Effect of Carotenoids on Aflatoxin B₁ Synthesis by *Aspergillus flavus*

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ABSTRACT

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Carotenes and xanthophylls occurring in yellow corn and related terpenoids were tested for their effect on growth and aflatoxin B₁ production by *Aspergillus flavus* NRRL 3357, using the suspended disc culture method. Aflatoxin synthesis was inhibited at concentrations of β -carotene, lutein, and zeaxanthin comparable to those found in the horny endosperm of mature corn. Usually growth was not significantly affected. Inhibition of aflatoxin biosynthesis was greater for compounds with an α -ionone-type ring (α -carotene, lutein, or α -ionone) compared with compounds with a β -ionone ring. The presence of hydroxy groups on the rings tended to decrease inhibition, but did not override the effect of the ring

type; lutein was similar to α -carotene and zeaxanthin was similar to β -carotene in inhibition. A mutant accumulating norsolorinic acid (NA), *A. parasiticus* SRRC 162, incubated with α -carotene produced reduced levels of both NA and aflatoxin, indicating that inhibition occurred before NA. Additional *A. flavus* strains tested against 50 μ g/ml of β -carotene had 89 to 96% inhibition, which was significantly more sensitive than NRRL 3357. *A. parasiticus* strains were less sensitive and generally had similar or lower inhibition than NRRL 3357. The results indicate that the presence of carotenoids in endosperm may decrease the amount of aflatoxin produced by *A. flavus*.

Additional keywords: aflatoxin inhibition, ionones.

Aspergillus flavus and *A. parasiticus* are preharvest pathogens of several important food crops including corn, cotton, peanuts, and several tree nut crops (15). Many strains of *A. flavus* and nearly all strains of *A. parasiticus* produce aflatoxin B₁ (19), a potent hepatotoxin (9) and carcinogen (36). There are stringent domestic and foreign regulations on the amount of aflatoxins allowed in food and feed grain that have significant economic results (28). As a result, there is growing interest in developing corn lines resistant to *A. flavus* infection or that inhibit aflatoxin production (11).

Kernel resistance to *A. flavus* can be due either to physical/structural factors (such as pericarp resistance to splitting) or to resistance that can arise from chemical effects on the fungus from metabolites in the various parts of the kernel. Chemical resistance can, in turn, result from inhibition of fungal growth or spore germination, with a corresponding reduction in aflatoxin, or from inhibition of aflatoxin synthesis, without an equivalent effect on growth. Although growth inhibition is the preferred form of resistance, a decrease in total aflatoxin B₁ at the same level of fungal growth would be beneficial for food safety and economical for the grower.

The constraints on resistance factors in edible plant parts are greater than for nonedible parts. Therefore, if naturally occurring components could be shown to control infection or toxin production at higher levels or with different tissue specificities than in current corn lines, then there would be less chance of encountering undesirable side effects than would be the case if a relatively unknown compound were to be introduced into the food. The studies reported here were undertaken with the objective of determining the effect on growth and aflatoxin B₁ (AFB₁) production by *A. flavus*

of one such group of compounds, the naturally occurring carotenoids of corn kernels and related compounds. Results for three interrelated aspects are presented: (i) the effect of carotenoids occurring in corn kernels and related compounds on growth and synthesis of AFB₁ by *A. flavus* NRRL 3357, (ii) the response of different *A. flavus* strains and *A. parasiticus* strains to β -carotene, and (iii) the effect on synthesis of norsolorinic acid (NA) and AFB₁ by a NA-accumulating mutant.

MATERIALS AND METHODS

Compounds. α - and β -carotene, α - and β -ionone, and α - and γ -tocopherol were obtained from Sigma Chemical Co., St. Louis. Lutein, zeaxanthin, β -cryptoxanthin, and canthaxanthin were a gift of Hoffmann-La Roche, Nutley, NJ. NA was a gift of S. McCormick. All compounds were used as supplied. Solutions were stored at -80°C when not in use.

Assays. All compounds were tested using suspended disc cultures as previously described (29). Briefly, the culture system was 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 was affixed. The disc contained the test solution and inoculum in medium and was humidified with 1 ml of sterile water in the bottom. Discs were cut from Extra Thick Glass Fiber Filters (Gelman Sciences Inc., Ann Arbor, MI) that had been washed successively with acetone, benzene, chloroform, and methanol. Carotenoids and tocopherols were dissolved in benzene, filter-sterilized, and pipetted onto the discs. 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 for carotenes were 6 mm in diameter and received 29 μ l of medium; those used for the α - and β -ionone were 1 cm in diameter and received 90 μ l of medium. Ionones were dissolved in ethanol at 100 $\times$ the final concentration and added to inoculum at a level to give a 1% ethanol concentration. Each experiment was the average of 10 replicates involving two separate incubations of five replicates at each concentration. Each incubation used conidia from different subcultures of fungus.

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Except as noted, *A. flavus* NRRL 3357, which produces AFB₁ and AFB₂ (42), was used for all experiments. Conidia were obtained from stock cultures as described previously (29). For disc cultures, stock solutions of conidia were diluted with sterile medium made with synthetic low salts (SL) medium salts (32) and 50 g of glucose made up to 1 liter with deionized water. The inoculum contained 10,000 conidia/ml. *A. parasiticus* SRRC 162, an aflatoxin pathway mutant that accumulates NA, was obtained from N. Keller, Texas A&M University, College Station. *A. flavus* strains not given NRRL identifiers were obtained from D. T. Wicklow, National Center for Agricultural Utilization Research (NCAUR), Peoria, IL. Strains with NRRL identifiers were obtained from USDA-ARS, NCAUR, Peoria, IL.

Growth determination for discs. After aflatoxin was extracted from discs with CHCl₃ (described below), 5 ml of benzene was added to the vials to extract any residual test compound. Benzene was removed, the discs dried in a fume hood, 5 ml of water was added, and the vials autoclaved for 15 min to extract any residual medium. The water was removed and the discs transferred to 24-well tissue culture plates (Corning Glass Works, Corning, NY), which had Teflon discs placed in the wells, and dried overnight at 95°C. The discs were weighed on an analytical balance (Mettler type M5, rated accuracy of ±0.002 mg; Mettler-Toledo, Inc., Hightstown, NJ), placed in 30-well ceramic spot plates (Fisher Scientific Co., Pittsburgh), and the organic material ashed in a muffle oven for 3 h at 650°C. Discs were reweighed after cooling overnight, and the difference between the two weights was corrected for the weight loss of similarly treated control discs inoculated with medium, but not spores. The difference was taken as the dry weight of extracted mycelium. Weights of control discs showed that no appreciable medium or test compound remained in the discs after extraction and autoclaving. Discs show a loss on combustion of about 2.5% even when nothing is added, due to combustion of binder in the discs.

Growth determination by ergosterol analysis. Cultures (10 replicates at each time) were harvested at 0, 24, and 48 h, and then at 6-h intervals until 96 h, and extracted and analyzed as discussed above, except that an aliquot of the chloroform extract used for aflatoxin analysis was analyzed for ergosterol by evaporating the chloroform and redissolving the residue in ethanol. Ergosterol was analyzed by high-performance liquid chromatography (HPLC) with Beckman System Gold equipment (Beckman Instruments, Inc., Fullerton, CA) and a 25 cm × 4.6-mm, 5-μm C₁₈ column (Microsorb-MV; Rainin Instrument Corp., Woburn, MA) under the following conditions: mobile phase was 100% methanol at 1.2 ml/min and detection was by UV diode-array detector (model 168; Beckman Instruments, Inc.) at 282 nm. Ergosterol (Sigma Chemical Co.) was recrystallized twice from ethanol and used for a standard curve at levels of 0.25, 1, 10, 100, and 1,000 μg/ml using a 50-μl injection loop. To determine the ratio of ergosterol extracted by chloroform and a standard ergosterol extraction, cultures were grown for 5 days on SL medium, and five replicates were each extracted using the following method adapted from Schwadorf and Muller (33): discs were extracted by refluxing 30 min at 80°C in a water bath in 2 ml of 40% ethanol in methanol in a test tube fitted with an air-cooled condenser. After cooling, 2 ml of water was added and the mixture extracted twice with 4 ml of hexane. The combined hexane extracts were dried with potassium sulfate, evaporated with nitrogen, made up to a suitable volume with ethanol, and analyzed by HPLC. Another set of cultures was saponified by refluxing in 2 ml of 40% ethanol in methanol containing 0.2 g/ml of KOH and extracted as before. A third set of discs was extracted as for aflatoxins. The calculated ergosterol per disc for Figure 1 was derived by multiplying the aflatoxin extract value by the ratio of the ergosterol found in unsaponified discs to that in the aflatoxin extracts.

Mycotoxin analysis. Aflatoxins were extracted twice by adding 2 ml of CHCl₃ to the disc in the vial, vortexing for 15 s, and allowing to set overnight. Extracts were combined, evaporated to dry-

ness with a stream of N₂ at room temperature, and the aflatoxins analyzed and quantitated by HPLC as described previously (29), except that analysis was isocratic using water/acetonitrile (69:31, vol/vol). Although the *A. flavus* strains produced small amounts of AFB₂ in addition to AFB₁, these data would not affect the interpretation of the data and were not tabulated. Because most of the compounds tested showed a linear relationship between inhibition and log concentration, the concentration required for 50% inhibition (I₅₀) was estimated by extrapolating between the log of the closest concentrations above and below I₅₀ using the linear regression function of a calculator. In the case of α-ionone, the I₅₀ value was extrapolated from the two concentrations below the 50% point.

NA was extracted twice by soaking discs overnight in 2 ml of acetone. The acetone was evaporated and the residue dissolved in 250 μl of acetone and then analyzed by HPLC according to the method of McCormick et al. (24), except that a 25 cm × 4.6-mm, 5-μm C₁₈ Microsorb-MV column (Rainin Instrument Co., Inc.) was used at a flow rate of 1.25 ml/min. NA from cultures eluted at 41.3 min, the same time as an authentic standard, and had an identical UV/Vis (visible) spectrum.

RESULTS

Effect of compounds on NRRL 3357. Fungal growth on suspended disc cultures had to be done indirectly, since it was not possible to get a direct gravimetric value. A simple and fast procedure, which was used for the work reported below, is to take the difference between the dry weight of the extracted cultures and the weight of the glass fiber disc remaining after the disc has been ashed in a muffle oven. An alternative method is to use ergosterol as a measure of growth (34). Preliminary extractions showed that saponified cultures gave 6.3% more ergosterol than unsaponified cultures, and the aflatoxin extract, which was used for ergosterol quantification, contained 25.9% of the ergosterol recovered from unsaponified cultures. Figure 1 shows the results for both methods of measuring growth for cultures grown over 4 days. In general, mycelium dry weight parallels ergosterol levels up to 72 h, when the values diverge. This divergence was due to the beginning of sporulation by the mycelium at this time and loss of the spores to the extracting solvent. Sporulation was normally complete by 90 to 96 h, and growth for 5 days gave a constant value. As shown, aflatoxin B₁ also plateaued at about 96 h. The spores could be recovered, weighed, and added to the mycelium value for a total weight. When this was done, growth over time reached a plateau at about 4 days, and spores accounted for 30 to 40% of the total weight (data not shown).

Structures of the compounds tested are shown in Figure 2 (all the C₄₀ carotenoids tested had the same conjugated backbone as β-

Fig. 1. Aflatoxin production and growth over 96 h of NRRL 3357; as measured by ergosterol and extracted mycelium weight. Vertical lines represent standard error.

carotene). The effects of the major carotenoids of corn, and related compounds, on growth and AFB₁ production by *A. flavus* NRRL 3357 are shown in Figure 3 and Table 1. α -Carotene and lycopene, the uncyclized biosynthetic precursor of β -carotene, are usually minor components of corn carotenoids. Canthaxanthin had not been reported from corn, but was included for structure/activity purposes. All the compounds tested showed inhibition over the range tested, and most produced a linear response when plotted against log concentration. The most inhibitory carotenoids were α -carotene and lutein (4,4'-dihydroxy- α -carotene), both of which have one β -ionone ring and one α -ionone ring (β,ϵ rings in carotenoid terminology). The amount required for I₅₀ by α -carotene was 3.3 μ g/ml. At the highest level tested, 1 mg/ml, AFB₁ was only 2% of the control. For lutein, the values were 0.62 μ g/ml and 4.3%, respectively (Table 1). Both of the corresponding β,β compounds, β -carotene and zeaxanthin, were less inhibitory at 1 mg/ml, and *A. flavus* produced about six times as much AFB₁ at 1 mg/ml as it did with the β,ϵ compounds (Table 1). The next most active carotenoid was β -cryptoxanthin (4-hydroxy- β -carotene), which had a I₅₀ value of 6.4 μ g/ml and at 1 mg/ml reduced AFB₁ accumulation to 11% of the control. The response curve for lycopene was steeper than for the other compounds, resulting in high inhibition at 1 mg/ml (93%), but requiring 130 μ g/ml to cause I₅₀. For canthaxanthin (3,3'-dioxo- β -carotene), the inhibitory effect plateaued at about I₅₀, and aflatoxin concentrations were not further reduced by the additions of 5.6 μ g/ml to 1 mg/ml of canthaxanthin. This type of response was also observed with abscisic acid (ABA) (Fig. 4B) and retinoic acid (data not shown). Growth was not appreciably

affected by any of the carotenoids or ionones except for zeaxanthin, which reduced growth of *A. flavus* by 30% at 0.032 μ g/ml, and canthaxanthin and lycopene, which stimulated growth about 40% at 1 mg/ml (Fig. 3).

The response of NRRL 3357 to α - and β -ionone is shown in Figure 4A. β -Ionone is a degradation product of β -carotene (37) and has been reported from corn husks (7) and tassels (8), but not corn kernels. The effect of β -ionone on aflatoxin production has been previously reported by Wilson et al. (43). α -Ionone has not been reported from corn, but provided a third comparison for the effect of the ring double-bond position. Less inhibition was shown by β -ionone than by α -ionone, with differences comparable to those shown by the related carotenes (β - and α -carotene) and the xanthophylls zeaxanthin and lutein (Fig. 3A and Table 1).

ABA is derived from violaxanthin or neoxanthin via xanthoxin (10) and has the same double-bond position as the ϵ -ring type. It is, however, considerably modified in being a carboxylic acid with a carbonyl group at the 4-position and an asymmetric hydroxy group at the 1-position. The effect of ABA is shown in Figure 4B. ABA had the least effect on AFB₁ level of any of the compounds tested, with only 53% inhibition at a concentration equimolar to the 1-mg/ml level of the C₄₀ carotenoids (Table 1).

The final group of compounds tested were tocopherols; they have some structural resemblance to carotenoids and similar antioxidant properties, but occur primarily in the germ of corn. The results for α - and γ -tocopherol, the major tocopherols of corn, are shown in Figure 5. The tocopherols had no effect on growth and stimulated AFB₁ production by about 20% at the highest level

Fig. 2. Compounds tested.

of 2 mg/ml. Concentrations of 162 and 378 $\mu\text{g/g}$ of dry weight for α - and γ -tocopherol, respectively, in the germ of a typical yellow dent corn have been reported, compared with 7.5 and 20.4 $\mu\text{g/g}$, respectively, for the pericarp fraction and 0.7 and 1.9 $\mu\text{g/g}$, respectively, of dry weight for endosperm (16).

Effect of β -carotene on other strains. The effect of β -carotene at 50 $\mu\text{g/ml}$ (the approximate I_{50} concentration) was tested on several other *A. flavus* and *A. parasiticus* strains to determine if NRRL 3357 was representative of *A. flavus* strains in its response. These results are shown in Table 2. Typical inhibition of aflatoxin biosynthesis for the *A. parasiticus* strains was about 30%. However, NRRL 6346 differed in that it showed no inhibition of AFB₁

biosynthesis, but AFG₁ synthesis was inhibited by 32%. Inhibition of aflatoxin biosynthesis greater than 90% was found for most of the *A. flavus* strains. NRRL 3357 was comparable to the *A. parasiticus* strains in sensitivity to β -carotene; this is an important consideration, since it implies that the results using this strain give an underestimate of the effect of the compounds tested compared with other *A. flavus* strains. Because corn kernels present a richer substrate that is more favorable to aflatoxin biosynthesis, inhibition is likely to be less in the kernel. Therefore, data obtained using NRRL 3357 is less likely to yield an overestimate of the effect of an inhibitor under field conditions.

Pathway site of inhibition. From the results obtained above, it was clear that a variety of carotenoids could strongly inhibit AFB₁ production in *A. flavus* and most of the *A. parasiticus* strains tested. The next question was at what point in the pathway was inhibition occurring. The value of the compounds as inhibitors would be greater if they inhibited early, rather than late, steps in this pathway, as late intermediates are toxic. No change in mycelium, spore color, morphology, or number was seen (16 $\times$ magnification) in even the most inhibited cultures, which, since most of the intermediates in aflatoxin biosynthesis are colored, suggested that inhibition was occurring at the beginning of the pathway. To determine the effect of inhibition by the carotenoids on NA, SRRC 162 was incubated with and without 1 mg/ml of α -carotene. SRRC 162 accumulates NA, which is the first isolatable metabolite in the aflatoxin biosynthetic pathway (39). If inhibition was before NA, then this should result in inhibition of both NA and AFB₁, but only inhibition of AFB₁ if the inhibited step is within the pathway. The immediate precursor to the inhibited step should accumulate as well. Two experiments were done: one set of incubations was given the normal 15 min/day of illumination and the other set was kept completely in darkness. At the end of the experiment, control discs were pink colored, whereas the α -carotene-treated discs were similar to their starting color. The results of HPLC analysis for the aflatoxins and NA content are shown in Table 3. As expected from previous experiments on the effect of light on *A. flavus* strains (R. Norton, unpublished data), toxin levels were somewhat higher for cultures given 15 min/day of light, but, overall, the same pattern of inhibition was found for both light and dark cultures, indicating that light is not involved in the mechanism of inhibition. Aflatoxins G₁ and G₂ (AFG₁ and AFG₂) had similar inhibition of about 95% except for AFG₂, which was undetectable in the dark culture with α -carotene. Aflatoxins B₁ and B₂ showed less inhibition—about 75% for AFB₁ and 60% for AFB₂. NA levels were markedly lower

Fig. 3. A, Effect of α -carotene, β -carotene, zeaxanthin, and lutein on growth and aflatoxin B₁ (AFB₁) synthesis by NRRL 3357. Concentration is a log scale except for the control point (0.0). **B,** Effect of lycopene, β -cryptoxanthin, and canthaxanthin on growth and AFB₁ synthesis. Vertical lines represent standard error. **A,** Controls for AFB₁ averaged 92.7 $\mu\text{g/ml}$ and ranged 66.6 to 132.6 $\mu\text{g/ml}$, for growth averaged 8.74 mg/ml and ranged 5.6 to 10.8 mg/ml. **B,** Controls were 91.3 and 73.0 $\mu\text{g/ml}$ for AFB₁ and 3.28 and 4.3 mg/ml for growth.

TABLE 1. Summary of inhibition by carotenoids and related compounds of aflatoxin B₁ (AFB₁) synthesis by NRRL 3357 and comparison with singlet oxygen-quenching values

Compound	MW ^a	I_{50}^b (μM)	I_{50}^b ($\mu\text{g/ml}$)	AFB ₁ ^c (% of control ^d)	K_q^e
α -Carotene	536.8	6.1	3.3	2.0	19
β -Carotene	536.8	119	64	14.5	14
Lutein	568.8	1.1	0.62	4.3	8
Zeaxanthin	568.8	56.2	32	25.1	10
α -Ionone	192.3	0.4	0.083	8.8	NA ^f
β -Ionone	192.3	37.4	7.2	27.4	NA
β -Cryptoxanthin	552.8	11.6	6.4	11	6
Lycopene	536.8	242	130	7.3	31
Canthaxanthin	564.8	425	240	39.4	21
Abscicic acid	264.3	2,870	760	53	NA
α -Tocopherol	430.7	NI ^g	NI	105.0	0.3

^a Molecular weight.

^b Concentration for 50% inhibition (I_{50}) of aflatoxin B₁ in μM and $\mu\text{g/ml}$.

^c AFB₁ production (% of control) following treatment with 1 mg/ml of carotenoids (358 and 490 $\mu\text{g/ml}$ for ionones and abscisic acid, respectively).

^d Average AFB₁ concentration for controls 92.7 $\mu\text{g/ml}$.

^e Singlet oxygen-quenching coefficient ($10^9 \text{ M}^{-1} \text{ s}^{-1}$) (12).

^f NA = data not available.

^g NI = not inhibited.

for the α -carotene group and inhibition was similar to AFG₁ and AFG₂. The results suggest that inhibition of aflatoxin biosynthesis is occurring before NA, either directly by affecting the polyketide synthase steps or indirectly by affecting general cell metabolism.

Possible mechanisms of inhibition. β -Carotene and α -tocopherol together have significantly greater antioxidative effect, quenching free radicals, than either of the compounds alone (30). It was possible that, although the tocopherols showed no inhibition by themselves, they might modify the effect of the carotenes by interfering with the mechanism of inhibition. To test this hypothesis, the effect of α -carotene and γ -tocopherol were determined separately and together. Aflatoxin B₁ production in the coinubation was comparable to that of α -carotene alone; γ -tocopherol added at 15.8 μ g/ml and α -carotene added at 31.6 μ g/ml gave values for AFB₁ of 90% of the control (106.5 μ g/ml) for γ -tocopherol alone, 23.5% for α -carotene alone, and 29.1% for both together. The value for γ -tocopherol was not significantly different from the control, and the two incubations with α -carotene were not significantly different from each other ($P < 0.05$). These results indicated that the mechanism of inhibition does not involve a free radical product of α -carotene. The ability of carotenoids to quench singlet oxy-

gen-mediated reactions suggested that this might be a feature of the compounds involved in inhibition. Bhatnagar et. al. (2) have suggested that a singlet oxygen reaction, catalyzed by an oxidase forming part of a multienzyme synthesis system, could be involved in the oxidation steps of aflatoxin biosynthesis. Singlet oxygen-quenching rates (K_q 10^9 M⁻¹ min⁻¹) for the C₄₀ compounds as reported by Di Mascio et al. (12) are listed in Table 1. Comparison of the K_q values with the I₅₀ and maximum inhibition values show no obvious correlation. The ionones and ABA should have negligible K_q values, since singlet oxygen-quenching is strongly dependent on the number of conjugated double bonds and more than seven bonds are required for effective quenching (20). Conversely, tocopherols are moderately effective singlet oxygen-quenchers, but showed no AFB₁ inhibition. Therefore, the antioxidative properties of the carotenoids do not appear to be directly involved in the mechanism of inhibition.

DISCUSSION

As shown in Figure 1, mycelium weight was more sensitive than ergosterol as an indicator of fungal growth, but this was partly a result of the efficiency of ergosterol extraction with chloroform, which only extracted 25.9% of that extracted by refluxing. Because the value for the binder lost while ashing discs must be estimated, this results in cultures with highly inhibited growth showing negative or falsely positive values due to deviations from this estimate, coupled with normal weighing errors. The result is that highly inhibited cultures have high coefficients of variation (CVs) and small negative or positive values for cultures with no growth, as seen in the variable and positive value for growth at 0 h in Figure 1. However, ergosterol levels after 48 to 54 h showed similar or greater variation than mycelium weight.

The results for the tested compounds show that carotenoids occurring in corn can markedly decrease aflatoxin levels and that those compounds containing the α -ionone ring are most effective. If carotenoid content and composition were to be considered in selecting corn lines for lower aflatoxin levels at harvest, then critical factors would be whether enough of the compounds occur in corn at the site of aflatoxin synthesis and at the appropriate stage of kernel maturity to be effective, and, if not, whether enough variability

Fig. 4. A, Effect of α -ionone and β -ionone on growth and aflatoxin B₁ (AFB₁) synthesis by NRRL 3357. Concentration is a log scale except for the control point (0.0). **B,** Effect of abscisic acid on growth and AFB₁ synthesis. Vertical lines represent 95% confidence interval. Control values for α - and β -ionone AFB₁ were 100.8 and 107.3 μ g/ml and for growth were 6.71 and 6.92 mg/ml. Average controls (one experiment) for abscisic acid AFB₁ was 88.0 μ g/ml and for growth was 8.0 mg/ml.

Fig. 5. Effect of α -tocopherol and γ -tocopherol on growth and aflatoxin B₁ synthesis by NRRL 3357. Concentration is a log scale except for the control point. Vertical lines represent 95% confidence interval. Controls for aflatoxin B₁ were 113.7 and 99.2 μ g/ml and for growth were 8.9 and 6.23 mg/ml.

exists for carotenoid composition and levels to allow selection of lines with higher inhibition potential than are now available.

Are there enough carotenoids in corn to affect aflatoxin synthesis? Carotenoid concentration is highest in the horny endosperm, which has approximately 5.4 times the level of germ (3). A survey of carotenoids in 125 corn inbreds found the level of total pigments ranged from 58 µg/g for Oh45 to 0.2 µg/g for K6, and the ratio of lutein to zeaxanthin ranged from 21.7 (for CI31A) to 0.37 (Kys); the inbred K770 contained 33.1 µg/g of lutein (31). However, a large percentage of the carotenoids in the highest inbreds consisted of phytoene and phytofluene, for which the inhibition potential is unknown. A survey of 10 inbred lines found the amounts of major carotenoids for whole grain varied from 0.09 to 72.0 µg/g (40). These values indicate that many corn lines have high enough levels of carotenoids to affect aflatoxin formation in the endosperm.

Is there evidence in the literature for a carotenoid effect? Studies of dry milled corn have shown that aflatoxin occurs predominantly in the germ and correlates positively with fat content in endosperm (4), and recent studies have confirmed that toxin production and fungal growth is significantly higher in the germ than in other kernel tissues (6,18). For this reason, the contribution of the endosperm to aflatoxin production by *A. flavus* will be less than its weight percent of the kernel, and aflatoxin levels for the whole grain would be higher than expected, on the basis of carotene levels. However, statistical comparison of the ratio of aflatoxin in endosperm and germ for a number of infected yellow and white lines should show an effect. Yellow lines as a group should have a lower ratio. This is illustrated by data from Brekke et al. (5), in which the ratio of aflatoxin levels for germ and grits of two white lines was 7.5; for a yellow line the ratio was 15. No examination of aflatoxin levels in related lines of white versus yellow corns, or surveys of lines grown under similar conditions, could be found in the literature. A few studies have reported toxin levels in several lines of white and yellow corns using the same analytical procedures. Some studies showed a possible effect (4), but others did not (22,35). There appears to be no clear evidence in the literature for or against a carotenoid effect on aflatoxin in corn.

Studies of other plants, however, suggest inhibition of aflatoxin by carotenoids. Capsanthin (3,3'-dihydroxy- α,γ -caroten-6'-one), which occurs in peppers, was tested for activity against *A. flavus* and found to limit the combined production of AFB₁ and AFG₁ to 58.8, 38.3, and 21.5% of control at 0.2, 0.6, and 1.0 mg/ml, respectively, for a 10-day incubation (23). However, growth was 77.3, 44.5, and 39.2%, respectively, of control, suggesting that the pentacyclic ring present in capsanthin is less inhibitory to AFB₁

production than ionone rings, but able to inhibit growth. Carrot root extract was found to inhibit aflatoxin formation by *A. parasiticus* NRRL 2999 over 99% at a level of 75 g of carrot extract to 2 ml of medium (1). This should represent over 1.25 mg/ml of carotenoids. There was no effect on growth from the extract, but it appeared to affect sporulation. The effect of β -ionone on AFB₁ production by *A. parasiticus* NRRL 2999 was reported by Wilson et al. (43), whose data yield an estimated I₅₀ of 79 µg/ml and 2.9% of control at 400 µg/ml (378 µg/ml, corrected for density). This compares with a I₅₀ of 7.2 µg/ml and 27% of control at 358 µg/ml for the current study. The limited amount of data available from related studies is, therefore, consistent with the results reported here.

Corn lines with higher α -ionone ring carotenoids should produce the most inhibition. The feasibility of developing corn lines with altered carotenoid ratios has been demonstrated in several studies (13,17,27). High xanthophyll corn lines were developed for use in chicken feeds, and lines with differing ratios of lutein to zeaxanthin were produced by selective crossing. In high lutein lines, the ratio was approximately 2:1, corresponding to 24 to 27 µg/g, with total carotenoid levels of 50 to 55 µg/g (13). Sweet corn has higher percentages of α -carotene and related compounds: the principal compounds are α -cryptoxanthin, β -cryptoxanthin, β -zeacarotene, γ -carotene, and 4 to 10% α -carotene (21). It appears, then, that selection of yellow dent corn with an endosperm carotenoid composition more favorable to aflatoxin inhibition is feasible.

An additional consideration is that the carotenoids need to be present at the time aflatoxin formation occurs. Little information, however, is available on carotenoid formation in corn as a function of ripening. One study found levels of total carotenoids at the waxy stage to be approximately one-tenth of the levels of mature corn (44), comparable to the levels found in sweet corn at the time of harvest (21). The site at which enhanced carotenoid synthesis would have the greatest effect is in the germ, but there is no information available on the level or composition of germ carotenoids from inheritance studies, therefore, its unclear if or how much levels could be raised by selection or how much existing lines vary in their levels of germ carotenoids. It appears that levels of carotenoids in the germ are at least an order of magnitude below levels that would be required to significantly inhibit aflatoxin. Whether selective breeding could increase this to significant levels is unclear.

The level of inhibitor in corn is only part of the problem of resistance; the range of fungal responses is equally important. Differences in inhibition of aflatoxin B₁ between *A. flavus* and *A. parasiticus* strains has previously been reported for chlobenthiazone (41). As shown above (Table 2), *A. flavus* NRRL 3357 showed inhibition by β -carotene similar to *A. parasiticus* NRRL 2999, but other *A. flavus* strains had greater aflatoxin inhibition, approximately 95% for two lines isolated from a corn field. NRRL 3357 appears to be less sensitive than other *A. flavus* strains and, therefore, typical field strains could be significantly more affected by the composition and levels of carotenoids found in corn, but confirmation of this would require examination of a broader range of strains isolated specifically from corn fields.

TABLE 2. Effect of β -carotene (β -C; 50 µg/ml) on aflatoxin production by strains of *Aspergillus flavus* and *A. parasiticus*

Strain	AFB ₁ ^a control	AFB ₁ + β -C % of control (CV ^b)	AFG ₁ ^c control	AFG ₁ + β -C % of control (CV)
<i>A. flavus</i>				
KS 7F 12/11	11.5	4.1 (33.5)	NP ^d	NP
KS 251 12/11	1.51	5.9 (88.2)	NP	NP
NRRL 6536	5.12	18.1 (27.1)	NP	NP
NRRL 6539	18.8	8.1 (20.1)	NP	NP
NRRL 3357	137	65.2 (7.2)	NP	NP
<i>A. parasiticus</i>				
NRRL 2999	51.7	69.2 (18.8)	37.4	87.2 (17.8)
NRRL 3145	56.9	70.9 (13.1)	82.4	63.7 (5.6)
NRRL 3240	142	70.5 (6.5)	208	62.2 (5.5)
NRRL 4123	70.0	83.1 (7.0)	110	83.8 (2.7)
NRRL 6346	66.5	120 (4.6)	189	67.8 (6.4)
NRRL 13004	27.6	63.4 (16.2)	58.7	64.5 (6.6)

^a Aflatoxin B₁ (µg/ml).

^b Coefficient of variation (SD/mean × 100).

^c Aflatoxin G₁ (µg/ml).

^d Not present.

TABLE 3. Effect of α -carotene (α -C; 1.0 mg/ml) and light or darkness on aflatoxins (AF) and norsolorinic acid (NA) production by *Aspergillus parasiticus* norsolorinic acid mutant SRRC 162

Treatment	NA ^a	AFB ₁	AFB ₂	AFG ₁	AFG ₂
Light	100	100 ^b	100 ^c	100 ^d	100 ^e
Light + α -C	8.3	22.3	35.1	3.5	7.9
Dark	76.3	92.3	74.5	95.7	62.8
Dark + α -C	4.0	29.3	43.8	29.3	<0.5

^a Numbers in table are % of control values for light cultures (15 min/day).

^b Aflatoxin B₁ control 9.22 µg/ml.

^c Aflatoxin B₂ control 0.09 µg/ml.

^d Aflatoxin G₁ control 15.5 µg/ml.

^e Aflatoxin G₂ control 0.13 µg/ml.

Comparison of the structures of all the compounds tested indicate that two facets of carotenoids are involved in inhibition: the conjugated tail and the double-bond arrangement of the ring. The length of the conjugated chain is not critical; the ionones are about as active as their corresponding carotenoids at the same molar concentration (Table 1), but the chain lengths are shorter by five atoms and two to three double-bonds for each ionone ring. The activity of lycopene shows that substantial inhibition occurs without a ring. Substituents on the ring are important, as shown by the relative effect of the carbonyl groups of canthaxanthin, the hydroxy groups of the xanthophylls, and the effect of ring substituents on ABA activity. One characteristic of the compounds that appears to be important and that differentiates the active compounds from the tocopherols is the presence of conjugated double bonds in the tail or central portion of the compound. Because of this feature, carotenoids can become incorporated into membranes and result in an increase in the transition temperature (decrease in fluidity) and a decrease in permeability in model membrane systems (25). The rings in the outer layers of the membrane (for the hydroxy and dioxo compounds) also appear to be involved in hydrogen-bond interactions with membrane phospholipid headgroups (26). A possibility suggested by the structure/activity data is that they may either modify cell membranes enough to indirectly affect the polyketide synthase, which is located in the cytosol (14), or they may specifically associate with hydrophobic domains of the synthase or aflatoxin pathway enzymes and, thereby, affect synthesis. Additional work is necessary to fully delineate the structural features producing inhibition and determine the mechanism of inhibition.

Given the effectiveness of carotenoids in inhibiting aflatoxin formation and their acceptability and desirability in food, the results of this study suggest that further investigations of the effect of these compounds in both endosperm and germ tissue on aflatoxin production is warranted. If carotenoids can produce inhibition in the very rich matrix of the germ, then evaluation of corn lines with increased levels of carotenes and xanthophylls in this tissue would certainly be called for. Recent reports (38) have suggested that β -carotene could have beneficial effects for human health beyond that of a vitamin A precursor. Therefore, increasing the level of this and other carotenoids in corn germ and the derived oil, might be desirable for reasons other than inhibition of aflatoxin biosynthesis.

In summary, we have shown that the major carotenes and xanthophylls occurring in corn endosperm significantly inhibit aflatoxin formation, without affecting growth, by most lines of *A. flavus* and *A. parasiticus* tested. The presence of an α -ionone ring is approximately 25 times more inhibitory than the β -ionone ring. Aflatoxin synthesis appears to be inhibited before the formation of NA. Further, with the exception of NRRL 3357, *A. flavus* strains appear to be significantly more sensitive to β -carotene than *A. parasiticus* strains.

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