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Possible Implications of Reciprocity between Ethylene and Aflatoxin Biogenesis in *Aspergillus flavus* and *Aspergillus parasiticus*

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***Aspergillus flavus* and *Aspergillus parasiticus* produced ethylene during early growth. However, the onset of toxin biosynthesis was marked by the absence of ethylene evolution. 2-Chloroethyl phosphonic acid, an ethylene-generating compound, inhibited aflatoxin biosynthesis in vivo. The reciprocal relationship between the production of aflatoxin and ethylene by the organism may indicate the involvement of the latter in the regulation of aflatoxin biogenesis.**

Aflatoxins are synthesized as secondary metabolites by strains of *Aspergillus flavus* and *Aspergillus parasiticus* (3). The events which trigger the biosynthesis of secondary metabolites in fungi are not yet understood (6). Therefore, there is a need to investigate the profound changes taking place in fungal physiology before and after the onset of aflatoxin biosynthesis. A number of fungi including certain aspergilli are also known to produce ethylene (4). The biological significance of fungal ethylene is obscure (1). The relationship between ethylene and aflatoxin biogeneses in aflatoxin-producing fungi was therefore studied. The use of 2-chloroethyl phosphonic acid (CEPA) as a convenient means of producing ethylene in vitro by a nonenzymatic reaction is known (11, 12). This system has been employed for assessing the role of ethylene in aflatoxin biogenesis by *A. parasiticus*.

MATERIALS AND METHODS

Organism and culture conditions. Aflatoxin-producing strains of *A. parasiticus* NRRL 3145, 2999, and 3000 and *A. flavus* NRRL 5565 and ATCC 15517 and nontoxigenic strains of *A. flavus* and *Aspergillus flavus-oryzae* were used in these studies. The recultivation of the stock cultures of the above fungi was carried out by streaking the slant material on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.) plates. Single colonies were picked up from the streaked plates and transferred to PDA slants. To maintain the cultures, regular transfers to fresh slants were carried out at 1-month intervals.

The spore suspensions of the above cultures were prepared by transferring slant material to Roux bottles containing PDA (pH 5.6, 200 ml), which were subsequently incubated for 10 days at ambient temperature (28 to 30°C). The harvesting of spores from Roux bottles and preparation of spore suspension were carried out as described earlier (8).

The synthetic growth medium (9) contained (per liter) 20.0 g of glucose, 6.0 g of $(NH_4)_2SO_4$, 5.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of glycine, 2.0 g of glutamic acid, 10.0 mg of $FeSO_4 \cdot 7H_2O$, 5.0 mg of $ZnSO_4 \cdot 7H_2O$, and 1.0 mg of $MnSO_4 \cdot H_2O$. The complete medium was prepared by mixing sterilized glucose solution with sterilized glucose-free salt solution. The pH of the medium was adjusted to 6.5. For inoculation, 1-ml aliquots of spore suspension were added to flasks containing 50 ml of medium in 100-ml conical flasks.

Measurement of growth. Mycelial dry weight was determined by filtering the contents of the flask through Whatman no. 541 filter paper, drying (85°C/6 h), weighing, and taking the average of at least three replicates.

Extraction and estimation of aflatoxin. The culture medium was separated from the mycelium, and the mycelium was ground separately with sand in a mortar and pestle. The ground mycelium was mixed with the culture filtrate, and the mixture was filtered through Whatman no. 541 paper. The resultant filtrate was treated with ferric gel (100 ml of water, 10 ml of 10% ferric chloride solution plus 15 ml of 4.8% sodium hydroxide solution) to remove interfering pigments. The mixture was filtered through filter paper (Whatman no. 1). The filtrate was extracted with chloroform (twice the volume of filtrate), and the extracts were evaporated on a water bath (80°C). The residue so obtained was preserved for the estimation of aflatoxin.

Total aflatoxin was estimated by using the microcolumn method of Velasco (10). The above residue was suitably diluted with chloroform to bring the reading within the 50-ppb range of the fluorotoxin meter. A portion (1 ml) of this sample was used for loading the microcolumns. The microcolumn was washed thrice with 1 ml of chloroform. The loaded microcolumn was inserted in the fluorotoxin meter (Velasco fluorotoxin meter; Neotec Instruments Inc., Silver Spring, Mo.), and a reading was taken. Another reading was taken by turning the microcolumn by 180°. The final aflatoxin reading was the average of the two.

Quantitation of individual aflatoxin was carried out by developing a portion of the residue from chloroform extract on thin-layer chromatographic plates. Thin-layer chromatography was carried out on precoated silica gel plates (TLP-102, Anasil; 250 μm; 5 by 10 cm; Analabs) (Foxboard Analytical, North Haven, Conn.). The plates were given a clean-up run in distilled peroxide-free diethyl ether. The R_f of aflatoxin in ether was zero, whereas most of the interfering pigments moved with the solvent. After the clean-up run, the plates were removed, dried, and developed in chloroform-acetone (9:1). Aflatoxins B_1, B_2, G_1, and G_2 were identified with reference standards of authentic aflatoxins (Maker Chemicals, Jerusalem, Israel) on viewing under UV light. The developed plates were scanned in a dual-wavelength thin layer chromatogram scanner (model CS910; Shimadzu Seisakusho Ltd., Kyoto, Japan). The instrument was equipped with a fluorescence detector.

Estimation of ethylene. For the estimation of ethylene in cultures, the following procedure was adopted. In the closed-

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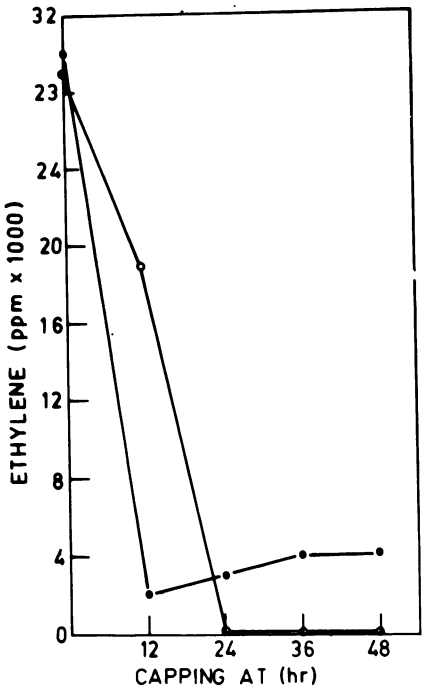

FIG. 1. Biogenesis of ethylene by toxigenic *A. parasiticus* NRRL 3145 (○) and nontoxigenic *A. flavus* (●) in an open system. The cultures were incubated for 72 h after capping. Details are given in the text. Headspace gases were withdrawn with the help of a syringe and injected into a gas chromatograph. Operating conditions were as follows: column, Porapak T (183 by 0.3 cm); carrier gas, nitrogen; flow rate, 30 ml min^{-1}; column temperature, 60°C; injection port temperature, 110°C; flame ionization detector temperature, 120°C.

system experiments, the flasks (100-ml capacity) containing synthetic glucose-salt medium (50 ml) were inoculated with the spores of the fungal strains and capped with sterilized silicone rubber septa. The flasks were subsequently incubated on a rotary shaker (150 rpm) at ambient temperature for 72 h. The flasks were withdrawn at regular intervals for estimation of ethylene in the headspace by withdrawing a measured amount of headspace gases with the help of a syringe. Headspace gases were analyzed for ethylene in a gas chromatograph (2). Standard ethylene was obtained from Matheson Gas Products, Rutherford, N.J. In open-system experiments, however, the flasks were inoculated and incubated with the cotton plugs on. The flasks were then capped with silicone rubber septa after definite periods of incubation, starting with 0 h. At the end of incubation for 72 h, headspace gases were withdrawn and analyzed as above. CEPA (Ethrel) was procured from Amchem Products Inc., Ambler, Pa. The solution was sterilized by passage through a Millipore filter.

RESULTS

Natural ethylene production by different *Aspergillus* strains. Figure 1 shows the biogenesis of ethylene by toxigenic *A. parasiticus* and nontoxigenic *A. flavus* during a 48-h incubation in an open-system experiment. In both strains, major ethylene evolution was confined to first 24 h of growth. The nontoxigenic strains, however, seemed to continue the biogenesis of ethylene beyond this period, although in low concentrations. In closed-system experiments, total ethylene production was lower in toxigenic strains as compared with nontoxigenic strains. Among the toxigenic strains, *A. flavus* ATCC 15517 produced the most ethylene, followed by *A. flavus* NRRL 5565, *A. parasiticus* NRRL 2999, and *A. parasiticus* NRRL 3145. However, a correlation between the yields of the toxin in toxigenic cultures and ethylene could not be obtained. Also, in general, ethylene biogenesis could not be related to spore number in the inoculum and growth in culture (Table 1). Among nontoxigenic cultures, *A. flavus* with a heavier inoculum and lower growth produced more ethylene as compared with *A. flavus-oryzae* with a low spore count in the inoculum and more growth. However, among toxigenic strains, *A. flavus* ATCC 15517, having the lowest spore count in the inoculum and maximum growth, produced maximum ethylene, whereas *A. parasiticus* NRRL 3145 with a heavier inoculum and the least growth showed the lowest yield of ethylene.

Effect of CEPA on *A. parasiticus*. The concentration-dependent effect of the ethylene-generating compound CEPA on growth and aflatoxin synthesis is shown in Fig. 2. The lower concentrations of CEPA seemed to stimulate growth of the organism, but the biogenesis of the toxin was drastically reduced. Higher concentrations of CEPA, however, inhibited both growth and toxin synthesis.

The yields of the four toxins, calculated from fluorodensitometric profiles, are shown in Table 2. A marked reduction in all four forms of the toxin was noted in the presence of CEPA. G_1 toxins seemed to disappear first. In shaken

TABLE 1. Ethylene biogenesis by nontoxigenic and toxigenic strains of *A. flavus* and *A. parasiticus*[a]

Culture	Inoculum (no. of spores)	Growth (mg [dry wt])	Total ethylene (ml)	Ethylene/spore (ml)	Ethylene/spore per g of mycelium (ml)
Nontoxigenic					
A. flavus oryzae	1.05×10^5	854	4.3	4.10×10^{-5}	4.80×10^{-5}
A. flavus	3.12×10^5	453	6.1	1.95×10^{-5}	4.30×10^{-5}
Toxigenic					
A. flavus ATCC 15517	1.08×10^5	1,036	3.5	3.24×10^{-5}	3.10×10^{-5}
A. flavus NRRL 5565	3.0×10^5	846	2.5	0.83×10^{-5}	0.99×10^{-5}
A. parasiticus NRRL 3145	2.9×10^5	583	2.1	0.73×10^{-5}	1.26×10^{-5}
A. parasiticus NRRL 2999	3.1×10^5	797	2.9	0.81×10^{-5}	1.01×10^{-5}

[a] Period of incubation, 72 h; temperature, ambient; shaken cultures in glucose-salt medium; shaker speed, 150 rpm. Spore counts were taken on PDA. Ethylene was measured by gas-liquid chromatography of the headspace of the flasks in a closed system as described in the text. Pure ethylene gas (0.1 ml) was injected into the chromatograph, and the peak height was noted; from the peak heights produced by ethylene in the headspace, the quantity of total ethylene was calculated, taking into account the volume of the flask.

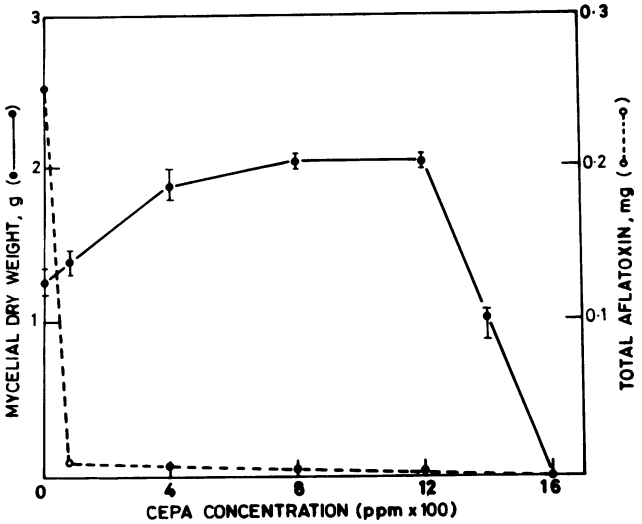

FIG. 2. Effect of CEPA on growth and toxin production of *A. parasiticus* NRRL 3145 in glucose-salt medium. Inoculum, ca. 10^5 spores; period of incubation, 7 days. Aflatoxin was determined by using the microcolumn method described in the text.

cultures, CEPA at 1,600 ppm (1.6 mg/ml) inhibited biogenesis of both B_1 and G_1, although B_2 and G_2 toxins were present in small amounts. No toxin could be detected in still cultures with 3,200 ppm (3.2 mg/ml) of CEPA in the medium and 2,400 ppm (2.4 mg/ml) in shaken cultures.

The growth and toxin yields in the presence of pure ethylene were poor, presumably because of anaerobiosis created by capping with silicone rubber septa. A general reduction in toxin levels, however, was observed in cultures with ethylene as compared with controls.

In another set of experiments, CEPA (3,200 ppm) was added to the cultures of *A. parasiticus* after different intervals of preincubation starting with 0 h. The flasks were then allowed to incubate for up to 72 h along with the controls, wherein CEPA was not added at any stage of incubation. It was observed that the cultures to which CEPA was added before 48 h of incubation did not show toxin formation at all. The toxin was, however, detected in those cultures where CEPA was added after 48 h of growth. The concentration of toxin in such cultures was much lower than that in the controls incubated for 72 h without CEPA. A comparison of the absorption spectra of CEPA-treated and untreated mycelia, which were ground with sand and extracted with chloroform, revealed a general reduction in the pigments absorbing in UV region (200 to 400 nm) in the presence of CEPA.

DISCUSSION

Evolution of substantial amounts of ethylene by aflatoxin-producing fungi in the early period of their growth has been observed. During this period, aflatoxin was not detectable in the cultures. The toxin synthesis started only when the fungus did not elaborate ethylene. We used CEPA to test this reciprocity between ethylene and aflatoxin biogenesis. The mechanism of release of ethylene from this compound has been described as a simple base-catalyzed reaction (12).

The experiments reported above indicated a profound correlation between the presence of ethylene and suppression of aflatoxin biogenesis in *A. parasiticus*. The stimulation or inhibition of growth depended essentially on the concentration of the plant growth hormone. It is worth noting that even at low levels of CEPA, aflatoxin formation was drastically reduced. The increased efficacy of CEPA in shaken cultures was presumably due to the improved contact between fungal cells and CEPA.

Addition of CEPA to the cultures at various points of incubation showed that the onset of aflatoxin synthesis (idiophase) started after around 48 h of incubation in still cultures at ambient temperature (28 to 30°C). The length of the trophophase and the onset of the idiophase could, however, vary with culture conditions such as inoculum size and aeration (8). CEPA could arrest the toxin synthesis even after the onset of the idiophase. Accumulation of some precursors during inhibited aflatoxin synthesis has been reported (5, 13). However, no known precursor of aflatoxin was found to accumulate in presence of CEPA as indicated during the preliminary experiments. This suggests that the action of the compound could be at an early stage of polyketide synthesis.

The study clearly brings out that ethylene has involvement in the regulation of secondary metabolite formation. The time course of ethylene and aflatoxin formation can be depicted as shown in Fig. 3. In the trophophase, when the secondary metabolite is not detectable in the medium, the fungus evolved ethylene. With the onset of idiophase, marked by the presence of detectable levels of secondary metabolite, biogenesis of ethylene was no longer detectable. It is possible that ethylene regulates the expression of the genes responsible for polyketide synthesis or inhibits enzyme(s) of the biosynthetic pathway of the toxin. There are several cases where expression of genes is regulated in response to presence or absence of certain molecules that exist in gaseous form. For example, ammonia and oxygen are known to prevent expression of nitrogen fixation genes (7). It is not clear whether such a situation arises in the present system.

Prevention of aflatoxin biogenesis by ethylene and ethylene-generating compounds could help in devising newer

TABLE 2. Aflatoxin formation by *A. parasiticus* NRRL 3145 in the presence of CEPA[a]

CEPA concn (ppm, 10^2)	Aflatoxin (μg/50 ml) in culture:									
	B_1		B_2		G_1		G_2		Total	
	Still	Shaken	Still	Shaken	Still	Shaken	Still	Shaken	Still	Shaken
0	205.2	75.6	129.6	172.8	324.0	18.0	80.0	32.0	738.8	298.4
8	108.0	32.4	168.0	86.4	115.2	7.2	56.0	8.0	447.2	134.0
16	13.4	ND	28.8	9.6	7.2	ND	8.0	4.0	57.4	13.6
24	2.7	ND	4.8	ND	ND	ND	1.2	ND	8.6	ND
32	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Inoculum, ca. 10^5 spores; period of incubation, 7 days at ambient temperature; shaker speed, 150 rpm. Aflatoxin detection was by fluorodensitometry with the following parameters: lamp, xenon; sample excitation wavelength, 365 nm; fluorescence emission wavelength, 450 nm; reference wavelength, 650 nm; recorder, Shimadzu U225 MCS; scan speed, 80 mm/min; chart speed, 40 nm/min.

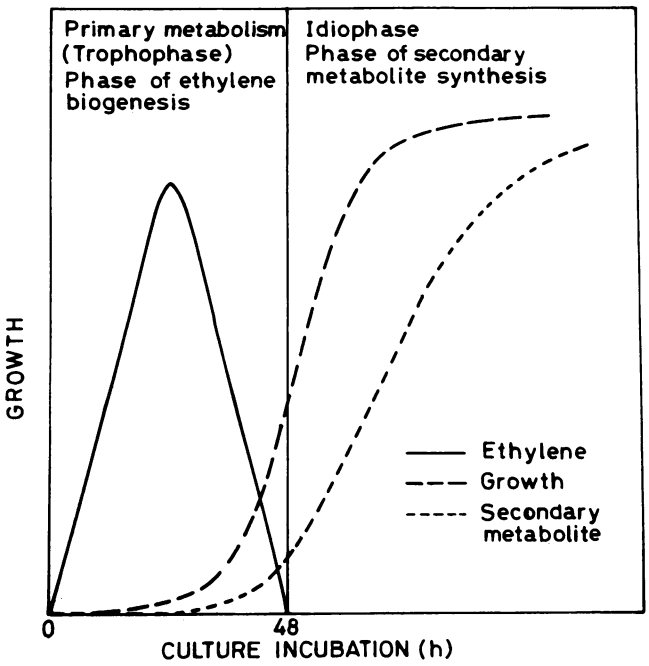

FIG. 3. Schematic relationship among ethylene biogenesis, growth, and elaboration of secondary metabolites based on the results (see the text).

methods of storage of agricultural commodities. Conditions are quite conducive to the growth of molds in humid and hot tropical climates. Large quantities of agricultural commodities face rejection because of aflatoxin contamination. The problem of aflatoxin has been a subject of increasing concern. The observations made above point to the utility of CEPA for this purpose. This compound is a known plant growth regulator and has been in use for controlled ripening of fruits (1).

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