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Effect of Water Activity and Temperature on Mycotoxin Production by *Alternaria alternata* in Culture and on Wheat Grain

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Both water activity (a_w) and temperature affected the production of altenuene (AE), alternariol (AOH), and alternariol monomethyl ether (AME) by *Alternaria alternata* on wheat extract agar and wheat grain. Greatest production of all three mycotoxins occurred at 0.98 a_w and 25°C on both substrates. At 0.98 a_w and 25°C, a single colony of *A. alternata* grown on wheat extract agar produced 807 μg of AOH, 603 μg of AME, and 169 μg of AE per ml in 30 days. However, production of all three mycotoxins at 0.95 a_w was less than 40% of these amounts. Little toxin was produced at 0.90 a_w. Changing temperature and a_w altered the relative amounts of the different toxins produced on agar. At 15°C and 0.98 a_w, maxima of 52 μg of AOH and 25 μg of AME per ml were produced after 15 and 30 days, respectively, whereas AE continued to increase and reached 57 μg/ml after 40 days. At 15°C and 0.95 a_w, production was, respectively, 62, 10, and 5 μg/ml after 40 days. All three metabolites were produced at 5°C and 0.98 to 0.95 a_w and at 30°C and 0.98 to 0.90 a_w. On wheat grain at 25°C and 0.98 to 0.95 a_w, more AME was produced than AOH or AE, but at 15°C there was less AME than AOH or AE. Only trace amounts of AE, AOH, and AME were found at 15 to 25°C and 0.90 a_w, but production of AME was inhibited at 30°C and 0.95 a_w or less.

The genus *Alternaria* is widely distributed in soil and on aerial plant surfaces, and many species are pathogenic to plants. Species can often grow at low temperature and may be associated with extensive spoilage of fruit and vegetables during refrigerated transport and storage (13). *Alternaria alternata* (Fr.) Keissler is ubiquitous and is abundant in the airspora, especially during ripening and harvesting of cereal crops (9). Ripening ears of wheat are colonized by *A. alternata* soon after emergence (18; N. Magan, Ph.D. thesis, Reading University, Reading, United Kingdom, 1982), and it is reported to be the most common subepidermal fungus of wheat grain (3, 19). *A. alternata* alone or with other fungi, e.g., *Alternaria triticina*, can cause a conspicuous black or brown discoloration of wheat kernels called black-point disease (1, 2, 22). This can result in decreased quality and yield of grain (10).

Because of the pathogenicity exhibited by many *Alternaria* spp., various workers have investigated the toxicity of crude preparations of cultures and infected substrates. Many isolates have been shown to be lethal to chickens, rats, and mice (6, 11, 12, 23). Extracts of *A. alternata* from cotton have been found to be highly toxic to brine shrimp, chicken embryos, and rats (18).

The most important secondary metabolites of known mammalian toxicity produced by *A. alternata* are the dibenzo-α-pyrones altenuene (AE), alternariol (AOH), alternariol monomethyl ether (AME), and a derivative of tetramic acid, tenuazonic acid (TZA) (16, 23, 27). *A. alternata* has been demonstrated to produce some or all of these mycotoxins in various concentrations on tomatoes (17), sorghum (30), and pecans (31).

Development of fungi is dependent on both abiotic and biotic variables (33). Perhaps the two most important environmental factors are substrate water activity (a_w) and temperature. Other important factors are pH of the substrate and gas composition of the surrounding atmosphere (22a, 22b, 34). Knowledge of the influence of environmental factors on growth and mycotoxin formation can be an important aid in predicting mycotoxin contamination of grain, both in the field and in storage. Further, the a_w and temperature limits for growth and mycotoxin production are sometimes markedly different (25).

This paper reports the effect of a_w and temperature on the production of AE, AOH, and AME by *A. alternata* growing on wheat extract agar and wheat grain.

MATERIALS AND METHODS

Culture methods. The isolate of *A. alternata* used in these studies was obtained from freshly harvested wheat grain directly plated on 2% malt agar in 1980 and chosen for its heavy sporulation. Cultures were stored on 2% malt agar at 5°C and subcultured when required for experiments onto freshly prepared 2% malt agar plates incubated at 25°C.

Wheat extract agar (pH 5.5) modified with glycerol to 0.98, 0.95, and 0.90 a_w was used as the test medium (22a). Petri dishes containing 13 ml of freshly prepared wheat extract agar were inoculated at the center with a needlepoint of spores obtained from the margin of 8- to 10-day-old colonies. Inoculated plates of the same a_w were sealed in sterile polyethylene bags, together with 3 or 4 uninoculated plates (15). The a_ws of representative samples of the media were checked with a dew point meter (Protimeter, Ltd., Marlow, Buckinghamshire, United Kingdom).

Duplicate sets of each treatment were incubated at 5, 15, 25, and 30°C. Every 5 days up to 30 days at 25°C and 40 days at 15°C, two petri dishes were removed from each treatment, colony diameters were measured, and then cultures were extracted and analyzed for AE, AOH, and AME. The amount of each mycotoxin produced by a single colony of *A. alternata* was then calculated.

Wheat grain experiments. Flasks containing 150 g of winter wheat (cv. Maris Huntsman) were sterilized by autoclaving at 1.1 kg/cm^2 (121°C) for 25 to 30 min. After autoclaving, the initial moisture content of the grain (usually 8 to 10%) was determined by using duplicate samples of ca. 5 g, oven dried at 105°C for 17 to 18 h (21). Sterile distilled water was then added to bring the moisture content to about 19, 22, and 26%, equivalent to 0.90, 0.95, and 0.98 a_w, respectively (24,

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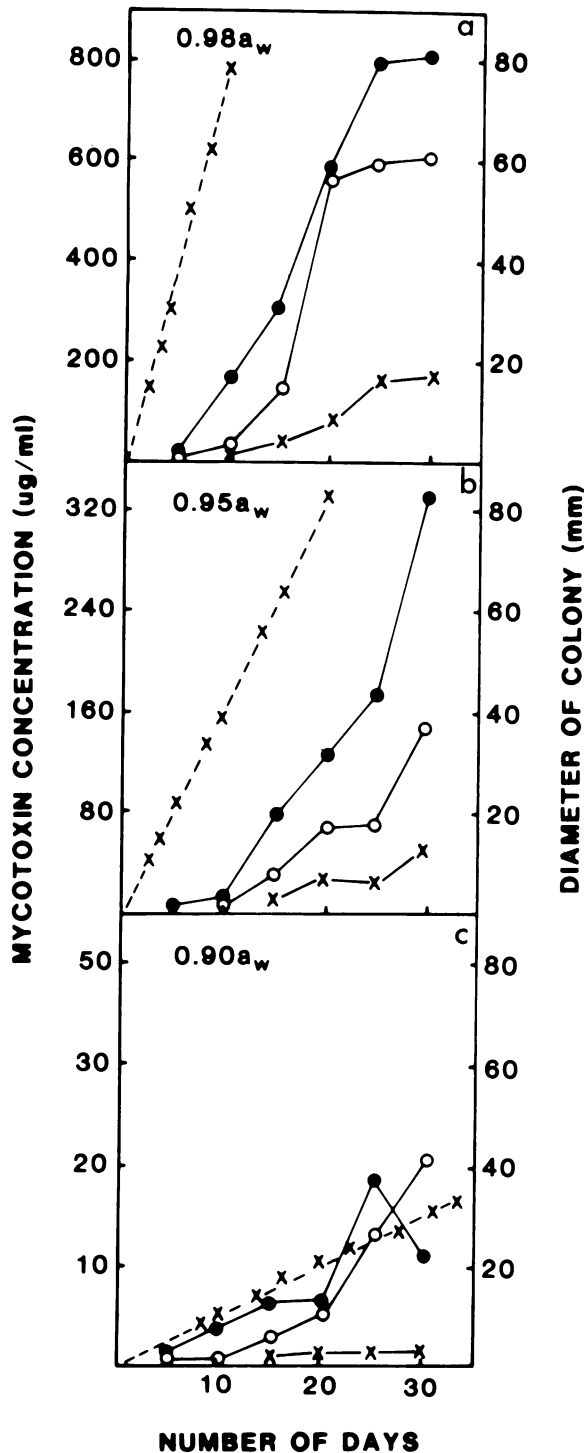

FIG. 1. The relationship between growth of *A. alternata* on wheat extract agar and mycotoxin production at 25°C and 0.98, 0.95, and 0.90 a_w. Symbols: x - - - x, colony diameter; ●, AOH; ○, AME; x——x, AE.

28). To allow equilibration of moisture content after adding water to the grain, the flasks were stored for 7 to 10 days at 5°C and shaken vigorously each day. At the end of this period, the moisture content was rechecked.

A single layer of sterile grain was placed on 7- to 10-day-old petri dish cultures of *A. alternata*, the lids were replaced, and the plates were shaken vigorously to coat the surfaces of the grain with spores and hyphae of the fungus. The inoculated grain was returned to the flask and thoroughly mixed with the sterile grain remaining. The inoculated grain was then placed in four perforated sterile foil containers, each holding ca. 35 to 40 g of grain. These were carefully placed on a wire gauze stand in a desiccator previously cleaned with alcohol and containing 250 ml of a glycerol-water mixture of the required a_w (8). The two halves of each desiccator were sealed with petrolatum. To allow air exchange, a small vent, plugged with cotton wool, was provided. Duplicate desiccators of each treatment were incubated for 3 weeks at 15, 25, and 30°C.

Extraction. Samples of grain (40 g) or wheat agar cultures (25 g) were homogenized in 100 ml of methanol (analytical grade) for 2 to 3 min. The filtered extract (30 ml) was mixed with 60 ml of 20% (wt/vol) ammonium sulfate in a 250-ml separating funnel. Distol grade hexane (30 ml) was added twice, and the funnel was shaken for 45 s before the layers were allowed to separate. The upper hexane layer was discarded, and the lower layer was transferred to a 100-ml separating funnel. This was extracted four times with 5 ml of dichloromethane. The extracts were passed through a column of anhydrous sodium sulfate (granules; 30 to 40 mm by 8 mm) into a conical flask. The combined extracts were evaporated under a gentle stream of nitrogen (16, 32). The residue was then dissolved in 1.2 ml of methanol, followed by 0.8 ml of 0.2 M ammonium acetate (pH 6).

Analysis. The high-pressure liquid chromatography system consisted of a column (250 by 4.6 mm) packed with 5 μm of Spherisorb ODS (Phase Separation, Ltd., Queensferry, United Kingdom). The eluent consisted of 70% methanol and 30% ammonium acetate (0.2 M) pH 5.9 with a flow rate of 1 ml/min. A Gilson pump (model 302) and manometric module (model 802) were used. The eluent was monitored by using a Perkin-Elmer 2000 fluorescence spectrophotometer set with an excitation wavelength of 315 nm and an emission cut-off filter of 430 nm (for AE, AOH, and AME) and an electrochemical detector (LC4A; Bioanalytical Systems, West Lafayette, Ind.) with a glassy carbon electrode set at an oxidation potential of 0.9 V (for AOH and AME). The amounts of the three metabolites in experimental samples were determined by comparison of their peak heights with those of known amounts of standards.

Standards. Samples of AE, AOH, and AME were kindly supplied by P. M. Scott (Health and Welfare Canada, Ottawa, Ontario, Canada), D. J. Harvan (National Institute of Health Research, Triangle Park, N.C.), and R. M. Eppley (Food and Drug Administration, Washington, D.C.).

RESULTS

Cultural studies on wheat extract agar. The colony growth of *A. alternata* at different a_w levels and temperatures was compared with its ability to produce AE, AOH, and AME. At 0.98 a_w and 25°C, colonies of *A. alternata* grew to 80 mm in 10 days. Both AOH and AME were first detected after 5 days of growth and reached maximum concentrations of over 800 and 600 μg/ml, respectively, after 30 days (Fig. 1a). Toxins were produced most rapidly during the period of 15 to 25 days after inoculation. AE was not detected until 10 days after inoculation and increased to 170 μg/ml after 30 days.

At 0.95 a_w and 25°C, colonies grew more slowly, not reaching a diameter of 80 mm until 20 days after inoculation.

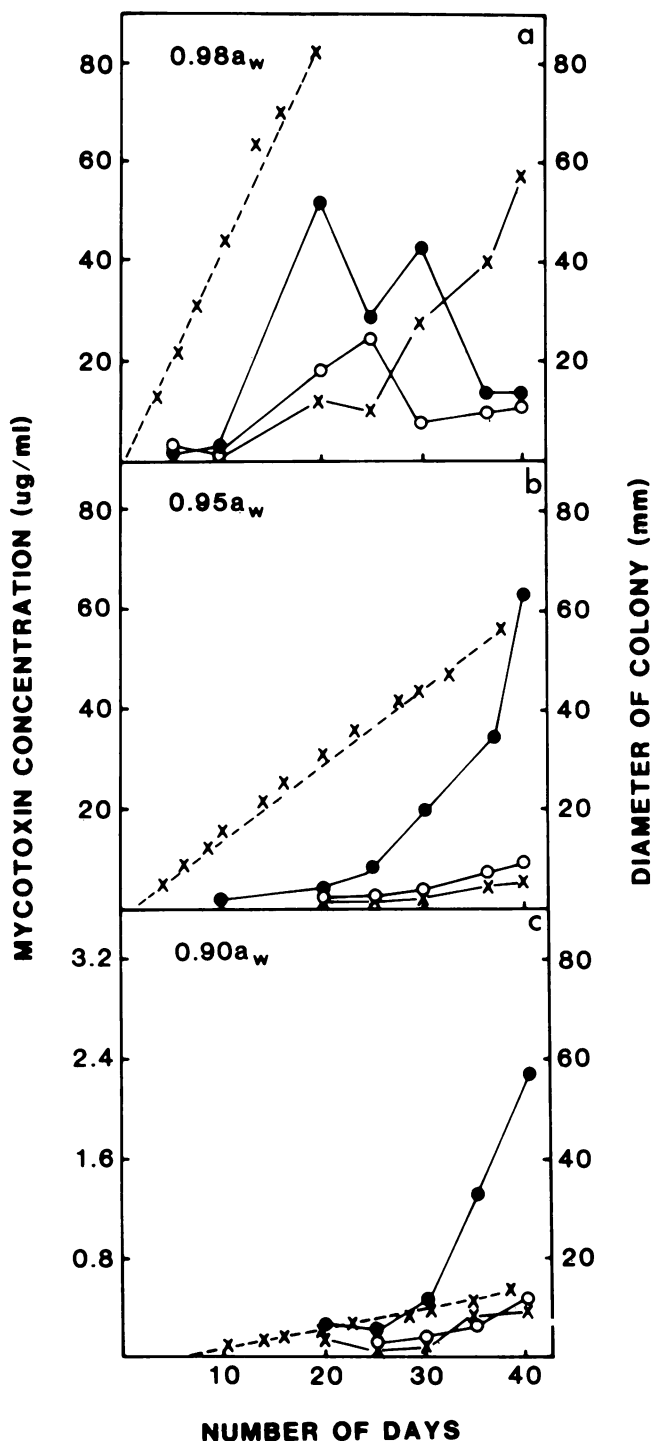

FIG. 2. The relationship between growth of *A. alternata* on wheat extract agar and mycotoxin production at 15°C and 0.98, 0.95, and 0.90 a_w. Symbols: x - - - x, colony diameter; ●, AOH; ○, AME; x——x, AE.

At this a_w the total concentration of all three mycotoxins after 30 days was less than 40% of that at 0.98 a_w (Fig. 1b). AOH was still produced in the largest total concentration after 30 days, followed by AME and AE.

At 0.90 a_w and 25°C, growth of *A. alternata* was even slower, reaching only about 30 mm in diameter after 30 days of growth. At this a_w, maximum concentration of AOH was produced after 25 days of growth (18 μg/ml), but this total decreased by half in the next 5 days. By contrast, total AME concentration increased continuously to a maximum of 22 μg/ml after 30 days. Very little AE was produced at this a_w and 25°C (Fig. 1c).

Altering the temperature to 15°C decreased the growth of *A. alternata* and altered the quantities of mycotoxins produced compared with those produced at 25°C. At 0.98 a_w, colonies grew to 80 mm in diameter within 20 days. Total concentration of AOH increased rapidly between 10 and 20 days of growth, reaching a maximum of 51 μg/ml before decreasing by 50% during the next 5 days. Further decreases, to 25% of the maximum, occurred during the next 15 days (Fig. 2a). Similarly, AME concentration increased to a maximum of 25 μg/ml after 25 days of growth before decreasing again to only 10 μg/ml after 40 days. By contrast, AE production increased continuously once it was first detected after 10 days of growth, to reach a maximum concentration of 57 μg/ml after 40 days.

At 0.95 a_w and 15°C, the isolate of *A. alternata* grew to a diameter of only 50 mm after 40 days. AOH was first detected after 10 days of growth and reached maximum concentration after 40 days. AME and AE, both detected first after 20 days of growth, increased only slowly during the next 20 days (Fig. 2b). At 0.90 a_w growth and mycotoxin production were further decreased. Total concentrations of all three mycotoxins were less than 5% of those at 0.95 a_w, with AOH again produced in the highest concentration, followed by AME and AE.

The effects of temperature on the total mycotoxins produced at different a_w levels after 30 days of growth are summarized in Table 1. Maximum concentrations of all three mycotoxins occurred at 25°C and 0.98 a_w. Changing temperature and a_w from 25°C and 0.98 decreased mycotoxin production, with the greatest decrease occurring when both were changed together. In general, AOH was produced in the greatest concentration, followed by AME and AE.

Experiments with wheat grain. Only small concentrations of AE were produced at 15°C, e.g., less than 0.5 μg/g of grain at 0.98 a_w. Production was greatest at 25°C, whereas at 30°C there was a decline to only 1.0 μg/g of grain. The effect of decreasing a_w on the concentrations of AE was most notable at 25°C. The amount of AE produced at 0.95 a_w was less than 5% of that at 0.98 a_w, whereas at 15 and 30°C changing the a_w from 0.98 to 0.95 decreased the amount of AE to about 25 and 10%, respectively, of that at 0.98 a_w (Table 2). Only traces of AE were found at 0.90 a_w regardless of incubation temperature.

AOH production was six times greater at 25°C than at 15°C (Table 2), but at 30°C was less than at 15°C. AOH responded to changes in a_w similarly to AE, with production declining

TABLE 1. Effect of temperature and a_w on production of mycotoxins by *A. alternata* on wheat extract agar (after 30 days of incubation)

Temp (°C)	Production of mycotoxin (μg/ml) at an a_w of: 0.98			0.95			0.90		
	AE	AOH	AME	AE	AOH	AME	AE	AOH	AME
5	2.7	16.5	1.5	0.3	1.2	0.2	NG[a]	NG	NG
15	27.9	42.1	7.8	2.0	19.2	3.0	0.1	0.6	0.2
25	169.2	807.0	603.0	55.3	331.5	158.1	1.1	11.1	21.8
30	31.4	48.7	32.7	4.4	19.9	8.5	5.3	3.5	5.9

[a] NG, No growth.

TABLE 2. Influence of temperature and a_w on the production of mycotoxins by *A. alternata* on wheat grain

Temp (°C)	a_w	Mycotoxin (μg/g of grain)[a]		
		AE	AOH	AME
15	0.98	0.45 ± 0.16	10.84 ± 1.24	0.59
	0.95	0.11 ± 0.04	0.73 ± 0.16	0.10 ± 0.02
	0.90	0.02	0.01	0.01
25	0.98	24.34 ± 5.22	63.67 ± 15.31	194.00 ± 14.99
	0.95	0.70 ± 0.06	5.80 ± 0.83	0.86 ± 0.04
	0.90	0.03	0.33	1.49 ± 0.24
30	0.98	0.95 ± 0.04	5.29 ± 0.85	10.11 ± 1.85
	0.95	0.07	1.32 ± 0.08	0
	0.90	0.14	1.08 ± 0.40	0

[a] Results are the mean of two replicates ± the standard deviation.

by 90% between 0.98 and 0.95 a_w. Only a trace was detected at 0.90 a_w and 15 and 30°C.

By increasing the temperature from 15 to 25°C, production of AME at 0.98 a_w was increased from 0.6 to 195 μg/g of grain, whereas at 30°C there was a marked decrease, with production only 5% of that at 25°C. Decreasing the a_w to 0.95 resulted in only a trace of AME being produced at 15°C, whereas production at 25°C was only 10% of that at 0.98 a_w. At 30°C, AME was not detected at either 0.95 or 0.90 a_w. Like AE and AOH, only a trace of AME was found at 15 or 25°C and 0.90 a_w.

As in experiments on wheat extract agar, the optimum conditions for production of all three mycotoxins on wheat grain were 25°C and 0.98 a_w. However, by contrast with wheat extract agar, at 25 and 30°C AME was produced in the largest concentration on wheat grain, followed in decreasing order by AOH and AE, whereas at 15°C AOH was produced in the largest concentration, followed by AME and AE.

DISCUSSION

The total amounts of AOH, AME, and AE produced by a single colony of the *A. alternata* isolate tested in this study were greatest at 25°C and 0.98 a_w. Further, all three metabolites were produced over the temperature range of 5 to 30°C and the a_w range of 0.98 to 0.90, although at marginal temperatures and 0.90 a_w little of any mycotoxin was produced. The minimum a_w allowing germination of *A. alternata* conidia is 0.85, whereas 0.88 a_w is necessary for growth on wheat extract agar at 25°C (22a). The limiting a_w for detectable mycotoxin production is thus slightly greater than that for growth, with optimum production occurring above 0.95 a_w. Differences between the conditions of a_w and temperature necessary for growth and those necessary for the production of aflatoxin B_1, patulin, penicillin acid, and ochratoxin A by *Aspergillus* and *Penicillium* species have been reported previously (25).

In culture, at all a_w values and temperatures with the exception of 0.98 a_w and 15°C and 0.90 a_w and 25°C, the increase in the diameter of the *A. alternata* colony was accompanied by a steady increase in production of AE, AOH, and AME until the end of the experiment. At 0.95 a_w and 15°C, maximum production of AOH and AME occurred after 20 to 25 days of incubation, when the colony had grown to about 60 mm in diameter. However, AE concentrations continued to increase until the end of the experiment (40 days). Aflatoxin B_1 production by *Aspergillus parasiticus* decreases similarly when incubated in submerged culture (26). It was also noticeable that a lag time of 5 to 10 days often elapsed between inoculation and AME and AE production, especially at lower a_w values (0.95 to 0.90 a_w). This was less pronounced with AOH production. Other studies on solid substrates have shown that mycotoxin production by some *Aspergillus* and *Penicillium* species occurs soon after mycelial growth commences, with lag times often less than 30 h (M. D. Northolt, Ph.D. thesis, Agricultural University of Wageninen, Wageningen, Holland, 1979). However, longer lag times have been reported in liquid culture media (29).

Although experiments with wheat grain were limited to 21 days, the optimum conditions for production of AE, AOH, and AME were the same as those on agar media, i.e., 25°C and 0.98 a_w. However, AME was produced in larger concentration than AOH at 25 and 30°C. Further, AME was not detected in wheat grain at 30°C and 0.95 to 0.90 a_w, indicating some differences from the production of this metabolite in agar. In the field, fluctuations of temperature, changes in relative humidity, and rainfall all influence the colonization of developing grain by *A. alternata*. a_w is particularly critical, and in the field there is a period of 10 to 20 days after anthesis when wheat grain is milky ripe (growth stage 70–79; reference 36) and the moisture content is about 70% (a_w = 1.00). During dough development (growth stage 80 onwards), the moisture content decreases rapidly to 20% (a_w = 0.92) or less as the grain ripens. While the moisture content is high, the a_w is close to 1.00 and ideal for germination and growth of *A. alternata* and mycotoxin production.

The only similar study with *Alternaria* spp. has been on the effect of temperature and moisture content on the production of TZA by *Alternaria tenuissima* isolated from cottonseed (35). Maximum production of TZA on cottonseed occurred at 20°C and 37.5% moisture content (1.00 a_w) with a minimum water content of 14.9% (0.85 a_w). Production of TZA was halved when a_w was decreased to 0.95. Young, Davis, and Diener (35) therefore suggested that a temperature of 20°C and an a_w value above 0.90 were required for TZA production. It is thus likely that the biosynthesis of different mycotoxins produced by *Alternaria* spp. is favored by different temperatures.

In our investigation, an isolate of *A. alternata* which spored heavily in culture and showed little aerial hyphal growth was used in both cultural and wheat grain experiments with little evidence of any loss in its ability to produce AE, AOH, and AME. However, there are frequent reports that *Alternaria* spp. can quickly lose their toxin-producing ability (14). Coombe et al. (7) found that subculturing the original culture twice resulted in dehydroaltenusin production by *A. alternata* (as *A. tenuis*) dropping to almost zero. Häggblom (20) found that high-mycotoxin-producing strains of *A. alternata* had little aerial mycelium and even this appeared only after 7 days, whereas low-mycotoxin-producing strains had much floccose mycelial growth after 2 days.

Christensen and Kaufmann (4, 5) considered that grain harvested with a high percentage of *Alternaria* contamination was sound. However, conducive environmental conditions and excessive rainfall, especially late in the growing season, may cause rapid development of *A. alternata* and increase not only black point of wheat grain but also the production of AE, AOH, AME, and TZA, which could present a hazard if the grain were used for human consumption or as animal feedstuff.

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