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Aspergillus carbonarius growth and ochratoxin A production on irradiated dried grapes under different water activity and temperature conditions

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Abstract

Grapes have different destinations. The most important in the national and international market is wine production, but another is dehydration to produce raisins. Dried vine fruits are at greater risk of ochratoxin A (OTA) contamination than wine grapes because the ratio of *Aspergillus carbonarius* to *Aspergillus niger* aggregate increases during drying. The growth of these species, and consequently OTA production, can be influenced by different environmental factors, the two most important being water activity (a_W) and temperature. The objective of the present work was to evaluate the lag phase, growth rate and OTA production by two *A. carbonarius* isolates on irradiated dried grapes at different a_W (0.910, 0.928, 0.955, 0.973 and 0.995), temperatures (15, 25 and 30 °C) and incubation times (7, 14 and 21 days). Growth was observed at all a_W and temperature ranges assayed. No significant differences between the growth rates reached at 25 °C and 30 °C by both isolates were observed. At the assayed conditions, OTA production occurred over the whole range of temperatures (15-30 °C), with the maximum at 25 and 30 °C depending on the a_W. In general, OTA concentration increased as a_W increased with no statistically significant differences at the tested incubation times. This work provides information that can be used by companies for the purpose of preventing *A. carbonarius* and OTA contamination during storage on this and other substrates (e.g. dried prunes, figs and apricots) destined for human consumption.

Keywords: *Aspergillus carbonarius*, ochratoxin A, dried grapes, environmental factors

1. Introduction

Grapes have different destinations. The most important in the national and international market is wine production. Another destination is dehydration to produce dried grape products (raisins) made from *Vitis vinifera* L. These can be used for direct consumption or as an ingredient in cereal bars, biscuits, cookies, puddings and breads, among other foods, many of which are consumed by children with the risk that this entails (Jordan, 2006). Some sweet wines are also made from grapes that are simply harvested later to contribute to high levels of both sugar and alcohol. Argentina occupies 10th place in global production and export of dried grapes, United States and Brazil being the major international buyers. Ninety five percent of the national production of dried grapes is sent to international markets and is concentrated in San Juan province. At present, climatic factors combined with little diversification in this sector and problems associated with the level of fungal contamination of raw materials, have resulted in a rather poor harvest, which could lead to problems of competitiveness abroad.

Ochratoxin A (OTA) is a very harmful chemical for human organisms. It exhibits hepatotoxic, nephrotoxic, teratogenic, mutagenic, immunotoxic and carcinogenic activity (category 2B) (IARC, 1993) and it is considered to be a natural contaminant in many countries. The production of this

toxin was originally associated with *Aspergillus ochraceus* strains in South Africa (Van der Merwe *et al.*, 1965). A few years later, *Penicillium verrucosum* was also associated with OTA production (Pitt, 1987). However, the latest evidence suggests that *Aspergillus carbonarius* isolated from grapes and dried fruits is the main ochratoxigenic fungus in several countries, as 75-100% of the isolates of this species were found to be OTA producers (Battilani *et al.*, 2006; Leong *et al.*, 2006; Magnoli *et al.*, 2004; Ponsone *et al.*, 2007; Romero *et al.*, 2005; Serra *et al.*, 2005, 2006; Téren *et al.*, 1996; Varga and Kozakiewicz, 2006; Varga *et al.*, 1996, 2006). Humidity between 16 and 19% (determined by the Dean Stara Method) and a maximum of 10 mg/kg of OTA are considered permissible by Secretaría de Agricultura, Ganadería, Pesca y Alimentación (2006). Thus, dried vine fruits are at greater risk of OTA contamination than wine grapes because the ratio of *A. carbonarius* to *Aspergillus niger* aggregate increases during drying (Valero *et al.*, 2005, 2007; Gómez *et al.*, 2006).

The method usually used to reduce the water activity (a_W) of the grapes consists mainly of drying them in sunshine in the open-air. Quality is thus dependent on weather conditions (Pateraki *et al.*, 2007). This substrate is exposed for prolonged periods of time to high temperatures and sunny radiation, and this fact determines the consequent contamination with different fungal species.

When intermittent sunshine and rain episodes occur, drying can be slowed down and this can lead to colonisation by *Aspergillus* section *Nigri* species, especially the ochratoxigenic species such as *A. carbonarius* (Magan and Aldred, 2005).

OTA in dried grapes and sweet wines is a matter of concern, considering the high levels and occurrence reported in several studies (Aksoy *et al.*, 2007; Bellí *et al.*, 2004a; Bircan, 2009; Blesa *et al.*, 2004; Lombaert *et al.*, 2004; López de Cerain *et al.*, 2002; MacDonald *et al.*, 1999; Magnoli *et al.*, 2004; Meletis *et al.*, 2007; Meyvaci *et al.*, 2005; Möller and Nyberg, 2003; Solfrizzo *et al.*, 2008; Stefanaki *et al.*, 2003; Valero *et al.*, 2008; Varga and Kozakiewicz, 2006; Zinedine *et al.*, 2007).

Growth of *Aspergillus* species and consequently OTA production can be influenced by intrinsic ecophysiological factors, such as moisture, pH, composition of the substrate, and extrinsic factors, e.g. temperature (Häggblom, 1982). *Aspergillus* growth is markedly affected by different environmental factors, a_W and temperature being the two most important (Magan and Lacey, 1984). Therefore, the determination of the optimal environmental conditions is essential for raw materials and finished food hygienic quality. At the present time, the effect of temperature and water availability on *A. carbonarius* growth and OTA production has been reported on synthetic medium (Bellí *et al.*, 2005; Esteban *et al.*, 2006; Leong *et al.*, 2006; Mitchell *et al.*, 2004; Pateraki *et al.*, 2007; Romero *et al.*, 2007; Tassou *et al.*, 2007a,b, 2009) and on other natural substrates (Bellí *et al.*, 2007; Joosten *et al.*, 2001; Marin *et al.*, 2008). Recently, only one study evaluating the *A. carbonarius* ecophysiology on dried grapes was carried out in Greece by Kapetanakou *et al.* (2009), but this fact has been not analysed in any of the American countries. Thus, the objective of the present work was to evaluate the lag phase, growth rate and OTA production of two Argentinean isolates of *A. carbonarius* on irradiated dried grapes at different water activities and temperatures.

2. Materials and methods

Fungal isolates

Two isolates of *A. carbonarius* (RCDG90 and RCDG92) isolated from Argentinean dried grapes were selected according to their capacity to produce OTA (Magnoli *et al.*, 2004). These isolates are kept in the National University of Río Cuarto, Argentina (RC) collection. Cultures were maintained in 15% glycerol at -80 °C.

Substrate conditions

Dried grapes (1 kg) packed in specially designed biaxial-oriented polypropylene bags (25 µm, World Plast S.A., Villamarina, Peru) were irradiated with 8-10 kGrays of gamma irradiation. The dried grapes were checked for sterility and absence of OTA and stored aseptically at 4 °C. The initial a_W of the grapes was 0.662. For all experiments, a_W was modified to 0.995, 0.973, 0.951, 0.928 and 0.910 by adding sterile distilled water using a previously defined moisture absorption curve. Finally, a_W levels were confirmed by using an Aqualab Series 3 water activity meter (Decagon Devices, Inc., Pullman, WA, USA).

Inoculation and incubation

Re-hydrated dried grapes were placed in sterile 9 cm Petri dishes to form a 20 g monolayer of fruit. Then a 4 mm diameter agar disk was taken from a 7 day old growing colony of each strain on Malt Extract Agar (MEA) at 25 °C and transferred to the centre of each plate containing the grapes. Petri dishes conditioned to the same a_W were placed in closed plastic containers together with beakers of glycerol-water solution of the same a_W in order to maintain the correct equilibrium of relative humidity inside the boxes. Containers were incubated at 15, 25 and 30 °C and the experiment was carried out in duple with three replicates per treatment.

Growth assessment

Growth was assessed every day during the incubation period. Dried grapes were examined using a binocular magnifier (10×), and two diameters of the growing colonies were measured at right angles in two directions until the colony reached the edge of the plate. The radii of the colonies were plotted against time, and a linear regression applied in order to obtain the growth rate as the slope of the line. Lag phase for growth was defined as the time (h) each colony reaches 5 mm of diameter for each treatment.

Ochratoxin A extraction

After 7, 14 and 21 days of incubation, three replicates per treatment were destructively sampled, and the OTA analysis were carried out according to the methodology described by Zimmerli and Dick (1996), with some modifications: a 5 g portion of a finely ground dried grape sample was added to a 250 ml Erlenmeyer flask along with 100 ml mixture of methanol : sodium bicarbonate at 1% (70:30). The mixture was shaken for 30 minutes and filtered to remove particulate matter. A 10 ml aliquot of the above extract was diluted with 40 ml of phosphate buffered saline (PBS) containing 0.01% Tween 20. It was filtered again through a microfibre filter and 10 ml of each diluted extract was taken and added to an immunoaffinity column (OchraTest^{TM}, Vicam, Digen Ltd., Oxford, UK). The column was washed with 10 ml of double distilled water at a flow rate of 1-2 drops per second. OTA was eluted from the column with methanol (HPLC grade) at a flow rate of 1-2 drops per second. The dry extract was kept at 4 °C. Each sample was analysed in triplicate.

Ochratoxin A detection

OTA detection was performed using high-performance liquid chromatography (HPLC), following the methodology proposed by Scudamore and MacDonald (1998), with some modifications. The HPLC apparatus used for OTA determination was a Hewlett Packard chromatograph (HP/Agilent, Santa Clara, CA, USA) with an injection loop of 50 µl, equipped with a spectrofluorescence detector (excitation: 330 nm; emission: 460 nm) and a C_{18} column (Supelcosil LC-ABZ, Supelco, Sigma-Aldrich, St. Louis, MO, USA; 150×4.6 mm, 5 µm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20×4.6 mm, 5 µm particle size). The mobile phase was pumped at 1.0 ml/min and consisted of an isocratic system: 57% acetonitrile, 41% water, and 2% acetic acid. OTA was quantified on the basis of HPLC fluorometric response compared with OTA standard (Sigma Aldrich, USA, purity >99%). The limit of detection was 1 ng/g. Each sample was analysed three times.

Assay of spiking and recovery of ochratoxin A

OTA-free sample (20 g) of dried vine fruits contained in a 250 ml Erlenmeyer flask was spiked with standard solutions of OTA, equivalent to 50, 100, 200 and 400 µg OTA/g. Spiking was carried out in duplicate and a single analysis of the blank sample was carried out. After leaving it for 16 h for the solvent to evaporate, extraction solvent was added and the OTA concentration was determined, using the protocol previously described. The recovery percentage was calculated.

Statistical analysis

The experimental design was performed as follows: five a_W × three temperatures × two isolates × three incubation times × three replicates for each parameters measured. Growth rate, lag phase and OTA accumulation produced by the *A. carbonarius* isolates were analysed statistically using PROC GLM in SAS program (SAS Institute Inc., Cary, NC, USA) by means of ANOVA. Means were compared by Fishers LSD test to determine the significant difference between the different treatments assayed (Quinn and Keough, 2002).

3. Results

Figure 1 shows the effect of a_W and temperature on lag phase (hours) and the growth rate (mm/day) of the *A. carbonarius* RCDG90 and RCDG92 isolates. Analysis of variance revealed that the factors a_W, temperature and their interaction had a significant influence on lag phase and mycelial growth of both isolates (P<0.0001). *A. carbonarius* isolates reached the maximum values of lag phases at the minimum temperature assayed (15 °C), which were similar for both isolates varying from 100 to 522 h at 0.995 and 0.910 a_W, respectively. Significant differences in lag phase values at this temperature were observed only at 0.910 a_W.

Growth was observed at all a_W and temperature ranges assayed. No significant differences between the growth rates reached at 25 °C and 30 °C by both isolates were observed. No significant differences in growth rate between the isolates tested were observed at 15 and 25 °C (data not shown) but the growth rates of RCDG92 isolate increased with the increment of temperature while the RCDG90 isolate reached the maximum growth rates at 25 °C and regardless of the assayed a_W.

OTA production occurred over all assayed temperatures with the maximum at 25 and 30 °C depending on the a_W. In general OTA concentration increased as a_W increased with no statistically significant differences at the tested incubation times (Table 1).

The data for the two *A. carbonarius* isolates were used to develop further contour maps in order to identify the

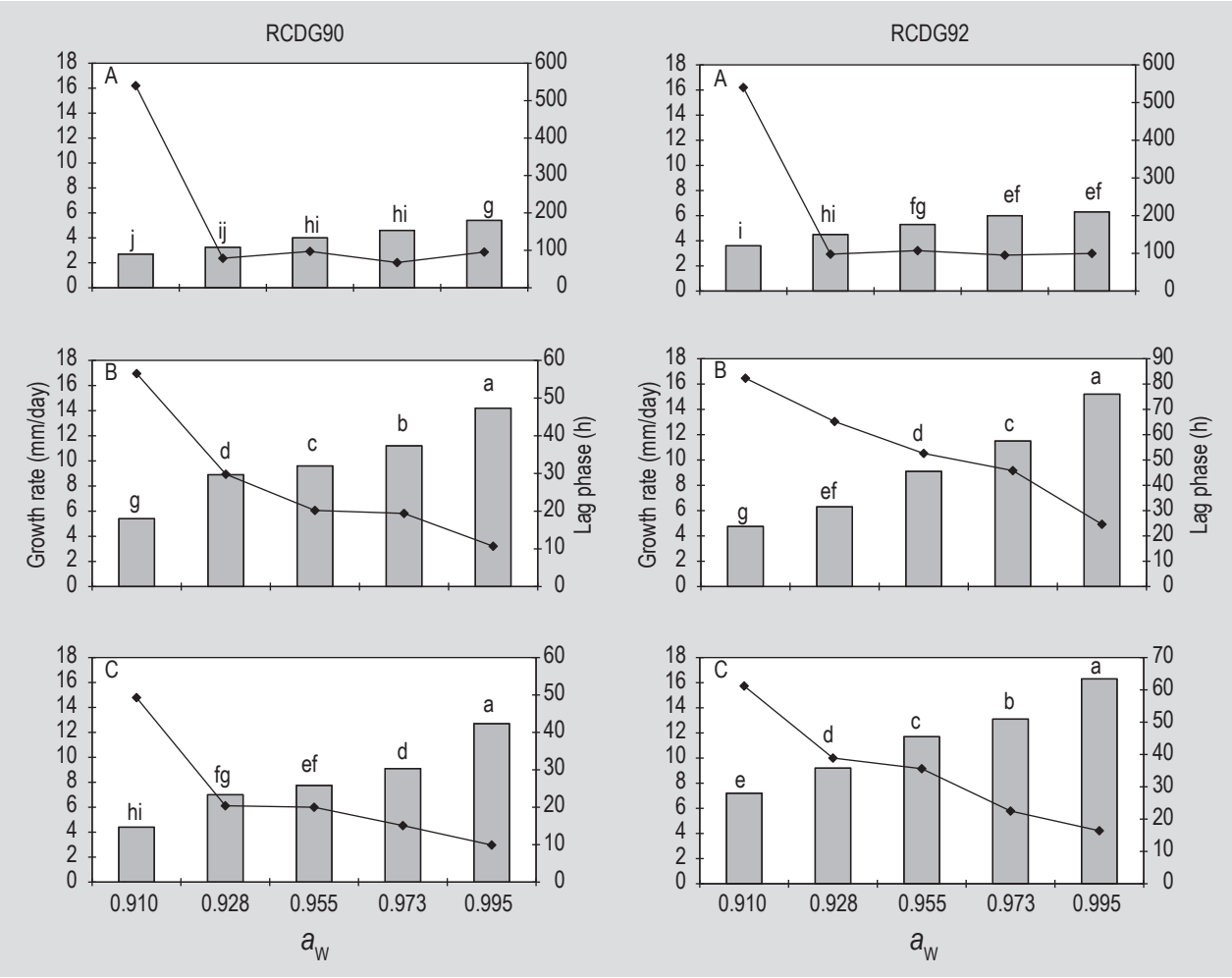

Figure 1. Effect of different water activities and temperatures on growth rate (bars) and lag phase (points) of *A. carbonarius* (RCDG90 and RCDG92) isolates at (A) 15 °C, (B) 25 °C and (C) 30 °C. Columns with the same superscript within each isolate indicate that they are not significantly different (*P*<0.001) according to Fisher LSD test.

influence of a range of a_W and temperature conditions on OTA production on irradiated dried grapes. With the conditions assayed in this study, the maximum OTA production was clearly observed at 0.995 a_W and 30 °C for both isolates, with mean toxin concentrations of 861.7 and 616 µg/g by RCDG90 and RCDG92 isolates, respectively (Figure 2). No significant differences (P<0.05) were observed among the incubation times for most treatments, except for RCDG92 isolate at 30 °C and 0.955 and 0.973 a_W (data not shown).

The evaluation of the results by ANOVA showed that the factors a_W and temperature were statistically significant in relation to the *in situ* OTA production by the *A. carbonarius* isolates analysed after the incubation period of 21 days (P<0.0001) (Table 2).

4. Discussion

In this study the influence of the a_W, temperature and incubation time on growth and OTA production by *A. carbonarius* isolates on dried grapes was determined. This study provides the first data about the impact of a_W and temperature regimes on growth rate and OTA production by isolates of *A. carbonarius* isolated from Argentina when grown on irradiated dried grapes. In previous work, Romero *et al.* (2007) studied the effect of a_W and temperature on the growth of ochratoxigenic isolates of *A. carbonarius* isolated from Argentinean dried vine fruits, but they determined these parameters on Czapek Yeast Extract Agar (CYA). In contrast to their results, mean growth rate of 14.6 mm/day at 0.995 a_W and 25-30 °C were observed, whereas they reported the maximum growth rate at different a_W and temperature conditions, reaching a mean growth rate of 17.46 mm/day at 0.955 a_W and 30 °C. This value is higher than ours and others found in the literature; Bellí *et al.* (2004b, 2005) reported a maximum

Table 1. OTA concentration of two isolates of *A. carbonarius* on irradiated dried grapes at different temperature, water activity and incubation time.

Isolates	a_W	OTA concentration (µg/g)[1,2] ± SD		
		7 days	14 days	21 days
15 °C				
RCDG90	0.91	27.9±7.4^{j}	21.9±3.3^{j}	22±3.6^{j}
	0.93	53.5±5.1^{h,i}	50.7±5.1^{h,i}	50.6±5.8^{h,i}
	0.95	96.0±19.2^{g,h}	97.8±22.7^{g,h}	92.7±21.0^{g,h}
	0.97	152.5±30.0^{g,h}	138.6±19.9^{g,h}	132.0±27.8^{g,h}
	0.99	200.6±22.0^{f,g}	185.5±33.2^{f,g}	165.0±32.9^{g,h}
RCDG92	0.91	13.0±2.6^{m,n}	19.0±2.4^{m,n}	17.3±4.1^{m,n}
	0.93	42.3±6.5^{l,m,n}	50.2±8.5^{l,m}	48.8±7.5^{l,m,n}
	0.95	87.6±4.4^{j,k}	99.3±15.0^{i,j,k}	90.9±13.4^{j,k}
	0.97	95.3±4.6^{i,j,k}	111.0±15.5^{h,i}	101.7±12.6^{i,j,k}
	0.99	150.3±19.8^{f,g}	170.8±14.2^{e,f}	152.4±4.9^{f,g}
25 °C				
RCDG90	0.91	31.3±5.8^{i,j}	31.0±5.6^{i,j}	30.6±7.0^{i,j}
	0.93	32.6±5.7^{i,j}	38.7±5.5^{i,j}	41.7±10.5^{i,j}
	0.95	84.7±16.2^{g,h}	85.3±6.5^{g,h}	77.4±3.2^{g,h}
	0.97	281.7±27.6^{d}	267.0±10.9^{d,e}	239.0±22.8^{d,e}
	0.99	374.5±102.4^{b,c}	435.7±14.2^{c}	411.6±8.7^{c}
RCDG92	0.91	12.3±2.5^{n}	17.0±4.3^{m,n}	20.1±1.0^{m,n}
	0.93	28.3±7.1^{m}	29.2±2.6^{m}	25.7±4.0^{m}
	0.95	73.7±13.0^{k,l}	73.4±12.5^{k,l}	69.4±9.5^{k,l}
	0.97	193.3±9.0^{d}	185.0±22.8^{d,e}	171.7±39.9^{e,f}
	0.99	284.3±23.4^{c}	276.7±16.3^{c}	270.8±30.9^{c}
30 °C				
RCDG90	0.91	84.7±9.8^{gh}	85.6±8.6^{gh}	81.5±12.0^{gh}
	0.93	100.0±17.9^{gh}	113.5±26.5^{gh}	111.6±27.3^{gh}
	0.95	226.5±48.8^{e}	222.2±23.8^{e}	205.3±9.2^{e,f}
	0.97	584.0±51.3^{c}	567.3±81.7^{c}	566.6±83.2^{c}
	0.99	861.7±115.9^{a}	840.6±109.2^{a}	814.6±101.4^{ab}
RCDG92	0.91	71.5±3.0^{kl}	74.6±2.6^{jkl}	78.9±1.4^{jkl}
	0.93	94.0±11.5^{jk}	88.4±11.9^{jk}	77.4±13.8^{jkl}
	0.95	173.9±18.5^{ef}	145.7±34.1^{g}	132.8±43.5^{gh}
	0.97	348.3±46.5^{b}	338.1±45.7^{b}	300.2±93.4^{c}
	0.99	616.0±29.3^{a}	607.9±4.9^{a}	595.8±4.5^{a}

[1] Mean levels of OTA; SD: standard deviation; limit of detection: 5 ng/g.
[2] Columns with the same superscript within each isolate indicate that they are not significantly different (P<0.001) according to LSD test.

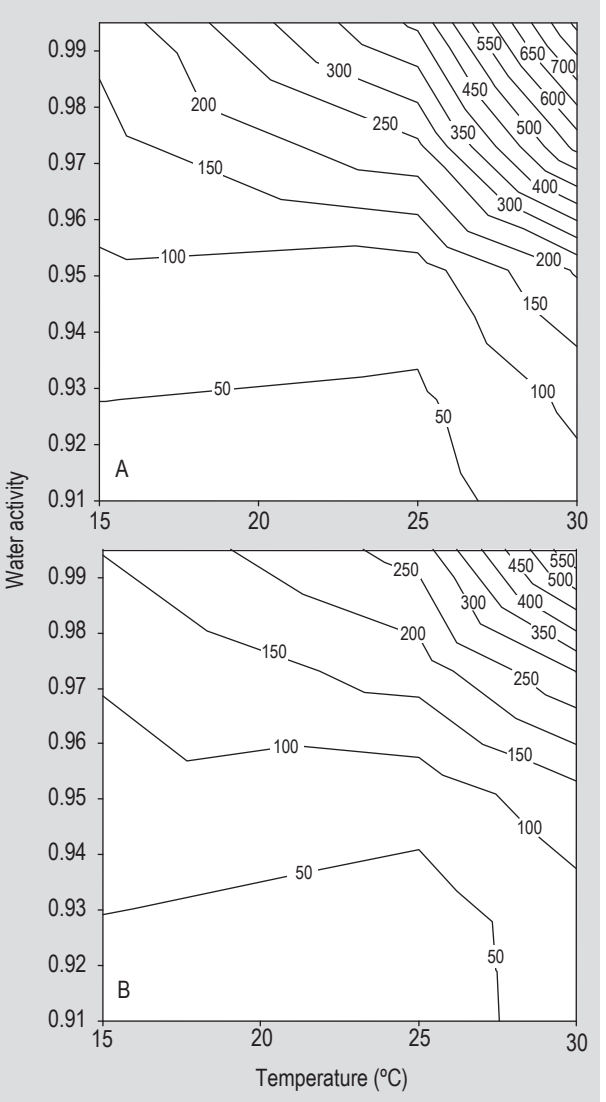

Figure 2. Contour maps for *A. carbonarius* isolates (A) RCDG90 and (B) RCDG92 in relation to water activity and temperature. The numbers on the contour lines refer to mean OTA concentrations (µg/g).

growth rate of 9.1 and 10.1 mm/day at 30 °C and 0.98-0.99 a_W for strains isolated from European and Spanish wine grapes, respectively in a Synthetic Nutrient Medium (SNM) with similar composition of grapes between *veraison* and ripeness. Leong *et al.* (2006) reported that the highest growth rate of Australian *A. carbonarius* strains occurred at 30 °C and a_W 0.965 on a simulated grape juice medium but the maximum growth rate registered was 7 mm/day. The different behaviour between the isolates can be attributed to culture media, intraspecific and regional variability.

These data indicate that each substrate greatly influences the behaviour of the isolates that develop in it, hence the importance of studies on natural substrates where environmental conditions are simulated.

The lag phases of the isolates assayed in this work were higher than those previously obtained on a media based on dried grapes (Astoreca *et al.*, 2007b). Furthermore, both isolates showed growth rates significantly higher than that reported in the *in vitro* assay (Astoreca *et al.*, 2007a). The optimal conditions for growth differed with respect to that obtained on dried grapes based medium, 0.995 being

Table 2. Variance analysis of the effect of water activity (a_W), temperature (T), incubation time (IT) and their interactions on OTA production (µg/g) of two *A. carbonarius* isolates on irradiated dried grapes.

Source	Df[1]	RCDG90		RCDG92	
		MS[2]	F[3]	MS[2]	F[3]
a_W	4	1,271,212.51	585.31*	446,920.76	845.09*
T	2	793,389.56	365.30**	349,248.36	660.40**
IT	2	2,409.65	1.11*	1,191.21	2.25*
a_W×IT	8	174,975.32	80.56*	279.75	0.53*
a_W×T	8	812.22	0.37***	71,969.55	136.09***
IT×T	4	222.89	0.10***	866.48	1.64**
a_W×T×IT	16	207.03	0.10***	133.46	0.25***

[1] Df: degrees of freedom.
[2] MS: Mean square.
[3] F: F-Snedecor. Significance: * = P<0.0001; ** = P<0.05 and *** = P<0.5.

the optimum a_W for growth. These results demonstrate that the natural substrate would be offering the isolates a greater nutritional contribution than the artificial media (Astoreca *et al.*, 2009a,b).

There are several studies in the literature that point to the crucial role of ecological factors, such as water activity and temperature, in strongly affecting OTA production by *A. ochraceus* and *A. carbonarius* (Esteban *et al.*, 2006; Leong *et al.*, 2006; Marín *et al.*, 2008; Mitchell *et al.*, 2003; Pardo *et al.*, 2004, 2006; Pateraki *et al.*, 2007; Tassou *et al.*, 2007a,b, 2009; Valero *et al.*, 2005).

On the other hand, Kapetanakou *et al.* (2009) evaluated the combined effect of a_W, pH and temperature on OTA production by *A. carbonarius* on culture medium and Corinth raisins. The present study is consistent with these authors since they reported a maximum OTA production at 25 °C for *A. carbonarius* and a decrease in OTA levels with a decrease in water activity. They also obtained the highest amounts of ochratoxin A produced at 0.99 a_W regardless of temperature, which was the highest a_W tested in our research.

These experiments showed a marked influence of a_W and temperature on lag phase, growth rate and OTA production on irradiated dried grapes. This study suggests that fungal growth could be prevented by an adequate control of these environmental factors. Values lower than 15 °C and 0.90 a_W could help in the prevention of OTA contamination in dried grapes.

This work provides information that can be used in the drying of dried grapes for the purpose of exporting dried grapes free of OTA and preventing *A. carbonarius* contamination, growth and OTA production on this and other substrates (e.g. dried prunes, figs, apricots) destined for human consumption.

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