International Journal of Food Microbiology 140 (2010) 277–281

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Ochratoxin A production by a mixed inoculum of *Aspergillus carbonarius* at different conditions of water activity and temperature

Stella M. Romero *, Virginia Fernández Pinto, Andrea Patriarca, Graciela Vaamonde

Laboratorio de Microbiología de Alimentos, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pab. II, 3° Piso (1428), Buenos Aires, Argentina

A R T I C L E I N F O

Article history:
Received 6 November 2009
Received in revised form 5 March 2010
Accepted 10 April 2010

Keywords:
Aspergillus carbonarius
Ochratoxin A
Mixed inoculum
Water activity
Temperature

A B S T R A C T

Growth rate, lag phase and OTA production of a mixed inoculum of four *Aspergillus carbonarius* strains were compared to the behaviour of the single strains at 30 °C on Czapek Yeast Extract Agar. Significant differences between radial growth rates and lag phases of the different isolates were observed; however, no significant differences between growth rates of each strain and the mixed inoculum were detected. When the four strains were inoculated simultaneously, the lag phase was the same than the higher value obtained for individual strains, suggesting mycelial interactions between the *A. carbonarius* isolates. The four strains differed in maximum OTA yield, and the toxin accumulation by the mixed inoculum showed intermediate levels at each time point. The effects of water activity (0.83, 0.85, 0.87, 0.89, 0.90, 0.93 and 0.95) and temperature (15, 20, 25, 30, and 35 °C) on OTA production by the mixed inoculum were studied at 7, 14, 21 and 28 days of incubation. The limiting water activity for OTA production by the mixed inoculum was 0.87, showing xerotolerant behaviour of the strains isolated from dried vine fruits. Results obtained were similar to those reported for single *A. carbonarius* strains from European countries, Israel, Australia and South America. The similar trend in the response of the different isolates to the variation of environmental parameters may be of interest for the building of predictive models.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, inmunosuppressive and teratogenic properties and has been recognized by the IARC (International Agency for Research of Cancer) as a possible human carcinogen (Ringot et al., 2006). Since *Aspergillus carbonarius* was reported as the main species responsible for OTA contamination in grapes and grape derived products (Cabañes et al., 2002) many studies on the ecophysiology of this mold have been carried out in different countries (Astoreca et al. 2007; Battilani et al. 2006; Bellí et al., 2005; Esteban et al., 2004; Leong et al., 2006; Marín et al. 2006; Mitchell et al., 2004; Palacios-Cabrera et al., 2005; Romero et al., 2007; Valero et al., 2005). All these research works provided very useful information to predict whether the fungus will grow and will be able to produce the toxin in the pre and post harvest of different commodities.

Black aspergilli and *A. carbonarius* in particular, are cosmopolitan fungi that have been isolated from vegetable foods in different regions around the world. Information on the ecophysiology of a wide variety of strains from different climatic regions is important in developing realistic forecasting systems for predicting risk of colonization by *A. carbonarius* and OTA production (Mitchell et al., 2004). The use of different strains for building a database to determine the conditions conducive to OTA contamination in different commodities and environments is needed.

The concept of using cocktail inocula was introduced for physiological studies on foodborne bacterial pathogens, particularly in acquisition of data for predictive modelling studies, as a way of determining the extremes of growth limits for particular species (Buchanan et al., 1993). Other authors (Gibson et al. 1987) suggested that the use of cocktail could minimize the variation that might be expected between different isolates of the same species. Determining the extremes of growth limits suggests finding the most tolerant or aggressive characteristics of the species, whereas minimizing the influence of the strain would imply obtaining an average tendency of strains behaviour within a species.

A cocktail inoculum was applied for the first time to studies on the a_w tolerances of fungi by Hocking and Miscamble (1995) and was considered as a legitimate method of achieving a "worst case" scenario, i.e. establishing the most extreme conditions under which a particular species is capable of growth. However, to our knowledge there are no studies comparing growth parameters and mycotoxin production by individual fungal strains with those of a mixed inoculum.

The aims of the present work were: a) to study the response of an *A. carbonarius* cocktail inoculum in comparison with the behaviour of each individual strain for growth and OTA production, and b) to

* Corresponding author. Tel/fax: +54 11 4576 3346.
E-mail address: smromero@qo.fcen.uba.ar (S.M. Romero).

0168-1605/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2010.04.013

apply the mixed inoculum technique as a practical method to determine the combined effect of temperature and water activity on OTA production by *A. carbonarius* isolated from Argentinean dried vine fruits.

2. Materials and methods

2.1. Fungal isolates

Four strains of *A. carbonarius* (A, B, C and D) isolated from dried vine fruits in a previous work (Romero et al. 2005) and studied for growth in Romero et al. (2007) were used in the present work. The strains named A, B, C and D are held in the BAFC (Buenos Aires Facultad de Ciencias) culture collection as BAFC 3392, BAFC 3393, BAFC 3394 and BAFC 3395 respectively.

2.2. Individual strains versus mixed inoculum comparison

2.2.1. Culture media

Growth and OTA production were determined on Czapek Yeast Extract (CYA) agar, which contained, per liter, 1 g of K_2HPO_4, 10 ml of Czapek concentrate, 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).

2.2.2. Inoculation and incubation

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

2.2.3. Growth measurement

The radial mycelial growth was determined by periodical measurement on a daily basis 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 for each individual strain and the mixed inoculum.

2.2.4. OTA analysis

Three plates inoculated with each strain and the mixed inoculum were analyzed after 7, 14, 21 and 28 days of incubation for OTA production 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 (CAMEO® 17 N 0.22 µm) directly into amber vials and analyzed by HPLC. OTA was detected and quantified using 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). The analytical column was Jupiter 4.6×250 mm 5 µm C18 (Phenomenex, USA). The mobile phase was acetonitrile, water and acetic acid (57:41:2) with a flow rate of 1 ml/min. A calibration curve was constructed for quantification purposes using the OTA standard (Sigma- Aldrich) 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 and quantification limit of the analyses were 0.02 and 0.05 µg/g, respectively.

2.3. Effect of water activity and temperature on OTA production by a mixed inoculum

2.3.1. Culture media

OTA production was determined on CYA at different water activity levels. To generate a_w of 0.83, 0.85, 0.87, 0.89, 0.90, 0.93 and 0.95, glycerol 87% (Merck Química, Argentina) was added in the amount of 525, 472, 430, 380, 355, 290 and 204 g respectively, after which the media was made up to 1 l and sterilized. Water activity was measured with a water activity meter (Aqualab, Decagon Devices CX3 02734) with an accuracy of ±0.002.

2.3.2. Inoculation and incubation

Inocula were prepared by growing each strain on MEA at 25 °C for 7 days. Spores of each strain were placed in an aqueous solution of 0.05% Tween 80 of a_w adjusted with glycerol. Suspension counting, mixed inoculum preparation, and plates inoculation were performed as in Section 2.2.2.

CYA plates inoculated with 1 µl of mixed inoculum were incubated at different temperatures (15, 20, 25, 30, and 35 °C) for 28 days. To maintain the a_w of the culture medium invariable during the whole experiment, plates corresponding to the same a_w level were placed in closed polythene bags (0.40 µm pore) in which relative humidity was controlled by including in the bag a recipient containing a glycerol–water solution adjusted to the corresponding a_w.

2.3.3. OTA analysis

OTA was determined after 7, 14, 21 and 28 days of incubation at each condition assayed as described in Section 2.2.4. Each set of conditions (a_w, temperature and time) was assayed in triplicate.

2.4. Statistical treatment of the results

Growth rate, lag phase and OTA concentrations detected in each treatment were evaluated by analysis of variance (ANOVA) using Statistix 8.1. Comparisons of means were conducted by Tukey's test of significant difference ($p<0.05$).

A fully randomized factorial design run in triplicate was used to determine the OTA production by the mixed inoculum at different temperatures and a_w levels. The effect of a_w, temperature, and their interactions were examined by ANOVA using Statistix 8.1 followed by Tukey's test of significant difference ($p<0.05$).

3. Results

3.1. Individual strains versus mixed inoculum

Radial growth rate and lag phase of each individual strain were measured in CYA at 30 °C. Results were compared with those obtained from the cocktail inoculum (Table 1). Analysis of variance (ANOVA) showed significant differences between radial growth rates and lag phases of the different isolates ($p<0.01$). The highest growth rate was 15.8 mm/day (strain C) and the lowest was 14.7 mm/day (strain A).

Table 1

Growth rate and lag phase of four *A. carbonarius* strains (A, B, C and D) and the mixed inoculum (A+B+C+D) were determined on CYA at 30 °C. Values with the same superscript are not significantly different ($p<0.05$).

	Growth rate (mm/day)	Lag (days)
A	14.7^{b}	0.26^{b}
B	15.2^{ab}	0.33^{ab}
C	15.8^{a}	0.45^{a}
D	15.0^{b}	0.28^{b}
A+B+C+D	15.4^{ab}	0.45^{a}