Mycotoxins in edible tree nuts

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Abstract

Tree nuts (almonds, pistachios, and walnuts) are an exceptionally valuable crop, especially in California, with an aggregate value approaching $3.5 billion. Much of this economic value comes from overseas markets, with up to 60% of the crop being exported. The product can be contaminated with aflatoxins or ochratoxins, with the former being of special concern because of the strict regulatory levels (4 ppb total aflatoxins) applied by the European Community (EC). Natural, consumer-acceptable control methods are therefore required to conform to such limits. Research has shown that aflatoxin production is markedly decreased by the presence of natural antioxidants that occur in tree nuts, including hydrolysable tannins, flavonoids and phenolic acids. *In vitro* testing of individual compounds showed that the antiaflatoxigenic effect correlated with the structure and concentration of such compounds in individual nut varieties and species. This lead to the hypothesis that aflatoxin biosynthesis is stimulated by oxidative stress on the fungus and that compounds capable of relieving oxidative stress should therefore suppress or eliminate aflatoxin biosynthesis. Oxidative stress induced in *A. flavus* by addition of *tert*-butyl hydroperoxide to the media stimulated peak aflatoxin production and maintained high levels over time. However, aflatoxin formation was significantly inhibited by incorporation into the media of the antioxidant, tannic acid. Measures to increase natural products with antioxidant properties in tree nuts may thereby reduce or eliminate the ability of *A. flavus* to biosynthesize aflatoxins, thus ensuring levels at or below regulatory limits and maintaining export markets for U.S. tree nuts.
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1. Introduction

Almonds, pistachios and walnuts, collectively defined as tree nuts, are major crops in California, both in terms of acreage cultivated and economic assets to the State. The aggregate income from these nut crops for the year 2005 was $3.46 billion (National Agriculture Statistics Service, USDA, 2006). Much of this value accrues from export markets around the world, which have been rapidly increasing over recent years. Depending on the tree nut variety, exports range from 40% to 60% of total crop production (Foreign Agricultural Service, USDA, 2006). In certain countries, nuts are consumed in place of snack foods such as popcorn and potato chips because of their high nutritional value and pleasant flavor. Per capita consumption is expected to increase both in the U.S. and abroad with continued promotion of their properties as healthy foods.

Tree nuts are subject to infection by a variety of microorganisms that can induce spoilage or produce metabolites that are toxic to humans, animals and birds. Although in many cases the sources of infections are not known, they are exacerbated by factors such as insect damage, drought and high temperatures. A survey of incidence established that the most frequently found genera were *Aspergillus*, *Rhizopus*, and *Penicillium* (Bayman et al., 2002a). Mycotoxigenic fungi of particular concern are *Aspergillus* species that produce hepatotoxic aflatoxins and nephrotoxic ochratoxins (Fig. 1). Screening of fungal isolates from tree nut orchards, nuts and figs in California showed that no field isolates of *A. ochraceus* and *A. melleus* produced detectable amounts of ochratoxin A but that all *A. alliaceus* isolates, which were obtained only from figs and not from nuts, produced the toxin (Bayman et al., 2002b). The EU has recently imposed new regulatory limits of 2–10 µg/kg (2–10 ppb) ochratoxin A in food commodities and processed foods (European Commission, 2005) but those of greatest concern are meats, cereals, beer, coffee, and grape products

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(wine, grape juice and dried vine fruits) (Jørgensen, 2005). There is little evidence of an ochratoxin contamination problem with tree nuts at the present time, although a single alert information notification was issued for ochratoxin A in pistachios from the U.S. in 2005 (European Commission, 2005). It has been hypothesized that the predominance of atoxigenic strains of *A. ochraceus* and *A. melleus* may account for the rare occurrence of ochratoxin in California tree nuts (Bayman and Baker, 2006).

In contrast, aflatoxins are a serious concern to exporters of California tree nuts. These compounds are metabolites of various strains of *A. flavus* and *A. parasiticus*, with the former producing only the aflatoxin B series and the latter both aflatoxins B and G (Fig. 1). Of greatest concern are aflatoxins B_1 and G_1, because they are procarcinogens due to the presence of the double bond in the terminal furan ring, which is oxidized by hepatic enzymes to an epoxide that can intercalate into DNA (Eaton and Gallagher, 1994). Metabolites such as aflatoxin B_2, in which the double bond is saturated, are a much less serious threat, although oxidation to the unsaturated compounds and subsequently the epoxides can be hypothesized. The acute toxicity of aflatoxins in developed countries is not a concern, especially in relation to tree nuts which are not a major component of the diet. However, the potential for such toxicity exists in countries such as Kenya, where consumption of contaminated maize, a staple food, recently led to 317 cases of poisoning and 125 deaths in rural areas (Lewis et al., 2005).

Due to their hepatocarcinogenic potential, especially in individuals infected with hepatitis B (Henry et al., 2002), aflatoxins are highly regulated in many countries around the world. In the United States the Food and Drug Administration has set a maximum guidance level limit for tree nuts intended for human consumption at 20 ng/g (20 ppb) (Food and Drug Administration, 1996), but in the European Community, a major importer of California tree nuts, the extremely low tolerance level of 2 ng/g for aflatoxin B_1 and 4 ng/g total

Table 1
European Commission Rapid Alert System for Food and Feed (RASFF) notifications on mycotoxins for 2005

Mycotoxin	Nuts and nut products	Fruits and vegetables	Herbs and spices	Other [a]	Total
Aflatoxins	827	66	48	6	947 U.S. 41 (4%)
Ochratoxin A	1	17	12	12	42 U.S. 1 (2%)
Patulin	0	0	0	6	6
Fumonisins	0	0	0	2	2

[a] Animal nutrition, cereal products, baby food, milk products, fruit juices.

aflatoxins has been applied (Commission of the European Community, 1998). In 2005, the European Commission Rapid Alert System for Food and Feed (RASFF) reported a total of 993 alerts or informational notifications for mycotoxins, of which 95% were for aflatoxins (European Commission, 2006) (Table 1). Most of these notifications (457, 46%) were for pistachio nuts originating from Iran but 28 notifications concerned almonds and almond products originating from the United States. Overall, almond and pistachio imports to the EU from the US in 2005 were subjected to 41 rapid alert and information notifications (European Commission, 2005), effectively amounting to a rejection or requirement for reprocessing of the shipment, costs which are further compounded by the affected shipper being subjected to much greater scrutiny of subsequent lots. This presents a serious economic threat for producers and exporters because of the need to ensure that shipments do not exceed regulatory limits, with the consequent potential for rejection or even destruction.

In contrast to many crops, tree nuts for export undergo minimal or very light processing, such as blanching, and the majority of the crop are consumed as whole nuts. Any subsequent processing, such as incorporation into baked goods or conversion into marzipan is performed by the importer or ultimate consumer after aflatoxin analysis has been performed. There is thus little opportunity to reduce aflatoxin levels by artificial means and natural, consumer-acceptable methods must therefore be found. The nature of tree nut harvesting and processing, which involves considerable potential for spreading of fungal spores and aflatoxins throughout the lots, mandates that the most effective method of control would be to prevent aflatoxin formation in the nuts themselves by enhancing natural resistance.

2. Resistance of tree nuts to aflatoxigenesis

There is considerable evidence that almonds, pistachios and walnuts show a differential resistance to contamination by aflatoxins. For example, in 2005, no walnut shipments to the EU were the subject of alerts or notifications for aflatoxins, although one shipment was the subject of an alert for mould. In contrast, 28 almond and 13 pistachio shipments were identified (European Commission, 2005). Although this is circumstantial evidence, it reflects market realities and conforms to the position of the industry groups, in which

Fig. 1. Structures of ochratoxin A and aflatoxins B_1 and G_1; aflatoxins B_2 and G_2 are saturated at the 8,9-double bond.

walnut producers are primarily concerned with spoilage microorganisms such as *Rhizopus*, *Penicillium* and *Aspergillus niger*, whereas almond and pistachio producers are greatly affected by the economic impact of aflatoxin contamination. Such differences cannot be accounted for merely by differences in physical protection of the nuts by the hulls or shells because both codling moth and navel orangeworm are major pests of almonds, pistachios and walnuts, wounding the external tissues and providing many avenues for entry of fungal spores into the edible portion.

Suppression of aflatoxin formation must therefore result from natural chemical or biochemical resistance factors inherent to each nut species. This has been confirmed by *in vitro* experiments in the laboratory, which showed that when 5% ground nut kernels were incorporated into agar media and inoculated with *A. flavus*, various walnut varieties produced 0–28 µg/plate and almond varieties produced 20–192 µg/plate. The only pistachio variety in commercial production in California, 'Kerman', was intermediate in value producing 40 µg/plate (Mahoney et al., 2003). The range of susceptibilities in almond and walnut varieties, respectively, showed that aflatoxigenesis must be controlled by specific constitutive compounds in the nut kernels. Even more significant was the fact that *A. flavus* grown on media incorporating the walnut cultivar 'Tulare' failed to produce any detectable level of aflatoxin. This provided an obvious starting point for investigation of specific factors associated with anti-aflatoxigenesis. Further experiments with this cultivar showed that all such compounds had to be located in the seed coat (pellicle) and none in the endosperm itself, since no inhibitory activity was associated with the kernel from which the pellicle had been removed. In fact, such kernel material promoted aflatoxin formation at higher concentrations, probably due to increased nutrient availability. In contrast, pellicle alone incorporated into PDA decreased aflatoxin biosynthesis to less than 3% of control at very low concentrations, and when added back to kernel media aflatoxin was decreased from

133 µg/plate for a control sample with no pellicle to below the detection limit of <20 ng/plate at 50 mg of added pellicle.

Sequential extraction of the seed coat with solvents of increasing polarity and testing of the individual fractions showed that all of the activity was associated with polar extractives and even that some of the activity was unextractable (Mahoney and Molyneux, 2004). Consideration of the known constituents of walnut pellicle lead to the conclusion that antiaflatoxigenic activity had to be related to hydrolysable tannins (Jurd, 1956; Fukuda et al., 2003). However, *A. flavus* is known to have tannase enzymes (Yamada et al., 1968) which are capable of hydrolyzing the tannins into their respective components, the core sugar (in the case of walnut tannins, glucose), gallic acid and ellagic acid (Fig. 2). Simple inspection of the pistachio-derived strain used in our experiments, *A. flavus* 4212 (NRRL 25347) growing on tannic acid/agar medium showed a visible zone of clearing, consistent with tannase production (Bradoo et al., 1996). This raises the question as to whether the anti-aflatoxigenic activity is due to the parent tannins or the hydrolytic products. However, analysis of levels of gallic and ellagic acids derived from the tannins by methanolysis of the seed coat under acidic conditions (Lei et al., 2001) showed that gallic acid levels were consistently higher in the variety 'Tulare' relative to the variety 'Chico', which is better able to support aflatoxin biosynthesis *in vitro*, whereas the ellagic acid levels in both varieties were quite similar (Mahoney and Molyneux, 2004). This does not resolve the question of whether free or bound gallic acid is more effective but it does indicate that overall gallic acid content is a determining factor.

3. Antiaflatoxigenic activity of tree nut phenolics

The exceptional diversity of hydrolysable tannin structural types in walnuts suggests that their ability to inhibit aflatoxin biosynthesis *in vitro* is related to their known antioxidative properties (Fukuda et al., 2003), rather than to any particular

Fig. 2. Structure of tellimagrandin I, representative of a typical walnut hydrolysable tannin, and its hydrolytic products, gallic acid, ellagic acid and glucose.

structural feature. This hypothesis is in accord with the demonstration that suppression of aflatoxin formation by the simple phenol, eugenol, is due to inhibition of lipid peroxidation, and led to the proposal that oxidative stress exacerbates aflatoxin production (Jayashree and Subramanyam, 1999, 2000). If this is the case, either the hydrolysable tannins themselves or their hydrolytic products, gallic and ellagic acids, should exhibit antiaflatoxigenic activity to a greater or lesser extent. Furthermore, whereas walnut phenolics consist almost entirely of tannins, pistachios and almonds have considerably more diversity in phenolic structural types.

Like walnuts, pistachio seed coats and hulls also contain hydrolysable tannins (unpublished results) but these are based on a quinic acid core and are much simpler in structure than the walnut tannins. The stereochemistry of quinic acid eliminates any possibility of dimerization of the gallic acid moieties which would lead to hexahydroxydiphenic acid and subsequently ellagic acid on hydrolysis; gallic and quinic acids are therefore the only possible hydrolysis products. In addition to these compounds, pistachio hulls contain anacardic acids, consisting of an *o*-dihydroxybenzoic acid moiety substituted with a series of lipophilic side chains (Yalpani and Tyman, 1983). The common plant constituent, caffeic acid, a known inhibitor of 5-lipoxygenase (IC_{50} 3.7 μM) and 12-lipoxygenase (IC_{50} 5.1 μM) is also present, together with its quinic acid ester, chlorogenic acid, a non-inhibitor of lipoxygenases. In contrast to walnuts and pistachios, almonds possess no hydrolysable tannins but instead contain simple phenolic acids (4-hydroxybenzoic, protocatechuic and vanillic acids) and flavonoids ((+)-catechin, (−)-epicatechin, and glycosides of quercetin, kaempferol and isorhamnetin, with the latter predominating). Depending on the individual variety, *ca.* 50–70% of the phenolics are located in the seed coat (Milbury et al., 2006).

Many of the polyphenolics present in tree nuts are quite common phytochemicals and readily available either from commercial sources or by extraction and isolation from the nuts themselves. A representative selection of these compounds was therefore tested for their ability to inhibit aflatoxin formation by *A. flavus* 4212 grown on a medium consisting of 5% ground pistachio kernels in agar. This medium was chosen because the fungal strain was originally isolated from pistachios and previous experiments had shown that aflatoxin production was much higher and more consistent than when the fungus was grown on artificial media such as PDA. The antioxidant compounds were added at 12 mM and aflatoxin production measured after culturing for 5 days at 30 °C, the period of maximum production; all experiments were performed in triplicate. The synthetic antioxidant, lauryl gallate, which has structural similarities to the anacardic acids and is approved for food use (GRAS) was also included in the evaluation. Table 2 shows the antiaflatoxigenic activity of these compounds. The most effective, with >99% inhibitory activity, were pentagalloyl glucose, the biosynthetic precursor of all hydrolysable tannins in walnuts, caffeic acid, and lauryl gallate. 3,4-Digalloyl quinic acid, representative of the pistachio tannins, was also highly effective (>98%

inhibition) and, somewhat surprisingly, also its hydrolysis product, quinic acid (>90%). The hydrolysis products of walnut tannins, gallic and ellagic acids, while still exhibiting considerable activity were less effective than the tannins, with the latter being the least inhibitory (59.5%) of all the compounds tested. These results indicate that the exceptional antiaflatoxigenic activity of walnuts relative to pistachios and almonds probably resides primarily in the tannins themselves, rather than their hydrolysis products. In general, the pistachio phenolics as a group were more effective than those from almonds. None of the compounds tested showed any effect on growth of the fungus, with the exception of the synthetic antioxidant, lauryl gallate. This indicates that the mechanism of action is linked directly to the biosynthesis of aflatoxins and is not an artefact of fungal growth inhibition.

4. Effect of oxidative stress on aflatoxin production

The ability of a structurally diverse suite of phenolic antioxidants to suppress aflatoxin production is confirmatory evidence for the hypothesis that aflatoxin biosynthesis is a response by the fungus to oxidative stress. Interaction of a pathogen with the cell wall of a host plant induces a cascade of reactions in the plasma membrane ultimately resulting in the formation of hydrogen peroxide and lipid peroxides (Lamb and Dixon, 1997). These products result in direct effects on the pathogen, sealing of the wound against further damage, and induced gene regulation resulting in defensive reactions such as formation of phytoalexins. In response, the pathogen (in this case *A. flavus*) initiates its own stress response to combat the defensive attack of the plant. This complex process is difficult to reproduce *in vitro* but the effect of oxidative stress on the fungus can be simulated by incorporation of an organic peroxide into the medium. One such candidate is *tert*-butyl hydroperoxide (*t*-BuOOH) which

Table 2

Inhibition of aflatoxin formation *in vitro* by phenolic constituents of tree nuts

Compound	% Inhibition [a]
Walnut constituents	
Pentagalloylglucose	99.8
Gallic acid	83.9
Ellagic acid	59.5
Pistachio constituents	
Caffeic acid	99.5
3,4-Digalloyl quinic acid	98.3
Quinic acid	90.2
Chlorogenic acid	88.5
Almond constituents	
Vanillic acid	85.6
4-Hydroxybenzoic acid	76.4
Protocatechuic acid	69.3
Catechin	69.0
Synthetic antioxidant	
Lauryl gallate	99.5

[a] Control (no antioxidant), 0%; all compounds incorporated into the media at 12 mM.

has sufficient balance of hydrophilicity and lipophilicity to simulate both hydrogen peroxide and more complex lipid peroxides.

The effect of culturing *A. flavus* on pistachio media with addition of 100 and 1000 μM *t*-BuOOH was therefore studied over a time-course of nine days. In comparison to control samples with no added peroxide, aflatoxin production was increased by 34% with 100 μM *t*-BuOOH at day 5. With 1000 μM *t*-BuOOH, there was a lag in aflatoxin synthesis, with the maximum of 14% above control being attained at day 6. However, both treatment samples ended the time course at day 9 with identical aflatoxin levels, 111% above control, showing that unstressed fungus had a decrease in aflatoxin at the maximum growth, whereas oxidatively stressed samples maintained aflatoxin production at a high level.

Similar experiments were conducted with respect to fungal weights with *A. flavus* grown on polycarbonate filters. When control and 100 μM *t*-BuOOH stressed cultures were incubated separately, the stressed sample had a fungal weights 12% and 23% less than the control on day 5 and day 9, respectively. It is therefore apparent that despite a significant decrease in fungal mass under oxidative stress, aflatoxin biosynthesis is not coupled to fungal growth.

5. Effect of relief of oxidative stress by tannic acid on aflatoxin production

If increased aflatoxin biosynthesis is a consequence of induced oxidative stress, then relief of such stress by an antioxidant should decrease aflatoxin production. This proved to be the case when *A. flavus* cultures were exposed to tannic acid as a surrogate for the walnut hydrolysable tannins. Tannic acid, or gallotannin, is a commercial product which is structurally heterogeneous but contains only gallic acid moieties (Hagerman, 2002). The fungal tannase present in *A. flavus* can therefore generate only gallic acid and not ellagic acid on hydrolysis. When incorporated into the media of an *A. flavus* culture exposed to oxidative stress induced by 100 μM *t*-BuOOH, tannic acid at a level of 0.4% by weight reduced aflatoxin production by 93% at day 5 and by 86% at day 9.

6. Functional genomics of aflatoxigenesis in relation to oxidative stress

The demonstration of enhanced aflatoxigenesis by induced oxidative stress and suppression by phenolic antioxidants provides the tools to investigate functional genomics of such relationships but the absence of a practical genetic analysis system has precluded direct elucidation in *A. flavus*. However, numerous stress response pathways have been characterized for the yeast, *Saccharomyces cerevisiae* (Toone and Jones, 1998) and this system has therefore be used as a model with hydrogen peroxide (H_2O_2) as a stressor (Kim et al., 2005) in order to evaluate phenotypic response to oxidative stress. Selected strains of *S. cerevisiae*, including mutants with single gene deletions were tested. Control colonies of serially diluted cells were all observable when grown in the presence of tannic acid or gallic acid at 0.4% w/v with H_2O_2 absent, establishing that these compounds alone were not toxic to the yeast. With the exception of undiluted ($\sim 10^6$) cells, growth was completely inhibited by 3.3 mM H_2O_2 for six 10-fold serial dilutions of wild-type *S. cerevisiae* cells, but this growth inhibition was overcome in a concentration dependent manner by addition of the antioxidants tannic acid or gallic acid over the range of 0.05–0.4%.

Similar results were obtained when oxidative stress was applied with 1.1 mM *tert*-butyl hydroperoxide (*t*-BuOOH). To identify the functional role of particular *S. cerevisiae* genes in antioxidative stress responses, cell-growth of 22 specific deletion mutants was examined under oxidative stress and potential alleviation. Particularly noteworthy was the recovery, on treatment with the antioxidants chlorogenic acid and gallic acid, of the mutants *yap1Δ*, defective in a transcription factor for gene regulation of the antioxidative stress response; *ahp1Δ*, deficient in alkyl hydroperoxide reductase; and *glr1Δ*, lacking glutathione oxidoreductase; in addition to the wild-type yeast. In correlation with the antiaflatoxigenic effects, chlorogenic acid was more effective than gallic acid in alleviating oxidative stress.

The relationship between oxidative stress and aflatoxin biosynthesis has been further established by identification of orthologs of 43 *S. cerevisiae* genes involved in gene regulation, signal transduction and antioxidation in the *A. flavus* Expressed Sequence Tag (EST) database (Yu et al., 2004). The validity of this procedure was established by successful functional complementation of the mitochondrial superoxide dismutase gene, *sodA*, an antioxidative stress gene from *A. flavus*, in a *sod2Δ* yeast mutant lacking the ortholog (Kim et al., 2005). This demonstrates the potential for *S. cerevisiae* to serve as a high throughput analysis model system to identify involvement of candidate genes from the *A. flavus* EST library in signaling pathways. The use of functional complementation analysis and knockout mutants should enable the relationship between aflatoxin biosynthesis and oxidative stress to be more completely elucidated.

Microarray analysis has recently shown, through differential expression, that treatment of *A. flavus* with caffeic acid results in dramatic down-regulation of genes in the aflatoxin biosynthetic gene cluster, except for the *aflR* regulatory gene. However, there is up-regulation of at least 16 other genes, including those involved in the antioxidative stress response (unpublished results). Further analysis using *A. flavus* genomic microarrays will enable identification of specific genes that are influenced by oxidative stress and provide specific targets for disruption of aflatoxin biosynthesis.

7. Future directions

The establishment of a definitive relationship between oxidative stress and aflatoxin biosynthesis provides a number of new basic research and practical directions for further exploration to reduce aflatoxin contamination of tree nuts. The fundamental question to be answered concerns the role of aflatoxin in the ecology of the fungus. The degree of

polymorphism in aflatoxin biosynthesis genes in *A. parasiticus* and *A. flavus* suggests that aflatoxin is important to the fungus but the fact that some atoxigenic strains are able to outcompete toxigenic strains (Garber and Cotty, 1997) indicates that they are not absolutely essential. The up-regulation of production by oxidative stress implies that aflatoxin mitigates such stress; however, this would be a simplistic conclusion. Examination of the molecular structure suggests that it would not function as a particularly effective antioxidant. Nevertheless, many of its biosynthetic precursors, from the initial aromatic metabolite norsolorinic acid, through sterigmatocystin, possess variable numbers of phenolic hydroxyl groups. It can be predicted that this structural feature would allow them to function as effective antioxidants, in just the same way as the phenolic phytochemicals in walnuts. Aflatoxin is therefore only one of many metabolites produced and its importance to the fungus should not be overestimated. Rather, the entire biosynthetic process must be considered, not merely the putative final product. For *Aspergillus* spp. that do not produce aflatoxins, alternative mechanisms to reduce oxidative stress may operate. The atoxigenic strain AF-36 has been shown to be defective in the polyketide synthase gene (*pksA*) required to initiate aflatoxin biosynthesis (Ehrlich and Cotty, 2004), however, this does not preclude the formation of other, as yet unidentified, metabolites.

In practical terms, the finding that specific antioxidants present in tree nuts can suppress aflatoxin formation indicates that manipulation of levels of such compounds by genetic methods or conventional breeding could limit contamination to acceptable levels. Furthermore there are no serious constraints to such an approach as the favorable health effects of high dietary levels of antioxidants have been widely promoted and their presence should prevent rancidity.

Acknowledgements

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