Fluorescence Produced by *Aspergillus flavus* in Association with Other Fungi in Autoclaved Corn Kernels

Donald T. Wicklow and C. W. Hesseltine

Research microbiologist and laboratory chief, Northern Regional Research Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, IL 61604.
The authors wish to credit G. L. Adams for technical assistance in this investigation.
Presented at the annual meeting of The American Phytopathological Society held at Tucson, AZ, 29 October–2 November 1978.
The mention of firm names or trade products does not imply endorsement or recommendation by the U.S. Department of Agriculture over other firms or similar products not mentioned.
Accepted for publication 12 December 1978.

ABSTRACT

WICKLOW, D. T., and C. W. HESSELTINE. 1979. Fluorescence produced by *Aspergillus flavus* in association with other fungi in autoclaved corn kernels. Phytopathology 69: 589-591.

Observation of a characteristic bright greenish-yellow fluorescence (BGYF) under ultraviolet light (λ = 365 nm) that forms within nonliving cereal grains when *Aspergillus flavus* is paired with other fungi is reported for the first time. Inoculation of autoclaved corn kernels simultaneously with *A. flavus* (NRRL 6412) and certain individual fungal isolates from corn (ie, *Alternaria alternata, Cladosporium cladosporioides, Curvularia lunata, Fusarium moniliforme, Penicillium variabile,* and an unidentified yeast), followed by 8 days of incubation at 28 C, resulted in BGYF. Fluorescence was not detected in kernels inoculated separately with either *A. flavus* or any one of the six fungal isolates that initiated BGYF when co-inoculated with *A. flavus*. The relationship of the BGYF response to the sequence in which the corn grains were inoculated with members of an interacting pair and their relative status as interference competitors (antagonists) within the microfloral community are considered. A discussion of the significance of these observations relative to the aflatoxin problem in cereal agroecosystems is presented.

The highly carcinogenic properties of aflatoxins, which are produced only by members of the *Aspergillus flavus* group (8), has resulted in an expanding research effort to detect these mycotoxins in animal feed and human food. A bright greenish-yellow fluorescence (BGYF) under ultraviolet light (λ = 365 nm) Mineralight (UVSL–25) Ultra-Violet Products Inc., San Gabriel, Calif. has been associated with the presence of aflatoxin produced either by *Aspergillus flavus* Link or *Aspergillus parasiticus* Speare, or both, in cotton fiber, cotton seed, corn, and various kinds of cereal grains including rice, wheat, oats, barley, and sorghum (1,2,4,5,10). The fluorescence is produced by the oxidative action of heat-labile enzymes (peroxidases) in living plant tissue on kojic acid, which is formed with aflatoxin by *A. flavus* and *A. parasiticus* (6,7). However, the exact nature of the fluorescent material has yet to be determined. Failure of a number of investigators to detect BGYF in autoclaved plant materials (ie, cotton fiber, cotton seeds, cereal grains, etc.) inoculated with *A. flavus* or *A. parasiticus,* even though fungal growth was extensive and aflatoxins may have been produced, has reinforced the theory that the enzymes of higher plant tissue are essential to the formation of BGYF (2–4,6). That significant amounts of aflatoxin may be detected following incubation of *A. flavus* on many of these same substrates after autoclaving is evidence that the fluorescent material is not a direct product of metabolism of the mold during aflatoxin biosynthesis.

The BGYF was detected regularly in nongerminated (dead?) kernels colonized by both *A. flavus* and *Fusarium moniliforme* Sheldon (Wicklow, *unpublished*). Moreover, the incidence of BGYF in germinated kernels containing both *A. flavus* and *F. moniliforme* was far more common than in germinated kernels in which *A. flavus* was the only mold detected or was associated with individual fungal species other than *F. moniliforme.* It appeared that *F. moniliforme,* and perhaps other fungi occurring in corn kernels, might be an important source of the enzymes (peroxidases) thought to be necessary in the formation of BGYF; and that the fluorescence may be produced in the absence of metabolically active plant tissues. The following research was designed to examine the latter possibility.

MATERIALS AND METHODS

Aspergillus flavus (NRRL 6412) and 13 prevalent species of fungi isolated from aflatoxin-contaminated corn sampled at harvest in 1977 from a field in North Carolina were examined for ability to produce BGYF in autoclaved corn kernels harvested from a field near Peoria, IL. Neither aflatoxin nor kernels with BGYF were

detected in the corn from Illinois. Cell and conidial suspensions of each fungal isolate, made from 14-day-old slants of either potato dextrose agar or corn meal agar, were used as inoculum. Twelve undamaged corn kernels were placed on a double layer of Whatman No. 1 filter paper moistened with 3.0 ml of distilled H_2O in individual glass petri dishes and autoclaved for 30 min at 121 C. After being cooled to room temperature, the kernels were inoculated according to the schedule given in Table 1 by evenly distributing 0.5 ml of a given cell and conidial suspension over the kernels in each petri dish. In one series of dishes, *A. flavus* was simultaneously inoculated onto kernels with each of the 13 fungal colonists. A second series consisted of incubation (5 days) of kernels inoculated with *A. flavus,* followed by inoculation with each of the 13 other fungi. The kernels were incubated for 8 days. In a third series, each of the 13 fungal species was incubated for 5 days before being inoculated with *A. flavus*. A final series in which kernels were separately inoculated with either *A. flavus* or one of the 13 other fungal colonists served as the control. Kernels were incubated at 28 C and examined for BGYF 8 days following the date of their dual inoculation. Incidence of BGYF was determined by slicing individual kernels in half with a scalpel and examining them under ultraviolet light (λ = 365 nm) (Mineralight [UVSL-25], Ultra-Violet Products, Inc., San Gabriel, CA). Sporulation (percent coverage) of *A. flavus* over kernel surfaces was recorded as an assessment of the relative dominance of this fungus in combinations with other fungi.

RESULTS

A characteristic BGYF was formed when autoclaved corn kernels were inoculated with *A. flavus* and one of the following fungi: *Alternaria alternata* (Fr.) Keissler, *Curvularia lunata* (Wakker) Boedijn, *Fusarium moniliforme, Penicillium variabile* Sopp, *Cladosporium cladosporioides* (Fresen.) de Vries, and *Nigrospora oryzae* (Berk. & Br.) Petch. A trace of BYGF was detected when *A. flavus* was combined with other species: *Acremonium strictum* W. Gams, *Aspergillus niger* V. Tiegh., *Candida guilliermondii* (Castellani) Langeron et Guerra, *Penicillium funiculosum* Thom (fasiculate and typical forms), and *Trichoderma viride* Pers. ex S. F. Gray. No fluorescence was obtained when *A. flavus* was inoculated along with: *Penicillium oxalicum* Currie and Thom. For a complete summary of the data, see Table 1.

Fluorescence was detected only once in co-inoculated kernels in which sporulation of *A. flavus* over kernel surfaces was 100% relative to the *A. flavus* control. This might well have been anticipated since in *A. flavus* the ability to form large numbers of conidiogenous cells is partially related to the mycelial biomass. Successful substrate invasion by a co-inoculated fungal species would be expected to reduce the amount of substrate available to *A. flavus,* thereby affecting sporulation. Significantly, BGYF was detected in kernels preinoculated with *Alternaria alternata* but showing no evidence of sporulation by *A. flavus*. Even though mycelial colonization of substrate by *A. flavus* and biosynthesis of kojic acid presumably had occurred, an abundance of surface vegetative mycelium produced by *A. alternata* prevented sporulation. Because all inoculations were performed with spore or cell suspensions, it is not surprising that a characteristic BGYF rarely was detected when the corn kernels were inoculated with *A. flavus* 5 days prior to inoculation with other fungi. In such instances, conidial germination and hyphal invasion would have to take place in the presence of a well-established and moderately aggressive saprophyte. All kernels preinoculated with *A. flavus* were entirely covered with sporulating heads at densities equal to that of the control. This was interpreted to mean that, with 5 days preincubation at 28 C, *A. flavus* competitively excluded or substantially limited the growth of fungi able to produce peroxidases. Frequently trace amounts of BGYF were detected when a microfungal species ranked as more aggressive than *A. flavus* was added to the kernels.

When corn grains were inoculated with *A. flavus* 5 days after the other fungal species used in these experiments, a characteristic BGYF usually was detected if *A. flavus* was the more aggressive colonist of the pair. In simultaneously inoculated kernels, a typical BGYF was formed with rapid-growing species such as *A. alternata, C. lunata,* and *F. moniliforme,* all of which were able to colonize the substrate in the presence of *A. flavus,* which also is a rapidly spreading saprophyte. It is interesting that only a trace of fluorescence was detected with slower growing weakly antagonistic species such as *C. cladosporioides*. However, given sufficient lead time (5 days), the latter promoted a typical BGYF.

Petri dishes containing malt extract agar buffered at pH 6.0 were co-inoculated (paired) with *A. flavus* and each of the 13 fungal isolates. The BGYF also was detected within the agar when *A. flavus* was paired with the same fungal species that gave a characteristic BYGF in autoclaved corn kernels. It was not determined whether the fluorescent material had originated from either member of the opposing pair or was formed at the point where it was detected in the agar.

TABLE 1. Effect of inoculation sequence on the incidence of a bright greenish-yellow fluorescence (BGYF) in autoclaved corn kernels co-inoculated with *Aspergillus flavus* (NRRL 6412) and other micro-fungal isolates from corn[a,b,c,d]

	BGYF and *A. flavus* sporulation on corn kernels inoculated (I) with *A. flavus*			
Co-occurring species	Preinoculation (I − 5 days)	Simultaneous inoculation	Delayed (I + 5 days) inoculation	Control
Candida guilliermondii (NRRL Y-11,624)	Tr (100%)	Tr (100%)	Tr (10%)	...
Cladosporium cladosporioides (NRRL 6421)	... (100%)	Tr (100%)	BGYF (10%)	...
Curvularia lunata (NRRL 6409)	... (100%)	BGYF (50%)	BGYF (5%)	...
Alternaria alternata (NRRL 6410)	... (100%)	BGYF (50%)	BGYF (0%)	...
Aspergillus niger (NRRL 6411)	... (100%)	Tr (50%)	... (5%)	...
Aspergillus flavus Link (NRRL 6412)	NA[e]	NA	NA	... (100%)
Fusarium moniliforme (NRRL 6413)	BGYF (100%)	BGYF (80%)	... (0%)	...
Nigrospora oryzae (NRRL 6414)	Tr (100%)	Tr (100%)	BGYF (5%)	...
Acremonium strictum (NRRL 6415)	Tr (100%)	Tr (100%)	Tr (5%)	...
Penicillium oxalicum (NRRL 6416)	... (100%)	... (50%)	... (5%)	...
Penicillium funiculosum I (NRRL 6417)	Tr (100%)	... (50%)	... (0%)	...
Trichoderma viride (NRRL 6418)	Tr (100%)	... (5%)	... (0%)	...
Penicillium variabile (NRRL 6419)	Tr (100%)	BGYF (20%)	... (0%)	...
Penicillium funiculosum (NRRL 6420)	Tr (100%)	Tr (20%)	... (0%)	...

[a]Species listed in descending order according to their increasing importance as antagonists within the community (D. T. Wicklow, *unpublished*) according to a formula devised by Wicklow and Hirschfield (11).
[b]Tr = trace of BGYF detected.
[c]All BGYF observations based on 13 days incubation after initial inoculation.
[d]Numbers in parentheses represent % sporulation of *A. flavus* covering kernel surfaces relative to the control.
[e]NA = not applicable since *A. flavus* was not paired with itself.

DISCUSSION

Twenty-three years ago, Marsh et al (4) first reported that *A. flavus* caused a cotton boll rot that was characterized by BGYF in the fibers attacked by the fungus. The fluorescence was reported to form in living cotton fibers incubated with *A. flavus*, but autoclaved fibers incubated with the fungus showed heavy fungal growth but no BGYF. It appeared that some heat-labile factor in the living fiber was necessary for the formation of BYGF. With the identification of the aflatoxin problem in the early 1960's, Ashworth and McMeans (1) wanted to determine whether the fluorescence in cotton fiber might be related to the presence of aflatoxin in the cotton seeds; if so, the fluorescence could serve as a simple method for identifying seedlots containing aflatoxin and for removing seeds that contain the toxins. They detected 400–2,300 times more aflatoxin in seeds from fluorescent bolls as compared to those from nonfluorescent bolls. When investigators at the Northern Regional Research Center observed a characteristic BGYF associated with *A. flavus* and possibly aflatoxin in mature corn kernels, it became apparent that the black light (UV) test for BGYF could be used as simple presumptive test for aflatoxin in grain samples (10). The test for BGYF has since become an increasingly important technique for the detection of *A. flavus* infection and aflatoxin contamination in corn (9). According to O. L. Shotwell *(personal communication)*, kernels or grain particles weighing more than 0.01 g that show BGYF contain detectable aflatoxin.

The observation, for the first time, that a characteristic BGY fluorescence can form within nonliving, mature corn kernels when *A. flavus* coexists with certain other fungi has significance for the following reasons. Any field or storage-related microenvironment suitable for fungal biosynthesis of kojic acid and peroxidases in cereal grains can result in the formation of BGYF. This precludes any requirement for a stage of seed development and environmental regimes that support high levels of metabolic activity within kernel tissues. Until now, microbiologists have not been able to explain how BGYF in mature cereal grains can increase in batches of nongerminating seed. The frequency of cooccurrence of particular fungi (ie, *F. moniliforme*, etc.) with *A. flavus* in individual kernels may determine, in part, the incidence of BGYF within grain samples. The ecological status and competitive potential of *A. flavus* relative to other members of the fungal community associated with corn kernels may determine the degree to which *A. flavus* becomes established in individual kernels and whether BGYF is formed. Generally, fungal community members that did not interfere with the development of *A. flavus* in tests on buffered [pH 6.0] malt extract agar (Wicklow, *unpublished*) were those which produced a characteristic BGYF in combination with *A. flavus* on autoclaved corn kernels. In only one instance was a characteristic fluorescence produced in combination with an isolate *(N. oryzae)* found to be antagonistic to *A. flavus*. The sequence of kernel invasion involving *A. flavus* and a potential peroxidase-producing fungal partner can determine whether the pair become established in the same kernel and whether a characteristic BGYF is formed. As we have noted above, it has been well documented that formation of BGYF and high aflatoxin levels in individual seeds or kernels are strongly correlated. The present study provides indirect evidence that fungal colonization patterns control the ability of *A. flavus* to colonize cereal grains in the field and to produce significant amounts of aflatoxin and kojic acid.

LITERATURE CITED

1. ASHWORTH, L. J., and J. L. McMEANS. 1966. Association of Aspergillus flavus and aflatoxins with a greenish yellow fluorescence of cotton seed. Phytopathology 56:1104–1105.
2. BOTHAST, R. J., and C. W. HESSELTINE. 1975. Bright greenish-yellow fluorescence and aflatoxin in agricultural commodities. Appl. Microbiol. 30:337–338.
3. HESSELTINE, C. W., and O. L. SHOTWELL. 1973. New methods for rapid detection of aflatoxin. Pure Appl. Chem. 35:259–266.
4. MARSH, P. B., K. BOLLENBACHER, J. P. SAN ANTONIO, and G. V. MEROLA. 1955. Observations on certain fluorescent spots in raw cotton associated with the growth of microorganisms. Text. Res. J. 25:1007–1116.
5. MARSH, P. B., M. E. SIMPSON, R. J. FERRETTI, T. C. CAMPBELL, and J. DONOSO. 1969. Relation of aflatoxins in cotton seeds at harvest to fluorescence in the fiber. J. Agric. Food Chem. 17:462–467.
6. MARSH, P. B., M. E. SIMPSON, R. J. FERRETTI, G. V. MEROLA, J. DONOSO, G. O. CRAIG, M. W. TRUCKSESS, and P. S. WORK. 1969. Mechanism of formation of a fluorescence in cotton fiber associated with aflatoxin in the seeds at harvest. J. Agric. Food Chem. 17:468–472.
7. PARRISH, F. W., B. J. WILEY, E. G. SIMMONS, and L. LONG, Jr. 1966. Production of aflatoxins and kojic acid by species of Aspergillus and Penicillium. Appl. Microbiol. 14:139.
8. RAPER, K. B., and D. I. FENNELL. 1965. The Genus Aspergillus. Williams and Wilkins Co., Baltimore, MD. 686 pp.
9. SHOTWELL, O. L. 1977. Aflatoxin in corn. J. Am. Oil Chem. Soc. 54:216A–224A.
10. SHOTWELL, O. L., M. L. GOULDEN, and C. W. HESSELTINE. 1972. Aflatoxin contamination: Association with foreign material and characteristic fluorescence in damaged corn kernels. Cereal Chem. 49:458–465.
11. WICKLOW, D. T., and B. J. HIRSCHFIELD. 1979. Competitive hierarchy in post-fire ascomycetes. Mycologia 71:47–54.