

# Environmental factors and tenuazonic acid production by *Alternaria* spp. isolated from sorghum

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## Abstract

*Sorghum bicolor* is one of the world's most important cereals. Ripening crops in damp, warm and humid conditions often develop characteristic head blights which can have significant effect on yield and quality of grain. Six *Alternaria* isolates (predominantly *A. alternata*) isolated from sorghum grain were screened for their ability to produce tenuazonic acid (TA) in vitro on a modified Fries medium. They all produced TA, but isolate 5 (Alt5) produced significantly ( $P=0.05$ ) greater quantities than the others over a 28-day incubation period. Studies with Alt5 showed that on this medium the isolate could produce TA over a wide range of temperatures (10–35°C) and water activities (0.998–0.93). Alt5 also produced TA over a range of water availabilities on 2% sorghum agar, and on sterile inoculated sorghum grain, although quantities produced were markedly less than those on richer laboratory media. This suggests that *Alternaria* spp. can rapidly colonise sorghum grain under conducive environmental conditions and produce significant amounts of toxic secondary metabolites.

## Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a very extensively grown dryland crop in many parts of the world and the fourth most important staple grain after wheat, rice and maize (FAO 1986). The hot, humid conditions and the fact that the ripening seed is exposed to the environment before harvest means that sorghum grain is particularly prone to weathering and attack by fungi and insects (Williams and Rao 1981). This often results in the development of characteristic head blight symptoms, markedly reducing quality and yield. There is evidence that pre-harvest infection of sorghum with *Aspergillus flavus* and *Fusarium* spp. can result in the production of aflatoxins and zearalenone (Connole and Hill 1970; McMillian et al. 1983). However, very little information is available on the potential for head blight fungi such as *Alternaria* spp. to grow and produce mycotoxins on ripening sorghum (Jewars and John 1990). This contrasts with other grains and perishables where detailed information is available on mycotoxin production by *Alternaria* spp. (Magan et al. 1986; Visconti et al. 1992; Vinas et al. 1992). This study was carried out to determine the ability of different *Alternaria* isolates from sorghum to produce tenuazonic acid (TA), to

quantify the effects of water availability, temperature and time on TA production in culture and on sorghum grain, and to assess potential for control using fungicides or grain preservatives.

## Materials and Methods

### Fungal isolates

These were isolated from sorghum grain (cv Maldandi M35-1, Maharashtra State, India) directly plated on 2% malt extract or potato dextrose agar, or after surface-sterilisation in sodium hypochlorite (5% available chlorine). Single spore isolates of six strains (Alt1–Alt6) were obtained, and stored at 5°C on 2% malt extract agar slopes until used.

### Media, inoculation and incubation

Experiments were carried out on two types of media: (a) a modified Fries medium (Lebrum et al. 1990), and (b) 2% milled sorghum agar. Both media were modified with the non-ionic solute glycerol to obtain water activities in the range 0.998–0.93 (Magan et al. 1986). Ten mL of the modified Fries medium was dispensed in Roux bottles; and 20 mL sorghum agar in 9 cm Petri plates.

The modified Fries liquid medium was inoculated with a 4 mm agar plug from the margin of actively growing culture and incubated at an angle of 10° to the horizontal. Agar media were inoculated centrally with a single 4 mm agar plug from a margin of a colony growing on 2% sorghum agar. Experiments were carried out to compare the six *Alternaria* isolates, and subsequently only with Alt5 to determine the effect of temperature and water activity ( $a_w$ ) on growth and TA production. All experiments were carried out with three replicates and repeated once.

Sorghum grain subsamples (20 g) were adjusted to 0.995, 0.98 and 0.95  $a_w$  by the addition of distilled water (More et al. 1992). After autoclaving, the water content was adjusted where necessary and 0.1 mL of Alt5 spore suspension ( $2.5 \times 10^6$ /mL) was added and the grain thoroughly mixed. Subsamples (2 g) were placed in sterile multi-welled chambers and incubated in sandwich boxes containing salt solutions of the required equilibrium relative humidity. Samples were taken after 7, 14 and 21 days and the TA concentration determined.

Benomyl (benlate, 50% a.i.) and iprodione (25% a.i.) were added to 10 mL Fries medium in the range 0.1–50 µg/mL before the addition of a 4 mm plug inoculum of Alt 5. Three replicates of each treatment were incubated at 25°C for up to 28 days. Similar experiments were carried out with potassium sorbate and sodium propionate on modified Fries medium modified to pH 4.6.

### Tenuazonic acid analysis

TA was quantified using the iron complexation method described by Lebrum et al. (1990). Liquid or homogenised agar cultures, or ground sorghum grain were acidified with 20

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$\mu\text{L}$  of 5M hydrochloric acid, treated with 400  $\mu\text{L}$  ferric chloride (2% in 1M hydrochloric acid) and 3 mL butanol and agitated with a Vortex mixer for 2 minutes. Solutions were centrifuged at 1500g for 15 minutes and stored overnight at 4°C. The absorbance of the upper phase was measured at 450 nm. Standard solutions of the sodium salts of TA (0.05–1.0 mM) in Fries and agar media at each  $a_w$  were used to plot calibration curves by linear regression. The  $r^2$  for all treatments were between 0.942–0.985. TA concentrations were estimated by interpolation from these curves. This assay method had a limit of detection of 0.05 mM concentration.

### Results and Discussion

Figure 1 compares the ability of six different *Alternaria* isolates to produce TA over periods of 28 days on a modified

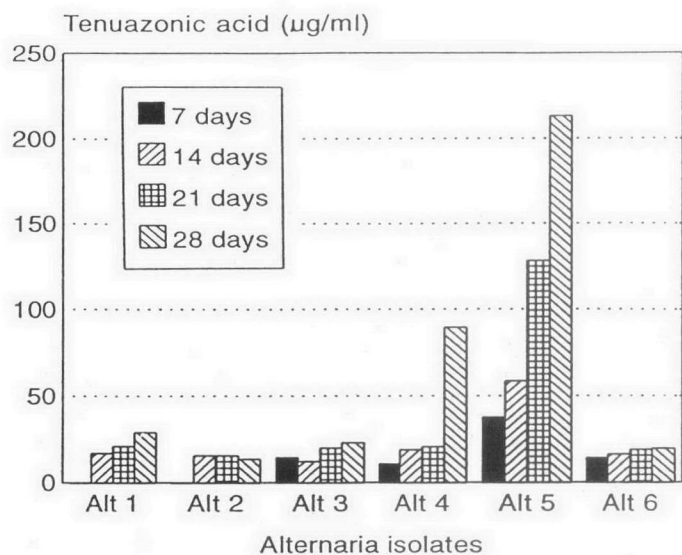


Fig. 1. Comparison of the ability of six *Alternaria* isolates (Alt1-Alt6) for production of tenuazonic acid ( $\mu\text{g ml}^{-1}$ ) on a modified Fries medium at 25°C over a period of 28 days.

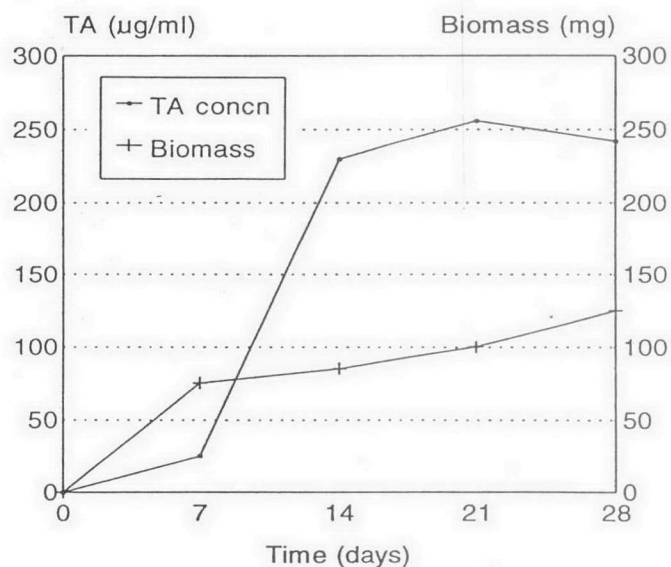


Fig. 2. Changes in mean biomass (mg dry weight) and tenuazonic acid (TA;  $\mu\text{g ml}^{-1}$ ) production by Alt5 on a modified Fries medium at 25°C.

Fries medium at 25°C. This shows that isolate Alt5 produced significantly more TA than the other strains obtained from sorghum grain. The changes in fungal biomass and TA production for Alt5 at 25°C are shown in Figure 2. Growth was most rapid during the first 7 days of incubation. However, most TA was produced subsequently during the period 7–14 days. Figure 3 compares the production of TA by Alt5 at 10–30°C. Production occurred over the whole temperature range tested, with optimum production at 15 and 25°C. However, time of incubation affected the amount of TA produced. Water activity also affected TA production with optimum being produced at 0.998  $a_w$ , but high concentrations also being produced at 0.995–0.95  $a_w$  (Fig. 4). At 0.93  $a_w$  there was very little TA produced initially because of the slow growth rate. However, after 21 days approx. 100  $\mu\text{g/ml}$  was present and this increased to over 150  $\mu\text{g/ml}$  after 28 days.

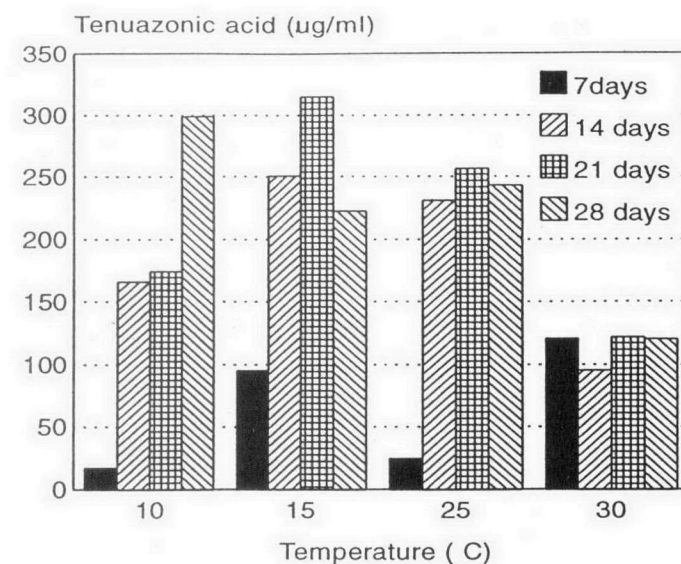


Fig. 3. Comparison of tenuazonic acid ( $\mu\text{g ml}^{-1}$ ) production at different temperatures by Alt5 on a modified Fries medium over a period of 28 days incubation.

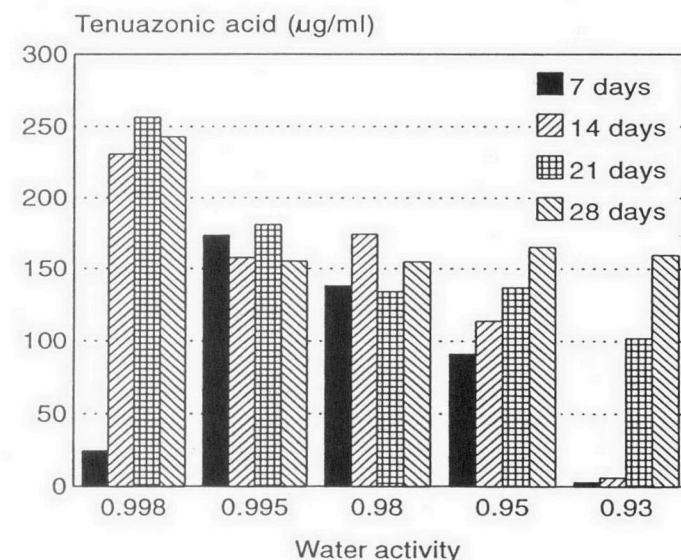


Fig. 4. Relationship between water activity and tenuazonic acid ( $\mu\text{g ml}^{-1}$ ) production by Alt5 on a modified Fries medium at 25°C over a period of 28 days.

Figure 5 shows that the amount of TA produced on 2% sorghum agar was much less than that on the richer laboratory nutrient medium. There was a general increase in TA production with time at all three  $a_w$  levels tested, but after 21 days incubation there was a slight reduction in concentrations, except at the lowest  $a_w$  (0.95) tested. Subsequent experiments on sterile sorghum grain showed that slightly greater amounts of TA were produced at similar  $a_w$  levels than on the 2% sorghum agar medium, over a 21 day incubation period (Fig. 6).

Subsequent studies have been concentrated on the possible effect of fungicides or preservatives for control of growth of Alt5 and TA production. This has so far suggested that up to 50  $\mu\text{g}/\text{mL}$  benomyl or iprodione had little effect on growth or TA production with up to 250  $\mu\text{g}/\text{mL}$  medium of TA being

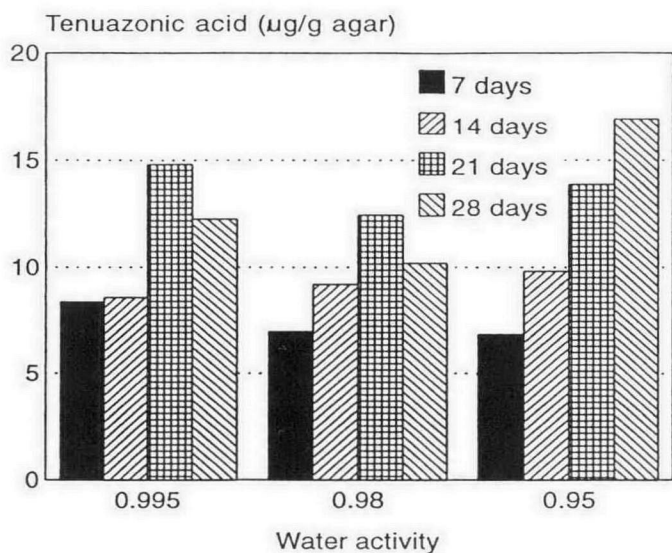


Fig. 5. Production of tenuazonic acid ( $\mu\text{g g}^{-1}$  agar) by Alt5 on a 2% sorghum agar medium at different water activities at 25°C.

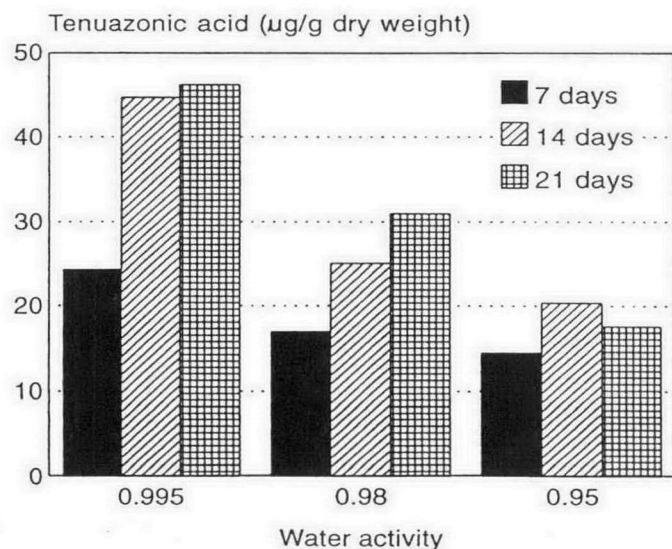


Fig. 6. Production of tenuazonic acid ( $\mu\text{g g}^{-1}$  dry weight) by Alt5 on sterile sorghum grain at different water activities and when stored at 25°C for up to 21 days.

produced. Studies with potassium sorbate and sodium propionate suggest that sorbate does inhibit growth and TA production at concentrations greater than 0.25%, while with propionate, greater than 0.5% is necessary for any inhibition of mycotoxin or growth to occur. However, there was no indication of any stimulation of TA production with either fungicides or preservatives.

This study has demonstrated that *Alternaria* strains from sorghum grain can produce significant quantities of TA when screened in the laboratory. The isolate producing the most TA is most probably an isolate of *A. alternata* and this is being confirmed at the present time. These results are in agreement with those of Bruce et al. (1984) who showed that on other small grains such as wheat, barley and rye, *A. alternata* was the predominant producer of TA, as well as alternariol, altenuene and alternariol monomethyl ether. The ability of Alt5 in this study to produce TA over a wider range of  $a_w$  levels and optimally at 25°C is similar to that found previously with *A. alternata* isolates from wheat and production of alternariol, altenuene and alternariol monomethyl ether (Magan et al. 1986).

There is only one comparable study of TA production in relation to water and temperature relations, and this involved *A. tenuissima* from cotton seed (Young et al. 1980). In that study, maximum TA was produced at 20°C and 37.5% water content (= 1.00  $a_w$ ) and minimum at 14.9% water content (0.85  $a_w$ ). However, these conditions were not accurately controlled, as normally *Alternaria* spp. seldom germinate or grow at 0.85  $a_w$ . Interestingly, in their study Young et al. (1980) found that TA production was almost halved at 0.95  $a_w$ . This suggests that the ability of different *Alternaria* species to produce TA may vary markedly in both growth and the production of mycotoxins. For example, isolates of *A. alternata* from oilseeds were found to vary considerably in their ability to produce TA (Visconti et al. 1992). However, the optimum concentrations produced were similar to those found in the present study.

Further study is now needed on whether varieties of sorghum with different resistances to head blight may influence not just preharvest colonisation but also the capability for mycotoxin production. More detailed information is also necessary on more effective fungicides for control of head blight pathogens and on their influence on growth of *Alternaria* spp., and on potential for mycotoxin production, especially in hot, humid preharvest conditions.

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