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Occurrence of Free and Conjugated 12,13-Epoxytrichothecenes and Zearalenone in Banana Fruits Infected with *Fusarium moniliforme*

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Three recognized 12,13-epoxytrichothecene mycotoxins, trichothecolone, diacetoxyscirpenol, and T-2 toxin, and a hyperestrogenic factor, zearalenone, together with the fatty acid esters of trichothecolone, scirpenetriol, T-2 tetraol, and zearalenone, were isolated from the flask culture extractives of *Fusarium moniliforme* Sheldon (IMI 225232) as well as from the fruit of banana (*Musa sapientum* L.) infected with the same fungus in the field and in storage. The total concentrations of these toxins in the naturally infected fruits were quite high (0.8 to 1.0 mg/g of fruit). *F. moniliforme* infections of banana fruits, being of wide occurrence in the world, could cause serious health problems in humans when the infected fruits are ingested for a prolonged period of time.

Our surveys over the past few years in Varanasi, India, and in adjoining areas of Uttar Pradesh, India, revealed a widespread incidence of fusarial infections in various agricultural products (6–9; S. Ghosal and D. K. Chakrabarti, Abstr. Int. Symp. Medicinal Aromatic Plants 1984, p. 43–44). We report here the occurrence of three 12,13-epoxytrichothecenes and zearalenone, along with their palmitate ester conjugates, in *Musa sapientum* L. (banana, fruit variety) infected with *Fusarium moniliforme* Sheldon. *F. moniliforme*-induced rotting of banana fruits has been found as a recurring phenomenon in this area throughout the year for several years. The infected fruits are unwittingly termed "Chittidar Kela," which bears the connotation a delicate variety. The infected fruits with the pinkish mycelium of *F. moniliforme* present at the tips and with a few olive-brown discolored spots on the skin are in great demand by the local populace and fetch more profit to dealers. One reason could be that the infection imparts a better taste and intense sweetness to the fruits, whereas the unstained (uninfected) fruits are neither as tasty nor as sweet as Chittidar Kela fruits.

The incidence of *F. moniliforme* infections in banana (fruit variety) is not restricted only to India (2, 4, 13, 14); the disease has also been reported in other parts of the world, e.g., Uganda (12), Trinidad (15), and Israel (3). Sometimes the infected fruits do not show any external symptoms. The symptoms in such cases become evident only when the fruits are cut open (4, 14).

As several strains of *F. moniliforme* prevailing in India were found to secrete mycotoxins in food and feeds (5, 8, 11), it was thought worthwhile to investigate the toxins secreted by *F. moniliforme* on Chittidar Kela fruits. The pathogen *F. moniliforme* Sheldon (IMI 225232) was isolated from infected banana fruits collected from the local market at different seasons. The disease was at its peak from July to September (temperature, 24 to 35°C; relative humidity, 51 to 92%) every year. An axenic culture of the pathogen was maintained on artificially inoculated sterile banana fruits stored at 5°C under aseptic conditions. Fresh cultures were initiated from these infected banana fruits.

To study the capacity of the fungus to produce mycotoxins in vitro, we cultured the fungus in Richard medium at 21 ± 2°C for 21 days. The culture filtrate and the mycelium were extracted with chloroform, and the chloroform extracts were subjected to chemical analysis by a procedure described earlier (6).

For the in vivo study (Fig. 1), healthy fruits were inoculated with an axenic culture of the fungus. A few light bruises were made on the sterilized (with a 0.1% aqueous solution of mercuric chloride) skin with a sterilized needle, and a droplet of the fungal spore suspension was placed over the bruise. The inoculated fruits were incubated at 21 ± 2°C for 1 week in a humid chamber. Typical olive-brown spots appeared on the skin of the banana fruits, and the pinkish myceli of the fungus grew over the bruises. The skins were peeled off, and the pulp was macerated in chloroform. The chloroform extracts were subjected to chemical analysis. Likewise, naturally infected fruits (Chittidar Kela) collected from the local fruit market were extracted with chloroform, and the constituents in the chloroform extract were chemically examined.

The total chloroform extractives from the in vitro (intra- and extracellular extracts) and in vivo cultures were subjected to phytotoxicity tests on safflower (*Carthamus tinctorius* L.) seedlings (1) and dermal toxicity tests on rabbit skin (in lieu of rat skin) as described before (6, 8).

For the phytotoxicity tests on safflower seedlings, the chloroform extractives from the in vitro and in vivo cultures were dissolved in Hoagland solution in two concentrations (0.028 and 0.28 μg/ml). Each of these solutions (20 ml) was placed in a culture tube, which was then wrapped with black paper; a disease-free safflower seedling was introduced into the culture tube. Only the roots were kept immersed in the solution. Hoagland solution was used as the control.

For the dermal toxicity tests, the residues from the chloroform extracts from the in vitro (0.1 to 0.2 mg/ml) and in vivo (0.1 to 0.2 mg/ml) cultures were applied (8) to the shaved skin of rabbits by means of a wire loop, and the effects were scored as described before (6, 8).

In a typical experiment for the chemical evaluation of the toxins, the chloroform extract of the fungus grown in vitro was washed with water, dried, and evaporated in vacuo to provide a brown oily substance. The substance was dissolved in methanol-water (3:1, vol/vol), and the lipids were separated by repeated shaking with *n*-hexane (fraction A). The methanol layer (fraction B) was processed as described

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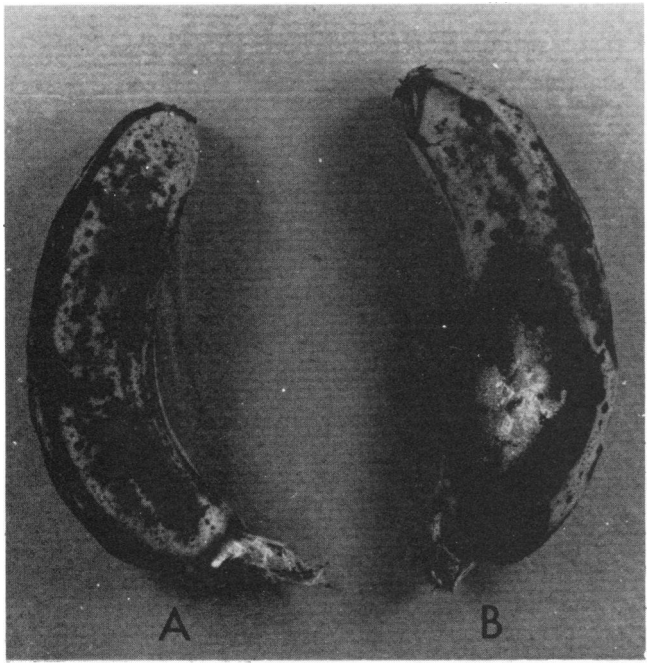

FIG. 1. Banana fruits infected with *F. moniliforme* Sheldon. (A) Naturally infected banana fruit collected from the local fruit market. (B) Banana fruit artificially infected with *F. moniliforme*; the fruit was incubated at 21 ± 2°C for 1 week in a humid chamber. Note the growth of the mycelia of the fungus on the infected fruits and the discolored spots on the skin.

before (6) to provide trichothecolone, diacetoxyscirpenol, T-2 toxin, and zearalenone (Table 1), along with some uncharacterized toxic materials as minor entities. Analytical high-pressure liquid chromatography (HPLC) model ALC 201 chromatograph; (Waters Associates, Inc.; M-6000 pump; U6K injector; C_{18} μBondapak analytical column [30 cm by 4 mm {inner diameter}]; CH_3OH-H_2O, 4:1) of fraction A revealed the presence of four major compounds. These were separated by preparative thin-layer chromatography (Silica Gel G [2-mm thickness]; $CHCl_3$-CH_3OH-CH_3COOH-H_2O, 90:8:1:1). Preparative thin-layer chromatography scrapings at R_f zones 0.1, 0.24, 0.3, and 0.45 yielded the four major compounds (C_1 to C_4). These compounds showed in their 90-MHz ^1H nuclear magnetic resonance spectra (in $CDCl_3$) features typical of fatty acid esters of 12,13-epoxytrichothecenes and a fatty acid ester (C_4) of zearalenone. The strong bands in the infrared spectrum in the ranges ν 1,720 to 1,730 and 1,240 to 1,228 cm^{-1} also suggested the presence of a fatty acid ester linkage. All of these compounds fragmented before exhibiting any molecular ion peak in their EI-MS, but the fragment ion peaks suggested the presence of palmitoyl (*m/z* 256, 239, 213, and 43) and stearoyl (*m/z* 284, 267, 241, 227, and 43) moieties along with those from the alcohols (6, 7, 10). The fragments arising from the stearoyl moieties were of minor abundance. In each case, CI-MS afforded identifiable quasi-molecular ion (MH^+) peaks, from which the molecular weights were obtained (C_1, 502; C_2, 520; C_3, 536 [also substantiated by FAB-MS on a glycerol matrix]; C_4, 556). Hydrolysis of the four different fractions separately with CH_3ONa-CH_3OH (11), followed by gas-liquid chromatography (3-m by 2-mm [inner-diameter] column of 10% diethylene glycol succinate on 100/120-mesh Gas-Chrom Q; operating temperature, 165°C; flow rate, 40 ml of N_2 min^{-1}) and EI-MS, established the presence of methyl palmitate and traces of methyl stearate from the acyl moiety. Analytical HPLC of the alcohols revealed the presence of four major compounds, which were separated by semipreparative HPLC and identified by the use of reference markers. The reference samples of scirpenetriol and T-2 tetraol were prepared, respectively, from diacetoxyscirpenol and T-2 toxin by careful hydrolysis with CH_3ONa-CH_3OH by a published procedure (11).

Compound C_1. The alcohol component from this compound was identified as triochotecolone. HPLC revealed the t_R (retention time in seconds) to be 170 s. Mass spectroscopy (MS) revealed *m/z* (relative percent intensity) 264 (M^+, 5), 247 (10), 246 (12), 233 (32), 165 (48), and 123 (100). The identity was confirmed by direct comparison (co-HPLC, MS, ^1H nuclear magnetic resonance spectroscopy) with a reference sample (10). Thus, compound C_1 was identified as palmitoyl trichothecolone. The point of ester linkage has not yet been settled.

Compound C_2. The alcohol component from this compound was identified as scirpenetriol. HPLC revealed the t_R to be 229 s. MS revealed *m/z* 282 (M^+, 2), 228 (48), 205 (23), 203 (12), 193 (52), 191 (7), 180 (18), 149 (22), and 93 (100). The identity was confirmed by direct comparison (co-HPLC, MS, ^1H nuclear magnetic resonance spectroscopy) with a reference sample prepared from diacetoxyscirpenol (8). Thus, compound C_2 was identified as palmitoyl scirpenetriol. The point of ester linkage is now being investigated.

Compound C_3. The alcohol component from this compound was identified as T-2 tetraol. HPLC revealed the t_R to be 270 s. MS revealed *m/z* 298 (M^+, 2.5), 280 (14), 250 (18), 249 (11), 221 (26), 203 (7), 191 (24), and 108 (100). The identity was confirmed by direct comparison (co-HPLC, MS, ^1H nuclear magnetic resonance spectroscopy) with a reference sample prepared from T-2 toxin (8). The point of ester linkage is now being investigated.

Compound C_4. The alcohol component from this compound was identified as zearalenone. HPLC revealed the t_R to be 318 s. λ_{max} (CH_3OH) nm (logϵ) 235 (4.47), 272 (4.14), and 314 (3.74). MS revealed *m/z* 318 (M^+, 55), 300 (12), 284

TABLE 1. Toxins produced by *F. moniliforme* on *M. sapientum*

Toxin	Amt of toxin produced in[a]:		
	Flask cultures (mg/200 ml)	Artificially infected fruits (μg/g)	Naturally infected fruits (μg/g)
Trichothecolone	22	27	11
Diacetoxyscirpenol	10	18	14
T-2 toxin	3	4	12
Zearalenone	14	26	17
C_1 (Palmitoyl trichothecolone)	122	250	204
C_2 (Palmitoyl scirpenetriol)	64	112	418
C_3 (Palmitoyl T-2 tetraol)	14	32	12
C_4 (Palmitoyl zearalenone)	78	118	76

[a] In vitro cultures were incubated at 21 ± 2°C for 21 days in a humid chamber; artificially infected fruits were incubated under similar conditions for 7 days; naturally infected fruits (with heavy mycelial infestation) were processed as such. The calibration curves for the four reference samples of toxins were obtained by plotting the peak height versus the concentration (1 to 6 ng; n = 6).

(5), 249 (6), 231 (4), 189 (62), and 188 (100). The identity was confirmed by direct comparison (co-HPLC, MS [the EI-MS were determined on an AEI-MS 902 spectrometer at an ionization potential of 70 eV; samples were introduced into the source via a direct insertion probe; the spectra were obtained at source temperatures ranging from 110 to 120°C; the CI-MS were obtained on the same instrument with a Chemospect CIS-2 chemical ionization-electron impact source with isobutane as the reagent gas]) with a reference sample (6). The point of ester linkage has not yet been settled.

The toxins from the chloroform extracts from infected (artificially and naturally) banana fruits were isolated as follows. Column chromatography on Sephadex LH-20 (11) of the chloroform extracts from artificially and naturally infected peeled fruits (ca. 500 g [wet weight] each) separated the chemical constituents into two distinct zones (fractions A and B). The compounds present in fraction A were organic in nature, whereas those present in fraction B were inorganic salts (of K^+, Ca^{2+}, Al^{3+}, and Fe^{3+}). Fraction A was partitioned between diethyl ether and water (2:1 [vol/vol]). In each case, the diethyl ether extract afforded a brown oily substance which was biologically active (phytotoxicity and dermal toxicity tests were performed as described above). The oily substance was processed as described for the in vitro culture extractives, and the amounts of the individual toxins were estimated by analytical HPLC with reference samples (Table 1).

The chloroform extracts from the in vitro and in vivo cultures produced typical scorching and necrotic symptoms of 12,13-epoxytrichothecenes on the leaf veins and petioles of safflower seedlings. In the dermal toxicity tests, the chloroform extracts produced severe edema accompanied by marked subdermal hemorrhaging in all the treated animals. The dermal toxicity of a mixture (1:1:1) of C_1, C_2, and C_3 was almost as potent as that of diacetoxyscirpenol (also tested in rabbits). Furthermore, the toxic effect in the former case persisted for a longer period of time (about 2 weeks), as revealed by the delay in the recovery of normal skin when the toxin was used at the lowest concentration.

The results clearly demonstrated that banana fruit infected with *F. moniliforme* contained several recognized 12,13-epoxytrichothecene mycotoxins, i.e., diacetoxyscirpenol, T-2 toxin, and trichothecolone, along with the hyperestrogenic factor zearalenone, in free and conjugated (fatty acid esters of trichothecolone, scirpenetriol, T-2 tetraol, and zearalenone) forms. The amounts of the 12,13-epoxytrichothecenes and zearalenone in fatty acid ester form were considerably higher than those in free form. The contribution of the lipid fraction extracted from an *F. moniliforme*-infested food sample to the risk of mycoses was demonstrated for the first time in this study. People in India and in other tropical countries consume sizable amounts of banana fruits as such and in the form of processed food. Prolonged ingestion of the moldy fruits (Chittidar Kela) thus presents a high toxin risk in humans.

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