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Influence of pH and Incubation Time on Ochratoxin A Production by *Aspergillus carbonarius* in Culture Media

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ABSTRACT

The effect of pH (2 to 10) and temperature (15 and 30°C) on growth and production of ochratoxin A (OTA) of six strains of *Aspergillus carbonarius* was studied in two culture media: Czapek yeast autolysate agar and yeast extract sucrose agar. Isolates were selected by their different source and different reported ability to produce OTA. Regardless of the initial pH or the temperature tested, Czapek yeast autolysate agar has been shown to be the best culture medium for OTA production by *A. carbonarius.* In this medium, OTA was produced from pH 2 to 10 at the two incubation temperatures tested. The results obtained show the ability of *A. carbonarius* to not only grow but also produce OTA over a wide pH range at high or low temperatures. This may help explain why this species is considered the main OTA source in some substrata.

Ochratoxin A (OTA) is a mycotoxin of growing importance in the last decade. The International Agency for Research on Cancer has rated OTA as a possible human carcinogen (category 2B). Although the role of OTA in human disease is still speculative, its acute nephrotoxicity, immunosuppressive actions, and teratogenic effects in animal models, coupled with its ability to be carried through the food chain, merit concern *(10, 18).* The European Commission has established limits for OTA of 10 μg/kg in dried vine fruit, 5 μg/kg in raw cereals, 3 μg/kg in cereal-derived products, 5 μg/kg for roasted coffee, 10 μg/kg for instant coffee, 2 μg/kg for wine, grape juice, and grape must, and 0.5 μg/kg for foods for infants and young children. By 30 June 2006, the European Commission will review these limits and consider the inclusion of maximum levels for OTA in green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices, and liquorice *(15–17).*

Although currently *Aspergillus ochraceus* and *Penicillium verrucosum* are considered typical OTA-producing species, they are unlikely to be significant sources of OTA in some substrates. Since the first description of OTA production by two species belonging to *Aspergillus* section *Nigri* (*Aspergillus niger* var. *niger (4)* and *Aspergillus carbonarius (20)*), the significance of these species as mycotoxin-producing fungi has increased *(1).* Recent surveys have shown that *A. carbonarius* is the main OTA source in wine, grapes, dried vine fruits, and probably coffee *(3).*

To prevent OTA contamination, it is necessary to determine the influence of environmental parameters on mycotoxin production. The effect of water activity *(9, 22),* temperature *(19, 24),* and incubation time *(32, 35)* on OTA production by some isolates of *A. carbonarius* have been recently reported. In one such study *(19),* we stated that *A. carbonarius* strains produced OTA from 15 to 35°C, achieving the highest levels in Czapek yeast extract agar (CYA) at 15 or 20°C. Similar results have been reported in synthetic grape juice medium *(24).* Because nothing is known about the effect of pH, the aim of this study is to determine the influence of pH on OTA production by *A. carbonarius* at two incubation temperatures (15 and 30°C) during a period of 30 days.

MATERIALS AND METHODS

Strains and preparation of inoculum. Six isolates of *A. carbonarius* were used in this study. Isolates were selected by their different source and different reported ability to produce OTA. The origin and OTA properties of the six strains studied are given in Table 1.

The strains were grown on malt extract agar *(28)* for 7 days at 25°C. Conidia suspensions of each isolate were prepared in an aqueous solution of 0.05% Tween 80. After filtering through sterile cheesecloth, they were adjusted to approximately 10^6 to 10^7/ml of conidia as determined by a counting chamber.

Growth media and incubation conditions. OTA production was determined in two basal culture media: yeast extract sucrose (YES) agar and CYA *(28).* The pH of the culture media was varied from 2 to 10. Adjustment of pH was performed before autoclaving by adding HCl (1 and 10 N) and NaOH (1 N). The pH value was measured using a pHmeter GLP21 (Crison Strumenti S.p.A., Carpi, Italy). Each plate was point inoculated with 1 μl of the adjusted suspension. Plates were incubated at two different temperatures: 15 and 30°C. Each assay was performed in duplicate.

OTA production and quantification. OTA production was analyzed after 5, 10, 15, 20, and 30 days of incubation at each pH value and temperature assayed following a previously described high-pressure liquid chromatography (HPLC) screening method *(11).* On each sampling occasion, three agar plugs were

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TABLE 1. Aspergillus carbonarius *strains used in this study*

Strain (source)[a]	OTA production reported (reference)[b]
A. carbonarius NRRL 67 (Brazil)	+ *(32)*
A. carbonarius CBS 127.49 (coffee)	+ *(22)*
A. carbonarius A-941 (grapes, CCFVB)	+ *(13)*
A. carbonarius M325 (apples, supplied by HMLJ Joosten)	− *(22)*
A. carbonarius CBS 110.49 (air)	+ *(22)*
A. carbonarius A-1082 (raisins, CCFVB)	− *(2)*

[a] CCFVB, Culture Collection of Veterinary Faculty of Barcelona, Bellaterra (Barcelona), Spain; CBS, Centralbalbureau voor Schimmelcultures, Utrecht, The Netherlands; and NRRL, Northern Agricultural Research Service Culture Collection, Peoria, Ill.
[b] +, positive; −, negative.

removed from different points of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. OTA detection and quantification were performed with a Waters LCM1 chromatograph (Waters Cromatografia, Barcelona, Spain) with a Waters 2475 fluorescence detector (excitation wavelength, 330 nm; emission wavelength, 460 nm) and with a C18 Spherisorb S5 ODS2 column (250 by 4.6 mm). Twenty microliters of each extract was applied. The mobile phase, with a flow rate of 1 ml/min, consisted of the following linear gradient: acetonitrile, 57%; water, 41%; and acetic acid, 2% *(8)*. The extracts with the same retention time as OTA (approximately 6.8 min) were considered positive. Confirmation was made through derivatization of OTA in its methyl-ester *(21)*. The detection limit of the extraction procedure and the HPLC technique was 0.02 ng of OTA, and the quantification limit of the HPLC technique with the extraction procedure was 0.05 μg/g for this mycotoxin.

Data analysis. Data obtained were analyzed statistically by means of one-way analysis of variance test and Student's *t* test. All statistical analyses were performed using SPSS software (version 10.0; SPSS Inc, Chicago, Ill.).

RESULTS AND DISCUSSION

All the studied strains grew in CYA and YES media adjusted from pH 2 to 10 and incubated at 15 and 30°C with only one exception: the strain CBS 110.49 did not grow in CYA medium adjusted at pH 10 when the incubation was performed at 15°C. The growth pH range for *A. carbonarius* has not been previously reported. Nevertheless, as expected, our results show a broad similarity to *A. niger* with established pH limits of 1.5 to 9.8 *(26)*.

Two of the six strains tested (CBS 110.49 and A-1082) did not produce detectable levels of OTA at any of the experimental conditions assayed. The strain CBS 110.49 was reported as a very weak OTA-producing strain, using coffee cherries as substrate *(22)*. The OTA-negative strain A-1082, isolated from dried vine fruits in a previous study *(2)*, is now under study, because its morphological and genetic characteristics differ from the remaining *A. carbonarius* strains studied *(7, 14)*.

In CYA medium, OTA was produced from pH 2 to 10 at the two incubation temperatures tested. In YES medium, the pH range for OTA production was sometimes narrower. Thus, at 15°C OTA production began at pH 3. At 30°C, the pH ranges were 2 to 10 (strains A-941 and M325), 3 to 9 (strain NRRL 67), and 3 to 10 (strain CBS 127.49).

Although the pH range for OTA production was similar in both media, the concentration produced in each medium was statistically different. The mean OTA concentration produced by the positive isolates in CYA medium (16.0 μg/g at 30°C and 19.2 μg/g at 15°C) was significantly higher ($P < 0.01$) than in YES medium (1.63 μg/g at 30°C and 2.69 μg/g at 15°C). So, regardless of the initial pH or the temperature tested, CYA has been shown to be the best culture medium for OTA production by *A. carbonarius* in agreement with previous reports *(11, 19)*.

OTA concentrations produced in CYA medium at each pH level tested and incubation time are given in Tables 2 (incubation temperature of 30°C) and 3 (incubation temperature of 15°C). Table 4 summarizes the maximum OTA level produced under the different assay conditions studied.

When CYA plates were incubated at 30°C, all the strains produced detectable levels of OTA after only five days of incubation, except for the strain NRRL 67 at pH 5 (Table 2). The optimum pH range for OTA production proved different for each strain. The strain A-941 isolated from grapes achieved a significantly higher OTA level at pH 2. In the same way, the strain M325 isolated from apples, which is a weak OTA-producing strain, produced the highest level after 5 days of incubation at pH 2. This strain was reported as OTA negative when coffee cherries were used as substrate *(22)*. The strain CBS 127.49 produced maximum OTA concentration in the pH range 4 to 7, and the strain NRRL 67 produced similar OTA levels at all the pH ranges tested.

When the plates were incubated at 15°C, OTA was detected after 10, 15, or 20 days of incubation, depending on the pH level tested (Table 3). The strains showed a similar trend to that observed at 30°C, but the highest OTA levels were obtained generally at a higher pH range (5 to 7). Nevertheless, the strain A-941 achieved the highest OTA level at pH 2 but after 30 days of incubation.

Incubation temperature also played an important role in the OTA concentration produced at the different pH levels tested. Although 30°C has been reported as an optimal growth temperature for *A. carbonarius (9, 23, 24)*, the optimum temperature range for OTA production in CYA medium has been established at 15 to 20°C *(19)*. When the plates were incubated at 15°C, OTA concentrations produced by the strains NRRL 67 and M-325 were significantly higher ($P < 0.01$). The strain A-941 also produced higher amounts of OTA at 15°C in the pH range of 3 to 10, but at pH 2 OTA concentration produced at 30°C was higher. The strain CBS 127.49 produced more OTA when incubated at 30°C ($P < 0.05$).

Our results show that *A. carbonarius* isolates are able to produce OTA at a wide range of pH values (2 to 10) on CYA medium, whereas other OTA-producing species such as *A. ochraceus* did not produce OTA outside the range of pH 5.5 to 8.5 on modified Adye-Mateles synthetic medium *(25)*. *P. verrucosum* was reported to produce OTA from pH

TABLE 2. *OTA concentration produced by the four OTA-producing strains of* A. carbonarius *in CYA medium at 30°C at each pH (2 to 10) and incubation time tested*

		OTA concentration (μg/g)[a]								
Strain	Days	2	3	4	5	6	7	8	9	10
NRRL 67	5	7.79 ± 2.23 AB	11.49 ± 3.80 B	1.66 ± 0 CD	ND	7.24 ± 0.41 ABC	8.43 ± 2.27 AB	5.63 ± 0.47 ACD	4.36 ± 0.28 ACD	2.66 ± 1.16 ACD
	10	11.21 ± 1.02 A	7.47 ± 0.32 BC	7.63 ± 0.20 BC	8.77 ± 2.19 AB	9.19 ± 1.03 AB	5.79 ± 0.63 BC	7.20 ± 0.88 BC	4.20 ± 0.59 C	4.34 ± 0.84 C
	15	4.16 ± 2.75 A	8.47 ± 1.78 A	10.54 ± 0.95 A	6.62 ± 0.10 A	8.29 ± 0.66 A	7.20 ± 2.50 A	5.97 ± 0.38 A	6.72 ± 5.16A	3.66 ± 1.17 A
	20	2.31 ± 0.22 A	8.11 ± 0.10 C	9.34 ± 3.68 C	16.52 ± 0.46 D	7.72 ± 0.07 BC	7.27 ± 1.69 BC	5.76 ± 1.40 ABC	7.29 ± 0.42 BC	2.92 ± 0.23 AB
	30	3.21 ± 2.55 A	6.16 ± 0.91 A	13.62 ± 2.93 B	20.45 ± 0.51 C	7.70 ± 1.18 A	9.34 ± 3.27 AB	6.64 ± 0.79 A	7.63 ± 0.88 A	5.08 ± 1.65 A
CBS 127.49	5	20.34 ± 2.06 A	47.73 ± 15.70 B	121.22 ± 11.22 C	18.29 ± 2.63 A	17.32 ± 2.98 A	10.08 ± 1.53 A	8.14 ± 0.43 A	8.51 ± 3.27 A	14.18 ± 3.25 A
	10	21.32 ± 1.48 A	36.63 ± 11.78 C	28.78 ± 5.94 BC	17.32 ± 0.94 AB	25.23 ± 6.87 ABC	15.21 ± 0.47 AB	12.02 ± 0.37 AB	6.68 ± 0.58 A	14.12 ± 3.46 AB
	15	14.85 ± 5.54 A	34.80 ± 8.56 AB	42.74 ± 8.87 B	42.99 ± 16.60 B	18.34 ± 2.94 A	16.57 ± 4.04 A	14.68 ± 1.25 A	10.72 ± 2.35 A	8.75 ± 0.67 A
	20	7.45 ± 3.04 A	27.50 ± 10.24 A	78.95 ± 31.90 B	29.72 ± 5.61 A	26.89 ± 0.59 A	35.60 ± 10.48 A	12.00 ± 1.06 A	19.02 ± 9.07 A	9.02 ± 0.52 A
	30	2.61 ± 2.51 A	22.42 ± 20.72 AB	44.62 ± 13.02 BC	61.13 ± 0.20 C	24.34 ± 2.79 AB	204.52 ± 13.19 D	17.01 ± 2.33 AB	25.22 ± 3.51 AB	12.68 ± 1.66 AB
A-941	5	158.74 ± 7.06 A	7.13 ± 1.23 B	1.85 ± 0.30 B	3.04 ± 1.61 B	1.21 ± 0.10 B	1.59 ± 0.33 B	3.11 ± 0.96 B	3.00 ± 0.98 B	0.63 ± 0.01 B
	10	230.33 ± 2.35 A	6.55 ± 2.35 B	3.51 ± 0.18 C	1.60 ± 0.16 C	1.18 ± 0.23 C	1.12 ± 0.33 C	1.96 ± 0.44 C	1.15 ± 0.02 C	0.74 ± 0.06 C
	15	289.99 ± 65.53 A	4.86 ± 1.14 B	2.62 ± 0.23 B	1.72 ± 0.11 B	1.62 ± 0.18 B	1.07 ± 0.06 B	2.84 ± 1.58 B	0.93 ± 0.27B	0.41 ± 0.03 B
	20	157.62 ± 32.17 A	4.83 ± 1.47 B	2.13 ± 0.98 B	4.87 ± 1.10 B	1.38 ± 0.22 B	1.79 ± 0.91 B	2.40 ± 0.83 B	1.55 ± 0.10 B	0.52 ± 0.04 B
	30	151.24 ± 2.75 A	5.20 ± 0.47 BC	2.74 ± 0 BCD	5.55 ± 0.22 B	1.46 ± 0.54 D	2.09 ± 0.39 CD	2.85 ± 0.95 CD	1.10 ± 0.12 D	0.78 ± 0.27 D
M325	5	25.35 ± 6.54 A	3.18 ± 0.10 B	4.32 ± 1.17 B	4.16 ± 0.01 B	1.09 ± 0.10 B	1.43 ± 0.33 B	2.53 ± 1.04 B	1.37 ± 0.57 B	0.58 ± 0.22 B
	10	7.25 ± 0.48 A	4.89 ± 1.44 AB	7.22 ± 3.18 A	2.74 ± 0.01 B	1.39 ± 0.43 B	1.02 ± 0.34 B	1.19 ± 0.08 B	1.04 ± 0.23 B	1.12 ± 0 B
	15	4.23 ± 0.61 A	6.00 ± 1.00 B	5.20 ± 0.12 AB	4.84 ± 0.13 AB	1.83 ± 0.76 C	1.07 ± 0.03 C	1.69 ± 0.15 C	0.82 ± 0.23 C	0.85 ± 0.28 C
	20	2.69 ± 1.22 AB	4.10 ± 0.30 B	7.37 ± 0.42 C	3.45 ± 0.47 B	1.54 ± 0.50 AD	1.50 ± 0.16 AD	2.81 ± 0.36 AB	1.04 ± 0.11 D	0.64 ± 0.20 D
	30	1.67 ± 1.07 A	6.59 ± 2.64 AB	8.42 ± 1.94 B	14.92 ± 4.04 C	1.70 ± 0.54 A	3.13 ± 0.80 A	2.13 ± 0.49 A	0.98 ± 0.37 A	1.36 ± 0.18 A

[a] Values are mean ± 1 standard error. Values with the same letter within each strain and incubation time are not significantly different ($P < 0.05$). ND, not detected (limit of detection, 0.05 μg/g).

TABLE 3. *OTA concentration produced by the four OTA-producing strains of* A. carbonarius *in CYA medium at 15°C at each pH (2 to 10) and incubation time tested*

Strain	Days	OTA concentration (μg/g)[a]								
		2	3	4	5	6	7	8	9	10
NRRL 67	5	ND	ND	ND	ND	ND	ND	NG	NG	NG
	10	ND	7.44 ± 0.36 A	14.38 ± 10.08 AB	21.40 ± 2.55 B	23.59 ± 4.31 B	5.38 ± 1.36 A	ND	ND	NG
	15	0.63 ± 0.69 A	22.85 ± 0.42 AB	21.52 ± 4.73 AB	45.23 ± 9.97 B	29.50 ± 3.34 B	24.51 ± 18.41 AB	24.12 ± 5.42 AB	ND	ND
	20	7.97 ± 2.07 A	23.31 ± 9.02 B	39.63 ± 2.98 B	35.30 ± 11.45 B	36.86 ± 0.23 B	41.85 ± 6.98 B	35.69 ± 1.18 B	4.30 ± 0.86 A	0.25 ± 0.12 A
	30	12.27 ± 0.30 A	19.65 ± 2.83 A	23.87 ± 3.13 AB	29.97 ± 3.92 AB	43.02 ± 9.88 AB	51.62 ± 21.19 B	30.25 ± 0.39 AB	28.65 ± 0.95 AB	26.51 ± 0.08 AB
CBS 127.49	5	ND	ND	ND	ND	ND	ND	ND	ND	NG
	10	ND	11.58 ± 6.87 A	12.58 ± 0.16 A	25.46 ± 1.77 BC	28.03 ± 5.10 C	16.65 ± 0.78 AB	18.81 ± 4.71 ABC	ND	ND
	15	0.22 ± 0.09 A	14.19 ± 2.82 AB	15.71 ± 4.86 AB	21.03 ± 6.59 AB	59.39 ± 30.61 AB	68.88 ± 28.96 B	43.57 ± 28.41 AB	7.99 ± 6.87 AB	1.76 ± 1.11 A
	20	2.51 ± 0.01 A	13.07 ± 4.20 A	17.10 ± 3.92 A	8.05 ± 5.49 A	38.57 ± 4.31 C	32.47 ± 9.81 BC	21.56 ± 2.47 AB	14.12 ± 4.12 A	13.12 ± 7.13 A
	30	9.42 ± 1.80 AB	10.57 ± 1.92 AB	18.79 ± 4.94 ABC	4.25 ± 4.36 A	32.19 ± 6.28 BC	39.13 ± 16.87C	20.76 ± 1.41 ABC	15.29 ± 1.22 AB	27.14 ± 0.94 ABC
A-941	5	ND	ND	ND	ND	ND	ND	ND	ND	NG
	10	ND	37.63 ± 2.90 A	17.65 ± 0.55 AB	30.53 ± 10.20 A	20.71 ± 4.08 A	39.60 ± 13.61 A	25.00 ± 0.35 A	ND	NG
	15	23.67 ± 3.28 A	51.14 ± 10.63 B	45.29 ± 0.31 B	53.84 ± 5.49 B	22.59 ± 0.94 A	45.90 ± 0.15 B	21.42 ± 1.57 A	2.30 ± 0.27 C	0.25 ± 0.17 C
	20	41.4 ± 3.14 A	39.69 ± 1.96 AB	36.91 ± 9.02 AB	32.47 ± 2.75 AB	21.37 ± 2.59 CD	28.64 ± 1.34 BC	14.85 ± 2.24 D	15.46 ± 0.04 D	15.07 ± 1.39 D
	30	101.57 ± 5.49 A	35.25 ± 16.09 B	34.69 ± 7.45 B	28.31 ± 2.35 B	23.14 ± 2.28 B	39.30 ± 6.60 B	21.07 ± 0.59 B	13.76 ± 0.16 B	20.54 ± 2.11 B
M325	5	ND	ND	ND	ND	ND	ND	ND	ND	NG
	10	ND	13.06 ± 4.28 AB	22.84 ± 0.04 BC	41.07 ± 6.28 C	34.97 ± 0.78 C	33.02 ± 16.87 C	ND	ND	NG
	15	31.24 ± 9.01 AB	38.02 ± 5.10 BC	13.32 ± 1.81 AB	46.90 ± 11.38 C	47.46 ± 9.8 C	33.30 ± 7.06 AB	25.82 ± 4.78 AB	10.65 ± 2.16 A	NG
	20	31.16 ± 1.22 A	14.43 ± 3.14 B	24.89 ± 5.06 A	31.64 ± 2.35 A	24.42 ± 0.78 A	29.89 ± 2.63 A	27.78 ± 1.37 A	12.60 ± 0.86 B	NG
	30	18.18 ± 0.67 AB	17.90 ± 4.43 AB	16.79 ± 1.53 AB	31.08 ± 7.85 BC	28.31 ± 3.13 BC	34.69 ± 10.59 C	16.10 ± 0.78 AB	14.10 ± 0.78 AB	5.39 ± 0.42 A

[a] Values are mean ± 1 standard error. Values with the same letter within each strain and incubation time are not significantly different ($P < 0.05$). ND, not detected (limit of detection, 0.05 μg/g); NG, no growth.

TABLE 4. *Maximum OTA concentration produced by each strain under the different assay conditions studied*

Strain	Maximum OTA production at 15°C				Maximum OTA production at 30°C			
	Culture media	pH	Days	Concentration (μg/g)	Culture media	pH	Days	Concentration (μg/g)
NRRL 67	CYA	7	30	51.6	CYA	5	30	20.5
	YES	6	20	9.4	YES	7	5	2.6
CBS 127.49	CYA	7	15	68.9	CYA	7	30	204.5
	YES	6	15	19.2	YES	9	5	16.2
A-941	CYA	2	30	101.6	CYA	2	15	290.0
	YES	4	30	5.4	YES	8	30	5.9
M-325	CYA	6	15	47.5	CYA	2	5	25.3
	YES	6	30	26.8	YES	4	30	7.5

4 to 8, with the greatest level at pH 5.6 *(27)* when growing on a bread analogue.

Strong evidence of the contribution of *A. carbonarius* to the OTA contamination of wine during a microvinification trial has been recently reported *(13)*. In the field, this species was recovered along the different developmental stages of berries, but the highest level of isolation was achieved at harvesting *(5, 6, 29)*. The pH of grapes increases during ripening, achieving a final pH range of 3.0 to 4.0 *(30)*, and dried vine fruits reported pH ranges of 3.8 to 4.1 *(34)*.

A. carbonarius has been isolated from coffee beans by several authors, but the time of invasion is not known. In Brazil, this species was isolated in samples from drying yard and storage on farm but not from the fruits obtained from trees *(31)*. The available data indicate that OTA is likely to be formed between the time of coffee cherry harvest and its arrival in the factory *(12)*. At that time, the pH of unfermented coffee beans can range from 5.4 to 6.4 *(33)*.

Our results show the ability of *A. carbonarius* to not only grow but also produce OTA over a wide pH range at high or low temperatures. This may help explain why this species is considered the main OTA source in some substrata. Further studies on the influence of different environmental conditions on growth and OTA production by *A. carbonarius* will lead to better knowledge of its ecology and will provide us with potential tools to reduce the risk of OTA contamination in foods.

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