Effects of Aflatoxin on Germination and Growth of Lettuce

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The relative susceptibility of 30 cultivars of lettuce to inhibition by aflatoxin was studied. Seed germination was not inhibited by concentrations as high as 1,000 µg/ml in cultivar Imperial 44 or by 100 µg/ml in the remaining cultivars. Hypocotyl elongation was inhibited by 46 to 68% at a concentration of 100 µg of aflatoxin per ml. Seedlings exposed to aflatoxin did not become chlorotic. The similarity between the morphological reaction of plants to coumarin and aflatoxin suggests a common mode of action, but further studies of the physiological basis for the inhibitory reactions induced by these compounds will be necessary before such conclusions will be valid.

Macroscopic observations show that aflatoxins affect certain plants by inhibition of seed germination (19), elongation of the hypocotyls or roots of developing seedlings, or both (5, 15, 16), and by interference with chlorophyll synthesis (5, 19, 20). Similar inhibitory activities have been attributed to coumarin (2, 10) and, since aflatoxins are derivatives of coumarin, analogous modes of action have been suggested for these substances (3).

The germination of lettuce seeds is particularly sensitive to coumarin inhibition. If aflatoxin and coumarin do function in a biologically analogous manner, lettuce should be equally sensitive to the mycotoxin. This is a report on the relative susceptibility of cultivars of lettuce to aflatoxin.

MATERIALS AND METHODS

Test organisms. Seeds of 28 cultivars of lettuce (Lactuca sativa L.) and two of cos or romaine lettuce (L. sativa var. longifolia Gam.) were obtained from W. Atlee Burpee Co., Philadelphia, Pa. The test organisms included both leaf and head lettuce (L. sativa var. capitata L.) as well as black- and white-seeded varieties (Table 1). One cultivar, Imperial 44, was used in specific tests to determine the effect of the test system and sample size on seed germinability and the effect of aflatoxin on the growth response.

Germination substrate. Seeds were germinated on a 2% washed agar substrate prepared from agar (Difco) by alternately soaking the dehydrated agar in water and 95% ethanol for 4-h periods at 25 C. After four extraction cycles, the agar was filtered and the filter cake was rinsed in situ with absolute ethanol, crumbled and air-dried on sheets of absorbent paper. Unwashed agar was unsatisfactory as a germination substrate since sufficient trace nutrients were present to support the growth of fungal contaminants which inhibited seed germination and subsequent seedling development. Filter paper could not be used as a germination substrate in these experiments since evaporation of the solvent and oxidation of the aflatoxin could change the effective concentration and result in unreliable data. Furthermore, filter paper has been reported to contain impurities which might inhibit seed germination (14).

Aflatoxin production and purification. The aflatoxin mixture used in this study was produced by Aspergillus parasiticus NRRL 2999 cultured on polished rice for 7 days, as previously reported (1). Chloroform extracts of the cultures were precipitated in 10 volumes of petroleum ether (bp 20–60 C), filtered, and reextracted with methanol, and the methanolic extract was evaporated to dryness in vacuo. This extract was redissolved in a minimal volume of chloroform and reprecipitated in petroleum ether to provide a purified toxin extract.

The potency of the purified toxin extract was determined on thin-layer chromatograms by visual comparison with a reference standard containing a known concentration of the four major aflatoxins obtained from L. A. Goldblatt, Southern Marketing and Nutrition Research Division, New Orleans, La. Equivalent concentrations of each aflatoxin in the purified extract and the reference standard were determined by observing the point of extinction of long-wave ultraviolet fluorescence. The purified toxin contained a mixture of aflatoxins B₁, G₁, B₂ and G₂ in a ratio of 1:1 (approximately 350 mg/g each), with only traces of aflatoxins B₃ and G₃ present. The remaining portion of the extract contained nonaflatoxin substances exhibiting little or no fluorescence.

Preparation of the test substrates. Chloroform solutions containing 0.1, 0.2, and 0.5 mg of aflatoxin mixture per ml were prepared by serial dilution from
a stock solution containing 1 mg of purified extract per ml (2.19 \times 10^{-4} \text{ M aflatoxin}). Toxic germination substrates were prepared from these solutions by adding portions of 1, 2, or 3 ml to individual test tubes to provide a concentration of 10 times the desired final concentration of aflatoxin mixture in each tube. The solvent was removed under a stream of N₂ while the tubes were rotated to form an even coating of toxin in the base of each tube. Ten milliliters of melted, 2% washed agar were added to each tube to adjust the final concentration of aflatoxin mixture to the desired concentration. The tubes were plugged, autoclaved at 121 C for 10 min, rapidly cooled to 45 C, and poured into individual, sterile, flat-bottomed petri dishes (9 cm; Corning no. 3162). Substrates for experimental controls were prepared in the same manner as above but using 3 ml of pure chloroform in place of the toxin solutions.

Under these conditions, the volume of agar was sufficient to dissolve the quantity of aflatoxin used in each test substrate. Although crystalline aflatoxin is heat sensitive, autoclaving did not significantly decrease the potency of the purified mixture used in this study.

Test system. A preliminary study using 25, 50, 75, 100, and 150 seeds per germination plate showed that within these limits the number of seeds did not affect the degree of germination or subsequent seedling development within 120 h. A sample size of 100 seeds was chosen to simplify data collection. Seed samples were counted and distributed evenly on the surface of the test substrates. A test series contained four plates of each test substrate, and each series was repeated a minimum of four times.

Seeded plates were incubated at 25 C in the dark to minimize the photodecomposition of aflatoxin and to minimize the inhibitory effect of light on the germination of light-sensitive cultivars. Plates were examined periodically, as indicated, and germination was considered positive after the radicle (root tip) had pierced the seed coat and extended at least 1 mm beyond the seed proper. The hypocotyl, that portion of the germinated seed extending from the point of maximum root hair development to the crook at the base of the cotyledons, was measured to the nearest millimeter. The mean and standard deviations were determined, and the latter fell within acceptable limits for biological data (P = 0.001).

Experiments conducted. The effect of aflatoxin on the germination of seeds of Imperial 44 was determined at concentrations of 0, 25, 100, and 1,000 \mu g of aflatoxin mixture per ml. The numbers of germinated seeds were recorded periodically between 12 and 48 h. The final percent germination in the controls and at each toxin concentration was recorded after 48 h.

The effect of aflatoxin on seedling growth was determined at a series of concentrations between 0 and 100 \mu g of aflatoxin mixture per ml. Plates were seeded with Imperial 44, four replicate plates were harvested, and the hypocotyls were measured after 48, 72, 96, and 120 h of incubation.

The inhibitory effect of aflatoxin on 30 cultivars of lettuce was studied to determine the degree of susceptibility of each variety to the toxin. Test substrates containing 100 \mu g of aflatoxin mixture per ml were seeded with each test organism the percent germination was determined, and hypocotyl measurements were made after 68 h.

| Variety; type* | Hypocotyl length (mm) | Percent inhibition |
|---------------|------------------------|------------------|
|               | Control                | Treated          |
| Bibb; H/bs    | 15.2 ± 2.6             | 6.0 ± 2.2        | 60.4 |
| Big Boston; H/ws | 14.0 ± 2.9             | 5.5 ± 1.0        | 60.9 |
| Black-seeded Simpson; L/bs | PG*                  | PG               |
| Burpee Bibb; H/bs | PG                   | PG               |
| Butter King; us | 13.5 ± 4.8             | 5.8 ± 3.1        | 57.2 |
| Buttercrunch; H/bs | 17.5 ± 3.4             | 6.2 ± 1.2        | 64.6 |
| Dark Green Boston; H/ws | 17.9 ± 3.3             | 6.7 ± 1.3        | 62.8 |
| Deer Tongue (Matchless); H/bs | 15.8 ± 2.1             | 5.7 ± 1.1        | 63.8 |
| Early Prizehead; L/ws | 13.9 ± 3.8             | 6.5 ± 2.1        | 53.4 |
| Fordhook; H/ws | 13.4 ± 3.9             | 6.6 ± 2.6        | 51.0 |
| Grand Rapids; L/bs | 15.1 ± 3.0             | 5.0 ± 1.3        | 67.3 |
| Great Lakes; H/ws | 21.7 ± 4.5             | 5.7 ± 1.4        | 55.3 |
| Great Lakes, no. 859 MT; H/ws | 16.8 ± 3.0             | 5.4 ± 1.3        | 67.9 |
| Greenhurt, Burpee; L/bs | 16.4 ± 4.2             | 6.5 ± 1.1        | 60.0 |
| Hanson; H/ws | 16.7 ± 2.8             | 7.7 ± 0.6        | 54.2 |
| Iceberg, Burpee's; H/ws | 17.6 ± 3.4             | 7.4 ± 2.9        | 57.7 |
| Imperial, no. 44; H/ws | 17.6 ± 2.4             | 7.3 ± 1.3        | 58.7 |
| Imperial, no. 456; H/ws | 17.7 ± 2.7             | 6.7 ± 1.3        | 62.0 |
| Imperial, no. 847; H/ws | 13.2 ± 3.7             | 6.6 ± 1.4        | 49.8 |
| New York, no. 12; H/ws | 11.5 ± 2.8             | 6.2 ± 1.8        | 46.0 |
| New York, no. 515; H/ws | PG                    | PG               |
| Oak Leaf; L/ws | 14.2 ± 4.8             | 7.0 ± 1.2        | 50.8 |
| Premier Great Lakes; H/ws | 17.0 ± 3.0             | 5.8 ± 1.4        | 65.7 |
| Ruby; L/ws | 14.3 ± 2.6             | 5.7 ± 1.4        | 59.8 |
| Salad Bowl; L/bs | 13.6 ± 2.1             | 5.3 ± 1.0        | 60.7 |
| Sibolt; L/bs | 13.4 ± 3.0             | 6.7 ± 1.5        | 49.9 |
| Wayhead, Burpee's; H/ws | 17.5 ± 2.6             | 6.8 ± 1.7        | 61.0 |
| White Boston; H/ws | 17.4 ± 3.2             | 7.2 ± 1.5        | 58.6 |
| Paris Island Cos; ws | 15.8 ± 2.8             | 6.4 ± 1.5        | 59.8 |
| Paris White Cos; ws | 14.0 ± 2.6             | 6.3 ± 1.4        | 55.1 |

* Abbreviations: H, heading; L, leaf; bs, black seeded; ws, white seeded; us, data on type unavailable.

PG, poor germination. Both controls and treated seeds showed less than 15% germination under these experimental conditions.

RESULTS

Inhibition of germination. Some seeds on each of the toxic and control substrates germinated within 18 h. After 24 h, 85 to 89% germination had occurred on all test substrates except those containing 1,000 \mu g of aflatoxin mixture per ml on which only 88% germination had occurred by this time. After 40 h, however, 90 to 94% germination was noted on all test substrates, including the highest concentration.
of aflatoxin. The maximum amount of germination, 95%, was observed on all test substrates within 48 h. Except for a slight initial lag in germination observed at a concentration of 1,000 µg/ml, aflatoxin did not inhibit the germination of Imperial 44.

**Growth response.** At concentrations of aflatoxin below 20 µg/ml, elongation of the hypocotyls in seedlings of Imperial 44 was only slightly inhibited. As the concentration of toxin was increased above 25 µg/ml, there was an increasing degree of inhibition of hypocotyl elongation. After 120 h, seedlings exposed to 100 µg of aflatoxin mixture per ml exhibited a 33% inhibition. The growth response of this cultivar of lettuce to concentrations of aflatoxin is shown in Fig. 1.

**VARIetal susceptibility to aflatoxin.** In all of the cultivars tested, the percentage of seeds germinating in the presence of aflatoxin was not significantly less than that of the controls. The seeds of three cultivars, Black-seeded Simpson, Burpee Bibb, and New York 515, exhibited poor germination in the presence or absence of toxin. Whether this reflected an inability to germinate under these test conditions or, more probably, the low viability of the specific seed samples, was not determined. These three cultivars were not studied further.

A concentration of 100 µg of aflatoxin mixture per ml inhibited hypocotyl elongation in all cultivars tested. The degree of inhibition ranged from 46 to 68% with an average of 58% (Table 1). There was no significant difference in the degree of inhibition exhibited between leaf or heading varieties or between those producing black or white seeds.

When compared visually, no differences could be distinguished between the coloration of treated and control seedlings. Surprisingly, the cotyledons of the test seedlings exhibited the same degree of coloration after incubation in the dark as did comparison seedlings incubated in the light. After 68 h of incubation, the seedlings did not appear etiolated.

**DISCUSSION**

Inhibition of seed germination and seedling elongation are the two physiological effects most often associated with coumarin activity. Aflatoxin does not affect seed germination but is inhibitory to hypocotyl elongation in lettuce. In comparing the inhibitory effect of coumarin and various coumarin derivatives on seed germination in lettuce, Mayer and Evenari (9) found the derivatives to be less inhibitory than the parent compound. They postulated that the inhibitory effect of coumarin is a function of the unsaturated lactone ring of the coumarin molecule. Goodwin and Taves (6) studied the effects of coumarin and coumarin derivatives on seed germination and root growth in *Avena*. Some derivatives were active inhibitors of root growth but were inactive as germination inhibitors, whereas other derivatives, which were very weak inhibitors of root growth, were as active as coumarin in inhibiting seed germination.

The aflatoxin molecule contains the unsaturated lactone ring structure postulated to be necessary for coumarin-like activity (9). The remaining portion of the aflatoxin molecule, however, is only distantly related to coumarin. This might explain aflatoxin's lack of inhibitory activity towards lettuce seed germination but does not explain the observation of Schoental and White (19) that aflatoxin inhibited seed germination in *Lepidium*. They found that 100 µg of aflatoxin per ml caused a 100% inhibition of germination. With lettuce, I found only a slight lag in the rate of seed germination at a concentration of 1,000 µg of aflatoxin mixture per ml, an effective toxin concentration of 700 µg/ml. More recently Reiss (16), also using *Lepidium*, was unable to repeat the observations of Schoental and White.

The effects of coumarin and aflatoxin on
elongation of hypocotyls and roots are more striking. Although inhibitory to the growth of *Avena* roots (6), coumarin stimulates hypocotyl elongation in *Helianthus, Avena, Pisum,* and *Phaseolus* (12). Coumarin derivatives can be either stimulatory or inhibitory. Lettuce hypocotyls exhibit a distinct sensitivity to aflatoxin. Aflatoxin, although varying in intensity, inhibited hypocotyl elongation in all cultivars tested in this study. The degree of inhibition appears to be directly related to the amount of toxin and, at high concentrations, the rate of hypocotyl elongation is diminished proportionately. Reiss (16) found both hypocotyl and root growth of *Lepidium* inhibited by aflatoxin B₁ with the root showing slightly more sensitivity to the toxin.

Aflatoxin has been associated with the development of albinism (virescence) in plants. Albinism has been induced in corn and citrus seedlings after infection with isolates of *Aspergillus flavus* of unknown toxigenicity (4, 8). Ryan et al. (18) and Joffe (7), however, were unable to induce albinism in citrus, tomato, and several legumes with toxigenic and nontoxigenic strains of *A. flavus.* Schoental and White (19) observed albinism in seedlings of *Lepidium* exposed to 10 μg of aflatoxin per ml. This “bleaching” phenomenon was further studied by Szwatitzki et al. (20) and was proposed as a bioassay method for aflatoxin M (11). Reiss (16) observed some lightening of the coloration of *Lepidium* exposed to 100 μg of aflatoxin per ml but did not observe complete loss of chlorophyll. Lettuce seedlings observed in this study did not exhibit albinism at concentrations as high as 1,000 μg/ml.

The relationships, if any, between the inhibitory effects of coumarin and aflatoxin on plants remain unclear. Although research on coumarin has provided much information concerning its scope of activity and its proposed mode of action, little is known about the physiological basis for aflatoxin inhibition in plants. The observed similarities in the phytomorphological effects of coumarin and aflatoxin are representative of the general type of inhibitory response characteristic of many toxic compounds. The fact that both coumarin (13) and aflatoxin (16, 17) exhibit auxin-like activities at very low concentrations may support the hypothesis that they share some common physiological activities. However, many toxic compounds, such as antibiotics, exert a stimulatory effect at sublethal concentrations. On the evidence presently available, it cannot be assumed that coumarin and aflatoxin share a common mode of action.

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**LITERATURE CITED**

1. Crisan, E. V., and E. Mazzucca. 1967. Separation of aflatoxin on selectively deactivated silicic acid. Contr. Boyce Thompson Inst. 29:361-365.
2. de Greef, J. A. 1964. Changes induced by coumarin and trans-cinnamic acid in desoxyribonucleic acid (DNA) content and growth of pea roots (*Pisum sativum* cultivar Rondo). Enzymologia 27:311-326.
3. Detroy, R. W., E. B. Lillehoj, and A. Cigler. 1971. Aflatoxin and related compounds, p. 3-175. In A. Cigler, S. Kadis, and S. J. Aji (ed.), Microbial toxins, vol. 6. Academic Press Inc., New York.
4. Durbin, R. D. 1969. The possible relationship between *Aspergillus flavus* and albinism in citrus. Plant Dis. Rep. 43:922-923.
5. El-Khadem, M., G. Menke, and F. Grossmann. 1966. Schädigung von Erdnusskeimlingen durch Aflatoxine. Naturwissenschaften 53:532.
6. Goodwin, R. H., and C. Teves. 1950. The effect of coumarin derivatives on the growth of *Avena* roots. Amer. J. Bot. 37:224-231.
7. Joffe, A. Z. 1969. Effects of *Aspergillus flavus* on groundnuts and some other plants. Phytopathol. Z. 64:282-285.
8. Koehler, B., and C. M. Woodworth. 1938. Corn-seedling virescence caused by *Aspergillus flavus* and *A. tamarii.* Phytopathology 28:811-823.
9. Mayer, A. M., and M. Evenari. 1952. The relation between the structure of coumarin and its derivatives, and their activity as germination inhibitors. J. Exp. Bot. 3:246-252.
10. Mayer, A. M., and A. Poljakoff-Mayber. 1961. Coumarins and their role in growth and germination, p. 735-749. In R. M. Klein (ed.), Plant growth regulation. Iowa State University Press, Ames.
11. Mayer, A. M., A. Poljakoff-Mayber, P. Robinson, and I. Slowatzky. 1969. A simple bioassay for detection of aflatoxin in milk. Toxicon 7:13-14.
12. Neumann, J. 1969. An auxin-like action of coumarin. Science 162:1675-1676.
13. Neumann, J. 1960. The nature of the growth-promoting action of coumarin. Physiol. Plant. 13:328-341.
14. Rehwaldt, C. A. 1968. Filter paper effect on seed germination of *Arabidopsis thaliana.* Plant Cell Physiol. 9:509-511.
15. Reiss, J. 1969. Hemmung des Sprosswachstums von *Caralluma freeri* Rowl. durch Aflatoxin. Planta 93:369-371.
16. Reiss, J. 1971. Hemmung der Keimung der Kresse (*Lepidium sativum*) durch Aflatoxin B₁ und Rubratoxin B. Biochem. Physiol. Pflanzen 162:363-367.
17. Reiss, J. 1971. Förderung der Aktivität von δ-Indolylessigsäure durch Aflatoxin B₁, Z. Pflanzenphysiol. 64:260-262.
18. Ryan, G. F., G. Greenblatt, and K. A. Al-Delaimy. 1961. Seedling albinism induced by an extract of *Alternaria tenuis.* Science 134:833-834.
19. Schoental, R., and A. F. White. 1966. Aflatoxin and 'albinism' in plants. Nature (London) 206:57-58.
20. Slowatzky, I., A. M. Mayer, and A. Poljakoff-Mayber. 1969. The effect of aflatoxin on greening of etiolated leaves. Isr. J. Bot. 18:31-36.