Aspergillus parasiticus Growth and Aflatoxin Synthesis on Florunner Peanuts Grown in Gypsum-Supplemented Soil

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ABSTRACT

Five levels of gypsum supplementation (0, 550, 1100, 2200, and 4400 kg ha−1) were applied to peanut fields 35 d after planting. After the growing season, peanuts were harvested, ground, and inoculated with 1 x 10⁷ Aspergillus parasiticus (NRRL 5139) conidia. After 14 d at 25°C, aflatoxin was extracted and quantified by thin-layer chromatography. Fungal growth was assayed using a modified chitin assay. Peanuts from gypsum-supplemented fields at each level of supplementation supported significantly less aflatoxin production when compared to control peanuts (no calcium supplementation). Results from the chitin assay showed a decrease in fungal biomass which correlated with the decreased aflatoxin synthesis.

Peanuts are a highly valuable commodity in the United States comprising a 6 billion dollar industry. Each year, millions of dollars are lost due to aflatoxin contamination (1). Aflatoxin, a carcinogenic mycotoxin, is produced on a variety of commodities when favorable environmental conditions are met (3,5). Prevention of fungal growth would eradicate the aflatoxin problem. Since commercial varieties of peanuts which are genetically resistant to fungal invasion have not yet been developed, other means to prevent contamination need to be established.

One potential approach for reducing aflatoxin-related problems is the use of agronomic practices which have the combined advantage of increasing product yield and quality while limiting aflatoxin contamination. One such practice commonly done with peanuts is the application of calcium, in the form of gypsum during peanut cultivation. This improves peanut quality and germination (2). Limited field trials indicated that the level of colonization by Aspergillus parasiticus and the production of aflatoxin by the mold may be reduced by applying gypsum to peanut fields (9,14). If true, application of gypsum would be a practical, cost-effective method to reduce aflatoxin contamination. It has been noted that some minerals enhance the synthesis of aflatoxin while others inhibit or reduce the biosynthesis of the toxin (7,12). This study was done to determine the relationship between the calcium content of peanuts, fungal growth, and the amount of aflatoxin synthesized.

MATERIALS AND METHODS

Peanut cultivation

The peanut cultivar ‘Florunner’ was planted May 5, 1989, at the University of Georgia’s Coastal Plain Experiment Station Gibb’s Research Farm near Tifton, Georgia. The soil was a Tifton loamy sand (fine loamy, siliceous, thermic Plinthic Paleudults). Soil tests taken 4 weeks before planting measured organic matter (g kg−1), pH, P (kg ha−1), K (kg ha−1), Ca (kg ha−1), and Mg (kg ha−1) at 7, 6, 65, 120, 520, and 87, respectively. Two weeks prior to planting 560 kg ha−1 of 3:9:18 (% elemental nitrogen: phosphorus as P2O5: potassium as K2O) analysis fertilizer was applied to the soil surface then deep turned (20 cm) into the soil.

Five gypsum (CaSO4) treatments (0, 550, 1100, 2200, and 4400 kg ha−1) were applied to plots (1.8 m x 9 m) 35 d after planting. The treatments were replicated four times in a randomized complete block design. Bimonthly applications of chlorothalonil were made starting 30 d after planting until harvest (8 applications). Other than seed treatments, no additional fungicides were applied to the crop. Digging date was determined by the hull-scrape method (4). Three days after digging, pods were combined and dried to a uniform moisture (90 g water kg−1 seed) and stored at 22°C until analyzed.

Preparation of fungal conidio suspension

Aspergillus parasiticus (NRRL 5139) colonies were maintained on potato dextrose agar and allowed to grow and sporulate. Conidia were harvested using phosphate buffer containing 0.02% Tween (0.1 M, pH 7.0) to wash colonies. The conidial suspension was adjusted to approximately 1 x 10⁷ conidia per ml as determined by direct microscopic count.

Preparation of growth substrate

Sound, in-shell Florunner peanuts (50 g) that visually were free of obvious fungal contamination or damage from each gypsum supplementation level were ground to a coarse meal (Sorvall Omnimixer speed 7, 20 s), and 5-g portions were placed in sterile 150-ml Erlenmeyer flasks. The ground meal was steamed in an autoclave (100°C) for 5 min to reduce any background contamination prior to inoculating with 1 x 10⁷ A. parasiticus conidia. Inoculated portions and uninoculated controls were incubated at 25°C for 14 d. After incubation, inoculated portions for each supplementation level were subdivided for either aflatoxin or chitin analysis. Uninoculated controls were analyzed for chitin content. There were duplicate samples for chitin analysis and aflatoxin analysis, and the experiment was repeated four times.
Aflatoxin determination

For each sample, 15 ml of chloroform was shaken with the peanut meal for 5 min on a rotary shaker. Leaving the meal in the flask, the chloroform was filtered through Na₂SO₄ (to remove H₂O) into a 100-ml round-bottom flask. This procedure was repeated two more times. The three combined chloroform portions were concentrated to 2 ml using a rotary evaporator. For thin-layer chromatography, 5-μl samples were spotted onto silica gel plates (Silica Gel G, Bodman Chemicals, Doraville, GA) which were developed in chloroform:acetone (9:1) for 30-45 min. The amount of aflatoxin B₁ and G₁ present was determined in relation to aflatoxin standards with the use of a scanning fluorodensitometer with an excitation wavelength of 365 nm and an emission wavelength of 440 nm.

Fungal growth determination

Inoculated peanut samples were frozen in liquid nitrogen, ground in a chilled Omnimixer (speed 7, 2 min), and then subjected to the procedure outlined by Ride and Drysdale (11). Chitin in the samples is chemically converted to glucosamine which was measured colorimetrically. Uninoculated controls were also analyzed for chitin content to correct for any interfering factors introduced by using peanuts as a substrate.

Mineral analysis of peanuts

Plasma emission spectroscopy was used to determine the elemental composition of the gypsum-supplemented peanuts by the method of Jones et al. (6).

RESULTS AND DISCUSSION

Calcium concentration of Florunner seeds and hulls increased with increasing gypsum supplementation concentration (Table 1). The increased uptake of calcium was most notable in the hulls. In contrast, zinc concentration decreased with the increased calcium levels, particularly in the hulls. There was a reduction in the amount of aflatoxin B₁ and G₁ synthesized by A. parasiticus grown on ground seeds and hulls from gypsum-treated plots compared to that synthesized on unsupplemented peanuts (Table 2). We used both hulls and kernels since a previous study found little difference in aflatoxin produced on each separately. The amount of glucosamine (as a measure of chitin content) present in the supplemented peanuts indicates that fungal growth was suppressed. No detectable fungal colonization was observed on the uninoculated control peanuts. Decreased growth of A. parasiticus resulted in decreased aflatoxin production.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chitin a</th>
<th>Aflatoxin B₁ b</th>
<th>Aflatoxin G₁ b</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 0</td>
<td>145.0 ± 10.1c</td>
<td>86.6 ± 10.6</td>
<td>112.3 ± 9.5</td>
</tr>
<tr>
<td>G 550</td>
<td>3.9 ± 0.8</td>
<td>9.6 ± 8.1</td>
<td>9.3 ± 1.3</td>
</tr>
<tr>
<td>G 1100</td>
<td>54.9 ± 7.6</td>
<td>4.0 ± 4.9</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>G 2200</td>
<td>2.7 ± 0.1</td>
<td>7.7 ± 2.5</td>
<td>10.7 ± 9.8</td>
</tr>
<tr>
<td>G 4400</td>
<td>3.9 ± 2.5</td>
<td>2.6 ± 0.03</td>
<td>15.1 ± 4.4</td>
</tr>
</tbody>
</table>

a Measured as μg glucosamine per 5 g ground peanuts (hulls and kernels).

b mg aflatoxin per 5 g ground peanuts (hulls and kernels).

c Mean ± SD.

Use of calcium supplementation in peanut production has been considered to reduce aflatoxin contamination levels. Early field trials hinted at this but were inconclusive (9,14). This study agrees with the idea that peanuts cultivated with calcium supplementation supports less aflatoxin synthesis, but it may be due to factors other than just increasing calcium levels. Our measured reduction of aflatoxin synthesis on supplemented peanuts may, in part, be due to alterations in the concentration of several key minerals.

Peanuts grown on gypsum-supplemented fields had less aflatoxin B₁ produced by A. parasiticus compared to those grown without gypsum supplementation. With the exception of the G 1100 treatment (1100 kg gypsum ha⁻¹), the aflatoxin content of the gypsum-supplemented peanuts was approximately the same. If the decrease in aflatoxin production due to the presence of calcium was a dose-response relationship, then the aflatoxin measured should have decreased proportionally to increasing calcium concentrations. It is possible that a constant response was attained once a threshold calcium concentration was exceeded and that this threshold coincides with a concentration attained with the lowest gypsum treatment. Another possibility is that other minerals may affect toxin synthesis either directly or indirectly. Manganese and zinc levels were greatest in the unsupplemented peanuts and were approximately the same for all four supplementation treatments. Thus these minerals may be important in aflatoxin biosynthesis on peanuts.

The literature concerning the effect of minerals on aflatoxin synthesis has many contradictions, but there is evidence that minerals play an important role. Of the minerals studied, zinc appears to be essential in that a variety of key enzymes related to aflatoxin production are zinc dependent (7,10). Zinc has been shown to stimulate aflatoxin production in peanuts grown without any significant increase in aflatoxin B₁ production. However, zinc supplementation may interfere with aflatoxin production as the threshold concentration of calcium is exceeded. Further research is needed to elucidate the role of minerals in aflatoxin synthesis.
Peanuts were found by Maggon and Venkitasubramian on harvested reduced the subsequent growth of *A. parasiticus* peanuts. To have high levels of aflatoxin present which they claimed culture media calcium in the peanuts to inhibit aflatoxin synthesis or in the toxin contamination in peanuts is not fully understood. Gypsum application may result in a sufficient concentration of other minerals in the seeds and hulls. The reason gypsum helps reduce aflatoxin biosynthesis. The reason gypsum helps reduce aflatoxin contamination in peanuts is not fully understood. Gypsum application may result in a sufficient concentration of calcium in the peanuts to inhibit aflatoxin synthesis or in the accumulation of other minerals in the seeds and hulls.

**ACKNOWLEDGMENTS**

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**REFERENCES**