Mechanism of Formation of a Fluorescence in Cotton Fiber Associated with Aflatoxins in the Seeds at Harvest

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A bright greenish yellow fluorescence, observed previously in cotton fibers infected in the living state in the field and in the laboratory with *Aspergillus flavus*, has now been produced experimentally in living tissues of several other plants that were mechanically injured and then incubated with the fungus. Evidence indicates that the fungus forms kojic acid, which is converted to the fluorescing substance under the influence of peroxidase in the plant. The relevance of these results to the use of the fluorescence to locate cotton seeds containing aflatoxins at harvest is discussed.

Fibers with a bright greenish yellow fluorescence under ultraviolet light were noted earlier in occasional samples of commercial cotton lint and were indicated to originate in a previously undescribed boll rot caused by *Aspergillus flavus* Link (Marsh *et al.*, 1955). A fluorescence of similar hue was produced in living cotton fiber incubated with *A. flavus* and the fluorescent substance was identified with the naturally occurring material by chromatography and through its absorption spectrum. The fluorescence was so easily detected as to suggest its use in locating fiber infected with *A. flavus* prior to harvest (Marsh and Simpson, 1968; Marsh and Taylor, 1958). Because of its bright greenish yellow color under an ultraviolet light, it was referred to as the "BGY" fluorescence. Data on the relation of fluorescence in the fiber to aflatoxin in the seed have been presented (Marsh *et al.*, 1969).

Although the BGY fluorescence formed readily in living cotton fibers incubated with *A. flavus*, fibers of the same kind which were autoclaved and subsequently incubated with the fungus showed no BGY fluorescence in spite of heavy growth on them (Marsh *et al.*, 1955). Some heat-labile factor in the living fiber seemed necessary for the formation of the fluorescence. The factor appeared to be present also in the living milk-stage seeds of corn (*Zea mays*) and in the living cells of the inner wall of a milkweed pod (*Asclepias syriaca*), since the BGY fluorescence could be produced in both tissues also by incubation with *A. flavus* (Marsh *et al.*, 1968). Again, no fluorescence was formed if the tissues had been autoclaved before incubation with the fungus, even though the fungus grew well.

MATERIALS AND METHODS

In the experiments of Table I, the tissues on which the fungus was grown were flamed in 95% ethanol, cut with a sterile scalpel in several places to cause mechanical injury, placed in previously autoclaved quart Mason jars containing a small amount of water to provide high humidity, inoculated by needle, and incubated at 30° C. Except as otherwise indicated, the fungus isolate used in all experiments was *Aspergillus flavus* NRRL 2999. In the incubation of individual cotton locks, nearly mature cotton bolls were taken from the greenhouse and flamed in 95% ethanol, and their locks removed aseptically. (A cotton boll is divided into four or five radial segments, each consisting of seeds and attached fiber, and termed a "lock.") The locks were then placed individually over water on a layer of glass beads in deep Petri dishes, inoculated by needle, and incubated at 30° C. Fluorescence was observed under a long-wave Blak-Ray ultraviolet lamp, Model B-100.

In producing the nonfluorescing culture filtrates of Tables II and III, the fungus was grown at 30° C.,

Table I. Plant Tissues That Formed Greenish Yellow Fluorescence during Incubation with *Aspergillus flavus* 2999 and R_f of Fluorescing Material

Plant	Part Incubated	Site of Fluorescence	R_f
Arachis hypogaea (peanut)	Stems and fruits	Stems, pegs, young fruit	0.36
Asclepias syriaca (milkweed)	Fruit	Inner wall of pod	0.36
Chenopodium album (lambs quarters)	Stem	Stem	0.39
Fragaria (strawberry)	Fruit	Fruit	0.36
Glycine soja (soybean)	Stem	Stem	0.39
Gossypium hirsutum (cotton)	Boll	Fiber	0.37
Panicum dichotomiflorum (panic grass)	Stem	Stem	0.37
Setaria sp. (foxtail)	Stem	Stem	0.35
Zea mays (corn)	Fruit	Seeds, leaf sheath bases	0.33

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Table II. Peroxidase Activity and Activity in Inducing Greenish Yellow Fluorescence in Presence of Peroxide

Organism	Part Tested	Activity in Peroxidase Test with Guaiacol	Activity in Inducing Greenish Yellow Fluorescence in: Filtrate from growth medium of *A. flavus*	Activity in Inducing Greenish Yellow Fluorescence in: Solution of kojic acid
Cabbage	Leaf (head)	High	High	High
Celery	Stalk	High	High	High
Corn	Seed, soft	High	High	High
Cotton	Fiber, near mature	High	High	High
Cow	Milk	High	High	High
Cucumber	Fruit	High	High	High
Radish	Root	High	High	High
Apple	Fruit	Medium	Low	Medium
Pear	Fruit	Medium	Low	Medium
Tomato	Fruit	Medium	Low	Medium
Grape	Fruit	V. low	V. low	V. low
Beef	Liver	V. low or none	V. low or none	V. low or none
Honeybee	Larvae and adults, brei	V. low or none	V. low or none	V. low or none
Mushroom	Sporophore	V. low or none	V. low or none	V. low or none
Yeast, baker's	Cell	V. low or none	V. low or none	V. low or none

without shaking, in 500-ml. Erlenmeyer flasks with 40 ml. of a modified Czapek's solution containing $NaNO_3$, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, KCl, $FeSO_4$, and glucose at concentrations of 3, 1, 0.5, 0.5, 0.01, and 30 grams per liter, respectively. After 4 to 5 days, the medium was filtered through a medium-porosity sintered glass crucible. The plant tissues of Table II were from fields around Beltsville, or from a local supermarket. Peanut plants were grown in the greenhouse at Beltsville and provided by W. K. Bailey, U. S. Department of Agriculture, Beltsville, Md. The honeybees were obtained from Hachiro Shimanuki, U. S. Department of Agriculture, Beltsville, Md. The yeast was Fleischmann's cake yeast.

Recommended safety procedures for working with *A. flavus* cultures were followed (Fischbach and Campbell, 1965; Stoloff and Trager, 1965).

Horseradish peroxidase came from Nutritional Biochemicals, Inc., catalase from Calbiochem, polyphenol oxidase (mushroom) from Worthington Biochemical Corp., hemoglobin from Mann Research Laboratories, and kojic acid from Matheson, Coleman, and Bell, Inc.

In the guaiacol test for peroxidase, the tissue to be tested was put into a Petri dish of 5-cm. diameter and wetted with a 5% solution of guaiacol in 60% ethanol. Then, 1 to 2 drops of 3% H_2O_2 were added and the tissue was observed for 1 to 10 minutes for the development of a brown color. The test for activity in inducing a greenish yellow fluorescence in filtrate from the growth medium of *A. flavus* was performed in the same manner, except that the filtrate was used in place of the guaiacol solution and the tissue was observed under the Blak-Ray lamp after 3 to 30 minutes. The test for inducing a greenish yellow fluorescence in a solution of kojic acid was the same, except that a 1% aqueous solution of kojic acid was used instead of the culture filtrate. Tests of the action of horseradish peroxidase upon kojic acid were in a volume of 10 ml. in a test tube. Visible fluorescence was observed at kojic acid, peroxidase, and peroxide concentrations of 1, 1, and 0.3 p.p.m. after a reaction period of 1 hour. Very pronounced fluorescence occurred within 15 minutes with 0.5% kojic acid, 0.001% peroxidase, and 0.0003% H_2O_2.

Table III. Role of Kojic Acid and Peroxidase in Formation of a Bright Greenish Yellow Fluorescing Material in Cotton Fiber Infected with *Aspergillus flavus*

	Material Tested	R_f Values	
1.	Water extract from live, never-dried cotton fiber incubated with *A. flavus*	0.33[a]	0.68[b]
2.	Water extract from never-dried fiber, autoclaved, then incubated with *A. flavus*	—	0.68
3.	Kojic acid	—	0.68
4.	Kojic acid pretreated with peroxidase-peroxide	0.33	0.68
5.	Filtrate from culture solution of *A. flavus* on glucose-mineral salts	—	0.67
6.	Same as 5, but filtrate pretreated with peroxidase-peroxide prior to chromatography	0.33	0.68

[a] BGY fluorescence on original chromatogram for spots in this column.
[b] Spots in this column all developed BGY fluorescence when sprayed with peroxidase-peroxide.

Plates for thin-layer chromatography (Tables I and III) were made with a 0.5-mm. layer of Avicel (1 to 1 mixture of superfine and technical grades). The solvent mixture contained formic acid, methyl ethyl ketone, *tert*-butyl alcohol, and water (3:6:8:3) (Fink *et al.*, 1963). A solvent system consisting of butanol–acetic acid–water (3:1:1), used with microcrystalline cellulose plates for most of the items listed in Table I, yielded closely matching R_f values approximating 0.39. The spectrophotometer was a Perkin-Elmer Model 202. Fluorescing solutions were chromatographed and eluted in water prior to use in this instrument. In the experiments of Table III, the peroxidase solution used to spray the chromatograms contained 10 mg. per 100 ml. of the enzyme in water. This was followed by spraying with 0.01% peroxide. Tests for kojic acid were performed

by addition of a few drops of 1% $FeCl_3$ to the solution or tissue in question, also by adding peroxidase and peroxide and observing production of the BGY fluorescence. Determinations of fluorescence-emission spectra were made on a Baird-Atomic Fluorispec.

RESULTS AND DISCUSSION

Production of Fluorescence in Living Plant Tissues Incubated with *Aspergillus flavus*. *A. flavus* appeared to be a weak invader of most plant tissues but, once a tissue had been mechanically damaged, in many cases the fungus produced the BGY fluorescence in it (Table I). The fluorescing material was similar in R_f value to that produced in cotton fiber (Table I). An absorption spectrum of the material produced in the peanut stems exhibited maxima at approximately 280 and 380 mμ, as in preparations from cotton fiber, corn seeds, and milkweed pods incubated with the same fungus (Marsh *et al.*, 1968).

Some living tissues infected with the fungus failed to develop fluorescence. This was true of the embryo of maturing cotton seeds from the nearly mature boll incubated as described for production of fluorescence in the fiber (Marsh *et al.*, 1955) and of mature cotton seeds surface-sterilized in chlorine gas (Marsh *et al.*, 1968) and then incubated and well infected with the fungus in pure culture in a humid atmosphere but in the absence of sufficient water to allow germination. Seeds with fluorescent fiber infected with *A. flavus* in the field also regularly failed to show BGY fluorescence in the living internal parts of the seeds, even when microscopic examination showed these seeds to be massively infected. Honeybees, subject to *A. flavus* infections in nature (Burnside, 1930), failed to exhibit fluorescence when incubated and infected in live condition in the laboratory, in either the larval or adult stage.

Production of Fluorescence by Enzymatic Reactions with an *A. flavus* Culture Filtrate. Evidence indicated that a peroxidase enzyme, or enzymes, was the heat-labile constituent of living plant tissues necessary for formation of the BGY fluorescence during incubation with *A. flavus*. A nonfluorescing filtrate from a culture of *A. flavus* on glucose and mineral salts developed a brilliant greenish yellow fluorescence when placed for a few minutes with any of several higher plant tissues in the presence of H_2O_2 (Table II). The capability of causing fluorescence development was not a characteristic of all living cells, but occurred specifically in cells exhibiting peroxidase activity (Table II). A test with cucumber tissue showed that the production of fluorescence was inhibited strongly by both cyanide and azide. A horseradish peroxidase preparation tested in the presence of H_2O_2 also caused rapid fluorescence development in the culture filtrate, whereas preparations of catalase, hemoglobin, and polyphenol oxidase (from mushroom) did not.

Production of Fluorescence by Enzymatic Reactions with Kojic Acid. A 1% aqueous solution of kojic acid, long known as a metabolite of *A. flavus* (Prescott and Dunn, 1959), developed a bright greenish yellow fluorescence within a few minutes in the presence of peroxide- and peroxidase-containing tissues, including living cotton fiber (Table II). The fluorescence was also produced in the presence of peroxide and peroxidase, but not peroxide and catalase, hemoglobin, or polyphenol oxidase. The living cotton fiber stimulated some fluorescence production with kojic acid, even in the absence of added peroxide, although the reaction was much faster when the peroxide was used.

The very young cotton seed developing in the boll exhibited strong enzymatic action in the kojic acid–fluorescence test of Table II, but lost this capability during development, so that the mature seed, either in the moist condition at boll opening or after drying, was inactive. Activity was not restored to mature seed by simple imbibition of water, but became strong at a later stage of germination in the radicle and hypocotyl, and eventually strong in the cotyledons after chlorophyll development. In this entire developmental sequence, the activities against kojic acid were paralleled by activity in the guaiacol test and in the test for formation of the BGY fluorescence in the *A. flavus* culture filtrate. Altschul *et al.* (1948) noted a rapid increase in peroxidase activity in cotton seeds during germination. The embryo of the very young peanut seed, like that of the cotton seed, exhibited high activity in the three enzyme tests of Table II, lost this activity during maturation, but exhibited it again in high degree during active growth at the time of germination. Mature lima beans, used in the three tests after imbibition of water, likewise gave very low or negative results, but strongly positive results in the growing root and shoot tissue of the germinating seedling.

The very young cotton fiber (22 days from flowering), when tested without drying, was strong in the three peroxidative enzyme activities of Table II. Unlike the seed to which it was attached, the moist, never-dried fiber did not lose these activities as it approached maturity, although initial drying of the mature fiber caused the disappearance of all three activities.

Further evidence on the roles of both kojic acid and peroxidase in the production of the BGY fluorescence in living cotton fiber infected with *A. flavus* may be seen in Table III. Item 1 represents R_f values of a water extract from live fiber incubated as a single lock with *A. flavus*. This incubation is considered to involve production of the BGY fluorescing material in a laboratory situation approximating that which occurs in nature. The R_f of 0.33 is for the greenish yellow fluorescing spot, whereas another spot, nonfluorescent and dark in color, was observed under short-wave ultraviolet at R_f 0.68. Item 2, a water extract of never-dried fiber incubated as in 1 but autoclaved before incubation, showed the presence of the dark spot, but not the greenish yellow fluorescing spot. Item 3 refers to kojic acid, with an R_f matching the spots of higher R_f in items 1 and 2. Item 4, from a kojic acid solution treated with peroxidase-peroxide before chromatographing, showed a bright greenish yellow fluorescent spot matching in R_f that of BGY in item 1, also a dark spot under short-wave ultraviolet representing residual kojic acid. Items 5 and 6 correspond to items 3 and 4, except that a nonfluorescing filtrate from a 5-day culture of *A. flavus* on mineral salts and glucose was used in place of the kojic acid solution. Again, the dark spot corresponding in R_f to kojic acid appeared in both cases and the greenish yellow fluorescing spot in item 6. All

of the spots with the higher R_f developed the greenish yellow fluorescence when the plate was sprayed with peroxidase and peroxide solutions, but no other spots or streaks with a greenish yellow color appeared at any other position after this spraying. Fiber incubated for item 1 exhibited a strong color reaction with ferric chloride, as with kojic acid. The autoclaved, never-dried fiber incubated with *A. flavus* developed no BGY fluorescence during incubation but did so conspicuously when supplemented with 100 μg. of peroxidase per lock prior to inoculation and incubation.

The kojic acid–peroxidase reaction product, eluted from a chromatogram with water, exhibited absorption peaks at approximately 280 and 380 mμ, closely matching a similar preparation from the *A. flavus*–live cotton fiber extract. Both preparations, when exposed in solution for 10 minutes under the Blak-Ray lamp to long-wave ultraviolet light, showed a distinct tendency for the 380 peak and most of the BGY fluorescence to disappear, while the 280 peak remained relatively unaffected. The BGY fluorescing product resulting from the reaction of horseradish peroxidase with kojic acid exhibited a fluorescence excitation maximum at 435 mμ and an emission maximum at 494, whereas the fluorescing material from a cotton fiber–*A. flavus* incubation showed the very similar corresponding values of 442 and 500.

The chemical structure of the bright greenish yellow fluorescing substance produced by *A. flavus* in a cotton fiber remains unknown. A material which matches the naturally occurring substance in appearance under ultraviolet light, in R_f, and in absorption spectrum may be produced by treatment of kojic acid at room temperature with $KMnO_4$. Beelik (1956) noted that oxidation of kojic acid at the hydroxymethyl group to form comenic acid has been attempted by several workers, but without success.

Production of Kojic Acid and BGY Fluorescence by *A. flavus* Growing on Defined Media. The BGY fluorescence was produced directly in cultures of *A. flavus* on certain liquid media in 4 to 5 days by incorporating peroxidase as an additive. The basal medium used in these experiments contained salts as indicated under "Materials and Methods." When this medium was supplemented with 100 μg. per flask of peroxidase and 3% of glucose, sucrose, or glycerol, a bright greenish yellow fluorescence developed and chromatograms showed a spot matching that from a culture of the fungus on living cotton fiber. Once produced, the BGY fluorescence in these flasks did not disappear during several weeks of further incubation, suggesting that the fluorescing substance was not readily degraded by the fungus that produced it. *A. flavus* USDA 248, like isolate NRRL 2999, also developed the fluorescence when incubated on the mineral salts–glucose–peroxidase medium. With long incubation, *A. flavus* NRRL 2999 produced BGY fluorescence on the mineral salts–glucose medium even without added peroxidase, possibly a result of a slow nonenzymatic conversion of kojic acid to BGY. The fluorescent material in these cultures matched in R_f that in the peroxidase-containing cultures and was presumed to be the same substance.

Several other fungi which were tested failed to produce the BGY fluorescence on the mineral salts–glucose–peroxidase medium, failed to produce kojic acid on the same medium in the absence or presence of the peroxidase, and failed to produce the BGY fluorescence when incubated on the living fiber. These included USDA isolates *Aspergillus niger* 401, *Alternaria* 345, *Nigrospora* 350, *Chaetomium globosum* 1042.4, and *Myrothecium verrucaria* 1334.2.

Evidence suggests that for the BGY fluorescence to form in a higher plant under the influence of *A. flavus*, at least three events must occur: *A. flavus* must infect the plant tissue and grow in it to some degree; kojic acid must be formed; and the kojic acid must be transformed into one or more greenish yellow fluorescing compounds, probably in a peroxidase type of reaction. With many plant tissues, invasion by the fungus may not occur. Inoculations on many higher plant tissues without prior mechanical damage have not resulted in infection. Even if infection of some damaged cells occurs, the fungus may not grow further into the surrounding healthy tissue. With cotton in the field, the fungus appears to gain entry through the cracking suture of the opening boll and then to grow around and among the fiber cells without penetrating into their lumens. The failure of the mature, moist but nongerminating cotton seed to produce the BGY fluorescence when infected with *A. flavus* may be due to more than a lack of peroxidase, since such infected seeds also failed to give positive tests for kojic acid with either $FeCl_3$ or peroxidase and peroxide. Kojic acid production occurred in both autoclaved and nonautoclaved peanut stems incubated with *A. flavus*, as shown by strong positive responses in both the $FeCl_3$ and peroxidase-peroxide tests.

Previous experience with cotton bolls might have led to a prediction that the BGY fluorescence would form mostly or entirely in the fiber of a milkweed pod incubated with *A. flavus*, but in actuality an intense fluorescence occurred in the spongy inner wall of the pod and very little or none in the fiber. Subsequent examination of these tissues in the unincubated pod by the three enzyme activity tests of Table II, however, offered a reasonable explanation of the culture results. All three activities were very high in the spongy wall tissue and very low or absent in the fiber.

No claim is made that the BGY fluorescence serves as a diagnostic marker to locate *A. flavus* infections in plants except for the specific case of cotton fiber at harvest, even though later evidence might warrant such claims for specific plants under defined conditions. Cotton fiber at harvest has a relatively restricted fungal population (Marsh and Bollenbacher, 1949) that sometimes includes *A. flavus*, but rarely if ever any of the several other fungi reported capable of producing kojic acid (Prescott and Dunn, 1959). The BGY fluorescence was produced with all of a group of *A. flavus* isolates taken from cotton fiber in the field and incubated in pure culture on the live fiber (Marsh *et al.*, 1968), but not with any of the other boll-rotting fungi common in U. S.–grown cotton (Marsh *et al.*, 1955). Bacteria could conceivably produce kojic acid and a resulting BGY fluorescence in the fiber, but there is no evidence that this occurs. Of 119 commercial fiber samples exhibiting BGY-fluorescing spots, 111 had *A. flavus* clearly present in the fluorescing fibers (Marsh *et al.*, 1955). In the case of *A. flavus* boll rot in the field,

internal infection of the seed frequently, but not invariably, accompanies infection of the fiber. Shuck leaves of both field and sweet corn have been observed with naturally occurring spots exhibiting a fluorescence similar in hue to the BGY in cotton fiber, but apparently without associated *A. flavus* infection. No reaction with $FeCl_3$ occurred with extracts of the affected tissue and the fluorescent material was not extracted with water.

In many living plant tissues incubated with *A. flavus*, a blue fluorescence was produced in addition to the BGY, but always before the BGY. In some tissues it was inconspicuous (cotton fiber), in others evident (milk-stage corn seeds), and in still others highly conspicuous (peanut stems and seeds, lima bean seeds). In no case was the blue fluorescence seen in tissues sterilized by autoclaving prior to incubation with the fungus. Its relation to kojic acid or BGY, if any, is not known, nor indeed is it known that it is a single material. Lima beans incubated in living condition over water with *A. niger* exhibited a strong blue fluorescence, indicating that this fluorescence is clearly not specific for *A. flavus*.

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