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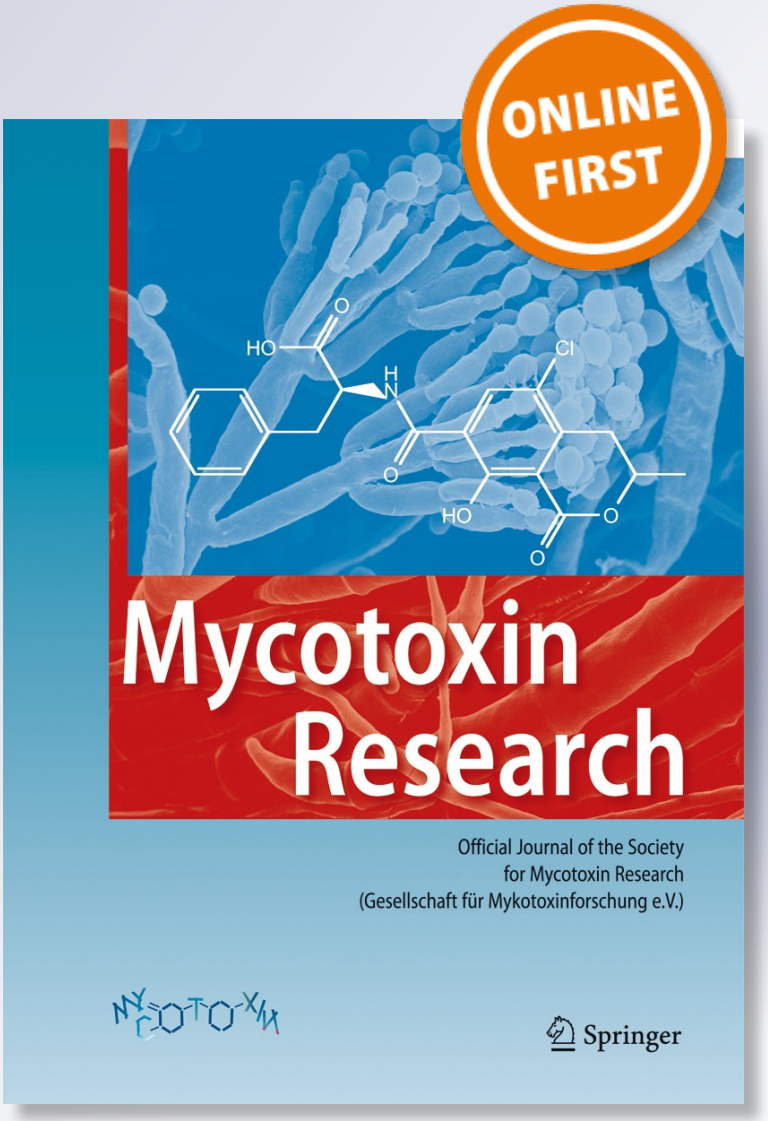

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ORIGINAL PAPER

Effect of indigenous mycobiota on ochratoxin A production by *Aspergillus carbonarius* isolated from soil

Ochratoxin in mixed cultures

Carla L. Barberis · Gabriela Pena · Cecilia Carranza · Carina E. Magnoli

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Abstract This study determined the biotic interaction between 30 non-toxigenic indigenous strains of *Aspergillus niger* aggregate, *Aspergillus flavus*, *Trichoderma* spp., *Mucor* spp., *Cladosporium* spp., *Ulocladium* spp., *Curvularia* spp., *Absidia* spp., *Geotrichum* spp. and *Acremonium* spp., isolated from soil destined for maize crops, with respect to their ability to prevent ochratoxin A (OTA) production by *A. carbonarius* on "in vitro" assay, on liquid and solid medium. OTA production was completely inhibited when *A. carbonarius* was inoculated in a interactive mixed culture with all *A. niger* aggregate strains assayed, a 80 % of *Trichoderma* spp. strains, a 40 % of *Cladosporium* spp. strains, *Acremonium* spp and *Geotrichum* spp; only one strain of *A. flavus* tested was able to completely inhibit the mycotoxin accumulation. OTA production increased when *A. carbonarius* ACS 8 was growing on liquid interactive mixed culture with *Mucor* spp strains. These results demonstrated that OTA production by *Aspergillua carbonarius* strain was significantly influenced by the presence of different non-toxigenic fungal strains when growing together on paired cultures.

Keywords *Aspergillus carbonarius* · Indigenous mycobiota · Ochratoxin A · Interacting mixed cultures

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Introduction

Argentina is the world's second biggest exporter of maize (*Zea mays* L.), and has been responsible roughly for 15 % of the world's maize exports in the last 3 years. During the harvest season 2012-2013, maize production reached 26.5 million tons (Secretaría de Agricultura Ganadería, Pesca y Alimentación 2013).

In soil destined for maize crops, and in the surrounding vegetation, the dominant fungi include species of *Aspergillus*, *Fusarium*, *Penicillum*, *Trichoderma, Cladosporium* and *Alternaria* (González et al. 1995). Throughout the world, maize and maize derivatives are frequently contaminated with a family of mycotoxins such as ochratoxins and ochratoxigenic fungi (Caldas et al. 2002; Magnoli et al. 2006a; Rosa et al. 2006; Fraga et al. 2007). In Argentina, *Aspergillus carbonarius*, *A. niger* aggregate species and uniseriates *Aspegillus* species such as *A. aculeatus* and *A. japonicus* have frequently been isolated from several agricultural products, e.g., coffee beans, red wine, dried vine fruits, maize and peanut kernels, and feeds (Dalcero et al. 2002; Magnoli et al. 2003, 2004, 2005, 2006a, b, 2007; Ponsone et al. 2007, 2009).

Magnoli et al. (2006a, b) evaluated ochratoxigenic mycoflora from 50 samples of corn kernels for human consumption corresponding to the 2003-2004 harvest season. These samples were obtained from stored plants located in Argentina. Among the *Aspergillus*, section *Nigri* were isolated in a 62 % of corn samples. Among 112 black *Aspergillus* strains, 30 (25 %) were OTA producers with levels ranging from 2 to 31.5 ng ml^{-1}. Potential OTA production was evaluated by Magnoli et al. (2007) from corn-based feeds (poultry, pig and rabbit feeds) who found that *A. niger* strains were isolated from 27.5 % and *A. awamori* strains were isolated from 17.5 % of poultry, pig and rabbit feeds. In another study from Argentina, black *Aspergillus* strains isolated from poultry, pig and rabbit feeds

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were tested for OTA production The highest percentage of ochratoxigenic strains was isolated from rabbit feeds with 100 % of *A. niger*-producing strains, and the natural incidence of OTA in corn-based feeds was found in 38 % of the poultry feed samples with levels ranging from 25 to 30 ng g^{-1}. From rabbit feed samples, 25 % contained OTA, on levels ranged between 18.5 and 25.5 ng g^{-1} (Dalcero A et al. 2002). Astoreca et al. (2007) found that ochratoxigenic *A. niger* strains were capable of producing OTA on corn-based medium under different environmental conditions, and reported OTA levels of up to 387.4 ng g^{-1}.

Ochratoxin A (OTA) is one of the most important fungal toxic metabolites of worldwide concern for human and animal health, due to its diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity, and immunotoxicity (O'Brien and Dietrich 2005). It has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC 1993; Pfohl-Leszkowicz and Manderville 2007). A maximum level for OTA (5 μg/kg) has been established for cereal and cereal products and dried vine fruit by European Community regulations (EC) 466/2006 (Commission of the European Communities 2006). Ochratoxin A has been detected in some of these substrates (Dalcero et al. 2002; Magnoli et al. 2003, 2004, 2005, 2006a, b, 2007; Ponsone et al. 2007, 2009).

A promising strategy to reduce mycotoxin accumulation in maize ears at harvest involved the biological interaction among toxigenic fungi and natural biocompetitive agents like non-mycotoxin-producing strains (atoxigenic strains) of the same species, which competitively exclude mycotoxin producers in agricultural environments and thereby reduce mycotoxin contamination (Garber and Cotty 1997; Dorner and Cole 2002; Dorner et al. 2003). The use of bacteria or yeast to control pre- and post-harvest pathogens and pests of agricultural commodities has already been studied (Milus and Rothrock 1996; McSpadden-Gardener and Fravel 2002; Pereira et al. 2007, 2008). In previous "in vitro" studies, bacteria and yeast isolates were selected to control *Aspergillus* section *Flavi* or *F. verticillioides* (Nesci et al. 2005; Cavaglieri et al. 2005a, b; La Penna et al. 2004; La Penna and Etcheverry 2006). In some of these studies, a negative correlation between *A. flavus* and *F. moniliforme* (now *verticilliodes*) species have been demonstrated in maize grains (Wicklow et al. 1988; Cuero et al. 1988; Widstrom et al. 1994; Yoshizawa et al. 1996; Torres et al. 1997; Picco et al. 1999). The ability of several fungal cultures to prevent aflatoxin B_1 synthesis in a liquid medium has been reported. Among these, *Phoma*, *Mucor*, *Rhizopus*, *Alternaria* and *Trichoderma* species consistently reduced aflatoxins by 90 % or more (Zuber and Lillehoj 1993; Calistru et al. 1997; Aziz and Shahin 1997; Shantha 1999). However, there have been no studies on the influence of indigenous mycobiota on OTA production by *A. carbonarius* species.

The aim of this study was to determine ochratoxin A biodegradation through a liquid medium assay and a biotic interaction of interactive mixed cultures in solid medium, between 30 indigenous strains of different fungal genera isolated from soil destined for maize crops, with respect to their ability to prevent OTA production by an *A. carbonarius* strain.

Materials and methods

Fungal strains

Aspergillus carbonarius (ACS 8) as an active producer of ochratoxin A (OTA), and 30 non-toxigenic tested strains of *Aspergillus niger* aggregate (5 strains), *A. flavus* (5 strains), *Trichoderma* spp. (5 strains), *Mucor* spp. (5 strains), *Cladosporium* spp. (5 strains), *Ulocladium* spp. (1 strain), *Curvularia* spp. (1 strain), *Absidia* spp. (1 strain), *Geotrichum* spp. (1 strain), and *Acremonium* spp. (1 strain), used as biocompetitive agents in this study, were isolated from soil samples destined for maize crops in Argentina, and were identified by classic taxonomy according to the methodology proposed by Samson et al. (2000) and Klich et al. (2002). The strains were maintained in glycerol (15 %; Sigma-Aldrich) at −80 °C and kept in the culture collection at the Department of Microbiology and Immunology, National University of Río Cuarto, Córdoba, Argentina.

Inoculum preparation

All strains were induced to sporulate on Malt Extract Agar (MEA) slants at 28 °C for 7 days. At the end of the incubation period, 5 ml of spore suspension of each fungal strain solution was added to the slants and harvested by vigorous agitation. The concentration of spores was measured with a Neubauer chamber. The volume of conidial suspension was adjusted to 10^6 spores/ml. The conidial viability was confirmed by the standard plate count method using MEA.

Liquid medium assay

Culture medium and inoculation

Interactive mixed cultures of *A. carbonarius* (ACS 8) and each of the tested fungal strains were co-inoculated in 50 ml of YES medium (2 % yeast extract and 15 % sucrose) (Magnoli et al. 2007) with 1 ml of each conidia suspension (of 10^6 conidia mL^{-1}). Flasks single-inoculated with *A. carbonarius* (ACS 8) were used as controls. Flasks were incubated for 10 days in dark at 30±2)°C, and were shaken every day. Four replicates per interactive mixed culture were used and all the experiments were repeated twice.

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Ochratoxin A production

OTA production was tested in all the interactive mixed cultures and was determined following the methodology described by Téren et al. (1996), with some modifications. After incubation, a portion of these culture media (1 ml) was mixed with 1 ml of chloroform and centrifuged at 4, 000*g* for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness, and re-dissolved in 0.5 ml of methanol.

Solid medium assay

Culture medium and inoculation

Yeast extract sucrose agar (YES) was prepared. The water activity (a_W) of the basic medium was adjusted to 0.980 and 0.930 with known amounts of glycerol (Barberis et al. 2009). The basic medium was autoclaved at 120 °C for 20 min before cooling to 50 °C and pouring into 90-mm sterile Petri dishes. Water activity of representative samples of each treatment was checked with an AquaLab Series 3 (Decagon Devices, WA, USA). In addition, control plates were prepared and a_W were measured at the end of the experiment in order to detect any significant deviation of a_W. Plates were inoculated centrally by needle single point with *A. carbonarius* strain, as controls, from 7-day-old cultures on MEA. Inoculated Petri dishes were sealed in polyethylene bags. Four replicate plates per treatment were used and incubated at 25 °C for 14 days; all the experiments were repeated twice.

Interactive cultures of *A. carbonarius* (ACS 8) and each of the tested indigenous fungal strains were co-inoculated on YES medium by needle single point, separated at a distance of 45 mm in every plate. Cultures were grown for 14 days at two a_W (0.98 and 0.93). Radii of colonies were recorded daily and OTA production was tested after 7 and 14 days of incubation at 4 cm from the inoculation point.

Ochratoxin A production

At 7 and 14 days of incubation, OTA was determined following the methodology proposed by Bragulat et al. (2001) with some modifications. From the plates of each treatment, three-agar plugs were removed from different points of the colony and extracted with 1 ml of methanol. The mixture was centrifuged at 14,000 rpm for 10 min. The solutions were filtered, evaporated to dryness, re-dissolved in mobile phase, and the extract injected into the HPLC.

Ochratoxin A detection

The detection of OTA was performed by HPLC, following the methodology proposed by Scudamore and MacDonald (1998). The HPLC apparatus used for determination of OTA was a Hewlett Packard chromatograph with a loop of 50 μl, equipped with a spectrofluorescence detector (excitation, 330 nm; emission, 460 nm) and a C18 column (Supelcosil LC-ABZ, Supelco; 150×4.6 mm, 5 μm particle size), connected to a precolumn (Supelguard LCABZ, Supelco; 20×4.6 mm, 5 μm particle size). The mobile phase was pumped at 1.0 ml min^{-1} and consisted of an isocratic system as follows: 57 % acetonitrile, 41 % water, and 2 % acetic acid.

Table 1 Ochratoxin A production by *Aspergillus carbonarius* (ACS 8) strain in single and interacting mixed cultures on liquid YES medium

Fungal strains	Concentration OTA (μg ml^{-1})	Inhibition %
A. carbonarius ACS 8[a]	139.38	0
A. carbonarius [a] – *A. niger* aggregate	nd*	100
A. carbonarius [a] – *A. niger* aggregate	nd*	100
A. carbonarius [a] – *A. niger* aggregate	nd*	100
A. carbonarius [a] – *A. niger* aggregate	nd*	100
A. carbonarius [a] – *A. niger* aggregate	nd*	100
A. carbonarius [a] – *A. flavus* (AFS 1)	18.38*	86.8
A. carbonarius [a] – *A. flavus* (AFS 2)	41.56*	70.1
A. carbonarius [a] – *A. flavus* (AFS 3)	nd*	100
A. carbonarius [a] – *A. flavus* (AFS 375)	80.78	42
A. carbonarius [a] – *A. flavus* (AFS 389)	58.18*	58.2
A. carbonarius [a] – *Trichoderma* spp.	87.38	37.3
A. carbonarius [a] –*Trichoderma* spp.	nd*	100
A. carbonarius [a] –*Trichoderma* spp.	nd*	100
A. carbonarius [a] –*Trichoderma* spp.	nd*	100
A. carbonarius [a] –*Trichoderma* spp.	nd*	100
A. carbonarius [a] – *Mucor* spp.	147.58	<5.8
A. carbonarius [a] – *Mucor* spp.	22.46*	83.8
A. carbonarius [a] – *Mucor* spp.	38.58*	72.3
A. carbonarius [a] – *Mucor* spp.	179.78	<28.9
A. carbonarius [a] – *Mucor* spp.	115.98	16.7
A. carbonarius [a] – *Cladosporium* spp.	32.22*	76.8
A. carbonarius [a] – *Cladosporium* spp.	32.24*	76.8
A. carbonarius [a] – *Cladosporium* spp.	39.18*	71.8
A. carbonarius [a] – *Cladosporium* spp.	nd*	100
A. carbonarius [a] – *Cladosporium* spp.	nd*	100
A. carbonarius [a] – *Ulocladium* spp.	28.34*	79.6
A. carbonarius [a] – *Curvularia* spp.	28.28*	79.7
A. carbonarius [a] –*A bsidia* spp.	13.96*	90
A. carbonarius [a] – *Acremonium* spp.	nd*	100
A. carbonarius [a] – *Geotrichum* spp.	nd*	100

Mean values based on quadruplicated data.

nd Not detected

[a] *A. carbonarius* (ACS 8), OTA producer

*Mean significantly different according to Bonferroni tets (p <0.0001)

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OTA was quantified on the basis of HPLC fluorometric response compared with an OTA standard (Sigma Aldrich, St. Louis, MO, USA; purity N99%). The lowest limit of detection was 1 ng g^{-1}. Each sample was analyzed three times.

Statistical analysis

Data analyses were performed by analysis of variance. Means were compared by Bonferroni test for liquid assay and Fisher's protected LSD test for solid assay to determine the significant differences between the control and the co-inoculated cultures. The Pearson correlation coefficient was used to evaluate the strength of the relationship between OTA levels produced by *A. carbonarius* (ACS 8) in interactive mixed cultures, with indigenous fungal strains, on liquid and solid media. The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC), USA (Quinn and Keugh 2002).

Results and discussion

Table 1 shows the ability of 30 indigenous fungal strains to degrading OTA production by *A. carbonarius* on liquid YES medium; all strains were isolated from soil destined for maize crops. The fungal strains were non-toxigenic belonging to the genera: *A. niger* aggregate (5 strains), *A. flavus* (5 strains), *Trichoderma* spp. (5 strains), *Mucor* spp. (5 strains), *Cladosporium* spp. (5 strains), *Ulocladium* spp. (1 strain), *Curvularia* spp. (1 strain), *Absidia* spp. (1 strain), *Acremonium* spp. (1 strain), and *Geotrichum* spp. (1 strain).

OTA production was completely inhibited when *A. carbonarius* was inoculated in a liquid interactive mixed culture with all *A. niger* aggregate strains, 80 % of *Trichoderma* spp. strains, and 40 % of *Cladosporium* spp. strains, *Acremonium* spp and *Geotrichum* spp; only one strain of *A. flavus* was able to completely inhibit the mycotoxin accumulation.

A. flavus strains reduced significantly OTA production by *A. carbonarius* in 71.4 % with OTA levels ranged from 18.38 to 80.78 μg/ml of culture medium (mean level: 49.72 μg/ml) ($p < 0.0001$).

Toxin accumulation was significantly diminished by all *Cladosporium* spp. strains tested; two of them completely inhibited OTA production, while the decrease reached to 75 % (OTA levels ranged from 32.22 to 39.18 μg/ml) for the rest of *Cladosporium* spp. strains assayed.

In interactive mixed cultures of *A. carbonarius* (ACS 8) and strains belonging to genera *Ulocladium* spp., *Curvularia* spp. and *Absidia* spp. a significant reduce on OTA production was observed ($p < 0.0001$).

OTA production increased from 139.38 μg/gml to 147.58 and 179.78 μg/ml (corresponding to 5.8 and 28.9 % respectively), when *A. carbonarius* ACS 8 was growing on interactive mixed culture whit *Mucor* spp strains.

The Bonferroni test of data shows the significant differences among control treatments and interacting mixed cultures. The statistical analysis of strains showed that all fungal strains analyzed influenced significantly OTA production ($p < 0.0001$) (Table 2).

This is one of the few "in vitro" studies developed to examine the effects of indigenous mycobiota strains isolated from soil on OTA production by *A. carbonarius* on interactive mixed cultures using YES medium. These results demonstrated that OTA production by *A. carbonarius* strain was significantly influenced by the presence of different non-toxigenic fungal strains when grown together on paired cultures. Toxin evaluation showed that OTA accumulation was completely reduced by *A. niger* aggregate, *Trichoderma* spp., *Acremonium* spp., and *Geotrichum* spp. strains on culture medium.

Table 2 Analysis of effect variance of different isolates (*I*), and their interactions on ochratoxin A production of *Aspergillus carbonarius* (c) strain

Source of variation	OTA production						
	Liquid medium assay			Solid medium assay			
	df[a]	MS[b]	*F*[c]	*df*[a]	MS[b]	*F*[c]	Pr > *F*
I	119	68.30	15,162.34*	78	72.33	13,977.5*	0.0001
C	3	219.94	48,822.78*	3	202.52	46,739.67*	0.0001
I x C	452	9.45	2,098.68*	399	8.31	1,997.97*	0.0001

[a] Degrees of freedom

[b] Mean square

[c] F Snedecor

*Significant $p < 0.0001$

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Varga et al. (2000) examined the OTA decomposing activities of a number of *Aspergillus* strains, OTA producers, and OTA nonproducers and found that none of the OTA producers could significantly lower the OTA content, *A. niger* strains were found to be able to degrade OTA in liquid YES media. Although Hwang and Draughon (1994) found that *A. niger* is unable to degrade OTA, an *A. niger* isolate was later reported to be able to convert OTA to ochratoxin α (Xiao et al. 1991). This work shows that *A. niger* aggregate strains completely inhibit OTA accumulation on liquid and solid assays, Varga et al. (2000) found that on solid medium OTA inhibition was higher than on liquid medium. The results reported in this work show that strains belonging to *Cladosporium* spp, and *A.* section *Flavi* strains significantly reduce OTA accumulation. Previous publications have reported that species of the genera *Alternaria, Cladosporium*, and *Trichoderma* are capable of degrading OTA, probably by means of carboxypeptidase A activity (Abrunhosa et al. 2002). Carboxypeptidase secreted by the *A. niger* strain could decompose OA to ochratoxin A and phenyl alanine. Carboxypeptidase A has earlier been found to be able to convert OTA to ochratoxin α (Deberghes et al. 1995; Varga et al. 2000, 2005).

In another recent study, Cvetnic et al. (2007) observed that growth of *A. flavus* NRRL 3251 was suppressed and AFB_1 production completely inhibited when incubated with *Alternaria* or *Cladosporium* strains in mixed liquid cultures in all combinations tested. A decrease in aflatoxin production was also observed in dual cultivation with *A. niger.* Cvetnic et al. (2007) found that five strains of *Mucor* spp. were the least antagonistic to the productive strain *A. flavus* NRRL 3251 when growing together on YES medium, reducing aflatoxin B_1 to levels from 18.7 to 9.3 μg/ ml (50 % of alfatoxin inhibition).

On solid medium assay, ten indigenous strains belonging to different fungal genera were tested to evaluate the influence on OTA accumulation by *A. carbonarius* (ACS 8) isolated from soil destined for maize crops on YES medium and 0.980 and 0.930 a_W; this data are detailed in Fig. 1. Toxin production on solid medium had, in general, the same pattern as the one observed on liquid medium. In general, the largest amount of OTA was observed (in control treatments) at 0.980 a_W and 7 days of incubation time. At both a_W assayed (0.980 and 0.930), *A. niger aggregate* spp, *Acremonium* spp., and *Geotrichum* spp. strains completely inhibit ochratoxin A

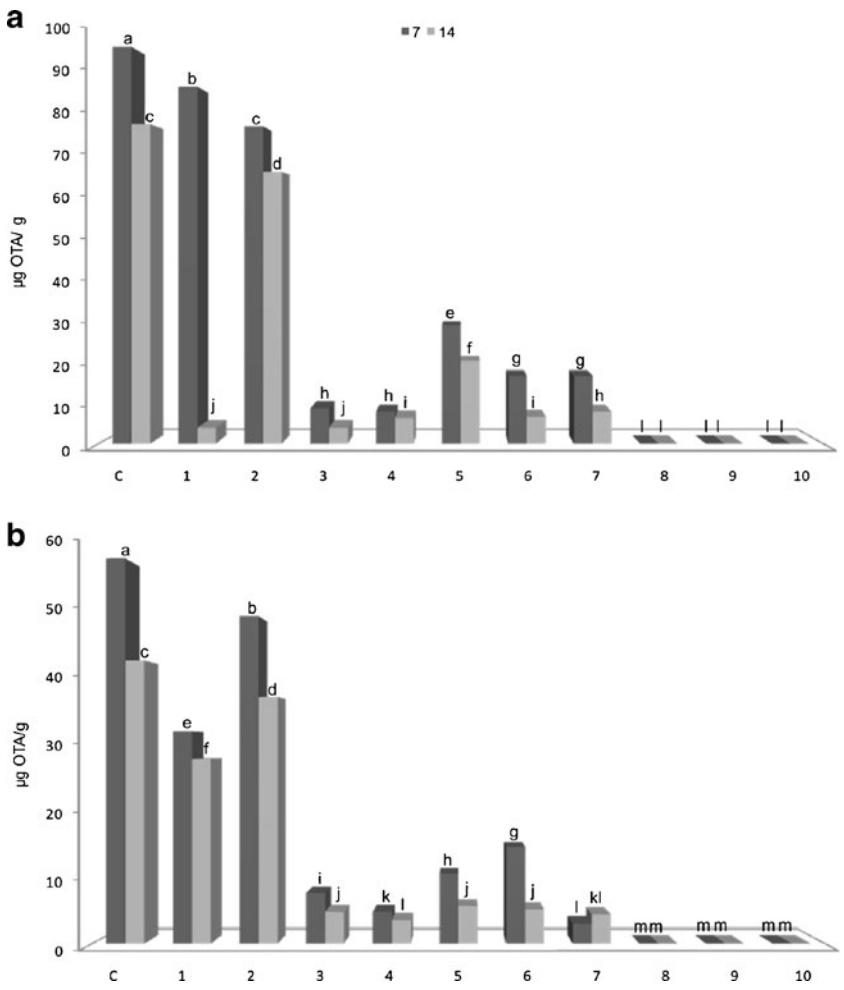

Fig. 1 Ochratoxin A production by *Aspergillus carbonarius* (ACS 8) strain on interacting mixed cultures on solid YES medium at 7 and 14 days of incubation time. **a** 0.980 a_W; **b** 0.930 a_W. *C* Control; *1 Absidia* spp.; *2 Mucor* spp.; *3 Trichoderma* spp.; *4 Cladosporium* spp.; *5 Ulocladium* spp.; *6 Curvularia* spp.; *7 A. flavus*; *8 A. niger* aggregate; *9 Acremonium* spp.; *10 Geotrichum* spp. Mean values based on quadruplicated data. Means in *columns* with a *letter* in common are not significantly different according to LSD test ($p < 0.0001$)

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accumulation by *A. carbonarius* (ACS 8) for all times of incubation ($p < 0.0001$).

The highest percentages of OTA inhibition at 0.980 a_W were observed on interactive cultures of *A. carbonarius* with *Trichoderma* spp., *Cladosporium* spp., *Curvularia* spp., and *A. flavus* strains (91.05, 91.98, 83.02, and 83.06 % respectively), and at 0.930 a_W, with the strains belonging to the genera *Trichoderma* spp. (86.97 %), *Cladosporium* spp. (81.94 %), and *A. flavus* (95.03 %) at both incubation times evaluated. On interactive mixed culture of *A. carbonarius* (ACS 8) with *Absidia* spp., at 0.980 a_W and 14 days of incubation, a significant reduction (95.02 %) in OTA accumulation was observed; this fact was not evident at 7 days of incubation ($p < 0.0001$). On solid medium assay, *Mucor* spp. strain assayed diminished OTA accumulation by 20 %, but this fact was not observed on liquid assay, in which two of the five strains assayed stimulated toxin production.

A decrease of 10.4 % was observed on the interactive mixed culture of *A. carbonarius* with the strain belonging to the genera *Absidia* spp. on solid assay, while the same interaction produced a 90 % of inhibition on OTA production when growing together on liquid YES medium. The LSD test of data showed the significant differences among control treatments and interacting mixed cultures. The statistical analysis of strains showed that all fungal strains analyzed significantly influenced OTA production ($p < 0.0001$) (Table 2). A significant ($p < 0.0001$) correlation ($r = 0.736$) between the OTA levels, roduced by *A. carbonarius* (ACS 8) on interactive cultures with no toxigenic indigenous fungal strains on liquid and solid medium assays was found (data not shown).

The results obtained in the present work agree with Valero et al. (2006) shown that *A. carbonarius* was the highest OTA producer in pure culture, followed by *A. niger* aggregate OTA-positive, with their mixed inoculum producing the least. These authors observed that, in general, when competing fungi were added to the *A. carbonarius* inoculum, the OTA content was reduced, mostly in combination with *A. niger* aggregate OTA-positive. Thus, intra-section fungal competence affects OTA accumulation. This reduction could be due (1) to the growth limitation of OTA producer strain, (2) to the using up of specific nutrients for OTA synthesis, (3) or to its degradation by competing fungi (Abrunhosa et al. 2002). Interacting fungi could even excrete substances that diffuse towards the *A. carbonarius* colony and block OTA synthesis. The growth and OTA production inhibition in dual cultures can be explained by the competition for specific nutrients, antimycotoxigenic metabolites produced by co-existing fungi which specifically inhibit ochratoxin A synthesis, biodegradation of this mycotoxin, and/or a combination of these factors (Shanta et al. 1990).

Wicklow et al. (2005) revealed that *Acremonium zeae* strains were capable of diminishing growth and aflatoxin production by *A. flavus* strains on maize grains. Valero et al. (2006) showed that higher OTA levels were obtained (in comparison with OTA accumulation produced by strains in single cultures) when combining *Eurotium amstelodami* or *Penicillium janthinellum* with *A. carbonarius* and *A. niger* aggregate OTA-producing strains; this was not observed in the present study.

The results of this study suggest that certain fungal strains isolated from soil could be effective OTA inhibitors, as they are able to completely reduce OTA production. Further studies could address the topic of OTA degradation with the aim of developing corrective techniques that reduce OTA accumulation as much as possible.

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Conflict of interest None

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