Differential Production of Aflatoxin on Natural and Heat-Treated Cocoa Beans

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ABSTRACT

Costa Rican-type cocoa beans were tested as a substrate for two known aflatoxigenic fungal species, Aspergillus flavus NRRL 3251 and Aspergillus parasiticus NRRL 2999. Natural floral growth in both cooked (autoclaved) and raw cultures failed to show aspergillic and aflatoxin as analyzed by thin layer chromatography. Raw, ground cocoa bean medium inoculated with both species of aspergilli had mycelial growth, sporulation, but no detectable levels of aflatoxin. Similarly treated but cooked (autoclaved) cocoa medium also provided for mycelial growth, and sporulation, but total aflatoxin levels were 388.1 µg/g of substrate for the A. parasiticus cultures and 2.2 µg/g of substrate for the A. flavus cultures. The general results appear to support the low level of a limited number of reports of toxin contamination in natural cocoa. The simplified extraction methods used herein provided results similar to other methods.

Chocolate manufacturers collect and store cocoa beans from many sources. Samples of beans that have been studied in relation to mycotoxins include types from the following countries: Nigeria, Ghana, New Guinea, Trinidad (14), and the Philippines (3). In 1965 the Tropical Products Institute reported aflatoxicosis (AFT) positives in cocoa bean (11). Campbell (3) in 1969 also reported finding up to 17 µg aflatoxin/kg in two of nine samples of cocoa from the Philippines. Following these reports there was an effort to develop better methods for analyzing large numbers of samples for AFTs. In 1971, Scott (13) and Scott and Przybylski (15) modified the CB method (Contaminants Branch Method, FDA) for use with cocoa beans. Collaborative tests were made and the technique was accepted as the Official AOAC First Action Method (15). A modification of the BF Method (Best Foods Method) using 3% silver nitrate solution in the extracting solvent mixture was reported in 1973 (14).

There seem to be few reported occurrences of aflatoxin contamination in cocoa and cocoa products. Limited occurrence could be due to the complexity of the test (10, 14) or limited testing. More recently, Yndestand and Underdal (19) surveyed Norwegian foods including cocoa and cocoa products. They found one positive in 40 samples of cocoa products.

The present study was undertaken to determine the potential of cocoa as a substrate for aflatoxigenic fungi under "near-ideal" laboratory growth conditions. Also, no studies have utilized Costa Rican cocoa beans.

MATERIALS AND METHODS

A one-kg sample of naturally dried cocoa beans, selected at random as a government inspection sample, from composite stocks, was collected from the Zent Region, Puerto Limon, Costa Rica. This sample served as the source for the experiments completed herein.

Preparation of cultures and inoculation

The cocoa beans were minced in a sterile blender for 45 sec. Five g of the blended substrate were placed in each of the culture vials with 7 ml of distilled water required to moisten the ground cocoa beans. Two-ounce, clear glass prescription vials having one flat side were plugged with cotton. They were sterilized (20 psi for 20 min) either with the ground cocoa plus distilled water to provide cooked cocoa or only with distilled water. In the latter case, the cocoa (uncooked) was added to the vials containing sterile water following their return to room temperature after autoclaving.

Eighteen vials were used in the natural flora portion of the study. An equal number of vials contained cooked (sterile, 20 psi for 20 min) or uncooked (non-sterile) cocoa.

Twenty-seven vials were used in the growth and toxin production aspect of the study. Fifteen vials containing water were prepared by sterilizing (cooking) the minced cocoa with the vials. Three of these vials served as controls and were not inoculated. The remaining vials containing sterile water received equal aliquots of uncooked cocoa. Six of these vials having cooked cocoa and six having uncooked cocoa were inoculated with Aspergillus parasiticus NRRL 2999 spores or Aspergillus flavus NRRL 3251 spores. Spores for both species were taken from potato-dextrose-agar slants maintained in our laboratory. Both of these species used were known producers of aflatoxins (6, 16).

Incubation procedures

All culture vials were placed in a growth chamber and observed regularly for 30 days. Environmental conditions included relative humidity at 45 ± 5% and temperature at 23 ± 2 C. The chamber was dark except when opened for observations.

Extraction and analyses

After 30 days all cultures were attenuated by addition of 30 ml of chloroform. The vials were capped and shaken gently for 1 h at room temperature. All clumps of cocoa were broken. The solids in the culture floated and the chloroform layer was removed with a syringe which penetrated the floating solid layer. Twenty-microliter samples were spotted on silica gel thin-layer plates (205-nm layer of Absorbosil-1 Applied Science Lab. Inc., State College, PA). Fluorescent spots were compared visually to reference samples (Applied Science Lab., Inc.,...
procedure for TLC. Quantitations were repeated in triplicate. Final aflatoxin concentrations per culture and then per gram of substrate were calculated based on these values and the 30 ml of chloroform first added.

RESULTS AND DISCUSSION

Natural flora cultures for both raw and cooked medium failed to develop Aspergillus-like fungal growth. Aflatoxin analyses were negative for these cultures as well as the cooked, control flasks. Even though in Costa Rica such beans are dried outdoors on movable wheel and rail beds about 10 m in square area, and usually on a small-scale basis by individual farmers, there was no aflatoxin contamination in the sample used in this study. At least for the present, there are no reports indicating that there is a significant aflatoxin problem. These flat-car beds are often located in the front yard of the rural or village farmer. When it rains they are usually pushed under a crude roof and stored in a layer-type fashion. In some instances a local farmer may serve as the dealer who collects and dries beans for further sale. Such small businessmen are often seen during the day rearranging the cocoa beans in the drying beds. Groundnuts are also grown in Costa Rica and they have been found to have the typical AFT problems found in the tropics (1, 12). Therefore, the fungal spores apparently are native, but could be regional or possibly Costa Rican cocoa beans are not susceptible to aflatoxin occurrence.

Additional results, as listed in Table 1, may present an interesting aspect of this problem in support of the non-susceptibility of Costa Rican cocoa beans. A. flavus NRRL 3251 and A. parasiticus NRRL 2999 both produced mycelia and sporulated on raw and cooked moist cocoa bean media. A. parasiticus followed its typical growth pattern and sporulated first. Spores were evident after day five and extensive by day 10. For A. flavus, spores were evident on day seven and extensive by day 14. Visual evaluations showed similar mycelial growth and sporulation for each individual species on both cooked and raw substrates. The AFT analyses were positive only for the cooked (sterilized) ground cocoa beans. This was true for both species tested. Concurrent tests using the same species on rice and shredded coconut were positive. For example, when A. parasiticus was inoculated into sweetened coconut flake cultures (20 g substrate plus 10 ml H2O and grown for 7 days at room temperature), they were found to contain 0.938 mg/g of substrate for AFB1, 19.715 mg/g of substrate for AFG1, 0.060 mg/g of substrate for AFG2, and no AFB2. It is unlikely that a change from a toxin-producer to a non-producer occurred in the species used. To date, we have no definite explanation for growth and sporulation but lack of detectable levels of AFT on these laboratory cultures of ground raw cocoa beans. Factors under consideration indicate that the cooking that occurred during the autoclaving process may have denatured a natural inhibitory agent, changed the dispersion of the nutrients, or altered favorably the nutritional composition of the ground cocoa beans. Also the absence of microbial competition may have played an important role in this situation.

Several extracts from the non-aflatoxin producing raw cultures in the present study were further concentrated and evaluated again for AFT. Non positives were found. The system for quantitation used herein is sensitive to approximately 2 ppb. Although the samples were not extracted following the official procedures the remaining test procedures were official AOAC procedures. To confirm the absence of aflatoxin, several cultures were extracted using the AOAC, IUPAC, and Costa Rican procedures (7, 8, 10) and no toxins were found.

In studying the quantities of toxin produced, A. parasiticus NRRL 2999, a known producer of AFT did

<table>
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<tr>
<th>Inoculation Treatments</th>
<th>Aflatoxin levels (Mean and standard deviationsb)</th>
<th>% Total (of total)</th>
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<tbody>
<tr>
<td>A. flavus</td>
<td>AFB1 (μg/g)</td>
<td>20.7 ±9.6</td>
</tr>
<tr>
<td>A. parasiticus</td>
<td>AFB1 (μg/g)</td>
<td>ND</td>
</tr>
<tr>
<td>(NRRL 3251, cooked)</td>
<td>ND</td>
<td>1.2 ±0.4</td>
</tr>
</tbody>
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A. flavus NRRL 3251 with raw cocoa; natural flora; both raw and autoclaved; and autoclaved control cultures contained no detectable levels of aflatoxin.

bMean and standard deviations are for six cultures in triplicate.

cNone detected. Lower level for detection is approximately 2 ppb.
well on the cooked cocoa having a mean level for AFB\textsubscript{1} of 206.3 \mu g/g of substrate; 180.1 \mu g/g of substrate for AFG\textsubscript{1}; and traces of AFG\textsubscript{2}. These values and especially the total of 388.1 \mu g/g are similar to that found for sunflower seed media (7). *A. flavus* NRRL 3251 produced significantly less AFB\textsubscript{1} and AFG\textsubscript{1} than *A. parasiticus* NRRL 2999 and no AFG\textsubscript{2} at all. There are indications that there are not only differential AFT levels produced on cooked and raw cocoa but also a preference of toxin production for fungal species or strains. The presence of the mold on cocoa is not an absolute case for aflatoxin occurrence. Due to the number of aflatoxigenic strains, it is suspected that some isolates could produce toxin on raw cocoa or possibly even the strains used herein may be producers under some environmental conditions. Additional aflatoxigenic strains, especially those native to Costa Rica should be evaluated.

Wildman et al. (19) appear to have made the only other mention of aflatoxin production under laboratory conditions on cocoa beans. Among the many foods they inoculated with *A. flavus* NRRL A 13794 (a known producer of 1000 \mu g AFB\textsubscript{1} per gram of moistened sterilized wheat biscuits) they report for cocoa beans, 4\mu g (total aflatoxin) per gram of media. There is no mention of the source of the cocoa bean or the culture medium preparations other than that the beans apparently were not sterilized. This low level of aflatoxin from a high producing strain supports Scott’s (14) inference and our present study that non-heat-treated cocoa tends to yield little or no aflatoxin.

Further work is planned to evaluate the strain-specific agent as it relates to aflatoxin production as well as the apparent natural resistance of Costa Rican cocoa beans and other types of cocoa beans to aflatoxin occurrence. The rapid and simple extraction technique used herein seemed adequate for aflatoxin analyses and produced results similar to other methods of extraction.

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**REFERENCES**


