellagic acid was the least effective at 60%; phenolic acids and catechin, typical almond constituents, fell between these two values. The fact that all of these compounds show a significant degree of antiaflatoxigenic activity is further circumstantial evidence that aflatoxin biosynthesis is somehow involved in fungal oxidative stress response, but studies at the genetic level are necessary to establish the precise nature of this effect.

6.5.2 FUNCTIONAL GENOMICS OF FUNGAL OXIDATIVE STRESS RESPONSE

The phenolic compounds shown to reduce aflatoxin production in response to oxidative stress have potential as tools to investigate functional elucidation of the genes involved but this is limited by the absence of a practical gene transformation system. In the absence of a direct approach with *A. flavus*, use can be made of the yeast *Saccharomyces cerevisiae*, for which numerous stress response pathways have been characterized [34]. Selected *S. cerevisiae* strains, including mutants with single gene deletions, have been used as a model to evaluate phenotypic response to oxidative stress induced by H_2O_2 [35].

With the exception of undiluted ($\sim 10^6$) cells, growth of wild-type *S. cerevisiae* cells was completely inhibited for 6- to 10-fold serial dilutions on exposure to 3.3 mM H_2O_2 , whereas under

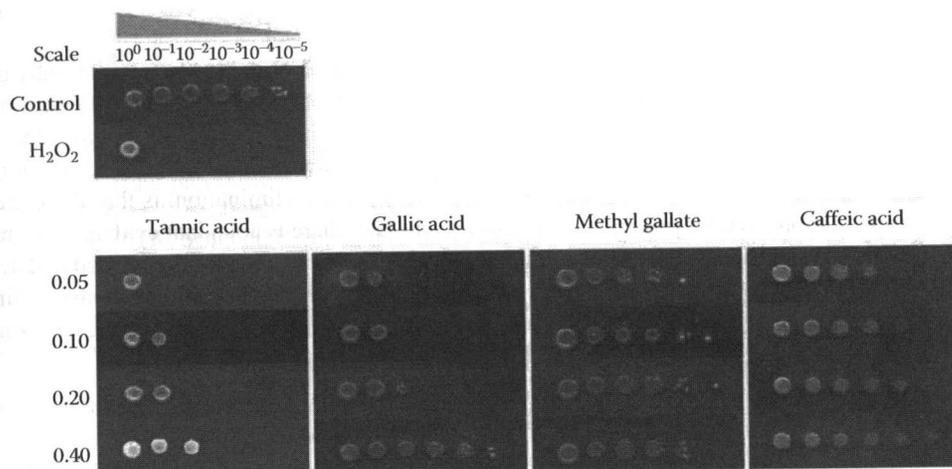


FIGURE 6.6 Effect of tannic acid, gallic acid, methyl gallate, and caffeic acid on growth recovery of serial dilutions of the yeast *S. cerevisiae* subjected to oxidative stress with H₂O₂.

control conditions in the absence of H₂O₂, all colonies from serially diluted cells were visible (Figure 6.6). When grown only in the presence of tannic acid, a hydrolysable gallotannin readily obtainable from commercial sources, or on gallic acid, at 0.4% (w/v), there was no inhibition of growth, demonstrating that these compounds alone are not toxic to the yeast. However, as shown in Figure 6.6, growth inhibition produced in the presence of H₂O₂ was overcome by culturing in the presence of the antioxidants tannic acid, gallic acid, methyl gallate, and caffeic acid in a concentration-dependant manner at 0.05%, 0.1%, 0.2%, and 0.4% incorporation, respectively. It is noteworthy that caffeic acid was much more effective than gallic acid, consistent with their differences in antiaflatoxic activity.

This approach was extended to the identification of the function of specific *S. cerevisiae* genes in antioxidative stress response using 22 deletion mutants. These encompassed strains defective in antioxidative stress response, gene regulation, DNA damage control, and signal transduction. When exposed to 2.5 mM H₂O₂ in the presence and absence of 0.4% antioxidant, the mutant *yap1Δ*, defective in a transcription factor for gene regulation of the antioxidative stress response, showed little appreciable recovery on treatment with the antioxidants, indicating that it is extremely sensitive to oxidative stress, while *rad54Δ*, deficient in DNA-dependent ATPase, showed partial recovery. In contrast, the strains *sho1Δ* and *cta1Δ*, deficient in transmembrane osmosensor signal transduction and catalase-dependent antioxidant stress response, respectively, exhibited complete recovery [35].

Using the expressed sequence tag (EST) database for *A. flavus* [36], 43 orthologs of *S. cerevisiae* genes involved in gene regulation, signal transduction, and antioxidation have been identified and the effect of oxidative stress on aflatoxin biosynthesis has been investigated in more detail. Functional complementation of the mitochondrial superoxide dismutase gene, *sodA*, an antioxidation stress gene from *A. flavus*, in a *sod2Δ* yeast mutant lacking the ortholog, demonstrated the utility of this approach [35]. The combination of knockout mutants and functional complementation analysis should thus enable the relationship between oxidative stress and aflatoxin biosynthesis to be elucidated. The recent availability of *A. flavus* genomic microarrays has enabled differential expression microarray analysis to show that treatment of *A. flavus* with antioxidants affects genes far upstream from the aflatoxin biosynthetic gene cluster (unpublished results). This approach will undoubtedly provide extremely detailed knowledge of the antioxidative stress response/aflatoxin biosynthesis relationship in the future.

6.6 CONCLUSION

It has been established that phenolic antioxidants and especially hydrolysable tannins, naturally present in tree nuts, can play an important role in preventing the formation of aflatoxins. Application of this knowledge should not only reduce exposure of consumers to these mycotoxins but also increase the marketability of tree nuts, especially through exports. A distinct advantage of enhancement of these natural products, as opposed to other approaches to aflatoxin elimination, is that there are no major contraindications to their presence in the product. In fact, there is abundant evidence that natural antioxidants in foods have significant health benefits. Not only can they reduce oxidative deterioration but hydrolysable tannins have also been reported to have antiviral, bacteriocidal, anthelmintic, and antihepatotoxic properties [37], while phenolic antioxidants in food may limit diseases associated with aging, such as CHD, neurological degeneration, and various types of cancer [38].

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