Mycotoxin Production by *Alternaria* Species Grown on Apples, Tomatoes, and Blueberries

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Known toxigenic strains of *Alternaria alternata*, *Alternaria tenuissima*,and *Alternaria solani*, isolated from a variety of plant materials, were cultured on apple and tomato slices. Wild strains of *Alternaria* sp. were isolated from commercial blueberries and tomatoes and reinoculated on their respective lightly crushed (blueberries) or sliced (tomatoes) host fruit in the laboratory. All fruits were heated under mild conditions to eliminate indigenous microorganisms prior to inoculation with *Alternaria*. After incubation for 21 days at 21 °C, the mycotoxin content of the cultured fruits was determined by reverse-phase LC. Most of the known toxigenic strains and wild isolates of *Alternaria* produced tenuazonic acid (TeA), alternariol (AOH), and alternariol monomethyl ether (AME). Many cultures also produced altenuene (ALT) and altertoxin I (ATX-I). Ranges of concentrations (mg of mycotoxin produced per 100 g of fruit substrate) were as follows: TeA, 0–137.30; AOH, 0–56.90; AME, trace–20.90; ALT, 0–2.06. ATX-I was not determined quantitatively but was detected in about one-half of the cultured fruits. Some of the wild isolates produced greater amounts of mycotoxins than the known toxigenic strains of *Alternaria*.

Molds of the genus *Alternaria* are widely distributed in the environment. The genus is indigenous to soil, and many species are plant pathogens that damage crops in the field or cause postharvest decay. Because the *Alternaria* grow well at low temperatures, they are associated with extensive spoilage of fruits and vegetables held under refrigeration. Many vegetables become particularly susceptible to *Alternaria* rot as a result of chilling injury (McCollough, 1962). Some species of *Alternaria* are host specific, such as *Alternaria citrii* (citrus fruits) and *Alternaria solani* (solanaceous plants), while others grow well on a number of hosts.

The occurrence of *Alternaria* on a myriad of fruits and vegetables and the wide diversity of possible growing, handling, and storage procedures suggest that the *Alternaria* may pose a hazard comparable to that from more widely studied mold genera such as *Aspergillus*, *Penicillium*, and *Fusarium*. The toxicity to mammals of some *Alternaria* metabolites has been documented.

Toxic strains of *Alternaria* were found in overwintered grain that caused human fatalities from alimentary toxic aleukia (ATA) in the U.S.S.R. in 1944. Although the weight of evidence indicates that *Fusarium*-produced tricothecenes, e.g., T-2, were responsible for ATA, a synergistic toxicity was observed in test animals between toxic strains of *Alternaria tenuis* and the *Fusaria* (Joffe, 1964). Ingestion of moldy feed by poultry caused 40% mortality in some flocks (Forgacs et al., 1962); *Aspergillus flavus* was the main causative fungus, but in addition toxic strains of *Alternaria* were isolated from the feed. Isolates of *Alternaria* from tobacco that were grown in culture were found to produce substances toxic to mice when administered orally, and smoke from cigarettes prepared from timothy hay contaminated with this organism caused carcinomas and emphysema in mice, while smoke from untreated timothy hay produced only chronic inflammation (Forgacs and Carll, 1966). Later investigation of 96 strains of *Alternaria* isolated from tobacco disclosed that 33% were toxic to chicks, while an additional 13% caused severely retarded growth (Doupnik and Sobers, 1968). Ninety percent of the cultures of *Alternaria* isolated from a number of food crops and grown on corn/rice substrate were lethal when the cultures were fed to rats (Christensen et al., 1968).

The *Alternaria* metabolites with demonstrated mammalian toxicity belong to three classes of compounds: tenuazonic acid (TeA), a tetramic acid; dibenzo-α-pyrones including alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT); altertoxins I and II (ATX-I and -II), which are toxic substances of unknown structure. ATX-II is thought to be a dehydro form of ATX-I. Toxicity to mice from specific amounts of these compounds has been established (Pero et al., 1973). Onyalai, a common hematologic disorder among African Blacks, is caused by salts of TeA from mold contamination of grain (Steyn and Rabie, 1976).

TeA is produced in culture by numerous strains of *Alternaria* isolated from a variety of sources (Meronuck et al., 1972; Davis et al., 1977; Kinoshita et al., 1972; Burroughs et al., 1976). It is considered a major toxin of *Alternaria* cultures but has been found in only two *Alternaria*-infected field crops, rice plant leaves (Umetsu et al., 1973) and tobacco (Mikami et al., 1971). A steady, high moisture content of the susceptible green plant has been suggested as a requirement for TeA production by *Alternaria* (Sauer et al., 1978).

The dibenzo-α-pyrones are abundant, comprising up to 10% of the dry weight of the mold mycelia, and have been isolated from naturally infected grain sorghum (Sauer et al., 1978) and pecans (Schroeder and Cole, 1977).

ATX-I and ATX-II have been reported in laboratory cultures of *Alternaria mali* (Roberts) (Slifkin et al., 1973) and *Alternaria alternata* (Pero et al., 1973), as well as in heavily weathered grain sorghum (Sauer et al., 1978).

Despite the common association of *Alternaria* with the spoilage of fruits and vegetables, there are no reports of mycotoxins being produced by *Alternaria* grown on these materials. These commodities have a high moisture content, conducive to TeA formation, unlike other commodities investigated such as nuts, grain, and cotton. Contamination of fruits and vegetables by *Alternaria* could result from indigenous *Alternaria* present at harvest or from contamination during transportation, processing, or storage. Since many of the known mycotoxin-producing strains of *Alternaria* belong to species having no specific host requirement, we decided to investigate the myco-

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toxin-producing potential of some of these *Alternaria* grown on apples and tomatoes. We also investigated the mycotoxin-producing potential of wild *Alternaria* species grown on tomatoes and blueberries, the fruits from which they had been isolated.

EXPERIMENTAL SECTION

Samples of AOH and AME were obtained from D. J. Harvan (National Institutes of Health, Research Triangle Park, NC). ALT and ATX-I were obtained from L. M. Seitz (U.S. Grain Marketing Research Laboratory, Manhattan, KS). Additional quantities were prepared by fermentation with *Alternaria* strains obtained from the Northern Regional Research Center, Peoria, IL (5255; donated by C. E. Main as M-1), and the American Type Culture Collection, Rockville, MD (34457; donated by Rosemary Burroughs as RL-8442-2). The organisms were cultured in flasks by the method of Burroughs et al. (1976) with rice media containing 35% moisture for 21 days. The flask contents were then homogenized in a Waring Blendor with a minimum amount of water, acidified to pH 2.0, and extracted twice with equal volumes of $CHCl_3$. The extracts were combined, dried over Na_2SO_4, and concentrated by evaporation under N_2. AOH, AME, and ALT were separated and eluted from a silicic acid column with a benzene/tetrahydrofuran gradient (Pero et al., 1971) and purified by recrystallization. The identity of AOH, AME, and ALT was confirmed by the mixture melting point, R_f on TLC plates (two solvent systems), and mass spectrometry (Dusold et al., 1978). TeA was purified by extracting a portion of the concentrate with aqueous 5% $NaHCO_3$, acidifying the aqueous extract to pH 2.0, and extracting with $CHCl_3$. TeA was precipitated and stored as the cupric salt to avoid decomposition (Rosett et al., 1957).

TeA was conveniently regenerated by shaking the copper salt with $CHCl_3$ and 2 N HCl until all of the blue-green color was in the aqueous layer. The $CHCl_3$ phase was removed, the aqueous phase was extracted again with $CHCl_3$, and the $CHCl_3$ phases were combined. The $CHCl_3$ solution was washed with 2 N HCl followed by water. The identity of the TeA was confirmed by TLC [toluene/ethyl acetate/88% formic acid, 6:3:1 (v/v), which produced a tan spot at R_f 0.5–0.6 which turned red when treated with $FeCl_3$/EtOH] and by mass spectrometry. The concentration of TeA in the solution was determined by UV absorption and the molecular extinction coefficient of 13 490 at 287 nm (Davis et al., 1977).

Two sets of experiments were conducted. The first set consisted of fermentations in which known toxigenic strains of *Alternaria* were grown on apples and tomatoes. The cultures used were the following: *A. tenuis* strain "M-1" (Pero and Main, 1970; Pero et al., 1973), obtained from the Northern Regional Research Center as NRRC 5255 [*A. tenuis* is now considered to be a form of *A. alternata* (Lucas, 1971)]; *A. alternata* strains RL-8442-2, RL-8442-3, and RL-671-2 (Burroughs et al., 1976; Sauer et al., 1978), obtained from the American Type Culture Collection, Rockville, MD, as ATCC 34956, 34957, and 34958; *A. alternata* strains 582, 584, and 938 (Diener et al., 1976; Davis et al., 1977), obtained from Dr. Diener of Auburn University; *Alternaria tenuissima* strain 843 (Diener et al., 1976; Davis et al., 1977), obtained from Dr. Diener; and *A. solani* strain 2168 from the Northern Regional Research Center. During single spore isolation, *A. alternata* 584 displayed two forms which were designated 584R (rough) and 584S (smooth). The 584R was closest in appearance to the original strain.

The second set of experiments was carried out with wild *Alternaria* species isolated from blueberries and tomatoes. Standard procedures of phytopathology were followed to isolate and identify the organisms. The blueberries were obtained directly from growers in New Jersey, and the tomatoes were obtained from retail stores in the New York metropolitan area. These cultures were inoculated into and grown on 200 g of the commodity from which they had been isolated.

Fresh whole apples and tomatoes were sliced $^1/_4$ in. thick. Fresh blueberries were lightly crushed so that the skin was broken but the berry was otherwise intact. For the purpose of destroying indigenous microorganisms, about 200 g of prepared fruit was introduced into a 500-mL flask plugged with cotton and heated with steam for 1 h on 3 successive days to minimize possible destruction of factors needed for *Alternaria* growth. The flask contents were inoculated and incubated in the laboratory for 21 days at 21 °C (room temperature). There was no growth in uninoculated flasks. At the end of this period, the fruit was homogenized in a Waring Blendor. The pH of each homogenate was adjusted to 2.0, and the homogenate was extracted by blending for 1 min in a Waring Blendor with 500 mL of $CHCl_3$. The contents of the Blendor jars were filtered, and the filtrates from each flask were combined and extracted with a second portion of $CHCl_3$.

The $CHCl_3$ phases were combined, dried over Na_2SO_4, concentrated to 25 mL (or 50 mL if a precipitate developed) by evaporation under a N_2 stream, and stored at 5 °C until analyzed.

The solutions were analyzed for AOH, AME, ALT, and TeA by reverse-phase LC on a μBondapak C_{18} (organosilane bonded to silica) column. Elution with acetone/H_2O, 65:35 (v/v), at a flow rate of 0.4 mL/min separated AOH, AME, and ALT. The same column was used to quantitate TeA by eluting with methanol/H_2O, 9:1 (v/v), at a flow rate of 2.0 mL/min. Detector wavelengths were 324 and 278 nm, respectively (Heisler et al., 1980). The presence of ATX-I was detected by TLC with $CHCl_3$/acetone, 9:1 (v/v), as a developing solvent and authentic ATX-I used as a standard. ATX-I appeared on the TLC plate at R_f 0.19 as a yellow fluorescent spot when examined under 364-nm ultraviolet light.

RESULTS AND DISUSSION

The mycotoxin contents of fruits inoculated with known toxigenic strains of *Alternaria* and wild strains isolated from tomatoes and blueberries are given in Tables I and II.

All of the known mycotoxin producers gave abundant mycelial growth on both apples and tomatoes. No apparent differences in vigor were noted between the various cultured strains and the wild strains isolated from blueberries and tomatoes, despite the fact that the cultured strains had been isolated from quite dissimilar sources—cottonseed, grain sorghum, and tobacco. Both 584R and 584S produced similar amounts and types of mycotoxins.

TeA is usually considered the most important toxic material produced by *Alternaria* based on the positive correlation between in vitro production of TeA and the toxicity of cultures to laboratory animals (Meronuck et al., 1972). Another study of the relative toxicities of the *Alternaria* toxins indicated that TeA was somewhat more toxic than the other compounds (Pero et al., 1973). The importance of TeA produced under natural conditions has never been established, since the only reported natural occurrences of TeA have been in rice plant leaves and tobacco and trace amounts in tomato paste (Scott and Kanhere, 1979).

Production of TeA has been reported for most of the organisms in Table I (Davis et al., 1977; Burroughs et al.,

Table I. Toxin Production by Known Toxigenic Strains of *Alternaria* Grown on Apple and Tomato Tissue

	apple substrate					tomato substrate				
	toxins,[a] mg/100 g of substrate					toxins,[a] mg/100 g of substrate				
organism	TeA	AOH	AME	ALT	ATX-I	TeA	AOH	AME	ALT	ATX-I
A. tenuis M-1	0.05	7.40	18.30	0	+	1.50	0.07	0.42	0	+
A. alternata										
RL-8442-2	1.62	6.50	7.50	0.35	+	56.40	2.40	4.35	0	++
RL-8442-3	0.03	9.82	5.18	0.20	–	0	0.95	0.35	0	–
RL-671-2	0.06	8.40	18.05	0.95	–	0	0.33	0.49	tr	–
582	0.18	4.54	6.76	0	+	0	0.49	0.30	0	+
584S	0.92	3.56	10.00	0	tr	21.85	1.68	1.14	0	tr
584R	1.75	3.53	14.00	0	–	18.25	0.59	0.53	0	–
938	0.04	0.12	0.08	0	–	0	0	0	0	–
A. tenuissima 843	0.06	0.09	0.30	0	–	1.65	0.10	0.05	0	–
A. solani 2168	0.04	3.20	16.40	0	–	0	0	0	0	–

[a] TeA = tenuazonic acid, AOH = alternariol, AME = alternariol monomethyl ether, ALT = altenuene, and ATX-I = altertoxin I. tr = trace.

Table II. Toxin Production by Wild Strains of *Alternaria* on Blueberries and Tomatoes, the Substrates from Which They Had Been Isolated

	toxins,[b] mg/100 g of substrate				
organism[a]	TeA	AOH	AME	ALT	ATX-I
BB 1	20.25	tr	0.40	tr	+
2	0	tr	0.12	tr	–
3	14.40	0.13	5.65	0.28	+
4	8.00	1.75	5.05	tr	+
5	17.40	7.25	20.90	tr	++
6	tr	0.33	0.97	0.54	+
7	tr	0.23	0.63	2.06	–
8	tr	0.025	0.43	0	++
9	tr	tr	0.13	0	–
TOM 1	137.30	8.10	1.14	0.56	++
2	28.30	1.68	0.42	tr	+
3	0.38	0.08	0.10	0	+
4	0.38	0.08	0.25	0	–
5	24.30	1.05	1.30	1.30	++
6	78.50	0.98	tr	0	++
7	36.80	45.20	13.20	1.60	–
8	0	0	0.18	0.38	–
9	56.50	4.12	5.24	0	++
10	tr	24.00	15.80	tr	–
11	0	2.63	2.85	0	+
12	10.50	56.90	17.10	tr	–

[a] Wild strains of *Alternaria* designated BB were isolated from and grown on blueberries; strains designated TOM were isolated from and grown on tomato tissue. [b] TeA = tenuazonic acid, AOH = alternariol, MAE = alternariol monomethyl ether, ALT = altenuene, and ATX-I = altertoxin I. tr = trace.

1976; Sauer et al., 1978), but the substrates on which they had been cultured were varied. We found that when grown on apples and tomatoes most of these organisms still produce TeA. These strains of *Alternaria* generally produced much higher yields of TeA on tomato than on apple. Both of these substrates are low in N, suggesting that other factors are responsible for differences in TeA production.

Of the organisms isolated from cotton, *A. tenuissima* 843 was reported as the highest TeA producer when cultured on yeast extract/sucrose medium or cottonseed (Davis et al., 1977). This organism was only a moderate producer of TeA on tomato or apple. Wild *Alternaria* from tomatoes and blueberries produced large amounts of TeA (Table II) and the other toxins. Strain 1 from tomato produced the highest yield of TeA found in this study, 137 mg of TeA/100 g of substrate. The tomato fermentations yielded larger quantities of TeA than the blueberry fermentations. Production of the dibenzo-α-pyrones AOH, AME, and ALT on tomatoes and apples was also influenced by substrate. The known toxigenic strains of *Alternaria* produced more of the dibenzo-α-pyrone toxins on apples than on tomato, the reverse of the TeA production. The wild strains from tomato generally produced more of the dibenzo-α-pyrones than the wild strains from blueberries or any of the known toxigenic strains. In many instances, production of TeA was greater than the combined quantities of the dibenzo-α-pyrones. Data from the tables suggest that the probability for natural occurrence of ALT in fruits and vegetables is small.

Two of the known toxigenic *Alternaria* sp. examined, M-1 and RL-8442-2, have been reported to produce ATX-I. These strains retained their ability to produce this toxin when cultured on apples and tomatoes. ATX-I was also produced by strains 582 and 584, for which this metabolite was a new reporting.

The *Alternaria* spp. isolated from blueberries and tomatoes produced abundant amounts of ATX-I when cultured on their respective hosts; 6 of 9 *Alternaria* spp. from blueberry and 7 of 12 *Alternaria* spp. from tomato produced prominent fluorescent spots matching known ATX-I. ATX-I has been mentioned as an important element in the toxicity of *Alternaria* (Slifkin et al., 1973; Sauer et al., 1978).

Our findings indicate that fruits contaminated with *Alternaria* may develop copious amounts of mycotoxins. The high moisture content of fruit may favor the production of all three groups of mycotoxins known to be produced by *Alternaria*. Additional work is needed to establish whether these are formed in intact fruits and whether these materials can be found in commercial raw or processed products. This work is in progress.

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Uptake and Metabolism of Aflatoxin by *Zea mays*

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Ten–twelve-day-old maize seedlings were grown for 7 days in Hoagland's solution adulterated with aflatoxin B_1 (AFB_1) plus uniformly ring-labeled [^{14}C]AFB_1. Seedlings were transferred to aflatoxin-free Hoagland's solution or soil to determine the concentration of toxin absorbed and retained within the tissue. Two days following the transfer there was a 75 and 50% reduction in concentration of AFB_1 in the root and leaf–stem tissue, respectively. The reduction may reflect toxin degradation, particularly in the leaf–stem tissue. After 4 days the concentration of toxin increased slightly, suggesting that when the seedlings were transferred to Hoagland's solution there was a desorption of toxin from the root tissue, followed by a reabsorption. Where labeled seedlings were transferred to soil for a period of 13 days and analyzed for aflatoxin there was an 80 and 86% reduction in the concentration of AFB_1 in the root and leaf–stem tissue, respectively. AFB_1 injected into the internode below the ear bearing node in maize showed that it was translocated to developing ears by recovery 33 days later.

Following the discovery that aflatoxins were synthesized by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*, it was generally assumed that the principal aflatoxin hazard occurred as a consequence of improper field-curing or storage of agricultural commodities, such as corn, peanuts, and cottonseed. However, it is now recognized that *A. flavus* and *A. parasiticus* can infect preharvested standing crops (Lillehoj and Zuber, 1975; Widstrom, 1979). In 1977, the corn crop in southeastern United States was so severely contaminated with aflatoxin caused by *A. flavus* infection that many fields were destroyed by incorporating the stover and grains back into the soil (McMillian et al., 1978). Furthermore, aflatoxin contaminated commodities have been from time to time returned to agricultural soils (Dean, 1979). If aflatoxin is not rapidly degraded by the soil microflora, it is possible that substantial amounts of toxin may be adsorbed by the root systems of subsequent crops and translocated to the foliage and developing fruits. Accumulation of aflatoxin by plants could not only represent a health hazard to the consumer, but may also seriously affect the growth, development, and productivity of plants. This investigation was undertaken to determine if aflatoxin can be absorbed by the root system of plant seedlings, and whether aflatoxin introduced into the stems of corn plants can be translocated to developing fruits.

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MATERIALS AND METHODS

Growth Conditions. Seeds from the maize (*Zea mays*) single cross Mo17 × FRN 28 were soaked in aerated distilled water for 8–12 h. The seeds were rolled in sterile filter paper and germinated at 28 °C for 3–4 days. The seedlings were transferred to large glass tubes (4 × 20 cm) and grown in aerated Hoagland's solution (Hoagland and Arnon, 1950). Following a 7-day growth period uniform seedlings were selected and transferred to Hoagland's solution containing aflatoxin B_1 (AFB_1) plus uniformly ring-labeled [^{14}C]AFB_1 obtained from Moravek Biochemicals (City of Industry, CA). Aflatoxin was introduced into the growth media dissolved in 50 μL of methanol. Following the uptake period the root system was rinsed in tap water, and seedlings were transferred to unlabeled Hoagland's solution or potting soil (peatmoss, perlite, and loam, 1:1:1) and grown for various periods of time before being extracted for the toxin. Three seedlings were extracted for each treatment. Plants were grown in a growth chamber at a constant temperature of 23 °C and a photoperiod of 13 h of light. The light intensity was 200 W/m^2 at the plant level. The growth tubes were wrapped in foil to protect the roots and the aflatoxin solution from light.

In a second study, in order to determine if aflatoxin can be translocated to developing fruits, a corn hybrid with a large kernel was obtained from Cornnuts, Inc. The large kernel facilitated the dissection of the kernel into the

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