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Effect of gallic acid on *Aspergillus carbonarius* growth and ochratoxin A production

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Abstract

The aim of the present study was to investigate the gallic acid influence on *Aspergillus carbonarius* growth and ochratoxin A (OTA) biosynthesis. A mixed inoculum was used in Czapek Yeast Extract Agar and growth and OTA production was observed at 100 to 500 mg/l of the phenolic compound. Slower colony growth rates and longer lag phases were observed with increasing concentration and 500 mg/l was totally inhibitory for *A. carbonarius* growth. OTA production decrease was observed even at the lower concentration assayed. Gallic acid could be used as a natural antimicrobial for the control of ochratoxigenic *A. carbonarius*.

Keywords: antifungal activity, black Aspergilli, mycotoxin inhibition, phenolic compound

1. Introduction

Plant phenolic compounds have been reported to have multiple biological effects such as antioxidant activity, anti-inflammatory action, inhibition of platelet aggregation and antimicrobial activities (Kanner *et al.*, 1994). The inhibitory effect of phenolic acids on bacteria that affect human health has been studied (Puupponen-Pimiä *et al.*, 2001; Rodríguez Vaquero *et al.*, 2007) as well as their effect on lactic acid bacteria of importance in winemaking, since grapes and wine contain a large array of phenolic compounds (Alberto *et al.*, 2002, 2004, 2007). Gallic acid is the major phenolic acid present in grapes. Several studies have shown that phenolic compounds can control the growth rate and spore germination time of spoilage fungi (Hope *et al.*, 2003; López-Malo *et al.*, 2005) and the biosynthesis of mycotoxins such as aflatoxins and fumonisins (Beekrum *et al.*, 2003; Norton, 1999; Samapundo *et al.*, 2007). Mahoney and Molyneux (2004) demonstrated that gallic acid had potent inhibitory activity toward aflatoxin biosynthesis and concluded that the resistance to aflatoxin contamination of certain walnut varieties could be essentially attributable to the high content of this phenolic acid.

To our knowledge, the effect of gallic acid on the production of other mycotoxins has not been extensively studied. Ochratoxin A (OTA) is receiving increasing attention worldwide because of the hazard it poses to human and animal health. OTA is a nephrotoxin with immunosuppressive and teratogenic properties and has been recognised by the IARC (International Agency for Research of Cancer) as a possible human carcinogen (Ringot *et al.*, 2006). This mycotoxin has been widely detected in food products and beverages including cereal grains, beer, coffee, nuts, grapes and its derived products (Chung *et al.*, 2009; Jorgensen, 1998; MacDonald *et al.*, 1999; MAFF, 1999; Pittet, 1998; Serra *et al.*, 2005; Zimmerli and Dick, 1996). The mould identified as responsible for such contamination is *Aspergillus carbonarius*, a 'black Aspergilli' commonly associated with spoilage of berries during pre- and post-harvest (Abarca *et al.*, 2003; Cabañes *et al.*, 2002). It can be hypothesised that phenolic antioxidants would affect OTA production in a similar way to that observed for aflatoxin. The objective of the present work was to determine the effect of gallic acid on *A. carbonarius* growth kinetics and OTA production.

2. 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 litre, 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 *A. carbonarius* (Bragulat *et al.*, 2001; Esteban *et al.*, 2004). Gallic acid (Merck, Buenos Aires, Argentina) was assayed at 100, 200, 300 and 500 mg/l. For each concentration, the phenolic compound was dissolved in absolute ethanol and the filter-sterilised solution was added to autoclaved base medium. The CYA medium with the same amount of absolute 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 an aqueous solution of 0.05% Tween 80. After homogenising, the suspension was counted using a Neubauer chamber and adjusted to 10^6 spores/ml. Mixed inoculum was prepared adding one millilitre of each spore suspension. CYA plates were inoculated centrally with a 1 μl calibrated loop of inoculum and were incubated at 30 °C.

Growth measurement

The radial mycelial growth was determined by periodical measurement of two right-angled diameters of the colonies. Colony diameters vs. 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 at each concentration assayed following the method described by 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 (25/0.2 μm Agilent Technologies, Amstelveen, the Netherlands) directly into amber vials and analysed by HPLC. 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 (λ_{exc} 330 nm; λ_{em} 460 nm). Acetonitrile:water:acetic acid (57:41:2) with a flow rate of 1 ml/min was the mobile phase and a C18 column (Waters Spherisorb 5μm, ODS2, 4.6×250 mm; Milford, MA, USA) was used. A calibration curve was constructed for quantification purposes using the OTA standard (Sigma-Aldrich, Buenos Aires, 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 detection limit was 0.02 ng OTA and the quantification limit was 0.05 μg/g.

Statistical treatment of the results

The effect of gallic acid on growth rate, lag phase and OTA concentrations was evaluated by analysis of variance (ANOVA) using Statistix 8.1 (Analytical Software, Tallahassee, FL, USA). Comparisons of means were conducted by Tukey´s test of honestly significant difference ($P<0.05$).

3. Results and discussion

The effect of gallic acid on *A. carbonarius* growth and OTA production was evaluated in a range of concentrations between 100 and 500 mg/l. The statistical treatment of the data by ANOVA showed that gallic acid had significant effects on lag phase and growth rate ($P<0.0001$). Longer lag phases and slower colony growth rates were observed with increases in gallic acid concentration, the highest concentration (500 mg/l) being totally inhibitory for *A. carbonarius* growth (Figure 1A and 1B). The extension of the lag phase in presence of gallic acid demonstrates the effectiveness of the treatment in preventing fungal contamination and consequent mycotoxin production.

OTA production was determined weekly over a period of 28 days (Figure 2). Analysis of variance demonstrated that gallic acid concentration had a significant influence on OTA production ($P<0.0001$) but not significant differences in incubation time and a concentration-incubation time interaction was observed. A marked effect of this compound on OTA production was observed even at the lowest concentration assayed (100 mg/l).

Palumbo *et al.* (2007) studied the effect of phenolic antioxidants, including gallic acid, on OTA production and fungal growth of several ochratoxigenic Aspergilli in a synthetic medium. With regard to *A. carbonarius*, gallic acid

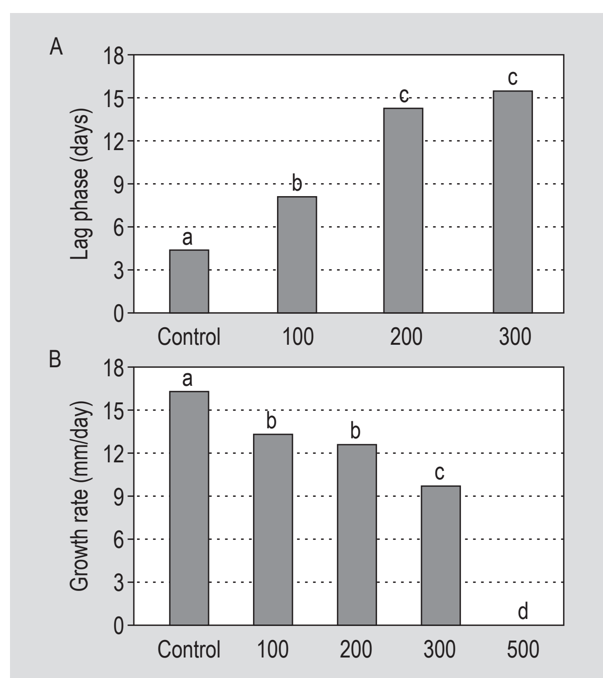

Figure 1. Effect of different concentrations (mg/l) of gallic acid on *Aspergillus carbonarius* lag phase (A) and growth rate (B). Bars with different letters are statistically different (*P*<0.05).

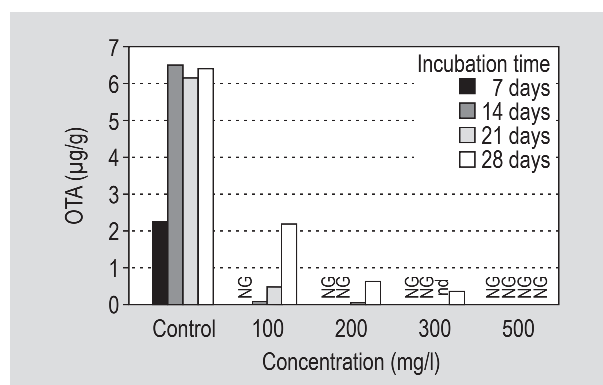

Figure 2. Effect of different concentrations of gallic acid (mg/l) on OTA production (µg/g) by *Aspergillus carbonarius*. NG: no growth; nd: not detected. Quantification limit: 0.05 µg/g.

did not affect growth but tended to inhibit OTA production. These authors reported a high variability in the response of *A. carbonarius* to the antioxidant treatment probably due to the use of strains that produced very low levels of OTA even in the control cultures. On the other hand, Barberis *et al.* (2009a,b) observed that other phenolic antioxidants of food grade (butylated hydroxyanisole and propyl paraben) inhibited OTA production by several strains of *Aspergillus* section *Nigri*.

The results of the present work show the inhibitory effect of gallic acid on OTA production by *A. carbonarius*, similar to those reported by Mahoney and Molyneux (2004) for aflatoxin production by *Aspergillus flavus*. The mechanisms by which this compound can suppress mycotoxin formation have not yet been established. Possible modes of action of gallic acid in inhibiting aflatoxin production have been proposed by Mahoney and Molyneux (2004). It has been suggested that gallic acid may be affecting genes that are known to control specific steps on aflatoxin biosynthesis encodes for a zinc-containing, DNA-binding protein. Gallic acid acts as a chelator of this metal ion through sequestration by catecholic hydroxyl groups. It can be pointed out that zinc is also an important ion for OTA biosynthesis (Mühlencoert *et al.*, 2004). Another hypothesis is that phenolic acids, being potent antioxidants and free radical scavengers, can counter the oxidative stress that triggers or enhances the toxin production by the moulds (Mahoney and Molyneux, 2004).

In the last few years, the possible role of natural phenolic compounds in the inhibition of growth and mycotoxin production has been considered as an alternative to the use of chemical fungicides. The results of the present work suggest that gallic acid could be used as a natural antimicrobial for the control of ochratoxigenic *A. carbonarius* in foods.

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