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Tenuazonic Acid Production by *Alternaria alternata* and *Alternaria tenuissima* Isolated from Cotton

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Cultures of *Alternaria alternata* (three isolates) and *Alternaria tenuissima* (three isolates) obtained from cottonseeds and bolls were toxigenic when cultured on various laboratory media. A mycotoxin was isolated and identified as tenuazonic acid by using solvent partition, thin-layer chromatography, and instrument analyses. Toxicity was monitored with brine shrimp and chicken embryo bioassays. All cultures except *A. alternata* 938 produced tenuazonic acid when grown on cottonseed and on yeast extract-sucrose broth. The most toxin (266 mg/kg) was produced by *A. tenuissima* 843 on cottonseed.

Tenuazonic acid appears to be a characteristic toxic metabolite of several *Alternaria* species associated with agricultural commodities (6). The compound is a known mycotoxin, phytotoxin, antitumor agent, and antibiotic. It has been implicated as a possible cause or contributing factor to Onyalai, a hematological disorder in man (13). The mycotoxicology and chemistry of tenuazonic acid have recently been reviewed (4, 13). Its mode of action is to inhibit incorporation of amino acids into protein, possibly interfering with the release of newly formed protein from ribosomes (12). Research has shown that toxigenic *Alternaria* species are commonly associated with cottonseed and bolls (3). This paper reports on production of tenuazonic acid by *Alternaria alternata* (three isolates) and *Alternaria tenuissima* (three isolates) isolated from cotton and grown on liquid media and cottonseed.

MATERIALS AND METHODS

Cultures. Fungi used in this investigation were isolated from cottonseed and bolls and maintained on agar slants as previously reported (3). Numbers following the name of each isolate are Auburn University culture collection numbers. Isolates used in this investigation were *A. tenuissima* 560, 589, and 843 and *A. alternata* 582, 584, and 938. Cultures of *A. tenuissima* (560 and 589) were previously reported as nonsporulating isolates of *Colletotrichum gossypii* (3). Exposure to ultraviolet radiation induced both cultures to sporulate, and they were subsequently identified as *A. tenuissima* by Morgan-Jones, who considers all of the long-beaked alternarias that bear their conidia in very short chains to be *A. tenuissima*, and they are so designated in this report. Isolates of *A. tenuissima* 560 and 589 are morphologically similar to *Alternaria longipes*, but we are recognizing the narrow delimitation of the latter as a host-specific pathogen of tobacco by applying the former name to them.

Cultivation. Fungi were grown for 28 days at 25°C as stationary cultures in 100 ml of 2% yeast extract-4% sucrose (YES) medium per 250-ml flask. *A. tenuissima* 560 was grown for 28 days in 4 liters of YES medium per 5-gallon (ca. 18.9-liter) carboy to provide larger quantities of toxin for chemicophysical and toxicological investigations. Cultures were also grown for 28 days at 25°C and 99 to 100% relative humidity as stationary cultures in 250-ml flasks containing 50 g of delinted cottonseed moistened with 35 ml of water and autoclaved at 121°C twice in 24 h. Seeds were delinted by placing them in 85% sulfuric acid for 1 to 2 min and washing them with water. Cultures were replicated three times, and results were averaged.

Biological assays. Toxigenicity studies on extracts of the various fungal cultures were previously reported (3). In the present investigations, the principal mycotoxin of *A. tenuissima* 560 was isolated and purified by using brine shrimp, chicken embryo, and rat bioassays as previously described (2, 3), except that extracts were prepared in 95% ethanol with 10 μl of extract injected per egg and 1 ml of extract used per brine shrimp test. One-day-old fertile eggs were used, and 36 were injected per treatment.

Mycotoxin analyses. The flow diagram for toxin extraction and purification is shown in Fig. 1. The toxicity of the various fractions was monitored by brine shrimp bioassay and confirmed by chicken embryo bioassays as previously described. High-resolution ^{1}H nuclear magnetic resonance (NMR) spectra were obtained by using a Varian EM-390 spectrometer. Infrared and ultraviolet absorptions were determined with Perkin-Elmer model 727B and model 200 spectrophotometers, respectively. Low-resolution mass spectra were obtained with a DuPont 21-490 mass spectrometer in our laboratory, whereas the high-resolution mass spectra of tenu-

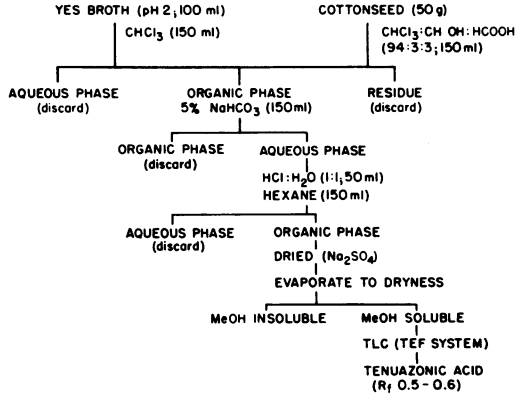

FIG. 1. *Flow diagram for extraction and purification of tenuazonic acid from YES broth and from cottonseed.*

azonic acid were made at the mass spectrometry laboratory of Florida State University, Tallahassee.

Thin-layer chromatography (TLC) analyses were with 450-μm silica gel G-HR plates developed in toluene-ethyl acetate-88% formic acid (6:3:1). The toxin was located initially by scraping 1-cm bands from developed preparative plates, eluting the bands with methanol, and bioassaying each band, using brine shrimp and chicken embryos. The toxin was found to occur at R_f 0.5 to 0.6, using the toluene-ethyl acetate-88% formic acid solvent system described. Initially, the toxic compound was detected on TLC analytical plates by spraying the developed plates with sulfuric acid-chromic acid (1:1) and charring for 15 min at 200°C. Later we found that the toxin developed a deep reddish-brown color when sprayed with ethanolic ferric chloride (14). The procedure for extraction of tenuazonic acid from cottonseed and YES broth is presented in Fig. 1. Quantitative analyses were made on methanol solutions with calculations based on a molecular weight of 197 and extinction coefficient of 13,490 at 287 nm after determining that no impurities present absorbed appreciably at this wavelength. Conversely, measurements at 218 nm were found unreliable because of interfering absorption due to impurities.

RESULTS AND DISCUSSION

The toxigenicity of *A. tenuissima* 560 was determined to be due primarily to a colorless viscous oil with an R_f of 0.5 to 0.6 in the toluene-ethyl acetate-88% formic acid solvent system. The 50% lethal dose of the toxin was approximately 50 mg/kg in chicken embryos at 10 days and 5 mg/kg at 18 days. The toxin gave a reddish-brown reaction to alcoholic ferric chloride. It was assigned the formula $C_{10}H_{15}NO_3$, based on high-resolution mass spectrometric analysis (197.1058 found, 197.1051 calculated). The base peak was 141.0425. The ultraviolet spectrum showed $\lambda_{max}^{MEOH,\ acidic}$ (log e) 218 nm (3.70) and 277.5 nm (4.10) and $\lambda_{max}^{MEOH,\ neutral,\ basic}$ 240 and 280 nm. The ^1H NMR spectrum ($CDCl_3$, tetramethylsilane) gave the following signals and assignments: 0.9 ppm (d + t, overlapping) of 2 × CH_3, 1.3 ppm (m) of CH_2, 2.0 ppm (m) of CH_2, 2.5 ppm (s) of $COCH_3$, 3.7 ppm (d) of $=C(O)-CH-N$, 6.8 ppm (broad s) of NH, and 9.3 ppm (broad s) of OH. The infrared spectrum of the toxin coated onto an NaCl block is presented in Fig. 2.

The toxic compound was identified as tenuazonic acid (Fig. 3) upon comparison of the above properties with those reported in the literature (5–7, 13, 14). Also, a ^{13}C NMR spectrum of the toxin (data not presented) was consistent with the structure proposed for tenuazonic acid by Stickings (14) and confirmed by Kaczka et al. (5).

After identification of tenuazonic acid as the principal toxic metabolite of *A. tenuissima* 560 and the development of procedures for qualitative and quantitative analyses, we found that all except one of the *Alternaria* cultures isolated from cotton produced tenuazonic acid in YES broth and also on cottonseed (Table 1). *A. tenuissima* 843 produced considerably more tenuazonic acid than did the other cultures. This fungus produced 266 mg/kg on cottonseed and 75.4 mg/kg in YES broth. *A. alternata* 938 did not produce the toxin on either substrate. *A. alternata* 584 produced the second-largest quantity of the mycotoxin on cottonseed, whereas *A. tenuissima* 589 produced the second-largest amount in YES medium. In medium-scale fermentations with *A. tenuissima* 560, approximately 20 mg of tenuazonic acid per kg was produced in 4 liters of medium per 5-gallon (ca. 18.9-liter) carboy. Extraction and purification procedures (Fig. 1) resulted in a relatively high-purity product. Final purifica-

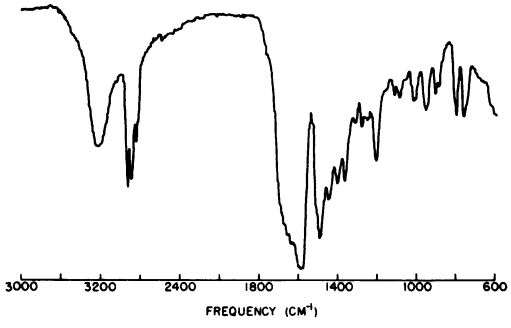

FIG. 2. *Infrared spectrum of tenuazonic acid coated onto an NaCl block.*

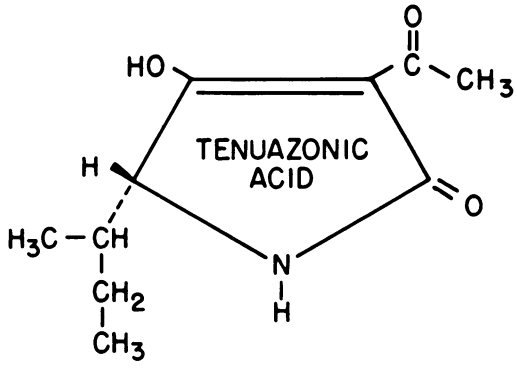

FIG. 3. *Structure of tenuazonic acid.*

TABLE 1. *Production of tenuazonic acid by Alternaria cultures growing in YES medium and on cottonseed at 25°C for 28 days*[a]

AUA culture no.	Fungus	Tenuazonic acid	
		YES medium (mg/liter)	Cottonseed (mg/kg)
	Uninoculated check	0.1[b]	0.4[b]
560	*A. tenuissima*	19.2	3.4
589	*A. tenuissima*	37.3	8.8
843	*A. tenuissima*	75.4	266.0
582	*A. alternata*	17.7	32.8
584	*A. alternata*	11.9	70.2
938	*A. alternata*	0.2[b]	3.2[b]

[a] Average of three replications; 100 ml of YES medium, 50 g of cottonseed.

[b] Negative for tenuazonic acid as determined by TLC analyses.

tion and stabilization are easily accomplished by preparation of the copper salt (10) in lieu of preparative TLC.

The cultures used in this investigation had previously been shown to be highly toxigenic (3). Since the cultures varied widely in their ability to produce tenuazonic acid and since culture *A. alternata* 938 did not produce this mycotoxin, it appears that mycotoxins other than tenuazonic acid must contribute to the overall toxicity of some of the cultures. This is not surprising, since *Alternaria* species are known to produce a large assortment of mycotoxins (1, 4, 8–11). It has not yet been determined which additional mycotoxins are produced by the *Alternaria* cultures reported in this investigation. Also, it has not yet been determined whether tenuazonic acid occurs naturally in cottonseed. However, all of the cultures examined in this investigation were originally isolated from cottonseed and bolls, and all grew well on cottonseed in the laboratory. All but one of the cultures produced tenuazonic acid on cottonseed. Therefore, it appears important to determine whether tenuazonic acid occurs in cottonseed under natural conditions. If so, it could have important implications with respect to use of cottonseed oil, meal, protein, etc., in the food chain of man as well as that of domestic animals.

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