Production of Aflatoxin on Soybeans

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Probable factors influencing resistance to aflatoxin synthesis in soybeans have been investigated by using cultures of Aspergillus parasiticus NRRL 3240. Soybeans contain a small amount of zinc (0.01 μg/g) bound to phytic acid. Autoclaving soybeans at 15 pounds (6803.88 g) for 15 min increases the aflatoxin production, probably by making zinc available. Addition of zinc to both autoclaved and nonautoclaved soybeans promotes aflatoxin production. However, addition of varying levels of phytic acid at a constant concentration of zinc depresses aflatoxin synthesis with an increase in the added phytic acid. In a synthetic medium known to give good yields of aflatoxin, the addition of phytic acid (10 mM) decreases aflatoxin synthesis.

Aflatoxins are hepatotoxic metabolites produced by certain strains of Aspergillus flavus and Aspergillus parasiticus. Production of aflatoxin by A. flavus strains on agricultural crops like groundnut, cottonseed, wheat, rice, barley, coconut, corn, dried peas, oat, sweet potato, millet, and cassava is well documented (4). In view of the wide acceptance of soybeans as an excellent source of nutrition to both man and poultry, due to its high protein content, the question of whether soybeans can become contaminated with aflatoxin assumes importance. There is little information on aflatoxin production in soybeans by A. parasiticus. Reports available are controversial. In a field study involving a survey of 866 commercial samples in the United States, Shotwell et al. observed that the incidence of aflatoxin contamination was only 0.8% although 50% of the samples were contaminated with A. flavus (16). Chang et al. (1) could not detect aflatoxin in moldy soybeans contaminated with toxigenic isolates of A. flavus though they were able to demonstrate aflatoxin production with A. parasiticus NRRL 2999. However, Davis and Diener (2) obtained considerable yields of toxin (48 to 138 μg/ml) on the Bragg variety of soybean after 21 days of incubation with a toxigenic strain of A. parasiticus. Nagarajan et al. (12) also found toxin production (0.12 to 31.25 μg/ml), using different isolates of A. flavus and A. parasiticus. Therefore, the present study was undertaken to find out the probable factors controlling the production of aflatoxin on soybeans.

MATERIALS AND METHODS

The Lee variety of soybean and the A. parasiticus strain NRRL 3240 used in the present study were obtained from the Indian Agricultural Research Institute, New Delhi, India, and the Northern Regional Research Laboratory, Peoria, Ill., respectively. Phytic acid was obtained from B.D.H., England.

Synthetic medium. Sucrose-low salt medium used in this study has the following composition: 85 g of sucrose, 10 g of asparagine, 3.5 g of (NH₄)₂SO₄, 10 g of KH₂PO₄, 2 g of MgSO₄. 6H₂O, 75 mg of CaCl₂, 2H₂O, 10 mg of ZnSO₄. 7H₂O, 5 mg of MnCl₂. 4H₂O, 2 mg of ammonium molybdate, 2 mg of Na₂B₄O₇, and 2 mg of FeSO₄. 7H₂O made up to 1,000 ml with double distilled water. The pH of the medium was adjusted to 4.5.

Twenty grams of finely powdered soybean was placed in each 500-ml conical flask and enough water (about 20 ml) was added to just moisten the powder. The flasks were divided into two groups. Group I was autoclaved at 15-pounds pressure for 15 min, and group II was not autoclaved. A spore suspension, prepared in sterile double distilled water from a 5- to 6-day-old culture of A. parasiticus grown on glucose-peptone agar, was distributed equally among the flasks (the inoculum usually contained 3 × 10⁶ to 3.5 × 10⁸ spores). The flasks were incubated at 26 ± 1 C for 8 days. At the end of the incubation period flasks were sprayed with 95% alcohol and dried overnight at 80 C as reported by Nagarajan et al. (12). The dried samples were first defatted with n-hexane and then extracted with chloroform. The chloroform extract was dried overnight over anhydrous sodium sulfate. The sodium sulfate was filtered off, and the extract was evaporated and made up to a known volume. Aflatoxins were separated by thin-layer chromatography using the solvent system toluene:iso-amyl alcohol:methanol (90:32:3, vol/vol/vol) (15). Individual aflatoxin bands were eluted with methanol and estimated by spectrophotometry using extinction coefficients reported by Nabney and Nesbitt (11).

Experiments were carried out in triplicate and data presented are the average of values from three separate flasks.
RESULTS AND DISCUSSION

Table 1 shows the effect of autoclaving on the production of aflatoxin in soybeans. Total aflatoxin production with nonautoclaved soybeans was only 0.34 mg per 100 g as compared to 6.85 mg per 100 g for autoclaved soybeans. This clearly indicates that exposure of soybeans to high temperature even for a short period promotes high production of aflatoxin. A similar observation has been made by Kratzer et al. (8) regarding the availability of dietary zinc for turkey poults. Many reasons have been advanced for the resistance to aflatoxin synthesis in soybeans by A. flavus. These are conditions of moisture unfavorable to the fungus at the time of soybean maturity, development of the seed in a closed pod, and the possibility of an inhibitor in soybeans preventing growth of the fungus (7). According to Nagarajan et al. (12), soybeans produce aflatoxin but the extent of production depends on the variety of soybean and the toxigenic potential of the isolate used. Soybeans are known to contain a high amount of phytic acid. In terms of phosphorous content, the phytic acid present constitutes 690 mg per 100 g of the edible portion as compared to 190, 390, 306, and 298 mg in rice, groundnut, wheat, and peas, respectively (5). Phytic acid is known to bind with zinc (13). It is likely that the negligible amount of aflatoxin synthesis observed with nonautoclaved soybeans was due to binding of zinc with phytic acid. Many workers have shown that swine develop parakeratosis, a zinc deficiency disease, when fed on soybean protein. O’Dell et al. (14) and Smith et al. (17) have reported that availability of zinc to animals fed plant protein was poor as compared to that in those fed animal protein. Zinc is known to have a pronounced stimulatory effect on aflatoxin production (3, 9, 10). Mateles and Adye (10) reported a reduction of 88% in toxin yields in the absence of zinc. The observed synthesis of high amounts of aflatoxin on autoclaved soybeans is probably due to the destruction of phytic acid by heat, resulting in the availability of zinc for aflatoxin synthesis.

Table 2 shows the effect of aflatoxin production by addition of zinc sulfate to nonautoclaved soybean. There is a gradual increase in aflatoxin formation with an increase in zinc sulfate from 1.0 to 5.0 g per flask. Addition of 5.0 g of zinc sulfate is observed to give a total yield of 9.60 mg of aflatoxin per 100 g of soybean. In the case of autoclaved soybeans, it was expected that it would increase the production of aflatoxin (from Table 1) because on high temperature the phytic acid structure is broken down and zinc would be released. To find out whether addition of zinc has any effect on autoclaved soybeans, only one concentration, i.e., 5.0 g of zinc sulfate, which gave maximum yield in the case of nonautoclaved soybeans, was studied. It was found that aflatoxin synthesis increased from 6.55 mg per 100 g of soybean to 9.60 mg per 100 g of soybean. This shows that on autoclaving the soybean zinc is released but it is not enough to synthesize maximum aflatoxin (Table 3).

Glick (6) has shown that the zinc content of soybeans is only 0.01 μg/g, whereas that of rice, barley, legumes, oats, and wheat is 18 to 19, 14 to 15, 22 to 23, 1 to 35, and 1 to 24 μg/g, respectively (6). The observation in the present study of a synthesis of aflatoxin on autoclaving soybeans and also on addition of zinc sulfate to both autoclaved and nonautoclaved soybeans can be explained on the basis of availability for aflatoxin synthesis of zinc, which under usual conditions is low in amount and bound to phytic acid.

This would lead to the conclusion that soybeans contain only a small amount of zinc which is present in a bound form with phytic acid. In view of the stimulation by zinc of aflatoxin production in soybeans, it was of interest to find out whether phytic acid blocks aflatoxin synthesis. The results are presented in Table 4. Addition of zinc sulfate increased total aflatoxin yield from 0.32 to 2.25 mg per 100 g of soybean. However, addition of increasing amounts of phytic acid, with the zinc sulfate level kept constant, results in a reduction of the total yield of toxin from 2.25 to 0.22 mg per 100 g of soybean. This shows clearly that zinc binds

| Condition       | Aflatoxin (mg/100 g of soybean powder) |
|-----------------|---------------------------------------|
|                 | B₁ + B₂ | G₁ + G₂ |
| Not autoclaved  | 0.125    | 0.210   |
| Autoclaved      | 1.900    | 4.950   |

| ZnSO₄ added (g) | Aflatoxin (mg/100 g of soybean powder) |
|-----------------|---------------------------------------|
|                 | B₁ + B₂ | G₁ + G₂ |
| None            | 0.130    | 0.210   |
| 1.0             | 0.700    | 1.650   |
| 2.0             | 1.600    | 4.500   |
| 5.0             | 2.300    | 7.300   |
with phytic acid and is essential for the formation of aflatoxin.

On the basis of the observation that phytic acid inhibits aflatoxin formation by combining with zinc, a further study was carried out to find out the effect of phytic acid on sucrose-low salt synthetic medium which gave good yields of aflatoxin. The results are presented in Table 5. Sucrose-low salt synthetic medium gives good yields of aflatoxin, about 24.0 mg per 100 ml of medium, but addition of 10 mM phytoic acid to the medium reduces aflatoxin production to 3.15 mg per 100 ml of medium, indicating that phytic acid binds with zinc which is an absolute requirement for aflatoxin synthesis.

Table 3. Effect of addition of ZnSO₄ to autoclaved soybeans on production of aflatoxin

| ZnSO₄ added (g) | Aflatoxin (mg/100 g of soybean powder) |
|----------------|----------------------------------------|
|                | B₁ + B₂                               | G₁ + G₂ |
| None           | 1.800                                  | 4.750   |
| 5.0            | 2.400                                  | 6.700   |

Table 4. Effect of addition of ZnSO₄ and ZnSO₄ plus different levels of phytic acid to nonautoclaved soybeans on production of aflatoxin

| Condition                  | Aflatoxin (mg/100 g of soybean powder) |
|----------------------------|----------------------------------------|
|                            | B₁ + B₂ | G₁ + G₂ |
| Soybean                    | 0.12    | 0.20    |
| Soybean + 1 g of ZnSO₄     | 0.65    | 1.60    |
| Soybean + 1 g of ZnSO₄ + 200 mg of phytic acid | 0.28    | 0.31    |
| Soybean + 1 g of ZnSO₄ + 400 mg of phytic acid | 0.16    | 0.15    |
| Soybean + 1 g of ZnSO₄ + 1,000 mg of phytic acid | 0.10    | 0.12    |

Table 5. Effect of addition of phytic acid to the synthetic medium

| Condition                  | Aflatoxin per 100 ml of medium (mg) |
|----------------------------|-------------------------------------|
|                            | B₁ + B₂   | G₁ + G₂   |
| Synthetic medium (100 ml)  | 15.210    | 1.937     |
| Synthetic medium (100 ml) + 10 mM phytic acid | 0.8928   | 0.6156    |

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