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

M.R. Bragulat, M.L. Abarca, G. Castellá*, F.J. Cabañes

*Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Spain.*

*Corresponding author:

Gemma Castellá

Grup de Micologia Veterinària

Departament de Sanitat i d'Anatomia Animals

Facultat de Veterinària, Universitat Autònoma de Barcelona

08193 Bellaterra (Barcelona), Spain.

Tel: +34 93 5811089

Fax: +34 93 5812006

e-mail: Gemma.Castella@uab.cat
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<sub>w</sub>) levels (0.90; 0.95 and 0.98-0.99), and three incubation temperatures (15ºC, 25ºC and 35ºC) on the growth and OTA production by 16 strains of *A. carbonarius*. The strains were selected on the basis of the geographical origin of the substrate and included strains from different climatic zones of Spain as well as from other countries with different climatology. 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<sub>w</sub> and at 0.95 a<sub>w</sub> after 10 days of incubation at 25ºC and 35ºC. None of the strains were able to grow at 0.90 a<sub>w</sub> and 15ºC in any culture media after 10 days of incubation. OTA concentration was statistically higher at 15ºC than at 25ºC or 35ºC. The highest significant OTA values were obtained at 0.98-0.99 a<sub>w</sub> 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 25ºC than at 15ºC. 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.

*Keywords: Aspergillus carbonarius, coffee, ecophysiology, grapes, raisins, ochratoxin A*
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, dry 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 responsible source of OTA in wine or dried vine fruits from main vinicultural 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 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 in 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 they are laborious and time-consuming methods.

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 suboptimal 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/25ºC; 0.90 aw/25ºC; 0.98 aw/37ºC) were tested. Using above data, García et al. (2012) mathematically assessed the minimum number of isolates that would lead to equivalent growth parameters estimates to those obtained with a high number of strains. They concluded that 12-17 isolates of *A. carbonarius* led to the same growth parameters as the total 30.
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*.

### 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 the geographical origin of the substrate and included strains from different climatic zones of Spain as well as from other countries with different climatology.

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, Biolink, Barcelona, Spain). The DNA was kept at -20 ºC 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 1,000 bootstrap replicates was used.

2.2. Inoculum preparation and verification

Spore concentration was adjusted to around $10^6$ 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 25ºC. After heavy particles were allowed to
settle for 10-15 minutes, 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: $K_2HPO_4$, 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, 70g; D(-) fructose, 30g; L(-)
tartaric acid, 7g; L(-) malic acid, 10g; (NH₄)₂HPO₄, 0.67g; KH₂PO₄, 0.67g; MgSO₄·7 H₂O,
NaCl, 0.15g; CaCl₂, 0.15g; CuCl₂, 0.0015g; FeSO₄·7 H₂O, 0.021g; ZnSO₄·7 H₂O,
0.0075g; (+) Catechin hydrate, 0.05g; pH adjusted with 10M NaOH to pH 4.0-4.2

(Mitchell et al., 2004). WGJ was prepared with 200ml of commercially sold white grape juice made from ecological 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 were determined after 1, 2, 4, and 10 days of incubation at three different temperatures (15, 25, and 35ºC).

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 530nm 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 -80ºC 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 µg/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 556.65T (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 ±
0.1 x 10^6 conidia/ml by microscopic enumeration with a cell-counting
haemocytometer. Mean colony counts of the above *A. carbonarius* suspensions were
0.3 ± 0.1 x 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 35ºC,
although not statistically different from those obtained at 25ºC. Values recorded at
0.98-0.99 *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 25ºC and 35ºC in CYB at 0.99 aw and at 0.95 aw.

In all culture media, initial growth in the microtiter wells could be visually detected at the naked eye, when absorbance value was greater than 0.1. According to this, none of the strains were able to grow at 0.90 aw and 15ºC, 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 0.98-0.99 aw.

At 25ºC, absorbance values statistically increased after 2-4 days (0.98-0.99 aw and 0.95 aw) or 10 days (0.90 aw) of incubation.

At 35ºC a statistically growth increase was observed after 2 days of incubation in the three culture media adjusted at 0.98-0.99 aw and 0.95 aw. At 0.90 aw, 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 15ºC than at 25ºC or 35ºC. The highest significant OTA values were obtained at 0.98-0.99 aw 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\textsubscript{w} and 15°C. The highest OTA concentration was recorded at 15°C after 10 days of incubation in CYB at 0.99 a\textsubscript{w}. It is worth to highlight that in those conditions of temperature and a\textsubscript{w}, all the strains produced detectable levels of OTA in the three culture media studied.

At 25°C and 0.98-0.99 a\textsubscript{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 0.98-0.99 a\textsubscript{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\textsubscript{w}, still some strains could produce detectable levels of OTA after 10 days of incubation.

Although taking into account all the results 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.

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 10^6 conidia/ml. The use of the portable photometer Densimat (BioMérieux) provides a substantial reduction in time 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 in solid culture media, A. carbonarius strains also showed optimal conditions range for OTA production narrower than that for growth when temperature, water activity or pH effects were evaluated (analyzed) (Esteban et al., 2004; 2005; 2006).
In agreement with our results, 15ºC or 15-20ºC 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 obtained. 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 15ºC, 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 15ºC, 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) reported 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 25ºC than at 15ºC or 35ºC. Most, if not all, current ecophysiological studies have been carried out with *A. carbonarius* strains isolated from grapes and derived products. Up 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 coffee Arabica produced more OTA at 20ºC than at 15ºC (Esteban et al., 2004). Kouadio et al. (2007) reported maximum OTA production at 0.99 a_w at a temperature range of 15-30ºC in one strain of *A. carbonarius* isolated from Robusta coffee beans. On irradiated coffee cherries, optimal conditions for OTA production was also reported at 25ºC and 0.99 a_w (Joosten et al., 2001). In the same way, on a green coffee-based medium the highest OTA production was obtained at 0.99a_w and 25ºC 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 temperature ranges of 35-40ºC and 17-27ºC respectively (Fig. 2b). The remaining *A.
carbonarius strains studied were isolated from areas with Mediterranean climate, with relatively mild winters and warm summers but reaching minimum temperatures of 4-18°C, lower 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 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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Figure captions

Fig. 1. Phylogenetic tree of *Aspergillus* section *Nigri* inferred from Neighbor-Joining analysis of partial calmodulin gene. Bootstrap values >70% in 1,000 replications are shown at nodes.

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).
Table 1.
List of *A. carbonarius*\(^a\) strains studied and their source and location.

<table>
<thead>
<tr>
<th>Strain number (^b)</th>
<th>Source, location</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-882</td>
<td>tomatoes, Morocco</td>
</tr>
<tr>
<td>A-884</td>
<td>coffee cherries, Thailand</td>
</tr>
<tr>
<td>A-1002</td>
<td>grapes, Spain</td>
</tr>
<tr>
<td>A-1004</td>
<td>grapes, Spain</td>
</tr>
<tr>
<td>A-1021</td>
<td>grapes, Spain</td>
</tr>
<tr>
<td>A-1625</td>
<td>grapes, Italy</td>
</tr>
<tr>
<td>A-1653</td>
<td>grapes, France</td>
</tr>
<tr>
<td>A-1687</td>
<td>grapes, Portugal</td>
</tr>
<tr>
<td>A-1749</td>
<td>grapes, Israel</td>
</tr>
<tr>
<td>A-1841</td>
<td>grapes, Greece</td>
</tr>
<tr>
<td>A-1995</td>
<td>grapes, Australia</td>
</tr>
<tr>
<td>A-2128</td>
<td>grapes, Spain</td>
</tr>
<tr>
<td>A-2275</td>
<td>grapes, Spain</td>
</tr>
<tr>
<td>A-2520</td>
<td>grapes, Spain</td>
</tr>
<tr>
<td>A-3615</td>
<td>raisins, Turkey</td>
</tr>
<tr>
<td>A-3905</td>
<td>raisins, Iran</td>
</tr>
</tbody>
</table>

\(^a\) Identification confirmed by sequencing of the calmodulin gene.

\(^b\) Culture Collection of the Veterinary Mycology Group, Universitat Autònoma de Barcelona, Spain.
Table 2.

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

<table>
<thead>
<tr>
<th></th>
<th>vs. EXP</th>
<th>vs. T (ºC)</th>
<th>vs. aw</th>
<th>vs. culture media</th>
<th>vs. strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS</td>
<td>0.078</td>
<td>0.000 (35º a &gt; 25º a &gt; 15º b)</td>
<td>0.000 (0.99 a &gt; 0.95 a &gt; 0.90 b)</td>
<td>0.000 (CYB a &gt; SGM b &gt; WGJ c)</td>
<td>0.999</td>
</tr>
<tr>
<td>OTA</td>
<td>0.257</td>
<td>0.000 (15º a &gt; 25º b &gt; 35º b)</td>
<td>0.000 (0.99 a &gt; 0.95 b &gt; 0.90 b)</td>
<td>0.000 (CYB a &gt; WGJ b &gt; SGM b)</td>
<td>0.265</td>
</tr>
</tbody>
</table>

Superscript values of variables with the same superscript are not significantly different (p > 0.05).

Abbreviations: EXP, experiment; T, temperature in ºC; aw, 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.

<table>
<thead>
<tr>
<th>culture media</th>
<th>days</th>
<th>T</th>
<th>15ºC</th>
<th>25ºC</th>
<th>35ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aw</td>
<td>0.99-0.98</td>
<td>0.95</td>
<td>0.90</td>
</tr>
<tr>
<td>CYB</td>
<td>1</td>
<td>0.0050 ± 0.0047</td>
<td>a 0.0009 ± 0.0084</td>
<td>a 0.00005 ± 0.0069</td>
<td>a 0.0467 ± 0.0203</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0106 ± 0.0075</td>
<td>a 0.0010 ± 0.0121</td>
<td>a 0.0016 ± 0.0056</td>
<td>b 0.7721 ± 0.3356</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.1478 ± 0.0597</td>
<td>a 0.0104 ± 0.0115</td>
<td>a 0.0036 ± 0.0034</td>
<td>c 2.8224 ± 0.1081</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.1620 ± 0.336</td>
<td>b 0.8526 ± 0.5574</td>
<td>b 0.0045 ± 0.0051</td>
<td>d 2.9535 ± 0.1252</td>
</tr>
<tr>
<td>SGM</td>
<td>1</td>
<td>0.00048 ± 0.0064</td>
<td>a 0.0017 ± 0.0093</td>
<td>a 0.0071 ± 0.0108</td>
<td>a 0.0028 ± 0.0175</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0035 ± 0.0081</td>
<td>a 0.0013 ± 0.0082</td>
<td>a 0.0069 ± 0.0105</td>
<td>a 0.0451 ± 0.0287</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.0349 ± 0.0186</td>
<td>b 0.0070 ± 0.0075</td>
<td>a 0.0124 ± 0.0092</td>
<td>b 0.5936 ± 0.3639</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.7549 ± 0.3014</td>
<td>c 0.2533 ± 0.1680</td>
<td>b 0.0105 ± 0.0084</td>
<td>b 1.0018 ± 0.4218</td>
</tr>
<tr>
<td>WGJ</td>
<td>1</td>
<td>0.0022 ± 0.0065</td>
<td>a 0.0023 ± 0.0058</td>
<td>a 0.0064 ± 0.0057</td>
<td>a 0.0491 ± 0.0150</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0191 ± 0.0110</td>
<td>a 0.0077 ± 0.0081</td>
<td>a 0.0054 ± 0.0053</td>
<td>a 0.1948 ± 0.0485</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.1242 ± 0.0279</td>
<td>c 0.0573 ± 0.0158</td>
<td>b 0.0065 ± 0.0067</td>
<td>c 0.3319 ± 0.0885</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.3095 ± 0.0628</td>
<td>d 0.1928 ± 0.0404</td>
<td>c 0.0265 ± 0.0089</td>
<td>d 0.4019 ± 0.1273</td>
</tr>
</tbody>
</table>

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 ºC; aw, water activity; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGJ, White grape juice.
Table 4.

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

<table>
<thead>
<tr>
<th>culture media</th>
<th>days</th>
<th>$a_w$</th>
<th>$0.99-0.98$</th>
<th>$0.95$</th>
<th>$0.90$</th>
<th>OTA (µg / ml) mean value ± δ (no. of OTA positive strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T 15ºC</td>
<td></td>
<td>0.99</td>
<td>0.95</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td></td>
<td>0.99-0.98</td>
<td>0.95</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.052 ± 0 *(1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.1965 ± 0.2893 *b *(8)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.2828 ± 0.5487 *(15)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.4784 ± 7.361 *b *(16)</td>
<td>0.6995 ± 1.30 *b *(14)</td>
<td>ND</td>
<td></td>
<td>0.2039 ± 0.2894 *b *(16)</td>
</tr>
<tr>
<td>T 25ºC</td>
<td></td>
<td>0.99</td>
<td>0.95</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.99-0.98</td>
<td>0.95</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.0762 ± 0.0532 *b *(7)</td>
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<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.0731 ± 0.0398 *c *(10)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.0775 ± 0.0285 *(15)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.1329 ± 0.0902 *b *(16)</td>
<td>0.0865 ± 0.0934 *b *(13)</td>
<td>ND</td>
<td></td>
<td>0.0758 ± 0.008 *(3)</td>
</tr>
<tr>
<td>T 35ºC</td>
<td></td>
<td>0.99</td>
<td>0.95</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.99-0.98</td>
<td>0.95</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.0724 ± 0.0315 *(15)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.0727 ± 0.027 *(15)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.0696 ± 0.0123 *(3)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.098 ± 0.0416 *b *(16)</td>
<td>0.0799 ± 0.0255 *b *(16)</td>
<td>ND</td>
<td></td>
<td>0.0735 ± 0.0343 *(15)</td>
</tr>
</tbody>
</table>

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 ºC; $a_w$, water activity; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGI, White grape juice.
A. carbonarius CBS 556.65\textsuperscript{T} (AJ964873)
A. sclerotiorum CBS 115572\textsuperscript{T} (EU163271)
A. ibericus CBS 121593 \textsuperscript{T} (AJ971805)
A. sclerotiacarbonarius CBS 121057 \textsuperscript{T} (EU159235)
A. brasiliensis CBS 101740 \textsuperscript{T} (AM295175)
A. niger CBS 554.65\textsuperscript{T} (JX500080)
A. lactofera CBS 101883\textsuperscript{T} (EU163270)
A. welwitschiae CBS 139.54\textsuperscript{T} (KC480196)
A. tubingensis CBS 134.48\textsuperscript{T} (AJ964876)
A. luchuensis CBS 205.80\textsuperscript{T} (JX500071)
A. piper CBS 112811\textsuperscript{T} (EU163267)
A. neoniger CBS 115656 \textsuperscript{T} (FJ491700)
A. eucalyptica CBS 122712 \textsuperscript{T} (EU482433)
A. floridica CBS 115574 \textsuperscript{T} (EU163268)
A. costaricaensis CBS 115574 \textsuperscript{T} (EU163268)
A. vadensis CBS 113365 \textsuperscript{T} (EU163269)
A. ellipticus CBS 482.65 \textsuperscript{T} (AM117809)
A. heteromorph CBS 117.55 \textsuperscript{T} (AM421461)
A. homomorph CBS 101889 \textsuperscript{T} (AM887685)
A. labrusci IBT 33586 \textsuperscript{T} (KT986008)
A. saccharolyticus CBS 127449 \textsuperscript{T} (HM853554)
A. indologenus CBS 114.60 \textsuperscript{T} (AM419750)
A. japonicus CBS 114.51 \textsuperscript{T} (AJ964875)
A. uvarum CBS 121591 \textsuperscript{T} (AM745755)
A. trinidadensis NRRL 62480 (HE984425)
A. aculeatus CBS 121060 \textsuperscript{T} (EU159241)
A. aculeatus CBS 172.66 \textsuperscript{T} (AJ964877)
A. floridica IHEM 21069 (HE818083)
A. aculeatus CBS 313.89 \textsuperscript{T} (FJ491695)
A. brunneviolaceus CBS 621.78 \textsuperscript{T} (EF661147)
Fig. 2

A-884 (in CYB)

A-1002 (in CYB)

A-2128 (in CYB)

Monthly maximum and minimum average temperatures in Mediterranean areas and Thailand

data obtained from https://weather-and-climate.com