

Intraspecific variability of growth and ochratoxin A production by *Aspergillus carbonarius* from different foods and geographical areas

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ABSTRACT

Ochratoxin A (OTA) is a nephrotoxic mycotoxin naturally found in a wide range of food commodities throughout the world. *Aspergillus carbonarius* is the most important source of OTA in food commodities such as wine, grapes and dried vine fruits and is also responsible for the formation of OTA in coffee.

The aim of this study was to determine the simultaneous effect of three culture media (Czapek Yeast Extract Broth (CYB); Synthetic Grape Juice Medium (SGM) and White grape juice (WGJ)) at three water activity (a_w) levels (0.90; 0.95 and 0.98–0.99), and three incubation temperatures (15 °C, 25 °C and 35 °C) on the growth and OTA production by 16 strains of *A. carbonarius*. The strains were mainly isolated from grapes from areas with a Mediterranean climate. All the strains were confirmed for identity by sequencing of the calmodulin gene. The assay was performed in microtiter plates, determining the absorbance at 530 nm and the concentration of OTA after 1, 2, 4 and 10 days of incubation.

No significant differences were observed in absorbance values between the strains. The highest absorbance values were recorded in CYB at 0.99 a_w and at 0.95 a_w after 10 days of incubation at 25 °C and 35 °C. None of the strains were able to grow at 0.90 a_w and 15 °C in any culture media after 10 days of incubation. OTA concentration was statistically higher at 15 °C than at 25 °C or 35 °C. The highest significant OTA values were obtained at 0.98–0.99 a_w and the best culture media for OTA production was CYB, followed by WGJ and SGM. While strains isolated from Mediterranean climate foods had a similar behavior despite being isolated from different geographical areas, OTA concentration produced by one Robusta coffee strain from Thailand was statistically higher at 25 °C than at 15 °C. This would suggest that the type of food matrices and consequently the adaptation of *A. carbonarius* strains to different climatic conditions would have a greater influence on the ecophysiology of the strains than only their geographical origin.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species of the genera *Penicillium* and *Aspergillus*. OTA is nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic in various animals *in vitro*, with renal toxicity and carcinogenesis being the key adverse effects (Heussner and Bingle, 2015). OTA contamination of food commodities, including cereals and cereal products, pulses, coffee, beer, grape juice, dried vine fruits and wine as well as cacao products, nuts and spices, has been reported from all over the world (EFSA, 2006). In the European diet, wine was identified as the second contributor to the human OTA exposure after cereals. Dried vine fruit and grape juice contributed to a significant extent to the OTA-exposure for specific groups of vulnerable groups of consumers such as children.

Coffee represented the third contributor to the mean European total dietary intake of OTA (European Commission, 2002). As a consequence of the possible health hazards related to ingestion of OTA, the European Union introduced maximum limits for OTA in a wide range of foodstuffs (Commission of the European Communities, 2006, 2010, 2012, 2015).

A large number of studies have shown that *Aspergillus carbonarius* is the main source of OTA in wine or dried vine fruits from main viticultural regions worldwide (Cabañes and Bragulat, 2018; Visconti et al., 2008). According to recent surveys, *A. carbonarius* is also the most important ochratoxin producer in Robusta coffee beans (Noonim et al., 2008).

Aspergillus carbonarius is very consistent in producing this mycotoxin, and non OTA-producing strains in this species are very rare (Cabañes et al., 2013). Contamination of grapes by this species can

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occur since the beginning of maturation stage but becomes more prominent near harvest time (Bau et al., 2005; Visconti et al., 2008). With respect to coffee, OTA contamination can be regarded as a post-harvest problem. The use of dry processing method, surface contact with dust and soil increase the chance of *A. carbonarius* contamination (Noonim et al., 2008).

The knowledge of the influence of environmental parameters on OTA production may contribute to prevention of OTA contamination in food commodities. In the last years many studies have been published on the effect of some environmental factors on *A. carbonarius* growth and to a lesser extent on its OTA production ability, as reviewed by several authors (Amézqueta et al., 2012; Battilani and Camardo, 2015; Magan et al., 2011). These kind of ecophysiological studies are routinely performed in Petri dishes and the number of isolates included is usually low because these methods are laborious and time-consuming.

In order to know the existence of intraspecific variability on growth and OTA production, several strains have to be included in ecophysiological studies. García et al. (2011) assessed the impact of sub-optimal environmental conditions on thirty isolates of *A. carbonarius*. Although they included a large number of isolates, only one culture medium and three water activity/temperature conditions (0.98 aw/25 °C; 0.90 aw/25 °C; 0.98 aw/37 °C) were tested. Using above data, García et al. (2012) mathematically assessed the minimum number of isolates needed to obtain a reasonable estimation of growth parameters when designing ecophysiological experiments. They recommended the use of 12–17 isolates of *A. carbonarius* in this kind of studies. Some studies, as Romero et al. (2010), used a cocktail inoculum of *A. carbonarius* strains. Using a mixed inoculum could be useful because it takes into account the fungal interactions which may take place among strains, but led to the loss of information about the behavior of individual strains.

In this study we have adapted a previously described method using microtiter plates (Abarca et al., 2014, 2019) to determine the simultaneous effect of three culture media at three water activity levels and three incubation temperatures on the growth and OTA production by 16 strains of *A. carbonarius*. This method allowed studying individually the behaviour of a large number of strains under different environmental conditions.

2. Materials and methods

2.1. Strains and molecular identification

Sixteen *A. carbonarius* strains, mainly isolated from grapes and raisins were studied (Table 1). All the strains were previously detected as OTA-producers in our laboratory and had been morphologically identified as *A. carbonarius*. The strains were selected on the basis of their geographical origin. They were isolated from areas of Spain and other countries with a Mediterranean climate with the exception of one strain isolated from Robusta coffee beans from Thailand, with a tropical climate.

All the strains were confirmed for identity by sequencing of the calmodulin gene. Briefly, DNA was extracted and purified from 48 h old cultures in malt extract broth according to the FastDNA Spin kit protocol with the FastPrep FP-24 instrument (MP Biomedicals, Bioblock, Barcelona, Spain). The DNA was kept at –20 °C until used as template for PCR amplification. Following the DNA extraction, the calmodulin gene was amplified and sequenced by using the fungal primers CL1/CL2A (O'Donnell et al., 2000). For the phylogenetic analyses, sequences obtained were aligned using Clustal X v2.0.12 (Larkin et al., 2007) and analyzed to generate a phylogenetic tree in Mega 6 software (Tamura et al., 2013). The Neighbor-Joining method based on the Tamura-Nei model (Tamura and Nei, 1993) with 1000 bootstrap replicates was used.

Table 1

List of *A. carbonarius*^a strains studied and their source and location.

Strain number ^b	Source, location
A-882	Tomatoes, Morocco
A-884	Coffee cherries, Thailand
A-1002	Grapes, Spain
A-1004	Grapes, Spain
A-1021	Grapes, Spain
A-1625	Grapes, Italy
A-1653	Grapes, France
A-1687	Grapes, Portugal
A-1749	Grapes, Israel
A-1841	Grapes, Greece
A-1995	Grapes, Australia
A-2128	Grapes, Spain
A-2275	Grapes, Spain
A-2520	Grapes, Spain
A-3615	Raisins, Turkey
A-3905	Raisins, Iran

^a Identification confirmed by sequencing of the calmodulin gene.

^b Culture Collection of the Veterinary Mycology Group, Universitat Autònoma de Barcelona, Spain.

2.2. Inoculum preparation and verification

Spore concentration was adjusted to around 10⁶ conidia/ml. Briefly, the inoculum suspensions were prepared in sterile saline (0.85%) containing 0.05% Tween 80 from 7-day-old cultures on malt extract agar at 25 °C. After heavy particles were allowed to settle for 10–15 min, the upper homogenous suspensions were transferred to sterile tubes and adjusted to 0.8 McFarland turbidity standard (Abarca et al., 2014) by using a photometric method (Densimat, BioMérieux). The inoculum size was confirmed by haemocytometer counting and quantitative colony counts.

2.3. Culture media and microtiter inoculation

Sterile 96-well flat-bottom microtiter plates were used. Three liquid culture media were assayed: Czapek Yeast Extract broth (CYB), used as a control, synthetic grape juice medium (SGM) representative of grape composition at mid-veraison (Mitchell et al., 2004), and white grape juice (WGJ).

CYB contained per liter: K₂ HPO₄, 1 g; Czapek concentrate, 10 ml; trace metal solution, 1 ml; yeast extract, 5 g; sucrose, 30 g; pH adjusted to 6.3 ± 0.2 (Pitt and Hocking, 2009). SGM contained per liter: D(+) glucose, 70 g; D(–) fructose, 30 g; L(–) tartaric acid, 7 g; L(–) malic acid, 10 g; (NH₄)₂HPO₄, 0.67 g; KH₂PO₄, 0.67 g; MgSO₄·7 H₂O, 1.5 g; NaCl, 0.15 g; CaCl₂, 0.15 g; CuCl₂, 0.0015 g; FeSO₄·7 H₂O, 0.021 g; ZnSO₄·7 H₂O, 0.0075 g; (+) Catechin hydrate, 0.05 g; pH adjusted with 10 M NaOH to pH 4.0–4.2 (Mitchell et al., 2004). WGJ was prepared with 200 ml of commercially sold white grape juice made from organic grapes and 800 ml of distilled water; pH adjusted at 4.0.

The initial a_w was 0.99 for CYB and WGJ media, and 0.98 for SGM. These initial values were modified to 0.95 a_w and 0.90 a_w by the addition of different amounts of glycerol. Media were autoclaved and the final a_w values were checked with LabMASTER-a_w (Novasina, Switzerland).

For each a_w level the adjusted fungal suspensions were diluted 1:100 in the culture medium assayed (CYB, SGM, WGJ). In each microplate column, five wells were inoculated with 200 µl of the diluted suspension of each strain and one well, used as a blank, was filled with 200 µl of un-inoculated culture media (CYB, SGM, WGJ).

Growth assessment and OTA production at each a_w level after 1, 2, 4, and 10 days of incubation at three different temperatures (15, 25, and 35 °C) were determined. Thus, each strain-a_w-level-temperature combination was repeated in 4 microplates, one for each sampling day.

For each sampling occasion and temperature assayed, microtiter plates with the same water activity level were enclosed in sealed polyethylene bags. The entire experiment was repeated twice on different days.

2.4. Growth measurement and OTA extraction procedure

For each culture media, a_w level and temperature assayed, growth was monitored by absorbance measurements at 530 nm using the Multilabel-Reader Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany) after 1, 2, 4 and 10 days of incubation. The absorbance of the corresponding uninoculated medium, used as blank was subtracted to the absorbance values of the inoculated media. After each reading, microplates were sealed and stored at -80°C until they were analyzed for OTA content.

OTA production was detected using a previously described high-pressure liquid chromatography (HPLC) screening method developed in our laboratory for fungi growing in microtiter wells (Abarca et al., 2014). On each sampling occasion, one of the five replicate wells inoculated for each strain, culture media, a_w level and incubation temperature, were randomly selected and their content was removed and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. The limit of quantification was $0.045\ \mu\text{g/ml}$ for this mycotoxin.

2.5. Statistical analysis

Data obtained from the different conditions tested were statistically analyzed by means of one-way analysis of variance test. All statistical analyses were performed using Minitab 17 statistical software (Minitab Inc., State College, Pennsylvania, USA).

3. Results

3.1. Molecular species identification

Based on the calmodulin sequences, all the strains were identified as *A. carbonarius*. The phylogenetic tree was reconstructed showing that the isolates grouped with *A. carbonarius* CBS 556.65^T (Fig. 1). Sequence analysis revealed that all the strains had the same sequence. The nucleotide sequence of one representative strain (A-1002) has been deposited in GenBank under accession number MK778845.

3.2. Inoculum standardization

Inocula adjusted to 0.8 McFarland turbidity standard provided suspensions of $1.1 \pm 0.1 \times 10^6$ conidia/ml by microscopic enumeration with a cell-counting haemocytometer. Mean colony counts of the above *A. carbonarius* suspensions were $0.3 \pm 0.1 \times 10^6$ cfu/ml.

3.3. Growth measurement

Table 2 shows results of one-way analysis of variance for the effects of the variables assayed (experiment, temperature, water activity, culture media and strain) on absorbance values. No significant differences were observed in absorbance values neither between the experiments, nor between the strains ($p > 0.05$). The remaining variables had a significant effect on growth of all the strains studied. Regardless a_w level or culture medium, the highest absorbance values were recorded at 35°C , although not statistically different from those obtained at 25°C . Values recorded at $0.98\text{--}0.99\ a_w$ and $0.95\ a_w$ were significantly higher than at $0.90\ a_w$. In relation to culture media, the highest significant absorbance values ($p < 0.001$) were recorded in CYB, followed by SGM and WGJ.

Mean absorbance values recorded in both experiments by all the studied strains at each condition and incubation time tested are shown

in Table 3. The highest absorbance values were recorded after 10 days of incubation at 25°C and 35°C in CYB at $0.99\ a_w$ and at $0.95\ a_w$.

In all culture media, initial growth in the microtiter wells was visible to the naked eye, when the absorbance value was > 0.1 . According to this, none of the strains were able to grow at $0.90\ a_w$ and 15°C , the most extreme conditions tested, in any culture media after 10 days of incubation. At this low temperature, a significant increase in absorbance values was detected after 4–10 days of incubation in the three culture media adjusted at 0.95 and $0.98\text{--}0.99\ a_w$.

At 25°C , absorbance values significantly increased after 2–4 days ($0.98\text{--}0.99\ a_w$ and $0.95\ a_w$) or 10 days ($0.90\ a_w$) of incubation.

At 35°C a significant growth increase was observed after 2 days of incubation in the three culture media adjusted at $0.98\text{--}0.99\ a_w$ and $0.95\ a_w$. At $0.90\ a_w$, this increase was recorded after 2–4 days in SGM and WGJ and after 10 days in CYB.

3.4. OTA production

No significant differences were found in OTA concentration neither between the experiments, nor between the strains ($p > 0.05$) (Table 2). Temperature, culture media and water activity had significant effect ($p < 0.01$) on OTA production. OTA concentration was statistically higher ($p < 0.01$) at 15°C than at 25°C or 35°C . The highest significant OTA values were obtained at $0.98\text{--}0.99\ a_w$ and the best culture media ($p < 0.01$) for OTA production was CYB, followed by WGJ and SGM.

Table 4 shows OTA concentration produced by all the strains at each condition assayed and incubation time. Results are expressed as mean value of both experiments as no statistically significant differences were observed ($p > 0.05$). As a general trend, OTA production increased over incubation time achieving the highest concentration ($p < 0.01$) after 10 days of incubation. None of the strains produced detectable levels of OTA in any culture media at $0.90\ a_w$ and 15°C . The highest OTA concentration was recorded at 15°C after 10 days of incubation in CYB at $0.99\ a_w$. It is worth to highlight that in those conditions of temperature and a_w , all the strains produced detectable levels of OTA in the three culture media studied.

At 25°C and $0.98\text{--}0.99\ a_w$, all the strains produced OTA after only 2 days (CYB, WGJ) or 4 days (SGM) of incubation. As water activity decreases, optimum temperature for OTA production moved to 25°C , although not all the studied strains were able to produce the mycotoxin.

At 35°C , and $0.98\text{--}0.99\ a_w$ some strains produced low levels of OTA after only 1 or 2 days of incubation. By decreasing the water activity, decreases the number of OTA-producing strains. At $0.90\ a_w$, still some strains could produce detectable levels of OTA after 10 days of incubation.

Although taking into account the results that overall no intraspecific differences were observed, the strain A-884 isolated from Robusta coffee cherries from Thailand, showed a different behavior to the remaining strains, mostly isolated from grapes. Thus, OTA concentration produced by this strain was statistically higher ($p < 0.05$) at 25°C than at 15°C or 35°C . Fig. 2a shows OTA values recorded in CYB medium by this coffee strain (A-884) in comparison to a high OTA-producing grape strain (A-1002) and a low OTA-producing grape strain (A-2128), both isolated from Spain, in each condition assayed. The monthly maximum and minimum average temperatures in Mediterranean areas and in Thailand are also shown (Fig. 2b).

4. Discussion

For each strain and reading day (1, 2, 4 and 10 days), the total number of conditions studied were 27 (3 culture media at 3 water activities and 3 incubation temperatures). The large number of isolates and conditions studied here will allow a more comprehensive overview of the ecophysiology of *A. carbonarius*, difficult when using laborious methods based on Petri dishes.

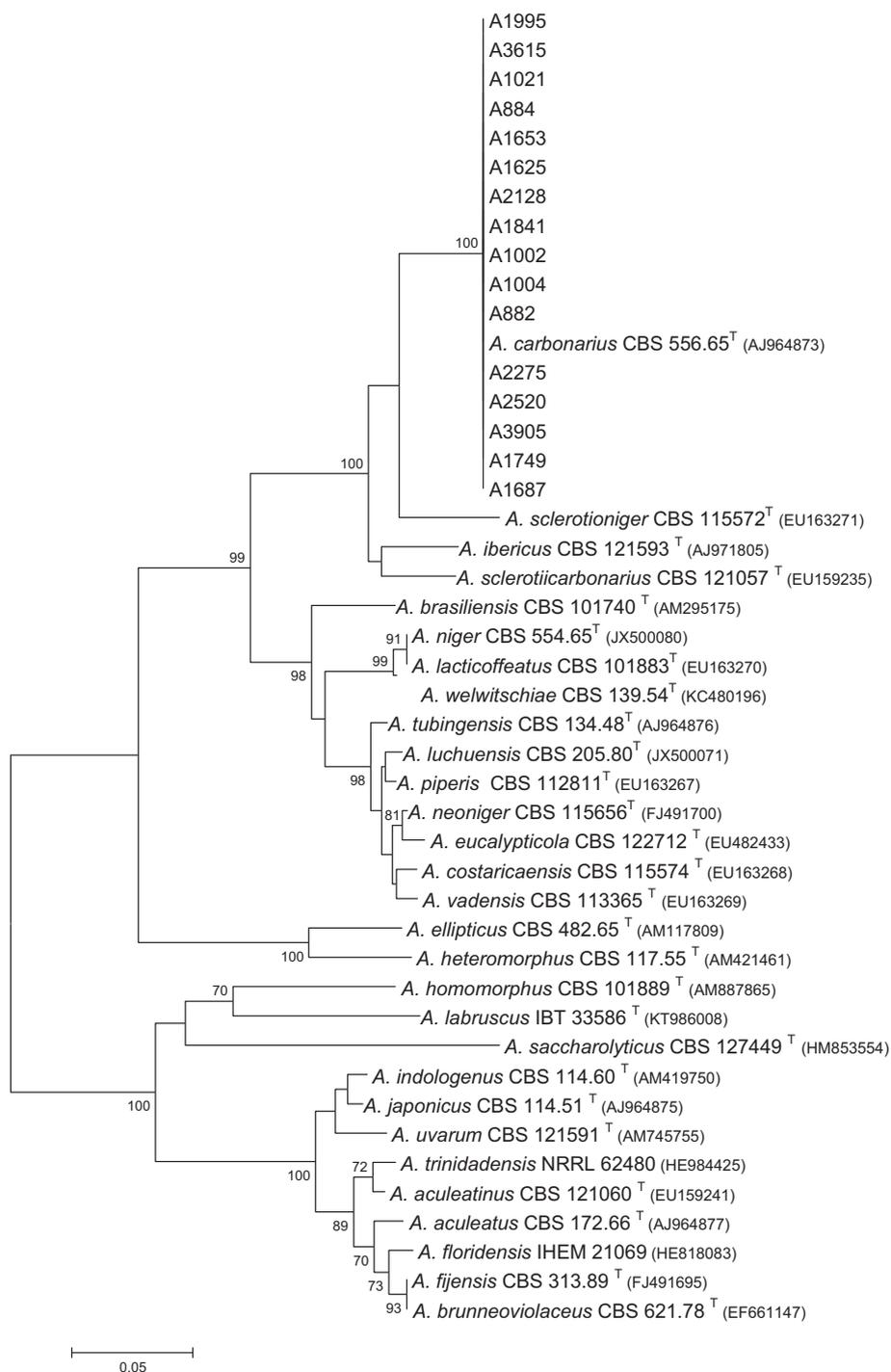


Fig. 1. Phylogenetic tree of *Aspergillus* section *Nigri* inferred from Neighbor-Joining analysis of partial calmodulin gene. Bootstrap values > 70% in 1000 replications are shown at nodes.

Table 2

One-way analysis of variance of absorbance (ABS) and ochratoxin A (OTA) values versus (vs.) each of the variables assayed.

	p value				
	vs. EXP	vs. T (°C)	vs. a _w	vs. culture media	vs. strain
ABS	0.078	0.000 (35 ^a > 25 ^a > 15 ^b)	0.000 (0.99 ^a > 0.95 ^a > 0.90 ^b)	0.000 (CYB ^a > SGM ^b > WGJ ^c)	0.999
OTA	0.257	0.000 (15 ^a > 25 ^b > 35 ^b)	0.000 (0.99 ^a > 0.95 ^b > 0.90 ^b)	0.000 (CYB ^a > WGJ ^b > SGM ^b)	0.265

^{a,b,c} Values of variables with the same superscript are not significantly different ($p > 0.05$).

Abbreviations: EXP, experiment; T, temperature in °C; a_w, water activity; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGJ, White grape juice.

Table 3
Mean absorbance (ABS) and standard deviation values in both experiments by all the studied strains of *Aspergillus carbonarius* at each condition and incubation time tested.

Culture media	Days	ABS ± δ												
		15 °C				25 °C				35 °C				
		0.95		0.99–0.98		0.95		0.99–0.98		0.95		0.99–0.98		
CYB	1	0.0050 ± 0.0047 ^a	0.0009 ± 0.0084 ^a	-0.00005 ± 0.0069 ^a	0.0035 ± 0.0054 ^a	0.0051 ± 0.0064 ^a	0.0577 ± 0.0323 ^a	0.0506 ± 0.0357 ^a	0.185 ± 0.0280 ^a	0.0035 ± 0.0084 ^a	0.0009 ± 0.0084 ^a	0.0577 ± 0.0323 ^a	0.0506 ± 0.0357 ^a	0.185 ± 0.0280 ^a
	2	0.0106 ± 0.0075 ^a	0.0010 ± 0.0121 ^a	0.0016 ± 0.0056 ^b	0.0455 ± 0.0181 ^b	0.0034 ± 0.0060 ^a	1.0636 ± 0.7917 ^b	0.372 ± 0.2816 ^b	0.0384 ± 0.0400 ^a	0.0016 ± 0.0056 ^b	0.0010 ± 0.0121 ^a	1.0636 ± 0.7917 ^b	0.372 ± 0.2816 ^b	0.0384 ± 0.0400 ^a
	4	0.1478 ± 0.0597 ^b	0.0104 ± 0.0115 ^a	0.0036 ± 0.0034 ^c	1.9008 ± 0.3453 ^c	0.0368 ± 0.0132 ^a	2.5320 ± 0.4117 ^c	2.5535 ± 0.3375 ^c	0.0842 ± 0.0673 ^a	0.0104 ± 0.0115 ^a	0.0036 ± 0.0034 ^c	2.5320 ± 0.4117 ^c	2.5535 ± 0.3375 ^c	0.0842 ± 0.0673 ^a
	10	2.1620 ± 0.336 ^c	0.8526 ± 0.5574 ^b	0.0045 ± 0.0051 ^c	2.9535 ± 0.1252 ^d	2.7043 ± 0.3234 ^b	2.7725 ± 0.4672 ^d	3.0476 ± 0.0878 ^d	1.5364 ± 0.4573 ^b	0.8526 ± 0.5574 ^b	0.0045 ± 0.0051 ^c	2.9535 ± 0.1252 ^d	2.7043 ± 0.3234 ^b	3.0476 ± 0.0878 ^d
SGM	1	-0.0048 ± 0.0064 ^a	0.0017 ± 0.0093 ^a	0.0071 ± 0.0108 ^a	0.0028 ± 0.0175 ^a	0.0143 ± 0.0140 ^a	0.0200 ± 0.0198 ^a	0.0080 ± 0.0109 ^a	0.0243 ± 0.0160 ^a	-0.0048 ± 0.0064 ^a	0.0017 ± 0.0093 ^a	0.0071 ± 0.0108 ^a	0.0028 ± 0.0175 ^a	0.0143 ± 0.0140 ^a
	2	-0.0035 ± 0.0081 ^a	0.0013 ± 0.0082 ^a	0.0069 ± 0.0105 ^a	0.0451 ± 0.0287 ^a	0.0388 ± 0.0298 ^a	0.2812 ± 0.2362 ^b	1.1593 ± 0.5971 ^d	0.2409 ± 0.2440 ^b	-0.0035 ± 0.0081 ^a	0.0013 ± 0.0082 ^a	0.0069 ± 0.0105 ^a	0.0451 ± 0.0287 ^a	0.0388 ± 0.0298 ^a
	4	0.0349 ± 0.0186 ^b	0.0070 ± 0.0075 ^a	0.0124 ± 0.0092 ^b	0.5936 ± 0.3639 ^b	0.1290 ± 0.1043 ^b	0.9074 ± 0.5209 ^c	0.7608 ± 0.5171 ^c	0.1571 ± 0.1582 ^c	0.0349 ± 0.0186 ^b	0.0070 ± 0.0075 ^a	0.0124 ± 0.0092 ^b	0.5936 ± 0.3639 ^b	0.1290 ± 0.1043 ^b
	10	0.7549 ± 0.3014 ^c	0.2533 ± 0.1680 ^b	0.0105 ± 0.0084 ^b	1.0018 ± 0.4218 ^c	0.2198 ± 0.0264 ^b	1.3746 ± 0.6066 ^d	1.1593 ± 0.5971 ^d	0.4964 ± 0.4964 ^d	0.7549 ± 0.3014 ^c	0.2533 ± 0.1680 ^b	0.0105 ± 0.0084 ^b	1.0018 ± 0.4218 ^c	0.2198 ± 0.0264 ^b
WGJ	1	0.0022 ± 0.0065 ^a	0.0023 ± 0.0058 ^a	0.0064 ± 0.0057 ^a	0.0491 ± 0.0150 ^a	0.0088 ± 0.0090 ^a	0.0803 ± 0.0365 ^a	0.0687 ± 0.0362 ^a	0.0109 ± 0.0073 ^a	0.0022 ± 0.0065 ^a	0.0023 ± 0.0058 ^a	0.0064 ± 0.0057 ^a	0.0491 ± 0.0150 ^a	0.0088 ± 0.0090 ^a
	2	0.0191 ± 0.0110 ^b	0.0077 ± 0.0081 ^a	0.0054 ± 0.0053 ^a	0.1948 ± 0.0485 ^b	0.0933 ± 0.0313 ^b	0.1591 ± 0.0710 ^b	0.3237 ± 0.1053 ^b	0.0511 ± 0.0251 ^b	0.0191 ± 0.0110 ^b	0.0077 ± 0.0081 ^a	0.0054 ± 0.0053 ^a	0.1948 ± 0.0485 ^b	0.0933 ± 0.0313 ^b
	4	0.1242 ± 0.0279 ^c	0.0573 ± 0.0158 ^b	0.0065 ± 0.0067 ^a	0.3319 ± 0.0885 ^c	0.2099 ± 0.0661 ^c	0.2182 ± 0.1050 ^c	0.5222 ± 0.1616 ^c	0.0934 ± 0.0934 ^c	0.1242 ± 0.0279 ^c	0.0573 ± 0.0158 ^b	0.0065 ± 0.0067 ^a	0.3319 ± 0.0885 ^c	0.2099 ± 0.0661 ^c
	10	0.3095 ± 0.0628 ^d	0.1928 ± 0.0404 ^c	0.0265 ± 0.0089 ^b	0.4019 ± 0.1273 ^d	0.2714 ± 0.1036 ^d	0.3469 ± 0.1842 ^d	0.6301 ± 0.1572 ^d	0.3845 ± 0.1406 ^d	0.3095 ± 0.0628 ^d	0.1928 ± 0.0404 ^c	0.0265 ± 0.0089 ^b	0.4019 ± 0.1273 ^d	0.2714 ± 0.1036 ^d

a,b,c,d In columns, values with the same superscript within each culture medium are not significantly different ($p > 0.05$).

Abbreviations: T, temperature in °C; a_w, water activity; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGJ, White grape juice.

Inoculum size is one of the technical variables that can influence the spectrophotometric tests outcome. Conidial suspensions adjusted to 0.8 McFarland yielded in all cases haemocytometer counting around 10⁶ conidia/ml. The use of the portable photometer Densimat (BioMérieux) provides a substantial reduction in workload as we have previously reported (Abarca et al., 2014, 2019; Cabañas et al., 2009). As no statistically significant differences were observed in absorbance and OTA values between the experiments, our method has proven to be an easy way to monitor growth and OTA production of *A. carbonarius* in ecophysiological studies.

Results obtained in this study confirm that *A. carbonarius* strains can grow at a wide range of temperatures (15–35 °C) and water activities (0.90–0.99). The optimum conditions of a_w and temperature for growth were in the range 25–35 °C and 0.95–0.99 a_w. The highest amount of OTA was obtained at 15 °C and 0.98–0.99 a_w. In previous studies on solid culture media, *A. carbonarius* strains also showed a narrower range of optimal conditions for OTA production than for growth, when temperature, water activity or pH effects were evaluated (Esteban et al., 2004, 2005, 2006).

In agreement with our results, 15 °C or 15–20 °C have been reported as the optimum temperatures for OTA production by *A. carbonarius* strains in different culture media (Esteban et al., 2004; Leong et al., 2006; Marin et al., 2006; Mitchell et al., 2004; Passamani et al., 2014; Romero et al., 2010; Tassou et al., 2007).

Although all the strains were able to produce OTA in the three culture media studied, the highest OTA concentration was achieved in CYB (final pH 6.3 ± 0.2). Czapek yeast extract agar (CYA) has been reported as a suitable medium to detect OTA-producing ability of *A. carbonarius* strains (Abarca et al., 2014; Bragulat et al., 2001; Esteban et al., 2004). In SGM and WGJ media, with a final pH of about 4, a decrease in both growth and OTA production was observed. The acidity of the growth medium could exert some influence on the growth and OTA production by *A. carbonarius*. In previous studies, *A. carbonarius* isolates were able to produce OTA at a wide range of pH values (2 to 10) on CYA medium. When CYA plates were incubated at 15 °C, the highest OTA levels were obtained generally at a higher pH range (5 to 7) (Esteban et al., 2005). In a similar way, the greatest OTA concentration was reported in semisynthetic grape culture medium at 15 °C, with pH above 6.0 and a_w of 0.99 (Passamani et al., 2014). In other studies, low pH level seemed optimal for maximum OTA production (Spadaro et al., 2010), while Kapetanakou et al. (2009) reported that pH (3.9–6.8) had no particular effect on OTA production by *A. carbonarius*.

In accordance with our results some studies have shown that *A. carbonarius* strains isolated from grapes from different geographical origins have a common pattern in growth and OTA production under variable conditions of temperature and a_w. Strains of *A. carbonarius* isolated from Tunisian grapes behave as those from Spanish grapes (Marin et al., 2006) and as those from Australian and European grapes as reported in the literature (Lasram et al., 2010). Leong et al. (2006) showed that trends for growth and OTA production were similar among Australian isolates and those from European grapes. Using a cocktail inocula of strains isolated from Argentinean dried vine fruits, similar results were obtained to those reported for single *A. carbonarius* strains from European countries, Israel, Australia and South America (Romero et al., 2010). Therefore, strains isolated from grapes seem to have a similar behavior despite being isolated from different geographical areas.

In our study, OTA concentration produced by the strain A-884 isolated from Robusta coffee cherries from Thailand was statistically higher at 25 °C than at 15 °C or 35 °C. Most, if not all, current ecophysiological studies have been carried out with *A. carbonarius* strains isolated from grapes and derived products. To date, there are few data available regarding the effect of environmental factors on *A. carbonarius* strains isolated from coffee. In a previous study, including strains from different substrates, *A. carbonarius* CBS 127.49 isolated from a seed of

Table 4
Mean OTA concentration values (in µg/ml) produced by *Aspergillus carbonarius* strains at each condition assayed and incubation time.

Culture media	Days	OTA (µg/ml) mean value ± δ (no. of OTA positive strains)											
		15 °C				25 °C				35 °C			
		a _w	0.99-0.98	0.90	0.95	0.99-0.98	0.90	0.95	0.99-0.98	0.90	0.95	0.99-0.98	0.90
CYB	1	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	0.052 ± 0 ^a (1)	ND ^a	ND ^a
	2	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	0.1965 ± 0.2893 ^{a,b} (8)	ND ^a	ND ^a
	4	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	0.2828 ± 0.5487 ^c (15)	0.1145 ± 0.0439 ^a (2)	ND ^a
	10	8.4784 ± 7.361 ^b (16)	0.6995 ± 1.30 ^b (14)	ND ^a	1.0181 ± 1.6597 ^b (10)	0.9958 ± 1.0135 ^c (16)	0.3883 ± 0.572 ^b (7)	0.2039 ± 0.2894 ^{b,c} (16)	0.0762 ± 0.0532 ^{b,c} (7)	ND ^a	ND ^a	0.1083 ± 0.1607 ^b (15)	0.069 ± 0.011 ^a (1)
SGM	1	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a
	2	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	0.0762 ± 0.0532 ^{b,c} (7)	ND ^a	ND ^a
	4	ND ^a	ND ^a	ND ^a	0.0907 ± 0.069 ^{a,b} (7)	ND ^a	ND ^a	0.0731 ± 0.0398 ^{b,c} (10)	ND ^a	ND ^a	0.061 ± 0.0057 ^{a,b} (3)	ND ^a	ND ^a
WGJ	10	0.1329 ± 0.0902 ^b (16)	0.0865 ± 0.0934 ^b (13)	ND ^a	0.122 ± 0.1276 ^b (13)	0.1756 ± 0.2119 ^b (16)	0.0694 ± 0.0304 ^a (4)	0.0775 ± 0.0285 ^c (15)	ND ^a	ND ^a	0.0613 ± 0.0126 ^b (6)	0.0586 ± 0.008 ^a (3)	
	1	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	
	2	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	0.0724 ± 0.0315 ^b (15)	0.0596 ± 0.0069 ^a (4)	ND ^a
	4	ND ^a	ND ^a	ND ^a	0.0626 ± 0.0159 ^b (11)	0.1175 ± 0.0489 ^c (16)	ND ^a	0.0727 ± 0.027 ^b (15)	ND ^a	ND ^a	0.0696 ± 0.0123 ^a (3)	ND ^a	ND ^a
10	0.098 ± 0.0416 ^b (16)	0.0799 ± 0.0255 ^b (16)	ND ^a	0.058 ± 0.0048 ^b (8)	0.0884 ± 0.0334 ^b (16)	0.0576 ± 0.0037 ^a (2)	0.0735 ± 0.0343 ^b (16)	0.0613 ± 0.0117 ^b (15)	0.0546 ± 0.0008 ^b (4)				

^{a,b,c} In columns, values with the same superscript within each culture medium are not significantly different ($p > 0.05$).
 ND, denotes not detected.
 Abbreviations: T, temperature in °C; a_w, water activity; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGJ, White grape juice.

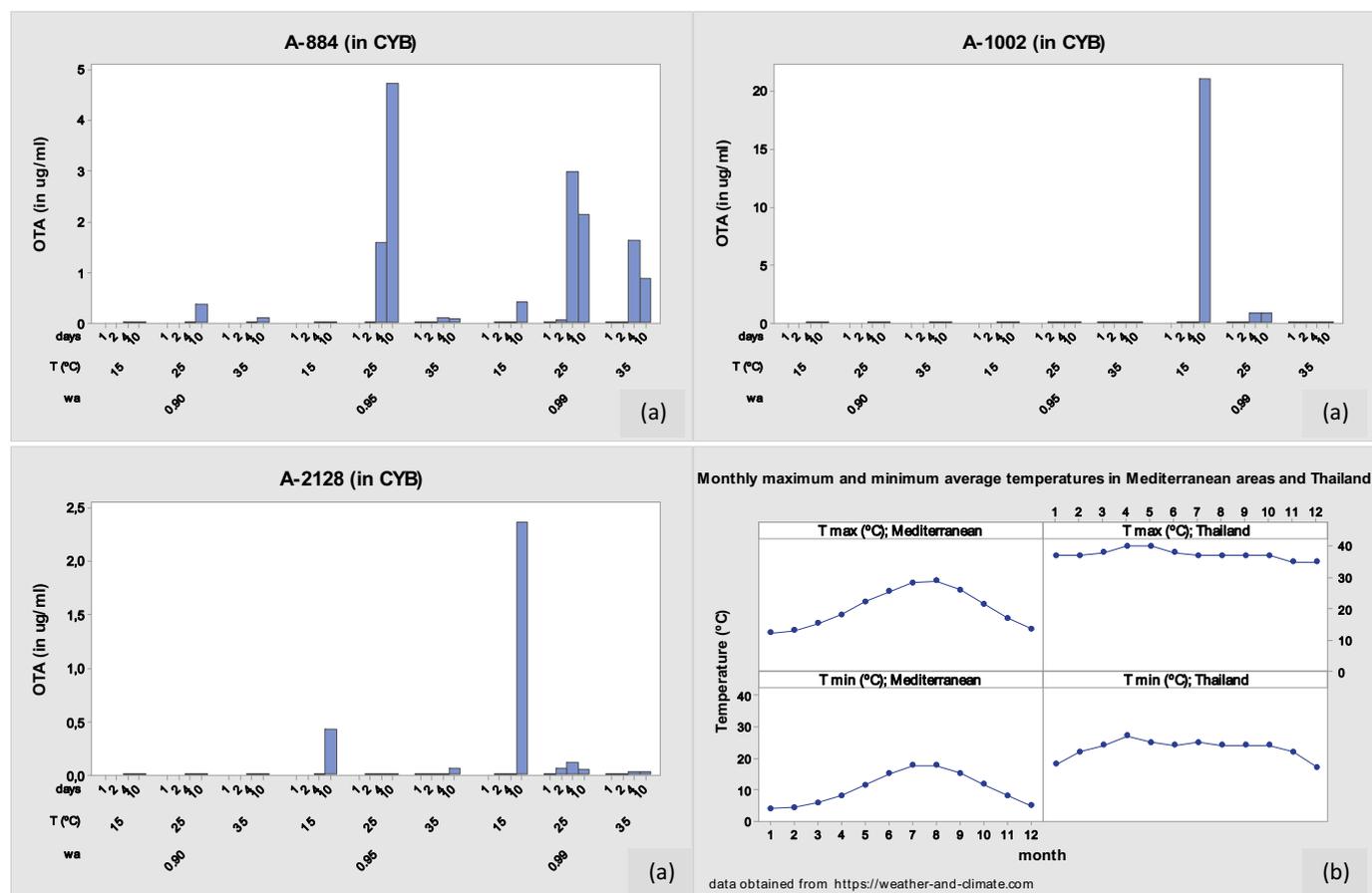


Fig. 2. OTA production of several *A. carbonarius* assayed strains: A-884 (Robusta coffee cherries, Thailand), A-1002 (grapes, Spain) and A-2128 (grapes, Spain) at each condition assayed (a) and monthly maximum and minimum average temperatures in Mediterranean areas and in Thailand (b). Data obtained from <https://weather-and-climate.com> on March 2019.

coffee Arabica produced more OTA at 20 °C than at 15 °C (Esteban et al., 2004). Kouadio et al. (2007) determined the effect of water activity (range 0.75–0.99) and temperature (range 5–45 °C) on OTA production in one strain of *A. carbonarius* isolated from Robusta coffee beans. The reported maximum OTA production was at 0.99 a_w at 15 °C although similar OTA concentration was obtained at 25 °C and 30 °C. On irradiated coffee cherries, Joosten et al. (2001) reported that optimal condition for OTA production was at 0.99 a_w (range 0.85–0.99). At this a_w maximum OTA production was achieved at 25 °C (range 20–37 °C). In the same way, on a green coffee-based medium the highest OTA production was obtained at 0.99 a_w (range 0.90–0.99) and 25 °C (range 20–37 °C) by the *A. carbonarius* strain studied, isolated from Arabica coffee beans (Akbar, 2015).

The strain A-884 was isolated from the South of Thailand (Joosten et al., 2001). In this tropical area the climate is hot all year round, with maximum and minimum temperatures much higher than those in the Mediterranean climate and with a lower temperature variation along the year (Fig. 2b). The remaining *A. carbonarius* strains studied were isolated from areas with Mediterranean climate, with lower minimum temperatures (4–18 °C) than those observed in tropical areas (Fig. 2b). This would explain that 15 °C be the optimum temperature for these strains, as OTA can be continuously produced in the field, in spite of there are large temperature variations between day and night.

Although very few coffee strains have been studied so far, this different pattern in relation to optimal temperature for OTA production could suggest that the type of food matrices (e. g. coffee beans) and consequently the adaptation of *A. carbonarius* strains to different climatic conditions would have a greater influence on the ecophysiology of the strains than only their geographical origin. Further studies are

needed including a great number of coffee isolates to corroborate it.

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References

- Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2014. A new in vitro method to detect growth and ochratoxin A-producing ability of multiple fungal species commonly found in food. *Food Microbiol.* 44, 243–248.
- Abarca, M.L., Bragulat, M.R., Castellá, G., Cabañes, F.J., 2019. Impact of some environmental factors on growth and ochratoxin A production by *Aspergillus niger* and *Aspergillus welwitschiae*. *Int. J. Food Microbiol.* 291, 10–16.
- Akbar, A.H., 2015. In: Cranfield University (Ed.), Growth and Ochratoxin A Production by *Aspergillus* Species in Coffee Beans: Impact of Climate Change and Control Using O₃. PhD. <http://dspace.lib.cranfield.ac.uk/handle/1826/9264>.
- Amézqueta, S., Schorr-Galindo, S., Murillo-Arbizu, M., González-Peñas, E., López de Cerain, A., Guiraud, J.P., 2012. OTA-producing fungi in foodstuffs: a review. *Food Control* 26, 259–268.
- Battilani, P., Camardo, M., 2015. OTA-grapes: a mechanistic model to predict ochratoxin A risk in grapes, a step beyond the systems approach. *Toxins* 7, 3012–3029.
- Bau, M., Bragulat, M.R., Abarca, M.L., Minguez, S., Cabañes, F.J., 2005. Ochratoxigenic species from Spanish wine grapes. *Int. J. Food Microbiol.* 98, 125–130.
- Bragulat, M.R., Abarca, M.L., Cabañes, F.J., 2001. An easy screening method for fungi producing ochratoxin A in pure culture. *Int. J. Food Microbiol.* 71, 139–144.
- Cabañes, R., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2009. In vitro activity of Imazalil against *Penicillium expansum*: comparison of the CLSI M38-A broth microdilution method with traditional techniques. *Int. J. Food Microbiol.* 129, 26–29.

- Cabañes, F.J., Bragulat, M.R., 2018. Black aspergilli and ochratoxin-producing species in foods. *Curr. Opin. Food Sci.* 23, 1–10.
- Cabañes, F.J., Bragulat, M.R., Castellá, G., 2013. Characterization of nonochratoxigenic strains of *Aspergillus carbonarius* from grapes. *Food Microbiol.* 36, 135–141.
- Commission of the European Communities, 2006. Commission regulation (EC) no 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union L364*, 5–24.
- Commission of the European Communities, 2010. Commission regulation (EU) no 105/2010 of 5 February 2010 amending regulation (EC) no 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A. *Off. J. Eur. Union L35*, 7–8.
- Commission of the European Communities, 2012. Commission regulation (EU) no 594/2012 of 5 July 2012 amending regulation (EC) 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foodstuffs. *Off. J. Eur. Union L176*, 43–45.
- Commission of the European Communities, 2015. Commission regulation (EU) 2015/1137 of 13 July 2015 amending regulation (EC) no 1881/2006 as regards the maximum level of Ochratoxin A in *Capsicum* spp. spices. L185 11–12.
- EFSA (European Food Safety Authority), 2006. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food. *EFSA J.* 365, 1–56.
- Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2004. Effects of temperature and incubation time on production of ochratoxin A by black aspergilli. *Res. Microbiol.* 155, 861–866.
- Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2005. Influence of pH and incubation time on ochratoxin A production by *Aspergillus carbonarius* in culture media. *J. Food Prot.* 68, 1435–1440.
- Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2006. Study of the effect of water activity and temperature on ochratoxin A production by *Aspergillus carbonarius*. *Food Microbiol.* 23, 634–640.
- European Commission, 2002. SCOOP task 3.2.7. Assessment of dietary intake of ochratoxin A by the population of EU member states. In: European Commission, Directorate-General Health and Consumer Protection, Reports on Tasks for Scientific Cooperation, . http://europa.eu.int/comm/food/fs/scoop/3.2.7_en.pdf.
- García, D., Ramos, A.J., Sanchis, V., Marín, S., 2011. Is intraspecific variability of growth and mycotoxin production dependent on environmental conditions? A study with *Aspergillus carbonarius* isolates. *Int. J. Food Microbiol.* 144, 432–439.
- García, D., Valls, J., Ramos, A.J., Sanchis, V., Marín, S., 2012. Optimising the number of isolates to be used to estimate growth parameters of mycotoxigenic species. *Food Microbiol.* 32, 235–242.
- Heussner, A.H., Bingle, L.E.H., 2015. Comparative ochratoxin toxicity: a review of the available data. *Toxins* 7, 4253–4282.
- Joosten, H.M.L.J., Goetz, J., Pittet, A., Schellenberg, M., Bucheli, P., 2001. Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. *Int. J. Food Microbiol.* 65, 39–44.
- Kapetanakou, A.E., Panagou, E.Z., Gialitaki, M., Drosinos, E.H., Skandamis, P.N., 2009. Evaluating the combined effect of water activity, pH and temperature on ochratoxin A production by *Aspergillus ochraceus* and *Aspergillus carbonarius* on culture medium and Corinth raisins. *Food Control* 20, 725–732.
- Kouadio, A.I., Lebríhi, A., 2007. Influence de l'interaction de la température et de l'activité de l'eau sur la croissance et la production de l'ochratoxine A par *Aspergillus niger*, *Aspergillus carbonarius* et *Aspergillus ochraceus* sur un milieu de base café. *Can. J. Microbiol.* 53, 852–859.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal wand clustal X version 2.0. *Bioinformatics* 23, 2947–2948.
- Lasram, S., Oueslati, S., Valero, A., Marin, S., Ghorbel, A., Sanchis, V., 2010. Water activity and temperature effects on fungal growth and ochratoxin A production by ochratoxigenic *Aspergillus carbonarius* isolated from Tunisian grapes. *J. Food Sci.* 75, M89–M97.
- Leong, S.L., Hocking, A.D., Scott, E.S., 2006. Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium. *Int. J. Food Microbiol.* 110, 209–216.
- Magan, N., Medina, A., Aldred, D., 2011. Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. *Plant Pathol.* 60, 150–163.
- Marin, S., Bellí, N., Lasram, S., Chevill, S., Ramos, A.J., Ghorbel, A., Sanchis, V., 2006. Kinetics of ochratoxin A production and accumulation by *Aspergillus carbonarius* on synthetic grape medium at different temperature levels. *J. Food Sci.* 71, M196–M200.
- Mitchell, D., Parra, R., Aldred, D., Magan, N., 2004. Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. *J. Appl. Microbiol.* 97, 439–445.
- Noonim, P., Mahakarnchanakul, W., Nielsen, K.F., Frisvad, J.C., Robert, A., Samson, R.A., 2008. Identification and toxigenic potential of ochratoxin A-producing *Aspergillus* species from coffee beans grown in two regions of Thailand. *Int. J. Food Microbiol.* 128, 197–202.
- O'Donnell, K., Nirenberg, H.I., Aoki, T., Cigelnik, E., 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. *Mycoscience* 41, 61–78.
- Passamani, F.R.F., Hernandez, T., Lopes, N.A., Bastos, S.C., Santiago, W.D., Cardoso, M.G., Batista, L.R., 2014. Effect of temperature, water activity, and pH on growth and production of ochratoxin A by *Aspergillus niger* and *Aspergillus carbonarius* from Brazilian grapes. *J. Food Prot.* 77, 1947–1952.
- Pitt, J.I., Hocking, A.D., 2009. *Fungi and Food Spoilage*, third ed. Springer, London.
- Romero, S.M., Fernandez-Pinto, V., Patriarca, A., Vaamonde, G., 2010. Ochratoxin A production by a mixed inoculum of *Aspergillus carbonarius* at different conditions of water activity and temperature. *Int. J. Food Microbiol.* 140, 277–281.
- Spadaro, D., Patharajan, S., Lore, A., Gullino, M.L., Garibaldi, A., 2010. Effect of pH, water activity and temperature on the growth and accumulation of ochratoxin A produced by three strains of *Aspergillus carbonarius* isolated from Italian vineyards. *Phytopathol. Mediterr.* 49, 65–73.
- Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10, 512–526.
- Tamura, K., Stecher, G., Peterson, D., Filipowski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729.
- Tassou, C.C., Natskoulis, P.I., Panagou, E.Z., Spiropoulos, A.E., Magan, N., 2007. Impact of water activity and temperature on growth and ochratoxin A production of two *Aspergillus carbonarius* isolates from wine grapes in Greece. *J. Food Prot.* 70, 2884–2888.
- Visconti, A., Perrone, G., Cozzi, G., Solfrizzo, M., 2008. Managing ochratoxin A risk in the grape-wine food chain. *Food Add. Contam.* 25, 193–202.