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Short communication

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

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ABSTRACT

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

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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}

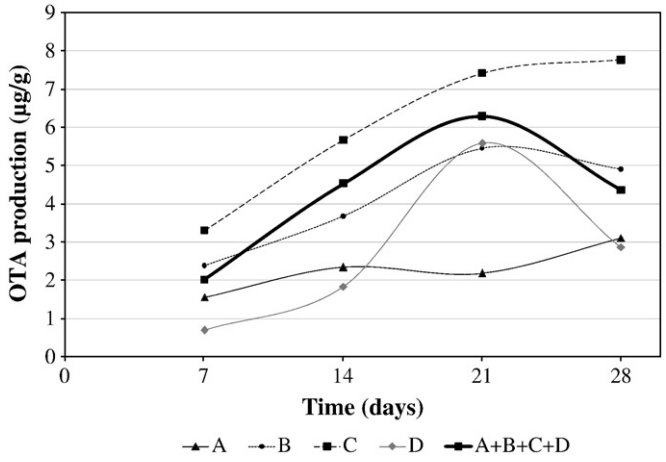

Fig. 1. OTA concentration produced by four *A. carbonarius* strains (A, B, C and D) and the mixed inoculum (A + B + C + D) at 7, 14, 21 and 28 days on CYA at 30 °C.

However, no significant differences between growth rates of each strain and the growth rate of the mixed inoculum were detected. Lag phases were lower for strains A and D and differed significantly of the value obtained with the mixed inoculum. When the four strains were inoculated simultaneously, the lag phase was the same than the higher value obtained for individual strains (0.45 days, strain C).

Statistical analysis of variance showed that the strain and time incubation had significant effect on OTA production ($p<0.0001$). Fig. 1 shows OTA production by each isolate and the mixed inoculum in CYA at 7, 14, 21 and 28 days of incubation at 30 °C. The four strains differed in maximum OTA yield. Strain C was the stronger OTA producer during the whole incubation period. Maximum OTA accumulation by this strain was 7.75 µg/g at 28 days. Strains B and D showed a maximum OTA accumulation at 21 days and the same behaviour was observed with the mixed inoculum. Maximum OTA production by strain A (3.1 µg/g after 28 days) was lower in comparison with those produced by the other strains. OTA accumulation by the mixed inoculum showed intermediate levels in each point of time.

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

The cocktail inoculum technique was used to study the effect of environmental parameters on growth and OTA production by *A. carbonarius*. Results on the effect of a_w and temperature on fungal growth with the same mixed strains have been published previously (Romero et al. 2007). Present work reports the OTA production at different water activities, temperatures and incubation times, over a period of 28 days.

Statistical analysis of variance (ANOVA) showed that the effect of a_w, temperature, incubation time and their interactions significantly influenced on OTA production by *A. carbonarius* ($p<0.0001$). The combined effects of a_w and temperature on OTA production are shown in Table 2. At high a_w (0.95 and 0.93) the mixed inoculum produced more toxin at 15 °C than at any other temperature. The highest accumulation of OTA was observed at 15 °C and a_w 0.95 after 28 days of incubation.

As a_w decreases, the range of temperatures allowing OTA production is more restricted. At 0.90 a_w the toxin was only detected at 20 °C. At 0.89 a_w the fungus was not able to grow at 15 and 20 °C. Fungal growth was observed at this a_w level and 25 °C or higher temperature but OTA production was to a large extent inhibited. At a_w 0.87 the toxin was detected at traces level, below the quantification limit of the method (0.05 µg/g), in only one replicate at 25 °C.

4. Discussion

In this work growth and mycotoxin production by individual strains were compared with those of a mixed inoculum. With respect to growth parameters, the mixed inoculum yielded an average growth rate, which represents the tendency of the individual strains behaviour in optimal growth conditions. This average data is the result of

Table 2
OTA concentration (mean and standard deviation) produced by a mixed inoculum of *A. carbonarius* in CYA medium at each incubation time, a_w and temperature tested.

a_w	Time	Temperature (°C)				
		35	30	25	20	15
0.95	7	+	n.d.	0.12 ± 0.10^{e}	0.37 ± 0.19^{de}	n.d.
	14	0.51 ± 0.24^{de}	0.10 ± 0.05^{e}	0.24 ± 0.07^{e}	1.58 ± 1.13^{de}	4.69 ± 1.91^{cd}
	21	0.69 ± 0.19^{de}	0.08 ± 0.07^{e}	1.14 ± 0.93^{de}	2.07 ± 1.66^{de}	15.08 ± 1.27^{b}
	28	0.85 ± 0.43^{de}	0.13 ± 0.07^{e}	0.23 ± 0.05^{e}	0.86 ± 0.10^{de}	21.04 ± 10.48^{a}
0.93	7	n.d.	n.d.	0.05 ± 0.03^{e}	+	NG
	14	0.03 ± 0.03^{de}	n.d.	0.05 ± 0.01^{e}	1.11 ± 0.42^{de}	NG
	21	0.56 ± 0.44^{de}	+	0.05 ± 0.03^{e}	1.96 ± 0.27^{de}	8.56 ± 3.24^{c}
	28	0.05 ± 0.00^{e}	0.04 ± 0.03^{e}	+	1.62 ± 1.25^{de}	4.63 ± 5.88^{cde}
0.90	7	n.d.	n.d.	n.d.	NG	NG
	14	n.d.	n.d.	n.d.	+	NG
	21	n.d.	n.d.	+	2.26 ± 0.71^{de}	NG
	28	n.d.	n.d.	n.d.	16.80 ± 3.30^{ab}	NG
0.89	7	n.d.	n.d.	n.d.	NG	NG
	14	n.d.	n.d.	n.d.	NG	NG
	21	n.d.	n.d.	$0.02 \pm 0.03^{e*}$	NG	NG
	28	n.d.	n.d.	n.d.	NG	NG
0.87	7	NG	NG	NG	NG	NG
	14	n.d.	n.d.	n.d.	NG	NG
	21	n.d.	n.d.	n.d.	NG	NG
	28	n.d.	n.d.	+	NG	NG
0.85	7	NG	NG	NG	NG	NG
	14	NG	NG	NG	NG	NG
	21	NG	n.d.	NG	NG	NG
	28	NG	n.d.	NG	NG	NG

+: traces (<limit of quantification) in at least one replicate. NG: no growth, n.d.: not detected. * In this condition OTA was detected in only one of the three plates at the quantification limit level.
Limit of detection 0.02 µg/g, limit of quantification 0.05 µg/g. Values with the same superscript are not significantly different ($p<0.05$). OTA concentrations are the mean and standard deviation from three replicates.

the complex interactions that occur between the strains on a substrate. As one of the most important aspects of model development is ensuring that predictions made by the model are applicable to real situations, the use of a mixed inoculum could be used to approach this objective. It should be pointed out that although the differences in growth rate between strains were statistically significant, the difference between the fastest and the slowest strain is not great, as the strains used in the present work did not show a great variability.

The lag phase for the mixed inoculum was the same that the higher obtained for individual strains, suggesting mycelial interactions between the *A. carbonarius* isolates. Intraspecific interactions were also observed by Morales et al. (2008) when two isolates of *Penicillium expansum* were inoculated together in wounded apples. Since the isolates occupy the same niche, interaction may be due to competition for resources, which is restricted to early stages of the community development (Cooke and Whipps, 1993).

These data, showing an average growth rate and a long lag phase for the mixed inoculum, did not represent the "worst case scenario" as if it had yielded the fastest growth rate and the shortest lag phase. The last situation would be preferable if the goal of modelling were risk assessment, where the characteristic of the fastest strain should be reflected by the predictions.

The amount of OTA produced by the mixed inoculum was an intermediate level from that synthesized by the four individual strains. This could be due to intraspecific interactions which have also been observed by Valero et al. (2006, 2007) in *Aspergillus* section *Nigri*. Remetabolism of OTA could explain the decrease in toxin observed between 21 and 28 days. It is possible that one strain in the mixture (D) that showed an average toxigenic potential but a high remetabolism capacity might degrade its own toxin and also the toxin produced by the other strains. These OTA degradation capabilities of black aspergilli were also observed by Abrunhosa et al. (2002) and Varga et al. (2000).

The employment of models may be useful to predict the potential level of contamination in a substrate. A mixed inoculum showing an average amount of toxin produced would be useful to this purpose as it takes into account the complex fungal interactions which may take place between strains in the substrate. The final amount of toxin produced by the cocktail would be the result of OTA accumulation by individual strains and its degradation by remetabolism.

Also, predictive models are developed for decision-making purposes to prevent/reduce yield losses and hazards for human and animal health. In this case, the knowledge of the extremes conditions for growth and toxin production of a species would be necessary. From this point of view the use a mixed inoculum could be criticized because of loss of information about the responses of individual strains.

Comparing the influence of environmental parameters on OTA production by the mixed inoculum to individual strains from literature, a similar trend was observed. *A. carbonarius* strains from different substrates and geographical origins have shown a common pattern in growth and OTA production under variable conditions of temperature and a_w. There are few studies in which a_w levels below 0.90 have been considered in regard to OTA production by *A. carbonarius*. Most work has been focused on the conditions conducive to OTA contamination of grapes and wines in vineyards during pre and post-harvest and in the winemaking process. The effect of lowering a_w levels is of interest in regard to strains isolated from raisins and other sun dried commodities. The 0.87 a_w level could be considered as the limitant a_w value for OTA production by the mixed inoculum because in only one replicate at 25 °C the toxin was detected at trace level. Valero et al (2007) demonstrated that sun-drying process may be conducive to OTA accumulation in dried grapes. Esteban et al. (2006) detected a weak OTA production (0.22 ± 0.31 μ/g) at a_w 0.86 and 30 °C by one *A. carbonarius* isolated from coffee. Astoreca et al. (2007) studied the OTA production by two strains isolated from dried grapes and reported toxin production at 0.85 and 30 °C by one of them and at 0.89 and 25–30 °C by the other one. These results and ours show that the *A. carbonarius* strains isolated from dried vine fruits exhibit a xerotolerant behaviour since they are able to grow up to 0.85 a_w and produce OTA at relatively low a_w levels (0.85–0.89). Implications of this fact on the quality of dried vine fruits and probably other dried fruit products have been pointed out (Magan and Aldred, 2005; Esteban et al. 2006; Astoreca et al. 2007; Romero et al. 2007), suggesting that drying step should be a critical control point in the production of fruits preserved by low a_w in which black aspergilli are usually present as part of their mycota.

The similar trend in the response of the isolates from different substrates and geographical origins to the variation of environmental parameters may be of interest for the building of predictive models. However, individual strains exhibit some differences in their growth rates and in the amount of OTA produced at optimal and marginal conditions. Given the different abilities to biosynthesise OTA by the different strains of the species, and their interactions, extrapolation of the models obtained with one individual strain might not be representative for the majority of the strains. The use of a cocktail inoculum could be a useful approach to obtain data for prediction of the final level of contamination and better organise post-harvest management.

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