EFFECT OF WATER ACTIVITY AND TEMPERATURE ON PRODUCTION OF AFLATOXIN AND CYCLOPIAZONIC ACID BY *ASPERGILLUS FLAVUS* IN PEANUTS

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1. INTRODUCTION

It is well known that some isolates of *Aspergillus flavus* are able to produce cyclopiazonic acid (CPA) in addition to aflatoxins (Luk et al., 1977; Gallagher et al., 1978). CPA producing strains of *A. flavus* are frequently isolated from substrates such as peanuts (Blaney et al., 1989; Vaamonde et al., 2003) and maize (Resnik et al., 1996), indicating that this toxin could be a common metabolite and thus is likely to be present in some aflatoxin contaminated foods. Natural co-occurrence of both toxins has been detected in peanuts (Urano et al., 1992; Fernández Pinto et al., 2001) and it has been hypothesized that the presence of both toxins in food and feeds may result in additive or synergistic effects.

CPA is toxic to poultry and may have contributed to the outbreak of the classic "Turkey X" disease which killed about 100,000 turkey poults in England in 1960 (Cole, 1986). Some disease outbreaks of unknown aetiology observed in chickens in Argentina could also be attributed to the presence of CPA in peanut meal used as a raw material in poultry feeds, as strains of *A. flavus* capable of producing

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high levels of CPA are frequently isolated from peanuts grown in this country (Vaamonde et al., 2003). CPA accumulates in the muscle of chickens following oral dosing (Norred et al., 1988) so that a potential for contamination of meat and meat products exists.

In view of the health hazards for animals and humans caused by the co-occurrence of aflatoxins and CPA, the production of these toxins in agricultural commodities must be controlled. Water activity (a_w) and temperature are the most important environmental factors preventing fungal growth and mycotoxin biosynthesis. Conditions for mycotoxin production are generally more restrictive than those for growth and can differ between mycotoxins produced by the same fungal species, as well as between fungi producing the same mycotoxin (Frisvad and Samson, 1991). Interactions between factors such as a_w, temperature and time are also important and should be taken into account in the design of experiments to study their effects on mycotoxin production (Gqaleni et al., 1997).

The effects of temperature and a_w on the production of aflatoxins by *A. flavus* have been widely studied (ICMSF, 1996) but very little is known about the effect of these factors on CPA production. Besides, most published studies on mycotoxin formation have been concerned with single mycotoxins. Few studies have examined how environmental factors can affect the simultaneous production of these two toxins on agar culture media (Gqaleni et al., 1997) and natural substrates (Gqaleni et al.,1996b).

In the present work an experiment with a full factorial design was used to study the effects of, and interactions among, temperature, a_w and incubation period on co-production of AF and CPA on peanuts inoculated with a cocktail of *A. flavus* strains. Peanuts were sterilized by gamma irradiation to keep, as closely as possible, to natural conditions for mycotoxin production. In this way, it was hoped that a different fungal response to environmental conditions in relation to biosynthesis of both secondary metabolites could be observed.

2. MATERIALS AND METHODS

2.1. Substrate

Samples of peanuts (*Arachis hypogaea* cv Runner) from the peanut growing area in the province of Córdoba, Argentina, were

used. Samples were analysed for aflatoxins and CPA and shown to be free of both toxins. Peanut kernels were distributed in plastic bags in portions of 2.5 kg of material and were treated by gamma irradiation (Cuero et al., 1986) to kill contaminant microorganisms. Preliminary experiments showed that a dosage rate of 6 kGy produced kernels free of viable microorganisms without adversely affecting seed germination. Irradiation was carried out at the Comisión Nacional de Energía Atómica, Buenos Aires, Argentina, using a ^{60}Co source.

Water activity was adjusted by adding sterile water according to data from the water sorption isotherms of peanuts (Karon and Hillery, 1949). The material was mixed and left to equilibrate at 7°C until measurements of a_w in three consecutives days showed variation <0.005. A Vaisala Humicap HMI31 hygrometer (Woburn, MA) was used to measure a_w at 25°C. Levels of a_w achieved were 0.86, 0.88, 0.92 and 0.94.

2.2. Microorganisms

Fungi used in this study were isolated from peanuts and identified as previously described (Vaamonde et al., 2003). Four *A. flavus* strains which were shown to produce both aflatoxin B_1 (AFB_1) and CPA were selected.

2.3. Inoculum and Incubation

A cocktail inoculum was prepared with the four selected strains. Spores of each strain from a 7 day old culture on Malt Extract Agar were harvested using glycerol solutions (2.5 ml) adjusted to the a_w appropriate for each sample of equilibrated peanuts. The four suspensions were mixed and diluted to obtain a solution of 10^6 spores/ml. The numbers of spores in the inoculating suspensions were counted in a haemocytometer. Samples of peanuts (25 g) equilibrated to the selected a_w were placed in Erlenmeyer flasks (250 ml) and inoculated with 1 ml of the spore suspension. The material was thoroughly mixed to obtain homogeneous distribution of the inoculum.

The Erlenmeyer flasks were incubated at 20°C, 25°C and 30°C for 7, 14, 21 and 28 d respectively under constant relative humidity. This was achieved by placing the flasks in polyethylene bags (gauge 0.04 mm) containing receptacles with glycerol solutions adjusted to

the corresponding a_w. Each experiment was carried out with three replicates.

2.4. Mycotoxin Analysis

Three flasks were removed weekly from each set of conditions and analysed for AFB_1 and CPA. Analysis of AFB_1 was carried out according to the BF method (AOAC, 1995). Peanut kernels (25 g) were extracted with methanol-water (125 ml; 55+45), hexane (50 ml) and NaCl (1 g) in a high-speed blender (1 min). The extract was filtered through Whatman No 4 filter paper and centrifuged at 2000 rpm (5 min). Aliquots of the aqueous phase (25 ml) were transferred to a separatory funnel and extracted with chloroform (25 ml) by shaking for 1 min. The chloroform extract was filtered through anhydrous Na_2SO_4 and evaporated to dryness. AFB_1 was detected by TLC on silica gel G60 plates using chloroform:acetone (90:10) as a developing solvent. AFB_1 concentrations were determined by visual comparison of fluorescence under UV light (366 nm) with standard solutions (Sigma Chemical, St. Louis, MO, USA) dissolved in benzene:acetonitrile 98:2.

CPA was analysed by the method of Fernández Pinto et al. (2001). Peanuts (25 g) were mixed with methanol:2% sodium hydrogen carbonate mixture (7:3; 100 ml). The mixture was blended at high speed for 3 min, centrifuged (1500 rpm) for 15 min and then filtered. Filtrate (50 ml) was defatted twice with hexane (50 ml) by shaking in a wrist action shaker (3 min). KCl solution (10%; 25 ml) was added to the sample layer and acidified to pH 2 with 6N HCl. The solution was transferred quantitatively to a separatory funnel, extracted twice with chloroform (25 ml), and filtered over anhydrous Na_2SO_4. This extract was evaporated to dryness and redissolved in chloroform (200 µl). CPA was detected by TLC separation on silica gel G60 plates. TLC plates were immersed completely in oxalic acid solution (2% wt/wt) in methanol for 10 min, heated at 100°C for 1 h and cooled. Extracts (2 µl) were applied to the plates with standard solution every fourth track. The plates were developed in ethyl acetate:2-propanol: ammonium hydroxide (40:30:20). After development, the plates were dried 5 min at 50°C to drive off ammonia and then sprayed with Erlich's reagent [4-dimethylaminobenzaldehyde (1 g) in ethanol (75 ml) and concentrated HCl (25 ml)]. Blue spots were analysed after 10 min by visual comparison with a standard CPA solution (Sigma Chemical Co., St. Louis, MO, USA). The detection limit of the method was 50 µg/kg.

3. RESULTS AND DISCUSSION

Figure 1 shows the influence of water activity and temperature on CPA accumulation over a 28-day period. CPA production was inhibited at the lowest a_w studied (0.86) at all temperatures. At higher a_w levels, CPA was detected after a lag period of between 7 and 14 d. The amount of CPA produced was determined by the complex interaction of a_w, temperature and incubation time. Measurements performed only at a fixed time could lead to misinterpretation of the results. For example, from the curve at a_w 0.92 (Figure 1) different conclusions could be drawn regarding the influence of temperature on CPA production by considering the accumulation after 14 or 28 d. Taking into account the whole incubation period at the various a_w levels, CPA reached a maximal accumulation (4469.2 µg/kg) after 28 d at 0.94 a_w and 25°C, but also a considerable production (2690 ppb) was detected at the lowest temperature studied (20°C) after 21 d at relatively high

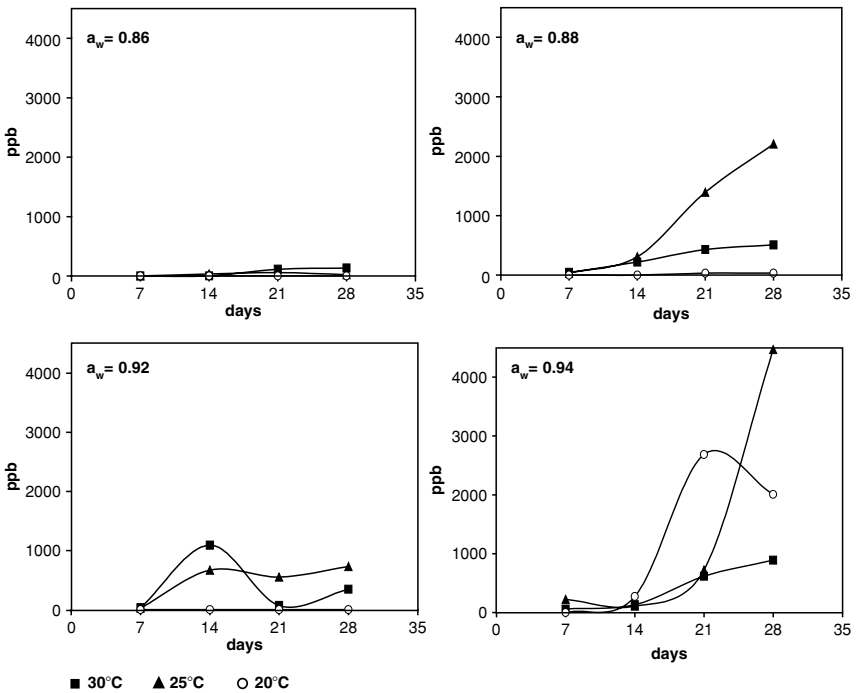

Figure 1. Interaction between a_w and temperature and their effect on cyclopiazonic acid production by *A. flavus* on peanuts over a 28 day period

a_w. These results are in concordance with those obtained by Gqaleni et al. (1996a) who reported that *A. flavus* produced higher levels of CPA at 20°C than at 30°C on maize grains held at constant a_w. The same effect was observed by Sosa et al. (2000) who found maximum CPA production by *Penicillium commune* at 20°C and 0.90 a_w in a medium based on meat extract. According to Gqaleni et al (1996b) CPA production proceeded in a similar pattern for *A. flavus* and *P. commune* with a combination of high a_w and low temperature favouring high CPA production and low a_w and high temperature supporting least CPA production.

AFB_1 was produced over the whole a_w range (Figure 2). AFB_1 concentrations were low after 7 d at all temperatures, but as time progressed, the maximal accumulation was observed at the lowest a_w (0.86) with temperatures of 25°C and 30°C, temperatures reported as favourable for aflatoxin production by other authors (ICMSF, 1996). A combined effect of a_w and temperature was observed as AFB_1 production was inhibited at low a_w (0.86) and low temperature (20°C). At

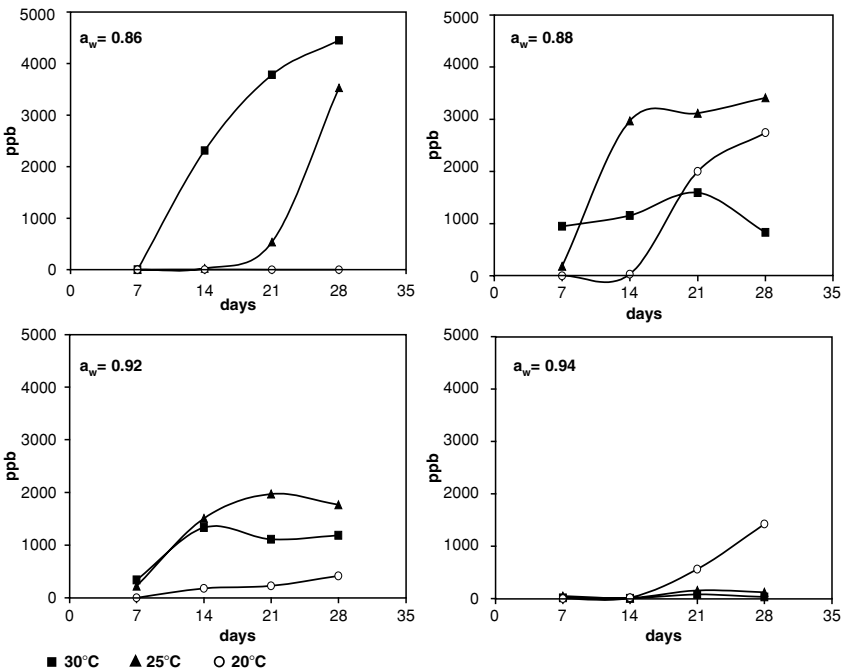

Figure 2. Interaction between a_w and temperature and their effect on aflatoxin B_1 production by *A. flavus* on peanuts over a 28 day period

higher a_w, AFB_1 production taken over the whole incubation period was lower than at low a_w. The lowest accumulation was detected at 0.94 a_w. At this a_w level the fungus grew vigorously at 25°C and 30°C but very low quantities of AFB_1 were detected in such conditions, while at 20°C slower growth was observed but a considerable amount of AFB_1 (1375 ppb) was produced after 28 d.

As mycotoxins are fungal secondary metabolites, production need not to be correlated with the growth of the fungus and factors such as induction, end product inhibition, catabolite repression and phosphate regulation can influence production (Tuomi et al., 2000). However, the results obtained for AFB_1 accumulation were unexpected and contradict data from previous studies which reported that aflatoxin production increases with increasing a_w (Diener and Davis, 1970; Montani et al., 1988; Gqaleni et al., 1996b).

Differences could be due to several factors. Gqaleni et al. (1996a), using several *A. flavus* strains known as co-producers of aflatoxins and CPA, reported that the pattern of co-production of these toxins by a particular isolate can vary widely depending on the type of cultural system. When they used yeast extract sucrose agar or a liquid medium, aflatoxins were produced in very low quantities over a period of 21 d of incubation at 30°C despite the high a_w of these substrates. When they used autoclaved maize grains (a_w 0.992) higher concentrations of both toxins were detected and the ratios between concentrations of aflatoxins and CPA were 16.6:1, 5.7:1 and 1.6:1 after 7, 15 and 21 d respectively (Gqaleni et al., 1996a). The heat sterilised maize grains used by Gqaleni et al. (1996a; 1996b) differed from the cultural system used in the present work, where living seeds were used. In this case, the substrate conditioned at higher a_w levels might perhaps have had some defence mechanism, e.g. synthesis of inhibitors of aflatoxin biosynthesis. The fungus might respond by increasing production of CPA since the biosynthetic pathways of the two toxins are different. CPA is derived from tryptophan (Holzapfel, 1971) whereas aflatoxins are decaketides (Smith and Moss, 1985). On the other hand, reduced metabolic activity of viable kernels associated with decreased a_w could increase susceptibility to *A. flavus* growth and aflatoxin production, an effect observed in the field with decreased pod moisture content under drought stress (Mehan et al., 1991).

Other factors such as the peanut variety, the size of inoculum and oxygen availability could also be responsible for the unexpected results obtained in the present work. The possibility of competitive growth of microorganisms that could have survived the disinfection treatment should be also considered since the radiation dose employed (6 kGy)

was relatively low when compared with that applied in other studies (Marín et al., 1999), although microbial contamination was not visually detected. Further experiments are currently in progress in order to clarify the possible influence of some of the above mentioned factors, e.g. to perform mycotoxin accumulation curves using *A. flavus* and *A. parasiticus* strains capable of producing only aflatoxins with other peanut varieties and different inoculum levels.

It is evident that accumulation of two (or more) toxins produced simultaneously by a fungus is affected by numerous factors, as well as their interactions, as the result of a very complex relationship between the microorganism, the substrate and the environment. The influence of each of these factors on the production of each toxin may be different. In fact, variation of the relative amounts of the toxins produced with changing a_w and temperature has been observed for other fungal species producing several toxins (Magan et al., 1984; Wagener et al., 1980). Biosynthesis of different mycotoxins produced by *Alternaria* spp. is favoured by different temperatures. Production of tenuazonic acid, a tetramic acid like CPA, occurred at 20°C (Young et al., 1980), and was affected in a very different way by environmental factors compared with other *Alternaria* toxins such as alternariol and alternariol monomethyl ether (Magan et al.,1984).

The conditions for maximum production of aflatoxins and CPA by *A. flavus* are different, according to results of the present work and those obtained by Gqaleni et al. (1996a; 1996b). Table 1 illustrates the relative concentrations of both toxins in some of the conditions used in our study. It can be pointed out that these conditions are different from those in most published studies on mycotoxin formation because a) a cocktail of native strains that co-produce aflatoxins and CPA was used; b) the substrate was not autoclaved and; c) the study covered sufficient time to observe the evolution of the interaction between the

Table 1. Mean levels of cyclopiazonic acid (CPA) and aflatoxin B_1 (AFB_1) produced by *Aspergillus flavus* in peanuts under conditions of controlled temperature, a_w and time

Water activity	Temperature (°C)	Time (days)	CPA (µg/kg)	AFB_1 (µg/kg)
0.94	25	28	4469.2[a]	118.7
0.94	25	14	109.8	14.8
0.92	25	21	550.2	1972.8
0.88	25	28	2205.7	3411.7
0.86	30	28	134.5	4450.0
0.86	20	28	ND[b]	0.4

[a] Optimal conditions for CPA production; [b]Limiting conditions for CPA production

fungus and the substrate under different environmental conditions. It can be seen that at the points at which one of the toxins reaches the highest concentration the other is accumulated in minimal quantities. While concentrations of both toxins in extreme conditions are quite different, at intermediate points the relative concentrations are more similar as can be observed in Table 1 for 0.88 a_w, 25°C and 28 d.

The ability to respond in a different way to environmental factors in relation to the biosynthesis of toxic secondary metabolites may aid survival of the fungus in a particular ecological niche allowing it to colonize the substrate more efficiently than other competitors.

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Editors' note

As the authors point out, the results of this work are unexpected, i.e. higher mycotoxin production at lower water activities. Of the explanations suggested by the authors, the most likely reason is limitation of oxygen during growth of the fungi, due to the use of Erlenmeyer flasks as experimental vessels. Oxygen limitation during growth of fungi in narrow necked flasks such as Erlenmeyers has long been known. Fungal growth under optimal conditions is characterised by very high oxygen consumption. The wide mouthed Fernbach flask (illustrated in Raper and Thom, *Manual of the Penicillia*, Williams and Wilkins, Baltimore, 1949, p. 91) was found to be superior for growth of fungi. Even with the use of wide mouthed flasks, care needs to be taken to use thin cotton wool closures, to permit the maximum possible diffusion of oxygen.

The view point that oxygen limitation produced these unusual results is supported by the data: at lower water activities where growth is slower, oxygen consumption is much lower, and sufficient for normal growth and mycotoxin production.