CHAPTER 9

DISCUSSION

A particular discussion of the results found in this work has been provided in the discussion section of each of the manuscripts presented in this thesis. However, a general discussion is presented afterwards, focussed to reflect the present state of the art of the occurrence of OTA in grape derivatives, especially in Spanish wines, the origin of OTA and the ecophysiological parameters influencing ochratoxigenic fungi.

9.1. OTA IN WINES, MUSTS AND GRAPE JUICES: OCCURRENCE, AND METHODS OF ANALYSIS

The main parameters of wine appreciation have been, throughout the years, colour, flavour and taste. However, the development of new agricultural practices and the sharpness of analytical quality control, particularly associated to the enhancement of instrumental resolution which allows the detection of more of the so called trace compounds and in smaller concentrations, have placed food safety as one of the main factors influencing the consumers' opinion. Thus, marketing strategies also begin to take such variable into account (Ratola et al., 2004). OTA is amongst several compounds with undesirable effects to human health which have been investigated in wines.

The natural occurrence of OTA, had been associated to a large number of food commodities but mainly with cereals and derived products, until 1995, when Zimmerli and Dick reported for the first time, the presence of OTA in 18 samples of wine (Zimmerli and Dick, 1995). They suspected that the consumption of wine, together with coffee and beer, could offer a possible explanation of why, when analysing the serum of a representative part of Switzerland population, men showed significantly higher concentrations of OTA than women. The discovery might also explain the higher concentrations of OTA in population from the south of the country, where wine was consumed more regularly than in north of the Alps. These suspicions encouraged the authors to investigate more intensively the presence of OTA in wine and its derivatives, and their possible contribution to the daily intake of this toxin. Thus, in 1996, the first two publications about the OTA occurrence in wines and grape juices appeared (Majerus and Otteneder, 1996; Zimmerli and Dick, 1996). Since then, an unceasing number of studies of OTA in these beverages, have revealed the presence of this toxin world-wide and increased the concern over wine, as it has been demonstrated one of the major sources of OTA in developed countries.

Thirteen studies published until 2001 were reviewed in this thesis (Bellí et al., 2002; see 6.3.4.2.). Results from different publications were not directly comparable principally because of the different methods of analysis used. In addition, the percentage of samples containing OTA depended on the detection limit (DL) value. The lower the DL, the higher the percentage of positive samples was, and therefore, higher DL values imply that probably many samples containing low values of OTA were not detected.

Amounts of OTA found in red wines were higher than those in rosé and white ones, as at the early steps of infection, the fungi grow outside the grape, and the longer contact of the must with the skins of the grapes in the production of red wines will extract more OTA.

The number of samples across the sampling strategy will not ever satisfy the statistician's criteria for statistical significance; in addition, the number of samples of red wine in all studies was much higher than the number of the others, perhaps contributing to distort the reported percentage of samples contaminated. Stander and Steyn (2002) suggested that when the grapes are in a more advanced stage of fungal infection, OTA occurs throughout the fruit, leading to similar levels in white and red wine. What is known, is that OTA is formed prior to alcoholic fermentation (Zimmerli and Dick, 1996); therefore, fungal contamination and OTA formation can occur in the vineyards or while the grapes are being transported or during storage. Further discussion on this topic, together with the possibility of a North-South gradient in the OTA contamination, regarding the geographical origin of the samples, and the agricultural and wine-making practices influencing OTA content of wines, was detailed in the review.

The accumulation of OTA in wine could depend also on other factors such as climate, and particularly the meteorological conditions of each vintage. This could be related to the theory supported by several authors that the more southerly in Europe the wine samples are originated, the higher the frequency of occurrence and concentration of OTA (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000). To some extent, climatic conditions influence the mycoflora composition of grapes.

In view of the significance of OTA contamination of grapes, the occurrence of this toxin in grape juices was also reviewed. All the studies highlighted the imperative importance of controlling OTA in these beverages, since the high levels detected and above all, the potential consumption of juices by children.

After the publication of the review presented in this thesis (2002), new methods to determine OTA in wine and modifications of the proposed ones have appeared. Most of these recent methodologies continue using the fluorescent properties of OTA for subsequent detection and quantification. Liquid chromatography (LC) combined either with electrospray ionisation tandem mass spectrometry (MS-MS) and RP-18 SPE (Leitner et al., 2002; Bacaloni et al., 2005), combined anion exchange/reversed-phase clean-up and LC-tandem-MS analysis (Reinsch et al., 2005), etc., were shown to be

alternatives to the already established and effective HPLC-FD protocols. Dall'Asta et al. (2003) proposed a new method for the detection of OTA in wine, by directly injecting the liquid in the chromatographic system without any extraction or clean-up, as they used an alkaline mobile phase which enhanced OTA fluorescence and facilitated its detection. Reduction of the use of solvents were also proposed by González-Peñas et al. (2004) by a liquid-liquid microextraction technique, consisting in extracting the mycotoxin with an organic solvent immobilized in the pores of a porous hollow fibre immersed in the donor phase, and by Serra et al. (2004), who used a hydrogen carbonate and PEG solution in the extraction step.

Wine contaminated with OTA continues to be a problem even with the recent-established tolerance levels for OTA in this beverage; thus new validated methods to determine this toxin in wine are needed for both surveillance and research.

9.2. OTA IN WINES, MUSTS AND GRAPE JUICES FROM SPAIN

The occurrence of OTA in Spanish wines and grape beverages has been determined in 240 samples, collected in 2002 (Bellí et al., 2004a; see 6.4.3.). The sampling plan of this study did not attempt a comprehensive coverage of the entire Spanish market, nonetheless the majority of the leading brands of four Designations of Origin were sampled. Since only one sample of each brand was taken, it is difficult to give any clear interpretation on the relative significance of the results for individual brands in this survey. However, from the finding that more than 17 % of the samples contained OTA, a significant problem seems to exist with some sources of wine, which affected most of the wine-producing companies.

The significance of the results was clearly explained and correlated to the colour and the origin of the wine, the beverage category, the aging wine received, etc. Legislation in Spain was then absent, hence OTA content of the samples was approximately evaluated according to non-official maximum levels proposed by several institutions. Nowadays, Spanish legislation fixes maximum limit of OTA in wines of 2 $\mu g\ l^{-1}$ (see 6.6.). In the survey, this level was exceeded by six samples: two red wines and four special wines, three of them dessert wines. Regarding the last ones, their consumption is occasional and the ingested quantities are generally limited, so although presenting the highest OTA levels, their contribution to daily OTA intake could be considered rather small.

As Spanish wine is to an increasing extent traded across borders, a wider analysis of Spanish grape-derived products is required to improve the knowledge about the OTA contamination of these products and to further protect the health of the consumers. Thus, several works have contributed to this issue. Data about the proportion of contaminated samples and the detected level of OTA, only from Spanish origin, was compiled and summarized in table 37. Red wines were mostly analysed by all authors, and our study

presented the lowest percentage of red samples contaminated (18 %), although the highest average level (0.55 μg l^{-1}), due to the unusually high level of OTA of a few samples.

OTA is of toxicological concern in dessert wines, where percentage of incidence of contamination was the highest (45-100 %), sometimes levels exceeding the legal established limits (Burdaspal and Legarda, 1999; Bellí et al., 2004a). This could be connected to the specific winemaking technique that requires overripening of the grape on the vine, which may increase the susceptibility of grapes to fungal attack.

In 2002, Mantle said that 'zero OTA is desirable: a trace is probably acceptable, but the size of the trace is the question in the current state of knowledge'. However, it is important not to exaggerate the degree of natural contamination either of wines or other foodstuffs. Generally the concentration of OTA in food does not exceed a few ppb. In very exceptional cases values of the order of ppm have been detected, but the increasingly sophisticated analytical methodology, has revealed trace amounts of OTA quite widely in foodstuffs (Mantle, 2002).

In practice, considering OTA tolerance at 5 ng kg^{-1} body weight day^{-1}, it can be calculated that a person weighing 60 kg should not have a daily intake exceeding 0.3 μg of OTA. This is roughly equal to the amount in a 0.75 litre bottle of wine containing 0.4 μg l^{-1} OTA, supposing that the person does not ingest OTA from other sources. In this context, the detrimental effects of ethanol present in wine will outstand the negative-health effects of OTA.

Table 37. OTA occurrence in Spanish wines and grape beverages.

Samples	Positives/ Total (% of +)	Mean (µg l^{-1})	Range (µg l^{-1})	Detection Limit (µg l^{-1})	Reference
Red wine Dessert wine	9/14 (64.2 %) 5/5 (100 %)	0.009 0.19	D.L.-0.022 D.L.-0.45	0.005	Zimmerli and Dick (1996)
Red wine	3/6 (50 %)	0.13	D.L.-0.19	0.01	Majerus and Otteneder (1996)
Red wines Rosé wines White wines Special wines Dessert wines Sparkling w.	66/72 (91.6 %) 24/26 (92.3 %) 35/43 (81.3 %) 23/27 (85.2 %) 13/14 (92.8 %) 10/12 (83.3 %)	0.04 0.03 0.04 0.05 1.06 0.01	D.L.-0.60 D.L.- 0.15 D.L.-0.27 D.L.-0.25 D.L.-2.54 D.L.-0.04	0.003	Burdaspal and Legarda (1999)
Red wines	3/6 (50 %)	-	0.10-0.50	0.002	Markaki et al. (2001)
Red wines White wines	13/28 (46.4 %) 7/12 (58.3 %)	0.146 0.188	0.056-0.32 0.154-0.21	0.05	López de Cerain et al. (2002)
Red wines White wines Sparkling w. Musts Grape juices Special wines	24/130 (18.4 %) 4/50 (8 %) 4/10 (40 %) 2/20 (10 %) 0/10 (0 %) 9/20 (45 %)	0.55 0.37 0.44 0.13 0 4.47	D.L.-4.24 D.L.-1.13 0.14-0.71 0.06-0.18 N.D. 0.09-15.25	0.05	Bellí et al. (2004a)
Red wines Rosé wines White wines Dessert wines	21/61 (34.4 %) 12/21 (57.1 %) 1/21 (4.7 %) 8/13 (61.5 %)	0.28 0.30 0.09 0.27	D.L.-0.28 D.L.-0.46 D.L.-0.09 D.L.-0.40	0.05	Blesa et al. (2004)

N.D., not detected; D.L., detection limit.

9.3. ORIGIN OF OTA

9.3.1. Mycobiota of grapes

Mould growth in wine is strongly inhibited by ethanol and the anaerobic conditions during the fermentation process (Otteneder and Majerus, 2000). Therefore, the origin of the production of OTA should be found in field. As OTA is a natural contaminant and its complete elimination is impossible, the major goal of wine producers should be to prevent and control OTA production in the field for human health safety. In consequence, it was important to find out the moulds responsible for the production of the toxin and determine their role among the mycoflora present in grapes. Several studies since 1997, have attempted to identify the characteristic mycoflora of grapes, screening for the presence of ochratoxigenic fungi (Table 38).

Table 38. Studies of grape mycoflora from different countries.

Code	Country	Year sampling	Vine-yards (bunches)	Samplings (growth stages)[1]	Reference
A	Portugal	1999	4 (-)	2 (r., h.)	Abrunhosa et al. (2001)
B[2]	Italy	1999 2000	9	2 (e.v., r.) 4 (s., b.i., e.v., r.)	Battilani and Pietri (2002)
C	Argentina and Brazil	1997- 1998	n.r. (10)	1 (h.)	Da Rocha Rosa et al. (2002)
D	Southern France	n.r.	n.r. (11)	n.r.	Sage et al. (2002)
E	Italy	1999 2000	9 (180) 9 (360)	2 (e.v., r.) 4 (s., b.i., e.v., r.)	Battilani et al. (2003a)
F	Argentina	2001	n.r. (50)	1 (h.)	Magnoli et al. (2003)
G[2]	Portugal	2001	11 (330)	3 (s, e.v., h.)	Serra et al. (2003)
H	Spain	2001	7 (280)	4 (s., b.i., v., h.)	Bau et al. (2005)
I	Portugal	2001 2002 2003	11 (330) 11 (330) 11 (330)	3 (s, e.v., h.) 3 (s, e.v., h.) 3 (s, e.v., h.)	Serra et al. (2005)
J	Spain	n.r.	12 (52)	1 (h.)	Medina et al. (2005)
K	Spain	2001	40 (1600)	4 (s., b.i., v., h.)	Bellí et al. (2004b)
L	Spain	2002 2003	40 (1200) 40 (1200)	3 (b.i., v., h.) 3 (b.i., v., h.)	Bellí et al. (2005a)

n.r. not reported; [1]Growth stages: s, setting; b.i., berry increase; e.v., early veraison; v, veraison; r, ripening; h, harvest; [2]Results of studies coded as B and G are enclosed in later publications E and J, respectively.

Table 39 shows the results of each of the mycoflora surveys mentioned above, with the last two columns showing those of the studies presented in this thesis. Similar genera were found in most of the studies despite the different origin of the samples, prevailing *Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp. and *Penicillium* spp., above all. Bunches collected in our studies were in general free of visual infection, except *Botrytis* mouldy appearing in some regions and near harvest (Figure 51). *Botrytis cinerea* is regarded as the highly desirable "noble rot" in certain wine grapes, but it is by far the most serious cause of spoilage in table grapes (Pitt and Hocking, 1987). *Botrytis* was also reported as a predominant genera in other countries such as France, Portugal, Argentina and Brazil.

Figure 51. *Botrytis* infection in a bunch of grapes.

Table 39. Mycoflora isolated from grapes of different countries. Cells in grey means that the genera prevailed in those regions.

Fungi	A[a]	B	C	D	E	F	G	H	I	J	K	L
Deuteromycetes												
Absidia spp.											x	
Acremoniella spp.									x			
Acremonium spp.				x				x	x	x		
Alternaria spp.	x		x	x		x		x	x	x	x	x
Arthrinium spp.									x			
Aspergillus spp.	x	x	x	x	x	x	x	x	x	x	x	x
Aureobasidium spp.	x			x					x			
Beauveria spp.									x			
Botrytis spp.	x		x	x				x	x			x
Chaetomium spp.									x			
Cladosporium spp.	x		x	x		x		x	x	x	x	x
Chrysonilia spp.									x			
Curvularia spp.				x					x			
Dendryphiella spp.									x			
Drechslera spp.				x					x			
Emericella spp.									x			
Epicoccum spp.				x					x		x	x
Eurotium spp.						x			x			
Fusarium spp.	x		x	x		x			x	x	x	x
Geotrichum spp.			x	x					x			
Gliocladium spp.				x					x			
Histoplasma spp.									x			
Harzia spp.				x								
Microdochium spp.				x								
Monilia spp.								x				
Moniliella spp.			x									

Mucor spp.									x			x
Neurospora spp.									x			
Nigrospora spp.									x			
Paecilomyces spp.									x			
Penicillium spp.	x	x	x	x	x	x	x	x	x	x	x	x
Periconia spp.									x			
Pestalotiopsis spp.									x			
Phoma spp.				x					x	x		x
Pithomyces spp.									x			
Phytophtora spp.			x									
Rhodotorula spp.				x								
Scopulariopsis spp.				x								
Scytalidium spp.									x			
Stemphylium spp.				x					x			
Syncephalastrum spp.									x			
Thricoderma spp.				x				x	x			x
Tricothecium spp.	x								x			
Truncatella spp.									x			
Ulocladium spp.				x	x				x		x	x
Verticillium spp.									x			
yeast			x	x								x
Ascomycetes												
Coniochaeta spp.				x								
Emericella spp.				x								
Neoosartorya spp.				x								
Sordaria spp.				x								
Zygomycetes												
Actinomucor spp.				x								
Rhizopus spp.				x					x	x	x	x

[a] A, Abrunhosa et al. (2001); B, Battilani and Pietri (2002); C, Da Rocha Rosa et al. (2002); D, Sage et al. (2002); E, Battilani et al. (2003a); F, Magnoli et al. (2003); G, Serra et al. (2003); H, Bau et al. (2005); I, Serra et al. (2005); J, Medina et al. (2005); K, Bellí et al. (2004b); L, Bellí et al. (2005a).

Reliable identification required considerable expertise and sometimes classification within genera was complex, due to the plasticity of species like *Aspergillus* and *Penicillium*. However, *Aspergillus* and *Penicillium* genera were identified at species level in some of the studies (Table 40 and 41). *A. aculeatus, A. carbonarius* and *A. niger* were also isolated from fresh, semi-dried and dried grapes, in a study carried out in 222, 199 and 385 bunches from Australian vineyards in 1998, 1999 and 2000, respectively (Leong et al., 2004).

Table 40. *Aspergillus* species isolated from grapes of several countries.

***Aspergillus* species**	**A[a]**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
A. aculeatus				x				x	x			
A. auricomus									x			
A. candidus						x			x			
A. carbonarius		x	x	x	x		x	x	x	x	x	x
A. carneus									x			
A. clavatus							x		x			
A. flavipes									x			
A. flavus			x			x	x	x	x	x		
A. foetidus						x						
A. fumigatus		x		x	x		x	x	x			
A. ibericus									x			
A. japonicus					x		x	x	x			
A. alliaceus							x		x			
A. melleus								x				
A. niger aggregate		x	x	x	x	x	x	x	x	x	x	x
A. ochraceus			x		x		x	x	x			
A. ostianus									x			
A. parasiticus								x				
A. tamarii								x				
A. terreus			x	x			x	x	x			
A. ustus			x	x			x	x	x			
A. variecolor								x				
A. versicolor							x	x	x			
A. wentii							x		x			
uniseriates (n.i.)											x	x

n.d. not identified at specie level; [a] [a] A, Abrunhosa et al. (2001); B, Battilani and Pietri (2002); C, Da Rocha Rosa et al. (2002); D, Sage et al. (2002); E, Battilani et al. (2003a); F, Magnoli et al. (2003); G, Serra et al. (2003); H, Bau et al. (2005); I, Serra et al. (2005); J, Medina et al. (2005); K, Bellí et al. (2004b); L, Bellí et al. (2005a).

Table 41. *Penicillium* species isolated from grapes of several countries.

***Penicillium* species**	**A[a]**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
P. altramentosum				x								
P. aurantiogriseum	x						x		x			
P. bilaiae									x			
P. brevicompactum	x			x			x	x	x			
P. canescens				x				x	x			
P. chrysogenum			x	x		x	x	x	x			
P. citrinum			x	x			x	x	x			
P. citreonigrum				x								
P. corylophilum							x	x	x			
P. crustosum						x	x		x			
P. decumbens			x					x				
P. echinulatum				x			x		x			
P. expansum	x			x			x		x			
P. fellutanum							x		x			
P. funiculosum					x		x		x			
P. glabrum	x		x	x	x	x	x	x	x			
P. griseofulvum				x				x	x			
P. implicatum							x		x			
P. italicum								x				
P. janczewskii							x		x			
P. lividum								x				
P. miczynskii				x			x		x			
P. minioluteum	x			x			x		x			
P. novae-zeelandiae									x			
P. olsonii									x			
P. oxalicum				x			x	x	x			
P. paxilli				x								
P. pinophilum		x					x	x	x			
P. purpurogenum				x			x	x	x			
P. raistrickii							x		x			
P. restrictum									x			

P. roquefortii							x		x			
P. rugulosum				x					x			
P. sclerotiorum							x	x	x			
P. simplicissimum							x		x			
P. solitum									x			
P. spinulosum	x			x	x							
P. thomii	x			x	x		x	x	x			
P. variabile							x	x	x			
P. verrucosum							x		x			
P. waksmanii									x			
Species not identified										x	x	x

n.d. not identified at specie level; [a] [a] A, Abrunhosa et al. (2001); B, Battilani and Pietri (2002); C, Da Rocha Rosa et al. (2002); D, Sage et al. (2002); E, Battilani et al. (2003a); F, Magnoli et al. (2003); G, Serra et al. (2003); H, Bau et al. (2005); I, Serra et al. (2005); J, Medina et al. (2005); K, Bellí et al. (2004b); L, Bellí et al. (2005a).

9.3.2. Black aspergilli distribution and OTA production

Black aspergilli species are ubiquitous saprophytes present in soils around the world (Klich and Pitt, 1988). They were often encountered in Spanish vineyards in a rough range of 5-10 % of the total mycoflora detected. Black spores offer these fungi protection from sunlight and UV light, providing a competitive advantage in such habitats (Pitt and Hocking, 1997). However, they are not primary invaders of grapes, growing only as the result of damage by plant pathogenic fungi, insects or of skin splitting due to for example rain. A fraction of the black species were randomly chosen to further identification, classification and OTA analysis, demonstrating that the 4.6 to near 30 % of the isolates were able to produce this toxin *in vitro* (Table 42).

However, black aspergilli occurrence changed significantly with the maturation stage of the berries. *Aspergillus* section *Nigri* were present on grape bunches early in the season and their frequency increased in later growth stages. Bellí et al. (2005a) suggested that late ripening marks a profund change in the ecological factors affecting fungal sporulation, dissemination of spores as well as microbial growth. Moreover, grapes are more susceptible to fungal infection when approaching harvest as sugar content increases and the berry texture softens (MAPA, 1998). Results for a four-year survey are summarized in table 43, where a representative part of the total black aspergilli was isolated, classified and tested for OTA. Results of 2004 were not directly comparable with the results of the previous years, as fewer vineyards were sampled that year. General trends, however, appeared to be the same.

Table 42. Black aspergilli isolated from grapes and their ability to produce OTA (see 7.8).

Year	Total[a]	% mycoflora[b]	Num. black aspergilli isolated	OTA +	% OTA +[c]
2001 A	n.r.	n.r.	386	18	4.6
2002 B	464	7.7	240	17	7
2003 B	648	10.8	401	101	25
2004	39	4.6	34	10	29

n.r. not reported; [a]Total number of black aspergilli detected in grapes; [b]percentage of black aspergilli from the total number of moulds visually counted in grapes; [c]percentage of OTA + referred to the number of isolated strains.

A: Bellí et al. (2004). Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *J. Sci. Food Agric.* 84, 541-546.

B: Bellí et al. (2005). Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. *Europ. J. Plant Pathol.* (in press).

Table 43. Black aspergilli distribution among regions, years and grape stage.

		Uniseriates (OTA+)				***A. niger* aggregate (OTA+)**				***A. carbonarius* (OTA+)**			
Region	S[a]	2001A	2002B	2003B	2004	2001	2002	2003	2004	2001	2002	2003	2004
P/CB	1	n.c. (0)	0 (0)	0 (0)	-[b]	n.c. (0)	1 (0)	2 (0)	-	n.c. (0)	0 (0)	0 (0)	-
UR		n.c. (0)	0 (0)	0 (0)	-	n.c. (0)	6 (0)	11 (0)	-	n.c. (0)	0 (0)	0 (0)	-
R		n.c. (0)	4 (0)	4 (0)	-	n.c. (0)	12 (0)	28 (0)	-	n.c. (0)	0 (0)	0 (0)	-
CS		n.c. (0)	0 (0)	12 (0)	-	n.c. (0)	2 (0)	6 (0)	-	n.c. (0)	0 (0)	0 (0)	-
P/CB	2	n.c. (0)	15 (0)	34 (0)	-	n.c. (0)	10 (0)	15 (1)	-	n.c. (0)	0 (0)	1 (1)	-
UR		n.c. (0)	24 (0)	14 (0)	-	n.c. (0)	71 (0)	33 (2)	-	n.c. (1)	1 (1)	0 (0)	-
R		n.c. (0)	0 (0)	0 (0)	-	n.c. (1)	0 (0)	4 (2)	-	n.c. (0)	0 (0)	0 (0)	-
CS		n.c. (0)	0 (0)	0 (0)	-	n.c. (1)	15 (0)	16 (1)	-	n.c. (2)	0 (0)	6 (0)	-
P/CB	3	n.c. (0)	1 (0)	0 (0)	0 (0)	n.c. (0)	53 (0)	58 (5)	10 (0)	n.c. (0)	1 (0)	0 (0)	4 (4)
UR		n.c. (0)	0 (0)	0 (0)	0 (0)	n.c. (1)	3 (1)	2 (-)	8 (0)	n.c. (0)	7 (6)	7 (7)	0 (0)
R		n.c. (0)	0 (0)	2 (0)	0 (0)	n.c. (4)	2 (1)	8 (-)	-	n.c. (0)	0 (0)	0 (0)	-
CS		n.c. (0)	1 (0)	6 (0)	0 (0)	n.c. (1)	6 (1)	28 (-)	6 (0)	n.c. (7)	8 (7)	104 (82)	6(6)
TOTAL		**n.c. (0)**	**45 (0)**	**72 (0)**	**0 (0)**	**n.c. (8)**	**181(3)**	**211 (11)**	**24 (0)**	**n.c. (10)**	**17 (14)**	**118 (90)**	**10 (10)**
% [c]		n.c.	18 %	18 %	0 %	n.c.	75 %	53 %	70 %	n.c.	6 %	29 %	30 %

[a] S, sampling; P/CB, Penedès/Conca-Barberà; UR, Utiel-Requena; R, La Rioja; CS, Costers del Segre; n.c. not classified; [b] - not tested; [c] % of each group from the total number of black aspergilli isolated.

A: Bellí et al. (2004). Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *J. Sci. Food Agric.* 84, 541-546.

B: Bellí et al. (2005). Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. *Europ. J. Plant Pathol.* (in press).

Some other field surveys carried out world-wide classified black aspergilli in the three groups (*A. carbonarius*, *A. niger* aggregate and uniseriate species) and also tested OTA production in a large number of isolates (Table 44). Most of the studies used Bragulat et al. (1998) methodology, since it was quick, easy, clean and economical with respect to reagents and time (Sage et al., 2002; Serra et al., 2003; Bau et al., 2005; Bellí et al., 2004b; 2005a). However, other authors preferred to screen the production of OTA by testing the characteristic fluorescence of this toxin on the growth medium (Da Rocha Rosa et al., 2002; Leong et al., 2004), or other methodologies such as the one proposed by Téren et al. (1996) had the same function (Magnoli et al., 2003).

Table 44. Occurrence of black *Aspergillus* species in grapes and their ability to produce OTA, reported by several authors.

Unis. species	OTA + (%)	*A. niger* aggr	OTA + (%)	*A. carb*	OTA + (%)	Reference
-	-	48[a] 53[b]	8 (17 %) 16 (30 %)	32	8 (25 %)	Da Rocha Rosa et al. (2002)
-	-	73	0 (0 %)	15	14 (93 %)	Sage et al. (2002)
108	3 (3 %)	270	14 (5 %)	86	52 (60 %)	Battilani et al. (2003a)
63[c] 26+ (41 %)[c]						Magnoli et al. (2003)
200	0 (0 %)	470	0 (0 %)	245	245 (100 %)	Leong et al. (2004)
5	0 (0 %)	474	0 (0 %)	101	101 (100 %)	Bau et al. (2005)
-	-	85	3 (3 %)	120	89 (74 %)	Medina et al. (2005)
-	-	571	4 (0.7 %)	68	68 (100 %)	Serra et al. (2005)

[a] Grapes from Argentina; [b] Grapes from Brazil; [c] Reported as *Aspergillus* section *Nigri*.

Comparing the totality of the studies, it is seen that *A. niger* aggregate was the dominant species in grapes. On average, this group represented the highest percentage among the black isolates collected [60 % (Battilani et al., 2003); 61 % (Magnoli et al., 2003), 53-75 % Bellí et al., 2004b; 2005a)], followed by *A. carbonarius* [19 % (Battilani et al., 2003); 6-30 % (Bellí et al., 2005a)], and variable percentage of uniseriate species [21 % (Battilani et al., 2003); 0-18 % (Bellí et al., 2005a)]. Species not belonging to the *Nigri* section represented low percentages of the *Aspergillus* isolates [0.04-0.5 % (Bau et al., 2005); < 3 % (Battilani et al., 2003)].

Within black aspergilli, *A. carbonarius* and members belonging to the *A. niger* aggregate are considered as clearly OTA-producing species. The possibility that OTA can be produced by *A. carbonarius* was discovered only in the past few years (Heenan et al; 1988; Horie, 1995; Varga et al., 1996). However, *A. niger* species are unlikely to be significant sources of OTA in grapes, as reported percentages of ochratoxigenic isolates belonging to this group (0-30 %) are much lower than in *A. carbonarius* (25-100 %). Misleading in classification and the unawareness of the new reported specie *A. ibericus*, could be the reason why some of the studies found percentages of *A. carbonarius* OTA+ lower than 100 %. Furthermore, *A. carbonarius* produced higher amounts of OTA than the other black aspergilli, when tested *in vitro* (Table 45). Finally, uniseriate species are discarded as responsible for the OTA levels in the surveyed areas, as only occasional strains were positives, but the vast majority did not have the ability to produce the toxin.

Table 45. OTA-producing ability of black aspergilli isolated from grapes.

Type	Range (μg g^{-1})	Medium	Reference
A. niger aggregate *A. carbonarius*	26-96 18-234	YES	Da Rocha Rosa et al. (2002)
A. carbonarius	0.5-87.5	YES + CYA	Sage et al. (2002)
A. carbonarius *A. niger* aggregate uniseriate	> 0.1 < 0.010 < 0.001	CYA + sucrose	Battilani et al. (2003)
A. section *Nigri*	2-24.5 μg l^{-1}	YES	Magnoli et al. (2003)
A. niger aggregate *A. niger* aggregate *A. carbonarius* *A. carbonarius*	0.137[a] 0.026[a] 1.129[a] 0.327[a]	CYA /YES GJ50[b] CYA /YES GJ50[b]	Serra et al. (2005)
A. carbonarius	1.92-195.5	CYA	Bau et al. (2005)
A. carbonarius *A. tubingensis*	1.2-3530 μg l^{-1} 46.4-111.5 μg l^{-1}	YES + bee pollen	Medina et al. (2005)

[a] Mean values; [b] Grape juice extract.

Although *A. ochraceus* and *P. verrucosum* were considered the classical OTA-producing species, they are rarely isolated from grapes, discarding their importance as a source of OTA in wine. However, their importance in other foodstuffs should not be mislead: *P. verrucosum* continues appearing in cool temperate regions and has been reported almost exclusively in cereal products, while *A. ochraceus* is found sporadically in different commodities in warmer and tropical climates (Pitt and Hocking, 1997).

9.3.3. OTA in musts

OTA was not detected in any of the musts analysed in 2002 and 2003, although in 2001, 15 % of the musts contained low amounts of OTA (0.09-0.81 μg l^{-1}) (Bellí et al., 2004b; 2005a). This could be attributed to the differences in meteorological conditions among these years. Moreover, as with other moulds and mycotoxins, it is crucial that sampling is carried out in a way that ensures that OTA in the analytical sample is truly representative of the consignment, and higher number of bunches used in the analysis may have improved the accuracy of the results.

Musts obtained after crushing grapes obtained from French vineyards, were also analysed (Visconti et al., 1999 methodology) by Sage et al. (2002). They found 8 contaminated musts out of 11 (73 %) with an OTA content that ranged from <10 to 461 ng l^{-1}. A strong correlation between the presence of OTA-producing strains on grapes and the finding of OTA in musts was also stated. The year of the study was not reported, but probably coincides with the most contaminated year in our surveys, 2001, as the study was published one year later.

9.3.4. Parameters influencing black aspergilli occurrence and OTA content

Meteorological conditions could be stated as the main parameter influencing black aspergilli occurrence and OTA content in grapes. Temperature and humidity differences between years and grape-growing areas are responsible for differences, together with other meteorological parameters. For example, vines under physiological stress due to drought, excessive water and cold weather, are also more susceptible to fungal infection (Stander and Steyn, 2002). This was also observed by Battilani et al. (2003b), who found significantly more OTA in 1999 than in 2000.

Different practices that are applied in grape cultivation, such as the use of pesticides and different cultivars, and in wine-making, time period and conditions of storage of the harvested grapes, type of maceration, time and temperature of fermentation, can influence the accumulation of mycotoxins (Zimmerli and Dick, 1996). Particularly, the use of pesticides has been studied both *in vitro* and *in vivo* to prevent *A. carbonarius* growth and OTA production (Bellí et al., 2005b; 2005c). Both infection and OTA production were reduced when using the mixture cyprodinil 37.5 %+fluodioxonil 25 % or azoxystrobin, tested on grapes *in vitro*. Penconazole 10 % also showed a clear reduction of the OTA production, meanwhile fenhexamid 50 %, mancozeb 80 % and copper 50 % enhanced both infection and toxin production. A field experiment showed that a mixture of cyprodinil 37.5 % and fluodioxonil 25 % applied at two different and particular periods, or combined with a second application of cyprodinil 50 %, were the most effective treatment to prevent black aspergilli in grapes. However, additional studies are required

to recommend effective fungicides, doses, application time and other related parameters against black aspergilli infection in vines.

Most of the species isolated from grapes, were obtained from berries which showed no symptoms. Black aspergilli are weak in penetrating the intact berry epidermis, thus fungal infection of intact grapes was hardly observed (Leong et al., 2004). Bellí et al. (2005d) demonstrated that any opening in the grape skin facilitated *A. carbonarius* colonization and OTA production in grapes. Thus, prevention of skin damage caused by insects, with biological or chemical methods, may be a useful tool to reduce fungal infection.

Variety could also be another factor influencing black aspergilli infection and OTA contamination, as different varieties have different susceptibility to diseases, sizes of the bunches, compactness, thickness of the berry skin, etc. Although several varieties were chosen in the studies of grape mycoflora, the experiments in this thesis were not designed to study the effect of the variety. However, a couple of studies in the literature dealt with this aspect. Battilani et al. (2004) found relevant differences among 12 red and white varieties tested, but it was not possible to correlate the susceptibility of each grape variety to *A. carbonarius* infection with the intrinsic characteristics of the grape. Similar conclusion was withdrawn by Medina et al. (2005), as they found that grape variety had a strong influence on the occurrence of black aspergilli, but the differences were uncorrelated to berry colour or other grape characteristics.

9.4. ECOPHYSIOLOGICAL PARAMETERS INFLUENCING OCHRATOXIGENIC FUNGI

Several parameters such as temperature, water availability, light, presence of preservatives, effect of vectors, etc. have been studied in this thesis, both in growth and in OTA production by black aspergilli, trying to simulate as far as possible what happens in the field. It is important to highlight that results obtained in culture media cannot easily be extrapolated to natural systems. Most of the parameters studied are not constant in the field, presenting above all, temporary (hours, days, season, year…) and geographical variations. Study of one or few parameters is neither reliable, as actually many factors influence, with connections among them. The same parameters are continuous in natural conditions, whereas in the laboratory, discontinuous values are used to carry out the studies. More difficulties are related to the media used in the experiments, as usually they are not the real substrate, but *in vitro* simulations, sometimes far from the reality. In addition, individual species are used in laboratory studies, meanwhile in the field, berries carry an inoculum of many species leading to many interactions among them. However, ecophysiological studies *in vitro* are not useless, as they provide an overall idea of the conditions governing the fungal development together with estimations of the levels favouring growth and mycotoxin production that although slightly differing from the field reality, could facilitate the establishment of preventive and control measures.

9.4.1. Growth of black aspergilli

Some authors have studied the growth of black aspergilli under different influencing parameters, and the main results are summarised in table 46, together with the results of the studies presented in this thesis.

Growth of black aspergilli correlated well to the a_w of the substrate. Lower a_w values adversely affected growth, with minimum reported in most of the studies around 0.90-0.93 a_w. Pandey et al. (1994) stated that decreased a_w of the substrate led to lower mass transfers and water availability for the microorganism and may thus be responsible for an incomplete conversion of the substrate to biomass. Values of a_w over 0.95 were usually favourable for growth, being the optimum between this value and maximum water availability: 0.995-1 a_w. Levels of a_w in grapes could vary because of many factors, such as variety, climate, sugar content increasing with ripening, etc. but at harvest, it is reported between 0.95-0.99 a_w (Bellí et al., 2005e). As natural habitat of spores and growing fungi on grapes is their surface, relative humidity of the surroundings determines the development rate of these fungi; as stated before, humid years, with high rainfalls or regions where watering is done by sprinkling or similar water-spraying systems are the more propitious for *Aspergillus* section *Nigri* infection.

Results from all the studies clearly demonstrate that black aspergilli are mesophilic, although different species showed different responses to temperature. Optimum temperatures for growth have been reported around 30 ºC for *A. carbonarius* and uniseriate species, sometimes 35 ºC for the first ones (Mitchell et al., 2003; 2004), and around 37 ºC for *A. niger* aggregate. Maximum temperatures reported for uniseriates and *A. carbonarius* growth were 37-42 ºC. Higher thermotolerance was showed by *A. niger* aggregate species, with faster growth at higher temperatures and ability to grow even at 42-45 ºC (Leong et al., 2004; Esteban et al., 2004). Minimum temperatures reported allowing growth were between 10 and 15 ºC; therefore, refrigeration temperatures can be a primary factor controlling all these fungi. At 7 ºC, no growth was observed the first 10 days of incubation independent of the a_w assayed (Marín et al., 2005). Lower temperatures conferred a strong competitive advantage on uniseriate species compared with the other two groups (Leong et al., 2004). For all that, the competence that uniseriates and *A. niger* aggregate species offered on *A. carbonarius* is high, as the first ones were dominant at lower temperatures and *A. niger* aggregate above 30 ºC. However, the occurrence of *A. carbonarius* in the vineyard environment suggests that factors other than temperature enable this species to compete effectively with the other two species (Leong et al., 2004). Accordingly, interactions between temperature and a_w were significant in most of the ecophysiological studies. Isolates were more tolerant to the adversity of one factor when the other was close to the optimum. This principle holds true for the interactions involving other parameters.

The effect of pH on the growth of black *Aspergillus* is significant, growth has been reported in the range 3.5-7.5 pH. 6.5 was the optimum pH reported for one strain of *A. niger* (Vats and Banerjee, 2002) and growth was faster at pH 4 than 7 or 2.6, regardless of the a_w level used in a study on synthetic grape medium (Mitchell et al., 2005). Growth was inhibited at pH 2.6 and 7.0 at 0.90 a_w, while at pH 4 growth was inhibited at 0.88 a_w. López-Malo et al. (1997) stated that low pH may sensitise the microorganisms, and thus they are more susceptible to other stress factors or hurdles.

The addition of some natural substances to the medium can reduce black aspergilli growth. López-Malo et al. (1997) found that the addition of vanillin to PDA medium, had a fungistatic effect on four *Aspergillus* species studied (*A. niger*, *A. ochraceus*, *A. flavus* and *A. parasiticus*). Vanillin produced an extension of the lag phase, which increased as temperature and pH decreased. It can be used to prevent mould spoilage in processes that combine the preservation effects of a_w, pH and storage temperature.

In general, growth rates of *A. niger* aggregate were similar to uniseriate species but significantly higher than *A. carbonarius* at the same conditions tested (Bellí et al., 2004c). In this last paper, a particularity with *A. carbonarius* 01UAs294 strain was observed, as it was detected a different behaviour in its growth pattern. At that time, this strain was classified as *A. carbonarius* OTA negative, but it presented some molecular differences in comparison with the other *A. carbonarius* (Dr. F.J. Cabañes, personal communication).

Nowadays this strain, which was isolated from Portuguese grapes, is reconsidered as *A. ibericus* (Serra, 2005), and more isolates have been found in grapes from this country (Serra et al., 2005). It is difficult to distinguish *A. ibericus* from the other black aspergilli by its morphological characteristics. Serra (2005) pointed out that the mean size of the *A. niger* aggregate spores is below 5 μm, the size of those of *A. ibericus* between 5 and 6 μm, while *A. carbonarius* spores are the biggest, with mean size above 6 μm. The reported size could be strain-variable among *A. carbonarius* and *A. niger* aggregate strains, while the size of *A. ibericus* spores remain constant. As the measure of the spores should be extremely precise since only differ in approximately one micrometer to each other, more characteristics such as OTA production, could facilitate to distinguish between *A. ibericus* species (non-producers) and *A. carbonarius* (OTA +). However, differentiation among the species belonging to the *A. niger* aggregate and *A. ibericus* can only be done by measuring the spores. Because of their accuracy, molecular methods should be emphasised in the classification of black aspergilli. Finally, the growth pattern of this new taxonomic species has not been studied yet. Further revision of other OTA negatives *A. carbonarius* used in the ecophysiological studies (93cr4, 36br4 and Mu644 isolates) is required.

Apart from growth and OTA production, Mitchell et al. (2005) examined the effect of a_w and temperature on germination and germ tube extension of *A. carbonarius*. Germination was very rapid (100 % of spores successfully germinating within 24 h) at 0.90-0.99 a_w and 25-35 ^oC. At 15 ^oC germination was significantly slower with 50 % after 36 h. At 0.88 a_w, there was a lag of about 18 h before germination occurred, with almost all spores germinating after about 60 h. Furthermore, germ tube extension was just over 100 μm after 72 h at 15 ºC, while at all other temperatures tested the germ tubes had reached about 300 μm in 24 h. At 0.85 a_w there was no germination in the time frame of the experiment. Other studies of germination of *A. niger* group species prior to the taxonomic knowledge of different species in the section *Nigri* and the ability to produce OTA have been carried out, although they may not be strictly comparable. Ayerst (1969) and Marín et al. (1998) showed that isolates from grain germinated at 0.82-0.83 a_w at optimum temperatures with mycelial growth occurring over the range 0.83-0.995 with optimum conditions at 0.995 and 35 ^oC.

Lag phases for growth were noticeably influenced by both temperature and a_w. Generally, the time required to reach the linear phase increased with decreasing temperatures and decreasing a_w (Bellí et al., 2004c).

Table 46. Summary of the different conditions studied by several authors on black *Aspergillus* growth; range of conditions allowing growth is in brackets and optimum conditions highlighted in grey.

Number and type of isolates	Substrate	a_w	T (ºC)	pH	Time experim (days)	Reference
1 *A. niger*	Wheat+ corn+ mineral solution	0.74, 0.82, 0.90, 0.94	30	-	5	Pandey et al. (1994)
1 *A. niger*	PDA	0.98	10, [15, 17.5, 25, 30]	[3, 3.5, 4]	60	López-Malo et al. (1997)
1 *A. niger*	PDA	n.r.	[25, 30, 37], 45, 50	[3.5, 4.5, 5.5, 6.5, 7.5]	30	Vats and Benerjee (2002)
9 *A. carb*	SNM	0.88, 0.90, [0.93, 0.95, 0.98, 0.99]	10, [15, 25, 35]	-	56	Mitchell et al. (2003)
8 *A. carb*	SNM	0.85, 0.88, [0.90, 0.93, 0.95, 0.98, 0.99]	10, [15, 20, 25, 30, 35,] 40]	-	56	Mitchell et al. (2004)
10 *A. nig* aggr	YES CYA	n.r.	5, [10, [15, 20, 25, 30, 35, 40, 45]	-	30	Esteban et al. (2004)
6 *A. carb*	YES CYA	n.r.	5, [10, [15, 20, 25, 30][a], 35, 40], 45	-	30	Esteban et al. (2004)
3 uniseriates 3. *A. niger* aggr 3. *A. carb*	CYA	n.r.	10, [15, 25, 30, 37], 42 10, [15, 25, 30, 37, 42] 10, [15, 25, 30, 37], 42	-	30	Leong et al. (2004)
8 uniseriates 10 *A. nig* aggr 8 *A. carb*	SNM	[0.90, 0.93, 0.95, 0.98, 0.995]	25	-	60	Bellí et al. (2004c, 1st part)

3 uniseriates 3 *A. nig* aggr 4 *A. carb*	SNM	[0.90, 0.93, 0.95, 0.98, 0.995]	[10, 15, 20, 30, 37] [10, 15, 20, 30, 37] [10, 15, 20, 30, 37]	-	60	Bellí et al. (2004c, 2nd part)
8 *A. carb*	SNM	[0.90, 0.93, 0.95, 0.99]	[15, 20, 30, 35, 37]	-	30	Bellí et al. (2005e)
4 *A. carb*	grapes	[80, 90, 100] % R.H.	[20, 30]	-	30	Bellí et al. (2005d)
4 *A. carb*	SNM	0.96	7, [15, 20, 25, 30, 35], 42	-	10	Marín et al (2005)
12 *A. carb*	SNM	0.85, 0.90, 0.93, 0.95, 0.99	10, 15, 20, 25, 30, 35, 37, 40	[2.6, 4, 7]	56	Mitchell et al. (2005)

n.r. not reported; [a]range for growth in CYA was narrower than that in YES medium.

9.4.2. OTA production of black aspergilli

OTA production was also studied in different media and it is clearly demonstrated it was growth condition-dependent as well as strain-dependent (Table 47).

High values of a_w (0.95-1 a_w), apart of favouring growth, favoured OTA production too. OTA was rarely detected below 0.90 a_w. Control of a_w could be used hence to modify metabolite production in most foodstuff (Gervais, 1990), but it is not possible an artificially modification of a_w of grapes during their development in the vine, without affecting wine quality.

In contrast, temperature ranges for OTA production were more restrictive than those for growth. 20 ºC was the most times reported optimum temperature for OTA production by *A. carbonarius* (Esteban et al., 2004; Mitchell et al., 2004; Bellí et al., 2005e; Marín et al., 2005), but the optimum range could be assumed between 15 and 25 ºC. Similar temperatures are reported as optimum for OTA production by *A. niger* aggregate isolates (Esteban et al., 2004). Only in one study carried out on grapes, *A. carbonarius* produced more OTA at 30 ºC than at 20 ºC, contrary to that reported on synthetic medium (Bellí et al., 2005d). The authors attributed the cause to the unavailability of nutrients, as grape skin acted as a barrier for the fungi to free access to the inside of the berry. Thus, when a breach or puncture was made in the skin, 20 ºC became again the optimum temperature for *A. carbonarius* OTA production. Thus, any damage on the skin of the berry may enable fungal invasion of grape and probably increase OTA production. Thus, the amount of OTA accumulated in grapes is clearly influenced by the status of the berries and both the infection and the OTA content are also favoured by high temperature and R.H.

Low temperatures stopped or minimized OTA production. However, neither is actually feasible the transport of the grapes from field to the wine cellar under refrigerated conditions, nor the realization of the initial must obtention steps under refrigeration.

The effect of pH on OTA production has been little studied. Mitchell et al. (2005) showed that more OTA was produced by *A. carbonarius* at pH 7, followed by pH 2.8, with the lowest amounts produced at pH 4 in a study at 25 ºC. This is in contrast to growth where very slow growth occurred at pH 2.8.

Composition of the medium is another factor that plays an important role in the amount of OTA produced by the fungi. The use of CYA rather than YES agar in studies of OTA was recommended by Abramson and Clear (1996). CYA (3 % sucrose) probably presents a less hydrophilic layer more permeable to lipophilic solvents compared to YES agar (15 % sucrose) (Bragulat et al. 2001) and it was also recommended by these authors in the extraction method they proposed. Addition of bee pollen to the culture medium was observed to stimulate OTA production by *Aspergillus ochraceus* (Medina et al., 2004) and later by *Aspergillus* section *Nigri* (Medina et al., 2005). Employing this substrate and

extending incubation time, Medina et al. (2005) found for the first time OTA positives isolates of *A. tubingensis*.

A. carbonarius produced more OTA than *A. niger* var. *niger* when tested on CYA, but less on YES medium (Bragulat et al., 2001). This was confirmed later in another study by the same authors (Esteban et al., 2004), where even in the respective optimal culture media, *A. carbonarius* produced higher mean amounts of OTA (0.05-150.08 μg g^{-1}) than *A. niger* aggregate (0.05-33.16 μg g^{-1}). When both groups were tested in the same medium (SNM), OTA accumulation was significantly higher again for *A. carbonarius* (Bellí et al., 2004d). However, OTA production is highly strain-dependant, and in this study, one of the strains of *A. carbonarius* tested produced less OTA than some *A. niger* aggregate isolates. Statistical differences in the optimum conditions for OTA production among strains have also been detected (Esteban et al., 2004; Mitchell et al., 2004). The different amounts of OTA found between species of the same group may be attributed to the genetic features of the strains as well as to their environment (Blumenthal, 2004). It is completely possible that non-toxic strains are selected as production organisms and vice versa, that some weak-producers loose their abilities under non-favourable conditions or after a long period of time without metabolic activity.

Because of the high a_w of the grape surface (0.95-0.99), high R.H. in the field and mean temperature levels (20 –26 ºC) in the Spanish vineyards during the months preceding harvest, with maximum temperatures surpassing 30 ºC, the probability for *A. carbonarius* growth and OTA production in grapes is high. Of course, other factors will influence positively or negatively in the real contamination by this mycotoxin.

Kinetics of OTA production was examined by few authors in black aspergilli species. In general, secondary metabolites are usually produced during the stationary phase of microbial culture growth, although exceptions do seem to occur (Kharchenko, 1999). Particularly, Calvo et al. (2002) associated mycotoxin production with sporulation in aspergilli. OTA production by black aspergilli has been shown for the first time to occur optimally after as little as five days on a grape-like medium, in a study carried out at 25 ºC (Bellí et al., 2004d). In a further study, the same authors found OTA production even before (2-4 days) (Marín et al., 2005). In both studies, OTA production was dependent on the levels of the other parameters tested, such as a_w and temperature. Esteban et al. (2004) confirmed 5 days as the optimum time for OTA production by both *A. carbonarius* and *A. niger* aggregate species.

The OTA production of most of the strains decreased sharply after incubation for long periods. Apart from *A. carbonarius*, this was also observed for other *Aspergillus* species such as *A. albertensis*, *A. melleus*, *A. ochraceus*, *A. foetidus* by Varga et al. (2001), who stated the possibility that the strains removed and assimilated the phenylalanine moiety from the OTA molecule, as other nitrogen sources of the culture became exhausted.

It has been shown that *A. carbonarius* growth is favoured by photoperiod. However, no significant differences on OTA production were detected due to photoperiod. It is accepted that black spores give protection from sunlight and UV light, providing a competitive advantage in such habitats (Pitt and Hocking, 1997). In field, apart of photoperiod, temperature also fluctuates daily and seasonally. Drastic changes in temperature may cause metabolic stress in fungi, which may result in higher or lower toxin production. However, the amount of OTA detected in an *in vitro* study was not statistically affected by alternating temperatures. Moreover, continuous presence of light did not reduce the yields of OTA, contrary to other mycotoxins behaviour.

Research to clarify the genetic background of OTA biosynthesis is in progress in several laboratories. Varga et al. (2001) concluded that OTA biosynthesis is differently regulated in different species, since they studied the effects of a variety of compounds known to affect ochratoxin biosynthesis, in two species: *A. ochraceus* and *A. albertensis*. Most of the compounds which were found to inhibit OTA biosynthesis in *A. ochraceus* had only limited or no effect on OTA production in *A. albertensis*.

Table 47. Summary of the different conditions studied by several authors on OTA production by black *Aspergillus*; range of conditions allowing OTA production is in brackets and optimum conditions highlighted in grey.

Number and type of isolates	**Substrate**	**a_w**	**T (ºC)**	**Extraction time (days)**	**Reference**
5 *A. niger* aggr 5. *A. carb*	CYA YES	n.r.	[25]	[7, 14, 21]	Bragulat et al. (2001)
9 *A. carb*	SNM	0.88, 0.90, [0.93, 0.95, 0.98, 0.99]	[20, 25]	[7, 14, 21, 28, 35, 42, 49, 56]	Mitchell et al. (2003)
8 *A. carb*	SNM	0.85, 0.88, [0.90, 0.93, 0.95, 0.98, 0.99]	10, [15, 20, 25, 30, 35], 40	[5, 10, 15, 20]	Mitchell et al. (2004)
10 *A. nig* aggr	YES CYA	n.r.	5, [10, 15, 20, 25, 30], 35], 40, 45[a] 5, 10, [15, 20, 25, 30], 35], 40, 45	[5, 10, 15, 20, 30]	Esteban et al. (2004)
6 *A. carb*	YES CYA	n.r.	5, [10, 15, 20, 25, 30, 35], 40, 45[b] 5, 10, [15, 20, 25, 30, 35], 40, 45	[5, 10, 15, 20, 30]	Esteban et al. (2004)
8 *A. carb*	SNM	[0.90, 0.93, 0.95, 0.99]	[15, 20, 30, 35, 37]	[7]	Bellí et al. (2005e)
4 *A. carb*	grapes	[80, 90, 100] % R.H.	[20, 30]	[7]	Bellí et al. (2005d)
2 *A. nig* aggr 2 *A. carb*	SNM	[0.90, 0.93, 0.95, 0.98, 0.995] [0.90, 0.93, 0.95, 0.98, 0.995]	25	[5, 10, 15, 20] [5, 10, 15, 20]	Bellí et al. (2004d)
4 *A. carb*	SNM	0.96	7, [15, 20, 25, 30, 35], 42	[2, 4, 6, 8, 10]	Marín et al. (2005)
12 *A. carb*	SNM	0.85, 0.90, 0.93, 0.95, 0.99	10, 15, 20, 25, 30, 35, 37, 40	[5, 10, 15, 20]	Mitchell et al. (2005)

n.r. not reported; [a]OTA production on YES > on CYA; [b]OTA production on CYA > on YES.

9.5. PREVENTION AND CONTROL OF OTA

Much research is focused on surveillance, occurrence and the development of new analytical methods, but equally important effort should be applied to the prevention of mycotoxin formation. It is impossible to provide adequate protection to the consumer by merely sampling and analysing the final product. Although under certain environmental conditions, contamination of grapes with toxigenic fungi and mycotoxins is unavoidable for producers, good practices would at least reduce as much as possible the toxin present. In general, the introduction of preventive measures at all stages of the food production and distribution chain, rather than only inspection and rejection at the final stage, makes better economic sense, because unsuitable products can be identified earlier along the chain (FAO/WHO, 2003).

Prevention could be divided in the following three stages. Several practices focused on the OTA problematic in grapes and wine, some of them derived from the studies presented in this thesis, are suggested afterwards:

- **Primary prevention.** This step should be initially carried out before the fungal infestation and mycotoxin contamination. This level of prevention is the most important and effective plan for reducing fungal growth and mycotoxin production. Several practices could be recommended to keep the conditions unfavourable for black aspergilli growth:

  - Making schedule for suitable pre-harvest and post-harvest practices. For example, avoid leaving the residues of infected plants in the field, in order to prevent any risk of contamination in succeeding vintages, adequate irrigation, proper plant nutrition, etc.

  - Use fungicides and/or preservatives to prevent the appearance of any mould in general, and against black aspergilli in particular. Anti-mildew or anti-*Botrytis* actions appear that could effectively reduce the source of the contamination in grapes (Anonymous, 2000).

  - Control field infestation by insects with approved insecticides.

  - Good practices at harvest, avoiding at maximum grape splitting. Harvest at night is recommended, apart from its vitivinicole advantages, because low temperatures will compensate the incontrollable damages caused in grape skin.

  - Fast transport of grapes from field to the wineries, especially with damaged and mouldy berries, avoiding leaving the grapes so much time at high temperature, and frequently clean and disinfect the transportation systems of the grapes. It has been demonstrated that a long time period of outdoor storage between harvest

and processing, dramatically increases the patulin levels in apple fruits (Sydenham et al., 1997), and probably will happen the same with OTA in grapes.

- Once the grapes are in the winery, control the storage-time period until the grapes processing, and maintain them in the most appropriate conditions of temperature and humidity.

▪ **Secondary prevention.** If the invasion of some fungi begins in commodities at an early stage, this level of prevention will then be required. The existing toxigenic-fungi should be eliminated or its growth should be stopped to prevent further deterioration and mycotoxin contamination. Several measures are suggested as follows:

- Use of anti-fungal treatments to attack the mould once it has grown, destroying it or minimizing its development. The use of other microorganisms that can compete with OTA-producing organisms is another possible method of preventing OTA formation, but it needs further investigation.

- Removal of contaminated grapes at harvest. Grape selection would be a very difficult practice, even impossible in mechanical harvest, contrary to what happens with other bigger fruits like apples, where the use of good manufacturing practices, such as proper fruit selection, handling, storage and washing, can reasonably assure that the obtained juices will not contain residual mycotoxin levels in excess of the maximum allowed limits (Delage et al., 2003).

- Employ of different practices in wine-making such as type of maceration or time and temperature of fermentation could also influence on the extraction of OTA (Zimmerli and Dick, 1996).

▪ **Tertiary prevention.** Once the products are heavily infested by toxigenic fungi, the primary and secondary preventions would not be then feasible. Any action would not be effective, since it will be quite late to completely stop toxic fungi and reduce mycotoxin formation. However, some measures should be done to prevent the transfer of fungi and their health hazardous toxins highly contaminated in products into our daily foods and environment. Only a few practices are recommended:

- Detoxification or destruction of OTA, once it is detected in wine. Several methods are under study, but much research is needed in this area.

- Complete destruction of the contaminated products. This would be economically unfeasible for the wine industry.

The more economic and effective prevention strategy is to entrust food producers and operators with primary responsibility for food safety and quality (FAO/WHO, 2003). They will be well educated about mycotoxins and encouraged to prevent and control the contamination of mycoflora and their health-hazardous mycotoxins in their commodities as much as possible.

Careful control of mycotoxins should be started and administered by the government of each country. Government regulators are then responsible for auditing performance of the food system through monitoring and surveillance activities and for enforcing legal and regulatory requirements.

International cooperation for the mycotoxin regulation in trading products or commodities is also needed. The countries should establish quality control limits for certain commodities intended for export or import. The producer countries would be stimulated to be aware of mycotoxin contamination in their exported susceptible commodities. Low-cost technology for assessment, prevention and control of environmental mycotoxins could be then transferred from developed countries to developing ones. Finally, conferences, symposiums, trainings and workshops on current information of mycotoxins should be promoted. All the interested parties, particularly consumer groups, should be kept informed of the situation.

9.6. REFERENCES

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