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# Effects of temperature and incubation time on growth and ochratoxin A biosynthesis by *Aspergillus carbonarius* and *Aspergillus ochraceus* in grain-based media

**Sirlei Cristina de Souza[1], Vanessa Maria Pereira[1], Fabiana Reinis Franca Passamani[1], Marcelo Angelo Cirillo[2], Rodrigo Luz da Cunha[3], Maria das Graças Cardoso[1], Wilder Douglas Santiago[1], Sara Maria Chalfoun de Souza[3] and Luís Roberto Batista[1]***

[1]Department of Food Science, Federal University of Lavras, Lavras, MG, Brazil.
[2]Department of Exact Science, Federal University of Lavras, Lavras, MG, Brazil.
[3]Agricultural Research Corporation of Minas Gerais, Lavras, MG, Brazil.

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**Ochratoxin A (OTA) has been frequently found as a food contaminant, and it is considered one of the mycotoxins most harmful to human health. In this context, this study was performed aiming to evaluate mycelial growth and ochratoxin A production from three isolates of *Aspergillus carbonarius* and *Aspergillus ochraceus*. The isolates were inoculated on culture media coffee, wheat and maize as well as YES medium and cultivated at 15 and 25°C. Measurement of colony diameters was performed every 48 h for growth description. OTA quantification was made with high-performance liquid chromatography (HPLC) three times (5th, 15th and 25th day). All the isolates presented higher growth rate in YES medium at 25°C; however, growth in every culture media at both tested temperatures was observed. The maize-based medium showed the lowest growth rate. YES medium induced the most OTA production. In grain-based culture media, *A. ochraceus* isolates produced lower quantities of OTA, reaching a maximum of 9.95 µg/g. However, *A. carbonarius* isolates produced much higher quantities, showing the *A. carbonarius* CCDCA10608 isolate, which produced 29.83 µg/g at 15°C in YES medium and 13.06 in wheat-based medium at 15°C. Therefore, among the tested conditions, those more and less favorable to OTA production were recognized.**

**Key words:** *Aspergillus*, food, high performance liquid chromatography, mycotoxin, ochratoxin A.

## INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin with a high nephrotoxicity potential, besides showing hepatotoxicity, teratogenicity, carcinogenicity, cytotoxicity, neurotoxicity and immunosuppressive properties (Woo et al., 2012; Stachurska et al., 2013; Solcan et al., 2013; von Tobel et al., 2014; Gayathri et al., 2015; Calado et al., 2015). They

*Corresponding author. E-mail: luisrb@dca.ufla.br. Tel/Fax: +55 35 3829-1407.

are detected in different foods such as coffee (Batista et al., 2009; Casal et al., 2014), grain, like wheat and maize and their derivatives (Juan et al., 2008; Gang et al., 2014; Al-Hadithi et al., 2015), wine (Terra et al., 2013; Giovannoli et al., 2014), powdered guarana (Martin et al., 2014), among others (Obanos et al., 2005; Vidal et al., 2015, Lippolis et al., 2014; Pacheco et al., 2015; Marino et al., 2014). At first, it was believed that OTA was produced only by *Aspergillus ochraceus* and *circumdati*-related species, as well as *Penicillium verrucosum*, though these species are not considered the main OTA sources in some food because OTA may also be produced naturally by *Aspergillus carbonarius* (Van der Merwe et al., 1965; Horie, 1995, Lund and Frisvad, 2003; Schabo et al., 2015; Kogkaki et al., 2015), since 75-100% of the isolates were found to be ochratoxigenic (Romero et al., 2005). Fungal growth and consequent OTA production are determined by a wide range of parameters, classified as physical, chemical and biological, as well as by interactions encompassing these factors (Nielsen et al., 2003). OTA biosynthesis may be influenced by eco physiological intrinsic factors, such as temperature, nitrogen shortage and pH conditions which also influence germination, growth and sporulation (Palacios-Cabrera et al., 2005; Garcia-Cela et al., 2014; Passamani et al., 2014). Under stress conditions, the signaling pathways certainly induce the production of mycotoxins (Choi et al, 2008; Kohut et al., 2009).Therefore, understanding the effects of these factors can help reduce mycotoxins, both in the field and during storage. Furthermore, interactions between mycotoxigenic species and other spoilage fungi can significantly influence ochratoxin A production (Lee and Magan, 2000).Several authors have studied the ability of *A. ochraceus* and *A. carbonarius* to produce OTA in coffee, maize and synthetic grape and barley medium under different environmental conditions (Pardo et al., 2005; Lee and Magan, 2000; Marín et al., 2006; Pardo et al., 2004; Garcia et al., 2011). Approximately, 25% of the entire annual grain production is affected by the presence of mycotoxins, leading to imminent risk of health problems associated with the ingestion of contaminated products, highlighting coffee, maize and wheat (Lawlor and Lynch, 2001). Each fungal strain has its own physiological peculiarities and hence different habitats. The water activity and temperature are very important because they affect the growth and production of OTA (Pardo et al., 2005; Kapetanakou et al., 2009).Thus, in this study, the authors aimed to investigate the necessary conditions for growth and synthesis of OTA by *A. carbonarius* and *A. ochraceus* as well as their concentration in different food-based culture media.

## MATERIALS AND METHODS

### Fungal strains and inoculation

Three *A. carbonarius* strains (CCDCA10610, CCDCA10609 and CCDCA10608) isolated from grape and three *A. ochraceus* strains (CCDCA10613, CCDCA10612, CCDCA10611) isolated from coffee, all ochratoxin A producers, were used in the study. They were provided by the Coleção de Cultura de microrganismos do Departamento de Ciências dos Alimentos – UFLA (CCDCA) (Culture Collection of Food Science Department – UFLA). The strains were transferred to Petri dishes containing Czapek Dox Agar (Sigma-Aldrich, St. Louis, MO) and were incubated at 25°C for 7 days. The spore suspension was prepared using 30 mL of sterile distilled water containing 0.05% Tween 80 (Sigma-Aldrich) and was then filtered through sterile gauze (Nexcare, 3M, São Paulo, Brazil). A 10 mL aliquot of the suspension was transferred to a Neubauer chamber (Sigma, São Paulo, Brazil) to determine the final spore concentration. Two microlitres of the spore concentration ($10^6$ spores per ml), were placed in the centre of the prepared plates and incubated at 15 and 25°C. Each strain was inoculated in three independent plates in each medium.

### Culture media

The strains were cultivated on five different agar culture media. Malt extract agar (MEA) and yeast extract sucrose (YES) media were purchased from Merck (Darmstadt, Germany) and prepared according to manufacturer suggestion. Coffee, wheat and maize were prepared as follows: coffee extract agar (CMEA), 175 g ground green coffee and 20 g agar were dissolved in 1 L distilled water (Pardo et al., 2005); Wheat Extract Agar (WMEA), 175 g wheat flour, 20 g agar were dissolved in 1 L of distilled water (Muñoz et al., 2011); Maize Agar (CMA), 30 g maize flour, 20 g agar were dissolved in 1 L distilled of water (Ramos et al., 1998). Flours and 1 L of water were placed in clean fabric and boiled at low heat for 60 min. The fabric was then wrung and the liquid filtered in hydrophilized gauze and the liquid completed to 1 L. Finally, the agar was added and the resulting product was sterilized at 121°C for 15 min in an autoclave.

### Assessment of strain growth and OTA extraction from cultures

Petri dishes were examined daily and the colony diameters were measured in perpendicular directions using a digital caliper on alternate days during the experiment. OTA was extracted according to the modified method of Bragulat et al. (2001). Three plugs of culture were removed from the center, middle and edge of each colony on the $5^{th}$, $15^{th}$ and $25^{th}$ day of the incubation period. The plugs were weighed in test tubes, and then 1 mL of methanol was added. The tubes were homogenized vigorously for 5 s and kept at 25°C for 60 min. The extracts were filtered through polytetrafluoroethylene membranes (0.22 µm; Millipore Corp., Billerica, MA) and were then analyzed using a Shimadzu high-performance liquid chromatograph coupled to two high-pressure pumps (model SPD-M20A), degasser DGU $20A_3$, interface CBM-20A, auto injector SIL-10AF, and RF-10 $A_{xl}$ fluorescence detector (Shimadzu, Kyoto, Japan). The Zorbax Eclipse XDB-C18 column (4.6 by 250 mm, 5 µm; Agilent Technologies, Palo Alto, CA) was used, connected to a Zorbax Eclipse XDBC18 4-pack precolumn (4.6 by 12.5 mm, 5 µm; Agilent). The chromatographic conditions for wavelength were 332 nm for excitation and 476 nm for emission. The flow used was 0.8 mL $min^{-1}$, and the injected volume of samples and standard was 20 µL. The elution was performed using an isocratic system of 35:35:29:1 (methanol–acetonitrile–water–acetic acid). The average retention time for OTA determination was 11 ± 0.1 min. The amount of OTA in the samples was determined using an analytical curve obtained by linear regression ($y$ = 1.11756 X $10^7x$ - 2,592.1485, where $y$ is the peak area and $x$ is the OTA concentration). The calculation defined the peak area versus the concentration of the respective standard solution, obtained by

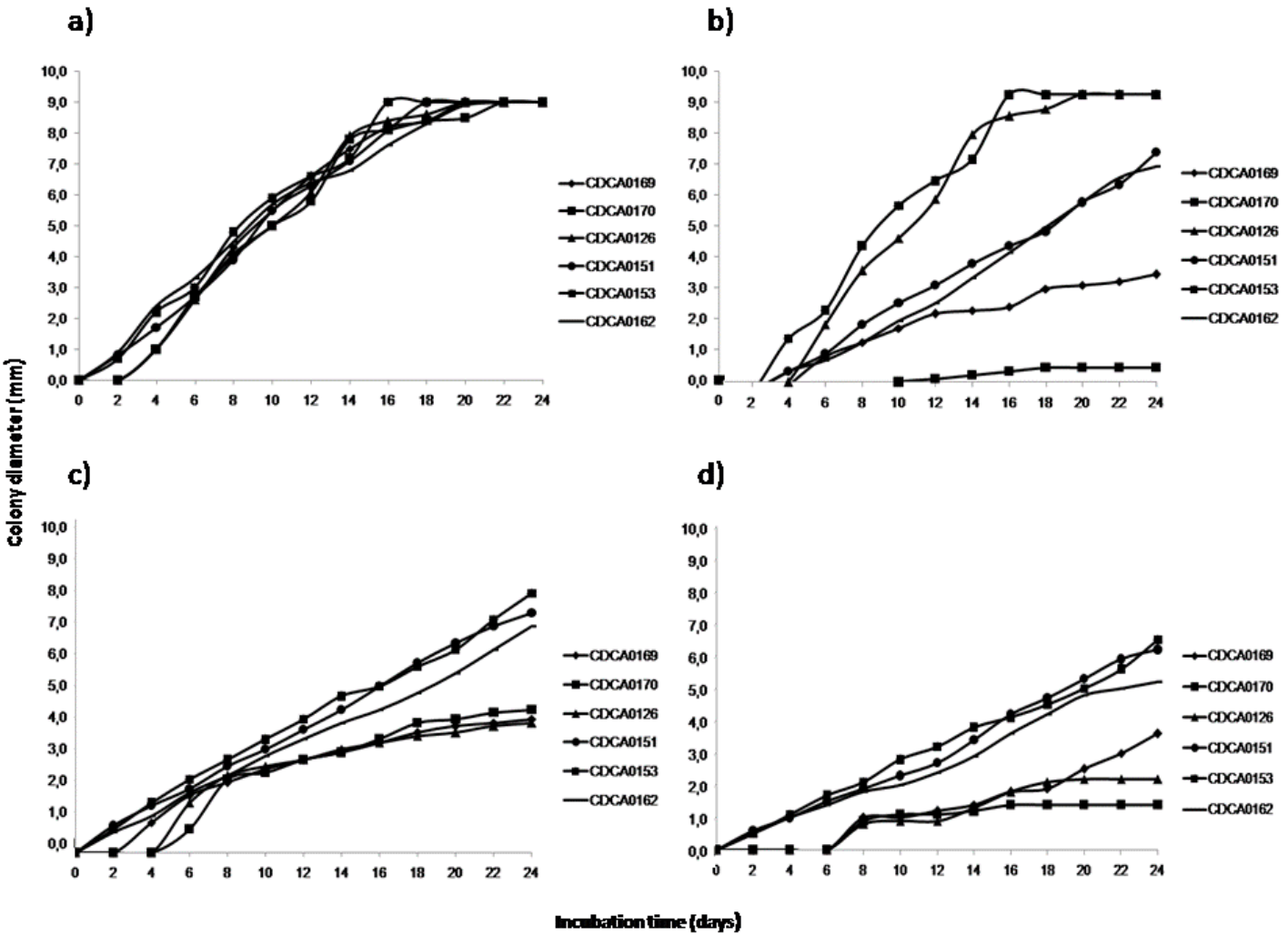


**Figure 1.** Growth of all *Aspergillus* isolates (6) at 15°C in different culture media: a) YES; b) CMEA; c) WMEA; d) CMA.

setting the coefficient of determination ($R^2$) at 0.9999. The detection limit (DL) and quantification limit (QL) were estimated through parameters obtained by the analytical curve and were calculated according to the following: DL=3 SD/*m* and QL=10 SD/*m* (where SD is the standard deviation and m is the angular coefficient of the linear regression) (Harris, 2008). The values obtained for DL and QL were 0.0004 and 0.0016 µg/g, respectively. All the samples were analyzed in duplicate, and the standard OTA solutions were assessed in triplicate.

### Recovery assays

Recovery assays were performed to ensure the analytical quality of the results. The semisynthetic culture medium was fortified with concentrations equal to 1.0, 3.0, and 6.0 µg/g in triplicate. The samples were extracted with methanol and analyzed according to the method of Bragulat et al. (2001).

The results of the recovery assays used were 82, 87, and 91%, respectively. These recoveries proved the remarkable reproducibility of the method and complied with Codex Alimentarius requirements for analytical methods (70 to 110% recovery) (Codex, 2008).

### Statistical analysis

Statistical analysIs for the determination of OTA were obtained with SISVAR computer software developed by Ferreira (2000), considering the split-plots as each investigated individual per species. Data from OTA production were analyzed considering time ($5^{th}$, $15^{th}$ and $25^{th}$ days), culture medium (YES, CMEA, WMEA and CMA) and temperature (15 and 25°C). Treatment means were compared by the Scott Knott test (1974) at 5% probability.

## RESULTS

### Mycelial growth

The growth of *A. carbonarius* and *A. ochraceus* isolates cultivated in different culture media, temperature and time is shown in Figures 1 and 2. All the isolates had similar growth in YES at 15°C, reaching a maximum on the plate on around the $18^{th}$ day. The same medium at 25°C temperature also showed maximum growth for all the

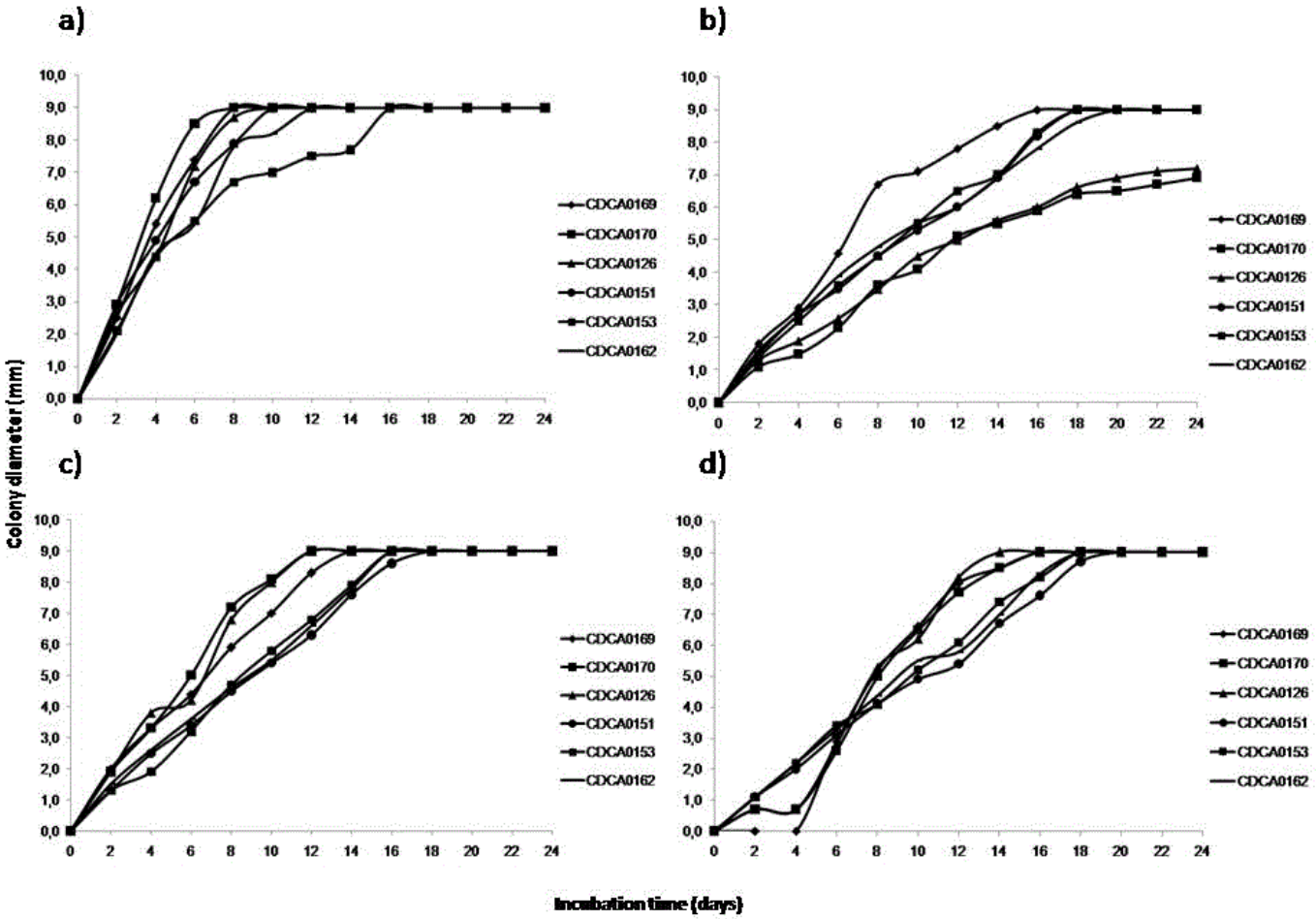


**Figure 2.** Growth of all *Aspergillus* isolates (6) at 25°C in different culture media: a) YES; b) CMEA; c) WMEA; d) CMA.

isolates, but this index was achieved at about 10 days.

On the coffee extract medium, the growth varied. *A. carbonarius* CCDCA10610 and *A. ochraceus* CCDCA10612 isolates had the highest growth rates for the 15°C temperature treatment with a maximum growth around the 17$^{th}$ day. *A. carbonarius* CCDCA10609, however, had a very slow growth and it did not exceed 2 cm diameter at the end of 24 days. In the treatments at 25°C temperature, *A. carbonarius* CCDCA10609 isolate also had a lower growth rate in comparison with the other fungi, attaining a maximum growth of 7 cm diameter.

The growth of isolates in maize and wheat based media at 15°C temperature were similar. *A. ochraceus* isolates grew relatively faster in comparison with *A. carbonarius* isolates. However, no isolate growth reached throughout the whole. *A. carbonarius* isolates achieved growth on the whole plate on the 11$^{th}$ day in wheat medium at 25°C, and *A. ochraceus* isolates on the 16$^{th}$ day. All isolates had maximum growth between the 15$^{th}$ and 18$^{th}$ day in maize medium at 25°C. The results reveal that the growth at 15°C was generally slower, regardless of medium and isolate. However, YES medium was markedly more favorable to fungal development at both temperatures.

*A. carbonarius* presented traditional morphological characteristics, such as white mycelium and black spores, even though each medium interfered in some colony features. *A. ochraceus* also displayed their common features, such as yellowish spores, although variations in each culture media have also been observed.

## Ochratoxin A production

OTA concentration values ranged from <QL - 30.42 µg/g. YES culture media stood out as the best substrate for OTA production in all treatments (Figure 3). Tables 1 and 2 show the mean OTA concentration obtained *in vitro* by isolates belonging to *A. ochraceus* and *A. carbonarius*, respectively, in relation to culture media, temperature and incubation time. *A. carbonarius* produced a greater amount of OTA in comparison with *A. ochraceus,* the *A.*

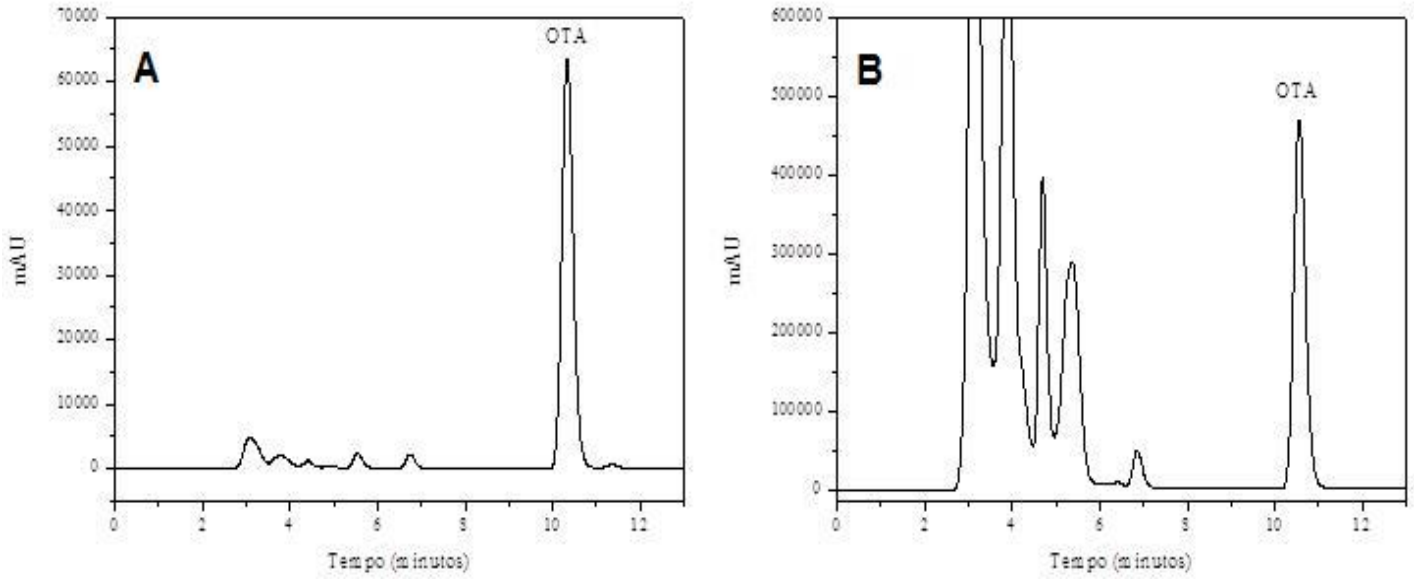


**Figure 3.** HPLC chromatograms of treatments in different factor combinations. (A) *A. carbonarius* CCDCA10608 in YES medium on the 15th day at 15°C; (B) *A. ochraceus* CCDCA10611 in YES medium on the 25th day at 25°C.

**Table 1.** Ochratoxin A concentration produced by three *A. ochraceus* isolates in different culture media, temperature and incubation time.

| Strain | Medium | OTA concentration (ug $g^{-1}$)* ± SD | | | | | |
|---|---|---|---|---|---|---|---|
| | | 15ºC | | | 25ºC | | |
| | | 5 days | 15 days | 25 days | 5 days | 15 days | 25 days |
| *A. ochraceus* CCDCA10613 | YES | 1.28 ± 0.22$^{aA}$ | 0.53 ± 1.66$^{aB}$ | 0.63 ± 0.23$^{aC}$ | 9.95 ± 2.70$^{aA}$ | 3.24 ± 1.75$^{aC}$ | 9.40 ± 2.03$^{aB}$ |
| | Coffee | 0.22 ± 0.38$^{bA}$ | 0.05 ± 0.32$^{bB}$ | 0.04 ± 0.02$^{bB}$ | 0.12 ± 0.77$^{bA}$ | 0.14 ± 0.08$^{bA}$ | 0.06 ± 0.02$^{bB}$ |
| | Wheat | 0.03 ± 0.02$^{cA}$ | 0.05 ± 0.31$^{bA}$ | 0.02 ± 0.34$^{cA}$ | ND | 0.02 ± 0.23$^{cB}$ | 0.04 ± 0.05$^{cB}$ |
| | Maize | 0.01 ± 0.02$^{cA}$ | ND | 0.01 ± 1.76$^{cA}$ | 0.01 ± 0.93$^{cA}$ | 0.03 ± 0.12$^{cA}$ | 0.07 ± 1.23$^{bB}$ |
| *A. ochraceus* CCDCA10612 | YES | 7.05 ± 1.35$^{aA}$ | 2.37 ± 1.98$^{aB}$ | 1.17 ± 0.75$^{aC}$ | 1.20 ± 3.61$^{aA}$ | 1.33 ± 0.98$^{aB}$ | 2.23 ± 0.84$^{aC}$ |
| | Coffee | 0.10 ± 0.99$^{bA}$ | 0.06 ± 0.87$^{bA}$ | 0.39 ± 2.08$^{bB}$ | 0.05 ± 0.04$^{bA}$ | 0.06 ± 0.01$^{cA}$ | 0.07 ± 0.98$^{bA}$ |
| | Wheat | 0.04 ± 1.90$^{cA}$ | 0.01 ± 0.87$^{cA}$ | 0.18 ± 0.98$^{cB}$ | 0.01 ± 0.91$^{bA}$ | 0.12 ± 1.09$^{bB}$ | 0.05 ± 0.02$^{bA}$ |
| | Maize | ND | ND | 0.03 ± 0.09$^{dA}$ | 0.01 ± 1.01$^{bA}$ | ND | 0.04 ± 2.87$^{bA}$ |
| *A. ochraceus* CCDCA10611 | YES | 0.17 ± 0.94$^{aA}$ | 2.83 ± 2.11$^{aB}$ | 8.44 ± 3.91$^{aC}$ | 7.19 ± 0.89$^{aA}$ | 4.61 ± 0.02$^{aB}$ | 24.01 ± 3.01$^{aC}$ |
| | Coffee | 0.10 ± 1.01$^{bA}$ | 0.08 ± 0.01$^{bA}$ | 0.10 ± 0.37$^{bA}$ | 0.23 ± 0.55$^{bA}$ | 0.33 ± 0.34$^{bB}$ | 0.05 ± 0.76$^{cC}$ |
| | Wheat | 0.01 ± 0.62$^{cA}$ | 0.04 ± 0.02$^{cA}$ | 0.01 ± 0.98$^{cA}$ | 0.01 ± 2.78$^{cA}$ | 0.07 ± 0.07$^{cB}$ | 0.14 ± 0.44$^{bC}$ |
| | Maize | ND | ND | 0.02 ± 0.04$^{cA}$ | 0.02 ± 0.29$^{cA}$ | 0.02 ± 1.02$^{dA}$ | 0.04 ± 1.01$^{cA}$ |

*Means followed by the same uppercase (in the row) or lowercase letter (in the column) do not differ from each other by the Scott Knott test at 5% significance. SD: standard deviation; ND: not detected.

*carbonarius* CCDCA10608 isolate standing out, which showed the highest OTA levels: 29.83 µg/g in YES medium at 15°C and 13.06 µg/g in wheat medium at 15°C. The *A. carbonarius* CCDCA10610 isolate presented a concentration of 8.3 µg/g in coffee extract medium at 15°C. Isolate *A. carbonarius* CCDCA10608 produced 5.07 µg/g in coffee extract medium at 25°C. Treatments in which it was not possible to detect any OTA level was about 12.5%. Of these undetected treatments, 61.1% are in maize media.

The highest concentrations produced by *A. ochraceus* were in YES medium. Maize culture medium in all treatments had low or no OTA concentration. *A. ochraceus* isolates produced much lower amounts in

**Table 2.** Ochratoxin A concentration produced by three *A. carbonarius* isolates in different culture media, temperature and incubation time.

| Strain | Medium | OTA concentration (ug $g^{-1}$)* ± SD | | | | | |
|---|---|---|---|---|---|---|---|
| | | 15ºC | | | 25ºC | | |
| | | 5 days | 15 days | 25 days | 5 days | 15 days | 25 days |
| *A. carbonarius* CCDCA10610 | YES | ND | 3.61 ± 1.90$^{bB}$ | 1.00 ± 0.09$^{bC}$ | 2.96 ± 1.33$^{aA}$ | 1.05 ± 0.67$^{cB}$ | 0.12 ± 0.09$^{cC}$ |
| | Coffee | 0.05 ± 0.02$^{aA}$ | 8.30 ± 2.59$^{aB}$ | 3.21 ± 1.32$^{aC}$ | 0.03 ± 0.01$^{cA}$ | 2.33 ± 1.83$^{bB}$ | 2.50 ± 0.78$^{bB}$ |
| | Wheat | 0.02 ± 0.01$^{aA}$ | 0.02 ± 0.01$^{cA}$ | 3.65 ± 0.99$^{aB}$ | 2.33 ± 1.21$^{bA}$ | 5.22 ± 3.17$^{aB}$ | 7.52 ± 2.88$^{aC}$ |
| | Maize | ND | 0.10 ± 0.09$^{cA}$ | 0.06 ± 0.01$^{bA}$ | ND | 0.87 ± 0.29$^{cA}$ | 0.03 ± 0.01$^{cB}$ |
| *A. carbonarius* CCDCA10609 | YES | 0.02 ± 0.01$^{aA}$ | 0.70 ± 0.09$^{bB}$ | 0.67 ± 0.08$^{bB}$ | 1.01 ± 0.48$^{aA}$ | 0.22 ± 0.09$^{cA}$ | 0.11 ± 0.06$^{cB}$ |
| | Coffee | ND | 0.06 ± 0.02$^{aA}$ | 0.11 ± 0.03$^{aA}$ | 0.75 ± 0.09$^{cA}$ | 0.53 ± 0.22$^{bB}$ | 0.26 ± 0.11$^{bB}$ |
| | Wheat | ND | 0.24 ± 0.13$^{cA}$ | 0.33 ± 0.04$^{aA}$ | 0.07 ± 0.06$^{bA}$ | 4.50 ± 1.02$^{aB}$ | 6.24 ± 2.97$^{aC}$ |
| | Maize | ND | 0.01 ± 0.01$^{cA}$ | 0.05 ± 0.02$^{bA}$ | 2.66 ± 0.83$^{cA}$ | 0.09 ± 0.03$^{cA}$ | 0.07 ± 0.03$^{cB}$ |
| *A. carbonarius* CCDCA10608 | YES | ND | 29.83 ± 1.78$^{aB}$ | 14.45 ± 4.08$^{aC}$ | 1.95 ± 0.30$^{bA}$ | 1.27 ± 0.94$^{bB}$ | 0.52 ± 0.30$^{bB}$ |
| | Coffee | ND | 4.05 ± 1.09$^{bB}$ | 3.15 ± 1.03$^{cC}$ | 5.07 ± 1.36$^{aA}$ | 0.53 ± 0.28$^{cA}$ | 0.15 ± 0.10$^{cB}$ |
| | Wheat | ND | 0.39 ± 0.25$^{cA}$ | 13.06 ± 1.92$^{bB}$ | 0.86 ± 0.23$^{cA}$ | 6.12 ± 2.90$^{aB}$ | 9.87 ± 3.04$^{aC}$ |
| | Maize | ND | 0.06 ± 0.02$^{cA}$ | 0.41 ± 0.39$^{dA}$ | 0.07 ± 0.01$^{dA}$ | 0.12 ± 0.03$^{dA}$ | 0.02 ± 0.01$^{cA}$ |

*Means followed by the same uppercase (in the row) - or lowercase letter (in the column) do not differ from each other by the Scott Knott test at 5% significance. SD: standard deviation; ND: not detected.

food-based culture media in comparison with *A. carbonarius* isolates with the maximum production of 0.39 µg/g in coffee extract at 25°C by the *A. ochraceus* CCDCA10612 isolate.

Among all the treatments, it was possible to verify that YES medium was the best substrate for OTA production. The most productive treatment was *A. carbonarius* CCDCA10608; YES; 15°C; 15$^{th}$ day. Regarding food-based culture media, *A. carbonarius* CCDCA10608; wheat medium; 15°C; 15$^{th}$ day stands out as well as the *A. carbonarius* CCDCA10610; coffee extract medium; 15°C; 15$^{th}$ day treatment.

## DISCUSSION

Ecological factors such as water activity, temperature and pH have affect OTA production by *A. ochraceus* and *A. carbonarius* (Pardo et al., 2004; Kapetanakou et al., 2011). Studies demonstrate that a higher temperature range allows *A. carbonarius* growth, as in similar studies for proposed optimal growth between 15 and 37°C. (Pitt et al., 2000; Kogkaki et al 2016; Ioannidis et al., 2015). In general, growth under temperatures below 15°C is not common and is only possible at high water activity levels (Bellí et al., 2004). Other studies suggest that the optimal temperature for *A. carbonarius* growth is between 25 and 30°C (Leong et al., 2004) or between 25 and 35°C depending on the isolate and the growth susbtrate used (Mitchell et al., 2003).

Studies conducted by Palacios-Cabrera et al. (2005) demonstrated that temperatures between 25 and 30°C favored *A. ochraceus* colony diameter growth, on the other hand, slower growth could be observed at 35°C and total inhibition at 41°C in all the tested culture media. Substrates directly affect fungal development. YES culture medium is generally considered to be a very favorable medium for OTA biosynthesis (Skrinjar and Dimic, 1992; Bragulat et al., 2001). YES is rich in sucrose, an energy-rich disaccharide with rapid metabolism which may explain the high growth rate of the six tested isolates. *Aspergillus* section *Circumdati* species produce higher amounts of toxin when the carbon source in the medium is sucrose and lower amounts when the sugar available is fructose (Mühlencoert et al., 2004; Medina et al., 2008; Gil-Serna et al., 2015).

Each culture medium may exhibit a different composition, therefore, varying growth rates. With *A. ochraceus,* for instance, the highest growth rates found in optimal conditions were different among culture media in different studies: *A. ochraceus* cultivated in maize-based agar was 3-4 mm/day (Marín et al., 1998), in barley-based medium, 4-5 mm/day, and up to 6mm/day in coffee extract agar medium (Pardo et al., 2005), whereas in the present study, the growth rate was 15 mm/day in YES medium (25°C), 10 mm/day in coffee extract medium (25°C), 10 mm/day in wheat medium (25°C) and 5 mm/day in maize medium. In semisynthetic grape culture medium, *A. carbonarius* showed the highest growth at temperatures of 20 to 33°C, between 0.95 and 0.98 aw, and pH levels between 5 and 6.5. The highest toxin concentration for *A. carbonarius,* 10 µg/g, was found at 15°C, 0.99 aw, and pH 5.35. However, the optimal conditions for toxin production are generally different from those optimal for fungal growth (Passamani et al., 2014).

The combination of factors that presented the highest OTA level was *A. carbonarius* CCDCA10610 in YES medium at 15°C and these data corroborate the results of Mitchell et al. (2004), Bellí et al. (2004) and Leong et al. (2004), who studied different *A. carbonarius* isolates in different interactions, such as the temperature effect, water activity and incubation time, on the production of OTA. Colony development does not follow the same production standards as OTA. For example, on the 5$^{th}$ day of incubation at 25°C colonies had already covered almost the entire length of the plates, while isolates incubated at 15°C were developing more slowly, even though the OTA production was generally higher at 15°C. According to Leong et al. (2006), OTA production by *A. carbonarius* and *A. niger* was not related to the growth length, colony size was strongly controlled by temperature, and the toxin was reduced when culture was kept at 30°C. Moreover, maximum OTA production may be related to a specific moment after innoculation due to the strong temperature effect on germination. Nutritional factors are closely related to the activation of genes encoding OTA production. Studies performed in 2009 by Abbas and partners assessed the effect of a wide variability of biotic factors based on nutritional differences regarding OTA and OTB production. Different carbon sources including glucose, sucrose, maltose, galactose, xylose and glycerol seemed to supress OTA production. In contrast, lactose appears to induce OTA production, since the addition of lactose and galactose to the restrictive medium (PDC) resulted in marked increases in OTA levels.

Yeast extract is considered an excellent source of vitamins, amino acids, small peptides, nucleotides, minerals and other nutrients (Zang et al., 2003). In this sense, it is clear that the production of OTA by *A. ochraceus* requires a medium nutritionally rich and appropriate. This validates the results obtained in this present study, where the media most responsive to OTA production by *A. ochraceus* were the Yeast Extract Agar (YES) and Coffee Extract Agar. The unroasted coffee has a wider variety of nutrients than wheat and maize flour, and features more than 300 different compounds, being rich in chlorogenic acids, glycosides, lipids, minerals, caffeine and other compounds (Flament, 2002; Clarke and Macrae, 1988).

In some treatments, there was a decrease in OTA detection from the 15$^{th}$ day on. This may occur because some ochratoxigenic fungi may use the OTA produced in the culture medium as substrate, after a certain cultivation time, when nutrients tend to become scarce, in an attempt to find an alternative carbon source. On account of this, strains remove and assimilate the phenylalanine moiety from the OTA molecule like other nitrogen sources in the culture media when they become exhausted (Téren et al., 1996; Varga et al., 2000; Lappa et al., 2015). According to Chalfoun et al. (2000), there was inhibition of OTA production as well as a sporulation decrease by *A. ochraceus* in YES culture media added different caffeine concentrations.

Abrunhosa et al. (2002), observed that most of the strains tested in their study were able to degrade ochratoxin A, whereas 51 strains (67% tested strains) growing in culture medium were able to degrade more than 80% ochratoxin A added to YES medium. In addition, isolates from the genus *Aspergillus (Aspergillus niger, Aspergillus clavatus, Aspergillus ochraceus, Aspergillus versicolor* and *Aspergillus wentii)* stood out as they degraded more than 95% ochratoxin A.

Lower OTA levels were found in treatments with maize-based culture media for both species under study, and the values did not exceed the limit proposed for grain-based food (10 mg/kg) (Agência Nacional De Vigilância Sanitária, 2011), although it is not possible to extrapolate the values of found in tests with culture media to food. These results are in agreement with that of authors who did not detect significant OTA levels in maize silage (Richard et al., 2009; El-Shanawany et al., 2005).

*Aspergillus* are not usually associated with the production of significant OTA quantities in wheat at lower temperatures (Magan and Aldred, 2007). However, *A. ochraceus* isolates CCDCA0151, CCDCA0153 and CCDCA0162 produced OTA at 25°C in wheat medium, the result corroborates the study carried out by Muñoz et al. (2011) which presented high OTA levels (> 50 µg/g agar) at 25°C in wheat-based culture medium.

The polyketide synthase gene (pks) required in the initial OTA production steps has been, in recent years, widely used in the elucidation of the factors that influence and induce the OTA production by some species of Aspergillus (Gallo et al., 2014). According to O'Callaghan et al. (2006), the culture medium is an important factor in the transcription of this gene and regulation of OTA production, furthermore, culture media supplemented with yeast extract exhibited an increase in PKS transcript levels and OTA accumulation of *A. ochraceus*. Results from the present study with ochratoxigenic strains from *Aspergillus* genus demonstrated a significant OTA production in YES culture medium, but they also produced smaller amounts in coffee extract and wheat-based culture media under the temperature and incubation time conditions tested in this study. *A. carbonarius* CCDCA 10608 isolate in YES medium at 15°C showed the highest OTA levels. Growth rates for all isolates were higher in YES medium at 25°C and the slowest growth took place in maize-based medium at 15°C. Therefore, it is possible to determine, among analyzed variables, the best conditions to avoid or at reduce to diminish OTA production by *A. carbonarius* and *A.ochraceus* in coffee beans, maize and wheat.

## Conflict of Interests

The authors have not declared any conflict of interests.

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