STUDY III

Ecophysiological characterization of *Aspergillus carbonarius*, *Aspergillus tubingensis* and *Aspergillus niger* isolated from grapes in Spanish vineyards

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ABSTRACT

The aim of this study was to evaluate the diversity of black aspergilli isolated from berries from different agroclimatic regions of Spain. Growth characterization (in terms of temperature and water activity requirements) of *A. carbonarius, A. tubingensis* and *A. niger* was carried out on synthetic grape medium. *A. tubingensis* and *A. niger* showed higher maximum temperatures for growth (>45 ºC versus 40-42 ºC), and lower minimum a_W requeriments (0.83 a_W versus 0.87 a_W) than *A. carbonarius*. No differences in growth boundaries due to their geographical origin were found within *Aspergillus niger aggregate* isolates. Conversely, *A. carbonarius* isolates from the hotter and drier region grew and produced OTA at lower a_W than other isolates. However, little genetic diversity in *A. carbonarius* was observed for the microsatellites tested and the same sequence of β-tubulin gene was observed; therefore intraspecific variability did not correlate with the geographycal origin or the isolates neither with they ability to produce OTA. Climatic change prediction appoints to drier and hotter climatic scenarios where *A. tubingensis* and *A. niger* could be even more prevalent over *A. carbonarius*, since they are better adapted to extreme high temperature and drier conditions.

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1. INTRODUCTION

Fungi classified within *Aspergillus* section *Nigri* (the black aspergilli) are ubiquitous saprophytes present in soils around the world, particularly in tropical and subtropical regions (Pitt and Hocking, 1997). Several field surveys have been published dealing with epidemiology, ecology and distribution of black aspergilli occurring on grapes worldwide (Bellí et al., 2004b, 2004c; Khoury et al., 2008; Lasram et al., 2012b; Leong et al., 2004; Magnoli et al., 2003; Rosa et al., 2002; Sage et al., 2002; Serra et al., 2005). These studies have clarified that the main ochratoxigenic black *Aspergillus* species occurring on grapes are the biseriate *Aspergillus carbonarius* and the so-called *Aspergillus niger aggregate*. In general, the reported percentages of ochratoxin A (OTA) producing strains in *A. carbonarius* are higher than those reported for members of the *Aspergillus niger aggregate* (Battilani et al., 2006; Bau et al., 2005; Guzev et al., 2006; Medina et al., 2005). By contrast, there is a higher incidence of species belonging to the *Aspergillus niger aggregate*, mainly *A. niger* and *A. tubingensis,* although other species have also been reported (Perrone et al., 2008, 2007b). In general*, A. niger* aggregate species predominate, followed *by A. carbonarius* and uniseriate species (Battilani et al., 2006). Species distribution resulting from several publications in 2006-2012 are: *A. tubingensis* (15.2-95.7%), *A. niger* (4.3-84.4%), and *A. carbonarius* (7.6-46.9%). Several studies have described separately the different species in the *Aspergillus niger aggregate* found in grapes, however, no general pattern can be derived from the existing reports (Table 1). A recent study has settled that *A. tubingensis* is the main species belonging to *Aspergillus niger aggregate* followed by *A. awamori*, and *A. niger* in dried vine fruits (Susca et al., 2013).

There is a controversy regarding the percentage of OTA producing strains within *A. carbonarius* isolated from grapes, Somma et al. (2012) concluded that close to 100% were OTA producers, based in literature published before 2006. Nonetheless, studies based on *A. carbonarius* identified by molecular techniques showed percentages under a 50% of producers (Martínez-Culebras and Ramón, 2007; Spadaro et al., 2012). Recently, an interesting study using morphology and genotypic methods have showed the existence of non ochratoxigenic *A. carbonarius* (Cabañes et al., 2013). In any case, all studies suggest that *A. carbonarius* is the main responsible for the OTA presence in wine since *A. carbonarius* showed higher OTA mean production than other species and a higher percentage of OTA producing strains compared to *A. tubingensis* (4.2 to 64.3%) and *A. niger* (3.1 to 40.6%) (**Table1**). Although *Aspergillus niger aggregate* may represent lower OTA risk in grapes than *A. carbonarius*, recent reports have confirmed the ability to produce FB_2 and FB_4 by *A. niger* and *A. awamori* strains

isolated from grape (Chiotta et al., 2011; Logrieco et al., 2009; Mogensen et al., 2010a; Varga et al., 2010). The impact of some environmental factors on growth and OTA production of *Aspergillus niger aggregate* strains from grape have been published (Bellí et al., 2004b, 2004b; Esteban et al., 2006, 2004; Selouane et al., 2009). However, few works have focused on *A. niger* and none in *A. tubingensis*. Therefore, it is important to know how the different environmental factors affect the presence and ability to compete of these species.

On the other hand, OTA contamination in wines from Europe is generally higher than contamination in wines from other wine-growing areas around the world as Chile or South Africa (Italian Health Superior Institute, 2002, Shephard et al., 2003; Vega et al., 2012). Additionally, a gradual increase of OTA contamination has been observed in Europe from North to South, with southern Europe presenting higher concentration of the toxin in its wines (Brera et al., 2008; Otteneder and Majerus, 2000). These results are in agreement with those published by the Italian Health Superior Institute (2002), which stated that the incidence and OTA levels are higher in Southern countries (72.3% and 0.64 μg/kg respectively) compared to those in Northern regions (50.3% and 0.18 μg/kg respectively). This could indicate that meteorological conditions can contribute to explain spatial distribution of black aspergilli (Battilani et al., 2006). In this sense, Blesa et al. (2006) considered that OTA contamination in grape, and consequently in wine, varies depending directly on the climatic conditions and indirectly on the latitude and the year of production. Recently, several studies have showed that the effect of specific geographic location and climate of the vineyards on the occurrence of ochratoxigenic moulds and OTA contamination of grape was significant (Lasram et al., 2012b; Serra et al., 2006b). This difference may also be attributed to a possible genetic diversity among strains from different regions. The genetic variability and the phylogenetic characterization of *Aspergillus* section *Nigri* isolated from vineyards have been assessed by using DNA fingerprints generated by PCR (Abed, 2008; Bau et al., 2006; Chiotta et al., 2011; Esteban et al., 2008, 2006; Martínez-Culebras and Ramón, 2007; Martínez-Culebras et al., 2009; Oliveri et al., 2008; Perrone et al., 2006b; Spadaro et al., 2012; Susca et al., 2013). Different molecular marker techniques such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), minisatellites or variable number tandem repeats (VNTRs) and microsatellites or simple sequence repeats (SSRs) were used in the aforementioned studies. However, most

Table 1 Distribution and OTA production capacity of *A. carbonarius*, *A. tubingensis* and *A. niger* isolates from berries.

Origin	Year		Total isolates identified	Species distribution											
				A. carbonarius				*A. niger*				*A. tubingensis*			
				% isolates	% OTA producers	Range OTA (µg/g)	Mean OTA (µg/g)	% isolates	% OTA producers	Range OTA (µg/g)	Mean OTA (µg/g)	% isolates	% OTA producers	Range OTA (µg/g)	Mean OTA (µg/g)
Italy[1]	2000-01	AFLP	58	39.7	95.6	0.01-7.5	0.6	25.9	20	0.250-0.360	0.307	34.4	25	0.002-0.13	0.033
France[2]	2001-02	RFLP	23					4.3	0			95.7	0		
Greece[2]	2001-02	RFLP	34					52.9	11.8	0.200-1	0.5	47.1	0		
Israel[2]	2001-02	RFLP	30					10.0	0			90.0	0		
Italy[2]	2001-02	RFLP	32					59.4	3.1		5.5	40.6	0		
Portugal[2]	2001-02	RFLP	32					84.4	40.6	0.200-10.5	4	15.6	0		
Spain[2]	2001-02	RFLP	22					27.3	9.1	0.100-10.5	0.5	72.7	0		
Spain[3]	2001	RFLP	92					47.8	6			52.2	0		
Spain[4]	2004	RFLP	209	21.5	44.4			15.8	0			62.6	4,2		
Tunisia[5]	07/06/2005	RFLP	21					38.1				61.9			
Italy[6]	2006	RFLP	172	18.6	34.4	0.3-3.0		65.1	0			16.3	21.4	0.050-0.08	
	2007	RFLP	160	46.9	46.7			25.6	0			27.5	11.4		
Argentine[7]	2008-09	AFLP	192	7.6	100	0.002-0.515	0.168	77.2	4.2	0.002-0.295	0.1	15.2	64.3	0.002-0.034	0.017

Data were obtained from the following references: (1) Perrone et al., 2006, (2) Bau et al., 2006, (3) Accensi et al., 2001, (4) Martinez-Culebras and Ramón, 2007, (5) Lasram et al., 2012b, (6) Spadaro et al., 2012, (7) Chiotta et al., 2011.
Ochratoxin A was determined in Czapek (1), YES (2, 3, 4 and 6) and CYA (5 and 7).

of them focused on genetic identification of black aspergilli species. In this sense, RFLPs, AFLPs, minisatelilles and microsatellites have shown to be efficient to distinguish among main black aspergilli species (Esteban et al., 2008; Martínez-Culebras and Ramón, 2007; Martínez-Culebras et al., 2009; Perrone et al., 2006b). In addition, multilocus sequence typing (MLST) based on four loci of nuclear DNA markers (calmodulin, β-tubulin, elongation factor 1-α and second largest subunit of RNA polymerase II) have been recently reported as practical tools for typing *Aspergillus* section *Nigri* (Susca et al., 2013). In this study, 62 haplotypes (H) from 18 species were identified when 230 isolates of black aspergilli isolated from five different countries were sequenced by MLST. 105 *A. carbonarius* from Italy evaluated by AFLP showed high genetic similarity (Perrone et al., 2006a); nevertheless, using the same methodology, strains from Southern Europe were clustered in nine subgroups which seemed to be correlated to their geographical origin (Perrone et al., 2006b).

In this study, an ecophysiological characterization (in terms of temperature and water activity requirements) of *A. tubingensis, A. niger* and *A. carbonarius* isolated from berries from Northeast and Southern Spain was carried out. Moreover, the genetic diversity of *A. carbonarius* was studied with four SSRs markers. In addition, partial sequences of the β-tubulin gene of *A. carbonarius* isolates from both regions were compared.

2. MATERIALS AND METHODS

2.1. Fungal isolates, origin and molecular identification

Isolates from two different Spanish wine-growing regions were used in this study (**Table 2**). The vineyards were located in Lleida and Sevilla, which are located in the Northeast and South of Spain, respectively. The climate in the vineyards sampled in the Northeast is defined as cold steppe (BSk) while in the South it is temperate with dry or hot summer (Csa) according to Köppen Climate Classification of the Iberian Peninsula Climate Atlas (Iberian Climate Atlas).

Black aspergilli isolates used were always from berries. The identification of isolates from Northeast and South was done by molecular characterization. Specific PCR assays were carried out using primers AcKS10R (5'-CCCTGATCCTCGTATGATAGCG-3') and

Table 2 *Aspergillus* section *Nigri* strains included in this study.

Strain	Isolates	Origin	OTA production	Reference
70-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
93-UdLTA [a]	*A. carbonarius*	Northeast	-	Present study
98-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
100-UdLTA [a]	*A. carbonarius*	Northeast	-	Present study
103-UdLTA [a]	*A. carbonarius*	Northeast	-	Present study
104-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
113-UdLTA [a]	*A. carbonarius*	Northeast	-	Present study
114-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
118-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
148-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
207-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
287-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
318-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
339-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
343-UdLTA [a]	*A. carbonarius*	Northeast	+	Present study
36br4 [b]	*A. carbonarius*	Northeast	-	Belli et al., 2004a
93cr4 [b]	*A. carbonarius*	Northeast	-	Belli et al., 2004a
W120 [b]	*A. carbonarius*	Northeast	+	Belli et al.; 2005
W128 [b]	*A. carbonarius*	Northeast	+	Belli et al., 2005
23N [b]	*A. carbonarius*	Northeast	+	Marn et al., 2006
234N [b]	*A. carbonarius*	Northeast	+	Marín et al., 2006
A-941 [b]	*A. carbonarius*	Northeast	+	Esteban et al., 2006
253-UdLTA [ab]	*A. carbonarius*	South	+	Present study
262-UdLTA [ab]	*A. carbonarius*	South	+	Present study
265-UdLTA [ab]	*A. carbonarius*	South	+	Present study
272-UdLTA [ab]	*A. carbonarius*	South	+	Present study
273-UdLTA [b]	*A. carbonarius*	South	+	Present study
275-UdLTA [a]	*A. carbonarius*	South	+	Present study
282-UdLTA [ab]	*A. carbonarius*	South	+	Present study
288-UdLTA [ab]	*A. carbonarius*	South	+	Present study
300-UdLTA [a]	*A. carbonarius*	South	+	Present study
304-UdLTA [a]	*A. carbonarius*	South	+	Present study
309-UdLTA [a]	*A. carbonarius*	South	+	Present study
311-UdLTA [ab]	*A. carbonarius*	South	+	Present study
3.122-UdLTA [b]	*A. carbonarius*	South	+	Valero et al., 2005,06,07,08

[a] Strains used in the study of genetic diversity.
[b] Strains used in the ecophysiological study.

Table 2 (Continued).

Strain	Isolates	Origin	OTA production	Reference
73-UdLTA [b]	*A. tubingensis*	Northeast	+	Present study
74-UdLTA [b]	*A. tubingensis*	Northeast	-	Present study
79-UdLTA [b]	*A. tubingensis*	Northeast	-	Present study
108-UdLTA [b]	*A. tubingensis*	Northeast	-	Present study
338-UdLTA [b]	*A. tubingensis*	Northeast	-	Present study
252-UdLTA [b]	*A. tubingensis*	South	-	Present study
274-UdLTA [b]	*A. tubingensis*	South	-	Present study
276-UdLTA [b]	*A. tubingensis*	South	-	Present study
296-UdLTA [b]	*A. tubingensis*	South	-	Present study
298-UdLTA [b]	*A. tubingensis*	South	-	Present study
84-UdLTA [b]	*A. niger*	Northeast	+	Present study
162-UdLTA [b]	*A. niger*	Northeast	+	Present study
190-UdLTA [b]	*A. niger*	Northeast	-	Present study
204-UdLTA [b]	*A. niger*	Northeast	-	Present study
321-UdLTA [b]	*A. niger*	Northeast	-	Present study
193-UdLTA [b]	*A. niger*	South	-	Present study
202-UdLTA [b]	*A. niger*	South	-	Present study
203-UdLTA [b]	*A. niger*	South	-	Present study
218-UdLTA [b]	*A. niger*	South	-	Present study
302-UdLTA [b]	*A. niger*	South	+	Present study

AcKS10L (5'-CCGGCCTTAGATTTCTCTCACC-3') for *A. carbonarius* (Selma et al., 2008), NIG1 (5'-GATTTCGACAGCATTT(CT/TC)CAGAA-3') and NIG2 (5'-AAAGTCAATCACAATCCAGCCC-3') for *A. niger* and TUB1 (5'-TCGACAGCTATTTCCCCCTT-3') and TUB2 (5'-TAGCATGTCATATCACGGGCAT-3') for *A. tubingensis* (Perrone et al., 2007b; Susca et al., 2007). A recent publication by Perrone et al. (2011) has emphasized that *A. niger* contains the cryptic phylogenetic species *A. awamori*; based on this and the fact that the primer NIG1-NIG2 has not been tested before in *A. awamori*, our *A.niger* isolates could be misidentified *A. awomari* isolates.

Morover, the ability of the isolates to produce OTA on CYA was confirmed following the method by Bragulat et al. (2001) with some modifications. In brief, three agar plugs (5 mm) were removed from the middle to the outer side of the colony and placed in a vial. Mycotoxins were extracted by adding 1 mL of methanol into the vials, which were shaken

for 5 s and allowed to rest. After 60 min, the vials were shaken again and the extracts filtered (OlimPeak filters by Teknokroma PVDF Filter, 0.45 μm, 13 mm D, Sant Cugat del Vallés, Barcelona, Spain) into another vial. Subsequently, the extracts was evaporated under a stream of nitrogen and stored at 4 ºC until HPLC analysis (Waters, Mildford, Ma, S.A.). Prior to HPLC injection, dried extracts were dissolved in 1 mL of methanol: water (50:50). A HPLC system (Waters 2695, separations module, Waters, Milford, USA) equipped with a fluorescence detector Waters 2475 module (Waters, Milford, USA) (λexc 330 nm; λem 460 nm), precolumn Waters Spherisorb 5 μm, ODS2, 4.6x10 mm and a C18 silica gel column (Waters Spherisorb 5 μm, ODS2, 4.6 x250 mm, Millford, MA, USA) kept at 40 ºC were used. Mobile phase (acetonitrile:water:acetic acid, 57:41:2) was pumped at 1 mL/min under isocratic conditions. Quantification was always achieved with a software integrator (Empower, Milford, MA, USA). Mycotoxins were quantified on the basis of the HPLC fluorimetric response compared with a range of mycotoxin standards. OTA retention time was 7 min and the detection limit was 0.01 ng OTA/g of SNM, based on a signal-to-noise ratio of 3:1.

2.2. Ecophysiological study

2.2.1. Data generation

Evaluation of the behaviour of *A. carbonarius* isolates from Northeast Spain was made by using previously published growth and OTA production data of *A. carbonarius* isolated from grapes of this region (Bellí et al., 2004b; Esteban et al., 2006; Marín et al., 2006; Valero et al., 2008, 2007b, 2006, 2005). However, not enough published data existed from South Spain, thus data were generated for eight *A. carbonarius* isolates from Southern Spain (**Table 3**). Additionally, as data on *A. tubingensis* and *A. niger* are scarce, newly generated data for both species (five strains isolated per region and species) were used. Tested conditions are shown in **Table 3**.

For generation of new data, the culture medium used was a synthetic nutrient medium (SNM) similar to grape composition between veraison and ripeness (Delfini, 1982). Water activity of the medium was modified to the required values by the addition of glycerol (g glycerol/L $=629.72+1813.44\ a_w-2426.08\ a_w^2$), as made before in the published results on *A. carbonarius*. The medium was autoclaved and poured into sterile petri dishes of 5 cm of diameter. Water

activity of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy ±0.003.

Table 3 Black aspergilli and tested conditions used in probabilistic models.

Strain	Origin	Isolates	Tested conditions			R
			a_w	T (ºC)	t (days)	
A. carbonarius	Northeast Spain	36br4 (1)	0.90, 0.93, 0.95, 0.98, 0.995	10, 20, 30, 37	60	Bellí et al. (2004)
		W120, 93cr4 (2)	0.90, 0.93, 0.95, 0.98, 0.995	25		
		W120, W128 (2)	0.90, 0.93, 0.95, 0.99	15, 20, 30, 35, 37	30	Bellí et al. (2005)
		23N, 234N (2)	0.96	7, 15, 20, 25, 30, 35	10	Marín et al., (2006)
		A-941(1)	0.86, 0.88, 0.90, 0.94, 0.98, 0.99	15, 30	30	Esteban et al. (2006)
	South Spain	3.122-UdLTA (1)	0.87, 0.92, 0.97	20, 30, 40	18	Valero et al. (2005)
		3.122-UdLTA (1)	0.92, 0.97	20, 30	18	Valero et al. (2006)
		3.122-UdLTA (1)	0.87, 0.92, 0.97	20, 30, 40	18	Valero et al. (2007)
		3.122-UdLTA (1)	0.97	25	21	Valero et al. (2008)
		253, 262, 265, 272, 273, 282, 288, 311-UdLTA (8)	0.84, 0.86, 0.88, 0.90, 0.92, 0.98	10, 15, 20, 25, 30, 37, 40	65	Present study
A. tubingensis	Northeast Spain	73, 74, 79, 108, 338-UdLTA (5)	0.84, 0.86, 0.88, 0.90, 0.92, 0.98, 0.99	10, 15, 20, 25, 30, 37, 40, 42, 44	65	Present study
	South Spain	252, 274, 276, 296, 298-UdLTA (5)	0.84, 0.86, 0.88, 0.90, 0.92, 0.98, 0.99	10, 15, 20, 25, 30, 37, 40, 42, 44	65	Present study
A. niger	Northeast Spain	84, 162, 190, 204, 321-UdLTA (5)	0.82, 0.84, 0.87, 0.90, 0.92, 0.98	10, 15, 20, 25, 30, 37, 40, 42, 44	65	Present study
	South Spain	193, 202, 203, 218, 302-UdLTA(5)	0.82, 0.84, 0.87, 0.90, 0.92, 0.98	10, 15, 20, 25, 30, 37, 40, 42, 44	65	Present study

Experiments were performed in Synthetic Nutrient Medium, except those from Esteban et al. (2006) in Czapek.
Total tested strains are shown in brackets. R: reference.

The isolates were sub-cultured on SNM plates and incubated at 25 ºC for 7 days to obtain heavily sporulating cultures. Following incubation, a sterile inoculation loop was used to remove the conidia which were suspended in Tween 80 (0.005%). After homogenizing, the suspensions were adjusted using a Thoma counting chamber to a final concentration of 1×10^4 spores/mL in Tween 80 (0.005%). Finally, 5 µL of the suspensions were centrally inoculated in SNM Petri dishes. Petri dishes with the same a_W were enclosed in sealed containers along with beakers containing water glycerol solution of the same a_W as the plates and incubated as detailed in Table 3.

The beakers were renewed periodically in order to maintain constant a_W (Dallyn, 1978). For each condition 5 replicates per isolate were carried out.

Plates were kept for a maximum of 65 days; within this period plates were regularly checked for growth occurrence. OTA production by *A. carbonarius* under all incubation conditions was determined once colony diameter reached 40 mm or on day 65 in the case of smaller diameter colonies. OTA production was not assayed for *A. tubingensis* and *A. niger* strains as they were mostly non-producers. OTA production was tested following the protocol detailed in section 2.1.

2.2.2. Modelling data

In order to present the pattern of behaviour for each species and origin, all strains within a species from each location were pooled and logistic regression was used to calculate the probabilities of growth as a function of temperature and water activity. For this purpose, both growth and OTA production data were converted into probabilities of growth by assigning the value of 1 in the case where visible fungal growth was evident (or OTA detected), and 0 in the case of absence of growth (or undetectable OTA) during the overall period of the experiment. The resulting data were fitted to a logistic regression model as described previously (Garcia et al., 2011):

$$logit\ (p) = In\left[\frac{p}{1-p}\right] = b_0 + b_1 a_W + b_2 T + b_{11} a_W^2 + b_{22} T^2 + b_{12} a_W T$$

Where p is the probability of growth (or toxin production), T is temperature in ºC, and bi are the coefficients to be estimated. The equation was fitted by using Statgraphics® Plus

version 5.1 (Manugistics, Inc, Maryland, USA) linear logistic regression procedure. The automatic variable selection option with a backward stepwise factor selection method was used to choose the significant effects ($p< 0.05$). The predicted growth/no growth interfaces for p=0.1, 0.5, and 0.9 by the three species, and predicted OTA production/no OTA production boundary for p=0.1, 0.5, and 0.9 by *A. carbonarius* was calculated using Microsoft Excel Solver.

2.3. Genetic diversity study within *A. carbonarius* isolates

2.3.1. DNA extraction

Cultures were grown for 2 days at 27 ºC on 500 µL of Czapek's yeast medium. Mycelium was recovered after 10 min of centrifugation at 17500 x g and 300 µL of extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. The mycelium suspension was vortexed with five 2.8 mm stainless steel beads (Precellys, Bertin Technologies) during 10 minutes. After centrifugation at 17500 × g for 10 min, 150 µL of 3 M sodium acetate (pH 5.2) were added to the supernatant. The supernatant was incubated at -20 ºC for 10 more minutes and centrifuged (17500 x g, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by adding 1 volume of isopropyl alcohol. After 5 minutes of incubation at room temperature the DNA suspension was centrifuged (17500 x g, 10 min). The DNA pellet was washed with 70% ethanol to remove residual salts. Finally, the pellet was air-dried and the DNA was resuspended in 50 µL of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

2.3.2. Ap-PCR amplification and analysis

To study the genetic diversity of 15 *A. carbonarius* isolates from Northeast and 11 from South, the following primers derived from 4 SSRs (ap-PCR), were used: GACGACGACGACGAC (GAC)5, GACAGACAGACAGACA (GACA)4, AGGAGGAGGAGGAGG (AGG)5 and AGGTCGCGGGTTCGAATCC (T3B) (Bahkali et al., 2012; Martínez-Culebras et al., 2009). DNA amplification was performed in a total volume of 25 µL containing 25 ng of DNA, 50 mM KCl, 10 mM Tris–HCl, 200 µM (each) dNTP, 0.6 µM of primer, 2.5 mM MgCl2 and 1 U of DNA polymerase (DFS-Taq DNA polymerase, BIORON, Germany). The reaction mixture was incubated in a thermalcycler (Applied Biosystem GeneAmp 2700) starting with 3 min of denaturation at 95 °C followed by 40 cycles consisting of 30 s at 95 ºC, 60 s at 60

ºC for (GAC)5, 52 ºC for (AGG)5 or 48 ºC for (GACA)4 and T3B, and 2 min at 72 ºC. The ap-PCR products were separated on 1.5% agarose gels with TAE buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 mg/ml), and the DNA bands visualized under UV light (**Figure. 1**). Sizes were estimated by comparison with a DNA standard length (100 bp DNA ladder, Invitrogen).

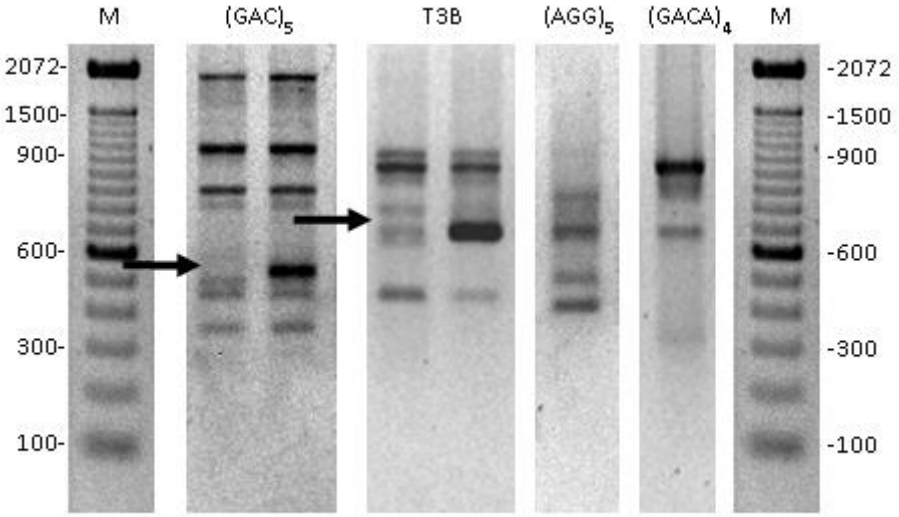


Figure 1 DNA fingerprinting profiles amplified from $(GAC)_5$, $(AGG)_5$, $(GACA)_4$ and T3B. M is the 100bp DNA ladder.

2.3.3. DNA sequencing

Pairs BT2A/BT2B (Glass and Donaldson, 1995) were used to obtain partial sequences of the β-tubulin gene of four *A. carbonarius* randomly selected from Northeast ones and four from South. Amplification reactions were carried out in volumes of 50 µL containing 50 ng of DNA, 50 mM KCl, 10 mM Tris–HCl, 250 µM (each) dNTP, 1 µM of each primer, 2 mM MgCl2 and 0.5 U of DFS-Taq DNA Polymerase (BIORON, Germany). PCR assays were conducted in a GeneAmp® PCR System 2700 (Applied Biosystems, USA) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 ºC (BT2A/BT2B), and extension at 72 °C for 60 s with a final extension of 10 min. PCR products were cleaned with the UltraClean PCR Clean-up DNA Purification kit (MoBio, USA). The PCR purified products were sequenced by the company Macrogen Europe (Amsterdam, The Netherlands). Finally, sequences were compared using the MEGA 5 software package (Sohpal et al., 2010).

3. RESULTS

3.1. Ecophysiological study

3.1.1. A. carbonarius

Growth response of *A. carbonarius* isolates from South Spain for each of the 5 fold repeated experiments were always similar. Although some strains grew earlier, at the end, all of them were able to grow under the same conditions (data no shown). Similarly, data of *A. carbonarius* isolates from Northeast Spain used from published results, showed little intraspecific variability in their response to temperature and a_W conditions (Bellí et al., 2004a).

A full second-order logistic regression model including all the linear, quadratic and interaction terms was generated for both fungal growth and OTA production (**Table 4**). Backward stepwise selection did not eliminate any of the linear or quadratic terms of the logistic model for growth, as all of them were statistically significant ($p<0.05$), thus the models consisted of 6 terms. Conversely, backward stepwise selection eliminated some linear and quadratic terms in the OTA model as some were not statistically significant ($p>0.05$).

As shown in **Figure 2**, strains from the South had optimal growth around 30 ºC, about 3 ºC higher than those from the Northeast, and also a higher minimum temperature for growth, suggesting a better adaptation to warmer temperatures, although maximum temperatures were similar. In addition, *A. carbonarius* isolates from Northeast grew at 10 ºC over 0.95 aw, while A. carbonarius isolates from South never grew at this temperature. Regarding water activity, the strains from the Northeast showed $p>0.5$ of growth between 23-33 ºC at 0.87 a_W, while those from the South grew between 20-37 ºC, a much wider interval. While Northeast strains did not grow at all at 0.85 a_W, those from the South reached a $p>0.4$, suggesting a better adaptation to dry conditions of these later strains.

At a given temperature, higher a_W was required for OTA production than for growth (**Figure 2**). Minimum temperature for OTA production was also higher for Southern strains, while optimum temperatures were similar (22-23 ºC). While at 0.89 a_W Northeast strains did not reach 0.05 probability of OTA production, Southern strains reached 0.7, and they were also able to produce OTA at 0.85 a_W. Finally, Northeast strains may grow and produce OTA at lower temperatures.

Table 4. Estimated parameters from logistic regression models and maximum adjusted r^2 for growth and ocrhatoxin A production.

		Region	**Northeast**	**South**
Growth model	**Intercept**	*A. carbonarius*	-4620.87±3606.93	-3406.56±351.67
		A. tubingensis	-1222.16±137.99	-1446.22±181.36
		A. niger	-1924.47±414.85	-1913.13±350.73
	$\mathbf{a_w}$	*A. carbonarius*	9114.18±7485.05	6629.23±729.63
		A. tubingensis	2442.26±274.39	2927.1±373.18
		A. niger	3806.45±840.15	3787.07±709.93
	T	*A. carbonarius*	20.95±12.35	20.53±2.52
		A. tubingensis	5.21±0.78	5.24±0.66
		A. niger	10.1±1.97	9.93±1.68
	$\mathbf{a_w^2}$	*A. carbonarius*	-4539.78±3916.74	-3301.91±390.51
		A. tubingensis	-1257.81±140.46	-1530.58±198.38
		A. niger	-1923.5±434.59	-1916.7±367.06
	$\mathbf{T^2}$	*A. carbonarius*	-13.61±9.23	-0.18±0.02
		A. tubingensis	-0.08±0.01	-0.09±0.01
		A. niger	-0.1±0.02	-0.1±0.02
	$\mathbf{a_w*t}$	*A. carbonarius*	-0.16±0.09	-11.58±1.98
		A. tubingensis	-0.96±0.38	
		A. niger	-4.63±10.21	-4.36±0.87
	$\mathbf{r^2}$	*A. carbonarius*	85.27	94.05
		A. tubingensis	77.52	81.85
		A. niger	84.34	83.85
OTA model	**Intercept**	*A. carbonarius*	-19.16±7.32	-48.3±5.39
	T		-2.04±0.81	
	$\mathbf{a_w^2}$		4.07±1.09	
	$\mathbf{T^2}$		-0.04±0.01	-0.09±0.01
	$\mathbf{a_w*t}$			4.83±0.55
	$\mathbf{r^2}$		58.14	56.05

Only significant parameters have been included in the table. Estimated value±standard error.
T: temperature

The predicted growth and OTA interface at probabilities of 0.1, 0.5 and 0.9 is shown in **Figure 3**. It is clearly shown that the interface is much wider in the case of OTA production than in the case of growth. As data from the different strains were pooled for the analysis, this suggests a wider intraspecific variability for OTA production conditions compared to that for growth.

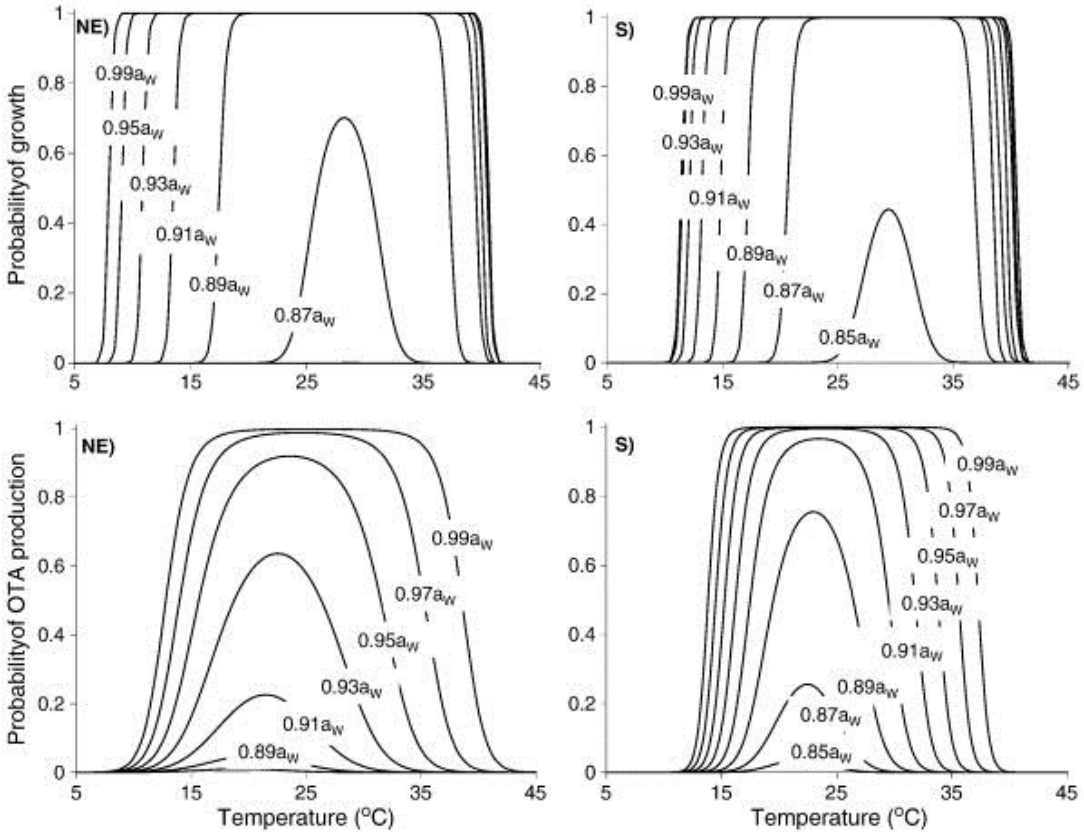


Figure 2 Effect of temperature and water activity on the predicted probability of growth and OTA production after 65 days of incubation of Aspergillus carbonarius strains isolated from Northeast (N) and South (S) Spain in synthetic nutrient medium (SNM).

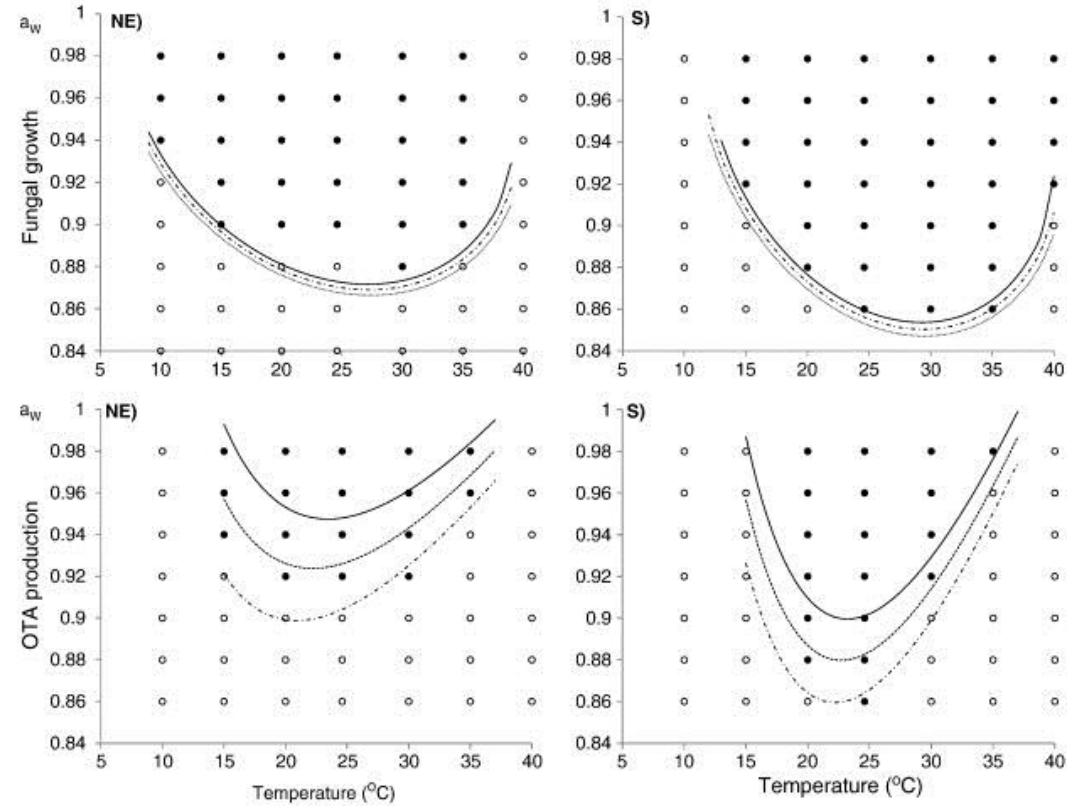


Figure 3 Fungal growth and ochratoxin A (OTA) production boundaries of *Aspergillus carbonarius* isolates from Northeast (N) and South (S) Spain in synthetic nutrient medium (SNM). Solid line indicates p=0.9; dotted line indicates p=0.5; dashed line indicates p=0.1.

3.1.2. A. tubingensis

Estimated coefficients and r^2 of logistic regression of binary growth data for *A. tubingensis* are shown in **Table 4**. As observed in **Figure 4**, strains from Northeast and South showed similar optimal, maximum and minimum temperatures for growth. Moreover, similar probabilities of growth were reached for a given a_W level. One isolate from Northeast grew at 10 ºC while none from South did; in addition, one isolate from South grew at 44 ºC, but these differences occurred in a single isolate and were not observed in the joint plots. High probability of growth at 0.85 a_W in the range 20-37 ºC was observed for isolates from both regions.

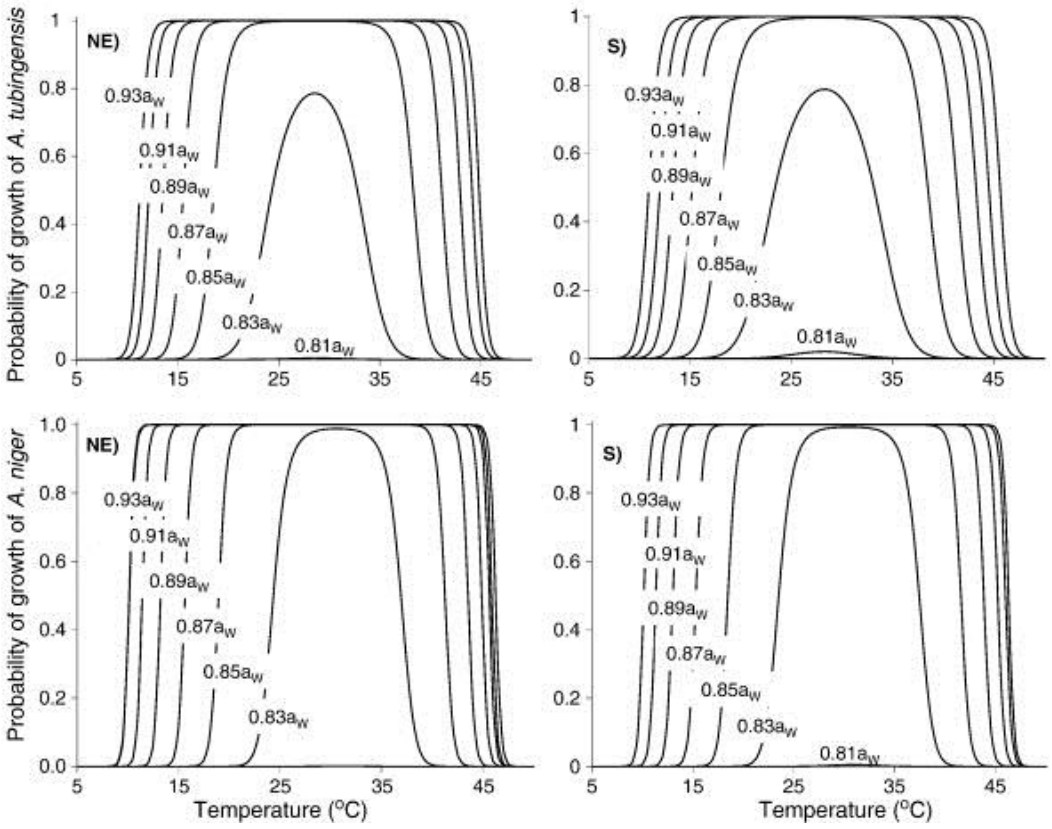


Figure 4 Effect of temperature and water activity on the predicted probability of growth and OTA production after 65 days of incubation of *Aspergillus tubingensis* and *Aspergillus niger* strains isolated from Northeast (N) and South (S) Spain in synthetic nutrient medium (SNM).

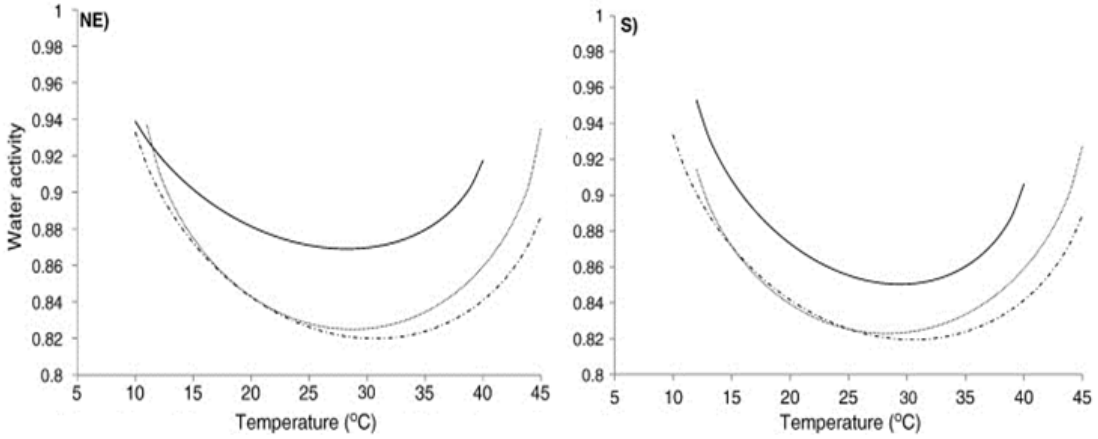
3.1.3. A. niger

Estimated coefficients and r^2 of logistic regression of binary growth data for *A.niger* are shown in table 4. As for *A. tubingensis*, strains from the two regions showed similar optimal, maximum and minimum temperatures for growth (**Figure 2**). Furthermore, similar

probabilities were obtained for a given a_W level. Isolates from both regions showed high probability of growth at 0.83 a_W in the range 25-30 ºC and at 0.85 a_W in the range 20-37 ºC, while differences among isolates were found at 0.86 a_W and 0.88 a_W and 42 ºC.

3.1.4. Comparison among species

Interestingly, *A. tubingensis* and *A. niger* showed higher maximum temperature for growth (>45 ºC versus 40-42 ºC), and lower minimum a_W requirements (0.83 a_W versus 0.87 a_W) than *A. carbonarius*, suggesting that these species may not need a further adaptation to stress conditions produced by high temperatures, as regardless of their origin they requirements are less strict than those of *A. carbonarius* strains from the South. *A. carbonarius* and *A. tubingensis* isolates from Northeast and all *A. niger* grew at 10 ºC, however *A. carbonarius* did so at lower aw than the others (0.95 a_W versus 0.98 a_W). It is worthy to mention that *A. niger* showed the widest growth range in terms of temperature and aw requirements of the black aspergilli tested (**Figure 5**). However, differences between *A. niger* and *A. tubingensis* were found only at extreme temperatures. Therefore strains of the three species isolated from Northeast could coexist in a range of 15 to 35 ºC and a_W higher than 0.88 a_W and higher than 0.86 a_W for the Southern strains. No differences in growth boundaries were observed between producer and non-producer isolates (data not shown).



Figue 5 Growth/no growth boundaries (p=0.5) of black aspergilli isolates from North East (N) and South (S) Spain in synthetic nutrient medium. Solid line indicates *A. carbonarius*; dotted line indicates *A. tubingensis*; dashed line *A. niger*.

3.2. Genetic diversity study within *A. carbonarius* isolates

The genetic diversity of 26 *A. carbonarius* isolates from grapes from two regions of Spain, which were previously identified at species level by species specific PCR primers and tested for OTA production, was examined by PCR using the primers $(GAC)_5$, $(AGG)_5$, $(GACA)_4$ and T3B. Low level of polymorphism was observed for the markers tested within the *A. carbonarius* isolates analyzed. A single banding pattern was observed when the DNA was amplified with the primers $(AGG)_5$ and $(GACA)_4$ (Figure 1). However, two different banding patterns were observed when the DNA was amplified with the primers $(GAC)_5$ and T3B. Two different banding patterns were observed in isolates from Northeast and South when they were amplified with $(AGG)_5$. In addition, two different banding patterns were observed in the isolates from Northeast when they were analyzed with the primer T3B. The β-tubulin gene showed exactly the same sequence. No correlation between the amplified sequences and geographic origin or capacity of OTA production was found.

4. DISCUSSION

Ecophysiological characterization of *A. carbonarius, A. tubingensis* and *A. niger* was carried out with probability models in two different regions. Probability models can provide useful information and assess fungal responses under boundary conditions of growth and toxin production (Tassou et al., 2009). In the present work, probability was not modeled as a function of time. Higher probabilities of growth and OTA production by *A. carbonarius* were predicted for 1 month compared to those after 1 week, but the probability data observed after 1 month were almost equal to those observed after 3 months (Marín et al., 2008; Tassou et al., 2009).

Growth of black aspergilli isolates from grapes of different parts of the world has been studied previously (Bellí et al., 2004b; Bellí et al., 2005; Esteban et al., 2006, 2004; Lasram et al., 2010; Leong et al., 2006; Mitchell et al., 2004; Romero et al., 2007; Selouane et al., 2009). Optimal growth conditions reported in those studies were similar for *A. carbonarius* isolates but in the case of *Aspergillus niger aggregate* isolates results were more divergent. These differences may be due not only to the different geographic areas of isolation, but also to the differences among the species belonging to the aggregate group. Therefore, growth differences observed under optimal conditions may not be relevant. Moreover, few studies have focused on suboptimal or extreme conditions.

In Spain, the Southern region is hotter than Northeast one, and it is common to exceed 40 ºC in summer. In this season, the mean temperature difference between regions fluctuates from 1 to 4 ºC. In addition, relative humidity is lower in the South and the rainfall is scarce. Our results showed differences in *A. carbonarius* maximum and minimal temperature and a_W conditions for fungal growth for isolates from Northeast and South Spain. Strains from Northeast were better adapted to colder temperatures while strains from South could grow under drier conditions. Similarly, when Greek isolates were incubated under a wider range of conditions (10-40 ºC and 0.85-0.96 a_W) differences in growth probability at 0.85 a_W among isolates were observed (p = 0.8-1.0) (Tassou et al., 2009). Also isolates from Argentina grew at 0.85 a_W at 25 and 30 ºC (Romero et al., 2007), whereas the probability of growth at this a_W by Spanish isolates was lower than 0.5. In relation to growth rate, *A. carbonarius* isolated from Tunisian hot and dry regions grew significantly faster than isolates from wetter regions (Lasram et al., 2012b). However, no significant differences were found among *A. carbonarius* isolates from different European regions (Bellí et al., 2005). This suggests that under conditions suitable for growth, most strains do not show differences in their growth rates, while they may differ in their ability to either grow or not under marginal growth conditions.

OTA production by *A. carbonarius* has been particularly studied since *A. carbonarius* is the most ochratoxigenic black aspergilli (Table 1). Optimum published temperatures for OTA production were about 22-23 ºC, and ochratoxigenic isolates can produce OTA in the range of 15 ºC to 35 ºC (Esteban et al., 2004; Leong et al., 2006; Mitchell et al., 2004; Selouane et al., 2009). Aforementioned works suggested 0.95-0.99 a_W as optimal for OTA production. Similarly, in our case, production probability at 0.95 a_W was over 0.8 in the range of 15-30 ºC. In addition, OTA accumulation of *A. carbonarius* isolated from vineyards of Europe was favoured by high a_W levels, while no OTA was detected at 0.90 a_W (Bellí et al., 2005). *A. carbonarius* strains from Greece produced OTA at lower a_W than ours, even comparing with isolates from South (Tassou et al., 2009). Considering p=0.5, *A. carbonarius* strains from Greece were able to produce OTA at 0.88 a_W after 25 days while *A. carbonarius* from Spain required 0.93 and 0.89 a_W for Northeast and South strains, respectively. OTA production has been rarely studied under extreme temperature and humidity conditions. Although low a_W levels seem to limit OTA production, low temperatures may not. This is very interesting since high OTA production has been observed at low temperature and high a_W (15 ºC/0.965 a_W) in Australia (Leong et al., 2006). In fact, nocturnal temperatures between 15-20 ºC are common during June and July in Spain, leading to a risk of OTA accumulation on grapes.

Environmental conditions required for growth of *A. tubingensis* and *A. niger* have also been considered in this study because species in the *Aspergillus niger aggregate* are more frequently isolated from vine than *A. carbonarius* (Somma et al., 2012). Published ecophysiological studies showed that *Aspergillus niger aggregate* is more tolerant than *A. carbonarius* to lower a_W (Bellí et al., 2004b; Leong et al., 2006; Valero et al., 2007b). In this sense, strains isolated from South Spain belonging to the *Aspergillus niger aggregate* grew at 40 ºC/0.87 a_W whereas only one of two *A. carbonarius* strains tested grew at 0.97 a_W at this temperature (Valero et al., 2007b). To our knowledge, only one work has studied the behaviour of *A. niger* and *A. tubingensis* but the data of both species were showed combined (Selouane et al., 2009). In our study, *A. tubingensis* and *A. niger* grew in a wide range of a_W and temperature, and minimal a_W for both species occurred at higher temperatures (25-35 ºC). Furthermore, few differences were found due to the geographical origin of the isolates. Nevertheless, *A. niger* grew at lower temperatures than *A. tubingensis*, and in a narrower a_W frame at 44 ºC. In addition, *Aspergillus niger aggregate* species have been shown to grow faster than *A. carbonarius* at temperatures higher than 25 ºC, when they were isolated from Spain and Australia, while no differences were found between species isolated from Morocco at this temperature (Bellí et al., 2004a; Leong et al., 2006; Selouane et al., 2009; Valero et al., 2005, 2007b). In addition, differences in optimal growth conditions were observed for *Aspergillus niger aggregate* species from Morocco which grew faster at 0.95 a_W /25 ºC while isolates from Europe and Australia did so at 0.98 a_W /30-37 ºC (Bellí et al., 2004b; Esteban et al., 2004; Leong et al., 2006; Selouane et al., 2009). Although the percentage of OTA producing strains in *Aspergillus niger aggregate* is not clear, optimal conditions for production have been reported to be equal or higher than 0.95 a_W (Bellí et al., 2004b; Esteban et al., 2004; Leong et al., 2006; Selouane et al., 2009). The adaptation of the species in the *Aspegillus niger aggregate* to a wider range of environmental conditions and their higher growth rates may determine their prevalence in the vineyards.

Water activity of ripening grapes is 0.95-0.98 a_W (Tassou et al., 2009), and temperature in Spanish vineyards may range from 17-18 ºC to 33-38 ºC in August. These conditions would be suitable for black aspergilli growth and therefore for OTA production. However, hotter and drier climate could promote the presence of *Aspergillus niger aggregate*. Interestingly, *Aspergillus niger aggregate* OTA-positive isolates showed higher colonization percentages than *A. carbonarius* when inoculated in healthy grapes (Valero et al., 2007b). Nevertheless, the balance of these species in vineyards is far from being elucidated since interaction between black aspergilli and other fungi present on grapes as *Alternaria alternata*, *Cladosporium herbarum*

and *Eurotium amstelodami*, showed that growth of *A. niger* was more inhibited by the interacting species than *A. carbonarius* (Valero et al., 2007b). Interestingly, when dried grapes were co-inoculated with *Aspergillus niger aggregate* OTA-negative and *A. carbonarius* OTA-positive, OTA production was reduced (Valero et al., 2007a).

To sum up, different a_W and temperature requirements may determine the geographical distribution of the species in the black aspergilli section, in terms of better adaptation and fungal interaction. Regarding the ecological profiles of black aspergilli, relevant differences due to geographic location and climate on the occurrence of ochratoxigenic moulds and OTA contamination of grape have been observed in Mediterranean countries (Battilani et al., 2006). In general, black aspergilli infection was higher in the hotter regions than in colder regions (Lasram et al., 2012a; Serra et al., 2006b). Moreover, *A. carbonarius* was more abundant reaching a 43% of mean infection, in three sampled years, in the most humid region studied in Tunisia (60-70% RH) (Lasram et al., 2012b). Similarly, in Spain the percentage of *A. carbonarius* decreased when RH decreased (unpublished data). However, this trend was not observed in Portugal (Serra et al., 2006). Unfortunately, few data about *A. tubingensis* and *A. niger* distribution in vineyards exist and it is therefore difficult to derive the relation with environmental conditions. However, several works pointed to *A. tubingensis* as the most prevalent black aspergilli species in vineyards (Chiotta et al., 2011; Susca et al., 2013).

This study is also focused on the evaluation of the genetic diversity of *A. carbonarius* from different origin of Spain. In previous studies, AFLP and RFLP primers have been used efficiently to discriminate among *A. carbonarius, A. tubingensis, A. niger* and *A. ja*ponicus (Bau et al., 2006, Culebras et al., 2007; Perrone et al., 2006). In addition, SSR markers have also discriminated between A. tubingensis and A. niger (Esteban et al., 2008). Similarly, $(GAC)_5$ and $(GACA)_4$ were effective in discriminating *A. carbonarius* from other black aspergilli species isolated from grapes (Martinez-Culebras et al., 2009). Moreover, T3B and $(AGG)_5$ amplified two polymorphic bands in *Fusarium graminearum* and *F. culmorum* (Bahkali et al., 2012). In the present study, although differences among the strains were observed in their response to a_W and temperature depending on their geographical origin, little genetic diversity at species level was observed for the microsatellites tested. Additionally, no differences in the sequence of the β-tubulin gene were observed. Therefore, intraspecific variability did not correlate with the isolate origin or ability of the strain to produce OTA (different A. carbonarius strains were used for both studies). Similarly, sequences of rRNA, calmodulin,

β-tubulin genes and ITS products obtained using the endonucleases HhaI, HinfI and RsaI in Italian strains of *A. carbonarius* were identical (Perrone et al., 2006a; Spadaro et al., 2012).

These results are in accordance with other studies conducted previously. Dachoupakan et al. (2009) found that clustering linked to RAPDs among *A. carbonarius* strains was not associated with the zone and harvest year, grape variety or chemical treatment, while OTA production of strains on culture medium seemed to better correlate with morphological characters as colour of colony, conidia density, wrinkle colony, reverse colony, umbilical colony, and aerial mycelium than with genotypic profiles. Similarly, no correlation was observed between the clusters and OTA production level or origin when black aspergilli clusters were analysed with AFLP (Oliveri et al., 2008). In addition, geographic differences in the haplotypes within the species were not detected when isolates from five countries (Chile, Iran, USA, China, South Africa) were included in the MLST analysis (Susca et al., 2013).

Nowadays*, A. carbonarius* is the main mycotoxigenic fungus found in vineyards, in terms of OTA positive strains and mycotoxin production levels, especially in the Mediterranean basin. Studies point to *A. tubingensis* as the most frequent black aspergilli species isolated in vineyards. Thus, fungal competition may mainly involve *A. tubingensis* and *A. carbonarius*. Climatic change prediction appoint to drier and hotter climatic scenarios where *A. tubingensis* could be even more prevalent over *A. carbonarius* since it is better adapted to extreme hot temperature and drier conditions. Such situation might result in a decrease in the OTA levels encountered in wine in the long term.

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