Universitat de Lleida
Escola Tècnica Superior d'Enginyeria Agrària

Ochratoxin A in overripe grapes, raisins and special wines.

In vitro and *in vivo* studies on fungi isolated from grapes and raisins as affected by physical, chemical and biotic agents.

Ana Valero Rello

2007

A mi familia,

los que están y los que no,

y a mis amigos

Universitat de Lleida

Escola Tècnica Superior d'Enginyeria Agrària

PhD candidate:

Ana Valero Rello

Thesis directors:

Vicente Sanchis Almenar

Sonia Marín Sillué

This work was performed in the Microbiology laboratories of the Food Technology Department at the University of Lleida, Spain. It was financially supported by the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2004-07549-05-01, grant BES-2003-2180 and Ramón y Cajal program) and byo the Catalonian Government (CeRTA strategic project 2005-2006 “Seguritat biòtica y abiòtica dels aliments”, Generalitat de Catalunya).

SUMMARY

Ochratoxin A (OTA) is a fungal secondary metabolite produced by *Aspergillus* and *Penicillium* species. It has been detected in a wide range of commodities, including cereals, coffee, grapes, raisins, must and wine. Within grape derivative products, the raisins, red wine and sweet wines have reported to contain the highest OTA levels. *Aspergillus* section *Nigri* (*A. niger* and *A. carbonarius*) are considered the OTA source in these commodities and they are commonly isolated among other fungi from grapes and raisins.

Starting from this basis the objectives of this thesis were focused into three main aspects: (1) Evaluation of the food products: vine dried fruits and special wines, concerning the mycobiota and OTA occurrence and incidence; (2) Ecophysiological studies of the ochratoxigenic fungi and accompanying mycobiota as affected by environmental conditions; (3) Control and preventive methods such as the evaluation of residual activity of pre-harvest fungicides during grape dehydration and the use of modified atmospheres.

Wine origin and winemaking procedure showed to be determinant for the final OTA content. All special wines analysed from northern European regions were negative for OTA while more than 50% of wines from warmer regions were positive for OTA contamination. The wines with higher OTA levels were fortified musts followed by those made from dried grapes. Acoholic and malo-lactic fermentations, biological 'crianza' (Flor yeast) and the action of *Botrytis cinerea* in noble rot of grapes may diminish the OTA levels in wine.

In grapes, the presence of *Aspergillus* section *Nigri* became predominant at harvest and mainly during sun-drying. Prevalence of *Aspergillus* section *Nigri* can be explained by their adaptation to environmental conditions of sun-drying, and by their ability to dominate other fungal species involved when coming into contact with them. Among the *Aspergillus* section *Nigri*, *A. niger* aggregate was dominant, although *A. carbonarius* increased its incidence in dehydrated grapes and showed a higher potential for OTA production, close to 100% of strains were ochratoxigenic, it being optimal at 20ºC and high water activity.
The complex fungal interactions studied *in vitro* and during grape dehydration may act as a control factor in some cases while other few fungal species stimulated OTA production. Integration of these findings on the effects of temperature, water activity and competitive abilities on the fungal spore resistance to ultraviolet radiation (UVC) provides a good explanation to the incidence of fungi found on both fresh and sun dried grapes.

The ultraviolet light could also be considered as an alternative control treatment given that 10 min of UVC irradiation on fungal spores were enough to reduce almost the 100% of their viability. Regarding the residual effect of pre-harvest fungicides, it was observed that they remained active in grapes during further dehydration, so they could be effective in reducing OTA production by black aspergilli. The application of modified atmospheres packaging showed differential results in controlling fungal growth and OTA production, combination of 15% CO_2 with 1% O_2 being the most effective treatment. However, to minimise the risk of OTA contamination during grape storage, modified atmospheres should be used in combination with low temperatures or with other preventive measures.

Finally, the application of chemical or biological treatments to grape products, other than those intrinsic to the winemaking process, should be considered as the last option. Attention should focus in preventive rather than in corrective actions. A suitable crop management followed by a smart post-harvest processing of grapes and also during winemaking should comprise enough cautions to obtain a safe final product.

RESUMEN

La ocratoxina A (OTA) es un metabolito fúngico secundario producido por especies de *Aspergillus* y *Penicillium*. Ha sido detectada en un amplio rango de productos alimenticios como cereales, café, uvas, pasas, mosto y vino. De entre los productos derivados de la uva; las pasas y los vinos tinto y dulce presentan los niveles más altos de OTA. Especies del grupo *Aspergillus* sección *Nigri* (*A. niger* y *A. carbonarius*) son frecuentemente aisladas de uvas y pasas y son considerados la fuente de contaminación por ocratoxina A.

Partiendo de esta base, los objetivos de la presente tesis se centraron en los siguientes aspectos: (1) Evaluación de los productos alimenticios: uvas pasificadas y vinos especiales, en relación a la contaminación fúngica y contaminación por ocratoxina A; (2) Estudios ecofisiológicos de los hongos ocratoxigénicos y micobiota acompañante bajo diferentes condiciones ambientales; (3) Métodos preventivos y de control: efecto residual de fungicidas, aplicados en campo, durante la deshidratación de las uvas y el uso de atmósferas modificadas.

El origen de los vinos y el proceso de vinificación fueron determinantes en el contenido de ocratoxina A. No se encontraron trazas de la toxina en ninguno de los vinos especiales europeos procedentes de las regiones productoras más septentrionales, sin embargo más del 50% de los vinos de zonas más cálidas estuvieron contaminados. Los vinos que registraron los mayores niveles de OTA fueron aquellos producidos mediante la fortificación del mosto base y elaborados a partir de uvas pasificadas. Tanto las fermentaciones alcohólica y maloláctica, como la crianza biológica bajo velo de Flor y la acción de *Botrytis cinerea* en la podredumbre noble de las uvas disminuyeron aparentemente la concentración de la toxina de estos vinos.

En uvas, la incidencia de *Aspergillus* sección *Nigri* aumentó con la maduración de las uvas y principalmente durante el posterior secado de las uvas al sol. La prevalencia de estos hongos puede ser explicada por su adaptación a las condiciones ambientales propias del secado al sol y por su capacidad de dominar a otras especies fúngicas en caso de interacción. Dentro de *Aspergillus* sección *Nigri*, el agregado *A. niger* fue predominante, aunque *A. carbonarius* aumentó su incidencia en uvas deshidratadas y mostró un mayor potencial ocracotixénico, siendo productores cerca del 100% de los aislados y 20º C y alta actividad de agua los óptimos de producción.

El complejo de las interacciones fúngicas estudiadas *in vitro* y durante la deshidratación de las uvas, podría actuar como agente de control en algunos casos, mientras que algunas especies

de hongos estimularon la producción de OTA. Integrando los resultados de los estudios ecofisiológicos, respecto a temperatura, actividad de agua y capacidad combativa con la resistencia de las esporas fúngicas a la radiación ultravioleta (UVC) se puede explicas en gran medida la incidencia de los hongos ensayados, tanto en uvas frescas como deshidratadas al sol.

La radiación ultravioleta podría ser considerada como un tratamiento de control alternativo dado que la irradiación de esporas de hongos con UVC durante 10 min fue suficiente para reducir hasta casi el 100% su viabilidad. En cuanto al efecto residual de los fungicidas aplicados en campo, se observó que éstos permanecieron activos durante la posterior deshidratación de las uvas, por lo que podrían ser efectivos para reducir la producción de OTA por *Aspergillus* sección *Nigri*. La aplicación de atmósferas modificadas produjo resultados variables en el control del desarrollo fúngico y de la producción de la toxina, siendo la combinación de 15% de CO_2 con 1% de O_2 el tratamiento más efectivo. Sin embargo, para minimizar el riesgo de contaminación por ocratoxina A durante el almacenado de la uva, la aplicación de atmósferas modificadas debería combinarse con el uso de bajas temperaturas u otros métodos de control.

Finalmente, el uso de tratamientos químicos o biológicos en la uva o derivados, aparte de aquellos propios del proceso de vinificación, deberían ser considerados como último recurso. De este modo, convendría centrar la atención en la prevención en lugar del empleo de acciones correctivas. Un manejo adecuado de la cosecha seguido de un apropiado procesado de la uva en postcosecha y en la vinificación deberían constituir precauciones suficientes para garantizar un producto final seguro.

RESUM

L'ocratoxina A (OTA) és un metabolit fúngic secundari produït per espècies d'*Aspergillus* i *Penicillium*. Ha estat detectada en un ampli rang de productes alimentaris com ara cereals, cafè, raïm, panses, most i vi. Dins els productes derivats del raïm; les panses i els vins negres i dolços presenten els nivells d'OTA més alts. Espècies del grup *Aspergillus* secció *Nigri* (*A. niger* i *A. carbonarius*) són frecuentment aïllades de raïm i panses i estan considerades la font de contaminació per ocratoxina A.

Partint d'aquesta base, els objectius de la present tesi es centraren en els següents aspectes: (1) Avaluació dels productes alimentaris: raïm passificat i vins especials, en relació a la contaminació fúngica i contaminació per ocratoxina A; (2) Estudis ecofisiològics dels fongs ocratoxigènics i micobiota acompanyant afectats per les diferents condicions ambientals; (3) Mètodes preventius i de control: efecte residual de fungicides, aplicats en el camp, durant la deshidratació del raïm i l'ús d'atmosferes modificades.

L'origen dels vins i el procés de vinificació són determinants per al contingut d'ocratoxina A. No es van trobar traces de la toxina en cap dels vins especials europeus procedents de les regions productores més Septentrionals, més del 50% dels vins de les zones més càlides, però, estaven contaminats. Els vins que registraren els majors nivells d'OTA van ser els produïts mitjançant la fortificació del most base i els elaborats a partir de raïm passificat. Tant les fermentacions alcohòlica i malolàctica, com la criança biològica sota tel de Flor i l'acció de *Botrytis cinerea* en la floridura noble del raïm van disminuir aparentment la concentració de la toxina d'aquests vins.

En el raïm, la incidència d'*Aspergillus* secció *Nigri* va augmentar amb la maduració del raïm i principalment durant el posterior assecat al sol. La prevalència d'aquests fongs pot ser explicada per la seua adaptació a les condicions ambientals pròpies de l'assecat al sol i per la capacitat de dominar altres espècies fúngiques en cas d'interacció. Dins d'*Aspergillus* secció *Nigri*, l'agregat *A. niger* va ser predominant, encara que *A. carbonarius* va augmentar la seua incidència en raïm deshidratat i va mostrar un major potencial ocratoxigènic, essent productors prop del 100% dels aïllats a 20º C i alta activitat d'aigua els òptims de producció.

El complexe d'interaccions fúngiques estudiades *in vitro* i durant la deshidratació del raïm, podria actuar com agent de control en alguns casos, mentre que algunes espècies de fongs van estimular la producció d'OTA. Integrant els resultats del estudis ecofisiològics, respecte a la temperatura, activitat d'aigua i capacitat combativa amb la resistència de les espores fúngiques

a la radiació ultravioleta (UVC) es pot explicar la incidència dels fongs assatjats, tant en raïm fresc com secat al sol.

La radiació ultraviolada podria ser considerada com un tractament de control alternatiu ja que la irradiació d'espores de fongs amb UVC durant 10 min va ser suficient per a reduïr fins casi el 100% la seua viabilitat. Pel què fa al efecte residual dels fungicides aplicats en el camp, es va observar que aquests van romandre actius durant la posterior deshidratació del raïm, pel què podrien ser efectius per reduir la producció d'OTA per *Aspergillus* secció *Nigri*. L'aplicació d'atmosferes modificades va produir resultats variables en el control del desenvolupament fúngic i de la producció de la toxina, la combinació de 15% de CO_2 amb 1% de O_2 essent el tractament més efectiu. Tot i així, per minimitzar el risc de contaminació per ocratoxina A durant el magatzamament del raïm, l'aplicació d'atmosferes modificades hauria de combinar-se amb l'ús de baixes temperatures o d'altres mètodes de control.

Finalment, l'ús de tractaments químics o biològics en el raïm o derivats, apart d'aquells propis del proces de vinificació, haurien de ser considerats com a últim recurs. D'aquesta manera, convindria centrar l'atenció en la prevenció en comptes de l'ús d'accions correctives. Un maneig adecuat de la collita seguit d'un apropiat processat del raïm en postcollita i en la vinificació haurien de constituir precaucions suficients per a garantir un producte final segur.

过熟葡萄，葡萄干和高糖度、高酒精度葡萄酒中的赭曲霉毒素 A（Ochratoxin A OTA）

从葡萄及葡萄干中分离的真菌受物理、化学和生物制剂影响的体内与体外研究

摘要

赭曲霉毒素A（Ochratoxin A OTA）是一种麴菌属（*Aspergillus*）和青徽菌属（*Penicillium*）真菌的二次代谢产物。它存在于许多农产品如谷类、咖啡、葡萄、葡萄干、葡萄榨汁和葡萄酒中。据报道，在葡萄及其产品中诸如葡萄干、红葡萄酒和甜葡萄酒中OTA的含量最高。黑麴菌属（碳黑曲霉（*A. carbonarius*） 和黑曲霉（*A. niger*））通常被认为是OTA的主要来源。OTA也可从葡萄及葡萄干中常见的其它真菌中分离出来。

本论文主要论述在三方面的问题：（1）食品的评价：对风干葡萄及高糖度、高酒精度葡萄酒中真菌及 OTA 的产生及影响范围。（2）产生赭曲霉毒素的真菌及其受环境条件影响的伴随系的生态生理研究（3）控制及防止真菌及 OTA 的方法，诸如收获前喷撒的杀真菌农药在葡萄脱水期间的残余活性的评价及运用不同的脱水气氛对真菌生长的抑制效应的评价。

葡萄酒的产地及制酒的工艺是产品中 OTA 含量的决定因素。所有产自北欧的葡萄酒都不含 OTA，而 50%以上产自较温暖地区的酒中都检出 OTA。由晾干的葡萄所制的葡萄汁加入酒精后进行发酵制造的葡萄酒中含有较高的 OTA。酒精性发酵及苹果酸性发酵、生物酵母发酵(Flor yeast)及在葡萄中大量滋生的灰葡萄孢菌（*Botrytis cinerea*）的作用可能会降低酒中 OTA 的含量。

黑曲霉(*Aspergillus* section *Nigri*)在葡萄的收获期逐渐出现，而在风干期大量繁殖。黑麴菌属(*Aspergillus* section *Nigri*)的大量繁殖是由于风干的环境适合其的生长，以及他们与其它的真菌种属相比能更加优先繁殖。在黑麴菌属(*Aspergillus* section *Nigri*)中，黑曲霉(*A. niger*)的聚集是最为主要的。尽管碳黑曲霉(*A. carbonarius*)也会在葡萄的风干过程中增加，加大了OTA 产生的可能性； 几乎 100%的菌株是能产生赭曲霉毒素，其最适宜的繁殖条件是 20ºC及高的水活性（相对湿度）。

以营养基模拟葡萄表面真菌生长及葡萄在脱水期间真菌生长的研究表明，多种真菌的的相互作用在通常情况下能抑制黑麴菌属(*Aspergillus* section *Nigri*)的生长，从而减少OTA的含量；而在另一些情况下，其它的种类的真菌会增加OTA的含量。综合所有这些因素诸如温度、水的活性（相对湿度）及真菌孢子对紫外线的抵御能力为真菌在新鲜及风干葡萄的发生率提供了良好的解释。

紫外线照射也可用作替换的的控制控制处理方法。10 分钟的紫外线辐射可几乎杀灭全部的真菌孢子。至于收获前所施用的杀真菌剂的残余作用方面，研究证明其在进一步的脱水过程中仍保持活性，能有效的抑制OTA的产生。不同的包装气氛控制在抑制真菌生长和OTA的产生方面显示了不同的结果。15%的CO_2与 1%O_2 的混合气体表现最为有效。但需要指出的是，为尽量减少葡萄在贮存过程中OTA污染的危险，包装气氛的控制应与低温等其它预防措施同时使用。

最后必须指出，良好的葡萄酒制造工艺应为首选，而使用化学或生物处理技术应作为最后的选择。应着重于事先的防范而非事后的补救。葡萄采收管理、采收后的处理工艺及葡萄酒的制造工艺应包括足够的措施以保证得到安全的成品。

(Thanks to David Xu for his translation)

Index

I. INTRODUCTION

1. OCHRATOXINS 3

1.1. Structure and Toxicity 3
1.2. Producing fungi 4
1.3. Percentage of OTA producer isolates 6
1.4. Requirements for fungal growth and ochratoxin A production 7
- Temperature
- Water availability
1.5. Competition between fungi 9
- Hyphal interactions
1.6. Distribution of *Aspergillus* section *Nigri* in European vineyards 11
1.7. Occurrence of OTA in foodstuffs 12
1.8. Incidence of OTA in grape products 13
- Dried vine fruits
- Wine
1.9. Law restrictions 16

2. WINE AND OTHER GRAPE PRODUCTS 17

2.1. Grapes 17
2.2. Raisins 18
2.3. Wine 19
2.4. Special wines 21
- Fortified musts and wines
- Sparkling wines
- Wines from overripe grapes

3. FATE OF OTA DURING WINEMAKING 24

4. CONTROL AND PREVENTIVE ACTIONS 26

4.1. Pre-harvest 27
- Agricultural practices
- Chemical and biological control
- Inducers of plant resistance
- Transgenic plant resistance

4.2. Post-harvest and storage 30
- General cautions
- Chemical and biological control
- Alternative methods

4.3. During winemaking 32
- Winemaking cautions
- OTA degradation by yeasts and LAB during fermentations

4.4. Decontamination of OTA in wine, grape juices and musts 33

II. OBJETIVES **37**

III. STUDIES **39**

1. Evaluation of the food products: vine dried fruits and special wines 39

Study I: 41
Ochratoxin A producing species in grapes and sun dried grapes and their relationship to ecophysiological factors. *Lett Appl Microbiol* (2005)

Study II: 53
Survey: Ochratoxin A in European special wines. *Int J Food Microbiol* (submitted)

2. Ecophysiological studies 69

Study III: 81
Kinetics and spatial distribution of OTA in *A. carbonarius* isolates. *Food Microbiol* (2006)

Study IV: 81
Studies on the interaction between grape-associated filamentous fungi on a synthetic medium. *Int J Food Microbiol* (2007).

Study V: 93
Effects of fungal interaction in ochratoxin A production by *A. carbonarius* at different temperature and a_w. *Int J Food Microbiol* (2006).

Study VI: 103
Effect of germicidal UV-C light on fungi isolated from grapes and raisins. *Lett Appl Microbiol* (in press).

Study VII: 113
Effect of *intra* and *inter*specific interaction on OTA production by *A*. section *Nigri* in grapes during dehydration. *Food Microbiol* (2007).

Study VIII: 125
Brief *in vitro* study on *Botrytis cinerea* and *Aspergillus carbonarius* regarding growth and Ochratoxin A. *FEMS Microbiol Lett* (in press).

3. Control and prevention 135

Study IX: 137
Effect of pre-harvest fungicides and interacting fungi on *Aspergillus carbonarius* growth and OTA synthesis in dehydrating grapes. *Lett Appl Microbiol* (in press).

Study X: 149
Mycelial growth and OTA production by *Aspergillus niger* and *A. carbonarius* on grape-like medium (SNM) at modified atmospheres. *J Appl Microbiol* (submitted).

IV. DISCUSSION 163

V. CONCLUSIONS 183

VI. FUTURE RESEARCH 187

VII. GLOSSARY OF USED TERMS 189

VIII. REFERENCES 191

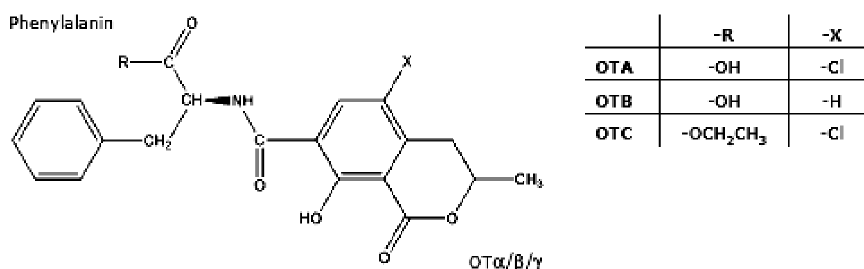
IX. ACKNOWLEDGEMENTS 209

INTRODUCTION

1. OCHRATOXINS

1.1. Structure and Toxicity

The chemical structure of ochratoxin A (OTA) contains a 7-carboxy-5-chloride-8-hydroxi-3,4 dihydro-(3R)-methylisocoumarine bound throughout a carboxyl group to a L-ß-phenylalanine (Fig. 1.1). There are many analogues to ochratoxin A, such as ochratoxin B and C, all them being fungal metabolites.



	-R	-X
OTA	-OH	-Cl
OTB	-OH	-H
OTC	$-OCH_2CH_3$	-Cl

Figure 1.1. Chemical structure of ochratoxins

OTA is a very stable compound that only can be fully hydrolyzed by heating under reflux for 48 h in 6 mol·l^{-1} acetic acid (van der Merwe *et al.,* 1965). The enzyme carboxypeptidase A, present in mammals has the property of hydrolyzing it to phenylanine and to the non-toxic ochratoxin R (OTR) (Pitout 1969; van der Westhuizen *et al.,* 2000).

Due to its physicochemical properties, OTA is easily absorbed from gastrointestinal tract, its bioavailability being above 50% in all mammals assayed. OTA shows a high affinity to plasmatic proteins, what entails a long persistency inside the organism. The toxin can be eliminated by renal and hepato-biliar way, and also through lacteal secretion (López de Cerain *et al.,* 2002).

The LD50 ranges from 0.2 to 58.3 mg·kg^{-1} among the assayed animals: dog, pig and chicken are more susceptible than rat and mouse. Chronic ingestion of OTA produces a renal toxic effect in all monogastric mammals tested. A number of descriptive studies have suggested a correlation between exposure to ochratoxin A and Balkan endemic nephropathy (Pavlovic *et al.,* 1979; Petkova-Bocharova and Castegnaro 1991; Pfohl-Leczkowicz and Manderville 2007).

In 1993 the International Agency for Research in Cancer (IARC) classified OTA as possible human carcinogen (2B group). Ochratoxin A also causes immunosuppression in several animal species, is genotoxic both in *vitro* and *in vivo*, but the mechanism of genotoxicity is still unclear. Because human exposure to ochratoxin A has been clearly demonstrated by its detection in blood and breast milk (Clark and Snedeker 2004), the presence of ochratoxin A in foodstuffs is clearly undesirable.

1.2. Producing fungi

Ochratoxin A was originally described as a metabolite of *Aspergillus ochraceus* in laboratory experiments (van der Merwe *et al.,* 1965). It was subsequently reported in several related *Aspergillus* and *Penicillium* species (Table 1.1 and 1.2). The former is predominant in tropical and warm climates while *Penicillium* is commonly isolated in temperate climates.

Recently, *A. carbonarius* was identified as one of the main OTA producers, with a low percentage of isolates of the closely related species *A. niger* (Abarca *et al.,* 1994; Téren *et al.,* 1996). Finally the OTA producing strains of the group A. section *Nigri* are considered responsible of the OTA detected in grapes, raisins and wine (Abarca *et al.,* 2003; Bellí *et al.*, 2004a).

Table 1.1. OTA producing *Aspergillus* spp.

Species	Group or Section	Reference
Aspergillus glaucus *A. repens*		Chelkowski *et al.*, 1987 El-Kady *et al.*, 1995
Eurotium amstelodami *E. herbariorum*	*Aspergillus*	Abarca *et al.*, 1997 Chelkowski *et al.*, 1987
A. auricomus *A. ochraceus* *A. petrakii* *A. sclerotiorum* *A. sulphureus* *A. melleus*	*Circumdati*	Varga *et al.*, 1996 Van der Merwe *et al.*, 1965 Ciegler 1972 Frisvad *et al.*, 2004
A. wentii	*Wentii*	Varga *et al.*, 1996
A. flavus *A. alliaceus*	*Flavi*	Czerwiecki *et al.*, 2002
A. fumigatus	*Fumigati*	Abarca *et al.*, 1997
A. terreus	*Terrei*	Ueno *et al.*, 1991
A. ustus	*Usti*	Ueno *et al.*, 1991
A. versicolor	*Versicolores*	Abarca *et al.*, 1997
A. carbonarius ***A. sclerotioniger***	***Nigri***	Horie 1995 Samson *et al.*, 2004
A. awamori ***A. foetidus*** ***A. lacticoffeatus*** ***A. niger*** ***A. tubingensis*** ***A. usamii***	***Nigri*** **(*A. niger* aggregate)**	Ono *et al.*, 1995 Téren *et al.*, 1996 Samson *et al.*, 2004 Abarca *et al.*, 1994 Medina *et al.*, 2005 Ono *et al.*, 1995

Table 1.2. OTA producing *Penicillium* spp.

Species	Section	Reference
P. spinulosum	*Aspergilloides*	Ciegler *et al.,* 1972
P. sclerotiorum		Ueno *et al.,* 1991
P. implicatum		
P. glabrum		Vázquez-Belda *et al.,* 1995
P. variabile	*Biverticillium*	Ciegler *et al.,* 1972
P purpugenum		Ueno *et al.,* 1991
P. verruculosum		
P. janczewski	*Furcatum*	Ueno *et al.,* 1991
P. raistrickii		
P. corylophilum		
P. hirayamae		
P. simplicissimum		
P. canescens		
P. verrucosum	*Penicillium*	Pitt 1987
P. expansum		Bridge *et al.,* 1989
P. aurantiogriseum		
P. brevicompactum		Kozakiewicz *et al.,* 1993
P. chrysogenum		Vázquez-Belda *et al.,* 1995

1.3. Percentage of OTA producer isolates

The biosynthetic pathway for OTA has not been completely established yet but it seems clear that requires the activity of a polyketide synthase (*pks*) and ochratoxin A synthetase (Harris and Mantle 2001; O'Callaghan *et al.,* 2003). Moreover, the gene encoding polyketide synthase appears to be very different between *Penicillium* and *Aspergillus* species (O'Callaghan *et al.,* 2003; Geisen *et al.,* 2004). In *A. ochraceus*, the gene of pks is expressed only under OTA permissive conditions and only during the early stages of the mycotoxin synthesis (O'Callaghan *et al.,* 2003).

Likewise, not all strains belonging to an ochratoxigenic species such as *A. carbonarius* or *A. niger* are capable for OTA synthesis. Furthermore ochratoxigenic strains do not produce the toxin in all substrates and can even loose their toxigenic potential.

The percentage of ochratoxigenic isolates within the black aspergilli, commonly found in grapes and raisins, are synthesised in the table 1.3.

Table 1.3. Proportion (%) of OTA producing isolates in *A. carbonarius* and in *A. niger* aggregate spp. isolated from grapes and grape derived products.

Fungus	Origin of strains	% OTA producers	References
A. carbonarius	grapes	76-82	Bellí *et al.,* 2005b
	dried grapes	100	Leong *et al.,* 2004
	drying grapes	91	Heenan *et al.,* 1998
	grapes	93	Sage *et al.,* 2002
	dried grapes	97	Abarca *et al.,* 2003
	grapes	58	Battilani *et al.,* 2003
A. niger aggregate	grapes	2-5	Bellí *et al.,* 2005b
A. niger var *niger*	grapes	43	Magnoli *et al.,* 2003
A. niger var *awamori*	grapes	33	
A. foetidus	grapes	25	
A. niger	raisins and grapes	77	Tjamos *et al.,* 2004

Between the 50-100% of *A. carbonarius* isolates are able to produce the toxin whereas less than 50% of the isolates belonging to *A. niger* aggregate (see table 1.1 for classification) shows to be ochratoxigenic, with the exception of those reported by Tjamos *et al.* (2004).

1.4. Requirements for fungal growth and ochratoxin A production

Temperature

Temperature is a critical parameter because it determines whether liquid water is present. Survival and growth at extreme temperatures is more widely found among microbes. Cold tolerant (at 0ºC) organisms are called psychrotolerant, and heat-tolerant (above 40ºC) called thermotolerant. Thermotolerant and psychrotolerant fungi can grow over a wide range of temperatures. Even so, few fungi can grow above 65ºC, and even fewer below -3ºC. The spores of some fungi also survive exposure to extreme temperatures. The capacity referred to as thermostability, which is widely found among the fungi (University of Sydney 2004).

As it can be observed in the table 1.4, *Aspergillus* species grow in a wide range of temperatures, up to 45 ºC, compared to *P. verrucossum*. Their growth optimum is also higher than those for *Penicillium*, they being well-adapted to temperatures around 30 ºC.

Regarding OTA, the production ranges for *A. ochraceus* and black aspergilli are very similar. However, optimum in *A. ochraceus* is about 5-10 ºC higher compared to black aspergilli.

In all cases, the optimal and maximal temperatures for OTA synthesis are lower than those for mycelial growth.

Table 1.4. Optimum temperatures and range for fungal growth and OTA production on synthetic medium.

Fungus	Origin of strain	Mycelial growth		OTA synthesis		References
		Optimum	Range	Optimum	Range	
A. ochraceus	?	20-25	10-45	25-30	15-30	Sansing *et al.,* 1973
	cereals	25-30	10-37	25-30	12-37	Ramos *et al.,* 1998
A. niger aggregate	grapes	-	10-45	25	10-35	Esteban *et al.,* 2004
	dried grapes	30-35	10-42	n.d.	n.d.	Leong *et al.,* 2004
A. carbonarius	grapes	-	10-40	15-20	15-35	Esteban *et al.,* 2004
	dried grapes	30	10-42	n.d.	n.d.	Leong *et al.,* 2004
	grapes	30-35	15-40	15-20	15-40*	Mitchell *et al.,* 2004
	grapes	30	15-37	20	15-37	Bellí *et al.,* 2005b
P. verrucossum	?	20	10-31	n.d.	n.d.	Pitt and Hocking 1997
	coffee	20	10-30	n.d.	n.d.	Pardo *et al.,* 2005

n.d.: no determined, *: maximum range obtained by modelisation, ?: unknown origin.

Water availability

Water is an excellent solvent for organic molecules and appears that liquid water is essential for life. If so, a variety of physical limits to life seem apparent. During the drying out process (desiccation), less available water forces substances to increase in their concentration. Such increases lead to stressful responses within a cell that are similar to those of a cell experiences when exposed to high salt environments (Rothschild 2002).

The ability to grow in conditions out of liquid water seems to be restricted in the microbes to the fungi. It is commonly observed in food spoilage that the first colonisers are normally fungi, especially of foods with reduced water activity as a means of preservation (jams, marmalades and similar preserves).

From the information given in the table 1.4, it can be observed that the three mentioned species and *A. niger* aggregate have similar a_w requirements and optimum for mycelial growth. The same similarities within these fungi were observed for OTA production, which requires higher a_w values than those for growing.

Table 1.5. Water activity optimum and range for fungal growth and OTA production on synthetic medium.

Fungus	Origin of strains	Mycelial growth		OTA synthesis		References
		Optimum	**Range**	**Optimum**	**Range**	
A. ochraceus	coffee	0.95	0.80-0.99	0.95	0.90-0.99	Suárez-Quiroz *et al.,* 2004
A. niger aggregate	grapes	-	0.82-0.99	0.96-0.99	0.90-0.99	Esteban *et al.,* 2006a
	?	0.97	0.80-0.99	n.d.	n.d.	Parra and Magan 2004
A. carbonarius	grapes	0.93-0.99	0.85-0.99	0.95-0.98	0.91-0.99	Mitchell *et al.,* 2004
	grapes	-	0.82-0.99	0.94-0.99	0.86-0.99	Esteban *et al.,* 2006b
P. verrucossum	?	-	0.79-?	0.95-0.99	-	Northolt *et al.,* 1979
	coffee	0.95-0.99	0.85-0.99	n.d.	n.d.	Pardo *et al.,* 2005

n.d.: no determined, ?: unknown origin.

1.5. Competition between fungi

Although soil is oligotrophic, up to 106 fungal propagules per g may be isolated from soil (University of Sydney 2004). Even higher densities can be found in substrates rich in organic matter. It is easy to argue that fungi compete in these circumstances. Competition studies are common in botany and zoology, and a vast body of research applies to fungi. After all, fungi require organic energy to survive, and by occupying space, they may increase their potential access to food.

However, interacting organisms do not need to compete if their food requirements are different. Indeed, one of the organisms may be the source of food for the other. The interactions between different organisms are varied and often complex.

Fungi have a variety of requirements for growing and reproduction. Though organic energy is of overriding importance to fungi, space, water, other nutrients and oxygen are also important. To survive through time, a fungus must reduce the effect of potential competitors or utilise effective competitive mechanisms.

The basic mechanisms used by microbes to compete include (University of Sydney 2004):

- Rapid recovery, growth and sporulation.
- Use of inhibitors
- Negation of inhibitors
- Special niche

Probably all fungi use each of these strategies to a greater or lesser extent. Studies in culture plates indicate some of these mechanisms. They can be summarised as indirect and direct interactions.

Hyphal Interactions

Indirect interactions would be where one fungus shows evidence of the presence of the other without any intervening chemical or stimulus. For example, one fungus may remove all nutrients from the zone between two fungi thus preventing the other from entering it. An indirect interaction would be particularly important where a resource is in very short supply, and one of the competitors has a high demand for the resource. Where the resource is abundant, then we would expect to see the hyphae of both fungi intermingling without apparent interaction (University of Sydney 2004).

Direct interactions can take place in the absence of any contact between the competitors. The production of inhibitors is widespread in the fungi, and these are expressed in culture. Many fungi produce secondary metabolites which either inhibit or kill competing fungi, some distance away. Antibiosis is often specific to groups of species, indicating that in nature, this interaction may be widespread. Antibiotics may be used to capture resources already occupied by a competitor or to secure a resource that may be under threat from a competitor (University of Sydney 2004).

Antibiotics may be volatile or nonvolatile compounds. The latter may be accumulated in media where they may induce autotoxicosis. These fungi are often seen growing in concentric rings on agar. Each new ring of the fungi arises from hyphae that have emerged from the agar beyond the zone of staling materials.

Several fungi have been found to produce a range of antibiotics, each produced under specific conditions. The specific action in nature of the diverse array of molecules is unclear in most cases. For instance, soil fungi such as *Trichoderma*, *Penicillium* and *Aspergillus* produce a diverse range of antibiotics. Specific compounds, however, appear to have specific targets. Plectasin is a defensin (peptide) with antibacterial activity. Aflatoxins have mammalian toxicity. Trichodermin may have broad antifungal activity.

Many compounds appear to have such broad potential activity that their role in the survival of the producer is unclear. Frequently, a fungus known to produce one important secondary metabolite

with bioactivity, will have a diverse array of secondary metabolites with unknown activity (and benefit) to the producer.

Contact phenomena are also important mechanisms of competition. The most obvious example of interaction is where the hyphae of one lyse the hyphae of a second fungus on contact. The antagonist may also coil around the target hyphae. Direct penetration is possible, though this is uncommon for necrotrophs. In some cases, the colonising fungus may form a biotrophic association with its host (University of Sydney 2004).

Competition may result from depletion of resources. Mechanisms include direct inhibition of the competitor. Death of the inhibitor without contact, or death of the competitor following contact are some possible consequences. Partitioning of resources may also take place.

1.6 Distribution of *Aspergillus* section *Nigri* in European vineyards

The *in vitro* and theoretical data formerly exposed, regarding the factors temperature, water activity and fungal interactions, would be expectable to agree with the geographical distribution of the fungal incidences according to these parameters. In several surveys and field studies, the results of fungal occurrences have been contrasted with the temperature and wetness of environment. However, there is lack of information about the effect of biotic interactions on the black aspergilli distribution.

Bellí *et al.* (2005a) found a positive correlation between the environmental temperature and the incidence of black aspergilli, mainly *A. niger* aggregate, in grapes sampled from vineyards in the North of Spain.

In a collaborative study that involved several European research groups (Battilani *et al.*, 2006) predictive models of *Aspergillius* section Nigri incidences in Southern European regions, depending on the temperature and environmental wetness, were designed by comparing the meteorological data with the real fungal incidences observed in a period of three years. Some results of this study can be observed in the Figure 1.2.

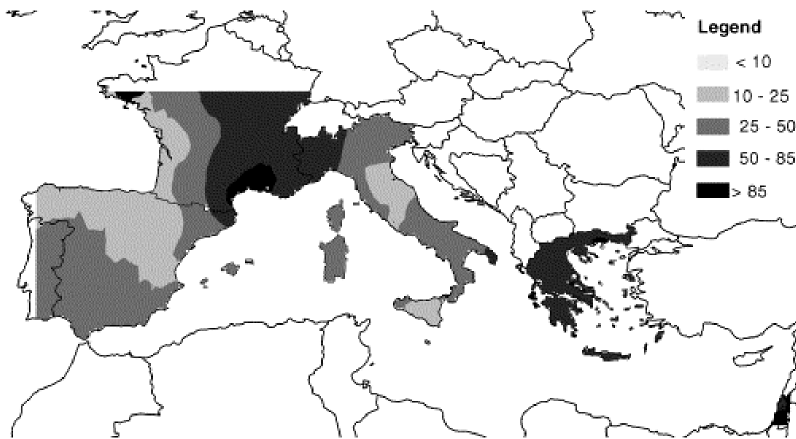


Figure 1.2. Prediction map of *Aspergillus* section *Nigri* incidence (%) at harvesting in 2003 (Battilani *et al.*, 2006).

At harvesting period, spatial variability of *Aspergillus* section *Nigri* is related to latitude and longitude, showing a positive West–East and North–South gradient. This indicates that meteorological conditions can contribute to explain spatial distribution variation of black aspergilli, within the Mediterranean basin at least (Battilani *et al.*, 2006).

As a result, crops located in areas with higher incidences of black aspergilli may lead to highly OTA-contaminated food products.

1.7. Occurrence of OTA in foodstuffs

OTA is a commonly detected as a contaminant in some foodstuffs such as coffee (Pardo *et al.,* 2004), grapes (Battillani and Pietri, 2002), raisins (MacDonald *et al.,* 1999), wine and musts (Bellí *et al.*, 2004a) among others.

The food products with the highest OTA occurrences are rye and derivatives and cocoa products (between 50-80%), followed by dried fruits (73%). About half out the total of commercialised wines contains OTA (Table 1.6).

Table 1.6. Occurrence of OTA in some products of European Union.

Foodstuff	Total samples	% positive samples
Olive oil	12	8.3
Baby foods	103	67.0
Rice	68	13.2
Oats	165	30.3
Cocoa and products	547	81.4
Coffee	1205	47.3
Green coffee	1761	36.7
Meat and products	1828	18.2
Barley	142	23.9
Rye	444	53.2
Rye and derivatives	242	88.0
Cereals and products	2212	69.8
Beer	496	32.7
Dried Fruits	**800**	**72.8**
Milk and derivatives	565	9.2
Maize	139	8.6
Malt	9	0.0
Millet	34	70.6
Millet and derivatives	81	8.6
Fruit products	353	41.6
Bran	53	35.8
Wheat	867	31.7
Wheat and derivatives	608	61.0
Wine	**1680**	**52.1**
Total	18599	48.8

DL: detection limit, QL: quantification limit

(Italian Health Superior Institute, 2002; Bellí *et al.*, 2004a; Pardo *et al.,* 2004)

1.8. Incidence of OTA in grape products

Dried vine fruits

Dried fruits and specially raisins are highly susceptible to be contaminated by OTA. In many cases, higher concentrations than those permissible by European Commission, set in 10 µg OTA · Kg^{-1}, were detected (Table 1.7) ((CE) nº 1881/2006).

Table 1.7. Incidence and OTA content in dried fruits.

Country	Dried fruits	No samples	% positive samples	OTA ($\mu g \cdot kg^{-1}$) Mean	Range
Finland	Raisins	31	71.0	1.34	<DL-7
France	Raisins	13	46.2	0.66	<QL-4.3
	Other	33	6.1	0.18	<QL-1.6
Germany	Sultanas	106	94.3	1.28	<DL-21.4
	Other	114	65.8	0.08	<DL-3.95
Greece	Sultanas	35	62.9	2.26	<QL-16.5
	Corinto raisins	47	48.9	1.69	<QL-12.38
UK	Corinto raisins	120	95.8	5.70	<DL-53.6
	Raisins	121	90.9	2.90	<DL-29.8
	Sultanas	120	86.7	3.65	<DL-25.1
	Dried peach	20	0.0	0.10	<DL
	Dates	20	5.0	0.10	<DL-0.2
	Figs	20	10.0	0.10	<DL-0.8
Europe		**800**	**32.3**	**2.30**	**<DL-53.6**

(Italian Health Superior Institute 2002)

Wine

According to the Italian Health Superior Institute (2002) (Table 1.8) the average of OTA concentration in wines from ten European countries is between 0.01–1.29 $\mu g \cdot kg^{-1}$, with 0.36 $\mu g \cdot kg^{-1}$ global average. However, the incidence and OTA levels are higher in Southern countries (72.3% and 0.64 $\mu g \cdot kg^{-1}$ respectively) compared to those in Northern regions (50.3% and 0.18 $\mu g \cdot kg^{-1}$ respectively).

Among the different wine types, red and sweet wines seem to be the most contaminated.

Table 1.8. Incidence and OTA content in European wines.

Country	Wine	No samples	% positive samples	OTA ($\mu g \cdot kg^{-1}$)	
				Average	Range
Finland (N)	Red	166	60.1	0.14	<DL-1.90
	White	10	70.0	0.14	<DL-0.39
	Fruity red	2	0.0	0.03	<DL
	Fruity white	1	0.0	0.03	<DL
France (N)	Local	34	41.2	0.05	<DL-0.36
	Imported	70	71.4	0.22	<DL-1.64
Germany (N)	Red	172	45.9	0.23	<DL-7.00
	Rosè	51	35.3	0.14	<DL-6.32
	White	56	21.4	0.10	<DL-1.36
	Sweet	1	100	1.04	1.04
Greece (S)	Red	38	55.3	0.16	<DL-2.61
	Rosè	5	60.0	0.07	<DL-0.13
	White	45	51.1	0.13	<DL-1.17
	Sweet	7	85.7	0.54	<DL-1.68
Italy (S)	Red	244	86.1	1.29	<DL-15.60
	Rosè	4	50.0	0.13	<DL-0.28
	White	20	35.0	0.59	<DL-8.86
	Sweet	15	60.0	0.74	<DL-3.86
Portugal (S)	Rosè	30	0.0	0.01	<DL
	Red	31	0.0	0.01	<DL
Spain (S)	Red	72	91.7	0.04	<DL-0.60
	Rosè	26	92.3	0.03	<DL-0.16
	White	43	81.4	0.03	<DL-0.27
	Sparkling	12	83.3	0.01	<DL-0.24
	Vermouth	27	85.2	0.06	<DL-0.25
	Sweet	16	81.3	1.09	<DL-2.54
Sweden (N)	Red	32	96.9	0.21	<DL-2.49
Netherlands (N)	Red	150	40.7	0.24	<DL-3.10
	White	20	10.0	0.16	<DL-2.10
UK (N)	Red	60	53.3	0.17	<DL-1.10
	White	10	0.0	0.10	<DL
Europe		**1470**	**59.3**	**0.36**	<DL-15.60
North Europe		**835**	**50.3**	**0.18**	<DL-7.00
South Europe		**625**	**72.3**	**0.64**	<DL-15.60

DL= detection limit; S=South; N=North. (Italian Health Superior Institute, 2002).

In general, OTA occurrence in wines is higher in sweet and red wines and higher means for OTA are frequently found in sweet wines. Among this type, those original from France, Hungary, Germany and Greece reported the lowest occurrences (Table 1.9).

Table 1.9. Occurrence of ochratoxin A in special wines

Type of wine	Origin	*n*	% positive samples	Range ($\mu g \cdot l^{-1}$)	Reference
Marsala	Italy	1	100	0.29	Visconti *et al.,* 1999
Dessert wine	Italy	15	40.0	0.001-3.856	Creppy *et al.,* 1991
Moscatel and Malaga	Spain	14	92.9	0.003-2.540	Burdaspal and Legarda 1999
Marsala wines	Italy	2	100	0.315-1.594	
Jerez and Montilla-Moriles wines	Spain	27	85.2	0.003-0.254	
Special wines	France	4	50.0	0.003-0.024	
Special wines	Italy	4	75.5	0.003-0.040	
Special wines	Germany	7	57.1	0.003-0.016	
Special wines	Portugal	4	75.0	0.003-0.029	
Special wines	Hungary	1	0.0	nd	
Sparkling wines	Spain	12	83.3	0.003-0.037	
Dessert wines	Greece	18	16.7	0.050-2.820	Stefanaki *et al.,* 2003
Retsina	Greece	8	25.0	0.050-1.750	
Port	Portugal	6	100	<0.003-0.017	Zimmerli and Dick 1996
Sherry	Spain	2	100	0.029-0.054	
Marsala	Italy	2	100	0.044-0.337	
Malaga	Spain	3	100	0.049-0.451	
Vermouth	Italy	2	100	<0.003	
Moscatel	Spain	7	42.9	0.050-0.400	Blesa *et al.,* 2004
Fondillón	Spain	6	50.0	0.050-0.380	

n= number of samples; nd= not detected (Blesa *et al.,* 2006)

1.9. Law restrictions

The FAO-OMS committee established the maximum weekly intake level tolerable, based on the OTA nephrotoxic effects, in 100 $ng \cdot kg^{-1}$ of body weight (JECFA 1995).

The European Commission of 19 December 2006 approved the Regulation (CE) nº 1881/2006 that widered the list of foods with a maximal permissible OTA level, thus wine and vine dried fruits were set in 2 and 10 $\mu g \cdot Kg^{-1}$ respectively (table 1.10).

Table 1.10. Maximum OTA levels allowed for different foodstuff.

Products	OTA maximum levels (µg kg^{-1})
Cereals and derived cereal products.	
• Unprocessed cereals	5.0
• All products derived from cereals (including processed cereal products and cereal grains intended for direct human consumption)	3.0
Dried vine fruit (currants, raisins and sultanas)	**10.0**
Roasted coffee beans and ground roasted coffee with the exception of soluble coffee.	5.0
Soluble coffee (instant coffee)	10.0
Wine (including sparkling wine, excluding liqueur wine and wine with alcoholic strength of not less than 15% vol.) and fruit wine.	**2.0**
Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails.	**2.0**
Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption.	2.0
Processed cereal-based foods and baby foods for infants and young children.	0.5
Dietary foods for special medical purposes intended specifically for infants.	0.5
Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices and liquorice.	Not established

(Commission Regulation 2006)

2. WINE AND OTHER GRAPE PRODUCTS

2.1. Grapes

Grape is the fruit of *Vitis vinifera*. There are three main groups of grapes depending on their use: vine grape, table grape and dried vine fruits. Thus, by crushing the vine grapes it is obtained the must and through its total or partial fermentation it is eventually obtained the wine.

Grapes are susceptible to be attacked by insects and moulds during ripening and after harvesting. At pre-harvest, *Alternaria*, *Botrytis*, *Cladosporium, Rhizopus* and *Aspergillus* are the most frequently isolated fungi (Sage *et al.,* 2002; Bellí *et al.,* 2004a; Serra *et al.,* 2006 a and b). *Penicillium* species apparently do not attack grapes before harvest (Pitt and Hocking 1997), but are prevalent in stored grapes where *P. expansum* is the most common contaminant species (Snowdon 1990). Other species isolated from stored grapes are *P. aurantiogriseum*, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. crustosum*, *P. decumbens, P. glabrum*,

Aspergillus niger and *Cladosporium herbarum* (Barkai-Golan 1980; Marois *et al*., 1992; Benkhemmar *et al.,* 1993).

2.2. Raisins

The preservation of grapes by drying is a major industry in many parts of the world. Drying practices for grapes are largely traditional and vary with geographical location and with the variety of grapes. In the traditional (open sun) drying method, the grape bunches are spread over either the ground or on a platform in a thin layer directly exposed to the sun (Figure 2.1.). The drying time is about 8-10 days. There is a risk of deterioration due to mould and insect infection.

Another improved traditional drying method, i.e. natural rack drying, is commonly used in some countries. In this method, the selected bunches of grapes are laid on long narrow wire screen racks under the shelter of an iron sheet roof. The main source of the heat required for drying, in this case, is the ambient air (Figure 2.2).

Some other methods for drying grapes have also been developed and improved:

- Sohagi-Hana type drier: used in Afghanistan for drying grapes in special houses (Figure 2.3). Because of no artificial heating and poor ventilation drying may take several months.
- Microwave vacuum drying associated with temperature control (Clary *et al.,* 2007)
- Hot air driers (Karathanos and Belessiotis 1999; Doymaz and Pala 2002)
- Natural convection solar drier, tunnel greenhouse and open sun (Fadhel *et al.,* 2005).

Figures 2.1, 2.2 and 2.3. Solar grape drying, shade drying and Soyagi-Hana dryer, respectively.

The drying techniques can influence on mycobiota and mycotoxin contamination of raisins. In the range of low and intermediate food a_w, yeasts and moulds are the common spoilage flora. Immediately after harvest, the dominant species are field fungi and during storage they are gradually substituted by storage fungi: *Eurotium*, *A. niger* and *Penicillium* (Wallace *et al.,* 1976). Dried fruits, which have low a_w, are generally resistant against microbial attack. Spoilage of dried vine fruits is mainly caused by black aspergilli (El Halouat and Debevere 1997; Abarca *et al.,* 2003) since they can grow to some extent during drying and are presumable highly resistant to drying conditions (Pitt and Hocking 1997). *Eurotium amstelodami* was also frequently isolated from dried fruits given that the minimum a_w permitting germination of this fungus was reported to be 0.75 a_w (Wheeler and Hocking 1988).

2.3. Wine

The main categories of wine are red and white. When red wine is being made, some of the stems, the skins and the pulp remain with the juice, forming what is known as the *must*. The alcohol in the fermenting juices helps extracting colour from the skins. The longer the juice is in contact with the skins, the darker the wine. Tannins and other substances such as OTA are also extracted into the wine during this process.

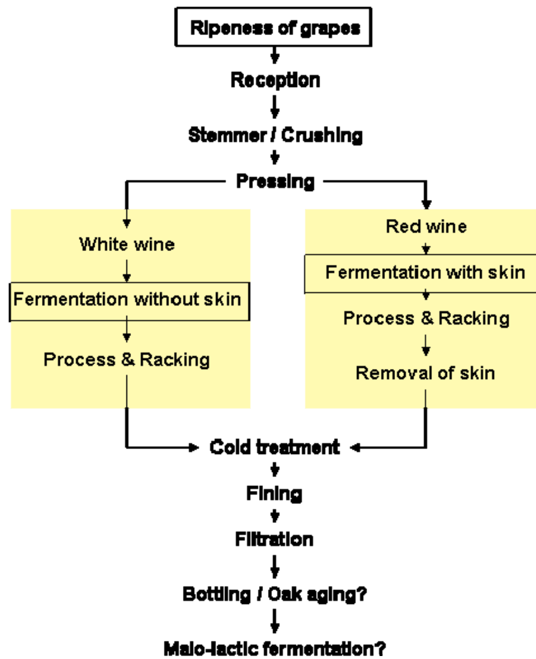


Figure 2.4. Flow chart of main winemaking steps.

As it is commonly known, alcohol in wine is produced by the fermentation of the sugar contained in the must by means of yeasts. In sweet wines it is therefore necessary that part of the sugar is transformed (fermented) into alcohol, whereas a certain quantity must be kept in order to give sweetness to the wine.

According to European Regulations (Council Regulation 1999) these are some types of wines and grape products:

- Fortified musts: Alcohol distillate is added to unfermented grape must (fortified).
- Table wine
- Liqueur wine: Wine that has been fortified to 15 to 22º.
- Sparkling wines (group of four types of wines)
- Fortified wine: Dry wine that has been fortified until between 18 and 24º.
- Wines from overripe grapes: Generally no alcohol is added but its alcohol percentage must be equal or higher than 15º.

2.4. Special wines

The aim of this thesis was the study of grape products derived from overripe grapes due to their higher OTA levels reported in previous works. In this direction, wines made from overripe grapes and several different types of sweet wines were selected for being studied. All them were divided into groups according to an arbitrary classification depending on their elaboration.

The aforementioned groups of wines are:

-Fortified musts
-Fortified wines (Liqueur wines included)
-Sparkling wines
-Dessert wines from overripe grapes

Several methods have been used throughout history to sweeten wine. The most common way was to harvest the grapes as late as possible. This method was advocated by Virgil and Martial in Ancient Roman times. In contrast the Ancient Greeks would harvest the grapes early, to preserve some of the grapes acidity, and then leave them in the sun for a few days to allow them to shrivel and concentrate their sugar. In Crete, a similar effect was achieved by twisting the stalks of the grape bunches to deprive them of the vine's sap and letting them dry on the vine-a method that produced Passum wines. Two other techniques popular in Ancient Rome were *defrutum*-reduction of the grape must by means of boiling, and *mulsum*-adding honey to the fermented wine. Stopping the fermentation also enhanced a wine's potential sweetness-the German method *süssreserve*. In ancient times this was achieved by submerging the amphorae in cold water till winter. A similar concept is achieved today with the use of modern technology in cod stabilisation (Wikipedia 2007).

<u>Fortified musts and wines</u>

The interruption of fermentation - in order to keep sugar and therefore sweetness - can be done by adding alcohol to the must (fortification), as well as lowering the temperature, adding sulphur dioxide (SO_2), treating the must with sterile filtering in order to completely eliminate yeast that would otherwise resume fermentation.

In the other hand, the fermentation of a must rich in sugar is very slow and sometimes in those having a very high concentration of sugar the fermentation could either not even start or produce a small quantity of alcohol.

Some examples of these types of wines are numbered as follows:

Fortified musts:	Muscats, Mistelles
Fortified wines:	Porto (Tawny, Ruby, Vintage), Sherry (Oloroso, Amontillado, Fino, Manzanilla), Marsala, Samos, Málaga.

Some fortified wines are made with must from sun-dried grapes such as Samos and Málaga.

Among Sherry wines, the Fino and Manzanilla, also experiment a secondary fermentation while aging in wooden barrels achieved by floating yeasts that are alcohol resistant, called Flor yeast.

Sparkling wines

Sparkling wine is a wine with significant levels of carbon dioxide in it making it effervescent. The carbon dioxide may result from natural fermentation, either in a bottle as with the 'méthode champenoise', or in a large tank designed to withstand the pressures involved, as in the Charmat process or as a result of carbon dioxide injection. If there is any injected gas the wine must be labeled as "Aerated Sparkling wine made with the addition of carbon dioxide" (Wikipedia 2007). The juice is allowed to ferment until it reaches approximately 5% to 6% alcohol by volume, at which point the temperature of the tank is lowered enough to cause the yeast to become dormant, which causes the fermentation process to halt.

Examples of some sparkling wines are:

Crémant (France), Champagne (France) Moscato d'Asti (Italy), Spumante (Italy), Sekt (Germany).

Wines from overripe grapes

There are numerous types and styles of non-fortified dessert wine. While none of the winemaking processes involves adding grape spirit to halt the fermentation process artificially, all of them require the premature cessation of the fermentation process, leaving behind varying residual sugar levels. The most common cause of fermentation cessation in non-fortified dessert wines is the extraordinary sugar levels of the fermenting grapes, which naturally drives the alcohol level above 15% by volume.

Dehydration by sunlight:

Harvested grapes are placed on esparto mats or other materials, on the ground or on higher structures, and exposed to sunlight for one-two weeks until grapes reach the desired sugar

content. During nights bunches are covered for being protected from the negative effect of high humidity.

Some wines made by sun-dehydrated grapes are:

Pedro Ximenez (Spain), Málaga (Spain), Passito (Italy), Fondillon (Spain), Samos (Greece), Commandaria (Cyprus).

Dehydration in dry closed places

Most grape drying for commercial purposes takes place in a winery loft, where windows may be opened to let in plenty of air (essential against the development of rot and mould). Bunches are hung up vertically (on hooks, or on long strings), or laid out horizontally on neutral dry materials for several weeks.

Few examples of this type of wines:

Garnatxa de l'Empordà (Spain), Vin de Paille (France), Vin Santo (Italy), Recioto (Italy), Amarone (Italy) (Asenjo 2001).

Dehydration by Noble rot

During the warm, damp autumn weather in some regions of France, Germany and Hungary the noble rot is encouraged to grow on the skin of selected varieties of grapes. Noble rot is produced by the development of the fungus *Botrytis cinerea* on the skin of healthy bunches of grapes. This process is only feasible in areas with brumes in early mornings followed by strong insolation. If the weather remains warm, but becomes dry, the rot flourishes with the beneficial effect of concentrating the sugar through dehydration of the water in the grape throughout the puncture of the grape skin by the *Botrytis* micelia. If the weather continues to be damp and turns cooler, the noble rot becomes the undesired gray rot and the crop is ruined as a result (The Winedoctor 2007b).

Some noble wines are as follows:

Sauternes, Barsac, Cadillac, Montbazillac, Alsace, Loire, etc. (France), Auslese, Beerneauslese, Trockenbeerenauslese (Germany), Tokaji (Hungary).

Late harvest

Other sweet dessert wines made from late harvest grapes do not necessarily have *Botrytis*. These wines are picked or harvested in stages, so by delaying the picking of the berries, grapes are allowed to shrivel on the vine. Consequently there are actually several grades of sweetness in the late harvest wines.

Few examples of late harvest wines and their origin:

Vendage Tardive (France and Switzerland), Tokaji (Hungary), Spatlese (Germany) (Asenjo 2001).

Dehydration by ice

The term Eiswein in German means “ice wine” and refers to the fact that Eiswein is made from grapes that have been frozen on the vine and pressed before they thaw. That is harvest of grapes is delayed until mid December, when weather becomes colder and temperatures remains below zero. The pre-thaw pressing causes the frozen water to be removed from the resultant grape must, which in turn super concentrates the sugar and acids in the juice. The grapes are not usually botrytis-affected.

Few types of ice wines according to their origin are as follows:

Icewine (Canada), Vi de Gel (Catalonia, Spain), Eiswein (Austria and Germany).

3. FATE OF OTA DURING WINEMAKING

Ochratoxin A is reduced in the different stages during the winemaking. The major reduction takes place during pressing where OTA bounds to the skins that are removed and only a small percentage is transferred to must (Figure 3.1). In case of red wines, that suffer a maceration of must with skins for longer time, for extracting tannins and other pigments and aromas, the toxin can also be extracted from skins into must, producing higher OTA levels in the must as a result (Logrieco 2004; Grazioli *et al.,* 2006).

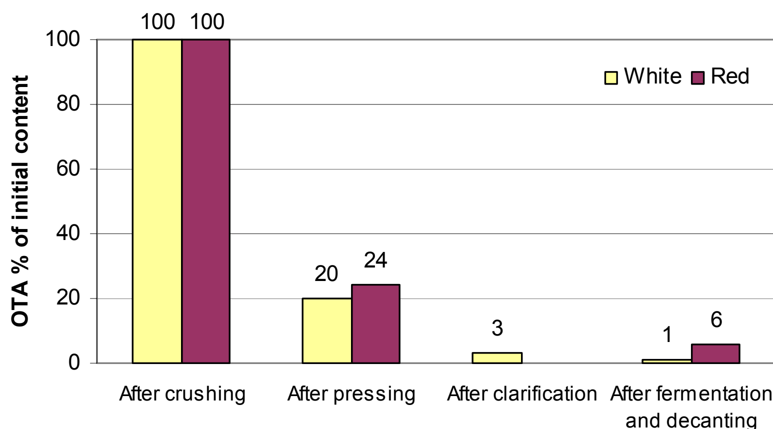


Figure 3.1. Fate of ochratoxin A during vinification of Semillon (white) and Shiraz (red) grapes inoculated with fungus in vineyard (Adapted from Leong *et al.,* 2006)

During fermentation there is also a reduction of OTA concentration in the must (Figure 3.2) as a consequence of:

- degradation of toxin by fermenter yeasts (Caridi *et al.,* 2006; Cechini *et al.,* 2006).
- adsorption mechanism onto yeast surface explained by the overall negative charge in the cell walls and the acidic nature of OTA (Castellari *et al.,* 2001) and further discarded during the fining process.

In every racking, filtering, fining, clarification or in any additional step where a solid phase is removed from the wine product, OTA can be also removed and discarded by adsorption. There also are many fining agents with different ability for adsorbing and removing OTA, up to 82% (Castellari *et al.,* 2001).

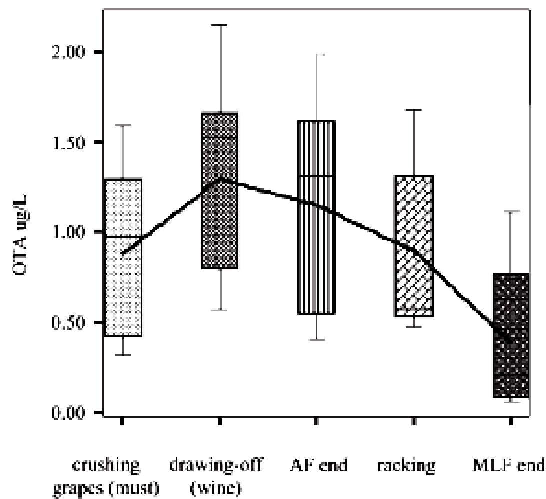


Figure 3.2. Trend of the toxin from crushing grapes (must) to wine at the end of the malo-lactic fermentation. The bold curve is the average trend of OTA (AF= alcoholic fermentation; MLF= malo-lactic fermentation) (Grazioli *et al.,* 2006).

Some wines aged in wooden barrels or in bottles for long periods of time can experiment oxidation and also a secondary fermentation by lactic acid bacteria, the so called malo-lactic fermentation. These biological or chemical actions have shown to reduce OTA (Grazioli *et al.,* 2006) (Figures 3.2 and 3.3).

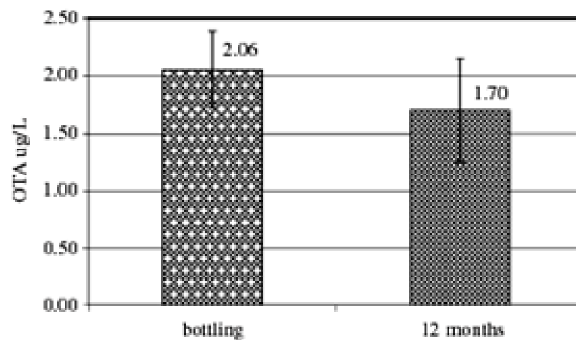


Figure 3.3. OTA reduction after 12 months of bottle-aging in relation to the OTA levels at bottling. Bottles were stored in the dark at 20 ºC (Grazioli *et al.,* 2006).

4. CONTROL AND PREVENTIVE ACTIONS

The available evidence indicates that *A. carbonarius* and *A. niger* are not pathogens on fruit, but saprophytes, and hence cannot gain entry to sound fruit. However, damage to fruit by any means, mechanical, chemical, or by disease microorganisms, may allow entry into fruit tissue, where the low pH, high sugar, and often warm temperature provide an ideal habitat for these species. This is especially true in grapes, which have very strong skins. When the skins are intact, they are resistant to attacks by these fungi, but ideal growth conditions prevail once the skin is disrupted (Bellí *et al.,* 2006a).
Fungicides are sometimes used against pathogenic fungi, but the effectiveness of such treatments is variable and depends on seasonal and geographic factors (Nair *et al.,* 1987; Snowdon, 1990).

Control of mechanical damage relies on good farm management. This is a serious problem when grapes are to be dried, as mechanical damage at harvest is difficult to avoid and the length of the drying process (2 weeks or more) provides ample time for the growth of *A. carbonarius* and ochratoxin A production. This is much less of a problem with wine grapes, which are usually crushed within a few hours of picking, and it is a reasonable assumption that the rapid establishment of anaerobic conditions prevents further growth of *A. carbonarius* or ochratoxin A production (Hall and Denning 1994).

Some cultivars, especially the sultana grapes used for drying, are susceptible to rain damage during the week preceding harvest, when turgor pressure inside the fruit is high and the skins often inflexible. Splitting around the neck of the grape below the stem provides an ideal

environment for invasion by *A. carbonarius*. Control is very difficult. A useful recommendation may be to discard those bunches that are visibly damaged and to start drying as quickly as possible.

4.1. Pre-harvest

Lesions on the berries by insects, warms, rots or sunburns that may favour the further fungal development must be prevented. On this purpose, it is advisable to apply specific phytoprotectants on grapevines in order to assess the control of oidium and acid rot following guidelines, dosage and withholding periods set by manufacturers.

<u>Agricultural practices</u>

The OIV-INCAVI (*Organisation International de la Vigne et du Vin-Institut Català de la Vinya i el Vi*) recommend setting the harvesting date depending on the ripeness of grapes, its sanitary level, the meteorological forecasts and evaluating the risk of contamination by ochratoxigenic fungi. In areas with high risk grapes should be harvested earlier (VITI-OENO 2005).

When grapes are heavily contaminated by fungus, they should not be intended for human consumption, neither as fresh product nor vine dried fruit nor to be transformed to concentrated must, grape juice, wine or vinegar. If it is finally used, it should be restricted for distillation.

<u>Chemical and biological control</u>

The application of chemical products has been the most widely method used for long time for controlling pests in crops, due to its easy and economic implementation. However, in recent years public concern over pesticide residues in the environment, foods and feeds has led to the development of alternative methods for controlling these pathogens and diseases, such as biological control (Tables 4.1 and 4.2).

Table 4.1. Some of the most used pesticides applied on grapevines, table and raisins grapes.

Chemical Name	Chemical Class	Uses
Azoxystrobin	Strobin	Fungicide
Benomyl	Benzimidazole	Fungicide
Captan	Thiophthalimide	Fungicide
Chlorpyrifos	Organophosphorus	Insecticide, Nematicide
Copper hydroxide	Inorganic-Copper	Fungicide, Nematicide
cypermethrin	Pyrethroid	Fungicide, Insecticide
Cyprodinil	Anilinopyrimidine	Fungicide
Dimethoate	Organophosphorus	Insecticide
Dinocap	Dinitrophenol derivative	Fungicide, Insecticide
Endosulfan	Organochlorine	Fungicide, Insecticide
Esfenvalerate	Pyrethroid	Insecticide
Fenarimol	Pyrimidine	Fungicide
Fludioxonil	Phenylpyrrole	Fungicide
Folpet	Thiophthalimide	Fungicide
Mancozeb	Dithiocarbamate, Inorganic-Zinc	Fungicide
Maneb	Thiocarbamate	Fungicide, Insecticide
Methalaxyl	Anilides	Fungicide
Methomyl	N-Methyl Carbamate	Insecticide, Breakdown product
Sulfur	Inorganic	Fungicide, Insecticide
Tebuconazole	Azole	Fungicide

Chemical Name	Class	Uses
Bacillus thuringiensis, subsp. Kurstaki, strain HD-1	Microbial	Insecticide
Myrothecium verrucaria, dried fermentation solids & solubles	Microbial	Insecticide, Nematicide

(Council Directive 1991; Pesticides Action Network 2006; Infoagro Systems 2007)

Many of the former pesticides are useful for controlling a wide range of pests, including insects, nematodes and fungi. On the other hand, by preventing grape damages by insects or nematodes, the black rot risk (*Aspergillus* section *Nigri*) may be reduced as a result.

Table 4.2. Biological control against fungi

Controller organism	Target	Mode of action	References
Yeasts non-*Sacharomyces* from grapes	*A. carbonarius* *A. niger*	Antagonism	Bleve *et al.,* 2006
Trichoderma spp.	Fungi	Antibiosis, lysis, antagonism, mycoparasitism, promotion of plan growth	Chet 1987 Elad 2001 Schirmbock *et al.,* 1994 Arisan-Atac *et al.,* 1995
Bacillus pumilus	*Aspergillus* *Penicillium* *Fusarium*	Synthesis of antifungal metabolites	Munimbazi and Bullerman 1998
Bacillus subtilis	Fungi	Synthesis of antifungal metabolite (rhizocticin A)	Kluger *et al.,* 1990
Aloe spp. extracts	*A. niger* *C. herbarum* *F. moliniforme*	Antifungal plant extracts	Ali *et al.,* 1999
Metsohnikowia fructicola	Antifungal	Antagonism	Keren Zur *et al.,* 2002
Soil bacteria	*B. cinerea*	Antagonism	Paul *et al.,* 1997 and 1998
P. chrysogenum glucose oxidase	*A. giganteus* *A niger* *A. terreus, etc.*	Synthesis of antifungal enzyme	Leiter *et al.,* 2004

Inducers of plant resistance

UV-C irradiation is a practical and reproducible method for inducing grapevine defense responses. Grapevine species respond to UV treatments by enhancement of defense mechanisms such as an ultraexpression of stilbene and resveratrol (Bonomelli *et al.,* 2004; Borie *et al.,* 2004).

B. cinerea has also been reported as an effective elicitor of resveratrol in infected grapes (Bavaresco *et al.,* 1997; Ureña *et al.,* 2003; Borie *et al.,* 2004).

Transgenic plant resistance

Plants are equipped with an array of defence responses to prevent their invasion by pathogens. Some of these defensive tools are already established in the plant, whereas others are inducible upon perception of the pathogen.

Some examples of studies made in plant genetics are mentioned below:

- Genetic transformation of grapevines with *Trichoderma harzianum* and antimicrobioal peptide genes for improvement of fungal tolerance (Hinrichsen *et al.,* 2005).
- Transgenic grapevine expressing a rice chitinase (Yamamoto *et al.,* 1999).
- Genetic transformation of grapevine with Magainins, short peptides with broad-spectrum antimicrobial activity (Vidal *et al.,* 2006).
- Transgenic grapevine expressing a chitinase gene, as well as other antifungal genes has shown to be effective in controlling powdery mildew and other fungal diseases (Kikkert *et al.,* 1997).

4.2. Post-harvest and storage

General cautions

The OIV recommends the following actions in case of grapes harvested intended for wine production are moderately contaminated by ochratoxigenic moulds.

- Grapes damaged by insects and/or by moulds should be removed before harvest or during harvesting.
- Grapes must be selected to discard damaged bunches or damaged portions of bunches. It is encouraged to remove all black moulds.
- Harvested grapes should be transported as fast as possible to cellar to avoid holding periods.
- Recipients for transportation shall be cleaned.

When grapes are intended for vine dried fruits elaboration, some further advices are given:

- To use solely healthy grapes, those not damaged by insects or moulds.
- To place grapes for drying in a single layer.
- To facilitate the progressive and homogeneous drying of bunches.
- Avoid the presence of flies such as drosophilae.
- In case of drying in exterior places, it is recommended choosing ventilated places and covering drying bunches during the night to avoid condensations.

Chemical and biological control

Although grapes were carefully picked and all cautions being warranted, they are still susceptible to insect attack and fungal contamination. Some chemical and biological agents are used and investigated on grapes after harvest as it can be observed in the table 4.3.

Table 4.3. Studies on post-harvest chemical and biological control of fungi.

Agent	Target	Mode of action	References
Sulphur dioxide	Fungi	Inhibition of growth	Ben Arie *et al.,* 1991
Methyl bromide Phosphine	Insects	Inhibition of growth	Soderstrom and Brandl 1984
Trans-Resveratrol	Wide range	Natural plant and fruit protective	Gonzalez Ureña *et al.,* 2003
Metschnikowia fructicola	Fungi	Antagonism by iron depletion	Keren Zur *et al.,* 2002 Sipiczki 2006
Grape fruit seed extract BC-1000	*B. cinerea*	Antioxidant phenolics	Esterio *et al.,* 1992
Aureobasidium pullulans	*B. cinerea* *Monilinia laxa*	Wide range of actions	Schena *et al.,* 2003
Candida guilliermondi *Acremonium cephalosporium*	*Botrytis* *Aspergillus* *Rhizopus*	Antagonism	Zahavi *et al.,* 2000
Soil bacteria	*B. cinerea*	Antagonism	Paul *et al.,* 1997 and 1998

Sulphur dioxide has been used for long time as a control agent in post-harvest management of grapes and raisins. However, due to its hazardous health effects for humans and environment (Ontario 2007) its usage is being reduced in foods intended for direct consumption.

Alternative methods

Alternative control methods have recently been developed with the aim of avoiding the residue problems in foods. Some of these techniques, mainly physical, are summarised in the table 4.4.

Table 4.4. Physical and alternative chemical methods for post-harvest pest management.

Method	Target	Mechanism of action		Reference
Ultraviolet radiation	Plant or fruit	Inducer of plant resistance.	Stimulates the biosynthesis of resveratrol and viniferins	Langcake and Pryce 1977 Cantos *et al.,* 2002
	B. cinerea *Monilia fructigena* *Aspergillus*	Germicide	Irreversible damages in fungal DNA.	Nigro *et al.,* 1998 Marquenie *et al.,* 2003 Jun *et al.,* 2003 Green *et al.,* 2004
Ionizing radiation	Insects	Insecticide	-	Cetinkaya *et al.,* 2006
Modified Atmospheres	*B. cinerea*	Prevent spores germination and fungal development		Zoffoli *et al.,* 1999
	Insects	Insecticide		Annis 1987
	Insects and Moulds	Prevent spores germination and fungal development. Insecticide		Tarr and Clingeleffer 2005
Cold storage (0-4 ºC)	Micro-organisms	Prevent spores germination and fungal development		

4.3. During winemaking

Winemaking cautions

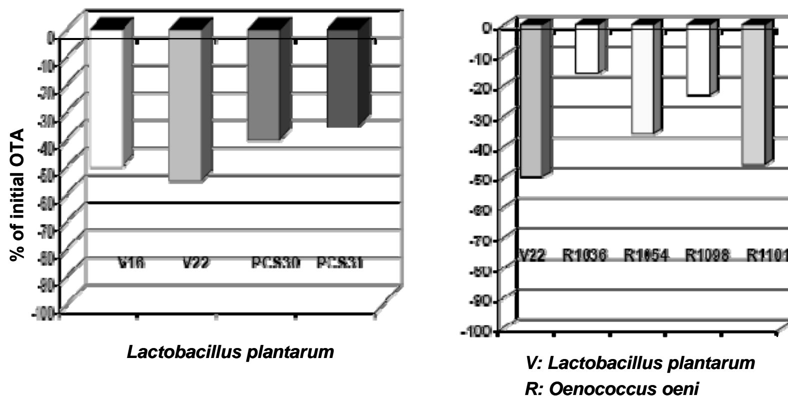
When a risk of OTA contamination is suspected, the analysis of the toxin in musts is encouraged and some practices are recommended as follows:

- Avoid the maceration with rachis or to reduce the maceration time.
- Fast and light crushing.
- Avoid the use of pectinolytic enzymes for the racking and maceration processes, it being preferable filtering, centrifugation or flotation.
- Grapes and must can be treated with enologic carbon rather than in the final wine.
- Use fermentators with smooth walls to facilitate the cleaning.
- Racking as soon as possible after fermentation is finished.
- Ageing with lees can be helpful for reducing OTA content.

OTA degradation by yeasts and lactic acid bacteria (LAB) during fermentations

Some oenological yeast have adsorbent properties for OTA removal (Bejaoui *et al.,* 2004; Caridi *et al.,* 2006; Cecchini *et al.,* 2006) during alcoholic fermentation.

Several strains of the LAB *Lactobacillus plantarum* and *Oenococcus oeni* have shown to lower OTA in must during malo-lactic fermentation (Silva *et al.,* 2003), *L. plantarum* being the most effective by achieving OTA reductions up to 50% of the initial content (Figure 4.1).



Figures 4.1. Differential OTA reduction by *Lactobacillus plantarum* and *Oenococcus oeni* during malo-lactic fermentation (Silva *et al.,* 2003).

4.4. Decontamination of OTA in wine, grape juices and musts

If the concentration of OTA in wine or in the final product is still high, it can be lowered by adding chemical or biological products that are commercialised for that purpose (Figure 4.2). Their mechanism of elimination is by binding to the toxin and to precipitate. The residue can be separated by filtering, centrifugation or other process, thus wine can be a secure product. Adsorbent products containing charcoal (Atos, Micosorb and Toxical) are efficient in decreasing the level of ochratoxin A in the wine, even above 90% of the initial OTA content when using Micosorb 0.3 $g \cdot l^{-1}$ (Silva *et al.,* 2003) (Figure 4.2).

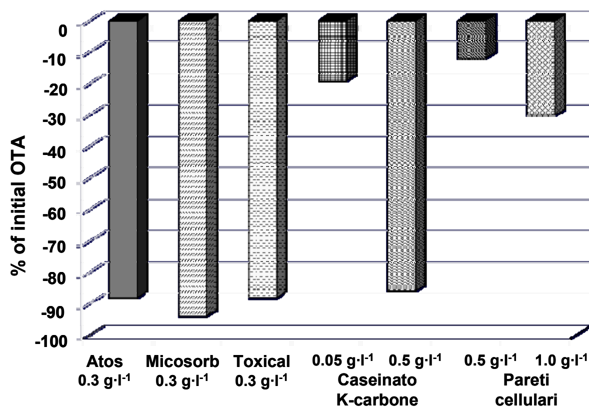


Figure 4.2. Differential OTA reduction by chemical adjuvants added to wine (Silva *et al.,* 2003).

The products used or proposed for reducing OTA in wine are so diverse that also include conidia of *Aspergillus* spp. as biological adsorbents. Adsorption phenomenon of OTA on dead conidia of black Aspergilli could be related to hydrophobic interactions. The global positive charge of OTA molecule in acid media (grape juices) could also interact with negatively charged molecules found on fungal conidia (Bejaoui *et al.,* 2006). However, the effectiveness of this method is variable depending on the initial OTA contamination of grape juice.

Although these products appear to be an easy and economical method for reducing OTA in wine, they can produce a detriment in the quality of wine what concerns to its organoleptic properties.

Finally, the application of chemical or biological additives to the grape product, excepting those intrinsic to the winemaking process, should be considered the last option and to focus the attention in preventive rather than to apply corrective actions.

OBJETIVES

II. OBJETIVES

This thesis starts with the results of the European project “Risk Assessment and Integrated Ochratoxin A (OTA) Management in Grape and Wine” (QLRT-2000-01761) and it is enclosed in the Spanish project “Evaluacion del Riesgo de Contaminación por ocratoxinas y tricotecenos en alimentos y desarrollo de métodos de control” (Evaluation of the contamination risk by ochratoxins and trichothecenes in foods and the development of control methods) (AGL2001-2974-C05-02).

Previous surveys studied the occurrence and incidence of OTA in wines and in other grape derived products and reported the highest OTA levels in sweet wines and in dried vine fruits.

Starting from this basis the following objectives, divided into three different points of view, were presented:

1. Evaluation of the food products: vine dried fruits and special wines

- To describe the mycobiota and ochratoxin A producing species present in grapes at the later ripe stages and further dehydration, including commercial raisins.
- To make an exhaustive analysis of the special wines from Europe and to evaluate the effect of wine origin and winemaking techniques used in their elaboration as regards OTA incidence and occurrence.

2. Ecophysiological studies

- To study the kinetics of OTA production by *Aspergillus* section *Nigri*.
- To study biotic and abiotic effects on growth and ochratoxin production of *A.* section *Nigri*.
- To understand why wines from botrytised grapes lack ochratoxin A.

3. Control and prevention

- To check the residual activity of fungicides applied before harvest on grapevines and interacting fungi on *Aspergillus carbonarius* growth and OTA synthesis in dehydrating grapes.
- To evaluate the effect of modified atmospheres packaging in the mycelial growth and OTA production by *Aspergillus niger* and *A. carbonarius in vitro*

STUDIES

Chapter I:

EVALUATION OF THE FOOD PRODUCTS: VINE DRIED FRUITS AND SPECIAL WINES

Study I:

Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors

Ana Valero, Sonia Marín, Antonio J. Ramos, Vicente Sanchis *

Food Technology Department, University of Lleida. Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

ABSTRACT

Aims: To explain the dominance of OTA-producing fungal species in sun-dried grapes for special wine production through an ecophysiological approach.

Methods and Results: Grapes at different ripening stages, sun-dried grapes and raisins were analysed for fungal presence, and isolates identified. *Aspergillus* section *Nigri* incidence in grapes increased with grape maturation. In the ecophysiological study five isolates (*Alternaria alternata*, *Cladosporium herbarum*, *Penicillium decumbens*, *Aspergillus carbonarius*, *A. niger* aggregate and *A.* section *Nigri* uniseriate) were inoculated in SNM medium at four a_w (0.82-0.97) and incubated at 20, 30 and 40 ºC for 18 days. Isolates were also inoculated in pairs to evaluate fungal interactions recording their growth rates and indexes of dominance. *A.* section *Nigri* grew in a wider range of temperature and a_w, had higher growth rates than the others under most of the conditions tested and showed behaviour usually dominant.

Conclusions: The presence of *A.* section *Nigri* is predominant in grapes during harversting and sun drying period likely due to a better adaptation to hot and humid environment.

Significance and Impact for the Study: The duration of the drying period should be reduced as much as possible without compromising the quality of the final product, or drying the grapes in controlled chambers with dry hot air flow.

Publication: *Letters in Applied Microbiology* **41** (2005) 196-201.

INTRODUCTION

In the latest years ochratoxin A (OTA) has attracted the attention from health authorities. This mycotoxin can be implicated in different toxic processes (Marquardt and Frohlich 1992) and has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC 1993). OTA occurs in a variety of plant products such as, coffee (Pardo *et al.* 2004), figs (Özay *et al.* 1995), grapes (Battillani and Pietri 2002; Bellí *et al.* 2004b) and dried vine fruits (Ostry *et al.* 2002; MacDonald *et al.* 1999; Abarca *et al.* 2003). OTA has also been detected in beverages such as grape juices, musts and wines (Bellí *et al.* 2004a). According to studies (Abarca *et al.* 2003; Bellí *et al.* 2004b), OTA-producing strains from the group *A.* section *Nigri* (*A. carbonarius* and *A. niger* aggregate) are the source of OTA in wines, grapes and dried vine fruits.

Among wines, sweet wines made with sun-dried grapes, and red wines, have the highest OTA contents, from 0.04 to 1.05 µg l^{-1} (Bellí *et al.* 2002) and up to 7.60 µg l^{-1} (Battilani and Pietri 2002). The highest OTA content, among grapes and its derivatives, has been reported in dried vine fruits, with OTA levels above 53 µg kg^{-1} (MacDonald *et al.* 1999).

Some Mediterranean countries make special sweet wines using dried grapes by direct exposition to sun. Fructose and glucose concentrations in grapes can be increased even more by sun-drying, and pressed when sugar levels reaches 300 g/ L. Contamination by different moulds can occur during preharvest, harvest and grape processing. *Botrytis*, *Alternaria*, *Cladosporium*, *Aspergillus*, *Eurotium*, and *Rhizopus* are regarded as the main natural contaminants in this sort of foodstuffs (Sage *et al.* 2002; Magnoli *et al.* 2003). Mycological studies have reported black aspergilli as the predominant fungi with occurrences between 33 to 100% in raisins (El Halouat and Debevere 1997; Abarca *et al.* 2003; Magnoli *et al.* 2004), and within them, *A. carbonarius* and *A. niger* have been recorded between 69 to 100% of vineyards in a study carried out in Australia (Leong *et al.* 2004).

Currently, several countries have specific regulations for OTA in various commodities, 10 ng OTA g^{-1} being the maximum level allowed for dried vine fruits in European Community, (EC) No. 472/2002 and 2 ng OTA g^{-1} for wine, grape must and grapes (EC) No.123/2005.

The aim of this study was to explain the dominance of OTA producing species through grape ripening and sun-drying process throughout an ecophysiological approach.

MATERIAL AND METHODS

Samples

Grapes and sun-dried grapes were supplied by wineries from south of Spain, included in the V climatic zone of Winkler classification (Hidalgo 1999). Sample size was three to six bunches, collected and packed individually. Samples provided were one in June, four from the period July

and August, six in September (at harvest) and 9 samples after sun-drying period. In addition, six samples of packed raisins (200-700g) were bought in supermarkets during 2003.

Mycobiota of samples. Isolation and characterization

Five berries were randomly taken from each bunch and directly plated onto Dichloran Rose Bengal Chloramphenicol medium (DRBC) in Petri dishes. All plates were incubated for 7 days at 25ºC. Moulds were identified to genus or specie, if required, in accordance with guidelines published by Pitt and Hocking (1997). Every black aspergilli was isolated and grown in Czapek-Dox medium (CZ) and MEA for further classification as *A. carbonarius*, *A. niger* aggregate group and uniseriate as recommended by Dr. Kozakiewicz guidelines (CABI Bioscience, Egham, UK) (personal communication).

The ability of OTA production by *A.* section *Nigri* was tested through HPLC (Waters, Milford, MA, USA) following the extraction and clean-up protocol detailed in Bragulat *et al.* (2001) with modifications.

Shannon-Weiner biodiversity index was calculated for each period. This index takes into account genus richness and proportion of each genus within a zone. It's given by the formula; $H = -\sum(Pi \log[Pi])$, where Pi means proportion of one genus within total of population.

Ecophysiological studies

From the isolated black aspergili, two isolates were chosen; *A. carbonarius* (OTA producer) and *A. niger* aggregate (OTA non-producer). The most common species among the remaining mycobiota were selected; *Alternaria alternata*, *Cladosporium herbarum* and *Penicillium decumbens.* Isolates are deposited in the Department of Food Technology, ETSEA, Universitat de Lleida, Spain.

The culture medium used was a synthetic nutrient medium (SNM) similar to grape composition between veraison and ripeness (Delfini *et al.* 1982), and was modified to adjust it to the different water activities. To prepare one litre of SNM medium at 0.82, 0.87 and 0.92 aw, were added 196.0 g of D(+)Glucose, 204.0 g of D(-)Fructose and 415.3, 288.5 and 143.2 g of Glycerol respectively. For the 0.97 aw medium, were mixed 135.2 g of Glucose and 140.8 g of Fructose.

Inoculums were prepared in suspensions of 10^6 spores ml^{-1}, and inoculated by needle single point. Firstly every micro-organism was grown under the four water activities and incubated at three temperatures (20º, 30º and 40º C). Radii of colonies were recorded daily until the 18th day of incubation. Secondly, moulds were grown in pairs on the SNM modified, inoculating one *A.* section *Nigri* faced to one of the three non-aspergilli at a distance of 45 mm between them, at 0.87, 0.92, 0.97 a_w and 20 and 30ºC. Growth radii in the line between both inoculation points were recorded daily for 18 days. In addition, each black aspergilli was given a numerical score

using the following scoring system: 1, mutual intermingling; 2, mutual inhibition on contact; 3, mutual inhibition at a distance; 4, dominance on contact; 5, dominance at a distance (Magan and Lacey 1984).

Statistical analyses

For singular cultures the radial extensions of colonies were plotted against time and the growth rates calculated using linear regression of the lineal phase. Daily measured radii were analyzed statistically by SAS (SAS Institute Inc., Cary, NC, USA) throughout a covariance analysis. Significance of the levels of T, a_w and fungal interaction were evaluated and Duncan means separation test was performed.

RESULTS

Mycobiota evolution in grapes during ripening and sun-drying

Seven main genera (*Aspergillus, Alternaria, Cladosporium, Epicoccum, Eurotium, Rhizopus* and *Penicillium*) were isolated in the samples from June to September, sun-dried grapes and raisins (Figure 1).

Alternaria was the main contaminant in June (80% grapes) while the remaining genera were present in 21.8% of the grapes. *Aspergillus* was present in 8%, *A.* section *Nigri* being 25% of total aspergilli. In July-August *Aspergillus* (100% black aspergilli) was found in 68.5% of the grapes, *Alternaria* decreased to 10.0% and *Epicoccum* was found in 30.0% of grapes. In September, *Alternaria* went on decreasing until 0.4 % of incidence and *Aspergillus* colonised the 75.9 % of grapes gathered (99% black aspergilli). *Penicillium* acquired a relevant incidence with 22.5 % of grapes contaminated. After the drying period, *Aspergillus* contaminated 84.8% (100% black aspergilli) of dried grapes, *Penicillium* decreased to 12.8% and *Alternaria* remained present with a percentage of 0.8. Finally, 79.3% of commercial raisins were mainly contaminated by *Aspergillus* (100% black aspergilli) and *Eurotium* in 12.7 %.

Regarding grapes, while biodiversity index was high after grape setting, it showed a decreasing trend throughout grape maturation and sun drying process (Figure 1).

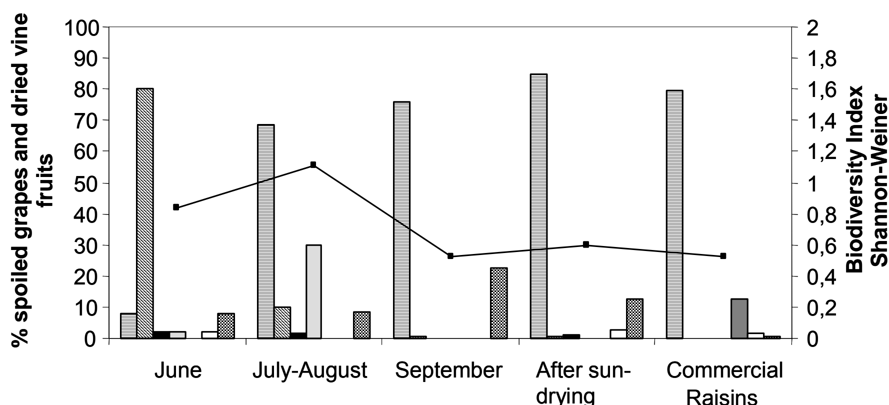


Figure 1 Percentages of grapes infected with moulds in four periods of sampling and its corresponding biodiversity index (Shannon-Weiner Index).

Aspergillus, *Alternaria*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Rhizopus*, *Penicillium*, Shannon-Weiner Index

A. section *Nigri* and OTA production ability

A. section *Nigri* isolates were randomly chosen from each period, classified into three groups: *A. niger* aggregate, *A. carbonarius* and uniseriates and tested for their OTA production ability (Figure 2). These were one in June (*A. carbonarius* OTA producer), 54 in July-August, 146 in September, 183 from sun-dried grapes and 97 from raisins samples. In July and August, *A. niger* aggregate was predominant with 88.9% of *A.* section *Nigri*. In September both black aspergilli increased their frequencies becoming equal with maturation and sun exposure. In commercial raisins *A. carbonarius* were the 32% and *A. niger* aggregate represented the 68% of black aspergilli. No uniseriates were found in the whole survey.

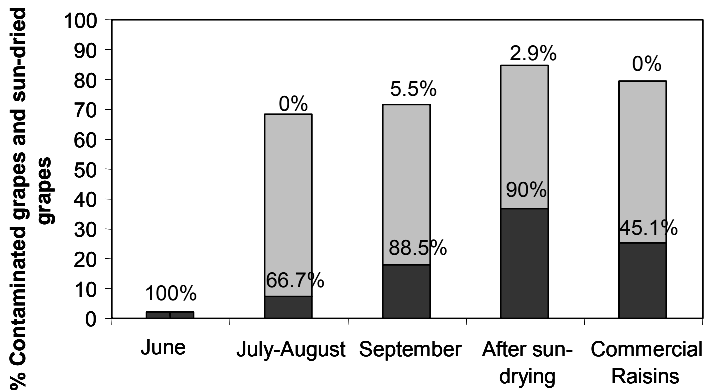


Figure 2 Percentages of grapes infected with *Aspergillus* section *Nigri*. %; percentages above bars correspond to OTA-producing strains within *A. niger* aggregate or *A. carbonarius*, ■*A. carbonarius*, ■ *A. niger* aggregate.

Ecophysiological approach

Main factors (temperature, water activity and interacting species) and their interactions had a significant effect on the growing colonies.

Table 1. Growth rates of pure cultures (mm*d^{-1})

		Temperature (°C)		
	a_w	20	30	40
A.carbonarius	0.87	0.019* ± 0.004	0.208 ± 0.015	n.g.
	0.92	0.457 ± 0.034	1.041 ± 0.032	0.015 ± 0.008
	0.97	3.124 ± 0.054	4.025 ± 0.123	0.203 ± 0.017
A. niger aggregate	0.87	0.577 ± 0.044	2.509 ± 0.077	0.212 ± 0.02
	0.92	1.635 ± 0.029	4.131 ± 0.083	2.899 ± 0.068
	0.97	2.981 ± 0.073	6.693 ± 0.236	10.450 ± 0.428
Alternaria alternata	0.87	n.g.	n.g.	n.g.
	0.92	0.019* ± 0.004	0.620 ± 0.014	n.g.
	0.97	3.201 ± 0.059	3.943 ± 0.108	n.g.
Cladosporium herbarum	0.87	n.g.	n.g.	n.g.
	0.92	0.259 ± 0.021	0.019* ± 0.004	n.g.
	0.97	1.458 ± 0.025	0.580 ± 0.011	n.g.
Penicillium decumbens	0.87	0.329 ± 0.025	0.169 ± 0.005	n.g.
	0.92	0.415 ± 0.013	0.834 ± 0.054	n.g.
	0.97	1.208 ± 0.040	2.217 ± 0.071	n.g.

n.g.: no growth, *; colony diameter = 1mm.

None of the isolates grew at 0.82 a_w, and neither *A. alternata* nor *C. herbarum* grew at 0.87 a_w, (Table 1). Optimal water activity was 0.97 for all of them, but *A. niger* aggregate isolate grew

faster than the other fungi. At 0.87 and 0.92 a_w, in general, *A.* section *Nigri* were also faster than the others. Among *A.* section *Nigri*, *A. niger* aggregate showed growth rates clearly higher than *A. carbonarius*. Regarding Temperature, high temperatures (40ºC) favoured *A.* section *Nigri* isolates in front of the others.

At 0.87 a_w *A. niger* aggregate´s growth was reduced by *P. decumbens*. At 0.92 a_w and 20ºC, both black aspergilli were reduced by *C. herbarum*. At the same temperature and 0.97 a_w, growth rates of *A.* section *Nigri* were inhibited by *A. alternata, C. herbarum* and *P. decumbens* (Table 2). At 30ºC *A. carbonarius* was stimulated by *A. alternata* and *C. herbarum*, and *A. niger* aggregate´s growth was reduced by *P. decumbens*. At increasing temperature and a_w, *A.* section *Nigri* showed high scores meaning dominance of these species over the others.

Table 2. Effect of fungal interaction on growth of *A.* section *Nigri* isolates (mean radii, mm) and its scores and indices of dominance against *A. alternaria*, *C. herbarum* and *P. decumbens.* Means with the same letter within each group are not significantly different according to Duncan Test.

	0.87		0.92				0.97			
	30ºC / SC		20ºC / SC		30ºC / SC		20ºC / SC		30ºC / SC	
A. carbonarius										
Single	0.69^{a}		2.17^{b}		8.08^{b}		21.56^{a}		24.44^{c}	
+A. alternaria	-		-		n.g.		17.97^{b}	4	34.76^{a}	4
+C. herbarum	-		3.84^{a}	n.g.	-		18.53^{b}	2	30.48^{b}	5
+P. decumbens	1.04^{a}	n.g.	4.46^{a}	n.g.	12.78^{a}	2	17.04^{b}	2	24.96^{c}	4
I_D		-		-		-		8		13
***A. niger* aggregate**										
Single	14.28^{a}		8.17^{b}		27.08^{a}		22.17^{a}		37.79^{ab}	
+A. alternaria	-		-		27.74^{a}	2	17.71^{c}	2	39.06^{a}	4
+C. herbarum	-		10.06^{a}	n.g.	-		20.19^{b}	5	35.55^{ab}	5
+P. decumbens	7.47^{b}	n.g.	7.69^{b}	n.g.	25.68^{a}	2	18.49^{c}	3	32.44^{b}	4
I_D		-		-		-		10		13

-; not tested, n.g.; not enough growth.

DISCUSSION

The area of origin of our samples has a Mediterranean climate with continental influence. Summers are prolonged, dry and warm, with diurnal temperatures over 40 ºC. It is remarkable the large number of hours of effective sun radiation, between 2800 and 3000 per year (http://www.juntadeandalucia.es/medioambiente/clima_atmosfera/indclimaatmosfera.html).

Samples collected in June exhibited the highest index of biodiversity, as environmental conditions probably fullfit growth requirements of many of soil micro-organisms. Relative moisture of grapes is high and temperature is between 20-30ºC approx., what may favour most of the fungi. *Alternaria* was the most common fungus, followed by *Penicillium*, *Aspergillus*, *Epicoccum*, *Cladosporium*, and *Rhizopus* as reported before (Sage 2002; Bellí *et al.* 2004b).

Both *Alternaria* and *Aspergillus* have a high growth rate at 0.97 a_w and 20ºC, but other factors may be involved to allow *Alternaria* to achieve higher concentration of spores on grape surface.
When temperatures raise to 40ºC, during July, August and September, black aspergilli, and mainly *A. niger* aggregate, become the predominant fungi as they are better adapted to high temperature and low a_w. *Penicillium* species were also found. Within this genus there are resistant species capable of growing at low water activities, between 0.79 and 0.85, and tolerating high temperatures (Smith and Onions 1994). Near harvest, moisture of grapes drops and temperatures are still high, conditions where *A.* section *Nigri* and *P. decumbens* isolates tested were the best suited to compete. This advantage of *A.* section *Nigri* for growing at hot and humid environmental conditions has previously been reported (Leong *et al.* 2004).
After harvest, grapes for sweet wines and raisins are exposed to sun light until they acquire the adequate sugar content, and a_w drops from about 0.95 to 0.75 a_w (unpublished data). In sun-dried grapes, *Aspergillus*, *Penicillium* and few isolates belonging to other genera were present. During the drying period that can last from 5 days to two weeks, only few micro-organisms such as black aspergilli, are capable to resist both germicidal UV light and the strong sunlight heating (Rotem and Aust 1991).
Black aspergilli, and mainly *A. niger* aggregate group was predominant from July-August till sun-drying. This may be due to *A. niger* aggregate is capable of faster growth at higher temperatures than *A. carbonarius* and in wider range of temperature and a_w.
As regards to commercial raisins *Aspergillus* was still predominant (80%), but *Eurotium* was also observed (12.7% of grapes). The composition of the mould flora colonizing the sun-dried grapes and raisins samples was similar; however, moulds were more frequently isolated from dried grapes than from raisins samples. The reason for this difference could be the presence of residual SO_2, which is usually added before raisins drying to prevent browning. SO_2 since it was reported to be an effective agent to control mould growth (Hocking, 1992). In a study carried in prunes and raisins, the predominant spoilage species that occurred were *E. amstelodami* and *A. niger*, followed by *P. chrysogenum* and *Fusarium* spp. (El Halouat and Debevere 1997).
A very small percentage of *A. niger* aggregate showed being OTA producers (1.4%), results that agree with previous surveys giving 1.7 to 30% (Abarca *et al.* 2003; Magnoli *et al.* 2004), whereas OTA production was detected in 72.6% of *A. carbonarius* isolates. OTA accumulation in sun-dried grapes and raisins likely takes place between the end of berry maturation (before harvest) and the first days of sun-exposure. The increase of black aspergilli occurrence during this period and the presence of OTA-producing strains, may explain the higher levels of OTA in wines elaborated from overmaturated and sun-dried grapes.
When water activity falls under 0.93, there is an inhibition of OTA production by *A.* section *Nigri* (Mitchell *et al.* 2004; Bellí *et al.* 2004c), so accelerating the drying process of vine dried fruits

should be a good point in reducing OTA concentrations both in vine dried fruits and sweet wines. Studies in optimizing figs drying techniques showed that drying fruit on trays inside a black painted chamber with solar heated air flow (60-65ºC), guaranteed to reduce microbial contamination level under the recommended limits and was helpful in reducing mycotoxins contamination (Özay *et al*. 1995).

ACKNOWLEDGEMENTS

The autors are grateful to the Spanish Government (CICYT, projects AGL 2004 07549-C05-01, AGL 2004 06413 and grant BES-2003-2180) for its financial support. We also thank M. R. Bragulat (Universitat Autónoma de Barcelona, Spain) for their help in fungal identification, and J. Moreno (Universidad de Córdoba, Spain).

REFERENCES

Abarca, M.J., Accensi, F., Bragulat, M.R., Castella, G. and Cabañes, F.J. (2003) Contamination in dried vine fruits from the spanish market. *Journal of Food Protection* **66** (3), 504-506.

Battilani, P. and Pietri, A. (2002) Ochratoxin A in grapes and wine. *European Journal of Plant Pathology* **108**, 639-643.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J. (2002) Review: Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. *Food Science Technology International* **8** (6), 325-335.

Bellí, N., Marín, S., Duaigües, A., Ramos, A.J. and Sanchis, V. (2004a) Ochratoxin A in wines, musts and grape juices from Spain. *Journal of the Science of Food and Agriculture* **84**, 591-594.

Bellí, N., Pardo, E., Marín, S., Farré, G., Ramos, A.J. and Sanchis, V. (2004b) Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *Journal of the Science of Food and Agriculture* **84**, 541-546.

Bellí, N., Ramos, A.J., Sanchis, V. and Marín, S. (2004c) Incubation time and water activity effects on ochratoxin a production by *Aspergillus* section *Nigri* strains isolated from grapes. *Letters in Applied Microbiology* **38**, 72-77.

Bragulat, M.R., Abaraca, M.L. and Cabañes, F.J. (2001) An easy screening method for fungi producing ochratoxin A in pure culture. *International Journal of Food Microbiology* **71**, 139-144.

Delfini, C. (1982) In *Tecnica di microbiologia eonological* ed. Scicalpi, L. Rome.

El Halouat, A. and Debevere, J.M. (1997) Molds and east isolated from hydrated prunes and raisins having different water activities. *Science of Aliments* **17**, 539-545.

Hidalgo, L. (1999) Grape production factors. *Viticulture manual.* Barcelona, Spain: Mundi-Prensa.

Hocking, A.D. (1992) Xerophilic fungi in intermediate and low moisture foods. In *Handbook of Applied Mycology, Foods and Feed*, Vol 3. ed. Arora, D.K., Mkerji, K.G., Marth, E.H. pp. 69-97. New-York: Marcel Dekker Inc.

http://www.juntadeandalucia.es/medioambiente/clima_atmosfera/indclimaatmosfera.html [Online] [Consulted: 10th January 2005] Clima y Atmósfera de Andalucía. Consejería de Medio Ambiente, Junta de Andalucía.

IARC (1993) Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Naturally Occurring substances: Food Items and Constituents. Heterocyclic Aromatic Amines and Mycotoxins, Vol. **56**, International Agency for Research on Cancer, Lyon, pp. 489-521.

Leong, S.L., Hocking, A.D. and Pitt, J.I. (2004) Occurrence of fruit rot fungi (*Aspergillus* section *Nigri*) on some drying varieties of irrigated grapes. *Australian Journal of Grape and Wine Research* **10**, 83-84.

MacDonald, S., Wilson, P., Barnes, K., Damant, A., Massey, R., Mortby, E. A and Shepherd, M.J. (1999) Ochratoxin A in dried vine fruit: method development and survey. *Food Additives and Contaminants* **16** (6), 253-260.

Magan, N. and Lacey, J. (1984) Effect of temperature and pH on water relations of field and storage fungi. *Transactions of the British Mycological Society* **82**, 71-81.

Magnoli, C., Violante, M., Combina, M., Palacio, G. and Dalcero, A. (2003) Mycoflora and ochratoxin–producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Letters in Applied Microbiology* **37**, 179-184.

Magnoli, C., Astoreca, A., Ponsone, L., Combina, M., Palacio, G., Rosa, C.A.R. and Dalcero, A.M. (2004) Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Letters in Applied Microbiology* **39** (4), 326-331.

Marquardt, R.R. and Frolich, A.A. (1992) A review of recent advances in understanding ochratoxicosis. *Journal of Animal Science* **70**, 3968-3988.

Mitchell, D., Parra, R., Aldred, D. and Magan, N. (2004) Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. *Journal of Applied Microbiology* **97**, 439-445.

Ostry, V., Ruprich, J. and Skarkova, J. (2002) Raisins, ochratoxin A and human health. *Mycotoxin Research* **18**A (2), 178-182.

Özay, G., Aran, N. and Pala, M. (1995) Influence of harvest and drying techniques on microflora and mycotoxin contamination of figs. *Die Nahrung* **39**, 156-165.

Pardo, E., Marín, S., Ramos, A.J. and Sanchis, V. (2004) Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different origins. *Food Science Technology International* **10**, 45-49.

Pitt, J.I. and Hocking, A.D. (1997) Fungi and food spoilage ed. Blackie Academic and Professional 2nd ed., pp.110-114. Cambridge: University Press.

Rotem, J. and Aust, H.J. (1991) The effect of ultraviolet and solar radiation and temperature on survival of fungal propagules. *Journal of Phytopathology* **133**, 76-84.

Sage, L. (2002) Fungal flora and ochratoxin A production in grapes and musts from France. *Journal of Agricultural and Food Chemistry* **50** (5), 1306-1311.

Smith, D. and Onions, A.H.S. (1994) *The preservation and maintenance of living fungi*. 2nd edn. Ed. International Mycological Institute. No. 2. pp. 17-20. Wallingford, UK: CAB International.

Study II:

Survey: Ochratoxin A in European special wines

Ana Valero, Sonia Marín, Antonio J. Ramos, Vicente Sanchis[a]

Food Technology Department, University of Lleida, UTPV-CeRTA, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

Tl. +34- 973702535, Fax +34-973702596

Abstract

121 representative special wines were analysed to know the Ochratoxin A (OTA) occurrence among the different winemaking techniques and from many European origins (A, B, CI, CII, CIII growing-zones from Northern to Southern regions according to (EC) No 1493/1999 and 1512/2005). There were not ochratoxin A detected in the three wine-growing zones A, B and CI while occurrences in CII and CIII zones ranged from 53.8 to 60%. CIII zone also registered higher OTA levels. The wine groups with higher OTA content and occurrence, above 90%, were those where the must was fortified before fermentation and those made from grapes dried by means of sun exposure. Wines affected by noble rot, late harvest wines and icewines did not have OTA. Fortified wines with long aging in wooden casks, sparkling wines and those made from grapes dried in fresh chambers were about 50% of samples contaminated, and OTA levels were lower, mainly in fortified wines which OTA concentrations did not reach 1.00 µg l^{-1}. Overall, 19.8% of wines contained OTA levels above the maximum permissible limit for European Union, set in 2 µg Kg^{-1}.

Publication: *International Journal of Food Microbiology* (submitted).

INTRODUCTION

Ochratoxin A (OTA) is a fungal metabolite with toxic properties produced by species belonging to genera *Aspergillus* and *Penicillium* (Van der Merwe 1965, Ciegler 1972, Ueno et al. 1991, Varga et al. 2000). It has been considered the causing agent of Balkan Endemic Nephropathy (BEN) (Marquardt and Frohlich 1992) and it has also been classified by the International Agency for Research on Cancer (IARC 1993) as a possible human carcinogen (group 2B).

OTA has been found in foodstuff such as cereals, coffee (Rafai et al. 2000, Accensi et al. 2004, Pardo et al. 2004), grapes (Abrunhosa et al. 2001) and in dried vine fruits (MacDonald et al. 1999, Ostry et al. 2002, Varga and Kozakiewicz 2006). OTA occurrence in wine achieves higher levels in red wine (Bellí et al. 2002, EU Report 2002) and in sweet and special wines (Zimmerli and Dick 1996, Burdaspal and Legarda 1999, Pietri et al. 2001, Bellí et al. 2004). In red wines the maceration of must with grape skins might favour OTA extraction from skins (Blesa et al. 2006). What concerns to sweet or special wines, oenological practices are very diverse and may result in different final OTA concentration (Gambuti et al. 2005, Ratola et al. 2005, Chiodini et al. 2006, Leong et al. 2006) but diverse studies have shown that their OTA content is higher than in dry wines (Zimmerli and Dick 1996, Burdaspal and Legarda 1999, Pietri et al. 2001, Stefanaki et al. 2003). Wines origin also is determinant for the final OTA content (Ottender and Majerus 2000, Varga and Kozakiewicz 2006) due to different climatic conditions that lead to a differential OTA producing fungi distribution (Battilani et al. 2006, Serra et al. 2006).

Despite many surveys have been made on wines from different origins, such as Italy, Spain, Greece, etc (Pietri et al. 2001, Soufleros et al. 2003, Bellí et al. 2004), there is no intensive study about OTA occurrence in sweet or special wines, also including wines made from overripe and botrytized grapes (noble rot). Currently, several countries have specific regulations for OTA in various commodities, 2 µg OTA kg^{-1} being the maximum level allowed for wine, grape must and grapes in European Union (Commission Regulation (EC) No.1881/2006).

Thus the aim of this study was to assess OTA occurrence in special wines from Europe obtained by different winemaking techniques.

MATERIAL AND METHODS

Samples

121 representative special wines from Europe were purchased from Spanish and Portuguese markets and from Italian and Spanish distributors. All wine-growing zones according to European regulations, A, B, CI, CII and CIII (Council Regulation (CE) No 1493/1999 and Corrigendum (CE) No 1512/2005) that are based in the production conditions, soil, region and climate have been sampled (Figure 1). Classification of the assayed wines is summarized in Table I.

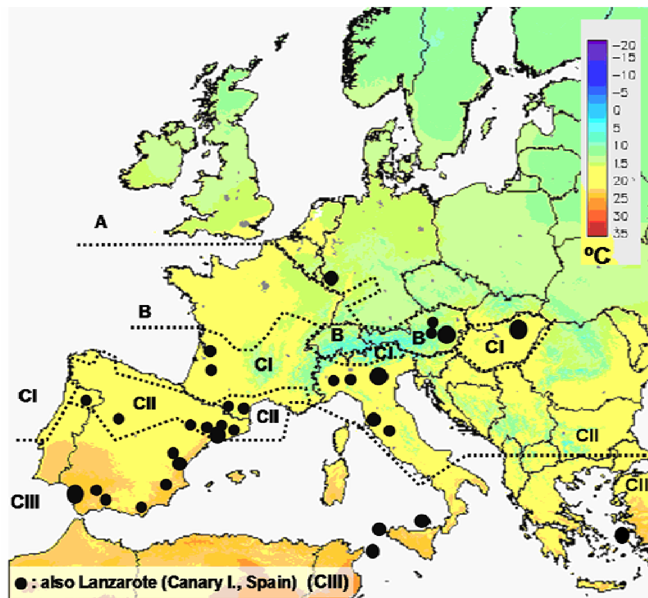


Figure 1. Thermo map of Europe showing the average of temperatures in September in grey scale and divided by dotted lines into wine-growing zones (A, B, CI, CII and CIII). Black points represent the origin of samples.

Table I. Classification of the assayed wines according to winemaking type.

Group		Wines (No samples)	Zones
Fortified must / early stopped fermentation		Mistelle (11), Muscat, (11)	CII, CIII
Fortified wines Long aging in wooden casks		Banyuls (1), Marsala (3), Maury (1), Oloroso (2), Porto (11), Sherry (7),	CII, CIII
Fortified wines, 2nd ferm. with Flor yeast, long aging in wooden casks		Fino (7), Manzanilla (5)	CIII
Sparkling wines		Muscat (1), Cava liquoureux (1)	CII
Late harvest		Fondillón (1), Spätlese (1), Tokaji (4), Others (2)	B, CI, CII,CIII
Dehydrated grapes by:	Sundrying	Fondillón (2), Málaga (5), Malvasía (1), Passito (2), Pedro Ximénez (10), Samos, Sherry (1), Other (1)	CIII
	Warm chamber	Passito (1), Vin Santo (2)	CII
	Fresh chamber	Amarone (2), Amarone Ripasso (1), Garnatxa de l'Empordà (3), Recioto (2)	CII
	Noble rot	Auslese (1), Beerenauslese (2), Trockenbeerenauslese (2), Sauternes (4), Montbazillac (1), Tokaji (3)	A, B, CI, CII
	Ice	Eiswein (3), Vi de gel (2)	B, CII

Reducing sugars content in the wine samples

For sugar analysis it was followed the guidelines given by the supplier. In an Erlenmeyer flask 10 ml of 0.168 mol l^{-1} cupric solution (Gab System. Olérdola, Spain), 5 ml of 0.886 mol l^{-1} alkaline solution (Potassium Sodium Tartrate, $KNaC_4H_4O_6 \cdot 4H_2O$. Gab System. Olérdola, Spain), a small pomez stone and 2 ml of wine, previously diluted if wine is suspected to be very sweet, were poured. Mixture was boiled for 90 seconds and cooled. Then, 10 ml of 1.6 mol l^{-1} iodide solution (Gab System. Olérdola, Spain), 10 ml mol l^{-1} soluble starch (Gab System. Olérdola, Spain) and 10 ml 16% sulphuric acidic (H_2SO_4. Gab System. Olérdola, Spain) were added. Finally sugar content was quantified through valuation with sodium thiosulphate (Gab system. Olérdola, Spain). The final point was determined by the change from dark brown-black to clear beige.

The reducing sugar content was calculated according to Equation 1 (R^2: 0.996):

$$\textit{Sugar content}\ (\text{g l}^{-1}) = -1.105 \cdot \textit{Valuation Solution}\ (\text{ml}) + 31.096 \qquad (1)$$

OTA analysis

OTA extraction method developed by Bezzo et al. (2000) for wine by HPLC was followed with little variations. In brief, 100 ml of wine were pH modified up to 7.40 with 4 mol l^{-1} NaOH and passed through filter paper number 1. Then the extract was cleaned-up by means of passing the sample through an immunoaffinity column (Ochraprep®, R-Biopharm Rhône LTD, Glasgow, Scotland) at a flow rate of 2-3 ml min^{-1}. The column was afterward washed with 20 ml bidistilled water and let to dry. OTA was finally eluted from the column with 3 ml methanol:acetic acid (98:2, v:v). The eluted extract was dried under N_2 flow at 40ºC and resuspended in 1 ml mobile phase (48% acetonitrile and 52% sodium acetate:acetic acid (19:1)). Separation and OTA quantification was performed using a high performance liquid chromatograph (Waters, Milford, MA, USA) with reverse-phase C18 silicagel column (Waters Spherisorb ® 5 µm ODS2 4.6x250 nm, Mildford, MA, USA) and detected by fluorescence. Exciting and emission wavelengths were set to 230 and 458 nm respectively. Flow rate of mobile phase was 1ml min^{-1} and the injection volume 25 µl. Retention time of OTA was 13 min (LOD=0.024 µg OTA l^{-1} wine; LOQ=0.081 µg OTA l^{-1} wine), identified according to a standard from Sigma (Steinheim, Germany).

Recovery rates of the method were calculated in for eight wines naturally containing increasing levels of sugar ranging from 1.3 to 449.8 g ml^{-1} and spiked with OTA at 2 µg l^{-1} and no spiked (control) (Table II).

Correlation between sugar content and recovery rate showed to be negative and significant (Pearson correlation coefficient (*ρ)* = -0.802, *P* = 0.017).

Table II. Recoveries for OTA in special wines according to its sugar content.

Sugar content ($g\ l^{-1}$)	Recovery %
1.3	96.2
2.9	89.7
87.1	86.3
123.8	83.6
206.7	85.2
224.1	71.8
394.6	77.4
449.8	75.8

The resultant Equation (2) (R^2 = 0.643) from lineal regression between sugar content and recovery percentages will be used for calculating the recovery percentage for each wine according to its sugar content.

$$\textit{Recovery percentage} = -\,0.038 \cdot \textit{Sugar content}\ (g\ l^{-1}) + 90.323 \qquad (2)$$

Another peak was usually detected at 8.2 retention time in all the analysed samples where OTA was present (Figure 2).

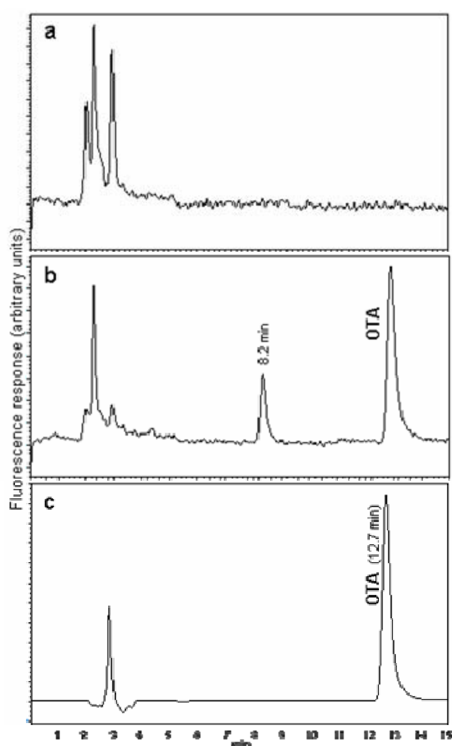


Figure 2. Chromatograms (HPLC; fluorescence detection) of : (a) wine sample negative for OTA; (b) wine sample positive for OTA; (c) OTA standard solution 2.24 μg ml^{-1}.

RESULTS

Occurrence of OTA in special wines

No ochratoxin A was detected in the three wine-growing zones A, B and CI while occurrences in CII and CIII zones ranged from 53.8 to 60% of samples (Table III). Higher OTA levels were also detected in CIII.

Table III. Occurrence and OTA levels in special wines from five European wine-growing zones.

Wine-growing zones	*n*	% positive samples	OTA (µg l^{-1})						
			Mean	Range	LOD-0.10	0.10-0.50	0.50-1.00	1.00-2.00	>2.00
A	2	0	n.d.	n.d.	0	0	0	0	0
B	7	0	n.d.	n.d.	0	0	0	0	0
CI	11	0	n.d.	n.d.	0	0	0	0	0
CII	26	53.8	2.01	n.d.-27.79	14	2	4	2	4
CIII	75	60.0	1.71	n.d.-15.62	29	10	12	4	20

n.d.: not detected

The wine groups with higher OTA content and occurrence, above 90%, were those in which must had been fortified before fermentation occurred and those made from grapevines dried by means of sun exposure (Table IV). Fortified wines with long aging in wooden casks, sparkling wines and those made from grapes dried in fresh chambers were found to be contaminated in 50% of cases, and OTA levels were lower, mainly in fortified wines in which OTA concentrations did not reach 1.00 µg l^{-1}. Among the fortified wines with a second fermentation with Flor yeast, only one from 12 samples was OTA contaminated (0.08 µg l^{-1}) and wines made from noble rot affected grapes, late harvest and icewines did not have OTA.

Table IV. Occurrence and OTA levels in special winemaking.

Group		n	% positive samples	(OTA ($\mu g\ l^{-1}$) Mean	Range	LOD-0.10	0.10-0.50	0.50-1.00	1.00-2.00	> 2.00
Fortified must / early stopped fermentation		22	90.9	4.48	n.d.-27.79	4	1	4	1	12
Fortified wines Long aging in wooden casks		25	52.0	0.24	n.d.-0.96	12	8	5	0	0
Fortified wines, 2nd ferm. with Flor yeast, long aging in wooden casks		12	8.3	0.01	n.d.-0.08	12	0	0	0	0
Sparkling wines		2	50.0	0.06	n.d.-0.13	1	1	0	0	0
Late harvest		8	0.0	n.d.	n.d.	0	0	0	0	0
Grapes dried by:	Sun	23	91.3	2.77	n.d.-15.62	2	2	6	3	10
	Warm chamber	3	33.3	0.58	n.d.-1.74	2	0	0	1	0
	Fresh chamber	8	50.0	1.35	n.d.-4.79	4	0	1	1	2
	Noble rot	13	0.0	n.d.	n.d.	0	0	0	0	0
	Ice	5	0.0	n.d.	n.d.	0	0	0	0	0
TOTAL		121	49.6	1.49	n.d.-27.79	63	12	16	6	24

n.d.: not detected

DISCUSSION

The extraction and clean-up procedure to analyze OTA from wines was checked for sweet wines and from our findings it could be advisable to take into account the interference of sugar in the recovery rate in order not to underestimate the OTA concentration, that could be up to 30% in some cases.

The unknown peak (8.2 min) found in all positive samples here analysed was also reported by Sáez et al. (2004) in wines, musts and beers at close retention time but not identified. Ochratoxin A is sometimes accompanied by the non-chlorinated analogue, ochratoxin B (WHO-IPCS, 1990) but it appears to be much less toxic than OTA (Mally et al. 2005). The identification of this peak was not our aim so we lack of suitable standard to confirm it but Varga et al. (1996) identified seven *Aspergillus* spp. OTB producer (all them OTA producer), and OTB peak appeared around two minutes before OTA peak.

The climatic and geographic differences influence mould development and OTA contamination of grapes, and consequently, the wine. All special wines analysed from A, B and CI wine-growing zones were negative for OTA. Other studies have reported OTA occurrences, in all types of wines, from 0 to 50% in these regions (Zimmerli and Dick 1996, Ottender and Majerus 2000, Berente et al. 2005) with medians between 0.01-0.10 μg OTA l^{-1} and maximum

concentrations ranging from 0.02 to 0.78 μg l^{-1}. It can be deduced that OTA-producing fungi are present in these latitudes and are able to grow and to produce OTA in these environmental conditions but some grape transformations occurred in special wines, such as noble rot, could reduce the growth of the OTA producer fungi and the toxin down to undetectable concentrations.
CII zones are located in warmer regions that may favour fungal development and OTA production by *A*. section *Nigri*, as reviewed and modelled by Battilani et al. (2006). The 54% of special wines from CII zone were positive for OTA with 15% of samples with OTA concentrations over 2 μg l^{-1}. Concentrations found in the present study are higher than those previously reported by some authors (Zimmerli and Dick 1996, Ottender and Majerus 2000, Pietri et al. 2001) where occurrences ranged from 53 to 100%, the means from 0,002 to 0.193 μg OTA l^{-1} and maxima between 0.041 and 2.55 μg OTA l^{-1}. It is important to point out that these values correspond to table wines, so once again, winemaking procedures involved in special wines may be affecting the OTA content of wines.
The majority of Mediterranean wine-growing regions are included in the CIII zone. Climates are hot and dry during summer and warm and wet in autumn. The 60% of wines sampled contained OTA concentrations above the detection limit (0.024 μg OTA l^{-1} wine) and the 27% higher than 2 μg OTA l^{-1}. Some authors have found between 80 and 100% of wines contaminated by OTA with mean values similar to those found in the present study (Zimmerli and Dick 1996, Ottender and Majerus 2000, Pietri et al. 2001), whilst maximum levels, between 0.220 and 3.856 μg l^{-1}, were much lower than that observed in this study.
What concerns to sweet or special wines, data in previous surveys is scarce but it can be assumed that OTA levels and occurrence are generally higher than those of table wines (Zimmerli and Dick 1996, Soufleros et al. 2003, Bellí et al. 2004).
Depending on wine-growing zones, the type of winemaking has different effect on OTA content in wines. Wines from dehydrated grapes obtained through five different techniques were studied in the present survey: sun exposure, storage in warm/dry and fresh/dry chambers, noble rot and late harvest until grapes freeze on the vineyard. In all cases the fermentation progresses slowly due to the high sugar content of musts and wines are aged in wooden barrels, with few exceptions, so variations in OTA content may depend mainly on the dehydrating procedure. Ochratoxin A contamination of dried vine fruits is usually much higher than that of wines or grape juices with the former reaching 50-70 μg l^{-1} values (Miraglia and Brera 2002), due to the action of black aspergilli in Europe (Abarca et al. 2003, Varga et al. 2005). More than 90% of wines made from sun-dried grapes were positive for OTA and levels were much higher in comparation with other wines. Other surveys reported occurrences in this type of wines between 57% to 100% and OTA concentrations were also high, up to 7.3 μg l^{-1} (Zimmerli and Dick 1996, JECFA 47 2001, Soufleros et al. 2003, Blesa et al. 2004, Hernández et al. 2006). Grapes are usually set on

esparto mats on the ground in the vineyard or on higher structures, but in all cases grapes are exposed to an open environment, to hot sun-drying during the day and to cool and wet night for 5-15 days. Under these conditions fungi find a nutrients substrate, and injured grapes likely due to harvesting, that eases fungal development and OTA production (Valero et al. 2005). This may occur during the first days of grape-drying that is before water activity of grapes drops down to values that are not conducive to OTA production (<0.90 a_w, *A. niger* aggregate spp.; <0.86 a_w, *A. carbonarius*) (Esteban et al. 2006a and b). Gomez et al. (2006) found a higher *A. carbonarius* occurrence in overripe grapes, left on the vine or exposed to sun-drying, than that at harvesting time confirming that *A. carbonarius* is the most important source of OTA in these liqueur wines.
Grapes that are dried out in warm and dry chambers (under sun-irradiated roofs) suffer a faster dehydration (three-four weeks) so that OTA producing fungi (*A*. section *Nigri*) has less available time to develop, also reduced by a dry environment. OTA occurrence on wines from these grapes is 33% and showing lower OTA levels with 0.58 µg l^{-1} mean and 1.74 µg l^{-1} maximum. In a study carried out by Larcher and Nicolini (2001) none of the Vin Santo wines analyzed had OTA.
By contrast, wines made from grapes dried in cool and dry chambers reported higher occurrence and OTA levels, up to 4.79 µg l^{-1}. This fact could be due to the longer periods of time about two-six months that are necessary for drying the grapes. Similarly to this procedure, some passito wines, not included in this study, are made from grapes dried in wet and cold rooms for 90-120 days. In a study that simulates this process (Torrelli et al. 2006) bunches from grapevine were kept in storage rooms for 20-25 days at temperatures between 14-18ºC and fungal mycobiota was identified. It is remarkable the fact that no *Aspergillus* spp. were detected in that study, but some isolated *Penicillium* spp. was able to produce OTA. *A*. section *Nigri* spp. (*A. niger* aggregate and *A. carbonarius*) are assumed to be the source of OTA in wines (Cabañes et al. 2002) but some winemaking techniques, such as fresh chamber for grape drying, could turn this trend. Gambuti et al. (2005) studied the effect of drying grapes in an aerated room for two months on OTA accumulation and found that wines made from these grapes had OTA levels more than twice higher than wines obtained from grapes at full maturity.
What concerns eisweins (icewines), noble wines (botrytized grapes) or late harvest wines, none of them contained ochratoxin A above the detection limit, in agreement with previous results published regarding noble rot affected wines (Kallay and Magyar 2000, Dumoulin and Riboulet 2002, Stander et al. 2002, Berente et al. 2005, Eder 2005) and ice wines (Eder 2005). These wines usually are made in Northern European regions or in colder climates that are favourable for noble rot rather than black rot (*A*. section *Nigri*). Noble rot is developed by the fungus *Botrytis cinerea* that colonizes and dehydrates the grape berry. Some studies performed in our laboratory (unpublished data) and also by Abrunhosa et al. (2003) have found that *B. cinerea*

isolates are able to degrade OTA spiked in agar medium. All together, in the even of black rot the little toxin produced could even be degraded by *B. cinerea*.

All fortified wines analysed here, included in this category, have been found to be contaminated by OTA in previous surveys with very variable occurrences ranging from 17% (Alves 2003), 20% (Ratola et al. 2005), 33% (Tateo et al. 2000) to 75% (Burdaspal and Legarda 1999) and higher than 90% (Zimerli and Dick 1996). Nevertheless OTA concentrations were very similar with maxima between 0.20 and 0.47 µg l^{-1} and agreeing with our results, with the exception of 1.59 µg l^{-1} found by Burdaspal and Legarda (1999) in Marsala wines.

In contrast, OTA levels detected in wines made by means of adding alcohol distillate to the sweet must are much higher (mean: 4.48 µg l^{-1}), OTA being detected in more than 90% of wines. Levels given by some authors are smaller compared to our data but still higher than fortified wines, with maxima ranging from 0.40 to 3.34 µg l^{-1} and occurrences from 0% to 57% and 75% (Tateo et al. 2000, Blesa et al. 2004, Hernández et al. 2006). It is not a surprising finding provided that fermentation is prevented or early stopped and, subsequently the OTA removal during this process reported by many authors (Ottender and Majerus 2000, Abrunhosa et al. 2003, Ratola et al. 2005, Grazioli et al. 2006, Leong et al. 2006) does not take place. In fortified wines, in contrast, initial must may be very similar in composition but it is fermented up to a certain alcoholic grade, fortified and further aged in wooden barrels for long periods of time. In addition to OTA reduction by fermentation, either alcoholic or malo-lactic (Abrunhosa et al. 2003, Grazioli et al. 2006), the oxidative process that occurs during aging could affect somehow the toxin, but it is uncertain.

Sparkling wines were similar in results to fortified wines and also what concerns to the uncompleted fermentation in their winemaking. Concerning the wines fermented by Flor yeast, OTA was detected in only one sample among 12. These wines suffer double fermentation, almost a full alcoholic fermentation followed by a secondary one, that may lead to a major OTA elimination.

Overall, 19,8% of wines contained OTA levels above the maximum permissible limit for European Union, set in 2 µg Kg^{-1} (Commission Regulation (CE) No 1881/2006).

Due to the humble amount of samples in some categories of wines, and considering our findings, it would be interesting to design further studies focussing into some types of wines in order to confirm our results and to identify the critical points where OTA is reduced.

Summarizing, ochratoxin A in wines is a matter of concern considering the high levels and occurrence reported in the present study. Origin of samples is determinant for fungal development and OTA contamination, favoured in Southern regions as consequence from the environmental conditions. Winemaking procedures also affect to OTA concentration in wines, it

being lower in wines with longer or double fermentation and in those made from botrytized grapes.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2004-07549-05-01, grant BES-2003-2180 and Ramón y Cajal program) and to the Catalonian Government (CeRTA strategic project 2005-2006 "Seguritat biòtica y abiòtica dels aliments", Generalitat de Catalunya) for their financial support.

REFERENCES

Abarca ML, Accensi F, Bragulat MR, Castella G, Cabañes FJ. 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. Journal of Food Protection 66: 504-506.

Abrunhosa L, Paterson RRM, Kozakiewicz Z, Lima N, Venâncio A. 2001. Mycotoxin production from fungi isolated from grapes. Letters in Applied Microbiology 32: 240-242

Abrunhosa L, Serra R, Venâncio A. 2002. Biodegradation of ochratoxin A by fungi isolated from grapes. Journal of Agricultural and Food Chemistry 50: 7493-7496.

Accensi F, Abarca ML, Cabañes FJ. 2004. Occurrence of *Aspergillus* species in mixed feeds and component raw materials and their ability to produce ochratoxin A. Food Microbiology 21: 623-627.

Alves A. 2003. Rastreio de ocratoxina A em uvas e vinhos portugueses. In: Cerdeira A, Venâncio A, Alves A, Barros P, Siamões T, editors. Impacto da Contaminação Fúngica obre a Competitividade de Vinhos-Ochratoxina A. Braga: Micoteca da Universidade do Minho.

Battilani P, Pietri A. 2002. Ochratoxin A in grapes and wine. European Journal of Plant Pathology 108: 639-643.

Battilani P, Barbano C, Marín S, Sanchis V, Kozakiewicz Z Magan N. 2006. Mapping of *Aspergillus* section *Nigri* in Southern Europe and Israel based on geostatistical analysis. International Journal of Food Microbiology 111: S72-S82.

Bellí N, Marín S, Sanchis V, Ramos AJ. 2002. Review: Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. Food Science and Technology International: 8, 325-335.

Bellí N, Marín S, Duaigües A, Ramos AJ, Sanchis V. 2004. Ochratoxin A in wines, musts and grape juices from Spain. Journal of the Science of Food and Agriculture 84: 591-594.

Berente B, Móricz A, H-Otta K, Záray G, Léko L, Rácz L. 2005. Determination of ochratoxin A in Hungarian wines. Microchemical Journal 79: 103-107.

Bezzo G, Maggiorotto G, Testa F. 2000. A method for the determination of specific mycotic contaminants randomly occurring in wines. FV 1097, 2706/070200. Paris: Office International de la Vigne et du Vin.

Blesa J, Soriano JM, Moltó JC, Mañes J. 2004. Concentration of ochratoxin A in wines from supermarkets and stores of Valencian Community (Spain). Journal of Chromatography A 1054: 397-401.

Blesa J, Soriano JM, Moltó JC, Mañes J. 2006. Factors affecting the presence of ochratoxin A in wines. Critical Reviews in Food Science and Nutrition 46: 473-478.

Bragulat MR, Abarca ML, Cabañes FJ. 2001. An easy screening method for fungi producing ochratoxin A in pure culture. International Journal of Food Microbiology,71: 139-144.

Burdaspal PA, Legarda TM. 1999. Ocratoxina A en vinos, mostos y zumos de uva elaborados en España y en otros países europeos. Alimentaria 36: 107-114.

Cabañes FJ, Accensi F, Bragulat MR, Abarca ML, Castella G, Minguez S, Pons A. 2002. What is the source of ochratoxin A in wine? International Journal of Food Microbiology 79: 213-215.

Caridi A, Galvano F, Tafuri A, Ritieni A. 2006. Ochratoxin A removal during winemaking. Enzyme and Microbial Technology 40: 122-126.

Chiodini AM, Scherpenisse P, Bergwerff AA. 2006. Ochratoxin A contents in wine: comparison of organically and conventionally produced products. Journal of Agricultural and Food Chemistry 54: 7399-7404.

Ciegler A. 1972. Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus* ochraceus group. Canadian Journal of Microbiology 18: 631-636.

Commission Regulation. 2006. (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union. Brussels: 20 December 2006.

Corrigendum to Commission Regulation. 2005. (EC) No 1512/2005 amending Regulation (EC) No 753/2002 laying down certain rules for applying Council Regulation (EC) No 1496/1999 as regards the description, designation, presentation and protection of certain wine sector products (OJ L 241, 17.9.2005). Brussels: 15 September 2005.

Council Regulation. 1999. (EC) No 1493/1999 On the common organisation of the market in wine. Office for Official Publications of the European Communities. Brussels: 17 May 1999.

Dumoulin M, Riboulet JM. 2002. Considerations on the presence of ochratoxin A in wines and grape juices. Revue des Oenologues 29: 11-13.

Eder R. 2005. Ochratoxin in Austrian wines? Der Winzer 61: 19-21.

Esteban A, Abarca MJ, Bragulat MR, Cabañes FJ. 2006a. Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregates species. International Journal of Food Microbiology 108: 188-195.

Esteban A, Abaca MJ, Bragulat MR, Cabañes FJ. 2006b. Study of the effect of water activity and temperature on ochratoxin A production by *Aspergillus carbonarius*. Food Microbiology 23: 634-640.

EU Report. 2002. Reports on tasks for scientific cooperation. Assessment of dietary intake of Ochratoxin A by the population of EU Member States. Directorate-General Health and Consumer Protection. Task 3.2.7. Brussels: January 2002.

Gambuti A, Strollo D, Genovese A, Ugliano M, Ritieni A, Moio L. 2005. Influence of enological practices on ochratoxin A concentration in wine. American Journal of Enology and Viticulture 56: 155-162.

Gómez C, Bragulat MR, Abarca ML, Mínguez S, Cabañes FJ. 2006. Ochratoxin A-producing fungi from grapes intended for liqueur wine production. Food Microbiology 23: 541-545.

Grazioli B, Fumi MD, Silva A. 2006. The role of processing on ochratoxin A content in Italian must and wine: A study on naturally contaminated grapes. International Journal of Food Microbiology 111: S93-S96.

Hernández MJ, García-Moreno MV, Durán E, Guillén D, Barroso CG. 2006. Validation of two analytical methods for the determination of ochratoxin A by reversed-phased high-performance liquid chromatography coupled to fluorescence detection in musts and sweet wines from Andalusia. Analytica Chimica Acta 566: 117-121.

IARC. 1993. (International Agency for Research on Cancer) Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Vol. 56.Lyon: International Agency for Research on Cancer. pp. 489-521.

Joint FAO/WHO Expert Committee on Food Additives (JECFA) 47. 2001. [internet]. Benford D, Boyle C, Dekant W, Fuchs R, Gaylor DW, Hard G, McGregor DB, Pitt JI, Plestina R, Shephard G, Solfrizzo M, Verger PJP, Walker R. Ochratoxin A. Available from: http://www.inchem.org/documents/jecfa/jecmono/v47je04.htm. Accessed 2007 February 25.

Kallay M, Magyar I. 2000. Occurrence of ochratoxin A under oenological conditions with special regard to the processing of Tokaj Aszú. In: Proceedings of Lippary J. Vas K. Scientific Symposium.(Budapest: Food Science. p. 15.

Larcher R, Nicolini G. 2001. Survey of ochratoxin A in musts, concentrated musts and wines produced or marketed in Trentino (Italy). Journal Commodity Science 40: 69-78.

Leong SL, Hocking AD, Varelis P. 2006. Fate of ochratoxin A during vinification of Semillon and Shiraz grapes. Journal of Agriculture and Food Chemistry 54: 6460-6464.

MacDonald S, Wilson P, Barnes K, Damant A, Massey R, Mortby EA, Shepherd MJ. 1999. Ochratoxin A in dried vine fruit: method development and survey. Food Additives and Contaminants 16: 253-260.

Mally A, Keim-Heusler H, Amberg A, Kurz M, zepnik H, Mantle P, Völkel W, Hard GC, Dekant W. 2005. Biotransformation and nephrotoxicity of ochratoxin B in rats. Toxicology and Applied Pharmacology 206: 43-53.

Marquardt RR, Frolich AA. 1992. A review of recent advances in understanding ochratoxicosis. Journal of Animal Science 70: 3968-3988.

Ottender H, Majerus P. 2000. Occurrence of ochratoxin A (OTA) in wines: influence of the type of wine and its geographical origin. Food Additives and Contaminants 17: 793-798.

Ostry V, Ruprich J, Skarkova J. 2002. Raisins, ochratoxin A and human health. Mycotoxin Research 18A: 178-182.

Pardo E, Marín S, Ramos AJ, Sanchis V. 2004. Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different origins. Food Science Technology International 10: 45-49.

Pietri A, Bertuzzi T, Pallaroni L, Piva G. 2001. Occurrence of ochratoxin A in Italian wines. Food Additives and Contaminants 18: 647-654

Rafai, P., Bata, A. and Jakab, L., 2000, Evaluation of mycotoxin-contaminated cereals for their use in animal feeds in Hungary. *Food Additives and Contaminants*, **17**, 799-808.

Ratola N, Abade E, Simões T, Venâncio A, Alves A. 2005. Evolution of ochratoxin A content from must to wine in Port wine microvinification. Analytical and Bioanalytical Chemistry 382: 405-411.

Sáez JM, Medina A, Gimeno-Adelantado JV, Mateo R, Jiménez M. 2004. Comparison of different sample treatments for the analysis of ochratoxin A in must, wine and beer by liquid chromatography. Journal of Chromatography A 1029: 125-133.

Serra R, Lourenço A, Alípio P, Venâncio A. 2006. Influence of the region of origin on the mycobiota of rapes with emphasis on *Aspergillus* and *Penicillium* species. Mycological Research 110: 971-978.

Soufleros EH, Tricard C, Bouloumpasi EC. 2003. Occurrence of ochratoxin A in Greek wines. Science of Food and Agriculture 83: 173-179.

Stander MA, Steyn PS. 2002. Survey of ochratoxin A in South African wines. South African Journal for Enology and Viticulture 23: 9-13.

Stefanaki I, Foufa E, Tsatsou-Drtisa A, Dais P. 2003. Ochratoxin A concentrations in Greek domestic wines and dried vine fruits. Food Additives and Contaminants 20: 74-83.

Torelli E, Firrao G, Locci R, Gobbi E. 2006. Ochratoxin A producing strains of *Penicillium* spp. Isolated from grapes used for the production of “passito” wines. International Journal of Food Microbiology 106: 307-312.

Ueno Y, Kawamura O, Sugiura Y, Horiguchi K, Nakajiima M, Yamamoto K, Sato S. 1991. Use of monoclonal antibodies, enzyme-linked immunosorbent assay and immunoaffinity column chromatography to determine ochratoxin A in porcines era, coffee products and toxin-producing fungi. In: Castegnaro M, Plestina R, Dirheimer G, Chernozemsky IN, Bartash H, editors. Mycotoxins endemic nephropathy and urinary tract tumors. Lyon: IARC Scientific Publications. pp. 71-75.

Valenta H. 1998. Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids. Journal of Chromatography A 815: 75-92.

Valero A, Marín S, Ramos AJ, Sanchis V. 2005. Ochratoxin A-producing species in grapes and sun-dried grapes and their relation to ecophysiological factors. Letters in Applied Microbiology 41: 196-201.

Van der Merwe KJ, Steyn PS, Fourie L. 1965. Mycotoxins. Part II. The constitution of ochratoxin A, B and C, metabolites of *Aspergillus ochraceus*. Journal of the American Chemical Society 1965: 7083 – 7088

Varga J, Kevei F, Rinyu E, Téren J, Kozakiewicz Z. 1996. Ochratoxin production by *Aspergillus* species. Applied and Environmental Microbiology 60: 4461–4464.

Varga J, Rigó K, Téren J. 2000. Degradation of ochratoxin A by *Aspergillus* species. International Journal of Food Microbiology 59: 1-7.

Varga J, Péteri Z, Tábori K, Téren J, Vágvölgyi C. 2005. Degradation of ochratoxin A and other mycotoxins by *Rhizopus* isolates. International Journal of Food Microbiology 99: 321-328.

Varga J, Kozakiewicz Z. 2006. Ochratoxin A in grapes and grape-derived products. Trends in Food Science 17: 78-81.

WHO-IPCS (World Health Organization-International Program on Chemical Safety). 1990. Selected mycotoxins: ochratoxins, trichothecenes, ergot. Environmental Health Criteria 105: 27-69.

Zimmerli B, Dick R. 1996. Ochratoxin A in table wine and grape-juice: occurrence and risk assessment. Food Additives and Contaminants 13: 655-668.

Chapter II:

ECOPHYSIOLOGICAL STUDIES

Study III:

Kinetics and spatial distribution of OTA in *Aspergillus carbonarius* cultures

Ana Valero, Joan R. Farré, Sonia Marín, Antonio J. Ramos, Vicente Sanchis*

Food Technology Department, University of Lleida. Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

ABSTRACT

The aim of this study was to characterize the OTA production by *A. carbonarius* under different environmental conditions, and to elucidate the diffusion capacity of ochratoxin A throughout synthetic medium.

One strain belonging to the specie *A. carbonarius* isolated from vine dried fruits was single point inoculated on SNM medium at two water activities (0.92 and 0.97) and two temperatures (20ºC and 30ºC) by triplicate. Daily radii were measured and OTA production was tested after 4, 7, 10, 14 and 18 days of incubation at four distances from the centre of colony (1-4 cm).

OTA production was detected mainly at 0.97 a_w. Earlier production was detected at 30ºC (optimum for growth), whereas maximum OTA concentrations were found at 20ºC. OTA production was detected from mycelium that was only a few days old and attained its optimum when mycelium was 4-7 days old at 0.97 a_w. OTA diffusion was observed at 0.92 a_w and 20ºC. Thus OTA production is discernable in young *A. carbonarius* mycelium and diffusion of the toxin has been shown to occur in a solid substrate.

Publication: *Food Microbiology* **23** (2006) 53-756.

INTRODUCTION

Ochratoxin A (OTA) is a widely distributed mycotoxin produced by several fungal species in *Penicillium* and in *Aspergillus* sections *Circumdati* and *Nigri*. OTA is a potent nephrotoxin, and possesses teratogenic, immunotoxic and possibly neurotoxic and genotoxic properties. Furthermore, it may play a role in the human disease Balkan Endemic Nephropathy (Marquardt and Frohlich 1992). In 1993, the International Agency for Research on Cancer classified OTA as a possible human carcinogen (group 2B) (IARC 1993).

Previous publications have reported the occurrence of this mycotoxin in foodstuffs and beverages such as grapes (Abrunhosa et al. 2001), dried vine fruits (MacDonald et al. 1999), and wines (Bellí et al. 2002; Cabañes et al. 2002). OTA-producing strains from *Aspergillus* section *Nigri* (*A. carbonarius* and *A. niger* aggregate) are considered the source of OTA in wines, grapes and dried vine fruits (Abarca et al. 2003; Bellí et al. 2004a), and mycological studies have found *A.* section *Nigri* and *Circumdati* as the predominant fungi, with occurrences between 33 and 100%, in raisins (El Halouat and Debevere 1997; Magnoli et al. 2004).

Depending on the kind of raw materials, OTA diffusion from infected to healthy areas can influence in OTA contamination in final products, as reported for patulin diffusion through the tissue of apples and pears (Taniwaki et al. 1992; Laidou et al. 2001). Studies in grapes (Battilani et al. 2001) showed that the skin of grape berries is potentially the major source of OTA in grape, although it was also detected in pulp. Considering that grape juices, musts and white wines are produced by pressing grapes and discarding the skin, understanding the ability of OTA to diffuse is important.

Previous ecophysiological studies (Bellí et al. 2004a and 2004b; Mitchell et al. 2004) in *A.* section *Nigri* species have shown the effect of water activity and temperature on their growth and OTA production, but accumulation of the toxin during mould growth and the phenomenon of diffusion has not been studied yet.

This study also aimed to understand OTA production and the diffusion capacity of OTA *in vitro* in synthetic medium by *A. carbonarius* under different environmental conditions.

MATERIALS AND METHODS

Isolate and media

The *A. carbonarius* strain used in this study (coded as 3-122) was isolated from dehydrated grapes collected from the south of Spain. It belongs to the collection of the Food Technology Department (Lleida University, Spain).

The growth medium was a synthetic nutrient medium (SNM; Delfini et al. 1982) similar to grape composition between veraison and ripeness. In order to adjust the medium to the different water activities assayed, it was modified by adding glycerol and varying its sugars content (Valero et

al. 2005). Water activity of media was tested with a a_w-meter (AquaLab CX-2, Decagon, Pullman, WA, USA).

Inoculation and incubation

For inoculation, a suspension of 10^6 conidia ml^{-1} was prepared in 0.005% Tween-80 distilled water from a seven day old culture grown in Czapek Yeast Autolysate Agar (CYA; Pitt, 1979). Fungus was single point inoculated with a needle in Synthetic Nutrient Medium; media at two water activity levels (0.92 and 0.97) and incubated at two temperatures (20 and 30 ºC) for 18 days. Daily radius measurements were carried out and OTA production was tested after 4, 7, 10, 14 and 18 days incubation (full factorial design, total number of Petri plates=2 a_w x 2T x 5 sampling times x 3 rep.). From each day of extraction two agar plugs (7 mm diameter) were taken at a 1, 2, 3 and 4 cm distance from the inoculation point.

The OTA production by *A. carbonarius* was determined using HPLC (Waters, Milford, MA, USA) following the extraction and clean-up protocol detailed in Bragulat et al. (2001).

Statistical analysis

Ochratoxin A accumulation by *A. carbonarius* at four distances from the inoculation point, extracted at five different days, was analysed statistically with SAS program (SAS Institute Inc., Cary, NC, USA) through an ANOVA. Significance of the effect of time, distance, temperature and a_w was evaluated and Duncan's multiple range test was performed for the different factors. Growth rates were obtained from Valero et al. (submitted). Pearson´s correlation coefficients between OTA production (average of all distances) and *A. carbonarius* colony radius were calculated.

RESULTS

Effects of temperature, water activity, T x a_w, distance and time were found to have a significant effect ($P<0.05$) on OTA accumulation by *A. carbonarius*. No OTA was detected at 0.92 a_w and 30ºC although the mould grew rapidilly. By contrast, at 0.92 a_w and 20ºC, growth was almost negligible but some OTA was detected at day 14-18 (Fig. 1). Mean OTA accumulation in medium was significantly higher at 0.97 a_w and 20ºC from the 10th to 18th day because growth occurred more slowly than at 0.97 a_w and 30ºC. Both at 20 and 30ºC maximum accumulation was reached at different times depending on the growth rates of the colonies, but, in general, a decline in OTA was observed in the following days (Fig. 2). In general, the shorter the distance from the inoculum point, the higher OTA concentrations detected for each period tested. Plotting OTA concentration in SNM in each of the four distances against age of mycelium in this point

(passed days since colony arrives at such point), we observed that OTA accumulation might be detected

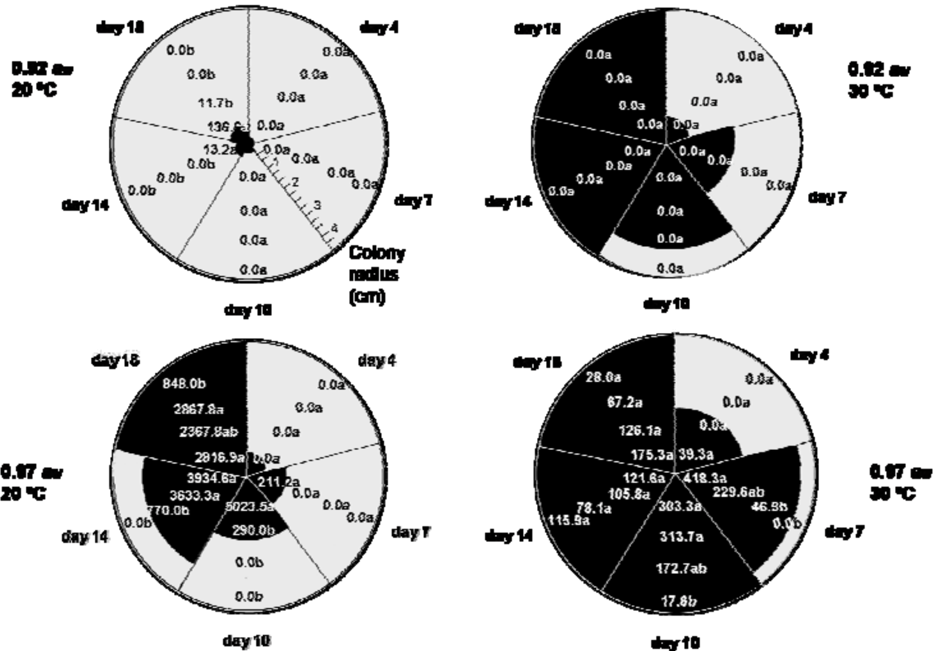


Figure 1. Growth (black area) and ochratoxin A production (values within each pie chart) by *A. carbonarius* in pure culture, at four distances from inoculum point. Mean values with the same letter are not significantly different according to Duncan test.

the first day and reached its maximum within 4-7 days. This effect occurred at 0.97 a_w and at both temperatures (Fig. 3).

OTA diffusion through medium agar was observed at 0.92 a_w and 20ºC at day 18, where OTA positive plugs were detected outside the colony margin (Fig. 1). For the remaining cases OTA was only detected in plugs were fungal growth had already taken place.

Correlation between colony radius and OTA production (average of all distances) was significant ($P<0.05$) at any condition with OTA production. Pearson coefficients obtained were 0.863, 0.945 and 0.543 for 0.92 a_w/20ºC, 0.97 a_w/20ºC and 0.97 a_w/30ºC, respectively.

DISCUSSION

Maximum mean OTA accumulation occurred at 0.97 a_w and 20ºC around the 10th day. Bellí et al. (2004c) found OTA production of two *A. carbonarius* isolates to be maximal at the fifth day of incubation at 25ºC and water activities between 0.93 and 0.98 on SNM; the shorter time may be attributed to the faster growth at 25ºC.

Taking into account OTA production depending on the age of mycelium, we observe that maximal accumulation occurs between 4-7 days, but OTA accumulation begins from the first day. However, the OTA accumulation detected the first day of mycelium age was due probably to diffusion from shorter distances rather than production in this point. The lapse till 4-7 days could represent the time mould needs for starting secondary metabolism and to produce OTA.

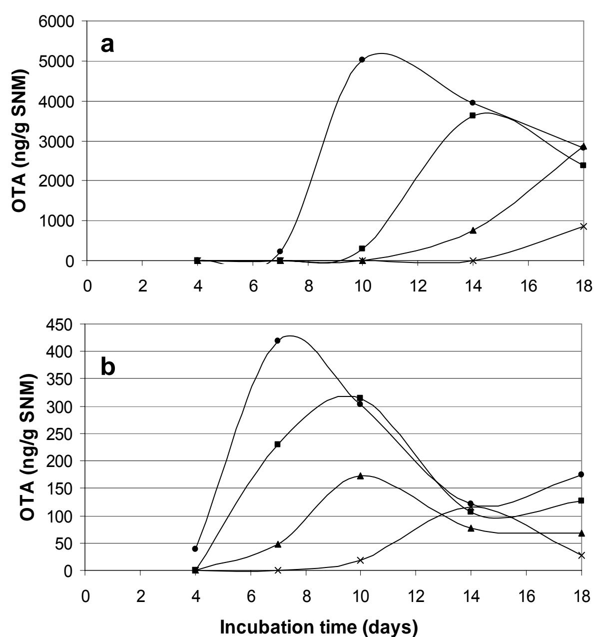


Figure 2. OTA accumulation in SNM with time at four distances from inoculum point. a, 0.97 a_w and 20ºC; b, 0.97 a_w and 30ºC. ●, 1 cm distance; ■, 2 cm; ▲, 3 cm and x, 4 cm.

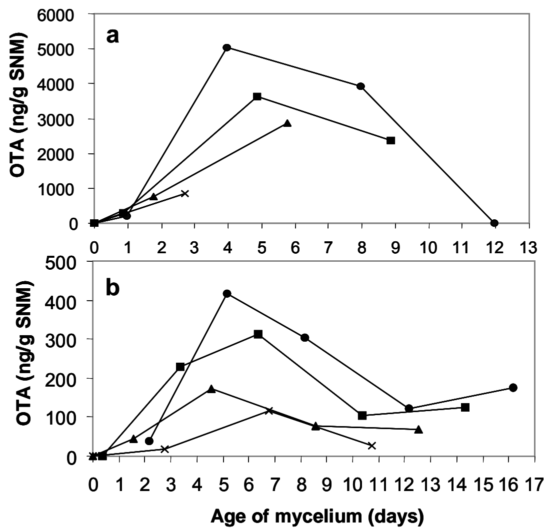


Figure 3. Effect of mycelium age in OTA concentration in SNM at four distances from inoculum point. a, 0.97 a_w and 20ºC; b, 0.97 a_w and 30ºC. ●, 1 cm distance; ■, 2 cm; ▲, 3 cm and x, 4 cm.

At 0.97 a_w, the decrease in OTA accumulation after the 7th day of age may be the result of a decreased or stopped and probably its degradation by the fungus as an alternative carbon source to maintain a high metabolic rate. Previous studies with *A. carbonarius* have shown its ability to degrade OTA into other breakdown products, such as ochratoxin alpha, likely achieved by a carboxipeptidase enzyme (Abrunhosa et al. 2002). Varga (2000) also studied this phenomenon with fungi belonging to the *A*. section *Nigri*, which degraded OTA into ochratoxin alpha within seven days.

The best conditions for growth were 0.97 a_w and 30ºC, whereas at 0.97 a_w and 20ºC OTA production was strongly higher, in accordance with previous publications (Bellí et al. 2004; Mitchell et al. 2004). Optimal growth rates for *A. carbonarius* isolates at temperatures between 30 and 35ºC and 0.95-0.98 a_w have been reported (Mitchell et al. 2003; Bellí et al. 2004b; Valero et al. [submitted]).

Diffusion of the toxin was observed at 0.92 a_w and 20ºC, when mycelial growth was slower. At the other conditions tested mycelium likely grew faster than OTA diffused, therefore diffusion phenomenon was not observable.

The mechanism of OTA synthesis could need some SNM compounds or metal ions, such chlorine or zinc, with high diffusion ability. This hypothesis would explain why OTA accumulation is maximal at the first centimetre of extraction, where the fungus may use fresher medium, and decreases with increasing distance from the inoculation point. There is not specific bibliography supporting this hypothesis. Nevertheless, a study done by Mühlencoert et al. (2004) investigated the effect of availability of some metal ions on *Aspergillus ochraceus* growth and on OTA synthesis, showing that Zn increased biomass and OTA production by 50%.

To sum up, OTA can diffuse through SNM agar, up to a distance higher than 1cm. Moreover OTA production occurs in a few days and its optimal around 20ºC thus we could expect that dehydrating grapes at temperatures above 30ºC, avoiding lower temperatures, and drying to a safe a_w in less than one week, could be a good preventive technique for reducing OTA content in vine dried fruits. However, further studies using grapes as growth substrate should be achieved to confirm or to refuse this hypothesis.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, projects AGL 2004-07549-05-01, AGL 2004-06413, grant BES-2003-2180 and Ramon y Cajal program) for its financial support.

REFERENCES

Abarca, M.J., Accensi, F., Bragulat, M.R., Castella, G. and Cabañes, F.J. (2003) Contamination in dried vine fruits from the spanish market. *Journal of Food Protection* 66, 504-506.

Abrunhosa, L., Paterson, R.R.M., Kozakiewicz, Z., Lima, N. and Venâncio, A. (2001) Mycotoxin production from fungi isolated from grapes. *Letters in Applied Microbiology* 32, 240-242.

Abrunhosa, L., Serra, R. and Venancio, A. (2002) Biodegradation of ochratoxin A by fungi isolated from grapes. *Journal of Agricultural and Food Chemistry* 50, 7493-7496.

Battilani, P., Giorni, P., Bertuzzi, T. and Pietri, A. (2001) Preliminary results on ochratoxin A production by fungi invading grape berries. Congress Proceeding. 11th Congress of the Mediterraean Phytopahtological Union and 3rd Congress of the Sociedade Portuguesa de Fitopatologia. Évora (Portugal).

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J. (2002) Review: Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. *Food Science Technology International* 8, 0325-11.

Bellí, N., Marín, S., Duaigües, A., Ramos, A.J. and Sanchis, V. (2004a) Ochratoxin A in wines, musts and grape juices from Spain. *Journal of the Science of Food and Agriculture* 84, 591-594.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J. (2004b) Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. *International Journal of Food Microbiology* 96, 19-27.

Bellí, N. Ramos, A.J., Sanchis, V. and Marín, S. (2004c) Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. *Letters in Applied Microbiology* 38, 72-77.

Bragulat, M.R., Abarca, M.L. and Cabañes, F.J. (2001) An easy screening method for fungi producing ochratoxin A in pure culture. *International Journal of Food Microbiology* 71, 139-144.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S. and Pons, A. (2002) What is the source of ochratoxin A in wine?. *International Journal of Food Microbiology* 79, 213-215.

Delfini, C. (1982) In *Tecnica di microbiologia eonological* ed. Scicalpi, L. Rome.

El Halouat, A. and Debevere, J.M., 1997. Molds and Yeast isolated from hydrated prunes and raisins having different water activities. *Science of Aliments* 17, 539-545.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Vol. 56, International Agency for Research on Cancer, Lyon, 1993, pp. 489-521.

Laidou, I.A., Thanassoulopoulos, C.C. and Liakopoulou-Kyariakides (2001) Diffusion of patulin in the flesh of pears inoculated with four post harvest pathogens. *Journal of Phytopathology* 149, 457-461.

MacDonald, S., Wilson, P., Barnes, K., Damant, A., Massey, R., Mortby, E. A and Shepherd, M.J. (1999) Ochratoxin A in dried vine fruit: method development and survey. *Food Additives and Contaminants* 16, 253-260.

Magnoli, C., Astoreca, A., Ponsone, L., Combina, M., Palacio, G., Rosa, C.A.R. and Dalcero, A.M. (2004) Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Letters in Applied Microbiology* 39, 326-331.

Marquardt, R.R. and Frolich, A.A. (1992) A review of recent advances in understanding ochratoxicosis. *Journal of Animal Science* 70, 3968-3988.

Mitchell, D., Aldred, D. and Magan, N. (2003) Impact of ecological factors on the growth and ochratoxin A production by *Aspergillus carbonarius* from different regions of Europe. *Aspects of Applied Biology* 68, 109-116.

Mitchell, D., Parra, R., Aldred, D. and Magan, N. (2004) Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. *Journal of Applied Microbiology* 97, 439-445.

Mühlencoert, E., Mayer, I., Zapf, M.W., Vogel, R.F., Niessen, L. (2004) Production of Ochratoxin A by some *Aspergillus ochraceus*. *European Journal of Plant Pathology* 00, 1-9.

Pitt, J.I. (1979) *Geosmitchia* gen. nov. for *Penicillium lavendulum* and related species. Canadian Journal of Botany 57, 2021-2030.

Taniwaki, M.H., Hoenderboom, C.J.M., de A.meida Vitali, A. and Vboldi Eiroa, M.N. (1992) Migration of patulin in apples. *Journal of Food Protection* 55, 902-904.

Valero, A., Marín, S., Ramos, A.J. and Sanchis, V. (2005) Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors. *Letters in Applied Microbiology* 41, 196-201.

Valero, A. Ramos, A.J., Sanchis, V and Marín, S. (submitted) Understanding the dominance of *Aspergillus* section *Nigri* in mycobiota succession in grapes and sun-dried grapes. *International Journal of Food Microbiology*.

Varga, J., Rigó, K. and Téren, J. (2000) Degradation of ochratoxin A by *Aspergillus* species. *International Journal of Food Microbiology* 59, 1-7.

Study IV:

Studies on the interaction between grape-associated filamentous fungi on a synthetic medium

Ana Valero, Vicente Sanchis, Antonio J. Ramos, Sonia Marín

Food Technology Department, University of Lleida, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

Abstract

Eleven fungi isolated from grapes and sun-dried grapes (*Alternaria alternata*, *Cladosporium herbarum*, *Eurotium amstelodami*, *Penicillium janthinellum*, *P. decumbens*, *Trichoderma harzianum*, *Candida* sp., *Aspergillus carbonarius* OTA-negative, *A. carbonarius* OTA-positive, *A. niger* var. *niger*. and *A. japonicus* var. *aculeatus*), were grown in SNM medium at different water activities (0.82-0.97) and temperature (20-40ºC) levels for 18 days. Pairs of one *Aspergillus* faced with one non-*Aspergillus* were grown at 0.87-0.97 a_w and at 20 and 30ºC. In single cultures, daily radii were recorded. In paired cultures radii were recorded and each *A.* section *Nigri* isolate was given a dominance score.

At high temperatures and low water activities, *Penicillium* isolates, *E. amstelodami* and *A. niger* var. *niger* showed higher growth rates, while *T. harzianum* only grew well at the highest water activity. In addition, *A.* section *Nigri* was dominant in most paired assays, being only surpassed by *T. harzianum* at 0.97 aw and 20ºC. Thus, prevalence of *A.* section *Nigri* in sun dried grapes can be explained by its adaptation to environmental conditions of sun-drying, and by its capability to dominate other fungal species when coming into contact with them.

Publication: *International Journal of Food Microbiology* **113** (2007) 271-27.

INTRODUCTION

Ochratoxin A (OTA) is a widely distributed mycotoxin produced by several fungal species belonging to the genera *Penicillium* and *Aspergillus* section *Circumdati* and *Nigri*. OTA is a potent nephrotoxin, and possesses teratogenic, immunotoxic and possibly neurotoxic and genotoxic properties. Furthermore, it is considered as a cause of human disease Balkan Endemic Nephropathy (Marquardt and Frohlich 1992; Pleština *et al*., 1996). In 1993, the International Agency for Research on Cancer classified OTA as a possible human carcinogen (group 2B) (IARC, 1993).

Previous publications reported the occurrence of this mycotoxin in foodstuffs and beverages such as coffee (Patel *et al*., 1997; Lombaert *et al*., 2002; Pardo *et al*., 2004), grape juices, musts and wines (Bellí *et al*., 2002; Cabañes *et al*., 2002; Soufleros 2003), grapes (Abrunhosa *et al*., 2001) and dried vine fruits (Ostry *et al*., 2002; MacDonald *et al*., 1999). The highest OTA content, among grapes and their derivatives, has been found in dried vine fruits, with OTA levels above 53 $\mu g\ kg^{-1}$ (MAFF, 1997; MacDonald *et al*., 1999; Battilani and Pietri, 2002). Mycological studies have reported *A.* section *Nigri* as the predominant fungi, with occurrences between 33 and 100%, in raisins (El Halouat and Debevere 1997; Abarca *et al*., 2003; Magnoli *et al*., 2004).

Contamination of grapes and their derivatives can occur during preharvest, harvest and grape processing. *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Fusarium* and *Rhizopus* are regarded as the main natural contaminants in these foodstuffs (Abrunhosa *et al*., 2001; Magnoli *et al*., 2003 and 2004; Bellí *et al*., 2003).

Previous ecophysiological studies (Bellí *et al*., 2004a and 2004b; Mitchell *et al*., 2004) in *A.* section *Nigri* species have shown the effect of water activity and temperature on their growth. Low moisture levels that typically occur during grapes sun-drying were not taken into account.

In this study we aimed to understand the mycobiota succession in grapes and sun-drying through parameters such as temperature, water activity and fungal interactions focusing on niche adaptation and antagonistic capability of black Aspergilli.

MATERIAL AND METHODS

Isolation of microorganisms

Microorganisms used in this study were *Alternaria alternata* (3.136), *Cladosporium herbarum* (3.129), *Eurotium amstelodami* (3.132), *Penicillium janthinellum* (3.126), *P. decumbens* (3.128), *Trichoderma harzianum* (3.134), *Candida* sp. (2.9), *Aspergillus carbonarius* OTA-negative (3.123), *A. carbonarius* OTA-positive (3.122), *A. niger* var. *niger* (3.124) and *A. japonicus* var. *aculeatus* (3.125). They were isolated from grapes and vine dried fruits collected from the South of Spain (Valero *et al*., 2004) Taxonomic identification of all isolates was achieved in accordance with guidelines of Pitt and Hocking (1997) and in particular, black aspergilli were identified

according to Al-Musallam (1980). The capability of OTA production by *A. carbonarius* OTA-positive isolate was tested through HPLC (Waters, Milford, MA, USA) following the extraction and clean-up protocol detailed in Bragulat *et al.* (2001).

The above mentioned strains are kept in the Department of Food Technology, ETSEA, Universitat de Lleida, Spain.

Growth in pure cultures

The culture medium used was a synthetic nutrient medium (SNM) similar to grape composition between veraison and ripeness (Delfin *et al.*, 1982). In order to adjust it to different water activities (0.82, 0.87, 0.92, and 0.97), it was modified adding glycerol and varying its sugars content (Valero *et al.*, 2004). Media were poured in 9 cm Petri dishes. For inoculation, suspensions of 10^6 conidia ml^{-1} were prepared in distilled water with 0.005% Tween-80. Each microorganism was single point inoculated with a needle in SNM media at the four a_w levels and incubated at three temperatures (20, 30 and 40 ºC) for 18 days. Daily radius measurements were carried out and every treatment was performed in duplicate.

Growth in paired cultures

Pairs were made with one *A.* section *Nigri* faced to one of the seven non-Aspergilli inoculated at a distance of 45 mm between them at three water activities (0.87, 0.92, and 0.97) and two temperatures (20 and 30ºC). Growth radii in the line between both inoculation points were recorded daily for 18 days. In addition, each fungus was given a numerical score using the following scoring system: 1, mutual intermingling; 2, mutual inhibition on contact; 3, mutual inhibition at a distance; 4, dominance on contact; 5, dominance at a distance (Magan and Lacey 1984). The addition of the scores obtained against the different species resulted in the calculated Index of Dominance (I_D).

Statistical Analysis

For single cultures, the radial extensions of colonies were plotted against time and the growth rates calculated using a linear regression. Data of daily lectures of radii were analyzed statistically by SAS (SAS Institute Inc., Cary, NC, USA) by a covariance analysis. Significance of the levels of T, a_w and fungal interaction and their interactions was evaluated and Duncan's multiple range test was performed.

For an easy overview of *A*. section *Nigri* behaviour, growth rates were modeled by polynomial multiple lineal regression (MLR): growth rate = $b_0 + b_1a_w + b_2T + b_{12}a_wT + b_{11}a_w^2 + b_{22}T^2$. The resulting response surface models (RSM) were obtained with the Unscrambler® software,

version 7.6 (CAMO ASA, Oslo, Norway), including the significant factors, interactions and quadratic terms.

RESULTS

Growth of pure cultures

The main effects (temperature, water activity and strain) proved to be statistically significant (P<0.001).

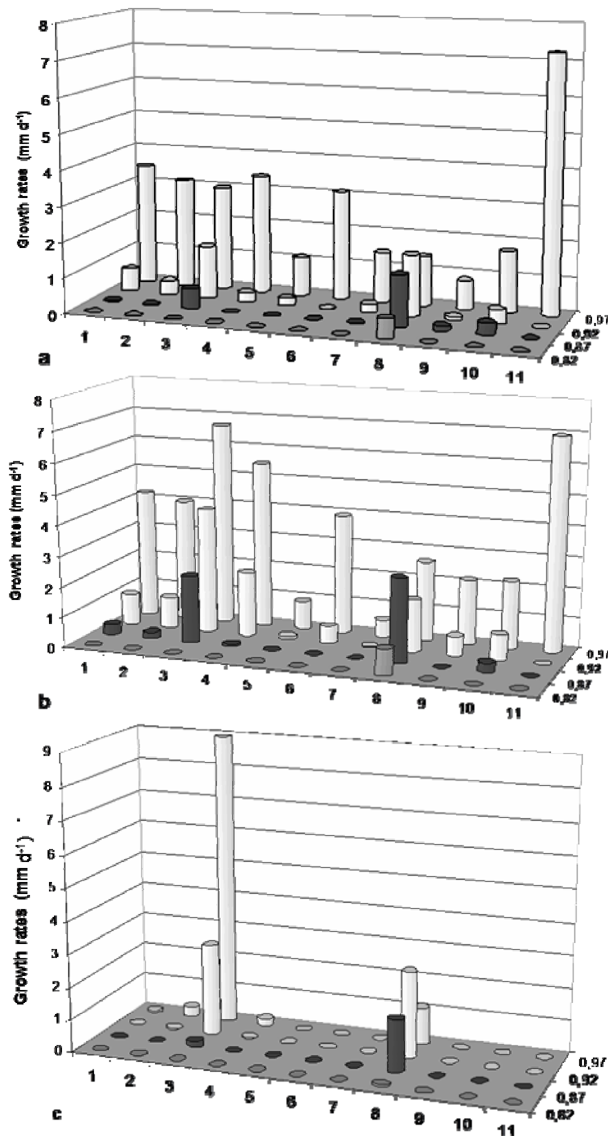


Figure 1. Growth rates (mm d^{-1}) of the different isolates as affected by a_w and T.

a, 20ºC; b, 30ºC and c, 40ºC. Isolates: 1, *A. carbonarius* OTA-negative; 2, *A. carbonarius* OTA-positive; 3, *A. niger* var. *niger*; 4, A. japonicus var. *aculeatus*; 5, *Candida* sp.; 6, *A. alternata*; 7, *C. herbarum*; 8, *E. amstelodami*; 9, *P. janthinellum*; 10, *P. decumbens*; 11, *T. harzianum*.

At 0.97 a_w and 20ºC all black Aspergilli, *T. harzianum* and *A. alternata* grew significantly faster (P<0.05) than the remaining species and at 40ºC only *A. niger* var. *niger*, *A. japonicus* var. *aculeatus*, *A. carbonarius* OTA-positive and *E. amstelodami* were able to grow. At 30ºC *A. niger* var. *niger*, *A. japonicus* var. *aculeatus*, *T. harzianum* and *A. alternata* had the highest growth rates. At 0.92 a_w both *E. amstelodami* and *A. niger* var. *niger* grew significantly faster than the other species. *A. niger* var. *niger* also grew well at 0.87 a_w and at higher temperatures (30 and 40ºC), while there were no significant differences among the remaining species. At low a_w (0.87-0.92) *P. decumbens* was capable of similar growth as *A. carbonarius* but did not tolerate 40ºC. At the lowest water activities (0.82 and 0.87 a_w) *E. amstelodami* grew significantly better (P<0.05) than the other fungi, being the only one capable to grow at 0.82 a_w (Fig. 1) and 30ºC, as optimum. In general *A. niger* var. *niger* species showed higher growth rates.

Paired cultures

Effects on black Aspergilli growth of temperature, water activity, paired species and all their interactions, proved to be significant (P < 0,001) (Table 1).

Table 1. Significance of the effect of a_w, T and competing species in mean radii of black Aspergilli (Duncan test, P<0.05).

	A. niger var. *niger*										
	0.87 a_w			0.92 a_w				0.97 a_w			
Fungi	20ºC	30ºC		20ºC		30ºC		20ºC		30ºC	
Single	-	14.28	A	8.44	B	26.89	A	22.87	B	38.44	A
A. alternata	-	-		-		26.93	A	17.19	D	33.77	AB
C. herbarum	-	-		9.76	A	-		20.19	C	35.55	AB
E. amstelodami	-	10.13	B	8.57	B	19.27	B	20.24	C	33.16	AB
P. decumbens	-	7.47	C	7.69	B	25.68	A	18.49	CD	32.16	B
P. janthinellum	-	-		-		26.47	A	20.49	C	30.00	B
T. harzianum	-	-		-		-		9.25	E	22.41	C
Candida sp.	-	-		-		-		25.43	A	35.59	AB

-, not tested; n.g., not growth

Table 1. (cont.)

	0.87 a_w			0.92 a_w				0.97 a_w			
A. carbonarius OTA-	20ºC	30ºC		20ºC		30ºC		20ºC		30ºC	
Single	-	1.84	B	4.30	C	7.85	D	22.22	BC	26.55	B
A. alternata	-	-		-		9.50	C	16.28	D	32.16	A
C.herbarum	-	-		7.62	A	-		23.13	BC	32.13	A
E.amstelodami	-	3.00	A	6.29	B	11.09	B	22.03	BC	31.72	A
P. decumbens	-	1.09	C	3.59	C	11.22	B	20.09	C	27.90	AB
P. janthinellum	-	-		-		13.93	A	23.87	B	23.27	B
T.harzianum	-	-		-		-		11.00	E	16.06	C
Candida sp.	-	-		-		-		29.73	A	32.36	A
A. carbonarius OTA+	20ºC	30ºC		20ºC		30ºC		20ºC		30ºC	
Single	-	1.38	A	2.24	B	8.08	B	20.82	B	23.88	ED
A. alternata	-	-		-		n.g.		17.44	C	30.56	AB
C.herbarum	-	-		3.84	A	-		18.53	BC	28.98	ABC
E.amstelodami	-	1.18	AB	4.24	A	7.34	B	19.09	BC	28.37	BCD
P. decumbens	-	1.04	B	4.46	A	12.78	A	17.04	C	24.96	CD
P. janthinellum	-	-		-		12.15	A	20.22	B	20.48	E
T.harzianum	-	-		-		-		10.46	D	16.15	F
Candida sp.	-	-		-		-		25.24	A	32.72	A
A. japonicus var. aculeatus	20ºC	30ºC		20ºC		30ºC		20ºC		30ºC	
Single	-	0.88	A	1.32	B	17.18	B	23.58	BC	30.97	A
A. alternata	-	-		-		14.94	C	20.88	C	25.71	B
C.herbarum	-	-		2.71	A	-		24.16	B	29.91	A
E.amstelodami	-	n.g.		2.69	A	13.28	C	22.22	BC	20.68	C
P. decumbens	-	n.g.		1.21	B	20.79	A	13.07	D	18.69	CD
P. janthinellum	-	-		-		19.29	AB	21.46	BC	16.31	DE
T.harzianum	-	-		-		-		11.75	D	12.98	E
Candida sp.	-	-		-		-		32.97	A	32.41	A

-, not tested; n.g., not growth

Among the black Aspergilli, *A. niger* var. *niger* was in general the more inhibited by the interacting species, while both *A. carbonarius* isolates growth was less affected and sometimes stimulated. At 0.97 a_w growth rates of all black Aspergilli were clearly inhibited by *T. harzianum*. At this water activity A*. niger* var. *niger* growth was reduced by almost all filamentous fungi, whilst *A. alternata* and *P. decumbens* were also usual inhibitors of the rest of black Aspergilli. At 0.92 a_w none of the species inhibited *A. carbonarius* growth, while they were stimulated by both Penicillia. *E. amstelodami* inhibited growth of both *A. japonicus* var. *aculeatus* and *A. niger* var. *niger* at 30ºC. At 0.87 a_w (30ºC) all black Aspergilli were strongly inhibited by *P. decumbens*.
At the remaining conditions either no effect or stimulation of growth was observed. Interestingly there was a consistent stimulation of black Aspergilli growth in the presence of *Candida* sp.
Regarding the Index of Dominance (Table 2), *A.* section *Nigri* isolates inhibited *Candida* sp. growth at a distance and surpassed it under all conditions assayed. Dominance was the most

common reaction of *A*. section *Nigri* against *A. alternata*, and mutual inhibition on contact or at a distance when paired with *T. harzianum* and *P. janthinellum*.

No correlation between growth rates and I_D was observed (Figure 2).

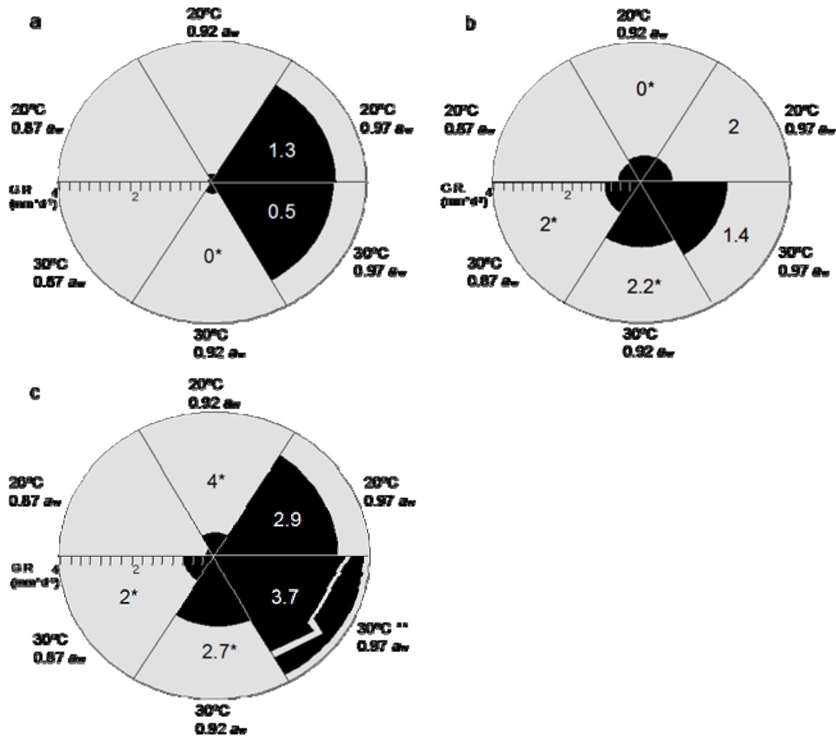


Figure 2. Comparison of effect of temperature and water activity on fungi growth rates represented as radii, and on fungi dominance indexes. *: I_D average of only pairs tested. **: G.R.= 5.12 mm*d^{-1}. a: global average of growth rates of *A. alternaria*, *C. herbarum*, *T. harzianum* and *Candida* sp., b: global average of growth rates of *P. decumbens*, *P. janthinellum* and *E. amstelodami*, c: global average of growth rates of both *A. carbonarius*, *A. niger* var. *niger* and A. japonicus var. *aculeatus*.

Table 2. Effect of water activity and temperature in growth and Indices of dominance (I_D)

a_w	Temp. (°C)	Scores of *A. japonicus* var. *aculeatus* paired with							I_D
		Candida sp.	*C. herb.*	*A. alt.*	*E. ams.*	*P. dec.*	*P. janth.*	*T. harz.*	
0.92	20	-	n.g.	-	n.g.	n.g.	-	-	
	30	-	-	n.g.	2	3	3	-	
0.97	20	5	2	2	2	3	3	2	19
	30	5	2	5	2	3	3	2	22

a_w	Temp. (°C)	Scores of *A. carbonarius* OTA+ paired with							I_D
		Candida sp.	*C. herb.*	*A. alt.*	*E. ams.*	*P. dec.*	*P. janth.*	*T. harz.*	
0.87	30	-	-	-	n.g.	n.g.	-	-	
0.92	20	-	n.g.	-	n.g.	n.g.	-	-	
	30	-	-	-	2	2	n.g.	-	
0.97	20	5	2	4	2	2	4	2	21
	30	5	5	4	4	4	3	4	29

a_w	Temp. (°C)	Scores of *A. carbonarius* OTA- paired with							I_D
		Candida sp.	*C. herb.*	*A. alt.*	*E. ams.*	*P. dec.*	*P. janth.*	*T. harz.*	
0.87	30	-	-	-	n.g.	n.g.	-	-	
0.92	20	-	n.g.	-	n.g.	n.g.	-	-	
	30	-	-	n.g.	2	3	3	-	
0.97	20	5	2	4	4	2	2	0*	19
	30	5	4	4	4	5	3	2	24

a_w	Temp. (°C)	Scores of *A. niger* var. *niger* paired with							I_D
		Candida sp.	*C. herb.*	*A. alt.*	*E. ams.*	*P. dec.*	*P. janth.*	*T. harz.*	
0.87	30	-	-	-	2	n.g.	-	-	
0.92	20	-	n.g.	-	4	n.g.	-	-	
	30	-	-	4	4	2	2	-	
0.97	20	5	5	2	2	3	3	2	22
	30	5	5	4	5	4	3	2	28

C. herb: C. herbarum; A. alt.: A. alternata; E. ams.: E. amstelodami; P. dec.: P. decumbens; P. janth.: P. janthinellum; T. harz.: T. harzianum.

-, Not tested; n.g., not growth enough; *, 0 is the score corresponding to *A. carbonarius* OTA-negative and 4 corresponds to *T. harzianum*.

1, mutual intermingling; 2, mutual inhibition on contact; 3, mutual inhibition at a distance; 4, dominance on contact; 5, dominance at a distance (Magan and Lacey 1984).

DISCUSSION

Grapes intended for raisins and sweet wines production are exposed to sun-drying during one or two weeks, where diurnal temperatures can be higher than 40ºC in areas with Mediterranean climate. Water activity of grapes drops gradually even under 0.75 a_w (Valero, unpublished data) due to their high sugar content and water loses. In such environmental conditions species of *Eurotium*, *Penicillium* and mainly *A.* section *Nigri* are prevalent (Valero *et al.*, in press).

Harvested grapes contain a diverse fungal inoculum coming from the vineyard, and the high moisture content of these berries may enable microorganisms to grow at high rates. In this study both *T. harzianum* and *A.* section *Nigri* showed the highest growth rates, but at 40ºC only species of *A.* section *Nigri* group were able to grow. In the competence assay *T. harzianum* reduced the growth of the four Aspergilli tested, and regarding the dominance indexes, black Aspergilli showed higher scores only being surpassed by *T. harzianum* at 20ºC. *Trichoderma* sp. is well known in biological pests control because of its ability to produce antifungal metabolites such as trichozianines, chitinase, proteases, ß-glucanase, trichodermin, harzianum A (Cook and Baker, 1989; Corley *et al.*, 1994; Egorov *et al.*, 1996; Küçuk and Kivanç 2004; Nampoothiri *et al.*, 2004). Also the presence of *A. alternata* and *P. decumbens* reduced growth rates of black Aspergilli. *A. alternata* produces antifungal metabolites like alternariol and several toxins with unknown antibiotic effects such as tenuazonic acid, altenuene, altertoxin I and altertoxin II (Ansari and Shrivastava, 1990; Centeno and Calvo, 2002).

However, high relative humidity, very favourable to *A. alternata* and to *T. harzianum,* occurs during a brief period at the beginning of the sun exposure and thereafter it drops rapidly limiting their growth and metabolic capacity. According to our results, at 0.92 a_w both *E. amstelodami* and *A.* section *Nigri* grew significantly faster, followed by both Penicillia. At this water activity*, E. amstelodami* reduced the growth of some black Aspergilli when grown in pairs. Nevertheless *A. niger* var. *niger* had the higher dominance scores.

While water content of SNM descends, only those fungi with higher growth rates at 0.92 a_w (*A.* section *Nigri* isolates, *E. amstelodami* and *P. decumbens*), might be able to grow at 0.87 a_w. In paired growth, rates of all black Aspergilli tested were reduced by *P. decumbens.* This *Penicillium* species can produce some metabolites with antibiotic activity such as decumbenone A and B and versiol (Fujii *et al.*, 2002; Stewart *et al.*, 2005). On the other hand these metabolites have not been tested on *Aspergillus* spp. yet and temperatures close to 40ºC inhibit the growth of the Penicillia tested.

During the drying period that can last from 5 days to two weeks, conidia of only few micro-organisms such as black Aspergilli, are capable to resist both germicidal UV light and the strong sunlight heating (Rotem and Aust 1991). Furthermore, *A. niger* can produce oxalic acid, with antifungal activity and other secondary metabolites such as malformins, naphto-r-pyrones, nigerazine B and nigragillin with unknown effect on fungi but probed to be toxic in plants, insects or mice (Blumenthal, 2003).

Microorganisms with higher growth rates are first in colonizing growth medium and consequently in primary resource capture (Cooke and Rayner, 1984). Other ecological strategies such as stress-tolerance or good enzymatic competence play an important role in substrate colonization, thus some fungi which are good at primary resource capture are not able to control the resource

when competing with a more combative fungus (Robinson *et al*., 1993). In field, contact competition of two fungi in the same berry is unlikely, so it is presumable that growing at high rates and being adapted to a wider range of conditions is crucial in grape colonization. To sum up, prevalence of *A*. section *Nigri* can be easily explained by its adaptation to environmental conditions of sun-drying, and by its capability to dominate some other fungal species involved when coming into contact with them.

Further studies are needed on the effect of this succession of mycobiota in sun-dried grapes on ochratoxin A accumulation by *A*. section *Nigri*.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2004-07549-05-01, grant BES-2003-2180 and Ramon y Cajal program) and to the Catalonian Government (CeRTA strategic project 2005-2006 "Seguridad biótica y abiótica de los alimentos", Generalitat de Catalunya) for their financial support.

REFERENCES

Abarca, M.J., Accensi, F., Bragulat, M.R., Castella, G. and Cabañes, F.J., 2003 *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. Journal of Food Protection 66, 504-506.

Abrunhosa, L., Paterson, R.R.M., Kozakiewicz, Z., Lima, N. and Venâncio, A., 2001. Mycoflora production from fungi isolated from grapes. Letters in Applied Microbiology 32, 240-242.

Al-Musallam, A. 1980. Revision of the Black *Aspergillus* Species. Ph.D. Thesis. Utrecht, Netherlands: University of Utrecht.

Ansari, A.A., and Shrivastava, A.K., 1990. Natural occurrence of *Alternaria* mycotoxins in sorghum and ragi from North Bihar, India. Food Additives and Contaminants 7, 815-820.

Battilani, P. and Pietri, A., 2002. Ochratoxin A in grapes and wine. European Journal of Plant Pathology 108, 639-643.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J., 2002. Review: Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. Food Science and Technology International 8, 0325-11.

Bellí, N., Pardo, E., Marín, S., Farré, G., Ramos, A.J. and Sanchis, V., 2003. Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. Journal of the Science of Food and Agriculture 84, 541-546.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J., 2004. Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. International Journal of Food Microbiology 96, 19-27.

Bellí, N. Ramos, A.J., Sanchis, V. and Marín, S., 2004. Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. Letters in Applied Microbiology 38, 72-77.

Blumenthal, CZ., 2003. Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regulatory Toxicology and Pharmacology 39, 214-228.

Bragulat, M.R., Abarca, M.L., Cabañes, F.J., 2001. An easy screening method for fungi producing ochratoxin A in pure culture. International Journal of Food Microbiology 71, 139-144.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S., Pons, A., 2002. What is the source of ochratoxin A in wine?. International Journal of Food Microbiology 79, 213-215.

Centeno, S., Calvo, M.A., 2002. Mycotoxins produced by fungi isolated from wine cork stoppers. Pakistan Journal of Nutrition 1, 267-269.

Cook, R.J., Baker, K.F., 1989. The nature practice of biological control of plant pathogens. APS Press, St. Paul, 539 p.

Cooke, R.C. and Rayner, A.D.M., 1984. Ecology of Saprotrophic Fungi. Longman, New York.

Corley, D.G., Miller-Wideman, M. and Durley, R.C., 1994. Isolation and structure of harzianum A: a new trichothecene from *T. harzianum*. Journal of Natural Products 57, 422-425.

Delfini, C., 1982. In *Tecnica di microbiologia eonological* ed. Scicalpi, L. Rome.

El Halouat, A. and Debevere, J.M., 1997. Molds and Yeast isolated from hydrated prunes and raisins having different water activities. Science des Aliments 17, 539-545.

Fujii, Y., Asahara, M., Ichinoe, M. and Nakajima, H., 2002. Fungal melanin inhibitor and related compounds from *Penicillium decumbens*. Phytochemistry 60, 703-708.

Küçuk, Ç., Kivanç, M., 2004. In vitro antifungal activity of strain of *Trichoderma harzianum*. Turkish Journal of Biology 28, 111-115.

Leong, S., Hocking, A.D. and Pitt, J.I., 2004. Occurrence of fruit rot fungi (*Aspergillus* section *Nigri*) on some drying varieties of irrigated grapes. Australian Journal of Grape and Wine Research 10, 83-88.

Lombaert, G.A., Pellaers, Pl, Chettiar, M., Lavalee, D., Scott, P.M., Lau, B.P.Y., 2002. Survey of Canadian retail coffees for ochratoxin A. Food Additives and Contaminants 19, 869-877.

MacDonald, S.J., Wilson, P., Barnes, K., Damant, A., Massey, R., Mortby, E. and Shepherd, M.J., 1999. Ochratoxin A in dried vine fruit: method development and survey. Food Additives and Contaminants 16, 253-260.

Magan, N., Lacey, J., 1984. Effect of water activity, temperature and substrate on interactions between field and storage fungi. Transactions of the British Mycological Society 82, 83-93.

Magnoli, C., Violante, M., Combina, M., Palacio, G. and Dalcero, A., 2003. Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. Letters in Applied Microbiology 37, 179-184.

Magnoli, C., Astoreca, A., Ponsone, L., Combina, M., Palacio, G., Rosa, C.A.R. and Dalcero, A.M., 2004. Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. Letters in Applied Microbiology 39, 326-331.

Marquardt, R.R. and Frolich, A.A., 1992. A review of recent advances in understanding ochratoxicosis. Journal of Animal Science 70, 3968-3988.

Mitchell, D., Parra, R., Aldred, D. and Magan, N., 2004. Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. Journal of Applied Microbiology 97, 439-445.

Ostry, V. Ruprich, J. and Skarkova, J., 2002. Raisins, Ochratoxin A and human health. Mycotoxin Research 18 A, 178-182.

Patel, S., Hazel, C.M., Winterton, A.G.M., Gleadle, A.E., 1997. Survey of ochratoxin A in UK retail coffees. Food Additives and Contaminants 14, 217-222.

Pardo, E., Marín, S., Ramos, A.J. and Sanchis, V., 2004. Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different origins. Food Science Technology International 10, 0045-5.

Pitt, J.I. and Hocking, A.D., 1997. Fungi and food spoilage ed. Blackie Academic and Professional 2nd ed. Cambridge: University Press.

Pleština, R., 1996. Nephrotoxicity of ochratoxin A. Food Additives and Contaminants 13, 49-50.

Robinson, C.H., Dighton, J. and Frankland, J.C., 1993. Resource capture by interacting fungal colonizers of straw. Mycological Research 97, 547-558.

Rotem, J. and Aust, H.J., 1991. The effect of ultraviolet and solar radiation and temperature on survival of fungal propagules. *Journal of Phytopathology* 133, 76-84.

Soufleros, E.H., Tricard, C. and Bouloumpasi, E.C., 2003. Occurrence of ochratoxin A in Greek wines. Journal of the Science of Food and Agriculture 83, 173-179.

Stewart, M., Capon, R.J., Lacey, E., Tennant, S., Gill, J.H., 2005. Calbistrin E and two other new metabolites from an Australian isolate of *Penicillium striatisporum*. Journal of Natural Products 68, 581-584.

Valero, A., Marín, S., Ramos, A.J., Sanchis, V., 2004. Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors. Letters in Applied Microbiology 41, 196-201.

Study V:

Effects of fungal interaction in ochratoxin A production by *A. carbonarius* at different temperatures and a_w

Ana Valero, Joan Ramón Farré, Vicente Sanchis, Antonio J. Ramos, Sonia Marín

Food Technology Department, University of Lleida. Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

Abstract

Ochratoxin A is a well-known mycotoxin produced by species of the genera *Penicillium* and *Aspergillus*. OTA-producing species from *A.* section *Nigri* are considered the source of OTA detected in grapes, dried vine fruits and wines. Other fungi present in grapes during their maturation can grow and interact with OTA-producing *Aspergillus* and affect OTA production. In this study seven fungi (*Alternaria alternata*, *Cladosporium herbarum*, *Eurotium amstelodami*, *Trichoderma harzianum*, *Penicillium decumbens, P. janhinellum* and *Candida* sp.) isolated from grapes and dried vine fruits were grown in SNM medium paired with OTA-positive *A. carbonarius* at two temperatures (20 and 30ºC) and at two water activities (0.92 and 0.97). OTA production was tested after 5, 7, 10, 14 and 18 days of incubation, at four distances (1, 2, 3 and 4 cm) from *A. carbonarius* inoculation point in the inter-colony axis. At 0.92 a_w OTA production was almost negligible. At 0.97 a_w and 30ºC OTA accumulation was reduced when *A. carbonarius* was grown in paired cultures, particularly with *A. alternata*, *C. herbarum*, *P. decumbens* and *P. janthinellum*. At 0.97 a_w and 20 ºC, there was not a clear effect of the interacting species on OTA accumulation; in general *E. amstelodami* and *Candida* sp. seemed to stimulate OTA production, while *T. harzianum* and *P. decumbens* reduced it. Competing Mycoflora acted as an additional control factor against OTA accumulation at 30ºC; 20ºC, where OTA production is optima, this did not happen. Thus keeping grapes at or above 30ºC during dehydration appears to be a good preventive control method against OTA accumulation in grapes.

Publication: *International Journal of Food Microbiology* **110** (2006) 160-164.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by fungal species belonging to the genera *Penicillium* and *Aspergillus* section *Circumdati* and *Nigri*. OTA is a potent nephrotoxin, and it is considered as a cause of human disease Balkan Endemic Nephropathy (Marquardt and Frohlich, 1992; Pleština *et al.*, 1996). In 1993, the International Agency for Research on Cancer classified OTA as a possible human carcinogen (group 2B) (IARC, 1993).

OTA has been widely detected in food products and beverages such as coffee (Pardo *et al.*, 2004), grapes (Abrunhosa *et al.*, 2001), grape juices, musts and wines (Bellí *et al.*, 2002; Cabañes *et al.*, 2002; Soufleros, 2003) and dried vine fruits (Ostry *et al.*, 2002; MacDonald *et al.*, 1999). Among wines, sweet wines made with sun-dried grapes, and red wines, have the highest OTA contents, from 0.04 to 7.60 µg l^{-1} (Battilani and Pietri, 2002; Bellí *et al.*, 2002). OTA levels above 53 µg kg^{-1} have been reported in dried vine fruits (MacDonald *et al.*, 1999).

Currently, several countries have specific regulations for OTA in various commodities, 10 ng OTA g^{-1} being the maximum level allowed for dried vine fruits in European Community, (EC) 472/2002 and 2 ng OTA g^{-1} for wine, grape must and grapes (EC) 123/2005.

OTA found in wines, grapes and dried vine fruits comes from OTA-producing strains from the group *A.* section *Nigri* (*A. niger* aggregate and mainly *A. carbonarius*) (Abarca *et al.*, 2003; Bellí *et al.*, 2004b) with occurrences that range from 33 to 100% in raisins (El Halouat and Debevere, 1997; Magnoli *et al.*, 2004).

Other fungi that have frequently been isolated in grapes and derivatives are species belonging to the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Fusarium* and *Rhizopus* (Abrunhosa *et al.*, 2001; Magnoli *et al.*, 2003 and 2004; Bellí *et al.*, 2003).

Previous ecophysiological studies (Bellí *et al.*, 2004a and 2004b; Mitchell *et al.*, 2004) in *A.* section *Nigri* species have shown the effect of water activity and temperature on their growth and OTA production, but effects produced by interacting fungi have not been considered.

In this study we aimed to understand the effect of fungal interactions at different environmental conditions in OTA production by *A. carbonarius*.

MATERIAL AND METHODS

Isolates and media

All microorganisms used in this study were isolated from grapes and vine dried fruits collected from the south of Spain and raisins bought in 2003, and they represent the most common species found in such foodstuffs. Selected fungi were *A. carbonarius* OTA producer, *Alternaria alternata*, *Cladosporium herbarum*, *Eurotium amstelodami*, *Trichoderma harzianum*, *Penicillium decumbens, P. janthinellum* and *Candida* sp. All of them belong to the collection of the Food Technology Department (University of Lleida).

The growing medium used for the ecophysiological assay was a synthetic nutrient medium (SNM) similar to grape composition between veraison and ripeness (Delfini, 1982). In order to adjust the medium to the different water activities required, it was modified adding glycerol and varying its sugars content (Valero *et al*., 2005).

Interactions effect on OTA production

The capability to produce OTA by *A. carbonarius* was tested through HPLC following the extraction and clean-up protocol detailed in Bragulat *et al*. (2001) with some modifications (d.l.=0.5 ng OTA/g SNM).

Single cultures of *A. carbonarius*, as controls, were grown in SNM medium at two water activities (0.92 and 0.97) and at two temperatures (20 and 30 ºC). Radii of colonies were recorded daily and OTA production was tested after 4, 7, 10, 14 and 18 days of incubation at 1, 2, 3 and 4 cm from the inoculation point.

Pairs consisted of *A. carbonarius versus A. alternata*, *C. herbarum*, *E. amstelodami*, *T. harzianum P. decumbens, P. janthinellum* or *Candida* sp. Inoculation points were placed, by needle single point, separated at a distance of 45 mm in every plate. Where conditions would lead to slow growth they were separated by 30 mm. Cultures were grown for 18 days at two water activities (0.92 and 0.97) and two temperatures (20 and 30 ºC). Diameters were recorded daily and OTA production was tested after 4, 7, 10, 14 and 18 days of incubation, at four distances along inter-colony axis (1, 2, 3 and 4 cm) from *A. carbonarius* inoculum point. All treatments were carried out in duplicate

Statistical analysis

The experiment followed a full factorial design, in which the assayed factors were a_w, temperature, incubation time and interacting species. Their effects in ochratoxin A production by *A. carbonarius* were statistically analyzed with SAS program (SAS Institute Inc., Cary, NC, USA) through an ANOVA. Significance was included at the P<0.05 level and Duncan’s multiple range test was performed. The Pearson´s correlation coefficients were also calculated between OTA production and the radii of *A. carbonarius* colonies grown in pairs and in single culture.

RESULTS

At 0.92 a_w and 30ºC no significant amount of OTA was detected in any of the treatments. At 0.92 a_w and 20 ºC significant amounts of OTA were only produced after 18 days but the production was prevented by *E. amstelodami* and *P. decumbens* (Table 1). At 0.97 a_w and 30ºC OTA contents were not significantly modified by the presence of interacting fungi till the 7th day being *A. alternata*, *C. herbarum*, *P. decumbens* and *P. janthinellum* the most inhibitory; from 10th day all species significantly inhibited OTA accumulation by *A. carbonarius* (Fig. 1 and 2). At 0.97 a_w

and 20 ºC, there was not a clear effect of the interacting species on OTA accumulation; in general *E. amstelodami* and *Candida* sp. seemed to stimulate OTA production while *T. harzianum* and *P. decumbens* to reduce it (Fig. 1 and 2).

Table 1. Ochratoxin A production (mean of four agar plugs from two replicates) by *A. carbonarius* grown in paired cultures. Mean values with the same letter are not significantly different according to Duncan test.

		Water activity and temperature of incubation			
Day	**Pairs**	**0.92 a_w 20 ºC**	**0.92 a_w 30 ºC**	**0.97 a_w 20 ºC**	**0.97 a_w 30 ºC**
4	Control	< d.l.	< d.l.	< d.l.	9.8^{abc}
	A alternata	n.t.	< d.l.	< d.l.	3.9^{bc}
	C. herbarum	< d.l.	n.t.	< d.l.	< d.l.^{c}
	Candida sp.	n.t.	n.t.	< d.l.	6.2^{abc}
	E. amstelodami	< d.l.	< d.l.	< d.l.	32.7^{a}
	P. decumbens	< d.l.	< d.l.	< d.l.	30.0^{ab}
	P. janthinellum	n.t.	< d.l.	< d.l.	5.6^{abc}
	T. harzianum	n.t.	n.t.	< d.l.	24.1^{abc}
7	Control	< d.l.	< d.l.	52.80^{bc}	174.1^{a}
	A alternata	n.t.	< d.l.	8.9^{c}	4.6^{c}
	C. herbarum	< d.l.	n.t.	13.1^{c}	14.3^{c}
	Candida sp.	n.t.	n.t.	148.3^{ab}	97.6^{b}
	E. amstelodami	< d.l.	< d.l.	73.6^{bc}	172.3^{a}
	P. decumbens	< d.l.	< d.l.	161.5^{a}	17.6^{c}
	P. janthinellum	n.t.	< d.l.	21.7^{c}	23.3^{c}
	T. harzianum	n.t.	n.t.	92.1^{abc}	156.9^{ab}
10	Control	< d.l.	< d.l.	1330.0^{ab}	201.8^{a}
	A alternata	n.t.	< d.l.	1507.7^{ab}	11.4^{b}
	C. herbarum	< d.l.	n.t.	481.7^{b}	28.1^{b}
	Candida sp.	n.t.	n.t.	1038.8^{ab}	53.0^{b}
	E. amstelodami	< d.l.	< d.l.	2325.1^{a}	52.8^{b}
	P. decumbens	< d.l.	< d.l.	1272.8^{ab}	14.8^{b}
	P. janthinellum	n.t.	< d.l.	1427.6^{ab}	39.2^{b}
	T. harzianum	n.t.	n.t.	1620.0^{ab}	81.5^{b}
14	Control	3.3^{a}	< d.l.	2084.5^{bc}	105.3^{a}
	A alternata	n.t.	< d.l.	2049.3^{bc}	1.4^{b}
	C. herbarum	< d.l.	n.t.	1827.8^{c}	7.3^{b}
	Candida sp.	n.t.	n.t.	3004.5^{a}	10.3^{b}
	E. amstelodami	< d.l.	2.4^{a}	2404.1^{b}	38.2^{b}
	P. decumbens	< d.l.	< d.l.	1239.6^{d}	2.2^{b}
	P. janthinellum	n.t.	2.3^{a}	1597.0^{cd}	19.6^{b}
	T. harzianum	n.t.	n.t.	223.9^{e}	40.5^{b}
18	Control	37.1^{a}	< d.l.	2225.1^{cd}	99.2^{a}
	A alternata	n.t.	< d.l.	3154.6^{bc}	3.3^{a}
	C. herbarum	16.9^{ab}	n.t.	1619.8^{d}	4.6^{a}
	Candida sp.	n.t.	n.t.	3850.9^{b}	7.3^{a}
	E. amstelodami	2.1^{b}	1.7^{a}	6820.6^{a}	15.0^{a}
	P. decumbens	6.8^{b}	3.6^{a}	1469.7^{de}	< d.l.
	P. janthinellum	n.t.	9.8^{a}	1138.8^{de}	3.5^{a}
	T. harzianum	n.t.	n.t.	341.6^{e}	27.8^{a}

n.t.; not tested, d.l.; detection limit.

Sometimes, compared to pure cultures, OTA was detected at shorter distances from the *A. carbonarius* inoculation point when paired with *Penicillium* species (Figures 1 and 2). When comparing the extension of *A. carbonarius* colonies with the extension of OTA production, it was observed that generally OTA was detected further on when paired with *T. harzianum*, *E amstelodami*, *Candida* sp. and *C. herbarum* even in the absence of *A. carbonarius* mycelium (Fig. 1 and 2).

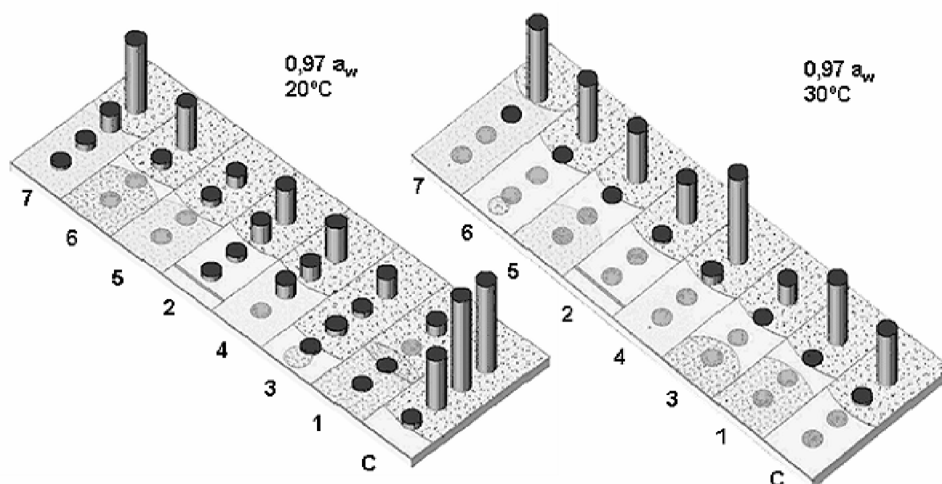


Figure 1. OTA production at four distances by *A. carbonarius* represented in bars at 0.97 a_w the tenth day of incubation.
C; *A. carbonarius* pure culture, 1; *A. alternata*, 2; *Candida* sp., 3; *C. herbarum*, 4; *E. amstelodami*, 5; *P. decumbens*, 6; *P. janthinellum*, 7; *T. harzianum*.
Maximum OTA contents: 8470,1 ppb at 20ºC (*versus E. amstelodami*, 1 cm), 313,7 ppb at 30ºC (Control, 2 cm). ○ ; OTA < d.l.,● ; OTA > d.l.

Pearson correlation coefficients were significant between OTA accumulation and *A. carbonarius* colony radius, both grown in single and paired culture, at 20ºC and 0.97 a_w (Table 2), with exception of when paired with *T. harzianum*, where no correlation was found. At 30ºC and 0.97 a_w a significant correlation was only found in pure culture and when paired with *P. decumbens*. No correlation was found at 0.92 a_w.

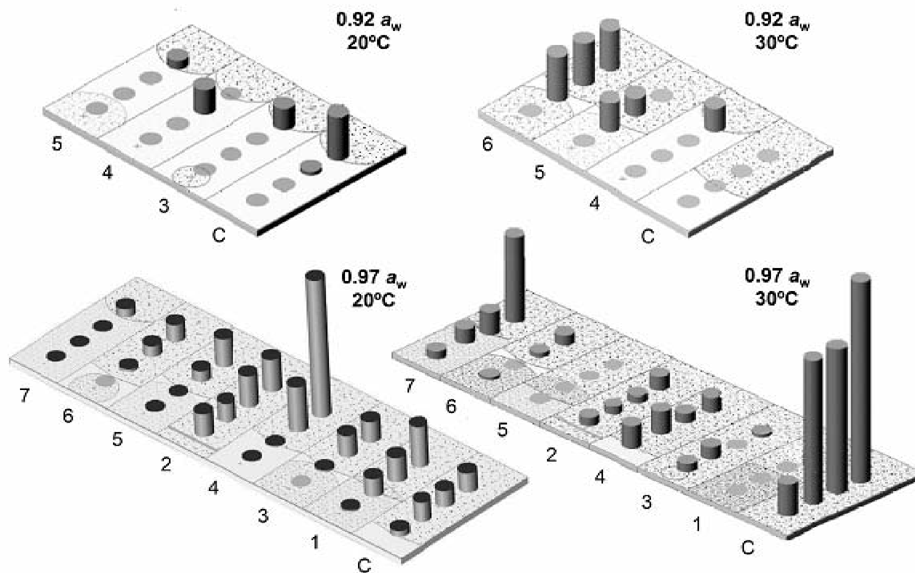


Figure 2. OTA production at four distances by *A. carbonarius* represented in bars at different conditions the 18th day of incubation. Comparison with fungi growth in SNM.

C; *A. carbonarius* pure culture, 1; *A. alternata*, 2; *Candida* sp., 3; *C. herbarum*, 4; *E. amstelodami*, 5; *P. decumbens*, 6; *P. janthinellum*, 7; *T. harzianum*.

Maximum OTA contents: 136,6 ppb at 20ºC and 0,92 a_w (Control, 1 cm), 13,7 ppb at 30ºC and 0,92 a_w (*versus P. janthinellum*, 3 cm), 20355,5 ppb at 20ºC and 0,97 a_w (*versus E. amstelodami*, 1 cm), 175,3 ppb at 30ºC and 0,97 a_w (Control, 1 cm). ○; OTA < d.l., ●; OTA > d.l.

DISCUSSION

This is the first *in vitro* study developed where biotic effects were considered in OTA production by *A. carbonarius*, using SNM medium. Inter-specific competence that occurs during grape ripening and sun-drying may affect the total OTA content in the final products.

At 0.97 a_w and 30ºC OTA accumulation was reduced when *A. carbonarius* was grown in paired cultures. This reduction may be due to: i) Limitation of *A. carbonarius* growth, what would generally lead into a reduction in OTA production.

Table 2. Pearson Correlation Coefficients between OTA accumulation and colony radii of *A. carbonarius*, grown in pairs and in single culture, at different temperatures and a_w.

	Water activity and temperature of incubation			
Pairs	**0.92 a_w 20 °C**	**0.92 a_w 30 °C**	**0.97 a_w 20 °C**	**0.97 a_w 30 °C**
Control	0.863**	-	0.945**	0.543*
A alternata	n.g.	n.g.	0.926**	-0.113
C. herbarum	0.700	n.g.	0.883**	0.344
***Candida* sp.**	n.g.	n.g.	0.953**	-0.132
E. amstelodami	0.529	0.402	0.786**	-0.271
P. decumbens	0.543	0.480	0.937**	-0.732*
P. janthinellum	n.g.	0.564	0.825**	0.360
T. harzianum	n.g.	n.g.	0.436	0.248

*; Correlation significant (*P*<0.05), ** Correlation significant (*P*<0.01), n.g.; not grown, -; lack of OTA production.

In such case correlation coefficient between growth and OTA accumulation would be positive and significant. This may have occurred for *P. janthinellum* and *T. harzianum*. ii) Antagonistic fungi consume specific nutrients necessary to synthesize OTA. iii) OTA degradation by the other fungi, since previous publications regard species of the genera *Alternaria*, *Cladosporium* and *Trichoderma* as capable to degrade OTA, probably by means of the carboxypeptidase A activity (Abrunhosa *et al*., 2002). In the last two cases, no significant correlation would be observed between growth and OTA accumulation, or coefficients would be negatives, as occurs with *A. alternata*, *Candida* sp. and *P. decumbens*. Seeing that none of them reduced *A. carbonarius* growth, and even increases it (Valero *et al*., submitted), we could assume that there is OTA degradation by interacting fungi, at least what concerns to *A. alternata*, since previous publications have found this ability in one *Alternaria* sp. isolate (Abrunhosa *et al*., 2002). iv) Interacting fungi could even excrete substances that arrive by diffusion to *A. carbonarius* colony and to block OTA synthesis. This hypothesis could be supported by a study made by Barr (1976) who showed that some volatile compounds produced by different bacterium had normally an inhibitory effect in mycotoxin production by an *Aspergillus* sp. isolate.

At 0.97 a_w and 20ºC, where the effect of the fungal interaction was not clear, all correlation coefficients were positive and significant meaning that *A. carbonarius* radius was closely linked to OTA production. There were significantly higher OTA concentrations with *Candida* sp. and *E. amstelodami*. *Candida* sp. also stimulated *A. carbonarius* growth while *E. amstelodami* did not. This last fungus has been previously reported as stimulator of OTA production by *A. ochraceus* when both them were grown in paired cultures (Lee and Magan, 2000). In contrast, OTA was reduced by *T. harzianum* but not as much as *A. carbonarius* growth. The latest could grow under

T. harzianum colony and produce OTA, what would explain why OTA was detected at further distance than 30 mm from *Aspergillus* colony edge.

At 0.92 a_w, OTA production was greatly reduced, thus the effect of fungal interactions on OTA production was negligible.

Given that at 0.92 a_w OTA production is slight, and that 0.97 a_w and 30ºC are not good conditions for OTA production, plus the OTA reduction by fungal interactions, these conditions do not imply a great risk for the final product regarding OTA content. It is at 0.97 a_w and 20ºC, where OTA production is important and fungal interactions do not exert any control, where preventive measures must be taken to prevent OTA accumulation. A good advice would be to avoid temperatures around 20ºC during grape dehydration by means of artificial dryers, keeping grapes at constant temperatures above 30ºC instead of sun-drying. Field conditions during grape maturation are, however, conducive to OTA accumulation.

Further studies could tackle the topic of OTA degradation by different microorganisms isolated from grapes, dried vine fruits or used in wine fermentation, with the aim of developing corrective techniques that reduce OTA accumulation as much as possible.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, projects AGL 2004-07549-05-01 and grant BES-2003-2180) for its financial support.

REFERENCES

Abarca, M.J., Accensi, F., Bragulat, M.R., Castella, G. and Cabañes, F.J., 2003. Contamination in dried vine fruits from the spanish market. Journal of Food Protection 66, 504-506.

Abrunhosa, L., Paterson, R.R.M., Kozakiewicz, Z., Lima, N. and Venâncio, A., 2001. Mycotoxin production from fungi isolated from grapes. Letters in Applied Microbiology 32, 240-242.

Abrunhosa, L., Serra, R., Venâncio, A., 2002. Biodegradation of Ochratoxin A by Fungi isolated from grapes. Journal of Agricultural and Food Chemistry 50, 7493-7496.

Barr, J.G., 1976. Effects of volatile bacterial metabolites on the growth, sporulation and mycotoxin production of fungi. Journal of the Science of Food and Agriculture 27, 324-330.

Battilani, P. and Pietri, A., 2002. Ochratoxin A in grapes and wine. European Journal of Plant Pathology 108, 639-643.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J., 2002. Review: Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. Food Science Technology International 8, 325-335.

Bellí, N., Pardo, E., Marín, S., Farré, G., Ramos, A.J. and Sanchis, V., 2003. Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. Journal of the Science of Food and Agriculture 84, 541-546.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J., 2004a. Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. International Journal of Food Microbiology 96, 19-27.

Bellí, N. Ramos, A.J., Sanchis, V. and Marín, S., 2004b. Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. Letters in Applied Microbiology 38, 72-77.

Bragulat, M.R., Abarca, M.L. and Cabañes, F.J. (2001) An easy screening method for fungi producing ochratoxin A in pure culture. International Journal of Food Microbiology 71, 139-144.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S. and Pons, A., 2002. What is the source of ochratoxin A in wine?. International Journal of Food Microbiology 79, 213-215.

Delfini, C., 1982. In Tecnica di microbiologia eonological ed. Scicalpi, L. Rome.

El Halouat, A. and Debevere, J.M., 1997. Molds and east isolated from hydrated prunes and raisins having different water activities. Science of Aliments 17, 539-545.

EC n. 472/2002 Reglamento de la Comisión. Diario Oficial de la Comunidad Europea 12 marzo 2002, L 75/18.

EC n. 123/2005 Reglamento de la Comisión. Diario Oficial de la Comunidad Europea 26 enero 2005, L 25/3.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Vol. 56, International Agency for Research on Cancer, Lyon, 1993, pp. 489-521.

Lee, H.B. and Magan, N. 2000. Environment factors influence in vitro interspecific interactions between *A. ochraceus* and other maize spoilage fungi, growth and ochratoxin production. Mycopathologia 146, 43-47.

MacDonald, S., Wilson, P., Barnes, K., Damant, A., Massey, R., Mortby, E. A and Shepherd, M.J., 1999. Ochratoxin A in dried vine fruit: method development and survey. Food Additives and Contaminants 16, 253-260.

Magnoli, C., Violante, M., Combina, M., Palacio, G. and Dalcero, A., 2003. Mycoflora and ochratoxin–producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. Letters in Applied Microbiology 37, 179-184.

Magnoli, C., Astoreca, A., Ponsone, L., Combina, M., Palacio, G., Rosa, C.A.R. and Dalcero, A.M., 2004. Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. Letters in Applied Microbiology 39, 326-331.

Marquardt, R.R. and Frolich, A.A., 1992. A review of recent advances in understanding ochratoxicosis. Journal of Animal Science 70, 3968-3988.

Mitchell, D., Parra, R., Aldred, D. and Magan, N., 2004. Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. Journal of Applied Microbiology 97, 439-445.

Ostry, V., Ruprich, J. and Skarkova, J., 2002. Raisins, ochratoxin A and human health. Mycotoxin Research 18A, 178-182.

Pardo, E., Marín, S., Ramos, A.J. and Sanchis, V., 2004. Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different origins. Food Science Technology International 10, 45-49.

Pleština, R., 1996. Nephrotoxicity of ochratoxin A. Food Additives and Contaminants 13, 49-50.

Soufleros, E.H., Tricard, C. and Bouloumpasi, E.C., 2003. Occurrence of ochratoxin A in Greek wines. Science of Food and Agriculture 83, 173-179.

Valero, A., Farré, J.R., Sanchis, V., Ramos, A.J., Marín, S. Understanding the dominance of *Aspergillus* section *Nigri* in mycobiota succession in grapes and sun-dried grapes. Food Microbiology (submitted).

Valero, A., Marín, S., Ramos, A.J., Sanchis, V., 2005. Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors. Letters in Applied Microbiology 41, 196-201.

Study VI:

Effect of germicidal UVC light on fungi isolated from grapes and raisins

Ana Valero[1], Mariam Begum[2], Su-lin L. Leong[2], Ailsa D. Hocking[2], Antonio J. Ramos[1], Vicente Sanchis[1], Sonia Marín[1]

[1]Food Technology Department, University of Lleida, UTPV-CeRTA. Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain
[2]CSIRO Food Science Australia, PO Box 52, North Ryde, NSW 1670, Australia

ABSTRACT

Aims: To examine how UVC affect the different genera of fungi commonly isolated from grapes, with the aim of understanding changes in mycobiota during grape ripening and possible applications for preventing grape decay during storage.

Methods and Results: Spores of *Aspergillus carbonarius*, *A. niger*, *Cladosporium herbarum*, *Penicillium janthinellum* and *Alternaria alternata* (between 100-250 spores / plate agar) were UVC irradiated for 0 (control), 10, 20, 30, 60, 300 and 600 seconds. Plates were incubated at 25ºC and colonies were counted daily up to 7 days. *A. alternata* and *A. carbonarius* were the most resistant fungi. Conidial germination in these species was reduced by approx. 25 % after 10 s of exposure, compared with greater than 70% reduction for the remaining species tested. *P. janthinellum* spores were the most susceptible at this wavelength. UVC exposures of 300 s prevented growth of all isolates studied, except for *A. alternata*.

Conclusions: UVC irradiation plays a major role in selecting for particular fungi that dominate the mycobiota of drying grapes

Significance and Impact of Study: The UVC irradiation of harvested grapes could prevent germination of contaminant fungi during storage or further dehydration.

Publication: *Letters in Applied Microbiology* (in press).

INTRODUCTION

Grapes are susceptible to insect attack and to fungal diseases, especially grey rot, downy mildew and black rot. Damaged grapes are vulnerable to further diseases such as summer bunch rot, which may be caused by *Aspergillus niger*, *Alternaria tenuis*, *Cladosporium herbarum*, *Rhizopus arrhizus*, *Penicillium* spp. and other fungi. Fungal invasion depends on grape maturity. *Alternaria*, *Cladosporium*, *Botrytis* and *Rhizopus* are common at early veraison, whereas *Aspergillus* and *Penicillium* are more frequently found at harvest and during sun drying (Magnoli *et al*. 2003; Bellí *et al*. 2004; Valero *et al*. 2005). *Aspergillus carbonarius* and *A. niger* which are know to produce ochratoxin A (OTA) in grapes and raisins (Abarca *et al*. 2003; Bellí *et al*. 2004).

Changes in the mycobiota of grapes are affected by weather, solar irradiation, rising temperatures, and decreases in the water activity of berries due to increasing berry sugar content. Grapes before harvest, and especially during drying, are exposed for long periods to sunlight. Ultraviolet radiation from the sun is divided into three wavebands, UVA, UVB and UVC.

Ultraviolet radiation has been known for many years to affect microorganisms. UVC (100- 280 nm) is highly germicidal and is commonly used for sterilisation of surfaces, water and air, however, quantification of these effects has been difficult. Levetin *et al*. (2001) reported that UVC radiation applied to air-conditioning systems significantly reduced the incidence of *Cladosporium* spp. and *Aspergillus versicolor*. Green *et al*. (2004) reported 35 and 54 mJ cm^{-2} (UV dose (mJ cm^{-2}) = Irradiance (μW cm^{-2}) · Irradiation time (s)·10^{-3}) as the doses of ultraviolet germicidal irradiation (225-302 nm) necessary to inactivate 90% of the spores of *Aspergillus flavus* and *A. fumigatus*, respectively. Jun *et al*. (2003) modelled the effect of pulsed UV-light on the inactivation of *Aspergillus niger* spores in corn meal and reported that for a 100 seconds treatment time, 3 cm of distance from the UV strobe, and with 3800 V input gave a 4·9 $\log_{10}$ reduction of *A. niger*. In contrast, Nigro *et al*. (1998) found that irradiating grape berries with UVC light had no effect on filamentous fungi and even increased the incidence of yeasts and bacteria.

The effect of solar radiation on some genera of airborne fungi, such as *Cladosporium*, *Penicillium*, *Alternaria*, *Epicoccum* or *Aspergillus* among others, was studied by Ulevičius *et al*. (2004), who found that *A. niger* was the species most commonly isolated after treatment. Given these findings, we examined how the most energetic wavelength from sunlight, ultraviolet C, could affect the different genera of fungi commonly isolated from grapes, with the aim of understanding changes in mycobiota during grape ripening and possible applications for preventing grape decay during storage.

MATERIALS AND METHODS

Fungi used in this study were isolated from grapes and dried vine fruits collected from Australia and Spain: *Aspergillus carbonarius* (UdLTA 3.122) capable of OTA production, *Cladosporium*

herbarum (UdLTA 3.129), *Penicillium janthinellum* (UdLTA 3.126), *A. niger* (FRR 5694) OTA positive, and *Alternaria alternata* (FRR 4780). UdLTA strains were from the culture collection of the Food Technology Department, Lleida University, Spain, and FRR strains were from the culture collection at CSIRO Food Science Australia, North Ryde, NSW Australia.

For spore production, each species was inoculated onto Malt Extract Agar (MEA; Raper and Thom 1949) and onto Dichloran Chloramphenicol Malt Agar for *A. alternata* (DCMA; Andrews 1992) and incubated for 7 days at 25 °C. Spores were harvested into aqueous Tween 80 (0·5%), quantified using a haemocytometer and diluted to give a final suspension of 10^3 spores ml^{-1}.

The growth medium was Synthetic Nutrient Medium (SNM), chosen because its composition is similar to that of grapes between veraison and ripeness (Delfini 1982), adjusted to 0.97 a_w (Valero *et al*. 2005). Aliquots (0.1 ml) of each 10^3 spore suspension were inoculated onto SNM by spreading uniformly on the agar surface and afterwards irradiated with UVC (254 nm), for 0 (control), 10, 20, 30, 60, 300 and 600 seconds. Plates without the lids were set upside at 10 cm from the lamp (G20T10 Sankyo Denki, Co., Ltd, Tokyo, Japan) in a closed chamber previously sterilised. Nominal lamp power was 19 Watts (W), and 75·8 μW cm^{-2} of irradiance (at 1 m of distance). Each combination of species and irradiation time had four replicates. Plates were incubated at 25 °C for 7 days and colonies counted daily up to 7 days.

Survival fractions (S) of the five microorganisms were plotted against UVC irradiation time and an equation describing the one stage exponential decay curve:

$$S = \exp(-k^*t)$$

where k = decay rate (s^{-1}); t = irradiation time

was generated using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). Covariance analysis of germination percentages *vs* time and related Duncan’s multiple range tests were performed using SAS (SAS Institute Inc., Cary, NC, USA).

RESULTS

A. alternata and *A. carbonarius* were the most resistant fungi when irradiated with UVC for 10 or 20 s (Fig. 1). Conidial germination in these species was reduced by approx. 25 % after 10 s of exposure, compared with greater than 70% reduction for the remaining species tested. For UVC exposures longer than 20 s, *A. alternata* was the only species capable of growing after 300 and 600 s exposure to UVC light. In contrast, *P. janthinellum* spores were the most susceptible at this wavelength, with total conidial inactivation after 30 s of exposure. UVC exposure of 60 s reduced survival of all fungi tested by more than 90 %.

The exponential decay of the survival fractions was modelled in order to generate a single equation describing the UVC resistance for each isolate against exposure time (Fig. 2). More resistant fungi showed lower decay rates (*k*).

UVC irradiation at a distance of 10 cm for 30 s prevented *Aspergillus* growth for 3 days, and UVC exposures of 300 s prevented growth of all isolates studied, apart from *A. alternata*, for longer than 7 days (Table 1).

The capacity to repair damaged spores was affected by UVC irradiation in all species tested, as longer incubation times were required for all viable spores to germinate when irradiated with higher cumulative UVC dosages. In fact all viable not irradiated

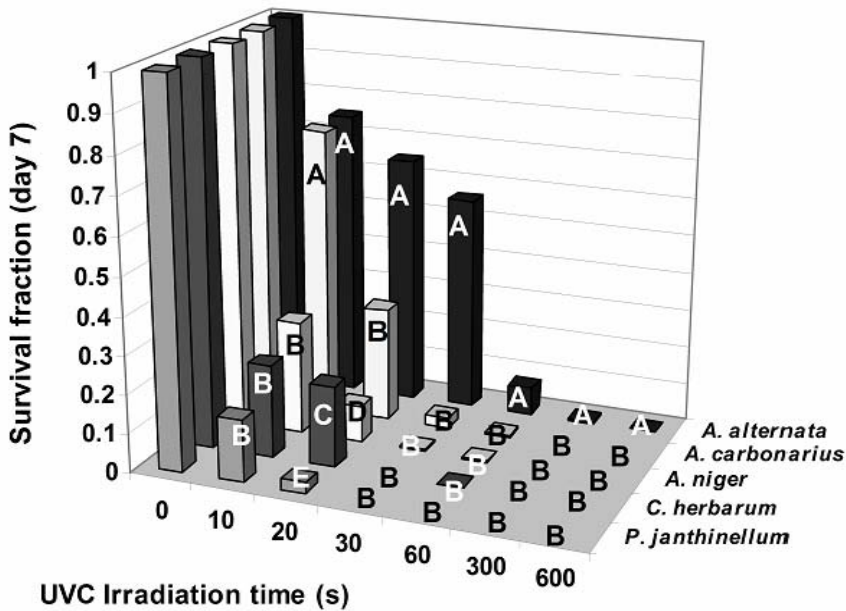
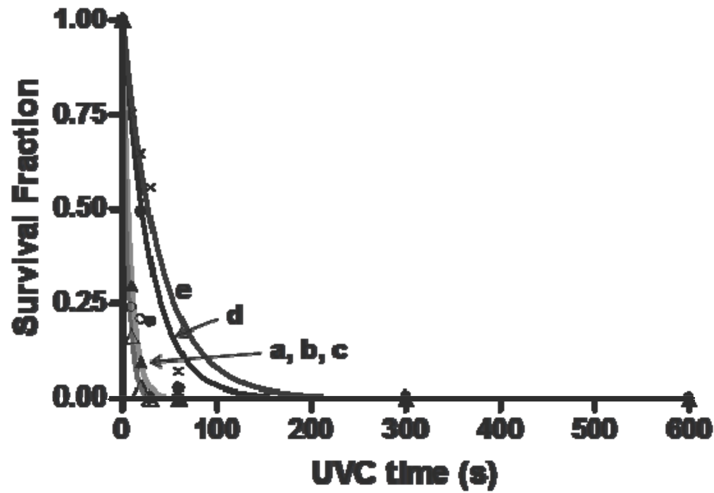


Fig. 1 Survival fraction of the five isolates (*Alternaria alternata*, *Aspergillus carbonarius*, *A. niger*, *Cladosporium herbarum* and *Penicillium janthinellum*) after incubation for 7 days (average of four replicates). For comparison of species within each time point, bars with different letters are significantly different ($P < 0.05$).



	Alternaria alternata	*Aspergillus carbonarius*	*Aspergillus niger*	*Cladosporium herbarum*	*Penicillium janthinellum*
k	0.0251	0.0340	0.1216	0.1195	0.1815
SE	0.0022	0.0056	0.0085	0.0090	0.0106
R^2	0.910	0.805	0.971	0.964	0.989

Fig. 2 Models for single stage exponential decay curves of five isolates as influenced by UVC irradiation. Exponential decay rates for each curve are detailed in table: *Survival fraction = exp (-k*t)*. *k*, decay constant (s^{-1}); t, UVC irradiation time (s). SE: standard error, R^2: regression coefficient. Isolates: a (Δ), *Penicillium janthinellum*; b (▲), *Aspergillus niger* ; c (o), *Cladosporium herbarum* ; d (●), *Aspergillus carbonarius* ; e (x), *Alternaria alternata*.

spores germinated between 2-3 days while the irradiated spores germinated differently, up to the 6th day, depending on the strains (Fig. 3). UVC irradiation for 10 s produced a significant reduction (P < 0.05) in germination of all fungi assayed, compared with germination of non-irradiated spores. Generally, subsequent exposures of 10 s led to successive significant reductions.

Table 1. Lag phase (days) of five isolates of fungi as influenced by duration of UVC irradiation.

	Duration of UVC irradiation (s)						
	0	10	20	30	60	300	600
Alternaria alternata	≤2	≤2	≤2	≤2	≤2	3	4
Aspergillus carbonarius	≤2	≤2	≤2	3	4	n.g.	n.g.
Aspergillus niger	≤2	≤2	≤2	4	3	n.g.	n.g.
Cladosporium herbarum	≤2	≤2	≤2	n.g.	5	n.g.	n.g.
Penicillium janthinellum	≤2	3	3	n.g.	n.g.	n.g.	n.g.

n.g., no growth

DISCUSSION

Integration of these findings on resistance to UVC with data on the effects of temperature and water activity on fungal growth and survival provides a good explanation of the incidence of fungi found on both fresh and sun dried grapes. *Alternaria* is the most common fungal genus found on grapes, followed by *Penicillium*, *Aspergillus*, *Epicoccum* and *Cladosporium* (Sage *et al.* 2002; Bellí *et al.* 2004; Valero *et al.* 2005). Leong *et al.* (2006) reported a decrease (10^5) in *A. carbonarius* spore viability on grapes after an exposure to UV irradiation equivalent to one week of high UV intensity with cloudless skies in Sydney, NSW Australia, a cumulative energy estimated in $3{\cdot}6\ 10^3$ Joules (10 mWh, milliwatts-hour). When grapes are dried in intense sunlight, the most commonly found fungi are *Aspergillus*, *Penicillium* and *Alternaria* (Romero *et al.* 2005; Valero *et al.* 2005). Likewise, Ulevičius *et al.* (2004) isolated fungal propagules from air on the Lithuanian coast at different hours during the day and found that *A. niger*, *A. alternata*, *Cladosporium* spp., *Arthrinium phaerosporum*, and dematiaceous sterile mycelium were prevalent after exposure to solar radiation.

Our data demonstrating the resistance of *A. alternata* to UVC agree with the findings of Rotem and Aust (1991), who noted that propagules of *Alternaria* sp. were the most resistant to ultraviolet radiation or sunlight, followed by *Mycosphaella* sp., *A. niger* and *Botrytis cinerea*. *A. alternata* has multi-celled spores with thick, melanised walls that are believed to confer tolerance to sunlight (Carzaniga *et al.* 2002). The photoprotective properties of melanin are believed to be important for the survival and longevity of fungal spores (Bell and Wheeler 1986), whereas non-melanin compounds are poor protectants against ultraviolet radiation (Durrell and Shields 1960). Grishkan et al. (2003) found a significant correlation between areas receiving high solar irradiation and the incidence of melanin-containing fungal species among soil microfungi isolated in Israel.

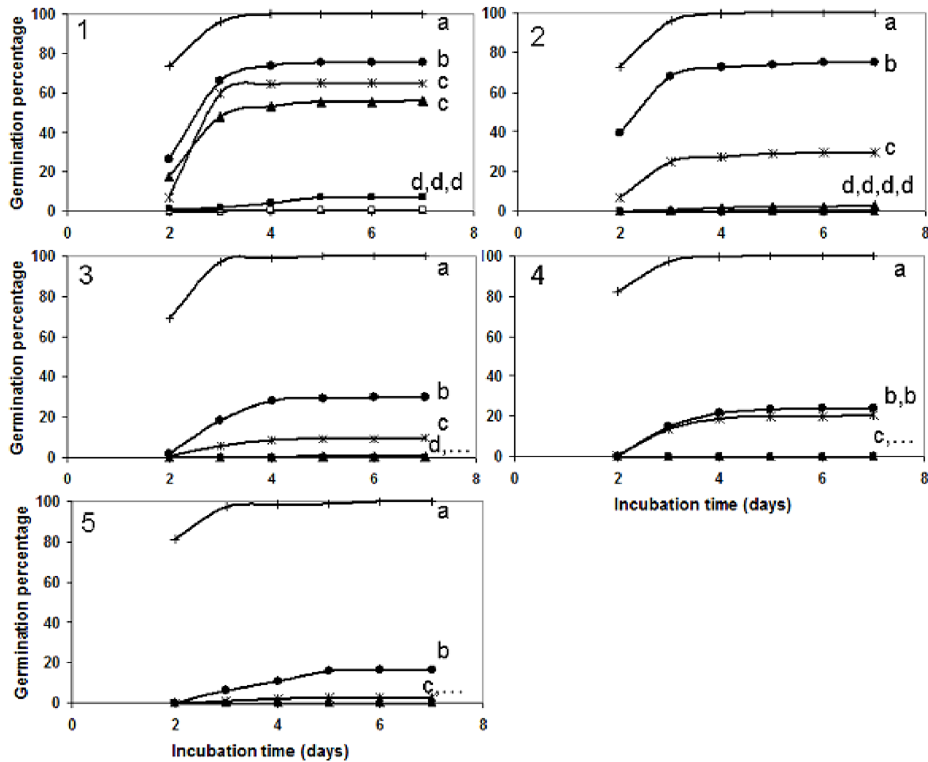


Fig. 3 Germination curves following radiation of different fungi genera. 1, *Alternaria alternata*; 2, *Aspergillus carbonarius*; 3, *Aspergillus niger*; 4, *Cladosporium herbarum*; 5, *Penicillilum janthinellum*. UVC irradiation times: +, 0 s; ●, 10 s; ⚹, 20 s; ▲, 30 s; ■, 60 s; □, 300 s; Δ, 600 s. Lower case letters denote final germination percentages that are significantly different ($P < 0.05$).

A. carbonarius and *A. niger* produce single-celled conidia with melanin and aspergilline in their cell walls (Duguay and Klironomos 2000; Babitskaya and Shcherba 2002), but differ in their UVC resistance and their incidence on grapes. *A. carbonarius* spores are thought to possess thicker walls than the 90-160 nm thick wall of *A. niger* (Tiedt 1993). The greater UVC resistance displayed by *A. carbonarius* spores than *A. niger* spores provides a logical explanation for the high numbers of *A. carbonarius* on grapes subjected to prolonged sun exposure. In a study of fungal contamination of grapes after sun drying, *Aspergillus* section *Nigri* spp. were found in more than 80% of dried grapes (Valero *et al.* 2005). *A. carbonarius* occurred in increased frequency compared with *A. niger,* on raisins and dried vine fruits (Leong *et al.* 2004; Romero *et al.* 2005; Valero *et al.* 2005; Gómez *et al.* 2006). This is somewhat surprising, as *A. niger* is more tolerant than *A. carbonarius* to low water activities (growth limits of 0·77 a_w and 0·87 a_w, respectively; Valero *et al.* 2007) and high temperatures (optimum growth at 35-40 °C and 30 °C,

respectively; Panasenko 1967; Palacios-Cabrera *et al*. 2005; Valero *et al*. 2007). Resistance to UVC provides a competitive advantage for *A. carbonarius* during grape drying that overrides the superior growth of *A. niger* in hot and dry conditions.

C. herbarum produces conidia with a melanin-like pigment (Margalith 1992). The thickness of its conidial wall is unknown, but the pigmented conidia allow *Cladosporium* to survive under high solar irradiation, which explains their commonly occurrence on grapes and raisins (Romero *et al*. 2005; Valero *et al*. 2005), even though its maximum growth temperature is approximately 32 ºC (Domsch *et al*. 1980; Valero *et al*. 2007).

Some *Penicillium* species are known to produce melanin-like compounds, however, there is a little information regarding *P. janthinellum* (Youngchim *et al*. 2004). This species produces single-celled slightly pigmented conidia and hyaline mycelium hence, has few protective mechanisms against UV radiation.

Both the spatial distribution of pigments and highly efficient DNA-repair mechanisms have a cumulative effect of ecological significance, conferring on those microorganisms an advantage in surviving on grape surfaces (Wynn-Williams *et al*. 2002; Ulevičius *et al*. 2004).

Our results show that UVC irradiation plays a major role in selecting for particular fungi that dominate the mycobiota of drying grapes. Temperature and water activity are also important. In addition, the UVC irradiation of harvested grapes could prevent germination of contaminant fungi during storage or further dehydration that may lead to production of undesired substances such as mycotoxins.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2004-07549-05-01, grant BES-2003-2180 and Ramón y Cajal program) and to the Catalonian Government (CeRTA strategic project 2005-2006 “Seguritat biòtica y abiòtica dels aliments”, Generalitat de Catalunya) for their financial support. We also thank the Mycology and Mycotoxins Section of Food Science Australia (CSIRO) for their financial support, collaboration and hospitality.

REFERENCES

Abarca, M.L., Accensi, F., Bragulat, M.R., Castella, G. and Cabañes, F. (2003) Contamination in dried vine fruits from the Spanish market. *J. Food Prot.* **66**, 504-506.

Andrews, S. (1992) Differentiation of *Alternaria* species isolated from cereals on dichloran malt extract agar. In *Modern Methods in Food Mycology* ed. Samson, R.A., Hocking, A.D., Pitt, J.I. and King, A.D. Amsterdam: Elsevier. pp. 61-65.

Babitskaya, V.G. and Shcherba, V.V. (2002) The nature of melanin pigments of several micro- and macromycetes. *Appl. Biochem. Microbiol.* **38**, 247-251.

Bell, A.A. and Wheeler, M.H. (1986) Biosynthesis and function of fungal melanins. *Ann. Rev. Phytopathol.* **24**, 411-451.

Bellí, N., Pardo, E., Marín, S., Farré, G., Ramos, A.J. and Sanchis, V. (2004) Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *J. Sci. Food Agri.* **84**, 541-546.

Carzaniga, R., Fiocco, D., Bowyer, P. and O'Connell, R.J. (2002) Localization of melanin in conidia of *Alternaria alternata* using phage display antibodies. *Mol. Plant-Microbe Interact.* **15**, 216-224.

Delfini, C. (1982) In *Tecnica di microbiologia enologica* ed. Scicalpi, L. Rome.

Domsch, K.H., Gams, W. and Anderson, T.H. (1980) Compendium of Soil Fungi. London: Academic Press.

Duguay, K.J. and Klironomos, J.N. (2000) Direct and indirect effects of enhanced UV-B radiation on the decomposing and competitive abilities of saprobic fungi. *Appl. Soil Ecol.* **14**, 157-164.

Durrell, L.D. and Shields, L.M. (1960) Fungi isolated in culture from soil of the Nevada test site. *Mycologia* **52**, 636-641.

Gómez, C., Bragulat, M.R., Abarca, M.L., Mínguez, S. and Cabañes, F.J. (2006) Ochratoxin-A producing fungi from grapes intended for liqueur wine production. *Food Microbiol.* **23**, 541-545.

Green, C.F., Scarpino, V.V., Jensen, P., Jensen, N.J. and Gibbs, S.G. (2004) Disinfection of selected *Aspergillus* spp. using ultraviolet germicidal irradiation. *Can. J. Microbiol.* **50**, 221-224.

Grishkan, I., Nevo, E., Wasser, S.P. and Beharav, A. (2003) Adaptative spatiotemporal distribution of soil microfungi in "Evolution Canyon" II, Lower Nahal Keziv, western Upper Galilee, Israel. *Biol. J. Linnean Soc.* **78**, 52-539.

Jun, S., Irudayaraj, J., Demirci, a. and Geiser, D. (2003) Pulsed UV-light treatment of corn meal for inactivation of *Aspergillus niger* spores. *Int. J. Food Sci. Technol.* **38**, 883-888.

Leong, S., Hocking, A.D. and Pitt, J.I. (2004) Occurrence of fruit rot fungi (*Aspergillus* section *Nigri*) on some drying varieties of irrigated grapes. *Aust. J. Grape Wine Res.* **10**, 83-88.

Leong, S., Hocking, A.D. and Scott, E.S. (2006) Survival and growth of *Aspergillus carbonarius* on wine grapes before harvest. *Int. J. Food Microbiol.* **111**, S83-S87.

Levetin, E., Shaughnessy, R., Rogers, C.A. and Scheir, R. (2001) Effectiveness of germicidal UV radiation for reducing fungal contamination within air-handling units. *Appl. Environ. Microbiol.* **67**, 3712-3715.

Magnoli, C., Violante, M., Combina, M., Palacio, G. and Dalcero, A. (2003) Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Lett. Appl. Microbiol.* **37**, 179-184.

Margalith, P.Z. (1992) *Pigmented Microbiology*. London: Chapman and Hall.

Nigro, F., Ippolito, A. and Lima, G. (1998) Use of UV-C light to reduce *Botrytis* storage rot of table grapes. *Postharvest Biol. Technol.* **13**, 171-181.

Palacios-Cabrera, H., Taniwaki, M.H., Hashimoto, J.M. and Menezes, H.C. (2005) Growth of *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* on culture media at different water activities and temperatures. *Braz. J. Microbiol.* **36**, 24-28.

Panasenko, V.T. (1967) Ecology of microfungi. *Botanical Review* **33**, 189-215.

Raper, K.B. and Thom, C. (1949) The Genus *Aspergillus*. Baltimore, Maryland: Williams and Wilkins.

Romero, S.M., Comerio, R.M., Larumbe, G., Ritieni, A., Vaamonde, G. and Fernández Pinto, V. (2005) Toxigenic fungi isolated from dried vine fruits in Argentina. *Int. J. Food Microbiol.* **104**, 43-49.

Rotem, J. and Aust, H.J. (1991) The effect of ultraviolet and solar radiation and temperature on survival of fungal propagules. *J. Phytopathol.* **133**, 76-84.

Sage, L. (2002) Fungal flora and ochratoxin A production in grapes and musts from France. *J. Agr. Food Chem.* **50**, 1306-1311.

Tiedt, L.R. (1993) An electron microscope study of conidiogenesis and wall formation of conidia of *Aspergillus niger*. *Mycol. Res.* **97**, 1459-1462.

Ulevičius, V., Pečiulytė, D., Lugauskas, A. and Andriejauskienė, J. (2004) Field study on changes in viability of airborne fungal propagules exposed to UV radiation. *Environ. Toxicol.* **19**, 437-441.

Valero, A., Marín, S., Ramos, A.J. and Sanchis, V. (2005) Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors. *Lett. Appl. Microbiol.* **41**, 196-201.

Valero, A., Sanchis, V., Ramos, A.J. and Marín, S. (2007) Studies on the interaction between grape-associated filamentous fungi on a synthetic medium. *Int. J. Food Microbiol* **113**, 271-276.

Wynn-Williams, D.D., Edvards, H.G.M., Newton, E.M. and Holder, J.M. (2002) Pigmentation as a survival strategy for ancient and modern photosynthetic microbes under high ultraviolet stress on planetary surfaces. *Int. J. Aerobiol.* **1**, 39-49.

Youngchim, S., Morris-Jones, R., Hay, R.J. and Hamilton, A.J. (2004) Production of melanin by *Aspergillus fumigatus*. *J. Med. Microbiol.* **53**, 175-181.

Study VII:

Effect of *intra* and interspecific interaction on OTA production by *A.* section *Nigri* in grapes during dehydration

Ana Valero, Sergio Oliván, Sonia Marín, Vicente Sanchis, Antonio J. Ramos

Food Technology Department, University of Lleida. CeRTA-UTPV, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

ABSTRACT

The aim of this study was to assess the colonising capability and OTA production of different populations of *A*. section *Nigri* spp. in grapes, as affected by the interactions with other fungi, during a simulated *in vitro* sun-drying. Mature white grapes were divided into two lots of healthy and artificially injured grapes and inoculated with *Aspergillus* section *Nigri* spp. (*A. carbonarius* OTA producer, *A. niger* aggregate OTA producer, *A. niger* aggregate OTA-non producer), *Eurotium amstelodami* and *Penicillium janthinellum*, in different combinations. The drying process was simulated adjusting water activity firstly at 0.98 a_w and gradually decreasing it to 0.76 a_w for a total of 20 days. Colonising grape percentages were recorded after 5, 10, 15 and 20 days of dehydration and OTA content was measured after 5, 7, 10, 12, 15, 17 and 20 days. Colonisation of grapes increased with time in all treatments. *A. niger* aggregate OTA-positive showed the highest colonisation percentage, followed by *A. carbonarius*, and finally their mixed inoculum. When the two OTA-producing strains were combined, addition of any other microorganism increased the percentage of infection by *A*. section *Nigri. A. carbonarius* was the highest OTA producer in pure culture, followed by *A. niger* aggregate OTA-positive. In general, when competing fungi were added to *A. carbonarius* inoculum, the OTA content was reduced. *E. amstelodami* was the only competing fungus which increased OTA accumulation.

The sun-drying process may be conducive to OTA accumulation in dried grapes. The complex fungal interactions which may take place during this process, may act as an additional control factor, given that the higher presence of *A. niger* aggregate OTA-negative inhibits OTA accumulation by OTA producing species.

Publication: *Food Microbiology* **24** (2007) 254-259.

INTRODUCTION

Ochratoxin A (OTA) is a very widely distributed toxin produced by species belonging to genera *Aspergillus* and *Penicillum*. In 1993, the International Agency for Research on Cancer classified OTA as a possible human carcinogen (group 2B) (IARC, 1993).

OTA has been detected in beverages as coffee, wine, grape juice (Bellí et al. 2002; Cabañes et al. 2002; Pardo et al. 2004) and foodstuffs like cereals, grapes and raisins (Abrunhosa et al. 2001; MacDonald et al. 1999; Ostry et al. 2002; Rafai et al. 2000). The highest OTA levels among grapes and their derivatives have been found in sweet wines (7.60 μg l^{-1}) and raisins (> 53 μg kg^{-1}) (Battilani and Pietri 2002; Bellí et al. 2002; MacDonald et al. 1999). OTA found in these products is produced by strains from the group *A.* section *Nigri* (*A. niger* aggregate and mostly *A. carbonarius*) (Abarca et al. 2003). *A. niger* aggregate groups biseriate strains of *A.* Section *Nigri,* excluding *A. carbonarius*, difficult to be identified at species level on based on their morphology (Cabañes et al., 2002).

Production of raisins and some sweet wines involves the dehydration of over-maturated grapes by means of different industrial or craft processing, depending on the area of origin. In some Mediterranean countries grapes are dried by sun exposure for periods of time that can last from 4-14 days. Environmental conditions required to achieve it, are very favourable for fungal growth, consequently species belonging to *Aspergillus* section *Nigri* and the genera *Eurotium* and *Penicillium* have been regarded as natural contaminants, with incidences in fresh and dehydrated grapes of about 80, 13 and 20% respectively (Magnoli et al. 2004; Valero et al. 2005). These conditions also enable OTA synthesis by *A.* section *Nigri* OTA-producing strains, which can be affected by the interacting mycoflora. In this work, we aimed to assess the colonising capability and OTA production of different strains of *A.* section *Nigri* spp. in grapes, as affected by the interactions with other fungi, during an *in vitro* sun-drying process.

MATERIAL AND METHODS

Design of the artificial drying process

Grapes used in this study were whole white table grapes var. Aledo, petiole included, externally sterilised through an immersion in Na-hypochloride (4%) (1 minute) and in ethanol (96%) (1 minute). Grapes were placed on racks inside lunchboxes previously surface-disinfected - firstly washed with 70% ethanol and secondly UV-C irradiated for 15 min.- and distributed in two lots; healthy and injured grapes (perforated with a scalpel). All grapes were incubated at 32ºC for nine hours under light conditions and 15 hours at 20ºC in the dark.

Different values of a_w (0.98, 0.92, 0.84 and 0.76 a_w) were selected to adjust saline solutions, on basis to water activities recorded in grapes at different dehydration grade by sun exposure (data not shown). A calibration curve was made to prepare all solutions containing NaCl as solute: (g

NaCl·100 ml^{-1} distilled water) = 151.19 – 150.76 · a_w (R^2 = 0.989). All solutions were sterilised by means of autoclaving at 121° C for 20 min. and stored at 5° C. Dehydration of grapes was helped by means of 200 ml of saline solution at the required a_w, poured inside the boxes and held for five days, in decreasing level. Thus the whole drying process lasted for 20 days.

Microorganisms and inoculation

Microorganisms used were *Aspergillus carbonarius* OTA-positive (3-122), *A. niger* aggregate sp. OTA-positive (3-155), *A. niger* aggregate OTA-negative (3-124), *Eurotium amstelodami* (3-132) and *Penicillium janthinellum* (3-126). The strains are kept in the Department of Food Technology, University of Lleida, Spain.

All microorganisms were isolated from grapes and dried vine fruits. The capability of OTA production by *A. carbonarius* and *A. niger* aggregate OTA-positive isolates was tested through HPLC (Waters, Milford, MA, USA) following the extraction and clean-up protocol detailed in Bragulat et al. (2001).

Inocula preparation and grape inoculation

Four inocula with *A. carbonarius* as OTA producer, in pure culture or combined with: (i) *A. niger* aggregate OTA-negative, (ii) *A. niger* aggregate OTA-negative and *E. amstelodami*, (iii) *A. niger* aggregate and *P. janthinellum*.

Four inocula with *A. niger* aggregate OTA-positive as OTA-producer, in pure culture or combined with: (i) *A. niger* aggregate OTA-negative, (ii) *A. niger* aggregate OTA-negative and *E. amstelodami*, (iii) *A. niger* aggregate OTA-negative and *P. janthinellum*.

Six inocula with both *A. carbonarius* and *A. niger* aggregate OTA-positive as OTA producers, alone or combined with: (i) *A. niger* aggregate OTA-negative, (ii) *E. amstelodami*, (iii) *P. janthinellum*, (iv) *A. niger* aggregate OTA-negative and *E. amstelodami*, (v) *A. niger* aggregate OTA-negative and *P. janthienllum*.

Pure and mixed spores suspensions at 10^6 ml^{-1} of distilled water (0.02 % Tween 80) were prepared from colonies of all isolates grown in Malt Extract Agar at 25ºC for seven days. Proportion within spores from different microorganisms in each inoculum were set in 1:1. Inoculation was performed by dropping 20 μl of the spore suspension on each grape, directly over stabbing when applicable.

All fungal combinations were inoculated in both healthy and injured grapes, with two replicates for each treatment.

Grape colonisation

Percentages of visually colonised grapes by *A.* section *Nigri*, were recorded after 5, 10, 15 and 20 days of grapes dehydration.

Analysis of OTA content

Samples (40 g) of grapes were collected, opening and closing boxes, the 5, 7, 10, 12, 15, 17 and 20th day of dehydration and were crushed and stored at -18ºC. OTA clean up and extraction was achieved following the protocol detailed in Pietri et al. (2001) with some modifications as follows: 30 g of each sample were minced and acidified to pH 2.4-2.6 using 85% orthophosphoric acid, then a liquid-liquid extraction was performed by adding 60 ml chloroform and stirred for 30 min. Phases were separated by centrifugation at 2500 rpm for 5 min. Extraction was repeated adding chloroform (20 ml). The two chloroform phases were combined and evaporated to dryness using a rotavapor at 35ºC; the residue was redissolved using ultrasonication with methanol (6 ml) and phosphate-buffered saline (44 ml) (PBS, Pietri et al. 2001). The final solution was filtered (Glass Fiber Binderless Filter, 1 µm, Osmonics Inc., Barcelona, Spain).

A C_{18} silicagel column (Bond Elut, Varian. Palo Alto, USA) was placed on a SPE vacuum manifold (Supelco. Bellefonte, USA), and the sample extract was applied to the column, followed by washing with acetonitrile (2 ml) and distilled water (2 ml). The OTA was then slowly eluted from the column with 6 ml methanol:acetic acid (99.5:0.5) into a glass vial. The eluate was evaporated to dryness under nitrogen stream and the residue redissolved in mobile phase (2 ml), composed by acetonitrile:water:acetic acid (99:99:2), by ultrasonication for a few seconds. The extract was then filtered (Millex-® HV, 0.45 µm, 13 mm, Millipore Corp. Bedford, USA), injected (50 µl) into reversed-phase HPLC (C_{18}, 5 µm, ODS2, 4.6 x 250 mm Waters). A fluorescence detector was used and mobile phase flow rate was 1 $ml \cdot min^{-1}$ (quantification limit= 5 ng OTA $\cdot$ g^{-1} grape). The recovery rates ranged from 80-108% in samples spiked with 2.5-5 ng OTA$\cdot g^{-1}$ grape

Statistical analysis

Variability of results of grape colonisation and OTA contents, due to different *A*. section *Nigri* inocula, presence of other moulds (*E. amstelodami* or *P. janthinellum*) and grape state (healthy or injured) was analysed by means of Generalised Lineal Model ($P < 0.05$) with time as covariance, through SAS (SAS Institute Inc., Cary, NC, USA) following a full factorial design with two replicates.

RESULTS

Grape colonisation

Grape colonisation increased with time in all treatments. In healthy grapes *A. niger* aggregate OTA-positive showed the highest colonisation percentage, followed by *A. carbonarius*, and finally their mixed inoculum, suggesting interference between them (Table 1).

The combination of *A. carbonarius* with the two *A. niger* aggregate and with or without *P. janthinellum* reduced *A*. section *Nigri* infective potential. Whilst, *A. carbonarius* co-inoculated with *A. niger* aggregate OTA-negative and *E. amstelodami* increased the global infection of *A*. section *Nigri*. The same behaviour was also observed when *A. niger* aggregate OTA-positive was co-inoculated with *A. niger* aggregate OTA-negative, *P. janthinellum* and *E. amstelodami*. When the two OTA-producing strains were combined, addition of *P. janthinellum* or *E. amstelodami* increased the percentage of infection by *A*. section *Nigri*.

In injured grapes, effects produced by inoculum were not statistically significant, the 34-100% of grapes being colonised on the fifth day of dehydration, and close to 100% of grapes, in all assays, on the tenth day of dehydration (data not shown).

It was also observed that grapes weigh did not decrease considerably during dehydration, due that similar amount of grapes were needed to reach 30 g necessary for each OTA analysis.

Table 1. Effect of inoculum in percentage of colonisation by *A*. section *Nigri* in healthy grapes. Mean percentage of colonisation values with different letters, within the same day, are statistically different, through a Duncan test (P<0.05).

Inoculum		Dehydration time (days)			
OTA producer fungi	Interacting fungi	5	10	15	20
A. carbonarius	none	26.7^{ab}	39.8^{bcde}	62.7^{bcd}	72.2^{ab}
	A.niger aggregate OTA(-)	13.0^{b}	31.0^{de}	44.7^{cde}	46.5^{bc}
	A.niger aggregate OTA(-) + *E.amstelodami*	63.2^{a}	64.6^{abcd}	71.5^{ab}	75.0^{ab}
	A.niger aggregate OTA(-) + *P.janthinellum*	10.8^{b}	16.7^{e}	21.9^{e}	29.2^{c}
A.niger aggregate OTA(+)	none	32.2^{ab}	62.8^{abcd}	65.1b^{c}	88.9^{a}
	A.niger aggregate OTA(-)	17.4^{b}	30.4^{de}	30.4^{e}	58.1^{b}
	A.niger aggregate OTA(-) + *E.amstelodami*	33.5^{ab}	72.5^{abc}	100^{a}	100^{a}
	A.niger aggregate OTA(-) + *P.janthinellum*	10.1^{b}	35.5^{cde}	37.0^{cde}	57.3^{b}
A.carbonarius + *A.niger* aggregate OTA(+)	none	16.7^{b}	39.8^{cde}	39.8^{cde}	58.0^{b}
	A.niger aggregate OTA(-)	12.4^{b}	32.4^{cde}	32.4^{de}	54.8^{bc}
	E.amstelodami	44.0^{ab}	91.0^{a}	100^{a}	100^{a}
	A.niger aggregate OTA(-) + *E.amstelodami*	31.0^{ab}	77.0^{ab}	100^{a}	100^{a}
	P.janthinellum	18.0^{b}	60.0^{abcd}	98.5^{a}	100^{a}
	A.niger aggregate OTA(-) + *P.janthinellum*	31.0^{ab}	57.0^{abcd}	94.7^{a}	97.6^{a}

OTA content

Healthy grapes

A. carbonarius was the highest OTA producer in pure culture (0.446 µg OTA · g^{-1}), followed by *A. niger* aggregate OTA-positive, with their mixed inoculum producing the least (Table 2). In general, when competing fungi were added to *A. carbonarius* inoculum, the OTA content was reduced; mostly in combination with *A. niger* aggregate OTA-positive. *E. amstelodami* was the only competing fungi which increased OTA accumulation.

OTA production by *A. niger* aggregate was reduced by all competing fungi. In contrast, when it was co-inoculated with *A. carbonarius*, despite the low OTA production, it was stimulated by both *P. janthinellum* and *E. amstelodami*.

In general, OTA accumulation increased till the 15th day, after that it increased in some cases and in some others decreased.

Injured grapes

In perforated grapes, *A. carbonarius* was also the highest OTA producer (Table 3). Less production was observed when co-inoculated with *A. niger* aggregate OTA-positive, and *A. niger* aggregate OTA-positive in pure culture produced the least.

Competence with other fungi appeared not to influence *A. carbonarius* OTA production, except for *A. niger* aggregate OTA-positive which made OTA content decrease OTA production by *A. niger* aggregate was reduced by all competing fungi OTA non producer, as it occurred in healthy grapes. Both OTA producing strains together were stimulated in their OTA production by all fungi, except for *A. niger* aggregate OTA-negative that reduced it.

In injured grapes, it was also observed a total OTA reduction after the 15th-17th days of dehydration, in case of high OTA concentrations detected. Whilst when *A. niger* aggregate OTA-positive was the unique producer, no clear trend was observed.

Table 2. Effect of different inoculum in OTA accumulation in healthy grapes during its dehydration. Mean OTA values with different capital letters are statistically different, through a Duncan test (P<0.05).

Inoculum		Dehydration time (days)						
OTA producer fungi	Interacting fungi	5	7	10	12	16	17	20
A. carbonarius	none	0.0^{a}	20.9^{a}	123.2^{ab}	91.0^{a}	-	446.1^{abc}	276.4^{abc}
	A.niger aggregate OTA(-)	0.0^{a}	0.3^{a}	4.9^{d}	0.7^{a}	64.7^{ab}	109.9^{cde}	273.4^{abcd}
	A.niger aggregate OTA(-) + *E.amstelodami*	0.4^{a}	22.5^{a}	138.4^{a}	117.5^{a}	467.4^{a}	377.6^{bcd}	-
	A.niger aggregate OTA(-) + *P.janthinellum*	0.0^{a}	0.7^{a}	0.0^{d}	0.0^{a}	55.2^{b}	0.0^{e}	222.6^{abcde}
A.niger aggregate OTA(+)	none	0.2^{a}	9.2^{a}	13.0^{d}	65.6^{a}	-	119.4^{cde}	$95.7b^{cde}$
	A.niger aggregate OTA(-)	0.0^{a}	0.0^{a}	2.9^{d}	0.0^{a}	0.0^{b}	0.0^{e}	6.0^{de}
	A.niger aggregate OTA(-) + *E.amstelodami*	1.9^{a}	35.0^{a}	6.9^{d}	64.6^{a}	11.7^{b}	65.5^{de}	23.4^{cde}
	A.niger aggregate OTA(-) + *P.janthinellum*	0.0^{a}	0.0^{a}	2.0^{d}	0.0^{a}	0.0^{b}	0.4^{e}	0.0^{e}
A. carbonarius + *A.niger* aggregate OTA(+)	none	0.0^{a}	0.0^{a}	0.6^{d}	20.6^{a}	-	9.6^{e}	24.6^{cde}
	A.niger aggregate OTA(-)	0.0^{a}	0.2^{a}	0.0^{d}	0.0^{a}	-	4.9^{e}	65.6^{bcde}
	E.amstelodami	7.7^{a}	2.3^{a}	91.9^{abc}	174.0^{a}	261.2^{ab}	504.9^{a}	214.6^{abcde}
	A.niger aggregate OTA(-) + *E.amstelodami*	1.6^{a}	39.6^{a}	33.1^{cd}	106.2^{a}	352.6^{ab}	201.6^{cde}	437.6^{ab}
	P.janthinellum	0.0^{a}	2.7^{a}	9.6^{d}	41.9^{a}	255.5^{ab}	164.0^{cde}	380.6^{a}
	A.niger aggregate OTA(-) + *P.janthinellum*	5.1^{a}	27.2^{a}	51.6^{bcd}	121.1^{a}	333.6^{a}	437.2^{ab}	411.6^{a}

-, missing data

Table 3. Effect of different inoculum in OTA accumulation in wounded grapes during its dehydration. Mean OTA values with different capital letters are statistically different, through a Duncan test (P<0.05).

Inoculum		Dehydration time (days)						
OTA producer fungi	**Interacting fungi**	**5**	**7**	**10**	**12**	**15**	**17**	**20**
A. carbonarius	none	2.5^{c}	44.2^{ab}	169.2^{bcd}	340.9^{ab}	725.9^{a}	491.3^{abcd}	102.3^{a}
	A.niger aggregate OTA(-)	14.9^{bc}	35.1^{ab}	170.4^{bcd}	271.9^{abcd}	542.8^{ab}	663.3^{ab}	277.8^{a}
	A.niger aggregate OTA(-) + *E.amstelodami*	13.0^{bc}	41.5^{ab}	167.1^{bcd}	357.3^{a}	380.3^{abc}	353.7^{abcde}	164.3^{a}
	A.niger aggregate OTA(-) + *P.janthinellum*	6.5^{c}	56.9^{ab}	162.2^{bcd}	207.3^{abcd}	546.6^{ab}	496.6^{abc}	231.3^{a}
A.niger aggregate OTA(+)	none	7.3^{c}	-	0.5^{d}	21.4^{d}	31.2^{c}	56.1^{e}	27.1^{a}
	A.niger aggregate OTA(-)	0.0^{c}	0.0^{c}	0.0^{d}	188.3^{abcd}	-	8.8^{e}	-
	A.niger aggregate OTA(-) + *E.amstelodami*	0.0^{c}	0.0^{c}	0.0^{d}	66.7^{bcd}	24.4^{c}	36.5^{e}	10.3^{a}
	A.niger aggregate OTA(-) + *P.janthinellum*	0.0^{c}	0.0^{c}	0.0^{d}	0.0^{d}	0.0^{c}	66.2^{de}	6.2^{a}
A. carbonarius + *A.niger* aggregate OTA(+)	none	0.0^{c}	20.7^{ab}	2.6^{d}	23.3^{d}	56.0^{c}	113.4^{cde}	230.1^{a}
	A.niger aggregate OTA(-)	0.0^{c}	6.5^{ab}	10.3^{d}	26.1^{d}	-	65.2^{de}	15.3^{a}
	E.amstelodami	14.5^{bc}	62.9^{a}	235.7^{b}	359.1^{a}	274.6^{abc}	164.3^{cde}	204.4^{a}
	A.niger aggregate OTA(-) + *E.amstelodami*	0.0^{c}	6.6^{ab}	26.9^{d}	59.1^{cd}	376.6^{abc}	357.4^{bcde}	162.6^{a}
	P.janthinellum	23.9^{ab}	51.6^{ab}	213.6^{bc}	169.6^{abcd}	235.1^{bc}	276.7^{bcde}	269.2^{a}
	A.niger aggregate OTA(-) + *P.janthinellum*	30.6^{a}	-	471.2^{a}	326.3^{abc}	362.7^{abc}	730.2^{a}	350.7^{a}

-, missing data

DISCUSSION

OTA producing strains of *A*. section *Nigri* are considered the source of OTA in grapes (Abarca et al. 2003). The incidence of these moulds is low at the beginning of grape maturation but it increases with time, favoured by high temperatures and decrease of a_w of grapes, as a result of its dehydration and sugars concentration (Bellí et al. 2004, Valero et al. 2005). Increasing percentages of infection by *A*. section *Nigri* were observed in this study with the drying process. Therefore, postharvest, and sun-drying process are auspicious periods for these fungi to develop easily and to produce OTA, mainly if grapes are injured during harvesting.

During this postharvest period *A*. section *Nigri* species may grow together with other species such as *Eurotium* sp. and *Penicillium* sp., which, as shown in this study, will be determinant in the final OTA content of dried grapes thought their interaction with OTA producers.

Percentage of colonisation in healthy grapes was higher when they were inoculated with *A. niger* aggregate in pure culture. In previous works concerning the enzymatic activity of *A.* section *Nigri*, cellulolytic activity was observed, which could help these fungi to penetrate into the berry (Seidle et al. 2005).

From previous works it was shown that incidence in grapes of *Aspergillus* spp., and *Penicillium* spp. were 10 % and 31 % respectively (Sage et al. 2002). These genera were also highly isolated from dried vine fruits with 50% and 14% incidence respectively, but also *Eurotium* spp. was frequently isolated in 21% of berries (Romero et al. 2005) and in 13% of commercial raisins (Valero et al. 2005).

Among genus *Aspergillus*, percentages of *A. carbonarius* OTA-producer, *A. niger* aggregate OTA-producer and *A. niger* aggregate OTA-non producer were 15, 3 and 48% of harvested grapes respectively (Valero et al. 2005). Thus, fungal competition may mainly involve *A. niger* aggregate OTA-negative and *A. carbonarius*. In this direction, it was observed that both *A. carbonarius* and *A. niger* aggregate OTA-positive infection were reduced by *A. niger* aggregate OTA-negative. *E. amstelodami* generally favoured grape colonisation by *A*. section *Nigri*, likely due to its enzymatic activity that could open an access through grape skin, or even help to degrade some grape compounds into nutrients that *A*. section *Nigri* strains could use. On the contrary, *P. janthinellum*, referred as a cellulolytic enzymes producer (Wang and Gao 2000), only favoured the colonising capability of *A. carbonarius* and *A. niger* aggregate OTA-positive combined. Despite these interesting results derived from the presence of other fungal species, it should be remarked that *A*. section *Nigri* spp. are the main contaminants in fresh and dehydrated grapes, and presence of *Penicillium* sp. and *Eurotium* sp. is lower and sometimes occasional (Valero et al. 2005).

The drying process led to an increasing level of OTA till about the 15th day, this may be due to OTA synthesis since decrease of grape weigh were not substantial. In this study *A. carbonarius*

was the highest OTA producer, both in healthy and injured grapes. When co-inoculating *A. niger* aggregate OTA-negative with *A. carbonarius*, OTA production was highly reduced. Thus intra section fungal competence affects to OTA accumulation. This reduction could be due (i) to the growth limitation of OTA producer strain, (ii) to the using up of specific nutrients for OTA synthesis, (iii) or to its degradation by competing fungi (Abrunhosa et al. 2002). In contrast, higher OTA levels were obtained when combining *E. amstelodami* or *P. janthinellum* with both OTA producing strains, agreeing with previous studies where effects of *E. amstelodami* competence with this species in OTA production were tested (Valero et al., in press).

In injured grapes, OTA accumulation was faster and higher at the beginning of the drying process, and decreased the latest days of dehydration to values below 0.4 µg OTA g^{-1} grape. This could be due to the fact that the depleting of nutrients occurs earlier in injured than in healthy grapes, and OTA could be degraded as a carbon or energy source. Previous studies showed OTA degradation capability in some *A. carbonarius* and *A. niger* aggregate strains, and producing OTA derivatives as ochratoxin α, probably through the action of the carboxypeptidase A enzyme (Abrunhosa et al. 2002; Varga et al. 2000).

The sun-drying process may be conducive to OTA accumulation in dried grapes. Addition of interacting fungi has also been proved to be an important factor to take into consideration. The complex fungal interactions which may take place during this process, may act as an additional control factor, given that the higher presence of *A. niger* aggregate OTA-negative inhibits OTA accumulation by OTA producing species.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2004-07549-05-01, grant BES-2003-2180, and Ramon y Cajal program) and to the Catalonian Government (CeRTA strategic project 2005-2006 “Seguretat biòtica i abiòtica dels aliments”, Generalitat de Catalunya) for its financial support.

REFERENCES

Abarca, M.J., Accensi, F., Bragulat, M.R., Castella, G., Cabañes, F.J. (2003) Contamination in dried vine fruits from the Spanish market. *J. Food Protect.* 66, 504-506.

Abrunhosa, L., Paterson, R.R.M., Kozakiewicz, Z., Lima, N., Venâncio, A. (2001) Mycotoxin production from fungi isolated from grapes. *Lett. Appl. Microbiol.* 32, 240-242.

Abrunhosa, L., Serra, R., Venâncio, A. (2002) Biodegradation of Ochratoxin A by Fungi isolated from grapes. *Journal of Agricultural and Food Chemistry* 50, 7493-7496.

Battilani, P., Pietri, A. (2002) Ochratoxin A in grapes and wine. *Eur. J. Plant Pathol.* 108, 639-643.

Bellí, N., Marín, S., Sanchis, V., Ramos, A.J. (2002) Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. *Food Sci. Technol. Int.* 8, 325-335.

Bellí, N., Marín, S., Sanchis, V., Ramos, A.J. (2004) Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. *Int. J. Food Microbiol. 96*, 19-27.

Bragulat, M.R., Abarca, M.L., Cabañes, F.J. (2001) An easy screening method for fungi producing ochratoxin A in pure culture. *Int. J. Food Microbiol*. 71, 139-144.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S., Pons, A. (2002) What is the source of ochratoxin A in wine? *Int. J. Food Microbiol*. 79, 213-215.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Vol. 56, International Agency for Research on Cancer, Lyon, 1993, pp. 489-521.

MacDonald, S., Wilson, P., Barnes, K., Damant, A., Massey, R., Mortby, E., Shepherd, M.J. (1999) Ochratoxin A in dried wine fruit: Method development and survey. *Food Add. Cont.* 16, 253-260.

Magnoli, C., Astoreca, A., Ponsone, L., Combina, M., Palacio, G., Rosa, C.A.R., Dalcero, A.M. (2004) Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Lett. Appl. Microbiol.* 39, 326-331.

Ostry, V., Ruprich, J., Skarkova, J. (2002) Raisins, ochratoxin A and human health. *Mycot. Res.* 18A, 178-182.

Pardo, E., Marín, S., Ramos, A.J., Sanchis, V. (2004) Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different origins. *Food Sci. Technol. Int*. 10, 45-49.

Pietri, A., Bertzzi, T., Pallaroni, L., Piva, G. (2001) Occurrence of ochratoxin A in Italian wines. *Food Addit. Cont.* 18, 647-654.

Rafai, P., Bata, A., Jakab, L. (2000) Evaluation of mycotoxin-contaminated cereals for their use in animal feeds in Hungary. *Food Addit. Cont*. 17, 799-808.

Romero, S.M., Comerio, R.M., Larumbe, G., Ritieni, A., Vaamonde, G., Fernández, V. (2005) Toxigenic fungi isolated from dried vine fruits in Argentina. *Int. J. Food Microbiol*. 104, 43-49.

Sage, L., Krivobok, S., Delbos, E., Seigle-Murandi, F., Creppy, E.E. (2002) Fungal flora and ochratoxin A production in grapes and musts from France. *J. Agric. Food Chem*. 50, 1306-1311.

Seidle, H.F., Huber, R.E. (2005) Transglucesidic reactions of the *Aspergillus niger* family 3 β-glucosidase: Qualitative and quantitative analyses and evidence that the transglucosidic rate is independent of pH. *Arch. Biochem. Biophys.* 436, 254-264.

Valero, A., Marín, S., Ramos, A.J., Sanchis, V. (2005) Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors. *Lett Appl. Microbiol.* 41, 196-201.

Valero, A., Farré, J.R., Sanchis, V., Ramos, A.J., Marín, S. (in press) Understanding the dominance of *Aspergillus* section *Nigri* in mycobiota succession in grapes and sun-dried grapes. *Int. J. Food Microbiol.*

Varga, J., Kevei, F., Hamari, Z., Tóth, B., Téren, J., Croft, J.H., Kozakiewicz, Z. (2000) Genotypic and phenotypic variability among black aspergilli, p. 397–411. *In* Samson, R.A. and Pitt, J.I. (eds.), Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood Academic Publishers, Amsterdam.

Wang, T., Gao, P. (2000) *In vitro* expresión of *Penicillium janthinellum* cellobiohydrolase I gene in a coupled transcription- translation system. *J. Biotechnol.* 81, 205-209.

Study VIII:

Brief in vitro study on *Botrytis cinerea* and *Aspergillus carbonarius* regarding growth and Ochratoxin A

Ana Valero, Vicente Sanchis, Antonio J. Ramos, Sonia Marín [a]

Food Technology Department, University of Lleida, UTPV-CeRTA, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

Abstract

The aim of this study was to evaluate the effect of *Botrytis cinerea* growth in OTA production by *Aspergillus carbonarius* and in its degradation. Five OTA-producing *A. carbonarius* and three *B. cinerea* were grown on grape-like medium at 20ºC for seven days. Radii of colonies were daily recorded and OTA analysed. In addition, each of the three *B. cinerea* isolates were inoculated on SNM paired with each of the five *A. carbonarius* at a distance of 45 mm. *B. cinerea* isolates were also grown in SNM spiked with OTA.

Growth rates of *B. cinerea* were much higher, about 20 mm·day^{-1} than *A. carbonarius* ones, around 7.5 mm·day^{-1}. Both fungal colonies stopped their growth when they contacted each other in paired cultures. OTA production by *A. carbonarius* in the contact area was affected by *B. cinerea* but no clear trend was observed.

The three *B. cinerea* isolates showed to degrade 24.2 and 26.7% of OTA from SNM medium, while their growth was unaffected.

The ecological advantage of *B. cinerea*, in terms of growth rate, versus OTA producing *Aspergillus* in some wine-growing regions and its ability to degrade OTA may explain the low levels of this toxin in noble wines.

Publication: *FEMS Microbiology Letters* 2007 (submitted).

INTRODUCTION

Grapes are susceptible to insect attack and to fungal diseases, especially grey rot (*Botrytis cinerea*), downy mildew (*Plasmopara viticola*) and black rot (*Guignardia bidwellii*). Damaged grapes are vulnerable to further diseases such as summer bunch rot, which may be caused by *Aspergillus niger*, *Alternaria tenuis*, *Cladosporium herbarum*, *Rhizopus arrhizus*, *Penicillium* spp. and other fungi (Hellman, 2004). Fungal invasion depends on grape maturity and environmental conditions. In warmer climates, *Alternaria*, *Cladosporium*, *Botrytis* and *Rhizopus* are common at early veraison, whereas *Aspergillus* and *Penicillium* are more frequently found at harvest and during sun drying (Magnoli *et al*., 2003; Bellí *et al*., 2004; Valero *et al*., 2005). In cooler climates the previously mentioned species at early veraison can remain until harvest favoured by climatic conditions.

Some species of *Penicillium* and *Aspergillus* are known to produce mycotoxins, mainly *A. carbonarius* and *A. niger* which are know to produce ochratoxin A (OTA) in grapes and raisins (Abarca *et al*., 2003; Bellí *et al*., 2004). This toxin has been considered the causing agent of Balkan Endemic Nephropathy (BEN) (Marquardt & Frohlich, 1992) and it has also been classified by the International Agency for Research on Cancer (IARC, 1993) as a possible human carcinogen (group 2B).

OTA incidence in wine achieves higher levels in sweet and special wines (Bellí *et al*., 2004, Burdaspal & Legarda, 1999, Pietri *et al*., 2001, Zimmerli & Dick, 1996). In this type of wines, oenological practices are very diverse and may result in different effects in OTA accumulation (Ratola *et al*., 2005, Gambuti *et al*., 2005, Leong *et al*., 2006, Chiodini *et al*., 2006). Noble wines are sweet wines that are made from dehydrated grapes through colonisation by *Botrytis cinerea*. This fungus requires cool temperatures to develop, high humidity during the mornings followed by dry and warm afternoons, thus grapes are left on the vines for longer periods of time, up to winter in some regions. *B. cinerea* dehydrates grapes by perforating grape skin with the mycelium development towards inside. Although *Botrytis* is the predominant genus present in botrytized grapes, other species of moulds belonging to the genera *Aspergillus* and *Penicillium* have also been isolated from botrytized grapes in Hungary (Bene & Magyar, 2004).

Ochratoxin A has previously been detected in 32% of table wines from Northern European regions (Austria, Hungary, Germany and North France) (Zimmerli & Dick, 1996; Lew, 2000; Majerus *et al*., 2000; Ottender & Majerus, 2000, Eder, 2002; Berente *et al*., 2005; Varga *et al.* 2005). In contrast, only three out of 89 noble and late harvest wines (some of grapes used may be botrytized) from the same regions and South Africa, contained ochratoxin A above the detection limit (Dumoulin & Riboulet, 2002, Eder *et al.,* 2002, Stander *et al*., 2002, Valero *et al*.,

2007). Additionally, Abrunhosa *et al*. (2002) found that one *B. cinerea* isolate was able to degrade more than 50% of OTA spiked in yeast extract sucrose medium.
All together, we wondered if the presence of *B. cinerea* in some grapevines can be the responsible for the absence of ochratoxin A in noble and late harvest wines. In this study the effect of *Botrytis cinerea* growth in grape-like medium in OTA production by *A. carbonarius* and in its degradation is evaluated.

MATERIAL AND METHODS

Moulds and growth medium

Five OTA-producing *Aspergillus carbonarius* (UdLTA 3.81, UdLTA 3.83, UdLTA 3.90, UdLTA 3.91, UdLTA 3.93) and three *Botrytis cinerea* (UdLTA 3.95, UdLTA 3.102, UdLTA 3.115) were isolated from grapes on dichloran rose Bengal chloramphenicol (DRBC) and identified to species level according to Pitt & Hocking (1997). All microorganisms were stored in the Food Technology Department collection in the University of Lleida, Spain. Growth medium used in all ecophysiological assays was grape synthetic medium agar, similar to grape composition developed by Delfini (1982) (0.99 a_w). Spore suspensions were prepared for each isolate from seven days cultures in sterile water with 0.02% Tween 80® at concentrations between 1.1-2.8 $\cdot 10^6$ spores$\cdot$ml^{-1}.

In vitro OTA degradation by *Botrytis cinerea*

Three *B. cinerea* isolates were single point inoculated from 10^6 spore solutions in the centre of SNM agar plates containing 1 µg OTA ml^{-1}. A volume of 500 µl of 1000 µg OTA$\cdot$ml^{-1} (in methanol) was added to 500 ml of sterile medium and then mixed before pouring it on plates. Four replicates for each isolate, four inoculated plates without OTA as positive controls and four uninoculated controls were incubated at 20ºC and at high relative humidity for seven days. Radii of colonies were daily recorded and at the end of the experiment OTA was analysed throughout HPLC. The protocol for OTA analysis is detailed in Bragulat *et al*. (2001). Briefly: three agar plugs (5 mm diameter) were randomly taken from agar plates and OTA was solid-liquid extracted with methanol 1 ml for one hour. A volume of this extract (50 µl) was injected into HPLC system.

B. cinerea paired with *A. carbonarius*

Each of the three *B. cinerea* isolates were inoculated on SNM paired with each of the five *A. carbonarius* at a distance of 45 mm. All fungi were also grown in pure culture on SNM as control of growth and OTA production in case of *A. carbonarius*. Four replicates for each combination and fungi in pure culture were incubated at 20ºC and high humidity relative for seven days. Radii

of colonies were daily recorded. OTA was analysed, from *A. carbonarius* pure cultures and from *A. carbonarius* paired with *B. cinerea* cultures. OTA extraction was performed between the 6-8th day (first day of contact between edges of colonies) by taking the three plugs at 15 mm distance from the center of *A. carbonarius* colonies (contact area between the two colonies) both for pure and paired cultures. OTA was analysed following methodology detailed in Bragulat *et al*. (2001).

Statistic analysis

Growth rates were obtained with software Statgraphics Plus (v. 5.1 Statistical Graphics Corp.) by means of using the Gompertz equation (1) as a model of growth.

$$\text{Diameter}=D_{max}\cdot \exp(-\exp(((K\cdot \exp(1/D_{max})\cdot(\text{Lag phase}-\text{time}))+1)) \quad (1)$$

Where D_{max}= Maximum diameter (mm)

K= growth rate ($mm\cdot day^{-1}$)

Lag phase (days)

Time=incubation time (days)

Analysis of the effect of competence in fungal growth and related Duncan's multiple range tests were performed using SAS (SAS Institute Inc., Cary, NC, USA).

RESULTS

OTA degradation by *B. cinerea*

The three *B. cinerea* isolates showed to degrade OTA from SNM medium (Figure 1). Degraded OTA ranged from 24.2 to 26.7% of the total spiked OTA (614.6 ± 43.8 ng OTA g^{-1}).

There were no differences between OTA-spiked and non-spiked medium in terms of *B. cinerea* growth (table 1).

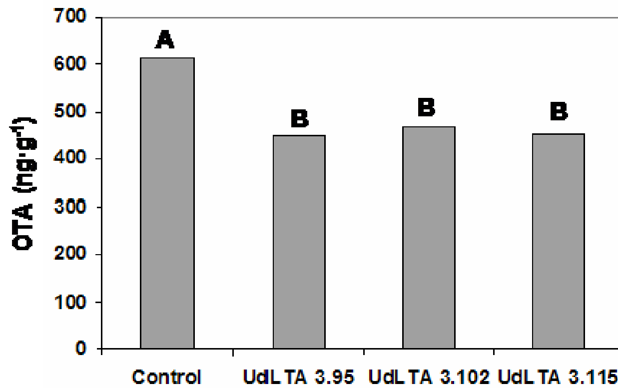


Figure 1. OTA concentration in SNM without inoculum (control) and inoculated with three isolates of *B. cinerea*. Bars with different capital letter are significantly different (Duncan test, $P < 0.05$).

Table 1. Growth of three *B. cinerea* isolates in SNM medium with or without OTA.

B. cinerea	Media	Lag phase (days)	*K* (mmd^{-1})	95% C.I. Min k	95% C.I. Max k	R^2
UdLTA 3.95	SNM	3.0	20.5	18.3	22.7	0.981
	SNM+OTA	3.0	20.4	17.4	23.3	0.972
UdLTA 3.102	SNM	3.2	20.3	18.9	21.7	0.992
	SNM+OTA	2.5	19.8	17.8	21.8	0.985
UdLTA 3.115	SNM	2.5	20.8	18.8	22.7	0.988
	SNM+OTA	2.3	21.2	18.5	23.9	0.983

C.I., confidence interval

B. cinerea **paired with** ***A. carbonarius***

Maximum growth rates of *B. cinerea* were much higher (20 mm·day^{-1}) than *A. carbonarius* ones, around 7.5 mm·day^{-1} (figure 2). No significant effect was observed in maximum growth rates of *A. carbonarius* as consequence of the presence of *B. cinerea* and *vice versa*. It was observed that both colonies stopped their growth as soon as they contacted each other.

Ochratoxin A production by *A. carbonarius* in the contact edge was affected by *B. cinerea* but a clear trend was not observed (figure 3).

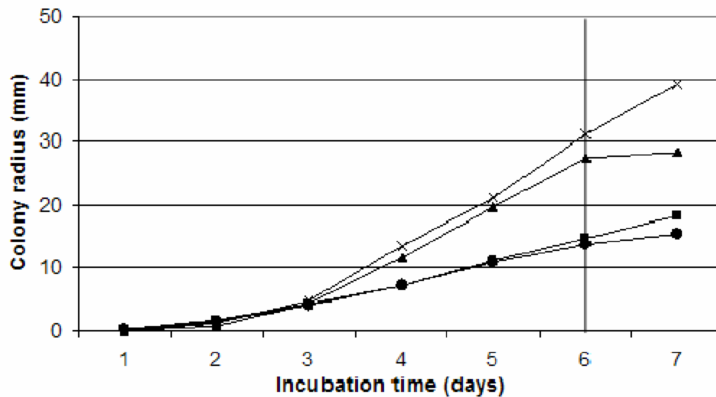


Figure 2. *A. carbonarius* and *B. cinerea* growth averages in pure and paired cultures. ●, *A. carbonarius* with *B. cinerea* (mean of 60 observations); ■, *A. carbonarius* alone (mean of 20 observations); ▲, *B. cinerea* with *A. carbonarius* (mean of 60 observations); x, *B. cinerea* alone (mean of 15 observations). Crossing line represents the day of contact between colonies.

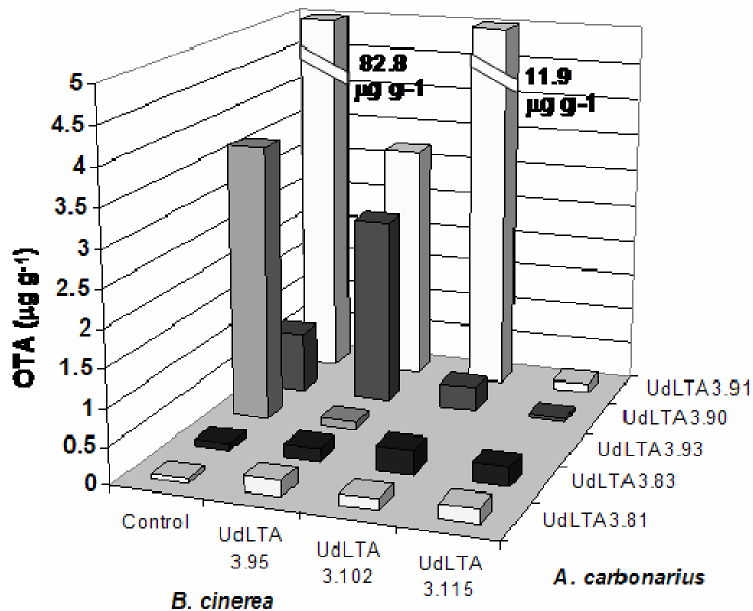


Figure 3. Ochratoxin A produced by *A. carbonarius* (five isolates) in SNM medium as affected by the presence of *B. cinerea* (three isolates).

DISCUSSION

B. cinerea is the most important cause of disease in grapes, both before harvest and in storage (Snowdon, 1990), but this fungus is also a highly desired agent in grapes that are intended for noble wines. Environmental conditions that are required for grapes to be botrytized are present in continental climates, with cool temperatures and moderate humidity. The a_w threshold for growth of *B. cinerea* has been reported around 0.93 (Snow, 1949; Rousseau & Donèche, 2001) or even at 0.90 (Jarvis, 1977) and optimal temperatures between 22-25ºC (Domsch *et al*., 1980). In this study growth rates of *B. cinerea* at 20ºC and 0.99 a_w were around 20 $mm \cdot day^{-1}$ agreeing with that previously reported by Vallejo *et al.* (2001). *A. carbonarius* showed a lower growth rate of 7 $mm \cdot day^{-1}$ under the same conditions. In case of co-contamination by both species, which has previously been proved by Bene & Magyar (2004), it has been observed that mutual inhibition in contact may occur. The co-contaminant mycobiota may have a strong impact on the quality of the botrytized grapes and wines. From the presence of mixed mould populations, with the special regard to *Aspergillus*, OTA contamination may occur. However, Kallay & Magyar (2000) reported that no significant levels of the former toxin were found in botrytized grapes. In addition, only three out of 89 noble wines and late harvest wines analysed from Northern European regions, France and South Africa contained ochratoxin A (Dumoulin & Riboulet, 2002; Eder *et al*., 2002; Stander *et al*., 2002; Valero *et al*., 2007).

In contrast, the 32% of 665 table wines analysed by several authors from Northern European regions, such as Hungary, North France, Austria and Germany, contained OTA (Zimmerli & Dick, 1996; Lew, 2000; Majerus *et al*., 2000; Ottender & Majerus, 2000, Eder, 2002; Berente *et al*., 2005; Varga *et al.* 2005). This fact may suggest a positive effect of *Botrytis* in preventing or reducing the OTA content in noble wines that enhances the characteristic low OTA contamination of wines from these regions.

Finally, in agreement with Abrunhosa *et al*. (2002) it has been observed that *B. cinerea* is capable of OTA degradation, that is, in case of black aspergilli colonised the grapes, the little toxin produced could be degraded and, consequently, the contamination of the noble wine be prevented. Melki Ben Fredj *et al.* (2007) also suggested that the highly co-contamination of ochratoxin-degrader (*Mucor* spp. and *Rhizopus* spp.) with ochratoxigenic fungal species (*Aspergillus* spp.) found in some grapes in Tunisia may explain the low OTA levels detected in such grapes.

To sum up, the ecological advantage of *B. cinerea* versus OTA producing *Aspergillus* in some wine-growing regions and its ability to degrade OTA may explain the low occurrence of this toxin in noble wines.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2004-07549-05-01, grant BES-2003-2180 and Ramón y Cajal program) for their financial support.

REFERENCES

Abarca ML, Accensi F, Bragulat MR, Castella G & Cabañes FJ (2003) *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *J Food Prot* 66: 504-506.

Abrunhosa L, Serra R & Venâncio A (2002) Biodegradation of Ochratoxin A by Fungi isolated from grapes. *J Agr Food Chem* 50: 7493-7496.

Bellí N, Marín S, Duaigües A, Ramos AJ & Sanchis V (2004) Ochratoxin A in wines, musts and grape juices from Spain. *J Sci Food Agr* 84: 591-594.

Bene Z & Magyar I (2004) Characterization of yeast and mould biota of botrytized grapes in Tokaj wine region in the years 2000 and 2001. *Acta Alimentaria* 33: 259-264.

Berente B, Móricz A, H-Otta K, Záray G, Léko L & Rácz L (2005) Determination of ochratoxin A in Hungarian wines. *Microchem J* 79: 103-107.

Bragulat MR, Abarca ML & Cabañes FJ (2001) An easy screening method for fungi producing ochratoxin A in pure culture. *Int J Food Microbiol* 71: 139-144.

Burdaspal PA & Legarda TM (1999) Ocratoxina A en vinos, mostos y zumos de uva elaborados en España y en otros países Europeos. *Alimentaria* 36: 107-114.

Chiodini AM, Scherpenisse P & Bergwerff AA (2006) Ochratoxin A contents in wine: comparison of organically and conventionally produced products. *J Agr Food Chem* 54: 7399-7404.

Delfini C (1982) In Tecnica di microbiologia eonological ed. Scicalpi, L. Rome.

Domsch KH, Gams W & Anderson T-H (1980) *Compendium of Soil Fungi*, 2 vols. Academic Press, London.

Dumoulin M & Riboulet JM (2002) Considerations on the presence of ochratoxin A in wines and grape juices. *Rev Fr Oenolog* 29: 11-13.

Eder R, Paar E, Edinger W & Lew H (2002) Investigations into the Ochratoxin A contents of Austrian wines with special respect to wines of the “Pradikatswein”-category. *Mitt Klosterneuburg* 52: 125-131.

Gambuti A, Strollo D, Genovese A, Ugliano M, Ritieni A & Moio L (2005) Influence of enological practices on ochratoxin A concentration in wine. *Am J Enol Vitic* 56: 155-162.

Hellman E (2004) Bunch rot and sour rot management. Texas Cooperative Extension. Department of Horticultural Sciences, Texas A&M University. [internet] Available from:

http://winegrapes.tamu.edu/grow/diseases/bunchrot.shtml. [Updated: 2004]. [Accessed: March 14 2007].

IARC (1993) Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Vol. 56, International Agency for Research on Cancer, Lyon, 1993, pp. 489-521.

Jarvis B (1977) *Botryotinia* and *Botrytis* species: taxonomy, physiology, and pathogenicity. Monograph 15, p. 195. Can. Dept Agric. Res. State, Harrow,

Kallay M & Magyar I (2000) Occurrence of ochratoxin A under oenological conditions with special regard to the processing of Tokaj Aszú. *Proceedings of Lippary J. Vas K. Scientific Symposium*, p. 15. Food Science, Budapest.

Leong SL, Hocking AD & Varelis P (2006) Fate of Ochratoxin A during vinification of Semillon and Shiraz grapes. *J Agr Food Chem* 54: 6460-6464.

Lew H (2000) Ochratoxin A in Traubensäften und Wein. Fachgruppe Wein und Obst (Specialized group in wine and fruit), Arbeitsgemeinschaft landwirtschaftlicher Versuchsanstalten (Working group of agricultural laboratories), ALVA-Annual Convention, pp. 89-90. Gmunden.

Magnoli C, Violante M, Combina M, Palacio G & Dalcero A (2003) Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Lett Appl Microbiol* 37: 179-184.

Majerus P, Bresch H & Ottender H (2000) Ochratoxin A in wines, fruit juices and seasonings. *Arch Lebensmittelhyg* 51: 9-97.

Marquardt RR & Frolich AA (1992) A review of recent advances in understanding ochratoxicosis. *J Anim Sci* 70: 3968-3988.

Melki Ben Fredj S, Chebil S, Lebrihi A, Lasram S, Ghorbel A, Mliki A (2007) Occurrence of pathogenic fungal species in Tunisian vineyards. *Int J Food Microbiol* 113: 245-250.

Ottender H & Majerus P (2000) Occurrence of ochratoxin A (OTA) in wines: influence of the type of wine and its geographical origin. *F Addit Contam* 17: 793-798

Pietri A, Bertuzzi T, Pallaroni L & Piva G (2001) Occurrence of ochratoxin A in Italian wines. *F Addit Contam* 18: 647-654

Pitt JI & Hocking AD (1997) *Fungi and food spoilage* ed. Blackie Academic and Professional 2nd ed., pp.110-114. University Press, Cambridge.

Ratola N, Abade E, Simões T, Venâncio A & Alves A (2005) Evolution of ochratoxin A content from must to wine in Port wine microvinification. *Anal Bioanal Chem* 382: 405-411.

Rousseau S & Donèche B (2001) Effects of water activity (a_w) on the growth of some epiphytic micro-organisms isolated from grape berry. *Vitis* 40: 75-78.

Snow D (1949) Germination of mould spores at controlled humidities. *Ann Appl Biol* 36: 1-13.

Snowdon AL (1990) General Introduction and Fruits. *A Colour Atlas of Post-harvest Diseases and Disorders of Fruits and Vegetables*. Vol. 1. Wolf scientific, London.

Stander MA & Steyn PS (2002) Survey of ochratoxin A in South African wines. *S Afr J Enol Vitic* 23: 9-13.

Valero A, Marín S, Ramos AJ & Sanchis V (2005) Ochratoxin A-producing species in grapes and sun-dried grapes and their relation to ecophysiological factors. *Lett Appl Microbiol* 41: 196-201.

Valero A, Marín S, Ramos AJ & Sanchis V (2007) Survey: Ocratoxin A in European special wines. *Food Addit Contam* (submitted).

Vallejo I, Rebordinos L, Collado IG & Cantoral Fernández JM (2001) Different behaviour of mycelial growth of several *Botrytis cinerea* strains on either Patchoulol- or Globulol-amended media. *J Phytopathol* 149: 113-118.

Varga J, Kiss R, Mátrai T, Mátrai T & Téren J (2005) Detection of ochratoxin A in Hungarian wines and beers. *Acta Alimentaria* 34: 381-392.

Zimmerli B & Dick R (1996) Ochratoxin A in table wine and grape-juice: occurrence and risk assessment. *Food Addit Contam* 13: 655-668.

Chapter III:

CONTROL AND PREVENTION

Study IX:

Effect of pre-harvest fungicides and interacting fungi on *Aspergillus carbonarius* growth and OTA synthesis in dehydrating grapes

Ana Valero, Sonia Marín, Antonio J. Ramos, Vicente Sanchis[a]

Food Technology Department, University of Lleida, UTPV-CeRTA, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

Aims: To evaluate the effect of pre-harvest grape pesticides in *Aspergillus* section *Nigri* infection in dehydrating grapes and final OTA content. Also the effect of co-inoculation of moulds frequently isolated from grapes and raisins on *Aspergillus* section *Nigri* infection was studied.

Methods and Results: Fungicide-treated grapes were inoculated with *A. carbonarius*, *A. niger* aggregate, *E. amstelodami* and *P. janthinellum* in different combinations then dehydrated by reducing a_w for 20 days. Percentages of colonised grapes treated with fungicides were, in general, lower, but no differences were observed among fungicides. Untreated grapes always showed higher concentrations of OTA, regardless the inoculum applied. In general, Chorus was the most effective antifungal treatment in reducing OTA accumulation in grapes during dehydration. *P. janthinellum* reduced *Aspergillus* section *Nigri* colonisation and OTA accumulation in grapes during dehydration.

Conclusions: The four pre-harvest fungicides studied reduced the *Aspergillus* section *Nigri* growth and OTA production by *A. carbonarius* during dehydration of grapes dehydration.

Significance and Impact of the study: Success of these chemical treatments could depend on mycobiota composition of grapes.

Publication: *Letters in Applied Microbiology* (in press).

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that was first described in 1965 as a secondary metabolite of *Aspergillus ochraceus* (van der Merwe *et al.* 1965). Currently, several *Penicillium* and *Aspergillus* species are known to produce OTA (Ciegler 1972; Ueno *et al.* 1991; Abarca *et al.* 2003; Bellí *et al*. 2004b). This toxin is chemically described as a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin, linked through its 7-carboxy group to L-β-phenylalanine by an amide bond (van der Merwe *et al.* 1965). OTA has been shown to be nephrotoxic, immunosuppressive, teratogenic and carcinogenic in many species (Boorman *et al.* 1984; Kittane et al. 1984; Gekle and Silberuagi, 1993). Some findings indicate that OTA may be implicated in the aetiology of Balkan endemic nephropathy (Pavlovic *et al.* 1979; Petkova-Bocharova and Castegnaro 1991; Pfohl-Leczkowicz and Manderville 2007).

OTA has been detected in foods from several origins including grapes and raisins (MacDonald *et al.* 1999; Abrunhosa *et al*. 2001; Ostry *et al*. 2002) and also in beverages such as grape juice, must and wine (Bellí *et al.* 2002; Cabañes *et al.* 2002).

Grapes are susceptible to insect attack and to fungal diseases, especially grey rot, downy mildew and black rot. Damaged grapes are vulnerable to further diseases such as summer bunch rot, which may be caused by *Aspergillus niger*, *Alternaria tenuis*, *Cladosporium herbarum*, *Rhizopus arrhizus*, *Penicillium* spp. and other fungi. Fungal invasion depends on grape maturity and environmental conditions. In warmer climates, *Alternaria*, *Cladosporium*, *Botrytis* and *Rhizopus* are common at early veraison, whereas *Aspergillus* and *Penicillium* are more frequently found at harvest and during sun drying (Magnoli *et al.* 2003; Bellí *et al.* 2004a; Valero *et al.* 2005).

Previous studies have compared the effectiveness of different pesticides such as sulphur, dinocap, captan, tebuconazole, azoxystrobin, penconazole in reducing grape infection by *Aspergillus* section *Nigri* (Lo Curto *et al.* 2004; Bellí *et al.* 2006). Other fungicides such as carbendazim, Switch (i.e. 25% fludioxonil and 37·5% cyprodinil) and Chorus (i.e. cyprodinil) have also ben studied for their activity against *Aspergillus* (Cabras *et al*. 1997; Cabras and Angioni, 2000; Tjamos *et al*. 2004). These studies demonstrated that grapes treated with Switch showed a reduction in sour rot by *Aspergillus* section *Nigri* (Tjamos *et al*. 2004; Bellí *et al.* 2007).

In grapes intended for the production of dried vine fruits, from harvest on and during further grape dehydration, environmental conditions may be very favourable for fungal growth. Species belonging to *Aspergillus* section *Nigri* and the genera *Eurotium* and *Penicillium* have been reported as natural contaminants with incidences in dehydrated grapes (and raisins) of about 41-95, 13-21 and 13%, respectivelly (Magnoli *et al.* 2004; Romero *et al.* 2005; Valero *et al.* 2005). As a result, the growth of *Aspergillus* section *Nigri* on dehydrating grapes may increase, causing an increase in the OTA levels during dehydration (Valero *et al*. 2007). The efficacy of sulphur

dioxide in preventing raisin decay is well known but there are no studies on the effect of field fungicide residues applied on grapes, on fungal development during the subsequent drying process. This is of interest, as it is possible that some pre-harvest pesticides may remain on grapes after oven and sun-drying (Cabras *et al.* 1998).

Thus, the aim of this study was to evaluate the effect of some pre-harvest grape pesticides in *Aspergillus* section *Nigri* infection and OTA content in final dehydrated grapes. The effect of co-inoculation of moulds frequently isolated from grapes and raisins on *Aspergillus* section *Nigri* strains was also analysed.

MATERIAL AND METHODS

Fungicide treatments

Field treated grapes var. Moscatel that had been subjected to the field treatments shown in Talbe 1 were supplied by Syngenta Agro S.A. Five to six bunches randomly distributed in each treated plot (30-70 m^2) were collected at harvest. As this is not a field study, the body of the experiment described here is constituted by the *in vitro* dehydrating process.

Table 1 Pre-harvest fungicide treatments, time of application and dose

Treatment	Active compound and dose
Control	-
Chorus (1 application) 21 days before harvest	cyprodinil (0.75 kg · ha^{-1})
Chorus (2 applications) 1st veraison 2nd 21 days before harvest	cyprodinil (0.75 kg · ha^{-1})
Switch (1 application) 21 days before harvest	(1 kg · ha^{-1}) 37.5% cyprodinil 25% fludioxonil
Switch (2 applications) 1st veraison 2nd 21 days before harvest	(1 kg · ha^{-1}) 37.5% cyprodinil 25% fludioxonil

Fungicides evaluated in this study were Chorus (cyprodinil) and Switch (37·5 % cyprodinil + 25% fludioxonil). Cyprodinil is an anilinopyrimidine class fungicide which is a methionine inhibitor with systemic action. Fludioxonil is a phenylpyrrole fungicide with systemic action that interferes with respiration on contact. They are registered for use as foliar and seed treatments in a number of countries including France, Italy, Spain and USA. As a result of combination of both fungicides, Switch inhibits mycelial growth and germination and reduces sporulation. At the dose recommended by the manufacturer the withholding period (WHP) for Switch is set at seven days

but some countries require a WHP of 28 days for cyprodinil and fludioxonil. The MRL (minimum residue limit) of these two compounds is 0·2 and 0·05 mg·kg^{-1} respectively.

Microorganisms, grapes inoculation and incubation

Four samples of about 200 g of grapes from each treatment were inoculated with five different inocula: (i) *Aspergillus carbonarius* OTA-producing strain, (ii) *A. carbonarius* OTA-positive combined with *Aspergillus niger* aggregate OTA-negative, (iii) *A. carbonarius* OTA-positive combined with *Eurotium amstelodami* and (iv) *A. carbonarius* OTA-positive combined with *Penicillium janthinellum,* (v) non-inoculated treatment. All microorganisms were isolated from grapes and raisins and are deposited to the culture collection of the Food Technology Department (Lleida University, Spain): *A. carbonarius* OTA-positive (UdLTA 3.122), *A. niger* var. *niger* OTA-negative (UdLTA 3.124), *E. amstelodami* (UdLTA 3.132) and *P. janthinellum* (UdLTA 3.126). Taxonomic identification of all isolates was achieved in accordance with guidelines of Pitt and Hocking (1997) and, in particular, black aspergilli were identified according to Al-Mussallam (1980). The capability of OTA production by *A. carbonarius* OTA-positive isolate was tested through HPLC (Waters, Milford, MA, USA) following the extraction and clean-up protocol detailed in Bragulat *et al.* (2001).

Inoculation was achieved by means of dropping 20 µl of a 10^6 spores ml^{-1} suspension (1:1 when co-inoculation of two microorganisms) onto the surface of each grape which were placed separately on racks. Racks containing 200 g of grapes (25-40 berries) with same inoculum were placed inside closed boxes and water activity modified by adding a saline solution (250 ml) adjusted firstly to 0·98 a_w and incubated at 25ºC. A simulated dehydration process was achieved as reported in Valero *et al.* (2007). Every five days the saline solution was changed to 0·92, 0·84 and 0·76 a_w, up to a total of 20 days in order to cause grape dehydration.

Mould colonisation, OTA content and statistical analysis

After 5, 10, 15 and 20 days of grape dehydration, percentages of visually colonised berries were recorded and OTA content analysed after 20 days using the extraction method detailed in Valero *et al*. (2007). Briefly, 30 g of grapes were minced, pH adjusted to 2·4-2·6 and OTA was twice liquid-liquid extracted with 60 and 20 ml of chloroform. Then extracts were evaporated under a nitrogen stream and redissolved in 6 ml methanol and 44 ml PBS (Phosphate Buffer Saline). OTA purification was performed using C-18 silicagel cartridges and eluted with 6 ml acidified methanol. OTA analysis was finally carried out through reverse HPLC with fluorescence detector.

The experiment followed a full crossed factorial design with 4 replicates. Analysis of variance of colonisation percentages and OTA content, as affected by antifungal treatment, inoculum

composition and time (if applicable), was carried out throughout a GLM analysis (SAS Institute Inc., Cary, NC, USA) and Duncan's multiple range tests were performed ($P<0{\cdot}05$).

RESULTS

Mould colonisation

The analysis of variance showed that fungicide treatments, co-inoculation, time and their interactions significantly affected grape colonisation by black aspergilli. In control grapes, the percentage of grape colonisation by *Aspergillus* section *Nigri* during dehydration rose above 50% by day 5 in almost all inocula and above 90% at the day 20 in all inocula (Fig. 1). The percentages of colonised grapes treated with fungicides also increased up to the end of the dehydration process, but they were always lower than 20% (a reduction of 96% of infection, after 5 days, and of 85% after 20 days, compared to the control treatment).

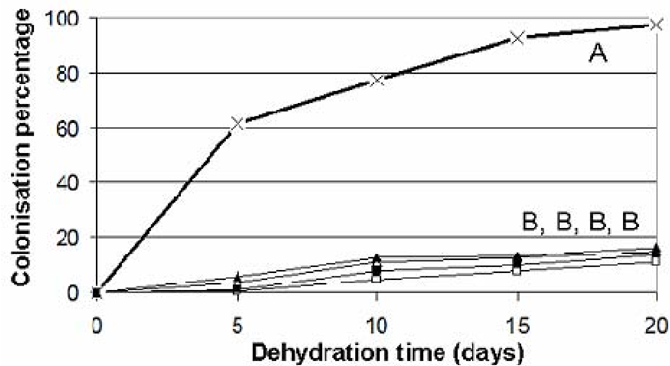


Fig. 1 Effect of four fungicide and control treatments in percentages of colonisation by *A.* section *Nigri* in grapes (mean of 5 inocula x 4 replicates). **x**, control grapes; □, Switch single application; ■, Switch double application; Δ, Chorus single application; ▲, Chorus double application.

Among fungicides, few differences were found, with Switch (5·74% mean infection) being slightly more efficient than Chorus (9·30% mean infection) (Table 2). No differences between single or double application of fungicides were observed. In general, non-inoculated grapes and grapes co-inoculated with *A. carbonarius* and *A. niger* had lower percentages of colonisation than the remaining inocula (Table 2).

Table 2 Mean grape infection percentages by *Aspergillus* section *Nigri* (average of 5 sampling times with 4 replicates) affected by the inoculum and fungicide. Different letters within columns mean significantly different infection percentages.

Fungicide	Control	*A. carb*	*A. carb + A. niger*	*A. carb + E. ams*	*A. carb + P. jan*
Control	64.1a	74.2a	84.6a	63.3a	59.1a
Chorus (1 application)	2.1b	2.9b	8.3b	24.3b	7.4b
Chorus (2 applications)	6.9b	19.9b	1.7b	9.1b	10.4b
Switch (1 application)	2.4b	3.7b	1.7b	11.4b	5.2b
Switch (2 applications)	2.5b	9.4b	6.1b	5.1b	9.4b

A. carb., A. carbonarius; E. ams., *E. amstelodami*; *P.jan.*, *P. janthinellum*

OTA content

Application of fungicides Chorus and Switch was effective in preventing OTA production both in inoculated and non-inoculated grapes (*P*<0·05) (Fig. 2). When grapes were only inoculated with *A. carbonarius*, a single application of Chorus was the sole treatment able to reduce the OTA content (Fig. 2c). Co-inoculation of *P. janthinelllum* resulted in a global OTA reduction in grapes regardless the antifungal treatments applied, but there were no significant differences among them (Fig. 2d).

Control treatments always showed the higher OTA concentrations, at any inoculum. In general, Chorus was the most effective antifungal treatment in reducing OTA content in grapes during dehydration.

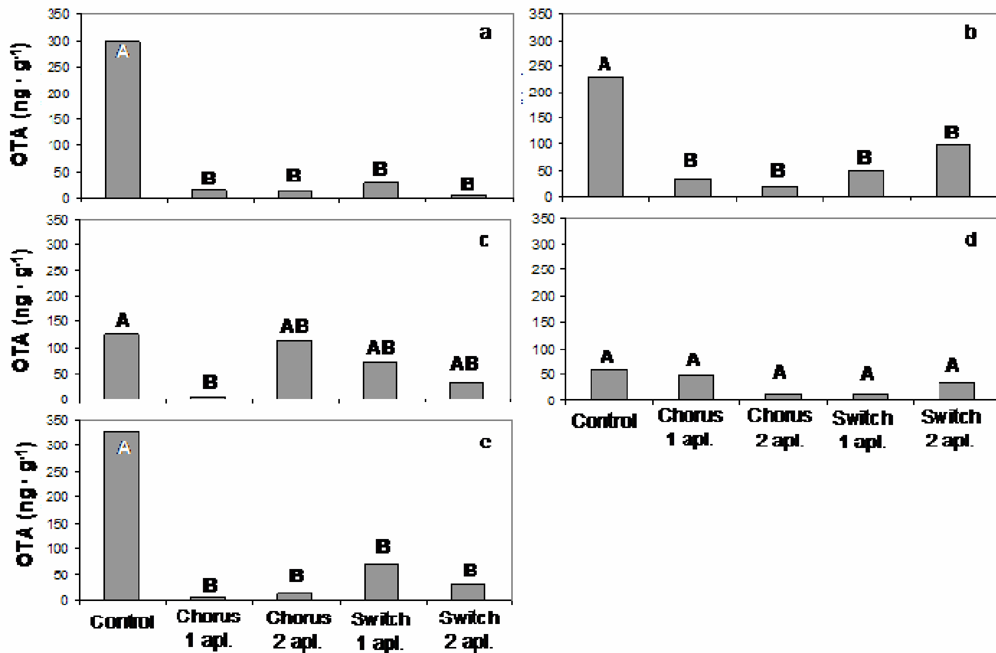


Fig. 2 Effect of four fungicide treatments and five inocula in final OTA content after grape dehydration. a, without inoculum; b, *Aspergillus carbonarius* with *Eurotium amstelodami*; c, *A. carbonarius*; d, *A. carbonarius* with *Penicillium janthinellum*; e, *A. carbonarius* and *A. niger* aggregate. Bars with the same letter inside a graph, are not statistically different.

DISCUSSION

Moscatel variety of the species *Vitis vinifera* is widely grown for production of wine and raisins. This variety of grape is grown around the world, including Romania, Italy, France, Greece, Australia, California, Hungary, Canada, but mainly in Portugal and Spain. Wines produced from this variety usually are sweet, which are known as Moscatel or Muscatel (Werner 2006).

Moulds and other rots can cause severe faults in wine that are not only detrimental to the organoleptic quality of wine quality but also to the sanitary quality. If wines contain higher levels of mycotoxins than those permitted for human consumption, their distribution may result compromised (Anon. 2005).

Fungicides are widely used to control decay of grapes, with Azoxystrobin, sulphur, fenarimol and kresoxim-methyl being some of the recommended ones (Adaskaveg *et al.* 2005). New pesticides such as fludioxonil and cyprodinil have been also recently used against black mould and grey rot in grapes. Their effectiveness has been previously tested on fresh grapes showing high efficacy

against *A. carbonarius* (Tjamos *et al.* 2004), *Penicillium expansum* and *Botrytis cinerea* (Frank *et al.* 2005).

In the present study fungicides applied to grapes before harvest were evaluated for their effect on grape colonisation by moulds and OTA production during dehydration. In all assays, the percentages of colonisation by *Aspergillus* section *Nigri* in grapes increased until the end of dehydration process, with control grapes showing the higher colonisation values (>90%). In general, all fungicides reduced the mean colonisation percentages below 20%, with Switch (single application) being the most useful. This finding agrees with that of Bellí *et al.* (2007) who tested the antifungal effect of Chorus and Switch *in vitro* against *Aspergillus* section *Nigri*. Thus, the present study shows that these two fungicides remain active on grapes during dehydration, effectively reducing OTA production by original mycobiota, present in non-inoculated lots, and also by the inoculated fungi.

Bellí *et al.* (2006 and 2007) found that the fungicide Switch to be the most effective in reducing infection of grapes by *A. carbonarius* and subsequent OTA production. Some other fungicides commonly used in grapes, such as azoxystrobin, dinocap and pencoazole, were also effective in reducing OTA accumulation (Lo Curto *et al.* 2004). In this study, all antifungal treatments to reduced the final OTA content in dehydrated grapes but there were no significant differences between them.

Success of these chemical treatments could depend on mycobiota composition of grapes. *E. amstelodami* has previously been shown to favouring OTA accumulation when interacting with *Aspergillus* section *Nigri* in grapes (Valero *et al.* 2007). In contrast, *P. janthinellum*, in accordance with Valero *et al.* (2006 and b), reduced the grape colonisation by *Aspergillus* section *Nigri* and OTA accumulation in grapes during dehydration.

In general, untreated grapes showed the highest percentages of colonisation and also higher OTA levels at any inoculum. There were no significant differences among the single or double application of Chorus or Switch. In conclusion, the four fungicides applied in field studied could reduce *Aspergillus* section *Nigri* growth and OTA production by *A. carbonarius* during grape dehydration.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2004-07549-05-01, grant BES-2003-2180 and Ramón y Cajal program) and to the Catalonian Government (CeRTA strategic project 2005-2006 “Seguritat biòtica y abiòtica dels aliments”, Generalitat de Catalunya) for their financial support. Authors also thank Syngenta Agro S.A. for its valuable collaboration.

REFERENCES

Abarca, M.J., Accensi, F., Bragulat, M.R., Castella, G. and Cabañes, F. (2003) Contamination in dried vine fruits from the Spanish market. *J Food Protect* **66**, 504-506.

Abrunhosa, L., Paterson, R.R.M., Kozakiewicz, Z., Lima, N. and Venâncio, A. (2001) Mycotoxin production from fungi isolated from grapes. *Lett Appl Microbiol* **32**, 240-242.

Adaskaveg, J.E., Holtz, B.A., Michailides, T.J. and Gubler, W.D. (2005) Fungicide efficacy and timing for deciduous tree fruit and nut crops and grapevines. Available: www.uckac.edu/plantpath/Fugicide-Efficacy/2004-EFFICACY-TIMING-3-04.pdf via the INTERNET. Accesed 2006 April.

Al-Mussallam, A. (1980) Revision of the Black *Aspergillus* Species. Ph.D. Thesis. Utrecht, Netherlands: University of Utrecht.

Anon. (2005) Commission regulation (EC) No 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2002 as regards ochratoxin A. Official Journal of the European Communities **L75**, 38-43.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J. (2002) Review: Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. *Food Sci Technol Int* **8,** 325-335.

Bellí, N., Pardo, E., Marín, S., Farré, G., Ramos, A.J. and Sanchis, V. (2004a) Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *J Sci Food Agr* **84,** 541-546.

Bellí, N., Ramos, A.J., Sanchis, V. and Marín, S. (2004b) Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. *Lett Appl Microbiol* **38**, 72-77.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J. (2006) Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes. *Food Addit Cont* **23**, 1021-1029.

Bellí, N., Marín, S., Argilés, E., Ramos, A.J. and Sanchis, V. (2007) Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes (*Vitis vinifera*). *J Food Protect* **70**, 157-163.

Boorman, G.A., Hong, H.L., Dieter, M.P., Hayes, H.T., Pohland, A.E., Staok, M. and Luster, M.I. (1984) Myelotoxicity and macrophage alteration in mice exposed to ochratoxin A. *Toxicol Appl Pharm* **72**, 304-312.

Bragulat, M.R., Abarca, M.L. and Cabañes, F.J. (2001) An easy screening method for fungi producing ochratoxin A in pure culture. *Int J Food Microbiol* **71**, 139-144.

Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castellá, G., Minguez, S. and Pons, A. (2002) What is the source of ochratoxin A in wine?. *Int J Food Microbiol* **79**, 213-215.

Cabras, P., Angioni, A., Garau, V.L., Melis, M., Pirisi, F.M., Minelli, E.V., Cabitza, F. and Cubeddu, M. (1997) Fate of some new fungicides (cyprodinil, fludioxonil, pyrimethanil, and tebuconazole) from vine to wine. *J Agr Food Chem* **45**, 2708-2710.

Cabras, P., Angioni, A., Garau, V.L., Melis, M., Pirisi, F.M., Cabitza, F. and Pala, M. (1998) Pesticide residues in raisin processing. *J Agr Food Chem* **46**, 2309-2311.

Cabras, P. and Angioni, A. (2000) Pesticide residues in grapes, wine, and their processing products. *J Agr Food Chem* **48**, 967-973.

Ciegler, A. (1972) Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. *Can J Microbiol* **18**, 631-636.

Franck, J., Latorre, B.A., Torres, R. and Zoffoli, J.P. (2005) The effect of preharvest fungicide and postharvest sulphur dioxide use on postharvest decay of table grapes caused by Penicillium expansum. *Postharvest Biol Tech* **37**, 20-30.

Gekle, M. and Silbernagi, S. (1993) Mechanism of ochratoxin A-induced reduction of glomerular filtration rate in rats. *J Pharmacol Exp Ther* **267**, 316-321.

Kittane, M., Stein, A.O., Berndt, W.O. and Phillips, T.D. (1984) Teratogenic effects of ochratoxin A in rats with impaired renal function. *Toxicology* **32**, 277-285.

Lo Curto, R., Pellicanò, T., Vilasi, F., Munafò, P. and Dugo, G. (2004) Ochratoxin A occurrence in experimental wines in relationship with different pesticide treatments on grapes. *Food Chem* **84**, 71-75.

MacDonald, S., Wilson, P., Barnes, K., Damant, A., Massey, R., Mortby, E.A. and Shepherd, M.J. (1999) Ochratoxin A in dried vine fruit: method development and survey. *Food Addit Cont* **16**, 253-260.

Magnoli, C., Violante, M., Combina, M., Palacio, G. and Dalcero, A. (2003) Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Lett. Appl. Microbiol.* **37**, 179-184.

Magnoli, C., Astoreca, A., Ponsone, L., Combina, M., Palacio, G., Rosa, C.A.R. and Dalcero, A.M. (2004) Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Lett Appl Microbiol* **39**, 326-331.

Ostry, V., Ruprich, J. and Skarkova, J. (2002) Raisins, ochratoxin A and human health. *Mycotoxin Res* **18**, 178-182.

Pavlovic, M., Plestina, R. and Krogh, P. (1979). Ochratoxin A contamination of foodstuffs in an area with Balkan (endemic) nephropathy. *Acta Pathol Micro Im B* **87**, 243-246.

Petkova-Bocharova, T. and Castegnaro, M. (1991) Ochratoxin A in human blood in relation to Balkan endemic nephropathy and urinary tract tumors in Bulgaria. *IARC Sci Publ* **115**, 135-142.

Pitt, J.I. and Hocking, A.D. (1997) *Fungi and food spoilage* 2nd edn. ed. Blackie Academic and Professional. pp.110-114. Cambridge: University Press.

Pfohl-Leszkowicz, A. and Manderville, R.A. (2007) Ochratoxin A: and overview on toxicity and carcinogenicity in animals and humans. *Mol Nutr Food Res* **51**, 61-99.

Romero, S.M., Comerio, R.M., Larumbe, G., Ritieni, A., Vaamonde, G., Fernández, V. (2005) Toxigenic fungi isolated from dried vine fruits in Argentina. *Int. J. Food Microbiol.* **104**, 43-49.

Tjamos, S.E., Antoniou, P.P., Kazantzidou, A., Antonopoulos, D.F., Papageorgiou, I. and Tjamos, E.C. (2004) *Aspergillus niger* and *Aspergillus carbonarius* in Corinth raisins and wine-producing vineyards in Greece: population composition, ochratoxin A production and chemical control. *J Phytopathol* **152**, 250-255.

Ueno, Y., Kawamura, O. and Sugiera, Y. (1991) Use of monoclonal antibodies, enzymelinked immunosorbent assay and immunoaffinity column chromatography to determine ochratoxin A in porcine sera, coffee products and toxin-producing fungi. In *Mycotoxins, endemic nephropathy and urinary tract tumours* ed. Castegnaro, M., Plestina, R., Dirheimer, G., Chernozemsky, I.N., Bartsch, H. pp. 71-75. Lyon: IARC Scientific Publication No. 115.

Valero, A., Marín, S., Ramos, A.J. and Sanchis, V. (2005) Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors. *Lett Appl Microbiol* **41**, 196-201.

Valero, A., Farré, J.R., Sanchis, V., Ramos, A.J. and Marín, S. (2006) Effects of fungal interaction in ochratoxin A production by *A. carbonarius* at different temperatures and a_w. *Int J Food Microbiol* **110**, 160-164.

Valero, A., Oliván, S., Marín, S., Sanchis, V. and Ramos, A.J. (2007) Effect of *intra* and interespecific competence in OTA production by A. section *Nigri* in grapes during dehydration. *Food Microbiol* **24**, 254-259.

Van der Merwe, K.J., Steyn, P.S. and Fourie, L. (1965) Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus*. Wilh. London: *Nature* **205**,1112-1113.

Werner, P.G. (2006) Muscat (grape and wine). Wikipedia. [internet] Available from: http://en.wikipedia.org/wiki/Muscat_%28grape_and_wine%29] Accesed:2006 August).

Study X:

Mycelial growth and OTA production by *Aspergillus* section *Nigri* on simulated grape medium in modified atmospheres

Ana Valero[1], Mariam Begum[2], Ailsa D. Hocking[2], Sonia Marín[1], Antonio J. Ramos[1], Vicente Sanchis[1]

[1]Food Technology Department, University of Lleida, UTPV-CeRTA. Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain

[2]Food Science Australia, PO Box 52, North Ryde, NSW 1670, Australia

ABSTRACT

Aims: To evaluate the impact of modified atmosphere packaging on *in vitro* growth of *A. carbonarius* and *A. niger*, and possible effects on ochratoxin A (OTA) biosynthesis.

Methods and Results: *A. carbonarius* and *A. niger* were grown on a synthetic grapejuice medium (SNM) and packaged in combinations of controlled O_2 (1 and 5%) and CO_2 levels (0 and 15%), and in air as a control. Colony diameters were recorded every three days up to 21 days and OTA was analysed after 7, 14 and 21 days. The greatest reductions in mycelial growth rate were observed at 1% O_2 followed by 1% O_2 / 15% CO_2, whereas 5% O_2 stimulated growth of all isolates. OTA production by *A. carbonarius* and *A. niger* isolates was minimised at 1% O_2 / 15% CO_2 and 1% O_2, respectively, after 7 days of incubation. Maximal OTA accumulation after 7 days was observed for all isolates in the control pack and at 5% O_2.

Conclusions: Of the atmospheres tested, only 1% O_2 combined with 15% CO_2 consistently reduced fungal growth and OTA synthesis by *A. carbonarius* and *A. niger*.

Significance and Impact of Study: Storage under modified atmospheres is unlikely to be suitable as the sole method for OTA minimisation and grape preservation; other inhibitory factors are necessary.

Publication: *Journal of Applied Microbiology* (submitted).

INTRODUCTION

In 1965, Van der Merwe et al. demonstrated that the fungus *Aspergillus ochraceus* produced a metabolite with toxic properties, ochratoxin A (OTA); this toxin has recently become of increasing relevance. In humans, dietary exposure to OTA has been associated with Balkan endemic nephropathy (BEN) (Marquardt and Frohlich 1992), a chronic kidney disease linked to tumours of the renal system. OTA has also been reported to be genotoxic, carcinogenic, teratogenic and immunosuppressive in a number of mammalian species (Ringot et al. 2006). In 1993, the International Agency for Research in Cancer classified OTA as a possible human carcinogen (group 2B) (IARC 1993).

OTA has been detected frequently in cereal products (Jørgensen 2005) and also in grapes, raisins and wine (Battilani and Pietri 2002; Bellí et al. 2004; Magnoli et al. 2004). Species belonging to *Aspergillus* section *Nigri*, particularly *A. carbonarius* and *A. niger*, have been reported to be the source of OTA in grapes and raisins (Abarca et al. 2003; Bellí et al. 2004). Bellí et al. (2005) found that 7.7–10.8% of the total mycobiota from grapes belonged to *Aspergillus* section *Nigri*, primarily comprising isolates of *A. carbonarius* (7–29% of section *Nigri* isolates) and the *A. niger* aggregate (53–75 %). *Aspergillus* spp. together with *Botrytis cinerea* (grey mould) and *Rhizopus* spp. are the primary postharvest pathogens of table grapes in many countries (Crisosto et al. 2002; Hoogerwerf et al. 2002)

Preharvest decay of grapes is typically minimised through use of fungicides (Franck et al. 2005; Bellí et al. 2007), some of which also successfully reduced growth of ochratoxigenic moulds and OTA synthesis (Lo Curto et al. 2004; Tjamos et al. 2004; Bellí et al. 2006). However, consumer demands for reduced use of chemical preservatives in food has prompted the search for alternatives to fungicides in the control of post-harvest diseases. Likewise, chemicals usually applied during grape storage, such as sulphur dioxide, carbonate and bicarbonate salts or disinfectants (Gabler et al. 2001; Franck et al. 2005) are becoming less favoured. New strategies to reduce decay of grapes during storage include enhancement of natural plant resistance by stimulating resveratrol synthesis with UV radiation, or the use of antifungal plant extracts (Ali et al. 1999; Gonzalez-Ureña et al. 2003).

Storage under controlled atmospheres is another method that does not leave residual products in food, and has also been used to good effect. Sarig et al. (1996) reported that ozone controlled postharvest decay of table grapes caused by *Rhizopus stolonifer*, and Zoffoli et al. (1999) used chlorine gas generators in modified atmosphere packaging to prevent *Botrytis cinere*a on table grapes.

Adjusting the concentrations of oxygen and carbon dioxide, in particular, elevated CO_2, has been shown to reduce mould growth in a number of commodities (Jacxsens et al. 2001; Taniwaki et al. 2001; Cairns-Fuller et al. 2005). However, Crisosto et al. (2002) found that controlling *Botrytis*

decay in table grapes solely through elevated CO_2 (≥ 15% CO_2) also reduced grape quality, through production of “off flavours” and rachis and berry browning. They suggested the use of controlled atmospheres of 15% CO_2 in combination with decreased O_2 (3%, 6% or 12%) to limit *Botrytis* rot development without adversely affecting quality. Similarly, Berry and Aked (1997) observed an inhibition of *Botrytis cinere*a by exposure to 15% CO_2 / 5% O_2. Such findings form the basis of recommendations for the postharvest management and storage of grapes under low O_2 (2–5%) in combination with medium CO_2 levels (1–3%) at 0ºC (Sydney Postharvest Laboratory 2006).

These and other studies on the application of modified atmospheres in preventing mould growth have not examined their effect on mycotoxin production.

This study evaluates the impact of low O_2 and elevated CO_2 in modified atmosphere packaging (MAP) on *in vitro* growth of ochratoxigenic fungi commonly isolated from grapes (*A. carbonarius* and *A. niger*), and possible effects on OTA biosynthesis.

MATERIAL AND METHODS

Microorganisms and growth medium

Two ochratoxigenic isolates of *A. carbonarius* from grapes and raisins collected in Spanish vineyards (UdLTA 3.122, UdLTA 3.90; accessioned into the collection of the Food Technology Department of the University of Lleida, Spain) and two ochratoxigenic *A. niger* isolates from grapes in Australian vineyards (FRR 5694, FRR 5695; Food Science Australia, North Ryde; Leong et al. 2006) were used in this experiment.

Fungi were grown on SNM (synthetic nutrient medium), modelled on grape composition between veraison and ripeness (Delfini 1982), but with glucose and fructose concentrations adjusted to reduce the a_w to 0.97 (Valero et al. 2005), similar to that of very ripe grapes. Plates were inoculated centrally with a single needle point dipped in a spore suspension (10^6 spores·ml^{-1}) prepared from cultures grown on CYA (Czapek Yeast Autholysate Agar) at 25ºC for 7 days.

Modified atmosphere packaging

Two concentrations of O_2 (1 and 5%) were tested in combination with two concentrations of CO_2 (0 and 15%), and the gas balance comprising N_2 (Gas Mixer KM 100-3M, Witt-Gasetechnik GmbH & Co KG. Sicherheitsarmaturen und apparatebau. Witten, Germany). Control packs contained air. Each treatment pack comprised single plates of each of the four strains and 2 l of the desired gas mixture inside plastic bags, thermally sealed (EKKA 840 volts, M. Eckersley Pty. Ltd. Melbourne, Australia). The packaging film used for this experiment was ethylene vinyl alcohol (EVOH) mixed (multi-laminated) high barrier vacuum film (Holmes Packaging, Cleveland, Qld, Australia). The oxygen transmission rate was <8 cc/m^2/24 hr/atm at 23ºC and 75% RH.

The moisture vapour transmission rate was <6 g/m^2/24 hr/atm at 38ºC and 90% RH. Four replicate packs of each treatment were prepared and incubated at 25ºC.

In a preliminary assay, packs (2 l capacity) were flushed with four initial gas mixtures (air, 5% O_2, 1% O_2, 5% O_2 / 15% CO_2), balanced with N_2, and fungal growth monitored at 25ºC for 24 days. After 12 days of incubation, the gas composition was tested and O_2 had been substantially or fully depleted (Table 1). Therefore, for the final experiment, gas mixtures in the sealed packs (2 l capacity) were monitored daily (Gaspace 2, Systech Instruments Ltd. Thame, Oxfordshire, UK) and the atmospheres maintained by re-flushing the packs with fresh gas mixture as necessary.

Table 1. Gas mixtures in the preliminary assay after 12 days. N_2 balance.

Packs		Day 0	Day 12
air (control)	% O_2	20.06	0.14
	% CO_2	0.00	19.76
5% O_2	% O_2	4.89	0.13
	% CO_2	0.00	8.73
1% O_2	% O_2	0.78	0.13
	% CO_2	0.00	2.15
5% O_2 / 15% CO_2	% O_2	4.90	0.00
	% CO_2	14.46	18.2

Mould growth and ochratoxin A analysis

Colony diameters were recorded every three days up to 21 days. OTA was analysed after 7, 14 and 21 days of incubation, from a total of 240 plates.

OTA was extracted as previously described (Bragulat et al. 2001), each extract (1 ml) was filtered (Millex-HV 0.45 µm 25 mm, Millipore Corporation. Bedford, U.S.A.) and a volume of 5 µl analysed by HPLC (Schimadzu Corporation, Japan). OTA was detected using a reverse-phase column (Ultracarb 5µm ODS (30) 250 x 4.60 mm. Phenomenex, Inc. Torrance, CA, U.S.A.) with mobile phase 55% acetonitrile and 45% aqueous acetic acid (2%), at a flow rate of 1 ml·m^{-1}, and with post column ammoniation (0.1 ml·m^{-1} of 12.5% aqueous ammonia). Excitation and emission wavelengths were set to 385 and 435 nm respectively (Leong et al. 2006). OTA was quantified by comparison with a calibration curve of OTA standards (Sigma-Aldrich, St Louis, MO, U.S.A) using the Shimadzu CLASS-VP software v. 6.14 SP2 (Shimadzu Corporation, Japan). OTA accumulated per colony was calculated multiplying OTA amount observed in an area unit under the colony (ng·cm^{-2}) by the area of colony (cm^2).

Statistical analysis

Periodical measures of colony radii were adjusted to the Gompertz equation:

Colony diameter (mm) = D_{max} * (-exp ((2.718 * K / D_{max}) (Lag - t) + 1)); where D_{max}, maximum colony diameter (mm); K, growth rate (mm · day^{-1}); Lag, lag phase (days); t, incubation time (days). Analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA).

Pearson correlation analysis between colony diameters and OTA accumulation, and related Duncan's multiple range tests of main effects and their interactions were performed using SAS software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Preliminary results: Closed packs

Growth of all microorganisms ceased when the O_2 was depleted to below 0.14%, a concentration that likely occurred at different time points, depending on the growth rates of the fungi (Table 1). Fungi in the control pack (20% O_2) grew more rapidly initially (data not shown), but growth ceased after 9 days. Fungi in 5% O_2 packs stopped growing after 10 days, and in 1% O_2 and 5% O_2 / 15% CO_2 packs, after 12 days. Higher O_2 concentrations promoted higher growth rates initially; consequently higher O_2 amounts were needed by the fungi to support this rapid growth, leading, in turn, to a faster depletion of O_2. Colony diameters attained after 12 days were smallest at 1% O_2 followed by 5% O_2 / 15% CO_2 (data not shown). OTA production by *A. carbonarius* isolates after 7 days generally appeared to decrease with decreasing O_2 concentration, and was further suppressed by 15% CO_2. Surprisingly, *A. niger* produced more OTA in 5% O_2 / 15% CO_2 than in the control (air) packs.

Mould growth

The main effects (strain, O_2, CO_2, time) and their interactions were significant (P < 0.05; Table 2). Growth of *A. carbonarius* appeared to be strongly influenced by O_2 concentration (P < 0.0001), whereas growth of *A. niger* was more sensitive to changes in CO_2 at the concentrations tested (P < 0.05).

Table 2. F values of main effects and their interactions in colony diameters and OTA accumulation of all isolates grouped (*Aspergillus* spp.) and the four strains separately.

	Colony diameter				
Effects	***Aspergillus* spp.**	***A. carbonarius* UdLTA 3.122**	***A. carbonarius* UdLTA 3.90**	***A. niger* FRR 5694**	***A. niger* FRR 5695**
Strain	47.85**	-	-	-	-
O_2	11.85**	97.34**	404.23**	1.22ns	1.74ns
CO_2	19.55**	7.82*	217.22*	4.21*	71.25**
Time	221.03**	316.01**	383.75**	21.69**	605.96**
Strain x O_2	2.94*	-	-	-	-
Strain x CO_2	3.04*	-	-	-	-
O_2 x CO_2	18.99**	28.86**	95.21**	4.90*	0.03ns
	ng OTA / colony				
Effects	***Aspergillus* spp.**	***A. carbonarius* UdLTA 3.122**	***A. carbonarius* UdLTA 3.90**	***A. niger* FRR 5694**	***A. niger* FRR 5695**
Strain	11.91**	-	-	-	-
O_2	10.30**	10.89**	6.32*	8.48*	5.09*
CO_2	25.25**	4.11*	34.29**	15.56*	2.91ns
Time	1.82ns	1.38ns	1.87ns	0.29ns	0.74ns
Strain x O_2	10.75**	-	-	-	-
Strain x CO_2	19.09**	-	-	-	-
O_2 x CO_2	4.04*	3.37ns	8.15*	3.86ns	0.17ns

*, *P* value ≤ 0.05; **, *P* value < 0.0001; ns, not significant.

After 21 days, *A. niger* isolates attained the maximum colony diameter (85 mm), however colonies of *A. carbonarius* only reached comparable dimensions in 5% O_2, 5% O_2 / 15% CO_2 and in air, but these results were strain-dependent (Table 3). Reductions in colony diameters (D_{max}) were most pronounced in 1% O_2. High CO_2 (15%), in combination with either 1% or 5% O_2, reduced growth rates (K) of all *Aspergillus* spp. (P < 0.05) and D_{max} of *A. carbonarius*, whereas it had no significant effect on D_{max} of *A. niger* (Table 3). Hypoxia effects varied with species; 1% O_2 reduced the D_{max} *A. carbonarius* and 5% O_2 stimulated growth rates (K) of all isolates.

The shortest *Lag* phase for *A. carbonarius* isolates was observed in 1% O_2 / 15% CO_2 and, for *A. niger* isolates, in control packs.

Table 3. Parameters in the Gompertz equation – Colony diameter (mm) = N_0 + D_{max}. * exp (($2.718 * K / D_{max}$)($Lag - t$) + 1) -. Dmax, maximum colony diameter (mm); *K*, growth rate (mm·day^{-1}); *Lag*, lag phase (days). Values represent the average ± standard error.

Fungi	MAP	Dmax (mm)	K (mm - day^{-1})	Lag (days)	R^2
A. carbonarius UdLTA 3.122	air	80.8 ± 2.4	12.16 ± 0.79	3.51 ± 0.28	0.986
	1% O_2	49.9 ± 1.9	12.09 ± 1.31	3.54 ± 0.28	0.974
	5% O_2	85.8 ± 1.8	18.49 ± 1.03	3.64 ± 0.16	0.993
	1% O_2 +15% CO_2	68.1 ± 8.1	4.69 ± 0.40	0.00 ± 1.41	0.969
	5% O_2 +15% CO_2	82.2 ± 3.9	10.02 ± 0.95	3.64 ± 0.52	0.969
A. carbonarius UdLTA 3.90	air	84.1 ± 1.7	14.42 ± 0.71	3.36 ± 0.18	0.993
	1% O_2	51.1 ± 1.9	10.35 ± 0.99	3.49 ± 0.29	0.978
	5% O_2	85.3 ± 1.5	22.23 ± 1.10	3.75 ± 0.13	0.995
	1% O_2 +15% CO_2	87.5 ± 29.1	4.27 ± 0.46	0.00 ± 2.77	0.941
	5% O_2 +15% CO_2	79.7 ± 3.7	9.37 ± 0.84	3.81 ± 0.52	0.972
A. niger FRR 5694	air	85.7 ± 1.0	19.50 ± 0.85	2.27 ± 0.11	0.997
	1% O_2	84.7 ± 2.8	24.89 ± 2.64	3.97 ± 0.25	0.982
	5% O_2	85.4 ± 0.9	23.28 ± 0.67	3.35 ± 0.06	0.998
	1% O_2 +15% CO_2	85.0 ± 2.1	15.90 ± 1.03	2.94 ± 0.20	0.989
	5% O_2 +15% CO_2	87.2 ± 2.5	16.98 ± 1.38	3.97 ± 0.26	0.990
A. niger FRR 5695	air	85.7 ± 1.1	18.95 ± 0.88	2.28 ± 0.12	0.997
	1% O_2	84.5 ± 5.9	18.57 ± 3.16	3.64 ± 0.52	0.935
	5% O_2	85.1 ± 0.8	42.32 ± 1.30	4.10 ± 0.06	0.999
	1% O_2 +15% CO_2	89.4 ± 4.0	9.05 ± 0.73	4.35 ± 0.53	0.979
	5% O_2 +15% CO_2	87.6 ± 2.6	15.33 ± 1.13	4.13 ± 0.28	0.986

OTA synthesis

The effect of various O_2 and CO_2 concentrations on OTA accumulation varied according to the strain (Table 2). There was less accumulation of OTA by *A. carbonarius* isolates in 1% O_2 / 15% CO_2 after 7 days than in other gas mixtures or at later sampling points (Figure 1). OTA production by *A. carbonarius* UdLTA 3.90 was also minimal in 5% O_2 / 15% CO_2 after 7 days. In contrast, the lowest OTA accumulation was observed for *A. niger* isolates in 1% O_2 after 7 days. At that sampling point, maximal OTA accumulation was observed for all isolates in the control pack (air gas mixture) or in 5% O_2.

In general, increasing OTA accumulation was observed over time in 1% O_2 and often also in 1% O_2 / 15% CO_2, paralleling the increase in colony diameter. Of the conditions tested, 1% O_2 / 15% CO_2 appeared to be most inhibitory to OTA production. At 5% O_2 / 15% CO_2, OTA concentrations were fairly stable over time, suggesting that poor production and/or degradation of OTA could be occurring simultaneously, as the colony was still increasing in diameter. Finally, in the air controls (20% O_2), OTA was greatest at the first sampling point then decreased with time.

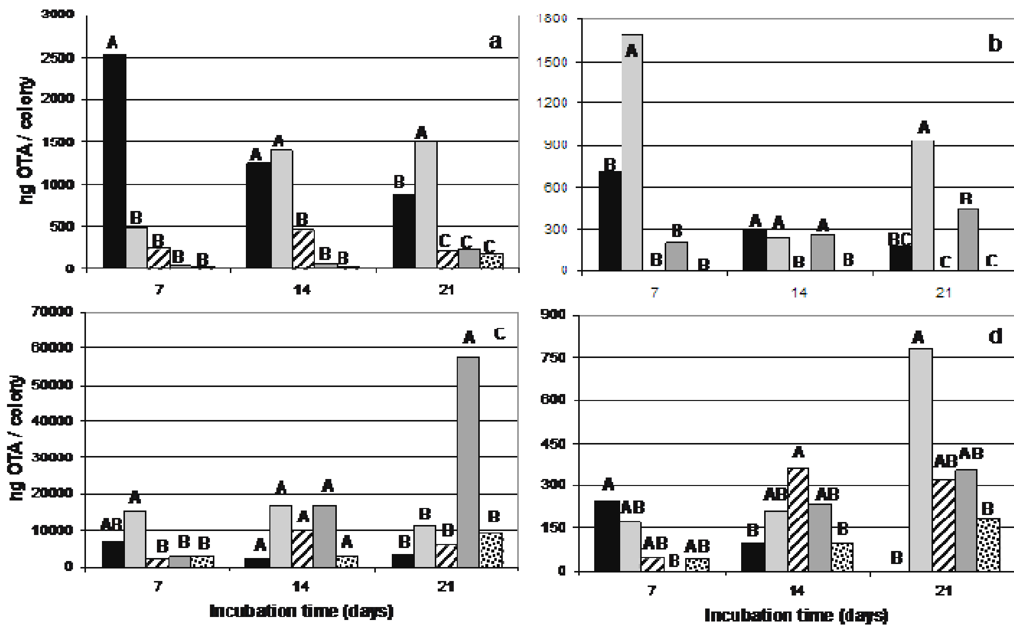


Figure 1. Ochratoxin A accumulation over time (7, 14 and 21 days) as affected by modified atmospheres: 20% O_2, 1% O_2, 1% O_2 / 15% CO_2, 5% O_2, 5% O_2 / 15% CO_2. a, *Aspergillus carbonarius* UdLTA 3.122; b, *A. carbonarius* UdLTA 3.90; c, *A. niger* FRR 5694; d, *A. niger* FRR 5695. Bars with same capital letter within each sampling day are not statistically different (*P* value < 0.05).

Correlation analysis

Fungi grown in 1% O_2 or in 1% O_2 / 15% CO_2 showed a positive and significant correlation between diameter of colonies on day 6 and OTA accumulation after 7 days (Pearson correlation coefficients, $P < 0.05$).

DISCUSSION

For aerobic fungi, O_2 depletion is a severe limiting factor for growth and metabolite production. *A. niger* isolates appeared to be slightly more resistant than *A. carbonarius* to the modified atmospheres tested here.

In this study the growth rates of all isolates were maximal in 5% O_2 / 95 % N_2 (in the preliminary experiment, the maximum diameters attained were similar in 5% or 20% O_2). Rahardjo et al. (2005) observed that O_2 concentrations at 5% had little effect on growth of *A. oryzae* relative to air; below 3%,a strong effect was observed, and this was even more pronounced at <1% O_2. Although some authors (Crisosto et al. 2002; Rahardjo et al. 2005) found that fungal behaviour

did not vary between 5% and 20% O_2, the observation in this study that maximum growth rate occurred in 5% O_2 is unique. One possible explanation for more rapid growth at 5% than at 20% O_2 could be that 5% O_2 is sufficient for primary metabolism, but is limiting for other undetermined metabolic pathways; thus resources and energy are solely channelled into vegetative development of the fungus. Another possible explanation is that *Aspergillus* species evolved at a time when atmospheric oxygen levels were closer to 5% than the current 21%. To investigate these theories this would require further work.

Effective concentration thresholds in O_2 needed to limit growth of black Apergilli appear to be below 1% O_2, as this concentration only reduced growth slightly and in a strain dependent manner; however, the maximum diameter attained by *A. carbonarius* isolates was somewhat reduced by 1% O_2. Hall and Denning (1994) reported that some representative *Aspergillus* species (*A. flavus, A. fumigatus*, *A. nidulans*, *A. niger*, and *A. terreus*) required a minimum of 0.1 and 0.5% O_2 for growth on Hall's medium and Potato Dextrose Agar, respectively. Such levels in grapes stored at in closed bags or in controlled atmospheres might hypothetically favour initiation of fermentations by yeasts, leading to rejection of the product.

High CO_2 partial pressures (15%) in combination with either 1% or 5% O_2 caused the four *Aspergillus* isolates tested here to grow 13–70% slower than in air packs. Pateraki et al. (2005) found that 50% CO_2 completely halted *A. carbonarius* growth after 5 days; however, after 10 days, growth was no longer controlled as effectively. Similarly, Paster et al. (1983) found that growth of *Aspergillus ochraceus* colonies was only partially inhibited by 60% CO_2, and no growth occurred at 80% or 100% CO_2. It has also been reported that concentrations above 15% CO_2 produce undesirable effects on quality of grapes (Crisosto et al. 2002). Hence, the combination of 15% CO_2 / 1% O_2 that was most effective in minimising growth of the black *Aspergillus* isolates would, hypothetically, be amenable to use for grapes.

Any proposed modified atmosphere regimen should also minimise OTA production by fungi in grapes. Maximal accumulation of OTA was generally observed for all isolates in 5% and 20% O_2 after 7 days, whereas after the same period, 1% O_2 / 15% CO_2 significantly reduce OTA production by *A. niger* and *A. carbonarius*. Paster et al. (1983) found no significant effect of low O_2 concentrations (1 and 5%) on OTA production by *A. ochraceus*, whereas the black *Aspergillus* spp. in this study were often stimulated to produce and accumulate OTA in these conditions. Paster et al. (1983) also reported that low O_2 levels in combination with high CO_2 levels (10 and 20%) reduced OTA synthesis up to 25% relative to the control, and even resulted in total inhibition at 1% O_2 / 20% CO_2 after 7 days, We observed a similar response in this study for *A. carbonarius* UdLTA 3.90. In contrast, in a study of *A. carbonarius* by Pateraki et al. (2007), high CO_2 concentrations (25% and 50%) in combination with standard O_2 levels (20%) did not significantly reduce OTA production.

In our study, OTA gradually accumulated during growth in 1% O_2, with or without increased CO_2, both in *A. carbonarius* and *A. niger*. However, at 20% O_2 (air), in general, and at 5% O_2, in some cases, the toxin content in the simulated grape medium decreased, possibly due to degradation by the fungus. Previous studies of the kinetics of ochratoxin production *in vitro* also noted a reduction in toxin after 7 days at 25ºC (Mitchell et al. 2003; Valero et al. 2006; Leong et al. 2006). OTA degradation has been previously reported by *A. niger* and *A. carbonarius* isolates (Stander et al. 2000; Varga et al. 2000; Bejaoui et al. 2006). Cairns-Fuller et al. (2005) observed a significant inhibition of OTA production by *P. verrucosum* by 50% CO_2, although toxin gradually accumulated over time and subsequent degradation was not observed. Our findings suggest that low O_2 concentrations (1% or below) inhibit or greatly reduce the ability of *A. carbonarius* and *A. niger* to degrade OTA, and this catabolic pathway may not be regulated by CO_2.

High CO_2 concentrations, in general, reduced OTA production by the isolates tested here, but at 7 days, differences between species were observed. It appears that toxin production by some species of *Penicillium* and *Aspergillus* can be controlled by elevated CO_2 levels; however, the inhibitory concentrations may differ among species (Paster et al. 1983) and strains, as observed in this study.

OTA is a fungal product from secondary metabolism, whereas growth is regulated in primary metabolism; thus, faster fungal growth may not necessarily result in greater OTA synthesis. In this study, 5% O_2 stimulated both *A. carbonarius* growth and OTA synthesis in some cases; however, a significant correlation between growth and OTA accumulation was not observed. It is noteworthy that modified atmosphere packaging in 1% O_2 combined with 15% CO_2 reduced both fungal growth and OTA synthesis by *A. carbonarius* and *A. niger*.

Overall, not all the modified atmospheres proposed solely to prevent grape decay would also inhibit OTA production by black *Aspergillus* spp. over an extended storage period. To minimise the risk of OTA contamination during grape storage, modified atmospheres, for instance 1% O_2 / 15% CO_2, could be used in combination with temperatures below 10ºC, *viz.* the temperature threshold for OTA production (Mitchell et al., 2004). Storage at low temperatures would also prevent undesirable fermentations at low O_2 concentrations and enzymatic deterioration of fruit (Sydney Postharvest Laboratory 2006; Deng et al. 2006).

REFERENCES

Abarca, M.J., Accensi, F., Bragulat, M.R., Castella, G. and Cabañes, F.J. (2003) Contamination in dried vine fruits from the Spanish market. *J Food Protect* **66**, 504-506.

Ali, M.I., Shalaby, N.M.M., Elgamal, M.H.A. and Mousa, A.S.M. (1999) Antifungal effects of different plant extracts and their major components of selected *Aloe* species. *Phytother Res* **13**, 401-407.

Battilani, P. and Pietri, A. (2002) Ochratoxin A in grapes and wine. *Eur J Plant Pathol* **108**, 639-643.

Bejaoui, H., Mathieu, F., Taillandier, P. and Lebrihi, A. (2006) Biodegradation of ochratoxin A by *Aspergillus* section *Nigri* species isolated from French grapes : a potential means of ochratoxin A decontamination in grape juices and musts. *FEMS Microbiol Lett* **255**, 203-208.

Bellí, N., Marín, S., Duaigües, A., Ramos, A.J. and Sanchis, V. (2004) Ochratoxin A in wines, musts and grape juices from Spain. *J Sci Food Agr* **84**, 591-594.

Bellí, N., Mitchell, D., Marín, S., Alegre, I., Ramos, A.J., Magan, N. and Sanchis, V. (2005) Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. *Eur J Plant Pathol* **113**, 233-239.

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J. (2006) Impact of fungicides on *A. carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes. *Food Addit Contam* **63**, 1021-1029.

Bellí, N., Marín, S., Argilés, E., Ramos, A.J. and Sanchis, V. (2007) Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes (*Vitis vinifera*). *J Food Protect* **70**, 157-163.

Berry, G. and Aked, J. (1997) Controlled atmosphere alternatives to the postharvest use of sulphur dioxide to inhibit the development of *Botrytis cinerea* in table grapes. In *Postharvest Horticulture Series* ed. Kader, A.A. No. 17, p. 100. CA 97 Proc., Vol. 3. Postharvest Outreach Program. Univ. Of California. Davis.

Bragulat, M.R., Abarca, M.L. and Cabañes, F.J. (2001) An easy screening method for fungi producing ochratoxin A in pure culture. *Int J Food Microbiol* **71**, 139-144.

Cairns-Fuller, V., Aldred, D. and Magan, N. (2005) Water, temperature and gas composition interactions affect growth and ochratoxin A production by isolates of *Penicillium verrucosum* on wheat grain. *J Appl Microbiol* **99**, 1215-1221.

Crisosto, C.H., Garner, D. and Crisosto, G. (2002) High carbon dioxide atmospheres affect stored 'Thomson Seedless' table grapes. *Hort Sci* **37**, 1074-1078.

Delfini, C. (1982) In *Tecnica di microbiologia enologica* ed. Scicalpi, L. Rome.

Deng, Y., Wu, Y. and Li, Y. (2006) Physiological responses and quality attributes of 'Kyoho' grapes to controlled atmospheres storage. *LWT-Food Sci Technol* **39**, 584-590.

Franck, J., Latorre, B.A., Torres, R. and Zoffoli, J.P. (2005) The effect of preharvest fungicide and postharvest sulphur dioxide use on postharvest decay of table grapes caused by *Penicillum expansum*. *Postharvest Biol Tec* **37**, 20-30.

Gabler, F.M. and Smilanick, J.L. (2001) Postharvest control of table grape grey mould on detached berries with carbonate and bicarbonate salts and disinfectants. *Am J Enol Viticult* **52**, 12-20.

Gonzalez-Ureña, A., Orea, J.M., Montero, C. and Jiménez, J.B. (2003) Improving postharvest resistance in fruits by external application of trans-resveratrol. *J Agr Food Chem* **51**, 82-89.

Hall, L.A. and Denning, D.W. (1994) Oxygen requirements of *Aspergillus* species. *J Med Microbiol* **41**, 311-315.

Hoogerwerf, S.W., Kets, E.P.W. and Dijksterhuis, J. (2002) High-oxygen and high-carbon dioxide containing atmospheres inhibit growth of food associated moulds. *Lett Appl Microbiol* **35**, 419-422.

IARC (1993) Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. In *Monographs on the Evaluation of Carcinogenic Risks to Humans*, Vol. 56, pp. 489-521. Lyon: International Agency for Research on Cancer.

Jacxsens, L., Devlieghere, f., Van der Steen, C. and Debevere, J. (2001) Effect of high oxygen modified atmosphere packaging on microbial growth and sensorial qualities of fresh-cut produce. *Int J Food Microbiol* **71**, 197-210.

Jørgensen, K. (2005) Occurrence of ochratoxin A in commodities and processed food. A review of EU occurrence data. *Food Addit Contam* **22**, 26-30.

Leong, S.L., Hocking, A.D. and Scott, E.S. (2006) Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium. *Int J Food Microbiol* **110**, 209-216.

Lo Curto, R., Pellicanò, T., Vilasi, F., Munfalò, P. and Dugo, G. (2004) Ochratoxin A occurrence in experimental wines in relationship with different pesticide treatments on grapes. *Food Chem* **84**, 71-75.

Magnoli, C., Astoreca, A., Ponsone, L., Combina, M., Palacio, G., Rosa, C.A.R. and Dalcero, A.M. (2004) Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Lett Appl Microbiol* **39**, 326-331.

Marquardt, R.R. and Frolich, A.A. (1992) A review of recent advances in understanding ochratoxicosis. *J Anim Sci* **70**, 3968-3988.

Mitchell, D., Aldred, D. and Magan, N. (2003) Impact of ecological factors on the growth and ochratoxin A production by *Aspergillus carbonarius* from different regions of Europe. *Aspects Appl Biol* **68**, 109-106.

Mitchell, D., Parra, R., Aldred, D. and Magan, N. (2004) Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. *J Appl Microbiol* **97**, 439-445.

Paster, N., Lisker, N. and Chet, I. (1983) Ochratoxin A production by *Aspergillus ochraceus* Wilhelm grown under controlled atmospheres. *Appl Environ Microbiol* **45**, 1136-1139.

Pateraki, M., Dekanea, A., Mitchell, D., Lydakis, D. and Magan, N. (2005) Potential for control of *A. carbonarius* strains from vine fruits using sulphur dioxide or controlled atmosphere storage. International Workshop: *Ochratoxin A in grapes and wine, prevention and control*. Marsala, Sicily, Italy.

Pateraki, M., Dekanea, A., Mitchell, D., Lydakis, D. and Magan, N. (2007) Influence of sulphur dioxide, controlled atmospheres and water availability on in vitro germination, growth and ochratoxin A production by strains of *Aspergillus carbonarius* isolated from grapes. *Postharvest Biol Technol* (in press) doi:10.1016/j.postharvbio.2006.11.016.

Rahardjo, Y.S.P., Sie, S., Weber, F.J., Tramper, J. and Rinzema, A. (2005) Effect of low oxygen concentrations on growth and α-amylase production of *Aspergillus oryzae* in model solid-state fermentation systems. *Biomol Eng* **21**, 163-172.

Ringot, D., Chango, A., Schneider, Y. and Larondelle, Y. (2006) Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chem-Biol Interact* **159**, 18-46.

Sarig, P., Zahavi, T., Zutkhi, Y., Yannai, S., Lisker, N. and Ben-Arie, R. (1996) Ozone for control of post-harvest decay of table grapes caused by *Rhizopus stolonifer*. *Physioll Mol Plant P* **48**, 403-415.

Stander, M.A., Bornscheuer, U.T., Henke, E. and Steyn, P.S. (2000) Screening of commercial hydrolases for the degradation of ochratoxin A. *J Agric Food Chem* **48**, 5736-5739.

Sydney Postharvest Laboratory and Food Science Australia (2006) Optimal Fresh : The Fruit, Vegetable and Fresh Produce Storage Expert System, CSIRO Publishing, Melbourne. http://www.postharvest.com.au/storage.htm

Taniwaki, M.H., Hocking, A.D., Pitt, J.I. and Fleet, G.H. (2001) Growth of fungi and mycotoxin production on cheese under modified atmospheres. *Int J Food Microbiol* **68**, 125-133.

Tjamos, S.E., Antoniou, P.P., Kazantzidou, A., Antonopoulos, D.F., Papageorgiou, I. and Tjamos, E.C. (2004) *Aspergillus niger* and *Aspergillus carbonarius* in Corinth raisin and wine-producing vineyards in Greece: population composition, ochratoxin A production and chemical control. *J Phytopathol* **152**, 250-255.

Valero, A., Marín, S., Ramos, A.J. and Sanchis, V. (2005) Ochratoxin A producing species in grapes and sun dried grapes and their relation to ecophysiological factors. *Lett Appl Microbiol* **41**, 196-201.

Valero, A., Farré, J.R., Sanchis, V., Ramos, A.J. and Marín, S. (2006) Kinetics and spatial distribution of OTA in *Aspergillus carbonarius* cultures. *Food Microbiol* **23**, 753-756.

Van der Merwe K.J., Steyn P.S., Fourie L., De Scott B. and Theron J.J. (1965) Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. *Nature* **56**, 1112–1113.

Varga, J., Rigó, K. and Téren, J. (2000) Degradation of ochratoxin A by *Aspergillus* species. *Int J Food Microbiol* **59**, 1-7.

Zoffoli, J.P., Latorre, B.A., Rodríguez, E.J. and Aldunce, P. (1999) Modified atmosphere packaging using chlorine gas generators to prevent *Botrytis cinerea* on table grapes. *Postharvest Biol Tec* **15**, 135-142.

DISCUSSION

IV. DISCUSSION

During the course of this thesis, many researches regarding ochratoxin A in foods or *in vitro* have been carried out. For a better understanding of the results, their discussion has been divided into four sections that address the four areas of interest.

1. Succession of fungi in field and dehydrating grapes as a function of environmental factors

Grapevines are susceptible to phytopathogenic fungi mainly since the leaves sprout. Grapes are also susceptible to fungal attack that increases during grape ripening. Berries are settled between May-June and start to accumulate sugar from veraison, in mid-July, up to harvest, between August-early September. Along these two-three months the grape fruit constitutes an exceptional growth substrate and the varying environmental conditions allow the fungal diversity and succession.

In our grape sampling in 2003 (Study I) the samples collected in June showed the highest fungal diversity. At that time moisture of grapes is high and temperatures are around 20-30 ºC. *Alternaria* was the most common fungus isolated, followed by *Penicillium*, *Aspergillus*, *Epicoccum*, *Cladosporium* and *Rhizopus*. In July, August and September day temperatures raise to 40 ºC, and black aspergilli (*A. carbonarius*, *A. niger* aggregate) become the predominant fungi followed by *Penicillium*, fungal diversity thus decreasing sharply.

After harvest, grapes intended for production of sweet wines and raisins are exposed to strong sun irradiation until they acquire the adequate sugar content, a_w dropping below 0.80 a_w. In sun-dried grapes, *Aspergillus*, and *Penicillium* were predominant. On the other hand the genera most commonly isolated in commercial raisins were *Aspergillus* and *Eurotium*. Previously published works also report similar fungal incidences in grapes and raisins (El-Halouat and Debevere 1997; Abarca *et al.* 2003; Magnoli *et al.* 2004). Among the *Aspergillus* section *Nigri* isolates, *A. niger* aggregate was prevalent in ripen grapes and in commercial raisins. Therefore *A. carbonarius* increased its proportion with grape maturity, mainly after sun-drying, and also in raisins in agreement with Tjamos *et al.* (2004).

From the fungal incidences in grapes at different ripe stages to dehydrating grapes, a succession in the fungal population could be observed. In order to understand the factors that play a major role in this succession, we analysed some abiotic factors, such as temperature,

water availability and ultraviolet radiation, and biotic factors such as the *intra* and *inter*-specific competence between fungi.

The capability of some representative grape fungi to grow at different temperatures and water activities (Domsch *et al*., 1980; Rousseau and Donèche 2001; Studies IV and VIII) is summarised in the figure IV. 1. High relative humidity (0.97 a_w) and moderate temperatures (20-30ºC), common in June and early-July, were very favourable to *A. alternata*, *B. cinerea* and to *T. harzianum*, followed by black aspergilli. The later grew faster at 30 ºC and were the only ones, together with *E. amstelodami*, capable of growing at 40 ºC, which constitutes a common day-temperature in the sampling region during August and early September. Leaving *T. harzianum* and *B. cinerea* aside, the evolution of the mentioned fungi in field and grape drying could be explained by their growth under the conditions tested in this study. As it will be afterward discussed, other factors may be involved in the fungal selection but a rapid mycelial development is also an effective strategy that enables the fungi to colonise the substrate.

After harvest, grapes can loose some water content through the respiration during storage and more rapidly by sun dehydration. Thus, water activity of dehydrating grapes drops quickly limiting the fungal growth. At 0.92 a_w both *E. amstelodami* and *Aspergillus* section *Nigri* grow well, followed by both penicillia compared to the remaining genera. While water content of grapes descends, only those fungi with higher growth rates at 0.92 a_w (*Aspergillus* section *Nigri* isolates, *E. amstelodami* and *Penicillium* sp.) and also *C. herbarum*, are able to grow (at 0.87 a_w). Once again, the adaptability of these fungi to grow and resist low water activities confers them a selective advantage in dehydrated products such as dried vine fruits.

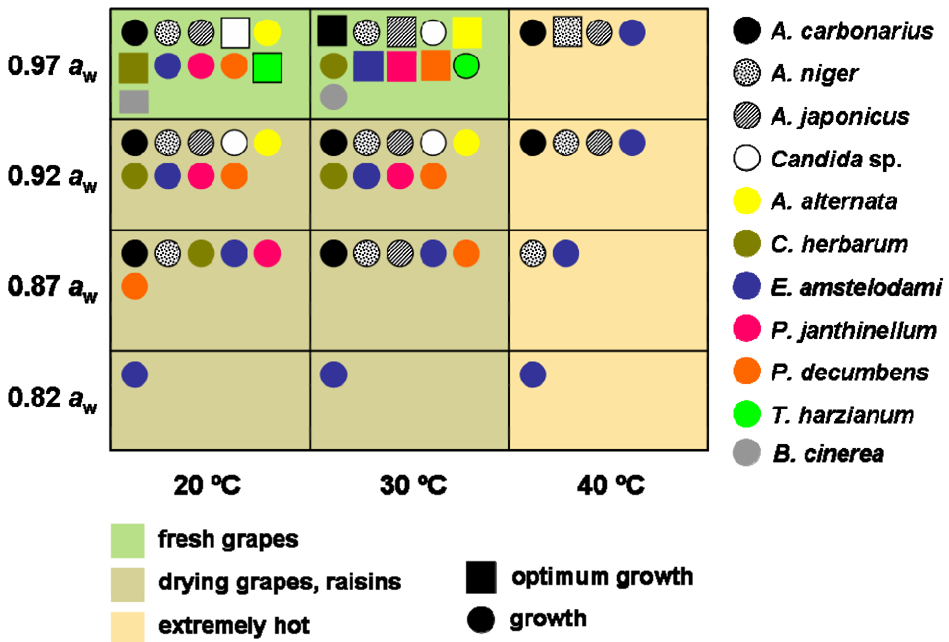


Figure IV. 1. Growth of fungi commonly isolated from grapes, dehydrating grapes and raisins. Colour of squares varies depending on the environmental conditions (temperature and a_w): typical of fresh grapes (green), dehydrating grapes and raisins (brown) and at extremely hot conditions (40 ºC) (pale orange).

Fungi with higher growth rates are first in colonizing growth medium and consequently in primary resource capture (Cooke and Rayner 1984). Other ecological strategies such as stress-tolerance or good enzymatic competence play an important role in substrate colonisation, thus some fungi which are good at primary resource capture may not be able to control the resource when competing with a more combative fungus (Robinson *et al.* 1993).

In the competence assay (Study IV) *T. harzianum* reduced the growth of the black aspergilli at 0.97 a_w, and regarding the dominance indexes, black aspergilli showed the highest scores only being surpassed by *T. harzianum* at 20ºC. *Trichoderma* sp. is well known as a biological control agent against pests due to its ability to produce antifungal metabolites (Cook and Baker 1989; Corley *et al.* 1994; Egorov *et al.* 1996; Nampoothiri *et al.* 2004).

At high water activities *A. alternata* and *P. decumbens* reduced growth rates of black aspergilli too. *A. alternata* produces antifungal metabolites and several toxins with unknown antibiotic effects (Ansari and Shrivastava 1991). At 0.92 a_w, *E. amstelodami* reduced the growth of some

black aspergilli when grown in pairs. Nevertheless the higher dominance scores corresponded to *A. niger* aggregate.

Aspergillus section *Nigri* spp. showed to grow well at a wide range of temperatures and water activities, and also displayed a high combative efficiency in case of contact with other fungi.

In field however, contact competition of two fungi in the same berry is unlikely, so it is presumable that growing at high rates and being adapted to a wider range of conditions is crucial in grape colonisation.

The third abiotic factor evaluated in this thesis was the ultraviolet radiation. Spores and mycelium are exposed to sun light for many hours per day, so these energetic radiations may affect the survival of the fungi. Sunlight is divided into infrared rays (> 700 nm wavelength), visible light (400-700 nm) and ultraviolet radiations (100-400 nm) (Figure IV.2). Within ultraviolet radiations, the ultraviolet C light (UVC) is the most energetic one and it is widely used as sterilising agent due to its germicidal properties. Ozone and clouds absorb the major part of the UVC radiation but some can reach the earth surface (TGL 2005).

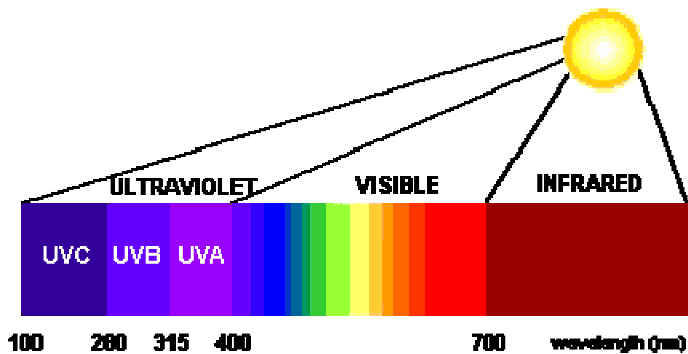


Figure IV.2. Divisions of sunlight according to wavelength.

The survival ability under UVC light of spores belonging to five fungal species isolated from grapes and raisins was evaluated (Study VI). It was observed that *A. alternata* was the most resistant followed by *A. carbonarius*, *C. herbarum*, *A. niger* and *P. janthinellum* in the last place. Ultraviolet resistance may confer the spores higher spreading and probability to germinate on a substrate. The fact that spores can survive due to either their thick cell walls or melanin pigments does not imply that the resulting hyphae have the same advantages given that some pigmented spores produce hyaline hyphae (Duguay and Klironomos 2000).

Alternaria is the most common fungal genus found on grapes, followed by *Penicillium*, *Aspergillus*, *Epicoccum* and *Cladosporium* (Sage *et al*. 2002; Bellí *et al*. 2004; Study I). When grapes are dried in intense sunlight, the most commonly found fungi are *Aspergillus*, *Penicillium* and *Cladosporium* (Ulevičius *et al*. 2004; Romero *et al*. 2005; Study I).

Our results regarding the resistance of *A. alternata* to UVC agree with the findings of Rotem and Aust (1991), who noted that propagules of *Alternaria* sp. were the most resistant to ultraviolet radiation or sunlight, followed by *Mycosphaella* sp., *A. niger* and *Botrytis cinerea*. *A. alternata* has multi-celled spores with thick and melanised walls that confer tolerance to sunlight (Carzaniga *et al*. 2002). *A. carbonarius* and *A. niger* produce single-celled conidia with melanin and aspergilline in their cell walls (Duguay and Klironomos 2000; Babitskaya and Shcherba 2002), but differed in their UVC resistance and their incidence on grapes. The greater UVC resistance displayed by *A. carbonarius* spores, that are thought to possess thicker walls than *A. niger* spores (Tiedt 1993), provides a logical explanation for the high numbers of *A. carbonarius* on grapes subjected to prolonged sun exposure. In the study of fungal contamination of grapes after sun drying (Study I), *Aspergillus* section *Nigri* spp. were found in more than 80% of sun-dried grapes and raisins. *A. carbonarius* occurred in increased frequency compared with *A. niger,* on raisins and dried vine fruits (Leong *et al*. 2004; Romero *et al*. 2005; Gómez *et al*. 2006; Study I). *A. niger* is more tolerant than *A. carbonarius* to low water activities (growth limits of 0·77 a_w and 0·87 a_w, respectively; Study IV) and high temperatures (optimum growth at 35-40 °C and 30 °C, respectively; Panasenko 1967; Palacios-Cabrera *et al*. 2005; Study IV). Thus, resistance to UVC provides a competitive advantage for *A. carbonarius* during grape drying that overrides the higher adaptability of *A. niger* to hot and dry conditions.

Integration of these findings on resistance to UVC with data on the effects of temperature and water activity on fungal growth and survival provides a good explanation of the fungal incidence of fungi found on both fresh and sun dried grapes.

To sum up, prevalence of *Aspergillus* section *Nigri* can be easily explained by its adaptation to environmental conditions of sun-drying, and by its capability to dominate some other fungal species involved when coming into contact with them.

2. Ochratoxin A production as affected by environmental conditions

The consequences derived from fungal contamination in foods involve the detriment of the food quality through the presence of bad odours, off-flavours, and also the contamination by toxins that are hazardous for health. The ochratoxin A, which is produced by *Aspergillus* spp. in grape products, is a contaminant commonly detected in grapes, raisins and wine. Its pattern of synthesis and the understanding of the factors that may affect it are a matter of great concern in foods where the presence of *Aspergillus* section *Nigri* is widely known and reported.

A very small percentage of *A. niger* aggregate isolated from grapes are OTA producers, between 1.4 -30 % (Abarca *et al.* 2003; Magnoli *et al*. 2004; Study I). In contrast, more than 70 % of the *A. carbonarius* isolates were able to produce OTA (Study I), in agreement with previous publications (Leong *et al*. 2004; Sage *et al*. 2004; Bellí *et al*. 2005; Perrone *et al.* 2006), *A. carbonarius* may be therefore considered the main source of ochratoxin A in grape products, thus further ecophysiological studies regarding the toxin production were conduced with *A. carbonarius.*

The optimum conditions for OTA production differred from that for growth. While optimum growth rates were at 30 ºC, the OTA production was maximal at 20 ºC (Study III). Under dry conditions, 0.92 a_w, the toxin production was highly reduced but still present at 20 ºC. In the same study it was also observed that OTA accumulation was maximal in 4-7 days aged mycelium, followed by degradation. A previous study with *A. carbonarius* showed its ability to degrade OTA into other breakdown products, such as ochratoxin alpha, likely achieved by a carboxipeptidase enzyme (Abrunhosa *et al.* 2002).

The effect of fungal competition in OTA production was also studied in paired cultures (Study V). At 0.97 a_w and 30 ºC *A. alternata, C. herbarum* and *Penicillium* spp. reduced OTA accumulation. After 10 days of incubation all species tested were able to reduce the toxin accumulation. At 20 ºC the effect of competence was more complex, OTA accumulation being stimulated by *E. amstelodami* and *Candida* sp. while reduced by *T. harzianum* and *P. decumbens*. At lower water activity (0.92 a_w) and 20 ºC OTA was reduced by all competitors while at 30 ºC it was solely produced by *A. carbonarius* grown in competence.

The reduction of OTA accumulation when *A. carbonarius* was grown in paired cultures at some conditions may be due to:

i) Limitation of *A. carbonarius* growth, what would generally lead into a reduction in OTA production.
ii) Toxin synthesis is reduced in behalf of primary metabolism as a result of stress by competence.
iii) Antagonistic fungi consume specific nutrients necessary to synthesise OTA.
iv) OTA degradation by the other fungi. Previous publications involve species of the genera *Alternaria*, *Cladosporium* and *Trichoderma* as capable to degrade OTA, probably by means of the carboxypeptidase A activity (Abrunhosa *et al*. 2002).
v) Interacting fungi could even excrete substances able to block OTA synthesis. This hypothesis could be supported by a study made by Barr (1976) who showed that some volatile compounds produced by different bacteria had normally an inhibitory effect in mycotoxin production by an *Aspergillus* sp. isolate.

A. alternata, *Candida* sp. and *P. decumbens* may exhibit the (ii), (iii), (iv) and (v) effects since no significant correlation, or even negative, were observed between growth of *A. carbonarius* and OTA accumulation when paired with them. The OTA reduction produced by other competing fungi was likely a direct consequence of limiting the growth of *A. carbonarius* since a positive correlation was observed between growth and OTA accumulation. *T. harzianum* seemed to stop the *A. carbonarius* growth at contact but OTA was detected even under the *T. harzianum* culture. This detected toxin might be produced by *A. carbonarius* growing intermingling with the *T. harzianum* colony.

B. cinerea was shown to degrade OTA although its effect as competitor against *A. carbonarius* was not clear (Study VIII).

Regarding the stimulation of OTA production, it may be due to cooperation between fungi in the use of substrate. When conditions are not favourable for all members of mycobiota, the enzymatic activity displayed by some fungi may favour the use of nutrients by other whose enzymes are not so active. Available nutrients can be then used by *A. carbonarius* to produce OTA. Apart from the field study (Study I) all the results above discussed were obtained from *in vitro* experiments in synthetic media, therefore some of those findings needed to be tested in an *in vivo* approach, by using grapes as a substrate.

The growth of *Aspergillus* section *Nigri* isolates and OTA production was studied on grapes affected by fungal competence in a simulation of a dehydrating process (Study VII). Ochratoxigenic and non-ochratoxigenic strains of *Aspergillus* species often co-contaminate the grapes surface but also *Penicillium* spp. and *Eurotium* are two genera highly isolated from dried vine fruits (Sage *et al.* 2002; Romero *et al.* 2005; Study I), so they were also included in the study.

The colonisation of healthy grapes by black aspergilli was higher when they were solely inoculated with *A. niger* aggregate. The cellulolytic activity observed in these species could help it to penetrate into the berry (Seidle *et al.* 2005). Interactions within *Aspergillus* section *Nigri* produced variable results. However, *E. amstelodami* and *P. janthinellum* generally favoured grape colonisation by *Aspergillus* section *Nigri*. This is likely due to their enzymatic activity that could favour the access to nutrients by *Aspergillus* section *Nigri* isolates.

What concerns OTA accumulation, it increased with the drying process up to a maximum decreasing after in some cases, mainly produced by *A. carbonarius*. In injured grapes, OTA accumulation was faster and higher at the beginning of the drying process, it achieving its maximum concentration the days 12-17. In contrast, in healthy grapes the OTA maximum is later observed, between the 17-20 days. This could be due to a faster depletion of nutrients in injured grapes; OTA could be then degraded by black aspergillli as an alternative source of carbon or energy. In healthy grapes the skin constitutes a physical and chemical barrier that delays the access to the nutrients, so keeping it undamaged may prevent many undesired contaminations and OTA accumulation.

When co-inoculating a non-ochratoxigenic *Aspergillus* section *Nigri* isolate with *A. carbonarius*, OTA production was highly reduced. Thus fungal competence between two fungi with a very similar ecological niche, affects negatively the OTA accumulation. This reduction could be due to one of the four different explanations formerly numbered (i-iv) or to the combination of few of them.

In contrast, higher OTA levels were obtained when co-inoculating *E. amstelodami* or *P. janthinellum* with both OTA producing strains, agreeing with the *in vitro* study (Study V) where *E. amstelodami* favoured the OTA accumulation.

The sun-drying process may be conducive to OTA accumulation in dried grapes. Interacting fungi has also been proved to be an important factor to take into consideration. The complex

fungal interactions which may take place during this process, may act as an additional control factor, given that the higher presence of non-ochratoxigenic *Aspergillus* section *Nigri* inhibits OTA accumulation by OTA producing species. Unfortunately, other fungi such as *Eurotium* sp. and *Penicillium* sp. should be prevented during grape dehydration given their stimulation of OTA production.

In summary, some of the main results observed in the formerly discussed studies have been synthesised in the table below.

Table IV.1. OTA production of *A. carbonarius* and *A. niger* under different environmental conditions.

GS	°C	OTA max. / final ($ng \cdot g^{-1}$)	Day max. OTA / Total	Inoculum	Other characteristics	Study
SNM	20	15362 / 2268	14 / 18	*A. carbonarius*	0.97 a_w	III
	30	418 / 175	7 / 18			
	25	3818 / 193	7 / 21	*A. carbonarius*	0.97 a_w (mean of two strains)	X†
		1908 / 267	7 / 21	*A. niger*		
	20	19761	18 / 18	*A. carbonarius* + *E. amstelodami*	0.97 a_w	V†
	30	643 / 11	7 / 18			
HG	25	446 / 278	17 / 20	*A. carbonarius*	Dehydrating grapes	VII
WG		728 / 102	15 / 20			
HG		119 / 98	17 /20	*A. niger*		
WG		58 / 27	17 / 20			
HG		487 / m.d.	15 / 20	*A. carbonarius* + *E. amstelodami*		
WG		380 / 164	15 / 20			
HG	25	126	20*	*A. carbonarius*	Dehydrating grapes	IX†
		230	20*	*A. carbonarius* + *E. amstelodami*		

GS, growth substrate; SNM, synthetic grape-like medium; †, some data are not shown in the mentioned study; HG, healthy grapes; WG, wounded grapes; -, not measured; m.d., missing data; *, single lecture.

It can be observed that the OTA accumulation produced by *Aspergillus* spp. in SNM is maximal at 20ºC, followed by 25 and 30ºC, in agreement with previous studies (Esteban *et al.*, 2004; Mitchell *et al.*, 2004; Bellí *et al.*, 2005).

Within fungi, *A. carbonarius* was able to produce higher amounts of OTA than *A. niger*, both *in vitro* and in grapes. It is well known the great ability of this species among *Aspergillus* for producing OTA (Abarca *et al.*, 1994 and 2003; Bellí *et al.,* 2004b).

At 25ºC, the toxin is highly produced on SNM, followed by wounded grapes and healthy grapes the last. Nutrients in grapes are mostly inside the vegetal cells, even if the grape skin is damaged, so when the fungus is grown on synthetic grape-like medium (SNM) it finds the nutrients free and is able to grow faster and produce more toxin as a result. Consequently, the maximal OTA production is earlier reached and next decreasing, firstly in SNM followed by wounded grapes and healthy grapes the last.

Since it has formerly been discussed, the addition of *E. amstelodami*, both *in vitro* or on grapes, stimulated the OTA accumulation, mainly in healthy grapes.

3. OTA occurrence and incidence in European special wines

This thesis includes the first exhaustive study about OTA occurrence in special wines made in Europe, with special emphasis in sweet wines (Study II).
The climatic and geographic differences influence mould development and OTA contamination of grapes, and the wine as a result. All special wines analysed from Northern European regions (A, B and CI wine-growing zones, (EC) No 1493/1999 and 1512/2005)) were negative for OTA while previous studies have reported OTA occurrences between 0 and 50% in the same regions in all types of wines (Zimmerli and Dick 1996, Ottender and Majerus 2000, Berente *et al.* 2005). Thus OTA-producing fungi are present in these latitudes and are able to grow and to produce OTA. However, some grape transformations occurring in special wines, such as noble rot, could reduce the growth of the ochratoxigenic fungi and thus the toxin to undetectable concentrations.

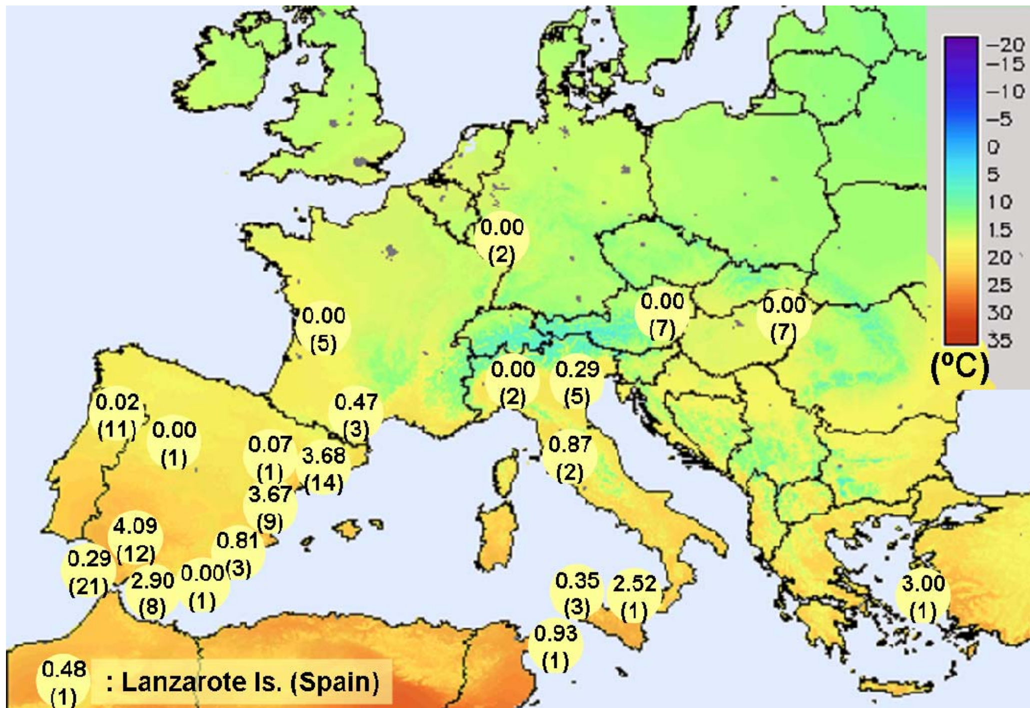


Figure IV.3. Thermomap of Europe indicating origin of samples with means of OTA concentration (μg·l^{-1}) and number of samples in parentheses.

Warmer regions (CII wine-growing zone) may favour fungal development and OTA production by *Aspergillus* section *Nigri*, as reviewed and modelled by Battilani *et al.* (2006). The 54% of special wines from CII zone were positive for OTA with 15% of samples over 2 μg l^{-1} (Study II), which is the maximum permissible concentration for OTA in wines (Commission Regulation (CE) No 1881/2006). The CIII growing-zone, that includes the majority of Mediterranean regions, has a hot and dry climate during summer and warm and wet in autumn. The 60% of wines sampled in this zone contained OTA, with the 27% of them reporting OTA concentrations above 2 μg OTA l^{-1}.

The OTA occurrences reported by previous surveys in all types of wines from CII and CIII zones, are very similar to our results, but the OTA levels were much lower than those found in this study focused into special wines (Zimmerli and Dick 1996, Ottender and Majerus 2000, Pietri *et al.* 2001; Study II).

Battilani *et al.* (2006) modelled the incidence of *Aspergillus* section *Nigri* in European countries at harvesting (see Introduction, Figure 1.2). Comparing the predictive map for the year 2003 with the Figure IV.3 it is observable a correspondence between areas with higher incidences of *Aspergillus* section *Nigri* and higher OTA levels reported in this thesis.

The different types of winemaking linked to different wine-growing zones have different effect on OTA content of wines. Wines from dehydrated grapes obtained through five different techniques were studied in this thesis (Study II):

i) Sun exposure
ii) Storage in warm and dry chamber
iii) Storage in fresh and dry chamber
iv) Noble rot
v) Freezing on the vineyard (eisweins)

Ochratoxin A contamination of dried vine fruits was usually much higher than that of wines or grape juices with the former reaching very high levels (50-70 μg l^{-1}), due to the action of black aspergilli (Miraglia and Brera 2002; Abarca *et al.* 2003, Varga *et al.* 2005).

More than 90% of wines made from sun-dried grapes were positive for OTA and levels were much higher (Figure IV. 4) than in other wines made from overripe grapes, in agreement with previous publications (Zimmerli and Dick 1996, JECFA 47 2001, Soufleros *et al.* 2003, Blesa *et al.* 2004, Hernández *et al.* 2006). For the elaboration of this type of wine, the grapes are exposed in an open environment, to sun-drying during the day and to cool and damp night, for 5-15 days. At these conditions microorganisms may reach injured grapes, as a result of harvesting process, and have plenty of time for fungal development and for OTA production (Study I). Grape sun-drying involves a decrease of water availability, so the OTA production may cease if a_w drops below values that are not conducive to OTA production (<0.90 a_w, *A. niger* aggregate spp.; <0.86 a_w, *A. carbonarius*) (Esteban *et al.* 2006a and b).

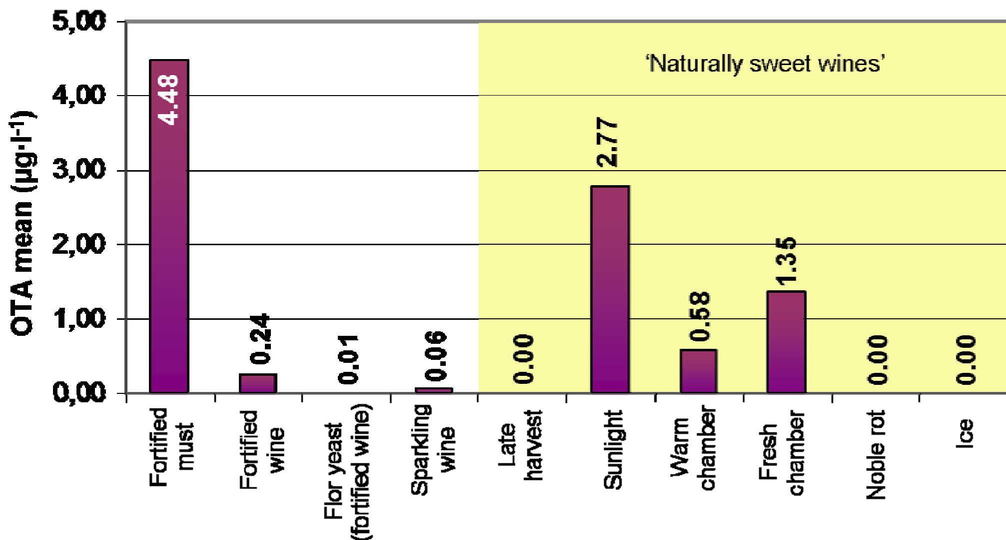


Figure IV. 4. OTA incidence in European special wines (Study II)

Grapes that are dried out in warm and dry chambers (under sun-irradiated roofs) undergo a fast and generally more continuous dehydration (three-four weeks). OTA was detected in 33% of these wines and toxin levels were lower than those from sun-dehydrated grapes. By contrast, wines made from grapes shrivelled in cool and dry chambers reported higher occurrence and OTA levels. This may be due to the longer periods of time, about two to six months, that are necessary for drying the grapes, although rotten bunches are accurately removed both in warm and fresh drying.

As regards eisweins (icewines), noble wines (botrytised grapes) or late harvest wines (a slight amount of grapes may be affected by *B. cinerea*), none of them contained ochratoxin A above the detection limit, in agreement with previous results published about noble rot affected wines (Kallay and Magyar 2000, Dumoulin and Riboulet 2002, Stander *et al.* 2002, Berente *et al.* 2005, Eder 2005) and eisweins (Eder 2005). These wines usually are made in Northern European regions or in colder climates that are favourable for noble rot rather than black rot (*Aspergillus* section *Nigri*). Noble rot is developed by the fungus *Botrytis cinerea* that colonises and dehydrates the grape berry.

The effect of *B. cinerea* on OTA and *A. carbonarius* was also studied (Study VIII) and found that *B. cinerea* isolates were able to degrade OTA from a spiked medium, agreeing with previous work published by Abrunhosa *et al.* (2002). All together, in the event of black rot development, the little toxin produced could be degraded by *B. cinerea*.

B. cinerea is a highly desired agent in grapes that are intended for noble wines but this fungus is also the most important cause of disease in grapes, both before harvest and in storage (Snowdon 1990). Environmental conditions that are required for grapes to be botrytised are common in continental climates, with cool temperatures and moderate humidity. The water activity threshold for growth of *B. cinerea* has been reported around 0.93 a_w (Snow 1949; Rousseau and Donèche 2001) or even at 0.90 a_w (Jarvis 1977) so this fungus is not expected to contaminate dried vine fruits but grapes. Its optimal temperature for growth is between 22-25ºC (Domsch *et al.* 1980). In a study achieved in this thesis (Study VIII) the growth rates of *B. cinerea* at 20ºC and 0.99 a_w were around 20 mm·day^{-1}, *A. carbonarius* however, showed a lower growth rate of 7 mm·day^{-1} under the same conditions. The effect of competence between these two species was also tested and mutual inhibition by contact was observed. Regarding OTA produced by *A. carbonarius* contacting with *B. cinerea*, it was affected without showing a clear pattern nevertheless the *B. cinerea* isolates proved to degrade the toxin in an OTA-spiked medium. To sum up, the possible effects of *B. cinerea* on *A. carbonarius* and OTA observed in this study that may explain the lack of this toxin in noble wines is the ability of *B. cinerea* to degrade OTA and its higher growth rates that prevent the grape colonisation by other fungi.
Fortified wines were also analysed and OTA was detected in 52 % of samples, which contained low-medium OTA levels (0.96 µg l^{-1} max. and 0.24 µg l^{-1} mean) in comparison with the remaining wines from this survey. In contrast, OTA levels detected in wines made by adding alcohol distillate to the sweet must were much higher (mean: 4.48 µg l^{-1}), OTA being detected in more than 90% of wines. In fortified wines the must is fermented up to a certain alcoholic grade, fortified and next aged in wooden barrels for long periods of time. In addition to OTA reduction by fermentation, either alcoholic or malo-lactic (Abrunhosa *et al.* 2005, Grazioli *et al.* 2006), the oxidative process that occurs during aging could affect somehow the toxin, but it has not been studied so far.

Within the wines aged by Flor yeast (biological 'crianza'), OTA was detected in only one sample out of the 12. These wines undergo double fermentation: almost a full alcoholic fermentation followed by biological 'crianza', which may lead to a further OTA reduction. Sparkling sweet wines analysed in this study were similar in OTA occurrence and incidence to fortified wines and also concerning their uncompleted fermentation. Given that only two samples of sparkling wines were analysed, a more exhaustive study would be needed to obtain more conclusive results.

Overall, the 19,8% of wines contained OTA levels above the maximum permissible limit for European Union, set in 2 µg Kg^{-1} (Commission Regulation (CE) No 1881/2006). Ochratoxin A in wines is a matter of concern considering the high levels and occurrences reported in the present

survey. Origin of samples is determinant for fungal development and OTA contamination, which is favoured by environmental conditions of Southern regions. The winemaking procedures also affected on OTA concentration in wines, it being lower in wines with longer or double fermentation and in those made from botrytised grapes.

4. Prevention and control

Fungicides are widely used to control grape and other commodities decay. In this thesis the residual effect of pre-harvest fungicides - Switch (a.i. 37.5% cyprodinil, 25% fludioxonil) and Chorus (a.i.: 50% cyprodinil) - was evaluated in grape colonisation by moulds and OTA production during dehydration (Study IX). Pre-harvest treated grapes were inoculated with *Aspergillus* section *Nigri* isolates and also with *E. amstelodami* and *Penicillium* sp. as common contaminants in dried vine fruits. In general, all fungicides reduced the mean colonisation percentages below 20% after dehydration, being Switch the most useful, agreeing with Bellí *et al*. (2006b), and also the final OTA content in dehydrated grapes without significant differences among them.

Thus, the present study proves that these two pre-harvest fungicides remain stable and active in grapes during further dehydration, so they can be effective in reducing OTA production by *A. carbonarius*.

Among the new generation of pesticides used in biological control, some fungi isolated from grapes, such as *T. harzianum*, *P. chrysogenum* and *Candida* spp., have shown antifungal properties (Chet 1987; Zahavi *et al*. 2000; Bleve *et al*. 2006). In the competence study made in the course of this thesis (Study IV) the fungi *A. alternata*, *T. harzianum* and *P. decumbens* proved to reduce both the *A. carbonarius* growth and OTA synthesis. This fact may suggest the possible application of these two new species, *A. alternata* and *P. decumbens* as biocontrol agents. In this direction, a more exhaustive study should be addressed in grape substrate.

T. harzianum stopped the growth of *A. carbonarius* at contact but OTA was detected in the area were *T. harzianum* had grown, suggesting that there was intermingling to some extent.

As to the application of physical methods in the control of grapes decay, the UVC irradiation may constitute a suitable alternative without residues problems. Only 10 min of UVC irradiation on fungal spores tested were enough for reducing almost in 100% their viability. Thus, the UVC irradiation of harvested grapes could prevent germination of contaminant fungi during storage or

dehydration thus preventing the production of undesired substances such as mycotoxins as a result. The application of this technique in a pre-harvest stage would result unviable given the numerous uncontrolled hazardous effects in the ecosystem due to its powerful mutagenic activity (Sage *et al.* 1996; Izumizawa *et al.* 2000).

Packaging or storing grapes under modified atmospheres is also used in controlling post-harvest grapes decay. Its effect in the mycelial growth and OTA production by *Aspergillus niger* and *A. carbonarius in vitro* was evaluated in the course of this thesis (Study X).

A. niger isolates were slightly more resistant than *A. carbonarius* to the modified atmospheres tested (air, 1% O_2, 5% O_2, 1% O_2 / 15% CO_2, 5% O_2 / 15% CO_2). In 5% O_2 the growth rates of all isolates were maximal. Similarly, other authors (Crisosto *et al.* 2002; Rahardjo *et al.* 2005) found that fungal behaviour did not vary between 5% and 20% O_2. A possible explanation is that primary metabolism may be favoured as a result of inhibition of other pathways by insufficient oxygen.

Effective concentration thresholds in O_2 needed to limit growth of black aspergilli appear to be below 1% O_2. Hall and Denning (1994) reported that some representative *Aspergillus* species (*A. niger* among others) required a minimum of 0.1-0.5% O_2 for *in vitro* growth. High CO_2 partial pressures (15%) in combination with either 1% or 5% O_2 caused the reduction of *Aspergillus* isolates growth. Previous studies reported the effectiveness of higher CO_2 pressures (50-100%) in controlling the growth of *Aspergillus* species (Paster *et al.* 1983; Pateraki *et al.* 2005). Concentrations above 15% CO_2 produce undesirable effects on quality of grapes (Crisosto *et al.* 2002), hence, the combination of 15% CO_2 / 1% O_2 that was the most effective in minimising growth of the black *Aspergillus* isolates would, hypothetically, be amenable for its use in grapes.

Leaving aside the practical results of this study that could derive into an implementation, some other interesting observations deserve to be discussed although they may belong to a basic research.

The metabolic pathways are very complex involving several steps, substrates and enzymes. All them are differentially affected by the modification of the atmosphere where the fungus grows.

Focusing into OTA kinetics, two different pathways may be affected by modified atmospheres:

- i) OTA anabolism
- ii) OTA catabolism

It was observed that OTA was gradually accumulated during growth at 1% O_2, with or without increased CO_2, in all *Aspergillus* spp. tested (Study X). The OTA levels observed under higher CO_2 concentrations were, in general, lower, *A. carbonarius* isolates being the most affected. Cairns-Fuller *et al.* (2005) observed a significant inhibition of OTA production by *P. verrucosum* by 50% CO_2, although toxin gradually accumulated over time and subsequent degradation was not observed.

In contrast, at 20% O_2 (air), in general, and at 5% O_2, in some cases, the toxin content showed a decreasing trend with time.

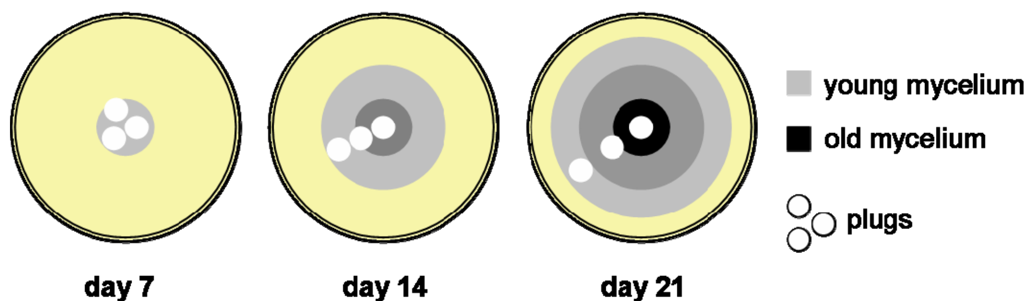


Figure IV.5. Plugs removal from *Aspergillus* section *Nigri* colonies at three times.

Considering the location where plugs were taken (Figure IV.5) to analyse OTA, there are two possible reasons that explain the OTA decrease with time:

- OTA production ceased immediately after 7 days of incubation. Thus, the plugs taken in following days would have diluted the total OTA concentration given that new mycelium would not synthesise toxin.
- OTA production kept constant but after few days the catabolism of the toxin was activated, thus reducing the OTA content from old mycelium. The plugs taken from old mycelium would have diminished the total OTA concentration as a result. Fast fungus growth lead to substrate depletion and the toxin could be assimilated as a carbon or energy source by old mycelium. This hypothesis may be considered the most probable given that these fungal species are able to degrade the toxin, as previously reported (Stander *et al.* 2000; Varga *et al.* 2000; Bejaoui *et al.* 2006), and there is not a current explanation for the hypothetical cease of OTA production while the fungi are growing on fresh medium.

To sum up:

i) It seems that toxin production by some species of *Aspergillus* can be prevented by elevated CO_2 levels; however, the inhibitory concentrations may differ among species (Paster *et al.* 1983) and strains, as observed in this study.

ii) Our findings might suggest that low O_2 concentrations (1% or below) inhibit or greatly reduce the ability of *A. carbonarius* and *A. niger* to degrade OTA, and this catabolic pathway may not be affected by CO_2. A more accurate research should be addressed to confirm or to refute these theories.

Overall, not all the modified atmospheres proposed solely to prevent grape decay would also inhibit OTA production by black *Aspergillus* spp. over an extended storage period. OTA is a fungal product from secondary metabolism, whereas growth is regulated in primary metabolism; thus, slower fungal growth may not necessarily result in a reduction of OTA synthesis. To minimise the risk of OTA contamination during grape storage, modified atmospheres, for instance 1% O_2 / 15% CO_2, could be used in combination with temperatures below 10ºC, *viz.* the temperature threshold for OTA production (Mitchell *et al.* 2004). Storage at low temperatures would also prevent undesirable fermentations at low O_2 concentrations and enzymatic deterioration of fruit (Sydney Postharvest Laboratory 2006; Deng *et al.* 2006).

Finally, the application of chemical or biological additives to the grape product, except for those intrinsic to the winemaking process, should be considered the last option. The attention should be focused in prevention rather than corrective actions. A suitable crop management followed by a smart post-harvest processing of grapes and also during winemaking may comprise enough cautions to obtain a safe final product.

CONCLUSIONS

V. CONCLUSIONS

1. Field study and ecophysiology

- The presence of *Aspergillus* section *Nigri* becomes predominant in grapes at harvest and mainly during sun-drying. Prevalence of *Aspergillus* section *Nigri* can be explained by their adaptation to environmental conditions of sun-drying, and by their ability to dominate other fungal species involved when coming into contact with them. Thus, the grape dehydrating process may be conducive to OTA accumulation in dried grapes.

- Among the *Aspergillus* section *Nigri*, *A. niger* aggregate is dominant, although *A. carbonarius* increases its incidence in dehydrated grapes. A very small percentage of *A. niger* aggregate showed to be ochratoxin A producers, whereas almost 100% of the *A. carbonarius* strains were OTA producers. OTA production by *A. carbonarius* occurs in few days of colony age and its optimum production is around 20ºC and high water activity (0.97 a_w).

- When *A. alternata*, *C. herbarum* and *Penicillum* spp. are grown *in vitro* with *A. carbonarius*, there is a reduction in OTA accumulation, while *E. amstelodami* and *Candida* sp. stimulated it. *T. harzianum* may be good biocontrol agent for *A. carbonarius* growth but not for OTA production. The complex fungal interactions during grape dehydration may act as an additional control factor, given that the higher presence of non-ochratoxigenic *Aspergillus* section *Nigri* inhibits OTA accumulation by OTA producing species. In contrast, *Eurotium* sp. and *Penicillium* sp. should be also avoided during grape dehydration given their stimulation of OTA production.

- *A. alternata* had the most resistant spores to UVC light followed in decrescendo by *A. carbonarius*, *C. herbarum* similar to *A. niger* and *P. janthinellum* the last. Ultraviolet resistance confers the spores higher spreading and probability to germinate on a substrate. Integration of these findings on resistance to UVC with data on the effects of temperature and water activity on fungal growth and survival provides a good explanation of the incidence of fungi found on both fresh and sun dried grapes.

2. OTA occurrence and incidence in European special wines

- All special wines analysed from northern European regions were negative for OTA. More than 50% of wines original from warmer regions were positive for OTA contamination.

- The wines with higher OTA levels were fortified musts followed by those made from sun-dried grapes, grapes dried in fresh chambers, grapes dried in warm chambers, fortified wines, sparkling wines and those affected by Flor yeast the last. Lateharvest wines, Icewines and noble rot affected wines did not have ochratoxin A. Both alcoholic and malo-lactic fermentations and also biological ‘crianza’, achieved by Flor yeasts, diminish the OTA levels in wine.

- Uncontrolled grape dehydration such as sun-drying, and drying during long periods of time may increase the OTA production by contaminating fungi.

3. Prevention and control

- The two pre-harvest fungicides tested (Chorus and Switch) remain active in grapes during further dehydration, so they can be effective in reducing OTA production by *A. carbonarius*.

- 10 min of UVC irradiation on fungal spores were enough for reducing almost the 100% of their viability. Thus, the UVC irradiation of harvested grapes could prevent germination of contaminant fungi during storage or during their dehydration and avoiding the production of undesired substances such as mycotoxins as a result.

- The combination of 15% CO_2 / 1% O_2 that was most effective in minimising growth of the black *Aspergillus* isolates and OTA production would, hypothetically, be amenable to use for grapes. To minimise the risk of OTA contamination during grape storage, modified atmospheres could be used in combination with temperatures below 10ºC, *viz.* the temperature threshold for OTA production.

- Finally, the application of chemical or biological additives to the grape products, other than those intrinsic to the winemaking process, should be considered the last option. Attention should focus in preventive rather than in corrective actions. A suitable crop management followed by a smart post-harvest processing of grapes and also during winemaking may comprise enough cautions to obtain a safe final product.

VI. FUTURE RESEARCH

1. To compare different drying techniques for grapes on mould development with emphasis on *Aspergillus* section *Nigri* and ochratoxin A production in order to find the most satisfactory.

2. In this direction some suggestions or modifications to traditional drying process could be designed so as to avoid contamination risks while preserving tradition.

3. Due the humble amount of samples in some categories of wines, and considering our findings, it would be interesting to design further studies focussing into some types of wines in order to confirm our results and to identify the critical points where OTA is reduced.

4. OTA degradation products could be pursued both in botrytised grapes and in noble wines to confirm the hypothesis of its degradation by *Botrytis cinerea*.

5. Studies at pilot plant level for implementation of UVC for disinfecting grapes for storage, exportation or drying. *Prior in vitro* study should be attained of its effectiveness against all contaminating fungi commonly found in storage rooms.

6. The consequences from application of some chemical and biocontrol agents should be checked with the aim of ensuring that their effectiveness in preventing moulds development also prevents toxin production, with special attention to *Trichoderma harzianum*

VII. GLOSSARY OF USED TERMS

Abiotic: Not associated with or derived from living organisms. Abiotic factors in an environment include such items as sunlight, temperature, wind patterns, and precipitation (The American Heritage 2007).

Acid rot: Disease that affects the grape bunch, and is caused by yeasts and bacteria that cause lactic fermentation in the grapes. The grapes take on such acidity that makes them impossible to be harvested (Diputación Foral de Bizkaia 2005).

Adjuvant: Anything that aids in removing or preventing a disease, esp. a substance added to a prescription to aid the effect of the main ingredient (Dictionary.co Unabridge 2007).

Adsorption: The process by which molecules of a substance, such as a gas or a liquid, collect on the surface of another substance, such as a solid. The molecules are attracted to the surface but do not enter the solid's minute spaces as in absorption (The American Heritage 2007).

Antagonism: A relation between two species of organisms in which the individuals of each species adversely affect the other, as in competition. The inhibition of the growth of one type of organism by a different type that is competing for the same ecological niche (Dictionary.co Unabridge 2007).

Biotic: Associated with or derived from living organisms. The biotic factors in an environment include the organisms themselves as well as such items as predation, competition for food resources, and symbiotic relationships (The American Heritage 2007).

Crushing: To extract or obtain by pressing or squeezing: *crush juice from a grape* (The American Heritage 2007).

Fining: A finishing process, performed before bottling. A coagulant such as bentonite, isinglass or egg white is added to the wine to collect proteins and other undesirable compounds (The Winedoctor 2007a).

Hyaline: Adjective that describes something that is both colourless and transparent, like clear untainted glass. The term hyaline is often used when a colour description is called for, to describe the "colour" of a clear, transparent object (Wikipedia 2007).

Lees: Heavy sediment consisting of dead yeast cells and other solid matter such a grape pulp, pips and so on (The Winedoctor 2007a).

Maceration: The period of time in which the grape skins remain in contact with the juice (Winegeeks 2006).

Oidium: A fungal disease, also known as powderly mildew. Like many wine diseases it thrives in damp conditions (The Winedoctor 2007a).

Phyllosphere: The phyllosphere is a term used in microbiology to refer to leaf surfaces or total above-ground surfaces of a plant as a habitat for microorganisms. The below-ground bacterial habitat (i.e. the root surfaces) are referred to as the rhizosphere. (Wikipedia 2007).

Rachis: The axis of an inflorescence when somewhat elongated as in a raceme (Dictionary.com Unabridge 2007).

Racking: The process of racking involves transfer of wine from one container, such as a barrel, to another. Carefully done, the lees may be left behind in the first barrel, resulting in a partial clarification of the wine (The Winedoctor 2007a).

Stemmer: A device for removing stems, as for tobacco, grapes, etc. (Dictionary.com Unabridge 2007).

Veraison: Young grapes are tiny, hard and green. As they swell and ripen they take on the colour they will be when they are harvested. Veraison is the point where the grapes just start to turn colour (Winedefinitions 2007).

Water activity: or a_w is the relative availability of water in a substance. It is defined as the vapour pressure of water divided by that of pure water at the same temperature; therefore, pure distilled water has a water activity of exactly one. RH (Relative Humidity) = a_w x 100 (Wikipedia 2007).

Withholding period: The withholding period (WHP) is defined as the period that must elapse between the last application of a chemical and: i) harvesting of plants; ii) grazing or cutting for stock food; iii) consumption by a human or animal after post-harvest use (NRA 1997).

VIII. REFERENCES

Abarca ML, Bragulat MR, Castellá G, Cabañes FJ (1994) Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. *Applied and Environmental Microbiology* **60**, 2650–2652.

Abarca ML, Bragulat MR, Castellá G, Accensi F, Cabañes FJ (1997) New ochratoxigenic species in the genus *Aspergillus*. *Journal of Food Protection* **60**; 1580–1582.

Abarca ML, Accensi F, Bragulat MR, Castellà G, Cabañes FJ (2003) *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection* **66**, 504–506.

Abarca ML, Accensi F, Cano J, Cabanñes FJ (2004) Taxonomy and significance of black aspergilli. *Antonie Van Leeuwenhoek* **86**, 33–49.

Abrunhosa L, Serra R, Venâncio A (2002) Biodegradation of Ochratoxin A by Fungi isolated from grapes. *Journal of Agricultural and Food Chemistry* **50**, 7493-7496.

Abrunhosa L, Fernandes A, Venâncio A (2005) Ochratoxin A removal during the wine making. In: *Actas do VII Encontro de Química dos Alimentos*. Instituto Superior Politécnico, Viseu, Portugal.

Ali MI, Shalaby NMM, Elgamal MHA, Mousa ASM (1999) Antifungal effects of different plant extracts and their major components of selected *Aloe* species. *Phytotherapy Research* **13**, 401-407.

Alves A. (2003) Rastreio de ocratoxina A em uvas e vinhos portugueses. In: Cerdeira A, Venâncio A, Alves A, Barros P, Siamões T, editors. *Impacto da Contaminação Fúngica obre a Competitividade de Vinhos-Ochratoxina A*. Braga: Micoteca da Universidade do Minho.

Annis PC (1987) Towards rational controlled atmosphere dosage schedules: a review of current knowledge. In: Donahaye E, Navarro S (Eds), *Proceedings of the Fourth International Working Conference on Stored-Product Protection*, Tel Aviv, Israel, September 21–26, 1986. Bet Dagen, pp. 128–148.

Ansari AA, Shrivastava AK (1991) The effect of eucalyptus oil on growth and Aflatoxin production by *Aspergillus flavus*. *Letters in Applied Microbiology* **13**, 75-77.

Arisan-Atac I, Heidenreich E, Kubicek ChP (1995) Randomly amplified polymorphic DNA fingerprinting identifies subgroups of *Trichoderma viride* and other *Trichoderma* sp. capable of chestnut blight biocontrol. *FEMS Microbiology Letters* **126**, 249-256.

Asenjo J (2001) Ante la definición euopea. Propuesta de clasificación de los vinos dulces y de licor. Cultura del vino. Mundovino. Available online: http://elmundovino.elmundo.es/elmundovino/noticia.html?vi_seccion=2&vs_fecha=2001

10&vs_noticia=1004043960. Last updated: October 25 2001. Date accessed: April 17 2007.

Babitskaya VG, Shcherba VV (2002) The nature of melanin pigments of several micro- and macromycetes. *Applied Biochemistry and Microbiology* **38**, 247-251.

Barkai-Golan R (1980) Species of *Penicillium* causing decay of stored fruits and vegetables in Israel. *Mycopathologia Mycologia Applicada* **54**, 141–145.

Barr JG (1976) Effects of volatile bacterial metabolites on the growth, sporulation and mycotoxin production of fungi. *Journal of the Science of Food and Agriculture* **27**, 324-330.

Battilani P, Pietri A (2002) Ochratoxin A in grapes and wine. *European Journal of Plant Pathology* **108**, 639-643.

Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P, Kozakiewicz Z (2003) Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. *Journal of Food Protection* **66**, 633–636.

Battilani P, Barbano C, Marín S, Sanchis V, Kozakiewicz Z Magan N (2006) Mapping of *Aspergillus* section *Nigri* in Southern Europe and Israel based on geostatistical analysis. *International Journal of Food Microbiology* **111**, S72-S82.

Bavaresco L, Pettegolli D, Cantú E, Fregoni M, Chiusa G, Trevisan M (1997) Elicitacion and accumulation of stilbene phytoalexins in grapevine berries infected by *Botrytis cinerea*. *Vitis* **36**, 77-83.

Bavaresco L, Vezzulli S, Battilani P, Giorni P, Pietri A, Bertuzzi T (2003) Effect of ochratoxin-A producing Aspergilli on stilbenic phytoalexin synthesis in grapes. *Journal of Agriculture and Food Chemistry* **51**, 6151–6157.

Bejaoui H, Mathieu F, Taillandier P, Lebrihi A (2004) Ochratoxin A removal in synthetic and natural grape juices by selected oenological *Saccharomyces* strains. *Journal of Applied Microbiology* ***97***, 1038-1044.

Bejaoui H, Mathieu F, Taillandier P, Lebrihi A (2006) Biodegradation of ochratoxin A by *Aspergillus* section *Nigri* species isolated from French grapes: a potential means of ochratoxin A decontamination in grape juices and musts. *FEMS Microbiology Letters* **255**, 203-208.

Bellí N, Pardo E, Marín S, Farré G, Ramos AJ, Sanchis V (2003) Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *Journal of the Science of Food and Agriculture* **84**, 541-546.

Bellí N, Pardo E, Marín S, Farré G, Ramos AJ, Sanchis V (2004a) Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *Journal of the Science of Food and Agriculture* **4**, 541-546.

Bellí N, Ramos AJ, Sanchis V, Marín S (2004b) Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. *Letters in Applied Microbiology* **38**, 72-77.

Bellí N, Mitchell D, Marín S, Alegre I, Ramos AJ, Magan N, Sanchis V (2005a) Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. *European Journal of Plant Pathology* **113**, 233-239.

Bellí N, Ramos AJ, Coronas I, Sanchis V, Marín S (2005b) *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape medium in relation to environmental conditions. *Journal of Applied Microbiology* **98**, 839-844.

Bellí N, Marín S, Coronas I, Sanchis V, Ramos AJ (2006a) Skin damage, high temperatures and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes. *Food Control* (submitted).

Bellí N, Marín S, Sanchis V, Ramos AJ (2006b) Impact of fungicides on *A. carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes. *Food Additives and Contaminants* **63**, 1021-1029.

Ben Arie R, Droby S, Zutkhi J, Cohen L, Weiss B, Sarig P, Zeidman M, Daus A, Chalutz E (1991) Preharvest and postharvest biological control of *Rhizopus* and *Botrytis* bunch rots of table grapes with antagonistic yeasts. In: Wilson, C., Chalutz, E. (Eds.), *Proceedings Workshop Biological Control of Postharvest Diseases in Fruits and Vegetables*. U.S. Dep. Agric. Agric. Res. Serv. Publ. 92, pp. 111–113.

Benkhemmar O, Lahlou H, Dupont J, Bompiex G, Boubekri C, EL-Mniai H (1993) Identification of different species of *Penicillium* causing deterioration of Moroccan table grapes during storage. *Mycopathologia* **124**, 27–30.

Berente B, Móricz A, H-Otta K, Záray G, Lékó L, Rácz L (2005) Determination of ochratoxin A in Hungarian wines. *Microchemical Journal* **79**, 103-103.

Blesa J, Soriano JM, Moltó JC, Mañes J (2004) The concentration of Ochratoxin A in wines from supermarkets and stores of Valencian Community (Spain). *Journal of Chromatography* A **1054**, 397-401.

Blesa J, Soriano JM, Moltó JC, Mañes J (2006) Factors affecting the presence of ochratoxin A in wines. *Critical Reviews in Food Science and Nutrition* **46**, 473-478.

Bleve G, Grieco F, Cozzi G, Logrieco A, Visconti A (2006) Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. *International Journal of Food Microbiology* **108**, 204-209.

Bonomelli A, Mercier L, Franchel J, Baillieul F, Benizri E, Mauro M-C (2004) Response of grapevine defenses to UV-C exposure. *American Journal of Enology and Viticulture* **55**, 51-59.

Borie B, Jeandet P, Parize A, Bessis R, Adrian M (2004) Resveratrol and stilbene synthase mRNA production grapevine leaves rated with biotic and abiotic phytoalexin elicitors. *American Journal of Enology and Viticulture* **55**, 60-64.

Bridge PD, Hawksworth DL, Kozakiewicz Z, Onions AHS, Paterson RRM, Sackin MJ, Sneath PHA (1989) A reappraisal of the terverticillate Penicillia using biochemical, physiological and morphological features. 1. Numerical taxonomy. *Journal of Genetics and Microbiology* **135**, 2941–2966.

Burdaspal PA, Legarda TM (1999) Ochratoxin A in wines and grape musts and juices produced in Spain and other European countries. *Alimentaria* **299**, 107-113.

Cabañes FJ, Accensi F, Bragulat MR, Abarca ML, Castellá G, Minguez S, Pons A (2002) What is the source of ochratoxin A in wine? *International Journal of Food Microbiology* **79**, 213–215.

Cairns-Fuller V, Aldred D, Magan N (2005) Water, temperature and gas composition interactions affect growth and ochratoxin A production by isolates of *Penicillium verrucosum* on wheat grain. *Journal of Applied Microbiology* **99**, 1215-1221.

Cantos E, Espín JC, Tomás-Barberán FA (2002) Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MS-MS. *Journal of Agriculture and Food Chemistry* **50**, 5691-5696.

Caridi A, Galvano F, Tafuri A, Ritieni A (2006) Ochratoxin A removal during winemaking. *Enzyme and Microbial Technology* **40**, 122-126.

Carzaniga R, Fiocco D, Bowyer P, O'Connell RJ (2002) Localization of melanin in conidia of *Alternaria alternata* using phage display antibodies. *Molecular Plant-Microbe Interactions* **15**, 216-224.

Castellari M, Versari A, Fabiani A, Parpinello GP, Galassi S (2001) Removal of ochratoxin A in red wines by means of adsorption treatments with commercial fining agents. *Journal of Agriculture and Food Chemistry* **49**, 3917-3921.

Cecchini F, Morassut M, Moruno EG, Di Stefano R (2006) Influence of yeast strain on ochratoxin A content during fermentation of white and red must. *Food Microbiology* **23**, 411-417.

Cetinkaya N, Ozyardimci B, Denli E, Ic E (2006) Radiation processing as a post-harvest quarantine control for raisins, dried figs and dried apricots. *Radiation Physics and Chemistry* **75**, 424-431.

Chelkowski J, Samson RA, Wiewiorowska M, Golinski E (1987) Ochratoxin A formation by isolated strains of the conidial stage *of Aspergillus glaucus* Link ex Grey (= *Eurotium herbariorum* Wiggers Link ex Grey) from cereal grains. *Die Nahmng* **31**, 267-270.

Chet I (1987) *Trichoderma* - application, mode of action and potential as a biocontrol agent of soil-borne plant pathogenic fungi. In: *Innovative Approaches to Plant Disease Control*, pp. 137-160 New York: Wiley.

Ciegler A (1972) Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus* ochraceus group. *Canadian Journal of Microbiology* **18**, 631-636.

Clark H, Snedeker S (2004) Ochratoxin A: Its cancer risk and potential for exposure. Cornell University. College of Veterinary Medicine. Program on: Breast Cancer and Environmental Risk Factors. Fact Sheet 51. December 2004. Available online: http://envirocancer.cornell.edu/FactSheet/general/fs51.OchratoxinA.cfm. Last updated: January 28 2005. New York.

Clary CD, Mejia-Meza E, Wang S, Petrucci VE (2007) Improving grape quality using microwave vacuum drying associated with temperature control. *Journal of Food Science* **72**, 23-28.

Commission Regulation (2005) (EC) No 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2001 as regards ochratoxin A. Official Journal of the European Union L25, 3–5.

Commission Regulation (2006) (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union. Brussels: 20 December 2006.

Cook RJ, Baker KF (1989) *The nature practice of biological control of plant pathogens.* APS Press, pp. 539. St. Paul.

Cooke RC, Rayner ADM (1984) *Ecology of Saprotrophic Fungi*. Longman, New York.

Corley DG, Miller-Wideman M, Durley RC (1994) Isolation and structure of harzianum A: a new trichothecene from *T. harzianum*. *Journal of Natural Products* **57**, 422-425.

Corrigendum to Commission Regulation (2005) (EC) No 1512/2005 amending Regulation (EC) No 753/2002 laying down certain rules for applying Council Regulation (EC) No 1496/1999 as regards the description, designation, presentation and protection of certain wine sector products (OJ L 241, 17.9.2005). Brussels: 15 September 2005.

Council Directive (1991) (EEC) No 414/1991 concerning the placing of plant protection products on the market. Consolidated text produced by the Consleg system of the Office for Official Publications of the European Communities. Brussels: 15 July 1991.

Council Regulation (1999) (EC) No 1493/1999 On the common organisation of the market in wine. Office for Official Publications of the European Communities. Brussels: 17 May 1999.

Creppy EE, Bedbeter AM, Gharbi A, Counord J, Castegnaro M, Bartsch H, Moncharmont P, Foiullet B, Chambon P, Dirheimer G (1991) Human ochratoxicosis in France mycotoxins, endemic nephropathy and urinary tract Tumors, Scientific Publication No

115, edited by M. Castegnaro, R. Plestina, G. Dirheimer, I.N. Chernozemsky and H. Bartsch. Lyon: International Agency for Research on Cancer, 145-151.

Crisosto CH, Garner D, Crisosto G (2002) High carbon dioxide atmospheres affect stored ‘Thomson Seedless’ table grapes. *Horticulture Science* **37**, 1074-1078.

Czerwiecki L, Czajkowska D, Witkowska-Gwiazdowska A (2002) On ochratoxin A and fungal flora in Polish cereals from conventional and ecological farms. Part 2: Occurrence of ochratoxin A and fungi in cereals in 1998. *Food Additives and Contaminants* **19**, 1051-1057.

Deng Y, Wu Y, Li Y (2006) Physiological responses and quality attributes of ‘Kyoho’ grapes to controlled atmospheres storage. *LWT-Food Science and Technology* **39**, 584-590.

Dictionary.com Unabridge (2007) Dictionary.com Unabridge (v 1.1) Random House, Inc. Available online: http://dictionary.reference.com. Last updated: March 29 2007. Accessed: March 29 2007.

Diputación Foral de Bizkaia (2005) Viticultura and Enology Bulletin No. 6. Available online: http://www.bizkaia.net/Nekazaritza/agricultura/boletines/in_septiembre_2005.pdf. Last updated: September 2005. Accessed: March 29, 2007.

Domsch KH, Gams W, Anderson TH (1980) *Compendium of Soil Fungi*. vol. 1 & 2. 405 pp. London: Academic Press.

Doymaz I, Pala M (2002). The effects of dipping pretreatments on air-drying rates of the seedless grapes. *Journal of Food Engineering* **52**, 413–417.

Duguay KJ, Klironomos JN (2000) Direct and indirect effects of enhanced UV-B radiation on the decomposing and competitive abilities of saprobic fungi. *Applied of Soil Ecology* **14**, 157-164.

Dumoulin M, Riboulet JM (2002) Considerations on presence of ochratoxin A in wines and grape juices. *Revue des Oenologues et des Techniques Vitivinicoles et Oenologiques* **104**, 11–13.

Eder R (2005) Ochratoxin in Austrian wines? *Der Winzer* **61**, 19-21.

Egorov T, Oditsova T, Musolyamov AK, Fido R, Tatham A, Shewry P (1996) Disulphide structure of a sunflower seed albumin: conserved and variant disulphide bonds in the cereal prolamin superfamily. FEBS Letters **396**, 285.288

El-Kady IA, El-Maraghy SS, Mostafa ME (1995) Natural occurrence of mycotoxins in different spices in Egypt *Folia Microbiology* **40**, 297-300.

El Halouat A, Debevere JM (1997) Molds and yeasts isolated from hydrated prunes and raisins having different water activities. *Sciences des Aliments* **17**, 539-545.

Elad Y (2001) TRICHODEX: commercialization of *Trichoderma harzianum* T39 – a case study. In: Jarvis P, ed. *Agro Report, Biopesticides: Trends and Opportunities.* Richmond, UK: PJB Publications Ltd, 45–50.

Esteban A, Abarca ML, Bragulat MR, Cabañes FJ (2004) Effects of temperature and incubation time on production of ochratoxin A by black aspergilli. *Research in Microbiology* **155**, 861–866.

Esteban A, Abarca ML, Bragulat MR, Cabañes FJ (2006a) Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species. *International Journal of Food Microbiology* **108**, 188-195.

Esteban A, Abarca ML, Bragulat MR, Cabañes FJ (2006b) Study of the effect of water activity and temperature on ochratoxin A production by *Aspergillus carbonarius*. *Food Microbiology* **32**, 634-640.

Esterio M, Auger J, Vasquez E, Scheelje F (1992) Efficacy of a grapefruit seed extract BC-100 for control of *Botrytis cinerea* in table grape in Chile. pp: 211-214. In: *Recent Advances in Botrytis Research*. Proceedings of the 10th International Symposium, Crete, Greece.

Fadhel A, Kooli S, Farhat A, Bellghith A (2005) Study of the solar drying of grapes by three different processes. *Desalination* **185**, 535-541.

Franck J, Latorre BA, Torres R, Zoffoli JP (2005) The effect of preharvest fungicide and postharvest sulphur dioxide use on postharvest decay of table grapes caused by *Penicillum expansum. Postharvest Biology and Technology* **37**, 20-30.

Frisvad JC, Frank JM, Houbraken JAMP, Kuijpers AFA, Samson RA (2004) New ochratoxin A producing species of *Aspergillus* section *Circumdati*. Studies in Mycology **50**, 23–43

Gambuti A, Strollo D, Genovese A, Ugliano M, Ritieni A, Moio L (2005) Influence of enological practices on ochratoxin A concentration in wine. *American Journal of Enology and Viticulture* **56**, 155-162.

Gómez C, Bragulat MR, Abarca ML, Mínguez S, Cabañes FJ (2006) Ochratoxin A-producing fungi from grapes intended for liqueur wine production. *Food Microbiology* **23**, 541-545.

Gonzalez-Ureña A, Orea JM, Montero C, Jiménez JB, González JL, Sánchez A, Dorado M (2003) Improving postharvest resistance in fruits by external application of trans-resveratrol. *Journal of Agriculture and Food Chemistry* **51**, 82-89.

Grazioli B, Fumi MD, Silva A (2006) The role of processing on ochratoxin A content in Italian must and wine: A study on naturally contaminated grapes. *International Journal of Food Microbiology* **111**, S93-S96.

Green CF, Scarpino VV, Jensen P, Jensen NJ, Gibbs SG (2004) Disinfection of selected *Aspergillus* spp. using ultraviolet germicidal irradiation. *Canadian Journal of Microbiology* **50**, 221-224.

Haenan CN, Shaw KJ, Pitt JI (1998) Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* and detection using coconut cream agar. *Journal of Food Mycology* **1**, 67–72.

Hall LA, Denning DW (1994) Oxygen requirements of *Aspergillus* species. *Journal of Medical Microbiology* **41**, 311-315.

Heenan CN, Shaw KJ, Pitt JI (1998) Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* and detection using coconut cream agar. *Journal of Food Mycology* 2, 67-72.

Hernández MJ, García-Moreno MV, Durán E, Guillén D, Barroso CG (2006) Validation of two analytical methods for the determination of ochratoxin A by reversed-phased high-performance liquid chromatography coupled to fluorescence detection in musts and sweet wines from Andalusia. *Analytica Chimica Acta* **566**, 117-121.

Hinrichsen P, Reyes MA, Castro A, Araya S, Garnier M, Prieto H, Reyes F, Muñoz C, Dell'Orto P, Moynihan MR (2005) Genetic transformation of grapevines with *Trichoderma harzianum* and antimicrobial peptide genes for improvement of fungal tolerance. ISHS *Acta Horticulture* **689**: VII International Symposium on Grapevine Physiology and Biotechnology. Davis, CA, USA.

Horie Y (1995) Productivity of ochratoxin A of *Aspergillus carbonarius* in *Aspergillus* section *Nigri. Nippon Kingakukai Kaiho* **36**, 73–76.

IARC (1993) (International Agency for Research on Cancer) Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Vol. 56.Lyon: International Agency for Research on Cancer. pp. 489-521.

Infoagro Systems (2007) AgroValdemécum. Infoagro Systems S.L. Available online: http://www.infoagro.com/agrovademecum/fito_plaga.asp. Last updated: 2001. Accessed: June 12, 2007.

Italian Health Superior Institute (2002) Assessment of dietary intake of ochratoxin A by the population of EU members states. Reports on Tasks for Scientific Cooperation. Report of Experts Participating in Task 3.2.7. January 2002, Rome.

Izumizawa Y, Yang S-J, Negishi T, Negishi K (2000) DNA lesion and mutagenesis induced in phageM13mp2 by UVA, UVB and UVC irradiation. *Nucleic Acids Symposium Series* **44**, 73-74.

Jarvis B (1977) *Botryotinia* and *Botrytis* species: taxonomy, physiology, and pathogenicity. Canadian Department of Agriculture Research. State, Harrow, Monograph 15. p. 195.

JECFA (1995) Joint FAO/WHO Expert Comittee on Food Additives, 44th Meeting. Technical Report Series Nº 859, Ginebra, OMS.

JECFA (2001) (Joint FAO/WHO Expert Committee on Food Additives). Safety evaluations of certain mycotoxins in food. In *WHO Food AdditiVes*; World Health Organization: Geneva, Switzerland, 2001; Series **27**.

Jun S, Irudayaraj J, Demirci A, Geiser D (2003) Pulsed UV-light treatment of corn meal for inactivation of *Aspergillus niger* spores. *International Journal of Food Science and Technology* **38**, 883-888.

Kallay M, Magyar I (2000) Occurrence of ochratoxin A under oenological conditions with special regard to the processing of Tokaj Aszú. In: *Proceedings of Lippary* J. Vas K. *Scientific Symposium*. Budapest: Food Science. p. 15.

Karathanos VT, Belessiotis VG (1999). Application of a thinlayer equation to drying data of fresh and semi-dried fruits. *Journal of Agricultural Engineering Research* **74**, 355–361.

Keren-zur M, Lazare M, Khusid A, Bercovitz A, Rebhun M, Cohen L, Weiss B, Daus A, Karabulut OA, Tezcan H (2002) Development and commercial testing of the yeast Metschnikowia fructicola for the control of pre and postharvest diseases. In: Proceedings: Seventh Meeting IOBC-EFPP, May 22-26. Elad Y, Kohl J, Delen N (eds). EBILTEM, Ege University, Kusadasi, Turkey. p. 69.

Kikkert JR., Ali GS, Striem MJ, Martens M-H, Wallace PG, Molino L, Reisch BI (1997) Genetic Engineering of Grapevine (*Vitis* sp.) for Enhancement of Disease Resistance. Proceedings: Third International Symposium on In Vitro Culture and Horticultural Breeding. ISHS. 16-21 June 1996. Jerusalem, Israel. Altman A, Ziv M (eds.) *Acta Horticulturae* **447**, 273-279.

Kluger M, Loeffler W, Rapp C, Kern A, Jung G (1990) Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: biological properties. *Archives of Microbiology* **153**, 276-81.

Kozakiewicz Z, Paterson RRM, Bridge PD (1993) Novel approaches to the identification of mycotoxin producing Penicillium species. In: Scudamore (Ed.). *Occurrence and significance of mycotoxins*. London, Central Science Laboratory, pp. 64-75.

Langcake P, Pryce RJ (1977) The production of resveratrol and the viniferins by grapevines in response to ultraviolet irradiation. *Phytochemistry* **16**, 1193–1196.

Larcher R, Nicolini G (2001) Survey of ochratoxin A in musts, concentrated musts and wines produced or marketed in Trentino (Italy). *Journal Commodity Science* **40**, 69-78.

Leiter E, Marx F, Pusztahelyi T, Haas H, Pócsi I (2004) *Penicillium chrysogenum* glucose oxidase-a study on its antifungal effects. *Journal of Applied Microbiology* **97**, 1201-1209.

Leong SL, Hocking AD, Pitt JI (2004) Occurrence of fruit rot fungi (*Aspergillus* section *Nigri*) on some drying varieties of irrigated grapes. *Australian Journal of Grape and Wine Research* **10**, 83–88.

Leong SL, Hocking AD, Varelis P (2006) Fate of ochratoxin A during vinification of Semillon and Shiraz grapes. *Journal of Agriculture and Food Chemistry* **54**, 6460-6464.

Logrieco A (2004) Communnication: Ochratoxin A in grapes and wine. GoodFood AmI Workshops. Florence, July 15-16 2004. Available online: http://www.goodfood-project.org/www/Works/Florence/presentations/Logrieco.pdf. Last accessed: March 27 2007.

López de Cerain A, Gónzalez-Peñas E, Jiménez AM, Bello J (2002) Contribution to the study of ochratoxin A in Spanish wines. *Food Additives and Contaminants* **19**, 1058–1064.

MacDonald SJ, Wilson P, Barnes K, Damant A, Massey R, Mortby E, Shepherd MJ (1999) Ochratoxin A in dried vine fruit: method development and survey. *Food Additives and Contaminants* **16**, 253-260.

Magnoli C, Violante M, Combina M, Palacio G, Dalcero A (2003) Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Letters in Applied Microbiology* **37**, 179-184.

Magnoli C, Astoreca A, Ponsone L, Combina M, Palacio G, Rosa CAR, Dalcero AM (2004) Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Leters in Applied Microbiology* **39**, 326-331.

Margalith PZ (1992) *Pigmented Microbiology*. London: Chapman and Hall.

Marois J, Bledsoe A, Bettiga L (1992) *Botrytis* Bunch Rot. Pages 63-68. In: *Grape Pest Management*, 2nd edition. University of California, Division of Agriculture and Natural Resources Publication 3343. California, Oakland, pp. 63-68.

Marquenie D, Geeraerd AH, Lammertyn J, Soonjens C, Van Impe JF, Michiels CW, Nicolai BM (2003) Combinations of pulsed white light and UV-C or mild heat treatment to inactivate conidia of *Botrytis cinerea* and *Monilia fructigena*. *International Journal of Food Microbiology* **85**, 185-196.

Medina A, Mateo R, López-Ocaña L, Valle-Algarra FM, Jiménez M (2005) Study of Spanish grape microbiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. *Applied and Environmental Microbiology* **71**, 4696–4702.

Miraglia M, Brera C (2002) 'Assessment of Dietary Intake of Ochratoxin A by the Population of EUMember States'. Reports on Tasks for Scientific Cooperation, Directorate-General Health and Consumer Protection, Task 3.2.7 ,Brussels.

Mitchell D, Parra R, Aldred D, Magan N (2004) Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. *Journal of Applied Microbiology* **97**, 439–445.

Munimbazi C, Bullerman LB (1998) Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. *Journal of Aplied Microbioogy* **84**, 959-968.

Nair NG, Emmett RW, Parker FE (1987). Programming applications of dicarboximides to control bunch rot of grapes of *Botrytis cinerea. Plant Pathology* **36**, 175-9.

Nampoothiri KM, Baiju TV, Sandhya C, Sabu A, Szakacs G, Pandey A (2004) Process optimization for fungal chitinase production by *Trichoderma harzianum*. *Process Biochemistry* **39**,1583–1590

Nigro F, Ippolitto A, Lima G (1998) Use of UV-C light to reduce *Botrytis* storage rot of table grapes. *Postharvest Biology and Technology* **13**,171–181.

Northolt MD, van Egmond HP, Paulsch WE (1979) Ochratoxin A production by some fungal species in relation to water activity and temperature. *Journal of Food Protection* **42**, 485-490.

NRA (1997) (National Registration Authority for Agricultural and Veterinary Chemicals). Ag Labelling Code: Code of Practices for Labelling Agricultural ChemicalProducts, NRA, Canberra.

O'Callaghan J, Caddick MX, Dobson DW (2003) A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. *Microbiology* **149**, 3845-3491.

Ono H, Kataoka A, Koakutsu M, Tanaka K, Kawasugi S,Wakazawa M, Ueno Y, Manabe M (1995) Ochratoxin A productibility by strains of *Aspergillus niger* group stored in IFO culture collection. *Mycotoxins* **41**, 47–51.

Ontario (2007) Sulphur Dioxide (SO_2). Air Quality Ontario. Ministry of Environment. Available online: http://www.airqualityontario.com/science/pollutants/sulphur.cfm. Last updated: 2007. Last accessed: April 11 2007.

Otteneder H, Majerus P (2000) Occurrence of ochratoxin A (OTA) in wines: Influence of the type of wine and its geographical origin. *Food Additives and Contaminants* **17**, 793–798.

Otteneder H, Majerus P (2001) Ochratoxin A (OTA) in coffee: nation-wide evaluation of data collected by German Food Control 1995–1999. *Food Additives and Contaminants* **18**, 431–435.

Palacios-Cabrera H, Taniwaki MH, Hashimoto JM, Menezes HC (2005) Growth of *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* on culture media at different water activities and temperatures. *Brazilian Journal of Microbiology* **36**, 24-28.

Pesticides Action Network (PAN) (2006) Orme S, Kegley S. Pesticide Database. Action Network, San Francisco, CA. Available from: http://www.pesticideinfo.org. Accessed March 2 2007.

Panasenko VT (1967) Ecology of microfungi. *Botanical Review* **33**, 189-215.

Pancotto VA, Sala OE, Cabello M, López NI, Robson TM, Ballaré CL, Caldwell MM, Scopel AL (2003) Solar UV-B decreases decomposition in herbaceous plant litter in Tierra del Fuego, Argentina: potential role of an altered decomposed community. *Global Change Biology* **9**, 1465-1474.

Pangavhane DR, Sawhney RL (2002) Review of research and development work on solar driers for grape drying. *Energy Converts Manage* **43**, 45-61.

Pardo E, Marín S, Ramos AJ, Sanchis V (2004) Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different origins. *Food Science Technology International* **10**, 0045-5.

Pardo E, Lagunas U, Sanchis V, Ramos AJ, Marín S (2005) Influence of water activity and temperature on conidial germination and mycelial growth of ochratoxigenic isolates of *Aspergillus ochraceus* on grape juice synthetic medium. Predictive models. *Journal of the Science of Food and Agriculture* **85**, 1681-1686.

Parra R, Magan N (2004) Modelling the effect of temperature and water activity on growth of Aspergillus niger strains and applications for food spoilage moulds. *Journal of Applied Microbiology* **97**, 429-438.

Paster N, Lisker N, Chet I (1983) Ochratoxin A production by *Aspergillus ochraceus* Wilhelm grown under controlled atmospheres. *Applied and Environmental Microbiology* **45**,1136-1139.

Pateraki M, Dekanea A, Mitchell D, Lydakis D, Magan N (2005) Potential for control of *A. carbonarius* strains from vine fruits using sulphur dioxide or controlled atmosphere storage. International Workshop: *Ochratoxin A in grapes and wine, prevention and control*. Marsala, Sicily, Italy.

Paul B, Girard I, Bhatnagar T, Bouchet P (1997) Suppression of *Botrytis cinerea* causing grey mould disease of grape vine (*Vitis vinfera*) and its pectinolytic activities by a soil bacterium. *Microbiological Research* **152**, 413-420.

Paul B, Chereyathmanjiyil A, Masih I, Chapuis L, Benoit A (1998) Biological Control of *Botrytis cinerea* causing grey mould disease of grapevine and elicitation of stilbene phytoalexin (resveratrol) by a soil bacterium. *FEMS Microbiology Letters* **165**, 65-70.

Pavlovic M, Plestina R, Krogh P (1979). Ochratoxin A contamination of foodstuffs in an area with Balkan (endemic) nephropathy. *Acta Pathologica, Microbiologica et Immunologica B* **87**, 243-246.

Perrone G, Mulè G, Susca A, Battilani P, Pietri A, Logriego A (2006) Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingiensis*, and *Aspergillus niger* strains isolated from grapes in Italy. *Applied and Environmental Microbiology* **72**, 680-685.

Petkova-Bocharova T, Castegnaro M (1991) Ochratoxin A in human blood in relation to Balkan endemic nephropathy and urinary tract tumors in Bulgaria. *IARC Scientific Publications* **115**, 135-142.

Pfohl-Leszkowicz A, Manderville RA (2007) Ochratoxin A: and overview on toxicity and carcinogenicity in animals and humans. *Moecular Nutrition and Food Research* **51**, 61-99.

Pietri A, Bertuzzi T, Pallaroni L, Piva G (2001) Occurrence of ochratoxin A in Italian wines. *Food Additives and Contaminants* **18**, 647–654.

Pitout MJ (1969) The hydrolysis of ochratoxin A by some proteolytic enzymes. *Biochemical Pharmacology* **18**, 485–491.

Pitt JI (1987) *Penicillium viridicatum*, *Penicillium verrucosum* and production of ochratoxin A. *Applied and Environmental Microbiology* **53**, 266– 269

Pitt JI, Hocking AD (1997) *Fungi and Food Spoilage*. Blackie Academic and Professional: London.

Rahardjo YSP, Sie S, Weber FJ, Tramper J, Rinzema A (2005) Effect of low oxygen concentrations on growth and α-amylase production of *Aspergillus oryzae* in model solid-state fermentation systems. *Biomolecular Engineering* **21**, 163-172.

Ramos AJ, Labernia N, Marín S, Sanchis V, Magan N (1998) Effect of water activity and temperature on growth and ochratoxin production by three strains of *Aspergillus ochraceus* on a barley extract medium and on barley grains. *International Journal of Food Microbiology* **44**, 133-140.

Ratola N, Abade E, Simões T, Venâncio A, Alves A (2005) Evolution of ochratoxin A content from must to wine in Port wine microvinification. *Analytical and Bioanalytical Chemistry* **382**, 405-411.

Robinson CH, Dighton J, Frankland JC (1993) Resource capture by interacting fungal colonizers of straw. *Mycological Research* **97**, 547-558.

Romero SM, Comerio RM, Larumbe G, Ritieni A, Vaamonde G, Fernández V (2005) Toxigenic fungi isolated from dried vine fruits in Argentina. *International Journal of Food Microbiology* **104**, 43-49.

Rotem J, Aust HJ (1991) The effect of ultraviolet and solar radiation and temperature on survival of fungal propagules. *Journal of Phytopathology* **133**, 76–84.

Rothschild L (2002) Life in extreme environments. NASA Ames Research Center. Available online: http://www.spaceref.com/news/viewnews.html?id=463. Last updated: June 18 2002. Accessed: March 26 2007.

Rousseau S, Donèche B (2001) Effects of water activity (a_w) on the growth of some epiphytic micro-organisms isolated from grape berry. *Vitis* **40**, 75-78.

Sage E, Lamolet B, Brulay E, Moustacchi E, Chateauneuf A, Drobetski EA (1996) Mutagenic specifity of solar UV light in nucleotide excision repair-deficient rodent cells. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 176-180.

Sage L, Krivobok S, Delbos E, Seigle-Murandi F, Creppy EE (2002) Fungal flora and ochratoxin A production in grapes and musts from France. *Journal of Agricultural and Food Chemistry* **50**, 1306–1311.

Sage L, Garon D, Seigle-Murandi F (2004) Fungal microflora and ochratoxin A risk in French vineyards. *Journal of Agriculture and Food Chemistry* **52**, 5764-5768.

Samson RA, Houbraken JAMP, Kuijpers AFA, Frank JM, Frisvad JC (2004) New ochratoxin A, sclerotium producing species in *Aspergillus* section *Nigri*. *Studies in Mycology* **50**, 45-61.

Sansing GA, Davis ND, Diener UL (1973) Effect of time and temperature on ochratoxin A production by *Aspergillus ochraceus*. *Canadian Journal of Microbiology* **19**, 1259-1263.

Schena L, Nigro F, Pentimone I, Ligorio A, Ippolito A (2003) Control of postharvest rots of sweet cherries and table grapes with endophytic isolates of *Aureobasidium pullulans*. *Postharvest Biology and Technology* **30**, 209-220.

Schirmbock M, Lorito M, Wang Y, Hayes ChK, Arisan-Atac I, Scala F, Harman GE, Kubicek ChP (1994) Parallel formation and synergism of hydrolytic enzymes and peptabol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Applied and Environmental Microbiology* **60**, 4364-4370.

Seidle HF, Huber RE (2005) Transglucesidic reactions of the *Aspergillus niger* family 3 β-glucosidase: Qualitative and quantitative analyses and evidence that the transglucosidic rate is independent of pH. *Archives of Biochemistry and Biophysics* **436**, 254-264.

Serra R, Lourenço A, Alípio P, Venâncio A (2006a). Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species. *Mycological Research* **110**, 971-978.

Serra R, Mendonça C, Venâncio A (2006b). Fungi and ochratoxin A detected in healthy grapes for wine production. *Letters in Applied Microbiology* **42**, 42-47.

Silva A, Galli R, Grazioli B, Fumi MD (2003) Metodi di riduzione di residui di ocratossina A nei vini. Symposium: La difensa antiparassitaria nelle industrie alimentari e la protezione degli alimenti. Piacenza, Italy, September 18-20 2002. In: *Industria delle Berande* **32**, 467-472.

Sipiczki M (2006) *Metschnikowia* strains isolated from botrytized grapes antagonize fungal and bacterial growth by iron depletion. *Applied and Environmental Microbiology* **72**, 6716-6724.

Snow D (1949) Germination of mould spores at controlled humidities. *Annual Applied Biology* **36**, 1-13.

Snowdon AL (1990) General Introduction and Fruits. In: *A Color Atlas of Post-Harvest Diseases and Disorders of Fruits and Vegetables* **1**. Wolfe: London.

Soderstrom EL, Brandl DG (1984) Low-oxygen atmosphere for post harvest insect control in bulk-stored raisins. *Journal of Economic Entomology* **77**, 440–445.

Soufleros EH, Tricard C, Bouloumpasi EC (2003) Occurrence of ochratoxin A in Greek wines. *Journal of Science and Food and Agriculture* **83**, 173-179.

Stander MA, Bornscheneuer UT, Henke E, Steyn PS (2000) Screening of commercial hydrolases for degradation of Ochratoxin A. *Journal of Agriculture and Food Chemistry* **48**, 5736-5739.

Stander A, Steyn PS (2002) Survey of ochratoxin A in South African wines. *South African Journal of Enology and Viticulture* **23**, 9-13.

Stefanaki I, Foufa E, Tsatsou-Drtisa A, Dais P (2003) Ochratoxin A concentrations in Greek domestic wines and dried vine fruits. *Food Additives and Contaminants* **20**, 74-83.

Suárez-Quiroz ML, González-Rios O, Barel M, Guyot B, Schorr-Galindo S, Guiraud JP (2004) Effect of chemical and environmental factors on *Aspergillus ochraceus* growth and toxigenesis in green coffee. *Food Microbiology* **21**, 629-634.

Sydney Postharvest Laboratory and Food Science Australia (2006) Optimal Fresh : The Fruit, Vegetable and Fresh Produce Storage Expert System, CSIRO Publishing, Melbourne. Available online: http://www.postharvest.com.au/storage.htm. Last updated: September 2005.

Tarr CR, Clingeleffer PR (2005) Use of an oxygen absorber for disinfection of consumer packages of dried vine fruit and its effect on fruit colour. *Journal of Stored Products Research* **41**, 77-89.

Tateo F, Bononi M, Lubian E (2000) Survey on ochratoxin A in wines. Data concerning the market of table wines in brik. *Bulletin O.I.V.* **73**, 773–783.

Téren J, Varga J, Hamari Z, Rinyu E, Kevei F (1996) Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathologia* **134**, 171–176

The American Heritage (2007) The American Heritage ® Science Dictionary ©. Available online: http://dictionary.reference.com. Last updated: March 29 2007. Accessed: March 29 2007.

TGL (2005) (The Green Land TM) UV index definition. Environment Canada's World Wide Web Site. Available online: http://es-ee.tor.ec.gc.ca/e/ozone/uv_index_definition.html. Last updated: November 2 2005. Last accessed: March 28 2007.

The Winedoctor (2007a) Glossary. Available online: http://thewinedoctor.com/glossary/glossary.shtml. Last updated: March 29 2007. Accessed: March 29 2007.

The Winedoctor (2007b) Sweet Wine: *Botrytis*. Available online: http://www.thewinedoctor.com/author/sweetnoble.shtml. Last updated: June 12 2007. Accessed: June 12 2007.

Tiedt LR (1993) An electron microscope study of conidiogenesis and wall formation of conidia of *Aspergillus niger*. *Mycological Research* **97**, 1459-1462.

Tjamos SE, Antoniou PP, Kazantzidou A, Antonopoulos DF, Papageorgiou I, Tjamos EC (2004) *Aspergillus niger* and *Aspergillus carbonarius* in Corinth raisin and wine-producing vineyards in Greece: population composition, ochratoxin A production and chemical control. *Journal of Phytopathol* **152**, 250-255.

Torelli E, Firrao G, Locci R, Gobbi E (2006) Ochratoxin A producing strains of *Penicillium* spp. Isolated from grapes used for the production of "passito" wines. *International Journal of Food Microbiology* **106**, 307-312.

Turner WB, Aldridge DC (1983) Fungal metabolites II. Academic Press: London.

Ueno Y, Kawamura O, Sugiura Y, Horiguchi K, Nakajiima M, Yamamoto K, Sato S (1991) Use of monoclonal antibodies, enzyme-linked immunosorbent assay and immunoaffinity column chromatography to determine ochratoxin A in porcines era, coffee products and toxin-producing fungi. In: Castegnaro M, Plestina R, Dirheimer G, Chernozemsky IN, Bartash H, editors. *Mycotoxins endemic nephropathy and urinary tract tumors*. Lyon: IARC Scientific Publications. pp. 71-75.

Ulevičius V, Pečiulytė D, Lugauskas A, Andriejauskienė J (2004) Field study on changes in viability of airborne fungal propagules exposed to UV radiation. *Environmental Toxicology* **19**, 437-441.

University of Sydney (2004) Fungal Strategies. Available online: http://bugsbio.usyd.edu.au/Mycology/Ecology/LifeStrategies.shtml#fungalStrategies. Last updated: June 2004. Accessed: March 26 2007.

Van der Merwe KJ, Steyn PS, Fourie L (1965) Mycotoxins. Part II. The constitutions of ochratoxins A, B and C metabolites of *Aspergillus ochraceus*. Wilh. *Journal of the Chemistry Society* 7083-7088.

van der Westhuizen TJ, Augustyn OPH, Khan W, Pretorius IS (2000) Seasonal variation of Indigenous *Saccharomyces cerevisiae* strains isolated from vineyards of the Western Cape in South Africa. *South African Journal of Enology and Viticulture* **21**, 10–16.

Varga J, Kevei F, Rinyu E, Téren J, Kozakiewicz Z (1996) Ochratoxin production by *Aspergillus* species. *Applied and Environmental Microbiology* **60**, 4461–4464.

Varga J, Rigó K, Téren J (2000) Degradation of ochratoxin A by *Aspergillus* species. *International Journal of Food Microbiology* **59**, 1-7.

Varga J, Péteri Z, Tábori K, Téren J, Vágvölgyi C (2005) Degradation of ochratoxin A and other mycotoxins by *Rhizopus* isolates. *International Journal of Food Microbiology* **99**, 321-328.

Vázquez-Belda B, Fente-Sampayo CA, Quinto-Fernández E, Franco-Abuin C, Rodriguez-Otero JL, Cepeda-Saez A (1995) Incidencia de hongos toxigénicos en queserías de la zona de Arzua (La Coruña, España). *Food Science and Technology International* **1**, 91-95.

Vidal JR, Kikkert J, Malnoy MA, Wallace PG, Barnard J, Reish BI (2006) Evaluation of transgenic 'Chardonay' (*Vitis vnifera*) containing Magainin genes for resistance to Crown Gall and Powdery Mildew. *Transgenic Research* **15**, 69-82.

Visconti A, Pascale M., Centonze G (1999) Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and HPLC. *Journal of Chromatography A* **864**, 89-101.

VITI-OENO (2005) Code of sound viticultural practices in order to mininise levels of ochratoxin A in vine-based products. Paris, October 24 2005. Available online: http://news.reseau_concept.net/images/oiv_uk/Client/VITI-OENO_1-2005_EN.pdf. Accessed: March 27 2007.

Wallace HAH, Sinha RN, Mills JT (1976) Fungi associated with small wheat bulks during prolonged storage in Manitoba. *Canadian Journal of Botany* **54**, 1332-1343.

Wheeler KA, Hocking AD (1988) Water relations of *Paecilomyces variatii*, *Eurotium amstelodami*, *Aspergillus candidus* and *Aspergillus sydowii*, xerophilic fungi isolated from Indonesian dried fish. *International Journal of Food Microbiology* **7**, 73-78.

Wikipedia (2007) Wikipedia the free encyclopedia. Available online: http://wikipedia.org. Accessed: March 29 2007.

Winedefinitions (2007) Winedefinitions.com. Available online: http://winedefintions/define/define.cfm. Last updated: March 29 2007. Accessed: March 29 2007.

Winegeeks (2006) Winegeeks.com Wine Glossary. Available online: http://winegeeks.com/resources/glossary/. Last updated: 2006. Accessed: March 29 2007.

Yamamoto T, Iketani H, Ieki H, Nishizawa Y, Notsuka K, Hibi T, Hayashi T, Matsuna N (1999) Fungal disease resistance in transgenic grapevine expressing a rice quitinase Plant ad Animal Genome VII Conference. San Diego, CA, January 17-21.

Youngchim S, Morris-Jones R, Hay RJ, Hamilton AJ (2004) Production of melanin by *Aspergillus fumigatus*. *Journal of Medical Microbiology* **53**, 175-181.

Zahavi T, Cohen L, Weiss B, Schena L, Daus A, Kaplunov T, Zutkhi J, Ben-Arie R, Droby D (2000) Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. *Postharvest Biology and Technology* **20**, 115-124.

Zimmerli B, Dick R (1996) Ochratoxin A in table wine and grape-juice: occurrence and risk assessment. *Food Additives and Contaminants* **13**, 655-668.

Zoffoli JP, Latorre BA, Rodríguez EJ, Aldunce P (1999) Modified atmosphere packaging using chlorine gas generators to prevent *Botrytis cinerea* on table grapes. *Postharvest Biology and Technology* **15**, 135-142.

IX. ACKNOWLEDGEMENTS

Quizás los agradecimientos deberían traducirse a más idiomas que el resumen mismo de la tesis. En estos cuatro años han cabido tantas experiencias, lugares y personas que difícilmente pueden resumirse brevemente.

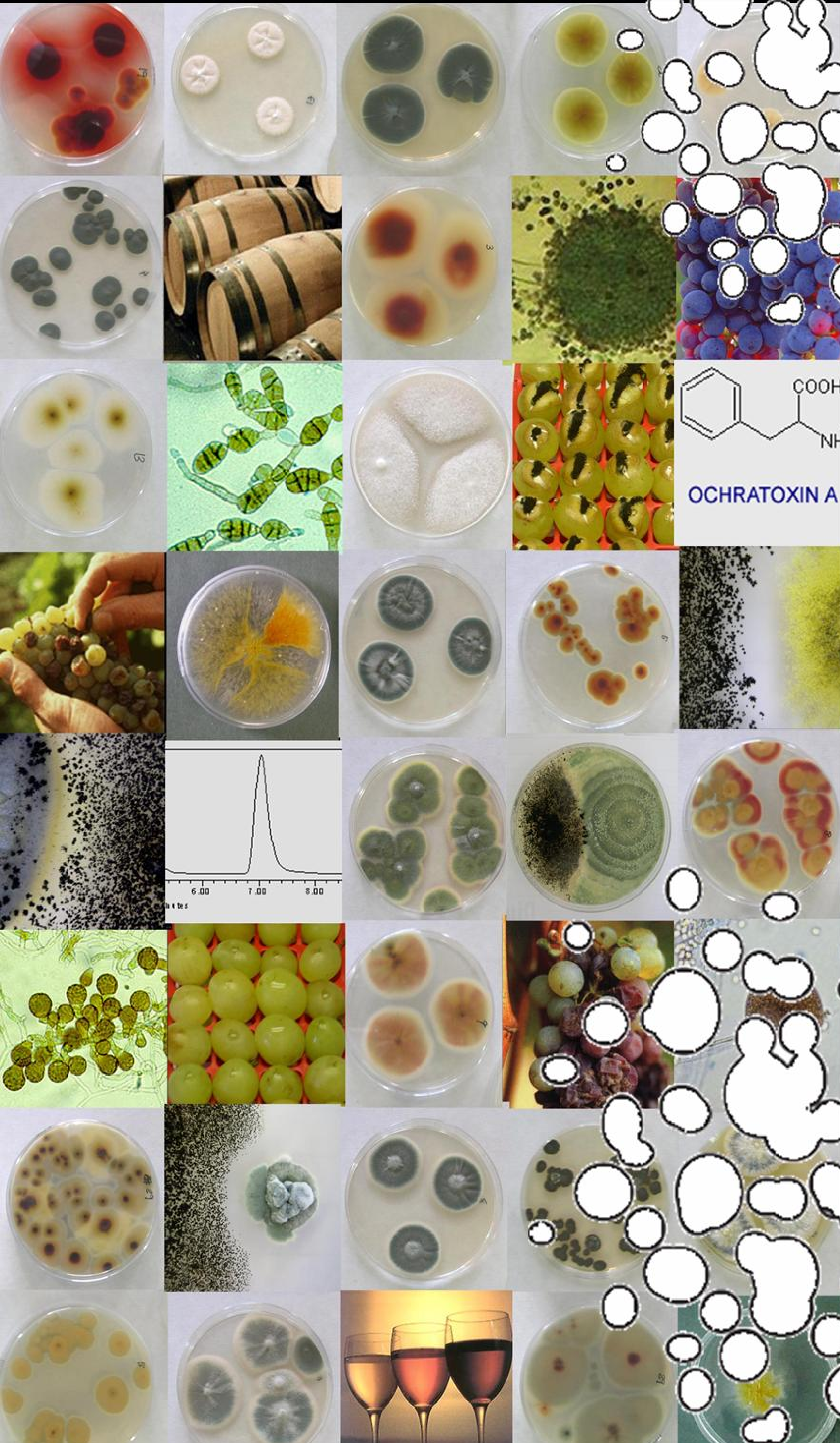
Loli García y Federico Uruburu me dieron la oportunidad de curiosear en el mundo de la microbiología, por primera vez, en la Colección Española de Cultivos Tipo hasta que Misi Jiménez me habló de Lleida. Confieso que la imaginé en la cima de los pirineos y con nieves perpetuas, luego descubrí que se trataba de la Seu con boira.

A Vicente Sanchis y Sonia Marín, mis directores, y a Antonio Ramos les agradezco su apoyo y guía durante este tiempo de trabajo y pequeñas batallas. Neus me presentó al Sr. Carbonarius y su pandilla, incluidos nuestro inglés más simpático; Sir David y a Su-lin, científica y cantante.

En el apartamento de micro hubo risas, y por supuesto trabajo, con Ester, Miren, Robert-el vecino, Montse-pelos, la Nuri y Hector- el hombre tranquilo, y siguiendo por el pasillo no puedo dejarme a Shakiro-Luz, Arantza-colores y Alejandra 4ª gama. Todos ellos fueron mi familia adoptiva durante este camino.

From my unforgettable austral journal, I will remember and thank to Ailsa, Su-lin, Mariam, Nick and Nai their teachings and friendship. All who I met made Sydney be my home while I was there: Taline, Raquel, Carina and specially my flatmaties Donnie and David Xu for many memories.

Por los innumerables buenos ratos con todos aquellos que he mencionado y algunos más, que por no convertir la tesis en biografía no puedo relatar, me voy con una maleta mucho más grande que con la que vine.


COOH
OCHRATOXIN A
6.00
7.00
8.00