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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

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Summary. *Aspergillus carbonarius* colonizes grapes and its derived products and produces ochratoxin A (OTA). In previous studies we screened 107 strains of *A. carbonarius* isolated from grapes for production of OTA, and we selected three high OTA-producing strains for this study (AC05, AC06, and AC07). The effect of different values of three conditions [temperature 15, 25, 30, 35°C; water activity (a_w) 0.98, 0.95, 0.90, 0.88; and pH 4.0, 7.0, 9.0, 10.0] on *A. carbonarius* growth and OTA production was examined. *A. carbonarius* AC07 produced higher levels of OTA than AC06 and AC05 at all the variables tested. At 30°C the strains of *A. carbonarius* produced more OTA than at the other temperatures. A water activity of a_w 0.98 produced the greatest mycelial growth and OTA accumulation for all three *A. carbonarius* strains. A pH of 4.0 produced the highest levels of OTA in all the strains. No growth was seen at a_w 0.88 or at pH 10.0 in any strain, except AC07, which grew at pH 10.0. The optimal conditions for growth and production of OTA by *A. carbonarius* strains were 30°C, a_w 0.98 and pH 4.0. When all the three factors (a_w, pH, T) were combined, a_w 0.98, pH 4.00 and 30°C produced the highest levels of OTA in all the strains. Maximum amounts of OTA were found 7–9 days after inoculation in all the strains. Temperature, water activity, and pH had a great impact on OTA production by *A. carbonarius* and these factors should be taken into account when developing management practices for future research programmes.

Key words: ecology, grapevine, HPLC, mycotoxin, wine.

Introduction

Black aspergilli are among the main ochratoxin A (OTA)-producing species encountered on different food matrices in warm countries (Kapetanakou *et al.,* 2009). OTA has been reported on foods such as cereals, wine, grapes, cocoa, coffee, spices and dried fruits (JEFCA, 2001; O'Brien and Dietrich, 2005). OTA is nephrotoxic, hepatotoxic, genotoxic, teratogenic and immunotoxic to animals and humans, and its carcinogenicity is well-established (Castegnaro *et al.,* 1998). It is one of the factors involved in causing Balkan endemic nephropathy and tumours of the human urinary tract (Radic *et al.,* 1997). The International Agency for Research on Cancer has classified OTA as a possible carcinogen to humans (group 2B; IARC, 1993). The contamination of grapes and their by-products with OTA has become a big health problem when these products are consumed by human beings (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000; Spadaro *et al.*, 2010). *Aspergillus* section *Nigri*, and in particular *A. carbonarius*, play a central role in producing OTA in grapes (Battilani and Pietri, 2002; Abarca *et al.*, 2003). Further reports from various parts of the world have shown that high levels of OTA are produced by different isolates of *A. carbonarius* on a variety of liquid or agar media (Cabañes *et al.*, 2002; Bellí *et al.*, 2005).

Fungal growth is strongly affected by a number of conditions, including pH (Esteban *et al.,* 2006), water activity (a_w) and temperature (Romero *et al.*,

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2007). These factors are not only important in fungal growth, but also in metabolite secretion. Interestingly, several researches found a strong correlation between the growth of OTA producing aspergilli (*A. ochraceus* and *A. niger*) and certain ecological conditions (Pardo *et al*., 2004a, b; Bellí *et al*., 2004a). Researchers from various countries have also studied the effect of water activity and temperature on the growth and OTA production of *A. carbonarius* strains isolated from grapes (Bellí *et al*., 2004b, Mitchell *et al*., 2004; Leong *et al*., 2006; Romero *et al*., 2007). However, only very few and scattered reports have examined the impact of ecological conditions on the growth and toxin production of Italian isolates of *A. carbonarius*. Information concerning the factors that cause production of OTA by *A. carbonarius* isolates is essential to develop realistic forecasting systems predicting the risk of colonization by *A. carbonarius* on grapes, and levels of OTA production. The main objective of the present study was to determine the effect of water activity, pH and temperature on the growth and OTA production of three strains of *A. carbonarius* (AC05, AC06, and AC07) isolated from grapevines in Liguria (northern Italy) and selected for high OTA production.

Materials and methods

Fungal cultures and selection of media

The three strains of *A. carbonarius* (AC05, AC06 and AC07) were isolated from grape berries in Liguria (Northern Italy) and identified by using a polyphasic approach (Oliveri *et al.*, 2008) based on ITS sequencing, calmodulin sequencing, and PCR-RFLP (Spadaro *et al.*, 2009). The ITS sequences of the three strains were deposited at the National Centre for Biotechnology Information (NCBI) GenBank, New York, NY, USA with the following accession numbers: GQ468223, GQ468224, and GQ468225. The strains were tested for their capacity to produce OTA on two agar media (Potato dextrose agar [PDA, 39 g L^{-1}, Merck, Darmstadt, Germany], and Yeast extract sucrose agar [YES-agar, 30 g L^{-1} yeast extract, 200 g L^{-1} sucrose, 0.5 g L^{-1} magnesium sulphate, and 20 g L^{-1} agar]), to select the best medium for further study.

Inoculum preparation

Inocula were prepared by growing each strain on PDA at 25°C for 7 days, and spore suspensions were prepared by adding 10 mL of sterile distilled water containing 0.05% Tween 80 to each Petri dish, and by scratching the colony surface with a sterile spatula. The conidial suspension was filtered through four layers of sterile cheese-cloth and brought to a final concentration of 1×10^6 conidia mL^{-1} using a haemocytometer. PDA and YES-agar plates were centrally point inoculated with 10 μL of the spore suspension and incubated at 25±1°C for 9 days. Five replications were done for each strain and each medium, and the experiment was carried out three times.

Effect of ecological conditions on the production of OTA by *A. carbonarius*

YES-agar media was selected to study the effect of the ecological conditions on the growth and OTA production by the *A. carbonarius* strains. To study the effect of temperature, Petri dishes containing YES-agar were centrally inoculated with 10 μL of the spore suspension (1×10^6 conidia mL^{-1}) of each of the three strains and incubated at 15, 25, 30 and 35°C. YES-agar had an initial a_w of 0.98, which was modified to a_w 0.95, 0.90 and 0.88 by adding glycerol, as reported by Pardo *et al*. (2005). YES-agar with different pH (4.0, 7.0, 9.0, and 10.0) were prepared by adding HCl (1 N) and NaOH (1 N). The plates were then inoculated with *A. carbonarius* as above and incubated at 30°C. Mycelial growth was determined by measuring colony diameters along two perpendicular axes, 3, 5, 7, 9 and 11 days after the inoculation. Five replications were done for each treatment and strain, and the experiment was carried out three times.

Experimental design comprising a_w, temperature and pH

A fully randomized factorial design was run in triplicate on the *A. carbonarius* strains to study the effect of temperature, a_w and pH on OTA production. The experiment consisted of 12 treatments: 4 temperatures, 4 water activities, and 4 pH levels. YES-agar was prepared at four water activities (a_w 0.98, 0.95, 0.90 and 0.88), and each a_w-modified YES-agar was further adjusted to four pH values (4.00, 7.00, 9.00, and 10.00). The *A. carbonarius* strains were centrally point inoculated with 10 μL of the adjusted spore suspension (1×10^6 conidia mL^{-1}) on YES-agar plates with the different a_w and pH values, and the plates were incubated at four temperatures (15, 25, 30, and 35°C). OTA was extracted 9 days after inoculation. The experiment was carried out twice.

The effect of a_w, pH, temperature, and their interactions were examined by multivariate analysis using SPSS software Release 17.0 (SPSS Inc, Chicago, IL). The interactions were analysed with a Duncan's multiple range test.

OTA extraction from agar media and HPLC analysis

OTA was extracted following the method of Bragulat *et al*. (2001). At 3, 5, 7, 9, and 11 days after incubation, three agar plugs (diameter 5 mm) were removed from the inner, middle and outer area of each colony. The plugs were weighed and placed into 3 mL vials. Then, methanol (1 mL) was added to the vials and they were shaken for 5 s (Autovortex SA6, Redhill, UK). After being left without shaking for 60 min, the extracts were shaken again, filtered through a 0.2 μm cellulose membrane (SPARTAN-Whatman) and stored at 4°C in HPLC vials for analysis.

OTA was analyzed following the method of Bragulat *et al*. (2001) in a HPLC Agilent series 1100 consisting of a degasser, an autosampler, a quaternary pump, a thermostat column and a fluorimeter. An analytical column RP-18 (150 mm × 4.6 mm i.d., 5 μm) with a pre-column was used. The mobile phase, eluting at 1 mL min^{-1}, consisted of an isocratic mixture of acetonitrile:water:acetic acid (45:45:10) run for 18 min. One hundred μL of the sample was injected into the HPLC column; the retention time of OTA was 6.15 min. The amount of OTA in the final solution was determined using a calibration graph of concentration versus peak area, and expressed as ng mL^{-1}, achieved by injecting 100 μL of standard solution of OTA (Sigma Chemical Co., St Louis, MO, USA) into the HPLC column. The standard solutions had concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 μg L^{-1} of OTA.

Recovery was determined on blank YES-agar medium spiked with three concentrations of OTA (1.0, 2.0 and 10.0 ng mL^{-1}). Each test was performed six times and the median recovery value at each OTA concentration was 90.6, 91.8 and 92.4% respectively. The repeatability, measured as reproducibility relative standard deviation (RSD_r), was 2.64, 2.71 and 2.82% respectively. The detection limit of the analysis was 0.01 ng OTA g^{-1} of YES-agar medium. The high value of the regression coefficient ($R^2 \geq 0.99$) obtained indicated a good linearity of the analytical response.

Results and discussion

Selection of media

The study examined the effect of some ecological conditions on the growth of strains of *A. carbonarius*, the main fungal species causing OTA accumulation in grapes and grape derived products. Three strains of *A. carbonarius* isolated from vineyards in Liguria (northern Italy) were tested for their ochratoxigenicity on two agar media. The *A. carbonarius* strains produced higher levels of OTA on YES-agar (ranging from 1.7 to 2.9 μg g^{-1}) than on PDA (0.5–1.0 μg g^{-1}). AC07 produced more OTA on YES-agar (2.9 μg g^{-1}) than AC05 and AC06 (Figure 1). The *A. carbonarius* strains produced less OTA on PDA, so that the YES-agar was chosen to study

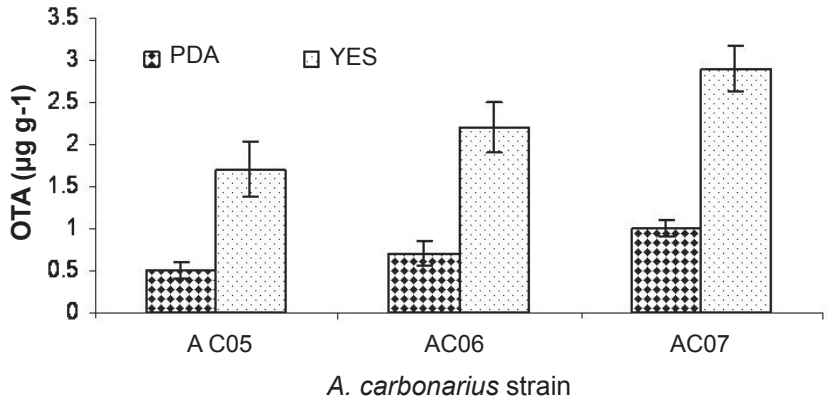

Figure 1. OTA production by *A. carbonarius* strains in two agar media after 9 days of incubation at 25°C.

OTA production. The results of the current study are consistent with O'Callaghan *et al*. (2006), who found a lower production of OTA by *A. ochraceus* on PDB. Al-Julaif (2003), examining optimal media for OTA production by *Eurotium* spp., found that less OTA was produced on PDA than on other media. This author had so chosen YES-agar to study the effect of the different ecological conditions on the fungus.

Effect of temperature on OTA production by *A. carbonarius* strains

The growth rate and the rate of OTA production by the *A. carbonarius* strains on YES-agar differed at different temperatures. *A. carbonarius* strains were able to grow at all temperatures tested, the strains of could grow starting from the lowest to the highest temperatures tested (from 15 to 35°C). The optimum temperature for both growth and OTA production was 30°C. Of the three strains, AC07 grew fastest at all the temperatures. However, the rate of OTA production was not related to the growth rate of the strain. Production of OTA for all strains peaked 9 days after inoculation. The highest levels of OTA were found after growth at 30°C, followed by growth at 25°C and 35°C. Conversely, OTA production after growth at 15°C was lower with all strains (Table 1). Bellí *et al*. (2004b) reported that 7 days of incubation were generally sufficient for OTA, but that some isolates required up to 14 days to reach their maximum OTA level. In our study, the greatest amounts of OTA were found at an earlier growth stage (after 7–9 days of incubation), showing that *in vitro* OTA was produced more rapidly. If the same rapid rate also occurred *in vivo*, these isolates would constitute a higher risk factor for OTA contamination in the vineyard. Of the three strains, AC07 produced the highest levels of OTA, ranging from 0.27 to 1.95 $\mu g\ g^{-1}$ at all temperatures tested. The other two strains produced lower levels of OTA, ranging from 0.18 to 1.42 $\mu g\ g^{-1}$. At 15°C, mycelial growth of the three strains was not seen until day 5 after inoculation. In YES-agar OTA levels increased for up to 9 days after incubation, and decreased after that (Table 1). The level of OTA in the medium is linked to the rates of OTA production and degradation. After day 9, the natural or fungal degradation of OTA probably began to be greater than its production, which was reduced by lower amounts of nutrients in the medium (Varga *et al.,* 2002). Similar findings were reported by Bellí *et al*. (2004a) for strains of *A. carbonarius* and *A. niger* isolated from Spanish grapes. Several researchers reported that the optimum temperature for *A. carbonarius* growth was 35°C, while no growth was seen at <15°C or >45°C (Battilani, *et al.,* 2004; Bellí *et al.,* 2004a; Mitchell *et al.,* 2004). Similar studies reported that significant amounts of OTA were produced after 5 days of incubation, but that at 25°C maximum OTA levels were not reached until after 7 to 14 days of incubation (Bellí *et al.,* 2004b; Esteban *et al.,* 2004). The differences found in the present study may be attributed to intra-specific and regional variations between strains: in northern Italy the contamination levels of OTA produced by black aspergilli are quite low (Spadaro *et al.,* 2006; Brera *et al.,* 2008).

Effect of a_w on OTA production by *A. carbonarius* strains

The growth rate and the amount of OTA produced by the *A. carbonarius* strains on YES-agar differed at different water activities at a given inspection date (Table 2). AC07 grew faster than the other strains at all the a_w tested. None of the strains grew at a_w 0.88. However, the amount of OTA produced varied according to the growth rate. OTA production fell when the a_w was lowered from 0.98 to 0.90 at all inspection dates. Of the three strains, AC07 produced most OTA, ranging from 0.27 to 3.33 $\mu g\ g^{-1}$, irrespective of the a_w (Table 2). OTA production of all strains peaked at a_w 0.98 and 0.95 after 9 days of incubation, and then gradually decreased. A high water activity was the optimal factor producing maximum OTA levels, while OTA decreased when water activity was lower (Bellí *et al.,* 2007; Kapetanakou *et al.,* 2009). Similarly, Mitchell *et al*. (2004) reported that growth of *A. carbonarius* increased when a_w was increased from 0.96 to 0.98 at 25–30°C. These authors also reported that a_w 0.83 and 0.87 strongly reduced mycelial growth and OTA production by *A. carbonarius* on a synthetic grape juice medium. In the present study, mycelial growth and OTA production by the *A. carbonarius* strains isolated from grapes were significantly influenced by a_w and by their interactions *in vitro* on agar media. Although the OTA production and the growth rates of all strains followed a similar pattern, the actual growth rates and levels of OTA produced varied extensively.

Table 1. Mycelial growth and production of OTA by three *A. carbonarius* strains on YES (yeast extract sucrose) agar at different temperature regimes.

Strain	No. days	15°C		25°C		30°C		35°C	
		Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]
A. carbonarius AC05	3	NG[b]	NT[b]	2.87±0.40	0.19±0.01	3.27±0.68	0.21±0.02	2.73±0.06	0.18±0.03
	5	1.1±0.36[c]	0.18±0.02	3.9±0.20	0.39±0.01	4.27±0.21	0.39±0.01	3.03±0.15	0.22±0.02
	7	1.53±0.51	0.23±0.02	5.27±0.21	0.59±0.01	5.57±0.23	0.59±0.01	4.5±1.11	0.49±0.01
	9	2.6±0.95	0.31±0.02	7.43±0.38	1.23±0.21	8.1±0.10	1.40±0.10	6.5±0.50	1.17±0.15
	11	3.33±0.59	0.29±0.01	8.67±0.29	1.20±0.17	9±0.00	1.10±0.01	6.9±0.10	1.03±0.06
A. carbonarius AC06	3	NG	NT	3.1±0.10	0.22±0.03	3.43±0.67	0.29±0.03	2.9±0.10	0.21±0.02
	5	1.2±0.10	0.20±0.01	4.23±0.25	0.37±0.03	4.53±0.55	0.40±0.02	3.87±0.35	0.30±0.02
	7	1.83±0.31	0.25±0.04	6.23±0.31	0.62±0.03	6.33±0.15	0.67±0.03	4.83±0.15	0.02±0.01
	9	2.73±0.40	0.32±0.02	8.27±0.21	1.23±0.21	8.43±0.21	1.42±0.10	6.9±0.10	1.30±0.10
	11	3.57±0.50	0.28±0.03	8.83±0.29	1.17±0.15	8.83±0.29	1.17±0.06	7.07±0.12	1.17±0.12
A. carbonarius AC07	3	NG	NT	3.47±0.25	0.24±0.03	3.93±0.38	0.28±0.03	3.07±0.15	0.23±0.01
	5	1.8±0.26	0.27±0.03	4.73±0.12	0.41±0.02	5±0.20	0.41±0.01	4.03±0.15	0.32±0.02
	7	2.13±0.32	0.30±0.01	6.17±0.12	0.63±0.03	6.4±0.10	0.79±0.02	5.27±0.38	0.61±0.03
	9	3±0.10	0.36±0.02	8.8±0.26	1.40±0.10	9±0.00	1.95±0.01	7.17±0.76	1.33±0.06
	11	4.03±0.15	0.30±0.01	9±0.00	1.13±0.06	9±0.00	1.23±0.06	7.7±0.26	1.07±0.12

[a] Values expressed as μg g^{-1} YES-agar medium.
[b] NG, no growth; NT, no toxin
[c] ±, Standard deviation.

Effect of pH on OTA production by *A. carbonarius* strains

The effect of different pH levels (4.0, 7.0, 9.0 and 10.0) on OTA production by *A. carbonarius* strains was investigated. Growth rates and OTA production levels differed between strains at different pHs (Table 3). AC07 grew faster than the other strains at all pH values. The highest levels of OTA were produced by all strains at pH 4.0, followed by pH 7.0 and pH 9.0. AC07 produced the highest amount of OTA at pH 4.0, ranging from 0.43 to 3.13 μg g^{-1} depending on the inspection date inoculation. Growth rates and the amount of OTA produced fell with increasing pH. At pH 10.0, AC05 and AC06 did not grow, while AC07 grew less and did not produce any OTA (Table 3). The greater production of OTA at low pH values could be related to the low pH of the ripened grapes, which ranges between 3.0 and 4.0 (Splittstoesser, 1987; US Food and Drug Administration, 2005). Other strains of *A. carbonarius* produce OTA at a wider pH range (Téren *et al.,* 1996; Abarca *et al.,* 2003; Battilani *et al.,* 2003).

Combined effect of a_w, temperature and pH

The study considered the combined impact of a_w, pH, and temperature on the production of OTA by three ochratoxigenic strains of *A. carbonarius*. To evaluate this combined effect, samples were collected after 9 days of incubation to measure the OTA level, because all the experiments carried out showed the

Table 2. Mycelial growth and production of OTA by three *A. carbonarius* strains on YES (yeast extract sucrose) agar at 30°C and at different water activity levels.

Strain	No. days	aw 0.98		aw 0.95		aw 0.90		aw 0.88	
		Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]
A. carbonarius AC05	3	2.77±0.31[c]	0.17±0.06	3.1±0.17	0.1±0.00	1±0	0.1±0.00	NG[b]	NT[b]
	5	3.77±0.31	0.57±0.15	3.77±0.25	0.27±0.06	1.67±0.21	0.17±0.06	NG	NT
	7	5±0.26	1.1±0.17	4.5±0.5	0.87±0.06	2±0	0.57±0.12	NG	NT
	9	7.37±0.32	2.17±0.29	5±0.00	1.17±0.29	2.77±0.25	0.73±0.15	NG	NT
	11	8.67±0.29	1.9±0.10	6.1±0.36	1.07±0.31	3.83±0.29	0.47±0.15	NG	NT
A. carbonarius AC06	3	3±0.10	0.27±0.06	3.5±0.00	0.17±0.06	1.17±0.29	0.13±0.00	NG	NT
	5	4.1±0.32	0.67±0.15	3.83±0.29	0.43±0.06	1.77±0.25	0.37±0.06	NG	NT
	7	6.17±0.31	1.3±0.26	4.63±0.23	1±0.10	2.07±0.12	0.67±0.12	NG	NT
	9	8.1±0.10	2.67±0.29	5.33±0.29	1.4±0.10	2.83±0.29	0.83±0.00	NG	NT
	11	8.8±0.29	2.23±0.25	6±0.00	1.23±0.25	3.93±0.12	0.53±0.06	NG	NT
A. carbonarius AC07	3	3.27±0.25	0.53±0.06	3.33±0.29	0.4±0.00	1.27±0.21	0.27±0.12	NG	NT
	5	4.67±0.15	1.1±0.17	3.9±0.17	0.83±0.06	1.67±0.49	0.53±0.06	NG	NT
	7	5.83±0.23	1.63±0.15	4.77±0.15	1.13±0.06	2.13±0.32	0.97±0.06	NG	NT
	9	8.8±0.26	3.33±0.29	5.3±0.29	1.5±0.29	2.67±0.29	1.17±0.15	NG	NT
	11	9±0.00	2.67±0.29	6.17±0.29	1.33±0.31	3.83±0.29	1.03±0.06	NG	NT

[a], [b], [c] See Table 1.

Table 3. Mycelial growth and production of OTA by three *A. carbonarius* strains on YES (yeast extract sucrose) agar at different pH levels.

Strain	No. days	pH 4.0		pH 7.0		pH 9.0		pH 10.0	
		Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]	Growth (cm)	OTA (μg g^{-1})[a]
A. carbonarius AC05	3	2.9±0.10[c]	0.23±0.06	3.5±0.45	0.20±0.00	1.0±0.00	0.13±0.06	NG[b]	NT[b]
	5	4.13±0.32	0.37±0.06	4.17±0.29	0.33±0.00	1.67±0.21	0.33±0.06	NG	NT
	7	4.77±0.25	1.03±0.06	5.3±0.10	1.03±0.06	2.0±0.00	0.73±0.15	NG	NT
	9	8.2±0.26	2.93±0.12	7.53±0.25	2.17±0.29	2.77±0.25	1.03±0.06	NG	NT
	11	9±0.00	2.13±0.15	9±0.00	2.07±0.06	3.83±0.29	0.83±0.06	NG	NT
A. carbonarius AC06	3	3.1±0.10	0.33±0.06	3.7±0.25	0.27±0.06	1.0±0.00	0.17±0.12	NG	NT
	5	4.13±0.32	0.57±0.06	4.8±0.29	0.43±0.06	1.77±0.25	0.50±0.10	NG	NT
	7	5.1±0.21	1.33±0.29	6.1±0.10	1.20±0.10	2.07±0.12	0.90±0.10	NG	NT
	9	8.5±0.00	3±0.10	8.5±0.00	2.23±0.21	2.83±0.29	1.07±0.12	NG	NT
	11	9±0.00	2.5±0.50	9.0±0.00	2.03±0.06	3.93±0.12	0.97±0.12	NG	NT
A. carbonarius AC07	3	3.5±0.35	0.43±0.06	3.3±0.21	0.30±0.00	1.07±0.06	0.13±0.06	NG	NT
	5	4.5±0.40	0.70±0.10	4.6±0.46	0.53±0.06	1.8±0.26	0.43±0.06	NG	NT
	7	6.33±0.45	1.70±0.20	6.1±0.31	1.40±0.10	2.3±0.35	1.13±0.12	1.2±0.29	NT
	9	8.8±0.29	3.13±0.12	8.3±0.26	2.57±0.06	2.67±0.29	1.63±0.23	1.5±0.29	NT
	11	9±0.00	2.97±0.06	9±0.00	2.20±0.10	3.83±0.29	1.07±0.06	2.5±0.29	NT

[a], [b], [c] See Table 1.

Table 4. Analysis of variance of the effect of temperature, pH and water activity on the production of OTA by three strains of *A. carbonarius* grown on YES (yeast extract sucrose) agar for 9 days.

Factor	*A. carbonarius* (AC05)				*A. carbonarius* (AC06)				*A. carbonarius* (AC07)			
	DF[a]	SS[a]	MS[a]	F[a]	DF	SS	MS	F	DF	SS	MS	F
Intercept	1	48.88	48.88	2508.75**	1	56.77	56.77	2174.28**	1	69.12	69.12	6077.04**
T	3	11.75	3.91	201.07**	3	12.22	4.07	156.08**	3	15.85	5.28	464.65**
pH	3	16.67	5.55	285.25**	3	19.33	6.44	246.81**	3	23.465	7.82	687.69**
a_w	3	22.75	7.58	389.30**	3	26.06	8.68	332.73**	3	30.40	10.13	891.06**
T × pH	9	4.01	0.44	22.88**	9	4.17	0.46	17.76**	9	5.43	0.60	53.11**
T × a_w	9	5.64	0.62	32.19**	9	5.40	0.60	22.99**	9	6.73	0.74	65.77**
pH × a_w	9	7.73	0.86	44.13**	9	8.89	0.98	37.84**	9	10.30	1.14	100.68**
T × pH × a_w	27	1.95	0.07	3.71*	27	1.87	0.06	2.65*	27	2.34	0.08	7.63*
Error	128	2.49	0.01	- NS	128	3.34	0.02	- NS	128	1.45	0.01	NS
Total	192	121.92	-	- NS	192	138.09	-	- NS	192	165.12	-	NS

[a] DF, degree of freedom; SS, sum of squares; MS, mean square; F, f-value.
** Significant (P<0.001); NS, not significant.

highest amounts of OTA at this time. Statistical analysis of variance (ANOVA) showed that all the single factors (T, pH, and a_w), as well as their two- and three-way interactions (T × pH, T × a_w, pH × a_w, and T × pH × a_w) had a significant effect on OTA production (Table 4). The effect of these factors and their interactions on the strains differed between strains. AC07 showed the highest accumulation of OTA regardless of a_w, pH or T, while AC06 and AC05 showed significantly lower OTA levels. Moreover, of all combinations of a_w, temperatures and pH, the highest levels of OTA (AC07: 2.23 μg g^{-1}; AC06: 2.03 μg g^{-1}; and AC05: 1.93 μg g^{-1}) were seen at a_w 0.98, pH 4, and 30°C. Similarly, Kapetanakou *et al.* (2009) found that a combination of a_w 0.99, pH 3.9 and 25–30°C produced the highest OTA levels in strains of *A. ochraceus* and *A. carbonarius*. Joosten *et al.* (2001) reported that *A. carbonarius* on coffee berries produced the highest level of OTA at 30°C and a_w 0.99, and that OTA production was strongly inhibited below 0.94 a_w. Pardo *et al.* (2005) found that water activity levels between 0.95 and 0.99 were optimal for germination and mycelial growth of *Aspergillus* strains and these levels were within the range of the a_w of grapes in the vineyard at harvest. Similar results were obtained by Bellí *et al.* (2004a; 2005) when they evaluated most of the *Aspergillus* section *Nigri* for growth under different water activity and temperature conditions. In general, for all strains, the effect of a_w on OTA production was greater than the effect of pH or temperature.

Conclusions

The study examined the combined effect of temperature, a_w and pH on OTA production by three strains of *A. carbonarius* isolated in Italy. Of all the combinations of a_w, pH and temperature tested, the highest levels of OTA in *A. carbonarius* were produced at a_w 0.98, pH 4, and 30°C. *A. carbonarius* can grow and produce OTA at wide ranges of temperature, pH and a_w, and this confirms that these environmental conditions strongly influence OTA production, both in the field and in the first hours after harvest. Further investigations are required to determine whether *A. carbonarius* also produces OTA when growing on a natural substrate such as grapes. Over long incubation periods, OTA levels may undergo a decrease due to natural degradation or a biodegradation caused by the *Aspergillus* that produced the OTA in the first phase (Bellí *et al.,* 2004b; Kapetanakou *et al.*, 2009).

The data obtained advance our understanding of the ecology of *A. carbonarius*. Indeed, the selection of highly ochratoxigenic strains from Italian vineyards could help to develop of management strategies to reduce OTA contamination on grapevine.

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