ANTIFUNGAL NATURAL CONSTITUENTS OF WALNUT VARIETIES

Noreen Mahoney, Russell Molyneux, Gale McGranahan, Chuck Leslie, and Jim McKenna

ABSTRACT

This interim report describes our progress in identifying natural antifungal constituents from walnuts. Our focus is on controlling the mold Aspergillus flavus and its metabolic byproduct aflatoxin. The presence of this carcinogen in foods is subject to government regulation both domestically and abroad. Walnuts are more resistant to the production of aflatoxin than either almonds or pistachios, and the walnut variety Tulare is particularly inhibitory. Tulare seed coat is not only a potent inhibitor of aflatoxin, but also restricts the mycelial growth and sporulation of A. flavus. We will continue to try to isolate and identify the chemical compounds from the seed coat responsible for this inhibition in A. flavus growth and aflatoxin production. Once identified we can test these compounds for antifungal activity against additional molds that can adversely affect walnut quality.

OBJECTIVES

The mold Aspergillus flavus is ubiquitous in California orchards and, being a wound pathogen, is usually associated with insect damaged kernels. This mold also produces the carcinogenic metabolite aflatoxin. In the U.S. the FDA has established guidelines that limit aflatoxin contamination in foods to 20ppb; however, implementation of stricter standards internationally has become a concern to the tree nut industry. New regulations by the European Union limit aflatoxin in foods to no more than 4ppb total aflatoxin and 2ppb aflatoxin B1. General concerns about food safety have brought the issue of aflatoxin to the attention of the European consumer.

Our research indicates that walnuts, and the variety Tulare in particular, are much more resistant to the production of aflatoxin than either almonds or pistachios. We would like to identify the chemical basis for this inhibition and determine if these compounds have antifungal activity against other molds that can contaminate walnuts. Our first experiments were to determine if the antifungal activity is associated with either the walnut fat or seed coat.

PROCEDURES

Aflatoxin production on various varieties of walnuts was tested under controlled laboratory conditions. In order to reduce variability that can affect aflatoxin production in individual nuts, 50 to 100 kernels of each variety were ground to a uniform size, thoroughly mixed and incorporated into a 1.5% agar medium, and poured into 60mm petri dishes. Each petri dish was inoculated with 200 A. flavus spores and incubated at 30°C for 7 days. Aflatoxin was extracted from each fungal mat with methanol and quantified by HPLC.
Additional experiments tested the effect of walnut seed coat, defatted nut meal, and unsaturated triglycerides on aflatoxin production. Various tree nuts were defatted with hexane and the resulting meal added to potato dextrose agar (PDA). Unsaturated triglycerides were added to VMN agar media. These plates were inoculated, incubated, and analyzed for aflatoxin as above.

RESULTS

Varietal differences in walnuts did affect aflatoxin production (Figure 1). The variety Tulare was unique among all tree nut varieties tested in that almost no aflatoxin was produced. Fungal growth and sporulation was also reduced. Walnuts in general supported much less aflatoxin production compared with almonds and pistachios. The overall average aflatoxin produced on the 26 walnut varieties tested was almost 10 times lower than the average aflatoxin produced on the Kerman pistachio, and almost 25 time lower than the average aflatoxin produced on 34 almond varieties (Figure 2).

In order to confirm that aflatoxin production on Tulare was inhibited and not just delayed, a time course of aflatoxin production was determined for Tulare and Chico walnuts, Kapareil and Mission almonds, and Kerman pistachios (Figure 3). Aflatoxin levels in all the varieties tested peaked at 5 to 6 days after inoculation. No aflatoxin was produced on Tulare up to 4 days after incubation, and only trace levels were measured after that. So the Tulare variety not only slows the growth of *A. flavus* but also almost completely inhibits the production of aflatoxin.

Since walnuts are much more resistant to aflatoxin production, we wanted to focus on differences between walnuts and the other tree nuts as the source of inhibition. One of the major dissimilarities between walnuts and the other tree nuts is fat content and composition. The average fat content of walnuts is about 70% compared with between 40 and 50% for almonds and pistachios (Figure 4). The fat in almonds and pistachios is primarily composed of the monounsaturated fatty acid oleic acid, while walnut fat is primarily composed of the polyunsaturated fatty acids linoleic and linolenic acids (Figure 5). Unsaturated triglycerides affected aflatoxin production depending on the level of unsaturation. Triolein, trilinolein, and trilinolenin (triglycerides consisting of 3 oleic acid, 3 linoleic acid, and 3 linolenic acid fatty acids, respectively) were added to media as sole carbon sources. Trilinolein supported only half as much aflatoxin production as triolein, while trilinolenin did not support any aflatoxin production (Figure 6). These results indicated that unsaturated fats found in walnuts could be a possible source of the aflatoxin inhibition. However, the effect of the defatted tree nut meals on aflatoxin production was similar to the aflatoxin produced on the whole kernels (Figure 7). Therefore, it appears that fat content and composition are not responsible for the low levels of aflatoxin produced in walnuts.

Tulare seed coat was very potent in inhibiting aflatoxin production on PDA media (Figure 8). Only 0.1% seed coat in this media inhibited aflatoxin by 64%, and 0.5% seed coat resulted in a 94% inhibition. A medium consisting of 5% Tulare kernels stripped of seed coat produced 186µg aflatoxin B1, while no aflatoxin was produced on the same medium.
with 1% seed coat added back. The presence of Tulare seed coat in both of these media resulted in decreased mycelia growth and sporulation by *A. flavus*.

**DISCUSSION**

Tulare seed coat not only contains powerful inhibitors of aflatoxin production, it also contains chemical compounds which adversely affect the growth and reproduction of *A. flavus*. We will chemically fractionate Tulare seed coat in order to try to isolate and identify the natural products responsible for this desirable characteristic of Tulare walnuts. Once identified we can also test these compounds for their effect on other fungi that adversely affect walnut quality. We also would like to develop a quantitative method of analysis such that the levels of these compounds can be measured in all walnut varieties. Due to the inhibition of aflatoxin by walnuts in general it seems likely that all varieties contain some of these bioactive natural products, but at concentrations lower than Tulare. In collaboration with the Walnut Improvement Program at UC Davis we would like to use this information to develop walnut varieties with increased resistance to fungal attack.
Figure 1. Aflatoxin produced on walnut varieties under laboratory conditions. The variety Tulare completely inhibited the production of aflatoxin.

Figure 2. Average aflatoxin produced on tree nuts under laboratory conditions. Averages represent 26 varieties for walnuts, 1 variety for pistachios, and 34 varieties for almonds. Walnuts are much more resistant to aflatoxin production than either pistachios or almonds.
Figure 3. Time course of aflatoxin production for various tree nuts. Aflatoxin is inhibited on the walnut variety Tulare for the entire life cycle of *A. flavus*.

![Graph showing aflatoxin production over time for different nuts.](image)

Figure 4. Fat content of various tree nuts.

![Bar chart showing fat content of different nuts.](image)
Figure 5. Fat composition of tree nuts. Walnuts have much higher levels of polyunsaturated fatty acids compared with pistachios and almonds.

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Figure 6. Effect of unsaturated triglycerides on aflatoxin production. The higher the level of unsaturation, the lower the aflatoxin produced.
Figure 7. Effect of defatted tree nut meal on aflatoxin production. Defatted tree nuts retained the same susceptibility and resistance to aflatoxin production as the whole kernels.

Figure 8. Effect of Tulare seed coat on aflatoxin production.