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Abiotic factors and their interactions influence on the co-production of aflatoxin B_1 and cyclopiazonic acid by *Aspergillus flavus* isolated from corn

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ABSTRACT

The objectives of this study were i) to determine the effects of the interactions of water activity, temperature and incubation time on the co-production of AFB_1 and CPA by isolates of *Aspergillus flavus* with different profile of mycotoxin production and ii) to identify the a_W and temperature limiting conditions for the production of both mycotoxins. Fungi used in this study were selected because they belonged to different chemotypes: chemotype I (AFB_1+/CPA+), III (AFB_1+/CPA−) and IV (AFB_1−/CPA+), respectively. Two culture media were used; Czapek yeast agar (CYA) and corn extract agar (CEM), at different incubated temperatures (10–40 °C) and a_W levels (0.80–0.98). AFB_1 and CPA production were analyzed after 7, 14, 21 and 28 days of incubation. Significant differences were observed with respect to mycotoxin production depending on the media evaluated. The AFB_1 production occurred more favorably on CYA while the highest CPA concentrations were recorded on CEM. Within the range of a_W evaluated in this study, 0.83 was the limiting level for both toxins production. The optimum conditions for AFB_1 production occurred at 0.96 a_W and 30 °C after 21 days of incubation, regardless of the media and isolate. Although different amounts of toxins were produced in each medium, the limiting and optimum conditions for their production were similar in both. No differences in the response of the three isolates to the abiotic factors discussed were observed despite belonging to different chemotypes. The determination of the thresholds of mycotoxins co-production, especially in the case of data obtained with the corn extract medium can be useful to avoid the conditions conducive to co-occurrence of these mycotoxins in corn.

1. Introduction

Aspergillus flavus is the predominant species of *Flavi* section associated to soil and vegetable products, mainly peanuts, maize and other crops in Argentina (Barros et al., 2003, 2005, 2006; Magnoli et al., 2006a,b; 2007a,b; Nepote et al., 1997; Nesci and Etcheverry, 2002; Novas and Cabral, 2002; Pildain et al., 2004, 2005; Resnik et al., 1996; Vaamonde and Varvsavsky, 1979; Vaamonde et al., 1995). *A. flavus* is one of the main producers of aflatoxins, highly toxic and carcinogenic compounds of concern in food safety (Horn and Dorner, 2001). Some *A. flavus* isolates are able to produce cyclopiazonic acid (CPA) in addition to aflatoxins (AFs) (Burdock and Flamm, 2000). Cyclopiazonic acid is an indole tetramic acid toxic to a variety of animals and has been implicated in human poisoning (Rao and Husain, 1985). Contamination of food commodities by *A. flavus* isolates capable to produce AFs and CPA simultaneously could result in the natural co-occurrence of these toxins as has been reported in previous studies (Lansden and Davidson, 1983; Martins and Martins, 1999; Vaamonde et al., 2003).

Cyclopiazonic acid effects could be masked by concurrent aflatoxicosis: for example CPA and AFs were isolated from peanut meal related to the turkey 'X' disease that caused the death of over 100,000 turkeys (Cole, 1986; Spensley, 1963). Although AFs were regarded as the main culprit, CPA likely contributed to some of the observed pathological clinical signs. Thus, the co-occurrence and possible toxic synergies between these two classes of mycotoxins are important to animal health and potentially to human food safety (Maragos, 2009).

Fernández Pinto et al. (2001) detected co-contamination with AFs and CPA in two out of 50 peanut samples analyzed in Argentina.

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Table 1
Chemotype patterns of *Aspergillus flavus* isolates based on aflatoxins and CPA production.

Isolates	Chemotype[a]	Mycotoxins				
		AFB_1	AFB_2	AFG_1	AFG_2	CPA
BAFC4274	I	+	−	−	−	+
BAFC4275	III	+	−	−	−	−
BAFC4273	IV	−	−	−	−	+

[a] According to Vaamonde et al., 2003.

The levels of these toxins found in the positive samples were 4300 and 493 μg kg^{-1} for CPA, 625 and 435 μg kg^{-1} for aflatoxin B_1 (AFB_1), and 625 and 83 μg kg^{-1} for aflatoxin G_1 (AFG_1), respectively. Another author, Amra (2009) obtained a higher percentage of positive samples but with lower levels of both mycotoxins from corn grown in Egypt: 75% of the samples were contaminated with both toxins with levels that ranged from 0.10 to 45.5 μg kg^{-1} and 1.2 to 56 μg kg^{-1} of AFs and CPA, respectively.

In view of the possible health hazards for animals and humans caused by the co-occurrence of AFs and CPA, the production of these toxins in agricultural commodities must be controlled. There are multiple factors involved in the development of *A. flavus* and in the biosynthesis of these secondary metabolites, such as humidity, temperature, presence of oxygen and carbon dioxide, development time, composition of the substrate, loss of integrity of the grains caused by insects or mechanical/thermal damage, fungal inoculum and the interaction between fungal species that share the same ecological niche. The effects of temperature and water activity (a_W) on aflatoxin production by *A. flavus* has been widely studied (Arrus et al., 2005; Ellis et al., 1993; Giorni et al., 2008; Molina and Giannuzzi, 2002; Ribeiro et al., 2006; Sanchis and Magan, 2004; Trenk and Hartman, 1970) but there is scarce information on the effect of these factors on the co-production of AFB_1 and CPA (Gqualeni et al., 1996, 1997; Vaamonde et al., 2006).

On the other hand, toxigenic isolates belonging to the same species differ in their toxin production profiles, ranging from nonproducing to highly producing of some, various or all the toxins registered for that species. Regarding *A. flavus*, considerable variability in their mycotoxin-producing potential has been found. Vaamonde et al. (2003) defined five *A. flavus* chemotypes based on their ability to produce aflatoxins of type B and G and CPA. Isolates able to produce simultaneously aflatoxins type B and CPA (chemotype I) are frequently detected in different substrates (Vaamonde et al., 2003; Resnik et al., 1996).

The objectives of this study were i) to determine the effects of the interactions of water activity, temperature and incubation time on the co-production of AFB_1 and CPA by isolates of *A. flavus* with different profile of mycotoxin production and ii) to identify the a_W and temperature limiting conditions for the production of both mycotoxins.

2. Materials and methods

2.1. Experimental design

A full factorial design was used in which five factors were assayed: isolate, media, water activity, temperature and incubation time. The water activity levels assayed were 0.83, 0.86, 0.90, 0.94, 0.96 and 0.98 and the incubation temperatures were 10, 15, 25, 30, 35 and 40 °C. Four replicates for each treatment were used. Two toxins (aflatoxin $B_!$ and cyclopiazonic acid) were measured at each condition after 7, 14, 21 and 28 days of incubation in two different culture media.

2.2. Fungal isolates

Fungi used in this study were isolated from corn used in the elaboration of poultry feeds (Astoreca et al., 2011). Based on their high capacity of aflatoxin B_1 and cyclopiazonic acid production, and considering the different chemotypes proposed by Vaamonde et al. (2003), three *A. flavus* isolates were selected each one belonging to chemotypes I (AFB_1+/CPA+), III (AFB_1+/CPA−) and IV (AFB_1−/CPA+). AFB_1 was the only aflatoxin produced by the isolates belonging to chemotypes I and III (Table 1).

2.3. Media

Czapek Yeast Agar (CYA) and a 3% corn extract (w/v) medium (CEM) were used in this study. The latter was made by boiling 30 g of corn in 1 L of distilled water for 45 min and filtering the resultant mixture through a double layer of muslin. The volume was made up to 1 L, and 1.5% of agar was added. The water activity of both media was modified by the addition of known amounts of glycerol to reach the desired a_W levels according to Dallyn and Fox (1980). The water activity of representative samples of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA). Additionally, control plates were prepared and measured at the end of the experiment in order to detect any significant deviation of a_W.

2.4. Inoculation and incubation conditions

The media for each treatment were centrally inoculated using 5 μl of a fungal spore suspension harvested from 7-day-old cultures on malt extract agar (MEA) using glycerol solutions adjusted to the a_W appropriate for each treatment. The suspensions were mixed and diluted to obtain a suspension of 10^6 spores ml^{-1} adjusted using a Thoma chamber. Inoculated Petri dishes of the same a_W were sealed in polyethylene bags. Four replicate plates per treatment were used and incubated at the selected temperatures for a maximum period of 28 days.

2.5. Mycotoxins extraction

Aflatoxin B_1 and cyclopiazonic acid production was analyzed after 7, 14, 21 and 28 days of incubation at each assayed medium, isolate, temperature and water activity. The methodology proposed by Bragulat et al. (2001) with some modifications was used in both cases. On each sampling occasion, three agar plugs were removed from inner, middle and outer points of the colony and extracted with 1 ml of chloroform for AFB_1 and methanol for CPA. The extract was centrifuged at 14,000 rpm during 10 min. The extracts were filtered (syringe nylon filtres, 0.45 μm, 13 mm, Advances Microdevices PVT, Ambala Cantt, India), evaporated to dryness and redissolved in methanol/water (50:50) to be analyzed by high performance liquid chromatography.

2.6. Mycotoxins quantification

The determination of AFB_1 and CPA was done using a Waters (Milford, MA, USA) chromatograph with a reverse-phase C_{18} silica gel column (Waters Spherisorb 3 μm ODS2 4.6 × 150 mm, Milford, MA, USA), followed by fluorescence detection (Waters 2475 fluorescence detector, Waters, Milford, MA, USA). Post-column derivatization was achieved by using a photochemical reactor for enhanced detection (PHRED) (LCTech UVE, Dorfen, Germany). For confirmation of the presence of both mycotoxins, the PHRED was switched off and the peak decreases were registered. Limit of detection (LOD) was defined as the system limit of detection for pure standard and limit of quantification (LOQ) was determined

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from a spiked sample. The estimated limit of detection (LOD) and limit of quantification (LOQ) for aflatoxin B_1 were 0.005 and 0.010 μg/g and 0.75 and 1.0 μg/g for cyclopiazonic acid, respectively. The column oven was set at 30 °C.

For AFB_1 determination, excitation and emission wavelengths were set at 333 and 460 nm, respectively. An isocratic mobile phase of water/acetonitrile/methanol (70:17:17, v/v/v) was used with a flow rate of 1.0 ml min^{-1} (AOAC International, 1995b).

For CPA determination, the mobile phase was pumped at 0.8 ml min^{-1} and consisted of an isocratic programmed as follows: acetonitrile/4 mM zinc sulfate (65:35, v/v) pH 5. An injection volume of 100 μl was used for both mycotoxins (Da Motta and Valente Soares, 2000).

2.7. Statistical analysis

Aflatoxin B_1 and cyclopiazonic acid production by the three *A. flavus* isolates were analyzed statistically using PROC GLM in SAS program (SAS Institute Inc., Cary, NC, USA) by means of ANOVA. Means were compared by Fisher LSD test to determine the significant differences among the different treatments assayed (Quinn and Keough, 2002). Finally, Spearman rank correlation coefficient was calculated in order to test the significance of the correlation between aflatoxin B_1 levels produced by different isolates, CPA levels produced by different isolates and aflatoxin B_1 and CPA levels produced simultaneously by the co-producing isolate.

3. Results

3.1. Effect of water activity, temperature, media and incubation time on aflatoxin B_1 production

Tables 2 and 3 shows the AFB_1 production by BAFC4274 and BAFC4275 isolates on both media at different water activity, temperature and incubation time.

The evaluation of the results by ANOVA showed that the factors a_W, temperature, medium and incubation time were statistically significant in relation to the AFB_1 production by the *A. flavus* isolates analysed ($P < 0.0001$).

The AFB_1 production occurred more favourably on CYA than on CEM for both isolates; in most environmental condition assayed, the toxin amounts detected in the former were significantly higher than in the latter ($P < 0.0001$).

AFB_1 was produced over the temperature range from 15 to 35 °C and a_W range from 0.86 to 0.98. This metabolite was not detected at 10 or 40 °C throughout the range of a_W tested. At 15 °C and $a_W \geq 0.94$, AFB_1 was produced only on CYA medium. Within the range of a_W evaluated in this study, 0.83 could be considered as the limiting value for AFB_1 production since at this a_W level only low amounts were detected during the incubation period at 30 °C (0.01 and 0.77 μg g^{-1} by BAFC4274 and BAFC4275 isolates, respectively).

In general, 7 days of incubation were sufficient for AFB_1 detection except at marginal conditions. Maximum AFB_1 amounts were detected at different days of incubation depending on the isolates, media and environmental conditions (Tables 2 and 3).

The conditions under which equivalent AFB_1 concentrations occurred were joined to produce contour lines and draw a map of the relative optimum and marginal AFB_1 production by the *A. flavus* isolates. As seen in Fig. 1, the two isolates showed very similar contour maps for production which represent conditions of similar production levels in each media. The optimum conditions for AFB_1 production occurred at 0.96 a_W and 30 °C after 21 days of incubation, for both isolates regardless the medium. On CYA the maximum toxin concentrations were 500.9 and 460.2 μg g^{-1} by BAFC4274 and BAFC4275 isolates, while on CEM the levels detected were significantly lower reaching 2.99 and 0.63 μg g^{-1} by BAFC4274 and BAFC4275 isolates, respectively (see Tables 2 and 3).

3.2. Effect of water activity, temperature, media and incubation time on cyclopiazonic acid production

The CPA production by BAFC4273 and BAFC4274 isolates on both media at different water activity, temperature and incubation time is shown in Tables 4 and 5, respectively. Analysis of variance

Table 2
Aflatoxin B_1 concentration (μg g^{-1}) ±SD produced by BAFC4274 isolate on CYA and corn extract media (CEM) at each temperature, water activity and incubation time assayed.

a_W	Days	CYA				CEM			
		15 °C	25 °C	30 °C	35 °C	15 °C	25 °C	30 °C	35 °C
0.83	7	ND	ND	ND	ND	ND	ND	ND	ND
	14	ND	ND	ND	ND	ND	ND	ND	ND
	21	ND	ND	0.01 ± 0.00	ND	ND	ND	ND	ND
	28	ND	ND	ND	ND	ND	ND	ND	ND
0.86	7	ND	ND	ND	ND	ND	ND	ND	ND
	14	ND	0.07 ± 0.02	0.02 ± 0.00	0.01 ± 0.00	ND	ND	ND	ND
	21	ND	0.03 ± 0.00	0.02 ± 0.00	ND	ND	ND	0.02 ± 0.00	0.02 ± 0.02
	28	ND	0.16 ± 0.06	0.04 ± 0.00	ND	ND	ND	ND	ND
0.90	7	ND	5.74 ± 1.20	25.71 ± 1.65	0.13 ± 0.08	ND	0.01 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
	14	ND	18.05 ± 3.63	39.85 ± 12.10	0.02 ± 0.00	ND	ND	0.02 ± 0.00	0.06 ± 0.00
	21	ND	11.75 ± 4.88	18.54 ± 4.13	0.08 ± 0.05	ND	0.02 ± 0.00	0.12 ± 0.02	0.01 ± 0.00
	28	ND	48.79 ± 14.61	22.43 ± 13.64	0.02 ± 0.00	ND	0.02 ± 0.00	ND	ND
0.94	7	ND	26.63 ± 2.38	39.31 ± 2.83	2.11 ± 0.73	ND	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00
	14	ND	37.01 ± 10.89	21.72 ± 8.05	0.02 ± 0.00	ND	0.19 ± 0.00	0.04 ± 0.00	0.03 ± 0.01
	21	0.03 ± 0.01	43.25 ± 6.63	50.96 ± 8.51	0.05 ± 0.00	ND	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
	28	ND	48.22 ± 11.75	11.84 ± 3.55	0.06 ± 0.00	ND	0.03 ± 0.00	0.03 ± 0.00	ND
0.96	7	ND	79.64 ± 14.38	243.61 ± 10.00	14.17 ± 1.98	ND	0.04 ± 0.00	0.06 ± 0.01	0.05 ± 0.04
	14	0.10 ± 0.08	129.20 ± 24.63	331.91 ± 85.77	128.93 ± 8.05	ND	0.13 ± 0.00	0.54 ± 0.06	0.40 ± 0.00
	21	0.18 ± 0.04	94.58 ± 24.44	500.92 ± 80.93	ND	ND	0.94 ± 0.24	2.99 ± 1.30	0.26 ± 0.00
	28	0.03 ± 0.00	42.13 ± 17.76	140.19 ± 23.16	ND	ND	0.02 ± 0.00	ND	ND
0.98	7	ND	115.60 ± 51.74	140.16 ± 13.67	42.91 ± 7.63	ND	0.01 ± 0.00	0.04 ± 0.00	0.02 ± 0.00
	14	0.41 ± 0.11	141.62 ± 9.74	255.43 ± 28.80	44.45 ± 19.09	ND	0.04 ± 0.00	0.02 ± 0.00	0.19 ± 0.06
	21	1.01 ± 0.00	91.86 ± 9.70	21.38 ± 8.90	61.28 ± 7.17	ND	0.03 ± 0.00	ND	0.02 ± 0.00
	28	0.22 ± 0.05	90.61 ± 8.52	13.94 ± 0.15	ND	ND	0.02 ± 0.00	0.02 ± 0.00	0.17 ± 0.06

SD: standard deviation; ND: not detected.

Table 3
Aflatoxin B_1 concentration (μg g^{-1}) ±SD produced by *A. flavus* BAFC4275 isolate on CYA and corn extract media (CEM) at each temperature, water activity and incubation time assayed.

a_W	Days	CYA				CEM			
		15	25	30	35	15	25	30	35
0.83	7	ND	ND	ND	ND	ND	ND	ND	ND
	14	ND	ND	ND	ND	ND	ND	ND	ND
	21	ND	ND	0.77 ± 0.00	ND	ND	ND	ND	ND
	28	ND	ND	0.49 ± 0.32	ND	ND	ND	ND	ND
0.86	7	ND	ND	ND	ND	ND	ND	ND	ND
	14	ND	1.00 ± 0.70	0.65 ± 0.28	0.09 ± 0.04	ND	ND	ND	ND
	21	ND	0.03 ± 0.00	0.12 ± 0.04	4.25 ± 3.78	ND	0.05 ± 0.02	0.04 ± 0.00	0.02 ± 0.00
	28	ND	0.60 ± 0.10	ND	9.71 ± 1.05	ND	ND	ND	ND
0.90	7	ND	26.35 ± 0.00	23.54 ± 8.60	0.03 ± 0.00	ND	ND	ND	0.01 ± 0.00
	14	ND	21.11 ± 10.26	33.32 ± 9.66	0.04 ± 0.00	ND	ND	0.03 ± 0.00	0.01 ± 0.00
	21	ND	4.96 ± 2.82	51.35 ± 10.29	0.04 ± 0.00	ND	6.37 ± 2.80	0.03 ± 0.02	0.04 ± 0.01
	28	ND	5.92 ± 1.81	11.34 ± 3.69	0.02 ± 0.00	ND	ND	ND	ND
0.94	7	ND	10.08 ± 1.55	11.31 ± 3.32	0.01 ± 0.01	ND	0.02 ± 0.00	0.05 ± 0.02	0.02 ± 0.00
	14	ND	46.97 ± 1.44	7.31 ± 1.55	0.48 ± 0.11	ND	0.05 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
	21	0.02 ± 0.01	ND	4.99 ± 1.13	0.03 ± 0.01	ND	ND	0.07 ± 0.03	0.05 ± 0.00
	28	0.03 ± 0.00	0.07 ± 0.01	27.91 ± 0.00	0.01 ± 0.00	ND	ND	ND	0.01 ± 0.00
0.96	7	ND	19.37 ± 7.47	172.03 ± 63.56	1.90 ± 1.00	ND	0.04 ± 0.03	0.11 ± 0.03	ND
	14	ND	166.60 ± 6.50	361.35 ± 12.30	20.40 ± 2.99	ND	0.06 ± 0.05	0.26 ± 0.03	0.03 ± 0.00
	21	0.84 ± 0.16	86.21 ± 12.40	460.20 ± 15.89	11.37 ± 10.40	ND	0.05 ± 0.00	0.63 ± 0.09	0.11 ± 0.00
	28	0.28 ± 0.07	24.71 ± 9.56	ND	19.65 ± 3.14	ND	ND	0.36 ± 0.00	0.17 ± 0.00
0.98	7	ND	0.01 ± 0.00	185.75 ± 9.83	5.34 ± 1.13	ND	0.03 ± 0.00	0.08 ± 0.02	0.01 ± 0.00
	14	ND	286.63 ± 28.45	130.91 ± 17.45	7.16 ± 0.00	ND	0.04 ± 0.00	0.05 ± 0.03	0.02 ± 0.00
	21	1.29 ± 0.25	255.22 ± 21.20	256.19 ± 49.83	6.44 ± 2.45	ND	0.03 ± 0.00	0.06 ± 0.01	0.03 ± 0.00
	28	8.36 ± 1.10	160.7 ± 77.33	ND	4.19 ± 2.86	ND	0.03 ± 0.00	ND	0.02 ± 0.00

SD: standard deviation; ND: not detected.

revealed that the factors water activity, temperature, medium and incubation time had a significant influence on CPA production by both isolates ($P < 0.0001$).

In contrast to the results obtained for AFB_1, the highest CPA concentrations were recorded on CEM for both isolates.

CPA was produced over the temperature range from 15 to 35 °C and a_W range from 0.86 to 0.98. At 15 °C and $a_W \geq 0.96$, CPA was produced on both medium by both isolates although the BAFC4273 was also able to produce low amounts on CYA at this temperature and 0.94 a_W. CPA was not detected at a_W 0.83 regardless neither the media nor isolate.

Data obtained for the two CPA producers (BAFC4273 and BAFC4274 isolates) were used to develop contour maps in order to identify the influence of a_W and temperature interactions on CPA production (Fig. 2). The maximum CPA amounts were registered on CEM at 0.96 a_W and 30–35 °C for BAFC4273 isolate and 0.98 a_W at 30 °C for BAFC4274 isolate, although this isolate produced similar concentration at 0.96 a_W/35 °C. The reached CPA concentrations at these optimum conditions were 1255 and 1091 μg g^{-1} of CPA for BAFC4273 and BAFC4274, respectively. In general, CPA concentrations decreased as a_W decreased.

The conditions for optimal CPA production by both isolates on CYA were different to those observed on CEM: the maximum CPA concentrations on CYA were achieved at 0.98 a_W/30 °C for BAFC4273 and 0.96 a_W/35 °C for BAFC4274 isolate (327 and 250 μg g^{-1} of CPA, respectively) (Tables 4 and 5).

In general, CPA production increased with incubation time for both *A. flavus* assayed showing the maximal production at 28 days of incubation; however, in some combinations of environmental conditions the maximum concentration was observed at 7 days of incubation (e.g. 0.94 a_W/25–35 °C and 0.98 a_W/30 °C for BAFC4273 on CYA).

3.3. Correlations between AFs and CPA production

A significative correlation between AFB_1 production by the two aflatoxigenic isolates was observed in both media with a Spearman coefficient of 0.72 for CYA and 0.57 for CEM. A higher correlation was found for CPA production by BAFC4273 and BAFC4274 isolates in CYA (Spearman coefficient = 0.92) and CEM (Spearman coefficient = 0.96). In both cases this confirmed that the conditions leading to either maximum levels of AF or CPA were very similar for both producing isolates.

As regard to the correlation between AF and CPA production by BAFC4274 isolate, the correlation between both toxin levels was weak, suggesting that maximum production of one of the toxins did not take place under the same conditions as the other toxin.

4. Discussion

Several previous works have pointed out that the obtained results on mycotoxins production on culture media cannot be directly extrapolated to the natural substrates (Bellí et al., 2004; Comerio et al., 1998; Garcia et al., 2011; Pardo et al., 2004). However, in view of the results of the present work, it could be suggested that corn extract added to CEM enhanced CPA production and diminished AFB_1 production and this effect was observed with all the studied isolates. Although different amounts of toxins were produced, the limiting and optimal conditions for their production were similar in both media.

Regardless of the influence of the substrate, the behavior of both aflatoxigenic isolates in relation to AFB_1 production was the same, since they presented identical limiting values and combinations of a_W, temperature and incubation time for maximum production. Optimum temperature for AFB_1 production obtained in this study was 30 °C confirming previous studies on aflatoxin production by *A. flavus* isolates (Abdel Hadi et al., 2012; Giorni et al., 2007, 2011; Klich, 2007; Mousa et al., 2011; Schindler et al, 1967). In the conditions of the present study, this toxin was optimally produced at 0.96 a_W, although 0.98 was also a favorable a_W level. It is generally accepted that AFB_1 production decreases with decreasing a_W. Limiting a_W value for AFB_1 production in the present work is also coincident with those reported by other authors since this toxin was not detected at 0.83

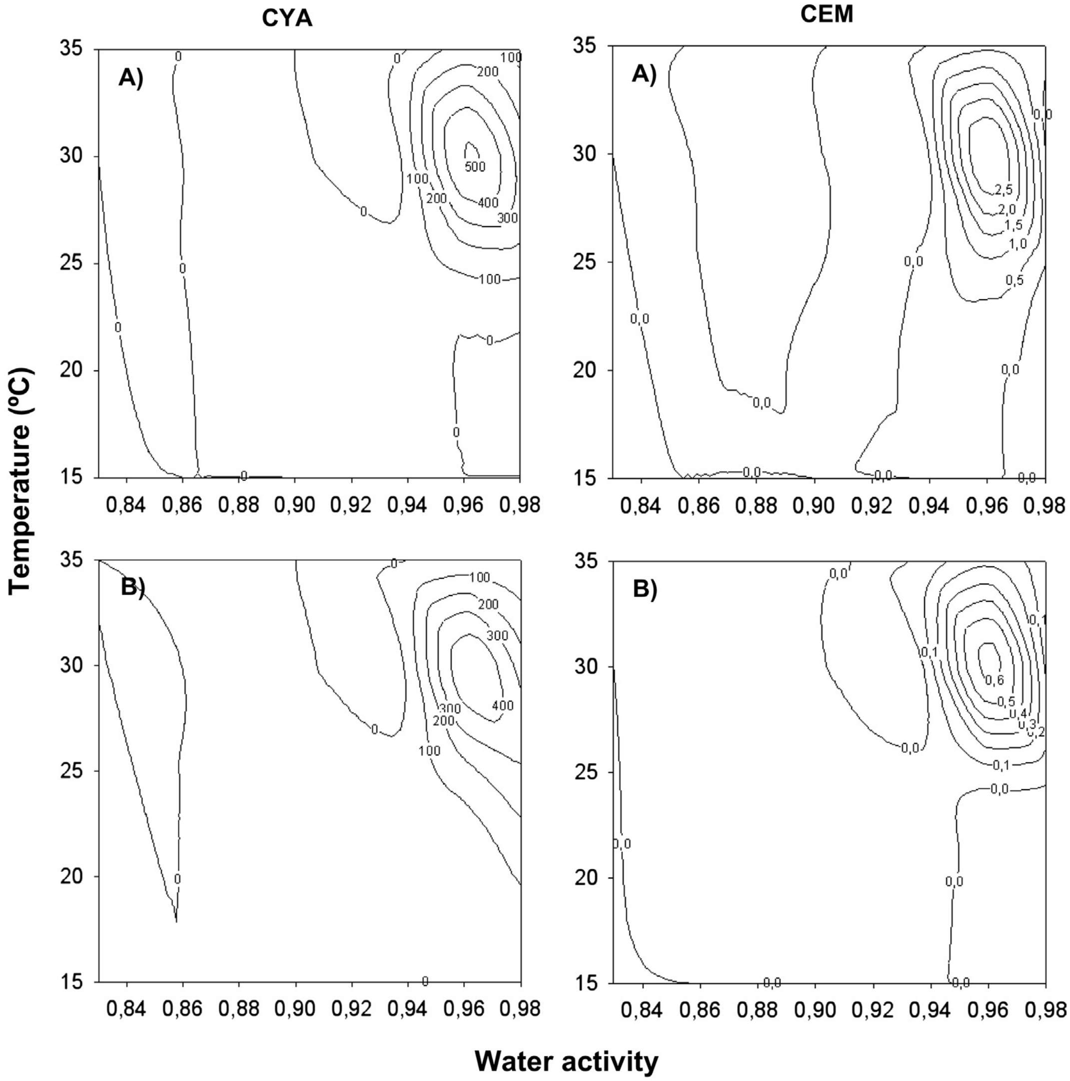

Fig. 1. Two dimensional contour maps of aflatoxin B_1 (AFB_1) production profiles of *Aspergillus flavus* isolates: A) BAFC4274 and B) BAFC4275 from corn in relation to temperature and water activity on CYA and CEM media. The numbers on the contour lines refer to mean AFB_1 concentrations (μg g^{-1}).

a_W and relatively low amounts were produced at 0.86 a_W in the range of favorable temperatures (25–30 °C). Similar results were obtained by Pitt and Miscamble (1995), Gqaleni et al. (2007) who reported minimal a_W values for AF production by *A. flavus* from 0.83 to 0.87.

Few published studies have examined how environmental factors can affect simultaneous production of two or more mycotoxins by a single isolate. Gqaleni et al. (1997) reported the interaction of temperature, a_W and time in determining the production of aflatoxins and CPA by a co-producing isolate of *A. flavus* on two agar media. They found that these toxins were not affected in the same way as they had different optimum temperature and minimum a_W values for production. In the present study limiting a_W and temperature values were the same for both toxins but conditions for maximum CPA production were slightly different from those observed for AF production. Although maximum CPA production was detected at different a_W, temperature and incubation time for each isolate, it can be concluded that optimum conditions for CPA production involve higher a_W levels (0.96–0.98) and temperatures (30–35 °C). These results are in contrast with those obtained by Gqaleni et al. (1996) who reported that 20 °C was a more favorable temperature than 25 or 30 °C for CPA production in culture medium. Vaamonde et al. (2006) reported maximum accumulation of CPA in peanuts at 25 °C but also a considerable production was detected at the lowest temperature studied (20 °C) and high a_W. Influence of temperature on CPA production by *Penicillium* species was also studied. Le Bars (1979) found optimal temperature of 25 °C for CPA production by *P. cammemberti* in Czapek agar. Gqaleni et al. (1996) reported that high a_W and low temperature favored high CPA production by *Penicillium commune*. However, Sosa et al. (2002) found maximum CPA production by the same species at 20 °C and 0.90 a_W in a medium based on meat extract.

According to our results it can be concluded that changes in temperature from 25 to 30 °C leads to no statistically differences in the maximum AFB_1 production and the same effect was observed for CPA production in the range from 30 to 35 °C. In contrast, water availability seems to be the more important factor in determining contamination levels with both toxins.

No differences in the response of the three isolates to the abiotic factors discussed were observed despite belonging to different chemotypes. The statistical analyses reflect that neither the isolate nor its interactions with other factors had a significant influence on

Table 4
Cyclopiazonic acid concentration (μg g^{-1}) ±standard deviation (SD) produced by *A. flavus* BAFC4273 isolate on CYA and corn extract media (CEM) at each temperature, water activity and incubation time assayed.

a_W	Days	CYA				CEM			
		15 °C	25 °C	30 °C	35 °C	15 °C	25 °C	30 °C	35 °C
0.86	7	ND	ND	ND	ND	ND	ND	ND	ND
	14	ND	1.41 ± 0.71	10.20 ± 2.30	10.57 ± 3.45	ND	ND	ND	ND
	21	ND	4.37 ± 1.96	16.91 ± 4.01	9.41 ± 2.42	ND	ND	121.46 ± 15.19	ND
	28	ND	8.30 ± 2.90	87.89 ± 12.61	51.00 ± 3.37	ND	29.39 ± 10.18	142.83 ± 62.40	104.78 ± 70.12
0.90	7	ND	2.35 ± 0.97	95.92 ± 16.80	82.02 ± 1.19	ND	ND	ND	ND
	14	ND	1.96 ± 0.28	7.36 ± 2.37	13.64 ± 6.58	ND	45.77 ± 6.21	69.34 ± 30.24	63.53 ± 15.48
	21	ND	7.44 ± 2.32	26.04 ± 9.30	21.19 ± 7.95	ND	47.72 ± 12.62	77.88 ± 26.29	138.89 ± 10.83
	28	ND	21.22 ± 3.41	15.49 ± 8.59	246.14 ± 41.65	ND	117.72 ± 28.74	131.63 ± 41.54	116.15 ± 21.25
0.94	7	ND	98.23 ± 14.26	272.27 ± 19.74	227.52 ± 72.07	ND	32.91 ± 10.62	104.20 ± 37.87	60.17 ± 6.10
	14	ND	6.47 ± 2.44	9.76 ± 5.01	22.22 ± 10.13	ND	163.58 ± 43.07	170.52 ± 74.65	138.41 ± 61.41
	21	1.24 ± 0.69	16.39 ± 4.84	25.87 ± 6.07	51.23 ± 11.43	ND	154.12 ± 16.83	397.48 ± 50.17	373.14 ± 76.23
	28	2.55 ± 1.07	19.46 ± 8.52	49.54 ± 9.29	115.52 ± 36.95	ND	255.91 ± 75.37	355.92 ± 61.61	126.62 ± 53.47
0.96	7	ND	96.20 ± 23.33	76.35 ± 26.55	174.61 ± 15.30	ND	52.03 ± 11.79	115.13 ± 74.87	81.88 ± 44.76
	14	6.39 ± 1.05	163.57 ± 34.05	24.72 ± 7.08	31.90 ± 10.95	ND	215.30 ± 90.31	194.89 ± 76.01	207.48 ± 70.20
	21	8.46 ± 2.66	25.62 ± 7.89	40.75 ± 11.84	48.62 ± 13.57	84.95 ± 24.95	264.95 ± 60.22	382.19 ± 17.95	584.51 ± 271.82
	28	11.53 ± 1.04	34.61 ± 7.49	86.17 ± 22.56	61.41 ± 16.38	86.69 ± 15.50	451.09 ± 71.67	1136.62 ± 82.70	1255.64 ± 280.23
0.98	7	ND	71.32 ± 6.12	327.11 ± 82.12	168.53 ± 8.91	ND	92.25 ± 0.04	132.97 ± 31.55	119.73 ± 47.96
	14	7.92 ± 1.39	16.77 ± 6.50	129.14 ± 17.80	26.87 ± 3.49	ND	421.20 ± 47.51	397.37 ± 33.25	275.17 ± 82.68
	21	9.63 ± 2.00	26.47 ± 12.09	46.28 ± 12.91	52.17 ± 12.24	77.04 ± 23.49	359.10 ± 70.27	540.59 ± 52.83	485.58 ± 94.65
	28	14.67 ± 3.25	124.01 ± 17.22	57.59 ± 16.28	201.94 ± 32.72	155.17 ± 48.86	616.75 ± 61.29	986.49 ± 57.50	614.06 ± 71.79

ND: not detected.

both mycotoxins production. Besides, the contour maps obtained from aflatoxigenic and CPA-producing isolates support this observation. The correlation analysis showed that each couple of isolates compared produced the toxins under similar conditions. Interestingly, in some combinations of environmental conditions in which the co-producing *A. flavus* isolate (BAFC4274) produced the highest concentration of CPA, AFB_1 was not detected (e.g. 0.96 and 0.98 a_W at 35 °C after 28 days of incubation in CYA and 0.96 a_W at 35 °C after 28 days of incubation in CEM). This point was supported by the correlation analysis.

The production of AFB_1 and CPA by *A. flavus* isolates from corn at the a_W and temperature combinations investigated suggests that their occurrence in this cereal could present particular risks to humans and animals, mainly in tropical and subtropical countries. Safe storage of corn is a major problem in these regions of the world. The determination of the thresholds of mycotoxins co-production especially in the case of data obtained with the corn extract medium can be useful to design control strategies of these mycotoxins. Results of this study demonstrated that AFB_1 and CPA production is inhibited at the same limiting levels of a_W and temperature. It can be concluded that conditions usually recommended for safe storage of corn as regard to aflatoxins contamination, particularly a_W lower than 0.85, would be also appropriate to protect this crop from CPA contamination. Close attention must be paid to the proper drying of grains at harvest followed by dry and cold storage.

Table 5
Cyclopiazonic acid concentration (μg g^{-1}) ±standard deviation (SD) produced by *A. flavus* BAFC4274 isolate on CYA and corn extract media (CEM) at each temperature, water activity and incubation time assayed.

a_W	Days	CYA				CEM			
		15 °C	25 °C	30 °C	35 °C	15 °C	25 °C	30 °C	35 °C
0.86	7	ND	ND	ND	ND	ND	ND	ND	ND
	14	ND	19.53 ± 5.46	4.47 ± 2.58	10.46 ± 1.01	ND	ND	ND	ND
	21	ND	3.58 ± 1.54	13.29 ± 1.71	20.76 ± 6.38	ND	ND	30.47 ± 12.65	ND
	28	ND	2.92 ± 1.68	25.82 ± 4.61	85.15 ± 23.29	ND	ND	72.74 ± 41.10	22.71 ± 9.53
0.90	7	ND	1.64 ± 0.07	21.03 ± 2.19	5.34 ± 1.89	ND	ND	ND	ND
	14	ND	4.88 ± 1.52	16.95 ± 2.63	14.38 ± 4.33	ND	45.32 ± 11.60	89.16 ± 7.26	39.00 ± 5.79
	21	ND	16.81 ± 2.66	36.64 ± 13.99	55.75 ± 12.21	ND	33.97 ± 15.85	145.17 ± 22.74	55.65 ± 11.10
	28	ND	14.09 ± 3.29	144.55 ± 15.45	157.76 ± 22.64	ND	105.6 ± 18.58	159.90 ± 11.65	200.02 ± 44.47
0.94	7	ND	7.20 ± 3.87	22.40 ± 6.04	8.88 ± 2.50	ND	27.14 ± 7.78	50.65 ± 2.95	38.45 ± 3.17
	14	ND	33.52 ± 1.37	46.68 ± 4.91	29.60 ± 3.65	ND	87.81 ± 3.48	82.10 ± 7.46	144.61 ± 43.18
	21	ND	35.82 ± 6.12	73.16 ± 8.71	62.85 ± 11.83	ND	113.79 ± 4.56	322.37 ± 11.09	249.88 ± 44.14
	28	ND	50.67 ± 6.85	154.85 ± 14.66	88.56 ± 18.43	ND	230.07 ± 9.21	497.86 ± 21.95	149.95 ± 25.88
0.96	7	ND	24.77 ± 8.12	26.67 ± 1.81	17.77 ± 4.88	ND	32.97 ± 7.42	88.52 ± 14.56	78.39±30.50
	14	ND	41.43 ± 5.00	66.60 ± 12.66	66.95 ± 11.92	ND	126.17 ± 6.13	194.14 ± 20.79	319.50 ± 38.38
	21	17.19 ± 6.82	56.18 ± 9.52	117.83 ± 17.12	102.47 ± 34.04	14.67 ± 3.69	177.38 ± 22.38	521.15 ± 18.82	597.10 ± 35.48
	28	21.39 ± 4.12	117.38 ± 26.73	80.82 ± 10.54	249.03 ± 19.11	92.90 ± 8.44	213.71 ± 37.53	724.96 ± 21.67	1044.75 ± 66.52
0.98	7	ND	18.94 ± 3.87	41.55 ± 5.63	14.81 ± 1.50	ND	55.95 ± 7.36	87.46 ± 22.06	96.25 ± 21.25
	14	ND	46.91 ± 1.65	39.99 ± 9.85	26.48 ± 5.69	ND	253.09 ± 19.52	243.34 ± 23.02	301.40 ± 18.22
	21	15.38 ± 1.72	47.57 ± 5.39	80.47 ± 8.77	119.59 ± 24.90	37.11 ± 7.78	232.36 ± 11.30	751.72 ± 18.11	587.84 ± 70.45
	28	16.80 ± 7.67	42.18 ± 2.69	174.64 ± 26.01	209.80 ± 8.71	54.03 ± 8.02	286.78 ± 25.16	1091.77 ± 32.98	519.38 ± 32.23

ND: not detected.

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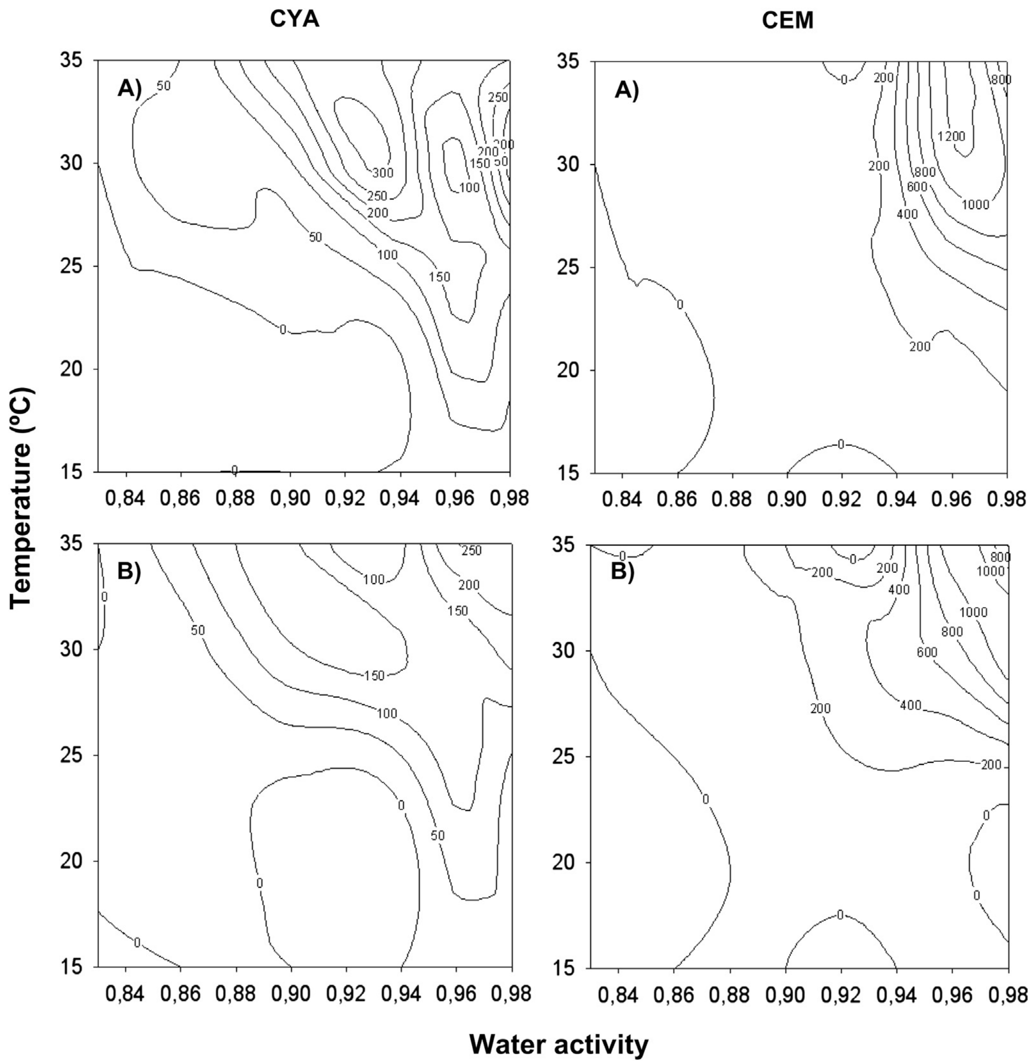

Fig. 2. Two dimensional contour maps of cyclopiazonic acid (CPA) production profiles of *Aspergillus flavus* isolates: A) BAFC4273 and B) BAFC4274 from corn in relation to temperature and water activity on CYA and CEM media. The numbers on the contour lines refer to mean CPA concentrations (μg g^{-1}).

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