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Growth and ochratoxin A production by *Aspergillus niger* group strains in coffee beans in relation to environmental factors

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Abstract

The effect of water activity (a_W), temperature and their interactions on lag phase, mycelial growth rate and ochratoxin A (OTA) production at 7, 14 and 21 days of incubation of two OTA-producer strains belonging to *Aspergillus niger* group on irradiated coffee beans was determined. Irradiated coffee beans were re-hydrated to 0.910-0.995 of a_W with sterile distilled water. The temperatures assayed were 15, 25 and 30 °C. Growth assessment was measured every day during the incubation period to calculate the growth rate. OTA production was examined at 7, 14 and 21 days by high-performance liquid chromatography. Optimal a_W for growth was 0.995 at 25 °C for RCC4 and RCC20 strains, being 1.10 and 2.36 mm/day, respectively. OTA concentration varied considerably depending on a_W, temperature and incubation time assayed. Maximum OTA production was obtained at 0.973 and 0.995 a_W at 30 °C for both strains. The results of the present work indicate that knowledge of the optimal and marginal conditions of black *Aspergillus* growth and OTA production allow methods to be established for preventing the development of these fungal and mycotoxin production on coffee beans. The data obtained provide useful information for predicting the risk factors for OTA contamination on coffee beans.

Keywords: *Aspergillus* section *Nigri*, coffee beans, growth rate, ochratoxin A, water activity, temperature

1. Introduction

Studies on the microbiology of coffee cherries and beans have shown that the main toxigenic fungal genera (*Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp.) are natural coffee contaminants and present from the field to the warehouse (Mislivec *et al*., 1983; Nakajima *et al*., 1997; Silva *et al*., 2000).

Ochratoxin A (OTA) has been extensively documented as a global contaminant of a wide variety of commodities. Cereal and legume seed commodities are particularly at risk of OTA contamination, while coffee beans are among other agricultural substrates susceptible to this contamination. Some authors have shown the presence of OTA-producing strains in coffee samples such as some species of *Aspergillus* and *Penicillium*, which are biodeterioration agents of carbohydrate-rich agricultural commodities from latitudes ranging from cool to tropical temperatures (Taniwaki *et al*., 2003; Urbano *et al*., 2001).

The presence of OTA in coffee beans is normally a result of badly controlled harvesting procedures, insufficient drying of the beans, and inadequate storage conditions, allowing the proliferation of toxigenic fungi (Urbano *et al*., 2001). The source of this toxin in coffee remains poorly understood, although its presence in commercial green, roasted and soluble coffee has been reported by several authors on variable levels (Batista *et al*., 2003, 2009; Fazekas *et al.,* 2002; Gopinandhan *et al*., 2008; Lombaert *et al*., 2002; Martins

et al., 2003; Pardo *et al.,* 2004). Worldwide it is assumed that the major source of OTA in coffee is *Aspergillus westerdijkiae*, belonging to the *Aspergillus ochraceus* group (Frisvad *et al*., 2004). Moreover, the discovery that *Aspergillus carbonarius* has the potential to produce OTA has prompted a more thorough investigation of this issue. This species and species belonging to the *Aspergillus niger* group have been isolated from coffee (Magnoli *et al*., 2008; Nakajima *et al*., 1997; Téren *et al*., 1997; Urbano *et al*., 2001), indicating that these latter are also potential sources of OTA in this substrate.

Argentina is not a coffee producing country but it is a country where consumption of this product is high. Therefore, small factories import the green coffee beans as raw materials and the roasting process is carried out in the country. In the factory the coffee remains in bags exposed to the environment. Sometimes, temperature, relative humidity and sanitary conditions are inadequate in these places. Temperature and a_W are the environmental risk factors for mould proliferation and OTA formation in coffee (Taniwaki *et al*., 2003). Awareness of these parameters allows the drying and processing stage of these grains to be optimised, thus maintaining their hygienic quality.

The combined effect of a_W and temperature on growth and OTA accumulation of ochratoxigenic strains have been studied on a synthetic medium (Belli *et al*., 2004, 2005; Esteban *et al*., 2006; Marín *et al*., 2006; Mitchell *et al*., 2004; Romero *et al*., 2007), on maize meal extract agar (Astoreca *et al*., 2007b; Lee and Magan, 2000; Marín *et al*., 1998), on barley meal extract agar (Pardo *et al*., 2004, 2005; Ramos *et al*., 1998) and on the natural substrate (Astoreca *et al*., 2009a,b; Pardo *et al*., 2005).

The aim of this study was to determine the effect of a_W, temperature and their interactions on lag phase, mycelial growth rate and OTA production at 7, 14 and 21 days of incubation by two OTA-producer strains on coffee beans.

2. Materials and methods

Fungal strains

Two strains belonging to the *A. niger* group (RCC4 and RCC20) isolated from Colombian coffee beans were selected according to their capacity to produce OTA (Magnoli *et al*., 2008). These strains were deposited in the National University of Río Cuarto, Argentina (RC) collection centre. Cultures were maintained in 15% glycerol at -80 °C.

Gamma sterilisation of coffee beans and adjustment of the water activity

Coffee beans (1 kg) packed in specially designed biaxial oriented polypropylene bags (BOPP, 25 μm) were irradiated with 8-10 kGrays of gamma irradiation to retain the coffee beans' germinating ability. They were checked for sterility and absence of OTA and stored aseptically at 4 °C. For all experiments, four hundred grams of irradiated coffee beans were weighed into sterile flasks and re-hydrated to the required a_W levels (0.995, 0.973, 0.951, 0.928 and 0.910) by adding sterile distilled water using a previously calculated moisture absorption curve. Flasks were subsequently refrigerated at 4 °C for 48 hours with periodic shaking to allow absorption and equilibrium. Finally, a_W levels were confirmed by using an Aqualab Series 3 (Labcell Ltd., Basingstoke, UK).

Inoculation and incubation

Re-hydrated coffee beans were placed in sterile 9 cm Petri dishes to form a monolayer. Then a 4-mm diameter agar disk was taken from the margin of a seven-day-old growing colony of each strain on malt extract agar (MEA) at 25 °C and transferred to the centre of each plate containing the coffee beans. Petri plates conditioned at the same a_W were placed in closed plastic containers together with beakers of glycerol-water solution of the same a_W in order to maintain the correct equilibrium of relative humidity inside the boxes. Containers were incubated at 15, 25 and 30 °C and the experiment was carried out twice with three replicates per treatment.

Growth assessment

Growth was assessed every day during the incubation period and the plates containing coffee beans were examined using a binocular magnifier. Two diameters of the growing colonies were measured at right angles in two directions until the colony reached the edge of the plate. The radii of the colonies were plotted against time, the growth rate was calculated by linear regression as the slope of the regression line. Lag phase was defined as the time (hours) at which each colony reaches 5 mm in diameter for each treatment since if the fungus is kept in latency, there would be no risk of mycotoxin production, and these are secondary metabolites formed in the stationary growth phase.

Ochratoxin A determination

OTA was extracted from the coffee beans as follows: at 7, 14 and 21 days of incubation, three replicates per treatment were destructively sampled, dried at 50 °C for 24 hours and stored at -20 °C until OTA analysis was carried out. The technique described by Joosten *et al*., (2001) was used. A 20 g portion of finely ground coffee beans was added to a 250 ml Erlenmeyer flask along with 100 ml mixture of methanol: 3% aqueous sodium hydrogen carbonate (50:50). The mixture was shaken overnight and filtered to remove particulate matter through a Whatman GF B^{-1} glass microfibre filter (Whatman, Maidstone, UK). The extract

was evaporated to dryness under a stream of nitrogen at 40 °C and re-dissolved in 150 µl mixture consisting of 45% acetonitrile : 55% 4 mM sodium acetate/acetic acid (19:1).

OTA detection in coffee beans was performed for each treatment by high-performance liquid chromatography (HPLC), following the methodology proposed by Scudamore and MacDonald (1998) with some modifications. The HPLC equipment used for OTA determination was a Hewlett Packard chromatograph (HP/Agilent, Santa Clara, CA, USA) with an injection loop of 50 µl, a spectrofluorescence detector (excitation, 330 nm; emission, 460 nm) and a C_{18} column (Supelcosil LC-ABZ, Supelco, Sigma Adrich, St. Louis, MO, USA; 150×4.6 mm, 5 µm particle size) and connected to a precolumn (Supelguard LC-ABZ, Supelco, Sigma-Aldrich; 20×4.6 mm, 5 µm particle size). The mobile phase was pumped at 1.0 ml/min and consisted of an isocratic system: 57% acetonitrile, 41% water, and 2% acetic acid. OTA was quantified on the basis of HPLC fluorometric response compared with OTA standard (Sigma Aldrich, USA, purity >99%). The lowest limit of detection was 1 ng/g. Each sample was analysed three times.

Assay of spiking and recovery of ochratoxin A

A stock solution of 4 ml of OTA in methanol with a concentration of 5 µg/ml was prepared for recovery determination. Each OTA-free sample of irradiated coffee beans (50 g) contained in a 250 ml Erlenmeyer flask was spiked with an equivalent of 2, 5 and 10 µg OTA/kg. Spiking was carried out in triplicate and a single analysis of the blank sample was carried out. After leaving it for 16 hours for the solvent to evaporate, extraction solvent was added and OTA concentration was determined, using the protocol previously described.

Statistical analysis

Lag phase, growth rate and OTA concentration at different temperature and a_W by two strains of *A. niger* group at 7, 14 and 21 days were analysed statistically using PROC GLM in SAS program (SAS Institute Inc., Cary, NC, USA) through an ANOVA. Means were compared by Fishers LSD test to determine the significant difference between the different treatments assayed (Quinn and Keogh, 2002).

3. Results and discussion

The effect of a_W, temperature on lag phase, mycelial growth rate and OTA production at 7, 14 and 21 days of incubation of two ochratoxigenic strains belonging to the *A. niger* group on irradiated Colombian coffee beans was evaluated.

Table 1 shows the lag phases reached by two strains belonging to the *A. niger* group on irradiated coffee beans under different conditions of a_W and temperature. The largest lag phases obtained by RCC4 and RCC20 strains were 240.6 and 208.4 hours at 0.955 of a_W at 25 °C and 30 °C, respectively. None of the assayed strains reached the exponential phase at 0.910 and 0.928 at any of the studied temperatures.

Table 1. Lag phase (hs) of two strains belonging to the *Aspergillus niger* group (RCC4 and RCC20) on irradiated coffee beans under different environmental conditions.

Strain	a_W	Temperature 25 °C		Temperature 30 °C	
		Lag phase (hs) ± SD	LSD[1]	Lag phase (hs) ± SD	LSD[1]
RCC4	0.910	>540	a	>540	a
	0.928	>540	a	>540	a
	0.951	240.0±52.1	b	>540	a
	0.973	128.5±21.4	d	189.5±22.8	c
	0.995	87.8±14.5	f	108.6±17.9	e
RCC20	0.910	>540	a	>540	a
	0.928	>540	a	>540	a
	0.951	176.6±21.7	c	208.4±36.7	b
	0.973	104.8±18.7	de	117.9±19.8	d
	0.995	72.4±8.9	f	81.1±9.8	f

[1] Values with the same superscript do not differ significantly (test of the minimum significant difference of Fisher LSD (P<0.0001). The data were transformed into $\log_{10}$ (hs). SD: standard deviation.

The effect of a_W and temperature on growth rate (mm/day) of two strains belonging to this *A. niger* group on the irradiated coffee beans is shown in Figure 1. Optimal a_W for growth was 0.995 at 25 °C for both RCC4 and RCC20 strains, being 1.10 and 2.36 mm/day, respectively. Significant statistical differences were only observed for the growth rates between the strains and the temperature at the highest a_W assayed (P<0.05). No growth was observed at 15 °C at any of the assayed water activities as well as at the lowest water activity at 25 and 30 °C during the tested period. Consequently, no OTA was detected under these conditions.

Astoreca *et al*. (2007a) studied the effect of a_W and temperature on growth parameters by *A. niger* group strains on coffee beans extract agar. Both strains grew under all temperature (15, 25 and 30 °C) and a_W (0.85, 0.89, 0.91, 0.94, 0.95, 0.97 and 0.995) conditions assayed. However, the growth rates were lower than those obtained with the same strains on coffee beans. This demonstrates that the natural substrate offer the strains a greater nutritional contribution than the artificial media.

In another study by Astoreca *et al*. (2009a,b) carried out with *A. niger* group strains on other natural substrates (peanut and corn grains) shorter lag phases than those

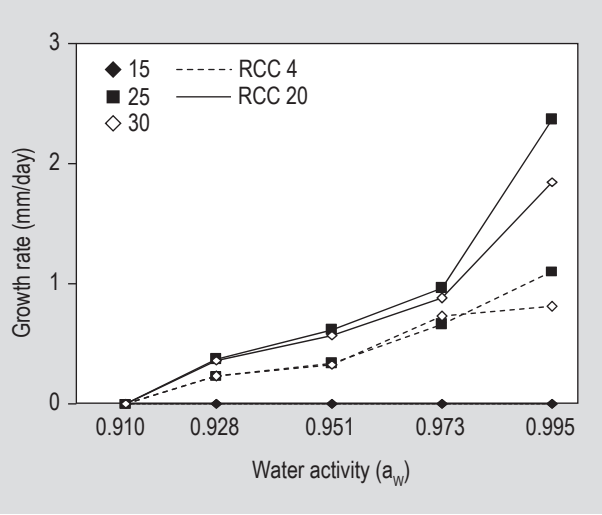

Figure 1. Effect of different a_W and temperatures on growth rate (mm/day) of two *A. niger* group strains on irradiated coffee beans.

obtained on coffee beans were obtained. On peanut grains, growth was observed under all environmental conditions assayed except at 0.910 and 15 °C, whereas on corn grains the fungal growth was observed at 25 and 30 °C at all a_W assayed but for 15 °C only at the highest a_W (0.973-0.995). These results are partially consistent with those obtained in the present study. Figure 2 shows the effect of the temperature (25 and 30 °C), the water activity (0.910, 0.928, 0.951, 0.973 and 0.995) and the incubation time (7, 14 and 21 days) on OTA production by the RCC4 and RCC20 strains on irradiated coffee beans. Ochratoxin A production was not detected at 15 °C regardless of the a_W and the incubation time. Ochratoxin A was produced at 0.973 to 0.995 a_W and 0.951 to 0.995 a_W for RCC4 and RCC20 strains, at 25 and 30 °C, respectively. Both strains produced the maximum OTA concentration under different environmental conditions. The highest OTA concentration produced by the RCC20 strain, (around 100 µg/g) was detected at 30 °C and 0.995 of a_W at 7 days of incubation. On the other hand, the RCC4 strain produced about 50 µg/g OTA at its optimal condition of 0.973 a_W at 7 and 14 days of incubation at the same temperature.

At a_W levels lower than 0.995 and the highest temperature assayed (30 °C), none of the strains were able to produce detectable OTA concentrations. At 25 °C the RCC4 strain produced OTA only at the highest a_W assayed, whereas detectable OTA concentrations were produced by RCC20 strain from 0.951 to 0.995 a_W.

The evaluation of the results of the variance analysis (ANOVA) showed that OTA production by both strains was not affected by the incubation time (P<0.05); and that for the RCC20 strain, the different temperatures did not statistically influence the detected OTA concentration (P<0.05) (Table 2).

The reported data about the influence of environmental factors on the ecophysiology of these species on coffee beans are scarce. Palacios-Cabrera *et al.* (2004) studied the production of OTA by ochratoxigenic *A. ochraceus* strains isolated from Brazilian coffee beans. This study was the only paper available on that topic with ochratoxigenic species on this substrate. The study was carried out in coffee beans at different a_W (0.80, 0.86 and 0.90) under alternating and constant temperatures. They found little or no OTA production at 0.80 at 25 °C whereas at 0.86 and 0.90 a_W OTA production increased as the incubation time increased reaching the maximum concentration at 60 days of incubation. Even though the species and the specific conditions used by these authors were different, the behaviour of the strains was similar to that observed in our study.

On the other hand, Astoreca *et al.* (2009a,b) found a similar OTA production pattern on peanut and corn grains. However, the OTA concentrations reached by other *Aspergillus* section *Nigri* strains were higher than those produced on irradiated coffee beans. These results suggest that the chemical composition of natural substrates determines growth rates values and the ability to produce OTA by these fungal species. In coffee beans, the inhibition of growth and OTA production may be due to caffeine or some other coffee component (Varga *et al.*, 2000).

The results of the present work indicate that the knowledge of the optimal and marginal conditions of black *Aspergillus* growth and OTA production allow methods to be established for preventing the development of these fungi and mycotoxin production on coffee beans.

It is important to emphasise that the strains did not reach the exponential phase during the entire testing time (21 days) at 25 °C and the lowest assayed a_W (0.928). Thus, we propose that maintaining the latter environmental conditions during storage of coffee beans should prevent the proliferation of these species, as well as the OTA production.

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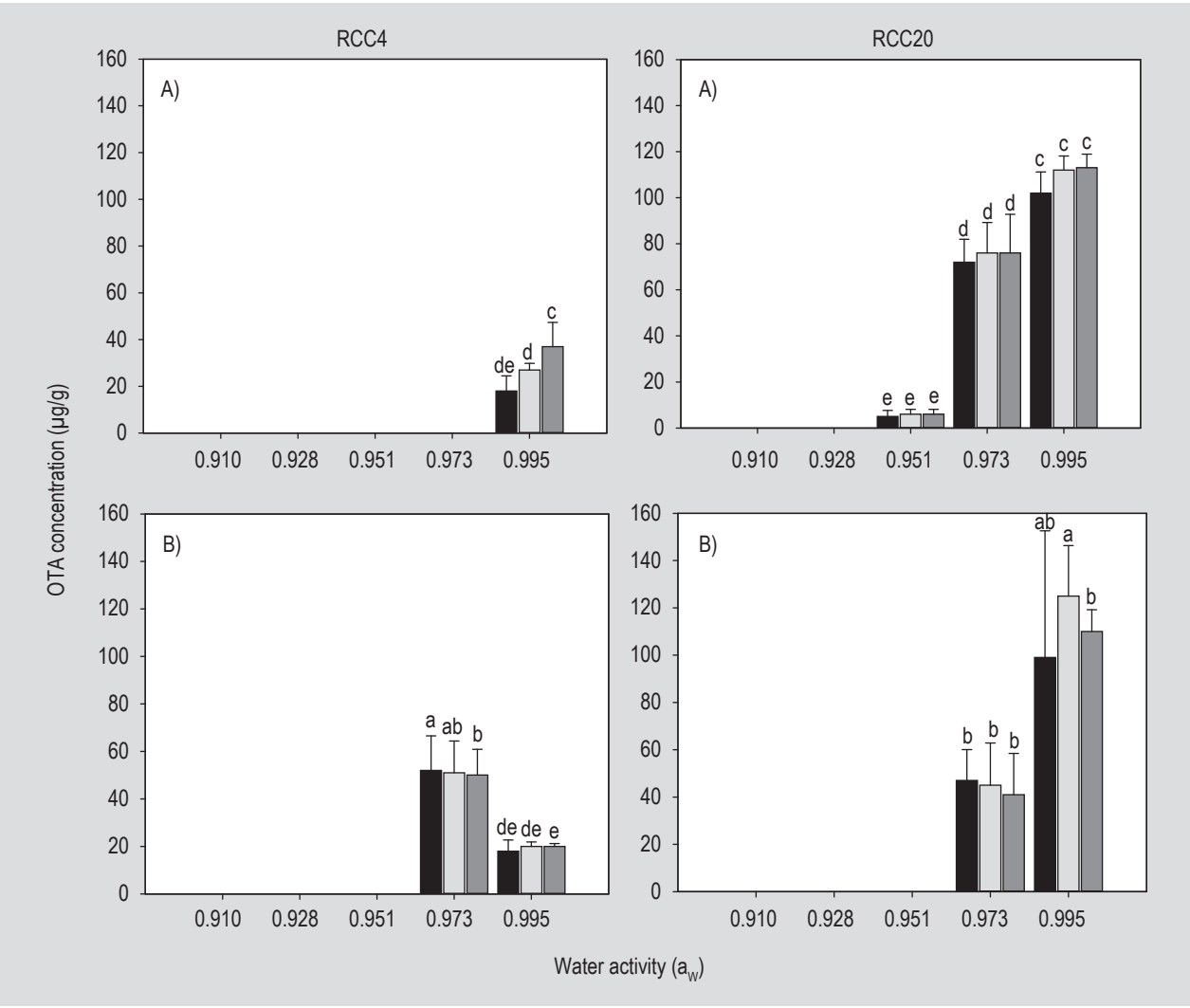

Figure 2. OTA concentrations (µg/g) produced by *Aspergillus niger* group strains (RCC4 and RCC20) on irradiated coffee beans at different water activity and incubation time (days) at (A) 25 °C and (B) 30 °C. Columns with no common letters are significantly different (*P*<0.0001) according to the LSD test.

Table 2. Variance analyses of water activity (a_W), temperature (T), incubation time (IT) and their interactions on OTA production (µg/g) of two strains belonging to the *Aspergillus niger* group (RCC4 and RCC20) in irradiated coffee beans.

Source of variation	df[1]	RCC4		RCC20	
		MS[2]	F[3,4]	MS[2]	F[3,4]
a_W	4	3,312.5	177.1*	46,002.7	438.8*
T	1	1,664.1	88.9*	360.0	3.4**
IT	2	10.1	0.5***	56.1	0.5***
a_W×T×IT	22	532.3	28.5**	214.7	2.1****

[1] df = Degrees of freedom.
[2] MS = Mean square.
[3] F = F-Snedecor.
[4] Significance: * *P<0.0001*; ** *P<0.001*; *** *P<0.01;* and **** *P*<0.5.

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