Ability of wild strains of Aspergillus niger to produce ocratoxin A in cracked corn

Capacidade de estirpes indígenas de Aspergillus niger para produzirem ocratoxina A em substrato natural (milho)

H. Marina Martins¹*, M. Lígia Martins¹, F. Bernardo² and Alberto Gimeno³

¹Laboratório Nacional de Investigação Veterinária, Serviço de Micología, Estrada de Bentica, 701, 1549-011 Lisboa, Portugal
²CIISA, Faculdade de Medicina Veterinária
³Special Nutrients, INC., 2766 Douglas Road, Miami, Florida, 33133 USA.

Summary: Aspergillus niger are worldwide distributed, occurring on a great variety of substrates, including grains, fresh and dried fruits (grapes) and many vegetables and food. Among the Aspergillus genus, the production of ochratoxin A (OTA) has been also referred by strains of Asp. niger. A total of 35 wild strains of Aspergillus niger were isolated from green coffee grains (22 strains) and from feedstuffs (13 strains) and screened for their ability to produce OTA “in vitro” in cracked corn (Zea mays), during 10 weeks at 25 ºC and aw =0.98. OTA production was determined weekly, using immunoaffinity columns and HPLC, with a quantification limit of 1µg/kg. The incidence of ochratoxigenic isolates in the Asp. niger, studied was very low: eight of the 35 isolates (22.9%) were ochratoxin producers. All positive strains tested had the maximum peak of OTA at 14th day (2º week), decreasing till 8º week of incubation, when OTA disappeared. Concerning the 22 strains isolated from green coffee grains, 5 were ochratoxinogenic (22.7%) with levels ranging from 1 to 3.5 µg/kg. Of the 13 strains of Asp. niger isolated from feedstuffs, three strains produced OTA (23,0%), with levels ranging from 1 to 3.2 µg/kg. These wild strains can be considered weak producers of OTA under the conditions assayed.

Key-words: Aspergillus niger, ochratoxin A, in vitro production.

Resumo: Aspergillus niger é um Ascomicete de distribuição cosmopolita, que é isolado com frequência em cereais, frutos secos e frescos (uvas), outras matérias vegetais e em géneros alimen-
tícios conservados. Recentemente, a capacidade de produção de ochratoxina A (OTA) foi identificada em estirpes de Aspergillus niger competentes. Com o objectivo de avaliar a capacidade de produção de OTA por Asp. niger, utilizaram-se um total de 35 estirpes indígenas a partir de grãos de café verde (22 estirpes) e de alimentos compostos para animais (13 estirpes). A capacidade de produção foi testada in vitro utilizando um substrato natural (milho triturado-Zea mays), incubado durante 10 semanas a 25 ºC com aw=0.98. A pesquisa de ochratoxina A foi efectuada semanalmente, utilizando colunas de imunoafinidade e por HPLC. O limite de quantificação da técnica é de 1µg/kg. A prevalência de Aspergillus niger ochratoxigênicos, revelou-se foi bastante baixa. Oito dos isolados eram produtoras de OTA (22,9%). Todas as estirpes positivas atingiram um ponto máximo de produção ao 14º dia de incubação (2ª semana), seguido de um declínio até à 8ª semana. No que se refere aos isolados a partir de grãos de café verde, 5 eram ochratoxigênicas (22,7%) com níveis de produção entre 1 a 3,5 µg/kg. Das estirpes de Asp. niger isoladas de alimentos compostos para animais 3 produziram ochratoxina A (23,0%), com níveis de 1 a 3,2 µg/kg. No modelo de ensaio desen
dado estas estirpes podem ser consideradas fracas produtoras de ochratoxina A.

Palavras-chave: Aspergillus niger, ochratoxina A, produção de in vitro

Introduction

The genus Aspergillus represents a very large proportion of all the moulds found in industrial food (Onions et al., 1981), and they have particular importance to humans and animals. Although many Aspergillus species are considered pathogens or spoilage organisms, some are beneficial. Some group of Aspergillus (black Aspergilli) are used for the production of gluconic acid, and of gallic acid from tannin (Onions et al., 1981). They have also particular importance as spoilage organ-
isms of food. Many species grow at very low water activity and are found attacking jam, cakes tobacco and are often the primary invaders in moulding of cereals and producing mycotoxins (Betina, 1989). Among the mycotoxins produced by species of this genus, ochra
toxin A (OTA) is receiving major attention for its nephrotoxic effect and its potential carcinogenic activity (Kuiper-Goodman and Scott, 1989). OTA is produced by several related Aspergillus species such as Aspergillus ochraceus, Asp. carbonarius with a low percentage of strains closely related with Aspergillus niger (FAO/WHO-JECFA, 2001) and by a single Penicillium species, P. verrucosum.

*Correspondência:
e-mail: marina.martins@lniv.min-agricultura.pt
Recently, it was referred that *Aspergillus niger* could be OTA producer (Abarca et al., 1994; Abarca et al., 1997). OTA is the most toxic mycotoxin in ochratoxin group (Cox and Cole, 1981). Its biosynthesis is determined by many factors, such as, temperature and water activity (aw) of the substrate, trace elements, cisteine and structurally related compounds and other nutrients (Bacon et al., 1973; Northolt et al., 1975). OTA is considered as a probable causative agent of diseases in human, pig and poultry (Gattier et al., 1981). It has been related as the most probable cause in nephrotoxic syndromes in pigs in Denmark and humans in Balkan countries, however, this link has not yet been definitively proven (Studer-Rohr et al., 1995).

In Portugal the presence of the *Aspergillus niger* are described in feedstuffs (Martins and Martins, 2001) in food (honey) and medicinal teas (Martins et al., 2001a; Martins et al, 2001b). Few studies are available on the capacity of *Aspergillus niger* to produce OTA. For this reason the aim of this study was to evaluate the occurrence of OTA-positive strains belonging to *Aspergillus niger*, isolated from food and feed from Portugal.

**Materials and methods**

**Strains**

A total of 35 strains of *Aspergillus niger* isolated from green coffee (22) and from feedstuffs (13) were screened for their ability to produce “in vitro” OTA in cracked corn (*Zea mays*), during 10 weeks at 25°C and *aw* = 0.98.

The strains of *A. niger* were picked to Czapek agar plates (Oxoid – CM 97, Portugal), and incubated at 25°C for 5 days. These isolates were identified considering both macroscopic and microscopic morphological aspects and compared to descriptions given by Raper and Fennell,(1965); Domsch, *et al.*, (1980) and Samson and Pitt, (1989).

**In vitro OTA production**

The study of OTA production by *A. niger*, was carried out in duplicate on Erlenmeyer flasks containing 25 g of sterilised cracked corn, adding 20 ml of distilled water and adjusting *aw* to 0.98. The corn contained neither fungal nor OTA contamination.

Autoclaved substrate was inoculated with 2 ml of the spore suspension, according to the following procedure: 5 ml of sterile distilled water was added to each slant of five days old culture and gently scraping the agar surface to give a turbid suspension, corresponding to 1x10⁶ spores/ml. Two ml of this suspension were added to the cracked corn. Inoculated flasks were shaken daily for the first three days. The incubation temperature was 25 °C, during 10 weeks. Cultures were examined for OTA determination, weekly.

**Ochratoxin determination and quantification by HPLC**

The ochratoxin determination was performed according to the method described by Entwistle *et al.* SMT4-CT96-2045). OTA was extracted from the 25 g of the sample with acetonitrile. The extract was cleaned up by passing through an immunofluor affinity column (OchrPrep, Code P 14B, Rhône-Diagnostics Technologies Ltd, Spain), and the OTA was eluted with methanol: water: acetic acid, and separated by reverse phase HPLC using a LiChrospher 100 RP-18, 5 µm column 25 x 4.6 mm EcoPack (Merck, Portugal), with fluorescence detector and computing integrator Merck Hitachi (Compaq Deskpro); excitation and emission wavelengths were 333 nm and 460 nm. The mobile phase was water-acetonitrile-acetic acid (102:96:2), filtered through a 0.22 µm filter membrane, and used at a flow rate of 1.0 ml/min. Working standards solutions for calibration curve, limit of detection and percentage recovery were determined according to the method previously referred. Standard of OTA was purchased from Sigma (Ref.O-1877). Samples with OTA level below 1 μg/kg were considered as negative, (inferior to quantification limit).

**Results**

Twenty seven of the 35 isolates of *Aspergillus niger* (77.1%) were negative for OTA and, only eight were ochratoxin producers (22.9%) (Table 1). All positive strains tested had a maximum peak of OTA at 14th day (2nd week), decreasing slightly till 8th week of incubation. At 9th and 10th week the presence of OTA was not detected, (less than detection limit). Concerning the 22 strains isolated from green coffee grains, 5 (22.7%) were ochratoxinogenic with levels ranging from 1 to 3.5 μg/kg, and 17 (77.3%) were negatives. Of the 13 strains of *Aspergillus niger* isolated from feedstuffs only three strains (23.0%) produced OTA, with levels ranging from 1 to 3.2μg/kg; 10 (77.0%) were negatives (Tables 1 and 2). The incidence of ochratoxigenic isolates was very low, and the levels of OTA produced by *Asp. niger* isolated from the two different sources (green coffee grains and feedstuffs) were quite similar.

**Table 1 - Frequency of Aspergillus niger strains in OTA producer**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Tested strains</th>
<th>OTA ≥ /N (%)</th>
<th>ND &lt; 1μg/kg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>coffee</td>
<td>22</td>
<td>5/22 (22.7)</td>
<td>17 (77.3)</td>
</tr>
<tr>
<td>feeds</td>
<td>13</td>
<td>3/13 (23.0)</td>
<td>10 (76.9)</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>8/35 (22.9)</td>
<td>27 (77.1)</td>
</tr>
</tbody>
</table>

*N*: nº of strains tested

ND- not detected, below the quantification limit (1.0 μg/kg)
Table 2 - Ochratoxin A (µg/kg) produced by Aspergillus niger in cracked corn

<table>
<thead>
<tr>
<th>Weeks</th>
<th>CS 1</th>
<th>CS 2</th>
<th>CS 3</th>
<th>CS 4</th>
<th>CS 5</th>
<th>FS 6</th>
<th>FS 7</th>
<th>FS 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>1.6</td>
<td>1.0</td>
<td>1.3</td>
<td>2.9</td>
<td>2.7</td>
<td>2.6</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>2nd</td>
<td>3.2</td>
<td>2.2</td>
<td>2.3</td>
<td>3.1</td>
<td>3.5</td>
<td>3.2</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>3rd</td>
<td>2.8</td>
<td>1.8</td>
<td>2.1</td>
<td>3.0</td>
<td>2.7</td>
<td>2.2</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>4th</td>
<td>2.8</td>
<td>1.8</td>
<td>2.0</td>
<td>2.7</td>
<td>2.1</td>
<td>1.6</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>5th</td>
<td>1.9</td>
<td>1.5</td>
<td>1.8</td>
<td>2.5</td>
<td>1.7</td>
<td>1.6</td>
<td>1.9</td>
<td>1.2</td>
</tr>
<tr>
<td>6th</td>
<td>1.4</td>
<td>1.2</td>
<td>1.8</td>
<td>1.7</td>
<td>1.5</td>
<td>1.3</td>
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<td>7th</td>
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<td>1.2</td>
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<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
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<td>1.0</td>
</tr>
<tr>
<td>8th</td>
<td>1.0</td>
<td>1.1</td>
<td>1.5</td>
<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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</tr>
</tbody>
</table>

Average of two determinations; * Not detected; nd = limit detection (1µg/kg). • CS1; CS2; CS3; CS4; CS5 - Strains of A.niger isolated from green coffee grains • FS6; FS7; FS8 - Strains of A.niger isolated from feed

Discussion

Heenan et al. (1998) tested OTA production in 115 *Asp. niger* isolates using two detection techniques: growth on coconut cream agar (CCA) and by thin layer chromatography (TLC). OTA production was detected in two (2%) of *Asp. niger* isolates examined by either technique.

In 2001, Urbano et al. evaluated the capacity of *Asp. niger* strains to produce OTA, obtaining positive results from 11.5% of the strains isolated from coffee beans.

In extensive review, Abarca et al. (2001), stated that the ability of *Asp. Niger* for OTA production was lower than *Asp. ochraceus* or *Penicillium verrucosum*.

Eventually the strains tested in this study can be considered weak OTA producers under conditions of this assay. Comparing the levels of this mycotoxin production with others obtained by different authors Heenan et al (1998), Urbano et al. (2001) and Abarca et al. (2001), the results were significantly lower.

In conclusion, the presence of *Asp. niger* can be relevant in terms of safety attending to its importance in the public health. The results of this study indicate that the potential production of OTA by *Asp. niger* is not a relevant problem.

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References


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