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Efficacy of different caffeine concentrations on growth and ochratoxin A production by *Aspergillus* species

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Running title: Caffeine inhibition of growth and toxin production

Significance and impact of the study: Arabic (0.6%) and Robusta coffee (4%) have significantly different amounts of endogenous caffeine. The growth of six ochratoxigenic fungi which contaminate coffee with ochratoxin A (OTA) had differential tolerance/sensitivity to concentrations of caffeine in vitro in this range. However, low concentrations of caffeine (<0.5%) was inhibitory to OTA production. These results are discussed in the context of the potential for using such information for the design of minimisation strategies to control mycotoxin production in such products.

Abstract
The objective of this study was to evaluate the effect of different caffeine concentrations (0-4%) on (a) lag phase prior to growth, (b) growth rates, and (c) ochratoxin A (OTA) production by strains from the *Aspergillus* sections *Circumdati* and Section *Nigri* groups, isolated from coffee, when grown on a conducive medium at 0.98 water activity and 30^{o}C. The lag phases prior to growth increased with caffeine concentration. A strain of *A. niger* and *A. carbonarius* were the most sensitive to caffeine with growth being inhibited by <1% caffeine. For strains of *A. westerdijkiae*, *A. ochraceus* and *A. steynii*, although growth was inhibited significantly, some growth (10-15% of controls) occurred in 4% caffeine. OTA production was significantly inhibited by only 0.5% caffeine for strains of *A. westerdijkiae*, *A. niger* and *A. carbonarius*. For *A. steynii* at least 1.5% caffeine was required to inhibit OTA production. In contrast, for the strain of *A. ochraceus* there was a stimulation of OTA at 3% with a reduction at 4% caffeine. These results are discussed in the context of the different concentrations of caffeine found in Arabica and Robusta coffee and the development of minimisation strategies.
Key words: Caffeine concentration, *Aspergillus*, lag phase, growth, ochratoxin A

Introduction

Caffeine (1,3,7-trimethylxanthine) has been shown to have an inhibitory effect on a number of mycotoxigenic *Aspergillus* and *Penicillium* species by reducing both growth and

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mycotoxin production such as aflatoxins, ochratoxin A, sterigmatocystin, patulin and citrinin (Buchanan *et al.* 1978; 1982; 1983; Nartowicz *et al.* 1979). The caffeine concentration endogenously present in coffee varies with the type of bean. Thus, Arabica coffee contains approx. 0.6% caffeine, while Robusta coffee about 4% (Oestreich-Janzen 2010). Therefore, the question arises as to whether caffeine concentration may influence the ability of ochratoxigenic fungi to colonise and produce ochratoxin A (OTA) in the presence of different concentrations of caffeine.

Some studies have suggested that caffeine plays a role in aflatoxin B_1 inhibition in coffee (Nartowicz *et al.* 1979). However, *Aspergillus flavus* colonisation of coffee and aflatoxin B_1 contamination is not as important as OTA contamination. Tsubouchi *et al.* (1985) reported inhibition of growth and OTA production by *A. ochraceus* IFM 0458 in a Yeast Extract Sucrose (YES) medium containing 1% caffeine. In contrast, the production of OTA was found to be higher in the presence of 0.5 and 1.0% caffeine in another strain of *A. ochraceus* IFM S-235. However, there are few detailed studies which have attempted to examine the efficacy of different concentrations of caffeine on growth and mycotoxin production by a range of mycotoxigenic species which are responsible for OTA contamination of coffee. A few studies have also suggested that some *Aspergillus* strains (e.g. *A. ochraceus, A. elegans* and *A. sclerotiorum*) all isolated from green coffee beans and grown on ground coffee-based media could degrade caffeine (Tsubouchi *et al.* 1985).

The objectives of the present study was to examine the effect of different concentrations of caffeine (0-4%) on (a) the lag phase prior to growth, (b) growth rates and (c) ochratoxin A (OTA) production by 6 strains from the *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* on a conducive YES-based medium at 0.98 a_w and 30°C. Species from these groups are responsible for contamination of coffee with OTA.

Results and Discussion

The effect of caffeine (0.5-4%) on the lag phase (λ, days), growth (mm day^{-1}) and OTA production (ng g^{-1}) of the different strains and species examined are shown in Figure 1a-1f. This clearly shows that for all the strains examined, there was an increase in the lag phase as the caffeine concentration was increased. In terms of growth rate *A. westerdijkiae* (B2; 121986), were reduced by almost 60% with 0.5% caffeine and by >90% in 4% caffeine concentration when compared to the untreated controls. However, for *A. niger* (A 1911) and *A. carbonarius* (ITAL 204) there was almost complete inhibition with >0.5% caffeine concentration. For *A. ochraceus* (ITAL 14) there was a decrease in growth by about 50% and 94% in 0.5 and 4% caffeine respectively. In the case of *A. steynii* (CBS 112814), growth was inhibited by almost 70% in 0.5% caffeine and reduced by >90% in 4% caffeine compared to control (see Figure 1).

In terms of OTA production, with the exception of *A. steynii* (CBS 112814) and *A. ochraceus* (ITAL 14), toxin production was completely inhibited by 0.5% caffeine. *A. steynii* (CBS 112814) was relatively tolerant to 0.5-1.0% caffeine with only a 24% reduction in OTA production. The only exception was *A. ochraceus* (ITAL 14) where OTA production was significantly increased in the presence of 2-4% caffeine. The highest OTA production by *A. ochraceus* (ITAL 14) reached 8.7 ng g^{-1} in 3% caffeine.

Statistically, the caffeine concentration had a significant effect (p<0.05) on the lag phases prior to growth, growth and OTA production (see Table 1). This confirms the increase in lag phase for all strains with increasing caffeine concentration. The growth of all strains was significantly (P <0.05) affected by caffeine concentration, with the highest growth in the

control and the 0.5% concentration for all strains and this was reduced with increasing caffeine concentration. Statistical analysis showed that, overall, the mean OTA productions were variable among all strains. A similar effect on OTA production was found in the two strains at *A. westerdijkiae* (B 2, CBS 121986), where a large reduction in OTA production occurred in all the caffeine concentrations. The highest OTA production was found in *A. carbonarius* (ITAL 204) and *A. steynii* (CBS 112814) at 0.5% caffeine concentration. The stimulation of OTA production by the *A. ochraceus* strain in the presence of 2-3% caffeine may be due to a combined stress response die to a_w x inhibitory compound. Previously, intermediate concentrations of aliphatic acid based preservatives (calcium propionate, potassium sorbate) were shown to stimulate OTA production by *Penicillium verrucosum*. The was supported by molecular analysis of the *otapks*PV gene expression involved in OTA biosynthesis as well as phenotypic toxin production (Schmidt-Heydt *et al.* 2007).

No previous studies have examined the effect of different concentrations of caffeine on lag phases prior to growth, growth and OTA production by strains/species of the *Aspergillus* sections *Circumdati* and *Nigri*. This study has shown that increasing concentrations of caffeine had a significant impact on both growth and OTA production. Overall, for strains of *A. niger* (A 1911) and *A. carbonarius* (ITAL 204) there was inhibition of both growth and OTA production by >1% caffeine concentration.

Overall, more *Arabica* (*Coffea arabica*) than *Robusta* (*Coffea canephora*) is consumed; with the former accounting for 75%, and the latter, 25% of the total world-wide market. Robusta coffee beans have a more concentrated amount of caffeine reaching 4–5% and is characterised by its bitterness, with less characteristic aroma compounds. Arabica coffee has more flavour and aroma and less than half the caffeine of Robusta beans (Kreicbergs *et al.* 2011). This could be important in terms of colonisation by ochratoxigenic fungi and OTA contamination in these two types of coffee beans. However, few studies have considered this aspect.

Previously, Buchanan *et al.* (1982) reported an inhibitory effect of caffeine on OTA biosynthesis and growth of *A. ochraceus* (= *A. westerdijkiae* based on the new classification; Abdel-Hadi and Magan 2009). This might be due to caffeine interfering with lipid accumulation. Some fungal species accumulate lipids, including *A. flavus* and *A. ochraceus* as a result of secondary metabolism (Gupta *et al.* 1970; Shin and Marth 1974). Previously, growth and OTA production by a strain of *A. ochraceus* was completely inhibited by caffeine at 1.0-2.0% on a YES medium after 3–21 days (Nehad *et al.* 2005). It may be possible that caffeine content affects the colonisation and production of OTA. Interestingly, *A. steynii* was more tolerant while *A. ochraceus* (ITAL 14) was stimulated to produce higher amounts of OTA at >2% caffeine concentrations. Previously, caffeine concentrations of 1-2% were found to completely inhibit the growth and OTA production by *A. ochraceus* on a conducive YES medium over 3-21 days incubation (Nehad et al., 2005). It appears that a higher caffeine concentration actually inhibits growth and mycotoxin production by some mycotoxigenic species, although there may be some exceptions. This area needs more investigation to better understand the relationship between caffeine content of coffee, the contamination with mycotoxigenic fungi and the interactions at a molecular and physiological level.

Material and methods

Fungal strains

One strain of *Aspergillus westerdijkiae* (B 2) was isolated from green coffee beans imported from Ethiopia. A second strain of the same species (*A. westerdijkiae*; CBS 121986) was from the CBS Culture Collection as was the strain of *A. steynii* (CBS 112814). Both were isolated

from coffee beans. These were kindly supplied by Dr. B. Patino (Complutense University, Madrid, Spain). A strain of *Aspergillus niger* (A 1911), *was* kindly provided by Prof. Paola Battilani (Università Cattolica del Sacro Cuore, Piacenza, Italy), and one each of *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14), isolated from Arabica coffee beans in Brazil were kindly provided by Dr. Marta Taniwaki (ITAL, Campinas, Brazil).

Media

The basic YES medium was prepared with 20 g yeast extract (Oxoid, Basingstoke, Hampshire, UK) and agar (Technichal agar No. 2; Oxoid Ltd), Sucrose 150 g and 0.5 g $MgSO_4.7H_2O$. This was made up to a litre with a Universal buffer mixture by combining 63.15 g of 0.2 M NA2HPO4 ml^{-1} and 36.85 g of 0.1 M Citric acid ml^{-1} (McIlvaine's buffer solutions) to obtain a pH value of 6.0. The solution was modified with glycerol (9.2 ml per 100 ml ml medium) to obtain a water activity of 0.98 (Abdel-Hadi and Magan 2009). After autoclaving the medium at 121^{o}C for 10 min the medium was allowed to cool to 45^{o}C. The caffeine concentrations (%) (Sigma-Aldrich) were then added in the range 0.5-4% by addition to the molten cooled medium. This was then shaken well before pouring into 9 cm Petri plates (15-16 ml per plate). After they had cooled and solidified they were stored in polyethylene bags at 4ºC until used. Before use they were allowed to equilibrate at 25^{o}C to prevent any condensation in the individual Petri plates.

Inoculum preparation

The inoculum was prepared by growing each strain on Malt Extract Agar (Oxoid Ltd, Basingstoke, Hampshire, U.K.) at 25°C for 7 days. Spore suspensions were prepared by agitating the colony surface with a sterile spatula in 9 mL of sterile distilled water containing 0.05% (w/v) Tween 80. The spore suspensions were standardised at 10^6 spores ml^{-1} and the media were centrally inoculated with 7 µl of the spore suspension. The treatments were incubated at 30°C for 9 days. The experiment was carried out with three replicates per treatment for each strain and repeated once.

Calculation of lag phase prior to growth and growth rate

The diameter of the colonies was measured in two directions at right angles to each other every day, until the colonies reached the Petri plate margin. The change in the colony radius (mm) vs time (days) for each strain was plotted in Microsoft Excel®. After data plotting, a linear model was used to calculate the relative growth rates (mm day^{-1}) and the lag phases prior to growth initiation. The growth rates (mm day^{-1}) were obtained as the slope of the line. By using the same equation, lag time (in days) were calculated by the regression line formula. The square of the linear correlation coefficients was ≥0.98.

Extraction, detection and quantification of OTA

Five plugs (4mm diameter) from three replicates were transferred after 9 days incubation at 30^{o}C on YES medium to 2ml Eppendorf tubes and weighed. 750µl methanol was added to each Eppendorf tube. The samples were then shaken using a KS 501 digital orbital shaker for 30 min and centrifuged for 10min at 15000xg. The supernatant was filtered directly into HPLC vials.

The HPLC system used for OTA analysis was an Agilent 1200 Series system (Agilent, Berks, UK) with a fluorescence detector (FLD), an auto sampler, autosampler thermostat, thermostated column compartment, on-line degasser, and binary pump. Initial comparisons were performed in the isocratic mode. The mobile phase was AcN:water:acetic acid (57:41:2,

v/v/v) using a flow rate of 1 ml min^{-1}. Separation was performed using an Agilent Poroshell 120 EC-C18 4.6 mm × 100 mm, 2.7 m analytical column. The column was preceded by the appropriate pre-column. FLD detection was performed using λ_{exc} 333nm and λ_{em} 460 ((excitation and emission wavelengths, respectively) for 12 mins. The injection volume was set at 20 µl. After analysis the signals were processed using Agilent ChemStation software Ver. B Rev: 03.01 [317] (Agilent Technologies, Palo Alto, CA, USA). Ochratoxin A standard containing 1.0 mg ml^{-1} in methanol was purchased from Sigma (Sigma–Aldrich, UK). The Limit of Detection and Limit of Quantification were 0.09 ng g^{-1} and 0.32 ng g^{-1}, respectively. This method has been detailed in Sultan *et al.* (2014).

Data analysis

A full factorial design with different caffeine concentrations was carried out in triplicate, and repeated once, for calculating the lag phases (prior to growth) and the relative growth rates. Normality was checked using the Kolmogorov-smirnov test. Analysis of data, the effects of a_w, temperature and their interaction were examined by the Kruskal-Wallis (non-parametric) where data was not normally distributed. For normally distributed data the data sets were analysed using Minitab® 16 package (Minitab Inc., 2010. State College, PA, USA). The statistically significant level was set at $P<0.05$ for all single and interacting treatments.

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Conflict of interest

The authors of this work declare that there is not conflict of interest.

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Table 1: Summary statistical table for the Lag phase (λ, days), growth rate and OTA production of 6 strains on caffeine concentration by using Kruskal-Wallis Test (non-normality data) and ANOVA (normality data).

	Factors	
Strains	**Caffeine concentration (%)**	**Response**
A. westerdijkiae (B 2)	**S** [b]	Lag time (λ, days)
	S [a]	Growth rate (mm day^{-1})
	S [a]	OTA (ng g^{-1})
A. westerdijkiae (CBS 121986)	**S** [b]	Growth rate (mm day^{-1})
	S [a]	Lag phase (λ, days)
	S [a]	OTA (ng g^{-1})
A. niger (A 1911)	**S** [a]	Growth rate (mm day^{-1})
	S [a]	Lag phase (λ days)
	S [a]	OTA (ng g^{-1})
A. carbonarius (ITAL 204)	**S** [a]	Growth rate (mm day^{-1})
	S [a]	Lag phase (λ, days)
	S [a]	OTA (ng g^{-1})
A. ochraceus (ITAL 14)	**S** [a]	Growth rate (mm day^{-1})
	S [a]	Lag phase (λ, days)
	S [a]	OTA (ng g^{-1})
A. steynii (CBS 112814)	**S** [a]	Growth rate (mm day^{-1})
	S [a]	Lag phase (λ, days)
	S [a]	OTA (ng g^{-1})

P values of 0.05 or less are often considered evidence that there is at least one significant effect in the model.

S Significant

[a] Kruskal-Wallis test.

[b] ANOVA.

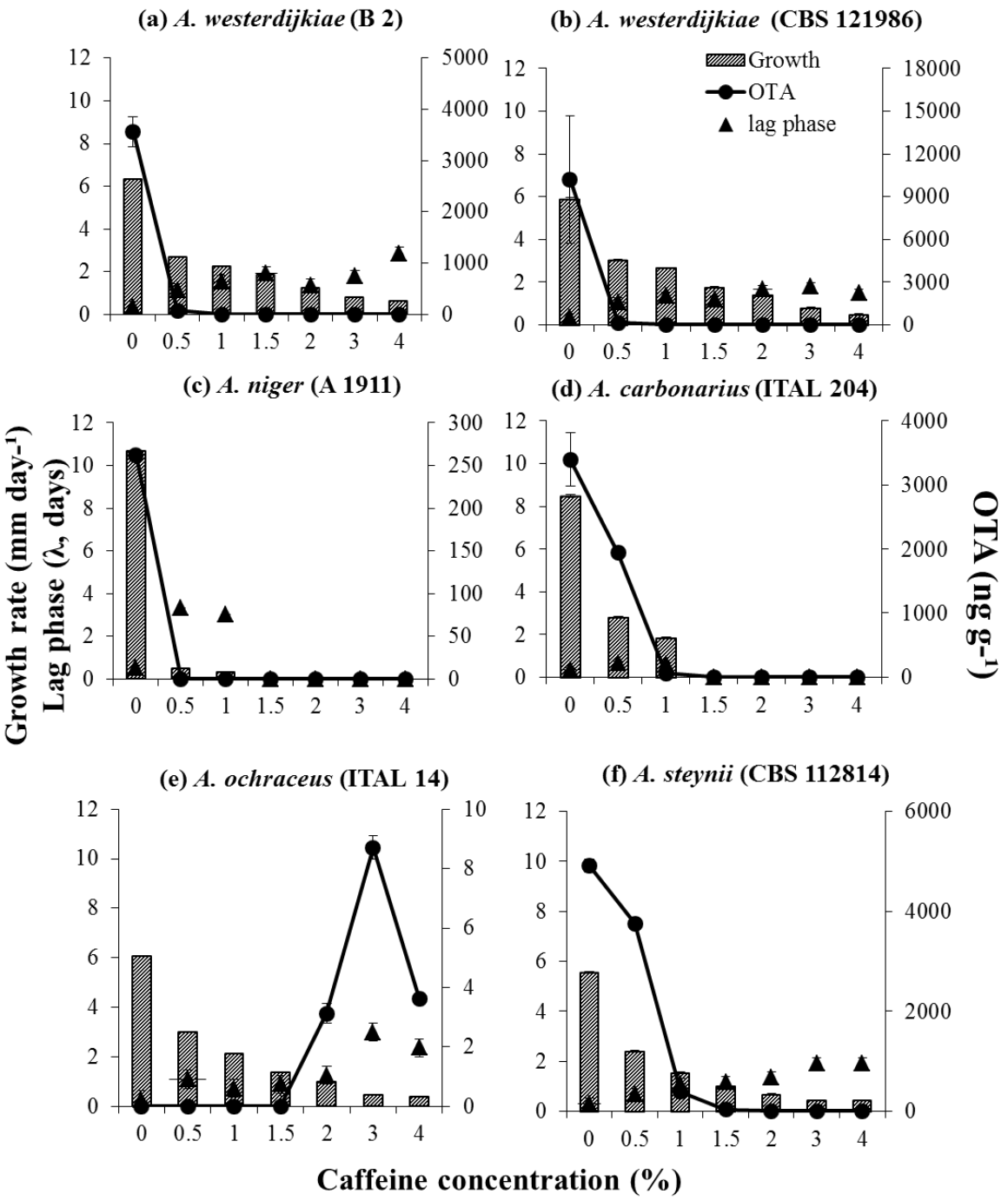


Figure 1 Effect of caffeine concentration (0.5-4%) on lag phase (λ, days), growth and ochratoxin a (OTA) production by (a) and (b) two strains of *A. westerdijkiae* (B2, CBS 121986), (c) *A. niger* (A 1911)**,** (d) *A. carbonarius* (ITAL 204), (e) *A. steynii* (CBS 112814) and (f) *A. ochraceus* (ITAL 14) on a 0.98 a_w YES medium at 30°C for 9 days. Bars indicate standard error of the mean.



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