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Growth and Ochratoxin A Production by *Aspergillus carbonarius* Isolated From Dried Figs In Aegean Region of Turkey Affected by Temperature and Water Activity

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Abstract

The impacts of temperature, water activity and their interactions on the growth rate and ochratoxin A (OTA) production by three *Aspergillus carbonarius* isolates obtained from dried figs in Aegean region of Turkey were investigated on Czapek yeast extract agar. The maximum specific radial growth rates at each set of conditions were obtained by using the primary model of Baranyi. Correlation coefficients were varied between 96% and 99%. Optimum growth of all isolates was at 30 °C and 0.97 a_w while optimum OTA production was observed at 15 – 20 °C and 0.97 - 0.99 a_w depending on the isolate. The assayed isolates showed varying abilities in growth rate and OTA production capacities as reported in the literature. The estimated optimum temperature and water activity values for growth and OTA production were in accordance with reported results for grape isolates. Temperature, water activity and their interactions significantly influenced the growth rate and ochratoxin A production by all tested isolates (P <0.05).

Keywords: Growth rate, ochratoxin A, dried figs, primary model of Baranyi.

1. INTRODUCTION

Ochratoxin A (OTA) is reported as a nephrotoxic mycotoxin which may contaminate various foods and feed products worldwide produced by some *Penicillium* and *Aspergillus* species [1]. Although, in *Penicillium* genus, *Penicillium verrucosum* and *Penicillium nordicum* are the two most important OTA producing species, the genus *Aspergillus* have more than twenty ochratoxigenic species [2]. In *Aspergillus* section *Nigri*, different studies have shown that *Aspergillus carbonarius* and

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Aspergillus niger are important sources of OTA in food commodities [1]. It was firstly reported by Abarca et al. [3] that *Aspergillus* section *Nigri* members are producers of OTA. *A. carbonarius* has high ochratoxigenic potential as 75-100% [4, 5]. *A. carbonarius* is considered as the major producer being responsible from OTA production in dried figs [6, 7], dried vine fruits and grapes [8-12].

Fig differs from other fruits with its fruit formation and properties. It has juicy and pulpy skin, and the cavity inside the fruit. Mould growth and mycotoxin production may occur on the outer surface or inside the cavity even if no damage occurs on the skin. Mycotoxin formation in fig fruits begins with the ripening. Apart from other fruits, figs are fully ripened and semi-dried right on the tree. Semi-dried figs remain on the tree until 30-50% moisture content. After they lose water, partially dry and shrivel, and the fig fruits naturally fall onto the ground/ special nets or collected from trees. The partially dried and shriveled figs are fully sun dried to approximately 20-22% moisture content [13].

Mold growth and OTA production are related to many environmental factors such as water activity, temperature, pH, substrate composition. In addition, the ability of *Aspergillus* species to develop in different climates and vegetation has gained worldwide significance. Therefore, it is important to determine the conditions that affect mold growth and toxin production in respect to food safety [14].

Baranyi model was previously reported as a well fitted model to predict bacterial growth at varying temperatures because of its dynamic nature [15]. This model was also selected as best primary model [16]. Baranyi model was also previously used to estimate the maximum growth rate and lag phase of *A. carbonarius* [17], *Aspergillus parasiticus* and *Aspergillus ochraceus* [18].

Although, temperature and water activity (a_w) were previously reported as the key environmental factors that influence both the rate of fungal spoilage and the production of mycotoxins [19], our previous study has investigated the effect of temperature on growth and OTA production of *A.* section *Nigri* obtained from Turkish dried figs, but only at constant a_w [7]. The aims of the present study were to describe the effects of temperature, water activity and their interactions on *in vitro* growth and OTA production by *Aspergillus carbonarius* isolated from dried figs obtained from Aegean region in Turkey on culture media.

2. MATERIAL AND METHOD

2.1. Fungal Isolates

Three ochratoxigenic isolates of *A. carbonarius* were selected to include different districts in Aegean Region and also according to their capacity to produce OTA. *A. carbonarius* isolates 5703D1, 3503X1 and 3904X4 were from Germencik-Aydın, Selcuk-Izmir and Soke-Aydın, respectively. All *A. carbonarius* isolates were previously screened for their OTA production abilities [20].

2.2. Chemicals and Media

Analytical, high performance liquid chromatography (HPLC) grade reagents and OTA standards were obtained from Merck (Darmstadt, Germany) and Sigma- Aldrich (Steinheim, Germany), respectively. Mould growth and OTA production were evaluated on Czapek yeast extract (CYA) agar. CYA was adjusted to five water activity values (0.85, 0.89, 0.93, 0.97 and 0.99) by addition of different amounts of glycerol. The final water activity (a_w) of the culture media was checked after solidification.

2.3. Inculation and Incubation

Each strain was grown on malt extract agar (MEA, Merck, Darmstadt, Germany) at 25 °C for 7 days. Spore suspensions were prepared in an aqueous solution of 0.05% Tween 80 and adjusted to 1-4 x 10^6 conidia/mL as determined by counting

chamber. Each CYA plate was centrally inoculated with 1 µL of the adjusted suspension. The plates were placed in closed polyethylene bags and incubated at eight different temperatures from 5 to 35 °C (at 5 °C intervals). The water activity of uninoculated plates incubated in similar conditions did not change during the experiment.

2.4. Growth Assessment

Mycelial growth was determined by measuring colony diameters along two perpendicular axes. The mean value of two diameters was used in calculations. Measurements were carried out for a maximum of 40 days. Estimates of the maximum specific radial growth rates (μ_{max}) were obtained by applying Baranyi's primary model by using an in-house Excel Add-in package "DMFit" (www.combase.cc) [21]. All the experiments were performed in quadruplicate.

The general form of Baranyi's primary model:

$$y = y_0 + \mu_{max} A - \ln\left\{1 + \frac{[\exp(\mu_{max} A) - 1]}{\exp(y_{max} - y_o)}\right\} \quad (1)$$

$$A = t + \left(\frac{1}{\mu_{max}}\right) ln[exp(-\mu_{max} t) + exp(-\mu_{max}\lambda) - exp(-\mu_{max} t - \mu_{max}\lambda)] \quad (2)$$

Where y is the colony radius or diameter (mm); y_0 is the initial colony radius or diameter (mm), usually zero; y_{max} is the maximum colony radius or diameter (mm) attained, asymptotic value; μ_{max} is the maximum specific growth rate (mm/day); λ is the lag period (day); and t is the time (day).

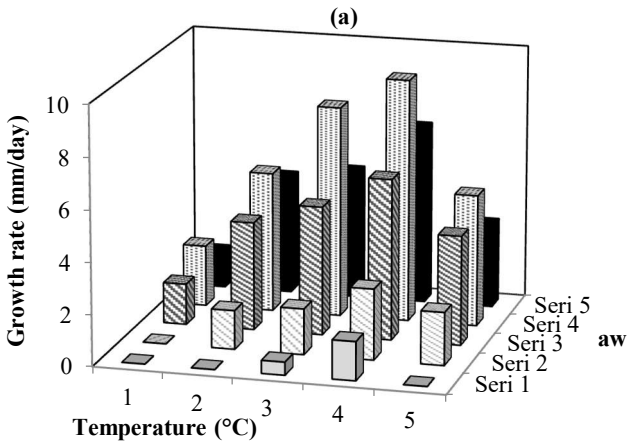
2.5. OTA Extraction and Analysis

OTA production was analyzed at each temperature and a_w assayed after 10 days of incubation. Extraction was carried out by using a previous method after slight modifications [22]. Three agar plugs were removed from the different parts of the colony, weighted, and vortexed with 0.5 mL of methanol. After 60 min, the extracts were filtered by using a centrifuge filter (Ultrafree®-MC, pore diameter of 0.45 µM, Merck Millipore, Billerica, MA, USA) at 10000 rpm and injected into HPLC.

The HPLC was an Agilent Technologies 1100 system equipped with a fluorescence detector set at an excitation wavelength of 333 nm and emission wavelength of 477 nm, quaternary pump, a vacuum degasser, the Rheodyne injector. The injection volume was 20 µL. Data were processed by Chemstation 3D software. The column was on Zorbax Eclipse XDB-C18 reversed phase column (Agilent, 150 mm×4.6 mm, 5 µm particle size). The separation was performed at 30 °C with 1 mL/min flow rate of mobile phase of acetonitrile–water–acetic acid (99:99:2, v/v/v) as mobile phase.

Recoveries were determined by analyzing spiked samples at 2.0 and 10.0 µg/kg of OTA. Samples were allowed to equilibrate for a night before extraction. The mean recoveries for spiked samples were 80% and 84%, respectively. The detection limit of the analysis was 0.1 ng OTA/g of medium.

2.6. Statistical Methods



The effect of temperature and a_w on growth rate and OTA production of isolates were statistically analyzed with SPSS Statistics 16.0 (Release 16.0.0) by analysis of variance (ANOVA). When analysis revealed statistically significant differences, comparison of means was conducted

by Duncan's Multiple Range Test. Statistical significance was judged at P <0.05 and P<0.0001.

3. RESULTS AND DISCUSSIONS

3.1. Water Activity and Temperature Effects on Radial Growth Rate

Statistical analysis of variance (ANOVA) showed that temperature, a_w, isolates, and their interactions significantly influenced on the growth rate of *A. carbonarius* (Table 1). Among the single factors tested, a_w had the greatest effect (F = 23,500). Growth of the *A. carbonarius* isolates in relation to a_w (0.89, 0.93, 0.97 and 0.99) and temperature treatments (15, 20, 25, 30 and 35 °C) are shown in Figure 1. Baranyi's model was used for the determination of the growth rate (mm/day) and lag phase (day) (data not shown) parameters. Correlation coefficients were varied between 96% and 99%.

Table 1. Variance analysis of the effects of water activity (a_w) and temperature (T) and their interactions on *in vitro* growth of three isolates of *A. carbonarius* on CYA.

Source of variation	DF[a]	MS[b]	F-value
T	4	218.18	23500*
a_w	4	333.94	35970*
Isolate	2	2340	252.09*
T × a_w	12	9.70	1045*
T × isolate	8	9.38	1010*
a_w × isolate	7	6.44	693.61*
T × a_w × isolate	23	2.83	304.58*

[a]DF: Degree of freedom
[b]MS: Mean square
*: Significant p <0.0001

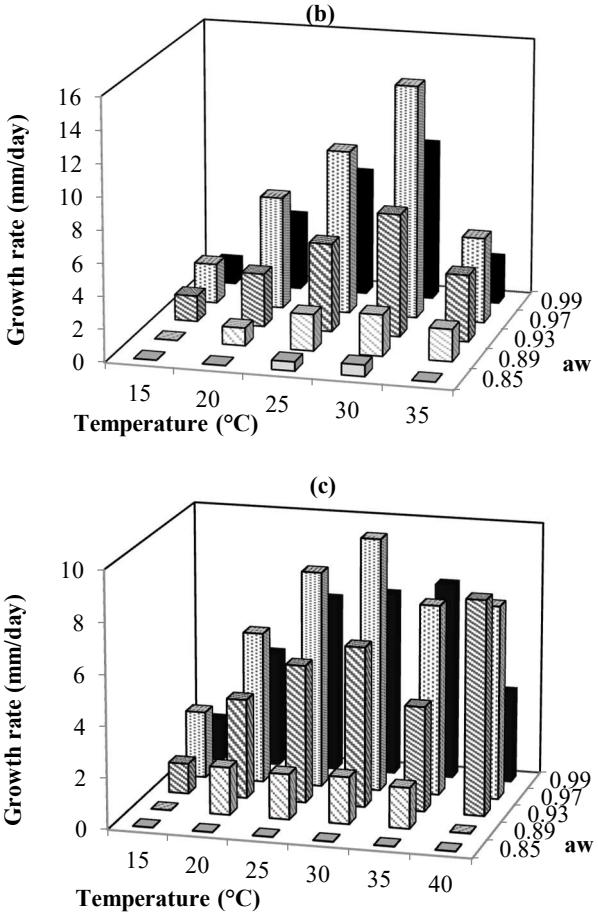


Figure 1. Effect of water activity and temperature on *in vitro* growth rates of three isolates of *A. carbonarius* on CYA; (a) *A. carbonarius* 3503X1, (b) *A. carbonarius* 3904X4, (c) *A. carbonarius* 5703D1.

The optimum growth of three *A. carbonarius* isolates on CYA was observed at 0.97 a_w and 30 °C. Similarly, other authors found optimum temperature for growth at 30 °C on CYA [23-25], dried grape extract medium [26] and synthetic nutrient medium (SNM) [27, 28]. For all a_w levels tested, maximum growth rates were observed at 30 °C and it was followed by 25 °C. Similar results were reported for isolates from Tunisian grapes [28] and Argentinian dried vine fruits [25].

For all the isolates and temperatures tested, growth rates increased with a_w reaching the optimum at 0.97 and decreasing at 0.99 a_w. The maximum radial growth rates achieved at 0.97 a_w and 30°C as 9.45, 14.33 and 9.92 mm/day for *A. carbonarius* 3503X1, 3904X4 and 5703D1, respectively. Similar growth rates with isolate 3904X4 were

reported for isolates from Tunisian grapes of about 16.46 mm/day at 30 °C and 0.99 a_w [28] and for isolates from Argentinian dried vine fruits of about 17.46 mm/day at 30 °C and 0.95 a_w [25].

On the other hand, lower growth rates were observed for other two isolates of dried figs. These values are also comparable with the growth rate of European isolates (10 mm/day at 30 °C and 0.995 a_w) and Turkish isolates (9.02 mm/day at 30 °C) which were detected by Belli et al. [27] and Mutlu-Ingok and Karbancioglu-Guler [7], respectively. These differences can be attributed to intraspecific and regional variability between isolates as suggested by other authors [25, 28].

None of the isolates grew at 5 and 10 °C for all a_w levels tested. These results are comparable with Tassou et al. [29], who reported no growth of two *A. carbonarius* strains from Greek wine grapes at 10 °C. No growth below 15 °C was also reported by Mitchell et al. [30].

In this study, minimum a_w for growth was 0.85 at levels of temperature 25 and 30 °C and 0.93 at 15 °C. Esteban et al. [24], found lower minimum water activity values of 0.82 for growth on yeast extract sucrose (YES) agar. On the other hand, similar minimum water activity level for growth of *A. carbonarius* were reported on CYA [24, 25], SNM [31] and dried grape extract medium [26]. Similarly, Romero et al. [25] observed that *A. carbonarius* did not grow below 0.85 a_w and at 0.85 a_w growth only determined at 25 and 30 °C. Intraspecific differences in relation to a_w and temperature were also found as reported by Garcia-Cela et al. [12] for *A. carbonarius* from Northeast Spain and Pardo et al. [32], *for A. ochraceus*. *A. carbonarius* 3503X1 and 3904X4 grew at 25 and 30 °C at 0.85 a_w and at 35 °C and 0.89 a_w but isolate 5703D1 did not grow at all.

3.2. Water Activity and Temperature Effects on OTA Production

The ANOVA for OTA *production* by three *A. carbonarius* isolates showed that all single factors (a_w, temperature, and isolate) and their two- and three-way interactions were highly significant at P <0.0001 (Table 2). Unlike the growth, temperature had the greatest effect on OTA production among the single factors tested.

Table 2. Variance analysis of the effects of water activity (aw) and temperature (T) and their interactions on *in vitro* OTA production of three isolates of A. carbonarius on CYA.

Source of variation	DF[a]	MS[b]	F-value
T	4	224.03	273.09*
a_w	4	85.38	104.07*
Isolate	2	32.91	40.11*
T × a_w	12	49.92	60.85*
T × isolate	8	38.22	46.59*
a_w × isolate	7	21.47	26.17*
T × a_w × isolate	23	23.19	28.26*

[a]DF: Degree of freedom,
[b]MS: Mean square
*: Significant p <0.0001

OTA production of the *A. carbonarius* isolates in relation to temperature and a_w treatments and statistical significances are shown in Table 3. The isolates tested showed variation in OTA production properties at different conditions.

The highest levels of OTA were produced at 15-20 °C and 0.97-0.99 a_w depending on the isolate. In accordance with our results, the maximum OTA production was detected at 0.98 a_w and 15 °C on CYA [24], at 0.98 a_w and 15-20 °C on SNM [31], at 0.99 a_w and 15 °C on semisynthetic grape culture medium (SGCM) [33] and at 0.98 a_w and 15 °C on maize kernels [34].

Table 3. Effect of temperature and a_w on OTA production of *Aspergillus carbonarius* isolates.

Isolate	a_w	OTA concentration (μg/g)				
		15 °C	20 °C	25 °C	30 °C	35 °C
A. carbonarius 3503X1	0.85	NG	NG	ND	ND	NG
	0.89	NG	0.012^{A}	ND	ND	ND
	0.93	ND	0.205^{bA}	0.009^{aA}	0.005^{aA}	0.014^{aA}
	0.97	4.060^{cB}	5.342^{dC}	0.133^{aB}	0.174^{aB}	1.530^{bC}
	0.99	1.874^{cA}	2.062^{cB}	0.105^{aB}	1.060^{bC}	0.947^{bB}
A. carbonarius 3904X4	0.85	NG	NG	ND	ND	NG
	0.89	NG	0.023^{A}	ND	ND	ND
	0.93	ND	0.638^{bA}	0.021^{aA}	0.004^{aA}	0.005^{aA}
	0.97	18.847^{cB}	6.837^{bB}	0.149^{aB}	0.101^{aB}	0.377^{aB}
	0.99	4.046^{bA}	9.142^{cC}	0.495^{aC}	0.437^{aC}	0.549^{aC}
A. carbonarius 5703D1	0.85	NG	NG	NG	NG	NG
	0.89	NG	ND	0.007^{A}	ND	ND
	0.93	0.024^{bA}	0.121^{cA}	0.004^{aA}	0.005^{aA}	0.002^{aA}
	0.97	10.983^{bB}	0.113^{aA}	0.069^{aB}	0.132^{aB}	0.150^{aB}
	0.99	14.129^{bB}	3.866^{aB}	$0.601a^{C}$	1.063^{aC}	$1.129a^{C}$

NG: No growth
ND: Not detected (Limit of detection: 0.1 ng/g)
$^{a\,b,c,d}$: Values with same superscript within each strain and water activity are not significantly different ($p > 0.05$)
A,B,C: Values with same superscript within each strain and incubation temperature are not significantly different ($p > 0.05$)

Moreover, different researchers also reported that optimum a_w and/or temperature for OTA production was dependent on the strain [27, 30]. Belli et al. [27], detected maximum amounts of OTA at 0.99 a_w for the most of isolates, at 0.95 for 2 out of the 8 isolates. On the other hand, Lasram et al. [28] reported there were no variation in optimum a_w levels (0.99) between all 8 Tunisian isolates after 10 days of incubation on SNM.

For all tested temperatures, maximum OTA levels were observed at 0.97 or 0.99 a_w. OTA was produced by all isolates at 0.93 a_w and 20 °C, lower amounts of OTA was detected at higher temperatures of this a_w. Very little or no OTA was produced at 0.89 a_w and below. No growth was observed at 5 and 10 °C after 10 days of incubation and consequently, no OTA was detected at these temperature levels.

The average minimum and maximum daily temperatures of the production area in Aegean region (Aydın and Izmir) during ripening, harvesting and sun drying periods (from June to September) are 16.6 - 22.3 °C and 29.1 - 36.0 °C, respectively [35]. According to our results, night temperature may be suitable for OTA production rather than day temperature. On the other hand, day temperature may be more suitable for mould growth and OTA production may also occur at low level especially in the initial step of drying.

The isolates assayed also showed varying abilities to produce OTA. There were significant differences (P <0.05) among OTA levels at the different conditions (Table 3). The maximum OTA production was determined as 5.342, 14.129 and 18.847 µg/g CYA for *A. carbonarius* 3503X1, 5703D1 and 3904X4, respectively. In accordance with our results, four *A. carbonarius* strains tested by Romero et al. [36] differed in maximum OTA yield on CYA.

Different OTA levels produced by *A. carbonarius* isolates have been reported in the literature. An Australian *A. carbonarius* isolate produced 21 µg/g on SNM at 0.95 a_w and 15 °C after 15 days [37]. Higher OTA levels have been determined on CYA by Esteban et al. [38] and Esteban et al. [24]. The maximum OTA production was 484.52 µg/g CYA at 20 °C after 10 days of incubation [38]. On the other hand, the isolates tested by Mitchell et al. [30] produced far less OTA (0.6-0.7 µg/g) on SNM.

Similar to this study, some studies pointed out that temperature and a_w range for OTA production were more restrictive than for growth and isolates can require different optimum conditions for growth and OTA production [24, 39]. Optimum growth of all isolates were at 30 °C and 0.97 a_w while optimum OTA production was observed at 15 – 20 °C and 0.97 - 0.99 a_w depending on the isolate. There was no correlation between growth rates and OTA production by *A. carbonarius*. For *A. carbonarius* 3904X4, growth rates were 2.4 mm/day at 0.97 a_w and 15 °C and 2.6 mm/day at 0.89 a_w and 30 °C, although OTA amounts accumulated were different in those conditions (18.847 µg/g and 0.01 µg/g, respectively). Moreover, OTA levels varied from 0.101 µg/g to 0.174 µg/g at the optimum growth conditions (30 °C and 0.97 a_w). As mentioned by Leong et al. [37] isolates with the most rapid growth rate did not necessarily produce the highest levels of OTA. Lasram et al. [28] also stated that no correlation could be established between colony size and OTA production of *A. carbonarius*.

Although there are no official regulations in Turkey regarding the maximum allowable OTA levels in dried figs, maximum levels for dried fruits like raisins (10 µg/kg) have been set [40]. Additionally, the European Union set maximum permitted level of OTA in dried fruits at 10 mg/kg [41]. Based on the findings of this study, OTA level in dried figs may exceed the maximum limits. Moreover, higher levels in dried figs contaminated with ochratoxin A than limits were also stated in the literature [42, 43].

4. CONCLUSIONS

Our results indicated that *A. carbonarius* isolated from Turkish dried figs may grow and produce OTA between 15 and 35 °C. The growth and OTA production were not affected from a_w, temperature and their interactions in the same way. The optimum temperature for OTA production was lower than optimum temperature for the growth of *A. carbonarius*, although both were optimum at high a_w levels. Minimum a_w level found as 0.85 for growth of *A. carbonarius* isolates and 0.89 for OTA production. The optimum temperature and a_w for growth and OTA production of Turkish dried fig isolates were similar with results for isolates from Australian, Tunisian, Argentinian and European grapes.

While night temperature was more suitable for OTA production, higher growth rates were obtained near the day temperature. Temperature fluctuations between day and night during harvesting and sun drying in the orchards may be crucial for OTA production. Growth and ochratoxin A production characteristics of *A. carbonarius* might be used for developing of safe drying and storage methods to control mould growth and to minimize the OTA content in dried figs after validation of results on dried figs. Moreover, these data may be used for developing predictive models of mould growth and OTA accumulation in Turkish dried figs. However, the isolates assayed showed varying abilities in growth rate and OTA production capacities as reported in the literature, further studies are needed to develop and validate models.

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