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ORIGINAL PAPER

# Inhibition of growth and ochratoxin A biosynthesis in *Aspergillus carbonarius* by flavonoid and nonflavonoid compounds

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**Abstract** The effect of naturally occurring phenolic compounds on *Aspergillus carbonarius* growth and ochratoxin A (OTA) production was studied. Caffeic acid and the flavonoids, rutin and quercetin, were added to Czapek Yeast Extract agar at concentrations ranging between 50 and 500 mg/l. All phenolic compounds had a significant influence on growth rate and lag phase of *A. carbonarius* at 250 mg/l. The growth was completely inhibited with 500 mg/l. In comparison with the control, a significant decrease in OTA production was observed with all phenolic compounds. In general, effect on growth was less evident than effect on toxin production. An inhibitory effect on growth and OTA production, as concentration was increased was observed in all cases. The response of *A. carbonarius* to the flavonoids, rutin and quercetin, was similar. The inhibitory effect of these natural phenolic compounds on fungal growth and OTA production could be an alternative to the use of chemical fungicides.

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## Introduction

Phenolic compounds are widespread plant secondary metabolites. In terms of chemical structures, there are three classes: non-flavonoids or phenolic acids, flavonoids, and tannins. Besides other biological characteristics, phenolic compounds showed antimicrobial properties against invading microorganisms. Since their contribution in defense mechanisms of plant tissues in response to infections or injuries is well established (Nychas 1995), they offer an alternative approach in food preservation as naturally occurring antimicrobials. Several studies have demonstrated the inhibitory effect of different types of phenolic compounds on bacteria that affect the human health (Puupponen-Pimiä et al. 2001; Rodríguez Vaquero et al. 2007). Of recent interest has been the possible role of natural phenolic compounds in inhibiting growth and toxin production by toxigenic fungi such as *Aspergillus flavus* and several *Fusarium* species (Beekrum et al. 2003; Mahoney and Molyneux 2004; Samapundo et al. 2007). Another interesting finding was the correlation between the resistance to fungal attack and the content of plant phenolic compounds in some cultivars of agriculturally important crops (Siranidou et al. 2002).

Grapes and wines contain a large array of phenolic compounds. The specific amounts and types of compounds depend on a number of factors, including variety, maturity, seasonal conditions, storage, and processing practices (Alberto et al. 2004). The effect of phenolics on the growth of lactic acid bacteria of importance in the winemaking process has been demonstrated (Alberto et al. 2001, 2004; Campos et al. 2003) but there is little information about the

effect of these compounds on the fungal population of grapes.

*Aspergillus* section *Nigri* have been reported as the predominant mycobiota of grapes from several regions (Da Rocha Rosa et al. 2002; Battilani et al. 2003). The "black aspergilli" (mainly *A. niger* aggregate and *A. carbonarius*) are not only responsible for spoilage of berries during the pre- and post-harvest but are also able to produce ochratoxin A (OTA), a nephrotoxin recognized by the IARC (International Agency for Research on Cancer) as a possible human carcinogen. OTA possesses teratogenic, inmunotoxic, and possibly genotoxic properties (Ringot et al. 2006). This mycotoxin has widely been detected in food products and beverages including cereal grains, beer, coffee, nuts, grapes, wine, and dried vine fruits (Zimmerli and Dick 1996; MAFF 1999). Some *Aspergillus* and *Penicillium* species, such as *A. ochraceus* and *P. verrucosum*, are considered typical OTA-producing fungi, but these are unlikely to be significant sources of OTA in some substrates. Recent surveys have shown that OTA in grapes, wine, and dried vine fruits comes from OTA-producing strains from *Aspergillus* section *Nigri*, and within this group, *A. carbonarius* is the main source of OTA in those products, especially because a high percentage of its isolates are able to produce the toxin (Bragulat et al. 2001; Battilani and Pietri 2002; Cabañes et al. 2002; Abarca et al. 2003).

A high rate of ochratoxigenic strains among *A. carbonarius* isolates from grapes and dried vine fruits from Argentina and natural occurrence of OTA in these products have been detected (Da Rocha Rosa et al. 2002; Magnoli et al. 2004; Romero et al. 2005). The aim of this study was to determine the effect of naturally occurring phenolic compounds in grapes on *A. carbonarius* growth and OTA production. A phenolic acid (caffeic acid) and two flavonoids, rutin and quercetin, were tested.

## Materials and methods

### Fungal isolates

Four strains of *A. carbonarius* (BAFC 3392, 3393, 3394, and 3395) isolated from dried vine fruits in a previous work (Romero et al. 2005) were used in this study. The strains are held in the BAFC (Buenos Aires Facultad de Ciencias) culture collection.

### Culture media

Growth and OTA production was determined on Czapek Yeast Extract (CYA) agar, which contained, per liter, 1 g of $K_2HPO_4$, 10 ml of Czapek concentrate with trace metals, 5 g of yeast extract, 30 g of sucrose, and 15 g of agar (Klich 2002). CYA was reported as the best culture medium for OTA production by *Aspergillus carbonarius* (Bragulat et al. 2001; Esteban et al. 2004). Caffeic acid, quercetin, and rutin (ICN, Argentina) were assayed at 50, 250, and 500 mg/l. Phenolic compounds were dissolved in absolute ethanol and the filter-sterilized solutions were added to the autoclaved base medium. The CYA medium with ethanol was used as a control.

### Inoculation and incubation

Inocula were prepared by growing each strain on malt extract agar at 25°C for 7 days to obtain heavily sporulating cultures. A mixed inoculum was prepared with the four strains according to Hocking and Miscamble (1995). Spores of each strain were placed in aqueous solution of 0.05% Tween 80. After homogenizing, the suspension was counted using a Neubauer chamber and adjusted to $10^6$ spores/ml. Mixed inoculum was prepared adding 1 ml of each spore suspension. CYA plates were inoculated centrally with a 1-µl calibrated loop of inoculum and were incubated at 30°C during 30 days.

### Growth measurement

The radial mycelial growth was determined by periodical measurement of two right-angled diameters of the colonies. Colony diameters versus time were plotted and radial growth rates (mm/day) were evaluated from the slope by linear regression. Lag phase was determined as the abscissa from growth rate curves. All the experiments were performed in quintuplicate.

### OTA analysis

OTA was determined by triplicate after 7, 14, 21, and 28 days of incubation for each phenolic compound at each concentration assayed following the method of Bragulat et al. (2001). Three agar plugs were removed from the centre of the colony and extracted with 0.5 ml of methanol. The extracts were filtered (Agilent technologies 25/0.2 µm NL) directly into amber vials and stored at 4°C until HPLC analysis was performed. OTA detection and quantification was made by a Shimadzu LC-CA liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Rheodyne sample valve fitted with a 20-µl loop and a spectrofluorometric detector Shimadzu RF-10Axl ($\lambda_{exc}$ 330 nm; $\lambda_{em}$ 460 nm). Acetonitrile, water and acetic acid (57:41:2) with a flow rate of 1 ml/min was the mobile phase and a C18 column (Waters Spherisorb 5 µm, ODS2, 4.6 x 250 mm) was used. A calibration curve was constructed for quantification purposes using the OTA standard (Sigma-Aldrich,

Argentina) and correlating peak area versus concentration. The extracts with the same retention time as OTA (around 5.8 min) were considered positive. The peak identity was confirmed by means of co-injection with the corresponding standard. The quantification limit was 0.05 µg/g of agar.

Statistical treatment of the results

The effect of phenolic compounds on growth rate, lag phase and OTA concentrations was evaluated by analysis of variance (ANOVA) using Statistix 8.1. Comparisons of means were conducted by Tukey´s test of honestly significant difference ($p<0.05$).

## Results

The effect of caffeic acid on *A. carbonarius* growth and OTA production was evaluated in a range of concentrations between 0 and 500 mg/l (Fig. 1). Statistical analysis of variance (ANOVA) showed that caffeic acid had significant effects on growth rate and lag phase ($p<0.0001$). In presence of 50 mg/l of caffeic acid the radial growth rate was slightly increased in comparison with the control (without phenolic compound) and at 250 mg/l was reduced by ca. 10%. *A. carbonarius* growth was completely inhibited with 500 mg/l (Fig. 1a). The lag phases increased with increasing concentrations of the compound (Fig. 1b).

OTA production was determined weekly during a period of 28 days. Analysis of variance showed significant effects of caffeic acid concentration and incubation time on OTA production ($p<0.01$). A significant reduction in OTA production was observed with caffeic acid at 250 mg/l (Fig. 1c) in comparison with the control. The mycotoxin production decreased by 63 and 60% at 21 and 28 days of incubation, respectively, in the presence of 250 mg/l of phenolic acid. Non-significant differences were observed using 50 mg/l. In general, OTA production increased with a larger incubation time, although a non-significant difference was observed between 21 and 28 days of incubation.

The effect of the flavonoids, rutin and quercetin, on *A. carbonarius* growth and OTA production was studied at concentrations of 50, 250, and 500 mg/l (Fig. 2). The statistical treatment of the data by ANOVA showed that the flavonoids had a significant influence on growth rate and lag phase ($p<0.0001$). It can be seen in Fig. 2a,b that the effect of both phenolic compounds on *A. carbonarius* growth rate was similar. Non-significant differences as compared to the control were observed in the presence of 50 mg/l. With 250 mg/l, the radial growth rate was reduced by 26 and 20% with rutin and quercetin, respectively. *A. carbonarius* growth was completely inhibited in the

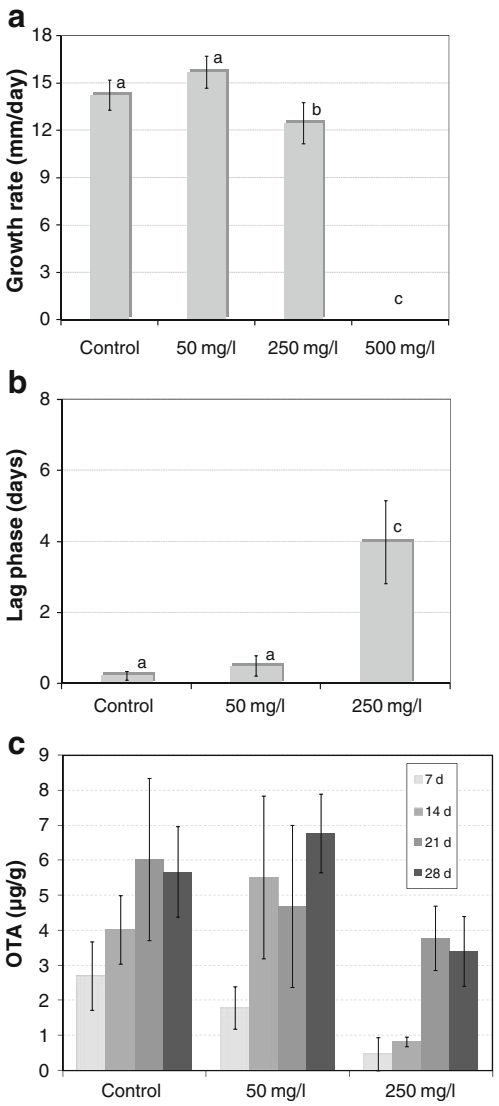


**Fig. 1** Effect of different concentrations (mg/l) of caffeic acid on *Aspergillus carbonarius* growth rate (**a**), lag phase (**b**) and OTA production (**c**). *Bars* with different *letters* are statistically different ($p<0.05$)

presence of 500 mg/l of both flavonoids. The effect on lag phase was observed at 250 mg/l, being affected more strongly by quercetin (Fig. 2c,d).

Figure 2e,f show OTA production by *A. carbonarius*, during a period of 28 days in the presence of rutin and quercetin, respectively. The ANOVA showed that the different concentrations and incubation time had a significant influence on OTA biosynthesis ($p<0.0001$). In general, OTA production increased to a larger incubation time up to 21 days of incubation. Non-significant differences were observed using rutin or quercetin. The analysis of variance of the effect of two-way interaction showed that all interactions were statistically non-significant (Table 1). An important inhibitory effect of these flavonoid concen-

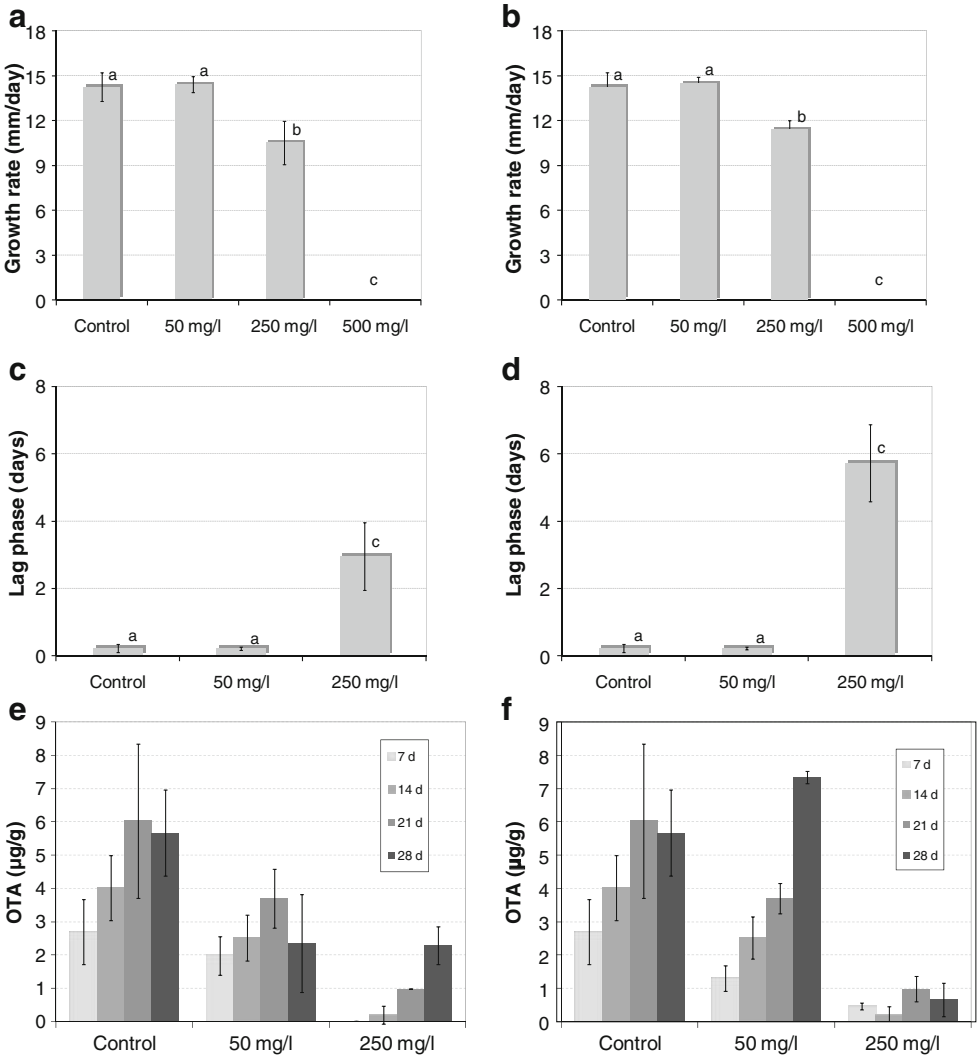


**Fig. 2** Effect of different concentrations (mg/l) of rutin (**a**,**c**,**e**) and quercetin (**b**,**d**,**f**) on *Aspergillus carbonarius* growth rate, lag phase, and OTA production. *Bars* with different *letters* are statistically different ($p<0.05$)

trations on OTA production was observed with 250 mg/l at all incubation times (Fig. 2e,f).

**Table 1** Analysis of variance of the effect of rutin and quercetin concentration, incubation time, and their interactions on the OTA production of *A. carbonarius*

| Source of variation | *F* |
|---|---|
| Phenolic compound | 0.13 $^{ns}$ |
| Concentration | 61.1* |
| Incubation time | 16.1* |
| Phenolic compound – concentration | 1.7 $^{ns}$ |
| Phenolic compound- incubation time | 1.5 $^{ns}$ |
| Concentration- incubation time | 1.4 $^{ns}$ |
| Concentration- phenolic compound- incubation time | 3.7** |

*F* F-Snedecor

*$p<0.0001$;,**$p<0.01$, $^{ns}$ not significant

## Discussion

The results of the present study confirm the inhibitory effect of different phenolic compounds (flavonoids and non-flavonoids) on *A. carbonarius* growth and OTA production. The effect on growth was less evident than the effect on toxin production. Our results are in agreement with the effects demonstrated by other authors in previous works related to other toxigenic fungi. Bisogno et al. (2007) reported that the MIC (minimal inhibitory concentration) of caffeic acid for *A. flavus*, *A. niger* and *A. terreus* was >250 mg/l. Phenolic extracts of callus tissues of olive, which mainly contains caffeic acid and to a lesser extent catechin and coumarins, was inhibited by 90% aflatoxin production without inhibiting the growth of *A. flavus* (Nychas 1995). Growth of *Aspergillus flavus* and *A.*

*parasiticus* was not affected while aflatoxin $B_1$ production by these fungal species was significantly reduced by caffeic acid (Samapundo et al. 2007). Palumbo et al. (2007) studied the effect of phenolic antioxidants, including caffeic acid, on OTA production and fungal growth of several ochratoxigenic *Aspergilli*. With regard to *A. carbonarius*, caffeic acid to 10 mM (ca. 1,700 mg/l) did not affect growth but tended to inhibit OTA production. However, Beekrum et al. (2003) and Guiraud et al. (1995) reported that caffeic acid has an inhibitory effect on both growth and mycotoxin production by fungi. Phenolic compounds have also been found to be inhibitory to the production of several mycotoxins, including fumonisins, tricothecenes, and aflatoxins (Chipley and Uraih 1980; Norton 1999; Bakan et al. 2003; Beekrum et al. 2003).

According to our results, the response of *A. carbonarius* growth and OTA production in the presence of the flavonoids, rutin and quercetin, was similar. Mallozzi et al. (1996) reported that the flavonoids quercetin, kaempherol, kaempheritrin, and naringinin at 300, 100, 300, and 125 ppm decrease 36, 40, 49, and 60% *A. flavus* growth in culture media, respectively. Against a range of fungi of the genus *Aspergillus*, Weidenbörner et al. (1990) reported little activity for quercetin and naringenin. Pereira et al. (2008) observed in A. *ochraceus* that the MIC value for rutin was 35 mg/l.

The specific amounts and types of phenolics present in grapes and wines depend on a number of factors including grape variety and the vinification process. The concentration of total phenols varied from 1,800 to 4,059 mg/l gallic acid equivalents (GAE) averaging 2,567 mg/l GAE for red wines and from 165 to 331 mg/l GAE averaging 239 mg/l GAE for white wines (Alberto et al. 2001). Phenols are responsible for red wine color, astringency, and bitterness, in addition to contributing somewhat to the olfactory profile of the wine (Alberto et al. 2004). The results of several surveys have demonstrated that OTA concentration is higher in red than in white wines (Zimmerli and Dick 1996; Otteneder and Majerus 2000). This trend to a higher content of OTA in red wines seems to be more related to the conditions of the winemaking process (Soufleros et al. 2003) than to their higher concentration in phenolic compounds.

The possible role of natural phenolic compounds in inhibiting growth and toxin production has been of recent interest as an alternative strategy to the use of chemical fungicides. The mechanisms by which these compounds can suppress mycotoxin formation are not yet established. Phenolic acids, being potent antioxidants and free radical scavengers, could counter the oxidative stress that triggers or enhances the toxin production by the molds (Mahoney and Molyneux 2004).

Consumer perception that the use of industrially synthesized food preservatives may be associated with potential toxicological problems has generated interest in the use of naturally occurring compounds (Sofos et al. 1998). Being widespread plant secondary metabolites found essentially in all plant material, phenolic compounds potentially provide an acceptable source of natural antimicrobials to prevent mycotoxin contamination in the pre- and post-harvest. The successful inhibition of both mycelial growth and release of ochratoxin A by the phenolic compounds used in the present study and normally present in grapes indicates the possibility of their use as plant fungicides, especially against the growth of ochratoxigenic *Aspergilli*. The effects of these compounds in combination with other antifungal agents or hurdles should be further investigated.

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