ATOXIGENICITY IN ASPERGILLUS FLAVUS INDUCED BY WALNUT SEED COAT CONSTITUENTS

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ABSTRACT

The ability of tree nuts to inhibit production of aflatoxins by Aspergillus flavus was investigated in vitro. Walnuts were much more resistant to aflatoxin formation than almonds or pistachios. The walnut cultivar 'Tulare' showed complete suppression of toxin production. Kernel without seed coat had no activity and the inhibitory factor(s) were shown to be specifically located in seed coat tissue. When seed coat was added back to 'Tulare' kernel media, aflatoxin was decreased to the detection limit or below. Aflatoxin production declined rapidly with seed coat maturity and was independent of growing location and rootstock. Chemical analysis of the seed coat has established that the inhibitory activity resides in a complex of hydrolyzable tannins common to all walnut cultivars. The 'Tulare' tannin completely suppressed growth of A. flavus at a concentration of 0.5% in the media, with no aflatoxin formed; at a concentration of 0.25%, fungal growth was retarded and aflatoxin was reduced to 0.06% of control.

Aflatoxin biosynthesis appears to be inhibited by gallic acid, produced from the tannin by the action of a tannase known to be present in A. flavus. Experiments in vitro with pure gallic acid showed that aflatoxin levels were reduced to ca. 12% of control at a concentration of 200 ppm in media consisting of either walnut or pistachio kernel without seed coat. Ellagic acid is concurrently produced and may also have an inhibitory effect, although its comparative activity cannot be determined because of its extreme insolubility. Treatment of walnut seed coat tissue with anhydrous methanolic HCl yields methyl gallate and ellagic acid, the levels of which can be measured by reverse phase HPLC. Gallic acid levels in seed coat of 'Tulare' and the variety 'Chico', which is susceptible to aflatoxin formation, were determined on a biweekly basis throughout the growing season. Levels in 'Tulare' were significantly higher, and were maintained throughout the growing season, whereas those in 'Chico' declined steadily as the nuts matured. At maturity, 'Tulare' had a gallic acid content of 3.3% (dry weight basis) while the level in 'Chico' was only 1.4%.

The evidence indicates that hydrolyzable tannins are capable of inhibiting growth of A. flavus and that atoxigenicity is phytochemically induced by biosynthesis and maintenance of high levels of tannins throughout the growing season. Gallic acid, produced in situ by a fungal tannase, is a specific tannin component responsible for suppression of aflatoxin biosynthesis by the fungus and should be amenable to enhancement of levels by conventional breeding or genetic manipulation. Breeding programs could increase resistance, enhancing marketability and safety of walnuts for human consumption.

OBJECTIVES

Infection of agricultural crops by various strains of the fungi Aspergillus flavus and A. parasiticus may result in production and accumulation of aflatoxins, which are a threat to food
safety and detrimental to quality. These mycotoxins are highly regulated in commodities marketed domestically and abroad because of their toxicity and potential carcinogenicity. Although the Food and Drug Administration has set a maximum guidance level limit of 20 ng/g (20 ppb) for tree nuts (shells included) intended for human consumption within the U. S., the European Community has recently imposed much more restrictive tolerance levels of 2 ng/g for aflatoxin B₁ and 4 ng/g total aflatoxins. As the largest importer of California tree nuts, rejection of shipments by the EC due to the presence of aflatoxins in excess of these amounts poses a serious economic threat to producers and exporters.

In contrast to other crops with the potential to be contaminated by aflatoxins, especially corn and peanuts, walnuts are well protected against fungal infection by the hull, shell, and the seed coat tissue surrounding the kernel. Nevertheless, all of these barriers can be penetrated by insect attack, providing an entrance for fungal spores. Since chemical treatments to prevent growth of microorganisms or to destroy aflatoxins are unlikely to be acceptable means to ensure that shipments are within tolerance levels, we have investigated the potential for factors naturally present in the crop to confer resistance to Aspergillus colonization and growth, or suppress aflatoxin biosynthesis, as a means to yield a product in conformance to regulations. If present, such factors offer the potential for enhancement through selection of cultivars and crop breeding programs. We have previously shown that naphthoquinones in walnut hulls can delay germination of A. flavus and affect levels of aflatoxin production and that natural products in plants and spices can inhibit biotransformation of aflatoxin B₁ to mutagenic compounds. The objectives of this study were to identify tree nut species with the greatest potential to resist aflatoxin contamination and to determine the localization of resistance factors within specific tissues, in order to provide fundamental information to direct the ultimate isolation and identification of the bioactive phytochemical constituents.

PROCEDURES

Materials
The 'Tulare' walnuts used to evaluate the effects of rootstock and geographical origin were collected from commercial orchards in the California counties of Kings, Sutter, Yuba, Yolo, and San Joaquin, growing on 'Paradox' and black walnut rootstock, respectively. All other walnuts, almonds, and pistachios were acquired from a germplasm collection in Winters, CA. Seed coat of 'Tulare' walnuts was separated from the remaining kernel by hand. Nut kernels and seed coat were ground to pass through a 1 mm screen with a mill.

Preparation and inoculation of media
Walnut, almond, or pistachio cultivar media consisted of 5 % ground nut kernels added to 1.5 % agar. Since plain agar was insufficient as a nutrient source to support fungal growth when seed coat alone was used, these samples were mixed with potato dextrose agar (PDA). Ground 'Tulare' seed coat, and kernel without seed coat, were added separately to PDA at 10, 25, 50, 100, 200, and 400 mg per 10 mL of media. Ground 'Tulare' seed coat collected at various stages of maturity was added to PDA at 50 mg per 10 mL of media. Control plates consisted of PDA with no walnut material incorporated. Ground 'Tulare' seed coat was added to a media of 5 % ground 'Tulare' kernel without seed coat in 1.5 % agar at 10, 20, 30, 40, 50, and 60 mg per 10 mL of media. Media were autoclaved and petri plates (10 mL of media per 60 mm diameter) were prepared in replicates of four for the comparison of tree nut cultivars and county/rootstock samples, and in triplicates for all others.
Preparation of Fungal Cultures
Spore suspensions were prepared from *Aspergillus flavus* NRRL 25347, which was originally collected from a pistachio orchard, grown on PDA for 7 days. This strain produces aflatoxin B₁, with only trace levels of aflatoxin B₂ (8,9-dihydroaflatoxin B₁) and no aflatoxins G₁ or G₂. 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 0.05% Tween 80) were inoculated in a single point onto each agar media Petri plate. Plates were incubated for 7 days at 30°C.

Analysis for Aflatoxin
The fungal mat, including spores, and media from each Petri dish were extracted with MeOH (50 mL). MeOH was removed from an aliquot (1 mL) by evaporation with N₂ 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 N₂ at 40°C and redissolved in H₂O-CH₃CN (9:1; 1 mL). Aliquots (20 μL) were analyzed for aflatoxin by reversed-phase HPLC and detected by fluorescence detection, with excitation at 365 nm and emission at 455 nm. The lower detection limit was 0.02 µg per Petri dish or 10 mL media. Aflatoxin B₂ was detected at levels which were insignificant relative to aflatoxin B₁ (ca. 0.1%) and was therefore not quantitated.

RESULTS AND DISCUSSION

Comparison of tree nut species
In order to compare susceptibility of the major California tree nut crops (almonds, pistachios and walnuts) to aflatoxin contamination, a diversity of germplasm, including plant introductions, selections, breeding lines and some of the most common cultivars were tested *in vitro*. These consisted of 34 samples of almonds and 26 of walnuts; only one pistachio sample was tested because commercial production in California is confined to the Kerman cultivar. Finely ground, mature nuts, consisting of both kernel and seed coat (testa) were incorporated at a level of 5% into 1.5% agar medium, inoculated with *A. flavus*, and the aflatoxin level measured after 7 days growth of the fungus; the lowest detectable level was 0.02 µg/plate. There was no apparent effect on the rate of growth of the fungus itself in this or any subsequent experiments. Table 1 shows the average aflatoxin levels obtain for each nut species. Aflatoxin levels were highest for almonds, being 2.6 times greater than for the Kerman pistachio and 25 times greater than for walnuts. With almonds, the aflatoxin levels ranged from 34 to 179 µg/plate for named cultivars and 20-192 µg/plate for the experimental crosses. However, the range for *J. regia* cultivars was even lower (non detected-28 µg/plate) whereas black walnut (*J. nigra*) had a comparatively high level of 45 µg/plate. Thus, the highest level detected for all *J. regia* walnut samples only slightly exceeded that of the most resistant of the almond breeding lines and was less than the lowest level measured for the commercial almond cultivars. These results indicated that the most promising choice for investigation of natural resistance factors in tree nuts was one of the walnut cultivars.

A comparison of nine walnut cultivars, of which four comprised the major proportion of commercial production, showed considerable differences in susceptibility (Figure 1). Thus, 'Chico' supported 1.5 times the aflatoxin production of 'Hartley' and 5 times that of 'Chandler'.
Most significantly, however, the 'Tulare' cultivar completely suppressed aflatoxin formation \textit{in vitro}.

These results established that aflatoxin-resistance is a unique feature of 'Tulare' and is the first time that complete suppression of aflatoxin by an agronomic crop plant known to be susceptible to contamination has been observed. 'Tulare' is derived from a controlled cross between the walnut cultivars 'Serr' and 'Tehama' and is a high yielding and precocious cultivar released in 1993. Differences in levels of aflatoxin production have been observed among almond genotypes but these did not correlate with mold expansion or kernel oil composition, which is also genetically controlled. Certain lines of corn and peanut have been bred for aflatoxin resistance but although the levels are generally lower than in commercial cultivars, the toxin is always produced to a greater or lesser extent.

\textbf{Effect of rootstock and growing location}

In order to confirm that the latter finding was a consistent feature, 'Tulare' walnuts from Kings, Sutter/Yuba border, and Yolo counties grown on either Paradox or black walnut rootstocks, and from San Joaquin county grown on black walnut rootstock, were tested under the same conditions as previously described. Aflatoxin was produced in only two of the seven samples in low but detectable levels; all other samples had no detectable levels of aflatoxin. The two positive samples were from Kings and Sutter/Yuba counties, respectively, both growing on Paradox rootstock. There did not appear to be a consistent relationship to type of rootstock, however, since the Yolo and San Joaquin county samples on Paradox had no detectable aflatoxin and neither did the black walnut rootstock samples.

\textbf{Localization of resistance factor}

The exceptional resistance of 'Tulare' walnut to aflatoxin contamination indicated that factors were present in the nuts that suppressed biosynthesis of the toxin, even though no appreciable retardation of fungal growth was observable. In order to determine whether or not these factors were distributed throughout the nut or localized in particular tissues, \textit{in vitro} experiments were conducted in which different amounts of either seed coat, or kernel from which the seed coat had been removed, were added to PDA inoculated with \textit{A. flavus}, and the aflatoxin produced was measured relative to control plates containing no nut material. As shown in Figure 2A, the addition of kernel without seed coat had no inhibitory effect on aflatoxin production. The addition of up to 100 mg of kernel without seed coat essentially maintained aflatoxin production at the same level as that of the control. The addition of amounts above 100 mg stimulated aflatoxin production significantly, with addition of 400 mg of kernel increasing aflatoxin content to four times that of the control. This increase is probably a function of increased nutrient availability in the media due to the additional material incorporated, even though no increase in rate or amount of fungal growth was apparent. Alternatively, there may be constituents in the kernel, such as lipids, that are capable of stimulating aflatoxin biosynthesis.

In contrast, the addition of seed coat had a marked inhibitory effect on aflatoxin production, which declined rapidly with increasing seed coat content. Seed coat contents of 100-400 mg in PDA decreased aflatoxin production to less than 3 \% of the control (Figure 2A). When seed coat was added back to 'Tulare' kernel media, aflatoxin levels were dramatically decreased, declining to below the detection limit (<20 ng) at 50 mg added seed coat compared with 133 \mu g for a
control kernel sample with no added seed coat. At a concentration of 30 mg added seed coat, approximately equivalent to the proportion by weight of seed coat to the whole kernel, aflatoxin production was just above the detection limit at 0.8% of control. Aflatoxin production was proportional to kernel incorporation over a measured range of 10 – 60 mg, as shown in Figure 2B.

Effect of walnut seed coat maturity
The localization of the resistance factor(s) in the seed coat required that the relationship of aflatoxin inhibition to maturity of the nut be established. Tulare walnuts were therefore collected each month from June-September, the seed coat removed and added to PDA to compare the effect of seed coat maturity on aflatoxin production. In June, when the kernels were at the "jelly" growth stage, the seed coat did not exhibit significant aflatoxin inhibitory activity, as the amount of aflatoxin produced was 94% of control. Aflatoxin production rapidly declined with each successive month of seed coat maturity, being reduced to only 0.6% of control close to harvest, in September (Figure 3).

The results of this study indicate that the 'Tulare' walnut possesses inhibitory factor(s) that suppress the formation of aflatoxin by A. flavus, in spite of the fact that growth of the fungus itself is not prevented. This suggests that the inhibition is a consequence of interruption or suppression of the aflatoxin pathway through regulation of specific genes in the gene cluster responsible for aflatoxin biosynthesis. The inhibitory components are specifically located in the seed coat and their activity increases with maturity of the walnut. Identification of the specific compounds present responsible for the observed activity should preferably be performed with seed coat from mature nuts. However, it is difficult to remove seed coat from the kernels at this growth stage, although it can be separated much more easily from immature kernels. In order to obtain sufficient amounts of seed coat material for bioactivity-directed fractionation and isolation, it may initially be necessary to use younger walnuts and then relate the specific components present to those in mature nuts by analytical chemistry techniques. The question of whether this increase in activity is related to an increase in concentration of the active component, or to a change in molecular structure to a more active form, can only be decided once the identity of the anti-aflatoxigenic factor has been established. The potential then exists to enhance the resistance factor through breeding programs, since the seed coat is maternally inherited and the parentage of 'Tulare' walnut is known.

Constituents of Walnut Seed Coat
Bioassay-directed fractionation and preliminary analysis of 'Tulare' seed coat has shown that the constituents are limited to complexes of gallic acid, primarily hydrolyzable tannins. It is known that Aspergillus flavus possesses a tannase that is capable of hydrolyzing the sugar linkages of such tannins to yield free gallic acid and possibly ellagic acid. Experiments have shown that gallic acid has strong activity in suppressing formation of aflatoxin in vitro. Measurement of the total gallic acid content of seed coat throughout the growing season in 2002 showed that levels in 'Tulare' were approximately twice those in 'Chico'. Furthermore, although maximum levels were achieved fairly early in nut formation, those in 'Chico' then started to decline whereas in 'Tulare' the levels remained constant to maturity. It appears that the ability of 'Tulare' to maintain high levels of gallic acid containing hydrolyzable tannins enables it to suppress formation of aflatoxins. These results need to be confirmed over several growing seasons and the levels in
other cultivars, including the parents of 'Tulare', must be measured in order to establish the inheritance pattern of the high tannin levels.

Table 1 - Average and range of aflatoxin production *in vitro* on ground kernels of almond, pistachio and walnut cultivars and breeding crosses inoculated with *Aspergillus flavus*.

<table>
<thead>
<tr>
<th>Species of tree nut</th>
<th>Average aflatoxin B₁ (mg/plate)</th>
<th>Range of aflatoxin B₁ (mg/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Almond (<em>Prunus dulcis</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 cultivars</td>
<td>91</td>
<td>34-179</td>
</tr>
<tr>
<td>11 breeding lines</td>
<td>136</td>
<td>20-192</td>
</tr>
<tr>
<td>Pistachio (<em>Pistacia vera</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 cultivar</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>Walnut, English (<em>Juglans regia</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 cultivars</td>
<td>4.2</td>
<td>n.d.-28</td>
</tr>
<tr>
<td>Walnut, black (<em>Juglans nigra</em>)</td>
<td>45</td>
<td>-</td>
</tr>
</tbody>
</table>

*All data points are averages of quadruplicates for each cultivar/breeding line. n.d.: <0.02 mg/plate*
Figure 1 - Comparison of amounts of aflatoxin B₁ produced *in vitro* by *Aspergillus flavus* NRRL 25347 grown on 5% ground nut kernels of various walnut cultivars added to 1.5% plain agar. Plates were incubated for 7 days at 30 °C. (All data points are averages of quadruplicates for each cultivar).
Figure 2 - (A) Aflatoxin B₁ produced in vitro by Aspergillus flavus NRRL 25347 grown on various levels of 'Tulare' walnut kernel and seed coat added to potato dextrose agar. Control plates consisted of PDA with no walnut material incorporated. (B) Aflatoxin B₁ produced in vitro by Aspergillus flavus NRRL 25347 grown on 'Tulare' seed coat incorporated at various levels into 5% 'Tulare' kernel media. Control plates consisted of kernel media with no seed coat incorporated. Plates were incubated for 7 days at 30 °C. (All data points are averages of triplicates for each concentration).
Figure 3 - Comparison of amounts of aflatoxin B₁ produced in vitro by Aspergillus flavus NRRL 25347 grown on 'Tulare' walnut seed coat added at 50 mg/10 mL PDA media for each month of the growing season. Plates were incubated for 7 days at 30 °C. (All data points are averages of triplicates of each collection).