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β-Carotene inhibition of aflatoxin biosynthesis among *Aspergillus flavus* genotypes from Illinois corn

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Thirty-nine *Aspergillus flavus* genotypes (DNA fingerprinting) isolated from corn grown in a field near Kilbourne, Illinois were evaluated for their sensitivity to β-carotene (50 μg/ml) inhibition of aflatoxin B_1 biosynthesis. Inhibition of aflatoxin was greater than 90% for 28 of the genotypes and >70% for 38 of the 39 genotypes. Five *A. flavus* strains (4 fingerprint groups) isolated from molded raw peanuts, NRRL 3239, NRRL 3357, NRRL 6514, NRRL 6515 and NRRL 13135, produced greater quantities of aflatoxin than all 39 genotypes isolated from corn, and were less sensitive to β-carotene inhibition. *Aspergillus flavus* NRRL 3357 is commonly used as inoculum in variety trials for aflatoxin resistance. Isolate identity and sensitivity to potential inhibitors in corn can be critical in assessing corn resistance to aflatoxin.

Key Words——aflatoxin; *Aspergillus flavus*; β-carotene; corn; genotype.

Carotenes and xanthophylls occurring in yellow corn (α-carotene, β-carotene, lutein, α-ionone, etc.) inhibited aflatoxin B_1 (AFB_1) production by *Aspergillus flavus* Link NRRL 3357 using the suspended disc culture method (Norton, 1997). When tested against 50 μg/ml β-carotene, NRRL 3357 showed 34% inhibition of AFB_1 production while four isolates of *A. flavus* from corn were more sensitive, having 82–96% inhibition. *Aspergillus flavus* NRRL 3357 is commonly used as inoculum in corn variety tests for resistance/susceptibility to *A. flavus* and aflatoxin (Payne, 1992; Zuber et al., 1987) but was originally isolated from molded peanuts (Hesseltine et al., 1970). Hesseltine and Shotwell initially recommended NRRL 3357 for aflatoxin studies because it proved reliable in producing quantities of aflatoxin in laboratory fermentations (Hesseltine et al., 1970; Vandegraft, 1975). ARS Culture Collection records show that NRRL 3357 was distributed to maize pathologists for use in aflatoxin resistance trials (e.g., O. H. Calvert, U. L. Diener, B. Fortnum, S. King, J. LaPrade, K. J. Leonard, E. B. Lillehoj, S. Marsh, G. Payne, J. Wallen, D. M. Wilson and N. Zummo, etc.). Because NRRL 3357 is less sensitive to aflatoxin inhibition by β-carotene and other corn metabolites (Norton, 1997), corn breeders and pathologists may have rejected relevant sources of resistant germplasm. Such rejections would have added meaning if the *A. flavus* population infecting pre-harvest corn is generally more sensitive to β-carotene and other corn metabolites than NRRL 3357. In this paper we evaluate β-carotene interference with aflatoxin production for a population of aflatoxin-producing *A. flavus* genotypes isolated from corn grown at the University of Illinois River Valley Sand Field, Kilbourne, Illinois. These results were contrasted with β-carotene inhibition of aflatoxin production in NRRL 3357 and four other *A. flavus* strains isolated from molded raw peanuts.

Materials and Methods

The *A. flavus* strains examined in this study were selected from a larger population isolated from corn sampled at harvest from an experimental planting (<1.5 acres) under continuous corn cultivation at the University of Illinois River Valley Sand Field (IRVSF), Kilbourne, Illinois, 1988–1991 (Wicklow et al., 1998). Individual single-spore isolates were first characterized for their ability to form sclerotia on Czapeks agar and produce aflatoxins in yeast extract soytone broth. The presence of aflatoxins was determined by thin-layer chromatography (Association of Official Analytical Chemists, 1984).

An evaluation was then made of the genotypic diversity (DNA fingerprinting) of the *A. flavus* population using a molecular hybridization probe pAF28 containing a chromosomal DNA insert from *A. flavus* NRRL 6541 that will hybridize to a homologous region of the *A. flavus* genome (McAlpin and Mannarelli, 1995). *Aspergillus flavus* genotypes (pAF28 fingerprints) were classified on the basis of the presence or absence of fragments, each of which is presumed to represent a single genetic locus. Isolates sharing more than 80% of the fragments in common were recognized as belonging to the same fingerprint group, while strains sharing 80% or fewer fragments were classified as separate fingerprint groups (Wicklow et al., 1998).

In the present study we examined 39 aflatoxin-producing strains of *A. flavus*, representing 35 different

fingerprint groups, for their sensitivity to β-carotene inhibition of aflatoxin biosynthesis. β-Carotene (Sigma) inhibition tests were performed using suspended disc cultures as previously described (Norton, 1995, 1997). Briefly, the culture system is composed of a 20 ml scintillation vial with an open type cap containing a thick Teflon-coated septum pierced by a pin on which a glass fiber disc is affixed. The disc contains the test solution and inoculum in medium and is humidified with 1 ml of sterile water in the bottom. Disks (10-mm in diam) were cut from Extra Thick Glass Fiber Filters (Gelman Sciences, Ann Arbor, MI) which had been washed successively with acetone, benzene, chloroform and methanol. β-Carotene was dissolved in benzene (50 μg/ml), filter sterilized, and 90 μl pipetted onto the discs. This is the approximate concentration required to inhibit AFB_1 forma-

Table 1. Effect of β-carotene (50 μg/ml) on aflatoxin production by *Aspergillus flavus* strains isolated from corn in a field near Kilbourne, Illinois.

RFLP Fingerprint group	Yr isolated	Fungal strain	AFB_1 (μg/ml)		%Inhibition
			Control	β-Carotene	
#02	1988	NRRL 27676	0.06	N/D[a)]	>70%
#60	1988	NRRL 27663	3.9	3.18	19%
#62	1988	NRRL 27671	4.5	0.50	89%
#49	1988	NRRL 27666	10.4	0.32	97%
#03	1988	NRRL 27674	27.5	0.43	98%
#07	1988	NRRL 27678	27.3	5.4	80%
#15	1989	NRRL 27831	0.15	N/D[a)]	>90%
#13	1989	NRRL 27832	0.12	0.02	83%
#12	1989	NRRL 27836	0.77	0.06	92%
#18	1989	NRRL 27834	0.98	0.01	99%
#38	1989	NRRL 27833	5.7	0.44	92%
#11	1989	NRRL 27825	5.0	0.42	97%
#19	1989	NRRL 27826	7.3	0.54	93%
#21	1989	NRRL 27837	46.0	2.2	95%
#25	1990	NRRL 26478	0.05	N/D[a)]	>70%
#63	1990	NRRL 26469	2.6	0.09	97%
#239	1990	NRRL 26480	2.6	0.15	94%
#64	1990	NRRL 26468	4.2	0.28	93%
#26	1990	NRRL 26474	4.9	0.39	92%
#32	1990	NRRL 26481	7.9	0.28	96%
#65	1990	NRRL 26473	14.6	1.8	88%
#27	1990	NRRL 26466	40.5	0.97	98%
#34	1990	NRRL 26467	42.5	1.09	97%
#42	1991	NRRL 26502	2.1	0.06	97%
#52	1991	NRRL 26483	3.3	0.21	94%
#233	1991	NRRL 26505	4.1	0.08	98%
#36	1991	NRRL 26496	6.7	0.13	98%
#46	1991	NRRL 26504	7.0	0.98	86%
#43	1991	NRRL 26510	8.7	0.17	98%
#37	1991	NRRL 26477	15.2	3.2	79%
#51	1991	NRRL 26482	18.6	0.37	98%
#36	1991	NRRL 26493	16.2	0.85	95%
#69	1991	NRRL 26501	20.4	0.39	98%
#36	1991	NRRL 26499	20.6	0.53	97%
#56	1991	NRRL 26509	22.3	4.1	82%
#47	1991	NRRL 26500	22.7	0.12	99%
#52	1991	NRRL 26507	37.9	4.38	88%
#36	1991	NRRL 26508	38.1	1.62	96%
#59	1991	NRRL 26503	45.9	1.9	96%

a) Not detected.

tion by 50% in *A. flavus* NRRL 3357 (Norton, 1997). Solvent was evaporated in a sterile desiccator under vacuum and medium with inoculum was applied at five points on the top and five points on the bottom of discs. Discs received 90 μl of SL medium salts (Reddy et al., 1971) containing 5% glucose, and included a suspension of *A. flavus* conidia (10,000 conidia/ml) representing a single strain. Tests for β-carotene interference with aflatoxin production consisted of one β-carotene treated disc and one control disc for each of the 39 *A. flavus* strains. Incubation was 5 d at 25°C. Visual assessment of sporulation served as an indicator of *A. flavus* growth. Norton (1997) has shown that growth of *A. flavus*, as measured indirectly by observations of sporulation abundance, estimates of extracted mycelium weight, and ergosterol quantification, was not appreciably affected by β-carotene.

Aflatoxins were extracted twice by adding 2 ml of $CHCl_3$ to the disc in the vial, vortexing for 15 s and allowing to set overnight. Extracts were combined, evaporated to dryness with a stream of N_2 at room temperature and the aflatoxins analyzed and quantitated by high performance liquid chromatography as described previously (Norton, 1995), except that analysis was isocratic using water: acetonitrile (69:31, v/v). Although some of the *A. flavus* strains produced small amounts of aflatoxin B_2 (AFB_2) in relation to AFB_1, these data would not affect the interpretation of the data and are not tabulated.

Results and Discussion

Each of the suspended disk cultures supported *A. flavus* growth (39 strains) as demonstrated by abundant sporulation for both β-carotene treated discs and controls. The effects of β-carotene at 50 μg/ml on AFB_1 production by these strains are shown in Table 1. Inhibition of aflatoxin biosynthesis greater than 90% was found for 28 of 39 *A. flavus* strains and greater than 70% for all but one (NRRL 27663) of the strains (Fig. 1). NRRL 27663 appears to be less sensitive to β-carotene, showing only 19% reduction in aflatoxin biosynthesis. Aflatoxin production for *A. flavus* controls ranged from 0.05 μg/ml to 46.0 μg/ml (Table 1), with individual strains being distributed as follows: 0.05–1.0 μg/ml=6 strains; 2.0–5.0 μg/ml=10 strains; 6.0–10 μg/ml=7 strains; 11–20 μg/ml=5 strains; 21–50 μg/ml=11 strains. On discs to which β-carotene was added, AFB_1 production ranged from 0.01 μg/ml to 5.4 μg/ml (Table 1), with the following strain distribution: 0.00–0.10 μg/ml=6 strains; 0.11–0.20 μg/ml=4 strains; 0.21–0.50 μg/ml=11 strains; 0.51–1.0 μg/ml=6 strains; 2.0–5.0 μg/ml=9 strains. In addition, inhibition by three strains could not be accurately quantified due to low aflatoxin values for the control cultures. Norton (1997) suggests that many corn varieties have high enough levels of carotenoids to affect aflatoxin formation in the endosperm but there is no clear evidence from the literature for or against a carotenoid effect on aflatoxin in corn.

The 39 aflatoxin-producing *A. flavus* strains (35 fingerprint groups) were selected from a population of 128 *A. flavus* strains (91 fingerprint groups) isolated from corn grown in an Illinois field (Wicklow et al., 1998). Forty-seven percent of 91 *A. flavus* fingerprint groups produced no aflatoxins (Wicklow et al., 1998) and the present results show that all but one (NRRL 27663) of the 39 aflatoxin-producing strains we tested were sensitive to β-carotene inhibition of aflatoxin biosynthesis. The colony appearance of NRRL 27663 on Czapeks agar is distinctly yellow green as in *A. flavus* but with conidial heads being consistently uniseriate as in *Aspergillus parasiticus* Speare. In addtion, NRRL 27663 produced AFB_1/B_2 but not aflatoxin G_1/G_2 as in *A. parasiticus*. While corn isolate NRRL 27663 showed little sensitivity to β-carotene inhibition of aflatoxin biosynthesis it ranked among the weaker aflatoxin producing strains (3.9 μg/ml).

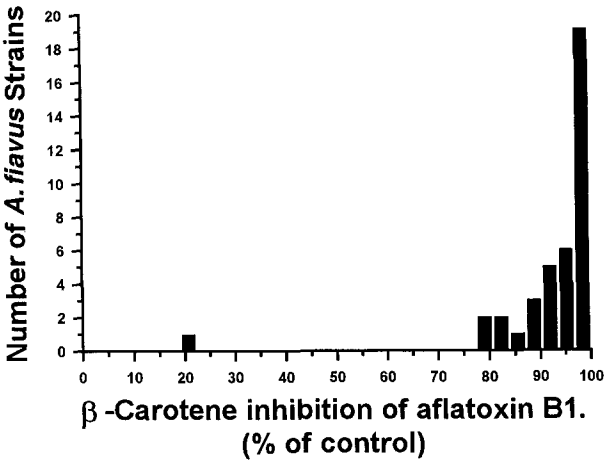

Fig. 1. Percent inhibition of AFB_1 biosynthesis by β-carotene (50 μg/ml) for 30 *Aspergillus flavus* strains isolated from corn in Illinois.

The present study has confirmed that typical field strains of *A. flavus* from corn at harvest in Illinois are significantly more affected by β-carotene than the peanut isolate NRRL 3357 (Norton, 1997). ARS Culture Collection records show that *A. flavus* NRRL 3357 (=NRRL A-12590) was received on October 4, 1963 as *A. flavus* No. M-52 from Dr. F. A. Hodges (Bureau of Biological and Physical Sciences, Department of Health and Welfare, Washington, D.C.). In his transmittal letter, Dr. Hodges notes that *A. flavus* isolate Nos. M-51 (=NRRL 6514), M-52 (=NRRL 3357), M-53 (=NRL 13135), M-54 (=NRRL 3239), and M-66 (NRRL 6515) were "obtained from mold growths on raw peanuts found on the surfaces of the cotyledon." Dr. Philip Mislevic (FDA) advised D. T. Wicklow (personal communication, October, 1981) that the M-52 isolate has an inventory number signifying domestic (U.S.A.) source of peanuts.

To determine if these five isolates from peanuts represent different genotypes, we obtained DNA fingerprints for each of these strains using the pAF28 molecular hybridization probe (McAlpin and Mannarelli, 1995). Additional shorter exposures enabled us to contrast band

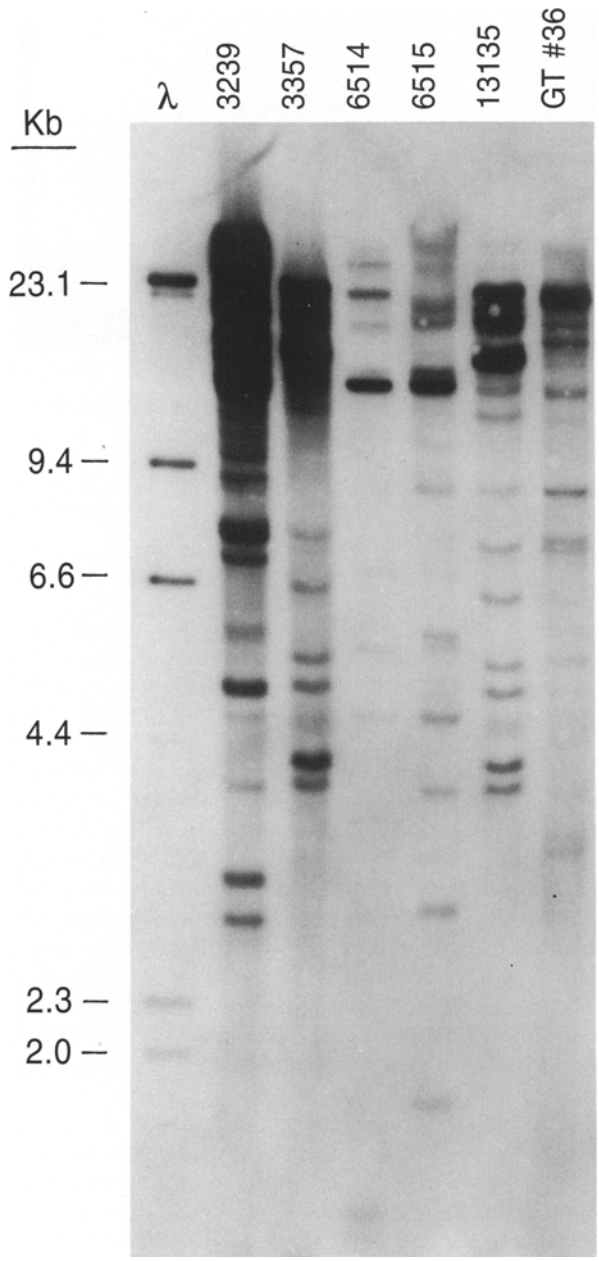

Fig. 2. DNA fingerprints of 5 *Aspergillus flavus* strains isolated from molded cotyledons of raw peanuts grown in U.S.A.

patterns in the 9 kb–21 kb range. Five distinct genotypes were determined, with two strains (NRRL 3357 and NRRL 13135) being classified in the same fingerprint group (Fig. 2). We wanted to learn if the other four *A. flavus* strains isolated from raw peanuts also show less susceptibility to β-carotene inhibition of aflatoxin. The effects of β-carotene inhibition of AFB_1 production by these strains are shown in Table 2. As a group, the *A. flavus* strains isolated from peanuts (Table 2) produced significantly more aflatoxin that was significantly less inhibited by β-carotene than the group of strains isolated from corn (Table 1). The average level of AFB_1 in controls of the former was 114.2 μg/ml and 14.1 μg/ml for the latter ($p<0.01$ by Kolmogorov-Sminov test); the 95% confidence intervals were 101–125 μg/ml and 9.44–18.7 μg/ml, respectively. For inhibition, the numbers were 36.3% inhibition and 91.2% ($p<0.01$ by t-Test); with 95% C.I. of 29–43.6% and 86.6–95.8%. These numbers include the outlier in the corn data.

Does the cultivation of peanuts contribute to the accumulation of *A. flavus* clonal population in field soil that (A) produce consistently elevated quantities of aflatoxins (Joffe, 1969; Schroeder and Boller, 1973) and (B) are less sensitive to natural sources of aflatoxin inhibitors (e.g., carotenes, xanthophylls, etc.)? In peanuts, unlike corn (Zsolt et al., 1963), carotenoids are highest in the youngest cotyledons and decline markedly as carotenoid formation is overtaken by triglyceride synthesis in the maturing kernel (Pattee et al., 1969). The principle carotenoid in peanuts is lutein (Pattee and Purcell, 1967) which was found to the most effective against aflatoxin formation (Norton, 1997). Therefore, it is possible that decreased sensitivity to carotenoids would enhance aflatoxin contamination of young peanuts. Peanut kernels, in addition, have a powerful set of fungal growth inhibitors in the form of stilbene phytoalexins, which appear to effectively protect kernels from fungal growth by *A. flavus* and *A. parasiticus* except under drought stress conditions (Dorner et al., 1991). *Aspergillus flavus* inoculum survives in field soild as sclerotia (Wicklow et al., 1993) and in peanut and corn crop residues (Griffin and Garren, 1976; Griffin et al., 1981; Zummo and Scott, 1990; Shearer et al., 1992) from which it becomes dispersed to corn as airborne conidia or by insects (Lussenhop and Wicklow, 1990; Olanya et al., 1997). In rota-

Table 2. Effect of β-carotene (50 μg/ml) on aflatoxin production by *Aspergillus flavus* strains isolated from molded raw peanuts.

FDA Code[a]	ARS Culture collection	AFB_1 (μg/ml)		% Inhibition[b]
		Control	β-Carotene	
M-51	NRRL 6514	81	59	27
M-52	NRRL 3357	135	55	59
M-53	NRRL 13135	105	86	18
M-54	NRRL 3239	96	57	41
M-66	NRRL 6515	154	98	36

a) Hesseltine et al. (1970).
b) 5 replicates.

tions with peanuts, corn would become infected with greater numbers of these more potent aflatoxin-producing *A. flavus* strains.

Fifteen yr ago King and Wallin (1983) obseved "Little attention has been given to inoculum, including the isolate of *A. flavus* used and inoculum concentration. Most researchers have used the NRRL 3357 isolate of *A. flavus* in an aqueous suspension ranging from 10^{-5} to 10^{-8} conidia/ml. Isolate identity and inoculum concentration are probably not areas of high priority now, but they should be addressed in the future." For their variety resistance trials conducted in Illinois, Campbell and White (1995) chose a mixed inoculum of four isolates including NRRL 6536, NRRL 6539, NRRL 6540 from corn at harvest in North Carolina (Wicklow et al., 1981) and an isolate from Illinois corn in 1988. The authors identified three aflatoxin resistant inbreds Tex6 (2, 9 ppb), C12 (14, 33 ppb) and OH516 (7, 23 ppb) among 37 inbreds tested with mean aflatoxin values of 322 ppb (1992) and 238 ppb (1993). Such contrasts are less apparent in variety trials using NRRL 3357 as inoculum, where resistant maize hybrids Mo18W × Mp313E and SC54 × Tx601 showed 625 and 972 ppb across seven locations while two susceptible hybrids had 1,757 and 2,040 ppb (Scott et al., 1991). The present study shows that *A. flavus* genotypes from corn harvested in Illinois are very sensitive to β-carotene inhibition of AFB_1. Isolate identity and sensitivity to potential inhibitors in corn can be critical in assessing corn resistance to aflatoxin.

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