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Production of the Mycotoxin Fumonisin B_1 by *Alternaria alternata* f. sp. *lycopersici*†

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The mycotoxin fumonisin B_1, originally described as being produced by *Fusarium moniliforme*, was detected in liquid cultures of *Alternaria alternata* f. sp. *lycopersici*, a host-specific pathogen of tomato plants. The metabolite was detected by high-pressure liquid chromatography and mass spectrometry. Its identity was confirmed by fast atom bombardment and ion spray mass spectrometry, as well as parent-daughter tandem mass spectrometry. In three separate experiments, the concentrations found ranged between 5 and 140 ppm (μg/ml).

Alternaria alternata f. sp. *lycopersici* is a host-specific pathogen that causes a stem canker disease on tomato plants (5). It produces a host-specific toxin called AAL, which is associated with its pathogenicity. Fumonisin B_1 (FB_1) is a mycotoxin produced by *Fusarium moniliforme* (1) that causes equine leukoencephalomalacia, as described by Marasas et al. (6). It is also associated with cancer of the esophagus in humans (9, 12) when contaminated corn is consumed. It was reported that FB_1 causes pulmonary edema in swine (10) and cancer in rats (4). *F. moniliforme* (presumably because of FB_1) was reported as producing cardiotoxicosis in avian species (3). FB_1 is structurally and functionally similar to AAL toxin and produces cankers on tomato plants similar to those caused by AAL toxin (7). During studies on the biosynthesis of AAL toxin, we decided to look for the presence of FB_1, which is the subject of this report.

An isolate of *A. alternata* f. sp. *lycopersici* (AS27-3P_2) was obtained and identified by D. G. Gilchrist (5) and stored in sterile soil in a freezer. An inoculum for liquid culture was prepared by growing the fungus on potato dextrose agar in 15-cm-diameter petri plates at 20 to 25°C for 7 days. The surface of the potato dextrose agar medium was washed with sterile water, and the suspension of spores was diluted to a concentration of 10^5 spores per ml.

A fumonisin-producing culture of *F. moniliforme* (NRRL 13569) obtained from fusarium-infected corn was used as a comparison for FB_1 production.

MATERIALS AND METHODS

Culture and growth. The liquid culture medium used in this study was that described by Clouse et al. (2) and contained 8.0 mM L-asparagine, 5.0 mM L-maleic acid, 1.7 mM NaCl, 8.0 mM dipotassium hydrogen phosphate, 2.0 mM magnesium sulfate, 8.0 mM calcium chloride, 0.5% yeast extract, and 0.12 M glucose. The pH was adjusted to 6, and the medium was seeded with a spore suspension which gave a final concentration of 10^3 spores per ml. The cultures were grown in 2-liter flasks in stationary culture at 22 to 25°C for 110 days with alternating dark-daylight periods of 10 and 14 h, respectively. Ten-milliliter samples of the culture were harvested at certain time intervals and analyzed for both AAL and FB_1.

Sample extraction and purification. Sample extraction and purification were done by the method of Ross et al. (11), with certain modifications. A C_{18} Sep Pak cartridge (lot no. P2079A1; Waters) was washed with 2 ml of acetonitrile and then 2 ml of water before loading of the sample. Ten milliliters of the mycelium-free culture filtrate was adjusted to pH 4 and then loaded directly onto the column. This was followed by rinsing with 6 ml of water and 2 ml of acetonitrile-water (15:85). The components of the sample were eluted with 2 ml of acetonitrile-water (70:30) and dried under nitrogen. The eluted samples were analyzed for FB_1, AAL, and other metabolites. Here we present only FB_1 analysis results.

Analytical detection and confirmation. The *o*-phthalaldehyde derivative (11) of FB_1 was analyzed by high-performance liquid chromatography (HPLC) by using a gradient program at a flow rate of 1 ml/min. Mobile phases A and B, respectively, were composed of acetonitrile-water-acetic acid (39:60:1 and 60:39:1, respectively). The prepared sample was reacted with *o*-phthalaldehyde in 0.1 M sodium borate buffer at pH 8.5 for 10 min and then diluted with 0.01 M boric acid-acetonitrile (3:2). Twenty microliters of the reaction mixture was injected into a Shimadzu HPLC apparatus through a C_{18} reverse-phase column (25 cm by 3.9 mm [inside diameter]; Waters μBondapak) and detected with a Shimadzu RF-530 fluorescence monitor at an emission wavelength of 440 nm after excitation at 335 nm. Quantitation was done by using peak height or area.

Detection and identification of FB_1 was done by continuous-flow secondary ion mass spectrometry (CFSIMS) by using a microcolumn of C_{18} (3-μm particle size [Spherisorb]; 320 μm [inside diameter] by 20 cm) and a solvent system of acetonitrile–water–0.1% trifluoroacetic acid (gradient of acetonitrile from 20 to 80%) and a flow rate of 3 μl/min. CFSIMS was done on a VG70SEQ tandem hybrid mass spectrometer at a resolution of 1,000. Analysis by fast atom bombardment by direct probe insertion was done on a VG7070EQ apparatus with glycerol. Analyses were also done by ion spray mass spectrometry (ISMS) on a Sciex Taga 6000 quadrupole mass spectrometer after resolution on a C_{18} reverse-phase column (25 cm by 3.9 mm [inside diameter]; Waters μBondapak) by using a solvent system of

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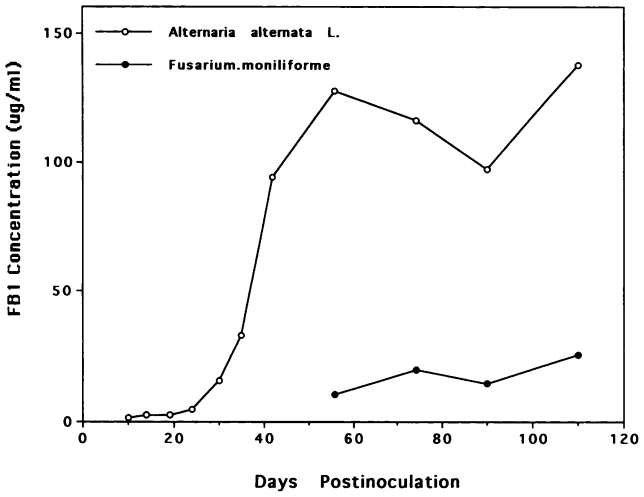

FIG. 1. Kinetic study of FB_1 production by *A. alternata* f. sp. *lycopersici* and a fumonisin-producing culture of *F. moniliforme* in liquid culture medium. Significant production of FB_1 by *A. alternata* occurred at 10 days, with ~8 ppm, and ranged up to 140 ppm at 110 days. *F. moniliforme* did not produce an appreciable amount of FB_1 under these conditions. Each value is an average of three determinations.

acetonitrile-water-acetic acid (55:45:1), a flow rate of 1 ml/min, and a split ratio of 1 to 9. ISMS was done in both the full-scan and the parent-daughter modes by using *m/z* 722 as the parent ion. The injection volume was 20 μl.

Additional confirmation of identity was done by hydrolyzing the sample with 2 M KOH for 1 h to obtain the corresponding pentolamine. The hydrolysate was cleaned on an Amberlite XAD-2 column as described by Plattner et al. (8). Trifluoroacetic acid (TFA) anhydride reagent was used to make the TFA ester derivatives of the pentolamine. The latter was analyzed by gas chromatography on a DB-5 bonded-phase capillary column (15 m by 250 μm [inside diameter]) from J&W Scientific, Folsom, Calif., interfaced to a VG7070EQ mass spectrometer in the electron impact and negative chemical ionization (methane) modes.

RESULTS AND DISCUSSION

Fumonisin was detected first by ISMS in 7-day-old stationary liquid cultures of *A. alternata* f. sp. *lycopersici* (Fig. 1). The concentration remained low (5 to 10 ppm) until 24 days and then increased rapidly to a maximum of 140 ppm (μg/ml). A fumonisin-producing strain of *F. moniliforme* was also cultured on a liquid medium, but its yield of fumonisin under conditions identical to those used for *A. alternata* was low.

FB_1 was detected first in culture by HPLC after 10 days of growth on a liquid medium (Fig. 1). Its identity was determined by both HPLC (*o*-phthalaldehyde derivative) and ISMS.

Confirmation of the identity of FB_1 was done (Fig. 2A) by ISMS analysis using parent-daughter analyses of extracts obtained from the liquid culture medium after 24 days of growth. The parent ion chosen was *m/z* 722 (protonated molecular ion), and after collision-activated decomposition at 26 eV in argon, daughter ions identical to those found in authentic FB_1 were found. The fragment ion at *m/z* 704 represents loss of water; *m/z* 546 is loss of the tricarballylic

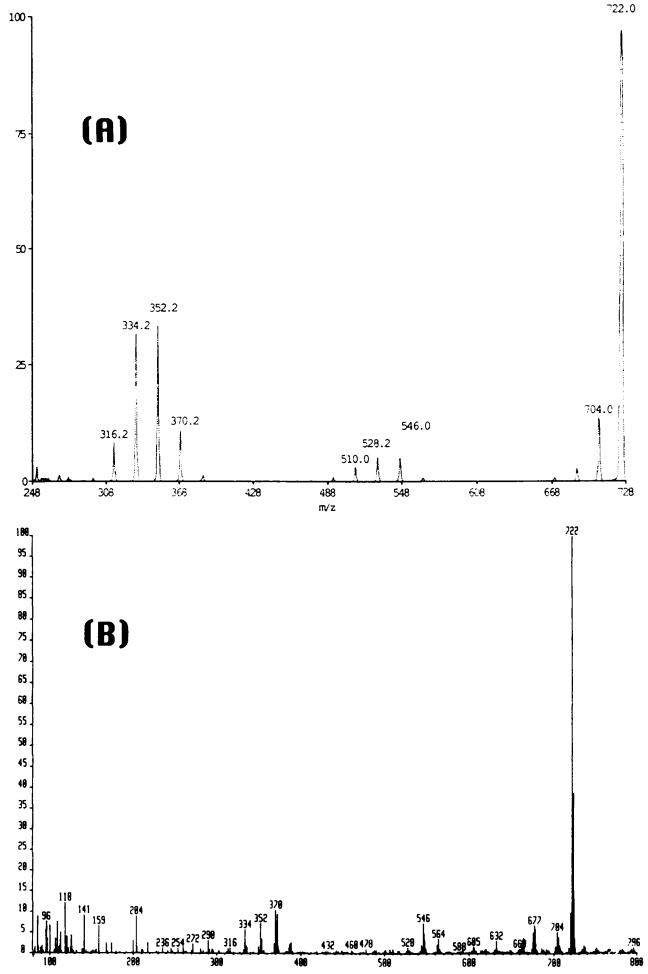

FIG. 2. (A) Parent-daughter mass spectrum of FB_1 done on a Sciex Taga 6000 triple tandem quadrupole mass spectrometer. Fumonisin was isolated from a culture of *A. alternata* f. sp. *lycopersici*. The protonated molecular ion (*m/z* 722) had undergone collision-activated decomposition to yield major daughter ions at *m/z* 546 and 370 which represent consecutive losses of two TCA groups. (B) Full-scan mass spectrum obtained by CFSIMS of fumonisin isolated from a culture of *A. alternata* f. sp. *lycopersici*. The protonated molecular ion is found at *m/z* 722, and loss of the TCA groups can be found at *m/z* 546 and 372.

(TCA) side group; *m/z* 528 and 510 are consecutive losses of water; *m/z* 370 is loss of a second TCA group; and *m/z* 352, 334, and 316 are consecutive losses of water. When full-scan ISMS was used, the molecular ion at *m/z* 722 was the predominant ion seen.

The mass spectrum (determined by CFSIMS on a VG70SEQ apparatus) of FB_1 was obtained after analysis of the culture extract taken from a 36-day-old culture of *A. alternata* f. sp. *lycopersici* (Fig. 2B). The constituents of the extract were resolved on a microcapillary column, and the retention time of FB_1 was determined by a mass-selective chromatogram of *m/z* 722. A full-scan mass spectrum of the FB_1 peak was taken, and after subtraction of the background a protonated molecular ion (*m/z* 721 plus 1) was found as the predominant species. Fragment ion clusters at *m/z* 546 and 547 represent loss of one TCA group, and the cluster at *m/z* 370, 371, and 372 represents loss of the second TCA group;

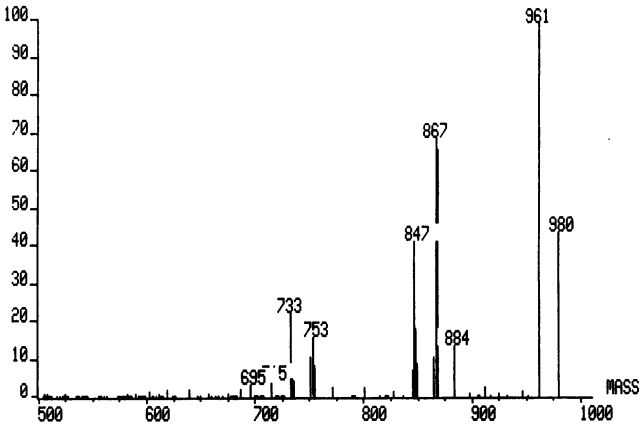

FIG. 3. Negative chemical ionization (in methane) mass spectrum of the TFA derivative of the hydrolysis product of FB_1 obtained from the *A. alternata* f. sp. *lycopersici* culture. The molecular ion $[M - H]^-$ was found at *m/z* 980, which compared favorably with the fumonisin standard.

m/z 704 is due to loss of water. The extract was also analyzed by fast atom bombardment by direct probe insertion with similar results, i.e., the protonated molecular ion (*m/z* 722) and the methyl ester (*m/z* 736, most likely found on the free carboxyl of the TCA moiety) were found. Results of analyses (electron impact) in which the base-hydrolyzed extract was reacted with TFA anhydride to produce the TFA derivative of the pentolamine showed prominent ion fragments at *m/z* 140, 180, 429, 527, 543, 556, 640, 736, and 754 (data not shown). In negative chemical ionization in methane, *m/z* 980, the molecular ion minus 1 was found (Fig. 3). Both *m/z* 981 and 980 were found in our analyses within the same run, indicating that both species M^- and molecular ion minus one occur in our sectoring species. We chose *m/z* 980 because it was more abundant.

HPLC analyses were also done by using the columns and conditions described in Materials and Methods (Fig. 4). Both AAL (R_f, 9.2) and FB_1 (R_f, 10.6) are shown. More than one metabolite of both AAL and FB_1 were found.

Partial confirmation of this report has come from an independent researcher (D. G. Gilchrist, University of California, Davis), who grew a culture of *A. alternata* f. sp. *lycopersici* (isolate AS27-12P_1) for 23 days (21°C with a 12-h–12-h light-dark regimen) on the same medium we describe here. That work was done in February 1986. After harvesting, the culture (pH 5) was stored at −20°C until mailed to us in July of 1991. We analyzed the culture in July 1992 and found FB_1 by using continuous-flow fast atom bombardment.

Evidence is herein provided for the biosynthesis of FB_1 by *A. alternata* f. sp. *lycopersici*. Although we present only data to support the presence of FB_1, other derivatives, such as FB_2, have been found and will be the subject of a kinetic study to be reported later. This is the first report of fumonisin production by *Alternaria* species. It is significant in that it allows us to reevaluate the concept of host-specific toxins in physiological plant pathology, i.e., production of host-specific toxins by nonpathogens.

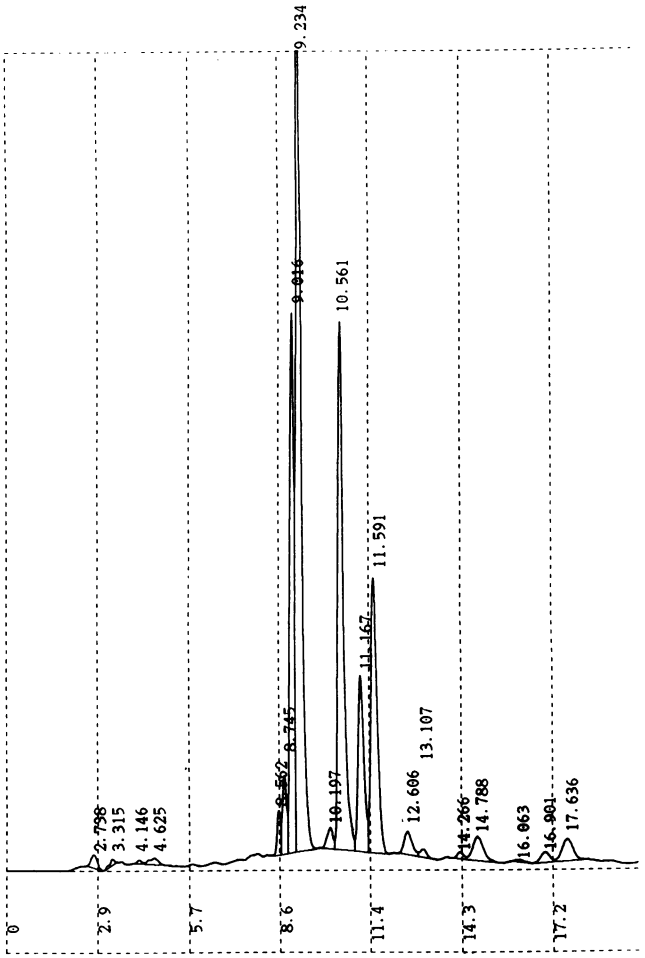

FIG. 4. Resolution by HPLC of the AAL (R_f, 9.2) and FB_1 toxin (R_f, 10.6) found in a 42-day-old liquid culture. The toxins are shown as the *o*-phthalaldehyde derivatives, and detection was by fluorescence.

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