Effect of Temperature and Moisture on Tenuazonic Acid Production by *Alternaria tenuissima*

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ABSTRACT

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The optimum temperature for tenuazonic acid (TZA) production by *Alternaria tenuissima* AUA 843 in 2% yeast extract–4% sucrose medium was 20 C. On cottonseed, maximum production of TZA also was obtained at 20 C. The limiting seed moisture content for mycelium growth and TZA production on cottonseed was approximately 14.9%, with highest production of mycelium and TZA occurring at 37.5% seed moisture content.

Alternaria spp. are ubiquitous fungi known to invade grains (10,11) as well as cottonseed and bolls during development in the field (5). The toxicity of *Alternaria* metabolites has been well established (2,4,6,8,10,11,14) and tenuazonic acid (TZA) has been reported as a toxic metabolite of several *Alternaria* spp. (10). Studies with rats, mice, dogs, and monkeys revealed that two salts of TZA produced two primary toxicoses, cardiovascular and emetic (13). Young chickens fed TZA orally showed decreased weight gain and lowered feed efficiency with the majority developing lesions of the spleen and gizzard as well as hemorrhages in the kidneys, liver, and on the surface of the heart (7). Steyn and Rabie (15) implicated TZA as the possible causal agent of a hematologic disorder called onyalai, which occurs widely in black populations in South Africa. TZA has been reported to inhibit protein synthesis by preventing the transfer of amino acids from tRNA to the acceptor site (12).

Cottonseed meal is an important animal feed supplement and is a potential source of protein in human diets (5). The production of mycotoxins by fungi in cottonseed would make it hazardous for animal as well as human consumption. Knowledge of the environmental factors influencing mycotoxin formation is important so that storage environments can be made unfavorable for fungus growth. Metabolites from several fungi (other than *Aspergillus flavus*) isolated from cottonseed were demonstrated to be toxic in laboratory bioassays (5). *Alternaria tenuissima*, which was highly toxic, was found to produce TZA (4). This paper reports the effect of temperature, time, and seed moisture content on TZA production by this fungus growing on a nutrient medium and on cottonseed.

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MATERIALS AND METHODS

Organism. *Alternaria tenuissima* (isolate #843) isolated by Diener et al (5) from Alabama cottonseed was used throughout this investigation. The isolate, which is maintained in the Auburn University culture collection, was identified by G. Morgan-Jones (5). Cultures were maintained on agar slants containing 2% dextrose, 0.7% yeast extract (Difco), 0.5% KH_2PO_4, and 2% agar.

Cultivation. Tests were conducted with 100 ml of 2% yeast extract–4% sucrose (YES) medium in 250-ml Erlenmeyer flasks stoppered with cheesecloth-covered cotton plugs and autoclaved at 121 C for 15 min. The medium in each flask was inoculated with 1 ml of a mycelium and spore suspension from a 7- to 10-day-old stationary culture of *A. tenuissima* grown on YES medium; the inoculum was prepared by blending the mycelial mat for 3–5 sec with the growth medium. Acid-delinted cottonseed (Deltapine-61) was obtained from Ring-Around-Products Inc., Montgomery, AL 36101. Erlenmeyer flasks (500 ml) containing 50 g of cottonseed and 30 ml of water were autoclaved twice in 24 hr at 121 C for 15 min. Inoculum was prepared by washing the mycelial mat with sterile distilled water, then blending it with 100 ml of sterile distilled water. The cottonseed in each flask was inoculated with 2 ml of a suspension of *A. tenuissima*.

Analytical methods. Fungus cultures grown in YES medium were filtered through rapid-flow filter paper. The mycelium was washed twice with distilled water, dried for 12 hr at 70 C, cooled to room temperature in a desiccator, weighed, and the dry weight of the mycelium was recorded. The filtrates were acidified to pH 2 with 50% HCl and extracted with 150 ml of chloroform. Each chloroform extract was taken to dryness under an air stream on a steam bath and the residue was resuspended in methanol. Appropriate dilutions were made to obtain UV absorption data on

a Perkin-Elmer Model 200 UV spectrophotometer at 278 μm.

Fungus cultures grown on cottonseed were extracted with 150 ml of chloroform-methanol-88% formic acid (94:3:3, v/v) by blending in an explosion-proof blender for about 30 sec. After filtration through rapid-flow filter paper, the filtrate was extracted with 200 ml of 5% sodium bicarbonate in a 500-ml separatory funnel. The aqueous phase was acidified with 50 ml of 50% HCl and extracted with 150 ml of hexane. The organic phase was taken to dryness under an air stream on a steam bath and taken up in methanol for measurement of UV absorption.

Identification of TZA was confirmed by thin-layer chromatography (TLC) using 20 × 20-cm glass plates coated with a 500-μm layer of Silica Gel GHR (Brinkmann Instruments, Westbury, NY 11590) developed in toluene-ethyl acetate-88% formic acid (6:3:1, v/v). The R_f of TZA was 0.5–0.6 and the identity of the toxin was confirmed by formation of a deep reddish brown color on chromatograms sprayed with ethanolic ferric chloride.

Time and temperature experiments. *Nutrient medium.* YES medium (100 ml) in 250-ml Erlenmeyer flasks was inoculated and incubated as stationary cultures at seven temperatures ranging from 5 to 35 C at 5-degree intervals. Duplicate flasks of medium were taken at each temperature and analyzed for TZA every 6 days for 52 days. The pH of the medium was determined before autoclaving, after autoclaving, and after incubation with a Corning Model 12 pH meter. The amount of fungus was determined by dry weight of mycelium. Two experiments were conducted and the data were averaged.

Cottonseed. Cottonseed (50 g) moistened with 30 ml of water in 500-ml Erlenmeyer flasks was inoculated with 2 ml of a suspension of spores and mycelium of *A. tenuissima* and incubated at seven different temperatures. The cottonseed from duplicate flasks was analyzed every 6 days for 60 days. The experiment was repeated and the data were averaged for the two experiments.

Relation of moisture. The relation of relative humidity (RH) and seed moisture content (SMC) to growth and TZA formation by *A. tenuissima* were studied in five Blue M Power-O-Matic 60 environmental cabinets that were adjusted to 20 C, the optimum temperature for TZA formation by this fungus on YES and cottonseed media. RH in the cabinets were adjusted to maintain the calculated moisture equilibria. The equilibrium SMC of cottonseed at various RH has been reported (9). The appropriate amount of water was added to culture flasks according to the equilibrium SMC at a specific RH. Cottonseed (50 g) in 500-ml flasks, containing the appropriate amount of water and plugged loosely with foam plugs, was autoclaved twice in 24 hr. Sterile water was added to compensate for water lost in autoclaving. Each flask was inoculated with 2 ml of a suspension of the fungus. After 24 days of incubation, cottonseed from duplicate flasks at each SMC was analyzed for TZA production. The SMC of cottonseed was determined at 101 C for 12–16 hr in a forced air oven as described in the AOCS method Aa 3-38 for cottonseed (1).

Data analysis. The data were analyzed by analysis of variance and general linear models procedure.

RESULTS

The effect of temperature and time on TZA production by *A. tenuissima* on YES medium is shown in Fig. 1. The optimum temperature for TZA production was 20 C with the maximum

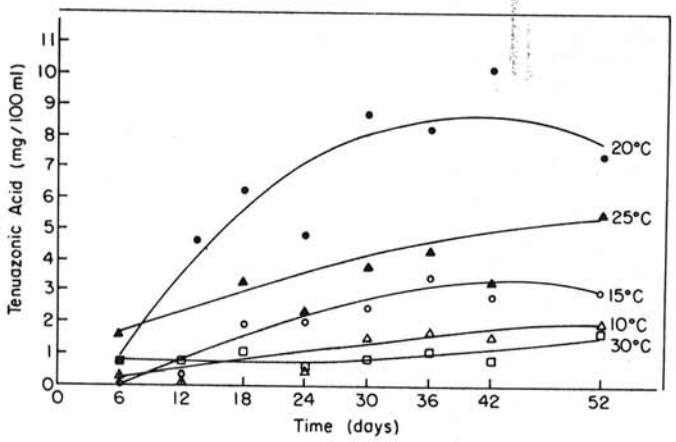

Fig. 1. Effect of temperature and time on tenuazonic acid production by *Alternaria tenuissima* in yeast extract—sucrose medium.

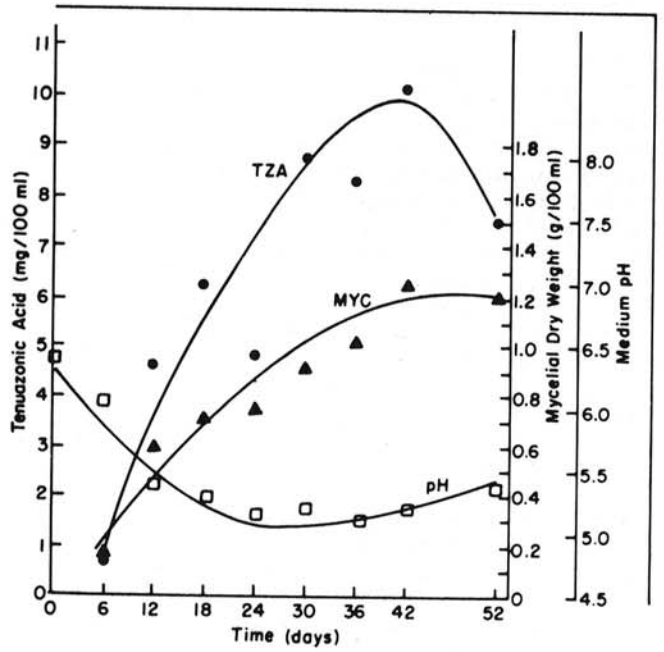

Fig. 2. Effect of time on tenuazonic acid (TZA) production, mycelium (MYC) growth, and medium pH change in *Alternaria tenuissima* cultures in yeast extract—sucrose medium at 20 C.

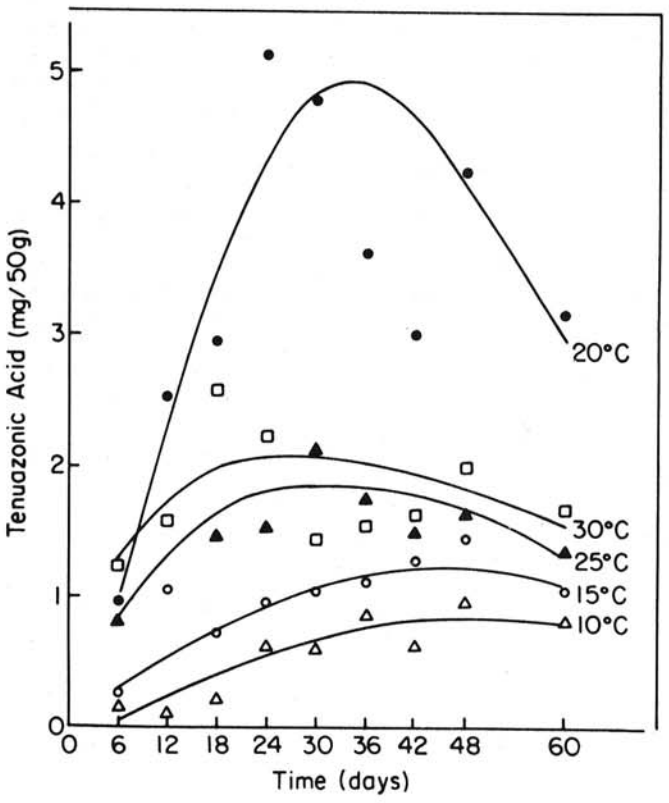

Fig. 3. Effect of temperature and time on tenuazonic acid production by *Alternaria tenuissima* in cottonseed.

TABLE 1. Effect of moisture in cottonseed on growth and tenuazonic acid production of *Alternaria tenuissima* at 20 C

Seed moisture (%)	Relative humidity (%)	Growth index[a]	Tenuazonic acid (mg/kg)
37.46	100	5	52.27 ± 0.96
28.91	98	5	49.67 ± 1.32
22.31	95	4	24.14 ± 2.02
19.16	90	2	7.37 ± 1.26
14.91	85	<1	<1.05
11.71	80	0	0

[a]Average of six flasks; visual index of 0–5 with 5 representing maximum mycelium growth and 0 representing none in 24 days of incubation.

amount of TZA being attained in 42 days. There was a noticeable drop in TZA formed at temperatures of 10 C or below as well as above 20 C. Over 10 mg of TZA was produced in 42 days at 20 C.

At 20 C, maximum TZA production of 10.2 mg/100 ml of YES medium was correlated with profuse mycelial growth (1.25 g/100 ml) as noted in Fig. 2. The pH of the medium decreased with time from 6.3 to about 5.2 in 30 days and then increased slightly to about 5.4 in 42 days of incubation.

The effect of temperature and time on TZA production by *A. tenuissima* in cottonseed is shown in Fig. 3. Optimum temperature for TZA formation in cottonseed was 20 C with the maximum amount of TZA (5.25 mg/50 g of seed) being attained after 24 days of incubation. Less than 2 mg of TZA was formed at temperatures of 10 C or below as well as above 20 C.

The effect of moisture in cottonseed on growth and TZA production by *A. tenuissima* at 20 C is shown in Table 1. After 24 days of incubation, maximum growth and TZA production was correlated with the highest SMC and RH maintained in the experiment.

DISCUSSION

Maximum TZA production at the optimum temperature of 20 C was correlated with maximum mycelium production in 42 days incubation. There was a notable drop in TZA production with continued incubation beyond 42 days. The decrease in pH of the YES medium during incubation probably was associated with the acidic properties of TZA (Fig. 2).

Maximum yields of TZA at 20 C were produced in 24 days in cottonseed compared with 42 days in YES medium. However, the amount of TZA formed in cottonseed was about one-half that obtained with the nutrient medium. The optimum temperature for TZA production by the fungus was 20 C in both media.

With cottonseed at various SMC, maximum yields of TZA and the greatest visible mycelium growth were obtained at 37.5% SMC (99% RH) and 20 C. Fungus growth and TZA formation decreased rapidly with decreasing moisture in the substrate and decreasing RH in the atmosphere around the substrate. At 19.2% SMC (90% RH) only 7 mg/kg of TZA was produced as compared to about 50 and 52 mg/kg at 29 and 37% SMC, respectively, a sevenfold decrease in TZA formation. From this moisture study on TZA formation in cottonseed by *A. tenuissima*, it was concluded that if TZA is to be actively produced in cottonseed in storage or in the field, it would most likely occur at SMC in equilibirum with RH greater than 90% and at temperatures around 20 C. Thus, significant production of TZA might occur naturally during a late fall when excessive rainfall delays the harvesting of cotton.

Forgacs and Carll (6) observed that species of *Alternaria* frequently lose the ability to maintain production of high levels of toxic substances after only a few transfers on laboratory culture media, and also on natural substrates. Coombe et al (3) found that after subculturing the original culture twice, the production of dehydroaltenusin by *A. tenuis* dropped almost to zero. In our investigations, although successively transferred cultures of *A. tenuissima* produced progressively lower amounts of TZA than originally reported (4), production never dropped to zero. This decline in production of TZA was reflected by greater variance in the statistical analyses of the data. However, our primary interest was the effect of temperature and moisture (RH) on toxin elaboration rather than on the production of high yields of toxin per se.

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