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The production of cyclopiazonic acid by *Penicillium commune* and cyclopiazonic acid and aflatoxins by *Aspergillus flavus* as affected by water activity and temperature on maize grains

N. Gqaleni[1,4], J.E. Smith[1], J. Lacey[2] and G. Gettinby[3]
[1]*Department of Bioscience and Biotechnology, University of Strathclyde, 204 George Street, Glasgow G1 1XW;* [2] *IACR-Rothamsted, Harpenden, Herts, AL5 2JQ;* [3]*Department of Statistics and Modelling Science, University of Strathclyde, 26 Richmond Street, Glasgow G1 1XH, U.K.* [4]*Present address: Department of Physiology, Medical School, University of Natal, Private Bag 7, Congella 4013, South Africa.*

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Abstract

The combined effects of water activity (a_w) and temperature on mycotoxin production by *Penicilium commune* (cyclopiazonic acid – CPA) and *Aspergillus flavus* (CPA and aflatoxins – AF) were studied on maize over a 14-day period using a statistical experimental design. Analysis of variance showed a highly significant interaction ($P \leq 0.001$) between these factors and mycotoxin production. The minimum a_w/temperature for CPA production (2264 ng g^{-1} *P. commune*, 709 ng g^{-1} *A. flavus*) was 0.90 a_w/30 °C while greatest production (7678 ng g^{-1} *P. commune*, 1876 ng g^{-1} *A. flavus*) was produced at 0.98 a_w/20 °C. Least AF (411 ng g^{-1}) was produced at 0.90 a_w/20 °C and most (3096 ng g^{-1}) at 0.98 a_w/30 °C.

Key words: Aflatoxins, cyclopiazonic acid, *Penicillium commune*, *Aspergillus flavus*, maize, factorial design

Introduction

The mycoflora of stored cereals commonly includes *Aspergillus flavus* and several species of *Penicillium* and *Fusarium* [1, 2, 3]. Some of these fungi produce mycotoxins that are detrimental to human and animal health. Cyclopiazonic acid (CPA) is unique in that it is the only mycotoxin that affects muscle tissue in chickens and other animals [4–6]. CPA, produced by *P. commune*, is being increasingly reported from foods and feeds [7–10]. One reason for this is the reclassification of all isolates earlier reported to produce CPA, i.e. *P. cyclopium* (*P. aurantiogriseum*), *P. puberulum*, and *P. viridicatum*, as *P. commune* [8, 11, 12]. This has made *P. commune* the predominant source of CPA among the penicillia [10].

Isolates of *A. flavus* have also been increasingly reported to produce CPA together with aflatoxins (AF) [13–16]. AF are toxic, carcinogenic, and probably immunosuppressive metabolites [17, 18]. However, it is interesting to note that *A. parasiticus*, which also produces AF, does not produce CPA [19].

AF and CPA have been found together as contaminants in different foodstuffs [20–22], and have been shown to cause health problems in animals and humans resulting in economic losses [17, 18, 21, 23]. It is, therefore, important that production of these toxins is controlled. To achieve this, it is necessary to understand fully the most important environmental factors affecting production and their interactions.

Temperature and water activity (a_w) are critical environmental factors affecting the production of mycotoxins [24–26]. The effects of temperature and a_w on aflatoxin production by *A. flavus* in maize have been determined and optimal production has been shown to occur at 30 °C and 0.98 a_w [20, 30]. The optimum temperature for CPA production by *Penicillium* isolates is 25 °C [20, 29], but little is known of the effect of a_w on CPA production. More information is required on the interaction between temperature and a_w on CPA production by *P. commune* and *A. flavus*.

Statistically valid experimental designs are being used increasingly in mycotoxin studies [31–33]. Factorial designs have been used particularly where the

magnitude of effects from changing the level of one factor depends on the levels of other factors used in treatment combinations [32]. This study demonstrates the effect of interaction between temperature and a_w on the co-production of aflatoxins and cyclopiazonic acid by an isolate of *A. flavus* and cyclopiazonic acid production by *P. commune* on maize using a full factorial designed experiment.

Materials and methods

Experimental design

To determine the effects of factors alone or in combination, a central composite design (full factorial design augmented by a central point) was used [34, 35]. Using this design, the factors were set at three levels, coded −1, 0, +1. The three temperatures (20, 25 and 30 °C) and three water activities (0.9, 0.95 and 0.98 a_w, corresponding to final water contents in maize of 20.5, 23.5 and 26.6%) used in this study, were based on previous studies with *A. flavus* [29, 32]. Measurements for all combinations of factors were carried out in triplicate.

Organisms and culture

Penicillium commune had previously been isolated from damp, mouldy dwellings in Scotland and shown to be cytotoxic to human cell lines [36]. The culture is retained in the departmental culture collection at the University of Strathclyde. *Aspergillus flavus* F2R4FP 1-5 was kindly supplied by Prof. R.J. Cole (Natural Peanut Research Laboratory, Dawson, U.S.A.) and known to co-produce AF and CPA. Stock cultures were maintained on malt extract agar slopes at 4 °C until required. Conidial suspensions were freshly prepared in sterile glycerol-water solution containing 0.1% Tween 80, with a_w adjusted to the same a_w as that of the maize culture following the method of Gervais *et al.* [37]. The a_w of representative samples of glycerol-water solutions and maize were measured with a dew point meter (Protimeter Ltd., U.K.). Concentrations of conidia were adjusted to 10^6 ml^{-1} using a Neubauer haemocytometer.

Conidial suspension (1 ml) was added to 50 g autoclaved yellow hybrid maize grain, previously adjusted to appropriate a_w by the method described by Pixton and Warburton [38], in 500-ml Erlenmeyer flasks. The maize was kindly supplied by Dr. Raul Cuero (Prairie View A & M University, Prairie View, Texas) and shown to be free of AF and CPA. The inoculated maize was then transferred to Valmic® microporous bags (20 × 14 cm, 0.3 μm diam. pore size, Van Leer, U.K., Ltd.) and grown for 14 days in Fisons Environmental Cabinets, with regular shaking to ensure complete mycelial colonisation [39].

Extraction and analysis of cyclopiazonic acid and aflatoxins

Maize cultures were extracted following the method for multimycotoxin analysis of Gorst-Allman and Steyn [40] using dichloromethane instead of chloroform. The dichloromethane extract containing AF and the aqueous layer containing CPA were separately rotary evaporated and concentrated under a gentle stream of nitrogen. The concentrated mycotoxins were stored in coloured vials at 4 °C until required.

The presence of AFB_1, AFB_2 and CPA was determined by thin layer chromatographic (TLC) separation on silica gel G60 plates (20 × 20 cm Merck). The plates were first dipped in a 10% (w/v) solution of oxalic acid in methanol for 2 min and after heating at 110 °C for 2 min and cooling, the plates were spotted with 50 μl of the respective extract and developed with a toluene : ethyl acetate : dichloromethane : formic acid (70 : 50 : 50 : 20) solvent system [41]. The developed plates were viewed under long wave UV light (366 nm). AF fluoresced blue while CPA developed a purple colour after spraying with Ehrlich's reagent (2.0 g *p*-dimethylaminobenzaldehyde in 100 ml HCl).

Quantitative determination of AF was achieved by pooling the fluorescent AF spots from the TLC plates and measuring their total fluorescence using a modified fluorometric determination method [42] with a Seqouia-Turner 450 digital fluorometer (360 nm excitation and 450 nm emission) after reacting 1 ml of sample with 1 ml of bromine solution (diluted 10^6 times with distilled H_2O). CPA was quantified using the spectrophotometric method of Chang-Yen and Bidasse [4].

Statistical analysis of data

The data were analysed using the ANOVA and GLM commands in the statistical software package Minitab® version 9.2. The completely randomised design was used to test for treatment effects amongst the five treatment combinations. Significance differences at the 5% level between means were further investigated using Turkey's multiple range test.

Table 1. Analysis of variance for CPA production by *Penicillium commune* in maize

Source	DF[a]	Mean squares	F[b]	p[c]
a_w-temperature combination	4	11233326	13.05	0.001
Error	10	861064		
Total	14			

[a] Degrees of freedom.
[b] Test statistic.
[c] Level of significance.

Table 2. Mean levels of cyclopiazonic acid produced by *Penicillum commune* after 14 days in maize culture under controlled water activity-temperature combinations

Water activity (a_w)	Temperature (°C)	Cyclopiazonic acid* (ng g^{-1} (s.d.)+
0.90	20	4435.0 (782)
0.98	20	7678.0 (722)
0.95	25	4054.0 (905)
0.90	30	2264.0 (517)
0.98	30	4761.0 (857)

*Values within columns with no common superscripts are significantly different ($p < 0.05$).
+ Standard deviations are shown in brackets.

Results

Effect of interaction of temperature and water activity on cyclopiazonic acid production by Penicillium commune

During 14 days' incubation at 0.98 a_w/20 °C, the *P. commune* isolate grew and conidiated vigorously but the grains were only covered with white mycelium at 0.95 a_w/25 °C and at 0.98 a_w/30 °C. Less growth was visible at 0.90 a_w than at higher a_w at both temperatures. The analysis of variance (ANOVA) for CPA (Table 1) showed that a_w and temperature had a highly significant (P = 0.001) effect on CPA production (Table 2). Significantly more CPA was produced at 0.98 a_w/20 °C while significantly less at 0.90 a_w/30 °C. At the following combinations: 0.95 a_w/25 °C, 0.90 a_w/20 °C and 0.98 a_w/30 °C, the mean concentrations of CPA produced ranged from 4054 ng g^{-1} to 4761 ng g^{-1} but were not significantly different. Generally at constant a_w, 20 °C appeared to favour greater CPA production than 30 °C, while at constant temperature mycotoxin production increased with increasing a_w.

Effect of interaction of temperature and water activity on cyclopiazonic acid production by Aspergillus flavus

Aspergillus flavus grew vigorously and produced many conidia at 0.98 a_w/30 °C but at 0.95 a_w/25 °C and at 0.98 a_w/20 °C the grains were covered only with white mycelium after 14 days' incubation. Less growth was visible at 0.90 a_w at both temperatures. ANOVA for CPA (Table 3) showed that the a_w and temperature combinations had highly significant ($P < 0.001$) effects on CPA production. The effects of a_w/temperature interactions on CPA production by *A. flavus* in maize culture are shown in Table 4. Significantly more CPA was produced at 0.98 a_w/20 °C while significantly less at 0.90/30 °C. At 0.95 a_w/25 °C, the mean concentration of CPA produced (1590 ng g^{-1}) was slightly less but not significantly different to that produced at 0.98 a_w/30 °C, the highest a_w and temperature combination used. CPA levels produced at 0.90 a_w/20 °C, the lowest a_w and temperature combination used were significantly higher than the lowest level of CPA production at 0.98 a_w/30 °C and lower than the other treatment combinations. In general, at constant a_w, 20 °C appeared to favour greater CPA than 30 °C while at constant temperature, mycotoxin production increased with increasing a_w.

Table 3. Analysis of variance for CPA production by *Aspergillus flavus* in maize culture

Source	DF[a]	Mean squares	F[b]	p[c]
a_w-temperature combination	4	605395	51.95	<0.001
Error	10			
Total	14			

[a] Degrees of freedom.
[b] Test statistic.
[c] Level of significance.

Effect of interaction of temperature and water activity on aflatoxin production by Aspergillus flavus

ANOVA for AF (Table 5) showed that the a_w and temperature had highly significant ($P < 0.001$) effects on AF production. The effects of a_w and temperature interactions on AF production by *A. flavus* in maize culture are shown in Table 4. All treatment combi-

Table 4. Mean levels of aflatoxins (AF) and cyclopiazonic acid (CPA) produced by *Aspergillus flavus* after 14 days in maize culture under controlled temperature – water activity conditions

Water activity (a_w)	Temperature (°C)	AF* (ng g^{-1}) (s.d.)	CPA* (ng g^{-1}) (s.d.)+
0.90	20	411.0 (65)	1252.0(66)
0.98	20	2030.0 (332)	1876.0(8.7)
0.95	25	1606.0 (308)	1590.0(50)
0.90	30	901.0 (119)	709.0(143)
0.98	30	3096.0 (537)	1614.0(175)

* Values within columns with no common superscripts are significantly different ($P < 0.05$).
+ Standard deviations are shown in brackets.

Table 5. Analysis of variance for aflatoxin production by *Aspergillus flavus* in maize

Source	DF[a]	Mean squares	F[b]	P[c]
a_w-temperature combination	4	3242183	31.31	<0.001
Error	10	103567		
Total	14			

[a] Degrees of freedom.
[b] Test statistic.
[c] Level of significance.

nations gave results which differed significantly from each other. The greatest concentration of AF was produced at 0.98 a_w/30 °C and the least at 0.90/20 °C. At 0.95 a_w/25 °C, the mean concentration of AF produced (1606 ng g^{-1}) was intermediate between the extreme a_w and temperature combinations. Generally at constant a_w, it appeared that 30 °C favoured greater AF production than 20 °C, whereas at constant temperature, AF production increased with increasing a_w.

Discussion

The present results demonstrate that correct experimental design can aid immensely towards understanding mycotoxin production by *P. commune* and *A. flavus*. *Aspergillus flavus* was able to produce AF and CPA in all a_w and temperature combinations investigated. Combinations of high a_w and low temperature favoured high CPA production, while low a_w and high temperature gave the smallest CPA concentration. By contrast the largest amounts of AF were produced with high a_w and high temperature, while low a_w and low temperature supported the least AF production. These results confirm previous studies [28, 29] on aflatoxin production by *A. flavus*.

The ratio of AF : CPA ranged from 1 : 3 at 0.90 a_w/20 °C to 2 : 1 at 0.98 a_w/30 °C. At the central point (0.95 a_w/25 °C) this ratio was close to 1 : 1. These results are consistent with the previous studies of Magan and Lacey [43] who showed that the relative amounts of altenuene (AE), alteriol (AOH), alternariol monomethyl (AME) co-produced by *Alternaria alternata* on wheat grain varied with changing a_w and temperature. At 25 °C/0.98–0.95 a_w, more AME was produced than AOH or AE but there was less AME than AOH or AE at 15 °C. At 0.90 a_w/15–25 °C, trace amounts of all the toxins were produced, but at 0.95 a_w/30 °C AME production was inhibited. A similar study by Wagener *et al.* [44] showed that temperature and relative humidity (RH) affected the production of penitrem A, and roquefortine by *P. commune*. Maximum mycotoxin production occurred after 28 days at 20 °C. Roquefortine was only produced at 99% RH whereas penitrem A was produced at 95–99% RH.

Penicillum commune was able to produce CPA in all a_w and temperature combinations investigated. CPA production proceeded in a similar pattern to that by *A. flavus* 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. CPA was produced in similar concentrations at extreme combinations of temperature and a_w as at the central point chosen, 0.95 a_w/25 °C, suggesting that this mycotoxin has the potential to occur widely in temperate and tropical regions of the world. Thus, if maize and its products are stored poorly, they are at risk of being contaminated with a CPA-producing *P. commune* strain and becoming contaminated with CPA.

In tropical and sub-tropical countries, safe storage of maize continues to be a major problem [45]. The production of AF and CPA at the a_w and temperature combinations investigated suggests that their occurrence in maize will continue to present particular health problems to humans and animals in these regions of the world. Therefore, there is a need for a concerted effort to ensure better storage conditions, especially in developing countries where acute toxicity by CPA and AF have been documented [21, 22]. Close attention must be paid to the proper drying of grains at harvest followed by dry and cool storage. Because of international trade between countries, these mycotoxins are likely to co-occur globally.

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Address for correspondence: Professor J.E. Smith, Department of Bioscience and Biotechnology, University of Strathclyde, 204 George Street, Glasgow G1 1XW.
Phone: (+44) 141 552 4400 ext. 2085; Fax: (+44) 141 553 1181.
E-mail: e.s.clements@strath.ac.uk.