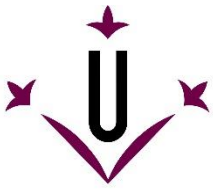
Challenges in management of aflatoxins and ochratoxin A in contaminated raw materials

Esther García Cela

Dipòsit Legal: L.145-2015
http://hdl.handle.net/10803/285374



Universitat de Lleida

Challenges in Management of Aflatoxins and Ochratoxin A in Contaminated Raw Materials

Esther García-Cela

Doctoral Thesis

Thesis supervisors

Antonio J. Ramos Girona, PhD.

Sonia Marín Sillué, PhD.

Dep. Tecnologia d'Aliments

Universitat de Lleida

Lleida 2014

Thesis submitted by Esther García-Cela to fulfil the requeriments of the degree of Doctor.

Thesis Reviewers

Armando Venâncio, PhD.
Universidade do Minho, Braga (Portugal).

Andrea Patriarca, PhD.
Universidad de Buenos Aires, Buenos Aires (Argentina)

Dissertation Committee

Armando Venâncio, PhD.
Universidade do Minho, Braga (Portugal)

Agustín Ariño, PhD.
Universidad de Zaragoza, Zaragoza (Spain)

Inmaculada Viñas, PhD.
Universitat de Lleida, Lleida (Spain).

Doctoral defense in Lleida, September 2014

Articles presented in this document are indexed in the following journals:

INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY
FOOD CONTROL
JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE

This research work was carried out in the Applied Mycology Unit of the Food Technology Department of the University of Lleida, Spain. This work was supported by the Spanish Government (project AGL2010-22182-C04-04), the BASELINE European project 22738, and the Mycored KBBE-2007-2-5-05 European project (222690).

The candidate to the degree of Doctor was financially supported in the first five months by a pre-doctoral grant of the University of Lleida, and the following three years by a pre-doctoral grant of the Agència de Gestiò d`Ajuts Universitaris de Recerca, funded by the Commisionat per a Universitats I Recerca del Departament d`Innovació, Universitats i Empresa of the Generalitat de Catalunya (Spain) and the European Social Fund.

A Predoctoral collaborating residence was carried out at Istituto di Entomologia e Patologia Vegetale of the Universitá Cattolica del Sacro Cuore, Piacenza (Italy) under the title Evaluation of atoxigenic isolates of *Aspergillus flavus* as a potencial biological control agents (2012).

AGRADECIMIENTOS

Es para mí un enorme placer el poder agradecer con todo mi corazón la ayuda prestada por todas las personas que, de alguna u otra forma, han colaborado para que este trabajo vea hoy la luz.

Es por esto que quisiera agradecer el apoyo y los conocimientos transmitidos por mis tutores, el Dr. Antonio Ramos y la Dra. Sonia Marín, gracias por darme la oportunidad de trabajar con vosotros, y por dejarme empaparme de vuestros conocimientos. No cabe decir que he aprendido muchísimo durante estos años en el grupo, y que, junto al Dr. Vicent Sanchis, habéis logrado dejar impronta en una de los periodos más importantes de mi vida profesional.

Gracias también al resto de personal del departamento, profesores y técnicos por haber compartido el día a día en las instalaciones y los equipos……. Especialmente a Jordi por las bonitas jornadas de vendimia que hemos pasado juntos.

Grazie alla professoressa Paola Battilani e al dottor Antonio Mauro, per la loro disponibilità e per aver permesso una proficua collaborazione tra le nostre università. Saluto con particolare affetto la mia amica Elisa e la sua famiglia, che mi hanno accolto come una di loro. Con grande affetto, saluto anche tutto il personale dell'Istituto di Patologia Vegetale: Simone, Giuseppe, Irene, Paola, Sara, Tito, Rosella, Giorgio, Marco, Giogio, e il professor Rossi, con i quali ho condiviso parte del mio soggiorno nella fredda, ma carina, città di Piacenza

Obrigada ao prof. Doutor Armando Venâncio pela correção do documento final da Tese e por todas as suas contribuições para melhorar-lo. Muchas gracias también, a la Dra. Andrea Patriaraca por su contribución en el documento final y por sus consejos desde el incio de esta tesis.

I also want to express my gratitude to the editors of the journals and specially the anonymous reviewers of the published articles of this work as well as to the external reviewers of the final document. Thank you for sharing your knowledge and giving suggestions to improve the manuscripts. Thanks also to the authors who answered my e-mails, from article requests to specific questions on their works. It was cheerful to experience such collaboration.

A los compañeros de la sala de becarios, con los que compartí horas de trabajo, stress y risas, y el intercambio de información multidisciplinario: gracias por ayudarme con vuestro conocimiento y puntos de vista diferentes! Agradezco en especial la compañía de mi familia mejicana, la cual no deja de crecer jejeje… Rogelio, Janet, Cyndia, Ines, Guillermo...al pequeño Dami que siempre hizo de nuestra sala un lugar acogedor con sus preciosos dibujos. A Paola, por compartir su particular forma de ver la vida, por su ilusión y por enseñarnos a todos que querer es poder y que todas las metas son alcanzables. A Maria, por su hombro para apoyarme siempre que fuera necesario, por las buenas horas dentro y fuera de la escuela, creo que nunca podre olvidar al moreno y al poni trotador, también a Rubén por compartir mi particular humor…. A Alejandra, por el coffee time, la sopa de caracol y por intentar mostrarme el lado bueno de las cosas. Aquellos que me han sufrido como asesora y con los que también he aprendido: Monica, Aleix, los Hector…Aquellos con los que he compartido laboratorio Amani, Abdelali, Andrea, Daiana…Buff, Marta, cuantas horas… por estar siempre, siempre ahí dispuestas a ayudar, por tus buenos consejos, por las visitas de los viernes,…A la ultima incorporación Irene animo que tu vales mucho!! En general a todos los compañeros del piso de abajo, por toda la ayuda en la etapa final de la tesis. Por la ayuda técnica, el conocimiento, por mostrarme otra forma de pensar.

Y por supuesto, gracias a todas las personas que, sin estar relacionadas con el departamento, han sido también una parte importante de mi etapa en Lleida.

A mi familia que aunque estemos lejos, siempre están presentes, mamá, Carolina, Rebeca, y mis sobrinitas Raquel y Eva por regalarme momentos mágicos y conseguir que me olvide de todo.

Aunque pudiera parecer poco lógico, deseo también mostrar mi agradecimiento a todas aquellas personas que me lo han puesto difícil, y que en más de una ocasión han sido una traba a la hora de alcanzar los retos y los objetivos marcados, ya que gracias a ellos he conseguido la tenacidad, la voluntad y la perserverancia necesarias para poder llevar a buen puerto los experimentos y lograr alcanzar mis objetivos.

Sobretodo, agradecerle a Nano su GRAAAANNNNNN PACIENCIA desde aquel día que nos conocimos, no solamente con la tesis, sino con todas mis locuras y caprichos. Por su apoyo incondicional, por abonarse a raynair, por comprender mis defectos, por compartir

mis fracasos, por celebrar mis logros, por ayudarme a mejorar a cada paso…..por ser el mejor compañero.

SUMMARY

Mycotoxins, toxic compounds produced by filamentous fungi in food and feed, are the chemical contaminants from biological origin that involve the highest risk in the food chain due to their widespread presence and the varied harmful effects on human beings and animals. Although enormous progress has been achieved in mycotoxin risk management, there are still major challenges in this regard, some of which have been considered in this Thesis.

In the case of pistachio the major concern is related to the presence of AFs, as the performed analysis in eight lots of raw and toasted pistachios showed a predominant presence of AFB_1. Suitability of the existing EU sampling plan (EC, 178/2010) and a simplified alternative were assessed. Variability was associated to all steps of the sampling process, particularly in the subfractioning where an increase in the number of the analyzed subfractions could be an alternative for reducing uncertainty. The alternative plan proposed resulted unappropriated as compared to the official one. Analytical results correction with measurement uncertainty (EC, 401/2006) increased the probability of rejection of most contaminated lots. The impact of sampling uncertainty on analytical results was demonstrated to be huge, thus new management tools are required to improve this point.

According to the initial and final values proposed by European legal limits (EC, 165/2010) for unprocessed and processed pistachio (either selection or selection plus toasting or just toasting), a decrease in 33% of the initial AFs concentration in the raw pistachio is expected. Industrial pistachio toasting (pre-toasting ≈135 ºC + toasting ≈165 ºC, during total time of 20 min) suggested that about a 75% of reduction may be achieved by the single toasting process, thus, under the hypothesis of raw pistachio compliance with maximum level, the toasted pistachio should be safe.

The impact of climate change has been identified as an emerging issue for food and feed safety. Focussing on ochratoxin A, fungal infection of grapes occurs in the field and therefore the climate conditions affect the fungal presence and consequently the mycotoxin contamination. In this Thesis the infection of berries by black aspergilli species in vineyards from two agroclimatic Spanish regions was significantly higher in the hotter and drought region in both sampled years. Although no clear trend could be identified about black aspergilli distribution, *A. tubingensis* seems to be the most widespread species in the Spanish

vineyards. Regarding to environmental growth boundaries *A. tubingensis* and *A. niger* were able to grow at higher temperatures (>45 ºC *versus* 40-42 ºC), and lower a_W (0.83 a_W *versus* 0.87 a_W) than *A. carbonarius*, except at 10 ºC where *A. carbonarius* grew at lower a_W than the other two species (0.95 a_W versus 0.98 a_W). Different ecophysiological profiles due to origin of the isolates were observed in *A. carbonarius*, showing than those from driest regions were more xerophilic, and therefore some adaptation to the environment was shown.

A. carbonarius was the main ochratoxigenic species either for the number of producing isolates or the amount produced. Additionally, contaminated musts were detected when more than 20% of the berries were infected by *A. carbonarius*. Mycotoxicological consequences in grapes and derivates resulting from a hypothetical warming climate could be a decrease in OTA risk due to the decreasing presence of *A. carbonarius* and an increase in FB_2 risk due to the promotion of the biseriate *A. niger*.

Aspergillus species showed different tolerance to UV radiation, hence an increase of it may modify the prevailing species present in field, and as a consequence the potential inoculum in the field may change, possibly favoring in the future an even higher predominance of black aspergilli that at present.

Although antifungal effectiveness showed differences among active ingredients in general they reduced the fungal growth although not always in a significant way, with the exception of thiophanate methyl which even stimulated it. The potential effect of changing climate conditions was different for different active ingredients and therefore a transition in the active ingredients may be required in the future as a result of changing climatic condition. OTA risk was not controlled by the only use of antifungals since in some cases, while they limiting fungal growth, induced a stress situation which triggered OTA production. Besides under predicted conditions, none of the antifungals was effective in preventing OTA production by any of the strains tested in both grapes and wheat. *Equisetum arvense* extract was applied as a natural alternative in grapes and wheat; it showed similar or enhanced effects than chemical antifungals in wheat, but lower doses stimulated mycotoxin production in grapes and its efficiency was strongly dose-dependent.

RESUMEN

Las micotoxinas, compuestos tóxicos producidos por mohos filamentos en alimentos y piensos, son los contaminantes químicos de origen biológico que suponen el mayor riesgo en la cadena alimentaria debido a su amplia presencia y a los múltiples efectos perjudiciales que producen en los seres humanos y en los animales. Aunque se han logrado enormes avances en la gestión del riesgo por micotoxinas, todavía existen retos importantes a este respecto, algunos de los cuales han sido considerados en esta Tesis.

En el caso del pistacho, la principal preocupación está relacionada con la presencia de aflatoxinas (AFs), ya que análisis realizados a ocho lotes de pistachos crudos y tostados han mostrado una presencia mayoritaria de aflatoxina B_1 (AFB_1). Se ha evaluado, la idoneidad de los planes de muestreo oficiales existentes en la UE (EC, 178/2010); así como una alternativa simplificada. Se encontró variabilidad asociada a todos los pasos del muestreo, particularmente en el submuestreo donde un aumento en el número de submuestras analizadas podría ser una alternativa para reducir la incertidumbre. El plan de muestreo alternativo propuesto resultó inapropiado comparado con el oficial. La corrección de los resultados analíticos mediante la medida de la incertidumbre (EC, 401/2006) incrementó la probabilidad de rechazo de la mayoría de los lotes contaminados. El impacto de la incertidumbre muestral sobre los resultados analíticos fue amplio, por lo que se requieren nuevas herramientas de gestión para mejorar este aspecto.

Conforme a los valores iniciales y finales propuestos por los límites legales europeos (EC, 165/2010) para pistachos crudos y procesados (selección, o selección y tostado, o solamente tostado), se espera una reducción de la concentración inicial de AFs del 33% en pistachos crudos. El tostado industrial de los pistachos (pre-tostado ≈135 ºC + tostado ≈165 ºC, durante un tiempo total de 20 min) sugiere que se podría alcanzar un 75 % de reducción solamente con el proceso de tostado, es decir, bajo la hipótesis de que los pistachos crudos cumplan con los máximos niveles permitidos, los pistachos tostados deberían ser seguros.

El impacto del cambio climático ha sido identificado como una nueva cuestión para la seguridad alimentaria tanto en alimentos como en piensos. Centrándonos en la ochratoxina A (OTA), la infección fúngica en las uvas ocurre en el campo y por lo tanto las condiciones climáticas afectan a su presencia y, consecuentemente, a la contaminación por micotoxinas. En esta Tesis la infección en uvas por especies de aspergilos negros en viñedos de dos

regiones agroclimáticas españolas fue significativamente superior en la región más cálida y seca en ambos años muestreados. Aunque no se identificó una tendencia clara en la distribución de aspergilos negros, *Aspergillus tubingensis* parece ser la especie más prevalente en los viñedos españoles. Respecto a las condiciones ambientales limitantes para el crecimiento de *A. tubingensis* y *A. niger,* estos mohos fueron capaces de crecer a temperaturas más altas (>45 ºC *versus* 40-42 ºC), y bajas a_W (0,83 a_W *versus* 0,87 a_W) que *A. carbonarius*, excepto a 10 ºC donde *A. carbonarius* creció a un valor de a_W inferior que las otras dos especies (0,95 a_W versus 0,98 a_W). Se observaron diferentes perfiles ecofisiológicos debido al origen del aislamiento en *A. carbonarius*, siendo los aislados de la región más cálida, y por lo tanto puede darse una posible adaptación al medio ambiente.

A. carbonarius fue la especie más ocratoxigénica tanto por el número de aislados productores como por la cantidad de toxina producida. Además, los mostos contaminados procedían de uvas con más de un 20% de infección de *A. carbonarius*. Consecuencias micotoxicológicas en uvas y derivados resultantes de un hipotético calentamiento del clima podrían ser una reducción del riesgo por OTA debido a la reducción de la presencia de *A. carbonarius*, y un incremento del riesgo de fumonisina B_2 (FB_2) debido al aumento de otros biseriados como *A. niger*.

Las especies de *Aspergillus* enseyadas mostraron diferente tolerancia a la radiación UV, por lo que un incremento de ésta podría afectar a la actual presencia de estas especies en campo y, consecuentemente, el potencial inóculo en campo podría cambiar, posiblemente favoreciendo en el futuro la presencia de aspergilos negros incluso más que en el presente.

Aunque la eficacia antifúngica de las materias activas fue diferente, en general todas redujeron el crecimiento fúngico, aunque no siempre lo hicieron de forma significativa, con la excepción del metil tiofanato que incluso lo estimuló. El potencial efecto del cambio de las condiciones climáticas fue diferente según las distintas materias activas consideradas y, por lo tanto, podría ser necesaria un cambio en las materias activas a emplear como resultados de los cambios climáticos. En la mayoría de los casos el riesgo de la presencia de OTA no fue controlado mediante el uso de fungicidas, ya que limitaron el crecimiento fúngico también indujeron a condiciones de estrés que incrementaron la producción de OTA. Ademas, bajo las condiciones predichas, ninguno de los fungicidas fue efectivo en la prevención de la producción de OTA en ninguna de las cepas ensayadas tanto en uvas como

en trigo. El extracto de *Equisetum arvense* se aplicó como una alternativa natural en uvas y trigo; obteniendo similares o mejores resultados que los obtenidos con antifungicos quimicos en trigo, sin embargo, bajas dosis estimularon la producción de micotoxinas en uvas por lo que su eficiencia fue claramente dosis-dependiente.

RESUM

Les micotoxines són compostos tòxics produïts per fongs filamentosos en aliments i pinsos, i són els contaminants químics d'origen biològic que suposen el major risc a la cadena alimentària a causa de la seva àmplia presència i als múltiples efectes perjudicials que produeixen en els éssers humans i en els animals. Encara que s'han fet avanços en la gestió del risc de micotoxines, encara hi ha reptes importants, alguns dels quals han estat considerats en aquesta tesi.

En el cas del festucs la principal preocupació està relacionada amb la presència de AFs, ja que les anàlisis realitzades en vuit lots de festucs crus i torrats van mostrar presència majoritària d'AFB_1. Es va avaluar la idoneïtat dels plans de mostreig oficials existents a la UE (EC, 178/2010) i també una alternativa simplificada. La variabilitat va ser associada a tots els passos del mostreig, particularment en el submostreig on un augment en el nombre de submostres analitzades podria ser una alternativa per reduir la incertesa. El pla alternatiu proposat resultà inadequat comparat amb l'oficial. La correcció dels resultats analítics mitjançant la mesura de la incertesa (EC, 401/2006) incrementà la probabilitat de rebuig de la majoria dels lots contaminats. L'impacte de la incertesa del mostreig sobre els resultats analítics va ser gran, per la qual cosa es requereixen noves eines de gestió per millorar aquest aspecte.

D'acord amb els valors inicials i finals proposats pels límits legals europeus (EC, 165/2010) per festucs crus i processats (selecció o selecció i torrat o només torrat), s'espera una reducció de la concentració inicial d'AFs del 33% en festucs crus. El torrat industrial de festucs (pre-torrat ≈ 135 º C + torrat ≈ 165 º C, durant un temps total de 20 min) va suggerir que es podria arribar a un 75% de reducció només amb el procés de torrat, és a dir, sota la hipòtesi que els festucs crus compleixin amb els màxims nivells permesos, els festucs torrats haurien de ser segurs.

L'impacte del canvi climàtic ha estat identificat com un nou condicionant per a la seguretat alimentària tant en aliments com en pinsos. Centrant-nos en la ocratoxina A, la infecció fúngica en el raïm es dona al camp i per tant les condicions climàtiques afecten la seva presència i conseqüentment a la contaminació per micotoxines. En aquesta Tesi la infecció en raïm per espècies d'aspergils negres en dues regions agroclimàtiques espanyoles va ser significativament superior a la regió més càlida i seca en els dos anys mostrejats. Encara que

no es va identificar una tendència clara en la distribució dels aspergils negres, *A. tubingensis* sembla ser l'espècie més prevalent en les vinyes espanyoles. Respecte a les condicions ambientals limitants per al creixement de *A. tubingensis* i *A. niger*, aquests van ser capaços de créixer a temperatures més altes (> 45 º C *versus* 40-42 º C), i baixes a_W (0,83 a_W *versus* 0,87 a_W) que *A. carbonarius*, excepte a 10 º C on *A. carbonarius* va créixer a a_W inferior a les altres dues espècies (0,95 a_W *versus* 0,98 a_W). En *A. carbonarius* es van observar diferents perfils ecofisiològics a causa de l'origen d'aïllament, on els aïllats de la regió més càlida van ser més xeròfils, i per tant es pot hipotetitzar una possible adaptació al medi ambient.

A. carbonarius va ser l'espècie més ocratoxigènica tant pel nombre d'aïllats productors com per la quantitat de toxina produïda. A més, els mostos contaminats procedien de raïm amb més d'un 20% d'infecció per *A carbonarius*. Les conseqüències micotoxicològiques resultants d'un hipotètic escalfament del clima en raïm i derivats podrien ser una reducció del risc d'OTA a causa de la reducció de la presència d'*A carbonarius*, i un possible increment del risc de FB_2 a causa de l'increment d'altres biseriats com *A. niger*.

Les espècies testades d'*Aspergillus* van mostrar diferent tolerància a la radiació UV, per tant un increment d'aquesta podria afectar l'actual distribució d'aquestes espècies en camp, i conseqüentment el potencial inòcul en camp podria canviar, possiblement afavorint en el futur la presència d'aspergils negres fins i tot més que en el present.

Encara que l'eficàcia antifúngica de les matèries actives va ser diferent, en general totes van reduir el creixement fúngic encara que no sempre ho van fer de manera significativa, amb l'excepció del metil tiofanat que fins i tot el va estimular. El potencial efecte del canvi de les condicions climàtiques va ser diferent en les diferents matèries actives i per tant podria ser necessari un canvi en les matèries actives utilitzades. El risc de la presència d'OTA no va ser controlat mitjançant l'ús de fungicides en la majoria dels casos. En limitar el creixement fúngic es poden induir condicions d'estrès que poden desencadenar la producció d'OTA. A més, sota les condicions predites, cap dels fungicides va ser efectiu en la prevenció de la producció d'OTA en cap de les soques testades tant en raïm com a blat. L'extracte d'*E arvense* es va aplicar com una alternativa natural, obtenint similars o millors resultats que els obtinguts amb antifúngics químics en blat. No obstant, les dosis baixes van estimular la producció de micotoxines en raïm pel que la seva eficiència va ser clarament dosi-depenent.

LIST OF ABBREVIATIONS

3ADON, 3-acetyl-deoxynivalenol

ADI, Acceptable Daily Intake

AEMET, State Meteorology Agency

AFB_1, Aflatoxin B_1

AFB_2, Aflatoxin B_2

AFB_{2a}, Aflatoxin B_{2a}

AFG_1, Aflatoxin G_1

AFG_2, Aflatoxin G_2

AFG_{2a}, Aflatoxin G_{2a}

AFM_1, Aflatoxin M_1

AFs, Aflatoxins

ALOP, Approppiate Level of Protection

a_w, Water activity

b.w., body weight

BEA, Beauvericin

CA, Cyclopiazonic acid

CAC, Codex Alimentarius Commission

CCCF, Codex Alimentarius on Contaminants in Foods

CIT, Citrinin

CM, Control Measure

DAS, Diacetoxyscirpenol

DFP, Destined For Further Processing

DON, Deoxynivalenol

DSS, Decision Support Systems

Ɛ, Molar Extinction Coefficient

EC, European Commission

EFSA, European Food Safety Authority

ENNs, Enniatins

EU, European Union

FAO, Food and Agriculture Organization

FB, Fumonisins

FB_1, Fumonisin B_1

FB_2, Fumonisin B_2

FSO, Food Safety Objective

FUS, Fusaproliferin

GAP, Good Agricultural Practice

GCMs, General Circulation Models

GHP, Good Hygiene Practices

GMO, Genetically modified organisms

GMP, Good Manufacturing Practices

GSP, Good Storage Practices

HACCP, Hazard Analysis and Critical Control Points

Ho, Initial level of the hazard

HPO, Hand Picked Out

HT2, HT-2 toxin

I, Increase of the hazard

IARC, International Agency for Research on Cancer

ICMSF, International Commission on Microbiological Specifications for Foods

IGN, National Spanish Geographic Institute

IPCC, Intergovernmental Panel on Climate Change

IPSM, Integrated Phytosanitary Management

JECFA, Joint FAO/WHO Expert Committee on Food Additive

KA, Kojic Acid

LOAEL, Lowest Observed Adverse Effect Level

m.c., Moisture content

MAS, Monoacetoxyscirpenol

MLs, Maximum Levels

MON, Moniliformin

MRLs, Maximum Residue Levels

NIV, Nivalenol

NOAEL, No Observed Adverse Effect Level

OC, Operating Characteristic

OTA, Ochratoxin A

OTB, Ochratoxin B

OTC, Ochratoxin C

OTα, Ochratoxin α

OTβ, Ochratoxin β

PAT, Patulin

PC, Performance Criteria

PcC, Process Criterion

PdC, Product Criterion

PMTDI, Provisional Maximum Tolerable Daily Intake

PO, Performance Objective

PTWI, Provisional Tolerable Weakly Intake

R, Reduction of the hazard

R.H., Relative Humidity

RASFF, Rapid Alert System for Food and Feed

RTE, Ready to Eat

SCF, Scientific Committee on Food

SPS, Sanitary and Phitosanitary Measures

SRES*, Special Report on Emissions Scenarios*

T, Temperature

T2, T-2 toxin

TDI, Tolerable Daily Intake

TWI, Tolerable Weakly Intake

U, Measurement uncertainty

UNEP, United Nations Environment Program

UV, Ultraviolet

WMO, World Meteorological Organization

WTO, World Trade Organization

ZEA, Zearalenone.

INDEX OF CONTENTS

I. MYCOTOXINS OVERVIEW 1
1. What are Mycotoxins 3
2. Which fungi produce mycotoxins? 4
3. Which commodities are often contaminated by mycotoxins? 5
4. Which health effects can cause their ingestion? 5
5. Which factors affect fungal growth and mycotoxin production? 6
6. When do the mycotoxins occur? Their presence in the food chain 8
7. How mycotoxins can be controlled in raw materials? 9
REFERENCES 13

II. OBJECTIVES and WORK PLAN 19

III. INTRODUCTION 25
1. Aflatoxins and ochratoxin A 27
1.1. Aflatoxins 27
1.2. Ochratoxin A 31
2. Management of mycotoxin presence in commodities 37
2.1. Case study I: Nuts-pistachio 38
2.2. Case study II: Cereals-Wheat 44
2.3. Case study III: Fruit-winemaking grapes 52
3. Challenges for food safety management 61
3.1. Sampling plans 61
3.2. Other fungal metabolites: what else is there in the food and feed? 63
3.3. Climate change 65
REFERENCES 69

IV. RESEARCH WORK 95

STUDY I: Risk management towards Food Safety Objective achievement regarding to mycotoxins in pistachio: the sampling and measurement uncertainty issue 99
1. INTRODUCTION 101
2. MATERIALS AND METHODS 104
2.1. Samples and sampling plans 104
2.2. Analytical methods 105
2.3. Measurement uncertainty calculation 107
2.4. Total uncertainty calculation 108
2.5. Statistical analyses 109
3. RESULTS 109
3.1. Presence of mycotoxins in the analysed pistachio lots 109
3.2. Comparison between sampling plans 110
3.3. Comparison between analytical methods (HPLC and ELISA) in sample plan B 111
3.4. Effect of roasting on naturally contaminated pistachios with mycotoxins 112
3.5. Analytical uncertainty ..... **¡Error! Marcador no definido.**

3.6. PO and FSO applied to the mycotoxin hazard in pistachio ..... **¡Error! Marcador no definido.**
3.7. Sampling uncertainty 116
4. DISCUSSION 121
5. CONCLUSION 124
ACKNOWLEDGMENTS 125
REFERENCES 125

STUDY II: Fungal diversity, incidence and mycotoxin contamination in grapes from two agro-climatic Spanish regions with emphasis on *Aspergillus* species 133
1. INTRODUCTION 134
2. MATERIALS AND METHODS 139
2.1. Field sampling and meteorological data 139
2.2. Mycobiota determination 143
2.3. DNA extraction, amplification and identification of Aspergillus species 143
2.4. DNA extraction, amplification and identification of Penicillium species 144
2.5. Biodiversity Indexes 145
2.6. Mycotoxins detection and quantification 146
2.7. Statistical analyses 147
3. RESULTS AND DISCUSSION 148
3.1. Mycobiota determination 148
3.2. Biodiversity Indexes 153
3.3. Mycotoxin production capacity of isolates and OTA presence in musts 154
3.4. Meteorological data ..... **¡Error! Marcador no definido.**
3.5. Relation between meteorological data and *Aspergillus* section *Nigri* infection 157
ACKNOWLEDGEMENTS 158
REFERENCES 159

STUDY III:Ecophysiological characterization of *Aspergillus carbonarius*, *Aspergillus tubingensis* and *Aspergillus niger* isolated from grapes in Spanish vineyards 165
1. INTRODUCTION 166
2. MATERIALS AND METHODS 169
2.1. Fungal isolates, origin and molecular identification 169
2.2. Ecophysiological study 172
2.3. Genetic diversity study within *A. carbonarius* isolates 175
3. RESULTS 177
3.1. Ecophysiological study 177
3.2. Genetic diversity study within A. carbonarius isolates 182
4. DISCUSSION 182
ACKNOWLEDGEMENTS 186
REFERENCES 186

STUDY IV: Conidia survival of *Aspergillus* section *Nigri*, *Flavi* and *Circumdati* under UV-A and UV-B radiation with cycling temperature/light regime 193
1. INTRODUCTION 194
2. MATERIALS AND METHODS 195

2.1. Isolates and inoculation 195
2.2. Incubating conditions 196
2.3. Statistical analysis 198
3. RESULTS AND DISCUSSION 198
ACKNOWLEDGEMENTS 205
REFERENCES 205

STUDY V:Effect of ultraviolet radiation A and B on growth and mycotoxin production by *Aspergillus carbonarius* and *Aspergillus parasiticus* in grape and pistachio media 211
1. INTRODUCTION 212
2. MATERIALS AND METHODS 215
2.1. Microorganisms, growth medium and inoculation 215
2.2. Experimental design and incubation conditions 215
2.3. Growth assessment: colony diameter and fungal biomass 218
2.4. Mycotoxin extraction and quantification 218
2.5. Statistical analysis 219
3. RESULTS 219
3.1. Effect of UV-A and UV-B radiation/darkness on *A. carbonarius* (exp.1) 219
3.2. Effect of UV radiation/light and dark cycles on *A. carbonarius* (exp. 2) 223
3.3. Effect of UV radiation/light and dark cycles on *A. parasiticus* (exp. 3) 225
4. DISCUSSION 228
ACKNOWLEDGMENTS 231
REFERENCES 231

STUDY VI: Ochratoxigenic moulds and effectiveness of grape field antifungalss in a climatic change scenario 235
1. INTRODUCTION 236
2. MATERIALS AND METHODS 237
2.1. Fungal isolates and preparation of spore suspensions 237
2.2. Antifungals preparation 238
2.3. Grape decontamination, inoculation, fumigation and incubation 238
2.4. Percentage of infection 240
2.5. OTA extraction and HPLC quantification 240
2.6. Statistical analysis 241
3. RESULTS 241
3.1. Effectiveness of antifungals against grape infection 241
3.2. Effectiveness of antifungals against OTA production 243
4. DISCUSSION 243
ACKNOWLEDGMENTS 248
REFERENCES 249

STUDY VI: Effect of preharvest anti-fungal compounds on *Aspergillus steynii* and A. *carbonarius* under fluctuating and extreme environmental conditions 251
1. INTRODUCTION 253
2. MATERIALS AND METHODS 254
2.1. Fungal isolates 254

2.2. Wheat preparation 255
2.3. Antifungals preparation 255
2.4. Phenolic compounds analysis in E. arvense extract (HPLC-DAD-ESI/MS) 256
2.5. Preparation of inocula and incubation 257
2.6. Growth determination 258
2.7. Quantification of fungal DNA 258
2.8. Ochratoxin A determination 259
2.9. Statistical analysis 260
3. RESULTS 260
3.1. Phenolic compounds in E. arvense extract 260
3.2. Impact of changing climatic conditions in fungal growth and OTA production 260
3.3. Effectiveness of antifungals against fungal growth in wheat 262
3.4. Effectiveness of antifungals against OTA production 264
4. DISCUSSION 269
ACKNOWLEDGEMENTS 273
REFERENCES 273

V. GENERAL DISCUSSION 281
1. SELECTION OF RAW MATERIALS 283
1.1. Samplings plans: effectiveness, uncertainty and alternatives 283
1.2 Analytical procedures 285
1.3. Toasting as process criteria (PcC) 286
2. CLIMATE CHANGE: 288
2.1. Increasing temperature and drought 289
2.2. Increasing solar ultraviolet radiation 294
2.3. Effect of environmental conditions on antifungal efficacy of preharvest fungicides 297
REFERENCES 300

VI. CONCLUSIONS and FUTURE RESEARCH 309
1. SELECTION OF RAW MATERIALS (PISTACHIO) 311
2. CLIMATE CHANGE 311

VII. ANEXOS 323

AnexoI
Review: Emerging risk management metrics in food safety: FSO, PO. How do they apply to the mycotoxin hazard? 325
1. INTRODUCTION 326
2. MYCOTOXINS: CHEMICAL HAZARDS 327
2.1. Aflatoxins 328
2.2. Ochratoxin A 328
2.3. Fumonisins 329
2.4. Patulin 329
2.5. Zearalenone 330
2.6. Trichothecenes 330
3. RISK ASSESSMENT FOR CHEMICAL HAZARDS 331

3.1. Hazard characterization 331
3.2. Exposure assessment 333
3.3. Risk characterization 333
4. RISK MANAGEMENT 335
4.1. The ALOP concept 336
4.2. The FSO concept, a food safety management metric 338
4.3. Meeting the FSO 339
5. CONCLUSIONS 351
ACKNOWLEDGEMENTS 351
REFERENCES 352

Anexo II
First report of field application of atoxigenic Aspergillus flavus as candidate biocontrol agents 325361

I. MYCOTOXINS OVERVIEW

1. What are Mycotoxins

Mycotoxins-from the Greek mykes, mukos "fungus" and toxikon "poison" are a large and growing family of secondary metabolites and hence natural products produced by fungi, in particular by moulds (Bräse et al., 2013). The most frequent mycotoxins found in foods and feeds are: aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1), citrinin (CIT), cyclopiazonic acid (CA), fumonisins (FB_1, FB_2 and FB_3), ochratoxin (OTA, OTB and OTC), patulin, trichothecenes (mainly nivalenol (NIV), deoxynivalenol (DON), T-2 toxin (T2) and HT-2 toxin (T2)) and zearalenone (ZEA) (van der Gaag et al., 2003).

Among food contaminants, mycotoxins have great consequences in terms of both human and animal health as well as economics. According to the annual report of the Rapid Alert System for Food and Feed (RASFF), in 2012 mycotoxins were the main hazard in border rejection notifications in the European Union (EU) (**Table 1**). The number of alert and information for attention notifications was also outstanding. Besides, they cause enormous economic losses annually to the grain trade and the marketing of foods and feeds (Windels, 2000).

Table 1 2012 Notifications by hazard category in the EU.

Hazard category	Alert	Border rejection	Information for attention	Information for follow-up
Allergens	64	3	17	1
Biocontaminants	6	9	26	2
Food additives and flavourings	10	59	23	47
Foreign bodies	24	61	26	47
GMO/novel food	2	52	14	22
Heavy metals	57	108	79	24
Industrial contaminants	16	9	18	14
Mycotoxins	38	425	53	9
Parasitic infestation	4	13	13	25
Pathogenic microorganisms	162	159	168	103
Pesticides residues	19	320	90	18
Residues of veterinary medicinal products	12	18	16	14

Rapid Alert System for Food and Feed (RASFF) Annual Report, 2012.

2. Which fungi produce mycotoxins?

These secondary metabolites are compounds biosynthesized and excreted through a set of metabolic pathways, but are not essential for growth or survival of the organism (Betina, 1989). Nowadays, the number of fungal toxic metabolites is unknown, the number of mycotoxins to be discovered being probably high. However, the number of mycotoxins which are often found in foods do not reach 30 different mycotoxins (Santos, 2011). Most of the known mycotoxins are produced by species of the fungal genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (**Table 2**). A certain mycotoxin can be produced by different species, and a certain strain can produce different mycotoxins.

Table 2 Main mycotoxins producer by *Aspergillus*, *Penicillium*, *Fusarium* and *Altenaria* species.

Genus	**Mycotoxin**	**Producer**
Aspergillus	Aflatoxins	section *Ochraceorosei*, *Nidulantes* and *Flavi*
	Cyclopiazonic acid	section *Flavi*
	Fumonisins	section *Nigri*
	Ochratoxins	section *Circumdati, Nigri*
	Patulin	section *Fumigati*
Penicillium	Cyclopiazonic acid	*P.commune*
	Ochratoxins	*P. nordicum*, P. *verrucosum*
	Patulin	*P.expansum, P. griseofulvum*
Fusarium	Beuavericin	*F. sporotrichioides, F. poae, F. langsethiae, F. avenaceum*, section *Liseola*
	Deoxynivalenol	*F. graminearum, F. culmorun*
	Enniatins	*F. avenaceum, F. tricinctum*
	Fusaproliferin	*F. poae, F. langsethiae, F. sporotrichiodes,F. proliferatum, F. subglutinans*
	Moniliformin	*F. avenaceum, F. tricinctum,* section *Liseola*
	Nivalenol	*F. graminearum, F. culmorun*
	Trichothecenes (T2, HT-2)	*F. sporotrichiodies, F. poae, F. equiseti*
	Zearalenona	*F. graminearum, F. culmorum, F. cerealis, F. equiseti, F. crookwellense, F. semitectum*
Alternaria	Alternariol	*A. alternate*
	Alternariol monomethyl ether	*A. alternata, A. solani*
	Tenuazonic acid	*A. alternate*
	Altertoxins	*A. tenuissima*
	Altenuene	*A. alternate*

(Ramos, 2011)

3. Which commodities are often contaminated by mycotoxins?

Foods associated with fungal spoilage are characterized by a relatively low water activity (a_W) or a low pH value, where filamentous fungi may dominate on the colonization over bacteria and yeasts. Therefore the main food groups contaminated by fungus are cereals and their derivatives, nuts and fruits (CAST, 2003). According to the last report of the RASFF, nuts, nut products and seeds, fruits and vegetables, herbs and spices, cereals/bakery products, and foodstuffs, were the most affected categories (**Table 3**).

Table 3 Mycotoxin notifications by products in the EU during 2012.

Product category	AFs	DON	FBs	OTA	ZEA
Cereals and bakery products	17	4	4	6	3
Feed	79				
Fruits and vegetables	137			19	1
Herbs and spices	33			4	
Nuts, nut products and seeds	204				
Other	14			3	
Total	484	4	4	32	4

Rapid Alert System for Food and Feed (RASFF) Annual Report, 2012.

4. Which health effects can cause their ingestion?

Exposure of humans to mycotoxins occurs mainly by the ingestion of contaminated foodstuffs from vegetal or animal origin. Contamination in the last case takes place when animals are fed with contaminated feed. An alternative way of exposure is the inhalation of contaminated dusts or skin contact. The diseases caused by mycotoxins in humans or animals are called "mycotoxicoses". While most animal mycotoxicoses have been experimentally confirmed, human mycotoxicoses are less well understood and not as clearly defined (Smith, 1985). The toxicity of a certain mycotoxin in an organism can be classified according to the exposure dose in acute or chronic. Acute toxicity refers to the ability of the compound to cause adverse effects within a short time of exposure, especially at high doses. Chronic toxicity stands for the effects of a prolonged exposure to small quantities of toxin (CAST, 2003). For certain chemicals, such as some mycotoxins, marine toxins, pesticides and veterinary drugs, mainly chronic health but also acute health effects need to be considered.

International agencies have studied the problem of mycotoxins in food in order to obtain guidelines regarding the limits of contamination in food and the tolerable intake of the toxins.

The International Agency for Research on Cancer (IARC) (IARC, 1998, 1993) has investigated the carcinogenic potential of most of these toxins (**Table 4**).

Table 4 Evaluation of carcinogenicity of some mycotoxins by IARC (1993 and 1998).

Mycotoxin	**Degree or evidence of carcinogenicity**		**Overall evaluation of carcinogenicity to humans**
	Human	**Animal**	
AFs, naturally occurring mixtures of	S	S	group 1
AFB_1	S	S	group 1
AFB_2		L	
AFG_1		S	
AFG_2		I	
AFM_1	I	S	group 2B
OTA	I	S	group 2B
Toxins derived from *F. gramineanun, F. culmorum* and *F. crookwellense*:	I		group 3
Zearalenone		L	
Deoxynivalenol		I	
Nivalenol		I	
Fusarenone X			
Toxins derived from *F. moniliforme*:	I	S	group 2B
Fumonisins, naturally occurring mixtures of	I		
Fumonisin B1		S	group 2B
Fumonisin B2		I	
Fusarin C		L	
Toxins derived from *F. sporotrichiodes:*	I[a]		group 3
T-2 toxin		L	

Degrees of evidence: S = sufficient; L = limited; I = inadequate; N = negative.
Evaluation of carcinogenicity: Group 1: Carcinogenic to humans, Group 2A: Probably carcinogenic to humans, Group 2B: Possibly carcinogenic to humans, Group 3: Not classifiable as to its carcinogenicity to humans, Group 4: Probably not carcinogenic to humans.
[a] No data available.

5. Which factors affect fungal growth and mycotoxin production?

Fungal growth and mycotoxin production are determined both by interaction of the fungal individual (strain variability, age…), as well as the intrinsic characteristics of the substrate colonized (a_W, hydrogen ion concentration (pH), nutritional composition, antifungal compounds…) and the environment in which it is growing (temperature (T), relative humidity (R.H.), light, gas composition, solar radiation...).

Genetic differences among species may contribute to the mycotoxin production, as some species may lack of functional genes needed for the production of certain mycotoxins

(Nicholson et al., 2003). In fact, it is possible to find producer and non-producer strains within the same species (Cabañes et al., 2013). Similarly, it is very common that different strains produce dissimilar amount of the same mycotoxin. For example, strains in a species isolated from different substrates or in different seasons may differ in their toxigenicity (Dobson et al., 2006).

The most important factors influencing fungal growth and mycotoxin production, and therefore the most studied, are T and a_W. Regarding T, the range usually reported for fungal growth is broad, 10-35 ºC, with a few species capable of growing below or above this range (Pitt and Hocking, 2009). Moulds are able to grow at a_W from 0.85 to 1 (Dantigny et al., 2002). In addition, spores of *Aspergillus* and *Penicillium* are able to survive at lower a_W for several years (Carlile and Watkinson, 1996). Besides, *Aspergillus* can also produce survival structures as sclerotia (McGee et al., 1996). It is worthy to mention that the presence of sclerotia *per se* does not seem to be related to AFs production, but the correlation between presence of small sclerotia (<400 μm) in *A. flavus* appears correlated with high AFs production (Cotty, 1989).

In general, T and a_W requirements for mycotoxin production are stricter than for fungal development and, interestingly, the optimum T for mycotoxin production is below the optimum for growth in some cases.

Other important intrinsic factor which can affect the growth and mycotoxin production is chemical composition and the pH of subtract. Most moulds tolerate a range pH from 4 to 9 but grow and sporulate maximally near neutrality (Cole, 1981). The impact of pH on the production of AFs, FBs, and trichothecenes appears simple: acidic conditions are conducive and alkaline conditions are repressive (Woloshuk and Shim, 2013). Not only the source of carbon and nitrogen but also the amount of them, even the presence of metals as zinc affect mycotoxin production (Buchanan and Stahl, 1984; Medina et al., 2008; Woloshuk and Shim, 2013).

As it has been already emphasized, the production of mycotoxins is influenced by the physiochemical environment in which the fungus is growing. Considering the effect caused by the atmosphere gases, although mycotoxigenic fungi have been considered to be aerobes, they could grow under facultatively anaerobic conditions (Taniwaki et al., 2009). Mycotoxin

formation can be controlled by enriching atmospheres with CO_2 or by decreasing O_2, while fungal growth may not be affected (Taniwaki et al., 2009). Light is a very important signal for fungi: it influences many different physiological responses such as pigmentation, sexual development, asexual conidiation, the circadian clock and secondary metabolism (Bayram et al., 2010). Particularly, white light reduced mycotoxin production respect to darkness (Crespo-Sempere et al., 2013).

Similar to light, the effect of ultraviolet (UV) radiation in fungal spores germination, growth and sporulation, showed that the effect is dependent of time, UV wavelength, and fungal species (Aylor and Sanogo, 1997; Cary and Ehrlich, 2006; Fourtouni et al., 1998; Moody et al., 1999; Nicot et al., 1996; Osman et al., 1988; Rotem et al., 1985). Concerning mycotoxins, it has been suggested that AFs production could be a strategy of fungi to prevent from UV damage (Cary and Ehrlich, 2006). Nevertheless, no scientific information exists about it.

The relevant biotic factor is the presence of other organisms, either by competition or by collaboration. Fungi have a variety of requirements for growth 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 utilize effective competitive mechanisms. Different ways of competition have been described: i) rapid growth, sporulation or stress recovery; ii) production of inhibitors against other species; iii) metabolization of inhibitors; and, iv) the colonization of a special niche (Dighton et al., 2005).

Biotic relation can also benefit one of the organisms involved. In this sense, it has been widely reported that insect presence could promote fungal infection and consequently the mycotoxin contamination. Insects can enhance the fungal infection process by carrying inoculum and causing damage in food and feed permitting the fungal entry (Dowd, 2003).

6. When do the mycotoxins occur? Their presence in the food chain

Mycotoxins are mainly present in raw materials. From the food safety point of view, only mycotoxins (as chemical hazards) are important, while moulds may cause spoilage but have no safety implications. However, presence of mycotoxin producer fungi in foods and feeds may indicate a risk of mycotoxins. Moreover, mycotoxins are resistant chemical compounds that can be present in processed food products even when the fungus is not present in the

commodity anymore. Additionally, wrong practices could cause subsequently fungal recontamination and therefore, mycotoxin risk is present along all the food chain. But the important matter is, how to avoid the mycotoxins presence in the raw materials.

In general, mycotoxigenic moulds are not aggressive pathogens, with an exception to *Fusarium* (Musa et al., 2007), but some species can invade and colonize plant tissues synthesizing mycotoxins during cultivation, harvesting, drying, transport and storage (Wagacha and Muthomi, 2008).

Regarding to fungi growing on the processed product, they may differ considerably from those occurring on the raw materials, due to changes in the composition of the substrate and the requirements of the producing fungi (Magan and Olsen, 2004).

7. How mycotoxins can be controlled in raw materials?

The ideal goal for controlling mycotoxins is to eliminate them from the food chain; however, on a practical level this is not possible. Regardless the specific fungi or the infection moment, the best way to reduce the mycotoxin content in food is preventing the development of mycotoxigenic fungi. Control of mycotoxin production includes pre and post harvests strategies, based on both preventative and curative measures. Food safety management approaches integrating agricultural management and postharvest control are potentially more economically rewarding than a simply regulation setting tolerance limits (Leslie et al., 2008).

The Codex Alimentarius (CAC) has set recommendations for prevention and reduction of mycotoxins in cereals, nuts, fresh fruits and other raw materials. These recommendations, also extensible to other crops, consist in good agricultural practices (GAP), followed by the implementation of good manufacturing practices (GMP) during the handling, storage (GSP) and processing (EC, 2003, 2006a; CAC, 2003). Emphasis should be placed on the fact that seeding, pre-harvest and postharvest strategies for a particular crop will depend on the climatic conditions of that particular year, taking into account the local crops, and traditional production conditions for that particular country or region (Kabak et al., 2006).

PRE-HARVEST

Environmental conditions as high R.H., rainfall and T favor fungal proliferation resulting in contamination in field (Bellí et al., 2006; Leong et al., 2004). It is obvious that the weather is a not controllable factor, nevertheless from field studies it is possible to determine the pathogen risk level in the crop associated to the climatic conditions (Battilani et al., 2006; Lindblad et al., 2012; van Asselt et al., 2012; Xu et al., 2013). Mechanistic predictive models can be used in the development of Decision Support Systems (DSS) to determine the level of risk for the accumulation of mycotoxins in fruits and grains. These models can be generated using methods based on information of fungal life (ecophysiology requirements) and meteorological data such as T, R.H., and precipitation rate. One of the most widespread applications of such models are advising farmers in the decision on fungal treatments or harvest time (Rossi et al., 2007). The DSS models are of great interest in all stages of production, thus farmers can benefit from the system at an earlier stage (for optimization of crop protection), traders (for purchase optimization) and/or processors (reorientation of product flows with different quality requirements). In addition to the implementation of a system for current cereal grain supply chains, this system can be used to evaluate future scenarios, for example, considering future climate scenarios (Fels-Klerx and Booij, 2010). With regard to mycotoxins, predictive models currently exist in different parts of the world. In Canada, DONcast provides advice to farmers on the application of fungicides to minimize the presence of DON from *Fusarium* spp. based on agronomic practices, the variety of wheat and other factors like the meteorological data (Schaafsma and Hooker, 2007). This application has been calibrated for use in other countries such as Uruguay and France (Schaafsma and Hooker, 2007; Schaafsma et al., 2006).

Regarding agronomical practices, soil preparation, crop rotation, choice of resistant varieties, avoiding plant stress (irrigation) and pest control (chemical pesticides and biological agents) are some of the main recommendations from the international agencies.

Crop residues are considered the major source of inoculum. Therefore, minimise it by plowing under or by destroying or removing residues that may have served as substrates for the growth of mycotoxin-producing fungi is desirable (CAC, 2003). However, the effect is not totally contrasted as it affects in different ways fungi and mycotoxin production and it is

strongly related to the species (Dill-Macky and Jones, 2000; Leplat et al., 2013; Parikka et al., 2008; Supronienė et al., 2012).

The effect of crop succession on the mycophaties is extremely variable. The main factor is if the alternating crops are hosts, not-hosts or alternative-host of a single pathogen, as it gives a chance of survival to previous diseases. For instance, attacks are less frequent and less intense in wheat following soybean, because soybean leaves little tailings (Broydé and Doré, 2013).

Understanding the life cycle of mycopathogens serves to determine the susceptible periods of infestation and allows the producers to select the most suitable varieties, and adjusting the seeding date to avoid the favorable conditions for infestation at the end of cycle (moisture at flowering for *Fusarium*, heat and drought for *Aspergillus*), and periods of susceptibility to insects in maize (Abbas et al., 2009; Blandino et al., 2009). Also, the selection of mycotoxin-resistant varieties has met diferent degrees of success for the diseases (Boutigny et al., 2008; Broydé and Doré, 2013). The development of transgenic cereals, through the genetic manipulation of defence-signalling pathways, can be an alternative strategy for reducing *Fusarium* head (ear) blight (Terzi et al., 2014). Moreover the reduction of the seeding doses also has been effective in control of *Fusarium* (Abbas et al., 2009; Blandino et al., 2009). For *Aspergillus,* various approaches have been suggested for genetic control of AFs contamination including the development and use of crops with resistance to insects and the resistance to plant stress (especially for tolerance to drought and high T) (Guo et al., 2008).

Weeds can be a source of inoculum, and therefore applying herbicides could reduce the risk of disease break-outs, as many fungal species including ochratoxigenic species were observed in weed of vineyards from Argentine (Ponsone et al., 2007). However, some glyphosate-base herbicides can stimulate *F. avenaceum* and *F. graminearum* growth, which leads to an increase in wheat stem-base and ear infection (Fernandez et al., 2009). So an accurate selection of the active principles is mandatory in order to prevent risks. However, the use of fungicides is the only efficient, cost-effective and often successful way to prevent mould growth (Munimbazi and Bullerman, 1997). In fact, the CAC includes the application of fungicides in GAP to reduce contamination in cereals (CAC,-2003). The effectiveness of diferent active principles against fungi and mycotoxin presence are reported in bibliography (Curto et al., 2004; Edwards and Godley, 2010; Haidukowski et al., 2005; Ioos et al., 2005; Ramirez et al., 2004;

Tjamos et al., 2004). Nevertheless, fungicides must be applied with care since some of them and under certain conditions, may even stimulate the production of mycotoxins (Simpson et al., 2001). Regarding pests, there is also a clear correlation between them and mycotoxin levels (Cozzi et al., 2006; Ostry et al., 2010). The reason is that insects act as wounding agents or as vectors spreading the fungus from origin of inoculum to plants, so targeting insects as a means for indirectly controlling mycotoxins is adviced (Dowd, 2003). Finally, there are a variety of bacteria, yeasts and moulds that can restrict fungal growth and also are able to degrade or remove mycotoxins from foods, these microorganisms may be used as biocontrol agents against mycotoxigenic fungi (Medeiros et al., 2012). Currently, non-aflatoxigenic *A. flavus* (*A. flavus* AF36 strain) and Afla-guard (*A. flavus* NRRL21882 strain) are commercialized and applied in corn, cotton and peanuts.

HARVEST

During harvest it is important to control, among other things, whether the crops ripened on time, avoiding the crop remaining in the field longer than necessary. To reduce or prevent production of mycotoxins, drying should take place soon after harvest and as rapidly as feasible. It is important to avoid damage before and during drying, and during storage (Chulze, 2010).

POST-HARVEST

Control of storage/transport conditions (T, a_W, gaseous atmosphere...) to avoid fungal development and mycotoxin production is critical. Safe conditions could be determined through the development of predictive models specific of fungi-mycotoxin/foodstuffs. They can be used in the implementation of Hazard Analysis and Critical Control Point (HACCP) plans (Marín et al., 2008).

For contaminated batches different physical, chemical, and biological detoxification methods have been proposed. Nevertheless, detoxification is less effective and sometimes restricted because of concerns of safety, possible losses in nutritional quality of the treated commodities and cost implications (Yang et al., 2014). Besides, chemical decontamination and mycotoxin dilution by mixing batches of product are specifically banned by European Commission (EC, 2006b).

REFERENCES

Abbas, H.K., Wilkinson, J.R., Zablotowicz, R.M., Accinelli, C., Abel, C.A., Bruns, H.A., Weaver, M.A., 2009. Ecology of *Aspergillus flavus,* regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. Toxin Rev. 28, 142–153.

Aylor, D.E., Sanogo, S., 1997. Germinability of *Venturia inaequalis* conidia exposed to sunlight. Phytopathology 87, 628–633.

Battilani, P., Barbano, C., Marin, S., Sanchis, V., Kozakiewicz, Z., Magan, N., 2006. Mapping of *Aspergillus* section *Nigri* in Southern Europe and Israel based on geostatistical analysis. Int. J. Food Microbiol. 111, S72–S82.

Bayram, Ö., Braus, G.H., Fischer, R., Rodriguez-Romero, J., 2010. Spotlight on *Aspergillus nidulans* photosensory systems. Fungal Genet. Biol. 47, 900–908.

Bellí, N., Bau, M., Marín, S., Abarca, M.L., Ramos, A.J., Bragulat, M.R., 2006. Mycobiota and ochratoxin A producing fungi from Spanish wine grapes. Int. J. Food Microbiol. 111, S40–S45.

Betina, V., 1989. Mycotoxins: Chemical, biological and environmental aspects. Bioact. Mol. 9, 114–150.

Blandino, M., Reyneri, A., Vanara, F., Tamietti, G., Pietri, A., 2009. Influence of agricultural practices on *Fusarium* infection, fumonisin and deoxynivalenol contamination of maize kernels. World Mycotoxin J. 2, 409–418.

Boutigny, A.-L., Richard-Forget, F., Barreau, C., 2008. Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. Eur. J. Plant Pathol. 121, 411–423.

Bräse, S., Gläser, F., Kramer, C., Lindner, S., Linsenmeier, A.M., Masters, K.-S., Meister, A.C., Ruff, B.M., Zhong, S., 2013. The Chemistry of Mycotoxins, Progress in the Chemistry of Organic Natural Products. Springer.

Broydé, H., Doré, T., 2013. Effects of cropping systems on food and feed contamination by *Fusarium* and *Aspergillus* mycotoxins. Cah. Agric. 22, 182–194.

Buchanan, R.L., Stahl, H.G., 1984. Ability of various carbon sources to induce and support aflatoxin synthesis by *Aspergillus parasiticus*. J. Food Saf. 6, 271–279.

Cabañes, F.J., Bragulat, M.R., Castellá, G., 2013. Characterization of nonochratoxigenic strains of *Aspergillus carbonarius* from grapes. Food Microbiol. 36, 135–141.

CAC, 2003. Code of practice for the prevention and reduction of mycotoxin contamination in cereals, including annexes on ochratoxin a, zearalenone, fumonisins and tricothecenes.

Carlile, M.., Watkinson, S., 1996. The fungi. Academ ic Press, Harcourt Brace and Com p any Publishers, London, UK.

Cary, J.W., Ehrlich, K.C., 2006. Aflatoxigenicity in *Aspergillus*: Molecular genetics, phylogenetic relationships and evolutionary implications. Mycopathologia 162, 167–177.

CAST, 2003. Mycotoxins: Risks in plant, animal and human systems. Task force report; Nº. 139. ISSN 0194-4088.

Chulze, S.N., 2010. Strategies to reduce mycotoxin levels in maize during storage: a review. Food Addit. Contam. Part A 27, 651–657.

Cole, G.T., 1981. Biology of Conidial Fungi. Academic Press, 1981.ISBN: 9780323138994

Cotty, P.J., 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79, 808.

Cozzi, G., Pascale, M., Perrone, G., Visconti, A., Logrieco, A., 2006. Effect of Lobesia botrana damages on black *aspergilli* rot and ochratoxin A content in grapes. Int. J. Food Microbiol. 111, S88–S92.

Crespo-Sempere, A., Marín, S., Sanchis, V., Ramos, A.J., 2013. VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J. Food Microbiol. 166, 479–486.

Curto, R.L., Pellicanò, T., Vilasi, F., Munafò, P., Dugo, G., 2004. Ochratoxin A occurrence in experimental wines in relationship with different pesticide treatments on grapes. Food Chem. 84, 71–75.

Dantigny, P., Soares Mansur, C., Sautour, M., Tchobanov, I., Bensoussan, M., 2002. Relationship between spore germination kinetics and lag time during growth of Mucor racemosus. Lett. Appl. Microbiol. 35, 395–398.

Dighton, J., White, J.F., Oudemans, P., 2005. The Fungal community: its organization and role in the ecosystem. Taylor & Francis, Boca Raton, FL.

Dill-Macky, R., Jones, R.K., 2000. The effect of previous crop residues and tillage on *Fusarium* head blight of wheat. Plant Dis. 84, 71–76.

Dobson, A.D.W., Ortoneda, M., O`Challagan, J., 2006. Advances in ochratoxin A biosynthesis. Book of Abstracts, International Conference of "Advances on genomics, biodiversity and rapid systems for detection of toxigenic fungi and mycotoxins" September 26-29, 2006. Monopoli (Bari), Italy 39

Dowd, P.F., 2003. Insect management to facilitate preharvest mycotoxin management. J. Toxicol. - Toxin Rev. 22, 327–350.

Edwards, S.G., Godley, N.P., 2010. Reduction of *Fusarium* head blight and deoxynivalenol in wheat with early fungicide applications of prothioconazole. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 27, 629–635.

EC, 2003. European Commission, Commission regulation (EC) Nº 2866/2003 of 11 August 2003 on the prevention and reduction of patulin contamination in apple juice and apple juice ingredients in other beverages. Official Journal of the European Union, L 203(2003), 54-59.

EC, 2006a European Commission Nº 583/2006 of 17 August 2006 on the revention and reduction of *Fusarium* toxins in cereals and cereal products. Official Journal of the European Union, L 234(2006), 35-40.

EC, 2006b. European Commission Nº 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L 364(2006), 5-24.

Fels-Klerx, H.J.V.D., Booij, C.J.H., 2010. Perspectives for geographically oriented management of *Fusarium* mycotoxins in the cereal supply Chain. J. Food Prot. 73, 1153–1159.

Fernandez, M.R., Zentner, R.P., Basnyat, P., Gehl, D., Selles, F., Huber, D., 2009. Glyphosate associations with cereal diseases caused by *Fusarium* spp. in the Canadian Prairies. Eur. J. Agron. 31, 133–143.

Fourtouni, A., Manetas, Y., Christias, C., 1998. Effects of UV-B radiation on growth, pigmentation, and spore production in the phytopathogenic fungus *Alternaria solani*. Can. J. Bot. 76, 2093–2099.

Guo, B., Chen, Z.-Y., Lee, R.D., Scully, B.T., 2008. Drought Stress and Preharvest Aflatoxin Contamination in Agricultural Commodity: Genetics, Genomics and Proteomics. J. Integr. Plant Biol. 50, 1281–1291.

Haidukowski, M., Pascale, M., Perrone, G., Pancaldi, D., Campagna, C., Visconti, A., 2005. Effect of fungicides on the development of *Fusarium* head blight, yield and deoxynivalenol accumulation in wheat inoculated under field conditions with *Fusarium graminearum* and *Fusarium culmorum*. J. Sci. Food Agric. 85, 191–198.

IARC, 1993. Monographs on the evaluation of carcinogenic risks to humans: some naturally occurring substances, food items and constituents, heterocyclic aromatic amines and mycotoxins.

IARC, 1998. Monographs on the evaluation of carcinogenic risks to humans: some traditional herbal medicines, some mycotoxins, naphthalene and styrene.

Ioos, R., Belhadj, A., Menez, M., Faure, A., 2005. The effects of fungicides on *Fusarium* spp. and *Microdochium nivale* and their associated trichothecene mycotoxins in French naturally-infected cereal grains. Crop Prot. 24, 894–902.

Kabak, B., Dobson, A.D.W., Var, I., 2006. Strategies to prevent mycotoxin contamination of food and animal feed: A review. Crit. Rev. Food Sci. Nutr. 46, 593–619.

Leong, S.-L., Hocking, A.D., 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.

Leplat, J., Friberg, H., Abid, M., Steinberg, C., 2013. Survival of *Fusarium graminearum*, the causal agent of *Fusarium* head blight. A review. Agron. Sustain. Dev. 33, 97–111. doi:10.1007/s13593-012-0098-5

Leslie, J.F., Bandyopadhyay, R., Visconti, A., 2008. Mycotoxins: detection methods, management, Public Health and Agricultural Trade. CABI.

Lindblad, M., Börjesson, T., Hietaniemi, V., Elen, O., 2012. Statistical analysis of agronomical factors and weather conditions influencing deoxynivalenol levels in oats in Scandinavia. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 29, 1566–1571.

Magan, N., Olsen, M., 2004. Mycotoxins in Food: Detection and Control. Edited by M Magan and M Olsen Woodhead.

Marín, S., Hodžić, I., Ramos, A.J., Sanchis, V., 2008. Predicting the growth/no-growth boundary and ochratoxin A production by *Aspergillus carbonarius* in pistachio nuts. Food Microbiol. 25, 683–689.

McGee, D.C., Olanya, O.M., Hoyos, G.M., Tiffany, L.H., 1996. Populations of *Aspergillus flavus* in the Iowa cornfield ecosystem in years not favorable for aflatoxin contamination of corn grain. Plant Dis. 80, 742–746.

Medina, Á., Mateo, E.M., Valle-Algarra, F.M., Mateo, F., Mateo, R., Jiménez, M., 2008. Influence of nitrogen and carbon sources on the production of ochratoxin A by ochratoxigenic strains of *Aspergillus* spp. isolated from grapes. Int. J. Food Microbiol. 122, 93–99.

Moody, S.A., Newsham, K.K., Ayres, P.G., Paul, N.D., 1999. Variation in the responses of litter and phylloplane fungi to UV-B radiation (290-315 nm). Mycol. Res. 103, 1469–1477.

Munimbazi, C., Bullerman, L.B., 1997. Inhibition of aflatoxin production of *Aspergillus parasiticus* NRRL 2999 by Bacillus pumilus. Mycopathologia 140, 163–169.

Musa, T., Hecker, A., Vogelgsang, S., Forrer, H.R., 2007. Forecasting of Fusarium head blight and deoxynivalenol content in winter wheat with FusaProg. EPPO Bull. 37, 283–289.

Nicholson, P., Chandler, E., Draeger, R.C., Gosman, N.E., Simpson, D.R., Thomsett, M., Wilson, A.H., 2003. Molecular tools to study epidemiology and toxicology of *Fusarium* head blight of cereals. Eur. J. Plant Pathol. 109, 691–703.

Nicot, P.C., Mermier, M., Vaissière, B.E., Lagier, J., 1996. Differential spore production by *Botrytis cinerea* on agar medium and plant tissue under near-ultraviolet light-absorbing polyethylene film. Plant Dis. 80, 555–558.

Osman, M., Mohamed, Y.A., el-Sayed, M.A., Abo-Zeid, A., 1988. Effect of UV-irradiation on some aspects of metabolic activities in *Aspergillus flavus* and *Penicillium notatum*. Microbios 56, 79–87.

Ostry, V., Ovesna, J., Skarkova, J., Pouchova, V., Ruprich, J., 2010. A review on comparative data concerning *Fusarium* mycotoxins in Bt maize and non-Bt isogenic maize. Mycotoxin Res. 26, 141–145.

Parikka, P., Hietaniemi, V., Rämö, S., Jalli, H., Vihervirta, T., 2008. The effect of reduce tillage on mycotoxin contents of oat and barley grain. NJF Semin. 418 New Insights Sustain. Cultiv. Methods Agric. 4.

Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage. 3nd Edtion. London; New York: Blackie Academic & Professional.

Ponsone, M.L., Combina, M., Dalcero, A., Chulze, S., 2007. Ochratoxin A and ochratoxigenic *Aspergillus* species in Argentinean wine grapes cultivated under organic and non-organic systems. Int. J. Food Microbiol. 114, 131–135.

Ramirez, M.L., Chulze, S., Magan, N., 2004. Impact of environmental factors and fungicides on growth and deoxinivalenol production by *Fusarium graminearum* isolates from Argentinian wheat. Crop Prot. 23, 117–125.

Ramos, A.J., 2011. Micotoxinas y micotoxicosis, España, AMV Ediciones.

RASFF, 2012. Rapid Alert System for Food and Feed http://ec.europa.eu/food/food/rapidalert/index_en.htm.

Rossi, V., Giosuè, S., Terzi, V., Scudellari, D., 2007. A decision support system for Fusarium head blight on small grain cereals. EPPO Bull. 37, 359–367.

Rotem, J., Wooding, B., Aylor, D.E., 1985. The role of solar radiation, especially ultraviolet, in the mortality of fungal spores. Phytopathology 75, 510–514.

Terzi, V., Tumino, G., Stanca, A.M., Morcia, C., 2014. Reducing the incidence of cereal head infection and mycotoxins in small grain cereal species. J. Cereal Sci. 59, 284–293.

Schaafsma, A.W., Hooker, D.C., 2007. Climatic models to predict occurrence of *Fusarium* toxins in wheat and maize. Int. J. Food Microbiol. 119, 116–125.

Schaafsma, A.W., Hooker, D.C., Pineiro, M., Díaz de Ackermann, M., Pereyra, S. Castaño, J.P. (2006). Pre-harvest forecasting of deoxynivalenol for regulatory action in wheat grain in Uruguay using readily available weather inputs Njapau (Ed.) et al., Mycotoxins and Phycotoxins: Advances in Determination, Toxicology and Exposure Management, Wageningen Academic Publishers of the Netherlands (2006), pp. 227–238

Simpson, D.R., Weston, G.E., Turner, J.A., Jennings, P., Nicholson, P., 2001. Differential control of head blight pathogens of wheat by fungicides and consequences for mycotoxin contamination of grain. Eur. J. Plant Pathol. 107, 421–431.

Smith, J.E., 1985. Mycotoxin hazards in the production of fungal products and byproducts. Pract of Biotech, Spec Prod and Serv Act..

Supronienė, S., Mankevičienė, A., Kadžienė, G., Kačergius, A., Feiza, V., Feizienė, D., Semaškienė, R., Dabkevičius, Z., Tamošiūnas, K., 2012. The impact of tillage and fertilization on *Fusarium* infection and mycotoxin production in wheat grains. Ž EmdirbystėAgriculture 99, 265–272.

Taniwaki, M.H., Hocking, A.D., Pitt, J.I., Fleet, G.H., 2009. Growth and mycotoxin production by food spoilage fungi under high carbon dioxide and low oxygen atmospheres. Int. J. Food Microbiol. 132, 100–108. doi:10.1016/j.ijfoodmicro.2009.04.005

Santos, 2011. Fungi and mycotoxins in *Capsicum* powder: occurrence, ecophisiology and control. Doctoral Thesis University of Lleida.

Tjamos, S.E., Antoniou, P.P., Kazantzidou, A., Antonopoulos, D.F., Papageorgiou, I., 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.

Van Asselt, E.D., Booij, C.J.H., van der Fels-Klerx, H.J., 2012. Modelling mycotoxin formation by *Fusarium graminearum* in maize in The Netherlands. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 29, 1572–1580.

Van der Gaag, B., Spath, S., Dietrich, H., Stigter, E., Boonzaaijer, G., van Osenbruggen, T., Koopal, K., 2003. Biosensors and multiple mycotoxin analysis. Food Control 14, 251–254.

Wagacha, J.M., Muthomi, J.W., 2008. Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. Int. J. Food Microbiol. 124, 1–12.

Windels, C.E., 2000. Economic and social impacts of *Fusarium* head blight: Changing farms and rural communities in the Northern Great Plains. Phytopathology 90, 17–21.

Woloshuk, C.P., Shim, W.-B., 2013. Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge. FEMS Microbiol. Rev. 37, 94–109. doi:10.1111/1574-6976.12009

Xu, X., Madden, L.V., Edwards, S.G., Doohan, F.M., Moretti, A., Hornok, L., Nicholson, P., Ritieni, A., 2013. Developing logistic models to relate the accumulation of DON associated with *Fusarium* head blight to climatic conditions in Europe. Eur. J. Plant Pathol. 137, 689–706. doi:10.1007/s10658-013-0280-x

Yang, J., Li, J., Jiang, Y., Duan, X., Qu, H., Yang, B., Chen, F., Sivakumar, D., 2014. Natural occurrence, analysis, and prevention of mycotoxins in fruits and their processed products. Crit. Rev. Food Sci. Nutr. 54, 64–83. doi:10.1080/10408398.2011.569860

II. OBJECTIVES and WORK PLAN

GENERAL OBJETIVE

This Thesis work is included in the main research line "Tools for the management of the Food Safety applied to mycotoxin risks" of the Applied Mycology Unit of the Food Technology Departament of the University of Lleida, and was supported by national and international projects:

- The MYCORED European project KBBE-2007-2-5-05: "New integrated strategies for reducing mycotoxins in the world in food and feed chains".

- The BASELINE European project KBBE-222738: "Selection and improving of fit-for-purpose sampling procedures for specific foods and risks".

- The Spanish project AGL-2010-22182-C04-04: "Climate change and new food habits: new scenarios challenging Food Safety Objectives for mycotoxin risk in Spain".

The general objective of this Thesis is to ensure the usefulness of the present methods of fungal control for an effective management, as well as to assess the possible Food Safety implications derived from climate change.

Specific objetives were also proposed:

- To identify the Performance Objectives (PO) for pistachio concerning the mycotoxin presence as chemical hazard of biological origin.

- To evaluate the suitability of existing sampling plans for the screening of pistachio lots for aflatoxins.

- To isolate and identify the mycobiota presence in grapes in two agroclimatic regions from Spain (Catalonia and Andalucia) for the evaluation of the mycotoxigenic fungi biodiversity in grapes and musts contamination.

- To compare the ecophysiological traits (in terms of temperature and water activity requirements) of *A. tubingensis*, *A. niger* and *A. carbonarius* isolated from berries from Catalonia and Andalucia.

- To evaluate the impact of ultraviolet radiation (UV) on the fungal development with a special attention to black aspergilli.

- To evaluate the climate change impact over the efficiency of natural and chemical antifungals applied for the control of the mycotoxigenic fungus on grapes and wheat.

WORK PLAN

In order to achieve the mentioned objectives, the following work plan was proposed:

Bibliographic revision

Research work:

Food Safety Management in mycotoxins issue	**PART I: SELECTION OF RAW MATERIALS**	SAMPLING PLANS Alternative sampling plans for raw and roasted pistachio (Study I) EFFECT OF PROCESSING Effect of industrial roasting of pistachio (Study I) SETTING PO IN INDUSTRIAL PISTACHIO PROCESSING Pistachio processing (Study I)
	PART II: CLIMATE CHANGE	FUNGAL PRESENCE AND ADAPTATION Isolation and identification of mycotoxigenic species from two agroclimatic regions (Study II) Ecophysiological profile of black aspergilli isolates from two agroclimatic regions (Study III) UV resistance of *Aspergillus* species (Study IV and study V) ANTIFUNGALS EFFECTIVENESS Effectiveness of natural and chemical antifungals on *Aspergillus* species in grapes and wheat (Study VI and study VII)

Preparation of the Thesis Document

III. INTRODUCTION

1. Aflatoxins and ochratoxin A

1.1. Aflatoxins

AFs were first discovered and characterized in the early 1960s after more than 100000 turkey poults died due to an apparent poisoning from mould contaminated peanut meal in England (Goldblatt, 1969). Nowadays, these mycotoxins are considered the most common naturally formed carcinogens (CAST, 2003).

Their chemical structure of AFs consists in a difuranocoumarin derivate produced by a poliketide pathway, and are classified in two groups (Figure 1 and **Table 5**).

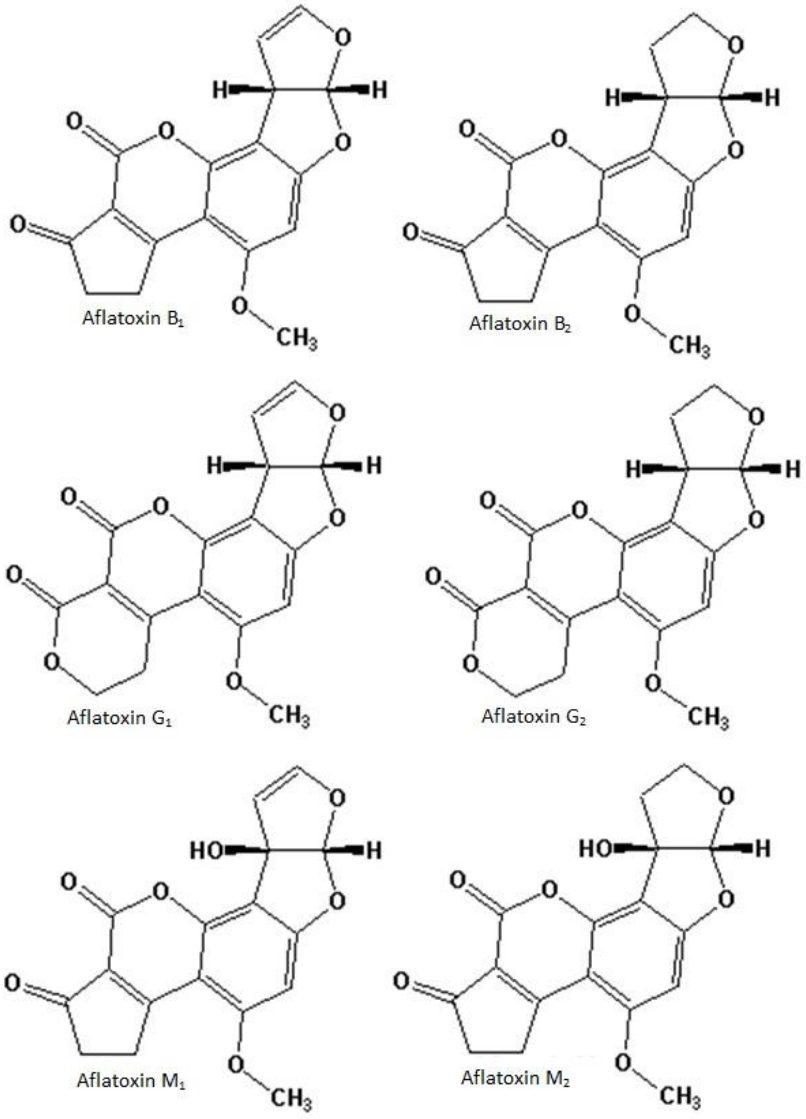


Figure 1 Structure of naturally occurring main AFs.

Table 5 Melting point and UV absorption of AFs.

Difurocoumarocyclopentenone series					
Common name	**Abbreviation**	**Melting point (ºC) crystal from**		**UV absorption (ethanol)**	
				λmax(nm)	**Ɛ(L/mol cm)**
Aflatoxin B_1	AFB_1	268-269	Chloroform	223	25600
				265	13400
				362	21800
Aflatoxin B_2	AFB_2	286-289	chloroform-pentane	265	11700
Aflatoxin M_1	AFM_1	299	Methanol	265	11600
				357	1900
Aflatoxin M_2	AFM_2	293	methanol-chloroform	221	20000
				264	10900
				357	21000
Difurocoumarolactone series					
Aflatoxin G_1	AFG_1	244-246	chloroform-methane	243	11500
				257	9900
				264	10000
				362	16100
Aflatoxin G_2	AFG_2	237-240	ethyl acetate	265	9700
				363	21000

Ɛ: molar extinction coefficient (Cole et al., 2003; IARC, 2002a)

Difurocoumarocyclopentenone series: polycyclic aromatic compounds containing a cyclopenten-2-one ring fused to the coumarin moiety of the difurocoumarin skeleton (AFB_1, AFB_2, AFB_{2A}, AFM_1, AFM_2, AFM_{2A} and aflatoxicol); and difurocoumarolactone series: polycyclic aromatic compounds containing a delta-valerolactone ring fused to the coumarin moiety of the difurocoumarin skeleton (AFG_1, AFG_2, AFG_{2A}, AFB_3). When mammals consume AFs-contaminated feeds, they metabolically biotransform AFB into a hydroxylated form called AFM. The physicochemical properties (description, solubility, stability, reactivity) of AFs have been described in published bibliography (Cole et al., 2003; IARC, 2002).

- Description: AFs are highly fluorescent substances in UV, emitting blue (AFB_1 and AFB_2) or green (AFG_1) and green-blue (AFG_2) fluorescence, from which the designations B and G were derived, or blue-violet fluorescence (AFM_1).

- Solubility: soluble in water, insoluble in non-polar solvents, freely soluble in moderately polar organic solvents (e.g., chloroform and methanol and especially in dimethyl sulfoxide).

- Stability: unstable to UV light in the presence of oxygen, to extreme pH (<3, >10) and to oxidizing agents.

- Reactivity: a solution prepared in chloroform or benzene is stable for years if kept cold and in the dark. The lactone ring makes them susceptible to alkaline hydrolysis. If alkaline treatment is mild, acidification will reverse the reaction to reform the original AF. In acid, AFB_1 and AFG_1 are converted to AFB_{2a} and AFG_{2a} by acid catalytic addition of water across the double bond of the furan ring. Oxidizing reagents react and the molecules lose their fluorescence properties.

1.1.1. Producing fungi and their requirements for growth and AFs production

Production of AFs has been reported from members of different groups of *Aspergilli*, as section *Ochraceorosei*, *Nidulantes* and *Flavi* (Frisvad et al., 2005; Varga et al., 2009). Nonetheless, the most important AF producers are members of *Aspergillus* section *Flavi* (Varga et al., 2009). Several species belonging to this section are important mycotoxin producers including AFs, CPA, OTA and kojic acid (KA) (Varga et al., 2011). Numerous authors have tested the AFs production capacity of numerous species belonging to this section (**Table 6**). Nevertheless, from a public health point of view *A. flavus* and *A. parasiticus* are the most significant. Considering their mycotoxigenic profile, it is usually accepted that not all *A. flavus* isolates produce AFs, and those that do usually produce only B-type AFs (and CPA), whereas almost all *A. parasiticus* isolates produce both B and G-type AFs, but not CPA (Giorni et al., 2007; Klich, 2007; Pildain et al., 2008; Rodrigues et al., 2009; Vaamonde et al., 2003). Furthermore, aflatoxigenic strains do not produce toxin in all substrates and can even lose their toxigenic potential (Wei and Jong, 1986). Similar incidence of both species has been reported in nuts (peanuts and almonds) and wheat (Rodrigues et al., 2009; Vaamonde et al., 2003), whereas *A. flavus* was clearly more dominant (93%) than *A. parasiticus* (7%) in maize (Giorni et al., 2007). With regard to T and a_W, a summary of the conditions that yield the highest AFs production for each species is presented in **Table 7.** *A. parasiticus* seems to be able to produce AFs under higher T than *A. flavus* nevertheless strains were tested in different culture medium.

Table 6 Species of *Aspergillus* section *Flavi* tested for AFs production.

Isolates	Aflatoxin production				References
	B_1	B_2	G_1	G_2	
A. arachidicola	+	+	+	+	(Gonçalves et al., 2012; Pildain et al., 2008)
A. avenaceus	-	-	-	-	(Pildain et al., 2008)
A. bombycis	+	+	+	+	(Frisvad et al., 2005; Peterson et al., 2002; Pildain et al., 2008)
A. caelatus	-	-	-	-	(Pildain et al., 2008)
A. coremiiformis	-	-	-	-	(Varga et al., 2011)
A. flavus	+/-	+	-	-	(Frisvad et al., 2005; Gonçalves et al., 2012; Pildain et al., 2008; Varga et al., 2009; Yazdani et al., 2011)
A. leporis	-	-	-	-	(Pildain et al., 2008)
A. minislerotigenes	+	+	+	+	(Gonçalves et al., 2012; Pildain et al., 2008)
A. nomius	+	+	+	+	(Peterson et al., 2002; Pildain et al., 2008)
A. novoparasiticus	+	+	+	+	(Gonçalves et al., 2012)
A. oryzae	-	-	-	-	(Yazdani et al., 2011)
A. parasiticus	+	+	+	+	(Araguás et al., 2005; Frisvad et al., 2005; Pildain et al., 2008)
A. parasiticus var. globosus	+	+	+	-	(Gonçalves et al., 2012)
A. parvisclerotigenus	+	+	+/-	+	(Frisvad et al., 2005; Pildain et al., 2008)
A. pseudocaelatus	+	+	+	+	(Varga et al., 2011)
A. pseudonomius	+	-	-	-	(Pildain et al., 2008; Varga et al., 2011)
A. pseudotamarii	+	+	-	-	(Frisvad et al., 2005; Ito et al., 2001)
A. sojae	-	-	-	-	(Gonçalves et al., 2012; Varga et al., 2011)
A. tamari	-	-	-	-	(Goto et al., 1996; Pildain et al., 2008; Yazdani et al., 2011)
A. terricola var. americans	-	-	-	-	(Gonçalves et al., 2012)
A. togoensis	+	-	-	-	(Rank et al., 2011)
A. toxicarius	+	-	+	+	(Gonçalves et al., 2012)

+, production; -, no production

Table 7 Summary of the conditions for AFs production by different fungi.

	A. flavus[a]	***A. parasiticus***[b]
Minimum T	15 ºC	15 ºC
Maximum T	37 ºC	40 ºC
Optimal T	30 ºC	37 ºC
Minimum a_w	0.86	<0.90
Optimal a_w	0.96	0.93-0.99
Minimum time	7 days	
Optimal time	21 days	

[a], medium based maize; [b], YES (Astoreca et al., 2014; Schmidt-Heydt et al., 2010)

1.1.2. Occurrence in foodstuffs

AFs have been found in a variety of agricultural commodities as cereals and their derivates as flour, breakfast cereals or beer (Aydin et al., 2008; Burdaspal and Legarda, 2013, 2013; Hassan and Kassaify, 2014; Rahmani et al., 2011; Roscoe et al., 2008). They are also frequently detected in nuts or nut products as peanut butter (Ezekiel et al., 2012; Jahanmard et al., 2014; Milhome et al., 2014; Yentür et al., 2006), cocoa and derivates (Turcotte et al., 2013), spices (Hammami et al., 2014), dried figs (Heperkan et al., 2012) and oil (Finoli et al., 2005). Since cereals are the base of feeds, AFs or their metabolites have been detected in meat (Aziz and Youssef, 1991), milk and dairy products (Gul and Dervisoglu, 2014; Hassan and Kassaify, 2014).

European Commission has established maximum levels for AFB_1 and AFs (AFB_1+AFB_2+AFG_1+AFG_2) in cereal products, nuts and dried fruit previous to sorting or other physical treatment, and lower values for direct consumption likewise contamination in spices (EC, 2010a, 2012). Furthermore AFM_1 contamination in milk, and AFB_1 and AFM_1 in infant and dietary foods for special medical purpose are also regulated (EC, 2010a). Besides AFB_1 maximum level is also legislated in feed (EU, 2003).

1.2. Ochratoxin A

OTA was discovered as a metabolite of *A. ochraceus* from corn meal intentionally inoculated with this fungus in South Africa, in 1965 (Van Der Merwe et al., 1965). OTA was described as one of the first fungal metabolites which was toxic to animals, which, with the AFs, launched the distinctive and individualised science of mycotoxicology in the 1960s (Zinedine et al., 2010).

Its chemical structure consists of a dihydroisocoumarin moiety coupled to L-β-phenylalanine by a peptide bond (Figure 2 and **Table 8**). The group comprises OTA, its dechloro analogue OTB, its ethyl ester ochratoxin C, and the hydroxylated forms. OT*a* and OT*β* are products of the hydrolysis of the peptide bound of OTA and OTB, respectively, and lack the phenylalanine moiety. OTA is the most important ochratoxin due to its incidence and toxicity.

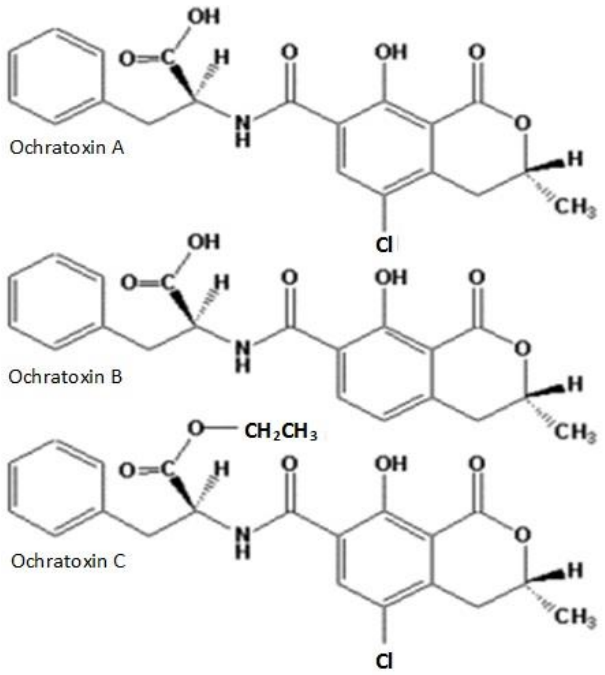


Figure 2 Structure of naturally occurring main ochratoxins.

The physiochemical properties (description, solubility, stability, reactivity) of OTA has been described in published bibliography (Deshpande, 2002; IARC, 1993; Valenta, 1998).

- Description: intense fluorescence in UV light, emitting green and blue fluorescence in acid and alkaline solutions, respectively.

- Solubility: it is soluble in polar organic solvents, slightly soluble in water and soluble in diluted aqueous bicarbonate solutions.

- Stability: the UV absorption spectrum varies with pH and solvent polarity. OTA is unstable to air and light, though ethanol solutions are stable for longer than one year if kept refrigerated and in the dark.

- Reactivity: partially degraded under cooking conditions. Solutions of OTA are completely degraded by treatment with an excess of sodium hypochlorite solution. Thermal stability of OTA varies according to the matrix where it is present, and it seems to be stable in some food matrices up to 180º C (Raters and Matissek, 2008).

Table 8 Melting point and UV absorption of ochratoxins.

Common name	Abbreviation	Melting point (ºC) crystal from		UV absorption (ethanol) λmax(nm)	Ɛ(L/mol cm)
Ochratoxin A	OTA	169	Benzene	333	6640
Ochratoxin B	OTB	221 °C		318	6900
Ochratoxin C	OTC	not availible		335	6200

Ɛ: molar extinction coefficient

1.2.1. Producing fungi and their requirements for growth and OTA production

Accumulation of ochratoxins has been reported from members of *Penicillia* and different groups of *Aspergilli.* Additionally, *Penicillium* spp. incidence is associated with colder temperate climates, while *Aspergillus* spp. is most frequently isolated from warmer and tropical parts of the world.

Several *Penicillium* spp. have been pointed as OTA producers. However, in a completed revision about extrolites produced by species in *Penicillium* subgenus *Penicillium* carried out by (Frisvad et al., 2004b) the authors considered than *P. verrucosum* and *P. nordicum* were the main OTA producer species, as many strains had been incorrectly identified or unidentified as producers.

Several sections within the *Aspergilli* group contain species capable to produce OTA. In this sense, the species belonging to section *Flavi* (*Petromyces alliaceus, P. albertensis,* and *A. lanosus*), section *Wentii* (*A. sepultus* and *A. dimorphicus*) section *Candidi* (*A. taichungensis* and *A. campestris*), section *Circumdati* (**Table 9**) and section *Nigri* have shown ability to produce OTA (Frisvad and Samson, 2000; Frisvad et al., 2004a; Medina et al., 2005; Zotti and Montemartini Corte, 2002).
Focusing on section *Circumdati* at least ten species have been reported as potential OTA producers (**Table 9**), although the most important species in this regard appear to be *A. ochraceus, A. westerdijkiae* and *A. steynii*, because they are very common and most of the isolates in these species produce large amounts of OTA (Frisvad et al., 2004a).

Table 9 Species of *Aspergillus* section *Circumdati* tested for OTA production.

Species	OTA	Reference
A. auricomus	-	(Frisvad et al., 2004a)
A. bridgeri	-	(Frisvad et al., 2004a)
A. cretensis	+	(Frisvad et al., 2004a)
A. elegans	-	(Frisvad et al., 2004a)
A. flocculosus	+	(Frisvad et al., 2004a
A. insulicola	-	(Frisvad et al., 2004a)
A. melleus	-*	(Frisvad et al., 2004a)
A. neobridgeri	-	(Frisvad et al., 2004a)
A. ochraceus	+/-	(Frisvad et al., 2004a)
A. ostianus	-*	(Frisvad et al., 2004a)
A. perseii	-*	(Frisvad et al., 2004a)
A. petrakii	-*	(Frisvad et al., 2004a)
A. pseudoelegans	+	(Frisvad et al., 2004a)
A. roseoglobulosus	+	(Frisvad et al., 2004a)
A. sclerotiorum	+/-	(Frisvad et al., 2004a)
A. steynii	+	(Frisvad et al., 2004a)
A. sulphurous	+	(Frisvad et al., 2004a)
A. westerdijkiae	+	(Frisvad et al., 2004a)

+, production; -, no production; *, produce trace amounts (Frisvad et al., 2004a).

Additionally, over the last two decades intense efforts have been directed to discriminate the species within section *Nigri* and metabolites production included OTA (**Table 10**).

As mentioned in the previous section, not all strains have the same capacity for OTA synthesis. Even the ochratoxigenic strains do not produce toxin in all substrates. With regard to T and a_W, a summary of the conditions that yield the highest OTA production for some species is present in **Table 11**.

Table 10 Species of *Aspergillus* section *Nigri* tested for OTA production.

Species	OTA	References
A. acidus	-	(Frisvad et al., 2011; Mogensen et al., 2009)
A. aculeatinus	-	(Frisvad et al., 2011; Noonim et al., 2008; Samson et al., 2007)
A. aculeatus	-	(Frisvad et al., 2011; Samson et al., 2007, 2004)
A. awamori	+	(Perrone et al., 2011)
A. brasiliensis	-	(Frisvad et al., 2011; Samson et al., 2007, 2004; Varga et al., 2007)
A. carbonarius	+/-	(Cabañes et al., 2013; de Vries et al., 2005; Frisvad et al., 2011; Samson et al., 2007, 2004; Serra et al., 2006b)
A. costaricaensis	-	(Frisvad et al., 2011; Samson et al., 2007; Varga et al., 2007)
A. ellipticus	-	(Frisvad et al., 2011; Samson et al., 2007, 2004)
A. foetidus	-	(de Vries et al., 2005; Samson et al., 2007, 2004; Varga et al., 2007)
A. heteromorhpus	-	(Frisvad et al., 2011; Samson et al., 2007, 2004)
A. homomorphus	-	(Frisvad et al., 2011; Samson et al., 2007)
A. ibericus	-	(Frisvad et al., 2011; Serra et al., 2006a)
A. japonicus	-	(Frisvad et al., 2011; Samson et al., 2007, 2004)
A. lacticoffeatus	+	(Samson et al., 2007, 2004; Varga et al., 2007)
A. niger	+/-	(de Vries et al., 2005; Frisvad et al., 2011; Perrone et al., 2011; Samson et al., 2007, 2004; Varga et al., 2007)
A. piperis	-	(Frisvad et al., 2011; Samson et al., 2007, 2004; Varga et al., 2007)
A. scleroticarbonarius	-	(Frisvad et al., 2011; Noonim et al., 2008; Samson et al., 2007)
A. sclerotioniger	+	(Frisvad et al., 2011; Samson et al., 2007, 2004; Serra et al., 2006a)
A. tubingensis	+/-	(de Vries et al., 2005; Frisvad et al., 2011; Medina et al., 2005; Samson et al., 2007, 2004; Varga et al., 2007)
A. uvarum	-	(Frisvad et al., 2011; Perrone et al., 2007b; Samson et al., 2007)
A. vadensis	-	(de Vries et al., 2005; Frisvad et al., 2011; Samson et al., 2007, 2004; Varga et al., 2007)

+, production; -, no production

Table 11 Summary of the conditions for OTA production by different fungi.

	P. verrucosum	***A. ochraceus***	***A. carbonarius***	***A. niger***
Minimum T	4–10 °C	5–10 °C	5–15 °C	10–15 °C
Maximum T	21–31°C	30–40 °C	30–45 °C	35–41 °C
Optimal T	24–25 °C	20–35 °C	15–30 C	15–35 °C
Minimum a_w	0.80–0.83	0.87–0.90	0.85–0.94	0.90–0.95
Optimal a_w	0.95–0.99	0.95–0.99	0.95–0.99	0.95–0.99
Minimum time	7 days	3 days	2–5 days	3–7 days
Optimal time	>14 days	9–21 days	10–15 days	5–30 days

(Amézqueta et al., 2012)

1.2.2. Occurrence in foodstuffs

OTA presence has been described in many commodities. It has been detected in cereals (Scudamore et al., 2003), grapes and derivates (Abrunhosa et al., 2001; Varga and Kozakiewicz, 2006; Zimmerli and Dick, 1996), coffee (Taniwaki et al., 2003), cocoa and derivates (Bonvehí, 2004), pork and poultry meat products (Beg et al., 2006; Matrella et al., 2006), milk (Boudra et al., 2007), spices(Aziz et al., 1998), nuts (Bayman et al., 2002), liquorice (Ariño et al., 2007) and vegetable oil (Ferracane et al., 2007). OTA contamination in meat and animal products is caused by the consumption of contaminated feedstuff (Curtui et al., 2001; Sabatini et al., 2007). In 2002 the European Food Safety Authority (EFSA) carried out a study for the assessment of OTA intake through the diet (Miraglia and Brera, 2002). It was estimated that at least 50% of OTA in the diet came from cereals, then, emphasized its importance in wine, as it is the second source of intake (13%).

In order to prevent human toxicological damage legal restrictions are applied to mycotoxins in food. In OTA, the Lowest Observed Adverse Effect Level (LOAEL) was 8 μg/kg body weight (b.w.) per day for early markers of renal toxicity in pigs; applying a composite uncertainty factor of 450 for the uncertainties due to the extrapolation of experimental data derived from animals to humans as well as for intra-species variability, a Tolerable Weekly Intake (TWI) of 120 ng/kg b.w was calculated (EFSA, 2006). The EC established maximum levels for OTA in cereals, dried vine fruits, coffee and wine in 2006. In the case of cereals, the legislation discriminated between unprocessed and intended for direct human consumption products. Subsequent studies found high OTA levels in spices and liquorice, considering appropriate to establish maximum limits of

contamination in these foods in 2010 (EC, 2006b, 2010b). Particularly, lower levels are set for baby and dietary foods.

2. Management of mycotoxin presence in commodities

Currently, risk management strategies applied to mycotoxins include, the establishment of science based regulatory limits likewise protocols development by the Internationally Agencies (CAC, FAO, European Commission…). Among these protocols, the HACCP system and pre-requisite programmes as well as GAP have clearly supplied a great improvement in the Food Safety Management.

Recently, the food safety management approach for microbiological risks has been completed and developed through the inclusion of other metrics like the Food Safety Objective (FSO) (ICMSF, 1998). The FSO specifies a goal which can be incorporated into the design of control measurements in the food chain corresponding with the maximum permissible level of a hazard in a food at the moment of consumption which leads to an Appropriate Level of Protection (ALOP). The acceptable level of risk is the level adopted following consideration of public health impact, technological feasibility, economic implications, and that which a society regards as reasonable in the context of and in comparison with other risks in everyday life (Schothorst, 1998).

In this context, the agro-food industry would use FSOs as means to co-ordinate risk management in the production process throughout the farm-to fork production chain (de Swarte and Donker, 2005). Maximum hazard levels at other steps along the food chain are called Performance Objectives (POs) (CAC, 2007).

The concept of FSO has mostly been applied to understanding the effects of handling and processing on levels of bacterial pathogens in foods, but it is also applicable to the formation and removal of mycotoxins (Pitt et al., 2013). The FSO is calculated from Equation 1.

$$Ho - \sum R + \sum I \leq FSO \quad \text{Equation 1}$$

Increase in mycotoxin levels may occur before or after harvest, during drying, or during storage ($\sum I$) while reduction in mycotoxin levels ($\sum R$) takes place during processing (ICMSF, 2002). Initial mycotoxin level (Ho) for food industries could be designed as the time of sale from the farm to

distributors or processors. In the case of mycotoxins, FSO, Ho, R and I are expressed in μg/kg. Recently, Pitt et al. (2013) described the time course of mycotoxin formation and reduction in major crops, with reference to the FSO using a graphical method.

Since this thesis has been focused on pistachio, wheat and grape, the occurrence and concentration of AFs and OTA, as well as the producing fungi and the infection moment in these substrates has been more extensively revised. Additionally, the effect of food processing (toasted pistachio, wheat flour and vinification) has been also considered, and proposed POs have been indicated in three suggested flow charts.

2.1. Case study I: Nuts-pistachio

Nuts present low a_W and, due to their intrinsic characteristics, fungi are the major microbiological contaminants. Some of these molds are mycotoxigenic, thus high levels of mycotoxins have frequently been reported in nuts from the orchards and from the market (Bayman et al., 2002; Fernane et al., 2010a).

AFs are often present in pistachio samples in the market (10-100%) while the incidence of OTA (2-3%) is generally low. The AFs contamination levels are widely variable with samples up to 2 ppm (**Table 12**). In most of the cases AFB_1 represented more than 90 % of the AFs.

Although OTA producers have been isolated from pistachio (Fernane et al., 2010a, 2010b; Marín et al., 2008), lower frequency and concentration of this toxin have been detected (

Table 13).

EU maximum limits for AFs in nuts have been recently modified (
Table 14) after EFSA reviewed the maximum limits and intake assessment for tree nuts concluding that there was no additional consumer concern at 4, 8, 10 or 15 μg/kg AFs (EFSA, 2007) in the context of exposure from all other sources and previous pertinent exposure assessments. Regarding to OTA, although its occurrence in nuts has been reported in several studies, its presence has

always been significantly lower than AFs, and indeed maximum levels have not been set by the EC in nuts.

Table 12 Occurrence and concentration of AFB_1 and AFs in pistachio.

Incidence rate	Contamination range (mean)		Reference
	AFB_1(μg/kg)	**AFs(μg/kg)**	
17/46 (37%)	–	0.5–289	(Abdulkadar et al., 2000)
48/101 (48%)	–	1.2–275	(Abdulkadar et al., 2000)
7/21 (33%)	0.005–1900(-)	0.01–2200(100)	(Thuvander et al., 2001)
8/52 (15%)	0.6–93.3(22.1)	0.6–106.9(25)	(Food Standards Agency, 2002)
12/24(50%)	0.57-98.5		(Burdaspal et al., 2005)
3/6(50%)		0.2-81.6	(Abdulkadar et al., 2004)
3699/10(36.7%)	n.d.->500(5.9)	n.d.->500(7.3)	(Cheraghali et al., 2007)
9/20(45%)	0.04-1430(158)	0.1-1450(163)	(Juan et al., 2008)
11/32 (34%)	0.12–0.29(0.17)		(Ariño et al., 2009)
7/72 (10%)	0.5–36.8	–	(Basaran and Ozcan, 2009)
95/105 (95%)	(185.9)	(215.0)	(Pour et al., 2010)
2/31 (6%)	–	0.4–0.7(0.5)	(Fernane et al., 2010a)
5/50 (10%)	2.2–1037.3(214)	3.04–1134.5(243.7)	
59/120 (49%)	0.1–4.1	0.07–7.72	(Set and Erkmen, 2010)
2/32 (6%)	0.2–0.4(0.3)	0.3	(Reinhold and Reinhardt, 2011)
1921/8203(23.4%)	n.d.-369.1(2.18)	n.d.-369.1(2.2)	(Dini et al., 2013)
9/9(100%)	1.9-411	3.3-422	(El tawila et al., 2013)
18/53(34%)		1.0-110(16.6)	(El tawila et al., 2013)
22/151(15%)	0.26-368(31.2)	0.26-385(37.9)	(Hepsag et al., 2014)
15/112(13%)		n.d.->5	(Jahanmard et al., 2014)

n.d.: no detected. Modified from (Marín et al., 2008)

Table 13 Occurrence and concentration of OTA in pistachio.

Incidence rate	Contamination range (mean) (µg/Kg)	Reference
0/6		(Abdulkadar et al., 2004)
1/32(3%)	170	(Fernane et al., 2010a)
1/50(2%)	0.67	(Fernane et al., 2010a)
2/70(2.9%)	0.134-0.321(0.228)	(Coronel et al., 2012)

Table 14 AFB_1 and AFs EU legal limits in pistachio.

	AFB_1 (µg/kg)	**AFs (µg/kg)**
Pistachios to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	12	15
Pistachios intended for direct human consumption or use as an ingredient in foodstuffs	8	10

(EC, 2010a).

Regarding the dominant mycobiota several studies have reported that *Aspergillus* spp. causes decay in pistachio nuts in different parts of the world, such as USA (Doster and Michailides, 1994), Iran (Mojtahedi et al., 1979), and Turkey (Denizel et al., 1976). Regarding *Aspergillus* species similar percentages of infection were observed in pistachio samples bought in Algeria and Spain: *A. flavus* (22-30%), and *Aspergillus* section *Nigri* (30-40%). Similarly, *A. niger* and *A. tubingensis* were the dominating species on Iranian pistachio (Sedaghati et al., 2011) and only one *A. carbonarius* was isolated from pistachios of Spanish market (Marín et al., 2008). Nevertheless, a relevant percentage of producers in *A. flavus* (>50%) and *Aspergillus* section *Nigri* (>30%)isolated from Spain was reported (Fernane et al., 2010a, 2010b). Moreover, *A. ochraceus* and *A. melleus* OTA producers were isolated from Iranians pistachios (Sedaghati et al., 2012).

The general trend in the time course of AFs development and reduction in pistachio is shown in (**Figure 3**). It is a qualitative approach, i.e. no weight is given to slopes of lines, so all have been drawn at the same angle. In practice, increases or decreases in mycotoxin levels in any commodity are strongly dependent on climate, storage and processing conditions. Any quantitative risk management framework for a particular situation would require the appropriate data to allow estimation of stochastic aspects at each stage (Pitt et al., 2013).

In pistachios, *Aspergillus* infects and decays kernels, while nuts are still on tree (Bayman et al., 2003). *A. flavus* is a weak plant pathogen which seems to lack the ability to penetrate the shell of nuts thus

the entry into the edible kernel usually depends on breaks caused by abrasion or insects. Several studies have correlated high levels of AFs with hull cracked ("early split") nuts, insect damaged and wounded nuts (Doster and Michailides, 1999, 1995; Georgiadou et al., 2012). Besides insects, water stress can lead to separation of hull exposing the pistachio to *Aspergillus* infestation, so high contamination was quantified in pistachios from non irrigated orchards and without plant protection in Greece (Georgiadou et al., 2012). Additionally, higher *Aspergillus* infection has been observed in mature stages near harvest.

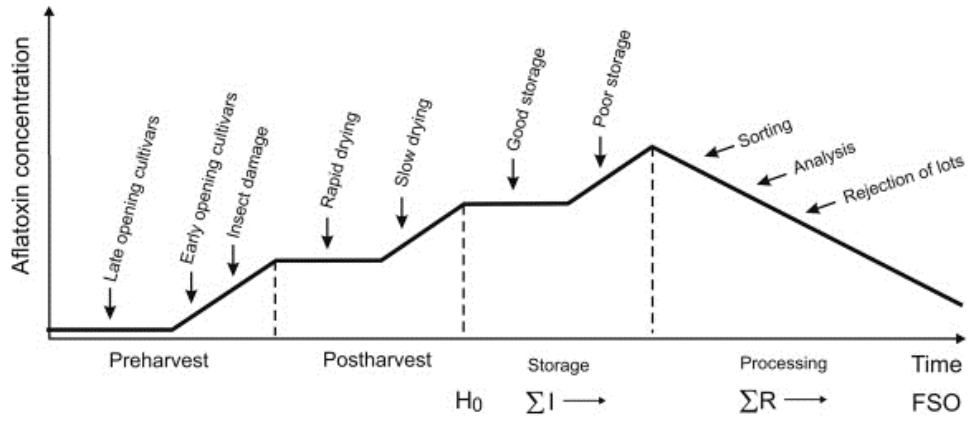


Figure 3 The time course of AFs formation and reduction in pistachio nuts, with reference to the FSO Modified from (Pitt et al., 2013).

In 2001, Food and Agriculture Organization of the United Nations (FAO) published the "Manual of the application of the HACCP system in mycotoxin prevention and control", considering two pistachio processing lines after harvest according to the different procedures applied in Asian producing countries. The fast dehulling process line involves fast dehulling (within 24 h after harvest) for preventing staining, floating segregation and quickly drying to 5-6% water content to prevent fungal development. This process is followed by the major producing countries (FAO, 2001). Subsequent steps include sorting, roasting, packaging and storage/shipping (
Figure 4). It is recognized that sorting and physical segregation significantly reduces the AFs content of consignments of nuts. High AFs levels are found in very small and insect damaged nuts, becoming the sorting an important step to reduce mycotoxin contamination (Schatzki and Pan, 1996). Physical cleaning, where mold-damaged kernels, seeds or nuts are removed from the intact commodity, may result in 40-80 % reduction AFs (Park, 2002). (Schatzki and Pan, 1996) related the AF reduction from pistachios previously partitioned by water flotation with the elimination of the stained nuts, which include the scalpers, the eye rejects, the hand pick out insects, the hand

pick out dye floaters, and the meat sinkers. Transport and storage steps are of particular risk regarding aflatoxigenic species growth and AFs accumulation.

Drying is one of the important steps in pistachio processing. In this step kernel m.c. is decreased from 50 to less than 5% which will result in suitable condition for storage (Set and Erkmen, 2010). During storage steps a zero increase of mycotoxins is desirable, in order to achieve this goal m.c. and T should be controlled. The influence of m. c. and T on fungal growth and mycotoxins production of *A. flavus* and *A. carbonarius* in pistachio was deeply studied by (Marín et al., 2012, 2008). Maximum fungal growth was between 30-35 ºC in both species, while optimum T for OTA production was lower (15-20 ºC) than for AFs production (25-35 ºC). The mycotoxin production in pistachio could be restricted under cool storage (<10 C) combined with an m.c. <20% (~0.92 a_W) or at low m.c (10%); alternatively, pistachios could be kept at 15% (~0.87 a_W) and T >15 C should be avoided (Marín et al., 2012, 2008). AFs was not detected in pistachio samples stored at controlled conditions (5-7 ºC, 45-60% R.H.) (Georgiadou et al., 2012).

Conflicting results have been published about the effect of the heat treatments on peanuts and pistachios (Ariño et al., 2009; Farah et al., 1983; Lee et al., 1969; Pluyer et al., 1987; Rustom, 1997; Waltking, 1971; Yazdanpanah et al., 2005). The percentage of reduction depends on T, time as well as the initial mycotoxin contamination. Temperatures between 200-400 ºC produce high mycotoxin reduction, but these T are higher from those actually used by the nuts industry (≅165 ºC).

Mycotoxin formation after roasting is unlikely, unless further fungal contaminations occurs afterwards. Packaged pistachios at ambient T (22-28 ºC) showed an increase in m.c. with the increase in storage time (Shakerardekani and Karim, 2013). Then, fungal contamination could take place so final product (bulk/packaged) should be storage in appropriate conditions as it was described previously. The most suitable packaging materials for maintaining the quality and safety of pistachio nuts is PET (polyethylene terephthalate) films followed by LDPE/PA (nylon), PA/PP (polyamide/polypropylene) and PVC (polyvinyl chloride). The shelf-life of pistachio can be extended from 2 months in LDPE (low density polyethylene) to 5 months when PET is used as the packaging material (Shakerardekani and Karim, 2013).

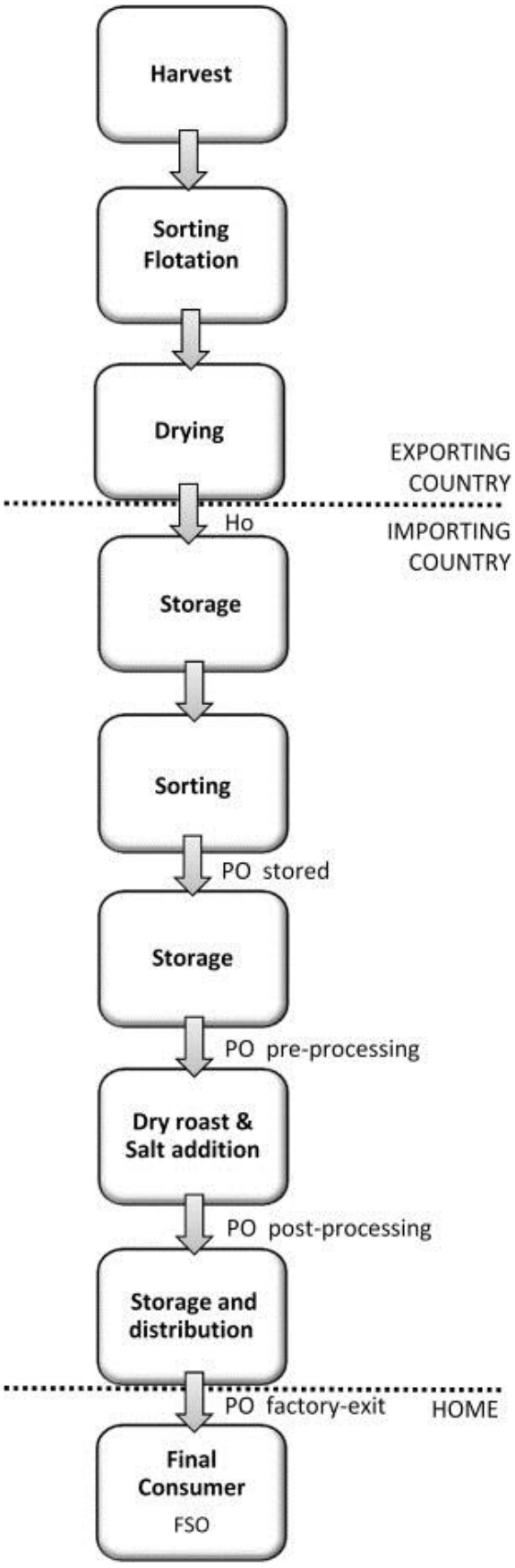


Figure 4 Flow chart toasted pistachio.

2.2. Case study II: Cereals-Wheat

Wheat is a cereal grain harvested from different species belonging to the *Triticum* genera, cultivated worldwide but originally from the Mediterranean and West Asia regions. The global production of this cereal reached the 670 millions of tonnes in 2012 (FAOSTAT, 2014). Aside from health risks, important economic and trade implications arise from fungal contamination as approximately 18% of wheat production is lost due to fungal invasion (Al-Hazmi and Gomaa, 2012).

In wheat the main efforts in the control of mycotoxins in the field have been directed towards species of *Fusarium*. However ochratoxigenic and aflatoxigenic fungus have been isolated in both pre-harvest and freshly harvested wheat suggesting that grain may be contaminated by AFs and OTA prior to storage (Elmholt and Rasmussen, 2005; Joubrane et al., 2011; Riba et al., 2010, 2008). In fact, AFs and OTA contamination both in grain and processed products have been reported in many studies. Regarding to AFB_1 and AFs, lower mycotoxin levels are detected in processed products, thus processing reduces the contamination (**Table 15**). Great incidence and mean contamination is observed in grain even overcoming the legal values.

No much information exists about AFs in bread, it is known that mycotoxins are reduced during the fermentation, but some studies showed an increase due to their release from the matrix and become detectable. Some strains of lactic acid bacteria (LAB) and bifidobacteria have shown ability to reduce the initial concentration of AFs from contaminated wheat flour during baladi breadmaking process (Elsanhoty et al., 2013). AF contents decreased significantly during prebaking and when the fermented dough was baked at 220°C for 35 min, the maximum reduction was 57.4% (AFG_1) at low toxin level and 55.7% (AFB_2) at high toxin level. Total losses of AFs during bread making process were 69.3% (AFG_1), 70.4% (AFB_2) at low level and 67.1% (AFB_1), 69.8% (AFG_1) at high level. These results indicate that fermentation and baking are effective in reducing AFs levels. However, approximately half of the spiked toxins still remained in the bread and therefore complete decomposition of AFs seems improbable during bread making process (Gumus et al., 2009).

Table 15 Occurrence and contamination of AFB_1 and AFs in wheat and wheat derivatives.

Commodity	Type	Incidence rate	Range (mean) (µg/kg)	Reference
Grain	AFs	0/4	-	(Abdulkadar et al., 2004)
Grain	AFB1	4/51(8%)	1.1-3.4(2.2)	(Ghali et al., 2008)
Grain	AFs	15/51(29%)	4-12.9(6.7)	(Ghali et al., 2008)
Grain	AFB1	23/50(46%)	0-7(1.9)	(Muthomi et al., 2008)
Grain	AFB_1	11/17(65%)	0.13-37.42	(Riba et al., 2010)
Grain	AFB1	17/28(61%)	0.21-13.96	(Riba et al., 2010)
Grain	AFs	119/156(76%)	n.d.-12.2	(Joubrane et al., 2011)
Grain	AFs	0/14	-	(Ibáñez-Vea et al., 2011)
Grain	AFs	13/19(68%)	0.5-2.6	(Al-Wadai et al., 2013)
Flour	AFs	0/6	-	(Abdulkadar et al., 2004
Flour	AFs	45/100 (45%)	0.05-14(0.79)	(Aydin et al., 2008)
Breakfast cereals	AFB_1	4/48(8%)	<0.01-0.02	(Tam et al., 2006)
Breakfast cereals	AFB_1	50%	<0.01-1	(Roscoe et al., 2008)

n.d. no detected

On the other hand, the use of propionic acid as preservative appeared to be more effective on the destruction of aflatoxins B_1 and G_1 than potassium sorbate in bread making (Amra et al., 1996).

Similarly to AFs, the OTA contamination seems to be reduced with milling, with the exception of the work by (Jørgensen and Jacobsen, 2002), who found higher contamination in flour than in grain (**Table 16**).

Table 16 Occurrence of OTA in wheat.

Commodity	Incidence rate	Contamination range (mean) (µg/kg)	Reference
Grain	32/201 (15.9%)	0.3–231	(Prickett et al., 2000)
Grain[a]	0/32(0%)	n.d.	(Czerwiecki et al., 2002a)
Grain[b]	3/39 (7.7%)	0.48–1.20 (0.83)	(Czerwiecki et al., 2002a)
Grain[a]	18/37 (48.6%)	0.60–1024 (267)	(Czerwiecki et al., 2002b)
Grain[b]	8/34 (23.5%)	0.8–1.60 (1.17)	(Czerwiecki et al., 2002b)
Grain[a]	217/405 (53.6%)	n.d.–32 (0.3)	(Jørgensen and Jacobsen, 2002)
Grain[b]	6/14 (42.9%)	n.d.–1.6 (0.3)	(Jørgensen and Jacobsen, 2002)

a: conventional; b:ecological/organic modified from (Duarte et al., 2010).

Table 16 (Continued).

Commodity	Incidence rate	Contamination range (mean) (µg/kg)	Reference
Grain	32/201 (15.9%)	0.3–231	(Prickett et al., 2000)
Grain[a]	0/32(0%)	n.d.	(Czerwiecki et al., 2002a)
Grain[b]	3/39 (7.7%)	0.48–1.20 (0.83)	(Czerwiecki et al., 2002a)
Grain[a]	18/37 (48.6%)	0.60–1024 (267)	(Czerwiecki et al., 2002b)
Grain[b]	8/34 (23.5%)	0.8–1.60 (1.17)	(Czerwiecki et al., 2002b)
Grain[a]	217/405 (53.6%)	n.d.–32 (0.3)	(Jørgensen and Jacobsen, 2002)
Grain[b]	6/14 (42.9%)	n.d.–1.6 (0.3)	(Jørgensen and Jacobsen, 2002)
Grain	6/70 (8.6%)	n.d.–1.4	(Palermo et al., 2002)
Grain	25/107 (23.4%)	n.d.–66 (19.6)	(Ayalew et al., 2006)
Grain	40%	n.d.–1.73 (0.42)	(Zinedine et al., 2007)
Grain	31/51(61)	0.7-24.3(2.9)	(Ghali et al., 2008)
Grain	42/110 (38%)	n.d.–250 (55)	(Zaied et al., 2009)
Grain	29/50(58%)	1.4-21.2	(Kumar et al., 2012)
Grain	9/26(35%)	3.88–11.3(6.39)	(Alexa et al., 2013)
Grain	24/26(35%)	2.67–25.70(5.71)	(Alexa et al., 2013)
Grain	30/50(60%)	2.6-12.1(8.2)	(Venkata Reddy et al., 2013)
Grain/bran	1/2(50%)	n.d.-1(1.0)	(Rodrigues and Naehrer, 2012)
Grain/bran	11/45(24%)	n.d.-43(29)	(Rodrigues and Naehrer, 2012)
Grain/bran	0/2(0)	0	(Rodrigues and Naehrer, 2012)
Grain/bran	5/22(23%)	n.d.-331(69)	(Rodrigues and Naehrer, 2012)
Grain/bran	1/13(8%)	n.d.-1(1.0)	(Rodrigues and Naehrer, 2012)
Grain/bran	11/37(30%)	n.d.-2.30(0.9)	(Vidal et al., 2013)
Flour[b]	108/156 (69.2%)	n.d.–16 (0.3)	(Jørgensen and Jacobsen, 2002)
Flour[a]	101/120 (84.2%)	n.d.–19 (0.5)	(Jørgensen and Jacobsen, 2002)
Flour	12/12 (100%)	(0.75)	(Baydar et al., 2005)
Flour	0/35 (0%)	n.d.	(Park et al., 2005)
Flour	28/50 (56%)	n.d.–0.48 (0.09)	(Kumagai et al., 2008)
Flour	21/30 (70%)	n.d.–2.1	(Vega et al., 2009)
Bread	252/252 (100%)	0.28	(Legarda and Burdaspal, 2001)
Bread	48/100 (48%)	0.14–149 (13)	(Zinedine et al., 2007)
Bread	4/31 (12.9%)	0.02	(Juan et al., 2008)
Bread	13/20 (65%)	n.d.–0.43 (0.3)	(Bento et al., 2009)
Bread	24/30 (80%)	n.d.–0.49 (0.2)	(Bento et al., 2009)
Bread	19/24 (79.2%)	n.d.–0.41 (0.21)	(Duarte et al., 2009)
Bread	14/67 (19%)	0.04–10.81	(González-Osnaya et al., 2007)

a: conventional; b:ecological/organic modified from (Duarte et al., 2010).

Table 16 (Continued).

Commodity	Incidence rate	Contamination range (mean) (μg/kg)	Reference
Breakfast cereals	11/29 (38%)	n.d.–0.64 (0.3)	(Roscoe et al., 2008)
Breakfast cereals	11/20(55%)	0.10–0.30(0.12)	(Nguyen and Ryu, 2014)
Breakfast cereals	2/3(67%)	0.24–1.50(0.87)	(Nguyen and Ryu, 2014)
Breakfast cereals	9/14(64%)	n.d.-1.12(0.43)	(Ibáñez-Vea et al., 2011)
Baking wheat	8.30%	0.12-0.5(0.29)	(Fazekas et al., 2002)
Feed wheat	26.70%	0.3-62.8(12.2)	(Fazekas et al., 2002)

n.d. no detected

Although the incidence in grain of both toxins was similar OTA amount was higher than AFs, surpassing 200 μg/kg in several surveys. Additionally, amounts detected in bran, flour and bread often exceeded the EU legal limits. The OTA presence in cereal products could be due to the mycotoxin's thermostability (Czerwiecki et al., 2002a). Interestingly, the amount detected in wheat breakfast cereals never exceeded the EU legal limits, and therefore processing could be an appropriate step to reduce the mycotoxin contamination in cereal products. No clear trend in OTA content due to the agricultural practices: conventional or ecological, was observed in grain or flour (Czerwiecki et al., 2002a, 2002b; Jørgensen and Jacobsen, 2002). Furthermore, another important issue is the co-occurrence of AFs and OTA since it was observed in 19.6% of samples (Ghali et al., 2008).

European Commission has set maximum levels for cereals, particularly in wheat maximum values are 2 and 4 μg/kg for AFB_1 and AFs respectively (**Table 17**). Legal limit for AFB_1 is reduced to 0.1 μg/kg in the case of wheat destined to baby or dietary foods. About OTA contamination maximum values are of 5 and 3 for unprocessed and destined to direct consumption products, respectively (EC, 2006, 2010a)(European Commission, 2010, 2006). As in the case of AFs, lower values (0.1 μg/kg) are permitted in baby or dietary foods.

Concerning mycotoxigenic fungi in wheat, *Aspergillus* section *Flavi* have been isolated from wheat seeds in different parts of the world. In addition, between 48-70% of isolates tested produced AFs (Al-Wadai et al., 2013; Joubrane et al., 2011; Riba et al., 2010). In the other hand, the presence of OTA contamination in cereals in Northwest Europe is mainly associated to the presence of *P. verrucosum* (Elmholt and Rasmussen, 2005). However pre-harvest mycobiota studies in Northern Africa pointed out than *Aspergillus* species might fit better than *Penicillium* species (Riba et al., 2008).

Regarding to *Aspergillus* isolates from pre-harvest wheat grain in Argelia and Lebanon, the occurrence of *Circumdati* section was lower than *Nigri* section but the isolates in the former section were higher OTA producers (Joubrane et al., 2011; Riba et al., 2008).

Table 17 AFB_1, AFs and OTA EU legal limits in wheat and derivatives.

	Foodstuffs	**µg/kg**
AFB_1/AFs	Wheat and all products derived from wheat, including processed wheat products	2/4
	Processed wheat-based foods and baby foods for infants and young children	0.1/-
	Dietary foods for special medical purposes intended specifically for infants	0.1/-
OTA	Unprocessed wheat	5
	All products derived from wheat including processed wheat products and wheat grains intended for direct human consumption	3
	Processed wheat-based foods and baby foods for infants and young children	0.5
	Dietary foods for special medical purposes intended specifically for infants	0.5

EC (2006b, 2010a)

Fungal infection starts in field, slow drying and/or raining during this stage could increase the mycotoxin presence, likewise inapropiate storage could be a critical step. Nevertheless, FSO could be successful through the mycotoxin reduction achieved during industrial processing. A qualitative approach in the general trend in the time course of mycotoxin levels progress in cereals is shown in **Figure 5**.

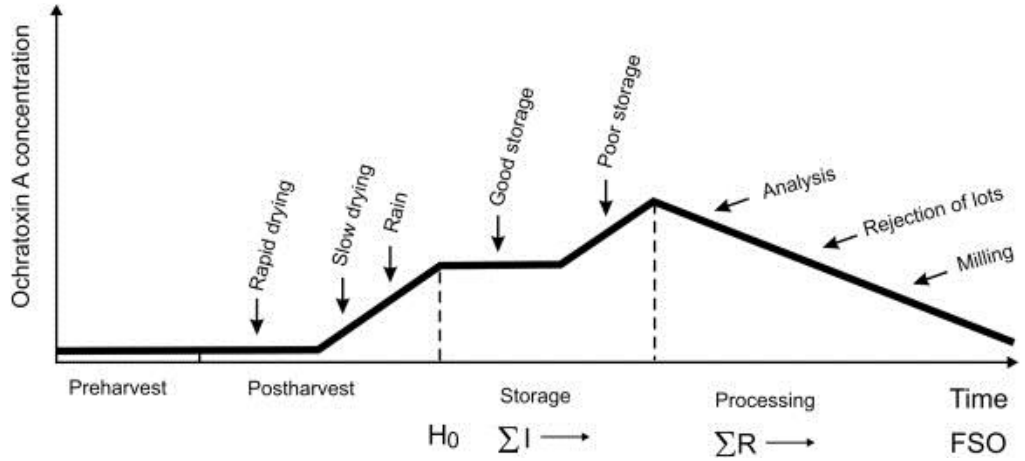


Figure 5 The time course of ochratoxin A formation and reduction in small grain cereals in Europe, with reference to the FSO (Pitt et al., 2013).

The wheat production from field to milling fractions is showed in **Figure 6.**

The external layers act as a coat protection from fungal invasion (Mohamed-Yasseen et al., 1994) consequently, it is easy to find fungal presence in the bran rather than in the endosperm or the germ (Barajas-Aceves et al., 2002; Brera et al., 2006; Hemery et al., 2007; Lancova et al., 2008). Hence, avoid mechanical damages to the grains keeping the coats unbroken prevent the fungal colonization inside the grain, which is recommended as a GAP (CAC, 2003).

Since the complex mycobiota found in wheat, the influence of a_W and T on fungal growth and mycotoxin production on wheat has been studied in several fungi. Respect to the main responsible for AFs and OTA in grain, the studies have targeted to the storage step and generally no fluctuating T has been tested (Cairns-Fuller et al., 2005; Ismail et al., 2012; Niles et al., 1985; Pardo et al., 2004).

Nowadays, wheat harvest is done worldwide by using combine harvesters. This step settles the first moment where a minimization of the mycotoxin content could occur. The combine is a machine system based on different physical and mechanical operations in where grains are separated from the stem and screened to certain level, throwing out dust, debris and other foreign materials, which present higher levels of fungal contamination (Pascale et al., 2011; Visconti et al., 2004), but it is not efficient with kernels presenting low levels of contamination.

This separation is achieved through a proper adjustment of the threshing drum and the wind stream, which drag out particles lighter than the kernels based on the different weight (Jouany, 2007). Several studies have described that highly contaminated grains are lighter than healthy ones so they can be separated from the healthy ones by this wind stream (Pascale et al., 2011; Visconti et al., 2004). However a possible recontamination can occur as the process is not as slow as it would be needed to accomplish a total clean up and the grain compartment is not sealed so dust particles could get into causing recontamination.

Once the wheat is harvested, grains are transported and stored into silos or other kind of warehouses. Wheat is harvested in the hotter months, so it is normally collected bellow the safety level settled at 14-15% of humidity (Magan and Aldred, 2007) and therefore drying at this moment is not necessary in most of the cases. In the cases where the humidity is higher than the recommended levels, the advice is to fast drying to avoid fungal development. Furthermore, since

the highest m.c. for trading in most markets is 15% fungal development can only occur before grains are dried to the safe m.c. These conditions can occur during: 1) ambient air drying 2) before drying due to harvest backlogs, 3) when surface grain absorbs moisture during the winter, or 4) during moisture translocation in un-cooled grain, owing to moisture in hot air rising from the bulk and then condensing on cold surface grain (Wontner-Smith et al., 2014).

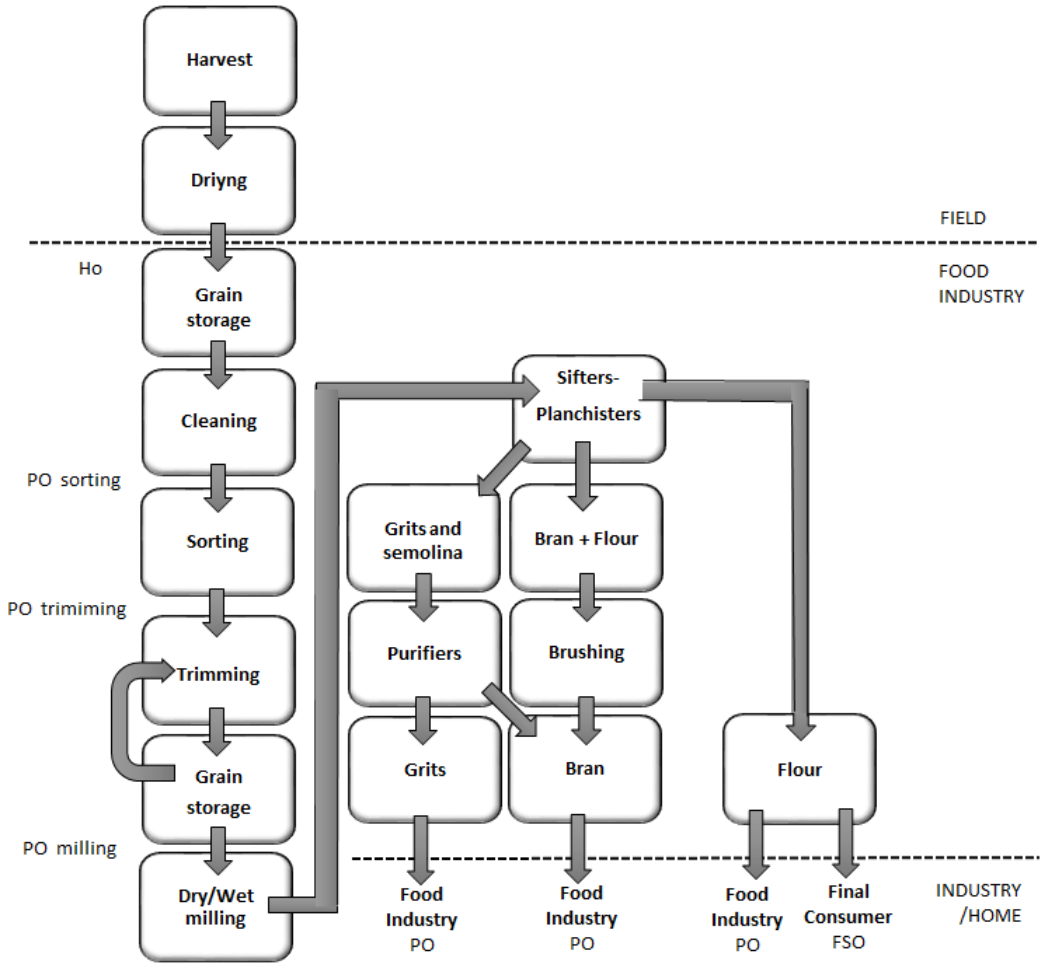


Figure 6 Flow chart for flour, brand and semolina production from raw wheat (own elaboration).

An excess of m.c. could lead to fungal development. Ecophysiological studies have shown that the optimum T for *A. flavus* growth and AFs production on wheat grain is 35 ºC and 25-35 ºC, respectively connecting to 0.975 a_W (Niles et al., 1985). Moreover, 15 ºC inhibited fungal growth and AFs production at 0.80 and 0.82 a_W. Respect to ochratoxigenic fungi the optimum T for *P. verrucosum* growth and OTA production was 25 ºC at 0.95 and 0.98 a_W, respectively (Cairns-Fuller et al., 2005). While 10 ºC and 0.85 a_W limited OTA production, a reduction to 0.80 a_W was needed

to inhibit fungal growth (Cairns-Fuller et al., 2005). The effect of T (5-40 ºC) in OTA production by *A. ochraceus* and *A. sulphureus* incubated in wheat, showed than 30 ºC was the most suitable T for mycotoxin production while at 5 ºC no OTA was detected (Ismail et al., 2012). Additionally, studies carried out in barley, also have established 30 ºC and the range 10-15 ºC as optimum and minima T for growth and OTA production of *A. ochraceus* (Pardo et al., 2004; Ramos et al., 1998). Optimum a_W for fungal growth and OTA production were 0.95 and 0.99 a_W whereas the minimum were 0.85 and 0.9 a_W, respectively (Pardo et al., 2004). Surprisingly, to our knowledge no ecophysiological studies of *Aspergillus* section *Nigri* inoculated on wheat exist. The mycotoxin production in grain could be restricted under cool storage (<10 C) combined with a low m.c. (~0.80-0.82 a_W) and T >15 C should be avoided.

After storage, and prior to milling the grains are cleaned. This step is based mainly in three operations: cleaning, sorting and trimming. Grains are subject to diverse physical and mechanical processes by removing kernels with extensive mould growth, broken kernels, fine materials, and dust that represent the screenings in which most of the toxins are accumulated (Bullerman and Bianchini, 2007; Hazel and Patel, 2004; Kushiro, 2008). This operation is generally carried out by the use of gravity separators (Hazel and Patel, 2004). Previous studies have reported reductions between 26-69% of FBs in corn (Sydenham et al., 1994), 5.5 -19% of DON in wheat (Abbas et al., 1985), 2-3% of OTA in barley (Scudamore et al., 2003) and 40-80% of AFs in seeds or nuts (Park, 2002). It is also described that washing grains with water under pressure reduces significantly the mycotoxin content (Wilson et al., 2004) both for foods and feeds (Fandohan et al., 2005). Furthermore, the initial condition of the grain, and extent of the contamination will have an effect on cleaning efficiency (Cheli et al., 2013). Once the wheat grains are cleaned and selected, they can be stored again before processing, depending on the industry demands.

Generally, the whole grain is milled to leave just the endosperm for white flour and obtaining different by-products as bran and grits or semolina. In this process mycotoxin is distributed in wheat milling fractions, minimizing the concentration in fractions used for human consumption, and concentrate it into fractions commonly used for animal feed (Cheli et al., 2013). The milling process usually starts with an operation called debraning, where the grains are dehulled and the external layers are separated. Thus, higher contamination levels where found in germen and bran than in flour (Saunders et al., 2001). In fact, reductions up to 34% of DON and ZEA have been reported when the outer layers where removed (Fandohan et al., 2005; House et al., 2003).

Usual processing stages for human consumption fractions are fermentation and thermal treatment (flour) or mixing and extrusion (semolina and bran) also decrease the mycotoxin contamination.

2.3. Case study III: Fruit-winemaking grapes

Wine is a beverage obtained from vine fruits (*Vitis vinifera*) through alcoholic fermentation. After cereals, wine is the second estimated source of OTA in the diet in Europe. Its consumption can represent up to 10% of the total OTA intake (E.C., 2002).

Since Zimmerli and Dick, (1996) described OTA contamination in wine for the first time, its presence has been frequently reported in grapes (Abrunhosa et al., 2001), dried vine fruits (Varga and Kozakiewicz, 2006), vinemarking must (**Table 18**) and wines (**Table 19, Table 20** and **Table 21**). Although OTA is detected in a high percentage of red wines, the levels rarely exceed the maximum level (2 μg/L) set by the EU Commission. OTA content in white and rose vines is lower than in red ones due to the different winemaking process.

Regarding AFs only one study in Lebanon has reported contamination in musts (40%), but at levels lower than 0.46 μg/L (Khoury et al., 2008; Magnoli et al., 2003; Medina et al., 2005; Sage et al., 2004; Serra et al., 2006b); moreover, no studies exist which reported them in wine. A recent study also has reported the incidence of FB_2 in wine in a range from 1 to 25 μg/L (Mogensen et al., 2010b). Currently, only legal limits for OTA are established in wine in the EU (**Table 22)**.

Several potentially toxigenic fungal species have been isolated from vineyards prior to harvest. *Aspergillus* and *Alternaria*, followed by *Penicillium*, are the most frequently reported genera on grapes. Focusing in *Aspergillus,* the section *Flavi* is rarely present in vineyards, only in a study in Lebanon, they were 43% of the total aspergilli and more than 40% of them produced AFB_1 (Khoury et al., 2008; Magnoli et al., 2003; Medina et al., 2005; Sage et al., 2004; Serra et al., 2006b). Similarly, low incidence of section *Circumdati* is described in Spain (2.5-6.6%) and these fungi were never isolated from Portuguese and French vineyards (Bellí et al., 2004c; Sage et al., 2002; Serra et al., 2005). Therefore, *Aspergillus* section *Nigri* is the most important mycotoxigenic (ochratoxigenic) fungi present on grapes (5-83% infected vine fruits). The main black aspergilli species occurring on grapes are biseriates, in particular *A. niger* aggregate. In more recent years, several authors have

proposed the division of the *A. niger* aggregate in four morphologically identical species: *A. niger*, *A. tubingensis*, *A. foetidus* and *A. brasiliensis* (González-Salgado, 2010).

Table 18 Occurrence and concentration of OTA in winemaking must.

Incidence rate	Contamination range (mean) (μg/L)	Reference
8/9(89%)	0.01-6.5 (2.1)	(Battilani et al., 2003)
11/60(18%)	0.01-0.43(0.06)	(Sage et al., 2004)
6/24(25%)	0.09-0.81(0.26)	(Bellí et al., 2004c)
6/11(55%)	0.01-0.46(0.18)	(Sage et al., 2002)
0/40(0%)		(Bellí et al., 2005a)
5/10(50%)	1.1-4.3(3.38)	(Fredj et al., 2007)
0/26(0%)		(Ponsone et al., 2007)
3/4(75%)	0.01-0.16	(Serra et al., 2005)
0/47(0%)		(Khoury et al., 2008)
13/24(54%)	0.06-1.88(0.38)	(Lasram et al., 2012)
16/24(67%)	0.05-5.45(0.98)	(Lasram et al., 2012)
10/24(42%)	0.11-5.85(1.3)	(Lasram et al., 2012)
22/50(44%)	0.1-1.20(0.13)	(Chiotta et al., 2009)
77/398(19%)		(Díaz et al., 2009)
62/204(30%)	0.003-2.0	(Lucchetta et al., 2010)

There is a controversy regarding the percentage of OTA producing strains within *A. carbonarius* isolated from grapes, Somma et al., (2012) concluded that close to 100% were OTA producers, based in literature published before 2006. Nonetheless, studies based on *A. carbonarius* identified by molecular techniques showed percentages under 50% of producers (Martínez-Culebras and Ramón, 2007; Spadaro et al., 2012). Recently, an interesting study using morphological and genotypic methods has proved the existence of non ochratoxigenic *A. carbonarius* (Cabañes et al., 2013).

Furthermore, recent reports revealed production of FB_2 and FB_4 by *A. niger* and *A. awamori* strains in culture medium, grapes or dried grapes (Chiotta et al., 2011; Logrieco et al., 2009; Mogensen et al., 2010a; Varga et al., 2010). Between 28-85 % of *A. niger* isolates collected from grapes of different origins were able to produce FBs (0.003-293 mg/g) in medium and on grapes (Abrunhosa et al., 2011; Chiotta et al., 2011; Logrieco et al., 2009; Mogensen et al., 2010a; Palumbo et al., 2011; Varga et al., 2010).

Table 19 Occurrence and concentration of OTA in red wine.

Incidence rate	Contamination range (mean) (μg/L)	Reference
40/89(45%)	n.d.–7.0	(Majerus and Otteneder, 1996)
62/79(78.5%)	n.d.–0.39(0.039)	(B. Zimmerli and Dick, 1996)
11/21(57%)	< 0.01–0.27(0.08)	(Ospital et al., 1998)
66/72(91.7%)	<0.003–0.60(0.038)	(Burdaspal and Legarda, 1999)
02/5(100%)	0.004–0.45(0.225)	(Burdaspal and Legarda, 1999)
06/8(75%)	<0.003–0.19(0.052)	(Burdaspal and Legarda, 1999)
37/38(97.4%)	<0.01–7.63(1.24)	(Visconti et al., 1999)
56/64(88%)	n.d.-5.3	(Majerus et al., 2000)
29/31(96.7%)	n.d.–3.80(0.94)	(Tateo et al., 2000)
19/23(86.9%)	n.d.-1.34(0.385)	(Cerutti et al., 2000)
6/6(100%)	0.14-2.93(1.802)	(Cerutti et al., 2000)
8/36(22.2%)	0.01-0.1(0.041)	(Larcher and Nicolini, 2001)
31/31(100%)	0.01-3.4	(Markaki et al., 2001)
82/96(85.4%)	<0.001–3.18(0.419)	(Pietri et al., 2001)
13/28(46.4%)	0.056–0.316(0.147)	(Cerain et al., 2002)
90/601(15%)	<0.02-1	(Hocking et al., 2003)
71/104(68.3%)	<0.05–2.69(0.34)	(Stefanaki et al., 2003)
96/580(16.6%)	0.051–0.200	(Soleas et al., 2001)
09/14(64.3%)	<0.02–2.51(0.68)	(Soufleros et al., 2003)
24/130(18.5%)	0.06–4.24(0.465)	(Bellí et al., 2004a)
21/61(34.4%)	0.06–0.53(0.281)	(Blesa et al., 2004)
08/9(40%)	(0.028)	(Rosa et al., 2004)
07/22(31.2%)	0.03–0.07(0.039)	(Rosa et al., 2004)
05/36(13.9%)	<0.01–0.39(0.024)	(Ng et al., 2004)
36/43(83.7%)	0.04–1.44(0.3)	(Bacaloni et al., 2005)
07/7(100%)	0.01–0.05(0.022)	(Domijan and Peraica, 2005)
35/35(100%)	0.04–1.92(0.728)	(Anli et al., 2005)
33/33(100%)	0.03–0.53(0.117)	(Varga and Kozakiewicz, 2006)
88/112(78.6%)	<0.01–4.93(0.64)	(Perrone et al., 2007a)
44/51(86.3%)	<0.01–0.82(0.11)	(Var and Kabak, 2007)
12/18(66.7%)	<0.01–0.46	(Belajová and Rauová, 2007)
535/773(69.2%)	<0.01–7.50(0.34)	(Brera et al., 2008)
08/10(80%)	<0.01–0.02(0.015)	(Flajs et al., 2009)

n.d.: no detected

Modified from (Bellí et al., 2002; Remiro et al., 2013)

Table 20 Occurrence and concentration of OTA in white wine.

Incidence rate	Contamination range (mean) (μg/L)	Reference
1/6(17%)	n.d.-0.73	(Majerus and Otteneder, 1996)
14/41(34%)	n.d.–1.2	(Majerus and Otteneder, 1996)
0/3(0%)	n.d.(0.116)	(B. Zimmerli and Dick, 1996)
8/24(33.3%)	n.d.–0.18(0.011)	(B. Zimmerli and Dick, 1996)
1/6(16.6%)	< 0.01–0.16(0.16)	(Ospital et al., 1998)
45/69(65.2%)	n.d.-0.27(0.02)	(Burdaspal and Legarda, 1999)
2/2(100%)	0.10–0.97(0.535)	(Visconti et al., 1999)
2/7(28.6%)	n.d.–0.06(0.045)	(Visconti et al., 1999)
9/18(50%)	n.d.-0.48(0.264)	(Cerutti et al., 2000)
5/10(50%)	n.d.-0.29(0.144)	(Cerutti et al., 2000)
21/27(78%)	n.d.-1.3	(Majerus et al., 2000)
14/58(24%)	n.d.-1.4	(Majerus et al., 2000)
1/6(17%)	n.d.-0.73	(Majerus and Otteneder, 1996)
14/41(34%)	n.d.–1.2	(Majerus and Otteneder, 1996)
0/3(0%)	n.d.(0.116)	(B. Zimmerli and Dick, 1996)
8/24(33.3%)	n.d.–0.18(0.011)	(B. Zimmerli and Dick, 1996)
1/6(16.6%)	< 0.01–0.16(0.16)	(Ospital et al., 1998)
45/69(65.2%)	n.d.-0.27(0.02)	(Burdaspal and Legarda, 1999)
2/2(100%)	0.10–0.97(0.535)	(Visconti et al., 1999)
2/7(28.6%)	n.d.–0.06(0.045)	(Visconti et al., 1999)
9/18(50%)	n.d.-0.48(0.264)	(Cerutti et al., 2000)
5/10(50%)	n.d.-0.29(0.144)	(Cerutti et al., 2000)
21/27(78%)	n.d.-1.3	(Majerus et al., 2000)
14/58(24%)	n.d.-1.4	(Majerus et al., 2000)
7/7(100%)	0.03-0.54(0.072)	(Filali et al., 2001)
2/27(7.4%)	0.01-0.02(0.015)	(Larcher and Nicolini, 2001)
9/15(60%)	< 0.01–3.86(0.736)	(Pietri et al., 2001)
14/362(3.9%)	0.051–0.100	(Soleas et al., 2001)
41/257(16%)	0.05-0.5	(Hocking et al., 2003)
7/13(53.8%)	<0.02-0.87(0.27)	(Soufleros et al., 2003)
55/118(46.6%)	0.05-1.72(0.25)	(Stefanaki et al., 2003)
4/24(16.7%)	0.05-0.76(0.41)	(Blesa et al., 2004)
10/43(23.3%)	<0.004-0.156	(Ng et al., 2004)
2/15(13.3%)	<0.021-0.0283(0.026)	(Rosa et al., 2004)
128/290(44.1%)	<0.005-1.9(0.08)	(Brera et al., 2008)
125/204(63.1%)	<0.0093-1.36(0.086)	(Spadaro et al., 2010)
1/7 (14%)	0.03	(Terra et al., 2013)
23/26(88.5%)	<n.d – 0.56(0.19)	(Sarigiannis et al., 2014)

n.d.: no detected. Modified from (Bellí et al., 2002; Remiro et al., 2013).

Table 21 Occurrence and concentration of OTA in rose wine.

Incidence rate	Contamination range (mean) (µg/L)	Reference
6/14(43%)	n.d.–2.4	(Majerus and Otteneder, 1996)
14/15(93.3%)	n.d.–0.12(0.025)	(B. Zimmerli and Dick, 1996)
1/2(50%)	< 0.01–0.11(0.11)	(Ospital et al., 1998)
29/32(90.6%)	n.d.-0.16(0.031)	(Burdaspal and Legarda, 1999)
11/11(100%)	0.46–4.72(1.185)	(Visconti et al., 1999)
2/2(100%)	0.41–0.64(0.525)	(Visconti et al., 1999)
0/1(0%)	n.d.	(Cerutti et al., 2000)
1/2(50%)	n.d.-1.35(1.348)	(Cerutti et al., 2000)
18/51(35%)	n.d.-2.4	(Majerus et al., 2000)
3/3(100%)	0.04-0.54(0.223)	(Filali et al., 2001)
0/1(0%)	n.d.	(Soufleros et al., 2003)
8/21(38.1%)	0.11-0.46(0.3)	(Blesa et al., 2004)
69/75(92%)	<0.005-4.07(0.5)	(Brera et al., 2008)
8/8(100%)	0,19–2.52(1.64)	(Sarigiannis et al., 2014)

n.d.: no detected. Modified from (Bellí et al., 2002; Remiro et al., 2013).

Table 22 OTA EU legal restriction in grape and derivatives.

Foodstuffs	OTA (µg/kg)
Dried vine fruit (Currants, raisins and sultanas)	10
Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption	2
Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % vol) and fruit wine	2
Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2

EC (2006b).

As previously mentioned, *Aspergillus* section *Nigri* is extensively isolated from vineyards. OTA presence in must has clarified that it is produced in field conditions. Hence, mycotoxin contamination in must and wine are mainly determined by climatic conditions and GAP applied along the crop cycle. Again the qualitative approach in the general trend in the time course of mycotoxin levels progress in grape is shown in **Figure 7**. Slow bunch transport and crushing, likewise onset of fermentation during transport could increase the mycotoxin contain. Nevertheless, initial contamination could be reduced during vinification process and FSO achieved.

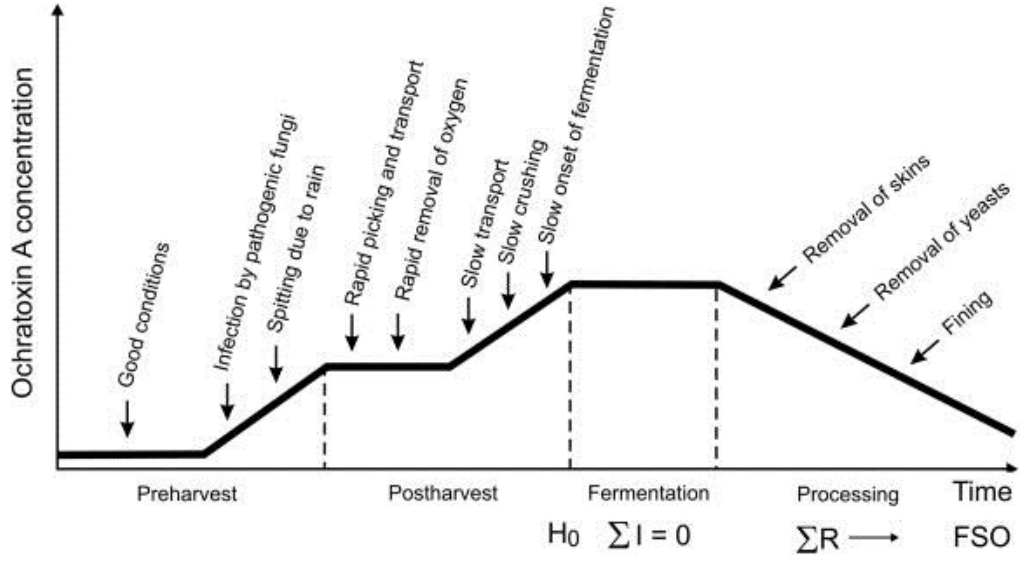


Figure 7 The time course of ochratoxin A formation in grapes and reduction durin wine manufacture, with reference to the FSO (Pitt et al., 2013).

Pre-harvest stage is determinant in wine contamination, and consequently, the relation between climatic conditions and fungal development and mycotoxins production has proven interest. The relationship between OTA contamination and the influence of a_W and T in some fungi has been assayed on both synthetic nutrient medium of similar composition to the grape and on grapes (Astoreca et al., 2010; Bellí et al., 2007b, 2004b; Marín et al., 2006; Mitchell et al., 2004; Pardo et al., 2005). *A. carbonarius* inoculated on grapes produced the maximum OTA level under high R.H. (100 % R.H.) and 30 ºC after 7 days (Bellí et al., 2007b; Pardo et al., 2005). Conversely, no significant differences in OTA production at different R.H. levels (80, 90 and 100% R.H.) were observed in *A. ochraceus* inoculated on grapes after 14 days (Bellí et al., 2007b; Pardo et al., 2005). Nonetheless, at 0.80 a_W/20ºC OTA production was clearly reduced in *A. carbonarius* and totally inhibited in *A. ochraceus* (Bellí et al., 2007b; Pardo et al., 2005). Moreover, no fungal growth and OTA production was recorded at 10 ºC by *A. ochraceus*. Additionally, significant differences in OTA contamination was observed in damaged and undamaged grapes infected by *A. carbonarius* (Bellí et al., 2007b; Pardo et al., 2005).

Possible red, white and rose winemaking processes are represented in
Figure **8.**

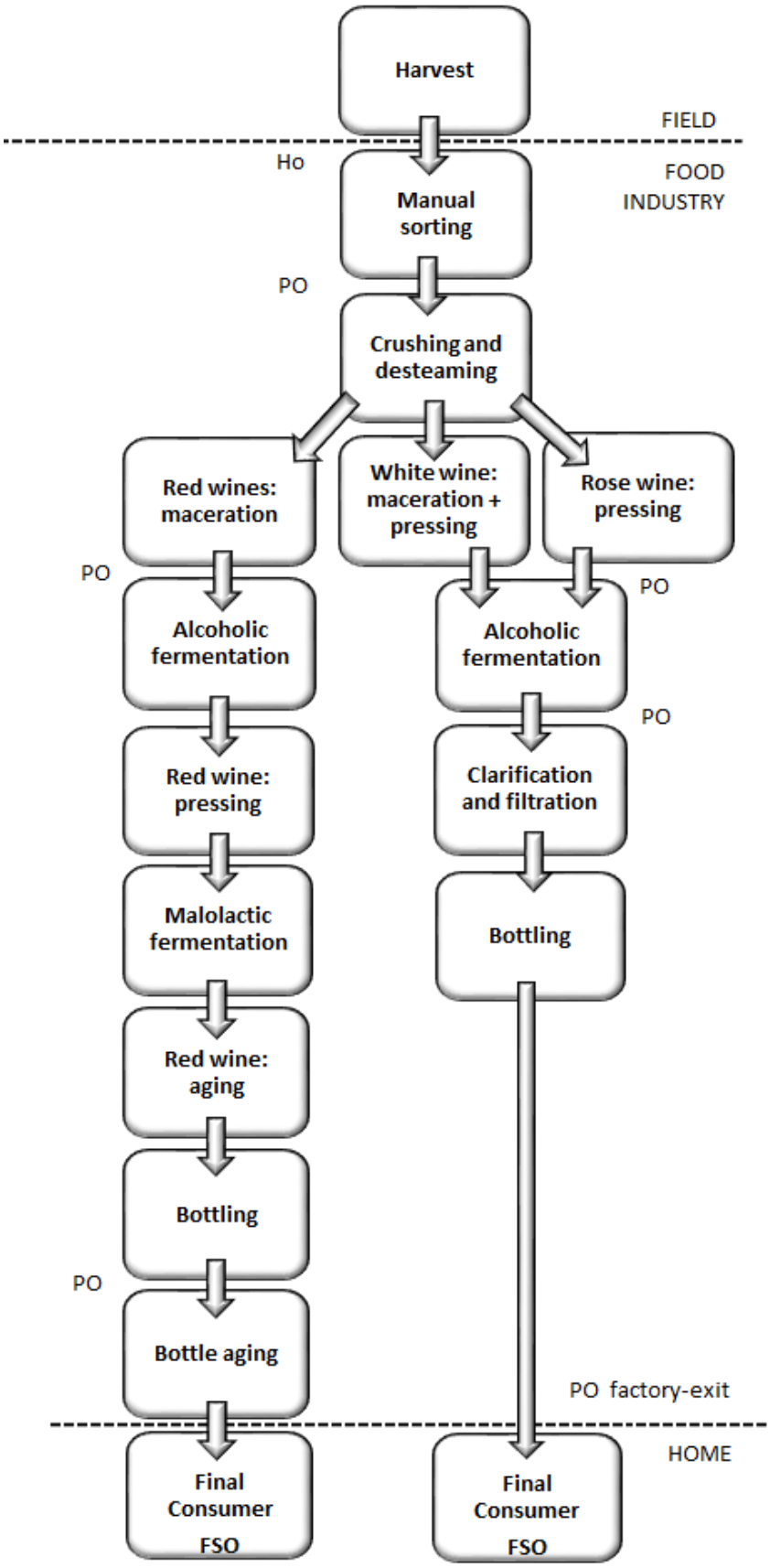


Figure 8 Flow chart of red, rose and white vinification (own elaboration).

Nowadays, bunches are collected manually or with grape harvesters, in the first case bunches are placed into plastic boxes, in both cases they are transported to the winery as fast as it is possible in order to prevent the degradation of the main compounds resulting in a decrease of the quality of musts (Li et al., 2013).

Once in the winery, a manual selection eliminating bunches and berries with evident signs of fungal contamination could reduce up to 98% of OTA (Rousseau, 2004). After the manual selection, bunches are destemmed and crushed and certain amount of the OTA present in the grapes is released into the must (Grazioli et al., 2006). Then, the must follows different pathways depending on the type of wine (
Figure 8). Red and white wines usually have a maceration step, which increases the OTA content due to the contact between must and other grape components (Grazioli et al., 2006).

OTA presence is higher in red wines where the whole berries are used in the fermentation in order to extract compounds like tannins and phenols, and lesser in white and rose wines where the must is fermented without seeds and skins (Brera et al., 2006). Several works described important reductions during the alcoholic and malolactic fermentation (Amézqueta et al., 2012; Bejaoul et al., 2004; Caridi et al., 2006; Cecchini et al., 2006; Fernandes et al., 2007; Lasram et al., 2008; Leong et al., 2006; Meca et al., 2010; Ratola et al., 2005). Reductions up to 68% in naturally contaminated and 78% in OTA added musts (Caridi et al., 2006) and up to 82% in synthetic grape medium (Petruzzi et al., 2014) have been reported during alcoholic fermentation. In vitro studies have demonstrated reductions between 2 and ≥95% in malolactic fermentation (Fernandes et al., 2007; Fuchs et al., 2008; Grazioli et al., 2006; Lasram et al., 2008; Mateo et al., 2010; Piotrowska and Zakowska, 2005). Mycotoxin degradation is explained because yeast and lactic acid bacteria cause the hydrolysis of the amine bond into non-toxic products as L-β-phenylalanine and OTα or the hydrolysis of the lactone ring (Quintela et al., 2012b). Besides, yeast cells adsorb OTA, achieving reductions up to 89%, 85% and 75% in red, rose and white wine due to a major contact between must and pomace (Csutorás et al., 2013).

Once fermentation has been carried out, wine is devatted and separated from the pomace and lees. It is reported that lees and pomace present higher amounts of OTA when compared to wine (Caridi et al., 2006; Cecchini et al., 2006; A. Fernandes et al., 2007), as only 4% of the OTA present in the

grapes remains in the wine, whereas 95% is retained in the pomace and 1% in the lees (Visconti et al., 2008).

Pressing could be an important step in mycotoxin reduction, it has been reported that OTA concentration is approximately four times smaller when the pomace is pressed at ≈ 8 atm instead of ≈ 80 atm (Gambuti et al., 2005). Pressing is normally followed by a filtration process in order to eliminate small particles. When the filtration is made over ligthtly contaminated pomaces a reduction up to 50-65% is achieved (Moruno et al., 2005; Solfrizzo et al., 2010) similarly when it is made through a filter of 0.45 μm, while filtration over a 10 μm membrane did not show a significant decrease in OTA (Gambuti et al., 2005). Commercial fining agents also have reduction properties, as they are adsorbent materials with the capacity to tightly bind and immobilize mycotoxins (Quintela et al., 2012b). Potassium caseinate removed up to 82% of OTA in wine when used at high dosages (Covarelli et al., 2012). Activated carbon showed a certain activity (Castellari et al., 2001; Quintela et al., 2012b; Varga and Kozakiewicz, 2006) but the efficiency depended on the type of activated carbon, toxin concentrations and incubation period varying from 13% to 98% of reduction (Gambuti et al., 2005; Olivares-Marín et al., 2009; Var et al., 2008; Visconti et al., 2008). However, active carbon is only authorized for white wines. Bentonite achieved up to total detoxification (Castellari et al., 2001; Gambuti et al., 2005; Kurtbay et al., 2008; Quintela et al., 2012b; Salaha et al., 2007; Var et al., 2008; Visconti et al., 2008). Chitin reductions ranged from 15 to 67% (Bornet and Teissedre, 2008; Quintela et al., 2012b). Chitosan achieved reductions from 3 to 100% (Bornet and Teissedre, 2008; Kurtbay et al., 2008; Quintela et al., 2012b). Egg albumin from 8 to 48% (Castellari et al., 2001; Quintela et al., 2012b) and polivinilpolipirrolidone (PVPP) showed up to 40% of reduction (Castellari et al., 2001; Gambuti et al., 2005; Quintela et al., 2012b). However, that most of these agents also have a negative effect on the organoleptic qualities of the wines.

Red wines usually have an ageing process in oak barrels, which also causes a decrease in OTA content (Ruíz Bejarano et al., 2010). Oak chips or powder may be used, achieving reductions up to 75% (Savino et al., 2007). Also reductions of 17% were observed in bottled ageing after 12 months of storage (Grazioli et al., 2006), conversely other study demonstrated that OTA is stable in wine for at least one year (Cerain et al., 2002).

3. Challenges for food safety management

3.1. Sampling plans

Nowadays, food safety management is based on the analysis of raw materials by comparing them to the legal limits established by the authorities. The concentration of a lot is usually estimated by analysing a sample of the lot. Then, based on the measured sample concentration, the quality of the safety of the lot is determined. If the sample concentration does not accurately reflect the lot concentration, then the lot may be misclassified and there may be undesirable health consequences (Magan and Olsen, 2004).

Chemical contaminant sampling plans would typically be applied in the control of incoming lots of raw material from suppliers and also in the verification of the compliance of limits for finished products. Appropriate sampling plans are essential to ensure that the analytically-derived mean concentration of a sample is representative of the true mean concentration of a lot. Sampling plans are particularly relevant in the area of mycotoxins where it is known that the contamination of a commodity can be heterogeneously distributed (Johansson et al., 2000; Schatzki, 1995). It is known that, as a general rule, the larger the individual particle or seed, the higher the sampling problem. Thus, there are foods in which mycotoxin contamination is usually distributed evenly, as liquid food powder or pasta, and meat products and eggs (Soriano del Castillo, 2007), but other commodities seem not to have the same trend, as for example peanuts, where Cucullu et al. (1966), showed that most individual nuts have undetectable AFs concentration, but occasionally a peanut may have an extremely high concentration. Several subsequent studies have also observed this tendency on aflatoxin distribution in other substrates as cottonseed, pistachios and corn (Cucullu et al., 1977; Johansson et al., 2000; Schatzki, 1995; Shotwell et al., 1974). Therefore, sampling and subsampling procedures should be designed according to the mode of transmission and distribution of mycotoxin.

Among other methods, a multistage sampling and analysis process consists of three distinct phases: sampling, sample preparation and analysis. However, there is great variability in each of these stages as among the measures of statistical variability only the variance is additive. Thus, it is assumed that the overall variance (VT) associated with a mycotoxin test procedure is the sum of the sampling (VS), sample preparation (VSS), and analytical variances (VA), that is VT = VA + VS + VSS

(Whitaker et al., 2006). In this same study Whitaker et al., (2006) independently analyzed each source of variation:

- VS was mainly due to the sample selection and the sample size so that sampling variance increases with an increase in concentration and decreases with an increase in sample size.

- The VSS is affected by the mill type particle size distribution. If the average particle size decreases (number of particles per unit mass increases), then the subsampling variance for a given size subsample decreases.

- VA is specific to each method of analysis.

Some authors studied the distribution of the variability associated to each sampling step among diferent comodities regarding to AFs, DON and FBs (Cheli et al., 2009; Whitaker et al., 2009) observing that the sampling step is the major source of variability. In general, mycotoxins distribution among diferent commodities present high heterogeneity although it is probable that OTA and FBs are less heterogeneously distributed than AFs, and therefore sampling procedures could be less difficult (Miraglia et al., 2005). Concerning DON, lower variability associated with the sampling was observed, but these results may be related to differences in seed size as well as to a less heterogeneous distribution of this mycotoxin (Whitaker et al., 2000).

A common practice to cope with the heterogeneous distribution of mycotoxins is through the formation of aggregated samples as the contaminated particles may not be distributed uniformly throughout the lot. Then, the sample should be an accumulation of many small portions taken from many different locations (Magan and Olsen, 2004). However, the information related to the spatial variability and distribution of the mycotoxin is lost (Casado et al., 2009). This information could be an important factor for designing of adequate sampling which leads to a more accurate decision in certain mycotoxins, as DON showed clear evidence of spatial structure while OTA did not (Casado et al., 2009; Macarthur et al., 2006). This difference may reflect the fact that DON is mainly produced in the field by a widespread organism, whereas OTA is typically produced in localized "hot spots" during storage. Once again, the mode of transmission and distribution of mycotoxin appears to be an essential factor.

Due to the variability among mycotoxin test results, two types of errors are associated with any mycotoxin-sampling plan: i) an underestimation could result in a risk for the buyer/consumer as a bad lot could be wrongly accepted and, ii) an overestimation means a risk for the seller/producer, as a good lot could be wrongly rejected (Miraglia et al., 2005). An Operating Characteristic (OC) curve is a decision tool which describes the probability of a lot acceptance as a function of its actual quality (CAC, 2004). The shape of the OC curve indicates the magnitudes of the buyers' and sellers' risks but is uniquely defined for a particular sampling plan, sample size, preparation, number of analysis and methodology. Since the slope of the OC curve has high economic and health relevance, it is crucial to increase the slope of the OC in order to reduce both risks when a sampling plan is developed (Cheli et al., 2008). Diverse OC curves have been drawn for several sampling plans for aflatoxins in shelled peanuts and almonds (Whitaker et al., 2010, 2007), OTA in green coffee (Vargas et al., 2005) and FBs in shelled corn (Whitaker et al., 2001).

Ir order to grant the achievement of these objectives the EC established the sampling methods for the official control of AFs, AFB_1, OTA and *Fusarium* toxins content in commodities by the regulation Nº 401/2006 (EC, 2006a). Four years later, the regulation Nº 178/2010 (EC, 2010c) modified the previous one for some foodstuffs like figs, peanuts, pistachios, Brazil nuts, apricot kernels, vegetal oils, coffee and liquorice and their derivates. Sample size has been reduced as the sampling plans required high economical and human efforts, as the mycotoxin quantification relies in destructive techniques.

3.2. Other fungal metabolites: what else is there in the food and feed?

Commonly, food contamination due to mycotoxins is low enough to ensure compliance with EU guidance values or maximum levels. However, co-contaminated samples with concentrations below guidance and maximum values might still exert adverse effects due to synergistic interactions of the mycotoxins (Schatzmayr and Streit, 2013). Additionally, the true mycotoxin contamination in food and feed could be underestimated due to masked, emerging mycotoxins or their co-ocurrence which should be taken into account.

Despite FBs, ZEA and tricothecenes are the most studied toxins produced by *Fusarium*, this genus is also producer of other bioactive compounds known as "emerging" mycotoxins as fusaproliferin (FUS), beauvericin (BEA), enniatins A, A1, B, B1 (ENNs) and moniliformin (MON) (Jestoi, 2008).

Regarding their presence in foods, high emerging mycotoxin contamination levels have been found in different commodities reaching values up to ppm (Mahnine et al., 2011; Ritieni et al., 1997; Serrano et al., 2012; Sifou et al., 2011; Uhlig et al., 2006).

The existing (mainly in vitro) data on biological activity of FUS, BEA, ENNs, and MON clearly indicate the possible toxicity of these fungal metabolites. However, there is a clear lack in the *in vivo* toxicity data and especially studies on the chronic effects are needed in order to dilucidate their importance (Jestoi, 2008).

In the other hand, masked mycotoxins are mycotoxins that are linked to other molecules such as a carbohydrates, amino acids or fatty acids (Coleman et al., 1997), comprising both extractable conjugated and bound (non-extractable) compounds (Berthiller et al., 2009). The former can be detected by appropriate analytical methods when their structure is known and analytical standards are available while the latter are not directly accessible and have to be liberated from the matrix prior to chemical analysis (Berthiller et al., 2009). These transformations are thought to take place during mold growth and mycotoxin formation in the field, with the plant upon which this is happening, effecting this conversion to reduce the toxicity of the mycotoxin and being subsequently stored in the cell vacuoles (Berthiller et al., 2013; Cole and Edwards, 2000; Cummins et al., 2011). The main concern about these transformations is that when the masked mycotoxin is consumed, usual passage through the mammalian and avian digestive tract may deconjugate the mycotoxin and therefore it will become toxic to the animal. (Berthiller et al., 2007).

Finally, the frequent detection of co-occurrence of AFs, ZEA, DON, FBs and OTA with each other and/or other "emerging" mycotoxins raises concern regarding possible synergistic or additive interactions of co-contaminants. In this sense, a study on the co-occurrence of *Fusarium* toxins in conventional and organic grains and derived products showed a co-occurrence of two or more mycotoxins in more than 50% of samples, being the most frequent combination DON + ZEA. Indeed, the correlation between the concentrations of T-2 and HT-2, DON and ZEA, as well as T-2 and ZEN was confirmed statistically, while none of the samples contained DAS (diacetoxyscirpenol), although NIV, MAS (monoacetoxyscirpenol) and 3ADON (3-acetyl-DON) concentrations were close to the detection limits (Błajet-Kosicka et al., 2014). Other study based on animal feeds showed that beauvericin and enniatins always occurred together and were found in 94% of the samples. Other frequently detected metabolites included ZEA (91% positives),

DON-3-glucoside, (a masked mycotoxin, 86%), culmorin (86%), tentoxin (86%), 15-hydroxyculmorin (77%), moniliformin (74%) and aurofusarin (71%) (Schatzmayr and Streit, 2013).

To sum up, the natural co-ocurrence of mycotoxins may result in synergistic, additive, or antagonistic interactions, which may make them harder to classificate and therefore, more research on the effects of co-occurring mycotoxins and also on the toxicological implications of the occurrence of emerging and masked mycotoxins is needed (Schatzmayr and Streit, 2013).

3.3. Climate change

In 1988, the World Meteorological Organization (WMO) and the United Nations Environment Program (UNEP) jointly established the Intergovernmental Panel on Climate Change (IPCC). The IPCC consists of a set of committees of leading scientists from all around the world whose task is to periodically review and report on the state of understanding of the climate problem. The 4th Assessment Report of the IPCC (IPCC 4AR) estimates global warming from different special report on emissions scenarios (SRES). These SRES are based on calculated greenhouse gas (GHGs) concentration pathways and consequent changes in radiative forcing as calculated by results of the most advanced coupled General Circulation Models (GCMs) as compiled in the IPCC-NCAR depository of GCM simulations. Subsequently, the EC has analyzed the results of this report establishing the most vulnerable areas (EC, 2007). Predictions for climate change indicate an annual increase of the global temperature of 0.03 ºC/year. Particularly, for Sourthern and South-Eastern Europe (Portugal, Spain, Southern France, Italy, Slovenia, Greece, Malta, Cyprus, Bulgaria, and Southern Romania) it may ecuate to an increase in the order of 4–5 ºC. With regard to water availability, this will be less, with the risk of hydropower disruption, particularly in summer. This effect combined with the rise of temperature could induce (i) decreased agricultural yields (in the range of 10–30% in many regions of the South), (ii) drought, (iii) heat waves, (iv) soil and ecosystem degradation, and (v) eventually desertification. The increase of violent rainfall will increase erosion and loss of organic matter from soil (EC, 2007).

In this new agricultural context, mycotoxin risk assessment should include a wider concept of risk evaluation, including emerging risks since new mycotoxins could arise for new fungus and plant associations making the occurrence of new mycotoxins or mycotoxins not yet considered as a new

potential human and animal health threat (Tirado et al., 2010). The impact of climate change has been identified as an emerging issue for food and feed safety (Miraglia et al., 2009), and its possible consequences on mycotoxins frequency in raw materials have been theorized by several researches (Magan et al., 2011; Miraglia et al., 2009; Paterson and Lima, 2011, 2010; Tirado et al., 2010; Wu et al., 2010).

Climatic differences among years in the same vineyard area sampled caused significant differences in fungal infection and mycotoxin contamination (Bellí et al., 2006). Generally crops are colonized by several species and little changes in environmental conditions causes the dominance of some species over others, affecting mycotoxin risks.

Bio-geographical differences in fungal profile infection have been observed in the world, clarifying that the climatic conditions are decisive in the colonization. In fact, it is largely accepted the relationship between mycotoxigenic species with certain latitudes. Nevertheless, warm European summers have seen the occurrence of the formely predominant species, *F. culmorum*, fall to be replaced by *F. graminearum*; both species producing ZEA and DON, but additionally *F. graminearum* produces nivalenol increasing the mycotoxin exposure (Miller, 2008). Moreover, in Italy *F. verticillioides*, the most diffuse maize pathogen, is favored by warm dry weather, while *A. flavus* tends to occur only in particularly hot summers, altering the maize contamination from FBs to AFs (Giorni et al., 2007). Besides, studies on AF-risk linked to climate change concluded that both cereal crops and mycotoxigenic fungi may move geographically as a result of changing conditions, thus some mycotoxigenic fungi may threaten those newly colonised areas (Battilani et al., 2012). Therefore, climate change increases the risk of migration of pathogens and therefore known fungal infections patterns could be affected and mycotoxin profile modified (Magan et al., 2011).

Interestingly, not only competition between different species has been observed, but also adapation to fungal species at new climate conditions. For instance, *A. flavus* isolated from maize in north Italy showed slightly different ecological profiles in terms of both optimal and marginal conditions for growth compared to other regions of the world (Giorni et al., 2007). Wu et al. (2010) pointed out that new and more aggressive strains could prevail.

Limiting and optimum conditions for CPA and AFs production by *A. flavus* were similar in both toxins (Astoreca et al., 2014). However, different environmental conditions could selectively promote the production of different toxins produced by the same strain.

In the same way as fungal invasion the agricultural practices are also climate-dependent and crops cultivation and yield vary from year to year depending on the weather; the agricultural sector is particularly exposed to climate change. It may directly influence host susceptibility through heat and/or drought stress. Periods of higher than average T and reduced annual rainfall increased rates of nut deformity and increased levels of AFs contamination (Tirado et al., 2010). Indirectly climate change may affect the seeding time of the crops, or change the geographical range of crops emerging new asociations host-pathogen, increasing mycotoxin contamination (Wu et al., 2010).

Moreover, the WMO has also highlighted that human emissions of the CFCs and other chemicals have an important role in the atmosphere changes by damaging the stratospheric ozone layer that filters out harmful ultraviolet radiation (UV) (WMO, 2013). Therefore, climate change and UV radiation increase are two phenomenon not only with an antropogenic origin but also with a big influence on each other (**Figure 9**).

- GHGs decreased temperatures in the stratosphere and accelerated circulation patterns, which tend to decrease total ozone in the tropics and increase total ozone at mid and high latitudes (WMO, 2010). Furthemore, changes in circulation induced by changes in ozone can also affect patterns of surface wind and rainfall (WMO, 2010).

- The UV increment at ground-level rise the tropospheric ozone, a significant GHGs and a major constituent of smog (Fergusson, 2001).

- The hydroxyl radical (OH), which is produced by the photochemical breakdown of ozone in the presence of water vapour, is an atmospheric scavenger that reacts with many pollutants and removes them from the atmosphere. The demands on the OH in a heavily polluted atmosphere may lead to a decline in OH concentrations and thus a reduction in the efficiency (Fergusson, 2001).

- The predicted decreases due to the climate change in mean cloudiness of the Mediterranean basin due to climate change may lead to increase in the UV radiation reaching Mediterranean ecosystems in the near future (WMO, 2010).

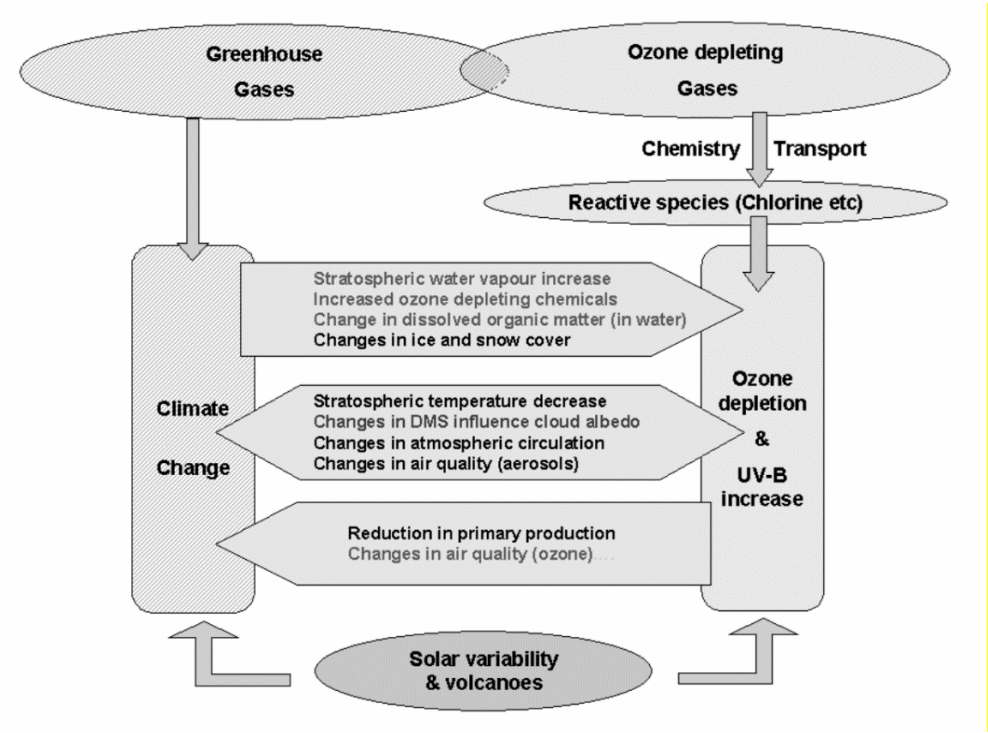


Figure 9 Interactions between ozone depletion and climate change. The sense of the interaction is given by the direction of the arrow (UNEP, 2002).

Increased UV-B radiation, interacting with other global change factors, may affect many of the important ecosystem processes and attributes, such as plant biomass production, plant consumption by herbivores including insects, disease incidence of plants and animals, changes in species abundance and composition, and mineral nutrient cycling, with importan implications for food security and food quality (WMO, 2010 UNEP 2002) (**Figure 10**).

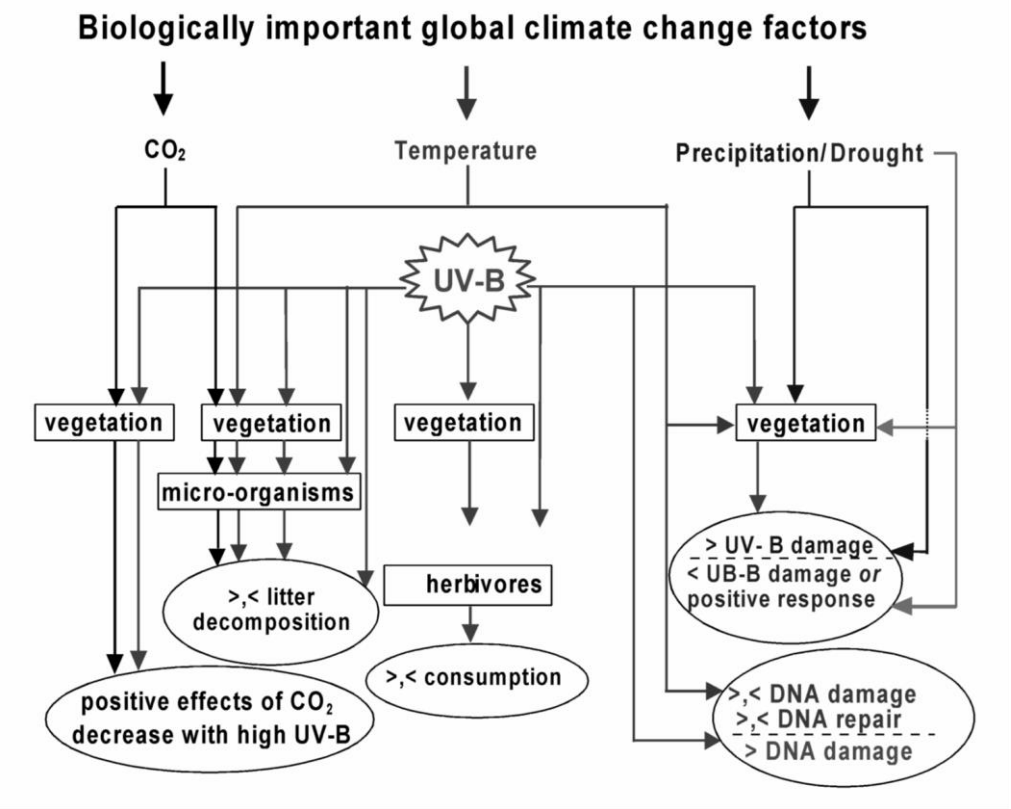


Figure 10 Major interactions of elevated UV-B with other climate changefactors in terrestrial ecosystems. Lines indicate influence of climate change factors on different trophic levels (in rectangles) that affect processes (in ovals) (UNEP, 2002).

Due to the lack of information about to effect to enhanced UV-B on fungi and bacteria the UNEP (2002) considering that the species composition and biodiversity of bacteria and fungi growing on plants can be changed by UVB and hence the biodiversity can be either increased or decreased. For pathogens, elevated UV-B can either increase or decrease the severity of disease development in plants.

To sum up, climate change may affect the mycotoxins presence in raw materials, and therefore it is necessary to know the potential risk in order to limit and manage the mycotoxin contamination since only a limited reduction is possible through food industrial processing.

REFERENCES

Abbas, H.K., Mirocha, C.J., Pawlosky, R.J., Pusch, D.J., 1985. Effect of cleaning, milling, and baking on deoxynivalenol in wheat. Appl. Environ. Microbiol. 50, 482–486.

Abdulkadar, A.H.W., Al-Ali, A., Al-Jedah, J., 2000. Aflatoxin contamination in edible nuts imported in Qatar. Food Control 11, 157–160.

Abdulkadar, A.H.W., Al-Ali, A.A., Al-Kildi, A.M., Al-Jedah, J.H., 2004. Mycotoxins in food products available in Qatar. Food Control 15, 543–548.

Abrunhosa, L., Calado, T., Venäncio, A., 2011. Incidence of fumonisin B2 production by *Aspergillus niger* in portuguese wine regions. J. Agric. Food Chem. 59, 7514–7518.

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.

Alexa, E., Dehelean, C.A., Poiana, M.-A., Radulov, I., Cimpean, A.-M., Bordean, D.-M., Tulcan, C., Pop, G., 2013. The occurrence of mycotoxins in wheat from western Romania and histopathological impact as effect of feed intake. Chem. Cent. J. 7.

Al-Hazmi, N.A., Gomaa, M.N., 2012. Alteration of fungal growth and toxigenicity due to the protective effect of cereal coats. Food Control 28, 299–303.

Altiokka, G., Can, N.O., Atkoşar, Z., Aboul-Enein, H.Y., 2009. Determination of Ochratoxin A in Turkish wines. J. Food Drug Anal. 17, 467–473.

Al-Wadai, A.S., Al-Othman, M.R., Mahmoud, M.A., El-Aziz, A.R.M.A., 2013. Molecular characterization of *Aspergillus flavus* and aflatoxin contamination of wheat grains from Saudi Arabia. Genet. Mol. Res. 12, 3335–3352.

Amézqueta, S., Schorr-Galindo, S., Murillo-Arbizu, M., González-Peñas, E., López de Cerain, A., Guiraud, J.P., 2012. OTA-producing fungi in foodstuffs: A review. Food Control 26, 259–268.

Amra, H.A., Mahmoud, S.A.Z., Tana, A.H., El-Azab, M.A., 1996. Destruction of aflatoxins B 1 and G 1 in bread making. Mycotoxin Res. 12, 73–78.

Anli, E., Çabuk, B., Vural, N., Başpinar, E., 2005. OchratoxinA in Turkish wines. J. Food Biochem. 29, 611–623.

Araguás, C., González-Peñas, E., Cerain, A.L.D., 2005. Study on ochratoxin A in cereal-derived products from Spain. Food Chem. 92, 459–464.

Ariño, A., Herrera, M., Estopañan, G., Juan, T., 2007. High levels of ochratoxin A in licorice and derived products. Int. J. Food Microbiol. 114, 366–369.

Ariño, A., Herrera, M., Estopañan, G., Rota, M.C., Carramiñana, J.J., Juan, T., Herrera, A., 2009. Aflatoxins in bulk and pre-packed pistachios sold in Spain and effect of roasting. Food Control 20, 811–814.

Astoreca, A., Vaamonde, G., Dalcero, A., Marin, S., Ramos, A., 2014. Abiotic factors and their interactions influence on the co-production of aflatoxin B_1 and cyclopiazonic acid by *Aspergillus flavus* isolated from corn. Food Microbiol. 38, 276–283.

Astoreca, A.L., Magnoli, C.E., Dalcero, A.M., 2010. Ecophysiology of *Aspergillus* section *Nigri* Species Potential Ochratoxin A Producers. Toxins 2, 2593–2605.

Aydin, A., Gunsen, U., Demirel, S., 2008. Total aflatoxin, aflatoxin B_1 and ochratoxin a levels in Turkish wheat flour. J. Food Drug Anal. 16, 48–53.

Aziz, N.H., Youssef, Y.A., 1991. Occurrence of aflatoxins and aflatoxin-producing moulds in fresh and processed meat in Egypt. Food Addit. Contam. 8, 321–331.

Aziz, N.H., Youssef, Y.A., El-Fouly, M.Z., Moussa, L.A., 1998. Contamination of some common medicinal plant samples and spices by fungi and their mycotoxins. Bot. Bull. Acad. Sin. 39, 279–285.

Bacaloni, A., Cavaliere, C., Faberi, A., Pastorini, E., Samperi, R., Laganà, A., 2005. Automated on-line solid-phase extraction-liquid chromatography-electrospray tandem mass spectrometry method for the determination of ochratoxin A in wine and beer. J. Agric. Food Chem. 53, 5518–5525.

Barajas-Aceves, M., Hassan, M., Tinoco, R., Vazquez-Duhalt, R., 2002. Effect of pollutants on the ergosterol content as indicator of fungal biomass. J. Microbiol. Methods 50, 227–236.

Basaran, P., Ozcan, M., 2009. Occurrence of aflatoxins in various nuts commercialized in Turkey. J. Food Saf. 29, 95–105.

Battilani P., Rossi V., Giorni P., Pietri A., Gualla A., van der Fels-Klerx H.J, Booij C.J.H., Moretti A., Logrieco A., Miglietta F, Toscano P., Miraglia M., De Santis B, Brera C., 2012. Question No EFSA-Q-2009-0081 [WWW Document]. URL http://www.efsa.europa.eu/fr/search/doc/223e.pdf (accessed 9.10.13).

Battilani, P., Pietri, A., Bertuzzi, T., Languasco, L., Giorni, P., Kozakiewicz, Z., 2003. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. J. Food Prot. 66, 633–636.

Baydar, T., Engin, A.B., Girgin, G., Aydin, S., Şahin, G., 2005. Aflatoxin and ochratoxin in various types of commonly consumed retail ground samples in Ankara, Turkey. Ann. Agric. Environ. Med. 12, 193–197.

Bayman, P., Baker, J.L., Doster, M.A., Michailides, T.J., Mahoney, N.E., 2002. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. Appl. Environ. Microbiol. 68, 2326–2329.

Bayman, P., Baker, J.L., Mahoney, N.E., 2003. *Aspergillus* on tree nuts: Incidence and associations. Mycopathologia 155, 161–169.

Beg, M.U., Al-Mutairi, M., Beg, K.R., Al-Mazeedi, H.M., Ali, L.N., Saeed, T., 2006. Mycotoxins in poultry feed in Kuwait. Arch. Environ. Contam. Toxicol. 50, 594–602.

Bejaoul, H., Mathieu, F., Taillandier, P., Lebrihi, A., 2004. Ochratoxin A removal in synthetic and natural grape juices by selected oenological *Saccharomyces* strains. J. Appl. Microbiol. 97, 1038–1044.

Belajová, E., Rauová, D., 2007. Determination of ochratoxin A and Its occurrence in wines of Slovakian retail. J. Food Nutr. Res. 46, 68–74.

Bellí, N., Bau, M., Marín, S., Abarca, M.L., Ramos, A.J., Bragulat, M.R., 2006. Mycobiota and ochratoxin A producing fungi from Spanish wine grapes. Int. J. Food Microbiol. 111, S40–S45.

Bellí, N., Marín, S., Coronas, I., Sanchis, V., Ramos, A.J., 2007. Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes. Food Control 18, 1343–1349.

Bellí, N., Marín, S., Duaigües, A., Ramos, A.J., Sanchis, V., 2004a. Ochratoxin A in wines, musts and grape juices from Spain. J. Sci. Food Agric. 84, 591–594.

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

Bellí, N., Mitchell, D., Marín, S., Alegre, I., Ramos, A.J., Magan, N., 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., Pardo, E., Marín, S., Farré, G., Ramos, A.J., Sanchis, V., 2004c. Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. J. Sci. Food Agric. 84, 541–546.

Bento, J.M.V., Pena, A., Lino, C.M., Pereira, J.A., 2009. Determination of ochratoxin A content in wheat bread samples collected from the Algarve and Bragança regions, Portugal: Winter 2007. Microchem. J. 91, 165–169.

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S.D., Haesaert, G., Karlovsky, P., Oswald, I.P., Seefelder, W., Speijers, G., Stroka, J., 2013. Masked mycotoxins: A review. Mol. Nutr. Food Res. 57, 165–186.

Berthiller, F., Schuhmacher, R., Adam, G., Krska, R., 2009. Formation, determination and significance of masked and other conjugated mycotoxins. Anal. Bioanal. Chem. 395, 1243–1252.

Berthiller, F., Sulyok, M., Krska, R., Schuhmacher, R., 2007. Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. Int. J. Food Microbiol. 119, 33–37.

Błajet-Kosicka, A., Twarużek, M., Kosicki, R., Sibiorowska, E., Grajewski, J., 2014. Co-occurrence and evaluation of mycotoxins in organic and conventional rye grain and products. Food Control 38, 61–66.

Blesa, J., Soriano, J.M., Moltó, J.C., Mañes, J., 2004. Concentration of ochratoxin a in wines from supermarkets and stores of Valencian Community (Spain). J. Chromatogr. A 1054, 397–401.

Bonvehí, J.S., 2004. Occurrence of ochratoxin A in cocoa products and chocolate. J. Agric. Food Chem. 52, 6347–6352.

Bornet, A., Teissedre, P.L., 2008. Chitosan, chitin-glucan and chitin effects on minerals (iron, lead, cadmium) and organic (ochratoxin A) contaminants in wines. Eur. Food Res. Technol. 226, 681–689.

Boudra, H., Barnouin, J., Dragacci, S., Morgavi, D.P., 2007. Aflatoxin M1 and ochratoxin a in raw bulk milk from french dairy herds. J. Dairy Sci. 90, 3197–3201.

Brera, C., Catano, C., De Santis, B., Debegnach, F., De Giacomo, M., Pannunzi, E., Miraglia, M., 2006. Effect of industrial processing on the distribution of aflatoxins and zearalenone in corn-milling fractions. J. Agric. Food Chem. 54, 5014–5019.

Brera, C., Debegnach, F., Minardi, V., Prantera, E., Pannunzi, E., Faleo, S., Santis, B.D., Miraglia, M., 2008. Ochratoxin A contamination in Italian wine samples and evaluation of the exposure in the Italian population. J. Agric. Food Chem. 56, 10611–10618.

Bullerman, L.B., Bianchini, A., 2007. Stability of mycotoxins during food processing. Int. J. Food Microbiol. 119, 140–146.

Burdaspal, P.., Legarda, T.., Iñigo, S., 2005. A survey of aflatoxins in peanuts and pistachios.Comparison with some data published in 1989 in Spain. Alimentaria 297, 151–156.

Burdaspal, P.A., Legarda, T.M., 1999. Ochratoxin A in wines and grape products originating from Spain and other European countries. Alimentaria 107–113.

Burdaspal, P.A., Legarda, T.M., 2013. Survey on aflatoxin in beer sold in Spain and other European countries. World Mycotoxin J. 6, 93–101.

Cabañes, F.J., Bragulat, M.R., Castellá, G., 2013. Characterization of nonochratoxigenic strains of *Aspergillus carbonarius* from grapes. Food Microbiol. 36, 135–141.

CAC, 2003. Code of practice for the prevention and reduction of mycotoxin contamination in cereals, including annexes on ochratoxin a, zearalenone, fumonisins and tricothecenes.

CAC, 2004. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission. Report of the 25th Codex Committee meeting of methods of analysis and sampling; March 8–12 2004; Budapest 29.

CAC, 2007. Principles and guidelines for the conduct of microbiological risk management (MRM). CACGL 63-2007.

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. J. Appl. Microbiol. 99, 1215–1221.

Caridi, A., Galvano, F., Tafuri, A., Ritieni, A., 2006. Ochratoxin A removal during winemaking. Enzyme Microb. Technol. 40, 122–126.

Casado, M.R., Parsons, D.J., Weightman, R.M., Magan, N., Origgi, S., 2009. Geostatistical analysis of the spatial distribution of mycotoxin concentration in bulk cereals. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 26, 867–873.

CAST, 2003. Mycotoxins: Risks in plant, animal and human systems. Task force report; Nº. 139. ISSN 0194-4088.

Castellari, M., Versari, A., Fabiani, A., Parpinello, G.P., Galassi, S., 2001. Removal of ochratoxin a in red wines by means of adsorption treatments with commercial fining agents. J. Agric. Food Chem. 49, 3917–3921.

Cecchini, F., Morassut, M., Moruno, E.G., Stefano, R.D., 2006. Influence of yeast strain on ochratoxin A content during fermentation of white and red must. Food Microbiol. 23, 411–417.

Cerain, A.L.D., González-Peñas, E., Jiménez, A.M., Bello, J., 2002. Contribution to the study of ochratoxin A in Spanish wines. Food Addit. Contam. 19, 1058–1064.

Cerutti, G., D'Amato, A., Zucchetti, M., 2000. Sulla presenza di ocratossina A, nitrato e nitrito nel vino. Imbottigliamento 23, 39–43.

Cheli, F., Campagnoli, A., Pinotti, L., Fusi, E., Dell'Orto, V., 2009. Sampling feed for mycotoxins: Acquiring knowledge from food. Ital. J. Anim. Sci. 8, 5–22.

Cheli, F., Pinotti, L., Campagnoli, A., Fusi, E., Rebucci, R., Baldi, A., 2008. Mycotoxin analysis, mycotoxin-producing fungi assays and mycotoxin toxicity bioassays in food mycotoxin monitoring and surveillance. Ital. J. Food Sci. 20, 447–462.

Cheli, F., Pinotti, L., Rossi, L., Dell'Orto, V., 2013. Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. LWT - Food Sci. Technol. 54, 307–314. doi:10.1016/j.lwt.2013.05.040

Cheraghali, A.M., Yazdanpanah, H., Doraki, N., Abouhossain, G., Hassibi, M., Ali-abadi S., Aliakbarpoor, M., Amirahmadi, M., Askarian, A., Fallah, N., Hashemi, T., Jalali, M., Kalantari, N., Khodadadi, E., Maddah, B., Mohit, R., Mohseny, M., Phaghihy, Z., Rahmani, A., Setoodeh, L., Soleimany, E., Zamanian, F., 2007. Incidence of aflatoxins in Iran pistachio nuts. Food Chem. Toxicol. 45, 812–816.

Chiotta, M.L., Ponsone, M.L., Combina, M., Torres, A.M., Chulze, S.N., 2009. *Aspergillus* section *Nigri* species isolated from different wine-grape growing regions in Argentina. Int. J. Food Microbiol. 136, 137–141.

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N., 2011. Phylogenetic characterization and ochratoxin A - Fumonisin profile of black *Aspergillus* isolated from grapes in Argentina. Int. J. Food Microbiol. 149, 171–176.

Cole, D.J., Edwards, R., 2000. Secondary metabolism of agrochemicals in plants. Metab. Agrochem. Plants 107–154.

Cole, R.J., Jarvis, B.B., Schweikert, M.A., 2003. 11 - Aflatoxins, in: Cole, R.J., Jarvis, B.B., Schweikert, M.A. (Eds.), Handbook of Secondary Fungal Metabolites. Academic Press, San Diego, pp. 545–569.

Coleman, J.O.D., Blake-Kalff, M.M.A., Davies, T.G.E., 1997. Detoxification of xenobiotics by plants: Chemical modification and vacuolar compartmentation. Trends Plant Sci. 2, 144–151.

Comuzzo, P., Rauhut, D., Werner, M., Lagazio, C., Zironi, R., 2013. A survey on wines from organic viticulture from different European countries. Food Control 34, 274–282.

Comuzzo, P., Rauhut, D., Werner, M., Lagazio, C., Zironi, R., 2013. A survey on wines from organic viticulture from different European countries. Food Control 34, 274–282.

Coronel, M.B., Marín, S., Cano-Sancho, G., Ramos, A.J., Sanchis, V., 2012. Exposure assessment to ochratoxin A in Catalonia (Spain) based on the consumption of cereals, nuts, coffee, wine, and beer. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 29, 979–993.

Covarelli, L., Beccari, G., Marini, A., Tosi, L., 2012. A review on the occurrence and control of ochratoxigenic fungal species and ochratoxin A in dehydrated grapes, non-fortified dessert wines and dried vine fruit in the Mediterranean area. Food Control 26, 347–356.

Csutorás, C., Rácz, L., Rácz, K., Futo, P., Forgó, P., Kiss, A., 2013. Monitoring of ochratoxin A during the fermentation of different wines by applying high toxin concentrations. Microchem. J. 107, 182–184.

Cucullu, A.F., Lee, L.S., Mayne, R.Y., Goldblatt, L.A., 1966. Determination of aflatoxins in individual peanuts and peanut sections. JAOCS J. Am. Oil Chem. Soc. 43, 89–92.

Cucullu, A.F., Lee, L.S., Pons, W.A., 1977. Relationship of physical appearance of individual mold-damaged cottonseed to aflatoxin content. J. Am. Oil Chem. Soc. 54, A235–A237.

Cummins, I., Dixon, D.P., Freitag-Pohl, S., Skipsey, M., Edwards, R., 2011. Multiple roles for plant glutathione transferases in xenobiotic detoxification. Drug Metab. Rev. 43, 266–280.

Curtui, V.G., Gareis, M., Usleber, E., Märtlbauer, E., 2001. Survey of Romanian slaughtered pigs for the occurence of mycotoxins ochratoxins A and B, and zearalenone. Food Addit. Contam. 18, 730–738.

Czerwiecki, L., Czajkowska, D., Witkowska-Gwiazdowska, A., 2002a. 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 Addit. Contam. 19, 1051–1057.

Czerwiecki, L., Czajkowska, D., Witkowska-Gwiazdowska, A., 2002b. On ochratoxin A and fungal flora in Polish cereals from conventional and ecological farms. Part 1: Occurrence of ochratoxin A and fungi in cereals in 1997. Food Addit. Contam. 19, 470–477.

De Swarte, C., Donker, R.A., 2005. Towards an FSO/ALOP based food safety policy. Food Control 16, 825–830.

De Vries, R.P., Frisvad, J.C., van de Vondervoort, P.J.I., Burgers, K., Kuijpers, A.F.A., Samson, R.A., Visser, J., 2005. *Aspergillus* vadensis, a new species of the group of black Aspergilli. Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol. 87, 195–203.

Denizel, T., Jarvis, B., Rolfe, E.J., 1976. A field survey of pistachio (Pistacia vera) nut production and storage in Turkey with particular reference to aflatoxin contamination. J. Sci. Food Agric. 27, 1021–1026.

Deshpande, S.S., 2002. Handbook of food toxicology. Marcel Dekker, New York.

Díaz, G.A., Torres, R., Vega, M., Latorre, B.A., 2009. Ochratoxigenic *Aspergillus* species on grapes from Chilean vineyards and *Aspergillus* threshold levels on grapes. Int. J. Food Microbiol. 133, 195–199.

Dini, A., Khazaeli, P., Roohbakhsh, A., Madadlou, A., Pourenamdari, M., Setoodeh, L., Askarian, A., Doraki, N., Farrokhi, H., Moradi, H., Khodadadi, E., 2013. Aflatoxin contamination level in Iran's pistachio nut during years 2009-2011.

Domijan, A.-M., Peraica, M., 2005. Ochratoxin A in wine. Arh. Hig. Rada Toksikol. 56, 17–20.

Doster, M.A., Michailides, T.J., 1994. *Aspergillus* molds and aflatoxins in pistachio nuts in California. Phytopathology 84, 583–590.

Doster, M.A., Michailides, T.J., 1995. The relationship between date of hull splitting and decay of pistachio nuts by *Aspergillus* species. Plant Dis. 79, 766–769.

Doster, M.A., Michailides, T.J., 1999. Relationship between shell discoloration of pistachio nuts and incidence of fungal decay and insect infestation. Plant Dis. 83, 259–264.

Duarte, S.C., Bento, J.M.V., Pena, A., Lino, C.M., 2009. Ochratoxin A exposure assessment of the inhabitants of Lisbon during winter 2007/2008 through bread and urine analysis. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 26, 1411–1420.

Duarte, S.C., Pena, A., Lino, C.M., 2010. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. Food Microbiol. 27, 187–198.

E.C., 2002. Reports on tasks for scientific cooperation. Report of experts participating in Task 3.2.7. Assessment of dietary intake of ochratoxin A by the population of EU member states. Directorate-General Health and Consumer Protection (2002) January 2002.

EC, 2006a. European Comimission, Nº 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union, L 70(2006), 12-34.

EC, 2006b. European Commission, Nº 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L 364(2006), 5-24.

EC, 2007. European Commision, 2007. Adapting to climate change in Europe: Options for EU Action. Green Paper from the Commission to the Council, the European Parlianment, the European Economic and Social Committee of the Regions, SEC (2007) Brusseles, Belgium.

EC, 2010a. European Commission, Nº 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuff as regards aflatoxins. Official Journal of the European Union, L 50(2006), 8-12.

EC, 2010b. European Commission, Nº 105/2010 of 5 February 2010 amending Regulation (EC) No1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A. Official Journal of the European Union, L 35(2006), 7-8.

EC, 2010c. European Commission, Nº 178/2010 of 2 March 2010 amending Regulation (EC) No 401/2006 as regards groundnuts (peanuts), other oilseeds, tree nuts, apricot kernels, liquorice and vegetable oil. Official Journal of the European Union, L 52(2006), 32-43.

EC, 2012. European Commission, Nº 1058/2012 of 12 November 2012 amending Regulation (EC) No 1881/2006 as regards maximum levels for aflatoxins in dried figs. Official Journal of the European Union, L (2012), 313-314.

EFSA, 2006. European Food Safety Authority, EFSA. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food. EFSA J 365, 1–56.

EFSA, 2007. European Food Safety Authority, EFSA. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products.

El tawila, M.M., Neamatallah, A., Serdar, S.A., 2013. Incidence of aflatoxins in commercial nuts in the holy city of Mekkah. Food Control 29, 121–124. doi:10.1016/j.foodcont.2012.06.004

Elmholt, S., Rasmussen, P.H., 2005. Penicillium verrucosum occurrence and Ochratoxin A contents in organically cultivated grain with special reference to ancient wheat types and drying practice. Mycopathologia 159, 421–432.

Elsanhoty, R.M., Ramadan, M.F., El-Gohery, S.S., Abol-Ela, M.F., Azeke, M.A., 2013. Ability of selected microorganisms for removing aflatoxins invitro and fate of aflatoxins in contaminated wheat during baladi bread baking. Food Control 33, 287–292.

Ezekiel, C.N., Sulyok, M., Warth, B., Odebode, A.C., Krska, R., 2012. Natural occurrence of mycotoxins in peanut cake from Nigeria. Food Control 27, 338–342.

Fandohan, P., Zoumenou, D., Hounhouigan, D.J., Marasas, W.F.O., Wingfield, M.J., Hell, K., 2005. Fate of aflatoxins and fumonisins during the processing of maize into food products in Benin. Int. J. Food Microbiol. 98, 249–259. doi:10.1016/j.ijfoodmicro.2004.07.007

FAO, 2001. Food and Agriculture Organization. Manual on the application of HACCP system in mycotoxin prevention and control. Food Nutr. (Roma) 73, 1–130.

FAOSTAT, Food and Agriculture Organization. http://faostat.fao.org/

Farah, Z., Martins, M.J.R., Bachmann, M.R., 1983. Removal of aflatoxin in raw unshelled peanuts by a traditional salt boiling process practiced in the north east of Brazil. Lebensm. Wiss. Technol. 16, 122–124.

Fazekas, B., Tar, A.K., Zomborszky-Kovács, M., 2002. Ochratoxin a contamination of cereal grains and coffee in Hungary in the year 2001. Acta Vet. Hung. 50, 177–188.

Fergusson, A., 2001. Ozone depletion and climate change: Understanding the linkages. Minister of Public Works and Government Services, Canada.

Fernandes, A., Ratola, N., Cerdeira, A., Alves, A., Venâncio, A., 2007. Changes in Ochratoxin A Concentration during Winemaking. Am. J. Enol. Vitic. 58, 92–96.

Fernandes, P.J., Barros, N., Câmara, J.S., 2013. A survey of the occurrence of ochratoxin A in Madeira wines based on a modified QuEChERS extraction procedure combined with liquid chromatography-triple quadrupole tandem mass spectrometry. Food Res. Int. 54, 293–301.

Fernane, F., Cano-Sancho, G., Sanchis, V., Marin, S., Ramos, A.J., 2010a. Aflatoxins and ochratoxin A in pistachios sampled in Spain: Occurrence and presence of mycotoxigenic fungi. Food Addit. Contam. Part B Surveill. 3, 185–192.

Fernane, F., Sanchis, V., Marín, S., Ramos, A.J., 2010b. First report on mould and mycotoxin contamination of pistachios sampled in Algeria. Mycopathologia 1–7.

Ferracane, R., Tafuri, A., Logieco, A., Galvano, F., Balzano, D., Ritieni, A., 2007. Simultaneous determination of aflatoxin B1 and ochratoxin A and their natural occurrence in Mediterranean virgin olive oil. Food Addit. Contam. 24, 173–180.

Ferracane, R., Tafuri, A., Logieco, A., Galvano, F., Balzano, D., Ritieni, A., 2007. Simultaneous determination of aflatoxin B1 and ochratoxin A and their natural occurrence in Mediterranean virgin olive oil. Food Addit. Contam. 24, 173–180.

Filali, A., Ouammi, L., Betbeder, A.M., Baudrimont, I., Soulaymani, R., Benayada, A., Creppy, E.E., 2001. Ochratoxin A in beverages from Morocco: A preliminary survey. Food Addit. Contam. 18, 565–568.

Finoli, C., Vecchio, A., Planeta, D., 2005. Mycotoxin occurrence in extra virgin olive oils and in olives. Presenza Micotoss8ine Oli Extra Vergini Oliva Ed Olive Mensa 44, 506–514.

Flajs, D., Domijan, A.-M., Ivić, D., Cvjetković, B., Peraica, M., 2009. ELISA and HPLC analysis of ochratoxin A in red wines of Croatia. Food Control 20, 590–592.

Food Standards Agency, 2002. Food Standards Agency, Contaminants Division. Survey of nuts, nut products and dried tree fruits for mycotoxins. Food Survey Information Sheet number 21, London, Uk.

Fredj, S.M.B., 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.

Frisvad, J.C., Frank, J.M., Houbraken, J.A.M.P., Kuijpers, A.F.A., Samson, R.A., 2004a. New ochratoxin A producing species of *Aspergillus* section *Circumdati*. Stud. Mycol. 50, 23–43.

Frisvad, J.C., Larsen, T.O., Thrane, U., Meijer, M., Varga, J., Samson, R.A., Nielsen, K.F., 2011. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS ONE 6.

Frisvad, J.C., Samson, R.A., 2000. Neopetromyces gen. nov. and an overview of teleomorphs of *Aspergillus* subgenus *Circumdati*. Stud. Mycol. 2000, 201–207.

Frisvad, J.C., Skouboe, P., Samson, R.A., 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus* rambellii sp. nov. Syst. Appl. Microbiol. 28, 442–453.

Frisvad, J.C., Smedsgaard, J., Larsen, T.O., Samson, R.A., 2004b. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. Stud. Mycol. 2004, 201–241.

Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M., Knasmüller, S., 2008. Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. Food Chem. Toxicol. 46, 1398–1407.

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.

Georgiadou, M., Dimou, A., Yanniotis, S., 2012. Aflatoxin contamination in pistachio nuts: A farm to storage study. Food Control 26, 580–586.

Ghali, R., Hmaissia-khlifa, K., Ghorbel, H., Maaroufi, K., Hedili, A., 2008. Incidence of aflatoxins, ochratoxin A and zearalenone in Tunisian foods. Food Control 19, 921–924.

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., Battilani, P., 2007. Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. Int. J. Food Microbiol. 113, 330–338.

Goldblatt, L.A., 1969. Aflatoxin: scientific background, control, and implications. Academic Press.

Gonçalves, S.S., Stchigel, A.M., Cano, J.F., Godoy-Martinez, P.C., Colombo, A.L., Guarro, J., 2012. Aspergi*llus novoparasiticus*: A new clinical species of the section *Flavi*. Med. Mycol. 50, 152–160.

González-Osnaya, L., Soriano, J.M., Moltó, J.C., Mañes, J., 2007. Dietary intake of ochratoxin A from conventional and organic bread. Int. J. Food Microbiol. 118, 87–91.

González-Salgado, A., 2010. Diagnóstico y control de especies de *Aspergillus* productoras de ocratoxina A. Dr. Tesis ISBN 978-84-693-2408-0.

Goto, T., Wicklow, D.T., Ito, Y., 1996. Aflatoxin and cyclopiazonic acid production by a sclerotium-producing *Aspergillus tamarii* strain. Appl. Environ. Microbiol. 62, 4036–4038.

Grazioli, B., Fumi, M.D., Silva, A., 2006. The role of processing on ochratoxin A content in Italian must and wine: A study on naturally contaminated grapes. Int. J. Food Microbiol. 111, Supplement 1, S93–S96.

Gul, O., Dervisoglu, M., 2014. Occurrence of aflatoxin M_1 in vacuum packed kashar cheeses in Turkey. Int. J. Food Prop. 17, 273–282.

Gumus, T., Arid, M., Daglioglu, O., Velioglu, M., 2009. Fate of aflatoxins during bread making. J. Food Sci. Technol. 46, 384–387.

Hammami, W., Fiori, S., Al Thani, R., Ali Kali, N., Balmas, V., Migheli, Q., Jaoua, S., 2014. Fungal and aflatoxin contamination of marketed spices. Food Control 37, 177–181.

Hassan, H.F., Kassaify, Z., 2014. The risks associated with aflatoxins M1 occurrence in Lebanese dairy products. Food Control 37, 68–72.

Hazel, C.M., Patel, S., 2004. Influence of processing on trichothecene levels. Toxicol. Lett. 153, 51–59.

Hemery, Y., Rouau, X., Lullien-Pellerin, V., Barron, C., Abecassis, J., 2007. Dry processes to develop wheat fractions and products with enhanced nutritional quality. J. Cereal Sci. 46, 327–347.

Heperkan, D., Güler, F.K., Oktay, H.I., 2012. Mycoflora and natural occurrence of aflatoxin, cyclopiazonic acid, fumonisin and ochratoxin A in dried figs. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 29, 277–286.

Hepsag, F., Golge, O., Kabak, B., 2014. Quantitation of aflatoxins in pistachios and groundnuts using HPLC-FLD method. Food Control 38, 75–81.

Hocking, A.D., Varelis, P., Pitt, J.I., Cameron, S.F., Leong, S.-L.L., 2003. Occurrence of ochratoxin A in Australian wine. Aust. J. Grape Wine Res. 9, 72–78.

House, J.D., Nyachoti, C.M., Abramson, D., 2003. Deoxynivalenol removal from barley intended as swine feed through the use of an abrasive pearling procedure. J. Agric. Food Chem. 51, 5172–5175.

IARC, 1993. International Agency for Research on Cancer. Monographs on the evaluation of the carcinogenic risk of chemicals to humans: some naturally occurring substances. Food items and constituents, heterocyclic aromatic amines and mycotoxins. Lyon, France. 56, 397–444.

IARC, 2002. International Agency for Research on Cancer. International Agency Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Some Natural Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mykotoxins. Lyon, France. 56, 26-32.

Ibáñez-Vea, M., Martínez, R., González-Peñas, E., Lizarraga, E., López de Cerain, A., 2011. Co-occurrence of aflatoxins, ochratoxin A and zearalenone in breakfast cereals from Spanish market. Food Control 22, 1949–1955.

ICMSF, 1998. International Commission on Microbiological Specifications for Foods. Microorganisms in Food 6: Microbial ecology of food commodities.London: Blackie Academic & Professional. ISBN: 041247350X

ICMSF, 2002. International Commission on Microbiological Specifications for Foods. Microorganisms in Food 7: Microbiological testing in food safety management. Kluwer Academic/Plenum Publishers, 2002. ISBN: 0306472627.

Ismail, R.A., M., Bhalli, J.A., Mobeen, A., Khan, Q.M., 2012. Effect of temperature on ochratoxin A production in common cereals by *Aspergillus* species. J. Anim. Plant Sci. 23, 1316–1320.

Ito, Y., Peterson, S.W., Wicklow, D.T., Goto, T., 2001. *Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section *Flavi*. Mycol. Res. 105, 233–239.

Jahanmard, E., Azarani, F., Sharifi, M., Esfandiari, Z., 2014. Aflatoxin in pistachio nuts used as ingredients in Gaz sweets produced in Isfahan, Iran. Food Addit. Contam. Part B Surveill. 7, 70–73.

Jestoi, M., 2008. Emerging *Fusarium*-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin - A review. Crit. Rev. Food Sci. Nutr. 48, 21–49.

Johansson, A.S., Whitaker, T.B., Giesbrecht, F.G., Jr, H.W.M., Young, J.H., 2000. Testing shelled corn for aflatoxin, part ii: modeling the observed distribution of aflatoxin test results. J. AOAC Int. 83, 1270–1278.

Jørgensen, K., Jacobsen, J.S., 2002. Occurrence of ochratoxin A in Danish wheat and rye, 1992-99. Food Addit. Contam. 19, 1184–1189.

Jouany, J.P., 2007. Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. Anim. Feed Sci. Technol. 137, 342–362.

Joubrane, K., Khoury, A.E., Lteif, R., Rizk, T., Kallassy, M., Hilan, C., Maroun, R., 2011. Occurrence of aflatoxin B_1 and ochratoxin A in Lebanese cultivated wheat. Mycotoxin Res. 27, 249–257.

Juan, C., Pena, A., Lino, C., Moltó, J.C., Mañes, J., 2008. Levels of ochratoxin A in wheat and maize bread from the central zone of Portugal. Int. J. Food Microbiol. 127, 284–289.

Khoury, A.E., Rizk, T., Lteif, R., Azouri, H., Delia, M.-L., Lebrihi, A., 2008. Fungal contamination and Aflatoxin B_1 and Ochratoxin A in Lebanese wine-grapes and musts. Food Chem. Toxicol. 46, 2244–2250.

Klich, M.A., 2007. *Aspergillus flavus*: The major producer of aflatoxin. Mol. Plant Pathol. 8, 713–722.

Kumagai, S., Nakajima, M., Tabata, S., Ishikuro, E., Tanaka, T., Norizuki, H., Itoh, Y., Aoyama, K., Fujita, K., Kai, S., Sato, T., Saito, S., Yoshiike, N., Sugita-Konishi, Y., 2008. Aflatoxin and ochratoxin A contamination of retail foods and intake of these mycotoxins in Japan. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 25, 1101–1106.

Kumar, R., Ansari, K.M., Saxena, N., Dwivedi, P.D., Jain, S.K., Das, M., 2012. Detection of Ochratoxin A in wheat samples in different regions of India. Food Control 26, 63–67.

Kurtbay, H.M., Bekçi, Z., Merdivan, M., Yurdakoç, K., 2008. Reduction of ochratoxin a levels in red wine by bentonite, modified bentonites, and chitosan. J. Agric. Food Chem. 56, 2541–2545.

Kushiro, M., 2008. Effects of milling and cooking processes on the deoxynivalenol content in wheat. Int. J. Mol. Sci. 9, 2127–2145.

Lancova, K., Hajslova, J., Kostelanska, M., Kohoutkova, J., Nedelnik, J., Moravcova, H., Vanova, M., 2008. Fate of trichothecene mycotoxins during the processing: Milling and baking. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 25, 650–659.

Larcher, R., Nicolini, G., 2001. Survey of ochratoxin A in musts, concentrated musts and wines produced or marketed in Trentino (Italy). J. Commod. Sci. 40, 69–78.

Lasram, S., Mani, A., Zaied, C., Chebil, S., Abid, S., Bacha, H., Mliki, A., Ghorbel, A., 2008. Evolution of ochratoxin A content during red and rose vinification. J. Sci. Food Agric. 88, 1696–1703.

Lasram, S., Oueslati, S., Mliki, A., Ghorbel, A., Silar, P., Chebil, S., 2012. Ochratoxin A and ochratoxigenic black *Aspergillus* species in Tunisian grapes cultivated in different geographic areas. Food Control 25, 75–80.

Lee, L.S., Cucullu, A.F., Franz, A.O.Jr, Walter, A.P.Jr, 1969. Destruction of aflatoxins in peanuts during dry and oil roasting. J. Agric. Food Chem. 17, 451–453.

Legarda, T.M., Burdaspal, P.A., 2001. Occurrence of ochratoxin A in samples of bread marketed in Spain and twelve other countries. Alimentaria 321, 89–96.

Leong, S.-L.L., Hocking, A.D., Varelis, P., Giannikopoulos, G., Scott, E.S., 2006. Fate of ochratoxin A during vinification of semillon and Shiraz grapes. J. Agric. Food Chem. 54, 6460–6464.

Li, C., Liu, Y., Weng, Z., Chen, Y.-N., 2013. The influence of storage and transport environment on grape quality. Mod. Food Sci. Technol. 29, 230–235.

Logrieco, A., Ferracane, R., Haidukowsky, M., Cozzi, G., Visconti, A., Ritieni, A., 2009. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. Food Addit. Contam. Part Chem. Anal. Control Expo. Risk Assess. 26, 1495–1500.

Lucchetta, G., Bazzo, I., Cortivo, G.D., Stringher, L., Bellotto, D., Borgo, M., Angelini, E., 2010. Occurrence of black *aspergilli* and ochratoxin A on grapes in Italy. Toxins 2, 840–855.

Macarthur, R., Macdonald, S., Brereton, P., Murray, A., 2006. Statistical modelling as an aid to the design of retail sampling plans for mycotoxins in food. Food Addit. Contam. 23, 84–92.

Magan, N., Aldred, D., 2007. Post-harvest control strategies: Minimizing mycotoxins in the food chain. Int. J. Food Microbiol. 119, 131–139.

Magan, N., Medina, A., Aldred, D., 2011. Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. Plant Pathol. 60, 150–163.

Magan, N., Olsen, M., 2004. Mycotoxins in Food: Detection and Control. Woodhead Pub.

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.

Mahnine, N., Meca, G., Elabidi, A., Fekhaoui, M., Saoiabi, A., Font, G., Mañes, J., Zinedine, A., 2011. Further data on the levels of emerging *Fusarium* mycotoxins enniatins (A, A1, B, B1), beauvericin and fusaproliferin in breakfast and infant cereals from Morocco. Food Chem. 124, 481–485.

Majerus, P., Bresch, H., Ottender, H., 2000. Ochratoxin A in wines, fruit juices and seasonings. Arch. Für Leb. 51, 81–128.

Majerus, P., Otteneder, H., 1996. Nachweis und vorkommen von ochratoxin A in wein und traubensaft. Dtsch. Lebensm.-Rundsch. 92, 388–390.

Marín, S., Bellí, N., Lasram, S., Chebil, S., Ramos, A.J., Ghorbel, A., Sanchis, V., 2006. Kinetics of ochratoxin A production and accumulation by *Aspergillus carbonarius* on synthetic grape medium at different temperature levels. J. Food Sci. 71, 196–199.

Marín, S., Hodžić, I., Ramos, A.J., Sanchis, V., 2008. Predicting the growth/no-growth boundary and ochratoxin A production by *Aspergillus carbonarius* in pistachio nuts. Food Microbiol. 25, 683–689.

Marín, S., Ramos, A.J., Sanchis, V., 2012. Modelling *Aspergillus flavus* growth and aflatoxins production in pistachio nuts. Food Microbiol. 32, 378–388.

Markaki, P., Delpont-Binet, C., Grosso, F., Dragacci, S., 2001. Determination of ochratoxin A in red wine and vinegar by immunoaffinity high-pressure liquid chromatography. J. Food Prot. 64, 533–537.

Martínez-Culebras, P.V., Ramón, D., 2007. An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes and wine. Int. J. Food Microbiol. 113, 147–153.

Mateo, E.M., Medina, A., Mateo, R., Jiménez, M., 2010. Effect of ethanol on the ability of Oenococcus oeni to remove ochratoxin A in synthetic wine-like media. Food Control 21, 935–941.

Matrella, R., Monaci, L., Milillo, M.A., Palmisano, F., Tantillo, M.G., 2006. Ochratoxin A determination in paired kidneys and muscle samples from swines slaughtered in southern Italy. Food Control 17, 114–117.

Meca, G., Blaiotta, G., Ritieni, A., 2010. Reduction of ochratoxin A during the fermentation of Italian red wine Moscato. Food Control 21, 579–583.

Medina, A., Mateo, R., López-Ocaña, L., Valle-Algarra, F.M., Jiménez, M., 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. Appl. Environ. Microbiol. 71, 4696–4702.

Milhome, M.A.L., Lima, C.G., de Lima, L.K., Lima, F.A.F., Sousa, D.O.B., Nascimento, R.F., 2014. Occurrence of aflatoxins in cashew nuts produced in northeastern Brazil. Food Control 42, 34–37.

Miller, J.D., 2008. Mycotoxins in small grains and maize: Old problems, new challenges. Food Addit. Contam. 25, 219–230.

Miraglia, M., Brera, C., 2002. Assessment of Dietary Intake of Ochratoxin A by the Population of EU Member States. Assess. Diet. Intake Ochratoxin Popul. EU Memb. States.

Miraglia, M., Brera, C., 2002. Assessment of Dietary Intake of Ochratoxin A by the Population of EU Member States. Assess. Diet. Intake Ochratoxin Popul. EU Memb. States.

Miraglia, M., Marvin, H.J.P., Kleter, G.A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., Santis, B.D., Dekkers, S., Filippi, L., Hutjes, R.W.A., Noordam, M.Y., Pisante, M., Piva, G., Prandini, A., Toti, L., Born, G.J. van den, Vespermann, A., 2009. Climate change and food safety: An emerging issue with special focus on Europe. Food Chem. Toxicol. 47, 1009–1021.

Miraglia, M., Santis, B.D., Minardi, V., Debegnach, F., Brera, C., 2005. The role of sampling in mycotoxin contamination: An holistic view. Food Addit. Contam. 22, 31–36.

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. J. Appl. Microbiol. 97, 439–445.

Mogensen, J.M., Frisvad, J.C., Thrane, U., Nielsen, K.F., 2010a. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. J. Agric. Food Chem. 58, 954–958.

Mogensen, J.M., Larsen, T.O., Nielsen, K.F., 2010b. Widespread occurrence of the mycotoxin fumonisin B_2 in wine. J. Agric. Food Chem. 58, 4853–4857.

Mogensen, J.M., Nielsen, K.F., Samson, R.A., Frisvad, J.C., Thrane, U., 2009. Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species. BMC Microbiol. 9.

Mohamed-Yasseen, Y., Barringer, S.A., Splittstoesser, W.E., Costanza, S., 1994. The role of seed coats in seed viability. BOT.REV. 60, 426–439.

Mojtahedi, H., Rabie, C.J., Lubben, A., 1979. Toxic *aspergilli* from pistachio nuts. Mycopathologia 67, 123–127.

Moruno, E.G., Sanlorenzo, C., Boccaccino, B., Di Stefano, R., 2005. Treatment with yeast to reduce the concentration of ochratoxin A in red wine. Am. J. Enol. Vitic. 56, 73–76.

Muthomi, J.W., Ndung'u, J.K., Gathumbi, J.K., Mutitu, E.W., Wagacha, J.M., 2008. The occurrence of *Fusarium* species and mycotoxins in Kenyan wheat. Crop Prot. 27, 1215–1219.

Ng, W., Mankotia, M., Pantazopoulos, P., Neil, R.J., Scott, P.M., 2004. Ochratoxin A in wine and grape juice sold in Canada. Food Addit. Contam. 21, 971–981.

Nguyen, K.T.N., Ryu, D., 2014. Concentration of ochratoxin A in breakfast cereals and snacks consumed in the United States. Food Control 40, 140–144.

Niles, E.., Norman, J.., Pimbley, D., 1985. Growth and aflatoxin production of *Aspergillus flavus* in wheat and barley. Trans Br Mycol Soc 84, 259–266.

Noonim, P., Mahakarnchanakul, W., Varga, J., Frisvad, J.C., Samson, R.A., 2008. Two novel species of *Aspergillus* section *Nigri* from Thai coffee beans. Int. J. Syst. Evol. Microbiol. 58, 1727–1734.

Olivares-Marín, M., Del Prete, V., Garcia-Moruno, E., Fernández-González, C., Macías-García, A., Gómez-Serrano, V., 2009. The development of an activated carbon from cherry stones and its use in the removal of ochratoxin A from red wine. Food Control 20, 298–303.

Ospital, M., Cazabeil, J.M., Betbeder, A.M., Tricard, C., Creppy, E., Medina, B., 1998. L'ochratoxine A dans les vins. Rev Fr Oenol 169, 16–18.

Palermo, D., Pietrobono, P., Palermo, C., Rotunno, T., 2002. Occurrence of Ochratoxin A in cereals from Puglia (Italy). Ital. J. Food Sci. 14, 447–453.

Palumbo, J.D., O'Keeffe, T.L., Mcgarvey, J.A., 2011. Incidence of fumonisin B2 production within Aspergillus section nigri populations isolated from california raisins. J. Food Prot. 74, 672–675.

Pardo, E., Marín, S., Sanchis, V., Ramos, A.J., 2005. Impact of relative humidity and temperature on visible fungal growth and OTA production of ochratoxigenic *Aspergillus ochraceus* isolates on grapes. Food Microbiol. 22, 383–389.

Pardo, E., Marín, S., Solsona, A., Sanchis, V., Ramos, A.J., 2004. Modeling of germination and growth of ochratoxigenic isolates of *Aspergillus ochraceus* as affected by water activity and temperature on a barley-based medium. Food Microbiol. 21, 267–274.

Park, D.L., 2002. Effect of processing on aflatoxin. Mycotoxins and Food Safety. Advances in Experimental Medicine and Biology, 504, 173-179.

Park, J.W., Chung, S.-H., Kim, Y.-B., 2005. Ochratoxin A in Korean food commodities: Occurrence and safety evaluation. J. Agric. Food Chem. 53, 4637–4642.

Pascale, M., Haidukowski, M., Lattanzio, V.M.T., Silvestri, M., Ranieri, R., Visconti, A., 2011. Distribution of T-2 and HT-2 toxins in milling fractions of durum wheat. J. Food Prot. 74, 1700–1707.

Paterson, R.R.M., Lima, N., 2010. How will climate change affect mycotoxins in food? Food Res. Int. 43, 1902–1914.

Paterson, R.R.M., Lima, N., 2011. Further mycotoxin effects from climate change. Food Res. Int. 44, 2555–2566.

Perrone, G., Nicoletti, I., Pascale, M., De Rossi, A., De Girolamo, A., Visconti, A., 2007a. Positive correlation between high levels of ochratoxin A and resveratrol-related compounds in red wines. J. Agric. Food Chem. 55, 6807–6812.

Perrone, G., Stea, G., Epifani, F., Varga, J., Frisvad, J.C., Samson, R.A., 2011. *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*. Fungal Biol. 115, 1138–1150.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakamchanakul, W., Samson, R.A., 2007b. Biodiversity of *Aspergillus* species in some important agricultural products. Stud. Mycol. 59, 53–66.

Peterson, S.W., Ito, Y., Horn, B.W., Goto, T., 2002. *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, A. nomius. Mycologia 93, 689–703.

Petruzzi, L., Corbo, M.R., Sinigaglia, M., Bevilacqua, A., 2014. Yeast cells as adsorbing tools to remove ochratoxin A in a model wine. Int. J. Food Sci. Technol. 49, 936–940.

Pietri, A., Bertuzzi, T., Pallaroni, L., Piva, G., 2001. Occurrence of ochratoxin A in Italian wines. Food Addit. Contam. 18, 647–654.

Pildain, M.B., Frisvad, J.C., Vaamonde, G., Cabral, D., Varga, J., Samson, R.A., 2008. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. Int. J. Syst. Evol. Microbiol. 58, 725–735.

Piotrowska, M., Zakowska, Z., 2005. The elimination of ochratoxin A by lactic acid bacteria strains. Pol. J. Microbiol. 54, 279–286.

Pitt, J.I., Taniwaki, M.H., Cole, M.B., 2013. Mycotoxin production in major crops as influenced by growing, harvesting, storage and processing, with emphasis on the achievement of Food Safety Objectives. Food Control 32, 205–215.

Pluyer, H.R., Ahmed, E.M., Wei, C.I., 1987. Destruction of aflatoxins on peanuts by oven- and microwave-roasting. J. Food Prot. 50, 504–508.

Ponsone, M.L., Combina, M., Dalcero, A., Chulze, S., 2007. Ochratoxin A and ochratoxigenic *Aspergillus* species in Argentinean wine grapes cultivated under organic and non-organic systems. Int. J. Food Microbiol. 114, 131–135.

Pour, R.S., Rasti, M., Zighamian, H., Daraei Garmakhani, A., 2010. Occurrence of aflatoxins in pistachio nuts in esfahan province of Iran. J. Food Saf. 30, 330–340.

Prickett, A.., MacDonald, S., Wildey, K.., 2000. Survey of mycotoxins in stored grain from the 1999 harvest in the U.K. Home-Grown Cereals Authority (HGCA).

Quintela, S., Villarán, M.C., López de Armentia, I., Elejalde, E., 2012a. Ochratoxin A in Spanish exportation wine market. Food Control 25, 501–504.

Quintela, S., Villarán, M.C., López de Armentia, I., Elejalde, E., 2012b. Ochratoxin A removal in wine: A review.

Rahmani, A., Soleimany, F., Hosseini, H., Nateghi, L., 2011. Survey on the occurrence of aflatoxins in rice from different provinces of Iran. Food Addit. Contam. Part B Surveill. 4, 185–190.

Ramos, A.J., 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. Int. J. Food Microbiol. 44, 133–140.

Rank, C., Nielsen, K.F., Larsen, T.O., Varga, J., Samson, R.A., Frisvad, J.C., 2011. Distribution of sterigmatocystin in filamentous fungi. Fungal Biol. 115, 406–420.

Raters, M., Matissek, R., 2008. Thermal stability of aflatoxin B_1 and ochratoxin A. Mycotoxin Res. 24, 130–134.

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.

Reinhold, L., Reinhardt, K., 2011. Mycotoxins in foods in Lower Saxony (Germany): Results of official control analyses performed in 2009. Mycotoxin Res. 27, 137–143.

Remiro, R., Irigoyen, A., González-Peñas, E., Lizarraga, E., López de Cerain, A., 2013. Levels of ochratoxins in Mediterranean red wines. Food Control 32, 63–68.

Riba, A., Bouras, N., Mokrane, S., Mathieu, F., Lebrihi, A., Sabaou, N., 2010. *Aspergillus* section *Flavi* and aflatoxins in Algerian wheat and derived products. Food Chem. Toxicol. 48, 2772–2777.

Riba, A., Mokrane, S., Mathieu, F., Lebrihi, A., Sabaou, N., 2008. Mycoflora and ochratoxin A producing strains of *Aspergillus* in Algerian wheat. Int. J. Food Microbiol. 122, 85–92.

Ritieni, A., Moretti, A., Logrieco, A., Bottalico, A., Randazzo, G., Monti, S.M., Ferracane, R., Fogliano, V., 1997. Occurrence of Fusaproliferin, Fumonisin B1, and Beauvericin in Maize from Italy. J. Agric. Food Chem. 45, 4011–4016.

Rodrigues, P., Venâncio, A., Kozakiewicz, Z., Lima, N., 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* isolated from Portuguese almonds. Int. J. Food Microbiol. 129, 187–193.

Rosa, C.A.R., Magnoli, C.E., Fraga, M.E., Dalcero, A.M., Santana, D.M.N., 2004. Occurrence of ochratoxin A in wine and grape juice marketed in Rio de Janeiro, Brazil. Food Addit. Contam. 21, 358–364.

Roscoe, V., Lombaert, G.A., Huzel, V., Neumann, G., Melietio, J., Kitchen, D., Kotello, S., Krakalovich, T., Trelka, R., Scott, P.M., 2008. Mycotoxins in breakfast cereals from the Canadian retail market: A 3-year survey. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 25, 347–355.

Ruíz Bejarano, M.J., Rodríguez Dodero, M.C., Barroso, C.G., 2010. Optimizing the process of making sweet wines to minimize the content of ochratoxin A. J. Agric. Food Chem. 58, 13006–13012.

Rusanova, T.Y., Beloglazova, N.V., Goryacheva, I.Y., Lobeau, M., Van Peteghem, C., De Saeger, S., 2009. Non-instrumental immunochemical tests for rapid ochratoxin A detection in red wine. Anal. Chim. Acta 653, 97–102.

Rustom, I.Y.S., 1997. Aflatoxin in food and feed: Occurrence, legislation and inactivation by physical methods. Food Chem. 59, 57–67.

Sabatini, A., Danieli, P.P., Bernabucci, U., Ronchi, B., 2007. Evaluation of mycotoxins contamination in intensive beef cattle production system. Ital. J. Anim. Sci. 6, 466–468.

Sage, L., Garon, D., Seigle-Murandi, F., 2004. Fungal microflora and ochratoxin A risk in French vineyards. J. Agric. Food Chem. 52, 5764–5768.

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.

Salaha, M.I., Metafa, M., Lanaridis, P., 2007. Ochratoxin a occurrence in greek dry and sweet wines. J. Int. Sci. Vigne Vin 41, 225–230.

Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F.A., Frank, J.M., Frisvad, J.C., 2004. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. Stud. Mycol. 50, 45–61.

Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J., 2007. Diagnostic tools to identify black aspergilli. Stud. Mycol. 59, 129–145.

Sarigiannis, Y., Kapolos, J., Koliadima, A., Tsegenidis, T., Karaiskakis, G., 2014. Ochratoxin A levels in Greek retail wines. Food Control 42, 139–143.

Saunders, D.S., Meredith, F.I., Voss, K.A., 2001. Control of fumonisin: Effects of processing. Environ. Health Perspect. 109, 333–336.

Savino, M., Limosani, P., Garcia-Moruno, E., 2007. Reduction of ochratoxin A contamination in red wines by oak wood fragments. Am. J. Enol. Vitic. 58, 97–101.

Schatzki, T.F., 1995. Distribution of aflatoxin in pistachios. 2. Distribution in freshly harvested pistachios. J. Agric. Food Chem. 43, 1566–1569.

Schatzki, T.F., Pan, J.L., 1996. Distribution of aflatoxin in pistachios. 3. Distribution in pistachio process streams. J. Agric. Food Chem. 44, 1076–1084.

Schatzmayr, G., Streit, E., 2013. Global occurrence of mycotoxins in the food and feed chain: facts and figures. World Mycotoxin J. 6, 213–222.

Schmidt-Heydt, M., Rüfer, C.E., Abdel-Hadi, A., Magan, N., Geisen, R., 2010. The production of aflatoxin B1 or G1 by *Aspergillus parasiticus* at various combinations of temperature and water activity is related to the ratio of aflS to aflR expression. Mycotoxin Res. 26, 241–246.

Schothorst, M.V., 1998. Principles for the establishment of microbiological food safety objectives and related control measures. Food Control 9, 379–384.

Scudamore, K.A., Banks, J., MacDonald, S.J., 2003. Fate of ochratoxin A in the processing of whole wheat grains during milling and bread production. Food Addit. Contam. 20, 1153–1163.

Sedaghati, E., Khodaygan, P., Kocsubé, S., Varga, J., Nadi, M., 2012. Evaluation of ochratoxin A production by *Aspergillus ochraceus* group isolated from pistachio orchards.

Sedaghati, E., Nikkhah, M.J., Zare, R., Fotuhifar, K.B., Kocsubé, S., Vágvölgyi, C., Varga, J., 2011. Molecular identification of potentialy mycotoxigenic black Aspergilli contaminating pistachio nuts in Iran. Acta Aliment. 40, 65–70.

Serra, R., Braga, A., Venâncio, A., 2005. Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. Res. Microbiol. 156, 515–521.

Serra, R., Cabañes, F.J., Perrone, G., Castellá, G., Venâncio, A., Mulè, G., Kozakiewicz, Z., 2006a. *Aspergillus ibericus*: A new species of section Nigri isolated from grapes. Mycologia 98, 295–306.

Serra, R., Lourenço, A., Alípio, P., Venâncio, A., 2006b. Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species. Mycol. Res. 110, 971–978.

Serrano, A.B., Meca, G., Font, G., Ferrer, E., 2012. Risk assessment associated to the intake of the emerging *Fusarium* mycotoxins BEA, ENs and FUS present in infant formula of Spanish origin. Food Control 28, 178–183.

Set, E., Erkmen, O., 2010. The aflatoxin contamination of ground red pepper and pistachio nuts sold in Turkey. Food Chem. Toxicol. 48, 2532–2537.

Shakerardekani, A., Karim, R., 2013. Effect of different types of plastic packaging films on the moisture and aflatoxin contents of pistachio nuts during storage. J. Food Sci. Technol. 50, 409–411.

Shotwell, O.L., Goulden, M.L., Hesseltine, C.W., Dickens, J.., Kwolek, W.F., 1974. Aflatoxin: distribution in contaminated corn plants. Cereal Chem 57, 206–208.

Sifou, A., Meca, G., Serrano, A.B., Mahnine, N., El Abidi, A., Mañes, J., El Azzouzi, M., Zinedine, A., 2011. First report on the presence of emerging *Fusarium* mycotoxins enniatins (A, A_1, B, B_1), beauvericin and fusaproliferin in rice on the Moroccan retail markets. Food Control 22, 1826–1830.

Soleas, G.J., Yan, J., Goldberg, D.M., 2001. Assay of ochratoxin A in wine and beer by high-pressure liquid chromatography photodiode array and gas chromatography mass selective detection. J. Agric. Food Chem. 49, 2733–2740.

Solfrizzo, M., Avantaggiato, G., Panzarini, G., Visconti, A., 2010. Removal of ochratoxin A from contaminated red wines by repassage over grape pomaces. J. Agric. Food Chem. 58, 317–323.

Somma, S., Perrone, G., Logrieco, A.F., 2012. Diversity of black aspergilli and mycotoxin risks in grape, wine and dried vine fruits.

Soriano del Castillo, J.M., 2007. Micotoxinas En Alimentos. Diaz de Santos Madrid, España.

Soufleros, E.H., Tricard, C., Bouloumpasi, E.C., 2003. Occurrence of ochratoxin A in Greek wines. J. Sci. Food Agric. 83, 173–179.

Spadaro, D., Lorè, A., Garibaldi, A., Gullino, M.L., 2010. Occurrence of ochratoxin A before bottling in DOC and DOCG wines produced in Piedmont (Northern Italy). Food Control 21, 1294–1297.

Spadaro, D., Patharajan, S., Lorè, A., Garibaldi, A., Gullino, M.L., 2012. Ochratoxigenic black species of *aspergilli* in grape fruits of northern Italy identified by an improved PCR-RFLP procedure. Toxins 4, 42–54.

Stefanaki, I., Foufa, E., Tsatsou-Dritsa, A., Dais, P., 2003. Ochratoxin A concentrations in Greek domestic wines and dried vine fruits. Food Addit. Contam. 20, 74–83.

Sydenham, E.W., Van Der Westhuizen, L., Stockenstrom, S., Shephard, G.S., Thiel, P.G., 1994. Fumonisin-contaminated maise: Physical treatment for the partial decontamination of bulk shipments. Food Addit. Contam. 11, 25–32.

Tam, J., Mankotia, M., Mably, M., Pantazopoulos, P., Neil, R.J., Calway, P., Scott, P.M., 2006. Survey of breakfast and infant cereals for aflatoxins B1, B2, G1 and G2. Food Addit. Contam. 23, 693–699.

Taniwaki, M.H., Pitt, J.I., Teixeira, A.A., Iamanaka, B.T., 2003. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. Int. J. Food Microbiol. 82, 173–179.

Tateo, F., Bononi, M., Lubian, E., 2000. Survey on ochratoxin A in wines. Data concerning the market of table wines in brik. Bull. OIV 73, 773–783.

Terra, M.F., Prado, G., Pereira, G.E., Ematné, H.J., Batista, L.R., 2013. Detection of ochratoxin A in tropical wine and grape juice from Brazil. J. Sci. Food Agric. 93, 890–894.

Terzi, V., Tumino, G., Stanca, A.M., Morcia, C., 2014. Reducing the incidence of cereal head infection and mycotoxins in small grain cereal species. J. Cereal Sci. 59, 284–293.

Thuvander, A., Möller, T., Enghardt Barbieri, H., Jansson, A., Salomonsson, A.-C., Olsen, M., 2001. Dietary intake of some important mycotoxins by the Swedish population. Food Addit. Contam. 18, 696–706.

Tirado, M.C., Clarke, R., Jaykus, L.A., McQuatters-Gollop, A., Frank, J.M., 2010. Climate change and food safety: A review. Food Res. Int. 43, 1745–1765.

Turcotte, A.-M., Scott, P.M., Tague, B., 2013. Analysis of cocoa products for ochratoxin A and aflatoxins. Mycotoxin Res. 29, 193–201.

Uhlig, S., Torp, M., Heier, B.T., 2006. Beauvericin and enniatins A, A1, B and B1 in Norwegian grain: A survey. Food Chem. 94, 193–201.

UNEP, 2002, United Nation Environment Programme. Environmental effects of ozone depletion and its interactions with climate change: 2002 Assessment. Nairobi, Kenia. ISBN 92-807-2312-X. Online document request: www.gcrio.org/ordering/

Vaamonde, G., Patriarca, A., Fernández Pinto, V., Comerio, R., Degrossi, C., 2003. Variability of aflatoxin and cyclopiazonic acid production by Aspergillus section flavi from different substrates in Argentina. Int. J. Food Microbiol. 88, 79–84.

Valenta, H., 1998. Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids. J. Chromatogr. A 815, 75–92.

Van Der Merwe, K.J., Steyn, P.S., Fourie, L., Scott, D.B., Theron, J.J., 1965. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. [20]. Nature 205, 1112–1113.

Var, I., Kabak, B., 2007. Occurrence of ochratoxin A in Turkish wines. Microchem. J. 86, 241–247.

Var, I., Kabak, B., Erginkaya, Z., 2008. Reduction in ochratoxin A levels in white wine, following treatment with activated carbon and sodium bentonite. Food Control 19, 592–598.

Varga, J., Frisvad, J.C., Samson, R.A., 2009. A reappraisal of fungi producing aflatoxins. World Mycotoxin J. 2, 263–277.

Varga, J., Frisvad, J.C., Samson, R.A., 2011. Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud. Mycol. 69, 57–80.

Varga, J., Kocsubé, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., Tóth, B., Bartók, T., 2010. Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin. Int. J. Food Microbiol. 143, 143–149.

Varga, J., Kocsubé, S., Tóth, B., Frisvad, J.C., Perrone, G., Susca, A., Meijer, M., Samson, R.A., 2007. *Aspergillus brasiliensis* sp. nov., a biseriate black *Aspergillus* species with world-wide distribution. Int. J. Syst. Evol. Microbiol. 57, 1925–1932.

Varga, J., Kozakiewicz, Z., 2006. Ochratoxin A in grapes and grape-derived products. Trends Food Sci. Technol. 17, 72–81.

Varga, J., Kozakiewicz, Z., 2006. Ochratoxin A in grapes and grape-derived products. Trends Food Sci. Technol. 17, 72–81.

Vargas, E.A., Whitaker, T.B., Dos Santos, E.A., Slate, A.B., Lima, F.B., Franca, R.C.A., 2005. Testing green coffee for ochratoxin A, part II: Observed distribution of ochratoxin a test results. J. AOAC Int. 88, 780–787.

Vega, M., Muñoz, K., Sepúlveda, C., Aranda, M., Campos, V., Villegas, R., Villarroel, O., 2009. Solid-phase extraction and HPLC determination of Ochratoxin A in cereals products on Chilean market. Food Control 20, 631–634.

Venkata Reddy, K., Naveen, K., Bhaskar Reddy, I., 2013. Incidence and molecular detection of ochratoxigenic fungi from Indian cereal grains. Int. J. Pharma Bio Sci. 4, B31–B40.

Vidal, A., Marín, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market. Food Chem. Toxicol. 53, 133–138.

Visconti, A., Haidukowski, E.M., Pascale, M., Silvestri, M., 2004. Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. Toxicol. Lett. 153, 181–189.

Visconti, A., Pascale, M., Centonze, G., 1999. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. J. Chromatogr. A 864, 89–101.

Visconti, A., Perrone, G., Cozzi, G., Solfrizzo, M., 2008. Managing ochratoxin A risk in the grape-wine food chain. Food Addit. Contam. Part A 25, 193–202.

Waltking, A.E., 1971. Fate of aflatoxin during roasting and storage of contaminated peanut products. J. Assoc. Off. Anal. Chem. 54, 533–539.

Wei, D.L., Jong, S.C., 1986. Production of aflatoxins by strains of the Aspergillus flavus group maintained in ATCC. Mycopathologia 93, 19–24.

Whitaker, T.B., Hagler Jr., W.M., Johansson, A.S., Giesbrecht, F.G., Trucksess, M.W., 2001. Distribution among sample test results when testing shelled corn lots for fumonisin. J. AOAC Int. 84, 770–776.

Whitaker, T.B., Jr, W.M.H., Giksbrecht, F.G., Johansson, A.S., 2000. Sampling, sample preparation, and analytical variability associated with testing wheat for deoxynivalenol. J. AOAC Int. 83, 1285–1292.

Whitaker, T.B., Slate, A., Birmingham, T., Adams, J., Jacobs, M., Gary, G., 2010. Correlation between aflatoxin contamination and various USDA grade categories of shelled almonds. J. AOAC Int. 93, 943–947.

Whitaker, T.B., Slate, A.B., Hurley, J.M., Giesbrecht, F.G., 2007. Sampling almonds for aflatoxin, part II: Estimating risks associated with various sampling plan designs. J. AOAC Int. 90, 778–785.

Whitaker, T.B., Slate, A.B., Jacobs, M., Hurley, J.M., Adams, J.G., Giesbrecht, F.G., 2006. Sampling almonds for aflatoxin, part I: Estimation of uncertainty associated with sampling, sample preparation, and analysis. J. AOAC Int. 89, 1027–1034.

Whitaker, T.B., Trucksess, M.W., Weaver, C.M., Slate, A., 2009. Sampling and analytical variability associated with the determination of aflatoxins and ochratoxin A in bulk lots of powdered ginger marketed in 1-lb bags. Anal. Bioanal. Chem. 395, 1291–1299.

Wilson, S.C., Brasel, T.L., Carriker, C.G., Fortenberry, G.D., Fogle, M.R., Martin, J.M., Wu, C., Andriychuk, L.A., Karunasena, E., Straus, D.C., 2004. An investigation into techniques for cleaning mold-contaminated home contents. J. Occup. Environ. Hyg. 1, 442–447.

WMO, 2010. World Meteorological Organization, Scientific Assessment of Ozone Depletion: 2010, Global Ozone Research and Monitoring Project-Report No. 52, 516 pp., Geneva, Switzerland, 2011.

WMO, 2013. World Meteorological Organization, The global climate 2001-2010 a decade of climate extremes Summary Report. Nº 1103.

Wontner-Smith, T.J., Bruce, D.M., Cardwell, S.K., Armitage, D.M., Jennings, P., 2014. Ochratoxin A production during ambient-air drying. J. Stored Prod. Res. 56, 1–7.

Wu, F., Bhatnagar, D., Bui-Klimke, T., Carbone, I., Hellmich, R., Munkvold, G., Paul, P., Payne, G., Takle, E., 2010. Climate change impacts on mycotoxin risks in US maize. World Mycotoxin J. 4, 79–93.

Yazdani, D., Abidin, M.A.Z., Tan, Y.H., Kamaruzaman, S., 2011. Molecular identification of *Aspergillus* and *Eurotium* species isolated from rice and their toxin-producing ability. Microbiology 80, 720–727.

Yazdanpanah, H., Mohammadi, T., Abouhossain, G., Cheraghali, A.M., 2005. Effect of roasting on degradation of Aflatoxins in contaminated pistachio nuts. Food Chem. Toxicol. 43, 1135–1139.

Yentür, G., Er, B., Gür Özkan, M., Bayhan Öktem, A., 2006. Determination of aflatoxins in peanut butter and sesame samples using high-performance liquid chromatography method. Eur. Food Res. Technol. 224, 167–170.

Zaied, C., Abid, S., Zorgui, L., Bouaziz, C., Chouchane, S., Jomaa, M., BACHA, H., 2009. Natural occurrence of ochratoxin A in Tunisian cereals. Food Control 20, 218–222.

Zimmerli, B., Dick, R., 1996. Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. Food Addit. Contam. 13, 655–668.

Zinedine, A., Blesa, J., Mahnine, N., El Abidi, A., Montesano, D., Mañes, J., 2010. Pressurized liquid extraction coupled to liquid chromatography for the analysis of ochratoxin A in breakfast and infants cereals from Morocco. Food Control 21, 132–135.

Zinedine, A., Brera, C., Faid, M., Benlemlih, M., Miraglia, M., 2007. Ochratoxin A and *Fusarium* toxins in cereals from Morocco. Cah. Agric. 16, 11–15.

Zotti, M., Montemartini Corte, A., 2002. Aspergillus persii: A new species in *Circumdati* section. Mycotaxon 83, 269–278.

IV. RESEARCH WORK

IV: RESEARCH WORK

PART I:

Selection of raw materials

STUDY I

Risk management towards Food Safety Objective achievement regarding to mycotoxins in pistachio: the sampling and measurement uncertainty issue

García-Cela E., Ramos A.J., Sanchis V. and Marin S.

Food Technology Department, Lleida University, XaRTA-UTPV, CRA, Rovira Roure 191, 25198 Lleida, Spain

ABSTRACT

The emerging risk management metrics, FSO, PO and PC, were applied to the aflatoxins (AFs) and ochratoxin A (OTA) determination in 8 commercial lots of pistachio. In order to determine the sampling uncertainty, two sampling plans (EU official samping and company plan) in quadruplicate, and two analytical methods (ELISA and HPLC), were considered in parallel. The combination of EU official sampling plan and HPLC proved to be the most appropriate option. The major variability was associated with the subfraction selection and, therefore, increasing the number of the analyzed subfractions could be an alternative for reducing uncertainty. AFs were present in all lots, mainly AFB_1 and AFB_2, while OTA was never detected. The effect of toasting on AFs presence in pistachio (a performance criteria, PC) was evaluated in order to achieve a given PO, taking into account the FSO, i.e., the EC limits. Percentages of AFs reduction were 87.62%±11.89, 81.05%±15.51 and 86.74%±11.31 for AFB_1, AFB_2 and total AFs, respectively. Given an initial AFB_1 and AFs level ≤ 12 μg/kg and ≤ 15 μg/kg, respectively, the toasting would ensure the AFB_1 and AFs legal limits compliance before human consumption (FSO).

Publication: Food Control, 31 (2013), 392-402.

1. INTRODUCTION

The Food Safety Objective (FSO) for a hazard is the maximum frequency and/or concentration of the hazard in a food at the time of consumption, and is preceded by the Performance Objective (PO), which is the maximum frequency and/or concentration of the hazard in a food at a specified step in the food chain before the time of consumption (ICMSF, 2002), that still provides or contributes to the achievement of an FSO or Appropriate Level of Protection (ALOP), as applicable. In the case of chemical hazards such as mycotoxins, the limits set by a country for mycotoxins in foods can be logically considered also to have the status of an FSO.

Nuts present low a_W and, due to their intrinsic characteristics, fungi are the major microbiological contaminants. Some of these moulds are mycotoxigenic, thus high levels of mycotoxins have frequently been reported in nuts from the orchards and from the market (Bayman et al., 2002; Fernane et al., 2010a). In pistachios, the dominant mycobiota are *Aspergillus* section *Nigri*, *Aspergillus flavus* and *Penicillium* spp. (Denizel et al., 1976, Fernane et al., 2010b). Several studies have reported that *Aspergillus* spp. causes decay in pistachio nuts at different parts of the world, such as California (USA) (Doster & Michailides, 1994), Iran (Mojtahedi et al., 1979), and Turkey (Denizel et al., 1976). The most important mycotoxins found in pistachio are aflatoxins (AFs), including aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) and ochratoxin A (OTA). The International Agency for Research on Cancer (IARC) classified AFs in group 1, as human carcinogens, and OTA in group 2B, as a possible human carcinogen (IARC, 2002).

On the regulatory side, legally binding, EU-wide maximum levels (MLs) for mycotoxins in food have been introduced by the European Commission and updated subsequently. Nowadays the Comission of the European Communities (EC) has established maximum levels of mycotoxins in nuts to be subjected to sorting, or other physical treatment. These established values should led processing companies to accept only those raw material batches which allow compliance with the final PO of the company in the final product. Regarding pistachio, toasting is the main way, together with physical separation, to reduce the levels of mycotoxins. Removal of highly contaminated pistachio nuts by sorting decreases AFs contamination by 2 to 4 times in processed pistachios compared to non-processed pistachios (Schatzki, 1996). However, conflicting results have been published about the effect of the heat treatment in AFs in pistachios (Ariño et al., 2009; Yazdanpanah et al, 2005).

In order to determine mycotoxin presence in foods, sampling and analysis are needed, despite a large variability and uncertainty is associated with these procedures. Mycotoxin determination is a multistage process and consists of three distinct phases: sampling, sample preparation and analysis. It is assumed that the total uncertainty associated with the AFs test procedure is the sum of the uncertainty associated with the three steps (Withaker et al., 2006). However most variability is due to sampling; in hazelnuts and almonds this step accounts for 96.2 to 99.4% of the total variability, respectively (Ozay et al., 2006, Whitaker et al., 2006). Also the Codex Alimentarius (CAC) proposed formula for calculating variances associated with the AFs test procedure for hazelnuts, almonds and pistachio (CAC, 2008).

Sampling and subsampling procedures should be designed according to the mycotoxin distribution. Mycotoxins are heterogeneously distributed, and the general rule is that the bigger individual particles or seeds the greater the sampling problems. Cucullu et al. (1966) reported that most individual peanuts have zero AFs concentration, but occasionally a peanut may have an extremely high concentration of AFs. Other studies also showed the heterogeneous distribution of AFs in other substrates such as cottonseed, pistachios and corn (Cucullu et al., 1977; Johanson et al, 2000; Schatzki, 1995; Shotwell et al., 1974). A common practice to reduce the heterogeneity of mycotoxins in commodities when sampling is through the formation of aggregate samples, thus, the sample should be an accumulation of many small portions taken from many different locations (Parker et al., 1982). However, as a result of this practice, the spatial information, variability and distribution of the mycotoxin is lost (Rivas et al., 2009).

For these reasons, harmonisation process for mycotoxin establishing maximum limits and sampling plans are necessary to protect consumer health and facilitate international trading. The Codex Committee on Contaminants in Foods (CCCF) has established maximum levels of AFs and sampling plans, where two 10 kg laboratory samples are needed in the case of Ready to Eat (RTE) lots, both containing less than 10 μg/kg AFs. In the case of further processing products (DFP); a single 20 kg laboratory sample taken from a lot must result in less than 15 μg/kg AFs in order to be accepted (CAC, 2008).

EU maximum limits for AFs in nuts have been recently changed (Commission Regulation 1881/2006 amended by Regulation 165/2010) after European Food Safety Agency (EFSA)

reviewed the maximum limits and intake assessment for tree nuts concluding that there was no additional consumer concern at 4, 8, 10 or 15 μg/kg AFs (EFSA, 2007) in the context of exposure from all other sources and previous pertinent exposure assessments. Regarding to OTA, although its occurrence in nuts has been reported in several studies, even by the Rapid Alert System for Food and Feed (RASFF, 2011), its presence has always been significantly lower than AFs, and indeed maximum levels have not been set by the European Commission in nuts. Consequently, taking into account the developments by the CAC and considering the recently established European maximum levels for mycotoxins in pistachios, the sampling procedure for tree nuts in Regulation (EC) No 401/2006 was afterwards amended (EC No 178/2010), maintaining the number of incremental samples but decreasing the weight of the incremental sample to 10 kg for lots higher than 15 t and between 1 and 10 kg for lots equal or less than 15 t. Also the number of laboratory samples from an aggregate sample decreased in lots higher than 5 t. Finally, when it is not possible to carry out the sampling method described above because of unacceptable commercial consequences resulting from damage to the lot (because of packaging forms, means of transport, etc.), an alternative method of sampling could be applied provided that it is as representative as possible and is fully described and documented.

However, while these plans aim to harmonize official sampling regimes, they have been criticized for the unrealistic need of workforce. In fact on the European cereal trading sector, 73-77% of the companies prefer their own sampling method for their quality control programs instead of the official method (Siegel & Babuscio, 2011). Commission Regulation 401/2006 allows the use of alternative sampling methods in cases of unacceptable commercial consequences or practical unfeasibility of the official method but only in case of quality control (Comission of the European Communities, 2006a).

Another important consideration in risk management is the analytical method used. Although the uncertainty is not as high as in the sampling, sensitive and reliable methods are required for mycotoxin detection. Companies require simple, fast and cheap methods, and ELISA and HPLC are the most demanded respectively for internal and external analyses (Siegel & Babuscio, 2011).

A recent review work García-Cela et al. (2012) highlighted the lack of existing information regarding performance criteria (PC) in pistachio processing as a key aspect in AFs risk

management. The aim of this study was to evaluate the PC or effect of toasting on mycotoxin in pistachio, in order to achieve a given PO. PC cannot be described if methods to assess sampling uncertainty are not in place, thus, in parallel, the impact of sampling and measurement uncertainty was also evaluated.

2. MATERIALS AND METHODS

2.1. Samples and sampling plans

Eight lots (n=8) of pistachio, weighted more than 15 t each, were sampled using two different sampling plans before and after industrial toasting. Industrial toasting included two main steps: pre-toasting (≈135 ºC) and toasting (≈165 ºC) during a total time of 20 min. However, the temperatures could change in a range of 6 ºC depending on the initial characteristics of the product.

Sampling plan A. Sampling of raw and toasted pistachio was made according to Commission Regulation (EC) No. 178/2010, and more specifically according to D.2. point, "method of sampling for groundnuts (peanuts), other oilseeds, apricot kernels and tree nuts". Twenty kg aggregate samples were obtained from 100 elemental samples of 200 g of each sampled lot. The composite samples were mixed and divided into two equal sub-samples of 10 kg (subsample) before grinding. To that purpose a Romer Analytical Sampling Mill (RAS® Mill, Coring-System Diagnostix GmbH, Gernsheim, Germany) was used. The RAS® Mill was specifically developed for products that are difficult to grind due to their hardness together with a high moisture and/or high oil content, like pistachios. Samples were kept at 4 ºC until analysis. After grinding, six sub-fractions of 50 g were taken from each sub-sample. Sub-fractions were stored at 4 ºC until analysis. Finally, two sub-fractions were analyzed in different days to account for the variability between days in duplicate (**Figure 1a**). The whole sampling plan was performed in quadruplicate.

Sampling plan B. Sampling of raw and toasted pistachio was made in this case following the current sampling plan used for the quality control of a Spanish nut processing company. Aggregate samples of 5 kg were obtained by pooling 20 elemental samples of 250 g from each sampled lot. The aggregate samples were mixed and 250 g (sub-sample) were taken and ground. After grinding, 10 g of each sub-fraction were analyzed in duplicate (**Figure 1b**). This sampling plan also was performed in quadruplicate.

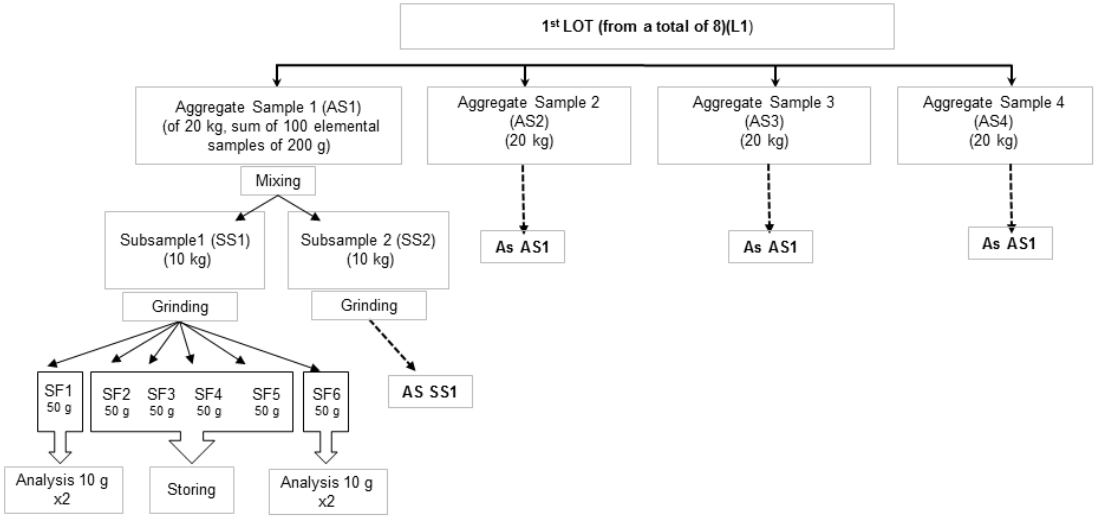


Figure 1a Design of the plan A from Official sample plan by quadruplicate.

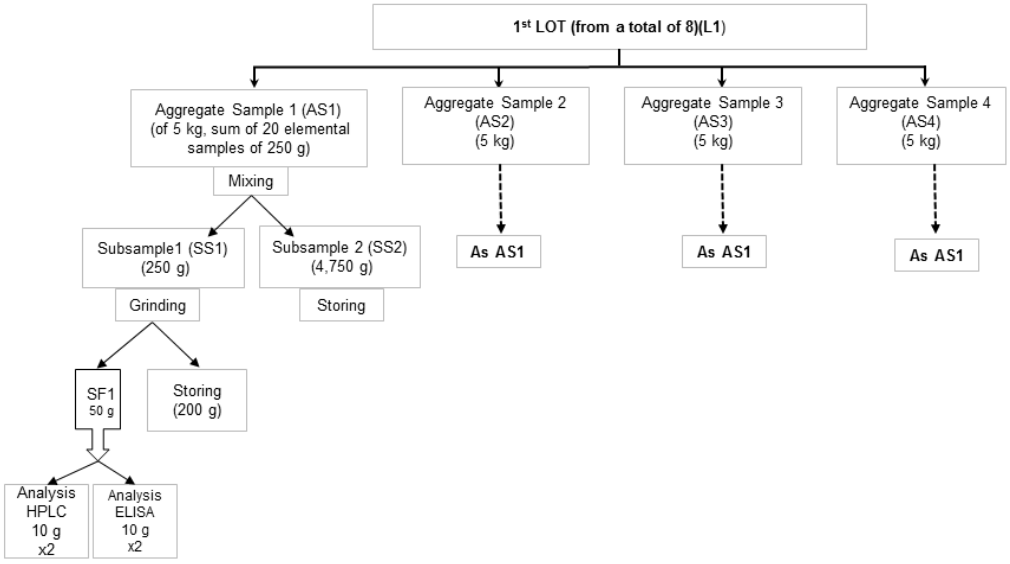


Figure 1b Design of the Plan B from Company sample plan by quadruplicate.

2.2. Analytical methods

For extraction and clean-up, an application note provided by R-Biopharm Rhône Ltd, Glasgow, UK, was used, slightly modified. Briefly, 10 g of ground pistachio were extracted using 40 mL of acetonitrile/water (60/40 v/v) by blending for 10 min; the extract was filtered through filter paper, and 2 ml of the filtrate were diluted with 18 mL of phosphate-buffered saline (PBS) pH=7.4. Then, the 20 mL diluted extract was purified by using an immunoaffinity chromatography column (Aflaochraprep®, R-Biopharm Rhône Ltd, Glasgow, UK) at a flow rate of 1-2 mL/min. Next, the column was washed with 20 mL of

PBS at 5 mL/min and dried by passing air through it. The AFs and OTA were eluted by gravity using 1 mL of HPLC grade methanol and applying backflushing three times to ensure the release of toxins into the solution. After that, 1 mL of Milli-Q water was passed through the column and collected in the same vial to give a total volume of 2 mL. The vials were sealed and kept under refrigeration (4 ºC) until quantification by HPLC. Simultaneous determination of AFs and OTA was done using HPLC (Waters, Milford, MA, USA), with a reverse-phase C18 silica gel column (Waters Spherisorb® 3 μm ODS2 4.6 × 150 mm, Milford, MA, USA) kept at 40 ºC, followed by fluorescence detection (Waters 2475 fluorescence detector, Waters, Milford, MA, USA). A post column photochemical derivatisation system (LC Tech detector, UVC 254 nm, Germany) was used for AFs.

The excitation and emission wavelengths were 360 and 455 nm (set from 0 to 17 min) for AFs and 335 and 460 nm (set from 17 to 20 min) for OTA. The injection volume was 100 μL. The mobile phase was pumped at a flow rate of 0.8 mL/min under the following gradient program: methanol, acetonitrile and acetic acid solution (0.1%), which started (0-10 min) with 27% methanol, 14% acetonitrile and 59% acetic acid (0.1%), then changed to gradient elution with 50% methanol and 50% acetonitrile (10-20 min), and finished with 27% methanol, 14% acetonitrile and 59% acetic acid solution for column re-equilibration (21-25 min). Under the conditions described retention time was 8.6, 9.9, 11.5, 13.5 and 18.3 min for AFG_2, AFG_1, AFB_2, AFB_1 and OTA, respectively.

The limit of detection (LOD), based on a signal to noise ratio of 3:1, was 0.312 μg/kg for OTA, 0.039 μg/kg for AFB_1 and AFG_1, and 0.020 μg/kg for AFB_2 and AFG_2. Recovery rates for OTA were 84%, 78% and 90% for 0.5, 8 and 12 μg/kg spiked levels. Precision was estimated by the relative standard deviation (RDSr), which was in the range of 8.39-15.48%. Recovery rates for 0.5 and 2.0 μg/kg spiked levels were 122% and 71% for AFB1 and 94% and 82% for AFG_1; and for 0.25 and 1.0 μg/kg were 120% and 73% for AFB_2 and 81% and 72% for AFG_2. RDSr for those concentrations were 7.99 and 12.8% for AFB_1, 12.04 and 11.26% for AFG_1, 22.20 and 3.01% for AFB_2 and 17.5 and 11.42% for AFG_2.

Additionally, ELISA was used for analysis of samples of sampling plan B. Competitive enzyme immunoassay kits from R-Biopharm AG (Darmstadt, Germany) were used (RIDASCREEN® Aflatoxin Total nº R4701 and RIDASCREEN® Ochratoxin A 30/15 nº R1311). The instructions given by the manufacturer were strictly followed. The results of the

analyses were obtained spectrophotometrically at 450 nm using a SEAC SIRIO S (Florence, Italy) microtitre spectrophotometer. Mycotoxin concentration in the samples was calculated using Ridasoft Win version 1.38 program. The ELISA kit measurable concentrations are from 1.25 to 45 μg OTA/kg and from 1.75 to 141.75 μg AFs/kg. The LOD were calculated based on the limits of detection given by the kit and the dilution factor of the method, and they were 1.25 and 1.75 μg/kg for OTA and AFs, respectively. The recovery rates for OTA were 107.9%, 84.6% and 72.8% for 2, 5 and 10 μg/kg and the RSDr was in the range of 8.27 to 18.87%. For AFs, recovery rates were 102.7%, 79.1% and 87.5% for 2, 5 and 20 μg/kg, with RSDr in the range of 0.55-7.19%.

Performance of HPLC and ELISA methods was in accordance to performance criteria established by Commission Regulation (EC) No. 401/2006 (Comission of the European Communities, 2006a), so these methods can be defined as acceptable.

Finally, the water activity (a_W) and moisture content (%) of each composite sample from sampling plan A were determined. Water activity was measured with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) and moisture content (%) (100 pistachio nuts) was calculated by sample weight difference before and after drying at 105 ºC overnight. A hundred whole nuts were taken at random separately from each composite, unshelled, and the percentage of edible part weight was determined. The calculated percentage of edible part was 55.36%, 57.09%, 56.14%, 56.23%, 56.64%, 55.94%, 55.53% and 56.26%, from lot 1 to lot 8, respectively.

In sections 3.1, 3.2, 3.3 and 3.4 AFs results are presented uncorrected by recovery and by % edible part.

2.3. Measurement uncertainty calculation

Measurement uncertainty was calculated according to the performance criteria for mycotoxin analysis (JCGM, 2008):

$$U = x_{corrected} \frac{RSD_R}{100} \times 1.96 \quad (1)$$

where U is the uncertainty associated with the analysis, RDSR was calculated as 1.5 times precision RSDr which is the relative standard deviation calculated from results generated under repeatability conditions (EC, 401/2006); $X_{corrected}$ is the mean value of the analyses corrected for the recovery rate and in the case of nuts also for percentage of shell; and 1.96 is the coverage factor for a level of confidence of approximately 95%. Two different values of RSDr and recovery rates were used according to the validated method for lower and higher than 1 μg/kg mycotoxin values.

2.4. Total uncertainty calculation

Total variability associated with estimation of the accurate mycotoxin concentration of a lot of in-shell nuts consists of sampling, sample preparation, and analytical step. Based on theoretical considerations and a model reported by Ozay et al. (2006), and assuming that a) each in-shell pistachio consists of a shell and an individual pistachio kernel, b) no mycotoxin contamination is associated with the shell, c) all shelled kernels have about the same mass and physical characteristics, and d) mycotoxin concentration varies from kernel to kernel, the variability among mycotoxin test results taken from the same lot can be represented by Equation 2.

$$\hat{C} = \mu + s + s_{ss} + s_{sp} + a \quad (2)$$

where $\hat{C}$ denotes the estimated mycotoxin concentration in a lot by measuring the mycotoxin concentration in a sample of individual nuts; μ, the accurate mycotoxin concentration in the lot being tested; s, ss, and ssp are random deviations of sample concentration around the true lot concentration due to sampling, subsampling and subsample preparation for laboratory analysis, with the expected value equal to zero and variances $\sigma^2_{\hat{C}(s)}$; $\sigma^2_{\hat{C}(ss)}$ and $\sigma^2_{\hat{C}(sp)}$; and a, random deviations of analytical assay with the expected value zero and variance $\sigma^2_{\hat{C}(a)}$. If independence among the random deviations in Equation 2 is assumed, the model for variance can be obtained by Equation 3.

$$\sigma^2_{\hat{C}(t)} = \sigma^2_{\hat{C}(s)} + \sigma^2_{\hat{C}(ss)} + \sigma^2_{\hat{C}(sp)} + \sigma^2_{\hat{C}(a)} \quad (3)$$

where $\sigma^2_{\hat{C}(t)}$ is the total variance associated with the measured mycotoxin concentration. The sampling variance, $\sigma^2_{\hat{C}(s)}$, represents the variability among aggregate sample concentrations taken from the same lot of nuts. Subsample variance, $\sigma^2_{\hat{C}(ss)}$, represents the variability among

replicate subsample concentrations taken from the same sample before grinding. Subfraction variance, $\sigma^2_{\hat{C}(sp)}$, represents the variability among replicate subfraction concentrations taken from the same comminuted subsample in a suitable mill. The analytical variance, $\sigma^2_{\hat{C}(a)}$, represents the variability among replicate analysis of a single subfraction.

The variance components, $s_{s+ss+sp}^2$ (sampling) and s_a^2 (analytical), for the total variance .s_t^2 (total) were estimated experimentally, through the nested design for each lot for both AFB_1 and AFs. Then sampling + subsample preparation and analytical variance components were plotted versus mycotoxin concentration and linear regressions in a full-log plot were calculated.

$$s_t^2 = s_{s+ss+sp}^2 + s_a^2 \quad (4)$$

$$s_{s+ss+sp}^2 = a' C^{b'} \text{ and } s_a^2 = a\, C^b \quad (5)$$

where a, a', b and b' are constants determined by the regression analysis and C is the mycotoxin concentration.

2.5. Statistical analyses

Kruskal-Wallis one-way analysis of variance was used for comparison of results obtained by the two sampling plans, and by analytical methods in sampling plan B ($p<0.05$). A non-parametric statistical hypothesis test, Wilcoxon signed-rank test, for paired samples was used to analyze the toasting effect in order to determinate if the process reduced or not the mycotoxins concentration ($p<0.05$). For statistical purposes undetected mycotoxin levels were considered as LOD divided by 2. Statistical tests were done with Statgraphics® Plus 5.1 program (Manugistics, Inc., Maryland, USA).

3. RESULTS

3.1. Presence of mycotoxins in the analysed pistachio lots

OTA was never detected in any of the lots tested while almost all the lots contained AFs. Regarding AFB_1 and AFB_2, they were present in all sampled lots (sampling plan A) of raw

pistachio and 5 lots of toasted pistachio (**Table 1**). Moreover, those lots more contaminated with AFB_1 were also the most contaminated with AFB_2, although AFB_1 contamination was always higher than that of AFB_2. AFG_1 and AFG_2 were only present in 2 lots of raw pistachio (plan A), their mean values being lower than 0.1 μg/kg for AFG_2 in both lots, and lower than 0.5 μg/kg for AFG_1 in lot 5 and 0.1 μg/kg in lot 8. Figure 2 shows AFB_1 and AFs mean values obtained per lot through HPLC, with undetected values replaced by LOD divided by 2.

Table 1 Individual aflatoxin contribution to the total contamination of the lot (%) in raw and toasted pistachio sample through plan A and raw pistachio sampled through plan B.

	Plan A								**Plan B**			
	Raw				**Toasted**				**Raw**			
	AFB_1	**AFB_2**	**AFG_1**	**AFG_2**	**AFB_1**	**AFB_2**	**AFG_1**	**AFG_2**	**AFB_1**	**AFB_2**	**AFG_1**	**AFG_2**
L1	94.5	5.5	0	0	0	0	0	0	0	0	0	0
L2	93.7	6.3	0	0	93.2	6.8	0	0	0	0	0	0
L3	97.9	2.2	0	0	0	0	0	0	0	0	0	0
L4	87	13	0	0	98.7	1.4	0	0	70.9	0	29.1	0
L5	91	5.6	2	1.4	79.7	20.3	0	0	0	0	0	0
L6	95.9	4.1	0	0	93.4	6.6	0	0	100	0	0	0
L7	89.4	10.6	0	0	0	0	0	0	100	0	0	0
L8	90.7	7.1	2.2	0	88.4	11.6	0	0	0	0	0	0

3.2. Comparison between sampling plans

Food companies often perform alternative mycotoxin sampling plans which reduce the number of samples and/or number of analysis by reducing the cost and time dedicated to the analysis, as a part of their quality control schemes. In order to compare the effectiveness of a simplified sampling plan, an alternative plan (sampling plan B) was conducted in parallel to official sampling plan (sampling plan A). However, the alternative proposed sampling plan resulted not appropriate as compared to the official one; it always led to significantly lower results (Kruskall-Wallis test, $p<0.05$) when comparing the AFB_1 and AFs mean values of raw ($p=0.0006$) and toasted ($p=0.0003$) lots obtained from the two sampling plans (**Figure. 2**), due to the reduced probability of taking a contaminated portion in the sampling.

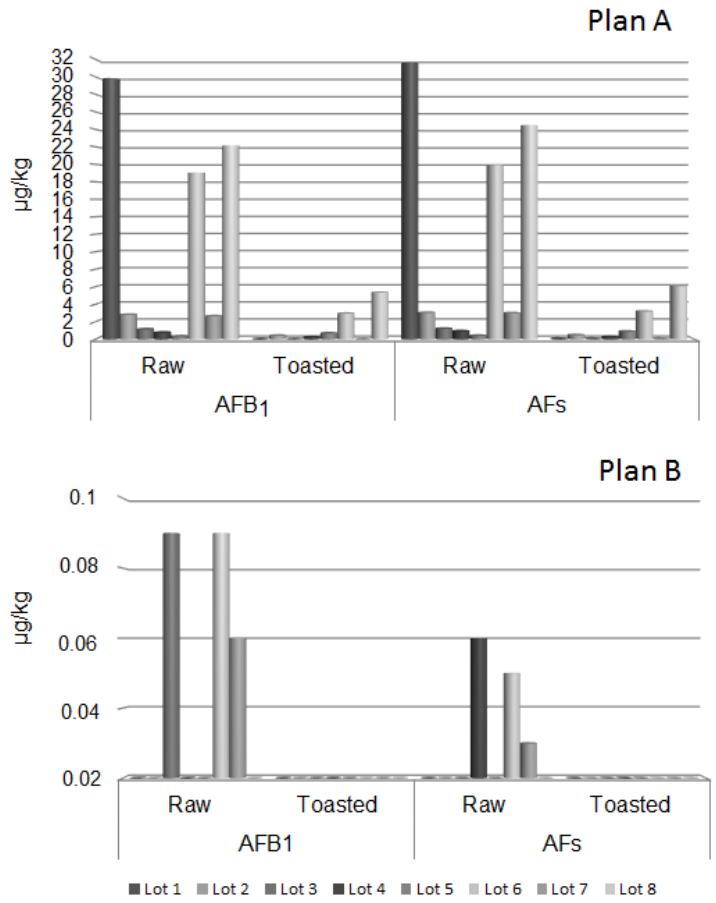


Figure 2 Mean values of AFB_1 and AFs in raw and toasted pistachio lots obtained from sampling plan A and B. Undetected values were replaced by LOD/2.

3.3. Comparison between analytical methods (HPLC and ELISA) in sample plan B

ELISA is a common analytical method used by companies. This test was carried out in parallel to HPLC analyses in sampling plan B. OTA was never detected by any of the methods while higher number of positive samples and higher concentration of AFs were detected by ELISA. Positive samples were only detected in raw lots number 1 and 2 by ELISA ranging from 2.54 to 2.05 µg/kg, respectively; AFs were only detected in lot 6 in one analysis by HPLC (0.58 µg/kg). AFs were detected by both techniques in lots 4 and 7; the same number of positive analyses was recorded for lot 7, while two-fold positive analyses were observed by ELISA compared to HPLC for lot 4. Mean positive values in these two lots were 1.86 and 3.75, respectively by ELISA and 0.59 and 0.31 µg/kg by HPLC. Regarding toasted lots, AFs positive samples were only detected by ELISA, in lots 1, 4 and 7 in a range

from 3 to 3.5 μg/kg, therefore this method may lead to false positive results. Consequently, significant differences (Kruskall-Wallis test, $p<0.05$) were found when comparing AFs mean values of raw ($p=0.0282$) and toasted ($p=0.0107$) lots analysed by the two different methods.

3.4. Effect of toasting on naturally contaminated pistachios with mycotoxins

For raw lots, a_W ranged from 0.31-0.44 corresponding to 2.19-3.54 % of moisture content (m.c.). In general after toasting the m.c. was reduced in a range or 17-70% except in the lots 4, 5 and 6 which presented an increased m.c., probably due to fast packing after toasting. The m.c. in lots 4, 5 and 6 were 3.75, 4.07 and 4.6 corresponding to 0.50, 0.51 and 0.55 a_W. These values are still below the minimum a_W required for fungal growth (about 0.70 a_W).

In order to examine the effect of toasting on the degradation of mycotoxins, lots were analyzed after commercial toasting (Figure 2). In all lots, except for lot 5 (very low concentration in the raw lot), the concentration of AFB_1, AFB_2 and AFs was lower after toasting (sampling plan A). Mean percentages of AFs reduction were 87.62%±11.89, 81.05%±15.51 and 86.74 %±11.31 for AFB_1, AFB_2 and AFs, respectively. Toasting effect was significant (Wilcoxon signed-rank test, $p<0.05$) when comparing mean values of raw and toasted lots obtained from sampling plan A. The p-values were 0.0209 for AFB_1 and AFs and 0.0423 for AFB_2. The percentage of reduction was not correlated with the initial AF contamination of raw pistachio lots. Conversely, the effect of toasting was not significant when comparing the lots before and after treatment in the plan B, probably as a consequence of the limitations of the sampling plan B, as suggested above.

3.5. Analytical uncertainty

Figures 3 and 4 show the mean AFB_1 and AFs value for each composite. The error bars in these figures represent analytical uncertainty as requested in EC 401/2006 (Equation 1). Positive values were corrected by the recovery rate and the shell percentage, while undetected values were replaced by LOD divided by 2. The AFB_1 and AFs legal limit (according to EC Regulation No. 165/2010) are 12 μg/kg and 15 μg/kg for raw pistachio and 8 μg/kg and 10 μg/kg for toasted pistachio, respectively. Regarding AFB_1 in raw pistachio, when comparing the mean corrected value of each composite in the same lots with the maximum limit the probability of rejecting the lot was 25% in lots 2, 6, 7 and 8 and 50 % in lot 1. However when the uncertainty measurement is also considered, the probability of rejecting the lots 2 and 7

was 50% and lots 1, 6 and 8, the most contaminated, was 100% (**Figure. 3**). Therefore considering the uncertainty measurement all the composites of these lots would be rejected. Comparing the mean corrected values of toasted pistachio versus the legal limit, lots 6 and 8 would be rejected with 25% of probability. The corrected mean of the sum of all AFs of each composite was also compared to the legal limits (**Figure. 4**). In this case the probability of rejecting lots number 2, 6, 7 and 8 was 25%, and 50% in lot 1. Once again, the probability of rejecting the lots when considering the uncertainty measurement increased, leading to 100% probability of rejection for lots 1, 6 and 8.

3.6. PO and FSO applied to the mycotoxin hazard in pistachio

During storage steps after processing and distribution, a zero increase of AFs should be possible with the application of appropriate environmental storage conditions. Therefore, aflatoxin-FSO value (EC maximum value) in pistachio could be equaled to the PO at the end of processing. To ensure such PO after toasting and taking into account the minimal toasting reduction of 75.76% and 76.41% for AFB_1 and AFs concentration, respectively, the mycotoxin concentration in the lots before thermal treatment should be bellow 32.96 and 41.66 μg/kg, respectively. The values exceed the limits established in the legislation for pistachios that will be subject to sorting or other physical treatment, before human consumption or use as an ingredient in foodstuffs, thus maximum established limits are in the safe side. This suggests that under the hypothesis of 75% of reduction (PC) achieved in the toasting, higher concentrations could be permitted in the raw material and still render a safe finished product (**Figure 5**).

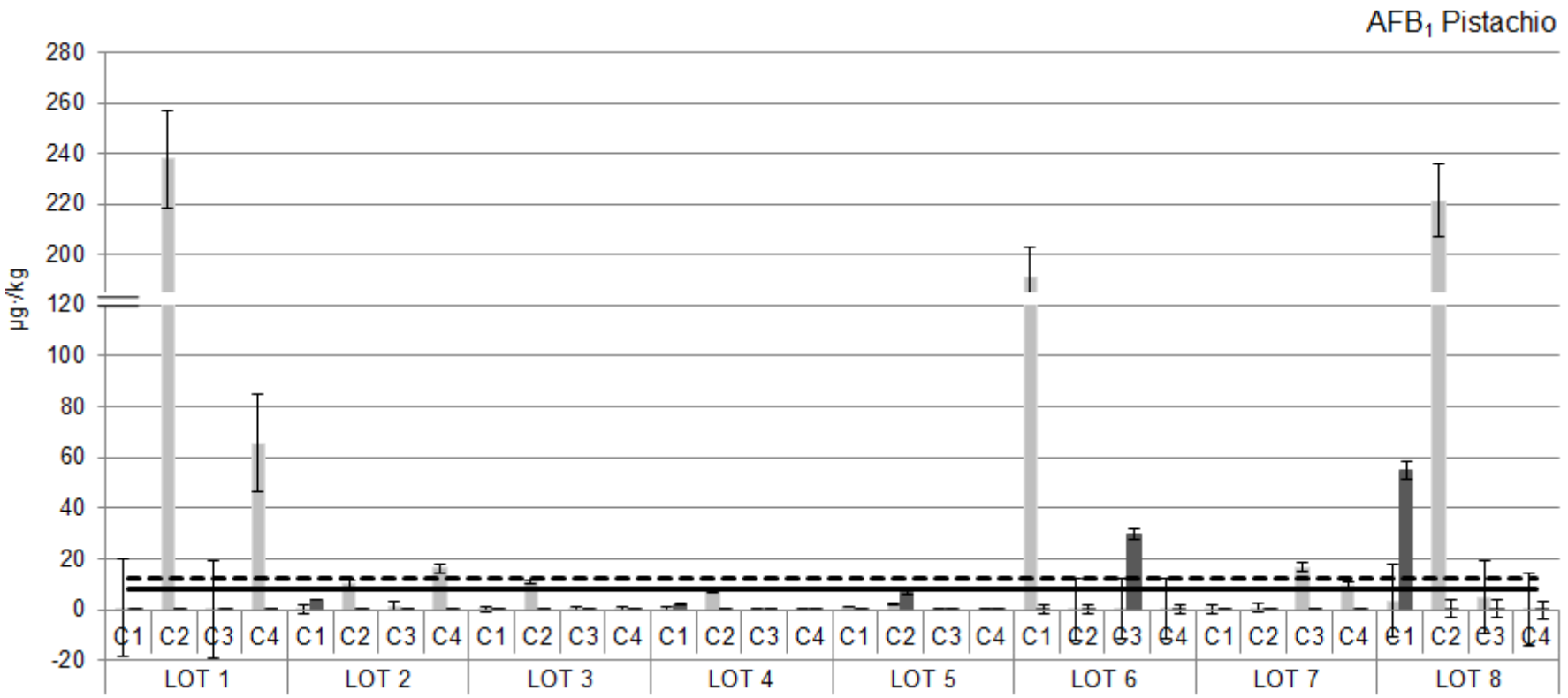


Figure 3 Columns (light grey raw pistachio; dark column toasted pistachio) indicate the average of 8 analysis of each composite in where undetected values were replaced by LOD/2. Bars indicate the measurement uncertainty in each lot calculated by Equation (1). Horizontal solid-line indicates the maximum European AFB_1 legal limit (12 μg/kg) for pistachios to be subject to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs (No. 165/2010). Horizontal dotted-line indicates the maximum European AFB_1 legal limit (8 μg/kg) for pistachios intended for direct human consumption or use as an ingredient in foodstuffs (No. 165/2010).

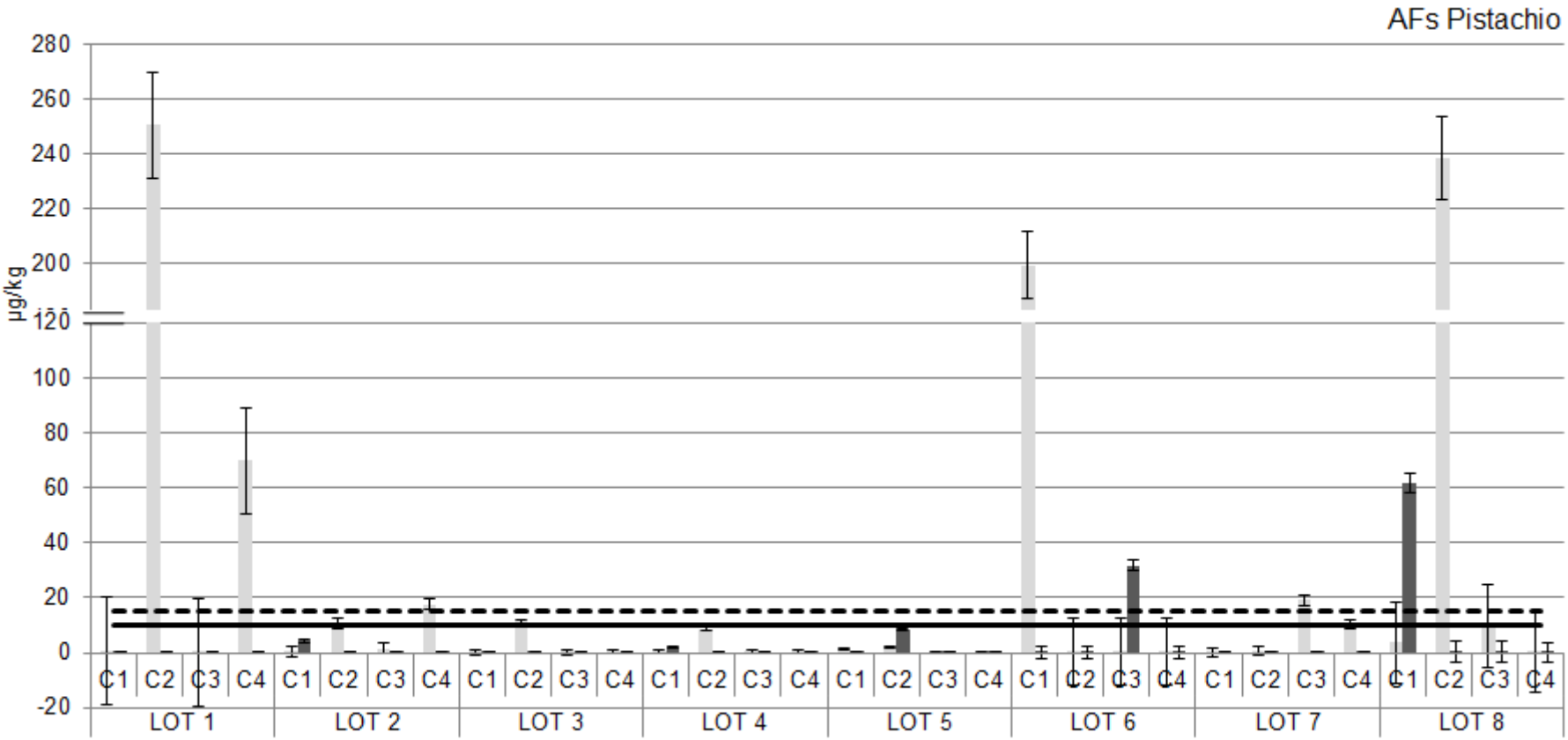
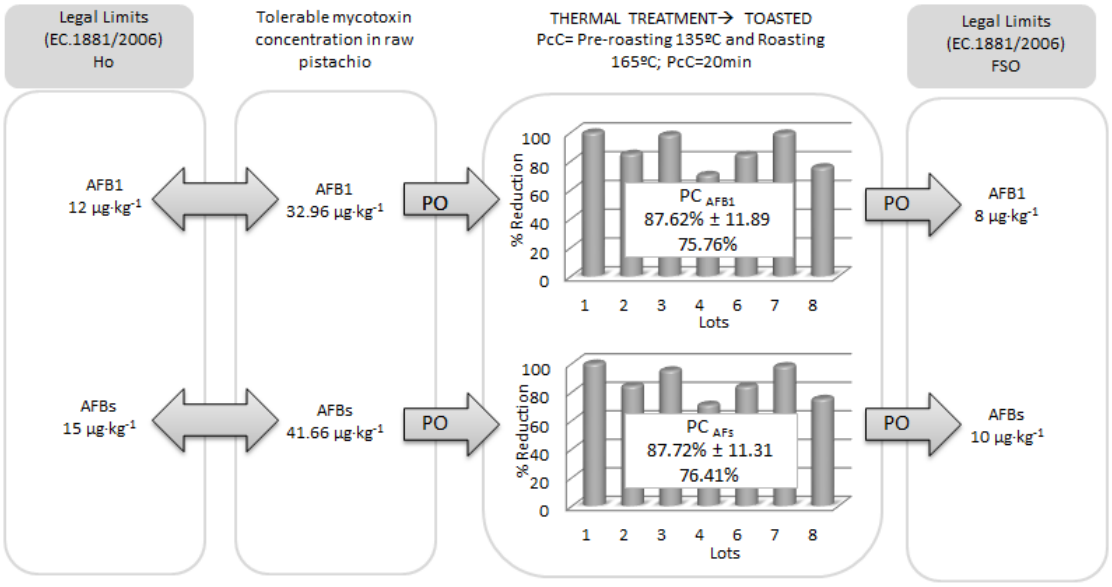


Figure 4 Columns (light grey raw pistachio; dark column toasted pistachio) indicate the average of 8 analysis of each composite in where undetected values were replaced by LOD/2. Bars indicate the measurement uncertainty in each lot calculated by Equation (1). Horizontal solid-line indicates the maximum European AFs legal limit (15 µg/kg) for pistachios to be subject to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs (No. 165/2010). Horizontal dotted-line indicates the maximum European AFs legal limit (10 µg/kg) for pistachios intended for direct human consumption or use as an ingredient in foodstuffs (No. 165/2010).



Ho: initial mycotoxin level; PO: Performance Objetive; PC: Performance Criteria; PcC: Process Criterion; FSO, Food Safety Object.

Figure 5 Aplication of risk mangement metrics, Ho, PO, PC, PcC and FSO in toasted pistachio, considering PC stored=0.

3.7. Sampling uncertainty

Sampling plans were performed by quadruplicate to account for sampling variability. For sampling plan A, the distribution of AFs concentration, from the 32 HPLC-analysis in each lot was very heterogeneous and the number of positive analyses in a lot was usually very low, although in some subfractions the contamination level was very high (above 300 µg/kg), mainly in AFB_1 in raw lots (**Table 2**). Moreover, all lots of raw pistachio except number 5 had at least one analysed subfraction higher than 10 µg/kg. However, the percentage of positive analytical results was not always correlated with the mean concentration of the lots. To explain the differences found within results from the same lot, variance components in the nested design were calculated for AFB_1 and AFs in raw pistachio, as suggested in Equation 1 (**Tables 3 and 4**). Total variance increased with an increase in mycotoxin concentration. The analytical variance in raw lots represented 0.28-40% of total variability, although the higher values of analytical variance were linked with the lowest contaminated lots. However, the most important variability of the results was always due to the subsample preparation, that is the grinding and selection of the analytical subfraction (0.4-99%). Grinding the sample increases the homogeneity, but more subfractions would be probably required to reduce uncertainty. The sampling and analytical variances in **Table 3 and 4** were plotted versus concentration in **Figures 6 and 7**.

Table 2 Incidence of positive results, by range, for AFB1 and AFB2 (μg/kg) in raw and toasted pistachio from sampling plan A.

	AFB_1	**ng/g**	**<LOD[a]**	**<0.5**	**<1**	**<2**	**<5**	**<10**	**<20**	**<50**	**<100**	**<200**	**<500**	**%Positive results**	**Lowest level[b]**	**Mean[b]**	**Highest level[b]**	**Mean[c]**
Aflatoxin	Raw	Lot 1	22	2	2	0	0	2	0	0	1	1	2	31.3	0.4	95.4	392.5	29.8
AFB_1	Pîstachio	Lot 2	23	4	0	1	1	0	0	3	0	0	0	28.1	0.2	3.4	30.9	2.8
		Lot 3	25	5	0	0	0	0	2	0	0	0	0	21.9	0.2	2.7	18.9	1.2
		Lot 4	21	6	3	0	0	1	1	0	0	0	0	34.4	0.2	1.0	11.3	0.8
		Lot 5	25	3	1	0	3	0	0	0	0	0	0	21.9	0.2	0.5	3.1	0.3
		Lot 6	15	15	0	0	0	0	0	0	0	0	2	53.1	0.2	18.5	313.9	19.0
		Lot 7	24	0	3	0	1	1	1	2	0	0	0	25.0	0.7	4.6	36.4	2.7
		Lot 8	19	4	1	1	4	0	1	0	0	0	2	40.6	0.0	29.9	388.9	22.2
	Toasted	Lot 1	32	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0
	Pistachio	Lot 2	30	0	0	0	1	1	0	0	0	0	0	6.3	4.7	4.0	8.0	0.4
		Lot 3	32	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0
		Lot 4	30	0	0	0	2	0	0	0	0	0	0	6.3	2.8	1.9	3.9	0.2
		Lot 5	31	0	0	0	0	0	0	1	0	0	0	3.1	21.3	21.3	21.3	0.7
		Lot 6	30	0	0	0	0	0	0	1	1	0	0	6.3	30.4	31.9	63.8	3.0
		Lot 7	32	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0
		Lot 8	21	6	3	0	0	0	0	1	0	1	0	34.4	0.2	11.2	123.1	5.4

[a] The symbol < in the heading indicates a range. Thus, <LOD. indicates 0 - <0.04 or 0 - <0.02 for AFB_1 and AFB_2 respectively, <0.5 is shorthand for 0.04-0.5 or 0.02-0.5 for AFB_1 and AFB_2 respectively, <1.0 for 0.051-1.0, etc. [b] Lowest, mean and highest values are calculated from positives samples .

Table 2. Continued

	AFB_1	ng/g	<LOD[a]	<0.5	<1	<2	<5	<10	<20	<50	<100	<200	<500	%Positive results	Lowest level[b]	Mean[b]	Highest level[b]	Mean[c]
Aflatoxin	Raw	Lot 1	24	4	0	0	0	2	1	1	0	0	0	25.0	0.1	2.8	22.2	1.7
AFB_2	Pîstachio	Lot 2	27	2	0	2	1	0	0	0	0	0	0	15.6	0.1	0.5	2.5	0.2
		Lot 3	28	3	1	0	0	0	0	0	0	0	0	12.5	0.0	0.2	0.6	0.0
		Lot 4	28	1	1	2	0	0	0	0	0	0	0	12.5	0.1	0.4	1.7	0.1
		Lot 5	29	3	0	0	0	0	0	0	0	0	0	9.4	0.1	0.1	0.3	0.0
		Lot 6	26	4	0	0	0	0	2	0	0	0	0	18.8	0.0	2.5	15.0	0.8
		Lot 7	26	2	1	2	0	1	0	0	0	0	0	18.8	0.0	1.0	6.1	0.3
		Lot 8	23	7	0	0	0	0	0	2	0	0	0	28.1	0.2	3.4	30.5	1.7
	Toasted	Lot 1	32	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0
	Pistachio	Lot 2	30	1	1	0	0	0	0	0	0	0	0	6.3	0.3	0.3	0.7	0.0
		Lot 3	32	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0
		Lot 4	31	1	0	0	0	0	0	0	0	0	0	3.1	0.1	0.1	0.1	0.0
		Lot 5	31	0	0	0	0	1	0	0	0	0	0	3.1	5.4	5.4	5.4	0.2
		Lot 6	30	0	0	0	2	0	0	0	0	0	0	6.3	2.3	2.2	4.4	0.2
		Lot 7	32	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0
		Lot 8	29	1	0	0	0	1	1	0	0	0	0	9.4	0.1	5.4	16.2	0.7

a The symbol < in the heading indicates a range. Thus, <LOD. indicates 0 - <0.04 or 0 - <0.02 for AFB1 and AFB2 respectively, <0.5 is shorthand for 0.04-0.5 or 0.02-0.5 for AFB1 and AFB2 respectively, <1.0 for 0.051-1.0, etc. b Lowest, mean and highest values are calculated from positives samples .

Table 3 Sampling, subsampling, subfractioning and analytical variances associated with estimating AFB_1 by sampling plan A in a 20 kg raw shelled pistachio sample by using grind sample preparation, a 10 kg subsample, 10 g subfractions and HPLC analysis.

Lot	AFB_1 Raw	Sample Variance		Subsample Variance		Subsample preparation		Analytical Variance		Total Variance
Lot 1	76.0	0.0	(0.0)	469.1	(0.8)	59637.4	(98.7)	347.5	(0.6)	60454.0
Lot 2	6.9	0.0	(0.0)	1.6	(0.4)	266.8	(60.0)	176.3	(39.7)	444.7
Lot 3	2.9	0.0	(0.0)	0.0	(0.0)	119.8	(98.8)	1.4	(1.2)	121.2
Lot 4	1.9	2.3	(5.2)	0.0	(0.0)	41.4	(93.1)	0.8	(1.7)	44.5
Lot 5	0.8	0.0	(0.0)	0.0	(0.0)	3.6	(67.7)	1.7	(32.3)	5.3
Lot 6	48.0	12.3	(0.0)	0.0	(0.0)	36311.7	(99.7)	100.4	(0.3)	36424.4
Lot 7	6.7	0.0	(0.0)	31.7	(7.7)	237.9	(58.0)	140.3	(34.2)	409.8
Lot 8	57.3	0.0	(0.0)	0.0	(0.0)	47633.4	(96.0)	1992.9	(4.0)	49626.3

Numbers in parentheses show the percentage

Table 4 Sampling, subsampling, subfractioning and analytical variances associated with estimating AFs by sampling plan A in a 20 kg raw shelled pistachio sample by using grind sample preparation, a 10 kg subsample, 10 g subfractions and HPLC analysis.

Lot	AFs Raw	Sample Variance		Subsample Variance		Subsample preparation		Analytical Variance		Total Variance
Lot 1	80.3	0.0	(0.0)	532.8	(0.8)	66361.9	(98.8)	306.0	(0.5)	67200.7
Lot 2	7.4	0.0	(0.0)	1.8	(0.4)	310.9	(61.5)	193.1	(38.2)	505.8
Lot 3	3.0	0.0	(0.0)	0.0	(0.0)	124.8	(99.2)	0.9	(0.8)	125.8
Lot 4	2.3	3.4	(5.9)	0.0	(0.0)	53.9	(92.3)	1.1	(1.9)	58.4
Lot 5	0.9	0.0	(0.0)	0.0	(0.0)	4.6	(71.7)	1.8	(28.3)	6.4
Lot 6	50.0	16.6	(0.0)	0.0	(0.0)	39390.5	(99.6)	137.6	(0.4)	39544.7
Lot 7	7.5	0.0	(0.0)	38.6	(7.3)	297.1	(56.4)	191.5	(36.3)	527.1
Lot 8	63.0	0.0	(0.0)	0.0	(0.0)	55334.6	(96.0)	2306.8	(4.0)	57641.4

Numbers in parentheses show the percentage

The plots for each variance are approximately linear in a log-log plot and equations 4 and 5 were calculated as $s^2_{s+sp} = 7.180C^{2.131}$ and $s_a^2 = 1.401C^{1.480}$ by AFB_1 and $s^2_{s+sp} = 6.998C^{2.131}$ and $s_a^2 = 1.261C^{1.514}$ by AFs. Then the mean corrected value of each lot was introduced in the equation 4 obtaining the estimated variance. After that the square root of estimated variance was multiplied by 1.96 to obtain the uncertainty (95% confidence). Relevant differences were

observed when comparing the analytical uncertainty results obtained here with those in section 3.5, the uncertainty calculated from linear regression being always higher (3-9 times, depending on the concentration of the lot).

Finally, the overall uncertainty was much higher than analytical uncertainty, if such uncertainty values are applied to the AFs results in this study, all lots, except lot 5, would surpasse the maximum levels as stated by the EC (Comission of the European Communities, 2010a).

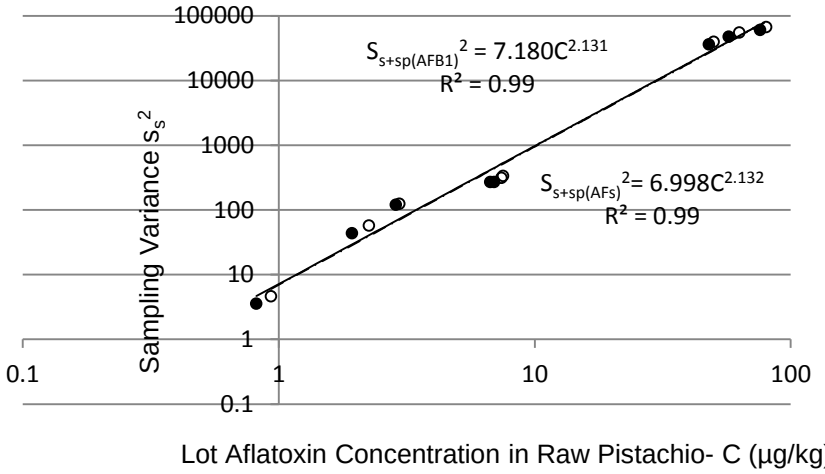


Figure 6 Full-log plot of sampling variance versus AFB_1 (black circles) and AFs (white circles) concentration. Linear regression AFB_1 (continuous line) and AFs (dotted line).

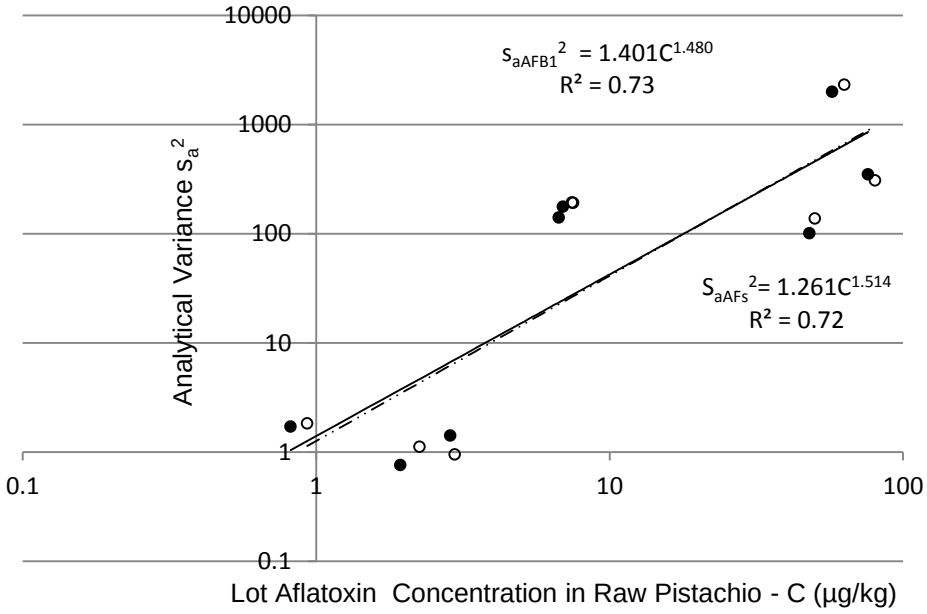


Figure 7 Full-log plot of analytical variance versus AFB_1 (black circles) and AFs (white circles) concentration. Linear regression AFB_1 (continuous line) and AFs (dotted line).

4. DISCUSSION

In the present study the emerging risk management metrics were applied. FSO was considered equivalent to the maximum EC limits (Comission of the European Communities, 2010a) and to the PO after processing. However, the determination and compliance with such levels is totally dependent on the validity of sampling and analytical procedures.

Aflatoxin results per composite sample were corrected with the percentage of shell, recovery and measurement uncertainty as required by European Regulation (Comission of the European Communities, 2006a). The application of measurement uncertainty increased the probability of rejection of the most contaminated lots. Under these conditions, in our study the probability of rejecting 2 of the 3 most contaminated lots increased to 25% when the uncertainty measurement in the corrected value was considered and compared with the legal limits.

Two analytical methods were tested in this study, ELISA showing always a higher number of positive samples. Griessler et al. (2010) quantified mycotoxins of several foods and feed commodities from Southern Europe by grinding and homogenizing each lot and then using HPLC and ELISA. Similar ranges of mycotoxin contamination were found, but no clear conclusion can be drawn from these data. Chun et al. (2007) analyzed AFs in nuts and derivative products consumed in South Korea by ELISA and HPLC; from their results it was concluded that ELISA was not suitable for quantification since the results were affected by the sample matrix and contamination was possibility overestimated at very low concentration. Similarly, our results showed higher detected concentration in samples analyzed by ELISA.

The distribution of the mycotoxin concentrations in products is an important factor to be considered when regulatory sampling criteria are established. In principle, lot distributions can be obtained by measuring mycotoxins level nut by nut despite it is not practical due the rarity of infested nuts. Studies on peanut kernels indicate that the percentage of contaminated kernels in a contaminated lot at 20 μg/kg is 0.095%, which is less than one contaminated kernel per 1,000 kernels (Whitaker et al., 1972). Same trend was observed in pistachio, where an infestation rate of only 1 nut per 10^4-10^6 nuts is typical (Schatzki et al., 1995). However these few contaminated particles can have extremely high levels of mycotoxins. Cucullu et

al. (1966, 1977) reported AFs concentrations in excess of 1,000,000 μg/kg for individual peanut kernels and 5,000,000 μg/kg for individual cottonseed. Therefore AFs can be found only in a small percentage of the kernels in the lot and the concentration in a single kernel may be extremely high. According to our results, analytical values above 300 μg/kg were detected in several lots while more than 60% of the analyses were below of the limit of detection in lots L1, L6 and L8. The difficulty in making precise estimations of mycotoxin concentrations in a large bulk of material, i.e., a lot or a truck, has been amply demonstrated for many agricultural products (peanuts, corn, soybean, cottonseed, pistachio, wheat, figs), mainly for AFs but also for deoxynivalenol (DON) in wheat and fumonisins in maize (Hart & Schabenberger, 1998; Johansson et al 2000; Ozay et al., 2006; Schatzki 1995; Vargas et al., 2004; Whitaker et al. 1974, 2000, 2003, 2006). Equations linking the sampling error of toxin estimation and the size of the aggregate sample have been derived for AFs, fumonisins, DON and OTA for several commodities (Whitaker et al. 1974; Johansson et al. 2000; Vargas et al., 2004, Ozay et al., 2006). The equations are specific for the mycotoxin type and the type of product studied, but generally show that sampling variance increases with an increase in concentration, and decreases with an increase in sample size. The sampling variability pattern is similar for the four previously cited mycotoxins (Larsen et al., 2004; Miraglia et al., 2005; Whitaker et al., 2000). Regarding to product characteristics, the AFs sampling variability was smaller for powdered ginger than seeded commodities such as, corn or peanuts, because of the particle size or the number of particles per unit mass (Whitaker et al., 1974; Johansson 2000). On the other hand, more aggregate samples for AFs analysis in groundnuts (20 kg) than for OTA or DON in cereals (10 kg) are required for the official control (98/53 EC amended by 2002/26 EC and 178/2010 EC).

In the present study the sampling step was divided into sampling (make the aggregate samples), subsampling (mix the aggregate sample and divide into two equal laboratory samples of 10 kg before grinding) and subfractioning (taking 10 g after grinding). Low variability was associated with the sampling step, probably because the size and number of elemental samples was enough; in fact, the last European Regulation about methods of sampling and analysis for the official control of mycotoxins levels in foodstuffs reduced the size of the aggregate sample from 30 to 20 kg, but maintains the analysis number (Comission of the European Communities, 2010b). On the other hand, a high percentage of the variability was attributed to the subfractioning step (subsample preparation), suggesting that either a better grinding and mixing could be achieved or more/bigger subfractions should be selected to reduce the uncertainty. Similar values of sampling and analytical variance were

observed in sampling almonds and hazelnuts for AFB_1 (Ozay et al., 2006, Whitaker et al., 2006). However, in those studies lower values of sample preparation variance were calculated, probably due to the higher size of samples for analysis used (50 to 100 g) and smaller milling particle size.

AFs have high decomposition temperatures, ranging from 237 to 306 ºC and AFB_1 is quite stable to dry heating (Betina, 1989; Rustom et al., 1997). Although these temperatures are higher from those actually used by the nuts industry, it is usually accepted that the heat treatment decreases the concentration of AFs to some extent. Toasted reduction percentages of 87.62%, 81.04% and 87.72% for AFB_1, AFB_2 and AFs were calculated in this study. However, these results were obtained based on 7 lots, because lot 5 did not present any mycotoxin reduction due to its low initial contamination, and just one process criteria (PcC), therefore more results would be required for a complete description of the PC. Nevertheless, conflicting results have been published about the effect of the heat treatments on peanuts and pistachios (Ariño et al., 2009; Farah et al., 1983; Lee et al., 1969; Ozkarsli et al., 2003; Pluyer et al., 1987; Rustom et al., 1997; Yazdanpanah et al. 2005). In general the extent of the destruction achieved was very dependent on the initial level of contamination, heating temperature, time and humidity. In naturally contaminated peanuts heat treatment by oven toasting at 150 ºC for 30 min caused a 30-45% AFB_1 reduction, while in artificially contaminated peanuts treated under the same conditions, the inactivation was around 48-61% (Pluyer et al., 1987). Degradation of AFs in peanuts toasted at 150 ºC for 30 min increased with the addition of ionic salts in a range from 38%, 41.5% and 47.6% in unsalted peanuts, and salted with 20 g/kg and 50 g/kg respectively (Ozkarsli et al., 2003). In pistachio, the results regarding degradation of AFs due to toasting are also contradictory. Yazdanpanah et al. (2005) studied the effect of toasting for 30, 60 and 90 min at different temperatures (90, 120 and 150 ºC). The milder treatment (90 ºC-30 min) reported slightest effect while the most extreme treatment resulted in a degradation of over 95% of AFB_1 but the pistachio showed a burned appearance. The toasting process at 150 ºC for 30 min showed significant reduction of AFB_1 and AFB_2 without any noticeable change in taste of sample. Also the rate of reduction was plotted against the initial amount and linear correlation was not found. On the other hand, Ariño et al. (2009) studied the effect of toasting on AFs: four commercial batches of raw pistachios in-shell from Iran were salted (1% salt content) and toasted at 120 ºC for 20 min in a toasting industry in Spain. This study did not show significant differences in relation with AFs reduction after toasting; however the level of contamination of the starting material was low, ranging from 0.12 to 0.18 μg/kg. Recently, García-Cela et al. (2012)

analyzed the effect of time and temperature in connection with the AFs degradation in nuts using published results, observing that with a thermal treatment of 135-165 ºC for 20 min a reduction of 15-35% in AFs could be achieved however in the present study higher reduction values were achieved in pistachio (57%).

An important side effect of toasting is the reduction of the m.c. The initial m.c. of raw pistachio was lower than 4% although 3 toasted lots presented a m.c. over than the initial one. However in both cases the m.c. was lower than 5%. Storage temperature and m.c. are important factors on fungal growth and mycotoxins accumulation in pistachio. For OTA prevention, pistachio should be kept under 12.6% m.c. (Marin et al., 2010), and lower m.c. (10%) is required for AFs prevention (unpublished data) in a range of temperatures of 10 to 42 ºC.

Regarding to OTA, several studies have reported the incidence of black aspergilli in pistachios and other tree nuts (Bayman 2002, Doster and Michailides, 1994, Fernane et al., 2010a,b). In fact, Fernane et al. (2010a,b) reported that more than 50% of black aspergilli isolates from Spanish and Argelian pistachios had OTA production capacity. Nevertheless, only one Spanish sample and another Argelian one contained 0.67 and 170 μg/kg OTA, respectively. However, OTA was never found in the 1,600 kg of sampled pistachio in the present work.

5. CONCLUSION

The absence of AFs in raw pistachio cannot be guaranteed, thus relying on industrial processes for a certain reduction is required. The food industry is responsible for setting up food safety management systems that deliver foodstuffs in compliance to the FSO. According to the initial and final values proposed by European legal limits, processing (either selection or selection plus toasting or just toasting) is expected to decrease in 33% the initial aflatoxin concentration in the raw pistachio. Our results suggest that about a 75% of reduction may be achieved by the single toasting process, thus under the hypothesis of raw pistachio compliance with maximum level, the toasted pistachio must be safe. The underlying problem is the uncertainty associated to the aflatoxin levels reported in the present work and any other existing works; the high uncertainties due to sampling and sample preparation procedures may lead to unrealistic results, and this is an issue that needs to be solved. In the present work the major variability was associated with the subfraction selection and therefore

increasing the number of the analyzed subfractions could be an alternative for reducing uncertainty.

ACKNOWLEDGMENTS

The authors are grateful to the Spanish government (project AGL2010-22182-C04-04) and EC, KBBE - Food, Agriculture and Fisheries and Biotechnology (project 222738- Selection and improving of fit-for-purpose sampling procedures for specific foods and risks) and Comissionat per a Universitats i Recerca del Departament d'Innovació Universitats i Empresa de la Generalitat de Catalunya (AGAUR) and European Social Fund for the financial support.

REFERENCES

Ariño, A., M. Herrera, G. Estopañan, M. C. Rota, J. J. Carramiñana, T. Juan, & A. Herrera (2009). Aflatoxins in bulk and pre-packed pistachios sold in Spain and effect of roasting. *Food Control,* 20(9), 811-814.

Bayman, P., J. L. Baker, M. A. Doster, T. J. Michailides, & N. E. Mahoney (2002). Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. *Applied and Environmental Microbiology,* 68(5), 2326-2329.

Betina, V. (1989). Mycotoxins: chemical, biological and environmental aspects. *Bioactive Molecules,* 9, 114-150.

CAC (Codex Alimentarius Commission) (2008). Codex Committee on Methods of Analysis and Sampling. Guidance on measurement uncertainty and uncertainty of sampling. Consulted: April 13, 2012. Available from: ftp://ftp.fao.org/codex/Meetings/CCMAS/ccmas29/ma29_09e.pdf

Chun, H. S., Kim, H. J., Ok, H. E., Hwang, J., Chung D. (2007). Determination of aflatoxin levels in nuts and their products consumed in South Korea. *Food Chemistry,* 102(1), 385-391.

Comission of the European Communities (1998). Commission Directive 98/53/EC of 16 July 1998 laying down the sampling methods and the methods of analysis for the official control of the levels for certain contaminants in foodstuffs. *Official Journal of the European Union* L201, 93-101.

Comission of the European Communities (2002). Commission Directive 2002/26/EC of 13 March 2002 amending Directive 98/53/EC laying down the sampling methods and the methods of analysis for the official control of the levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, L75, 38-43.

Comission of the European Communities (2006a). Commission Regulation (EC) 401/2006 of 23 February 2006, laying down the methods of sampling and analysis for the

official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Union,* L70, 12-34.

Comission of the European Communities (2006b). Commission Regulation (EC) 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union,* L364, 5-24.

Comission of the European Communities (2010a). Commission Regulation (EU) No 165/2010 of 26 February 2010, amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxin. *Official Journal of the European Union*, L50, 8-12.

Comission of the European Communities (2010b). Commission Regulation (EC) 178/2010 of 2 March 2010, amending Regulation (EC) No 401/2006 as regards groundnuts (peanuts), other oilseeds, tree nuts, apricot kernels, liquorice and vegetable oil. *Official Journal of the European Union,* L52, 32-43.

Cucullu, A. F., L. S. Lee, & W. A. Pons (1977). Relationship of physical appearance of individual mold-damaged cottonseed to aflatoxin content. *Journal of the American Oil Chemists' Society,* 54(3), A235-A237.

Cucullu, A. F., L. S. Lee, R. Y. Mayne, & L. A. Goldblatt (1966). Determination of aflatoxins in individual peanuts and peanut sections. *JAOCS, Journal of the American Oil Chemists' Society,* 43(2), 89-92.

Denizel, T., B. Jarvis, & E. J. Rolfe (1976). A field survey of pistachio (*Pistacia vera*) nut production and storage in Turkey with particular reference to aflatoxin contamination. *Journal of the Science of Food and Agriculture,* 27(11), 1021-1026.

Doster, M. A., & T. J. Michailides (1994). *Aspergillus* molds and aflatoxins in pistachio nuts in California. *Phytopathology,* 84(6), 583-590.

European Food Safety Authority (EFSA) (2007). Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. *The EFSA Journal*, 446, 1-127.

Farah, Z., M. J. R. Martins, & M. R. Bachmann (1983). Removal of aflatoxin in raw unshelled peanuts by a traditional salt boiling process practiced in the north east of Brazil. *Lebensmittel Wissenschaft und Technologie,* 16, 122-124.

Fernane, F., G. Cano-Sancho, V. Sanchis, S. Marin, & A. J. Ramos (2010). Aflatoxins and ochratoxin A in pistachios sampled in Spain: Occurrence and presence of mycotoxigenic fungi. *Food Additives and Contaminants: Part B Surveillance,* 3(3), 185-192.

Fernane, F., V. Sanchis, S. Marín, & A. J. Ramos (2010). First report on mould and mycotoxin contamination of pistachios sampled in Algeria. *Mycopathologia,* 1-7.

García-Cela, E., A. J. Ramos, V. Sanchis, & S. Marin (2012). Emerging risk management metrics in food safety: FSO, PO. How do they apply to the mycotoxin hazard? *Food Control,* 25(2), 797-808.

Griessler, K., I. Rodrigues, J. Handl, & U. Hofstetter (2010). Occurrence of mycotoxins in Southern Europe. *World Mycotoxin Journal,* 3(3), 301-309.

Hart, L. P., & O. Schabenberger (1998). Variability of vomitoxin in truckloads of wheat in a wheat scab epidemic year. *Plant Disease,* 82(6), 625-630.

IARC (International Agency for Research on Cancer) (2002). IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Vol. 82, 171-300. IARC Press, Lyon, France.

ICMSF (International Commission on Microbiological Specifications for Foods). (2002). Microorganisms in Foods 7: Microbiological Testing in Food Safety Management, Kluwer Academic/Plenum Publishers, New York.

Johansson, A. S., T. B. Whitaker, W. M. Hagler Jr., F. G. Giesbrecht, J. H. Young, & D. T. Bowman (2000). Testing shelled corn for aflatoxin, Part I: estimation of variance components. *Journal of AOAC International,* 83(5), 1264-1269.

Joint Committee for Guides in Metrology (JCGM) (2008). Evaluation of measurement data: Guide to the expression of uncertainty in measurement. Consulted: April 13, 2012. Available from: http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf.

Larsen, J. C., J. Hunt, I. Perrin, & P. Ruckenbauer (2004). Workshop on trichothecenes with a focus on DON: summary report. *Toxicology letters,* 153(1), 1-22.

Lee, L. S., A. F. Cucullu, A. O. Franz Jr., & W. A. Pons Jr. (1966). Destruction of aflatoxins in peanuts during dry and oil roasting. *Journal of Agricultural and Food Chemistry,* 17(3), 451-453.

Marín, S., I. Hodžić, A. J. Ramos, & V. Sanchis (2008). Predicting the growth/no-growth boundary and ochratoxin A production by *Aspergillus carbonarius* in pistachio nuts. *Food Microbiology,* 25(5), 683-689.

Miraglia, M., B. De Santis, V. Minardi, F. Debegnach, & C. Brera (2005). The role of sampling in mycotoxin contamination: an holistic view. *Food Additives and Contaminants,* 22(SUPPL. 1), 31-36.

Mojtahedi, H., C. J. Rabie, & A. Lubben (1979). Toxic aspergilli from pistachio nuts. *Mycopathologia,* 67(2), 123-127.

Ozay, G., F. Seyhan, A. Yilmaz, T. B. Whitaker, A. B. Slate, & F. Giesbrecht (2006). Sampling hazelnuts for aflatoxin: uncertainty associated with sampling, sample preparation, and analysis. *Journal of AOAC International,* 89(4), 1004-1011.

Ozkarsli, M, (2003). Effect of traditional-roasting and microwave-roasting on aflatoxin B_1 in peanuts. MSc Thesis, Cukurova University Institute of Natural and Applied Sciences, Adana, Turkey.

Parker, P.E., G.R. Bauwin & H.I. Ryan. (1982). Sampling, inspection and grading of grain. In: *Storage of Cereal Grains and Their Products* (C.M. Christensen, ed). American Association of Cereal Chemists, St. Paul, Minnesota, pp. 1-35.

Pluyer, H. R., Ahmed, E. M., & Wei C. I. (1987). Destruction of aflatoxins on peanuts by oven- and microwave-roasting. *Journal of Food Protection,* 50(6), 504-508.

RASFF (Rapid Alert System for Food and Feed). (2011). Consulted: April 13, 2012. Available from: http://ec.europa.eu/food/food/rapidalert/index_en.htm

Rivas Casado, M., D. J. Parsons, R. M. Weightman, N. Magan, & S. Origgi (2009). Geostatistical analysis of the spatial distribution of mycotoxin concentration in bulk cereals. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment,* 26(6), 867-873.

Rustom, I. Y. S. (1997). Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. *Food Chemistry,* 59(1), 57-67.

Schatzki, T. F. (1995). Distribution of aflatoxin in pistachios. 2. Distribution in freshly harvested pistachios. *Journal of Agricultural and Food Chemistry,* 43(6), 1566-1569.

Schatzki, T. F., & J. L. Pan (1996). Distribution of aflatoxin in pistachios. 3. Distribution in pistachio process streams. *Journal of Agricultural and Food Chemistry,* 44(4), 1076-1084.

Shotwell, O.L. Goulden, M.L. & Hesseltine C.W. (1974). Aflatoxin: distribution in contaminated corn. *Cereal Chemistry*, 51, 492-499.

Siegel, D., & T. Babuscio (2011). Mycotoxin management in the European cereal trading sector. *Food Control,* 22(8), 1145-1153.

Vargas, V. A., T. B. Whitaker, E. A. Santos, A. B. Slate, F. B. Lima, R. C. A. & Franca. (2004). Testing green coffee for ochratoxin A, Part I: estimation of variance components. *Journal of AOAC International*, 87(4), 884-891.

Whitaker, T. B. (2003). Detecting mycotoxins in agricultural commodities. *Applied Biochemistry and Biotechnology - Part B Molecular Biotechnology,* 23(1), 61-71.

Whitaker, T. B., A. B. Slate, M. Jacobs, J. Michael Hurley, J. G. Adams, & F. G. Giesbrecht (2006). Sampling almonds for aflatoxin, Part I: estimation of uncertainty associated with sampling, sample preparation, and analysis. *Journal of AOAC International,* 89(4), 1027-1034.

Whitaker, T. B., J. W. Dickens, & R. J. Monroe. (1974). Variability of aflatoxin test results. *Journal of the American Oil Chemists' Society*, 51(4), 214-21.

Whitaker, T. B., J. W. Dickens, R. J. Monroe, & E. H. Wiser. (1972). Comparison of the observed distribution of aflatoxin in shelled peanuts to the negative binomial distribution. *Journal of the American Oil Chemists' Society*, 49 (10), 590-593.

Whitaker, T. B., M. W. Trucksess, A. S. Johansson, F. G. Giesbrecht, W. M. Hagler Jr., & D. T. Bowman (1998). Variability associated with testing shelled corn for fumonisin. *Journal of AOAC International,* 81(6), 1162-1168.

Whitaker, T. B., W. M. Hagler Jr., F. G. Giksbrecht, & A. S. Johansson (2000). Sampling, sample preparation, and analytical variability associated with testing wheat for deoxynivalenol. *Journal of AOAC International,* 83(5), 1285-1292.

Yazdanpanah, H., T. Mohammadi, G. Abouhossain, & A.M. Cheraghali (2005). Effect of roasting on degradation of aflatoxins in contaminated pistachio nuts. *Food and Chemical Toxicology,* 43(7), 1135-1139.

IV: RESEARCH WORK

PART II:

Climate change

STUDY II

Fungal diversity, incidence and mycotoxin contamination in grapes from two agro-climatic Spanish regions with emphasis on *Aspergillus* species

García-Cela, E.[a], Crespo-Sempere, A.[a], Gil-Serna, J.[b], Porqueres, A.a, Marin, S.[a]

[a]Applied Mycology Unit, Food Technology Department, University of Lleida, UTPV-XaRTA, Agrotecnio Center, Av. Rovira Roure 191, 25198 Lleida, Spain

[b]Microbiology III Department, University Complutense of Madrid, Jose Antonio Nováis 12, 28040 Madrid, Spain.

ABSTRACT

Fourteen vineyards from two different agro-climatic regions in Spain were sampled in two consecutive years in order to determinate the grape mycobiota and diversity indexes with the final aim to define the potential mycotoxigenic species from both regions and their relationship. The most common fungal Genera encountered were *Aspergillus* (30.0%), *Alternaria* (53.2%), *Cladosporium* (11.9%) and *Penicillium* (2.9%). Black aspergilli presence in the hotter region (South) was significantly higher ($p<0.05$) than in Northeast in both years. Among black aspergilli, *A. tubingensis* seemed to be the better adapted species to environmental conditions, while *A. carbonarius* was the main potentially ochratoxigenic specie in both regions and years, owing to the most relevant percentage of ochratoxigenic isolates. OTA positive musts were only detected from Southern vineyards, although contamination was always lower than 0.1 μg/L. Finally, none of black aspergilli tested produced FBs on CYA, while 63% of *A. niger* tested produced FB_2 when inoculated on CYA20S, reaching the 100% of isolates from South.

Climate change scenarios in South Europe point to an increase of temperature and drought. This could promote particularly adapted species such as *A. niger*, decreasing OTA risk, but this could lead to an increase of FB_2 presence.

Accepted Journal of the Science of Food and Agriculture.

1. INTRODUCTION

Aspergillus and *Alternaria*, followed by *Penicillium*, are the most frequently reported Genera on grapes. *Aspergillus* spp. are ubiquitous saprophytes present in soils around the world, particularly in tropical and subtropical regions (Pitt and Hocking, 2009). Many field surveys have been published dealing with epidemiology, ecology and distribution of aspergilli occurring on wine grapes worldwide (**Table 1**). Some species of *Aspergillus* are aflatoxins (AFs), ochratoxin A (OTA) and fumonisin B_2 and B_4 (FB_2 and FB_4) producers, thus their presence in vineyards could result in mycotoxin contamination of grapes and grape-products, such as wine. *Aspergillus* section *Flavi* is rarely present in vineyards, only in a study in Lebanon, they were 43% of the total aspergilli and more than 40% of them produced aflatoxin B_1 (AFB_1), thereby more than 40% of musts contained this mycotoxin but at levels lower than 0.46 μg/L (Khoury et al., 2008; Magnoli et al., 2003; Medina et al., 2005; Sage et al., 2004; Serra et al., 2006b); moreover, no studies exist which reported AFs in wine. Similarly, low incidence of section *Circumdati* is described in Spain (2.5-6.6%) and these fungi were never isolated from Portuguese and French vineyards, and therefore their contribution to OTA in grape is not relevant (Bellí et al., 2004c; Sage et al., 2002; Serra et al., 2005).

Undoubtedly, *Aspergillus* section *Nigri* are the most important mycotoxigenic (ochratoxigenic) fungi present on grapes (5-83% infected berries, **Table 1**). The main black aspergilli species occurring on grapes are biseriates, in particular *A. niger* aggregate. In more recent years, several authors have proposed the division of the *A. niger* aggregate in four morphologically identical species: *A. niger, A. tubingensis, A. foetidus* and *A. brasiliensis* (González-Salgado, 2010). *A. carbonarius* showed the highest percentage of OTA producing strains in Europe and Tunisia (60-100%), conversely in Argentina only 5.9% were OTA positive (**Table 1**). Although uniseriates presence was rare in most reports, studies carried out in Argentina and Italy detected higher number of uniseriates than *A. carbonarius* isolates. Additionally, a high percentage of OTA-producing uniseriates was found in Argentina (45.2%). *A. niger* aggregate was always the most abundant group among *Aspergillus* (56-97%) although the percentage of OTA producing isolates was very variable (0-73%) (Table 1). Finally, recent reports revealed production of fumonisin B_2 (FB_2) and fumonisin B_4 (FB_4) by *A. niger* and *A. awamori* strains on culture medium, grapes or dried grapes (Chiotta et al., 2011; Logrieco et al., 2009; Mogensen et al., 2010a; Varga et al., 2010).

The highest levels of OTA in winemaking musts have been detected in Italy, Lebanon, and Tunisia (**Table 2**). In this sense, Battilani et al. (2006), observed that incidence of black aspergilli was significantly related to latitude and longitude, showing a positive West-East and North-South gradient. Recent studies have also evidenced the influence of geographic location and climate on the occurrence of ochratoxigenic moulds and OTA contamination of grape (Lasram et al., 2012b; Lucchetta et al., 2010; Serra et al., 2006b). In Spain, although wide surveys were carried out in the past (**Table 1**), no North-South trend was observed along the Mediterranean coast. Moreover, no recent works on fungal diversity on vineyards have been published. Specifically in Catalonia, the last field survey published was conducted 9 years ago (Bellí et al., 2005). According to climatic change scenarios predicted for Spain, it is expected that temperature and drought will increase (EC, 2007). Thus, future climate conditions in Northern Spain could be those currently found in Southern Spain.

Warm European summers have observed a decrease in the occurrence in wheat of the formerly predominant species, *F. culmorum*, which has been replaced by *F. graminearum*. Both species produce ZEA and DON, but additionally *F. graminearum* produces nivalenol increasing the mycotoxin exposure (Miller, 2008). Moreover, in Italy *F. verticillioides*, the most diffuse maize pathogen, is favored by warm dry weather, while *A. flavus* tends to occur only in particularly hot summers, altering the maize contamination from FBs to AFs (Giorni et al., 2007). Besides studies on AF-risk linked to climate change concluded that both cereal crops and mycotoxigenic fungi may move geographically as a result of changing conditions, thus some mycotoxigenic fungi may threaten those newly colonised areas (Battilani et al., 2012). Therefore, climate change increases the risk of migration of pathogens and therefore known fungal infections patterns could be affected and mycotoxin profile modified.(Magan et al., 2011)

Considering that in both Northern and Southern Spain there are wide winemaking areas, the aims of this work were: a) determine the grape mycobiota and biodiversity indexes in these two agroclimatic regions of Spain; b) determine the *Aspergillus* section *Nigri* and *Circumdati* distribution in both regions; c) determine the potential mycotoxigenesis of *Aspergillus* section *Nigri* and *Circumdati* from both regions; d) determine the OTA contamination in musts from both regions; d) establish the relationship between mycobiota, OTA presence and the climatic conditions.

Table 1 Summary of several published reports (last decade) on *Aspergillus* section *Nigri* isolation and ochratoxin A production on berries.

Sampling country	Year[a]	N	% of berries contaminated by *A.* section *Nigri*	Section *Nigri* distribution (%)		OTA producers (%)	LOD (ng/g or ng/mL)	Mean[e] (ng/g or ng/mL)	Range[e] (ng/g or ng/mL)
Italy[1b]	1999-2000	9	9.0-15	Uniseriate	18.5-23.8				
				Aggregate *niger*	56.2-60.6				
				A. carbonarius	25.3-15.6				
France[2b]	2000	60		Uniseriate	9.3				
				Aggregate *niger*	67.4				
				A. carbonarius	23.3	10/11(90.9%)		0.29	0.01-1.90
Spain[3c]	2001	40	4.9	*A.* section *Nigri*	100	18/386(4.6%)	0.02	1420	0.02/2820
Spain[4]	2001	7	20.7	Uniseriate	1	0/[f]			
				Aggregate *niger*	81.6	0/[f]			
				A. carbonarius	17.4	101/101(100%)			1920-195460[d]
Argentina[5]	2001	50	14	*A. niger* var. *niger*	74	20/44(45%)	1	13	2.0-24.5
				A. niger var. *awamori*	14	5/15(33%)	1	15	3.0-20.0
				A. foetidus	12	1/4(25%)	1	2	2
Portugal[6]	2001-2002-2003	11	31	*A. ibericus*	1.6				
				Aggregate *niger*	83.9				
				A. carbonarius	14.9				
Spain[7d]	2002-2003	40	10.5-17	Uniseriate	18-18	0/45(0%)-0/72(0%)	0.01		
				Aggregate *niger*	73-53	3/181(1.6%)-11/211(5.2%)			
				A.carbonarius	7-29.0	14/17(82.4)-90/118(76.3%)			
Greece[8d]	2002-2003	16	30.6-28.8	Aggregate *niger*	35	62/85(73%)	1		LOD ->25
				A. carbonarius	65	39/50(78%)	1		LOD ->25

References:1 Battilani et al., 2003; 2 Sage et al., 2004; 3 Belli et al., 2004b; 4 Bau et al., 2005; 5 Magnoli et al., 2003; 6 Serra et al., 2006; 7 Belli et al., 2005; 8 Tjamos et al., 2006; 9 Bejaoui et al., 2006; 10 Ponsone et al., 2007; 11 Serra et al., 2005; 12 Lasram et al., 2012b; 13 Chiotta et al., 2009; 14 Díaz et al., 2009 and 15 Lucchetta et al., 2010.

[a] when no sampling period was reported, the publication year was reported. Values were calculated from: [b] 2 samplings, [c] 4 samplings and [d] 3 sampling taken at different stages.

[e] mean and range and maximum were calculated from positive samples. [f] number of isolates tested does not appear in the publication.

Table 1 (Continued)

Sampling country	Year[a]	N	% of berries contaminated by *A.* section *Nigri*	Section *Nigri* distribution (%)		OTA producers (%)	LOD (ng/g or ng/mL)	Mean[e] (ng/g or ng/mL)	Range[e] (ng/g or ng/mL)
France[9]	2001-2002-2003	40		Uniseriate	8.6				
				Aggregate *niger*	54.7				
				A. carbonarius	36.7				
Argentina[10]	2003-2004	26		Uniseriate	33	28/62(45.2%)	1	3	3.0-6.0
				Aggregate *niger*	48	37/132(28.0%)	1	13.18	1.3-50.0
				A. carbonarius	19	0/7(0%)	1		
Portugal[11]	2005	4	17	Aggregate *niger*	97	0/33(0%)			
				A. carbonarius	3	1/1(100%)			
Tunisia[12]	2005-2006-2007	24	_	Uniseriate	2.3	0/29(0%)	0.02		
				Aggregate *niger*	76	29/931(3.2%)	0.02	1450	<100-5000
				A. carbonarius	21.7	268/270(99.3%)	0.02	70	<100-1000
Argentina[13]	2006-2007	50	32.5	Uniseriate	8	0/[f](0%)	0.05		
				Aggregate *niger*	81	63/230(27.4%)	0.05	2.24	0.5-17.50
				A. carbonarius	8	28/54(5.9%)	0.05	202	1.2-1285
Chile[14]	2006-2007-2008	398		Aggregate *niger*	78				
				A. carbonarius	22				
Italy[15]	2003-2007	204	64.8-82.5	Uniseriate	31.4				
				Aggregate *niger*	60.8				
				A. carbonarius	7.8				

Table 2 Summary of several published reports (last decade) on ochratoxin A contamination in winemaking musts.

Sampling country	Year[a]	N	N positives / (%)	LOD (µg/L)	Mean of positives (µg/L)	Range of positives (µg/L)	References
Italy[b]	1999	9	8 (89%)	0.01	2.1	0.01-6.5	Battilani et al., 2003
France	2000	60	11 (18%)	0.01	0.06	0.01-0.43	Sage et al., 2004
Spain	2001	24	6 (25%)	0.07	0.26	0.09-0.81	Belli et al., 2004a
France	2002	11	6 (55%)		0.18	0.01-0.46	Sage et al., 2002
Spain[c]	2002	40	0 (0%)	0.05			Belli et al., 2005
	2003	40	0 (0%)	0.05			
Tunisia	2003	10	5 (50%)	0.01	3.38	1.1-4.3	Fredj et al., 2007
Argentina[c]	2003-2004	26	0 (0%)				Ponsone et al., 2007
Portugal[d]	2005	4	3 (75%)	0.004		0.01-0.16	Serra et al., 2005
Lebanon	2005	47	0 (0%)	0.01			Khoury et al., 2008
Tunisia	2005	24	13 (54%)	0.05	0.38	0.06-1.88	Lasram et al., 2012b
	2006	24	16 (67%)	0.05	0.98	0.05-5.45	
	2007	24	10 (42%)	0.05	1.30	0.11-5.85	
Argentina	2006-2007	50	22 (44%)	0.01	0.13	0.1-1.20[d]	Chiotta et al., 2009
Chile	2006-2007-2008	398	77 (19%)	0.4			Díaz et al., 2009
Italy	2003-2007	204	62 (30%)			0.003-2.0	Lucchetta et al., 2010

Legal limit EC (1881/2006) 2 µg/L

[a] when no sampling period was reported, the publication year was reported.

Values were calculated from: [b] 2 samples and 4 [c] samples taken at different stages.

[d] µg/kg grape.

2. MATERIALS AND METHODS

2.1. Field sampling and meteorological data

Two different agro-climatic regions from Spain were sampled in two consecutive years (2011 and 2012). Ten fields were located in the Northeast (41º 41'N 0º 28' E) and four in the South (37º 35' N 05º 04' W), with 740 km of distance between regions (Fig. 1). Climate in these regions was defined as cold steppe (BSk) and temperate with dry or hot summer (Csa) respectively, according to Köppen Climate Classification of the Iberian Peninsula Climate Atlas (**Figure 1**).(AEMET, 2011).

Phenological growth stages of the grape vine covers the period between dormancy and leaf fall. In Spain, this period starts in May or June with flowering and studded, followed by veraison in July and ripening in August or September, depending of the variety and latitude. All fields were sampled at harvest, between August and September. Sampling was carried out through a diagonal in each vineyard and 10 bunches were randomly collected from 10 vines (one bunch per vine) along the diagonal from each field as described by Battilani et al. (2006) Bunches were collected in paper bags and kept at 4 ºC until laboratory analysis.

Different grape varieties were sampled in each region: 5 red varieties (Pinot, Cabernet Sauvignon, Tempranillo, Syrah and Merlot) and 2 white varieties (Chardonnay and Xarel•lo) in the Northeast, while in the South only 4 white varieties were present: Zalema, Chelva and Palomino in 2011, and Zalema, Chelva, Pedro Ximénez, and Montepila in 2012.

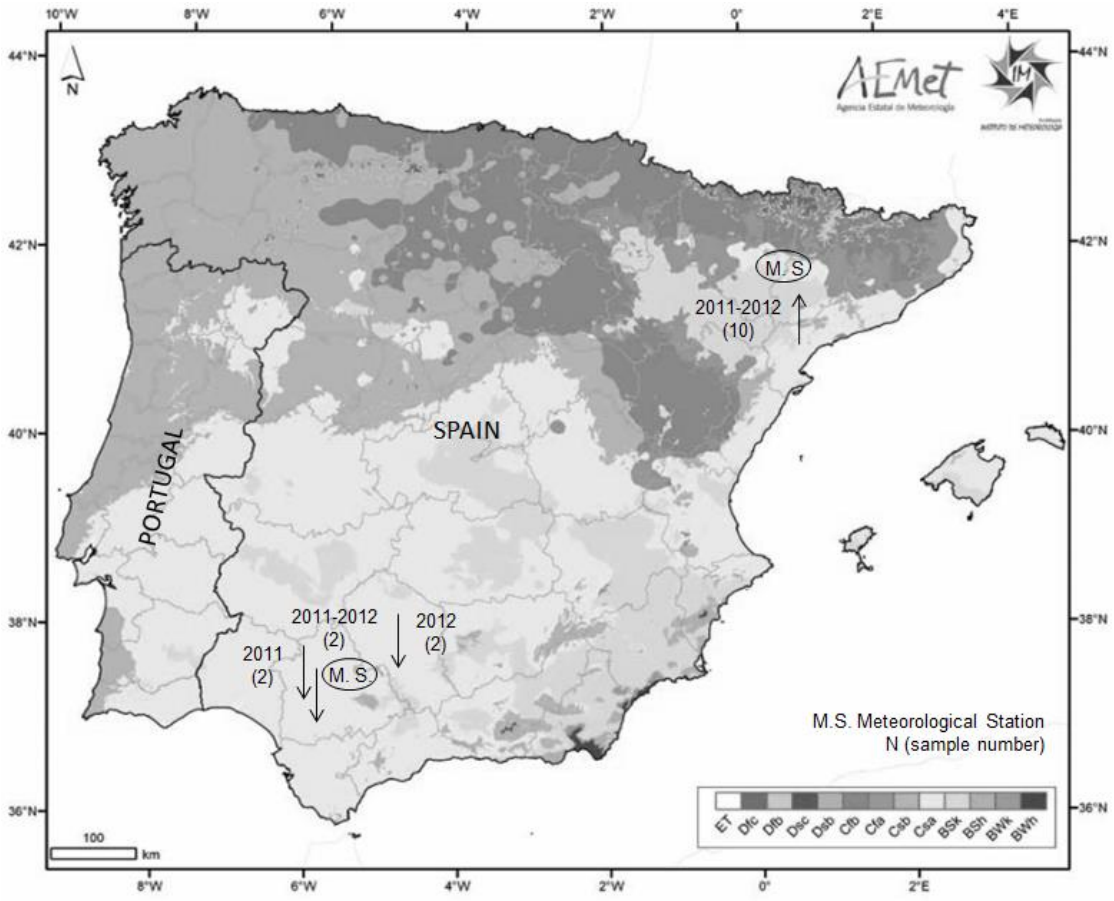
Regarding the cropping system, vines in Northeast were grown on trellises with localised drip irrigation, while in the South the common system was goblet vines without irrigation. Both locations had systematic fungicide application programs based on common active ingredients (copper, sulphur, triadimenol), besides, metrafenone, myclobutanil and strobilurin were used in the Northeast and cyproconazole in the South until three weeks before harvest. Regarding pests, sexual confusion was used and occasionally chemical pest treatments were applied when necessary.

2.2. Meteorological data

Meteorological data (from April to September) for each sample region were obtained from the weather network of Catalunya (Servei Meteorò␣gic de Cataluyna) and Andalucía (Estaciones agroclimáticas de Andalucía).Regarding mean temperatures, both 2011 and 2012 were above the values registered in the last 40 years, being extremely hot in the case of 2012, up to 10% higher than the mean values in both regions. In the same way the precipitation rate was under average in both regions and years, being among the driest years.

Daily mean relative humidity (RH), maximum (T_{max}), mean (T_{mean}) and minimal (T_{min}) temperatures and rainfall from April to September were plotted every 10 days in order to describe the environmental conditions during the grape cycle and fungal infection (**Figure 2**).

Regarding Northeast sampling area, a gradual increase of T_{mean} from 16 ºC was observed since May. This trend continued until mid-August reaching mean values of 25-26 ºC, although lower T_{mean} were registered in early June in 2011. Regarding to daily T_{max}, in the period between May to August 30 ºC were reached in a total of 45 and 61 days, of which 23 and 27 days were in August, in 2011 and 2012, respectively. The maximum T_{max} reached was 37 ºC, in August. Mean RH from June to August, was higher (60.3% RH) in 2011 than in 2012 (57.6 % RH) despite the accumulated rain in 2012 was 2.6 times higher (85.0 mm vs 32.1 mm). However the main difference between both years was a strong summer storm on 5th August 2012 in which 35.3 mm of rain were registered in 2 h. In the Southern region the T_{mean} in May also exceeded 18 ºC. The T_{mean} increased until mid-August reaching mean values of 27-29 ºC. Regarding maximum daily T_{max} between May to August in 2011, 30 ºC were reached in 94 single days, 37 ºC in 26 days and in 3 days the T_{max} was over 40 ºC. Year 2012 had higher maximum temperatures than previous one, in where a total of 108 days attained 30 ºC, and 48 and 14 days reached the T_{max} of 37 and 40 ºC, in the same period of time than in year 2011. In summary, a difference of 1-4 ºC in mean temperature was observed between both regions and inversely to temperature, the RH was lower in the South, as mean RH in June and August was of 52.7 and 44.9 respectively. Moreover scarce rainfall was recorded in both years (7.4 and 0 mm). Altogether, Southern sampling area was hotter and drier than Northeastern one.



Legend: ET (tundra), Dfc (cold with a dry season and fresh summer), Dfb (cold without dry season and temperate summer), Dsc (cold with dry and fresh summer), Dsb (cold with temperate and dry summer), Cfb (temperate with a dry season and temperate summer), Cfa (temperate with a dry season and hot summer), Csb (temperate with dry or temperate summer), Csa (temperate with dry or hot summer), BSk (clod steppe), BSh (hot steppe), BWk (cold desert), BWh (hot desert).
Source: Iberian Climate Atlas. Air temperature and precipitation (1971-2000). Agencia Estatal de Meteorología Ministerio de Medio Ambiente y Medio Rural y Marino © Instituto de Meteorologia de Portugal ©

Figure 1 Location of vineyards sampled in Spain in 2011 and 2012 and meteorological station from which climatic data were obtained.

.

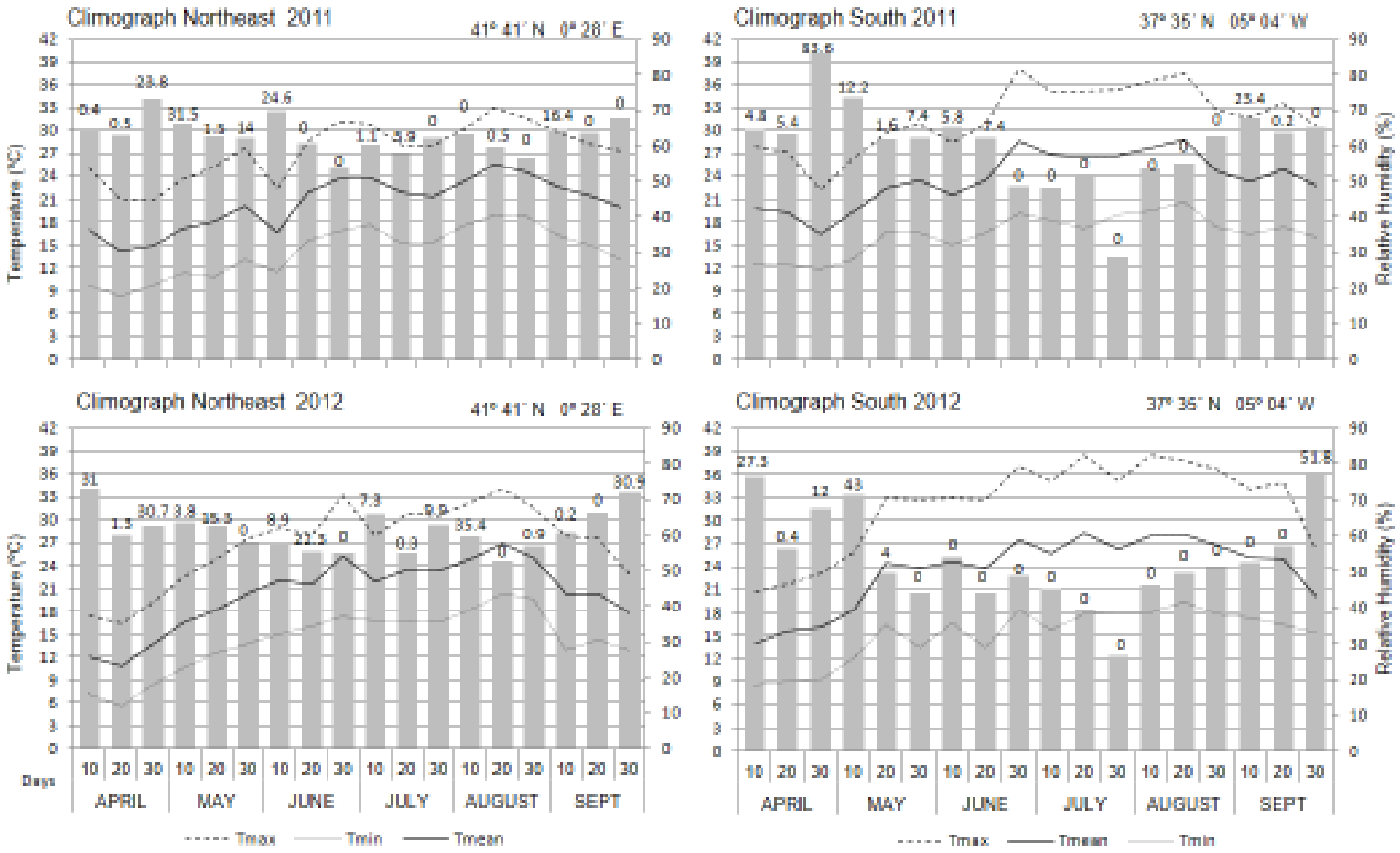


Figure 2 Meteorological data (temperature, relative humidity and rainfall) from April to September in both regions and years. Numbers in parentheses are accumulated rainfall in 10 days. Meteorological Station coordinates.

2.3. Mycobiota determination

Fifty berries were randomly chosen from each bunch and plated directly in groups of five in Petri dishes with Dichloran Rose Bengal Chloramphenicol medium (DRBC) under sterile conditions. Plates were incubated for 7 days at 25 ºC and colonies of developing fungi were examined and classified into Genera according to Pitt and Hocking, 2009. Then, samples of each Genera were inoculated separately using the appropriate media and identified to species level according to Pitt and Hocking, 2009; *Fusarium* species were inoculated in CLA and PDA and incubated under alternate dark/light cycles (12 h) at 25 ºC during 15 days, then, *Fusarium* species were identified (Leslie and Summerell, 2007). Concerning to *Aspergillus* section *Nigri*, microscopic observation and identification in uniseriate, biseriate and *A. carbonarius* isolates was conducted. Species that could not be identified by their cultural characteristics or by microscopy were identified using molecular biology techniques. All *Aspergillus* section *Flavi*, *Circumdati* and a representative sample of isolates from section *Nigri* were identified by PCR through specific primers of the most frequently reported species (*A. flavus, A. parasiticus, A. ochraceus, A. westerdijkiae, A. steynii, A. carbonarius, A. tubingensis,* and *A. niger*). In the case of *Penicillium*, isolates were incubated at 5, 25 and 37 ºC on CYA, and at 25 ºC on MEA, G25N and SCN, and then grouped by macroscopic morphological criteria. One isolate of each group was further identified by DNA sequencing.

2.4. DNA extraction, amplification and identification of Aspergillus species

DNA extraction were performed using the protocol described elsewhere (Querol et al., 1992). DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). All PCR assays were performed using an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Hamburg, Germany). Amplification reactions were carried out in volumes of 25 µL containing 200 ng of template DNA, 1 µL of each primer (20 µM), 2.5 µL of 10× PCR buffer, 1 µL of MgCl2 (50 mM), 0.2 µL of dNTPs (100 mM) and 0.15 µL of Taq DNA polymerase (5 U/µL) supplied by the manufacturer (Bio- tools, Madrid, Spain).

After DNA extraction, genomic DNAs were tested for suitability for PCR amplification using primers 5.8S1/5.8S2 (Gil-Serna et al., 2009a). Specific PCR protocols previously described were used to detect OTA-producing *Aspergillus* species the *A. carbonarius* (Selma et al., 2008), *A. niger* and *A. tubingensis* (Perrone et al., 2007b; Susca et al., 2007), the most

important *Aspergillus* section *Circumdati* species (*A. westerdijkiae, A. ochraceus and A. steynii*) (Gil-Serna et al., 2009b) as well as aflatoxin- producing *Aspergillus* species: *A. flavus* (González-Salgado et al., 2008) and *A. parasiticus* (Sardiñas et al., 2010). PCR products were detected in 2% agarose ethidium bromide gels in TAE 1× buffer (Tris-acetate 40 mM and EDTA 1.0 mM). A 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) was used as molecular size marker.

2.5. DNA extraction, amplification and identification of Penicillium species

The DNA extraction protocol described by Cenis, (1992) was followed with minor modifications. Cultures were grown in 500 μL of malt extract broth (2% w/v malt extract, 0.1% w/v peptone, 2% w/v glucose) for 2 days at 26 ºC. The mycelial extract was recovered after 10 min of centrifugation at 17500 x g and 300 μL of DNA extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS) was added. The mycelial suspension was lysed by vortexing with five 2.8 mm Precellys metal beads (Bertin Technologies, France) for 10 min. After centrifugation at 17500 x g for 10 min, 150 μL of 3 M sodium acetate (pH 5.2) was added to the supernatant. The supernatant was stored at -20 ºC for 10 min and then centrifuged (17500 x g, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by addition of 1 volume of isopropanol. After a 5-minute incubation time at room temperature, the DNA suspension was centrifuged (17500 x g, 10 min). The DNA pellet was washed with 70% ethanol to remove residual salts. Finally, the pellet was air-dried and the DNA was resuspended in 50 μL of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

To identify *Penicillium* isolates, primer pairs BT2A/BT2B (Glass and Donaldson, 1995) and EF-1/EF-2 (O'Donnell et al., 1998) were used to obtain partial sequences of the beta-tubulin and elongation factor genes. Amplification reactions were carried out in volumes of 50 μL containing 50 ng of DNA, 50 mM KCl, 10 mM Tris–HCl, 250 μM (each) dNTP, 1 μM of each primer, 2 mM MgCl2 and and 0.5 U of DFS-Taq DNA Polymerase (BIORON, Germany). PCR assays were conducted in a GeneAmp® PCR System 2700 (Applied Biosystems, USA) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 ºC (BT2A/BT2B) or 53 ºC (EF-1/EF-2) for 45 s, and extension at 72 °C for 60 s with a final extension of 10 min. PCR products were cleaned with the UltraClean PCR Clean-up DNA Purification kit (MoBio, USA). The PCR purified products were sequenced by the company Macrogen

Europe (Amsterdam, The Netherlands). Blast n-searches in GenBank permitted the identification of the level of identity with known sequences, and subsequently the species corresponding to the isolates.

2.6. Biodiversity Indexes

Biodiversity is defined by both species richness (the number of species present) and species evenness (how equally abundant the species are). Biodiversity indexes combine specific richness and evenness resulting in a single value. A given diversity index value can be obtained not only by a community with low richness and higher evenness but also from a community with high richness and lower evenness. Therefore, the value of evenness should be also considered together with the biodiversity index.

For each vineyard, data of abundance and diversity of fungal species were integrated to calculate the specific richness (Margalef, 1958), diversity Shannon (Shannon and Weaver, 1949) and evenness Shannon indexes (Pielou, 1969), and diversity and evenness (Simpson, 1949) indexes. Minimal value for diversity is 1 for Simpson index and 0 for Shannon index. Therefore the Inverse Simpson index was used to obtain a direct comparison to biodiversity.

Specific richness, D_{Mg}

$$D_{Mg} = \frac{S - 1}{lnp_i}$$

Diversity Shannon index, H

$$H = -\sum_{i=1}^{S} p_i \, lnp_i$$

Evenness Shannon index, J

$$J = \frac{H}{H_{max}} = \frac{H}{lnS}$$

Inverse Simpson index, D

$$D = \frac{1}{\sum_{i=1}^{S}(p_i)^2}$$

Evenness Simpson index, E

$$E = \frac{D}{D_{max}} = \frac{D}{S}$$

$$D = \frac{1}{\sum_{i=1}^{S}(p_i)^2}$$

Where pi is the proportion of abundance of the species and S is the number of species.

2.7. Mycotoxins detection and quantification

2.7.1. General description of the equipment

Mycotoxins were detected and quantified separately by using a HPLC system (Waters 2695, separations module, Waters, Milford, USA) and a C18 column (5 µm Waters Spherisorb, 4.6×250mm ODS2). For fluorescence detection a Waters 2475 module (Waters, Milford, USA) was used. Mycotoxins were quantified on the basis of the HPLC fluorimetric response compared with a range of mycotoxin standards and the detection limit was established based on a signal-to-noise ratio of 3:1. The analysis was performed under isocratic conditions at a flow rate of 1 mL/min. Quantification was achieved with a software integrator (Empower, Milford, MA, USA).

2.7.2. OTA and fumonisin B_1 and B_2 production by Aspergillus section Nigri

Production by *Aspergillus* section *Nigri:* OTA production of 168 isolates of *Aspergillus* section *Nigri* (113 in 2011 and 55 in 2012) incubated for 7 days at 25 ºC onto Czapek Yeast Extract agar (CYA) was tested. Moreover, FBs production was tested on 55 black aspergilli isolates taken at random from 2011 and 2012 on CYA and, among them, 19 *A. niger* were tested on CYA20S (20% sucrose) under the same incubation conditions as for OTA production. After incubation, 3 agar plugs (5mm) were removed from the middle to the outer side of the colony and placed in a vial. Mycotoxins were extracted by adding 1 mL of methanol into the vials, which were shaken for 5 s and allowed to rest. After 60 min, the vials were shaken again and the extract filtered (OlimPeak filters by Teknokroma PVDF Filter, 0.45 µm, 13 mm D, Sant Cugat del Vallés, Barcelona, Spain) into another vial. Subsequently, the extract was evaporated under a stream of nitrogen and stored at 4 ºC until HPLC analysis (Waters, Mildford, Ma, S.A.). Prior to HPLC injection, dried extracts were dissolved in 1 mL of methanol: water (50:50), and FBs were manually derivatized with OPA (Sydenham et al., 1996). OTA and FBs were detected by fluorescence at the following wavelengths: λexc 330nm; λem 460 nm and λexc 335 nm; λem 440 nm, respectively. For OTA, the mobile phase

used was acetonitrile: water: acetic acid (57:41:2) and the detection limit was 0.01 μg/kg. For FBs, the mobile phase used was methanol: 0.1 M sodium dihydrogen phosphate (77:23), solution adjusted to pH 3.35 with orthophosphoric acid. The detection limit was 3 ng/g agar for FB_1 and 1.5 ng/g agar for FB_2.

2.7.3. OTA in musts

After the study on mycobiota, the ten bunches collected from each vineyard were crushed and the resulting musts (n = 14 musts each year) were analysed for OTA using the method of the Office International de la Vigne et du Vin (Bezzo, G., Maggiorotto, G.& Testa, 2000). Berries were weighed and crushed with a hand blender machine, and then the must obtained was macerated for two hours, and centrifuged (Hettich Zentrifugen EBA 12, Germany) at 3900 g for 10 min and filtered (Whatman No. 1) under vacuum. Must pH was modified up to 7.4 with NaOH (4 M) and was filtered again (Whatman No. 1). Undiluted must was cleaned-up by means of immunoaffinity columns (Ochraprep, R-Biopharm Rhône LTD, Glasgow, Scotland) at a flow rate of 2-3 mL/min. Columns were afterwards washed with 20 mL of distilled water and let to dry. Desorption was carried out with 3 mL of methanol/acetic acid (98/2) solution, slowly passed through the column; during desorption backflushing was applied twice. The eluate was then evaporated to dryness at 40 ^{o}C under a stream of nitrogen and redissolved in 2 mL of mobile phase (48% acetonitrile and 52% sodium acetate /acetic acid (19/1)). A 100 μL aliquot of each final sample was injected into the HPLC system (as previously described, but at λexc 333 nm; λem 443 nm) with a detection limit of 0.01 μg OTA/L of must.

2.8. Statistical analyses

All statistical analyses were performed with Statgraphics® Centurion XVI (USA, 2010). The distribution of variables was tested for normality using the Kolmogorov-Smirnov test. Mann-Whitney (Wilcoxon) W-test was used to evaluate whether significant differences existed between biodiversity indexes and between *Aspergillus* section *Nigri* incidence on vineyards from the different regions. The statistical analysis performed were considered significant when $p<0.05$.

3. RESULTS AND DISCUSSION

Different grape varieties were sampled in Nothern and Southern regions, thus no conclusions can be drawn regarding varieties susceptibility to fungal infection. As a general rule, Zalema and Chelva varieties, both grown in the South, presented higher *Aspergillus* infection, while Pinot showed the lowest. Similarly, Medina et al. (2005) found that Pinnot and Chardonnay were low *Aspergillus* infected varieties. Although most of the studies detailed the varieties sampled, comparison between studies is difficult because the varieties rarely coincided.

Besides varieties, agronomical practices are confounding factors. Similar agronomical practices, including chemical treatments were described by vine growers, with an exception to cropping system and irrigation: vertical trellises were present in the North, while goblet system was used in the South. Higher contamination by *A. carbonarius* and OTA accumulation was observed in vertical trellises and goblet formation than in cordon and horizontal trellises systems.(Cozzi et al., 2007) No irrigation was applied in the South region thus reinforcing the drought in this region; this could induce plant stress and enable fungal infection (Chen et al., 2004).

3.1. Mycobiota determination

The incidence of the different fungal Genera in the analysed berries is shown in table 3. The most common mycobiota isolated in both years were: *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium*. Colonies belonging to *Aureobasidium*, *Botrytis*, *Eurotium*, *Epicoccum*, *Fusarium*, *Mucor* and *Trichoderma* were occasionally observed in the samples. However, *Alternaria* and *Aspergillus* together represented 80% of isolates present in both regions although great variability was found between vineyards (**Table 3**). *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium* and *Mucor* were the dominant Genera isolated from grapes also in surveys in France, Spain, Portugal and Tunisia (Bellí et al., 2005; Fredj et al., 2007; Medina et al., 2005; Sage et al., 2002; Serra et al., 2006b, 2005). *Alternaria* colonized 68-97% of sampled berries. A high infection by *Alternaria* was also reported in Spain (70%) and Argentina (80%), nevertheless only 36% of Portuguese grapevines were contaminated by this genus (Bellí et al., 2005a; Magnoli et al., 2003; Serra et al., 2005).

Large differences were found in the number of berries colonized by *Aspergillus* between years and regions (20-71%) (**Table 3**). These values are higher than those previously reported in Spain and Portugal (Bellí et al., 2005a; Serra et al., 2006b). The most common species isolated within this genus, in decreasing order, were those in section *Nigri*, *Flavi* and *Circumdati* (96, 2 and 2% in the Northeast area and 95, 3 and 1% in the South area). Other species isolated in this genus were *A. candidus, A. fumigatus, A. terreus, A. ustus* and *A. wentii,* which have been previously reported in vineyards (Bau et al., 2005; Magnoli et al., 2003; Sage et al., 2004, 2002; Serra et al., 2006b).

Regarding *Aspergillus* section *Flavi*, over 92% of the isolates were *A. flavus* while the rest were *A. parasiticus*. Interestingly, all isolates from the South belonged to *A. flavus*, in both seasons. *A. flavus* has been isolated in Argentina, Lebanon, Spain, Portugal and *A. parasiticu*s in Portugal, but both species have never been reported together in the same survey (Bau et al., 2005; Khoury et al., 2008; Magnoli et al., 2003; Serra et al., 2006b, 2005). In relation to *Aspergillus* section *Circumdati*, most of the isolates were *A. ochraceus* in 2011 and *A. westerdijikiae* in 2012. However, in agreement with previous studies, low incidence of *Aspergillus* section *Circumdati* was observed (Battilani et al., 2003a; Bellí et al., 2004a; Sage et al., 2002; Serra et al., 2005).

Table 3 Mycobiota, percentage of infected berries, number of colonies, and frequency of distribution of fungi isolated from berries.

		% of infected berries						Nº of colonies						% of each genera					
		Northeast[a]			South[b]			Northeast[a]			South[b]			Northeast[a]			South[b]		
Year 2011	*Aspergillus*	20.1	±	16.92	64.1	±	21.2	100.7	±	84.6	320.4	±	106.2	13.5	±	10.7	34.9	±	10.1
	Alternaria	96.9	±	4.41	80.9	±	10.5	484.7	±	22.1	404.3	±	52.7	66.6	±	10.3	45.2	±	8.6
	Aureobasidium	0.2	±	0.5	0.1	±	0.2	0.9	±	2.5	0.5	±	1	0.1	±	0.4	0.1	±	0.1
	Botrytis	0	±	0	1.7	±	3.3	0	±	0	8.3	±	16.5	0	±	0	1.1	±	2.2
	Cladosporium	25.5	±	19.39	26	±	9.6	127.7	±	96.9	130	±	47.8	16.3	±	10.3	14.2	±	4.4
	Epicoccum	0.3	±	0.41	0	±	0	1.3	±	2.1	0	±	0	0.2	±	0.2	0	±	0
	Eurotium	0	±	0	0.2	±	0.3	0	±	0	0.8	±	1.5	0	±	0	0.1	±	0.1
	Fusarium	0.4	±	0.32	0.4	±	0.5	1.8	±	1.6	1.8	±	2.4	0.3	±	0.2	0.2	±	0.3
	Hypopichia	0.1	±	0.17	0	±	0	0.5	±	0.8	0	±	0	0.1	±	0.1	0	±	0
	Mucorales	0.1	±	0.27	0.6	±	1.1	0.7	±	1.3	3	±	5.4	0.1	±	0.2	0.3	±	0.5
	Penicillium	4.6	±	9.22	7.3	±	6.8	23.1	±	46.1	36.3	±	34.2	2.6	±	4.4	3.9	±	3.5
	Thichoderma	0.4	±	1.13	0.2	±	0.3	2.3	±	5.9	1	±	1.4	0.3	±	0.9	0.1	±	0.1
Year 2012	*Aspergillus*	40.1	±	14.6	71.3	±	7.6	200.6	±	73	356.3	±	38.2	25.7	±	10.4	45.7	±	2.9
	Alternaria	89.7	±	16.2	68.4	±	8.9	448.3	±	81.1	341.8	±	44.5	56.9	±	9.9	43.9	±	5.1
	Aureobasidium	0	±	0	0.2	±	0.4	0	±	0	1.3	±	2.3	0	±	0	0.1	±	0.3
	Botrytis	0.4	±	0.8	0.1	±	0.1	2	±	4	0.3	±	0.5	0.2	±	0.4	0	±	0.1
	Cladosporium	21.1	±	9.9	6.5	±	6.1	105.3	±	49.6	32.7	±	30.3	12.9	±	4.6	4.1	±	3.8
	Epicoccum	1.1	±	1	0.7	±	0.4	5.7	±	5.1	3.5	±	2.2	0.7	±	0.6	0.4	±	0.2
	Eurotium	0	±	0.1	0.6	±	0.6	0.1	±	0.3	2.8	±	3	0	±	0	0.3	±	0.3
	Fusarium	0.5	±	0.7	1.2	±	0.7	2.4	±	3.7	5.8	±	3.3	0.3	±	0.5	0.7	±	0.4
	Neosartorya	0	±	0.1	0	±	0	0.3	±	0.5	0	±	0	0	±	0.1	0	±	0
	Mucorales	2.2	±	1.4	0.8	±	0.6	11.2	±	7.1	3.9	±	3.1	1.4	±	0.9	0.5	±	0.4
	Penicillium	2.1	±	1.2	5.8	±	1.5	10.4	±	6.2	28.9	±	7.3	1.4	±	0.9	3.7	±	1.1
	Thichoderma	0.6	±	0.7	0.5	±	0.5	3.1	±	3.3	2.3	±	2.6	0.4	±	0.4	0.3	±	0.4

[a]Mean of 10 vineyards; [b]Mean of 4 vineyards; Mean of each region ± deviations between vineyards. Each vineyard 500 berries were sampled.

Aspergillus section *Nigri* was the main section of *Aspergillus* isolated in all vineyards and years sampled. While in Argentina and Lebanon this section represented 60-64% of total aspergilli, this percentage always was higher than 90% in Europe (Bau et al., 2005; Khoury et al., 2008; Magnoli et al., 2003; Serra et al., 2005). In our study, the percentage of infected grapes by *Aspergillus* section *Nigri* in the South was similar in both years (63-67%) and it almost doubled those in the Northern fields (19-38%), where the highest rate occurred in 2012 probably due to a hailstorm one week before harvest. Black aspergilli presence was significantly higher in the South both years ($p<0.05$, **Table 4**). Within black aspergilla species, *A. carbonarius* accounted for 14.2%-44%, while *A. tubingensis* and *A. niger* toghether accounted for the largest group (**Table 5**). Previous surveys in Spain showed 17-29% *A. carbonarius* (Table 1). Similarly, the present results show that *A. tubingensis* may be better adapted to Spanish environmental conditions, as it was the dominant species in all vineyards sampled (Table 5). However *A. carbonarius* was the main potentially ochratoxigenic species in both regions and years, with the highest percentage of ochratoxigenic isolates observed. Overall, the incidence of both *A. tubingensis* and *A. niger* was higher in the South, while the presence of *A. carbonarius* depended on the year tested.

Table 4 Mann-Whitney (Wilconxon) W-test for % of black aspergilli to total isolates and for colonised berries on regions sampled (Northeast and South)

Year	% black aspergilli isolates		% infected berries by black aspergilli isolates	
2011	NE (11.9)	S (35.0)	NE (16.9)	S (69.5)
W	37		39	
p-value	0.02		0.01	
2012	NE (24.2)	S (43.0)	NE (39.5)	S (62.2)
W	36		40	
p-value	0.03		0.01	

W-test to compare the medians of the two samples.
NE. Northeasth; S. South.
Numbers between parenthesis are median values.

Considering other genus, *Eurotium* was rarely isolated from the South (<1%), as previously reported in Portugal and Argentina (Magnoli et al., 2003; Serra et al., 2006b). The number of *Penicillium* isolates was higher in the South than in the North in both years. Due to the difficulty in *Penicillium* identification, molecular techniques were used. In 2011 the identified species in this genus were *P. angulare, P. aurantiogriseum, P. crustosum, P. erythromellis, P. expansum, P. glabrum, P. nothofagi, P. oxalicum, P. purpurogenum, P. ramulosum, P. simile, P. vasconiae, P. westlingii*

and Talaromyces trachyspermus; while in 2012 *P. brevicompactum, P. citrinum, P. glabrum, P. griseofulvum, P. mariae-crucis, P. minioluteum P. olsonii, P. oxalicum, P. pinophilum, P. purpurogenum, P. sizovae,* and *Talaromyces* sp. were identified. Largeincidence and diversity in the *Penicillium* genus was also observed in previous works. Ochratoxigenic *Penicillium* species were never found in Spanish, French or Portuguese vineyards (Bau et al., 2005; Sage et al., 2004, 2002; Serra et al., 2006b, 2005). Additionally, *P. expansum* was found in this study and in French vineyards (Sage et al., 2004, 2002). Nevertheless, no potential patulin problems have been detected in the past due to the low percentage of producer strains isolated from grape (Bragulat et al., 2008).

Table 5 Ochratoxin A production by *Aspergillus* Section *Nigri* in both regions and years sampled.

		% Infected berries	Species distribution	% OTA-positive	OTA range[a]	OTA mean[a]
				Year 2011		
Northeast	*A. carbonarius*	3.1	16.3(15/92)	73.3 (11/15)	180-9364	1561
	A. niger	3.7	19.6(18/92)	25.0 (4/16)	8-119	38
	A. tubingensis	12.0	64.1(59/92)	1.8 (1/57)	11	12
South	*A. carbonarius*	27.8	44 (11/25)	100 (11/11)	14-6345	1713
	A. niger	5.1	8 (2/25)	50 (1/2)	32	32
	A. tubingensis	30.3	48 (12/25)	0 (0/12)	-	-
				Year 2012		
Northeast	*A. carbonarius*	13.6	35.3 (12/34)	83.3 (10/12)	4-18696	2568
	A. niger	3.4	8.8 (3/34)	0 (0/3)	-	-
	A. tubingensis	21.5	55.9 (19/34)	16.7 (3/19)	5-475	159
South	*A. carbonarius*	9.3	14.2 (3/21)	66.7 (2/3)	55-470	263
	A. niger	28.0	42.9 (9/21)	0 (0/9)	-	-
	A. tubingensis	28.0	42.9 (9/21)	0 (0/9)	-	-

LOD 0.01 ng/g. CYA medium; a, (ng/g agar)

Limited incidence of *Fusarium* genus was reported in both regions, the number of infected berries being slightly higher in the South in 2012. The species identified in this genus *were F. anthophilum, F. armeniacum, F. graminearum, F. oxysporum, F. proliferatum, F. semitectum, F. solani, F. subglutinans and F. verticilloides* in 2011 and *F. equiseti, F. graminearum, F. poae, F. solani, F. subglutinans* and *F. verticilloides* in 2012. Although *Fusarium* was isolated in other vineyards in different parts of the world, its presence was always limited and only described at species level in a French study (Bellí et al., 2005a; Fredj et al., 2007; Magnoli et al., 2003; Sage et al.,

2004, 2002; Serra et al., 2006b, 2005). As in our study, different species were identified were identified also in French surveys, including *F. moniliforme* and *F. oxysporum* in 2002 and *F. culmorum* and *F. lateritium* in 2004.

Despite the different agronomical cultural practices between both regions could affect the mycobiota contamination, in particular irrigation, only the statistical effects of environmental conditions were considered.

3.2. Biodiversity Indexes

Shannon and Inverse Simpson indexes have been used to describe fungal diversity in vineyards (Bellí et al., 2006; Valero et al., 2005). Bellí et al., (2006) and Valero et al., (2005), observed that fungal diversity increased from flowering to harvest in all vineyards sampled, in a range from 1.6 to 3.7 and 1.4 to 1.8 for Inverse Simpson and Shannon indexes, respectively. In both works, observed an increase of Shannon index from June to August, but it decreased at harvest (**Figure 3**).

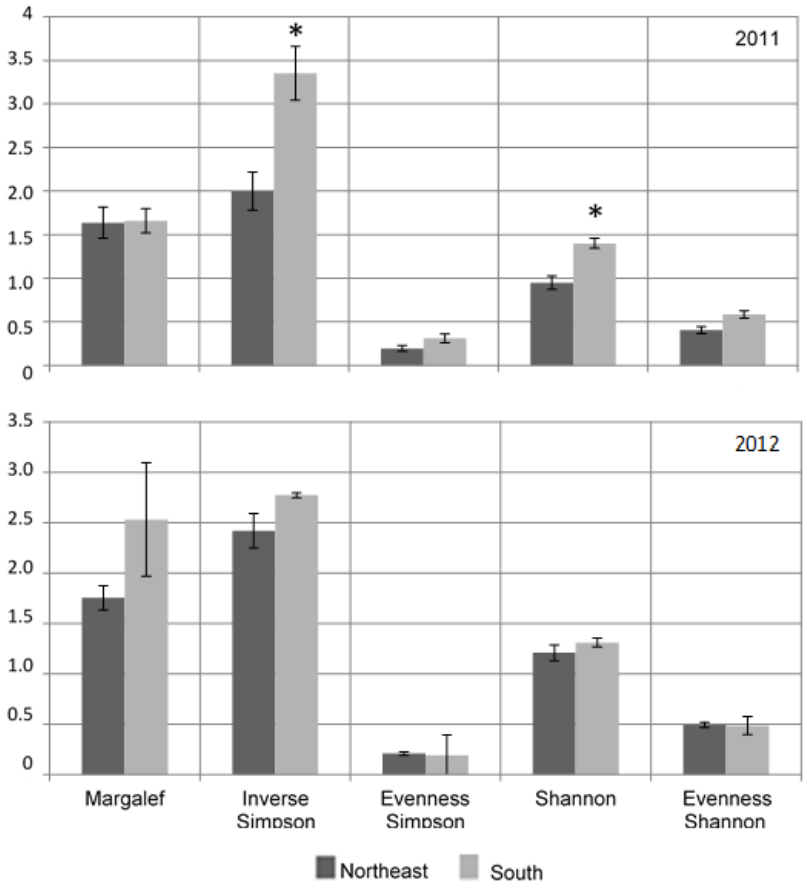


Figure 3. Diversity of the species found in the vineyards sampled in 2011 and 2012.

While no differences in richness were found between years in Northeast, an increment of richness was observed in 2012 (when hotter conditions occurred) in the South region with regards to data obtained in 2011. The surveyed area in the Northeast was smaller than the area in the South and this could increase the probability of finding fewer differences. Diversity indexes and evenness were always higher in the South, indicating a homogeneous distribution of the species in the ecosystem. In fact, significant differences were found between regions in 2011. However, the evenness was always far from 1, indicating the dominance of some species over others. The lowest diversity in the North was attributed to the predominance of *Alternaria* and *Cladosporium*, while the coexistence of black aspergilli, plus *Alternaria* and *Penicillium* resulted in a higher evenness and consequently diversity in the South ecosystem. As a result, higher diversity in this case is indicative of a higher OTA risk, assuming that the baseline mycobiota in grape is mainly composed by *Alternaria* and *Cladosporium*.

Most existing studies did not find any differences in the overall fungal contamination or diversity among the different regions sampled (Bellí et al., 2006; Fredj et al., 2007; Lasram et al., 2012b; Serra et al., 2006b), which may be due to the short distance between vineyards. At a bigger scale, Battilani et al., (2006) applied a geostatistical analysis to the incidence of *A.* section *Nigri* and particularly *A. carbonarius* presence in grapes in South Europe and Israel. Spatial variability of black aspergilli was significantly related to latitude and longitude, showing a positive West-East and North-South gradient.

3.3. Mycotoxin production capacity of isolates and OTA presence in musts

OTA production capacity of all the isolates of *Aspergillus* section *Circumdati* and a representative sample of *Aspergillus* section *Nigri* was determined in vitro in both years. No isolate from *A.* section *Circumdati* produced OTA in 2011, while 4 producers were found in 2012 (one *A. ochraceus* and one *A. westerdijikiae* isolated from Northeast and two *A. westerdijikiae* isolated from South). The *A. ochraceus* isolate produced 0.35 μg/g agar while the *A. westerdijikiae* isolates were higher producers (5.02 to 11.12 μg/g agar). Few works have tested OTA production by isolates of *A.* section *Circumdati* from grapes, probably due to the minor incidence observed (Bau et al., 2005; Bellí et al., 2004a). Interestingly, high producers were found in this section, as *A. melleus* and *A. ochraceus* produced OTA at levels of 7.82 and 73.81 μg/g agar, respectively (Bau et al., 2005). Regarding *A.* section Nigri, a high percentage of *A. carbonarius* isolates were OTA positive in both regions (66.7-100%) and showed also a

wide concentration range (0.01-18.70 µg/g agar) (Table 5). These values are similar to those observed in Europe, where *A. carbonarius* isolates able to produce 87.5 and 195.5 µg/g agar were found in France and Spain respectively (Bau et al., 2005; Sage et al., 2002). Furthermore, OTA producers were found among *A. tubingensis* (0-17%) and *A. niger* (0-50%) even though fewer isolates were tested (171). All ochratoxigenic *A. tubingensis* (4) were isolated from Northeast and no *A. niger* producers were isolated in 2012. Similar percentages of OTA producing *A. tubingensis* and *A. niger* isolates have been reported: 0-25% and 0-40%, respectively (Bau et al., 2005; Perrone et al., 2006a). A recent vine survey in the Alpine region of Northern Italy (Trentino) showed 1 out of 66 A. niger OTA producing isolates and none *A. tubingensis* (0/57) producer; however most *A. niger* isolates (87%) produced fumonisins (Storari et al., 2012). CYA was used as preferred medium to test OTA production within the isolates, as the first choice in most previously existing works (Belli, 2006; Chiotta et al., 2009; Magnoli et al., 2003; Ponsone et al., 2007; Serra et al., 2005). The culture medium used could affect both the number of mycotoxigenic isolates detected and mycotoxin amount produced. For example, OTA production were tested in parallel on CYA and GJ50 (Grape Juice) all black aspergilli isolates being OTA-positive in both media, however, lower levels of OTA and FBs were detected on GJ50.(Abrunhosa et al., 2011; Serra et al., 2005).

In our case, FB_1 and FB_2 production was analyzed in some *Aspergillus* section *Nigri* isolates from both years in which OTA production was tested. In the present survey no *Aspergillus* section *Nigri* tested produced FB_1 and FB_2 when grown on CYA, while 53% of *A. niger* isolates tested produced FB_2 when inoculated on CYA20S. Interestingly, 100% (9/9) of *A. niger* isolates tested from South in both years produced FB_2, while 1 isolate out of 10 from the Northeast region produced FB_2. Mean FB_2 production in the South isolates was 0.02 µg/mL, as well as the Northeast isolate. Similarly, 85 % of *A. niger* isolates from grape produced FB_2 and FB_4 in levels between 0.223-17.45 µg/mL and 0.069-6.955 µg/mL respectively using CYA20S (Chiotta et al., 2011). Moreover, Varga et al. (2010) detected FBs on CYA20S inoculated with *A. niger* in a range from 0.017 to 19 µg/g. CYA was previously used to check FBs production by Abrunhosa et al. (2011), although CYA20S is the most common medium used for this purpose (Abrunhosa et al., 2011; Chiotta et al., 2011; Varga et al., 2010).

Besides OTA and fumonisin producers, a wide range of potential mycotoxin producers have been isolated from vineyards and the presence of patulin, citrinin, AFs and *Alternaria* toxins

in must, wine and in dried wine fruits has been described in a recent review by (Somma et al., 2012). Authors concluded that, despite these toxins occur in grapes and wine, their low frequency does not appear to represent a human risk.

Although 10.7% of the analyzed musts contained OTA, the contamination was always below 0.1 μg/L. In 2011, OTA was detected in 3 out of 4 samples in the South, the most contaminated must containing 0.094 μg/L, with mean concentration for positive samples of 0.05 μg/L. In this region, that year, the highest *A. carbonarius* infection (27.8%) occurred, together with the higher rate of ochratoxigenic isolates (100%), which explains OTA contamination in the resulting musts. 15% of the musts surveyed in Spain in 2001 contained OTA in a concentration ranging from 0.091 to 0.813 μg/L, but OTA was never detected in musts of the vineyards sampled in Spain in 2002 and 2003 (Bellí et al., 2005a, 2004a). As in our case, Battilani et al. (2003) found a high percentage of positive samples (88%) in Italy but as in our case, they did not observe significant correlation between the number of samples colonized by black aspergilli and the OTA content in berries, although the correlation was significant when only samples colonized by OTA producing fungi were considered. In the same way, Sage et al. (2002) have pointed to a possible correlation between the presence of ochratoxin-producing strains on grapes and the presence of OTA. Although OTA risk is linked to the presence of ochratoxigenic fungi, previous studies have isolated ochratoxigenic strains while the OTA levels detected in grape and wine samples were low or undetectable (Belli et al., 2005; Chiotta et al., 2013). OTA production in berries may be different to that on medium (CYA), this could be attributed to the nutrient availability, commodity structure as well as environmental conditions. FBs contamination was not analyzed in the musts as the levels found in wine are of low concern (between 1 and 25 μg/L) (Mogensen et al., 2010b).

The initial pH of musts was between 2.9-3.9 and 3.1-4.1 in 2011 and 2012, respectively, probably due to the prominent temperatures registered in 2012 (Figure. 2). The pH of OTA contaminated musts was never lower than 3.5, but no correlation between the presence of *Aspergillus* section *Nigri* and pH of the musts was found. Several authors pointed to pH 4 as the most suitable pH for *A. carbonarius* growth and a decrease of OTA production in medium with low pH (2-2.5) (Esteban et al., 2005; Lasram et al., 2012a; Spadaro et al., 2010b) Conversely, Kapetanakou et al., (2009) did not find any specific effect of OTA production due to the medium pH (3.9-6.8).

3.4. Relation between meteorological data and *Aspergillus* section *Nigri* infection

In the Northeast, high temperatures were registered, especially during August, but never higher than 37 ºC. (Valero et al., 2005) reported growth of *Aspergillus* section *Nigri* up to 30-40 ºC, therefore these temperatures were suitable for colonization. The Southern region was hotter and drier than Northeast, and in both regions 2012 was hotter than 2011. In Northeast, the great storm which occurred in 2012 damaged the grapes favoring fungal infection; in fact, in this year the black aspergilli incidence was twice higher than the previous year (Table 5). Similarly, in Australia overall infection levels in 1998 were poor, but rain prior to harvest in 1999 and 2000 caused significant berry splitting, which allowed invasion of black aspergilli (Leong et al., 2004).

On the other hand, temperature and rainfall values recorded in August were used to calculate the thermo-wetness values proposed by Battilani et al., (2006). In this month, degree day (ºC) was 765.7 and 783.8 in the Northeast and 844.8 and 866.9 in the South, in 2011 and 2012, respectively. Rain accumulation (mm) was 0.5 in 2011 and 36.3 in 2012 in the Northeast, and no rainfall was recorder in the South (Figure. 2). From these values, high risk of black aspergilli where predicted according to Battilani's approach in both regions. Moreover, irrigation was applied in the Northeast and not in the South, thus Southern vines would be facing much drought conditions, resulting in a higher infection by black aspergilli. Positive correlation between temperature and black aspergilli incidence on grapes was confirmed in many studies (Bellí et al., 2005a, 2004a; Leong et al., 2004). However no positive correlation between RH and black aspergilli infection was found in other studies (Bellí et al., 2006, 2005). Irrigation was applied in the Northeast and not in the South, thus Southern vines would be facing much drought conditions, resulting in a higher infection by black aspergilli. In particular, the reduction of drought stress by irrigation reduced the AFs contamination in maize and drought tolerant maize cultivars results in significantly less AFs contamination in the field under drought conditions (Chen et al., 2004).

Optimal and maximum growth temperatures for *A. niger* were 35–37 °C and 45–47 °C while *A. carbonarius* showed optimum growth between 25–30 °C with an upper limit between 37–42 °C (Leong et al., 2004). In addition, strains isolated from South Spain belonging to the *Aspergillus niger* aggregate grew at 40 ºC/0.87 a_w whereas only one *A. carbonarius* strain tested grew at 0.97 a_w at this temperature (García-Cela et al., 2013). In 2012 summer in the South

was extremely hot and dry, resulting in lower levels of *A. carbonarius* compared to other biseriates, probably due to the better adaptation to hotter conditions of *A. niger* and *A. tubingensis*. Similar results were observed in Tunisia, where *A. carbonarius* was rarely isolated from the Regueb vineyards, region characterized by higher temperatures than Raf-Raf, where a large number of *A. carbonarius* was isolated (Lasram et al., 2012b).

Considering published studies in the same Northeast sampling area over the years 2001, 2002 and 2003 the percentage of infected berries by black aspergilli were 10, 6 and 19%, respectively. Therefore, minimal infection of berries took place in the most temperate and wetter year, 2002, when August Tmean was 21.3 ºC. Years 2001, 2003 and 2011 with similar Tmean in the month of August (24.5-25.5 ºC) resulted in a 10-20% of infected berries.

In conclusion, the coexistence of fungal species in vineyards, the intimate nature of competition among them and the combination of environmental conditions could affect the balance of species infecting grapes. Nevertheless, an increase in the presence of *A.* section *Nigri* may not imply greater presence of OTA in musts since this is primarily related to the presence of *A. carbonarius*. Climate change scenarios point to an increase of temperature and drought; while in not extreme climate conditions (like Northeast area in our study) this could lead to increasing black aspergilli populations, including *A. carbonarius*, under extreme conditions (like in the South in our study) this could promote the prevalence of particularly adapted species such as *A. niger*, decreasing OTA risk. FB_2 production by *A. niger* might represent an additional risk in hotter areas. As the presence of FBs in grapes has been only recently reported, ecophysiological profile of FBs production by *A. niger* is unknown, and therefore it is not possible to relate it to environmental factors. Future researches should be conducted to determine not only the distribution of FBs producing black aspergilli, but also to determine the environmental conditions that could stimulate production.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (AGL2010-22182-C04-01) and Comissionat per a Universitats i Recerca, del Departament d`Innovació, Universitats i Empresa de la Generalitat de Catalunya (AGAUR). Jéssica Gil-Serna is supported by a research grant for young scientist awarded by the Institute DANONE. The authors are also grateful to Dra. García, D. and Reyes, M., for their help in the identification of *Fusarium* and

Penicillium species, and Jordi Vidal, Josep Ramón Civit and Jose María Bidegain, for their collaboration during the harvest.

REFERENCES

Abrunhosa, L., Calado, T., Venâncio, A., 2011. Incidence of fumonisin B2 production by *Aspergillus niger* in portuguese wine regions. J. Agric. Food Chem. 59, 7514–7518. doi:10.1021/jf202123q

AEMET., 2011. Iberian Climate Atlas. Air temperature and precipitation (1971-2000).

Battilani, P., Barbano, C., Marin, S., Sanchis, V., Kozakiewicz, Z., Magan, N., 2006. Mapping of *Aspergillus* section *Nigri* in Southern Europe and Israel based on geostatistical analysis. Int. J. Food Microbiol. 111, S72–S82.

Battilani, P., Giorni, P., Pietri, A., 2003. Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. Eur. J. Plant Pathol. 109, 715–722.

Battilani P., Rossi V., Giorni P., Pietri A., Gualla A., van der Fels-Klerx H.J, Booij C.J.H., Moretti A., Logrieco A., Miglietta F, Toscano P., Miraglia M., De Santis B, Brera C., 2012. Question No EFSA-Q-2009-0081 [WWW Document]. URL http://www.efsa.europa.eu/fr/search/doc/223e.pdf (accessed 9.10.13).

Bau, M., Bragulat, M.R., Abarca, M.L., Minguez, S., Cabañes, F.J., 2005. Ochratoxin A producing fungi from Spanish vineyards, Advances in Experimental Medicine and Biology.

Bejaoui, H., Mathieu, F., Taillandier, P., Lebrihi, A., 2006. Black aspergilli and ochratoxin A production in French vineyards. Int. J. Food Microbiol. 111, S46–S52.

Belli, N., 2006. Ochratoxin A and ochratoxigenic moulds in grapes musts and wine. Ecophysiological studies. Dr. Tesis.

Bellí, N., Bau, M., Marín, S., Abarca, M.L., Ramos, A.J., Bragulat, M.R., 2006. Mycobiota and ochratoxin A producing fungi from Spanish wine grapes. Int. J. Food Microbiol. 111, S40–S45.

Bellí, N., Marín, S., Duaigües, A., Ramos, A.J., Sanchis, V., 2004a. Ochratoxin A in wines, musts and grape juices from Spain. J. Sci. Food Agric. 84, 591–594.

Bellí, N., Mitchell, D., Marín, S., Alegre, I., Ramos, A.J., Magan, N., 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., Pardo, E., Marín, S., Farré, G., Ramos, A.J., Sanchis, V., 2004b. Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. J. Sci. Food Agric. 84, 541–546.

Bezzo, G., Maggiorotto, G. and Testa, F., 2000. A method for the determination of specific mycotic contaminants random occurring in wines. Office International de la Vigne e du Vin, Paris.

Bragulat, M.R., Martínez, E., Castellá, G., Cabañes, F.J., 2008. Ochratoxin A and citrinin producing species of the genus *Penicillium* from feedstuffs. Int. J. Food Microbiol. 126, 43–48.

Cenis, J.L., 1992. Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Res. 20, 2380.

Chen, Z.-Y., Brown, R.L., Cleveland, T.E., 2004. Evidence for an association in corn between stress tolerance and resistance to *Aspergillus flavus* infection and aflatoxin contamination. Afr. J. Biotechnol. 3, 693–699.

Chiotta, M.L., Ponsone, M.L., Combina, M., Torres, A.M., Chulze, S.N., 2009. *Aspergillus* section *Nigri* species isolated from different wine-grape growing regions in Argentina. Int. J. Food Microbiol. 136, 137–141.

Chiotta, M.L., Ponsone, M.L., Sosa, D.M., Combina, M., Chulze, S.N., 2013. Biodiversity of *Aspergillus* section *Nigri* populations in Argentinian vineyards and ochratoxin A contamination. Food Microbiol. 36, 182–190.

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N., 2011. Phylogenetic characterization and ochratoxin A - Fumonisin profile of black Aspergillus isolated from grapes in Argentina. Int. J. Food Microbiol. 149, 171–176.

Cozzi, G., Perrone, G., Epifani, F., Pascale, M., Visconti, A., 2007. Epidemiology of ochratoxin A producing fungi in Apulian vineyards. XII Int. IUPAC Symp. Mycotoxin Phycotoxins Poster 1422.

Díaz, G.A., Torres, R., Vega, M., Latorre, B.A., 2009. Ochratoxigenic Aspergillus species on grapes from Chilean vineyards and Aspergillus threshold levels on grapes. Int. J. Food Microbiol. 133, 195–199. doi:10.1016/j.ijfoodmicro.2009.04.018

EC, 2007. GREEN PAPER The European Research Area: New Perspectives.

Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2005. Influence of pH and incubation time on ochratoxin A production by *Aspergillus carbonariu*s in culture media. J. Food Prot. 68, 1435–1440.

Fredj, S.M.B., 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.

Gil-Serna, J., González-Salgado, A., González-Jaén, M.T., Vázquez, C., Patiño, B., 2009a. ITS-based detection and quantification of *Aspergillus ochraceus* and *Aspergillus westerdijkia*e in grapes and green coffee beans by real-time quantitative PCR. Int. J. Food Microbiol. 131, 162–167.

Gil-Serna, J., Vázquez, C., Sardiñas, N., González-Jaén, M.T., Patiño, B., 2009b. Discrimination of the main Ochratoxin A-producing species in *Aspergillus* section *Circumdati* by specific PCR assays. Int. J. Food Microbiol. 136, 83–87.

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., Battilani, P., 2007. Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. Int. J. Food Microbiol. 113, 330–338.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

González-Salgado, A., 2010. Diagnóstico y control de especies de *Aspergillus* productoras de ocratoxina A. Dr. Tesis ISBN 978-84-693-2408-0.

González-Salgado, A., González-Jaén, T., Vázquez, C., Patiño, B., 2008. Highly sensitive PCR-based detection method specific for *Aspergillus flavu*s in wheat flour. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 25, 758–764.

Kapetanakou, A.E., Panagou, E.Z., Gialitaki, M., Drosinos, E.H., Skandamis, P.N., 2009. Evaluating the combined effect of water activity, pH and temperature on ochratoxin A production by *Aspergillus ochraceu*s and *Aspergillus carbonarius* on culture medium and Corinth raisins. Food Control 20, 725–732.

Khoury, A.E., Rizk, T., Lteif, R., Azouri, H., Delia, M.-L., Lebrihi, A., 2008. Fungal contamination and Aflatoxin B1 and Ochratoxin A in Lebanese wine-grapes and musts. Food Chem. Toxicol. 46, 2244–2250.

Lasram, S., Barketi, A., Mliki, A., Ghorbel, A., 2012a. Growth and ochratoxin A production by *Aspergillus carbonarius* at different pHs and grape maturation stages. Lett. Appl. Microbiol. 54, 418–424.

Lasram, S., Oueslati, S., Mliki, A., Ghorbel, A., Silar, P., Chebil, S., 2012b. Ochratoxin A and ochratoxigenic black *Aspergillus* species in Tunisian grapes cultivated in different geographic areas. Food Control 25, 75–80.

Leong, S.-L., Hocking, A.D., 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.

Leslie, J.F., Summerell, B.A., 2007. The *Fusarium* Laboratory Manual.

Logrieco, A., Ferracane, R., Haidukowsky, M., Cozzi, G., Visconti, A., Ritieni, A., 2009. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 26, 1495–1500.

Lucchetta, G., Bazzo, I., Cortivo, G.D., Stringher, L., Bellotto, D., Borgo, M., Angelini, E., 2010. Occurrence of black aspergilli and ochratoxin A on grapes in Italy. Toxins 2, 840–855.

Magan, N., Medina, A., Aldred, D., 2011. Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. Plant Pathol. 60, 150–163.

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.

Margalef, R., 1958. Temporal succession and spatial heterogeneity in phytoplankton. Perspect. Mar. Biol. Buzzati-Traverso Ed Univ Calif Press Berkeley 323–347.

Medina, A., Mateo, R., López-Ocaña, L., Valle-Algarra, F.M., Jiménez, M., 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. Appl. Environ. Microbiol. 71, 4696–4702.

Miller, J.D., 2008. Mycotoxins in small grains and maize: Old problems, new challenges. Food Addit. Contam. 25, 219–230.

Mogensen, J.M., Frisvad, J.C., Thrane, U., Nielsen, K.F., 2010a. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. J. Agric. Food Chem. 58, 954–958.

Mogensen, J.M., Larsen, T.O., Nielsen, K.F., 2010b. Widespread occurrence of the mycotoxin fumonisin B_2 in wine. J. Agric. Food Chem. 58, 4853–4857.

O'Donnell, K., Kistler, H.C., Cigelnik, E., Ploetz, R.C., 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. 95, 2044–2049.

Perrone, G., Mulè, G., Susca, A., Battilani, P., Pietri, A., Logrieco, A., 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensi*s, and *Aspergillus niger* strains isolated from grapes in Italy. Appl. Environ. Microbiol. 72, 680–685.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakamchanakul, W., Samson, R.A., 2007. Biodiversity of *Aspergillus* species in some important agricultural products. Stud. Mycol. 59, 53–66.

Pielou, E.C., 1969. An Introduction to Mathematical Ecology. Wiley-Intersci. John Wiley Sons 285.

Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage. Springer.

Ponsone, M.L., Combina, M., Dalcero, A., Chulze, S., 2007. Ochratoxin A and ochratoxigenic Aspergillus species in Argentinean wine grapes cultivated under organic and non-organic systems. Int. J. Food Microbiol. 114, 131–135.

Querol, A., Barrio, E., Huerta, T., Ramon, D., 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. Appl. Environ. Microbiol. 58, 2948–2953.

Sage, L., Garon, D., Seigle-Murandi, F., 2004. Fungal microflora and ochratoxin A risk in French vineyards. J. Agric. Food Chem. 52, 5764–5768.

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.

Sardiñas, N., Vázquez, C., Gil-Serna, J., González-Jaén, M.T., Patiño, B., 2010. Specific detection of *Aspergillus parasiticus* in wheat flour using a highly sensitive PCR assay. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 27, 853–858.

Selma, M.V., Martínez-Culebras, P.V., Aznar, R., 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. Int. J. Food Microbiol. 122, 126–134.

Serra, R., Braga, A., Venâncio, A., 2005. Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. Res. Microbiol. 156, 515–521.

Serra, R., Lourenço, A., Alípio, P., Venâncio, A., 2006. Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species. Mycol. Res. 110, 971–978.

Shannon, C.E., Weaver, W., 1949. The Mathematical Theory of Communication. Univ. Ill. Press Urbana IL.

Simpson, E.H., 1949. Measurement of Diversity. Nature, 163: 688. Nature 163–688.

Somma, S., Perrone, G., Logrieco, A.F., 2012. Diversity of black aspergilli and mycotoxin risks in grape, wine and dried vine fruits.

Spadaro, D., Patharajan, S., Lore, A., Gullino, M.L., Garibaldi, A., 2010. Effect of pH, water activity and temperature on the growth and accumulation of ochratoxin A produced by three strains of *Aspergillus carbonarius* isolated from Italian vineyards. Phytopathol. Mediterr. 49, 65–73.

Storari, M., Broggini, G.A.L., Bigler, L., Cordano, E., Eccel, E., Filippi, R. de, Gessler, C., Pertot, I., 2012. Risk assessment of the occurrence of black aspergilli on grapes grown in an alpine region under a climate change scenario. Eur. J. Plant Pathol. 134, 631–645.

Susca, A., Stea, G., Mulè, G., Perrone, G., 2007. Polymerase chain reaction (PCR) identification of Aspergillus niger and Aspergillus tubingensis based on the calmodulin gene. Food Addit. Contam. 24, 1154–1160.

Sydenham, E.W., Shephard, G.S., Thiel, P.G., Bird, C., Miller, B.M., 1996. Determination of Fumonisins in Corn: Evaluation of Competitive Immunoassay and HPLC Techniques. J. Agric. Food Chem. 44, 159–164.

Tjamos, S.E., Antoniou, P.P., Tjamos, E.C., 2006. Aspergillus spp., distribution, population composition and ochratoxin A production in wine producing vineyards in Greece. Int. J. Food Microbiol. 111, S61–S66.

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.

Varga, J., Kocsubé, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., Tóth, B., Bartók, T., 2010. Fumonisin contamination and fumonisin producing black aspergilli in dried vine fruits of different origin. Int. J. Food Microbiol. 143, 143–149.

Servei Meteorò␣gic de Cataluyna. Avaliable from http://www.meteo.cat/xema/AppJava/Detall24Estacio.do

Estaciones agroclimáticas de Andalucía. Avaliable from http://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria/servlet/FrontController.

STUDY III

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

García-Cela E., Crespo-Sempere A., Ramos A.J., Sanchis V. and Marin S.

Applied Mycology Unit, Food Technology Department, University of Lleida, UTPV-XaRTA, Agrotecnio Center, Av. Rovira Roure 191, 25198 Lleida, Spain

ABSTRACT

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

Publication: International Journal of Food Microbiology, (2014) 173, 89-98.

1. INTRODUCTION

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

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

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

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

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

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

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

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

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

2. MATERIALS AND METHODS

2.1. Fungal isolates, origin and molecular identification

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

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

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

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

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

Table 2 (Continued).

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

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

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

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

2.2. Ecophysiological study

2.2.1. Data generation

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

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

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

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

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

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

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

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

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

2.2.2. Modelling data

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

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

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

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

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

2.3.1. DNA extraction

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

2.3.2. Ap-PCR amplification and analysis

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

by 40 cycles consisting of 30 s at 95 ºC, 60 s at 60 ºC for (GAC)5, 52 ºC for (AGG)5 or 48 ºC for (GACA)4 and T3B, and 2 min at 72 ºC. The ap-PCR products were separated on 1.5% agarose gels with TAE buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 mg/ml), and the DNA bands visualized under UV light (**Figure. 1**). Sizes were estimated by comparison with a DNA standard length (100 bp DNA ladder, Invitrogen).

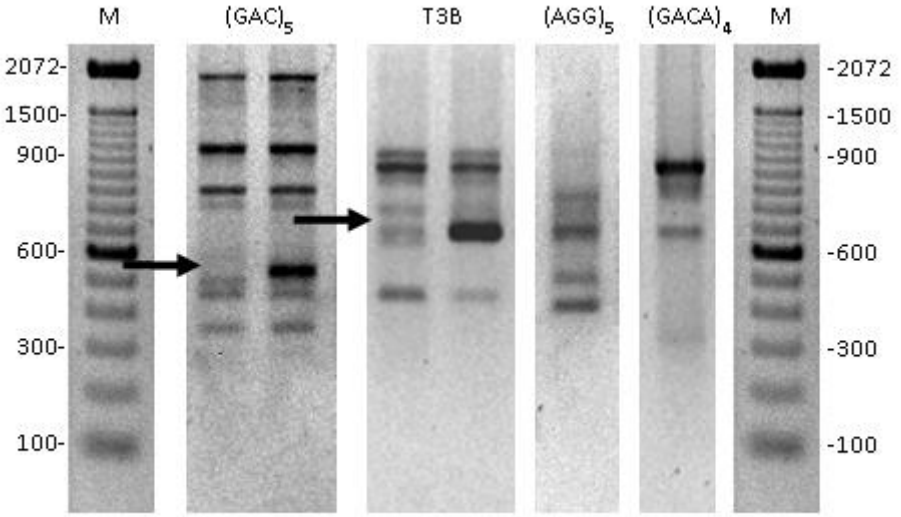


Figure 1 DNA fingerprinting profiles amplified from $(GAC)_5$, $(AGG)_5$, $(GACA)_4$ and T3B. M is the 100bp DNA ladder.

2.3.3. DNA sequencing

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

3. RESULTS

3.1. Ecophysiological study

3.1.1. A. carbonarius

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

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

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

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

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

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

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

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

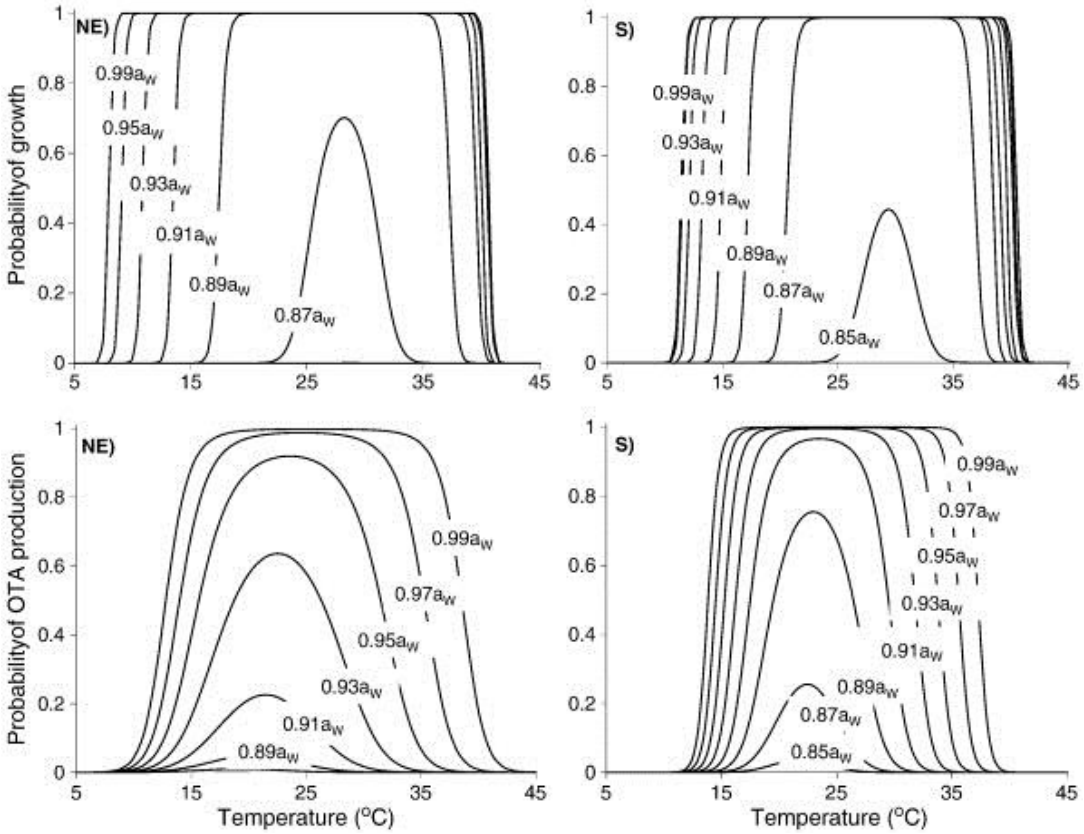


Figure 2 Effect of temperature and water activity on the predicted probability of growth and OTA production after 65 days of incubation of Aspergillus carbonarius strains isolated from Northeast (N) and South (S) Spain in synthetic nutrient medium (SNM).

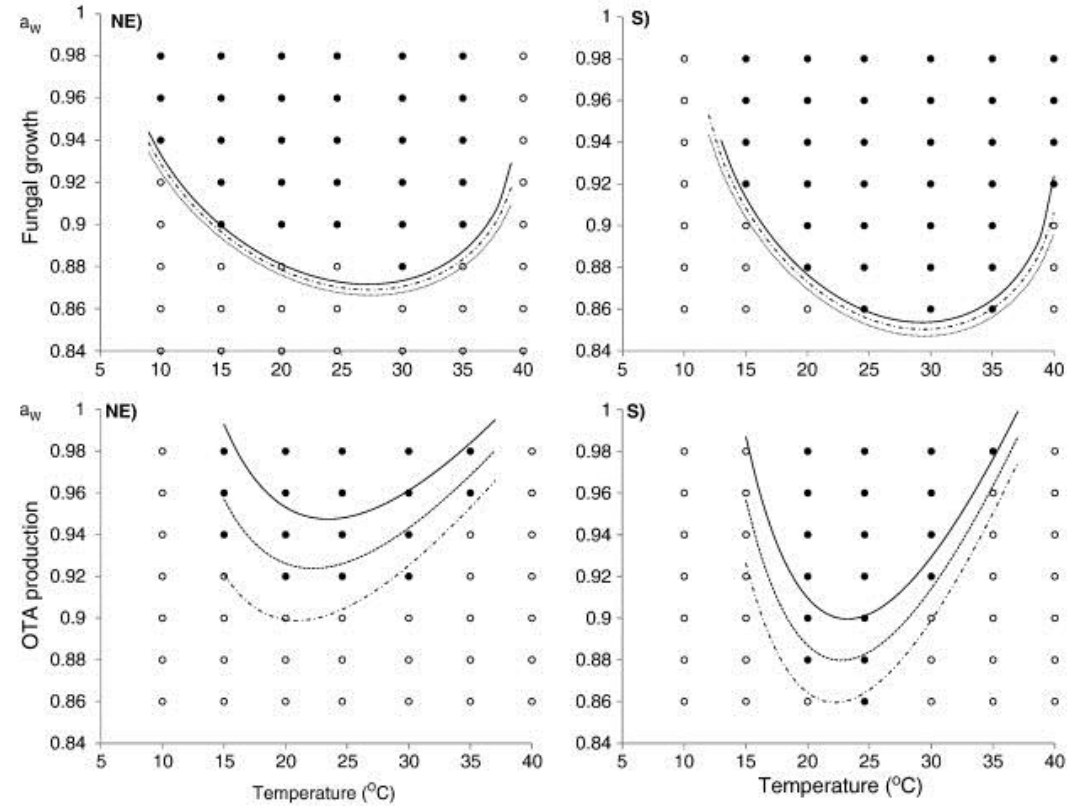


Figure 3 Fungal growth and ochratoxin A (OTA) production boundaries of *Aspergillus carbonarius* isolates from Northeast (N) and South (S) Spain in synthetic nutrient medium (SNM). Solid line indicates p=0.9; dotted line indicates p=0.5; dashed line indicates p=0.1.

3.1.2. A. tubingensis

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

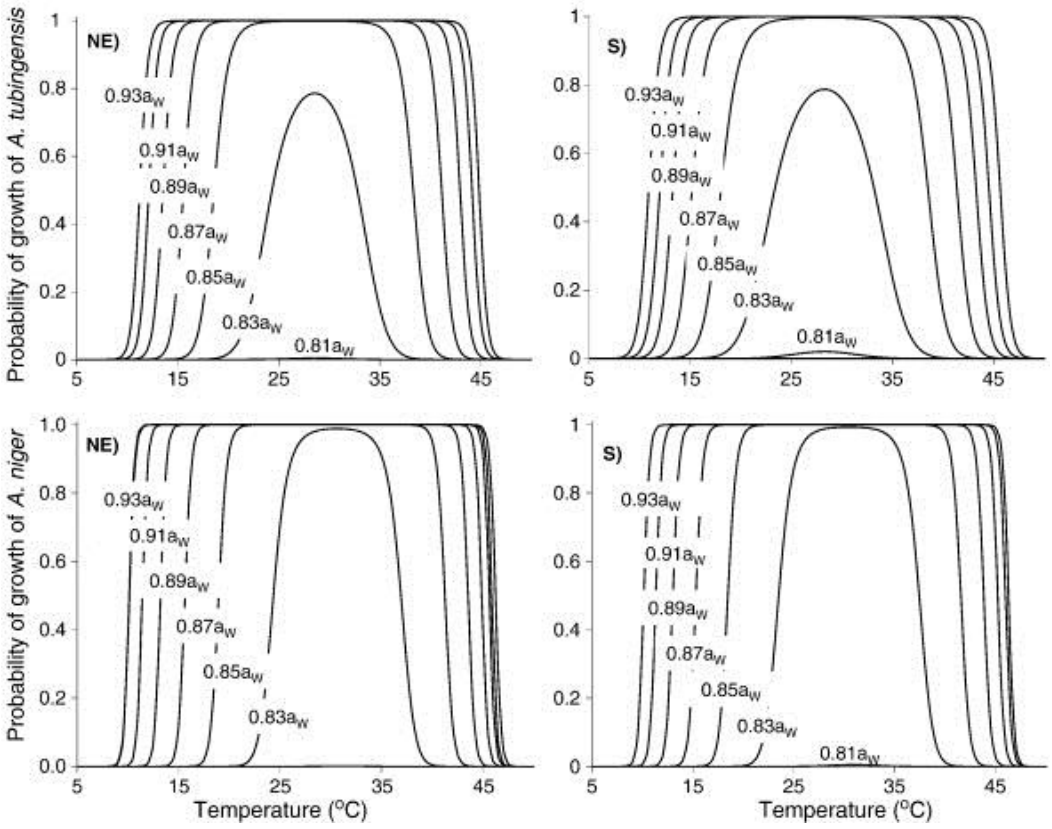


Figure 4 Effect of temperature and water activity on the predicted probability of growth and OTA production after 65 days of incubation of *Aspergillus tubingensis* and *Aspergillus niger* strains isolated from Northeast (N) and South (S) Spain in synthetic nutrient medium (SNM).

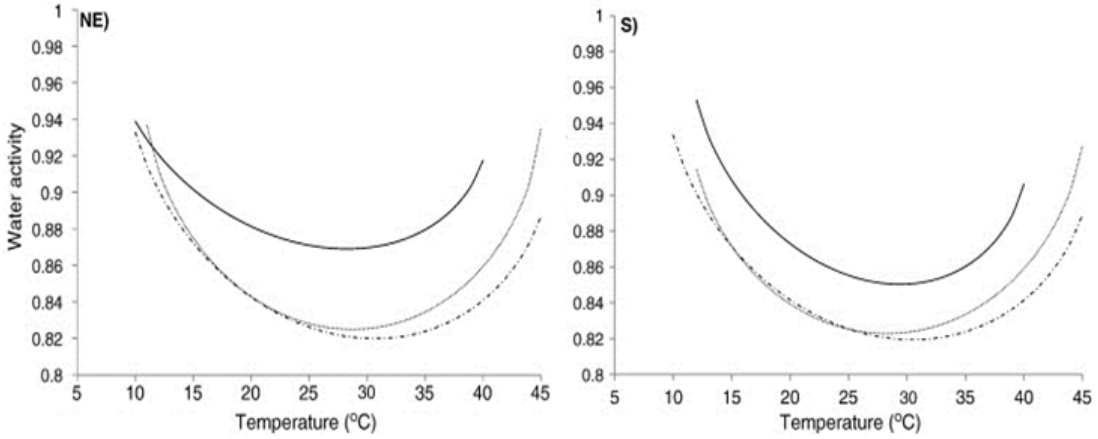
3.1.3. A. niger

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

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

3.1.4. Comparison among species

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



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

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

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

4. DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (AGL2010 - 22182-C04-01), the Comissionat per a Universitats i Recerca, del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (AGAUR).

REFERENCES

Abed, K.F., 2008. Differentiation of *Aspergillus niger* by random amplification of polymorphic DNA. J. Ind. Microbiol. Biotechnol. 35, 1027–1032. doi:10.1007/s10295-008-0378-x

Bahkali, A.H., Abd-Elsalam, K.A., Guo, J.-R., Khiyami, M.A., Verreet, J.-A., 2012. Characterization of novel Di-, Tri-, and tetranucleotide microsatellite primers suitable for genotyping various plant pathogenic fungi with special emphasis on fusaria and mycospherella graminicola. Int. J. Mol. Sci. 13, 2951–2964.

Battilani, P., Barbano, C., Marin, S., Sanchis, V., Kozakiewicz, Z., Magan, N., 2006. Mapping of Aspergillus Section Nigri in Southern Europe and Israel based on geostatistical analysis. Int. J. Food Microbiol. 111, S72–S82.

Battilani, P., Chiusa, G., Cervi, C., Trevisan, M., Ghebbioni, C., 1996. Fungal growth and ergosterol content in tomato fruits infected by fungi. Ital. J. Food Sci. 8, 283–289.

Bau, M., Bragulat, M.R., Abarca, M.L., Minguez, S., Cabañes, F.J., 2005. Ochratoxin A producing fungi from Spanish vineyards, Advances in Experimental Medicine and Biology.

Bau, M., Castellá, G., Bragulat, M.R., Cabañes, F.J., 2006. RFLP characterization of *Aspergillus niger aggregate* species from grapes from Europe and Israel. Int. J. Food Microbiol. 111, S18–S21.

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

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

Bellí, N., Ramos, A.J., Coronas, I., Sanchis, V., Marín, S., 2005. *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. J. Appl. Microbiol. 98, 839–844.

Blesa, J., Soriano, J.M., Moltó, J.C., Mañes, J., 2006. Factors affecting the presence of ochratoxin a in wines. Crit. Rev. Food Sci. Nutr. 46, 473–478.

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

Brera, C., Debegnach, F., Minardi, V., Prantera, E., Pannunzi, E., Faleo, S., Santis, B.D., Miraglia, M., 2008. Ochratoxin A contamination in Italian wine samples and evaluation of the exposure in the Italian population. J. Agric. Food Chem. 56, 10611–10618.

Cabañes, F.J., Bragulat, M.R., Castellá, G., 2013. Characterization of nonochratoxigenic strains of *Aspergillus carbonarius* from grapes. Food Microbiol. 36, 135–141. doi:10.1016/j.fm.2013.05.004

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N., 2011. Phylogenetic characterization and ochratoxin A - Fumonisin profile of black *Aspergillus* isolated from grapes in Argentina. Int. J. Food Microbiol. 149, 171–176.

Dallyn, H., 1978. Effect of substrate water activity on growth of certain xerophilic fungi. PhD Thesis South Bank Univ. Lond.

Delfini, C., 1982. Tecnica di microbiologia enologica. Luigi Scialpi, Roma.

Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2004. Effects of temperature and incubation time on production of ochratoxin a by black aspergilli. Res. Microbiol. 155, 861–866.

Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2006. Study of the effect of water activity and temperature on ochratoxin A production by *Aspergillus carbonarius*. Food Microbiol. 23, 634–640.

Esteban, A., Leong, S.L., Hocking, A.D., Abarca, M.L., Cabañes, F.J., Tran-dinh, N., 2008. Utility of Microsatellite Markers and Amplified Fragment Length Polymorphism in the Study of potentially ochratoxigenic black Aspergilli. Curr. Microbiol. 57, 348–55. doi:http://dx.doi.org/10.1007/s00284-008-9201-1

Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2011. Modelling the effect of temperature and water activity in the growth boundaries of Aspergillus ochraceus and Aspergillus parasiticus. Food Microbiol. 28, 406–417.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

Guzev, L., Danshin, A., Ziv, S., Lichter, A., 2006. Occurrence of ochratoxin A producing fungi in wine and table grapes in Israel. Int. J. Food Microbiol. 111, S67–S71.

Pitt, J.I, Hocking, D.A., 1997. Fungi and food spoilage. Lond. N. Y. Blackie Acad. Prof. 2nd ed.

Khoury, A.E., Rizk, T., Lteif, R., Azouri, H., Delia, M.-L., Lebrihi, A., 2008. Fungal contamination and Aflatoxin B1 and Ochratoxin A in Lebanese wine-grapes and musts. Food Chem. Toxicol. 46, 2244–2250.

Lasram, S., Barketi, A., Mliki, A., Ghorbel, A., 2012a. Growth and ochratoxin A production by *Aspergillus carbonarius* at different pHs and grape maturation stages. Lett. Appl. Microbiol. 54, 418–424.

Lasram, S., Oueslati, S., Mliki, A., Ghorbel, A., Silar, P., Chebil, S., 2012b. Ochratoxin A and ochratoxigenic black *Aspergillus* species in Tunisian grapes cultivated in different geographic areas. Food Control 25, 75–80.

Lasram, S., Oueslati, S., Valero, A., Marin, S., Ghorbel, A., Sanchis, V., 2010. Water activity and temperature effects on fungal growth and ochratoxin A production by ochratoxigenic *Aspergillus carbonarius* isolated from Tunisian grapes. J. Food Sci. 75, M89–M97.

Leong, S.. L., Hocking, A.D., 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.

Leong, S.L., Hocking, A.D., 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.

Logrieco, A., Ferracane, R., Haidukowsky, M., Cozzi, G., Visconti, A., Ritieni, A., 2009. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 26, 1495–1500.

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.

Marín, S., Bellí, N., Lasram, S., Chebil, S., Ramos, A.J., Ghorbel, A., Sanchis, V., 2006. Kinetics of ochratoxin A production and accumulation by *Aspergillus carbonarius* on synthetic grape medium at different temperature levels. J. Food Sci. 71, M196–M199.

Marín, S., Hodžić, I., Ramos, A.J., Sanchis, V., 2008. Predicting the growth/no-growth boundary and ochratoxin A production by *Aspergillus carbonarius* in pistachio nuts. Food Microbiol. 25, 683–689.

Martínez-Culebras, P.V., Crespo-Sempere, A., Sánchez-Hervás, M., Elizaquivel, P., Aznar, R., Ramón, D., 2009. Molecular characterization of the black *Aspergillus* isolates responsible for ochratoxin A contamination in grapes and wine in relation to taxonomy of *Aspergillus* section *Nigri*. Int. J. Food Microbiol. 132, 33–41. doi:10.1016/j.ijfoodmicro.2009.03.015

Martínez-Culebras, P.V., Ramón, D., 2007. An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes and wine. Int. J. Food Microbiol. 113, 147–153.

Medina, A., Mateo, R., López-Ocaña, L., Valle-Algarra, F.M., Jiménez, M., 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. Appl. Environ. Microbiol. 71, 4696–4702.

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. J. Appl. Microbiol. 97, 439–445.

Mogensen, J.M., Frisvad, J.C., Thrane, U., Nielsen, K.F., 2010. Production of fumonisin B_2 and B_4 by A*spergillus niger* on grapes and raisins. J. Agric. Food Chem. 58, 954–958.

Oliveri, C., Torta, L., Catara, V., 2008. A polyphasic approach to the identification of ochratoxin A-producing black *Aspergillus* isolates from vineyards in Sicily. Int. J. Food Microbiol. 127, 147–154.

Otteneder, H., Majerus, P., 2000. Occurrence of ochratoxin A (OTA) in wines: Influence of the type of wine and its geographical origin. Food Addit. Contam. 17, 793–798.

Perrone, G., Mulè, G., Susca, A., Battilani, P., Pietri, A., Logrieco, A., 2006a. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Appl. Environ. Microbiol. 72, 680–685.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakamchanakul, W., Samson, R.A., 2007. Biodiversity of *Aspergillus* species in some important agricultural products. Stud. Mycol. 59, 53–66.

Perrone, G., Susca, A., Epifani, F., Mulè, G., 2006b. AFLP characterization of Southern Europe population of *Aspergillus* section *Nigri* from grapes. Int. J. Food Microbiol. 111, S22–S27.

Perrone, G., Varga, J., Susca, A., Frisvad, J.C., Stea, G., Kocsubé, S., Tóth, B., Kozakiewicz, Z., Samson, R.A., 2008. *Aspergillus uvarum* sp. nov., an uniseriate black *Aspergillus* species isolated from grapes in Europe. Int. J. Syst. Evol. Microbiol. 58, 1032–1039.

Romero, S.M., Patriarca, A., Pinto, V.F., Vaamonde, G., 2007. Effect of water activity and temperature on growth of ochratoxigenic strains of *Aspergillus carbonarius* isolated from Argentinean dried vine fruits. Int. J. Food Microbiol. 115, 140–143.

Rosa, C.A.D.R., Palacios, V., Combina, M., Fraga, M.E., Rekson, A.D.O., Magnoli, C.E., Dalcero, A.M., 2002. Potential ochratoxin A producers from wine grapes in Argentina and Brazil. Food Addit. Contam. 19, 408–414.

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.

Selma, M.V., Martínez-Culebras, P.V., Aznar, R., 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. Int. J. Food Microbiol. 122, 126–134.

Selouane, A., Bouya, D., Lebrihi, A., Decock, C., Bouseta, A., 2009. Impact of some environmental factors on growth and production of ochratoxin A of/by *Aspergillus tubingensis, A. niger,* and *A. carbonarius* isolated from moroccan grapes. J. Microbiol. 47, 411–419.

Serra, R., Braga, A., Venâncio, A., 2005. Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. Res. Microbiol. 156, 515–521.

Serra, R., Lourenço, A., Alípio, P., Venâncio, A., 2006. Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species. Mycol. Res. 110, 971–978.

Shephard, G.S., Fabiani, A., Stockenström, S., Mshicileli, N., Sewram, V., 2003. Quantitation of ochratoxin A in South African wines. J. Agric. Food Chem. 51, 1102–1106.

Sohpal, V.K., Dey, A., Singh, A., 2010. MEGA biocentric software for sequence and phylogenetic analysis: a review. Int J Bioinforma. Res Appl 6, 230–240. doi:10.1504/IJBRA.2010.034072

Somma, S., Perrone, G., Logrieco, A.F., 2012. Diversity of black aspergilli and mycotoxin risks in grape, wine and dried vine fruits.

Spadaro, D., Patharajan, S., Lorè, A., Garibaldi, A., Gullino, M.L., 2012. Ochratoxigenic black species of aspergilli in grape fruits of northern Italy identified by an improved PCR-RFLP procedure. Toxins 4, 42–54.

Susca, A., Perrone, G., Cozzi, G., Stea, G., Logrieco, A.F., Mulè, G., 2013. Multilocus sequence analysis of *Aspergillus* section *Nigri* in dried vine fruits of worldwide origin. Int. J. Food Microbiol. 165, 163–168.

Susca, A., Stea, G., Mulè, G., Perrone, G., 2007. Polymerase chain reaction (PCR) identification of *Aspergillus niger* and *Aspergillus tubingensis* based on the calmodulin gene. Food Addit. Contam. 24, 1154–1160.

Tassou, C.C., Natskoulis, P.I., Magan, N., Panagou, E.Z., 2009. Effect of temperature and water activity on growth and ochratoxin A production boundaries of two *Aspergillus carbonarius* isolates on a simulated grape juice medium. J. Appl. Microbiol. 107, 257–268.

Valero, A., Begum, M., Hocking, A.D., Marín, S., Ramos, A.J., Sanchis, V., 2008. Mycelial growth and ochratoxin A production by *Aspergillus* section *Nigri* on simulated grape medium in modified atmospheres. J. Appl. Microbiol. 105, 372–379.

Valero, A., Farré, J.R., Sanchis, V., Ramos, A.J., Marín, S., 2006. Effects of fungal interaction on ochratoxin A production by *A. carbonarius* at different temperatures and a_W. Int. J. Food Microbiol. 110, 160–164.

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

Varga, J., Kocsubé, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., Tóth, B., Bartók, T., 2010. Fumonisin contamination and fumonisin producing black aspergilli in dried vine fruits of different origin. Int. J. Food Microbiol. 143, 143–149.

Vega, M., Ríos, G., Baer, D.V., Mardones, C., Tessini, C., Herlitz, E., Saelzer, R., Ruiz, M.A., 2012. Ochratoxin A occurrence in wines produced in Chile. Food Control 28, 147–150.

STUDY IV

Conidia survival of *Aspergillus* section *Nigri*, *Flavi* and *Circumdati* under UV-A and UV-B radiation with cycling temperature/light regime

García-Cela, E*., Reyes, M., Marín, S., Sanchis, V. and Ramos A. J.

Applied Mycology Unit, Food Technology Department.University of Lleida. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure 191. 25198 Lleida. Spain.

ABSTRACT

The impact of climate change has been identified as an emerging issue for food and feed safety. Moreover, the United Nations Environment Programme has noted the lack of information regarding the enhanced effect of UV-B on fungi and bacteria. In this study the effect of ultraviolet radiation (UV) on conidia of six mycotoxigenic *Aspergillus* species (*A. carbonarius, A. tubingensis, A. flavus, A. parasiticus, A. westerdijkiae* and *A. ochraceus*) was assessed. Conidia were incubated for 15 days under light/dark cycles and temperatures between 20 to 30 ºC per day. Additionally, six hours of exposure to UV-A or UV-B radiation per day were included in the light exposure. UV doses used were 1.7 ± 0.2 mW/cm^2 of UV-A (peak 365 nm) and 0.10 ± 0.2 mW/cm^2 of UV-B (peak 312 nm), slightly higher than present UV sunlight mean values. The intrinsic decrease of viability of conidia along time was accentuated when they were UV irradiated. UV-B radiation was more harmful than UV-A. Conidial resistance to UV light was more marked in *Aspergillus* section *Nigri*, followed by *Flavi* and *Circumdati* and hence the pigmentation of the conidia could be related to UV sensitivity. Interestingly, different resistance was observed within species belonging to sections *Flavi* and *Nigri*. As conclusion, a possible increase in UV radiation related with climatic change could lead to a reduction in the inoculum present in the field and even it could exert a selective selection on fungal species.

Submitted to Fungal Ecology

1. INTRODUCTION

Aspergillus can contaminate agricultural products at different stages including pre-harvest, harvest, storage, processing and handling. *Aspergillus* species have been isolated in field surveys from several commodities such as corn, rice, wheat, Brazil nuts, peanuts, pistachios, figs, grapes, onions, pepper, coffee and cocoa (Baquião et al., 2012; Copetti et al., 2011; Da Silveira Campos et al., 2009; Denizel et al., 1976; Freire et al., 2000; Giorni et al., 2007; Heperkan et al., 1994; Makun et al., 2011; Özer et al., 2009; Riba et al., 2008; Sweany et al., 2011; Zorzete et al., 2011). Fungal infection of crops can result in food secondary rots, with the possible accumulation of mycotoxins (Perrone et al., 2007). Moreover, climatic conditions directly affect the fungal infection and the subsequent mycotoxin contamination in foods and raw materials.

Recently, the Intergovernmental Panel on Climate Change (IPCC) has published the last report (AR5), in which it was concluded that the climate warming is 'unequivocal', and that it is likely that anthropomorphic greenhouse gas emissions contribute to current warming trends (IPCC, 2013). The impact of climate change has been identified as an emerging issue for food and feed safety (Miraglia et al., 2009), and its possible consequences on mycotoxins frequency in crops have been theorized by several researches (Magan et al., 2011; Miraglia et al., 2009; Paterson and Lima, 2011, 2010; Tirado et al., 2010; Wu et al., 2010). Moreover, the World Meteorological Organization (WMO) has also highlighted that human emissions of the chlorofluorocarbons (CFCs) and other chemicals have an important role in the atmosphere changes by damaging the stratospheric ozone layer that filters out harmful ultraviolet radiation (UV) (WMO, 2013). Increased UV-B radiation, interacting with other global change factors, may affect many of the important ecosystems, with important implications for food security and food quality (UNEP, 2002; WMO, 2010).

Although the information about the effect of UV-B on fungi and bacteria is scarce, studies on microfungal communities in soils of extreme habitats, as desert (very high solar radiation, drought, and extreme temperatures), suggest the dominance of dark-coloured microfungi with large multicelled conidia (Grishkan et al., 2007, 2003). Particularly, species of the genus *Aspergillus* (mainly *A. fumigatus*) and teleomorphic ascomycetes accounted for the thermotolerant mycobiota obtained at a temperature of 37 ºC (Grishkan et al., 2007). The main airborne fungal species identified after exposure to solar radiation were predominantly:

Aspergillus niger, Alternaria alternate, Cladosporium cladosporoides and *Arthrinium phaerosporum* (Ulevičius et al., 2004).

The impact of UV radiation in fungal spore germination, growth and sporulation depends on time exposure, UV wavelength and fungal species (Aylor and Sanogo, 1997; Fourtouni et al., 1998; Moody et al., 1999; Nicot et al., 1996; Osman et al., 1988; Rotem, et al., 1985).

The main goal of our investigation was to evaluate the survival and adaptation to UV radiation of conidia from six species belonging to *Aspergillus* Genus. Also the effect of isolate origin and pigmentation of conidia on resistance to UV light was assessed.

2. MATERIALS AND METHODS

2.1. Isolates and inoculation

All isolates used in this study were isolated from vineyards located in the Northeast and South Spain during the 2011 and 2012 vintages (**Table 1**).

Table 1 *Aspergillus* Spanish isolates included in this study.

Reference	Section	Specie	Isolated from	Year
AC14-UdLTA	*Circumdati*	*A. westerdjikiae*	South	2012
AC18-UdLTA	*Circumdati*	*A. westerdjikiae*	South	2012
AC7-UdLTA	*Circumdati*	*A. ochraceus*	Northeast	2012
AC16-UdLTA	*Circumdati*	*A. ochraceus*	South	2012
AF51-UdLTA	*Flavi*	*A. flavus*	South	2012
AF34-UdLTA	*Flavi*	*A. flavus*	Northeast	2012
AF41-UdLTA	*Flavi*	*A. parasiticus*	Northeast	2012
AF16-UdLTA	*Flavi*	*A. parasiticus*	Northeast	2012
311-UdLTA	*Nigri*	*A. carbonarius*	South	2011
287-UdLTA	*Nigri*	*A. carbonarius*	Northeast	2011
276-UdLTA	*Nigri*	*A. tubingensis*	South	2011
68-UdLTA	*Nigri*	*A. tubingensis*	Northeast	2011

Isolates were grown in different culture media: those from section *Nigri* in synthetic nutrient medium of grape, section *Flavi* in pistachio based medium, and section *Circumdati* in wheat based medium, for seven days at 25 ºC in the dark to enable significant sporulation. After this, spores were removed from Petri dishes and suspended in 80 mL of sterile water containing 0.05% (w/v) Tween-80® to reach a final concentration of 10^5 conidia/mL.

Aliquots (5 mL) of conidial suspensions were filtered under vacuum onto individual sterile filter membranes (pore size 0.45 μm, 25 mm diameter, cellulose acetate filter) (Sartorius Biolab Products Göttingen Germany). A total of twelve membranes for each isolate were used (3 replicated x 2 membranes per replicate x 2 irradiated/control). Membranes were enclosed in pairs in 5 cm diameter plastic Petri dishes and dried at 37 °C in microbiology incubators overnight (Leong et al., 2006). Spores deposited onto filter membranes were exposed to radiation and temperature cycles for 15 days as described latter.

2.2. Incubating conditions

Photoperiod and temperatures were chosen concurring with grape ripening in Spain (August). Photoperiod values were obtained from the National Spanish Geographic Institute (IGN), while temperatures were obtained from the State Meteorology Agency (AEMET). With the aim to simulate dawn and dusk and the consequent gradient of temperature between night and day, the incubators were set up in a temperature gradient mode based on temperature increasing period (dawn) and a temperature decreasing period (dusk) linked by two constant periods simulating day and night temperatures (**Figure. 1**).

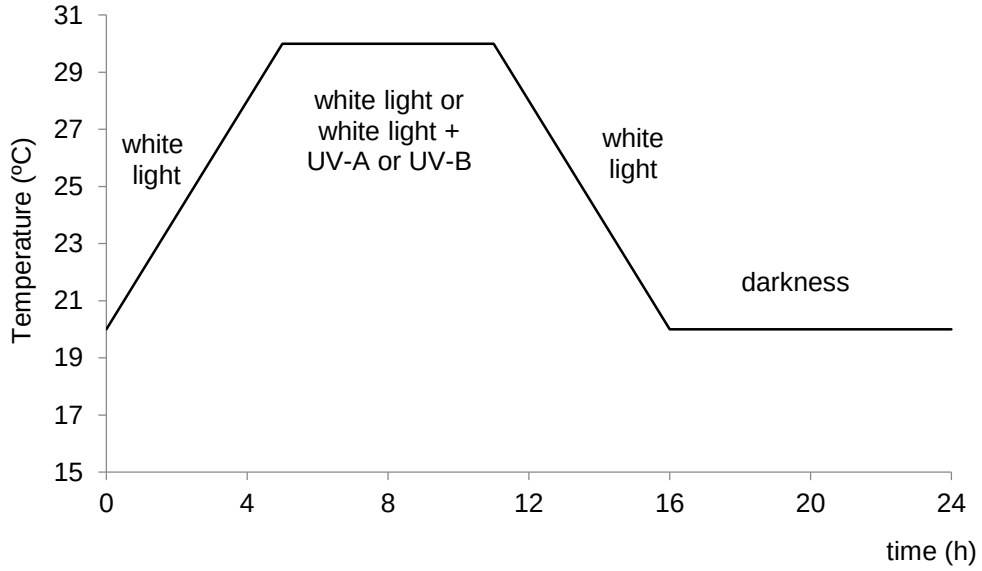


Figure. 1 Daily incubation conditions of *Aspergillus* conidia on filter membranes for 15 days. UV-A irradiation: 1.7±0.2 mW/cm²; UV-B irradiation: 0.1±0.2 mW/cm².

UV irradiated and non-irradiated Petri dishes were incubated in parallel under the same conditions in two cooled incubators (Memmert ICP-600, United Kingdom). Daylight was simulated with four cold white fluorescent lights (standard illuminant D65, 6,500 K) located in the incubators. UV irradiation was generated with a Vilber Lourmat lamp VL-215.LM

(Germany). The lamp includes two fluorescent tubes of 15 W each one and a filter that minimizes light interferences. UV-A extends from 320 to 400 nm with an energy peak at 365 nm and UV-B runs from 280 to 370 nm with an energy peak at 312 nm (**Figure. 2**).

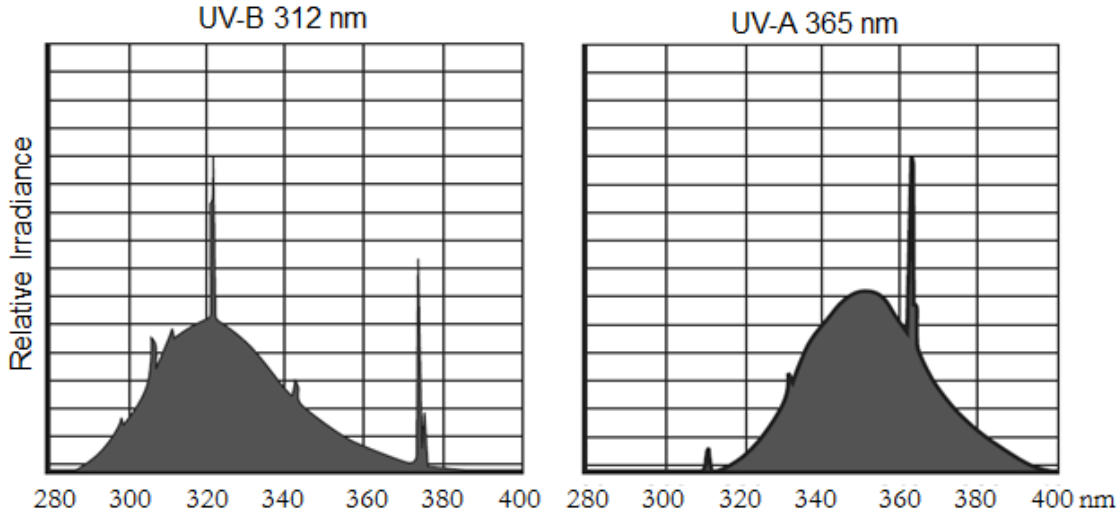


Figure. 2 Ultraviolet emission spectra for the UV lamps.

Irradiated treatments were located at 8 and 32 cm of distance from the UV-A and UV-B lamps, resulting in an irradiation of 1.7±0.2 mW/cm^2 and 0.10±0.2 mW/cm^2 for UV-A and UV-B, respectively. The irradiation was measured with a portable UV light meter (UVA-UVB PCE-UV34, PCE Iberica S.L, Spain). Values of radiant energy were chosen taking into account annual values from the South of Spain, which has a high number of annual hours of sun. UV irradiation was measured for 11 daily hours (from 7:00 to 18:00 hours) for five years. (Ortega et al., 2001). The maximum and minimum UV irradiation values were measured in July and in December, respectively. Moreover, the lower UV irradiation values were recorded between 17 to 18 h while the higher values were between 12 to 14 h GMT (GMT: Greenwich Mean Time). Annual values for UV-A ranged from 0.06 mW/cm^2 to 2.49 mW/cm^2 with a mean value of 1.17 mW/cm^2, while for UV-B they were from <0.001 mW/cm^2 to 0.23 mW/cm^2 with a mean value of 0.09 mW/cm^2. Considering the doses and the exposure time, the daily accumulated UV-A and UV-B radiation in the experiments was of 0.367 MJ/m^2 and 0.022 MJ/m^2, respectively. Ortega et al. (2001) reported a mean annual daily accumulated UV-A of 0.464 MJ/cm^2, and focusing on grape harvest month (August) the value was 0.632 MJ/cm^2. The UV-B mean daily accumulated annual value was 0.035 MJ/cm^2 while in August was 0.050 MJ/cm^2. As consequence, direct UV doses used in the study were slightly higher than the global (direct + diffuse) UV mean values recorded in South Spain, but daily accumulated irradiation was lower due to the lesser number of hours of exposure.

Each five days (5th, 10th and 15th day) conidia were dislodged from two control and two irradiated membranes into 100 mL sterile peptone (0.1% w/v) solution using a stomacher. Subsequently, a seriate dilution was carried out and 100 μL of each dilution were plated onto Dichloran Rose Bengal Chloramphenicol agar (DRBC). DRBC plates were incubated at 25 ºC in darkness for two days in the case of *Aspergillus* sections *Nigri* and *Flavi*, and three days for *Aspergillus* section *Circumdati*. Afterwards the colonies were counted. The number of viable conidia (N) was calculated through the sum of colonies counted (C) in two consecutive dilutions considering the number of plates counted at first (n_1) and second dilution (n_2), taking into account the volume dispensed to each plate and the dilution from which first count was obtained (d) (Eq. 1). Only Petri dishes with 15-150 colonies forming units (CFUs) were considered and limit of detection of the whole procedure was 10^4 ufc.

$$N = \frac{\sum C}{V\,(n_1 + 0.1\,n_2)\cdot d} \quad \text{(Eq. 1)}$$

Logarithmic reductions were calculated for each time (Eq. 2) as the difference between logarithmic of colonies in non-irradiated filters (CFU_C) and log of colonies in irradiated filters (CFU_I).

Logarithmic reduction = Log (CFU_C) - Log (CFU_I) (Eq. 2)

2.3. Statistical analysis

As an equal number of conidia was irradiated for all isolates across the experiment ($5\cdot10^6$ conidia in each membrane), analysis of variance (ANOVA) was applied directly to log CFU data in order to establish the significance of the geographical origin of the isolates, *Aspergillus* section, irradiation treatment and time on conidial counts. Subsequently, significant effects were analyzed by the Tukey´s honestly significant different test (HSD). Significance was defined as $p<0.05$.

3. RESULTS AND DISCUSSION

The effect of UV-A and UV-B radiation was tested separately on conidial survival of different *Aspergillus* spp. Both types of radiation (UV-A and UV-B) caused significant effect on

conidial survival compared to non-irradiated conidia, with a significant effect of the interaction between irradiation treatment and *Aspergillus* section conidia and time of exposure ($p<0.001$) (**Table 2**).

Table 2 The significance of the geographical origin, *Aspergillus* section, time, irradiation and their interactions on conidia survival.

Source	DF	Sum of Squares		F Ratio			
		UV-A	UV-B	UV-A		UV-B	
Origin	1	0.66	45.61	2.64	ns	1.14	ns
Section	2	48.43	30.37	96.93	**	174.52	**
Time	3	40.45	20.93	53.98	**	77.46	**
Irradiation	1	22.73	0.65	90.99	**	160.13	**
Origin x Section	2	5.99	0.18	11.99	**	2.49	ns
Origin x Time	3	1.92	0.02	2.56	ns	0.47	ns
Origin x Irradiation	1	0.15	22.63	0.61	ns	0.12	ns
Section x Time	6	20.29	6.05	13.54	**	28.86	**
Section x Irradiation	2	6.05	10.36	12.12	**	23.16	**
Time x Irradiation	3	7.75	45.61	10.34	**	26.42	**

**, p-value 0.001; *, p-value 0.05; ns, not significant.

As irradiance values measured in the Northeast region are usually lower than in the Southern region, the possible isolate adaptation as a consequence of their geographical origin was assessed. However, in general isolates from the South were more sensitive to UV radiation. Nevertheless, previous works had shown slightly different ecological profiles of temperature and a_W in terms of both optimal and marginal conditions for growth of *Aspergillus* due to the origin of isolation (García-Cela et al., 2014; Giorni et al., 2007).

In general, a decrease in viability of conidia was observed along the time for all isolates tested (**Figure 3**). *Aspergillus* section *Circumdati* showed the greatest loss of conidial viability both in UV irradiated and in control treatments; moreover, earlier (5 days) significant differences in viability due to UV radiation were observed in this section. *Aspergillus* sections *Nigri* and *Flavi* showed reduced viability after 10-15 days, although the relative decrease in viability after 15 days was less than 2 log cycles for all sections. Some authors suggested that conidia pigmentation could represent an important protection against UV radiation (Duguay and Klironomos, 2000; Grishkan et al., 2007; Moody et al., 1999; Osman et al., 1989; Rotem and Aust, 1991; Ulevičius et al., 2004; Valero et al., 2007). Three differently coloured conidia of *Aspergillus* were tested in our experiment; yellow, green and black, belonging to sections

Circumdati, *Flavi* and *Nigri*, respectively. Undoubtedly, conidia belonging to section *Circumdati,* with the lightest pigmentation, showed greater loss of viability, in both irradiated and non-irradiated treatments. Nevertheless, at the 5th day, when no significant differences compared to the first day were observed in the controls, only significant differences due to irradiation were noticeable in section *Circumdati* under UV-A and section *Circumdati* and *Flavi* under UV-B. The photo-protective potential under UV-C radiation of fungal pigments of three *A. niger* isolates possessing the same genetic background, but differing in their degree of pigmentation have been studied recently by Esbelin et al. (2013). The authors reported that spores of *A. niger* with a fawn and a white pigmentation were more sensitive to continuous UV-C radiation than the wild-type *A. niger* isolate with dark pigmentation. Dominance of dark-coloured microfungi is characteristic for almost all mycologically studied desert soils (Halwagy et al., 1982; Mulder and El-Hendawy, 1999; Ranzoni, 1968). Therefore, dark coloured spores could confer more protection against UV-B radiation.

Furthermore, data were analyzed separately for each *Aspergillus* section in order to establish significant differences among species (**Table 3**). Within the section *Flavi*, *A. parasiticus* was significantly more affected than *A. flavus*, similarly within the section *Nigri, A. tubingensis* was more affected than *A. carbonarius* but only when they were irradiated under UV-B, and no significant differences were observed within the section *Circumdati* (**Figure 4**). Therefore, differences in conidial survival can not be only attributed to the pigmentation, and other physical characteristics should be taken account. The projected surface area-to-volume ratio (SAV) of spores or wall thickness have also been emphasized as responsible for conidial survival to radiation (Moody et al., 1999; Rotem and Aust, 1991; Valero et al., 2007). In fact, the projected SAV of spores is an important factor on UV sensitivity, as the lower sensitivity of spores was related to lower SAV values (Moody et al., 1999). The spores of *Aspergillus* species tested are spherical, and therefore lower SAV corresponds to bigger radius. Simões et al. (2013) described the structural diversity of spores of black aspergilli. Interestingly, *A. carbonarius* showed the higher diameters (7.658 μm) among all the section *Nigri*, compared to other species like *A. tubingensis* (3.972 μm) or *A. niger* (3.340 μm). This could be the cause of the significant differences between these species when irradiated with UV-B. Similarly, *A. carbonarius* was significantly more resistant than *A. niger* when it was irradiated during short time under UV-C (Valero et al., 2007). The authors also suggested that the resistance of *A. carbonarius* was due to the thicker conidia wall.

Table 3 Effect of specie, time, irradiation and their interactions on conidia survival within each *Aspergillus* section.

	Source	Df	Sum of Squares		F-Ratio			
			UV-A	UV-B	UV-A		UV-B	
Aspergillus section *Nigri*	Species	1	0.01	0.09	0.12	ns	1.42	ns
	Time	3	1.34	0.66	5.52	*	3.29	*
	Irradiation	1	0.70	1.30	8.63	*	19.47	**
	Species x time	3	0.01	0.09	0.05	ns	0.46	ns
	Species x Irradiation	1	0.00	0.50	0.02	ns	7.45	*
	Time x Irradiation	3	0.96	1.06	3.94	*	5.3	*
Aspergillus section *Flavi*	Species	1	1.67	0.60	5.99	*	11.39	*
	Time	3	11.01	3.02	13.17	**	18.97	**
	Irradiation	1	9.09	4.69	32.59	**	88.32	**
	Species x time	3	3.17	0.41	3.79	*	2.6	ns
	Species x Irradiation	1	0.46	0.23	1.67	ns	4.35	*
	Time x Irradiation	3	7.09	1.57	8.47	**	9.84	**
Aspergillus section *Circumdati*	Species	1	0.39	0.04	1.51	ns	0.32	ns
	Time	3	51.03	51.28	65.47	**	124.21	**
	Irradiation	1	19.42	21.33	74.76	**	155.02	**
	Species x time	3	0.91	0.22	1.17	ns	0.54	ns
	Species x Irradiation	1	0.39	0.03	1.51	ns	0.23	ns
	Time x Irradiation	3	11.15	15.22	14.31	**	36.86	**

**, p-value 0.001; *, p-value 0.05; ns, not significant.

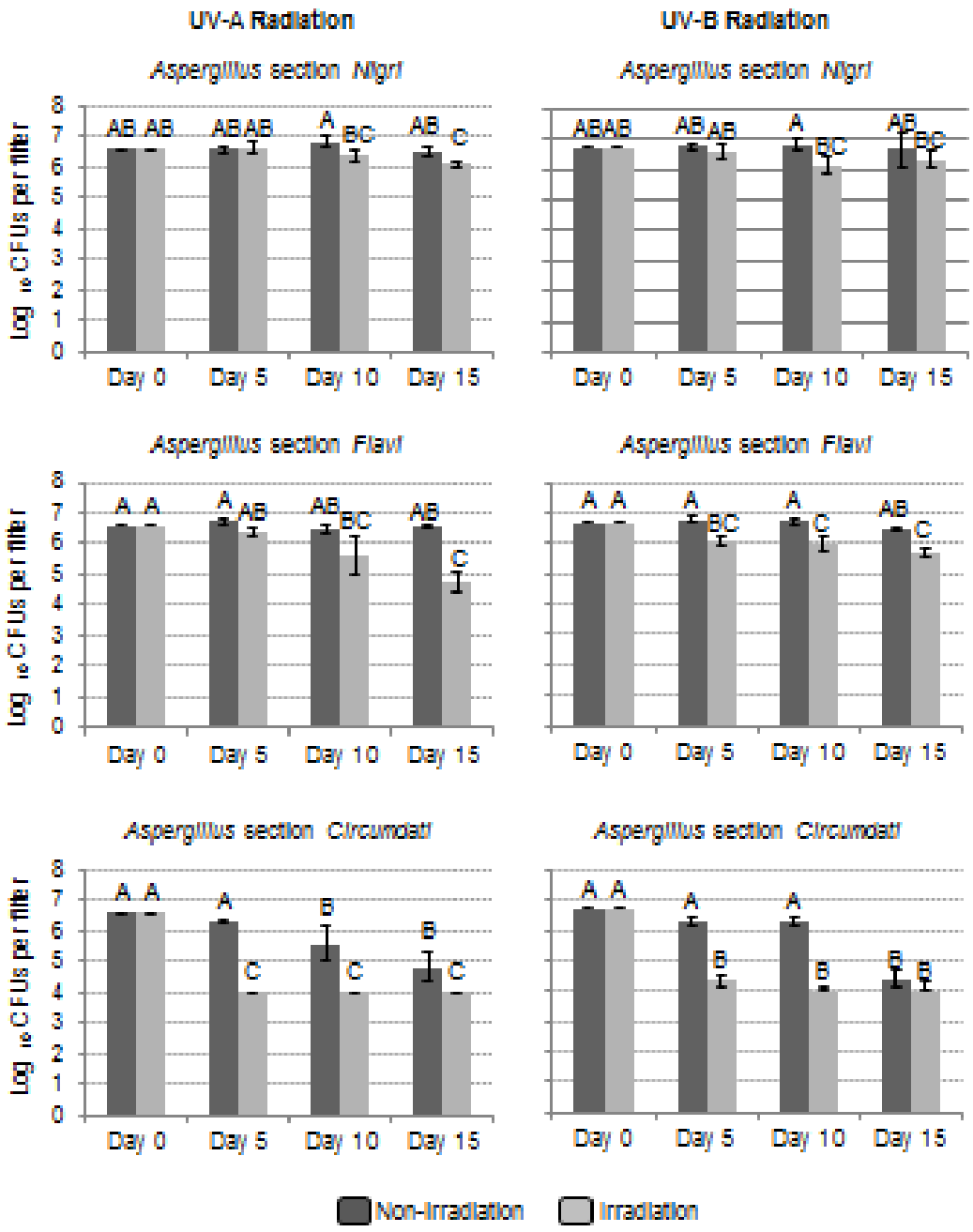


Figure. 3. Mean survival of UV-exposed Aspergillus conidia along time. Error bars denote standard deviation of eight 25 mm diameter filter membranes. Letters indicate homogeneous groups within the same treatment (Tukey HSD, $p<0.05$).

On the other hand, light is a very important signal for fungi: it influences many different physiological responses such as pigmentation, sexual development, asexual conidiation, the circadian clock and secondary metabolism (Bayram et al., 2010). For this reason the experimental design consisted of alternating periods of dark and white light with UV radiation exposure included in the light periods, while this approach was not used in previous experiments where only UV light was tested (Fourtouni et al., 1998; Moody et al., 1999).

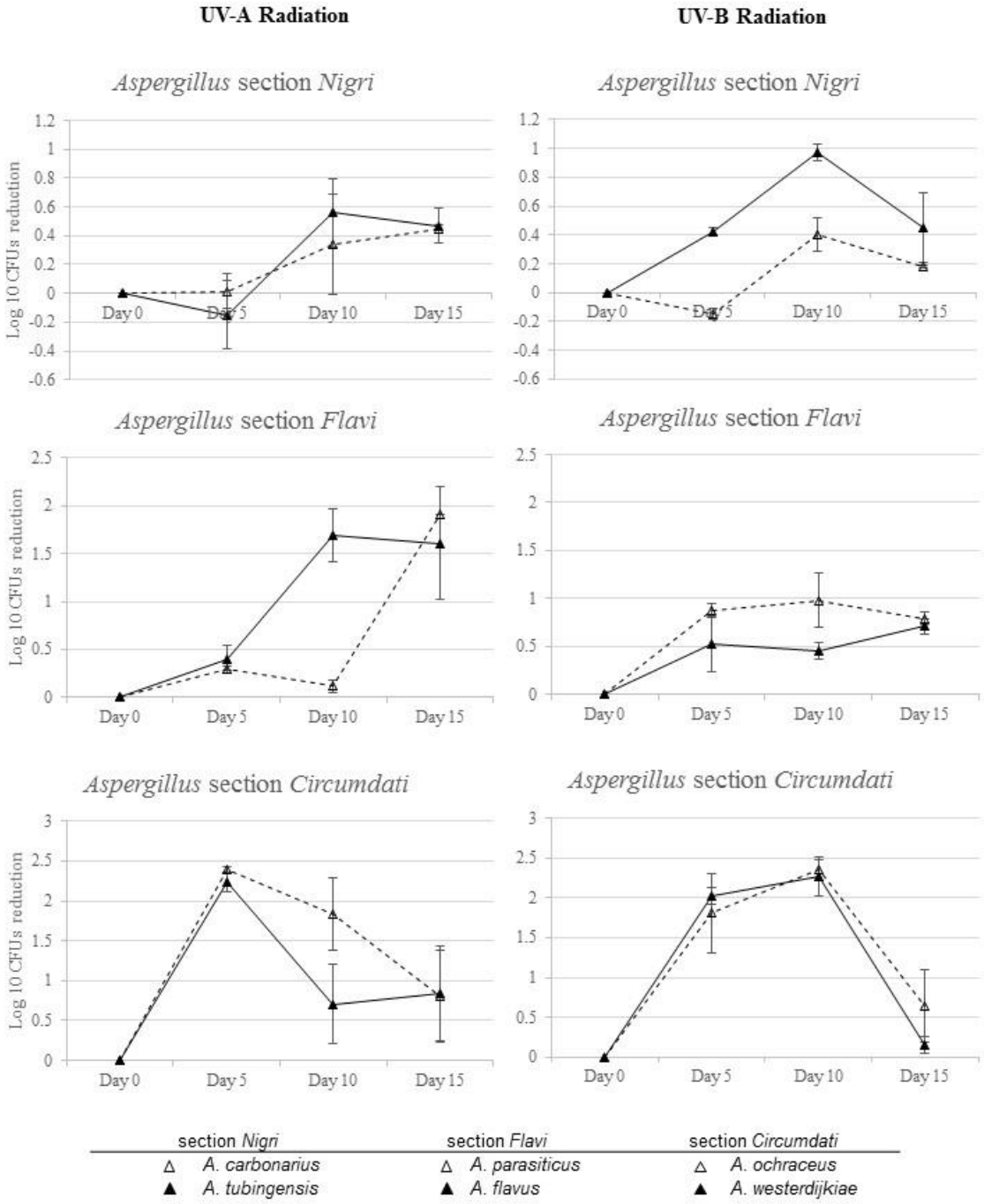


Figure. 4. Percentage of reduction of viability of *Aspergillus* conidia on filter membranes along time. Mean results of four 25 mm diameter filter membranes were used for percentage calculation.

Focusing on UV-A, we found a relevant mortality of conidia due to the exposure to 1.7 mW/cm^2 (1836 kJ/m^2) around the 5th day on section *Circumdati* and the 10th day on *Aspergillus* sections *Flavi* and *Nigri* (3672 kJ/m^2). Double time of exposure (12 h/day) but lower irradiance 0.60-1.250 mW/cm^2 (43.2-540 kJ/m^2·day) during one day did not cause any significant difference on germination percentage of sporangia of *Bremia lactucae* compared to

dark incubation (Wu et al., 2000). Shorter exposure times (3 h/day) but under higher irradiance doses (3.056-5.556 mW/cm^2) for 16 days (330-600 kJ/m^2·day) caused different effects on germination depending on the species tested (Moody et al., 1999): it was enhanced in *Penicillium purpurogenum*, but reduced in *Cladosporium cladosporoides*.

Under UV-B radiation (0.1 mW/cm^2 for 6 h/day, 21.6 kJ/m^{2}day), a significant reduction was also observed on conidia viability around the 5th day on *Aspergillus* sections *Circumdati* and *Flavi* and 10th day on *Nigri*. As for UV-A radiation, irradiation for 16 days at <0.001-0.019 mW/cm^2 for 3 h/day (0-2.1 kJ/m^2·day) led to divergent effects depending on the species (Moody et al., 1999). Exposure of *B. lactucae* to doses of 0.150 and 0.700 mW/cm^2 in a range from 2 to 12 hours (10.8-302.4 kJ/m^2·day) (Wu et al., 2000) resulted in highly reduced germination (71-100%) compared to white light and darkness.

The effect of UV-A and UV-B was not compared as both radiations were applied at different levels of intensity; however, as comparable levels of survival were observed and UV-B was applied at a lower dose, it is clear that UV-B produces more deleterious effects due to its shorter wavelength.

Field studies with sunlight exposure have shown greater conidia mortality than laboratory works (Rotem et al., 1985) for *Alternaria solani* (20%), *Uromyces phaseoli* (40%), *Peronospora tabacina* (93%) and *Venturia inequalis* (95%) (Aylor and Sanogo, 1997; Ben-Yephet and Shtienberg, 1994; Leong et al., 2006; Rotem et al., 1985). Exposure to direct sunlight showed a higher reduction on the viability of *A. carbonarius* conidia supported on filter membranes after aprox. one week than in our case, although a small part of this decrease (aprox. 15 %) was attributable to the wind which could have blown some spores form the filter membranes (Leong et al., 2006). Similarly, no disease was observed in carnation inoculated with *Fusarium wilt* grown under direct solar radiation, whereas severe disease was observed in plants under 85% shade cover (Ben-Yephet and Shtienberg, 1994). The effect of solar irradiance in the field cannot be assessed independently of other physical variables, especially temperature (Aylor and Sanogo, 1997) or protection by the infected host tissue (Rotem et al., 1985). For instance, spores on bunches could be somewhat shielded from sunlight, depending on the bunch and canopy architecture (Leong et al., 2006).

In conclusion, an increase in UV radiation may unbalance the surviving spore species present in vineyards, and as a consequence the potential inoculum in the field may change, possibly favoring in the future an even higher predominance of black aspergilli that at present. Moreover, the overall spore inoculum present in the field may decrease. Additionally, spore survival does not guarantee efficient germinative tube emergence and hyphal extension under UV exposure. Thus, germination and mycelium resistance should be evaluated, since the fact that spores can survive does not imply that the resulting hyphae have the same advantages (Duguay and Klironomos, 2000).

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (AGL2010-22182-C04-01) and Comissionat per a Universitats i Recerca, del Departament d`Innovació, Universitats I Empresa de la Generalitat de Catalunya (AGAUR).

REFERENCES

AEMET, 2012. Agencia Española de Meteorología. Atlas de radiación solar en España utilizando datos del SAF de clima de EUMETSAT. Agencia Estatal de Meteorología. Available from: http://www.aemet.es/documentos/es/serviciosclimaticos/datosclimatologicos/atlas_radiacion_solar/atlas_de_radiacion_24042012.pdf Consulted 03/2013

AEMET Agencia Española de Meteorología. Climate Dates. Available from: http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos Consulted 03/2013

Aylor, D. E. and Sanogo, S. (1997) Germinability of *Venturia inaequalis* conidia exposed to sunlight. *Phytopathology* **87**, 628–633.

Baquião, A. C., Zorzete, P., Reis, T. A., Assunção, E., Vergueiro, S. and Correa, B. (2012) Mycoflora and mycotoxins in field samples of Brazil nuts. *Food Control* **28**, 224–229.

Bayram, Ö., Braus, G. H., Fischer, R. and Rodriguez-Romero, J. (2010) Spotlight on *Aspergillus nidulans* photosensory systems. *Fungal Genetics and Biology* **47**, 900–908.

Copetti, M. V., Iamanaka, B. T., Frisvad, J. C., Pereira, J. L. and Taniwaki, M. H. (2011) Mycobiota of cocoa: From farm to chocolate. *Food Microbiology* **28**, 1499–1504.

Denizel, T., Jarvis, B. and Rolfe, E. J. (1976) A field survey of pistachio (*Pistacia vera*) nut production and storage in Turkey with particular reference to aflatoxin contamination. *Journal of the Science of Food and Agriculture* **27**, 1021–1026.

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. *Applied Soil Ecology* **14**, 157–164.

Esbelin, J., Mallea, S., Ram, A. F. J. and Carlin, F. (2013) Role of pigmentation in protecting *Aspergillus niger* conidiospores against pulsed light radiation. *Photochemistry and Photobiology* **89**, 758–761.

Fourtouni, A., Manetas, Y. and Christias, C. (1998) Effects of UV-B radiation on growth, pigmentation, and spore production in the phytopathogenic fungus *Alternaria solani*. *Canadian Journal of Botany* **76**, 2093–2099.

Freire, F., Kozakiewicz, Z. and Paterson, R. (2000) Mycoflora and mycotoxins in Brazilian black pepper, white pepper and Brazil nuts. *Mycopathologia* **149**, 13–19.

García-Cela, E., Crespo-Sempere, A., Ramos, A. J., Sanchis, V. and Marin, S. (2014) Ecophysiological characterization of *Aspergillus carbonarius*, *Aspergillus tubingensis* and *Aspergillus niger* isolated from grapes in Spanish vineyards. *International Journal of Food Microbiology* **173**, 89–98.

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T. and Battilani, P. (2007) Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. *International Journal of Food Microbiology* **113**, 330–338.

Grishkan, I., Beharav, A., Kirzhner, V. and Nevo, E. (2007) Adaptive spatiotemporal distribution of soil microfungi in "Evolution Canyon" III, Nahal Shaharut, extreme southern Negev Desert, Israel. *Biological Journal of the Linnean Society* **90**, 263–277.

Grishkan, I., Nevo, E., Wasser, S. P. and Beharav, A. (2003) Adaptive spatiotemporal distribution of soil microfungi in "Evolution Canyon" II, Lower Nahal Keziv, western Upper Galilee, Israel. *Biological Journal of the Linnean Society* **78**, 527–539.

Halwagy, R., Moustafa, A. F. and Kamel, S. M. (1982) Ecology of the soil mycoflora in the desert of Kuwait. *Journal of Arid Environments* **5**, 109–125.

Heperkan, D., Aran, N. and Ayfer, M. (1994) Mycoflora and aflatoxin contamination in shelled pistachio nuts. *Journal of the Science of Food and Agriculture* **66**, 273–278.

IPCC (2013) Climate Change 2013: The Physical Science Basis, IPCC Working Group I Contribution to AR5, [online] Available from: http://www.climatechange2013.org/.

Leong, S. l. L., Hocking, A. D. and Scott, E. S. (2006) Survival and growth of *Aspergillus carbonarius* on wine grapes before harvest. *International Journal of Food Microbiology* **111**, 83–S87.

Magan, N., Medina, A. and Aldred, D. (2011) Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. *Plant Pathology* **60**, 150–163.

Makun, H. A., Dutton, M. F., Njobeh, P. B., Phoku, J. Z. and Yah, C. S. (2011) Incidence, Phylogeny and Mycotoxigenic Potentials of Fungi Isolated from Rice in Niger State, Nigeria. *Journal of Food Safety* **31**, 334–349.

Miraglia, M., Marvin, H. J. P., Kleter, G. A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., Santis, B. D., Dekkers, S., Filippi, L., Hutjes, R. W. A., Noordam, M. Y., Pisante, M., Piva, G., Prandini, A., Toti, L., Born, G. J. van den and Vespermann, A. (2009) Climate change and food safety: An emerging issue with special focus on Europe. *Food and Chemical Toxicology* **47**, 1009–1021.

Moody, S. A., Newsham, K. K., Ayres, P. G. and Paul, N. D. (1999) Variation in the responses of litter and phylloplane fungi to UV-B radiation (290-315 nm). *Mycological Research* **103**, 1469–1477.

Mulder, J. L. and El-Hendawy, H. (1999) Microfungi under stress in Kuwait's coastal saline depressions, *Kuwait. Journal of Science and Engineering* **26**, 155–171.

Nicot, P. C., Mermier, M., Vaissière, B. E. and Lagier, J. (1996) Differential spore production by Botrytis cinerea on agar medium and plant tissue under near-ultraviolet light-absorbing polyethylene film. *Plant Disease* **80**, 555–558.

Ortega, M., Gutiérrez Martín, F. J. and Camacho (2001) Medidas de la radiación solar ultravioleta en Sevilla. *Actas Dermosifiliogr* **92**, 271–275.

Osman, M., Elsayed, M. A., Mohamed, Y. A. H. and Abo-Zeid, A. M. (1989) Effect of ultraviolet irradiation on germination and growth in *Aspergillus flavus* and *Penicillium notatum*. *Mycological Research* **92**, 293–296.

Osman, M., Mohamed, Y. A., el-Sayed, M. A. and Abo-Zeid, A. (1988) Effect of UV-irradiation on some aspects of metabolic activities in *Aspergillus flavus* and *Penicillium notatum*. *Microbios***56**, 79–87.

Özer, N., Koç, M. and Der, B. (2009) The sensitivity of *Aspergillus niger* and *Fusarium oxysporum* f. sp. cepae to fungistasis in onion-growing soils. *Journal of Plant Pathology*, **91**, 401–410.

Paterson, R. R. M. and Lima, N. (2011) Further mycotoxin effects from climate change. *Food Research International* **44**, 2555–2566.

Paterson, R. R. M. and Lima, N. (2010) How will climate change affect mycotoxins in food?. *Food Research International* **43**, 1902–1914.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J. C., Meijer, M., Noonim, P., Mahakamchanakul, W. and Samson, R. A. (2007) Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology* **59**, 53–66.

Ranzoni, F. V. (1968) Fungi isolated in culture from soils of the Sonoran desert. *Mycologia* **60**, 356–371.

Riba, A., Mokrane, S., Mathieu, F., Lebrihi, A. and Sabaou, N. (2008) Mycoflora and ochratoxin A producing strains of *Aspergillus* in Algerian wheat. *International Journal of Food Microbiology* **122**, 85–92.

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.

Rotem, J., Wooding, B. and Aylor, D. E. (1985) the role of solar radiation, especially ultraviolet, in the mortality of fungal spores. *Phytopathology* **75**, 510–514.

Da Silveira Campos, R., Freitas-Silva, O., Da Cunha, F. Q., De Lourdes Mendes De Souza, M. and De Freitas, S. C. (2009) Micotoxigenic fungi and ochratoxin a in coffee fruits with prolonged stay on the tree and on the soil in the Brazilian Cerrado. *Coffee Science* **4**, 136–148.

Simões, M. F., Santos, C. and Lima, N. (2013) Structural diversity of *Aspergillus* (section *Nigri*) spores. *Microscopy and Mycroanalysis* **19**, 1151–1158.

Sweany, R. R., Damann Jr., K. E. and Kaller, M. D. (2011) Comparison of soil and corn kernel *Aspergillus flavus* populations: Evidence for niche specialization. *Phytopathology* **101**, 952–959.

Tirado, M. C., Clarke, R., Jaykus, L. A., McQuatters-Gollop, A. and Frank, J. M. (2010) Climate change and food safety: A review. *Food Research International* **43**, 1745–1765.

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

UNEP (2010) Scientific Assessment of Ozone Depletion: 2010, World Meteorological Organization Global Ozone Research and Monitoring Project—Report No. 52 Scientific Assessment of ozone Depletion : 2010 Pursuant to Article 6 of the Montreal Protocol on Substances that Deplete the Ozone Layer National Oceanic and Atmospheric Administration National Aeronautics and Space Administration United Nations Environment Programme World Meteorological Organization European Commission, [online] Available from: http://www.esrl.noaa.gov/csd/assessments/ozone/2010/chapters/chapter2.pdf.

UNEP, 2002 (2002) UNEP, Environmental Effects of Ozone Depletion: 2002 Assessment, United Nations Environment Programme, ISBN 92-807-2312-X.

Valero, A., Begum, M., Leong, S. L., Hocking, A. D., Ramos, A. J., Sanchis, V. and Marín, S. (2007) Effect of germicidal UVC light on fungi isolated from grapes and raisins. *Letters in Applied Microbiology* 45, 238–243.

WMO (2013) The global climate 2001-2010 a decade of climate extremes Summary report, World Meteorological Organization, [online] Available from: http://library.wmo.int/pmb_ged/wmo_1119_en.pdf (Accessed 25 September 2013).

Wu, B. M., Subbarao, K. V. and Van Bruggen, A. H. C. (2000) Factors affecting the survival of *Bremia lactucae* sporangia deposited on lettuce leaves. *Phytopathology* **90**, 827–833.

Wu, F., Bhatnagar, D., Bui-Klimke, T., Carbone, I., Hellmich, R., Munkvold, G., Paul, P., Payne, G. and Takle, E. (2010) Climate change impacts on mycotoxin risks in US maize. *World Mycotoxin Journal* **4**, 79–93.

Ben-Yephet, Y. and Shtienberg, D. (1994) Effects of solar radiation and temperature on *Fusarium* wilt in carnation. *Phytopathology* **84**, 1416–1421.

Zorzete, P., Reis, T. A., Felício, J. D., Baquião, A. C., Makimoto, P. and Corrêa, B. (2011) Fungi, mycotoxins and phytoalexin in peanut varieties, during plant growth in the field. *Food Chemistry* **129**, 957–964.

STUDY V

Effect of ultraviolet radiation A and B on growth and mycotoxin production by *Aspergillus carbonarius* and *Aspergillus parasiticus* in grape and pistachio media

García-Cela E., Marin S., Sanchis V., Crespo-Sempere A. and Ramos A. J.

Applied Mycology Unit, Food Technology Department. University of Lleida. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure 191. 25198 Lleida. Spain.
esther.garcia@tecal.udl.cat

ABSTRACT

The effects of two exposure times per day (6 and 16 hours) of UV-A or UV-B radiation, combined with dark and dark plus light incubation periods during 7-21 days on fungal growth and mycotoxins production of Aspergillus species were studied. *A. carbonarius* and *A. parasiticus* were inoculated on grape and pistachio media under diurnal and nocturnal temperatures choosing light photoperiod according to harvest conditions of these crops in Spain. Ultraviolet irradiation had a significant effect on *A. carbonarius* and *A. parasiticus* colony size (diameter, biomass dry weight and colony density) and mycotoxin accumulation, although intraspecies differences were observed. Inhibition of *A. carbonarius* fungal growth decreased when exposure time was reduced from 16 h to 6 h, but this was not always true for ochratoxin A (OTA) production. OTA reduction was higher under UV-A than UV-B radiation and the reduction increased along time conversely to the aflatoxins (AFs). Aflatoxin B_1 (AFB1) was the main toxin produced by *A. parasiticus* except in the UV-B light irradiated colonies which showed a higher percentage of AFG than AFB. Morphological changes were observed in colonies grown under UV-B light.

Submitted toFungal Biology.

1. INTRODUCTION

The last report from the World Meteorological Organization (WMO) highlighted that human emissions of chlorofluorocarbons (CFCs) and other chemicals have an important role in the atmosphere changes by damaging the stratospheric ozone layer that filters out harmful ultraviolet radiation (UV) (WMO, 2013). The ozone depletion has a strong link with climate change, as the physics and chemistry of the Earth's atmosphere largely determine our climate, inasmuch changes in ozone can induce changes in climate, and vice versa (McKenzie et al., 2011). For example, changes in atmospheric circulation resulting from climate change can induce regional differences in ozone, leading to increase in UV radiation in some regions and reduction in others (Hegglin and Shepherd, 2009). The United Nations Environment Programme reported that the average of total ozone values for 2006–2009 of about 3.5% and 2.5% below than the 1964-1980 averages, for 90°S-90°N and 60°S-60°N, respectively (UNEP, 2010). Ground-based UV reconstructions and satellite UV retrievals, supported in the later years by direct ground-based UV measurements, show that erythemal ("sunburning") irradiance over midlatitudes has increased since the late 1970s, which is correlated with the observed decrease in column ozone (UNEP, 2010). Solar UV radiation transmitted through the earth's atmosphere has three primary streams of incoming radiant flux depending on their wavelength range: (i) UV-C (100–280 nm) is the higher energetic portion of the UV spectrum, which does not reach the ground surface as it is completely absorbed by the ozone layer and other atmospheric constituents; (ii) UV-B (280–315 nm) still reaches ground level but it is strongly absorbed by stratospheric ozone; (iii) UV-A (315–380 nm) is only slightly absorbed by ozone layer making up most of the UV irradiance at the ground level (CIE, 1987).

Certain groups of filamentous fungi can produce harmful secondary metabolites called mycotoxins. The major groups of mycotoxins, derived from polyketide metabolism, are present in a wide range of foodstuffs: aflatoxins (AFs), fumonisins (FBs), ochratoxin A (OTA) and zearalenone (Gallo et al., 2013). Although the ecological role of mycotoxins is far from being elucidated, several studies indicate the mycotoxin biosynthesis is induced under certain stress conditions (Schmidt-Heydt et al., 2008). Moreover, Cary and Ehrlich (2006) suggested that AFs production could be a strategy of fungi to prevent from UV damage. Also, citrinin has been considered as a light protectant, since citrinin producing colonies grew better under red and blue light than non-producing colonies (Schmidt-Heydt

et al., 2011). However, the effect of UV radiation in mycotoxin biosynthesis is unknown, and to our knowledge there are no publications on this topic.

Some previous works have studied the effect of UV radiation on fungal spore germination, growth and sporulation, showing that the effect is dependent of time and wavelenght of UV exposure (**Table 1**) (Fourtouni et al., 1998; Moody et al., 1999; Osman et al., 1989; Wu, Subbarao et al., 2000). Fungal spores of *Aspergillus flavus* and *Penicillum notatum* are much more resistant to the lethal effects of UV than the vegetative mycelium (Osman et al., 1989). UV-A irradiation stimulated fungal growth of several species while in others species it had no influence on radial growth or dry mass (Fourtouni et al, 1998; Moody et al., 1999; Osman et al., 1989). The UV-B irradiation not only reduced not only reduced the germination and sporulation in most of the fungi tested but also reduced de colony diameter (Fourtouni et al., 1998; Moody et al., 1999). This contrast between the responses of fungi to these two different parts of the UV region can be explained the fact that shorter wavelength radiations are more deleterious to biologicals systmes as they carry more energy per photon than longer wavelengths (Moody et al., 1999).

The aim of this study was to assess the effect of UV-A and UV-B radiation on fungal growth and mycotoxin (OTA/AFs) production of two *Aspergillus* species commonly isolated in foodstuffs: the OTA producer *Aspergillus carbonarius* which is isolated mainly in vineyards around the world and the AFs producer *Aspergillus parasiticus* which is frequently isolated from tree nuts, as pistachio (Denizel et al., 1976; Jamali et al., 2012). For that purpose three experiments were carried out focusing on i) evaluation of the effect of cycles of UV radiation/darkness on *A. carbonarius*; ii) the effect of cycling UV radiation/ white light/darkness on *A. carbonarius*; iii) the effect of cycles of UV radiation/white light/darkness on *A. parasiticus*. Experiments ii) and iii) were launched trying to simulate field temperature and photoperiod conditions.

Table 1 Previously published studies regarding the effects of UV-A and UV-B radiation in fungi in laboratory condition

Microorganism	Wavelength (nm)		Irradiance (mW/cm^2)	Exposure time per day	Irradiance ($KJ/m^2 \cdot day$)	Days of exposure	T (°C)	Culture médium	R
	Range	Peak							
Aspergillus flavus *Penicillium notatum*		366	0.04	20-40-60- 120-240 min	0.5-5.8	1	24-25	Czapek	1
Aspergillus fumigatus *Cladosporium cladosporioides* *Leptosphaeria coniothyrium* *Marasmius androsaceus* *Mucor hiemalis* *Penicillium hordei* *Penicillium janczewskii* *Penicillium purpurogenum* *Penicillium spinulosum* *Trichoderma viride* *Ulocladium consortiale* *Verticillium state* *Alternaria alternate* *Botytis cinerea* *Cochliobolus sativus* *Epicoccum nigrum* *Khuskia oryzae* *Ulocladium botytis*	315-400 292-350		3.056-5.556 <0.001-0.019	3 h	330-600 0-2.1	16	20	PDA	2
Alternaria solani	315-360	366	0.051-0.167	12 h	21.9-72.3	7	25	PDA enriched with glucose	3
	290-315	313	<0.001-0.194	12 h	0.3-83.6	7	25		
Bremia lactucae	200-400	340-350	0.600-1.250	2-4-8-12 h	43.2-540	1		Lettuce leaves	4
	280-315	305-310	0.150-0.700	2-4-8-12 h	10.8-302.4	1			

PDA Potato Dextrose Agar

2. MATERIALS AND METHODS

2.1. Microorganisms, growth medium and inoculation

This work was carried out on three *A. carbonarius* (311, 318, 287-UdLTA) isolates isolated from grapes and one *A. parasiticus* (3.18-UdLTA) from the culture collection of the Food Department of Lleida University. Different culture media were used for each species simulating commodities from which these species are commonly isolated: Synthetic nutrient medium of grape (SNM) and pistachio based medium (PBM), respectively. Composition of SNM is similar to grape composition between veraison and ripeness (Delfini, 1982). For PBM preparation, 30 g of pistachio were ground and boiled in 1L of distilled water during 30 min. Subsequently, the extract was filtered with a gauze made up to one litre with water. Additionally, 15f of agar were added. After that, the medium was autoclaved for 15 min at 121 ºC. For each experiment, the isolates were sub-cultured on SNM or PBM plates and incubated at 25 ºC for 7 days to obtain heavily sporulating cultures. Following incubation, a sterile inoculation loop was used to remove the conidia, suspending them in Tween 80 (0.005%). After homogenizing, the suspensions were adjusted using a Thoma counting chamber to a final concentration of 10^5 conidia/mL in Tween 80 (0.005%) and 5 μL of suspension were inoculated in the middle of the Petri dishes.

2.2. Experimental design and incubation conditions

Two different combinations of UV radiation time exposure (16 and 6 h) were assessed on *A. carbonarius*. Additionally the shortest period was also studied on *A. parasiticus*. Firstly, in order to determine the effect of UV radiation on three *A. carbonarius* isolates (287-UdLTA, 311-UdLTA, 318-UdLTA), inoculated Petri dishes were incubated at 25 ºC for 21 days. Control Petri dishes were incubated under darkness while irradiated Petri dishes were incubated under a photoperiod of 16 h of UV radiation (UV-A or UV-B) and 8 h of darkness. Fungal diameter of six Petri dishes of each isolate was measured every 7 days and then three of them were used for biomass weight determination and the other three for OTA production analysis. Additionally, second experiment was carried out with the same *three A. carbonarius* isolate but incubated for seven days under the photoperiod and temperature conditions described in **Figure 1A**. At the end of the incubation period colony diameters were measured and *A. carbonarius* colonies from three Petri dishes of each isolate were divided in two equal parts for quantification of biomass weight and OTA production. In a third

experiment, *A. parasiticus* (3.18-UdLTA) was incubated for 7 days under the photoperiod and temperature conditions described in the Figure. 1B, plus full dark incubation as a control. Colony diameters were measured in two Petri dishes on days 3, 5 and 7. Subsequently, colonies were divided in two equal parts, one for fungal biomass and one for AFs determination. Photoperiod and temperatures were chosen concurring with grape (August) and pistachio (September) ripening in Spain. Photoperiod values were obtained from the National Spanish Geographic Institute (IGN), while temperatures were obtained from the Station Meteorology Spanish Agency (AEMET). With the aim to simulate dawn and dusk and the consequent gradient of temperature between night and day, the incubators were set in a temperature gradient mode based on temperature increasing period (dawn) and a temperature decreasing period (dusk) linked by two constant periods simulating day and night temperatures (**Figure 1A-B**).

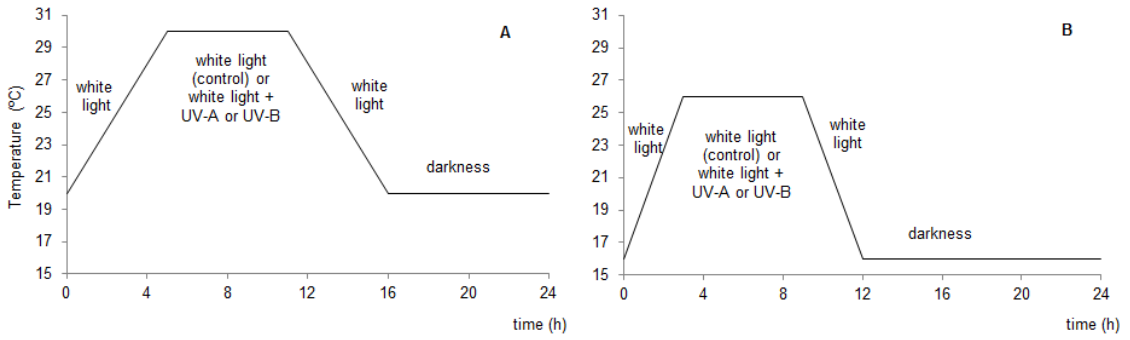


Figure 1 Incubation conditions for (A) *A. carbonarius* on SNM (synthetic nutrient grape) and (B) A. parasiticus on PBM (pistachio based medium). UV-A irradiation 1.7±0.2 mW/cm² and UV-B irradiation 0.1±0.2 mW/cm².

Irradiated and non-irradiated Petri dishes were incubated in parallel under the same conditions of time and temperature in two incubators (Memmert ICP-600, United Kingdom). Diurnal illumination was simulated with four cold white fluorescent lights (standard illuminant D65, 6,500 K) located in the incubators. UV irradiation was generated with a Vilber Lourmat lamp VL-215.LM (Germany). The lamp includes two fluorescent tubes of 15 W each one and a filter that minimizes light interferences. UV-A extends from 320 to 400 nm with an energy peak at 365 nm and UV-B runs from 280 to 370 nm with an energy peak at 312 nm (**Figure. 2**). Irradiated Petri dishes were located at 8 and 32 cm of distance from the UV-A and UV-B lamps, resulting in an irradiation of 1.7±0.2 mW/cm^2 and 0.1±0.2 mW/cm^2 for UV-A and UV-B, respectively. The irradiation was measured with a portable UV light meter (UVA-UVB PCE-UV34, PCE Iberica S.L, Spain).

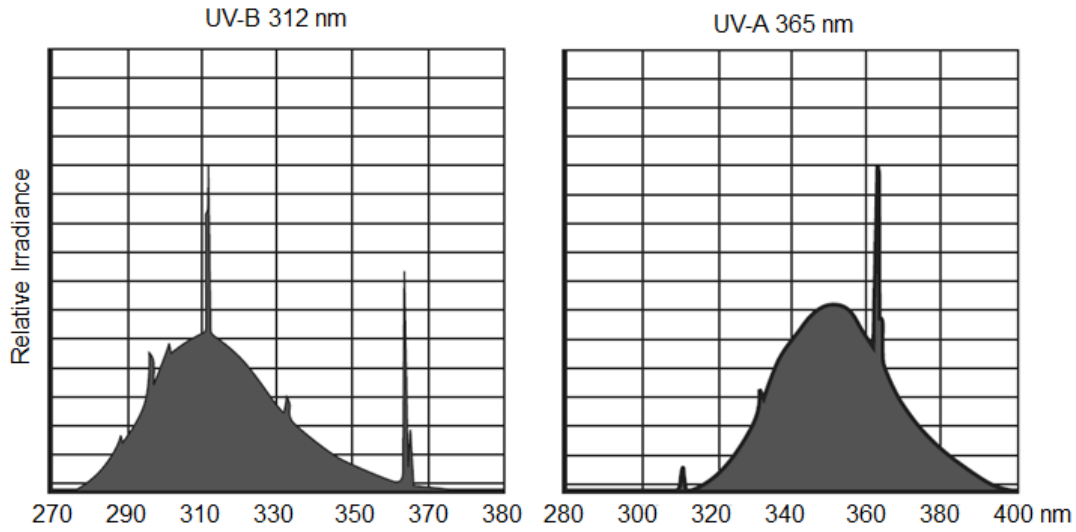


Figure 2 Ultraviolet emission spectra for the UV lamps.

Values of radiant energy were chosen taking into account annual values from the South of Spain, which has a high number of annual hours of sun. UV was measured for 11 daily hours (from 7:00 to 18:00 hours) for five years (Ortega, Martín, & Camacho, 2001). Monthly measures of maximum, mean and minimal UV-A and UV-B global (direct and diffused) radiation in hourly intervals (1991-1995) in South Spain and experimental irradiation used in this study are showed in **Figure 3**. The maximum and minimum UV irradiation were measured in July and in December, respectively. Moreover, the lower UV irradiation values were recorded between 17 to 18 h while the higher values were between 12 to14 h GMT (GMT: Greenwich Mean Time). Annual values for UV-A ranged from 0.06 mW/cm2 to 2.49 mW/cm^2 with a mean value of 1.17 mW/cm^2, while for UV-B they were from <0.001 mW/cm^2 to 0.23 mW/cm^2 with a mean value of 0.09 mW/cm^2. Considering the doses and the exposure time, the daily accumulated UV-A and UV-B radiation in the experiments was of 0.367 MJ/m^2 and 0.022 MJ/m^2 respectively. Whereas the mean daily accumulated annual UV-A radiation was 0.464 MJ/cm^2, and focusing on harvest months values were 0.632 MJ/cm^2 in August and 0.539 MJ/cm^2 in September. The UV-B mean daily accumulated annual values were 0.035 MJ/cm^2 and 0.050 MJ/cm^2 in August and 0.045 MJ/cm^2 in September. In conclusion, direct UV doses used in the study were slightly higher than the global (direct + diffuse) UV mean values recorded in South Spain, but daily accumulated irradiation was lower due to the less number of hours of exposure.

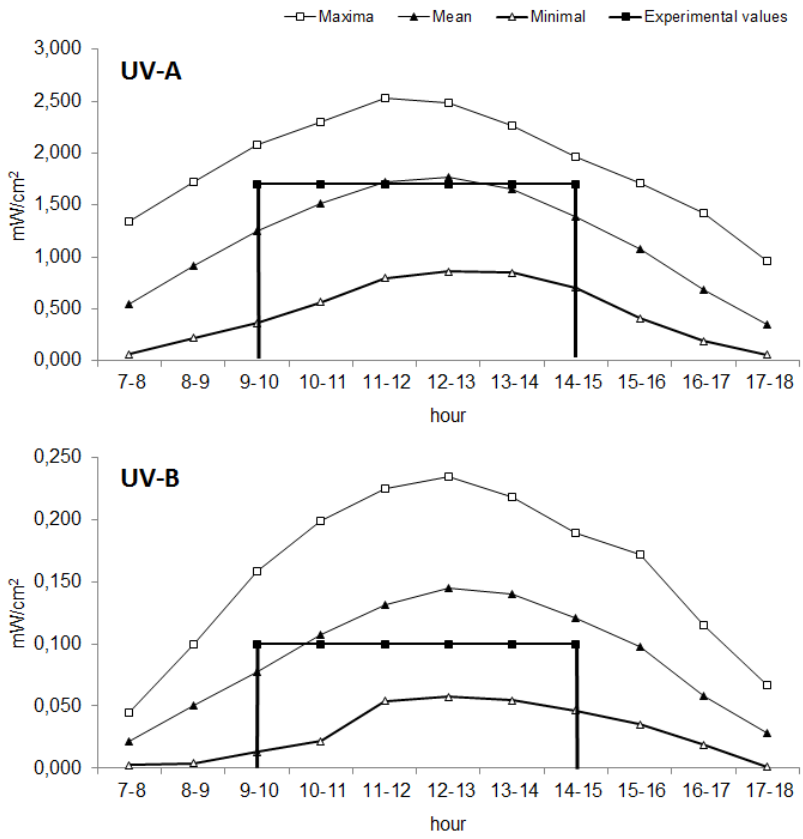


Figure 3 Monthly measures of maximum, mean and minimal UV-A and UV-B global (direct and diffused) radiation in hourly intervals (1991-1995) in Seville and experimental irradiation used in this study.

2.3. Growth assessment: colony diameter and fungal biomass

Fungal growth was determined by measuring two perpendicular diameters for each colony. Mycelium dry weight was measured as mentioned in Taniwaki et al. (2006) on culture medium. Briefly, colonies were cut from the medium, transferred to a beaker containing distilled water (100 mL approximately), and heated in a streamer for 30 min for agar melting. The intact mycelium was collected and transferred to a dried, weighed filter paper and dried at 80 ºC for 18 h. Then the filter paper plus de colony were weighed and the dry weight of biomass was calculated by difference.

2.4. Mycotoxin extraction and quantification

Agar plugs (5 mm) were removed from the middle to the outer part of the colony and placed in a vial. Mycotoxins were extracted by adding 1 mL of methanol into the vials, shaken for 5 s and allowed to rest. After 60 min, the vials were shaken again and the extract filtered

(OlimPeak filters by Teknokroma PVDF Filter, 0.45 μm, 13 mm D, Sant Cugat del Vallés, Barcelona, Spain) into another vial. Subsequently, the extract was evaporated under a stream of nitrogen and stored at 4 ºC until HPLC analysis (Waters, Mildford, Ma, S.A.). Prior to HPLC injection, dried extracts were dissolved in 1 mL of methanol: water (50:50). A HPLC system (Waters 2695, separations module, Waters, Milford, USA) equipped with a fluorescence detector Waters 2475 module (Waters, Milford, USA), precolumn Waters Spherisorb 5 μm, ODS2, 4.6x10 mm and a C18 silica gel column (Waters Spherisorb 5 μm, ODS2, 4.6 x250 mm, Millford, MA, USA) kept at 40 ºC were used. For AFs a post column photochemical derivatization system (LC Tech detector, UVC 254 nm, Germany) was used. Mobile phases were acetonitrile: water: acetic acid (57:41:2) for OTA and water: methanol: acetonitrile (70:17:17) for AFs, and were pumped at 1 mL/min under isocratic conditions. Mycotoxins were quantified on the basis of the HPLC fluorimetric response (OTA: λexc 330 nm; λem 460 nm; AFs: λexc 365 nm; λem 455 nm) compared with a range of mycotoxin standards. Detection limits of 0.01 ng/g for OTA, of 0.02 ng/g for aflatoxins B_2 and G_2, and of 0.04 ng/g for aflatoxins B_1 and G_1, were established based on a signal-to-noise ratio of 3:1. Quantification was achieved with a software integrator (Empower, Milford, MA, USA).

2.5. Statistical analysis

Results were analyzed by one-way ANOVA followed by the Tukey´s honestly significant different test (HSD), using Statgraphics® Centurion XVI (USA). The level of significance was defined as $p<0.05$.

3. RESULTS

3.1. Effect of UV-A and UV-B radiation/darkness on *A. carbonarius* (exp.1)

In this experiment, three A. carbonarius isolates were incubated at 25 ºC for 21 days under darkness or under 16 h of UV radiation/8 h darkness. Under UV-A radiation/darkness cycles, colony diameters, biomass dry weight and OTA production were reduced compared to dark treatment both on day 14 and 21 ($p<0.05$) (**Figure 4, Table 2**). Colony density was calculated dividing biomass dry weight by colony area for each time period. Density values ranged from 0.04 to 0.09 mg/mm^2 in the control treatment and from 0.03 to 0.16 mg/mm^2 in irradiated colonies with the exception of the isolate 318-UdLTA which reached 0.61 mg/mm^2 under UV-A radiation. Control colonies were less dense than the irradiated ones

on day 14, but this difference was reduced after 21 days. Significant differences were found among strains in colony diameter and OTA production but not in biomass dry weight. Besides, OTA concentration decreased with the time. The mean percentages of reduction for colony diameter, biomass dry weight and OTA production were 78.9%, 75.5% and 89.1% when isolates were cultivated under UV-A radiation for 14 days and 38.4%, 59.3% and 96.9% when the incubation period was 21 days. The isolate 287-UdLTA was less affected by the UV-A radiation than the other two isolates of A. carbonarius tested, in terms of colony diameter and biomass dry weight. Besides, OTA production by this isolate after 14 days was significantly higher than the others.

Table 2 F values of main effects and their interaction in colony diameters, fungal dry weight biomass, OTA accumulation and density of three *A. carbonarius* (287, 311 and 318-UdLTA) incubated on SNM (synthetic nutrient medium) for 14 and 21 days.

Raditation	Effects	Colony diameter (mm)	Biomass dry weight (mg)	OTA (ng/g)	Colony density (mg/mm^2)
UV-A	Strain	5.28 *	0.03 ns	21.8 **	1.18 ns
	Time	10 *	0.04 ns	13.7 **	4.17 ns
	Treatment	84 **	37.8 **	46.3 **	4.31 *
	Strain x Time	0.79 ns	1.41 ns	12.6 **	1.36 ns
	Strain x Treatment	5.28 *	2.67 ns	18.5 **	1.14 ns
	Time x Treatment	10 *	2.59 ns	15.5 **	3.87 ns
	Strain x Time x Treatment	0.79 ns	2 ns	14.1 **	1.23 ns
UV-B	Strain	1.03 ns	1.29 ns	1.5 ns	2.25 ns
	Time	2.3 ns	1.4 ns	0.81 ns	2.32 ns
	Treatment	26.1 **	5.57 **	5.77 **	2.14 ns
	Strain x Time	0.51 ns	0.55 ns	0.3 ns	2.39 ns
	Strain x Treatment	1.03 ns	0.32 ns	0.83 ns	2.26 ns
	Time x Treatment	2.3 ns	0.33 ns	0.91 ns	2.1 ns
	Strain x Time x Treatment	0.51 ns	0.07 ns	0.17 ns	2.36 ns

**, p-value 0.001; *, p-value 0.05; ns, not significant.

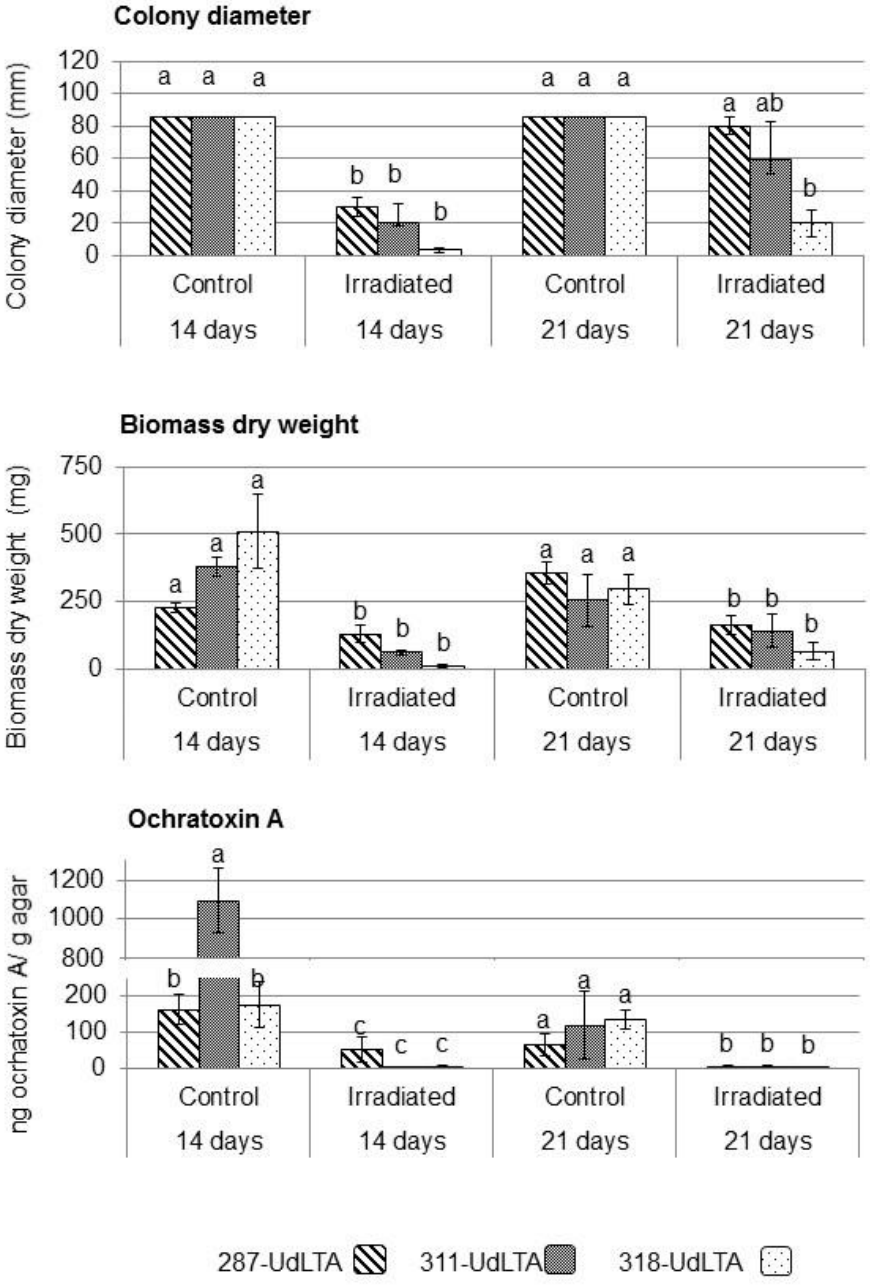


Figure 4 Colony diameters, biomass dry weight and OTA (ochratoxin A) production of *A. carbonarius* (287-UdLTA, 311-UdLTA and 318-UdLTA) on SNM (synthetic nutrient medium) at 25 ºC. Control Petri dishes were incubated under darkness while irradiated Petri dishes were incubated under photoperiod of 16 h of UV-A radiation (1.7±0.2 mW/cm²) and 8 h of darkness. Different letters mean significant differences according to Tukey (HSD) test.

Regarding UV-B light, colony diameters, biomass dry weight and OTA production were reduced, compared to the control treatment (darkness incubation) while incubation time and isolate differences had no significant impact ($p<0.05$) (**Figure. 5, Table 2**). Colony density values ranged from 0.03 to 0.05 mg/mm^2 in non-irradiated and 0.01-0.09 mg/mm^2 in irradiated colonies except for the isolate 318-UdLTA under UV-B light which was significantly denser than the other isolates (1.84 mg/mm^2). The mean percentages of

reduction caused by UV-B radiation were 37.7%, 52.3% and 70.5%; and 20.4%, 55.7% and 82.1% for colony diameter, biomass dry weight and OTA production, respectively at 14 and 21 days.

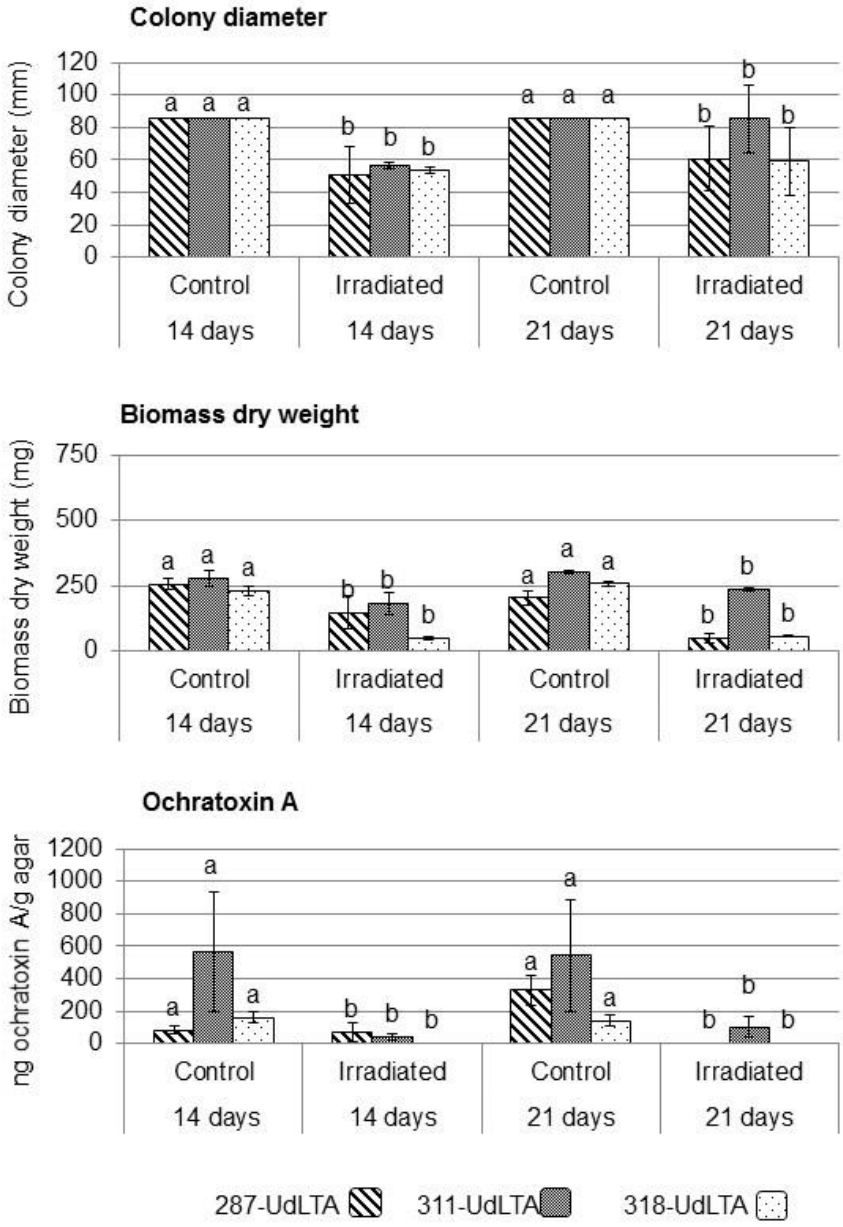


Figure 5 Colony diameters, biomass dries weight and OTA (ochratoxin A) production of *A. carbonarius* (287-UdLTA, 311-UdLTA and 318-UdLTA) in SNM (synthetic nutrient medium) at 25 ºC. Control Petri dishes were incubated under darkness while irradiated Petri dishes were incubated under photoperiod of 16 h of UV-B radiation (0.1±0.2 mW/cm²) and 8 h of darkness. Different letters mean significant differences according to Tukey (HSD) test.

Bigger colonies and higher mycotoxin production were observed after incubation under UV-B radiation than under UV-A radiation. Contrary to UV-A radiation the isolate 311-UdLTA was the less affected. Therefore, the isolate sensitivity would depend on UV wavelength. UV-

B irradiation caused milder inhibitory effects than UV-A irradiation, however, it affected the colony morphology. Sporulation was only observed in the center of the colonies, in fact, this part was harder and more compact than in non-irradiated colonies. Besides, under the microscope, neither conidia nor conidiophores were observed in the surrounding growing colony area.

3.2. Effect of UV radiation/light and dark cycles on *A. carbonarius* (exp. 2)

Three *A. carbonarius* isolates were incubated for 7 days under the photoperiod and temperature cycles described in Figure. 1. Irradiation had a significant effect on diameter, biomass dry weight and OTA accumulation, but intraspecies differences affected colony size and OTA production (**Table 3**). Significant differences on density were only observed under UV-B irradiation, where density mean value in control Petri dishes was of 0.10 mg/mm2 against mean value of 0.02 mg/mm^2 on irradiated ones. Percentages of reduction were 35.3%, 53.3% and 97.0% for UV-A, and 16.8%, 77.16% and 81.9% for UV-B in terms of colony diameter, biomass dry weight and OTA production, respectively (**Figure. 6**). UV-B radiation affected the colony morphology as observed in experiment 1. Sporulation was observed only in the center of colonies, which was harder and more compacted than the rest of the colony.

Comparing with the previous experiment, colony diameter and fungal biomass dry weight of *A. carbonarius* UV irradiated for 16 h and incubated at 25 ºC, did not exceed 10 mm and 40 mg under UV-B radiation (data not shown), respectively, after 7 days. Hence, a decrease in the UV time exposure reduced also the deleterious effects on A. carbonarius. Moreover, when comparing the controls of both experiments, that is, incubation under full darkness (exp. 1) and darkness and light cycles (exp. 2), higher values of fungal growth and OTA production were reached when white light was included. However it should be taken into account that, different temperature regimes were applied and therefore these differences could not only be attributed to light conditions but also to temperature or the combination of both variables.

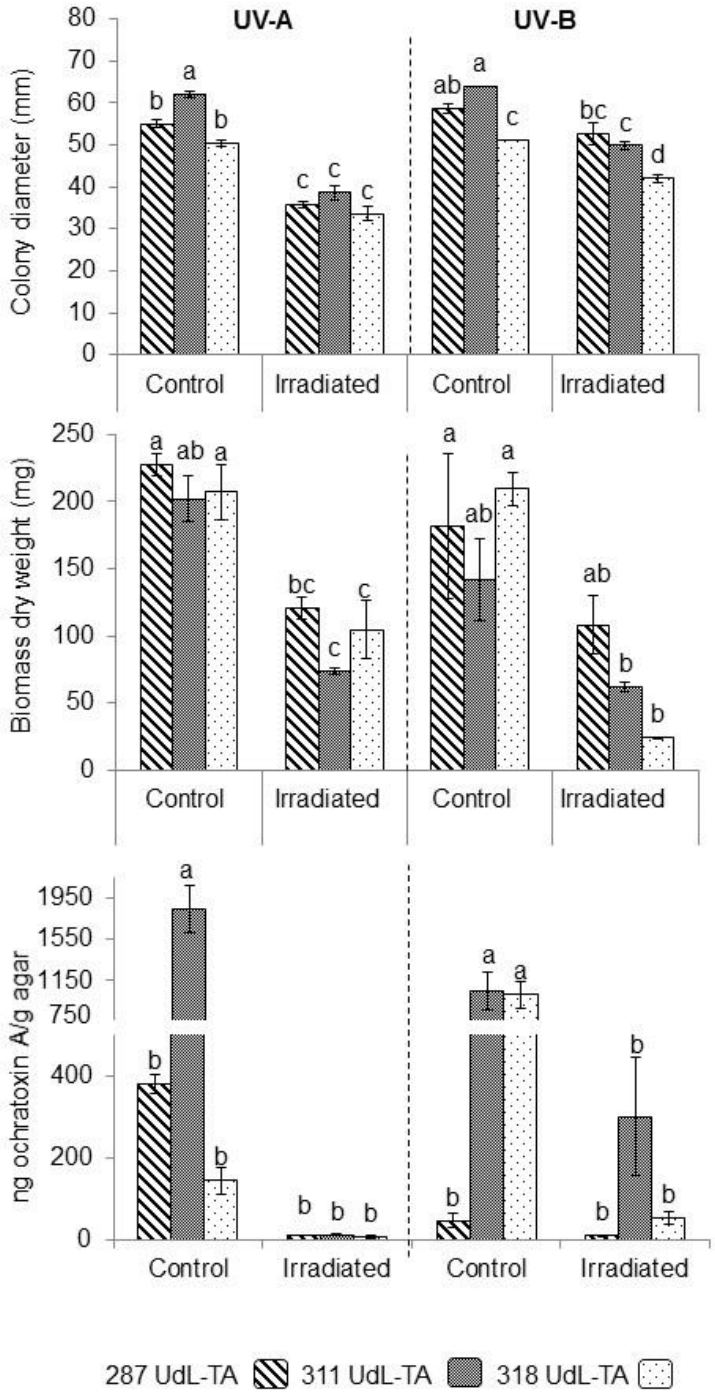


Figure. 6 Colony diameters, biomass dry weight and OTA (ochratoxin A) production by *A. carbonarius* (287-UdLTA 311-UdLTA and 318-UdLTA) on SNM (synthetic nutrient medium) after seven days under incubated conditions presented in Figure. 1. Irradiation doses are 1.7±0.2 mW/cm^2 and 0.10±0.2 mW/cm^2 for UV-A and UV-B. Different letters mean significant differences according to Tukey (HSD) test.

Table 3 F values of main effects and their interactions in colony diameters, fungal biomass, OTA accumulation, and density of three *A. carbonarius* (287, 311 and 318-UdLTA) incubated on SNM (synthetic nutrient medium) for 14 and 21 days.

Raditation	Effects	Colony diameter (mm)	Biomass dry weight (mg)	OTA (ng/g)	Colony density (mg/mm^2)
UV-A	Strain	19.59 **	2.16 ns	34.16 **	12.88 **
	Treatment	326.64 **	64 **	73.08 **	1.61 ns
	Strain x Treatment	3.13 ns	0.32 ns	33.88 **	1.01 ns
UV-B	Strain	29.93 **	1.85 ns	14.41 **	5.43 *
	Treatment	67.93 **	27.77 **	31.64 **	31.53 **
	Strain x Treatment	3.82 ns	1.28 ns	7.27 **	4.53 **

**, p-value 0.001; *, p-value 0.05; ns, not significant.

3.3. Effect of UV radiation/light and dark cycles on *A. parasiticus* (exp. 3)

One *A. parasiticus* isolate (3.18-UdLTA) was incubated on PBM for 7 days under the temperature cycles described in Figure. 1, plus full dark incubation as an additional control. UV-A irradiation had a significant effect on colony diameter compared to full darkness. In the case of AFs production significant differences were found between irradiated and non-irradiated treatments (**Table 4, Figure. 7**). In addition, biomass dry weight differences were also significant from day 5 in the case of UV-B. Nevertheless, no significant differences were observed in colony density due to the UV radiation, with mean values of 0.05 mg/mm^2. The present experiment included the comparison between full dark period and light/dark without UV light; the results showed no differences between these two treatments, this fact suggests that the differences shown can be fully attributed to UV-light and not to white light.

Comparing both wavelengths, higher percentages of reduction were observed in colonies irradiated with UV-B than UV-A radiation compared to non-irradiated ones (Figure. 7). Moreover, under UV-A light, percentages of reduction of colony diameter (from 21.43 to 5.41 %) and biomass dry weight (from 36.51 to 9.60 %) decreased along the time. Conversely, under UV-B radiation percentages of reduction of colony diameter (from 48.48 to 73.68 %) and biomass dry weight (from 26.98 to 96.02 %) increased with time. These results could indicate some adaptation to UV-A, whilst UV-B radiation would practically stop fungal metabolism.

Table 4 F values of main effects and their interactions in colony diameters, fungal biomass dry weight, AFs accumulation and density of *A. parasiticus* (3.18-UdLTA) incubated on PBM (pistachio based medium) for seven days.

Radiatation	Effects	Colony diameter (mm)	Biomass dry weight (mg)	OTA (ng/g)	Colony density (mg/mm^2)
UV-A	Strain	337.73 **	56.42 **	21.81 **	0.21 ns
	Treatment	10.43 *	0 ns	27.92 **	1 ns
	Strain x Treatment	0.82 ns	0.51 ns	10.96 **	0.34 ns
UV-B	Strain	52.14 **	39.43 **	36.62 **	0.08 ns
	Treatment	105.9 **	41.22 **	72.66 **	0.03 ns
	Strain x Treatment	12.79 **	13.09 **	8.53 **	0.42 ns

**, p-value 0.001; *, p-value 0.05; ns, not significant.

Interestingly, high significant differences due to the UV radiation were found for AFs production (**Figure. 7**). Kinetics of AFs production without UV-light showed the maximum production in the 5-7th day, while under UV-A AFs concentration did not change with time and under UV-B the maximum AFs level was recorded in the 7th day. AFB_1, AFB_2, AFG_1 and AFG_2 contribution to total AFs was quite constant except under UV-B (**Figure. 8**). In general, AFB_2 and AFG_2 were produced in lower amount while AFB_1 always presented values over 65% of the total AFs production. However, under UV-B light a higher percentage of AFG than AFB was recorded.

As observed, for *A. carbonarius,* mycelium morphology changed under different light conditions. Six hours of UV-B radiation practically inhibited the aerial mycelium, and conidia were not observed after seven days. Colonies under UV-A radiation showed a dense centre, high development of aerial mycelium on the periphery of colony and tiny areas without visible growth inside the colony. Colonies incubated under cycles of light/darkness were more heavily sporulated and appeared more coloured than those grown under darkness or UV-A light/darkness.

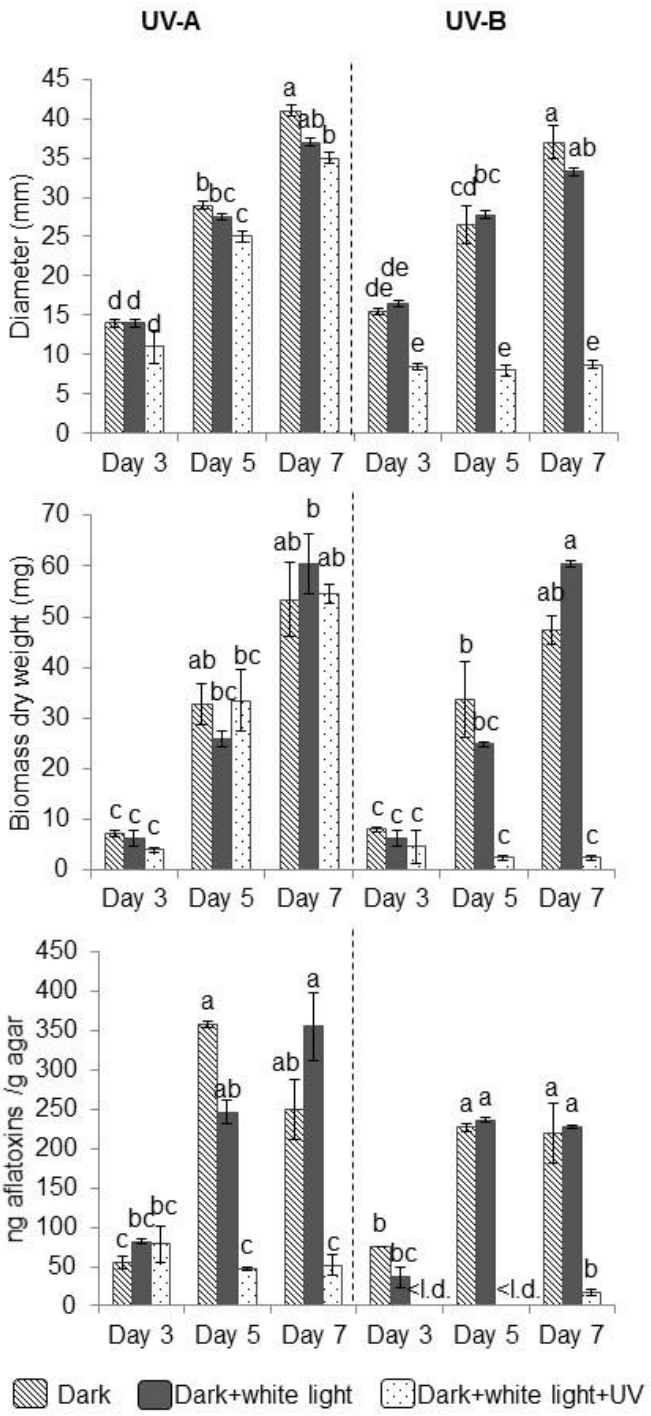


Figure. 7 Colony diameters, biomass dry weight and aflatoxins production of *A. parasiticus* (3.18-UdLTA) on PBM (pistachio based medium) under full dark (24 h), dark (12 h) and white light (12 h) and incubated conditions presented in

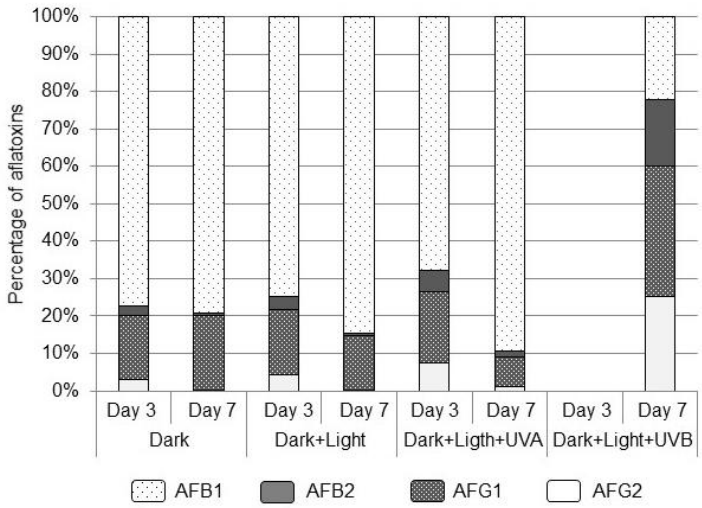


Figure 8 Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) distribution produced by *A. parasiticus* 3.18-UdLTA on PBM (pistachio based medium) after three and seven days under full dark (24 h), dark (12 h) and white light (12 h) and incubated conditions presented in Figure. 1.

4. DISCUSSION

The biological consequences of ozone depletion, mediated through an increase in UV-B radiation, have been cause for concern for many years. However, there is not much information about the ecological response to the hypothetically increasing solar UV radiation in fungi and specifically in mycotoxigenic fungi. Spain is not only the European country that receives the greater amount of radiation but also the country that shows the greatest contrast and radiative gradients and complexity in the distribution of the radiative energy (AEMET, 2012). Values used in this study concerned to mean values of UV-A and UV-B global (direct and diffused) radiation measured in Seville, since this city has one of the greatest intensity of radiation and number of hours of exposure values. In this work, the effect of UV-A (365 nm) and UV-B (312 nm) radiation on fungal growth and mycotoxin production of A. carbonarius and A. parasiticus, which are frequently isolated from crops, has been studied. Crop simulation media were chosen since irradiation has been shown to have different effects depending on the microbial growth media due to the potentially protective nutrient presence (Osman et al., 1989).

The UV-A radiation (315-380 nm) is only slightly absorbed by the ozone layer, it reaching most of ground level (CIE, 1987). In fact, UV-A radiation is an important environmental factor for sporulation in many fungi (Elad, 1997; Fourtouni et al., 1998; Osman et al., 1989; Nicot et al., 1996). Irradiance at 0.04 mW/cm^2 (366 nm) decreased the germination but

increased colony radial growth rate of *Penicillium notatum* after up to 20 min of exposure, while longer periods of irradiation reduced colony development (Osman et al., 1989). By contrast, 12 h exposure (0.051 to 0.167 mW/cm^2) for seven days had no influence on radial growth or dry mass weight in *Alternaria solani* despite spore production increased significantly (Fourtouni et al., 1998). Similarly, 3 h photoperiod ranging from 3.056-5.556 mW/cm^2 at a wavelength of 315-400 nm caused a significant enhancement of germination of *Penicillium purpurogenum* spores while significantly reduced germination of conidia of *Cladosporium cladosporoides* (Moody et al., 1999). Sporangia exposed to irradiance of 1.25 mW/cm^2 of UV-A (340-350 nm) for 8 h showed reduced germination compared to the sporangia that remained in the dark (Wu et al., 2000). In the present work, we have observed for *Aspergillus* a significant reduction on mycelium and mycotoxins when irradiated for 6 h at 1.7 mW/cm^2. Sixteen hours of exposure at the same irradiation reduced significantly the OTA contamination detected along the time.

Although UV-B radiation is for less than 1% of the total energy of the electromagnetic spectrum, it is a highly active component of the solar radiation that can porduce chemical modifications in DNA changing its molecular structure by the formation of dimers (Rastogi et al., 2010). Therefore, this radiation may directly damage the exposed tissues of plant pathogens, including spores during dispersal (Rotem et al., 1985; Wu et al., 2000). As for UV-A, different impact of UV-B on fungi are found in the literature. Exposure to 0.019 mW/cm^2 (292-350 nm) consistently reduced spore germination and mycelial extension rates in *Aspergillus fumigatus, Penicillium hordei, P. janczewskii, P. spinulosum* and *P. purpurogenum*, while inhibitory effects were lower in *Mucor hiemalis, C. cladosporioides, Leptosphaeria coniothyrium, Nectria inventa, Trichoderma viride, Ulocladium consortiale* and *Marasmius androsaceus* (Moody et al., 1999). *Bremia lactucae* was exposed to two elevated irradiation doses, 0.150 and 0.700 mW/cm^2, in a range from 2 to 12 hours (Wu et al., 2000). Both irradiations reduced significantly the percentage of germination of the incubated isolates under white light (0.4 mW/cm^2) or under complete darkness. Moreover, after 8 h of exposure to the highest irradiation the germination was practically inhibited. We observed than 0.1 mW/cm^2 reduced all fungal parameters studied on *Aspergillus*. Additionally, an increase on the UV-B radiation exposure time on *A. carbonarius*, increased the deleterious effects. Although different media and temperature regimes were used, *A. carbonarius* showed higher resistance to UV-B irradiation than *A. parasiticus*.

Different tolerances to the deleterious effect of solar UV radiation on fungi have been reported before. Rotem et al. (1985) found that mortality due to solar UV radiation increased from *A. solani* to *Ulocladium phaseoli* to *Peronospora tabacina*. Osman et al. (1989) suggested that differences in sensitivity may be attributed to spore colour. These authors pointed out that the resistance of pigmented conidia to the lethal effects of UV irradiation may be due to the action of pigments as quenchers to singlet oxygen produced by photosensitive compounds in fungal cells. Furthermore, 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.

It is worthy to mention that mycelium morphology changed due to UV-B exposure, as the colonies produced pigmented compact mycelium in the center of the colony and submerged mycelium at the periphery. This tendency was also observed in *A. solani* by Fourtouni et al. (1998), where the authors suggested that the fungi employs mainly a morphological (i.e., increased density) rather than chemical (i.e., UV-B absorbing compounds) protective strategy against UV-B radiation damage. Therefore, the submerged growth could be a fungal strategy against radiation since minimal doses of UV-B penetrate into the medium (Fourtouni et al., 1998).

To sum up, UV radiation is an interesting abiotic natural factor which could affect not only survival and growth of fungi but also secondary metabolites production. In this study, A. carbonarius showed a great UV resistance even during prolonged periods of direct UV exposure of 12 h (exp 1). This provides a logical explanation for the high numbers of A. carbonarius on grapes subjected to prolonged sun exposure in countries with high UV irradiance as Spain, Italy or Greece (Battilani et al.,2006; Tjamos et al., 2006).

On the other hand, it is widely accepted that stress conditions could promote the mycotoxin production. However, from our results UV radiation always reduced OTA and AFs contamination compared to non-irradiated colonies probably as a result of a decreased growth. Nonetheless, the possible degradation to other compounds was not evaluated, as it occurs on maize, where a natural transformation from trans-zearalenone (ZEN) to cis-ZEN after 24 h of UV-A irradiation (3.2 mW/cm^2) can occur (Brezina et al., 2013). Similarly, incubation of *A. carbonarius* under two simulated climatic conditions characterized by alternating temperature cycles (10/25 ºC and 15/35 ºC) with photoperiod (14/10 h

lightness/darkness), and two moisture levels (40 and 25%) for 21 days, showed that the extreme conditions tested caused a significant OTA reduction contamination (García-Cela et al., 2012). Therefore, *Aspergillus* presence on crops under future climate conditions proposed by Southern Europe (EC, 2007), could not be compromised; even the mycotoxin risk in vivo derived from predicted climatic conditions characterized by high temperatures, drier conditions and increased UV could be reduced. It must be noted that in these in vitro studies, plant stress is not taken into account, and this stress situation could led to an increased fungal colonisation. Nevertheless, fungal presence per se represents an important risk, because favourable conditions for toxin production can occur in the following postharvest stages.

ACKNOWLEDGMENTS

The authors are grateful to the Spanish Government (AGL2010-22182-C04-01) and Comissionat per a Universitats i Recerca del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (AGAUR). The authors are also grateful to Fernando Rubio and Hector Castan by the laboratory support.

REFERENCES

Agencia Española de Meteorología (AEMET), 2012. Atlas de radiación solar en España utilizando datos del SAF de clima de EUMETSAT. Agencia Estatal de Meteorología. http://www.aemet.es/documentos/es/serviciosclimaticos/datosclimatologicos/atlas_radiacion_solar/atlas_de_radiacion_24042012.pdf

Agencia Española de Meteorología (AEMET). Datos climáticos. Available from: http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos

Aylor, D. E., Sanogo, S., 1997. Germinability of Venturia inaequalis conidia exposed to sunlight. Phytopathology, 87(6), 628-633.

Battilani, P., Barbano, C., Rossi, V., Bertuzzi, T., Pietri, A., 2006. Spatial distribution of ochratoxin A in vineyard sampling design to assess must contamination. Journal of Food Protection, 69(4), 884-890.

Brezina, U., Kersten, S., Valenta, H., Sperfeld, P., Riedel, J., Dänicke, S., 2013. UV-induced cis-trans isomerization of zearalenone in contaminated maize. Mycotoxin Research, 29(4), 221-227.

Cary, J. W., Ehrlich, K. C., 2006. Aflatoxigenicity in *Aspergillus*: Molecular genetics, phylogenetic relationships and evolutionary implications. Mycopathologia, 162(3), 167-177.

CIE. 1987. International Lighting Vocabulary. 4th edition. Commission Internationale de L' Eclariage, 379 pp.

Delfini, C., 1982. Tecnica di microbiologia enologica. Roma: Luigi Scialpi.

Denizel, T., Jarvis, B., Rolfe, E. J., 1976. A field survey of pistachio (*Pistacia vera*) nut production and storage in Turkey with particular reference to aflatoxin contamination. Journal of the Science of Food and Agriculture, 27(11), 1021-1026.

Elad, Y., 1997. Effect of filtration of solar light on the production of conidia by field isolates of *Botrytis cinerea* and on several diseases of greenhouse-grown vegetables. Crop Protection, 16(7), 635-642.

European Commission, (2007). Adapting to Climate Change in Europe – Options for EU Action. Green Paper from the Commission to the Council, the European Parliament, the European Economic and Social Committee of the Regions, SEC 849, COM 354 Final. European Commission, Brussels (2007).

Fourtouni, A., Manetas, Y., Christias, C. 1998. Effects of UV-B radiation on growth, pigmentation, and spore production in the phytopathogenic fungus *Alternaria solani*. Canadian Journal of Botany, 76(12), 2093-2099.

Gallo, A., Ferrara, M., Perrone, G., 2013. Phylogenetic study of polyketide synthases and nonribosomal peptide synthetases involved in the biosynthesis of mycotoxins. Toxins, 5(4), 717-742.

García-Cela, E., Gil-Serna, J., Marín, S., Acevedo, H., Patiño, B., Ramos, A. J., 2012. Effect of preharvest anti-fungal compounds on *Aspergillus steynii* and *A. carbonarius* under fluctuating and extreme environmental conditions. International Journal of Food Microbiology, 159(2), 167-176.

Grishkan, I., Nevo, E., Wasser, S. P., Beharav, A., 2003. Adaptive spatiotemporal distribution of soil microfungi in «Evolution Canyon» II, Lower Nahal Keziv, western Upper Galilee, Israel. Biological Journal of the Linnean Society, 78(4), 527-539.

Hegglin, M. I., Shepherd, T. G., 2009. Large climate-induced changes in ultraviolet index and stratosphere-to- troposphere ozone flux. Nature Geoscience, 2(10), 687-691.

IGN, Spanish Geographic National Institute Available from: http://www.fomento.es/MFOM/LANG_CASTELLANO/DIRECCIONES_GENERALES/INSTITUTO_GEOGRAFICO/Astronomia/publico/efemerides/salida_puesta_sol.htm.

Jamali, M., Ebrahimi, M.-A., Karimipour, M., Shams-Ghahfarokhi, M., Dinparast-Djadid, N., Kalantari, S., Pilehvar-Soltanahmadi, Y., Amani, A. Razzaghi-Abyaneh, M., 2012. An insight into the distribution, genetic diversity, and mycotoxin production of *Aspergillus* section *Flavi* in soils of pistachio orchards. Folia Microbiologica, 57(1), 27-36.

McKenzie, R. L., Aucamp, P. J., Bais, A. F., Björn, L. O., Ilyas, M., Madronich, S., 2011. Ozone depletion and climate change: Impacts on UV radiation. Photochemical and Photobiological Sciences, 10(2), 182-198.

Moody, S. A., Newsham, K. K., Ayres, P. G., Paul, N. D., 1999. Variation in the responses of litter and phylloplane fungi to UV-B radiation (290-315 nm). Mycological Research, 103(11), 1469-1477.

Nicot, P. C., Mermier, M., Vaissière, B. E., Lagier, J., 1996. Differential spore production by *Botrytis cinerea* on agar medium and plant tissue under near-ultraviolet light-absorbing polyethylene film. Plant Disease, 80(5), 555-558.

Ortega, M., Martín, F.J., Camacho, F., 2001. Medidas de la radiación solar ultravioleta en Sevilla. Actas Dermosifiliográficas, 92, 271-275.

Osman, M., Elsayed, M.A., Mohamed, Y.A.H., Abo-Zeid, A.M., 1989. Effect of ultraviolet irradiation on germination and growth in *Aspergillus flavus* and *Penicillium notatum*. Mycological Research, 92(3), 293-296.

Rastogi, R. P., Richa, Kumar, A., Tyagi, M. B., Sinha, R. P., 2010. Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. Journal of Nucleic Acids, 2010. doi:10.4061/2010/592980

Riba, A., Mokrane, S., Mathieu, F., Lebrihi, A., Sabaou, N., 2008. Mycoflora and ochratoxin A producing strains of *Aspergillus* in Algerian wheat. International Journal of Food Microbiology, 122(1-2), 85-92.

Rotem, J., Wooding, B., Aylor, D. E., 1985. The role of solar radiation, especially ultraviolet, in the mortality of fungal spores. Phytopathology, 75, 510-514.

Schmidt-Heydt, M., Magan, N., Geisen, R., 2008. Stress induction of mycotoxin biosynthesis genes by abiotic factors. FEMS Microbiology Letters, 284(2), 142-149.

Schmidt-Heydt, M., Rüfer, C., Raupp, F., Bruchmann, A., Perrone, G., Geisen, R., 2011. Influence of light on food relevant fungi with emphasis on ochratoxin producing species. International Journal of Food Microbiology, 145(1), 229-237.

Schmidt-Heydt, M., Cramer, B., Graf, I., Lerch, S., Humpf, H.-U., Geisen, R., 2012. Wavelength-dependent degradation of ochratoxin and citrinin by light in vitro and in vivo and its implications on *Penicillium*. Toxins, 4(12), 1535-1551.

Taniwaki, M.H, Pitt, J.I., Hocking, A.D., Fleet, G.H., 2006. Comparison of hyphal length, ergosterol, mycelium dry weight and colony diameter for quantifying growth of fungi from foods. In: Hocking, A.D., Pitt, J.I., Samson, R.A., Thrane, U. (Eds.), Advances in Food Mycology. Springer, New York, pp. 49 – 67.

Tjamos, S. E., Antoniou, P. P., Tjamos, E. C., 2006. *Aspergillus* spp., distribution, population composition and ochratoxin A production in wine producing vineyards in Greece. International Journal of Food Microbiology, 111, S61-S66.

UNEP. (2010). Scientific Assessment of Ozone Depletion: 2010. World Meteorological Organization Global Ozone Research and Monitoring Project—Report No. 52 Scientific Assessment of ozone Depletion : 2010 Pursuant to Article 6 of the Montreal Protocol on Substances that Deplete the Ozone Layer National Oceanic and Atmospheric Administration National Aeronautics and Space Administration United Nations Environment Programme World Meteorological Organization

European Commission Available from: http://www.esrl.noaa.gov/csd/assessments/ozone/2010/chapters/chapter2.pdf.

WMO. (2013). The global climate 2001-2010 a decade of climate extremes Summary report. World Meteorological Organization. Available from http://library.wmo.int/pmb_ged/wmo_1119_en.pdf

Wu, B. M., Subbarao, K. V., Van Bruggen, A. H. C. (2000). Factors affecting the survival of *Bremia lactucae* sporangia deposited on lettuce leaves. Phytopathology, 90(8), 827-833.

STUDY VI

Ochratoxigenic moulds and effectiveness of grape field antifungals against in a climatic change scenario

García-Cela E., Ramos A.J., Sanchis V. and Marin S.

Food Technology Department, Lleida University, XaRTA-UTPV, Rovira Roure 191, 25198 Lleida, Spain

ABSTRACT

The presence of ochratoxin A (OTA) in grape and its derivates has been reported mainly in the Mediterranean area. Consequently, great efforts are being made to prevent the growth of *Aspergillus* on grapes. However the European Commisision suggests that climate change may results in increase temperatures and longer drought period in Southern Europe. Therefore, the aim of this study was to investigate how ochratoxin fungal growth and the efficiency of fungicides used at present may be affected by environmental conditions predicted with climate change. The effectiveness of grape field antifungals (Switch®, Flint Max® and *Equisetum arvense* extract) under two alternating temperature, photoperiod and relative humidity (R.H.) scenarios (current: 20/30 ºC, 16 h light/8 h darkness, 80% R.H.; predicted: 25/37 ºC, 16 h light/8 h darkness, 75% R.H.) on the growth and OTA production of two *Aspergillus carbonarius* isolates and one *Aspergillus ochraceus* isolate on grapes was investigated. Predicted conditions reduced *A. carbonarius* and limited *A. ochraceus* growth. Antifungals reduced the fungal infection (by 40 to 84%), although no correlation between climate conditions and effectiveness of the antifungals was found. However, Switch® always showed the greatest reduction and *E. arvense* (0.02%) the least. Higher temperatures affected OTA production by the isolates in different ways. In general, Switch and Flint Max® reduced OTA production while *E. arvense* stimulated it.

Publication: Journal of the Science of Food and Agriculture (2012) 92,1455-1461.

1. INTRODUCTION

Mycotoxins, naturally occurring substances produced by toxigenic fungi that commonly grow on a number of crops, cause adverse health effects when consumed by human and animals (Tirado et al., 2010). Ochratoxins were first identified in 1965 and have been suggested as a factor involved in Balkan endemic nephropathy (Krogh 1977, Smith and Moss, 1985). Naturally occurring ochratoxins are ochratoxin A (OTA), the most prevalent and relevant fungal toxin of this group, as well as ochratoxin B and ochratoxin C exist, with are less important and pose a lower risk to human and animal health. The International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen in group 2B based on the evidence from diverse studies (IARC, 2002). Filamentous fungi belonging to the genus *Penicillium,* mainly *Penicillium verrucosum,* and *Aspergillus* sections *Circumdati* and *Nigri* are recognized as source of OTA. In the *Circumdati* section, *Aspergillus westerdijkiae* and *Aspergillus steynii* have recently acquired more relevance owing to their higher OTA production than *Aspergillus ochraceus* (Gil-Serna et al., 2010). *A. steynii* has been revealed as the main cause of OTA contamination (Abarca et al., 2004).

Regarding mycotoxigenic moulds on grapes, several studies had pointed to black aspergilli, particularly *A. carbonarius*, as been mainly responsible of the presence of OTA in grapes (Battilani et al., 2004; Cabañes et al., 2002) and, to a lesser extent, *Aspergillus* section *Circundati* (Battilani et al., 2003a; Belli et al., 2006). Various researches have reported the presence of OTA in grapes (Abrunhosa et al., 2001), dried vine fruits (Varga and Kozakiewicz, 2006), grape juice (Burdaspal and Legarda, 1999; Zimmerli andand Dick, 1996) and wines (Cerutti et al., 2000; Filali et al., 2001; Visconti et al., 1999).

Prevention of the growth of mycotoxin-producing fungi is the most effective strategy for controlling the presence of mycotoxins in foods; in many cases the use of fungicides is the only efficient, cost-effected and often successful way to prevent mould growth (Munimbazi et al., 1997). Various fungicides have been tested in the field and laboratory against *A. carbonarius* on grapes (Belli et al., 2006b; Lo Curto et al., 2004; Tjamos et al., 2004). However, legal restrictions on the maximum residue limits (MRL), and consumer demands for healthier products are necessitating the use of safer and more environmentally friendly antifungals. Therefore it is important to seek alternatives such as plant extracts as new sources of antimicrobial or antimycotoxigenic compounds (Mitscher et al., 1987). Extracts of several plants have been tested against *Aspergillus* species (Rasooli and Owlia, 2005; Rasooli et al.,

2006) including those of *Equisetum* spp. (commonly know as field horsetail) (Milanovic et al., 2007; Radulovic et al., 2006).

Belli et al. (2006a) identified the ochratoxigenic microbiota of grapes from vineyards located in three Spanish winemaking regions during three consecutive growing seasons (2001-2003) and found thath most of the ochratoxigenic fungi belonged to *Aspergillus* section *Nigri*. *A. ochraceus* was present in 0.16% of samples on average, with no differences among years, regions and dates of sampling. Previous ecophysiological studies simulating field conditions on growth and mycotoxin production revealed that *A. carbonariu*s growth and OTA production were determined by environmental factors such as temperature, water availability and photoperiod (Belli et al., 2006c; Oueslati et al., 2010). These studies were carried out on a synthetic nutrient medium (SNM) simulating grape composition. Moreover, Belli et al. (2005) demonstrated that the incidence of black aspergilli increased in years with very hot summers, while humidiy was less relevant.

According to climatic change scenarios predicted for Spain, it is expected that temperature and drought will increase (European Commission, 2007). The efficiency of fungicides used at present might be affected by environmental conditions predicted with climate change. The aim of this study was to test the impact of changing climatic conditions in Spain on ochratoxigenic mould development on grapes and on the effectiveness of syntethic and natural antifungals.

2. MATERIALS AND METHODS

2.1. Fungal isolates and preparation of spore suspensions

Two *A. carbonarius* strains (3.83 and 3.168) and one *A. ochraceus* strain (3.66) held in the culture collection of the Food Technology Department, Lleida University, Spain, were used in this study. All three isolates were isolated from naturally infected Spanish grapes and were previously found to be OTA producers. Spore suspensions of each isolate (10^3 spores/ml) were prepared, from colonies previously grown on synthetic nutrient medium (SNM) (Delfini *et al.*, 1982) for seven days at 25ºC, in distilled water containing 0.005% Tween 80.

2.2. Antifungals preparation

One natural and two commercial fungicides (Switch ® and Flint Max ®) were used. The natural antifungal was obtained from *E. arvense* collected in Alpicat, Lleida (Spain), washed with water and dried at 40 ºC for two days until a constant weight was achieved. The dry plant material was macerated in 70% etanol (99.6% purity) in a ratio of 5:95 (v/v) at 25ºC under agitation and darkness for 2 days. Then the extract was filtered under vacuum and the plant material was removed, leaving a dark green hidroalcoholic extract. The ethanol was evaporated in a rotary evaporator. The extract was cold stored until use, when it was mixed with distilled water to a final concentration of 2%. The commercial fungicides were applied in the dosages recommended by the manufacturers, namely 1 g/L for Switch ® (Syngenta Agro S.A. Madrid, Spain) and 0.025 g/L for Flint Max® (Bayer CropScience S.L., Valencia, Spain). Switch ® is a preformulated mixture containing cypronidil (37.5%) and fludioxonil (25%). Cypronidil is an anilinopyrimidine fungicide with some systemic properties that is taken up into the cuticle and waxy layers of leaves and fruits. Fludioxonil is a phenylpyrrole fungicide that remains on the leaf and fruit surfaces to provide contact activity. Switch is registered in Spain against *Botrytis* and *Sclerotinia* in several crops and against *Botrytis* spp. and *Aspergillus* spp. in grapes. Flint Max is a mesostemic fungicide containing tebuconazole (50%) and trifloxistrobin (25%). Although it is still in process of registration in Spain, other countries such France, Croatia and Slovenia have already approved it for the control of vineyard diseases like *Uncinula necator*, *Guignardia bidwellii*, and *Pseudopeziza tracheiphila,* but not *Aspergillus* spp. Tebuconazole affects sterol biosynthesis in membranes while trifloxistrobin is a respiration inhibitor for foliar application.

2.3. Berry decontamination, inoculation, fumigation and incubation

Red table grape berries (var. Red Globe) without physical damage were used in this study. The berries were separated from the bunches by cutting the stem with scissors at approximately 0.5 cm from each grape. They were surface-sterilized by dipping them first in NaClO solution (0.01% Cl) for 2 min and then in sterile distilled water for 2 min. Excess water was removed by placing the berries on a laminar flow bench for 10 min. For each treatment, 20 berries were arranged on top of a grate (to prevent contact between the berries) and then placed in a plastic box containing 300 ml of glycerol-water solution to assure the R.H. of the treatment (Dallyn, 1978). The water activity of glycerol-water solutions was checked through three readings on an AquaLab Series 3 meter (Decagon Devices, Inc., WA,

USA) with an accuracy of ±0.003. Grates and plastic boxes were previously disinfected with ethanol (70%) and placed under ultraviolet light on a laminar flow bench for 15 min.

Grape berries were sprayed with either the desired antifungal or sterile distilled water (control treatment). Each spray dose was equivalent to 5 ml. Then all grapes were wounded to facilitate fungal infection, and immediately afterwards a 10^3 spores/ml suspension was sprayed for each plastic box. The filled containers were hermetically sealed and subjected to a photoperiod of 16 h light/8 h dark. To simulate light conditions, white light was used (Mazda, 23W Eureka3 Electronic bulbs; 230-240 V; 50-60 Hz; 1500 lumen; 175 mA). The heat emitted by the bulb did not increase the temperature proposed for incubation.

Berries were incubated at different combinations of temperature and R.H. for seven days, simulating a climatic change scenario in Northern Spain. Temperatures of 30 and 20 ºC for light and dark periods were chosen as current climatic conditions in August in Northern Spain. Temperatures of 37 and 25 ºC for light and dark periods respectively were chosen as predicted climatic conditions that might be expected to occur in the future, based on the current conditions in Southern Spain (INM, Instituto Nacional de Meteorología). Moreover, a 5% R.H. decrease was simulated by incubating the first treatment at 80% R.H. and the second at 75% R.H. Each treatment was carried out in triplicate (**Table 1**).

Table 1 Environmental conditions of the different treatments

Treatment	Environmental Conditions		
	R.H.	Temperature	Hours
Control Switch ® Flint Max ® *Equisetum arvense*	75	37ºC 25ºC	16 h light 8 h dark
Control Switch ® Flint Max ® *Equisetum arvense*	80	30ºC 20ºC	16 h light 8 h dark

2.4. Percentage of infection

At the end of incubation, berries were observed, visible fungal growth was assessed and infection percentage was calculated. Fungal infection with each fungicide treatment was compared with that of the control under the same environmental conditions.

2.5. OTA extraction and HPLC quantification

After 7 days of incubation the OTA extraction method developed by Bezzo *et al.* (2000) with HPLC was applied with slight modification. Each batch of 20 berries was weighed and then crushed with a hand blender (Opticlick Pro, Moulinex, France). The must obtained was macerated for 2 h. Samples were stored in sealed centrifuge tubes until their extraction. The must was centrifuged (Hettich Zentrifugen EBA 12, Germany) at 3900g for 10 min and filtered (Whatman no 1) under vacuum. The pH was adjusted to 7.4 with NaOH (4 M) and the must was filtered again (Whatman no. 1). Initial pH values of filtered musts were 3.47-4.16. Undiluited must (75-100 mL) was cleaned-up by means of immunoaffinity columns (Ochraprep, R-Biopharm Rhône LTD, Glasgow, Scotland) at a flow rate of 2-3 ml/min. The columns were subsequently washed with 20 ml of distilled water and left to dry. Desorption was carried out by slowly passing 3 mL of methanol/acetic acid (98:2 v/v) solution through the column; during desorption, backflushing was applied twice. The eluate was then evaporated to dryness at 40 ºC under a stream of nitrogen and redissolved in 2 mL of mobile phase (48% acetonitrile and 52% sodium acetate /acetic acid (19/1)). A 25µl aliquot of each final sample was injected into the HPLC system, wich was equipped with a fluorescence detector (Waters 474) (λ_{exc} 333 nm; λ_{em} 443 nm) and a C18 column (Waters Spherisorb 5 µm, ODS2, 4.6x250 mm, Milford, Massachusetts, USA). The analysis was performed under isocratic conditions at a flow rate of 1 mL/min. The detection limit was 0.75 ng OTA/ml, based on a signal to noise ratio of 3:1. OTA was quantified by external standard method. OTA standard was purchased from Sigma-Aldrich (Steinheim, Germany), acetonitrile from Merck (Darmstadt, Germany) and sodium acetate and acetic acid from Prolabo (Briare, France). The retention time of OTA under the conditions described was approximately 12 min.

2.6. Statistical analysis

Data on fungal infection percentage and and OTA production were subjected to analysis of variance using JMP® 8.0.1 (SAS Institute Inc, Cary, NC, USA). Significance of the effects was evaluated and Student`s test was applied to the different factors.

3. RESULTS

3.1. Effectiveness of antifungals against grape infection

After 7 days of incubation, fungal infection percentage was recorded. Skin is a natural protection against fungal entry, so fungal infection was observed mainly around the wounds made on berries. In most of samples, fungal colonisation was lower than 50% of the berries surface. Both environmental conditions (temperature and R.H.) and antifungal treatments significantly affected ($p<0.001$) fungal infection for all isolates (**Figure.1**).

For berries inoculated with *A. ochraceus* 3.66, a 5% decrease in R.H. plus a increase in temperatures led to a significant decrease in berry infection, regardless of the antifungal treatment; hence, under current conditions, 42% infection was observed in the control, while no infection was observed under the predicted conditions. Reduction of the infection percentage for each treatment was determined by comparison with the control infection. All antifungals reduced the infection significantly under current conditions ($p<0.05$, 40 to 84% reduction). The most effective was Switch ®, while the least effective was *E. arvense* extract. Low variation was found among repeated experiments (SD between 0 and 2).

As *A. ochraceus* 3.66 infection by *A. carbonarius* took place around the wounds made on berroes, although *A. carbonarius* 3.83 also showed fungal infection around the pedicel. Fungal colonisation was lower than 50% of the grapes surface. *A. carbonarius* 3.83 led to higher infection levels under all test conditions. In general, a 5% decrease in R.H. and a shift to higher temperatures led to a significant decrease in the infection on berries. Under current conditions, fungal infections observed in the controls were 53 and 27%, while under predicted conditions they were 48 and 8%, for isolates 3.83 and 3.168, respectively. On the other hand the use of Switch ® significantly reduced the percentage of infection for both isolates and both conditions (% inhibition between 60-100%) while *E. arvense* and Flint Max ® had no effect on *A. carbonarius* 3.83 grown under current conditions. No significant effect

of these two antifungals was observed for *A. carbonarius* 3.168, while for *A. carbonarius* 3.83 all antifungals led to significant inhibition of infection under predicted adverse conditions, in the order Switch ® (82.75%) > Flint Max ® (62.75%) > *E. arvense*. (34.48%). The synthetic fungicides applied at the recommended doses showed higher infection reduction than *E. arvense* extract at 2%. Moreover, the *E. arvense* results showed higher variation among replicates (SD between 2 and 4.5).

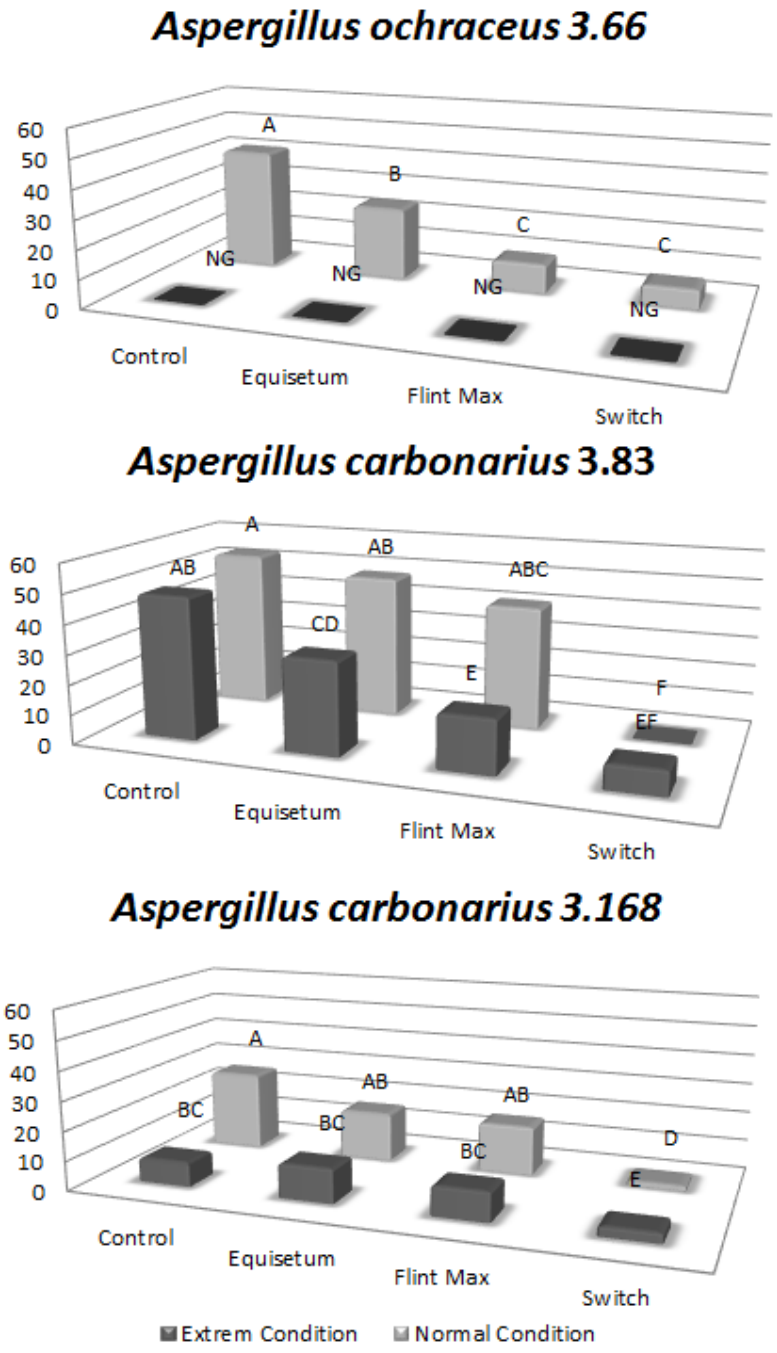


Figure 1 Effects of antifungal treatments and environmental conditions on infected berries(%) by ochratoxigenic species. alternating temperature 20/30ºC, 80% R.H.; alternating temperature 25/37ºC, 75% R.H. Different letters over bars indicate significant differences among values for each isolate (Student´t test, with a level of significance of 0.05).

3.2. Effectiveness of antifungals against OTA production

For *A. ochraceus* 3.66, OTA production was only analyzed under current condition, because no growth (NG) was observed under predicted conditions. No significant differences ($p<0.05$) in OTA production due to antifungals were observed. Although Flint Max ® and Switch ® reduced OTA production (by 45% and 94% respectively), the effect was not significant owing to the high variation among replicates, while *E. arvense* significantly timulated OTA production from 0.164 ppb to 0.555 ppb (**Figure 2**).

In relation to *A. carbonarius,* under current conditions, isolate 3.83 was a higher OTA producer than isolate 3.168 (3.398 and 0.702 ppb respectively); however, under predicted conditions isolate 3.168 produced more OTA than isolate 3.83 (1.258 and 0.047 ppb, respectively). In general, low OTA levels were recorded under predicted climatic conditions. Therefore, in the case of isolate 3.83, the most favorable climatic conditions for OTA production were the same as those for fungal growth; by contrast, for isolate 3.168, growth was favoured under current conditions, while no significant diference was observed in OTA production. Regarding antifungals, only Flint Max ® and Switch ® reduced OTA production (by 78 and 89% respectively) by *A. carbonarius* 3.83 under current climatic conditions, while for the remaining isolates and conditions no significant effects of antifungals were observed. On the other hand, application of *E. arvense* extract enhanced OTA production of isolate 3.168 under current conditions but had no effect in the remaining cases.

4. DISCUSSION

Since Zimmerli, andand Dick, (1996) described OTA contamination in wine for the first time, its presence has been frequently reported and has led the EU to set a maximum level of 2 ppb for grapes derivatives such as wine, sparkling wine, aromatized wine and grape juice (European Commission 2006). The origin of OTA in these products is field fungal-contaminated grapes, so it is important to avoid fungal infection on berries and consequently reduce the probability of OTA production. Some studies have suggested that the month preceding ripening is crucial for OTA contamination (Battilani et al., 2006a,b), as the sugar content increases and the berry texture softens (MAPA 1998).

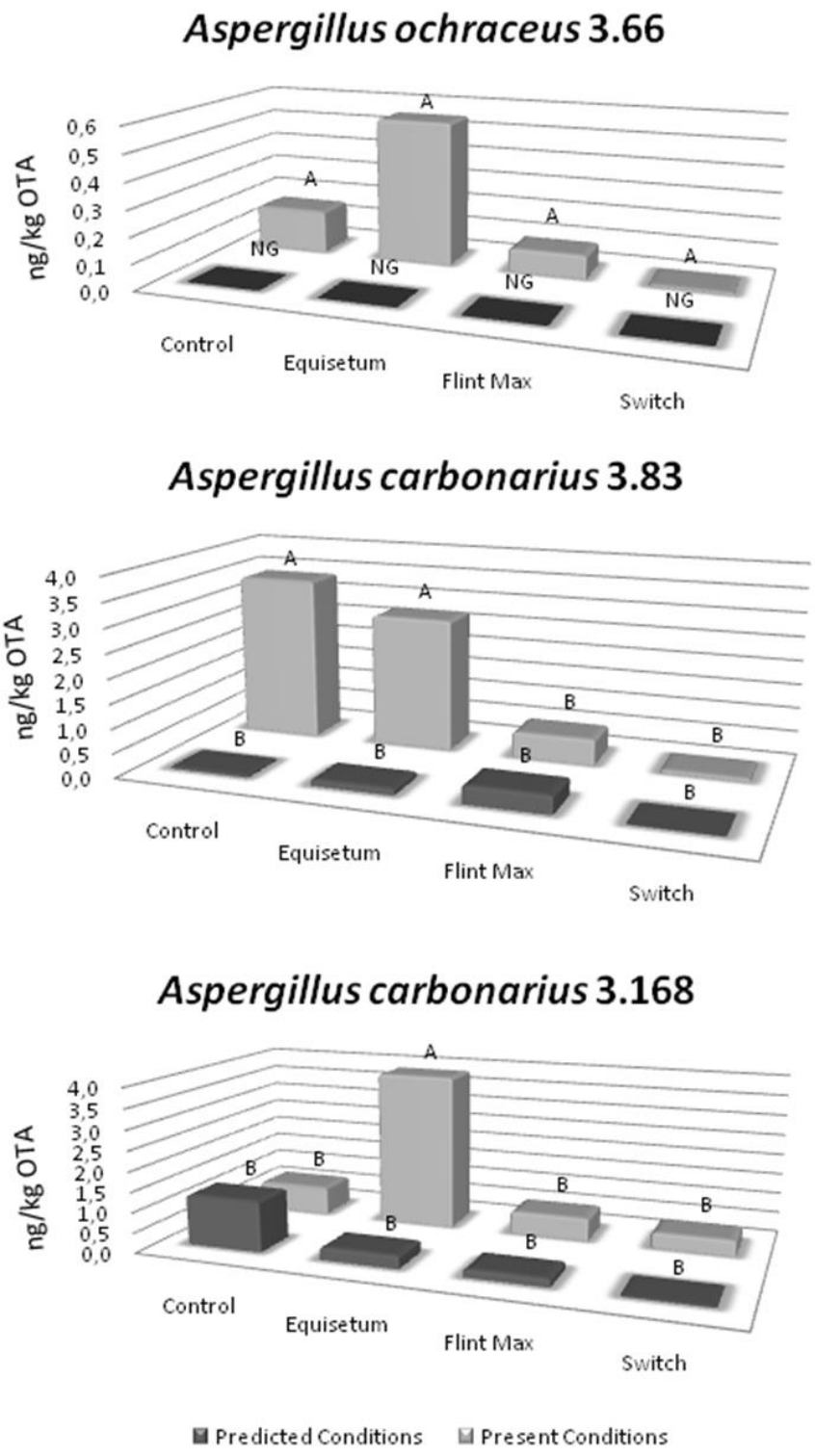


Figure 2 Effects of antifungal treatments and environmental conditions on OTA produced (ng/kg) by ochratoxigenic species. alternating temperature 20/30ºC, 80% R.H.; alternating temperature 25/37ºC, 75% R.H. Different letters over bars indicate significant differences among values for each isolate (Student´t test, with a level of significance of 0.05).

Climate change is generating growing insecurity about the future temperature and rainfall regimes. The most vulnerable areas in Europe are southern Europe and the entire

Mediterranean basin, owing to the combined effect of high temperature increases and reduced precipitation in areas already having to cope with water scarcity (European Commission, 2007). Temperature and R.H. are suggested to be the main factors determining fungal infection; besides, different geographic areas have been distinguished in the Mediterranean basin as a function of these factors (Battilani et al., 2003b). The European Commission suggests that, in southern Europe, changes may equate to temperature increase of 4-5 ºC coupled with longer drought periods (European Commission, 2007). In this study, climatic conditions were chosen in order to simulate actual mean day and night temperatures and R.H in the month preceding harvest in northern Spain; additionally, an experimental condition was considered including a temperature increase of 5-7 ºC and R.H decrease of 5%. Decreased H.R. is not synonymous with drought conditions, because drought would also imply a stress condition in the plant, which may affect fungal growth positively and OTA production positively or negatively, as has been shown for cereals and aflatoxins and *Fusarium* toxins; this point was not assessed in our laboratory trial. Our work shows that increased temperature regimes reduced or even prevented fungal growth compared with moderate conditions. Battilani et al. (2006b) studied the risk associated with various combinations of temperature and precipitation and observed a high risk of OTA accumulation in grapes during August in northern hemisphere with temperatures between 5-35 ºC and rainfall of 20-25 mm. Also, a positive correlation was found between the number of black aspergilli isolated from grapes and the temperature in the field: the most contaminated grapes were those isolated in the warmest year and from the warmest regions (Battilani et al., 2003b, 2006a). Moreover, a sampling was carried out from 2001 to 2003 in Spain, showing a higher presence of black aspergilli in 2003 than in the two previous years. The authors related the increased presence of *Aspergillus* to extreme temperatures, as 2003 was very hot year in Spain (Belli et al., 2005a). However, the high presence of *Aspergillus* inocula in the field may not have a direct correlation with the ability of such spores to germinate and grow on the grapes. *A. ochraceus* has a reduced presence in Spanish vineyards (Belli et al., 2006a), and our study suggests that it might be further precluded as a result of changing climate conditions.

All isolates showed higher infection under current climate conditions in all treatments. Both on SNM and on berries, growth was maximum at around 30 ºC at high R.H. (Belli et al., 2007; Pardo et al., 2005b), although *A. ochraceus* grew faster between 25-35 ºC while *A. carbonarius* did so between 30-37 ºC (Belli et al., 2004b; Pardo et al., 2005a). Fungal infection by three isolates of *A. ochraceus* on healthy berries did not show differences among isolates;

the maximum visible growth was observed at levels of 90-100% R.H, while at 80% R.H. the percentage of berries with visible fungal growth was significantly lower than 90-100% (40-80% *versus* nearly 100%) (Pardo et al., 2005b). Similar experiments on damaged grapes with four isolates of *A. carbonarius* showed higher fungal infection at 90-100% R.H (80-100%) than at 80% R.H. (50%) (Belli et al., 2007). Those studies showed higher percentages of infection, but in both cases the lower R.H. tested was 80% and temperature and light cycles were not used.

In the present experiment higher amounts of OTA were produced by one *A. carbonarius* isolate, which also gave the highest percentage of infected berries. The diferent isolates showed diferent responses to environmental conditions. Thus *A. ochraceous* 3.66 and *A. carbonarius* 3.83 produced higher amount of OTA under non-adverse conditions, while for *A.carbonarius* 3.168 there was no diference. Pardo et al. (2005b) quantified OTA accumulated in 14 days by three isolates of *A. ochraceus* isolated from grapes, and found levels ranging from 9.27 ng/g at 100% R.H. and 30ºC to near the limit of detection at 80% R.H. On the other hand, Belli et al. (2007) quantified OTA on berries inoculated with four *A. carbonarius* isolates and found a range of concentration between 0.06 and 15.60 g/L of must, with maximum values being obtained 100% R.H. More OTA was found in undamaged berries at 30 ºC than at 20º C, contrary to previous works on synthetic medium, where temperatures of 15-20ºC were reported as optimal for *A. carbonarius* OTA production (Belli et al., 2007; Mitchell et al., 2004).

A. carbonarius 3.83 was more adapted to grow under predicted conditions than *A. carbonarius* 3.168; however, OTA production was sharply reduced in these conditions, while *A. carbonarius* 3.168 was unaffected. Recent studies suggest that secondary metabolites may play a role in competitive interactions between xerophilic fungi in extreme dry conditions (Leong et al., 2010).

In a climatic change scenario, fungal metabolism could be affected by other factors such as increased CO_2 concentration and ultraviolet radiation (UV). Magan et al. (2010) suggested that slightly increased CO_2 concentrations and interactions with temperature and water availability may stimulate the growth of some mycotoxigenic species, especially under water stress. Recentlystudies found that up to 50% CO_2 had only a slight impact on OTA production by *A. carbonarius* over a range of a_W conditions, with a_W being a more important

factor than CO_2 (Pateraki et al., 2007). Valero et al. (2007) observed that *Alternaria alternata* and *A. carbonarius* in SNM were more resistant than other fungi isolated from grapes when they were irradiated with UVC light. Therefore studies considering the maximum number of factors (a_W, t, UV light, $C0_2$...) simultaneously are required to understand the implications of climate change on fungal metabolism and its consequences on food safety.

The potential impact of climate change on the effectiveness of fungicide treatments was also assessed. Chemical fungicides were used at the doses recommended by the manufacturers for the control of target moulds. Although neither of the commercial fungicides used here were recommended for *Aspergillus,* Switch ® showed high efficiency under both environmental conditions tested, significantly inhibiting growth and reducing OTA production in certain cases. Other works have shown the efficiency of this fungicide in SMN and grapes against *A. carbonarius* (Belli et al., 2006b); moreover its efficacy has been demonstrated in field treatments (Tjamos et al., 2004; Valero et al., 2007). In addition, a slight improvement in the control exerted by this fungicide was observed under predicted environmental conditions. In relation to active compounds, penconazole (100 g/kg), cypronidil (375 g/kg) plus fluodioxonil (250 g/kg) and mancoceb (800 g/kg) completely inhibited *A. carbonarius* on SNM but on grapes a mixture of cypronidil (375 g/kg) and fluodioxonil (250 g/kg) seemed, together with penconazole (100 g/kg) and azoxystrobin (250 g/kg), the best fungicides to control *A. carbonarius* grow and OTA production (Belli et al., 2006). Other studies also described the suitability of penconazole: a reduction of around 90% was found in the level of OTA in wines made from grapes treated with this fungicide (Lo Curto et al., 2004). Tjamos et al. (2004) observed higher efectiveness of fludioxonil than of fludioxonil plus cypronidil, pointing out fludioxonil as being responsible for the effectiveness; besides, they recommended late fungicide applications owing to their greater efficiency than early applications.

On the other hand, Flint Max ® was less effective, as found in other studies (Belli et al., 2006b), although its effectiveness was in some cases enhanced by predicted environmental conditions.

The general public perceives risks related to pesticides as posing a greater hazard than mycotoxins (Williams and Hammitt, 2001). Thus the use of plant extracts could be an alternative to chemicals for fungal prevention, because they are biodegradable, do not leave

residues, do not pollute the environment and, above all, are obtained naturally. Nonetheless, *E. arvense* at 2%, although previous studies had reported its antifungal properties (Milovanović *et al.,* 2007 Radulović et al., 2006), was not been effective in controlling ochratoxigenic fungal growth and OTA production in grapes. Probably the dosage tested in our experiment was not high enough for mould control. A recent study has proven the effectiveness of *E. arvense* extracts (3%) against different strains of *Aspergillus*, including *A. carbonarius, A. westerdijkae*, on maize agar medium. (Garcia el al., 2011). The results were dose-dependent, since high levels reduced growth byt low levels could stimulate it under some conditions. Growth rate of these species where 93-100 % inhibited at 3% agar medium. At 2% agar medium the growth rate of *A. carbonarius* was not reduced, although growth rate was delayed, while for *A. westerdijkae* a 37-100 % of reduction in growth rate was observed. Finally, the extract at 1% agar medium stimulated growth of all *Aspergillus* strains tested. Regarding OTA production, extract at 3% inhibited growth and therefore OTA production; however, at lower concentration, stimulation of OTA production occurred under some conditions (Garcia et al., 2011). In our case, spraying of *E. arvense* extract (2%), although slightly reduced fungal growth, stimulated OTA production.

As conclusion, the climatic scenario affected growth and OTA production of different strains. Conditions simulating a climatic change scenario involving higher temperatures and lower RH led to a reduced infection and consequent OTA accumulation, although previous studies have shown that such conditions could favour a higher presence of ochratoxigenic inocula in vineyards. *A. ochraceus* was more sensitive to predicted climatic conditions than *A. carbonarius,* therefore *A. carbonarius* could be more adaptable to the climate scenarios predicted. Magan et al. (2011) suggested that a climate change towards hot temperatures and drought could increase the risk of migration of pathogens, which might occur as a result of a shift in response to warmer, drought-like climatic conditions. On the other hand, the effect of fungicides depends mainly on the active ingredient, the results being only weakly related to changing environmental conditions.

ACKNOWLEDGMENTS

The authors are grateful to the Spanish government (AGL2010-22182-C04-01), EU (Mycored KBBE-2007-2-5-05 project), Comissionat per a Universitats i Recerca, del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (AGAUR)

and European Social Fund for the financial support. We also thank Bayer CropScience, S.L for the supply of the antifungal and the laboratory staff for their collaboration.

REFERENCES

Abarca, M. L., Accensi, F., Cano, J., Cabañes, F. J., 2004. Taxonomy and significance of black aspergilli. Antonie Van Leeuwenhoek, International Journal of General and Molecular Microbiology, 86(1), 33-49.

Abrunhosa, L., Paterson, R. R. M., Kozakiewicz, Z., Lima, N., Venâncio, A., 2001. Mycotoxin production from fungi isolated from grapes. Letters in Applied Microbiology, 32(4), 240-242.

Battilani, P., Giorni, P., Pietri, A., 2003. Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. European Journal of Plant Pathology, 109(7), 715-722.

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(4), 633-636.

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. International Journal of Food Microbiology, 96(1), 19-27.

Burdaspal, P. A., Legarda, T. M., 1999. Ochratoxin A in wines and grape products originating from spain and other european countries. Alimentaria, , 107-113.

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(3), 213-215.

Dallyn, H., 1978. Effect of substrate water activity on growth of certain xerophilic fungi. PhD Thesis South Bank Univ. Lond.

European Commission. (2006). Commission regulation (EC) no 1881/2006 of 19 december 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L364, 5-24.

Filali, A., Ouammi, L., Betbeder, A. M., Baudrimont, I., Soulaymani, R., Benayada, A., Creppy, E. E., 2001. Ochratoxin A in beverages from Morocco: A preliminary survey. Food Additives and Contaminants, 18(6), 565-568.

Krogh, P., Hald, B., Plestina, R., Ceovic, S., 1977. Balkan (endemic) nephropathy and foodborn ochratoxin A: Preliminary results of a survey of foodstuffs. Acta Pathologica Et Microbiologica Scandinavica - Section B Microbiology and Immunology, 85(3), 238-240.

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(2), 439-445.

Mitscher, L. A., Drake, S., Gollapudi, S. R., Okwute, S. K., 1987. A modern look at folkloric use of anti-infective agents. Journal of Natural Products, 50(6), 1025-1040.

Rasooli, I., Owlia, P., 2005. Chemoprevention by thyme oils of *Aspergillus parasiticus* growth and aflatoxin production. Phytochemistry, 66(24), 2851-2856.

Rasooli, I., Rezaei, M. B., Allameh, A., 2006. Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from *Thymus eriocalyx* and *Thymus x-porlock*. Food Control, 17(5), 359-364.

Tjamos, S. E., Antoniou, P. P., Kazantzidou, A., Antonopoulos, D. F., Papageorgiou, I., 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. Journal of Phytopathology, 152(4), 250-255.

Varga, J., Kozakiewicz, Z., 2006. Ochratoxin A in grapes and grape-derived products. Trends in Food Science and Technology, 17(2), 72-81.

Visconti, A., Pascale, M., Centonze, G., 1999. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. Journal of Chromatography A, 864(1), 89-101.

Williams, P. R. D., Hammitt, J. K., 2001. Perceived risks of conventional and organic produce: Pesticides, pathogens, and natural toxins. Risk Analysis, 21(2), 319-330.

Zimmerli, B., Dick, R., 1996. Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. Food Additives and Contaminants, 13(6), 655-668.

STUDY VII

Effect of preharvest anti-fungal compounds on *Aspergillus steynii* and *A. carbonarius* under fluctuating and extreme environmental conditions

García-Cela, E.[a], Gil Serna, J. [b], Marín, S. [a], Acevedo, H. [a], Patiño, B. [b] and Ramos, A. J. [a]

[a]Food Technology Department, Lleida University, UTPV-XaRTA-Agrotecnio, Rovira 191, 25198 Lleida, Spain

Roure

[b]Department of Microbiology III, Faculty of Biology, University Complutense of Madrid, José Antonio Novais 2, 28040 Madrid, Spain

ABSTRACT

Ochratoxin A (OTA) has been found in pre-harvest and freshly harvested wheat. Spanish climatic conditions point to *Aspergillus* species as the probable responsible for this OTA presence. In this study the effectiveness of 5 non-specific chemical wheat field antifungals (25.9% tebuconazole + 60.0% N,N-capramide dimethyl; 12.70% tebuconazole + 12.7% prothioconazole + 59.5%N,N- amide dimethyldecane; 12.5% epoxiconazole; 12.5% tetraconazole; and 70% thiophanate methyl) and an extract from *Equisetum arvense* was investigated *in vitro* on wheat by recording growth (colony size and DNA concentration) and OTA production of two ochratoxigenic isolates of *Aspergillus carbonarius* and three of *A. steynii*, simulating current and extreme climatic conditions. Inoculated wheat was incubated under two alternating temperature cycles (35/15 ºC and 25/10 ºC) with photoperiod (14/10 h lightness/darkness), and two moisture levels (40 and 25%). The *Aspergillus* species tested seemed to be able to persist in the future coming conditions, in particular, *A. steynii*, a high OTA producer. Azoles were effective in controlling the growth of A. carbonarius and A. steynii, and this effectiveness may not be compromised by the increase in temperature and

decrease of humidity. However, azoles are not useful for the prevention of OTA accumulation, which could be only reduced in *A. carbonarius* under non-extreme conditions. Although some adjustment will probably be required, further studies should be conducted in the field, since the antifungals used in this study are applied at flowering and not directly on the grain. Moreover, timing of antifungal application may need to be optimized. Finally, *Equisetum* extract showed promising results as an antifungal, however further work to adjust the applied concentrations is required,

Publication: International Journal of Food Microbiology (2012), 159(2), 167-176.

1. INTRODUCTION

Due to the global importance of cereals in the diet and, in certain climatic conditions, their susceptibility to invasion by molds, mycotoxin production is a concern that can from crops to the final products (Molinié et al., 2005). Consumption of food contaminated by mycotoxins has been associated with several cases of human poisoning or mycotoxicoses, sometimes resulting in death (Molina and Giannuzzi, 1999).

In wheat the main efforts in the control of mycotoxins in the field have been directed towards species of *Fusarium*. However, presence of ochratoxin A (OTA) in wheat and wheat products in several parts of the world makes necessary to focus also to ochratoxigenic fungi (Duarte et al., 2010).

In northwest Europe OTA presence in cereals is mainly associated to the presence of *Penicillium verrucosum*, but warmer countries *Aspergillus* spp. may be the most likely responsible. In Algeria more *Aspergillus* (55%) than *Penicillium* (6%) species were isolated from freshly harvested wheat grain, both increasing during storage (Riba et al., 2008). However, in Lebanon more *Penicillium* (50%) than *Aspergillus* (31%) species were isolated preharvest, although only 57% of *Penicillium* spp. compared with 80% of *A. ochraceus* produced OTA, at maximum concentrations of 53 and 65 μg/kg, respectively, with no *A. niger* aggregate isolates producing OTA (Joubrane et al., 2011). In Spain, Cabañes et al. (2008) observed high occurrence of *Aspergillus* and *Penicillium* spicies in retail wheat flours from supermarket, but only 17 isolates of *P. verrucosum* were OTA producers. By contrast, Mateo et al. (2011) pointed to Aspergillus species as the main fungi responsible for the presence of OTA inbarley grain from Spain, since A. steynii, A. ochraceus and A. carbonarius were detected in most of the samples contaminaeted with OTA. Soldevilla et al. (2005) found that 37-55% and 45-76% of samples from Spain soft wheat and Spring barley contained *A. fumigatus* and *A. niger* var. *niger,* respectively. Likewise, Riba et al. (2008) and González-Salgado (2009) found *A. carbonarius* in fresh wheat. Most strains *A. carbonarius* are OTA producers.

The presence of black aspegilli in pre-harvest grain samples in Spain and Algerian studies indicates an adaptation of such species to warmer weather. Magan et al. (2011) suggested that a climate change towards hot temperatures and drought could increase the risk of migration of pathogens as a result of a shift to warmer, drought like conditions. For example, *Fusarium verticillioides*, the most widespread maize pathogen, is favored by warm dry weather, while *A.*

flavus tends to occur only in a particularly hot summer. Under warmer conditions a clear modification of mycotoxin association on maize was observed: more fumonisins and aflatoxins, less DON and zearalenone. Additionally, recent studies of strains of *A. flavus* isolated from maize in North Italy showed slightly different ecological profiles in terms of both optimal and marginal growth conditions compared to other regions of the world (Giorni et al*.,* 2007).

Prevention o f growth and mycotoxin-producing fungi is the most effective strategy for controlling the presence of mycotoxins in crops. The *Codex Alimentarius* (CAC) includes the application of fungicides in Good Agricultural Practices to reduce contamination in cereals (CAC/RCP 51-2003). However care should be taken since continuous and indiscriminate use of these compounds could lead to toxic effects for consumers and to the development of resistant microorganisms (López-Malo et al., 2000). The EU has set the maximum residue level (MRL) on wheat at 0.1 μg/kg for prothioconazole and tetraconazole, 0.2 μg/kg for epoxiconazole and tebuconazole, and 0.05 mg/kg for thiophanate methyl (European Union Pesticides database 2010; EU Nº 559/2011). Legal restrictions on MRL and consumer demand for healthier products requires the use of safer and more environmentally friendly antifungals. Plant extracts have been tested against *Aspergillus* species (Rasooli and Owlia, 2005; Rasooli et al., 2006) including those of *Equisetum* spp., commonly known as field horsetail (Garcia et al., 2011, 2012; García-Cela et al., 2011; Milovanović et al 2007, Radulovic et al., 2006).

It is important to evaluate the effectiveness of agricultural practices, including application of field fungicides under different climate scenarios. For this reason, the aim of the present study was to test in vitro the impact of extreme fluctuating environmental conditions in development of ochratoxigenic molds on wheat and effectiveness of synthetic and natural antifungals.

2. MATERIALS AND METHODS

2.1. Fungal isolates

One strain of *A. carbonarius* (3.265 UdLTA) and 3 strains of *A. steynii* (3.263 UdLTA, 3.264 UdLTA and 3.266 UdLTA) isolated from Spanish barley, and one *A. carbonarius* (3.83 UdLTA) isolated from Spanish grapes were subcultured on Yeast Extract Agar (CYA) plates

and incubated at 25 ºC for 7 days to enable significant sporulation. Following incubation, a sterile inoculation loop was used to remove the conidia from CYA plates and they were suspended in Tween 80 (0.005%). After homogenizing the suspensions were adjusted using a Thoma counting chamber to final concentrations of 1×10^6 spores/mL in Tween 80 (0.005%) and in water modified with glycerol to provide 0.98 a_W. Previously, the OTA-producing capacity of the isolates was assessed using the method of Bragulat et al. (2001).

2.2. Wheat preparation

Fourteen 1-L bottles were filled with 400 g of wheat and autoclaved at 121 ºC for 20 min. Due to the low initial water content, no softening was observed in the grains due to autoclaving. From the 14 bottles, seven were adjusted to 40% (a_W =0.99) and seven to 25% (a_W =0.98) moisture content level (m.c.). Moisture content was adjusted by aseptically adding amounts of sterile distilled water to wheat. The bottles were cooled down to 4 ºC for 48 hours with periodic hand-shaking during this time. The amount of water necessary to reach the different m.c. levels was determined by using to relation between wheat m.c. and water initially determined subtracting the volume of antifungal to be added.

2.3. Antifungals preparation

One plant extract (*Equisetum arvense*) and five non-specific wheat commercial antifungals for *Aspergillus* (Folicur ®, Prosaro ®, Lovit ®, Domark evo ®, and Enovit®) were used at the doses described in **Table 1**. Doses of liquid antifungals were calculated from recommendations by manufacturers (L/ha) divided between average field efficiency per ha in unirrigated land in Spain (2128 kg/ha) as an approximation. Then, antifungals were mixed with distilled water until final volumes of 1.5 ml to facilitate the dispersion in the wheat grain. The antifungal powder was mixed in a rate of 0.075% w/v. The plant extract was obtained from the aerial parts of *E. arvense* (collected in fields from Lleida, Spain, 2010-2011), washed with water and dried at 40 ºC. Dried vegetal material (25 g) was macerated in 500 mL of 70% ethanol (99.6% purity) at 25 ºC during 7 days. The extract was then filtered under vacuum and the plant material was removed, leaving a dark green hydroalcoholic extract. The ethanol and part of water were then evaporated by rota-evaporation at 40 ºC to obtain a 19% (w/v) concentration of plant extract which was stored at 4 ºC until use. The plant extract and antifungals were aseptically incorporated into the wheat bottles and shaked vigorously. Then 20 g of wheat were incorporated into the Petri dishes. Final a_W values of seeds were checked

with and AguaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of ±0.003, before, during and at the end of the experiment.

Table 1 Antifungal dose and active ingredients

Supplier	Antifungal	Active ingredient	Presentation	Doses
Bayer CropScience S.L.	(F1) Folicur 25® Ew	25.9 %Tebuconazole 60.0%N,N-capramida dimethyl	Liquid	0.47[A]
Bayer CropScience S.L.	(F2), Prosaro®	12.70%Tebuconazole 12.7%Prothioconazole 59.5%N,N-amidedimetildecano	Liquid	0.47[A]
Basf Española S.L.	(F3) Lovit®	12.5%Epoxiconazole	Liquid	0.38[B]
Sipcam Inagra S.A.	(F4) Domark Evo®	12.5%Tetraconazole	Liquid	0.35[C]
Sipcam Inagra S.A.	(F5) Enovit Metil®	70%Thiophanate methyl	Powder	0.375[D]
	(F6) E. arvense	Table 2	Liquid	15.6[E]

Doses (ml antifungal/100g wheat) A: From recommended doses of 1L/ha; B: From recommended doses of 0.8L/ha; C: From recommended doses of 0.75L/ha; D: From 0.075% of powder antifungal solution; E: From 19% acuose plant extract of *E. arvense*.

2.4. Phenolic compounds analysis in E. arvense extract (HPLC-DAD-ESI/MS)

A lyophilized *E. arvense* extract was analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C8, 3 μm (4.6 mm × 150 mm) column thermostated at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% B over 5 min, 15-20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% B over 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. Spectra were recorded in negative ion mode between m/z 100 and 1000. EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) of the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V.

2.5. Preparation of inocula and incubation

Petri dishes with 20 g of grain wheat were inoculated centrally with 5 µl of each spore suspension of 10^6 spores/mL. Plates with the same a_W were enclosed in sealed containers along with beakers containing water glycerol solutions of the same a_W as the plates in order to maintain the a_W. Petri dishes were incubated at different combinations of temperature and R.H. for 21 days simulating a possible extreme climatic change scenario. A simulated photoperiod of fourteen sunlight hours and ten hours of darkness per day was applied. To simulate light conditions, white light was used (Mazda 23 WEureka3 Electronic bulbs, 230–240 V, 50–60 Hz, 1500 lumen, 175 mA). The heat emitted by the bulbs did not increase the temperature proposed for incubation. Mean temperatures of 25 and 10 ºC for day and night, respectively, were chosen as current climatic conditions in May ("present" conditions), antifungal pre-harvest application time in Northern Spain. Mean temperatures of 35 and 15 ºC for day and night respectively were chosen as extreme predicted climatic conditions which could occur in the expected future ("extreme" conditions), based in the current conditions in Southern Spain (INM, Instituto Nacional de Meteorología, Spain). Also, 40% m.c. was used for the first treatment simulating the m.c. of the crop at flowering (Gooding et al., 2003), and 25% m.c. was used for the second treatment simulating a decrease caused by drought in a climate change scenario. Each treatment was carried out by triplicate.

2.6. Growth determination

Mycelial extension rates were measured over time. Two perpendicular diameters of the growing colonies were measured daily until 21 days or when the colony reached the edge of the Petri dish (85 mm). The slope of the line obtained by linear regression of colony radius against time was used to determine growth rates (mm/day).

2.7. Quantification of fungal DNA

DNeasy Plant Mini Kit (Qiagen, Valencia, Spain) was used according to manufacturer's instructions to obtain genomic DNA from inoculated wheat samples. After 21 days of incubation, the 20 g of inoculated wheat was finely milled with a grinder. 100 mg of each wheat sample in all conditions were used to carry out DNA extraction. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

Since inoculation was performed in sterile wheat grains, quantification of fungal DNA in all samples analyzed was performed using the primer set 5.8S1/5.8S2 which amplifies a fragment of 90 bp of the 5.8S region of the ribosomal DNA of all *Aspergillus* species. The real-time PCR protocol used was described previously in our group (Gil-Serna et al., 2009) but including some modifications. Real-time PCR assays were performed and monitored in an ABI PRISM 7900HT system (Applied Biosystems, Madrid, Spain) in the Genomic Unit of the Complutense University of Madrid, Spain. The reaction mixture composition in a final volume of 10 µl was: 5 µl SYBR® Green PCR Master Mix (Applied Biosystems, Madrid, Spain), 0.6 µl forward primer 5 µM, 0.6 µl reverse primer 5 µM, 2.5 µl DNA template in suitable concentration (30 ng/µl) and 1.3 µl molecular biology water (MO-BIO, Carlsbad, USA). qPCR assays were carried out using a standard program: 95 ºC for 10 min, 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. All reactions were carried out by duplicate in MicroAmp 384-well plates (Applied Biosystems, Madrid, Spain).

This is the first time this protocol was used to quantify genomic DNA of *A. carbonarius* and *A. steynii*; therefore, the efficiency of the method was evaluated using ten-fold serial dilutions of DNA from *A. steynii* 3.264 UdLTA and *A .carbonarius* 3.83 (from 50 to $5x10^{-3}$ ng/µl) as template in the reactions to create the standard curve. C_T values were plotted against the logarithm of starting quantity of template for each dilution. Then, amplification efficiency

was calculated from the slope of the standard curve (Kubista et al, 2006). The standard curve generated using DNA from *A. steynii* was C_T = -3.31log[DNA] + 20.22 and the amplification efficiency was 100%. In the case of *A. carbonarius* the standard curve was C_T = -3.30log[DNA] + 18.96 and the amplification efficiency calculated was 101%.

C_T values obtained after evaluating DNA extracted from inoculated wheat samples were interpolated in their corresponding standard curve to calculate the relative amount of DNA present in them.

2.8. Ochratoxin A determination

Extraction: After 21 days of incubation, fungal growth was registered for the last time and the wheat samples were stored at -17 ºC until analyzed. Prior to OTA extraction, samples were finely milled with a grinder (Braun Aroma Gourmet, KSM2, Kronberg Germany). Next, 10 g of ground wheat were weighed in an amber flask and 40 mL of 60% acetonitrile/water solution were added. The mixture was blended for 10 min by means of a magnetic stirrer. The extract was filtered by gravity (Whatman No 1 filter).

Clean-up by immunoaffinity chromatography columns (IAC): 2 mL of the filtrate were mixed with 22 mL of PBS (phosphate buffered saline, solution containing 0.2 g KCl, 0.2 g KH_2PO4, 1.16 g Na_2HPO_4, 8 g NaCl and 900 mL H_2O, pH = 7.4). The diluted extract was loaded onto the IAC column (Ochraprep, R-Biopharm, Rhône LTD) and allowed to pass through it by gravity. After washing the column with 20 mL PBS and drying it with air, OTA was eluted with 1.5 mL desorption solution (methanol: acetic acid, 98:2) in an amber vial. Backflushing was done three times. Finally, 1.5 mL Milli-Q water was passed to obtain a total volume of 3 mL.

Chromatographic analysis: High performance liquid chromatography (HPLC) was performed on the cleaned-up extract on a Waters 2695 Separations Volume (Alliance) coupled to a Waters 2475 Multi λ fluorescence detector. Waters Spherisorb ODS2 C18 column (5 µm, 4.6 × 150 mm) equipped with a Waters Spherisorb ODS2 guard column (5 µm, 4.6 × 10 mm) (Waters, Ireland) was used. Mobile phase consisted of acetonitrile:Milli-Q water:acetic acid (51:47:2). Flow-rate was 1 mL/min. Injection volume was 100 µL. Excitation and emission wavelengths were 333 nm and 443 nm, respectively. Temperature of column and guard column was maintained at 40 °C. Retention time for OTA was 5.8 min.

The integration software used was Empower 2 (2006 Waters Corporation, Database Version 6.10.00.00).

Quantification: OTA quantification was based on the external standard calibration method. The limit of detection (LOD) of the analysis was 0.5 μg/kg based on a signal to noise ratio of 3:1.

2.9. Statistical analysis

Analysis of variance of growth rates and diameter of the colony after 7, 14 and 21 days, , DNA concentration and OTA production was used in order to assess significant differences ($p<0.05$) due to climatic conditions and assayed antifungals. Post-hoc analyses were performed using the Tukey-honestly significant differences (Tukey-HSD) test. To test the possible correlation between the production of OTA and the growth responses (diameters, growth rate and DNA), the Spearman correlation coefficient was calculated. For statistical purposes undetectable OTA levels were considered LOD value. Statgraphics ® Plus 5.1 (Manugistics, Inc., Maryland, USA) was employed.

3. RESULTS

3.1. Phenolic compounds in E. arvense extract

Phenolic compounds in the *E. arvense* extract belonged to families of hydroxycinnamic (phenylpropanoids) and methoxycinnamic acids and flavonls (**Table 2**). The main phenolic compounds were kaempferols belonging to flavonols.

3.2. Impact of changing climatic conditions in fungal growth and OTA production

The analysis of variance revealed that all factors (strains, environmental conditions and antifungal treatment) and their two and three-way interactions had a significant effect on growth responses ($p<0.05$), while for OTA production, the environmental conditions only had a significant effect linked to antifungal treatment.

Under the conditions tested, *A. carbonarius* isolates grew faster than *A. steynii* reaching greater colony diameters after 7, 14 and 21 days; in fact *A. carbonarius* isolates colonized the whole 9-cm Petri dish before 14 days in "extreme" conditions and before 21 days under "present" conditions (**Table 3).** Growth rates in *A. carbonarius* were higher in 'extreme' conditions (7 mm/day vs 5 mm/day), while few differences were found in *A. steynii* due to the assayed climatic conditions (2–3 mm/day).

Table 2 Retention time, maximum wavelength in the visible region (λ_{max}), mass spectral data and identification of plant phenolic compounds in *E. arvense* extract.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (m/z)	Tentative identification	µg/mg
1	7.0	354	787	625, 463, 301	Quercetin 3-*O*-rutinoside-7-*O*-rhamnoside[1]	0.77 ± 0.01
2	7.7	344	771	609, 447, 285	Kaempferol-3-*O*-rutinoside-7-*O*-glucoside[2]	10.84 ± 0.21
3	8.9	358	787	625, 463, 301	Quercetin trihexoside[1]	0.14 ± 0.01
4	9.2	328	355	193	Ferulic hexoside acid[3]	0.14
5	12.5	330	179	135	Caffeic acid[4]	1.82 ± 0.09
6	15.1	372	423	261	Equisetumpyrone	-
7	15.7	352	625	463,301	Quercetin 3-*O*-sophoroside[1]	0.85 ± 0.01
8	16.7	328	309	193,178,149,135	Ferulic acid derivative[3]	0.26 ± 0.04
9	17.9	348	609	447, 285	Kaempferol dihexoside[2]	7.48 ± 0.12
10	18.5	328	193	178, 149, 135	*trans*-Ferulic acid[3]	1.19 ± 0.03
11	20.5	356	463	301	Quercetin 3-*O*-glucoside[5]	0.46 ± 0.02
12	21.8	354	505	463,301	Quercetin acetyl hexoside[5]	0.55 ± 0.01
13	22.6	352	447	285	Kaempferol 3-*O*-glucoside[6]	0.15 ± 0.01

*mean ± standard deviation (n=2).
[1] Están expresados como mg quercetina 3-*O*-rutinósido/mg planta.
[2] Están expresados como mg kaempferol 3-*O*-rutinósido/mg planta.
[3] Están expresados como mg ácido ferúlico/mg planta.
[4] Están expresados como mg ácido cafeico/mg planta.
[5] Están expresados como mg quercetina 3-*O*-glucósido/mg planta.
[6] Están expresados como mg kaempferol 3-*O*-glucósido/mg planta

Under "present" conditions, *A. carbonarius* (3.265 UdLTA) grew initially faster ($p<0.05$) than *A. carbonarius* (3.83 UdLTA), but no significant differences were found in their growth after

14 and 21 days, nor in their growth rate (Table 3). In *A. steynii* no significant differences were found among isolates regarding growth kinetics. By contrast, under "extreme" conditions, at 7 days, colony diameter of *A. carbonarius* (3.83 UdLTA) was significantly higher than *A. carbonarius* (3.265 UdLTA)*;* however as in the "present" condition this difference disappeared later (Table 3). In *A. steynii*, although all isolates grew at the same rate, 3.266 UdLTA isolate reached a larger size after 21 days. DNA concentration paralleled colony size results; correlation analysis showed a significant positive correlation with diameter at 7, 14 and 21 days, as well as with growth rate. The effect caused by climatic conditions on OTA production was only significant when considered along with the other factors, strain and antifungal treatment. In general, the *A. steynii* isolates were higher OTA producers than *A. carbonarius* under both conditions tested (Table 3). Although 'present' conditions were more favorable for OTA production in *A. carbonarius*, no significant differences were observed in *A. steynii*, although the observed means were higher under 'extreme' conditions.

3.3. Effectiveness of antifungals against fungal growth in wheat

Significant differences ($p<0.001$) were found among antifungal treatments; their interactions with environmental conditions and strains tested were also significant.

3.3.1. Aspergillus carbonarius

After 7 days, fungal growth in the untreated controls was clearly visible, and had already reached the limit of the Petri dish after 21 days (**Table 4**). Under "present" conditions antifungals coded F1, F2, F3, F4 and F6 significantly inhibited fungal growth, although the effect could not be observed after 21 days, when the controls reached the limit of the Petri dish. Under "present" conditions, the percentage of reduction in growth rate showed similar values in F1, F2, F3 and F4 (19-41%) although higher variability in the effect was observed under "predicted" conditions (5-73%), due mainly to the increased effectively of F1 and F2, and the decreased activity of F4. Fungicide F5 either had no effect or stimulated the growth (**Figure 1**). Finally, F6 was the most efficient under all conditions (92-94% growth rate reduction under "present" conditions, 75-91% under "extreme" conditions).

Table 3 Significance of the effects of enviromental conditions in colony diameter (mm) at 7, 14 and 21 days, growth rate (mm/day) and OTA (μg/g) production of *Aspergillus* spp. in untreated trials. Different capital letters mean significant differences between strains within a given condition. Different lower case letters mean significant differences for a given strain among conditions (Tukey test, $p<0.05$).

	PRESENT CONDITIONS				
	7 days	**14 days**	**21 days**	**GROWTH RATE**	**OTA PRODUCTION**
3.830 UdLTA	33.33 ± 1.50 Ab	71.17 ± 1.20 Ab	85.00 ± 0.00 Aa	5.15 ± 0.04 Ab	356.58 ± 85.25 Ba
3.265 UdLTA	39.67 ± 0.80 Ba	69.83 ± 1.20 Ab	85.00 ± 0.00 Aa	4.82 ± 0.24 Ab	241.14 ± 52.75 Ba
3.266 UdLTA	10.83 ± 1.60 Cb	35.50 ± 2.80 Ba	56.50 ± 3.90 Ba	3.07 ± 0.38 Ba	778.19 ± 124.97 Aa
3.263 UdLTA	10.83 ± 1.30 Cb	31.67 ± 4.60 Ba	48.17 ± 7.30 Ba	2.67 ± 0.21 Ba	391.61 ± 70.91 Ba
3.264 UdLTA	12.75 ± 2.30 Cb	36.00 ± 8.50 Ba	49.75 ± 3.20 Ba	2.71 ± 0.93 Ba	466.60 ± 149.60 Ba
	PREDICTED CONDITIONS				
	7 days	14 days	21 days	GROWTH RATE	OTA PRODUCTION
3.830 UdLTA	52.25 ± 3.90 Aa	85.00 ± 1.70 Aa	85.00 ± 0.00 Aa	7.04 ± 0.45 Aa	27.00 ± 17.27 Cb
3.265 UdLTA	39.67 ± 1.80 Ba	85.00 ± 0.00 Aa	85.00 ± 0.00 Aa	7.19 ± 0.05 Aa	55.32 ± 9.00 Cb
3.266 UdLTA	16.75 ± 2.20 Ca	38.00 ± 3.00 Ba	56.50 ± 2.10 Ba	2.83 ± 0.10 Ba	1004.99 ± 70.07 Aa
3.263 UdLTA	19.75 ± 3.20 Ca	38.25 ± 4.30 Ba	51.50 ± 3.00 BCa	2.68 ± 0.44 Ba	518.06 ± 72.95 Ba
3.264 UdLTA	19.00 ± 1.70 Ca	37.33 ± 1.30 Ba	48.50 ± 2.30 Ca	2.30 ± 0.15 Ba	265.56 ± 228.82 BCa

In general, under both climatic conditions treated wheat samples, except those treated with F5, showed lower DNA concentration than the untreated wheat. However these reductions were not significant. As with colony size, F6 always reported the lowest values of DNA concentration. On the other hand the DNA level in F5 treated wheat was significantly higher than in the untreated control except in *A. carbonarius* 3.265 under "present" conditions.

3.3.2. Aspergillus steynii

Due to the slow growth rate of the isolates the colonies never reached the edge of the Petri plates. As a consequence, few differences were observed among antifungals after 7 days of incubation. Under 'present' conditions, after 14–21 days the most effective antifungals were F3, F4 and F6 showing a diameter reduction after 21 days of 27–62%, although the effectiveness of the antifungal varied among isolates; for *A. steynii* (3.266 UdLTA) the most effective was F3 (50%), while for the others it was F6 (**Table 4**). Regarding growth rate, F3 and F6 achieved the higher reduction in a range of 51 to 59% and 44 to 76%, respectively.

None of the antifungals reduced significantly DNA concentration under "present" conditions. Under "extreme" conditions F2, F3 and F6 reduced the DNA concentration in all *A. steynii* inoculated samples but the reduction was only significant in 3.263 UdLTA (81-94%) and 3.266 UdLTA (68-93%) (**Figure. 3**). Once again, higher DNA concentration was observed in F5 treated samples under both climatic conditions.

3.4. Effectiveness of antifungals against OTA production

Antifungal treatments had a significant impact on OTA production, as well as their interactions with strains and conditions tested ($p<0.05$).

3.4.1. Aspergillus carbonarius

Under 'present' conditions, all antifungals significantly reduced OTA production in *A. carbonarius* in a range from 56 to 98.28% and 73 to 99% in *A. carbonarius* 3.83 UdLTA and *A. carbonarius* 3.265 UdLTA, respectively. However, F6 and F2 were the most effective in both isolates.

Table 4 Signficance of the effects of enviromental conditions in mean diameter (mm) at 7, 14 and 21 days. Level not connected by same capital letters are significantly different in the same conditions and same lowercase letters are significantly different between conditions (Tukey test, $p<0.05$).

		PRESENT CONDITIONS						PREDICTED CONDITIONS					
		7 days		14 days		21 days		7 days		14 days		21 days	
A. carbonarius 3.830 UdLTA	C	33.33	B	71.17	A	85.00	A	52.25	A	85.00	A	85.00	A
	F1	23.33	CD	50.33	B	74.00	A	17.00	C	32.50	C	40.00	C
	F2	20.00	D	43.50	B	74.17	A	14.67	C	34.33	C	42.33	C
	F3	27.33	BC	50.17	B	78.67	A	30.75	B	60.25	B	72.25	B
	F4	25.50	CD	54.00	B	76.83	A	36.67	B	77.00	A	85.00	A
	F5	41.00	A	73.83	A	85.00	A	35.75	B	80.75	A	85.00	A
	F6	7.00	E	10.50	C	11.00	B	0.00	D	10.17	D	13.00	D
A. carbonarius 3.265 UdLTA	C	39.67	A	69.83	B	85.00	A	39.67	A	85.00	A	85.00	A
	F1	25.83	B	50.17	CD	68.33	BC	11.50	D	32.17	CD	43.17	BC
	F2	24.83	B	43.50	D	62.83	C	21.00	C	42.00	BC	57.00	B
	F3	24.67	B	50.50	CD	71.50	BC	27.75	B	63.50	AB	81.50	A
	F4	25.83	B	55.33	C	74.33	B	31.00	B	79.00	A	85.00	A
	F5	46.25	A	83.75	A	85.00	A	24.83	BC	71.50	A	85.00	A
	F6	9.00	C	10.33	E	10.67	D	0.00	E	15.33	D	21.67	C
A. steynii 3.266 UdLTA	C	10.83	BC	35.50	B	56.50	B	16.75	AB	38.00	AB	56.50	A
	F1	8.33	C	23.50	C	38.33	C	11.00	BC	24.50	BC	31.25	B
	F2	14.17	B	36.67	B	57.33	B	7.17	C	13.83	C	15.16	C
	F3	7.75	C	18.50	C	28.00	D	8.25	BC	23.75	BC	33.00	B
	F4	9.00	BC	21.50	C	41.00	C	20.67	A	42.50	AB	56.00	A
	F5	28.50	A	56.33	A	74.17	A	15.50	ABC	45.50	AB	64.00	A
	F6	12.67	BC	24.83	C	35.33	CD	5.00	C	27.50	ABC	39.25	B
A. steynii 3.263 UdLTA	C	10.83	B	31.67	B	48.17	B	19.75	A	38.25	A	51.50	A
	F1	6.17	C	22.67	BC	36.17	BC	14.00	ABC	23.00	BC	28.00	C
	F2	10.50	BC	26.50	BC	41.50	BC	7.25	BC	13.25	C	15.00	D
	F3	7.00	BC	18.50	C	26.00	CD	5.33	C	13.67	C	21.33	CD
	F4	8.33	BC	20.33	C	30.83	CD	17.33	AB	32.50	AB	42.83	B
	F5	27.00	A	49.33	A	65.00	A	13.75	ABC	38.50	A	51.50	A
	F6	11.33	B	15.00	C	18.83	D	6.00	BC	13.00	C	17.50	D
A. steynii 3.264 UdLTA	C	12.75	B	36.00	B	49.75	AB	19.00	A	37.33	A	48.50	A
	F1	7.25	CD	19.25	CD	32.75	BC	8.00	B	16.50	B	23.33	B
	F2	12.00	BC	30.00	BC	46.50	AB	5.33	B	9.67	B	13.17	B
	F3	4.67	D	13.83	D	20.67	C	6.75	B	15.50	B	19.25	B
	F4	9.17	BC	21.67	CD	31.83	BC	16.00	A	37.00	A	55.67	A
	F5	28.17	A	48.33	A	71.00	A	9.00	B	35.00	A	52.50	A
	F6	8.50	BCD	13.00	D	20.67	C	0.00	C	12.67	B	19.83	B

C, control, F1, Folicur 25® Ew, F2, Prosaro® , F3, Lovit®, F4, Domark Evo®, F5, Enovit Metil®, F6, *E. arvense*.

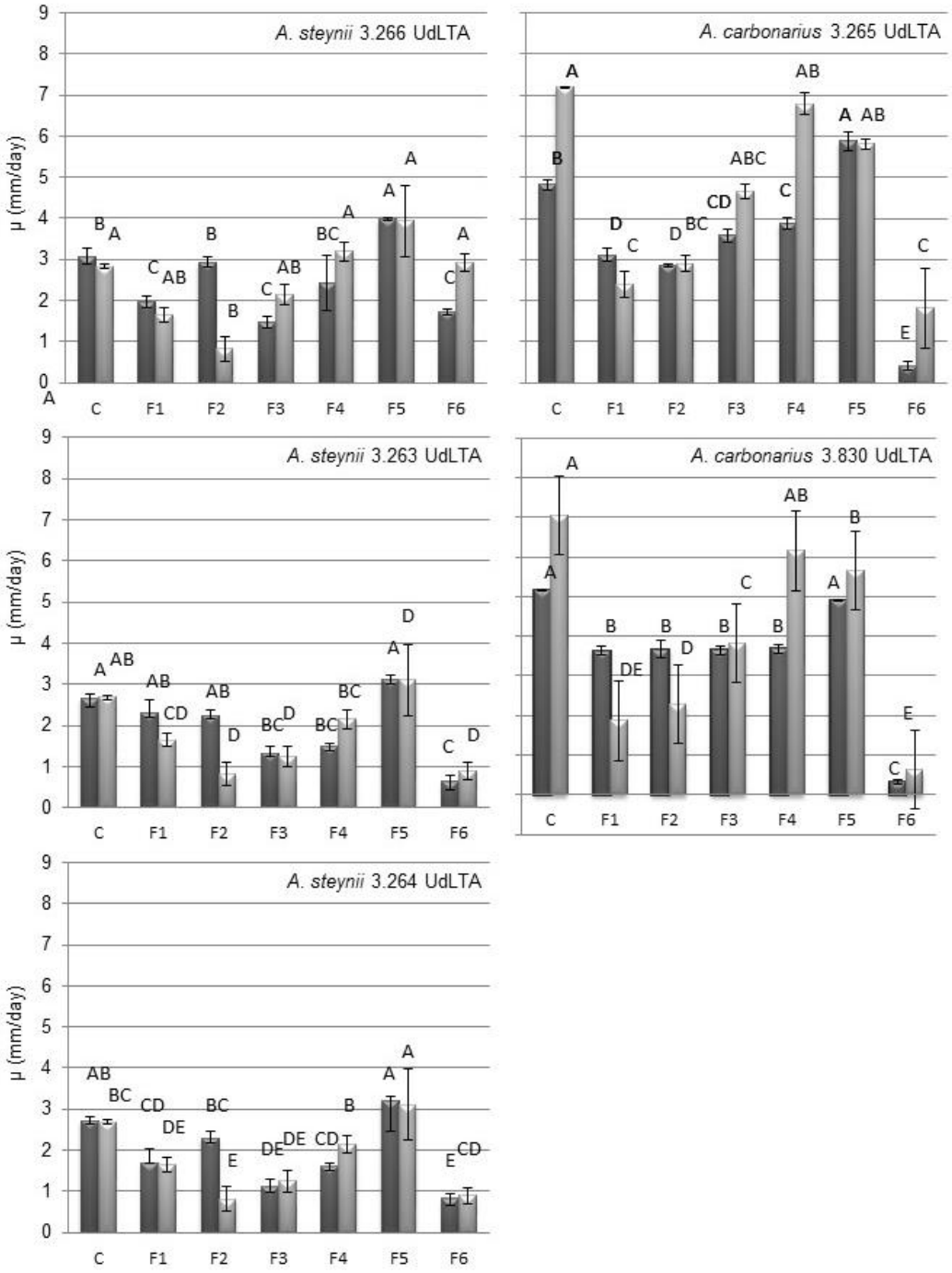


Figure 1 Effect of antifungal treatments (C: Control, F1: Folicur 25® Ew,F2: Prosaro®, F3: Lovit®,F4: Domark Evo®, F5: Enovit Metil® and F6: *E. arvense*) on growth rate (mm/day) of *Aspergillus* species on wheat under different climatic conditions: black bars, 'present' conditions (temperature cycle of 10/25°C, 40% RH) and gray bars, 'extreme' conditions (temperature cycle of 15/35°C, 25% RH). Different letters over bars indicate significant differences among values for each isolate (Tukey *t* test at $p < 0.05$).

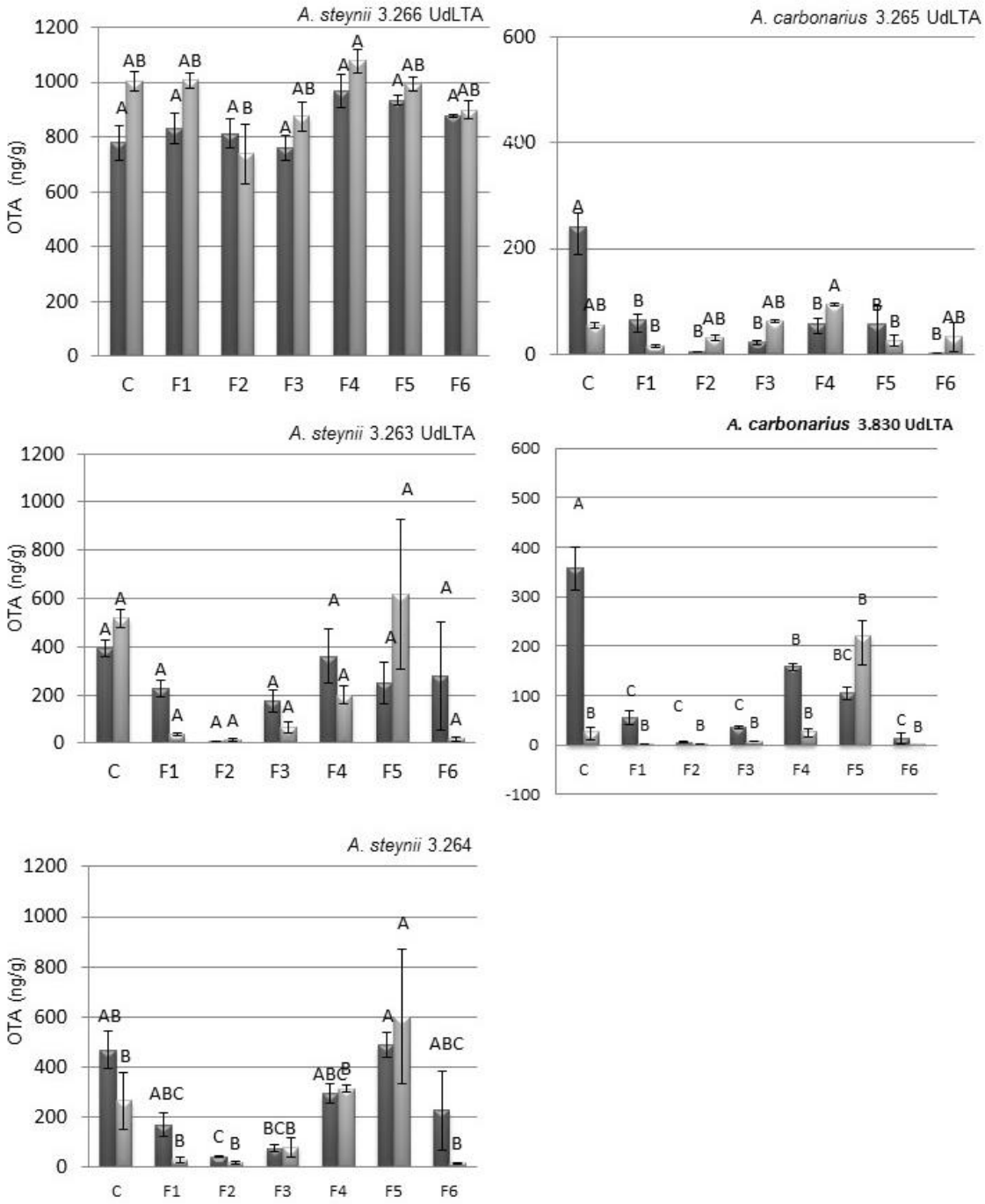


Figure 2. Effect of antifungal treatments (C: Control, F1: Folicur 25® Ew,F2: Prosaro®, F3: Lovit®, F4: Domark Evo®, F5: Enovit Metil® and F6: *E. arvense*) on OTA production (µg/g) on wheat by *Aspergillus* species under different climatic conditions: 'present' conditions (temperature cycle of 10/25 °C, 40% RH) and 'extreme' conditions (temperature cycle of 15/35 °C, 25% RH). Different letters over bars indicate significant differences among values for each isolate (Tukey *t* test at $p<0.05$).

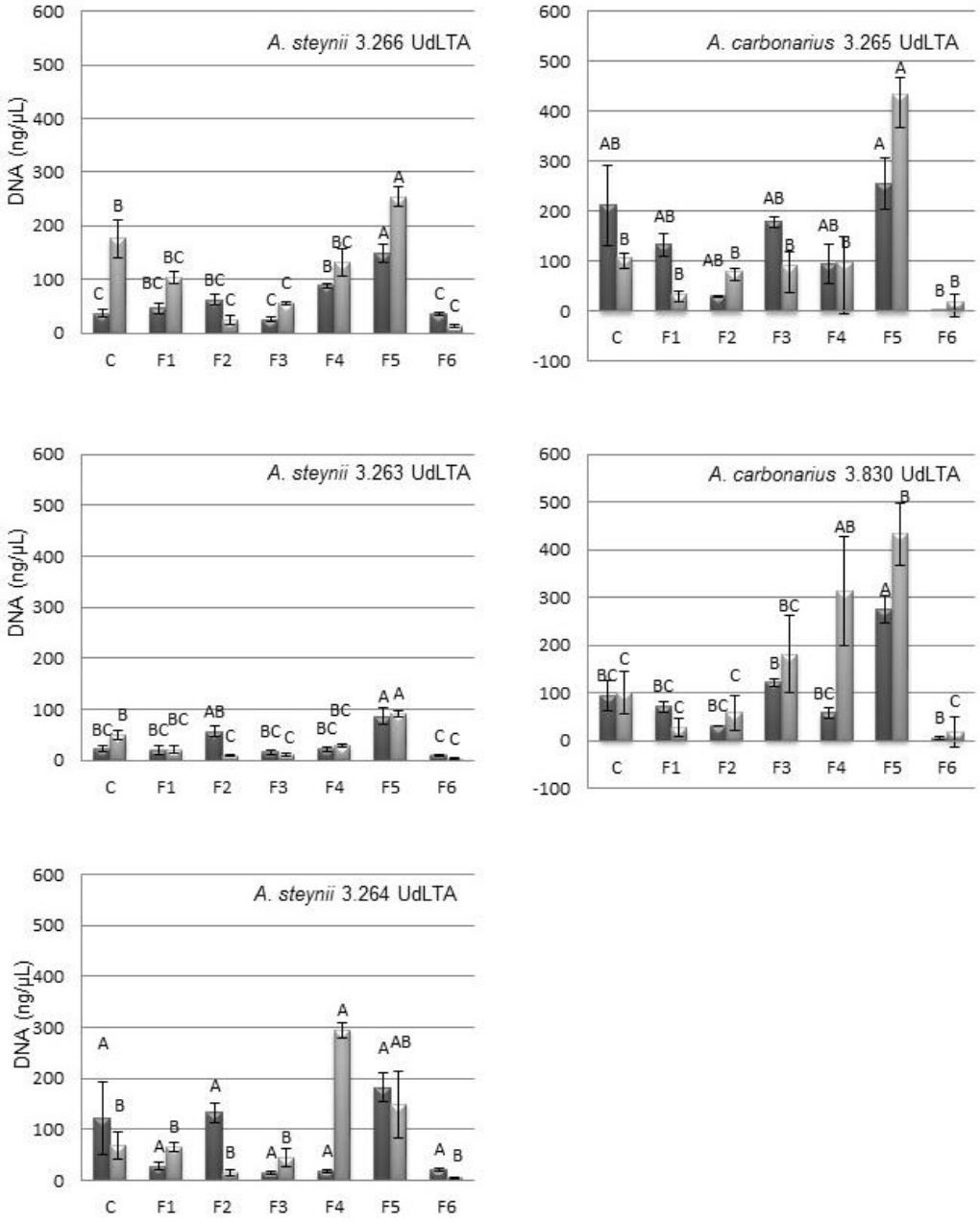


Figure 3. Effect of antifungal treatments (C: Control, F1: Folicur 25® Ew,F2: Prosaro®, F3: Lovit®, F4: Domark Evo®, F5: Enovit Metil® and F6: *E. arvense*) on DNA concentration (ng/µL) on wheat by *Aspergillus* species at 21 days under different climatic conditions: black bars, 'present' conditions (temperature cycle of 10/25 °C, 40% RH) and gray bars, 'extreme' conditions (temperature cycle of 15/35 °C, 25% RH). Different letters over bars indicate significant differences among values for each isolate (Tukey *t* test at $p<0.05$).

Under 'extreme' conditions, no significant reduction in OTA production due to antifungals was observed. F5 stimulated OTA production in *A. carbonarius* 3.83 UdLTA by more than 700%.

3.4.2. Aspergillus steynii

No significant effect on OTA production by *A. steynii* could be attributed to antifungal application, neither in 'present' or 'extreme' conditions, with the exception of fungicide F2 for *A. steynii* 3.264 UdLTA under 'present' conditions. In fact, although no significant differences could be observed due to the high variability of OTA results, fungicide F2 reduced OTA production in *A. steynii* 3.263 UdLTA in 99% and under 'present' conditions. On the other hand, reductions of 90–93%, 93–98%, and 94–97% by F1, F2 and F6 were observed for these same strains under 'extreme' conditions. Finally, *A. steynii* 3.266 UdLTA, which was the highest producer, seemed to be unaffected by antifungal application in its OTA production.

Finally, a positive correlation was observed between growth and OTA production after 21 days in the controls for 'present' conditions and a negative correlation was observed under 'extreme' conditions, suggesting that stress conditions triggered OTA production. The differentiated impacts of antifungals against growth compared to OTA production led to an absence of correlation between both variables in the treated experiments.

4. DISCUSSION

Recent studies found ochratoxigenic fungi in pre-harvest and freshly harvested wheat suggesting that grain may be contaminated prior to storage (Čonková et al., 2006; Elmholt and Rasmussen, 2005; Joubrane et al., 2011; Riba et al., 2008). Although Spain has a certain climatic diversity, it is considered as a temperate-warm country. However, climate predictions for a medium-term future point to warmer conditions similar to those in Northern Africa. According to mycobiota studies in wheat pre-harvest in Northern Africa, *Aspergillus* species might fit better than *Penicillium* species to such environmental conditions (Riba et al., 2008). In pre-harvest wheat in Argelia and Lebanon, the occurrence of *Circumdati* section was lower than *Nigri* section but the isolates in the former section were higher OTA producers (Joubrane et al., 2011; Riba et al., 2008).

In our work the behavior of *A. steynii* and *A. carbonarius* in two different simulated climatic conditions was tested. Cycling temperatures were chosen in order to simulate actual mean day and night temperatures in May, when antifungal pre-harvest application takes place in Northern Spain; additionally, an experimental condition was considered including a

temperature increase of 5-10 ºC and a decrease of 15% of the moisture content of wheat. *A. steynii* belongs to *Aspergillus* section *Circumdati*, characterized by the golden brown colored conidia, and has only been reported in coffee, grape, paprika and barley grain, probably due to its recent description (Gil-Serna et al., 2011; Leong et al., 2007; Sardiñas et al., 2011). Other OTA producing species in this section are *A. ochraceus, A. elegant* and *A. westerdijkiae.* However, Gil-Serna et al. (2011) considered *A. steynii* a major risk for OTA contamination due to its high OTA production. In fact in our study *A. steynii* isolates showed high OTA production under both conditions. Abdel-Hadi and Magan (2009) work is probably the only existing study on the ecophysiology of *A. steynii.* Our results suggest that in a predictable scenario characterized by temperature increase and humidity decrease, *A. steynii* could be considered an important OTA-producer; growth rate of *A. steynii* 3.264 was reduced while the OTA production was not affected by any of the antifungal treatments. These results also suggest that for an appropriate evaluation of antifungal agents it is necessary to take into account both effects on growth and mycotoxin production and that several strains per species should be considered.

In the present study, *A. carbonarius* isolates, despite their different origin, had similar behaviour. *A. carbonarius* is the main OTA producer in the *Nigri* section followed by species belonging to *A. niger* aggregate (Abarca et al., 2004). Most of the ecophysiology studies on *A. carbonarius* have been carried out on grapes, where optimal temperature of growth is between 25-35ºC and 0.95-0.99 a_W (Astoreca et al., 2010). However lower temperatures 15-20ºC and 15-30ºC were reported as optimal for growth on MEA by *A. carbonarius* and *A. niger*, respectively (Amézqueta et al., 2012). Temperature is an important factor affecting growth and toxin production by fungus. Effect of temperature cycling on rice of *F. graminearum* only showed significant differences during the second 2-week of incubation but not during the third 2-week of incubation period. However incubation at 15ºC followed by incubation at 25ºC for 2 weeks resulted in significantly higher production of deoxinivalenol and zearalenone (Ryu and Bullerman, 1999). Daylight temperatures of 35 ºC lead to a faster growth rate, while daylight temperatures of 25 ºC are associated with higher *A. carbonarius* OTA production in grapes (Belli et al., 2006). In the same line, the present study concludes that warmer future conditions may trigger *A. carbonarius* growth and infection, while OTA production may be reduced, compared to milder "present" conditions.

In our work DNA fungal concentration was considered, together with colony diameters, as other growth measurement showing a positive correlation between both measurements. However OTA production after 21 days only showed correlation with growth when no antifungal were not added. This correlation was also observed when *A. carbonarius* grew in synthetic medium agar, however disappeared when it grew paired with others species or alone in modified atmosphere (Valero et al., 2006a; 2006b; 2008). Therefore the effect in fungal growth and mycotoxin production under stress conditions may be different.

The qPCR assay is a sensitive and quantitative detection assay which could be useful in epidemiological studies and to assess mycotoxin contamination in wheat seeds. Nevertheless no correlation was found between OTA production and this parameter. Positive significant correlations can be found between *A. carbonarius* or *A. ochraceus* DNA content and OTA concentration, when they are quantified in natural, uninoculated samples of grapes (Atoui et al., 2007; Mulé et al., 2006) or green coffee (Schmidt et al., 2004). In the case of barley high correlation between *F. graminearum* DNA level and DON content in barley in North America was found, whereas the correlation was not evident in barley from Finland with naturally low DON and *F. graminearum* DNA levels (Sarlin et al., 2006).

Other authors also have set correlations between growth rates and lag phases estimated either through colony size measurement or ergosterol content (Marin et al., 2006, 2009). In this way different growth measurements should be correlated. Growth is regulated by primary metabolism whereas OTA is a fungal product from secondary metabolism thus, faster fungal growth may not necessarily result in greater OTA synthesis.

The potential impact of climate change in the effectiveness of non specific *Aspergillus* antifungals was also assessed. Chemical antifungals were used at the doses recommended by the manufacturers for the control of the target moulds and the main active ingredients used in this study were azoles and thiophanate methyl. Thiophanate acts on tubulin metafase mitosis causing cell death. Azoles are sterol demethylase inhibitors that interfere with the biosynthetic pathway of ergosterol, an essential component of the fungal cell membrane. There is scarce information on the effectiveness of azoles in *Aspergillus* spp. growth and OTA production. Concerning growth control, the azoles (Folicur®, F1; Prosaro ®, F2; Lovit®, F3 and Domark Evo ®, F4) were more effective than thiophanate methyl (F5) that generally did not affect fungal growth or even stimulated it. Respect to the azoles, epoxiconazole

(Lovit®, F3) and tetraconazole (Domark Evo ®, F4) were effective against both ochratoxigenic species, although their effectiveness may be compromised under extreme conditions. By contrast, tebuconazole (Folicur®, F1 and Prosaro ®, F2) increased their effectiveness under warmer and drier conditions. Thus a transition in the active ingredients may be required in the future as a result of changing climatic conditions. All antifungals reduced OTA production for *A. carbonarius* under "present" conditions, tebuconazol being the most effective. However under "extreme" conditions, none of the antifungals was effective in preventing OTA production by any of the strains tested. This means that in some cases, antifungals, while limiting fungal growth, induced a stress situation which triggered OTA production. Previous *in vitro* studies showed that azoles (fluquinconazole, tebuconazole, thiabendazole) reduced the growth and DON production in *F. graminearum* when applied at high concentration, but sub-lethal levels enhanced mycotoxin production (Matthies et al., 1999).

Finally, in this study the effectiveness of a plant extract was also tested as an alternative to application of chemicals. Plant extracts contain various antioxidant compounds such as polyphenols, phenols, flavonoids, etc. which could be the bioactive basis of their antimicrobial properties (Ebana and Madunagu, 1993). Quercetine and kaempferol were the main phenolic compounds in our *E.* extract. Similarly, several studies have confirmed the presence of phenolic acids, flavonoids and terpenes in *E arvense* extracts (Milovanović et al., 2007; Mimica-Dukic et al. 2008; Radulovic et al.; 2006, Sandhu et al.; 2010). In general *E. arvense* (F6) showed similar or enhanced effect than chemical antifungals, although higher doses of it were applied. The highest effectiveness was against *A. carbonarius* growth and OTA production under "present" conditions. By contrast, while growth of *A. steynii* was controlled, OTA production was not so. A slight reduction of fungal growth and stimulation of OTA production by *A. ochraceus* and *A. carbonarius* was observed on grapes treated with a hydroalcoholic *E. arvense* extract at 2% (García-Cela et al., 2012). *E. arvense* extract showed dose dependent results against some *Aspergillus* species on maize agar medium; high levels reduced the growth but low levels could stimulate it in some conditions (Garcia et al*.,* 2011). Besides of the doses, environmental conditions like temperature and a_W have been proven determinant in the effectiveness of antifungals. *E. arvense* (3%) was effective at 0.95 a_W against *A. flavus* and *F. graminearum* in maize; but not clear results were observed at lower a_W (Garcia et al., 2012).

Growth of mycotoxigenic fungi and mycotoxins production are largely dependent on climatic factors. *Fusarium, Aspergillus* and *Penicilium* species differ in their climatic distribution as a result of their different optimum conditions for growth, persistence and mycotoxin production. The *Aspergillus* species tested here seem to be able to persist in the future coming conditions, in particular, *A. steynii*, a high OTA producer. Azoles (F1, F2 and F3) were effective in controlling growth of *A. carbonarius* and *A. steynii*, and this effectiveness may not be compromised by the increase in temperature and decrease of humidity. However, they are not useful for the prevention of OTA accumulation, which could be only reduced in *A. carbonarius* under non-extreme conditions. Although some adjustment will probably be required, further studies should be conducted in the field, since the antifungals used in this study are applied at flowering and not directly on the grain. Moreover, antifungal application timing may need to be optimized. Finally, *Equisetum* extract showed promising results as antifungal, however further work to adjust the applied concentrations is required.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (AGL2010- 22182-C04-01), the EU (Mycored KBBE-2007-2-5-05 project), the Comissionat per a Universitats i Recerca, del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (AGAUR) and the European Social Fund for financial support. The authors also are grateful for the support by a research grant for young scientist awarded by the Institute DANONE. We also thank Bayer CropScience, S.L, Sipcam Inagra S.A and Basf Española, S.L. for supplying of the antifungals. Authors are also grateful to Dr. Rivas-Gonzalo (Grupo de Investigación en Polifenoles, Facultad de Farmacia, Universidad de Salamanca, Spain) for the analysis of *E. arvense* extract.

REFERENCES

Abarca, M.L., Accensi, F., Cano, J., Cabañes, F.J., 2004. Taxonomy and significance of black aspergilli. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 86, 33-49.

Abdel-Hadi, A., Magan, N., 2009. Influence of physiological factors on growth, sporulation and ochratoxin A/B production of the new *Aspergillus ochraceus* grouping. World Mycotoxin Journal 2, 429-434.

Astoreca, A., Barberis, C., Magnoli, C., Dalcero, A., 2010. *Aspergillus carbonarius* growth and ochratoxin A production on irradiated dried grapes under different water activity and temperature conditions. World Mycotoxin Journal 3, 175-182.

Astoreca, A., Barberis, C., Magnoli, C., Dalcero, A.,2010. *Aspergillus carbonarius* growth and ochratoxin A production on irradiated dried grapes under different water activity and temperature conditions. World Mycotoxin Journal 3, 175-182.

Atoui, A., Mathieu, F., Lebrihi, A.,2007. Targeting a polyketide synthase gene for Aspergillus carbonarius quantification and ochratoxin A assessment in grapes using real-time PCR. International journal of food microbiology 115, 313-318.

Battilani, P., Barbano, C., Marin, 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., Ramos A.J., Coronas I., Sanchís V., Marín S., 2005 *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. Journal Applied Microbiology. 98, 839–844.

Bellí, N., Marín, S., Coronas, I., Sanchis, V., Ramos, A.J.,2007. Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes. Food Control 18, 1343-1349.

Bellí, N., Mitchell, D., Marín, S., Alegre, I., Ramos, A.J., Magan, N., Sanchis, V., 2005. Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. European Journal of Plant Pathology 113, 233-239.

Bellí, N., Pardo, E., Marín, S., Farré, G., Ramos, A.J., Sanchis, V., 2004. 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., Marín, S., 2006. Effect of photoperiod and day-night temperatures simulating field conditions on growth and ochratoxin A production of *Aspergillus carbonarius* strains isolated from grapes. Food Microbiology 23, 622-627.

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 Jood Food Microbiology 71, 139-144.

Cabañas, R., Bragulat, M.R., Abarca, M.L., Castellá, G., Cabañes, F.J., 2008. Occurrence of *Penicillium verrucosum* in retail wheat flours from the Spanish market. Food Microbiology 25, 642-647.

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.

CAC (Codex Alimentarius Commission), 2003. Code of Practice for the Prevention and Reduction of Mycotoxin Contamination in Cereals, including Annexes on Ochratoxin A, Zearalenone, Fumonisins and Tricothecenes CAC/RCP 51.

Comission of the European Communities, 2006a. Commission Regulation (EC) No. 576/2006 of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Official Journal of the European L229, 7-9.

Comission of the European Communities, 2006b. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union L364, 5-24.

Comission of the European Communities, 2007. Green Paper from the Commission to the Council, the European Parliament, the European Economic and Social Committee of the Regions, Adapting to Climate Change in Europe-Options for EU Action. SEC 849, COM 354 Final. European Commission, Brussels.

Comission of the European Communities, 2011. Commission Regulation (EC) No. 559/2011 of 7 June 2011 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for captan, carbendazim, cyromazine, ethephon, fenamiphos, thiophanate-methyl, triasulfuron and triticonazole in or on certain products. Official Journal of the European Union L152: 1-21.

Čonková, E., A. Laciaková, I. Štyriak, L. Czerwiecki, and G. Wilczyńska (2006). Fungal contamination and the levels of mycotoxins (DON and OTA) in cereal samples from Poland and East Slovakia. Czech Journal of Food Sciences, 24(1), 33-40.

Duarte, S.C., Pena, A., Lino, C.M., 2010. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. Food Microbiology 27, 187-198.

Ebana, R.U.B., V.E. Madunagu, 1993. Antimicrobial effect of Strophanthus hispidus and Secamone afzelii on some pathogenic bacteria and their drug resistant strains Nigerian Journal of Botany, 6, pp. 27-31.

Edwards, S.G., Godley, N.P., 2010. Reduction of *Fusarium* head blight and deoxynivalenol in wheat with early fungicide applications of prothioconazole. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 27, 629-635.

Elmholt, S., Rasmussen, P.H., 2005. *Penicillium verrucosum* occurrence and ochratoxinA contents in organically cultivated grain with special reference to ancient wheat types and drying practice. Mycopathologia 159, 421e432

European Union Pesticides database [Internet]. 2010. Brussels (Belgium): Directorate General for Health and Consumers; [cited 2010 Sept 5]. Available from: http:// ec.europa.eu/sanco_pesticides/public/index.cfm/

Fernane, F., Sanchis, V., Marín, S., Ramos, A.J., 2010. First ron mould and mycotoxin contamination of pistachios sampled in Algeria. Mycopathologia 170, 423-429.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2008. Safety Evaluationof Certain Mycotoxins in Food. WHO Food Additives Series 59. RetrievedNovember 18, 2009 from http://whqlibdoc.who.int/publications/2008/9789241660594_eng.pdf.

Garcia, D., Garcia-Cela, E., Ramos, A.J., Sanchis, V., Marín, S., 2011. Mould growth and mycotoxin production as affected by *Equisetum arvense* and *Stevia rebaudiana* extracts. Food Control 22, 1378-1384.

Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2012. Effect of *Equisetum arvense* and *Stevia rebaudiana* extracts on growth and mycotoxin production by *Aspergillus flavus* and *Fusarium verticillioides* in maize seeds as affected by water activity. International Journal of Food Microbiology 153, 21–27.

García-Cela, E., Ramos, A.J., Sanchis, V., Marin, S., 2012. Ochratoxigenic moulds and effectiveness of grape field antifungals in a climatic change scenario. Journal of the Science of Food and Agriculture 92, 1455-1461.

Gil-Serna, J., González-Salgado, A., González-Jaén, M.T., Vázquez, C., Patiño, B., 2009. ITS-based detection and quantification of *Aspergillus ochraceus* and *Aspergillus westerdijkiae* in grapes and green coffee beans by real-time quantitative PCR. International Journal of Food Microbiology 131, 162-167.

Gil-Serna, J., Vázquez, C., Sardiñas, N., González-Jaén, M.T., Patiño, B., 2011. Revision of ochratoxin A production capacity by the main species of *Aspergillus* section *Circumdati*. *Aspergillus steynii* revealed as the main risk of OTA contamination. Food Control 22, 343-345.

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., Battilani, P., 2007. Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. International Journal of Food Microbiology 113, 330-338.

Gooding, M.J., Ellis, R.H., Shewry, P.R., Schofield, J.D., 2003. Effects of restricted water availability and increased temperature on the grain filling, drying and quality of winter wheat. Journal of Cereal Science 37, 295-309.

Haidukowski, M., Pascale, M., Perrone, G., Pancaldi, D., Campagna, C., Visconti, A., 2005. Effect of fungicides on the development of *Fusarium* head blight, yield and deoxynivalenol accumulation in wheat inoculated under field conditions with *Fusarium graminearum* and *Fusarium culmorum*. Journal of the Science of Food and Agriculture 85, 191-198.

Hajjaji, A., El Otmani, M., Bouya, D., Bouseta, A., Mathieu, F., Collin, S., Lebrihi, A., 2006. Occurrence of mycotoxins (ochratoxin A, deoxynivalenol) and toxigenic fungi in Moroccan wheat grains: Impact of ecological factors on the growth and ochratoxin A production. Molecular Nutrition and Food Research 50, 494-499.

IARC (International Agency for Research on Cancer), Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Humans: Some Traditionally Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. IARC Press, Lyon (2002).

Ioos, R., Belhadj, A., Menez, M., Faure, A., 2005. The effects of fungicides on *Fusarium* spp. and *Microdochium nivale* and their associated trichothecene mycotoxins in French naturally-infected cereal grains. Crop Protection 24, 894-902.

Joubrane, K., El Khoury, A., Lteif, R., Rizk, T., Kallassy, M., Hilan, C., Maroun, R., 2011. Occurrence of aflatoxin B_1 and ochratoxin A in Lebanese cultivated wheat. Mycotoxin Research 27, 249-257.

Kubista, M., Andrade, J.M., Bengtsoon, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Stahlberg, A., Zoric, N., 2006. The real-time polymerase chain reaction. Molecular Aspects of Medicine 27, 95-125.

Leblanc, J.., Tard, A., Volatier, J.., Verger, P., 2005. Estimated dietary exposure to principal food mycotoxins from The First French Total Diet Study. Food Additives and Contaminants 22, 652-672.

Leong, S.L., Hien, L.T., An, T.V., Trang, N.T., Hocking, A.D., Scott, E.S., 2007. Ochratoxin A-producing *Aspergilli* in Vietnamese green coffee beans. Letters in Applied Microbiology 45, 301-306.

López-Malo, A., Alzamora, S.M., Guerrero, S., 2000. Natural antimicrobials from plants. (Eds.), Minimally Processed Fruit and Vegetables. Fundamental Aspect and Application, AP, Aspen Publishers, Gaithersburg, pp. 237-264.

Magan, N., Medina, A., Aldred, D., 2011. Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. Plant Pathology 60, 150-163.

Magnoli, C., Astoreca, A., Ponsone, M.L., Fernández-Juri, M.G., Barberis, C., Dalcero, A.M., 2007a. Ochratoxin A and *Aspergillus* section *Nigri* in peanut seeds at different months of storage in Córdoba, Argentina. International Journal of Food Microbiology 119, 213-218.

Magnoli, C.E., Astoreca, A.L., Chiacchiera, S.M., Dalcero, A.M., 2007b. Occurrence of ochratoxin A and ochratoxigenic mycoflora in corn and corn based foods and feeds in some South American countries. Mycopathologia 163, 249-260.

Mateo, E.M., Gil-Serna, J., Patiño, B., Jiménez, M., 2011. Aflatoxins and ochratoxin A in stored barley grain in Spain and impact of PCR-based strategies to assess the occurrence of aflatoxigenic and ochratoxigenic *Aspergillus* spp. International Journal of Food Microbiology 149, 118-126.

Matthies, A., Buchenauer, H., 2000. Effect of tebuconazole (Folicur®) and prochloraz (Sportak®) treatments on Fusarium head scab development, yield and deoxynivalenol (DON) content in grains of wheat following artificial inoculation with *Fusarium culmorum*. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 107, 33-52.

Matthies, A., Walker, F., Buchenauer, H., 1999. Interference of selected fungicides, plant growth retardants as well as piperonyl butoxide and 1-aminobenzotriazole in trichothecene production of *Fusarium graminearum* (strain 4528) in vitro. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 106, 198-212.

Milovanović, V., Radulović, N., Todorović, Z., Stanković, M., Stojanović, G., 2007. Antioxidant, antimicrobial and genotoxicity screening of hydro-alcoholic extracts of five Serbian *Equisetum* species. Plant Foods for Human Nutrition 62, 113-119.

Mimica-Dukic, N., Simin, N., Cvejic, J., Jovin, E., Orcic, D., Bozin, B., 2008. Phenolic compounds in field horsetail (Equisetum arvense L.) as natural antioxidants. Molecules 13, 1455-1464.

Miraglia, M., Brera, C., 2002. Assessment of Dietary Intake of Ochratoxin A by the Population of EU Member States. Reports on Tasks for Scientific Cooperation. Reports of experts participating in Task 3.2.7. Directorate-General Health and Consumer Protection, Rome, Italy.

Molina, M., Giannuzzi, L., 1999. Combined effect of temperature and propionic acid concentration on the growth of *Aspergillus parasiticus*. Food Research International 32, 677-682.

Molinié, A., Faucet, V., Castegnaro, M., Pfohl-Leszkowicz, A., 2005. Analysis of some breakfast cereals on the French market for their contents of ochratoxin A, citrinin and fumonisin B_1: Development of a method for simultaneous extraction of ochratoxin A and citrinin. Food Chemistry 92, 391-400.

Mounjouenpou, P., Gueule, D., Fontana-Tachon, A., Guyot, B., Tondje, P.R., Guiraud, J., 2008. Filamentous fungi producing ochratoxin A during cocoa processing in Cameroon. International Journal of Food Microbiology 121, 234-241.

Mulè, G., Susca, A., Logrieco, A., Stea, G., Visconti, A.,2006. Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. International Journal of Food Microbiology 111, S28-S34.

Munimbazi, C., Saxena, J., Tsai, W.-J., Bullerman, L.B., 1997. Inhibition of production of cyclopiazonic acid and ochratoxin A by the fungicide iprodione. Journal of Food Protection 60, 849-852.

Muñoz, K., Vega, M., Rios, G., Geisen, R., Degen, G.H., 2011. Mycotoxin production by different ochratoxigenic *Aspergillus* and *Penicillium* species on coffee- and wheat-based media. Mycotoxin Research 27, 239-247.

Radulović, N., Stojanović, G., Palić, R., 2006. Composition and antimicrobial activity of Equisetum arvense L. essential oil. Phytotherapy Research 20, 85-88.

Ramirez, M.L., Chulze, S., Magan, N., 2004. Impact of environmental factors and fungicides on growth and deoxinivalenol production by *Fusarium graminearum* isolates from Argentinian wheat. Crop Protection 23, 117-125.

Rasooli, I., Owlia, P., 2005. Chemoprevention by *thyme* oils of *Aspergillus parasiticus* growth and aflatoxin production. Phytochemistry 66, 2851-2856.

Rasooli, I., Rezaei, M.B., Allameh, A., 2006. Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from Thymus eriocalyx and *Thymus* x-porlock. Food Control 17, 359-364.

Reyneri, A., 2006. The role of climatic condition on micotoxin production in cereal. Veterinary Research Communications 30, 87-92.

Riba, A., Mokrane, S., Mathieu, F., Lebrihi, A., Sabaou, N., 2008. Mycoflora and ochratoxin A producing strains of *Aspergillus* in Algerian wheat. International Journal of Food Microbiology 122, 85-92.

Sandhu, N.S., Kaur, S., Chopra, D., 2010. *Equisetum arvense*: Pharmacology and phytochemistry - a review. Asian Journal of Pharmaceutical and Clinical Research 3, 146-150.

Sardiñas, N., Gil-Serna, J., Santos, L., Ramos, A.J., González-Jaén, M.T., Patiño, B., Vázquez, C., 2011. Detection of potentially mycotoxigenic *Aspergillus* species in Capsicum powder by a highly sensitive PCR-based detection method. Food Control 22, 1363-1366.

Sarlin, T., Yli-Mattila, T., Jestoi, M., Rizzo, A., Paavanen-Huhtala, S., Haikara, A.,2006. Real-time PCR for quantification of toxigenic *Fusarium* species in barley and malt. European Journal of Plant Pathology 114, 371-380.

Schmidt, H., Taniwaki, M.H., Vogel, R.F., Niessen, L.,2004. Utilization of AFLP markers for PCR-based identification of *Aspergillus carbonarius* and indication of its presence in green coffee samples. Journal of applied microbiology 97, 899-909.

Taniwaki, M.H., Pitt, J.I., Teixeira, A.A., Iamanaka, B.T., 2003. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. International Journal of Food Microbiology 82, 173-179.

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.

Zaied, C., Abid, S., Zorgui, L., Bouaziz, C., Chouchane, S., Jomaa, M., Bacha, H., 2009. Natural occurrence of ochratoxin A in Tunisian cereals. Food Control 20, 218-222.

Zinedine, A., Brera, C., Faid, M., Benlemlih, M., Miraglia, M., 2007. Ochratoxin A and *Fusarium* toxins in cereals from Morocco. Cahiers Agricultures 16, 11-15.

V. GENERAL DISCUSSION

PAGINA BLANCO

GENERAL DISCUSSION

Traditionally, fungi have been used as food (edible mushroom), in fermented foods (cheese, beer), to cure diseases (*penicillin*) and even in religious ceremonies. However, at the beginning of the 1960s with the discovery of AFs the attitude towards the presence of filamentous moulds started to change (Goldblatt, 1969). Since then, the knowledge of major mycotoxin producing genera, the most susceptible foods, the most suitable production conditions, as well as toxicological implications, has expanded considerably in order to protect human health. Nevertheless, although enormous progress has been achieved in mycotoxin risk management, there are still major challenges in this regard, some of which have been considered in this Thesis. Firstly, an already existing challenge in mycotoxin management, which is the need to properly quantify and apply sampling uncertainty. Secondly, the potential impact of some simulated conditions linked to hypothetical climate change in the mycotoxin issue.

For a better understanding of the General Discussion Section, this part of the Thesis has been divided into two subsections that address the two areas of interest as it was done in the Research Work section.

1. SELECTION OF RAW MATERIALS

In the first part of this Thesis, emerging microbiological risk management metrics were applied to AFs in pistachio nuts. For selection of incoming raw materials, the maximum UE regulatory limit for nuts to be subjected to further physical treatment must be used as food safety management tool. In this case, the toasting was, after sorting, the most important step in the processing and its effect on mycotoxin contamination was analized. However, compliance with such regulatory levels is totally dependent on the validity of sampling and analytical procedures.

1.1. Samplings plans: effectiveness, uncertainty and alternatives

The distribution of the mycotoxin concentration in products is an important factor to be considered when regulatory sampling criteria are established. Mycotoxins are heterogeneously distributed, and then, specific sampling and subsampling procedures should be designed for each kind of mycotoxin and food. Probably, the design of accurate sampling

plans that provide reliable results is one of the most important challenges in mycotoxin management. For this reason, International Agencies work in the harmonization process of sampling plans in order to protect consumer health and to facilitate the international trading. However, these plans have been criticized for the unrealistic need of workforce. In particular, the EC allows the use of alternative sampling methods in cases of unacceptable commercial consequences or practical unfeasibility of the official method but only in case of quality control (EC, 2006a).

In pistachio, as in other nuts, the mycotoxins contamination rate is low, where only 1 nut per 10^4-10^6 nuts is common (Schatzki, 1995), although great mycotoxin contamination could be detected in a single contaminated pistachio. In this Thesis the EU official sampling plan (EC, 2010b) was applied in quadruplicate in different pistachio lots in order to determine the sampling uncertainty. Interestingly, low variability was associated with the initial sampling step, probably because the size and number of elemental samples was enough; in fact, the last European Regulation about methods of sampling and analysis for the official control of mycotoxins levels in foodstuffs reduced the size of the aggregate sample from 30 to 20 kg, but maintained the analysis number (EC, 2010b).

Nevertheless, the underlying problem is the uncertainty associated to the AFs levels reported in the present work and any other existing works; the high uncertainties due to sampling and sample preparation procedures may lead to unrealistic results, and this is an issue that needs to be solved. A recent work, have drew the OC curves for various sampling plans involving one, two or three 10 kg samples (**Figure 1**) (Wesolek et al., 2014). The EU sampling plan consisting of testing two samples of 10 kg gave a consumer risk with a probability of acceptance at 5% for a lot mean concentration of 75.34 μg/kg and a producer risk with a probability of acceptance at 95% for a lot mean concentration of 1.62 μg/kg (Wesolek et al., 2014).

On the other hand, a high percentage of variability was attributed to the subfractioning step (subsample preparation), suggesting that either a better grinding and mixing could be achieved or more/bigger subfractions should be selected to reduce the uncertainty. Similar values of sampling and analytical variance were observed sampling almonds and hazelnuts for AFB_1 (Ozay et al., 2006; Whitaker et al., 2006). However, in those studies lower values of

sample preparation variance were calculated, probably due to the higher size of samples for analysis used (50 to 100 g) and the lower milling particle size.

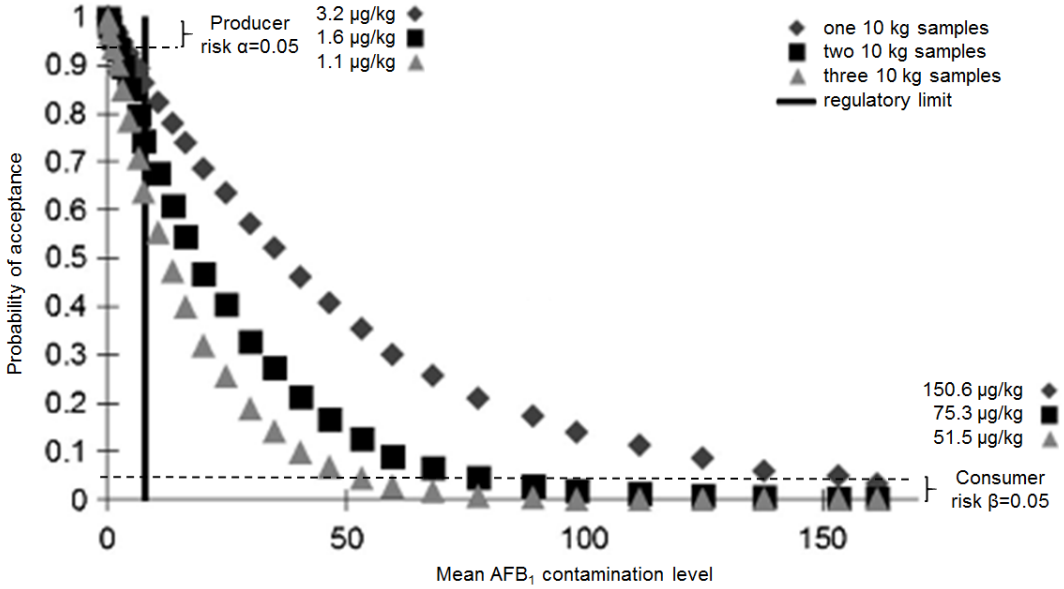


Figure 1 OC curves for various sampling plans involving one, two or three 10 kg samples.

Nowadays, only measurement uncertainty is to be used for correction of analytical results according to EC 401/2006 (EC, 2006a). The application of measurement uncertainty increased the probability of rejection (EC, 2006b) of the most contaminated lots. In our study the probability of rejecting 2 of the 3 most contaminated lots increased to 25% when analytical results were corrected for measurement uncertainty and compared to the legal limits. If given the existing sampling plan, the calculated sampling uncertainty was applied for correction of analytical results, this would lead to rejection of a high percentage of lots, unless sampling plans and procedures are revised in depth.

Food companies often perform alternative mycotoxin sampling plans which reduce the number of samples and/or number of analysis, reducing the cost and time devoted to the analysis, as a part of their quality control schemes. In order to assess the effectiveness of alternative plans, a simplified sampling plan was also applied to the same lots. However, it resulted obviously less suitable than the official one, due to the reduced probability of taking a contaminated portion in the sampling.

1.2 Analytical procedures

Most mycotoxins are toxic at very low concentrations and require sensitive and reliable methods for their detection. There are many methods used, of which many are lab-based but there is no single technique that stands out above the others (Turner et al., 2009). In addition, it is impossible to use one single method for analysis of all mycotoxins due to the variety of chemical structures (Yazdanpanah, 2011). Therefore, development of multi-mycotoxin methods in a single matrix are needed since mycotoxin co-ocurrence could produce unknow toxicological effects. Food is not necessarily safe, just because of the ruling out of the presence of well-known mycotoxins, as they might still be there in conjugated form, including masked or bound mycotoxins (Yazdanpanah, 2011). To sum up, the demand for fast, simultaneous and accurate determination of multiple mycotoxins as well as other hazards, along with the heterogeneity of food matrices, creates extreme challenges for routine analysis.

ELISA (enzyme-linked immunosorbent assay) is a common usually single-toxin analytical method used by food and feed companies. Therefore, it was considered interesting to analyze in parallel to HPLC the pistachio samples from the alternative simplified plan proposed in the study I. OTA was never detected by any of the methods while higher number of positive samples and higher concentration of AFs were detected by ELISA, confirming that this analytical procedure may lead to false positive results. Griessler et al. (2010) quantified mycotoxins in several food and feed commodities from Southern Europe using HPLC and ELISA. Similar ranges of mycotoxin contamination were found, but no clear conclusion could be drawn from their data. Chun et al. (2007) analyzed AFs in nuts and derivative products consumed in South Korea by ELISA and HPLC; from their results it was concluded that ELISA was not suitable for quantification since the results were affected by the sample matrix and contamination was possibly overestimated at very low concentration.

1.3. Toasting as process criteria (PcC)

Finally, even if the huge uncertainty linked to sampling is not solved, there is a need for food operators to know the potential of their processing steps (PcC) to either increase or reduce the levels of mycotoxins initially present in their raw materials, once accepted for processing according to raw materials control plans. In any case, the final product should not reach the maximum level which guarantees a safe food. In our case, the FSO was considered equivalent to the regulatory maximum EC limits (EC, 2010a) and to the PO after processing, assuming that further AFs production does not occur after toasting and packaging. It must be noted

that our results came from means obtained through application of the EC sampling plan by quadruplicate independently, and therefore they are expected to be reliable.

In a proactive approach to mycotoxin control each industry should determine the effect derived from its technological process and therefore accept only those lots of raw materials in which the initial level of contamination can be reduced to safe levels through processing. The effect of industrial toasting (pre-toasting ≈135 ºC + toasting ≈165 ºC, during total time of 20 min) on mycotoxin contamination in pistachio was assessed. Raw lots revealed AFs presence and OTA absence in all lots sampled. AFB_1 was detected in a range of 0.32- 392.5 μg/kg and was the major aflatoxin (70-100%).

Temperatures achieved during toasting eliminate the fungi and reduce the m.c. generating a biologically safe product. Nevertheless, mycotoxins tend to be stable compounds that are difficult to remove once formed; in particular they survive to many of the processing stages involved in food manufacture. Particularly, the AFs decomposition have been described in a range from 237 to 306 ºC (Betina, 1989; Rustom, 1997). Although these T are higher from those actually used by the nuts industry, it is usually accepted that the heat treatment decreases the concentration of AFs to some extent. Conflicting results have been published about the effect of the heat treatments on peanuts and pistachios (Ariño et al., 2009; Farah et al., 1983; Lee et al., 1969; Ozkarsli, 2003; Pluyer et al., 1987; Rustom, 1997; Yazdanpanah et al., 2005). In general, the extent of the reduction achieved was very dependent on the initial level of contamination, heating T, time and humidity. In pistachio, as in other nuts, the results regarding degradation of AFs due to toasting are also contradictory. Great reduction of initial AFs contamination was achieved after industrial toasting in analyzed lots reaching reductions of 87.62%, 81.04% and 87.72% for AFB_1, AFB_2 and AFs, respectively. Yazdanpanah et al. (2005) tested different T and time of toasting in pistachio, and a degradation of over 95% of AFB_1 was achieved with 150 ºC/90 min but the pistachios showed a burnt appearance, while 150 ºC/30 min showed significant reduction of AFB_1 and AFB_2 without any noticeable change in taste of sample. Interestingly, no linear correlation was found when the rate of reduction was plotted against the initial amount. Conversely, no significant reduction of the initial AFs contamination was observed at 120 ºC/20 min, although the contamination level of the starting material was low (0.12-0.18 μg/kg) (Ariño et al, 2009).

The food industry is responsible for setting up food safety management systems that deliver foodstuffs in compliance to the FSO. According to the initial and final values proposed by

European legal limits, processing (either selection or selection plus toasting or just toasting) is expected to decrease in 33% the initial AF concentration in the raw pistachio. Our results suggest that about a 75% of reduction may be achieved by the single toasting process, thus under the hypothesis of raw pistachio compliance with the maximum level, the toasted pistachio should be safe.

2. CLIMATE CHANGE:

Filamentous fungi and their mycotoxins are often found as contaminants in agricultural products, before or after harvest as well as during transportation and storage. Different strategies to prevent mould and mycotoxin contamination have been developed and generally are divided into pre-harvest and post-harvest strategies. The most common approach in pre-harvest is soil preparation, crop rotation, use of resistant varieties, herbicides, insecticides and biological and chemical antifungals. However, the efficacy of these techniques is strongly influenced by environmental conditions in field. Nonetheless, weather conditions in field are very variable due to both natural changes and those caused by the anthropomorphic greenhouse gas emissions which contribute to current warming trends (EC, 2007).

Understanding the potential impacts on agriculture of a warming climate has thus become increasingly important and it is of primary concern particularly to ensure the sustainability of agricultural systems as well as for policy-making purposes (Howden et al., 2007). In this new agricultural context, mycotoxin risk assessment should include a wider concept of risk evaluation, including emerging risks since new mycotoxins could arise for new fungus and plant associations making the occurrence of new mycotoxins, or mycotoxins not yet considered, a new potential human and animal health threat (Tirado et al., 2010). Probably the main questions are: how climate conditions could affect fungal infection and mycotoxin occurrence?, and consequently, are we prepared to manage the mycotoxin risk in this framework?.

The first question is difficult to answer because there is not always correlation between mycotoxin contamination and fungal infection. In addtion, the optimum environmental conditions for growth may not match those for optimum toxin production. For example, ecological data on *A. carbonarius* have suggested that T levels which favour growth, and cause

high fungal incidence, are different from those which are optimum for OTA production (Bellí et al., 2005b; Mitchell et al., 2004).

Regarding the second question, several authors highlighted the effect on the effectiveness of current GAP under the new climate scenarios (Magan et al., 2011; Miraglia et al., 2009). Focussing on antifungal compounds, loss of their effectiveness under different environmental conditions (T and a_W) has been observed (Medina et al., 2007a, 2007b). Moreover, sublethal dosis could reduce fungal growth but at the same time increase mycotoxin production (Garcia et al., 2011).

2.1. Increasing temperature and drought

Regionalized climatic change scenarios developed by the National Meteorological Agency for the following three periods of time 2011-2040; 2041-2070 and 2071-2100, pointed out that the maximum daily temperature will increase between 1 and 2 ºC, between 3 and 5 ºC and between 5 and 8 ºC, respectively. Also the minimum daily temperature could be reduced by 2 ºC, resulting in greater daily temperature fluctuations (AEMET, 2009).

The first stage of this work was focused in the effect of climate in fungal species distribution and strains adaptation in vineyards. Following this objective the distribution of the major fungal genera and the *Aspergillus* species with natural presence in the vineyards of two diferent agroclimatic regions from Spain (South and Northeast zones) spaced by more than 700 km were studied. In Spain, the Southern region is hotter than the Northeast, and it is common to exceed 40 °C in summer. In this season, the mean temperature difference between regions fluctuates from 1 to 4 °C. In addition, R.H. is lower in the South and the rainfall is scarce (**Table 1).** Therefore the actual conditions of the warmer zone (South) could resemble the future conditions of the Northeast zone.

It has been concluded that *Aspergillus* section *Nigri* is the major responsible for mycotoxin risk in this crop (Bellí et al., 2005a; Fredj et al., 2007; Medina et al., 2005; Sage et al., 2002; Serra et al., 2006b, 2005). In our study, the percentage of infected grapes by *Aspergillus* section *Nigri* in the South was similar in both years (63-67%) and it almost doubled those in the Northeast fields (19-38%). The remarkable difference in the percentage of infection between years in the Northeast region was attributed to a hailstorm one week before harvest in which

35.3 mm of rain in only 2 h was recorded. Rainfall occuring on the days before harvest triggers skin splitting and black aspergilli infection (Leong et al., 2006).

Table 1 Meteorological data from June to August obtained from weather stations near the sampling areas.

		T min (ºC)	T mean (ºC)	T max (ºC)	HR (%)	Rain (mm)	Infected berries[a]
N	2011	16.3 ± 2.3	22.5 ± 2.6	29.3 ± 3.1	60.3 ± 4.6	32.1	19%
	2012	17.4 ± 1.7	23.8 ± 1.8	30.8 ± 2.2	57.6 ± 4.2	85	38%
S	2011	18.0 ± 1.7	26.0 ± 2.4	34.4 ± 3.2	52.8 ± 10.9	13.2	63%
	2012	17.2 ± 1.7	26.5 ± 1.6	36.0 ± 2.2	45.0 ± 8.2	0	65%

Values obtained from: Servei Meteorò␣gic de Cataluyna and Estaciones agroclimáticas de Andalucía. N: Northeast, S: South
a, % infected berries by black aspergilli.

Black aspergilli presence was significantly higher in the hotter region (South) in both years ($p<0.05$). A positive correlation between T and black aspergilli incidence on grapes has been confirmed in many studies (Bellí et al., 2005a, 2004a; Leong et al., 2004). Battilani et al. (2006) observed that black aspergilli presence was significantly related to latitude and longitude in maturing grapes, showing a positive West-East and North-South gradient. Interestingly, recent studies have also evidenced that the influence of specific geographic location and climate of the vineyards on the occurrence of ochratoxigenic moulds and OTA contamination of grape was significant (Lasram et al., 2012; Lucchetta et al., 2010; Serra et al., 2006b).

Figure 2 indicates the percentage of infected berries by *A. carbonarius*, *A. tubingensis* and *A. niger*. Results show that *A. tubingensis* seems to be the black aspergilli species better adapted to Spanish environmental conditions, as it was the dominant species in all vineyards sampled, although no general pattern could be defined between years and regions. Extreme conditions promoted the *A. niger* presence since the higher infection was linked to the hotter summer (South, 2012), when 108, 48 and 14 days reached 30, 37 and 40 ºC repectively from May to June; moreover 40 ºC were reached in June, July and August. A recent study has also emphasized that *A. tubingensis* is the main species belonging to *Aspergillus niger* aggregate followed by *A. awamori*, and *A. niger* in dried vine fruits (Susca et al., 2013). Species distribution resulting from several publications in 2006-2012 are: *A. tubingensis* (15.2-95.7%), *A. niger* (4.3-84.4%), and *A. carbonarius* (7.6-46.9%) (Bau et al., 2006; Chiotta et al., 2009; Lasram et al., 2012; Martínez-Culebras and Ramón, 2007; Perrone et al., 2006; Spadaro et al., 2012).

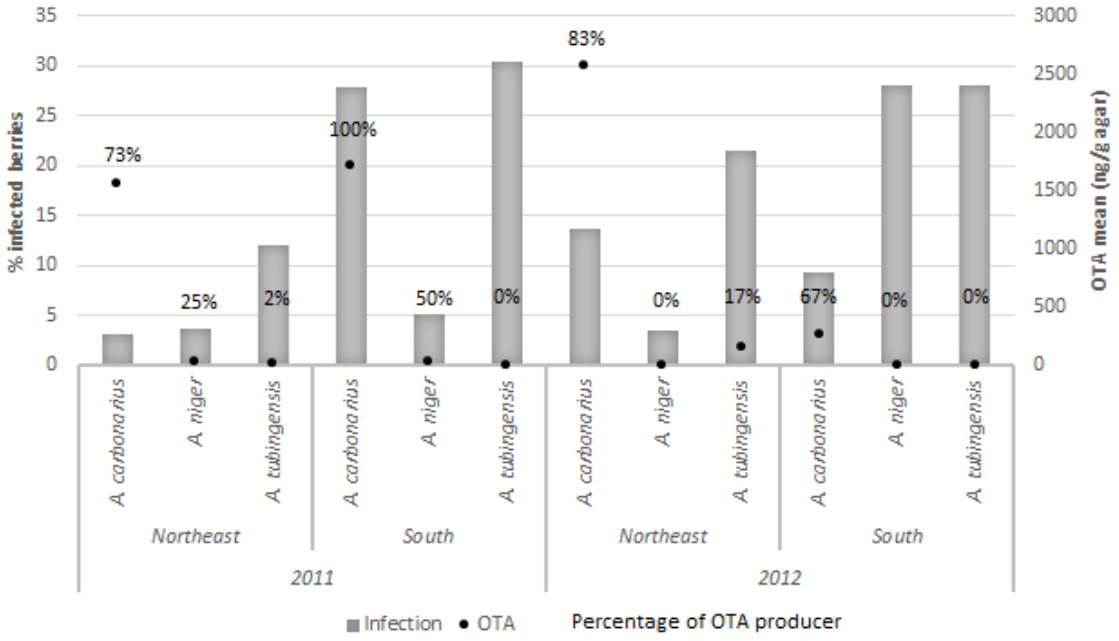


Figure 2 Percentage of berries infected by black aspergilli, percentage of producers and mean OTA (ng/g agar) produced by each species in CYA.

A higher percentage of OTA producing isolates was found in *A. carbonarius* (66.7-100%) than in *A. tubingensis* (0-17%) and *A. niger* (0-50%) in both regions (Figure 1). Moreover, *A. carbonarius* produced higher amount of OTA than the other species. Percentages of infection and toxin production agree with other studies (Bau et al., 2005; Sage et al., 2002), confirming that the source of OTA in these products is *A. carbonarius* (and to a much less extent, *A. tubingensis* and *A.niger*).

Despite the high infection observed, few musts contained OTA (<10.7%). This situation has been underlined in previous surveys in Spain in 2001 when only 15% of the sampled musts contained OTA (0.091 to 0.813 µg/ L), and OTA was not detected in musts of the vineyards sampled in Spain in 2002 and 2003 (Bellí et al., 2005a, 2004a). Positive musts (3 out of 4) were noticed in the South in 2011, in this region on that year T recorded were high but not extreme for *A. carbonarius* growth since only 3 days exceeded the 40 ºC from May to August. In this period, the higher *A. carbonarius* infection (27.8%) occurred together with the higher rate of ochratoxigenic isolates (100%), which explains the OTA contamination in the resulting musts. Battilani et al. (2003) did not observe significant correlation between the number of samples colonized by black aspergilli and the OTA content in berries, but the correlation was significant when only samples colonized by OTA producing fungi were considered.

Optimal environmental conditions for development of isolates of the same species are generally similar. However, it has been speculated that strains could show different ecophysiological profiles especially under marginal conditions of growth. In fact, *A. flavus* isolated from maize in north Italy showed slightly different ecological profiles in terms of both optimal and marginal conditions for growth compared to other regions of the world (Giorni et al., 2007). Our results showed differences only in *A. carbonarius* maximum and minimal T and a_W conditions for fungal growth in isolates from Northeast and South Spain (**Table 1**). Strains from the Northeast were better adapted to colder T while strains from the South could grow under drier conditions. Moreover, strains for South were also able to produce OTA at lower a_W. Probability of OTA production at 0.95 a_W was over 0.8 in the range of 15-30 ºC for isolates from both regions. Contrary, few differences were found due to the geographical origin of the isolates in *A. tubingensis* and *A. niger*, although both species grew in a wide range of a_W and T, and minimal a_W occurred at higher T (25-35 ºC). Nevertheless, *A. niger* grew at lower temperatures than *A. tubingensis*, and in a narrower a_W frame at 44 ºC.

On the other hand, recent reports revealed production of FB_2 and FB_4 by *A. niger* and *A. awamori* strains on culture medium, grapes or dried grapes (Abrunhosa et al., 2011; Chiotta et al., 2011; Logrieco et al., 2009; Mogensen et al., 2010a; Palumbo et al., 2011; Varga et al., 2010). *A. niger* isolates in this study only produced FB_2 when they were inoculated on CYA20S; the higher frequency of FB_2 producers was found in the South region but, isolates from both regions produced FB_2 in low amounts so little contamination in wine might be expected. In fact, the levels of FB_2 found in wine are of low concern (between 1 and 25 μg/L) (Mogensen et al., 2010b).

Climate change scenarios point to an increase of T and drought; while in non extreme climate conditions (like Northeast area in our study) this could lead to increasing black aspergilli populations, including *A. carbonarius*, under extreme conditions (like in the South in our study) this could promote the prevalence of particularly adapted species such as *A. niger*. As the presence of FBs in grapes has been only recently reported, ecophysiological profiles of FBs production by *A. niger* are unknown, and therefore it is not possible to relate them to environmental factors. However, mycotoxicological consequences derived this prediction in hotter areas could be a decreasing OTA risk and increasing FB_2 risk in grapes and derivates

On the other hand, the effect of increasing T and decreasing humidity was also evaluated *in situ* on grapes and on wheat. Laboratory trials were carried out trying to simulate the current environmental field conditions close to harvest date for grapes (August) and flowering date for wheat (May) in the Northeast Spain. Additionally, hypothetical extreme conditions (higher T and reduced R.H.) were also tested (Table 2).

Table 2 Environmental conditions tested

			T (ºC)	Photoperiod (h, light/darkness)
Grapes	Current	RH (%)80	30/20	16/8
	Extreme	RH (%)75	37/25	16/8
Wheat	Current	m.c. (%)40	25/10	14/10
	Extreme	m.c. (%)25	35/15	14/10

Current: environmental conditions similar to current
Extreme: possible predicted conditions

In grapes, infection and OTA production under current conditions was higher than under extreme conditions, where only *A. carbonarius* produced OTA while *A. ochraceus* was not able to grow. Decrease of R.H. also caused a minor reduction of grape infection and OTA production (Bellí et al., 2007b; Pardo et al., 2005), however in both cases the R.H. tested were superior than in our case and T and light cycles were not used. On the other hand, more OTA was found in undamaged grapes at 30 ºC than at 20 ºC (Bellí et al., 2007b), contrary to a prior work on synthetic medium, where 15-20 ºC was reported as the optimum T for *A. carbonarius* OTA production (Mitchell et al., 2004). Regarding the effect of photoperiod in OTA production, (Bellí et al., 2006b) observed that it could affect fungal growth, but they did not find a direct effect on OTA production. However, (Oueslati et al., 2010) observed that OTA production was affected by the alternating T and that the highest OTA amount was produced when incubated at 11 and 13 hours of light and darkness, respectively.

In wheat, *A. steynii* and *A. carbonarius* grew and produced OTA levels after 21 days of incubation. In general, and opposite to what happened with grapes, fungal growth was enhanced under extreme conditions, especially in section *Circumdanti*. These differences could be due to the commodity but also to the higher T tested in the grape study. Present conditions were more favorable for OTA production in wheat in *A. carbonarius*, and no significant differences were observed in *A. steynii*, although the observed means were higher under extreme conditions. Moreover, the *A. steynii* isolates tested were higher OTA producers than *A. carbonarius* ones under both conditions tested.

High T reduced OTA production by *A. carbonarius* in both commodities studied, and therefore and in agreement with observations in field, warming conditions could reduce OTA contamination, not only due to dominance by other black aspergilli better adapted to heat, but also because higher T are farther from the optimum conditions for OTA production by *A. carbonarius*. Moreover, *A. ochraceus* was totally inhibited under extreme conditions, and consequently no OTA contamination was found in must from inoculated grapes. Conversely, *A. steynii* was not affected under extreme conditions tested in wheat, nonetheless the T tested were lower in this commodity. It is worthy to mention that *A. steynii* produced high amount of OTA even under extreme conditions.

2.2. Increasing solar ultraviolet radiation

Changes in UV radiation in the future are estimated by model simulations that are based on the projected changes in ozone and clouds, which are the most important factors that are known to influence UV (UNEP, 2010). Although there is great uncertainty on future UV-B intensities, most of the studies consider that they are likely to remain significantly higher than pre-1980 values for the next few years at least (UNEP, 2010). The global mean irradiance values averaged for Europe in the period 1983-2005 showed much higher irradiances in the Mediterranean basis than the rest of the continent, being extremely higher under the parallel 40 N. In particular, Spain is not only the European country that receives the greater amount of radiation but also the country that shows the greatest contrast and radiative gradients and complexity in the distribution of the radiative energy (AEMET, 2012). The international agencies considered that variations in UV-B radiation can have large effects on plant interactions with pests, with important implications for food security and food quality (WMO, 2010).

For mycotoxins in particular, it has been suggested that AFs production could be a strategy of fungi to prevent from UV damage (Cary and Ehrlich, 2006). If this statement is correct a possible UV increase could promote the mycotoxin presence in crops. Therefore, the lack of information about this regard demands more studies about this topic.

The effect of UV-A and UV-B radiation was tested separately both on conidial survival and in mycelium development of surviving spores for diferent *Aspergillus* spp. in two different environmental conditions. Conidia could survive and colonize soil and organic debris associated with plant residues and later start the infection cycle on new host plants (Battilani

et al., 2012). Life cycle could be divided into two major phases: the colonization of plant residues in soil and the infection of crop tissue. In the first study conidia were dislogged onto membranes, and no source of water or nutrients was available, in the second experiment conidia were deposited on culture medium, which can provide both nutrients and protection. Direct UV doses used in this study (1.7 mW/cm^2 for UV-A and 0.1 mW/cm^2 for UV-B) were slightly higher than the present global UV mean values recorded in South Spain. Additionally, fluctuating T and white light were included in the period of incubation in order to simulate field conditions.

As a result of the first study, a decrease in viability of conidia was observed along time for all isolates tested. This phenomena was more marked when the isolates were subjected to cycles of UV radiation. *Aspergillus* section *Circumdati* showed the greatest loss of conidial viability both in UV irradiated and in control treatments. In 5 days significant differences in viability due to UV radiation were observed in this section, while reduced viability of *Aspergillus* sections *Nigri* and *Flavi* were observed after 10-15 days. Furthemore, interspecific differences were observed within the sections *Nigri* and *Flavi*. Reduction of spore germination due to UV radiation has been also observed in other fungal species (Moody et al., 1999; Wu et al., 2000).

Isolates tested were obtained from two vineyard areas of Spain. As the irradiance values measured in the Northeast region are usually lower than in the Southern region, the possible isolate adaptation as a consequence of their geographical origin was assessed. However, isolates isolated from the South were in general more sensitive to UV radiation, thus the hypothesis was not confirmed.

The effect of UV-A and UV-B was not compared as both radiations were applied at different levels of intensity; however, as comparable levels of conidia survival were observed and UV-B was applied at a lower dose, it is clear that UV-B produces more deleterious effects due to its shorter wavelength.

Additionally, the UV radiation affected germination and mycelial growth of *A. carbonarius* and *A. parasiticus* on nutrient media. Concerning *A. carbonarius*, UV-A and UV-B radiation caused always significant reduction of colony size and OTA production, although higher reduction of both parameters were reached under UV-A. Remarkably, intraspecific

differences were more frequent under UV-A radiation, being observed only after short periods of incubation (7 days) under UV-B. A decrease in the UV exposure time reduced also the deleterious effects on *A. carbonarius*. UV-B radiation affected colony morphology, sporulation was observed only in the center of colonies, which was harder and more compact than the rest of the colony.

Regarding *A. parasiticus,* UV (A and B) radiation had also significant effect on colony size and AFs production. Conversely to *A. carbonarius* higher reduction of these parameters was reached under UV-B. In fact, UV-B radiation practically stopped hyphal extension. Moreover morphological differences were observed under different light conditions. AFs were detected in UV-A radiated colonies in all tested times, representing AFB_1 more than 60% of total AFs, while AFG_2 never overcome a 10%. Contrary to UV-A, AFs were only detected after 7 days in colonies growing under UV-B, and in this case AFG_1 was predominant and AFG_2 attained a 20%.

Duguay and Klironomos, (2000) suggested that despite spores can survive under certain conditions this does not imply that the resulting hyphae have the same advantages. From our results species with more resistant conidia to UV radiation also had more unaffected mycelia.

In both studies *Aspergillus* section *Nigri* were more tolerant to UV radiation. Different tolerances to the deleterious effect of solar UV radiation on fungi have been reported before (Rotem et al., 1985). Some authors suggest that diferent UV tolerance levels could be due to the pigmentation or to the physical characteristics of conidia, as projected surface area-to-volume of conidia or thicker wall conidia (Moody et al., 1999; Rotem and Aust, 1991; Valero et al., 2007a).

Studies on microfungal communities in soils of extreme habitats, as desert (very high solar radiation, drought, and extreme T), showed dominance of dark-coloured microfungi with large multicelled conidia is characteristic (Grishkan et al., 2007, 2003). Particularly, species of the genus *Aspergillus* (mainly *A. fumigatus*) and telemorphic ascomycetes comprised a basic part of the thermotolerant mycobiota obtained at a temperature of 37 ºC (Grishkan et al., 2007).

Aspergillus species showed different tolerance to UV radiation, hence an increase of it may modify the prevailing species present in field, and as a consequence the potential inoculum in the field may change, possibly favoring in the future an even higher predominance of black aspergilli that at present. However, the overall spore inoculum present in the field may decrease, as well as the potentially produced OTA and AFs, once conidial germination and mycelium growth has occurred. Mycotoxin degradation to other metabolites due to UV irradiation was not considered in this work. Finally, conidia survival and therefore the fungal presence *per se* represents an important risk, because favourable conditions for toxin production can occur in the following postharvest stages.

2.3. Effect of environmental conditions on antifungal efficacy of preharvest fungicides

Nowadays, the application of fungicides during pre-harvest is one of the most widely used agricultural practices. However, it is important to note that partial inhibition of fungal growth could enhance mycotoxin production as a response of the mould to stress (da Cruz Cabral et al., 2013). Furthermore, attention should be given to the influence of changing climate conditions on the use of pesticides according to GAP, as the current system may not be suitable in the future crop status (Magan et al., 2011; Miraglia et al., 2009). In fact, loss of effectiveness on antifungals tested on *A. carbonarius* and OTA production has been reported under different T and a_W conditions (Medina et al., 2007a, 2007b).

On the other hand, the indiscriminate and excessive use of fungicides in crops has been the major cause of the development of resistant pathogen populations, resulting in the use of higher concentrations of these antifungals and the consequent increase in toxic residues in food products. For example, acquired resistance by *Penicillium italicum* and *P. digitatum* to many synthetic fungicides currently used on citrus fruit has been demonstrated (Fogliata et al., 2001). Additionally, some antifungals, especially at sublethal doses can stimulate the production of mycotoxins by a secondary activation of biosynthetic genes (Magan, 2006; Schmidt-Heydt et al., 2008).

The general public perceives risks related to pesticides as posing a greater hazard than mycotoxins (Williams and Hammitt, 2001). Currently there is a "social pressure" for reducing the use of chemical additives in the food industry. Today's consumers are increasingly demanding food without preservatives or chemically synthesized antimicrobial substances.

At the same time, the EC set Maximum Residue Levels (MRLs) for pesticide residues in or on food or feed to ensure the lowest possible consumer exposure based on properties of the active ingredients and residue levels resulting from the GAP defined for the treated crops.

At the same time several researches are investigating natural alternatives for controlling fungal growth and mycotoxin production. Focusing on *Aspergillus* species many plant extracts have been tested (Garcia et al., 2011; Milovanović et al., 2007; Rasooli and Owlia, 2005; Rasooli et al., 2006).

In this Thesis different active ingredients dosed following manufacturer recommendations (**Table 3**) as well as a plant extract were tested *in situ* under different environmental conditions (current and extreme) in order to evaluate: i) their efficiency against *Aspergilllus* species development and OTA production and, ii) the potential impact of climate change in the effectiveness of antifungals evaluated (**Table 2**).

Under current conditions and concerning to growth control, all active ingredients (cypronidil + fluodioxonil, tebuconazol + trifloxistrobin, tebuconazole +prothioconazole, epoxiconazole and tetraconazole) were more effective than thiophanate methyl that generally did not affect fungal growth or even stimulated it. In the case of grapes the best results were obtained with Switch®. Several works have shown its effectiveness on ochratoxigenic spicies in SNM and in grapes (Bellí et al., 2006a), but also in field treatments (Tjamos et al., 2004; Valero et al., 2007b). In relation to the active compound, Tjamos et al., (2004) pointed out fludioxonil as responsible for the effectiveness. On the other hand, Flint Max ® was less effective, like in other studies (Belli et al., 2006a).

In the same way as fungal growth, antifungals tested reduced the OTA production although it was not always significantly. Particularly, in *A. carbonarius*, tebuconazole was the most effective in wheat whereas no significant differences between tebuconazole + prothioconazole and cypronidil + fludioxonil where found in grapes. (Bellí et al., 2007a; Bellí et al., 2006a) found that cypronidil + fluodioxonil was the most effective in reducing OTA production by *A. carbonarius*. Other active ingredients commonly used in grapes, such as azoxystrobin, dinocap and pentaconazole, were also effective in reducing OTA accumulation (Curto et al., 2004). The growth rate of one *A. steynii* tested was reduced while, the OTA production was not affected by any of the antifungals suggesting that an appropriate

screening for antifungal agents requires to take into account both effects on growth and mycotoxin production.

Table 3 Chemical antifungals, active ingredients and main mechanism of action

	Supplier	Brand	Active ingredient	Action mechanism
Grape	Syngenta	Switch®	37.5% Cypronidil	Interferes membrane processes damaging the plasmatic membrane
			25.0% Fluodioxonil	Blocks the enzymatic action affecting the regulation of the synthesis of glycerol producing cell hypertrophy
	Bayer CropScience S.L.	Flint Max®	50% Tebuconazole	Affects sterol biosynthesis in membranes
			25% Trifloxistrobin	Mitocondrial respiration inhibition
Wheat	Bayer CropScience S.L.	Folicur 25® Ew	25.9% Tebuconazole	Affects sterol biosynthesis in membranes
	Bayer CropScience S.L.	Prosaro®	12.70% Tebuconazole	Affects sterol biosynthesis in membranes
			12.7% Prothioconazole	Affects sterol biosynthesis in membranes
	Basf Española S.L.	Lovit®	12.5% Epoxiconazole	Affects sterol biosynthesis in membranes
	Sipcam Inagra S.A.	Domark Evo®	12.5%Tetraconazole	Affects sterol biosynthesis in membranes
	Sipcam Inagra S.A.	Enovit Metil®	70%Thiophanate methyl	Acts on tubulin metaphase mitosis causing cell death

To sum up, the active ingredients tested, under current conditions, seemed to reduce fungal growth but they were not efficient in the OTA production control of *A. steynii*.

An interesting work about the efficacy of natamycin for control of growth and OTA production by *A. carbonarius* under different environmental conditions showed that the inhibition of mycelial growth was influenced by a_W and T (Medina et al., 2007a). Moreover, a higher amount of the antifungal was required in the most suitable conditions for growth and production (Medina et al., 2007a). Therefore, the potential impact of climate change in the effectiveness of antifungals tested was also evaluated.

Respect to the azoles, epoxiconazole (Lovit ®) and tetraconazole (Domark Evo ®) were effective against both ochratoxigenic species under current conditions despite their effectiveness may be compromised under predicted conditions. By contrast, tebuconazole (Flint Max ®, Folicur ® and Prosaro ®) increased their effectiveness under warmer and drier conditions. Thus a transition in the active ingredients may be required in the future as a result of changing climatic conditions. Under predicted conditions, none of the antifungals was effective in preventing OTA production by any of the strains tested in both grapes and wheat. This means that in some cases, antifungals, while limiting fungal growth, induced a stress situation which triggered OTA production.

On the other hand, consumer demands reduced use of chemicals in food products and therefore the use of safer and more environmentally friendly antifungals. The effectiveness of *E. arvense* extracts was tested as an alternative to chemical applications. Previous work pointed to the antioxidant compounds such as phenols as the bioactive basis of the antimicrobial propierties of plant extracts (Ebana and Madunagu, 1993). Analysis of phenolic compounds of the extract by HPLC/DAD/ESI/MS revealed quercitine and kaempferol as main phenolic compounds present in our extract.

Under current conditions, *E. arvense* extract (3%) showed similar or better effects than chemical antifungals in wheat, although higher doses of it were applied. As for chemical antifungals, the extract was not useful against OTA production by *A. steynii*. Conversely, *E. arvense* (2%) caused a slight reduction of fungal growth but stimulated OTA production. These contradictory results could be explained by the different doses applied. On maize agar medium, high dosis of *E. arvense* reduced the growth of *Aspergillus* species, while low doses could stimulate it in some conditions (Garcia et al., 2011). From our studies the effectiveness of *E. arvense* extract seemed not to be affected by different environmental conditions. However, environmental conditions have been proven to be determinant in the effectiveness of *E. arvense* (3%) since it was effective at 0.95 a_W against *A. flavus* and *F. graminearum* in maize; but not clear results were observed at lower a_W (Garcia et al., 2012).

REFERENCES

Abrunhosa, L., Calado, T., Venâncio, A., 2011. Incidence of fumonisin B_2 production by *Aspergillus niger* in portuguese wine regions. J. Agric. Food Chem. 59, 7514–7518.

AEMET, 2009. Agencia Española de Meterologia Generación de escenarios regionalizados de cambio climático para España. http://www.aemet.es/documentos/es/serviciosclimaticos/cambio_climat/datos_diarios/Informe_Escenarios.pdf

AEMET, 2012. Agencia Española de Meterologia. Atlas de radiación solar en España utilizando datos del SAF de clima de EUMETSAT. http://www.aemet.es/documentos/es/serviciosclimaticos/datosclimatologicos/atlas_radiacion_solar/atlas_de_radiacion_24042012.pdf

Ariño, A., Herrera, M., Estopañan, G., Rota, M.C., Carramiñana, J.J., Juan, T., Herrera, A., 2009. Aflatoxins in bulk and pre-packed pistachios sold in Spain and effect of roasting. Food Control 20, 811–814.

Battilani, P., Barbano, C., Marin, S., Sanchis, V., Kozakiewicz, Z., Magan, N., 2006. Mapping of *Aspergillus* section *Nigri* in Southern Europe and Israel based on geostatistical analysis. Int. J. Food Microbiol. 111, S72–S82.

Battilani, P., Pietri, A., Bertuzzi, T., Languasco, L., Giorni, P., Kozakiewicz, Z., 2003. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. J. Food Prot. 66, 633–636.

Battilani P., Rossi V., Giorni P., Pietri A., Gualla A., van der Fels-Klerx H.J, Booij C.J.H., Moretti A., Logrieco A., Miglietta F, Toscano P., Miraglia M., De Santis B, Brera C., 2012. Question No EFSA-Q-2009-0081 [WWW Document]. URL http://www.efsa.europa.eu/fr/search/doc/223e.pdf (accessed 9.10.13).

Bau, M., Bragulat, M.R., Abarca, M.L., Minguez, S., Cabañes, F.J., 2005. Ochratoxin A producing fungi from Spanish vineyards, Advances in Experimental Medicine and Biology.

Bau, M., Castellá, G., Bragulat, M.R., Cabañes, F.J., 2006. RFLP characterization of *Aspergillus niger* aggregate species from grapes from Europe and Israel. Int. J. Food Microbiol. 111, S18–S21.

Bellí, N., Marín, S., Argilés, E., Ramos, A.J., Sanchis, V., 2007a. Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes (Vitis vinifera). J. Food Prot. 70, 157–163.

Bellí, N., Marín, S., Coronas, I., Sanchis, V., Ramos, A.J., 2007b. Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes. Food Control 18, 1343–1349.

Bellí, N., Marín, S., Duaigües, A., Ramos, A.J., Sanchis, V., 2004. Ochratoxin A in wines, musts and grape juices from Spain. J. Sci. Food Agric. 84, 591–594.

Bellí, N., Marín, S., Sanchis, V., Ramos, A.J., 2006a. Impact of fungicides on Aspergillus carbonarius growth and ochratoxin a production on synthetic grape-like medium and on grapes. Food Addit. Contam. 23, 1021–1029.

Bellí, N., Mitchell, D., Marín, S., Alegre, I., Ramos, A.J., Magan, N., Sanchis, V., 2005a. Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. Eur. J. Plant Pathol. 113, 233–239.

Bellí, N., Ramos, A.J., Coronas, I., Sanchis, V., Marín, S., 2005b. Aspergillus carbonarius growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. J. Appl. Microbiol. 98, 839–844.

Bellí, N., Ramos, A.J., Sanchis, V., Marín, S., 2006b. Effect of photoperiod and day-night temperatures simulating field conditions on growth and ochratoxin A production of Aspergillus carbonarius strains isolated from grapes. Food Microbiol. 23, 622–627.

Betina, V., 1989. Mycotoxins: Chemical, biological and environmental aspects. Bioact. Mol. 9, 114–150.

Cary, J.W., Ehrlich, K.C., 2006. Aflatoxigenicity in *Aspergillus*: Molecular genetics, phylogenetic relationships and evolutionary implications. Mycopathologia 162, 167–177.

Chiotta, M.L., Ponsone, M.L., Combina, M., Torres, A.M., Chulze, S.N., 2009. *Aspergillus* section *Nigri* species isolated from different wine-grape growing regions in Argentina. Int. J. Food Microbiol. 136, 137–141.

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N., 2011. Phylogenetic characterization and ochratoxin A - Fumonisin profile of black *Aspergillus* isolated from grapes in Argentina. Int. J. Food Microbiol. 149, 171–176.

Curto, R.L., Pellicanò, T., Vilasi, F., Munafò, P., Dugo, G., 2004. Ochratoxin A occurrence in experimental wines in relationship with different pesticide treatments on grapes. Food Chem. 84, 71–75.

Da Cruz Cabral, L., Fernández Pinto, V., Patriarca, A., 2013. Application of plant derived compounds to control fungal spoilage and mycotoxin production in foods. Int. J. Food Microbiol. 166, 1–14.

Duguay, K.J., 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.

Ebana, R.U.B., Madunagu, V.E., 1993. Antimicrobial effect of Strophanthus hispidus and Secamone afzelii on some pathogenic bacteria and their drug resistant strains. Niger. J. Bot. 6, 27–31.

EC, 2006a. European Comimission, Nº 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union, L 70(2006), 12-34.

EC, 2006b. European Commission, Nº 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L 364(2006), 5-24.

EC, 2007. European Commision, 2007. Adapting to climate change in Europe: Options for EU Action. Green Paper from the Commission to the Council, the European Parlianment, the European Economic and Social Committee of the Regions, SEC (2007) Brusseles, Belgium: European Commission

EC, 2010a. European Commission, Nº 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuff as regards aflatoxins. Official Journal of the European Union, L 50(2006), 8-12.

EC, 2010b. European Commission, Nº 178/2010 of 2 March 2010 amending Regulation (EC) No 401/2006 as regards groundnuts (peanuts), other oilseeds, tree nuts, apricot kernels, liquorice and vegetable oil. Official Journal of the European Union, L 52(2006), 32-43.

Farah, Z., Martins, M.J.R., Bachmann, M.R., 1983. Removal of aflatoxin in raw unshelled peanuts by a traditional salt boiling process practiced in the north east of Brazil. Lebensm. Wiss. Technol. 16, 122–124.

Fogliata, G.M., Torres, L.G.., Ploper, L.., 2001. Detection of imazalil-resistant strains of *Penicillium digitatum* Sacc. in citrus packing houses of Tucumán Province (Argentina) and their behaviour against current employed and alternative fungicides. Rev. Ind. Agríc. Tucumán 77, 71–75.

Fredj, S.M.B., 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.

Garcia, D., Garcia-Cela, E., Ramos, A.J., Sanchis, V., Marín, S., 2011. Mould growth and mycotoxin production as affected by *Equisetum arvense* and *Stevia rebaudiana* extracts. Food Control 22, 1378–1384.

Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2012. Effect of Equisetum arvense and Stevia rebaudiana extracts on growth and mycotoxin production by Aspergillus flavus and Fusarium verticillioides in maize seeds as affected by water activity. Int. J. Food Microbiol. 153, 21–27.

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., Battilani, P., 2007. Studies on Aspergillus section Flavi isolated from maize in northern Italy. Int. J. Food Microbiol. 113, 330–338.

Grishkan, I., Beharav, A., Kirzhner, V., Nevo, E., 2007. Adaptive spatiotemporal distribution of soil microfungi in "Evolution Canyon" III, Nahal Shaharut, extreme southern Negev Desert, Israel. Biol. J. Linn. Soc. 90, 263–277.

Grishkan, I., Nevo, E., Wasser, S.P., Beharav, A., 2003. Adaptive spatiotemporal distribution of soil microfungi in "Evolution Canyon" II, Lower Nahal Keziv, western Upper Galilee, Israel. Biol. J. Linn. Soc. 78, 527–539.

IPCC, 2007. Intergovernmental panel on climate change. Contribution of working groups I, II and III to the Fourth Assessment Report. Core writing team, Pachauri, R.K. and Reisinger, A (Eds.) IPCC, Geneva, Switzerland.

Howden, S.M., Soussana, J.-F., Tubiello, F.N., Chhetri, N., Dunlop, M., Meinke, H., 2007. Adapting agriculture to climate change. Proc. Natl. Acad. Sci. U. S. A. 104, 19691–19696.

Lasram, S., Oueslati, S., Mliki, A., Ghorbel, A., Silar, P., Chebil, S., 2012. Ochratoxin A and ochratoxigenic black Aspergillus species in Tunisian grapes cultivated in different geographic areas. Food Control 25, 75–80.

Lee, L.S., Cucullu, A.F., Jr, A.O.F., Jr, W.A.P., 1969. Destruction of aflatoxins in peanuts during dry and oil roasting. J. Agric. Food Chem. 17, 451–453.

Leong, S.-L., Hocking, A.D., 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.-L.L., Hocking, A.D., Varelis, P., Giannikopoulos, G., Scott, E.S., 2006. Fate of ochratoxin A during vinification of semillon and Shiraz grapes. J. Agric. Food Chem. 54, 6460–6464.

Logrieco, A., Ferracane, R., Haidukowsky, M., Cozzi, G., Visconti, A., Ritieni, A., 2009. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. Food Addit. Contam. - Part Chem. Anal. Control Expo. Risk Assess. 26, 1495–1500.

Lucchetta, G., Bazzo, I., Cortivo, G.D., Stringher, L., Bellotto, D., Borgo, M., Angelini, E., 2010. Occurrence of black aspergilli and ochratoxin a on grapes in Italy. Toxins 2, 840–855.

Magan, N., 2006. Mycotoxin contamination of food in Europe: Early detection and prevention strategies. Mycopathologia 162, 245–253.

Magan, N., Medina, A., Aldred, D., 2011. Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. Plant Pathol. 60, 150–163.

Martínez-Culebras, P.V., Ramón, D., 2007. An ITS-RFLP method to identify black Aspergillus isolates responsible for OTA contamination in grapes and wine. Int. J. Food Microbiol. 113, 147–153.

Medina, Á., Jiménez, M., Mateo, R., Magan, N., 2007a. Efficacy of natamycin for control of growth and ochratoxin A production by *Aspergillus carbonarius* strains under different environmental conditions. J. Appl. Microbiol. 103, 2234–2239.

Medina, A., Mateo, R., López-Ocaña, L., Valle-Algarra, F.M., Jiménez, M., 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. Appl. Environ. Microbiol. 71, 4696–4702.

Medina, Á., Mateo, R., Valle-Algarra, F.M., Mateo, E.M., Jiménez, M., 2007b. Effect of carbendazim and physicochemical factors on the growth and ochratoxin A production of *Aspergillus carbonarius* isolated from grapes. Int. J. Food Microbiol. 119, 230–235.

Milovanović, V., Radulović, N., Todorović, Z., Stanković, M., Stojanović, G., 2007. Antioxidant, antimicrobial and genotoxicity screening of hydro-alcoholic extracts of five Serbian *Equisetum* species. Plant Foods Hum. Nutr. 62, 113–119.

Miraglia, M., Marvin, H.J.P., Kleter, G.A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., Santis, B.D., Dekkers, S., Filippi, L., Hutjes, R.W.A., Noordam, M.Y., Pisante, M., Piva, G., Prandini, A., Toti, L., Born, G.J. van den, Vespermann, A., 2009. Climate change and food safety: An emerging issue with special focus on Europe. Food Chem. Toxicol. 47, 1009–1021.

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. J. Appl. Microbiol. 97, 439–445.

Mogensen, J.M., Frisvad, J.C., Thrane, U., Nielsen, K.F., 2010a. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. J. Agric. Food Chem. 58, 954–958.

Mogensen, J.M., Larsen, T.O., Nielsen, K.F., 2010b. Widespread occurrence of the mycotoxin fumonisin B2 in wine. J. Agric. Food Chem. 58, 4853–4857.

Moody, S.A., Newsham, K.K., Ayres, P.G., Paul, N.D., 1999. Variation in the responses of litter and phylloplane fungi to UV-B radiation (290-315 nm). Mycol. Res. 103, 1469–1477.

Oueslati, S., Lasram, S., Ramos, A.J., Marin, S., Mliki, A., Sanchis, V., Ghorbel, A., 2010. Alternating temperatures and photoperiod effects on fungal growth and Ochratoxin A production by Aspergillus carbonarius isolated from Tunisian grapes. Int. J. Food Microbiol. 139, 210–213.

Ozay, G., Seyhan, F., Yilmaz, A., Whitaker, T.B., Slate, A.B., Giesbrecht, F., 2006. Sampling hazelnuts for aflatoxin: Uncertainty associated with sampling, sample preparation, and analysis. J. AOAC Int. 89, 1004–1011.

Ozkarsli, M., 2003. Effect of traditional-roasting and microwave-roasting on aflatoxin B_1 in peanuts. MSc Thesis, Cukurova University Institute of Natural and Applied Sciences, Adana, Turkey.

Palumbo, J.D., O'Keeffe, T.L., Mcgarvey, J.A., 2011. Incidence of fumonisin B2 production within *Aspergillus* section *Nigri* populations isolated from California raisins. J. Food Prot. 74, 672–675. doi:10.4315/0362-028X.JFP-10-412

Pardo, E., Marín, S., Sanchis, V., Ramos, A.J., 2005. Impact of relative humidity and temperature on visible fungal growth and OTA production of ochratoxigenic *Aspergillus ochraceus* isolates on grapes. Food Microbiol. 22, 383–389.

Perrone, G., Susca, A., Epifani, F., Mulè, G., 2006. AFLP characterization of Southern Europe population of *Aspergillus* section *Nigri* from grapes. Int. J. Food Microbiol. 111, S22–S27.

Pluyer, H.R., Ahmed, E.M., Wei, C.I., 1987. Destruction of aflatoxins on peanuts by oven- and microwave-roasting. J. Food Prot. 50, 504–508.

Rasooli, I., Owlia, P., 2005. Chemoprevention by thyme oils of *Aspergillus parasiticus* growth and aflatoxin production. Phytochemistry 66, 2851–2856.

Rasooli, I., Rezaei, M.B., Allameh, A., 2006. Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from Thymus eriocalyx and Thymus x-porlock. Food Control 17, 359–364.

Rotem, J., Aust, H.J., 1991. The Effect of Ultraviolet and Solar Radiation and Temperature on Survival of Fungal Propagules. J. Phytopathol. 133, 76–84.

Rotem, J., Wooding, B., Aylor, D.E., 1985. the role of solar radiation, especially ultraviolet, in the mortality of fungal spores. Phytopathology 75, 510–514.

Rustom, I.Y.S., 1997. Aflatoxin in food and feed: Occurrence, legislation and inactivation by physical methods. Food Chem. 59, 57–67.

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.

Schatzki, T.F., 1995. Distribution of aflatoxin in pistachios. 2. Distribution in freshly harvested pistachios. J. Agric. Food Chem. 43, 1566–1569.

Schmidt-Heydt, M., Magan, N., Geisen, R., 2008. Stress induction of mycotoxin biosynthesis genes by abiotic factors. FEMS Microbiol. Lett. 284, 142–149.

Serra, R., Braga, A., Venâncio, A., 2005. Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. Res. Microbiol. 156, 515–521.

Serra, R., Lourenço, A., Alípio, P., Venâncio, A., 2006. Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species. Mycol. Res. 110, 971–978.

Spadaro, D., Patharajan, S., Lorè, A., Garibaldi, A., Gullino, M.L., 2012. Ochratoxigenic black species of aspergilli in grape fruits of northern Italy identified by an improved PCR-RFLP procedure. Toxins 4, 42–54.

Susca, A., Perrone, G., Cozzi, G., Stea, G., Logrieco, A.F., Mulè, G., 2013. Multilocus sequence analysis of Aspergillus Sect. Nigri in dried vine fruits of worldwide origin. Int. J. Food Microbiol. 165, 163–168.

Tirado, M.C., Clarke, R., Jaykus, L.A., McQuatters-Gollop, A., Frank, J.M., 2010. Climate change and food safety: A review. Food Res. Int. 43, 1745–1765.

Tjamos, S.E., Antoniou, P.P., Kazantzidou, A., Antonopoulos, D.F., Papageorgiou, I., 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.

Turner, N.W., Subrahmanyam, S., Piletsky, S.A., 2009. Analytical methods for determination of mycotoxins: A review. Anal. Chim. Acta 632, 168–180.

UNEP, 2010. Scientific Assessment of Ozone Depletion: 2010.

Valero, A., Begum, M., Leong, S.L., Hocking, A.D., Ramos, A.J., Sanchis, V., Marín, S., 2007a. Effect of germicidal UVC light on fungi isolated from grapes and raisins. Lett. Appl. Microbiol. 45, 238–243.

Valero, A., Marín, S., Ramos, A.J., Sanchis, V., 2007b. Effect of preharvest fungicides and interacting fungi on *Aspergillus carbonarius* growth and ochratoxin A synthesis in dehydrating grapes. Lett. Appl. Microbiol. 45, 194–199.

Varga, J., Kocsubé, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., Tóth, B., Bartók, T., 2010. Fumonisin contamination and fumonisin producing black aspergilli in dried vine fruits of different origin. Int. J. Food Microbiol. 143, 143–149.

Wesolek, N., Ramirez-Martinez, A., Roudot, A.-C., 2014. Mathematical approach for sampling plan performance assessment for aflatoxin B1 in pistachios. Food Res. Int. 62, 448–455.

Whitaker, T.B., Slate, A.B., Jacobs, M., Hurley, J.M., Adams, J.G., Giesbrecht, F.G., 2006. Sampling almonds for aflatoxin, part I: Estimation of uncertainty associated with sampling, sample preparation, and analysis. J. AOAC Int. 89, 1027–1034.

Williams, P.R.D., Hammitt, J.K., 2001. Perceived risks of conventional and organic produce: Pesticides, pathogens, and natural toxins. Risk Anal. 21, 319–330.

Wu, B.M., Subbarao, K.V., Van Bruggen, A.H.C., 2000. Factors affecting the survival of Bremia lactucae sporangia deposited on lettuce leaves. Phytopathology 90, 827–833.

Yazdanpanah, H., 2011. Mycotoxins: Analytical Challenges. Iran. J. Pharm. Res. IJPR 10, 653–654.

Yazdanpanah, H., Mohammadi, T., Abouhossain, G., Cheraghali, A.M., 2005. Effect of roasting on degradation of Aflatoxins in contaminated pistachio nuts. Food Chem. Toxicol. 43, 1135–1139.

VI. CONCLUSIONS and FUTURE RESEARCH

CONCLUSIONS

The results presented in this Thesis work led to the following conclusions:

1. SELECTION OF RAW MATERIALS (SAMPLING OF RAW PISTACHIO FOR TOASTING)

Analysis of raw lots revealed OTA absence, while AFB_1 was not only detected in a range of 0.32- 392.5 μg/kg but also accounted for 70-100% of total AFs. Therefore AFB_1 represented the main mycotoxicological risk in pistachio.

High uncertainties due to initial sampling and sample preparation procedures may lead to unrealistic results, and this is an issue that needs to be solved. In the present work the major variability was associated with the subfractioning selection and therefore increasing the number of the analyzed subfractions could be an alternative for reducing uncertainty.

Expensive and complex sampling plans are defined by the EC for nuts. A simplified alternative plan proposed resulted inappropriate as compared to the official one, due to the reduced probability of taking a contaminated portion in the sampling.

Using the existing EU sampling plan, and applying the sampling uncertainty for correction of analytical results, a high percentage of lots could be rejected. Therefore sampling plans and procedures must be revised in depth.

Industrial toasting of pistachio resulted in a higher percentage of reduction than that suggested by the existing European legal limits, and therefore under the hypothesis of raw pistachio compliance with maximum level, the toasted pistachio should be safe.

2. IMPACT OF CLIMATE CHANGE IN *ASPERGILLUS* SPECIES

Black aspergilli presence was significantly higher in the hottest region sampled, although species distribution in both regions did not show a clear trend, *A. tubingensis* seemed to be the most prevalent species in Spanish vineyards.

A. niger and *A. tubingensis* are able to growth in a wider range of environmental conditions than *A. carbonarius*, mainly at lower a_W and higher T, indicating that these species could be

adapted to the climate scenarios predicted for Southern Europe. However, *A. carbonarius*, showed a possible adaptation to hostile environmental conditions since isolates from South grew at lower a_W.

A. carbonarius was the main ochratoxigenic species either for the number of producing isolates or the amounts of toxin produced; in fact, contaminated must was linked to highly infected vineyard by *A. carbonarius*. Mycotoxicological consequences in grapes and derivates resulting from a hypothetical warming scenario may involve a decreased OTA risk due to the decreasing presence of *A. carbonarius* and an increasing of FB_2 risk due to the promotion of *A. niger*.

UV irradiation affected to the conidia viability. The overall spore inoculum present in the field may decrease, as well as the potentially produced OTA and AFs, once conidial germination and mycelium growth has occurred. Particularly, black aspergilli showed the highest resistence of UV irradiation within aspergilli tested.

In general, antifungal tested reduced the fungal growth, although difference among them were found. Conversely, OTA risk was not controlled even some of them triggered its production. The antifungal effectiveness was affected by environmental conditons and therefore a transition in the active ingredients may be required in the future as a result of changing climate conditions.

CONCLUSIONES

Los resultados presentes en esta Tesis han dado lugar a las siguientes conclusiones.

1. SELECCIÓN DE MATERIAS PRIMAS (SELECCIÓN DE PISTACHO)

En el caso del pistacho, el análisis de los lotes de materia prima reveló la ausencia de OTA, mientras que la AFB_1 no solo fue detectada en un amplio intervalo de concentraciones (0,32-392,5 μg/kg), sino que también constituyó del 70 al 100% del total de AFs. Así pues, la AFB_1 representa el principal riesgo micotoxicológico en pistacho.

En relación al muestreo, y debido a la incertidumbre inherente al mismo, el muestreo inicial y a la preparación de la muestra pueden proporcionar resultados poco fiables, y éste es un asunto que necesita ser resuelto. En el presente trabajo la mayor variabilidad de resultados fue asociada con la selección de la submuestra, y por lo tanto, el aumento del número de submuestras analizadas podría ser una alternativa para reducir la incertidumbre.

Los planes de muestreo definidos por la EC para frutos secos son complejos y costosos. No obstante, el plan alternativo propuesto en esta Tesis resultó ser inapropiado en comparación con el oficial, debido a la escasa probabilidad de obtener una muestra contaminada durante el muestreo.

El plan de muestreo oficial obliga a corregir los resultados analíticos teniendo en cuenta la incertidumbre, lo que puede dar lugar a un elevado porcentaje de rechazo de los lotes, a menos que los planes de muestreo y los procedimientos sean revisados en profundidad.

El tostado industrial del pistacho causó una reducción superior a la propuesta por los límites legales europeos y por lo tanto, bajo la hipótesis de que el pistacho crudo no supere los límites máximos admisibles, el pistacho tostado puede considerarse seguro.

2. IMPACTO DEL CAMBIO CLIMÁTICO EN ESPECIES DE *ASPERGILLUS*

La presencia de aspergilos negros fue significativamente superior en la región más cálida muestreada y, aunque la distribución de especies en ambas regiones no mostró una tendencia clara, *A. tubingensis* parece ser la especie más prevalente en los viñedos españoles.

A. niger y *A. tubingensis* pueden crecer en un rango más amplio de condiciones ambientales que *A. carbonarius*, principalmente a menor a_W y mayor T, indicando que estas especies podrían estar mejor adaptadas a los escenarios climáticos predichos para el sur de Europa. Sin embargo, *A. carbonarius*, mostró una possible adaptación frente a condiciones ambientales desfavorables ya que los aislados del sur de España crecieron a bajas a_W.

A. carbonarius fue la principal especie ocratoxigénica tanto en el número de aislados productores de OTA como en las cantidades producidas; de hecho, los mostos contaminados con OTA provenían de viñedos con una elevada infección por *A. carbonarius*. De este estudio se derivan algunas consecuencias micotoxicológicas, como que el incremento de la temperatura ambiente puede conllevar una disminución del riesgo por OTA debido a una menr presencia de *A. carbonarius* y un incremento del riesgo por FB_2 debido al aumento en la presencia de *A. niger*.

La radiación UV afectó a la viabilidad de los conidios. El incremento en la irradiación podría afectar al inóculo global presente en el campo, que podría disminuir, como también a las especies potencialmente productoras de OTA y AFs, una vez que se haya producido la germinación de los conidios y el crecimiento del micelio. Particularmente, los aspergilos negros mostraron la mayor resistencia a la radicación UV dentro de las especies de *Aspergillus* estudiadas.

En general, los fungicidas ensayados redujeron el crecimiento fúngico, aunque se observaron diferencias de efectividad entre ellos. Paradójicamente, no controlaron el riesgo por OTA, e incluso algunos de ellos estimularon su producción. La eficacia de los fungicidas se vio afectada por las condiciones ambientales y, por lo tanto, en el futuro podría ser necesario un cambio en las materias activas empleadas como consecuencia de los cambios en las condiciones climáticas.

CONCLUSIONS

Els resultats presentats en aquest Tesi han donat lloc a les següents conclusions.

1. SELECCIÓ DE LES MATÈRIES PRIMERES (SELECCIÓ DELS FESTUCS)

En el cas dels festucs, la anàlisi dels lots de les matèries primeres va revelar l'absència d'OTA, mentre que la AFB_1 no va només detectada en un interval ampli de concentracions (0,32-392,5 μg/kg), sinó que també va representar del 70 al 100% del total d'AFs. Així doncs, la AFB_1 representa el principal risc micotoxicològic en els festucs.

En relació al mostreig, i degut a la incertesa inherent al mateix, el mostreig inicial i la preparació de la mostra poden proporcionar resultats poc fiables, i aquest és un assumpte que necessita ser resolt. En aquest treball la major variabilitat de resultats va ser associada amb la selecció de la submostra i per tant, l'augment del nombre de submostres analitzades podria ser una alternativa per reduir la incertesa.

Els plans de mostreig definits per la EC per fruits secs són complexos i costosos. El pla alternatiu proposat en aquesta Tesi va resultar ser inadequat en comparació amb l'oficial, degut a l'escassa probabilitat d'obtenir una mostra contaminada durant el mostreig.

El pla de mostreig oficial obliga a corregir els resultats analítics tenint en compte la incertesa, el que pot donar lloc a un elevat percentatge de rebuig dels lots, a menys que els plans de mostreig i els procediments siguin revisats en profunditat.

El torrat industrial dels festucs va causar una reducció superior a la proposada pels límits legals europeus, i per tant, sota la hipòtesi de que els festucs crus no superin els límits màxims admissibles, els festucs torrats poden considerar-se segurs.

2. IMPACTE DEL CANVI CLIMÀTIC EN ESPÈCIES D'*ASPERGILLUS*

La presència d'aspergils negres va ser significativament superior a la regió més càlida mostrejada i, encara que la distribució de les espècies en ambdues regions no va mostrar una tendència clara, *A. tubingensis* sembla ser l'espècie més prevalent a la vinya espanyola.

A. niger i *A. tubingensis* poden créixer en un rang més ampli de condicions ambiental que A. carbonarius, principalment a una menor aw i una major T, indicant que aquestes espècies podrien estar millor adaptades als escenaris climàtics predits per al sud d'Europa. No obstant, *A. carbonarius* va mostrar una possible adaptació front a condicions ambientals desfavorables, ja que els aïllats del sud d'Espanya van créixer a a_W baixes.

A. carbonarius va ser la principal espècie ocratoxigènica tant en el nombre d'aïllats productors d'OTA com en les quantitats produïdes; de fet, els mosts contaminats d'OTA provenien de vinyes amb una elevada infecció per *A. carbonarius*. D'aquest estudi es deriven algunes conseqüències micotoxicològiques, com que l'increment de la temperatura ambient pot comportar una disminució del risc per OTA degut a una menor presència d'A. carbonarius i un increment del risc per FB_2 degut a l'augment en la presència d'*A. niger*.

La radiació UV va afectar la viabilitat dels conidis. L'increment en la irradiació podria afectar a l'inòcul global present en el camp, que podria disminuir , així com a les espècies potencialment productores d'OTA i AFs, un cop que s'hagi produït la germinació dels conidis i el creixement del miceli. Particularment, els aspergils negres van mostrar una major resistència a la radiació UV entre les espècies d'*Aspergillus* estudiades.

En general, els fungicides assajats van reduir el creixement fúngic, encara que es van observar diferències d'efectivitat entre ells. Paradoxalment, no van controlar el risc per OTA, i fins i tot alguns d'ells van estimular la seva producció. L'eficàcia dels fungicides es va veure afectada per les condicions ambientals i, per tant, en el futur podria ser necessari un canvi en les matèries actives utilitzades com a conseqüència dels canvis en les condicions climàtiques.

Conclusions

FUTURE RESEARCH

In the case of pistachio there is a need to reduce sampling uncertainty, as inapproppriate decisions may be derived from analytical results, therefore future research should be conducted to minimize the uncertainty through improved sampling operations.

Fumonisins risk has been identified in vineyards, and hence not only the presence and distribution of FBs producing black aspergilla FBs producers in this crop should be assessed, but also the environmental conditions that stimulate toxin production should be determined.

More studies are needed in order to clarify the effect of UV light in mycotoxin production and degradation to other metabolites. Studies *in vivo* would take into account the possible plant stress and therefore a more realistic situation would be described.

Several antifungals were efficient against fungal growth, nevertheless OTA production is poorly controlled. Moreover, *A. steynii* was a great OTA producer, but it was less affected by antifungal treatments and consequently, more studies about active ingredients and doses should be carried out in order to control it.

E. arvense extracts could have some potentiality as natural antifungals, nevertheless more studies on dosing are required.

FUTURAS INVESTIGACIONES

En el caso de los pistachos existe la necesidad de reducir la variabilidad en el muestreo, ya que se pueden tomar decisiones erróneas derivadas de los resultados analíticos. Por lo tanto, futuras investigaciones deben dirigirse a identificar la incertidumbre de los planes de muestreo.

El riesgo de fumonisinas ha sido identificado en viñedos, y por lo tanto no solo se debe determinar la presencia y distribución de las especies de aspergilos negros productoras de FBs en este cultivo, sino también las condiciones ambientales que podrían estimular su producción.

Se debería realizar un estudio más extenso para clarificar el efecto de la luz UV en la producción de micotoxinas y en la degradación de otros metabolitos. Además, deberían realizarse estudios *in vivo* teniendo en cuenta el posible estrés de la planta, a fin de describir situaciones más cercanas a la realidad.

Aunque varios fungicidas fueron eficaces en el control del crecimiento fúngico, la producción de OTA fue escasamente controlada. Principalmente*, A. steynii* fue un gran productor de OTA, pero fue muy poco afectado por los tratamientos antifúngicos estudiados. En consecuencia, se debería realizar más estudios acerca de las materias primas con el objetivo de estableces medidas para el control de las principales especies ocratoxigénicas.

Se ha observado que los extractos de *E. arvense* podrían tener un uso potencial como fungicidas naturales, sin embargo, más estudios serían necesarios en lo referente a su óptima dosificación.

FUTURES INVESTIGACIONS

En el cas dels festucs existeix la necessitat de reduir la variabilitat en el mostreig, ja que es poden prendre decisions errònies derivades dels resultats analítics. Per tant, les futures investigacions han de dirigir-se a identificar la incertesa dels plans de mostreig.

El risc de fumonisines ha estat identificat en la vinya, i per tant no només s'ha de determinar la presència i distribució de les espècies d'aspergils negres productores de FBs en quest cultiu, sinó també les condiciones ambientals que podrien estimular la seva producció.

S'hauria de realitzar un estudi més extens per aclarir l'efecte de la llum UV en la producció de micotoxines i en la degradació d'altres metabòlits. A més, s'haurien de realitzar estudis in vivo tenint en compte el possible estrès de la planta, amb la finalitat de descriure situacions més properes a la realitat.

Encara que varis fungicides van ser eficaços en el control del creixement fúngic, la producció d'OTA va ser escassament controlada. Principalment, A. steynii va ser un gran productor d'OTA, però va ser molt poc afectat pels tractaments antifúngics estudiats. En conseqüència, s'haurien de realitzar més estudis sobre les matèries primeres amb l'objectiu d'establir mesures pel control de les principals espècies ocratoxicogèniques.

S'ha observat que els extractes d'E. arvense podrien tenir un ús potencial com fungicides naturals; no obstant, serien necessàris més estudis per establir la seva dosificació òptima.

VII. ANNEX

Review

Emerging risk management metrics in food safety: FSO, PO. How do they apply to the mycotoxin hazard?

García-Cela E., Ramos A.J., Sanchis V. and Marin S.

Food Technology Department, Lleida University, XaRTA-UTPV, Rovira Roure 191, 25198 Lleida, Spain

ABSTRACT

This review focuses on risk management issues applied to mycotoxins and, in particular, the Codex Alimentarius recommendations for microbiological hazards are considered. Mycotoxins are chemical hazards from microbiological origin, thus some parallelisms can be found. Firtsly, a revision of main points regarding risk assessment is done. Then, the existing control measures for risk management of mycotoxins are reviewed and ALOP, FSO and PO concepts are introduced. Finally, an example of the application of these metrics is included: the processing of roasted pistachio is considered. The starting point was the maximum levels in Commission Regulation 1881/2006 for total aflatoxins. Having these values in mind, the process steps were individually considered and PCs determined when required. Moreover, according to these PCs, possible PcC and PdC were calculated, using previously published results. The present study demonstrates that the emerging risk management metrics, FSO, PO and PC, might be also applied to the mycotoxin hazard. The example here presented underlines the need for better and more structured information on the impact of the storage and processing steps on mycotoxins accumulation. Moreover, the problem of the impact of uncertainty in checking PO and FSO compliance was brought up.

Publication: *Food Control, 25(2012), 797-808*.

List of abbreviations

ADI, Acceptable Daily Intake; **AFs**, Aflatoxins; **ALOP**, Approppiate Level of Protection; a_W, Water activity; **CAC**, Codex Alimentarius Commission; **CM**, Control Measure; **DON**, Deoxynivalenol; **EC**, European Commission; **EU**, European Union; **FAO**, Food and Agriculture Organization; **FB**, Fumonisins; **FSO**, Food Safety Objective; **GAP**, Good Agricultural Practice; **GHP**, Good Hygiene Practices; **HACCP,** Hazard Analysis and Critical Control Point; **Ho**, Initial level of the hazard; **HPO**, Hand Pick Out; **HT2**, HT-2 toxin; **I**, Increase of the hazard; **IARC**, International Agency for Research on Cancer; **IPSM**, Integrated Phytosanitary Management; **JECFA,** Joint FAO/WHO Expert Committee on Food Additive; **m.c**., Moisture content; **NOAEL**, No Observed Adverse Effect Level; **OTA**, Ochratoxin A; **PAT**, Patulin; **PC**, Performance Criteria; **PcC,** Process Criterion; **PdC**, Product Criterion; **PMTDI**, Provisional Maximum Tolerable Daily Intake; **PO**, Performance Objective; **PTWI**, Provisional Tolerable Weakly Intake; **R**, Reduction of the hazard; **R.H.**, Relative Humidity; **RASFF**, Rapid Alert System for Food and Feed; **SCF**, Scientific Committee on Food; **SPS**, Sanitary and Phitosanitary Measures; **T2**, T-2 toxin; **TDI**, Tolerable Daily Intake; **U**, Measurement uncertainty; **WTO**, World Trade Organization; **ZEA**, Zearalenone.

1. INTRODUCTION

Food-borne risks to human health can arise from hazards that are biological, chemical or physical in nature. Food safety generally refers to the prevention of illnesses resulting from the consumption of contaminated food (Akkerman et al., 2010).

A key discipline for further reducing food-borne illness and strengthening food safety systems is risk analysis. During the last several decades, risk assessment, risk management and risk communication have been formalized and incorporated into the specific discipline known as food safety risk analysis. This approach has now gained wide acceptance as the preferred way to assess possible links between hazards in the food chain and actual risks to human health, and takes into account a wide range of inputs to decision-making on appropriate control measures. When used to establish food standards and other food control measures, risk analysis fosters comprehensive scientific evaluation, wide stakeholder participation, transparency of process, consistent treatment of different hazards and systematic decision-making by risk managers. Application of harmonized risk analysis

principles and methodologies in different countries also facilitates trade in foods (FAO, 2006).

World Trade Organization (WTO) members are bound by the provisions of the Sanitary and Phitosanitary (SPS) Agreement, which places risk assessment within a coherent SPS system for developing and applying standards for food in international trade. The scope of the SPS Agreement covers risks to human life and health, and requires that WTO members: i) shall ensure that any measure is applied only to the extent necessary to protect human life and health; ii) shall base their measures on risk assessment, taking into account the techniques developed by the relevant international organizations; iii) may implement a measure that differs from international norms where a higher "appropriate level of health protection" is a legitimate goal; iv) shall apply the principles of equivalency where a different measure in an exporting country achieves their appropriate level of protection (ALOP) (FAO, 2006).

Today in place systems like Hazard Analysis and Critical Control Point (HACCP) are developed to manage food safety, based on risk management principles and cover a range of biological, chemical and physical hazards. The basis idea behind a HACCP system is to provide a structured way to identify food safety risks and reduce or eliminate them (Akkerman et al., 2010). Recently the food safety management approach has been completed and developed through the inclusion of other metrics like the Food Safety Objective (FSO) (ICMSF, 1998). The FSO specifies a goal which can be incorporated into the design of control measurements in the food chain corresponding with the maximum permissible level of a hazard in a food at the moment of consumption which leads to an ALOP. Maximum hazard levels at other levels along the food chain are called Performance Objectives (POs) (CAC, 2007). The application of these food safety approaches to the mycotoxin hazard will be discussed in this review.

2. MYCOTOXINS: CHEMICAL HAZARDS

Mycotoxins are natural contaminants in raw materials, foods and feeds. Some mycotoxins can cause autoimmune illnesses, have allergenic properties and some of them are teratogenic, carcinogenic, or mutagenic (CAST, 2003).The conditions for mycotoxin production by fungi vary widely, but in general, it depends on nutrients availability, moisture level, pH, temperature, strain, and presence or absence of specific gases. Therefore, the presence of potentially toxigenic fungi does not imply the presence of mycotoxins. In addition, the

finding of mycotoxins does not prove that a particular fungal species was or is present (Fung and Clark, 2004).

Foods associated with fungal alterations are characterized by a low value of water activity (a_W) or a low pH value, where fungi may be imposed on the colonization of bacteria and yeasts. Therefore the main food groups contaminated by fungus are cereals and their derivatives, nuts and fruits (CAST, 2003). On the other hand the major mycotoxin-producing fungal genera are *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*. Nonetheless, although thousands of mycotoxins exist, the most important for public health are aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT), fumonisins (FB), zearalenone (ZEA), and trichothecenes.

2.1. Aflatoxins

In relation with the effects produced in the human health, the most dangerous mycotoxins are the AFs. AFs were identified in the early 1960s, and are mainly produced by *Aspergillus flavus* and *A. parasiticus*. Crops usually affected are corn, cotton, peanuts, and certain tree nuts (CAST, 2003). Naturally occurring AFs are AFB_1, AFB_2, AFG_1 and AFG_2, being AFB_1 the most abundant, toxic and carcinogenic (IARC, 2002b). AFM_1 and AFM_2 are respectively the hydroxilation products of AFB_1 and AFB_2 where M denotes milk or mammalian metabolites. They are found in milk and dairy products in different countries (Cano-Sancho et al., 2010; Prandini et al., 2009; Rahimi et al., 2010). International Agency for Research on Cancer (IARC) classified naturally-occurring AFs as human carcinogens based on the evidence from animal studies, epidemiological studies in exposed populations and mechanistic data. In experimental animals, liver is the predominant tumour site in rats, mice, hamsters, trout, salmon, ducks, tree shrews and monkeys. Tumours at other sites, e.g. kidney, have been observed but are much less common (Wild and Gong, 2009). Exposure to AFs is typically by ingestion of contaminated foodstuff. Dermal exposure results in slow and insignificant absorption (Riley et al., 1985).

2.2. Ochratoxin A

Ochratoxins were identified in 1965. Filamentous fungi belonging to the genera *Penicillium,* mainly *Penicillium verrucosum,* and *Aspergillus* sections *Circumdati* and *Nigri* are recognized as the source of OTA. In the *Circumdati* section *Aspergillus westerdijkiae* and *Aspergillus steynii* have

acquired more relevance than *Aspergillus ochraceus,* considered for a long time as the main source of OTA (Gil-Serna et al., 2011) and in the *Nigri* section, *Aspergillus carbonarius* is the main OTA producer followed by species belonging to the *A. niger* aggregate (Abarca et al., 2004). The mainly contaminated crops are cereals, as well as coffee, wine grapes and dried grapes (Coronel et al., 2011; Hussein and Brasel, 2001; Manning and Wyatt, 1984). In the group of ocratoxins ocratoxin B and C also exist, however OTA is the most prevalent and relevant fungal toxin. IARC classified OTA as a possible human carcinogen on group 2B based on the evidence from diverse studies (IARC, 2002b). Often, a single mycotoxin can cause more than one type of toxic effect. The common organ affected by OTA toxicity in all mammalian species tested is the kidney, where lesions can be produced by both acute and chronic exposure (Harwig et al., 1983), although it affects liver, fat and muscle tissues too (Krogh et al., 1974). Much has been written regarding the possible role of OTA in etiology of these phenomena and detailed reviews on OTA toxicology have been published (Mantle, 2002).

2.3. Fumonisins

FBs were identified in 1988. They are produced by different strains of *Fusarium* and to a lesser extent by *Alternaria*. Recently, *A. niger* has been reported as FB_2 productor (Frisvad et al., 2007). They affect a wide range of foodstuffs specially maize (Fung and Clark, 2004). In total, 16 different types of fumonisins have been isolated and characterized, however, in naturally contaminated samples, FB_1 accounts for 70% of fumonisin presence (Plattner et al., 1992). IARC classified FB_1 as a possible human carcinogen in group 2B based on the evidence from diverse studies (IARC, 2002b). Several studies have described the toxic effects of fumonisins in animals like equineleukoencephalomalacia (ELEM), hepatotoxic syndrome in horses (Butler, 1902; Kellerman et al., 1972), and pulmonary edema in pigs (Kriek et al., 1981). In humans, fumonisins have been associated with an increased risk of esophageal carcinoma in certain areas (Chu and Li, 1994).

2.4. Patulin

PAT was discovered in 1940s in UK, as a possible treatment against flu. Its production has been detected in genera like *Byssochlamys, Aspergillus* and *Penicillium*, however *P. expansum* is the main responsible of the accumulation of this toxin in food (Betina, 1989).The foodstuffs affected are mainly apples and pears, but also cereals, nuts and roots or rhizomes (Soriano

and Dragacci, 2007). Patulin is a common contaminant of apple juice, concentrated juice, puree, and unfermented cider (Cano-Sancho et al., 2009; Stoloff, 1975). (Viñas et al., 1993) observed that almost 50% of the apples from fruit cold stores with evidences of blue rotcontained patulin. Therefore, wounded apples maybe contain and patulin and this shoulbe taken into account when they are used for juices, concentrated juices or subproducts (Baert et al., 2006; Boonzaaijer et al., 2005; Gökmen and Acar, 1998). Despite it has been classified as Group 3 (IARC, 1999), the chronic toxicity caused by patulin includes neurotoxic, immunotoxic, genotoxic, teratogenic and possibly carnicogenic effects (Hopkins, 1993; Pfeiffer et al., 1998; Wichmann et al., 2002).Patulin is not found in either alcoholic fruit beverages or vinegars produced from fruit juices, thus it is reported to be destroyed by fermentation. However, patulin survives pasteurization processes that cause only moderate reductions in patulin levels (Harrison, 1989; IARC, 1986; McKinley and Carlton, 1991; WHO/IARC, 1990).

2.5. Zearalenone

ZEA is a non-steroidal estrogenic mycotoxin produced by several *Fusarium* species. It is found around the world in a wide number of cereal crops, ZEA producing species are the major causative fungi of head blight of wheat, barley, and maize (Kawashima and Valente Soares, 2006; Kuiper-Goodman et al., 1987; Tanaka et al., 1988) and their food products, such bread, pastry and bakery products (Aziz et al., 1997). IARC classified ZEA in group 3 (IARC, 2002b). ZEA has been implicated in numerous incidents of mycotoxicosis in farm animals, especially in swine, causing infertility, abortion or other breeding problems (Kanora and Maes, 2009; López et al., 1988).Like other mycotoxins, it can be excreted from mammalians which were nourished with contamitated feed as alpha-zearalenol and beta-zearalenol metabolites. It can also be present in the beer made with contaminated grains (Chen et al., 2000).

2.6. Trichothecenes

Trichothecenes are produced mainly by several species of *Fusarium* but also by *Stachybotrys*, *Trichoderma*, and *Trichothecium*. They are the largest group of mycotoxins, consisting of more than 150 chemically-related toxic compounds classified in four groups,HT-2 toxin (HT2), T-2 toxin (T2) and deoxynivalenol (DON) being the most common. They are usual contaminants of cereals like wheat, barley, oats and maize.Thus, a wide range of cereal-based

foods have been confirmed to be contaminated by these toxins ratifying that food processing methods do not completely remove these mycotoxins from the matrix (Hazel and Patel, 2004; JECFA, 2001). Trichothecenes are strong inhibitors of protein synthesis in mammalian cells causing a wide range of toxic effects in animal and humans such as feed refusal, vomiting, diarrhea, hemorrhage, anemia and immunosuppression (Hussein and Brasel, 2001). Compared to some of the other mycotoxins such as AFs, the trichothecenes do not appear to require metabolic activation to exert their biological activity. Although DON is not as toxic as other trichothecenes such as T2 or HT2, this mycotoxin is one of the most common contaminants of cereals worldwide (Jelinek et al., 1989; Scott et al., 1989).

3. RISK ASSESSMENT FOR CHEMICAL HAZARDS

Chemical hazards in foods include food additives, environmental contaminants such as mercury and dioxins, natural toxicants in food, such as glycoalkaloids in potatoes and aflatoxins in peanuts, acrylamide, and residues of pesticides and veterinary drugs. As opposed to microbiological hazards, chemical hazards usually only enter foods in the raw food or ingredients, or through certain processing steps, and the level of hazard present in a food after the point of introduction often does not significantly change. Moreover, health risks may be acute but are generally chronic, and types of toxic effects are generally similar from person to person, but individual sensitivity may differ (FAO, 2006). In the particular case of mycotoxins, as chemical hazards from microbiological origin, they may increase in concentration through the processing steps, if conditions are conducive for fungal growth.

3.1. Hazard characterization

During hazard characterization, risk assessors describe the nature and extent of the adverse health effects known to be associated with the specific hazard. This includes consideration of mechanistic aspects (e.g. whether the mechanism of action of the chemical observed in often high-dose experimental studies is also relevant to human exposure at lower levels). If possible, a dose-response relationship is established between different levels of exposure to the hazard in food at the point of consumption and the likelihood of different adverse health effects. Adverse health effects are usually predicted for long-term exposure to chemicals. For certain chemicals, such as some mycotoxins, marine toxins, pesticides and veterinary drugs, both acute and chronic health effects need to be considered.

In cases where the toxic effect results from a mechanism that has a threshold, hazard characterization usually results in the establishment of a safe level of intake, an acceptable daily intake (ADI), or tolerable daily intake (TDI) for contaminants (FAO, 2006). Most mycotoxins are considered to act through a non-genotoxic mechanism. This allows the assumption of a practical biological threshold of effect, and consequently the derivation of a tolerable intake level via the determination of a no observed adverse effect level (NOAEL) for a surrogate biological endpoint and the application of factors to ensure the safety. Tolerable intake, which can be expressed in daily, weekly or monthly basis, is an estimate of the amount of a contaminant that can be ingested over a lifetime without appreciable risk.

Estimation of the ADI or TDI (provisional tolerable weakly intake, PTWI) includes the application of default "uncertainty factors" to a no-effect-level or low-effect level observed in experimental or epidemiological studies, to account for uncertainties inherent in extrapolating from an animal model to humans and to account for inter-individual variability. Safe levels of intake for the more frequent non genotoxic mycotoxins occurring in food are shown in **Table 1**.

Table 1 Safe levels of intake for the more frequent non genotoxic mycotoxins occurring in food. (Source of date: Opinion of the Scientific Committee on Food on Deoxynivalenol (1999), Patulin (2000a), Zearalenone (2000b), T-2 toxin and HT-2 toxin (2002), Nivalenol (2000c) and Fumonisin B_1, B_2 and B_3 (2003) and Summary and Conclusions of the Sixty-eight Meeting of the JEFCA (2007).

Mycotoxin	**Safe level of intake**	**References**
OTA	PTWI=100	JECFA (2007)
FBs	TDI=2	SCF (2003)
PAT	PTDI=0.4	SCF (2000a)
ZEA	PTDI=0.2	SCF (2000b)
DON	TDI=0.4	SCF (1999)
T2	PTDI=0.06	SCF (2002)
HT2	PTDI=0.06	SCF (2002)
NIVALENOL	PTDI=0.7	SCF (2000c)

Toxicological reference values used by different authorities for (genotoxic) carcinogenic chemicals vary. Some are based on a combination of epidemiological and animal data, some may be based on animal data alone, and different mathematical models may be used to

extrapolate risk estimates to low doses. These differences can lead to significant variability in cancer risk estimates for the same chemical (FAO, 2006). As regards AFs, the Scientific Committee on Food (SCF) expressed in its opinion of 23 September 1994 that AFs are genotoxic carcinogens (SCF, 1999)F, 1996).

3.2. Exposure assessment

Exposure assessment describes the exposure pathway or pathways for a chemical hazard and estimates total intake. For some chemicals, intake may be associated with a single food, while for others the residue may be present in multiple foods. Exposure assessment characterizes the amount of hazard that is consumed by various members of the exposed population(s). The analysis makes use of the levels of hazard in raw materials, in food ingredients added to the primary food and in the general food environment to track changes in levels throughout the food production chain. These data are combined with the food consumption patterns of the target consumer population to assess exposure to the hazard over a particular period of time in foods as actually consumed. For chemicals, exposure assessment often uses values at certain points on the continuum of exposure, such as the mean or the 97.5th percentile (FAO, 2006)

3.3. Risk characterization

The outcome of the exposure assessment is compared to the TDI in order to determine whether estimated exposures to the chemical in foods are within safe limits.

Risk characterization for chronic exposure to chemical hazards does not typically include estimates of the likelihood and severity of adverse health effects associated with different levels of exposure. A "notional zero risk" approach is generally taken and where possible the goal is to limit exposure to levels judged unlikely to have any adverse effects at all.

For example, considering exposure to OTA, it seems to be in most cases quite below the TDI (14 ng/kg bw/day). Nevertheless, some countries appear to be under a more relevant exposure especially if specific group of consumers are considered, as shown for UK population in the range of 1.5-4.5 years, which overpasses the TDI (JECFA, 2007).

Regarding PAT exposure reports seem to be quite below the provisional maximum tolerable daily intake (PMTDI) (0.4 μg/kg bw/day). Nevertheless, some countries seem to be suffering from a more relevant contamination, still under the PMTDI, especially in a worst case situations and if specific group of consumers especially small children are considered (SCF, 2000).

Exposure to *Fusarium* toxins studied was found to be considerably below the TDI values. Higher intakes and a transgression of the TDI values were observed for the group of infants and children. Intakes higher than the TDI were noted for the sum of T-2 and HT-2. For DON, the average intake level did not exceed 46.1% of the TDI of 1 μg/kg bw/day. However, for young children the intake might approach the TDI (SCF, 2002).

Quantitative risk assessment methodologies have only rarely been applied for chemical hazards thought to pose no appreciable risk below certain very low levels of exposure, probably because the approach described above has generally been considered to provide an adequate margin of safety without a need to further characterize the risk (FAO, 2006).

In contrast, quantitative risk assessment models have been applied by some governments as well as by international expert bodies (JECFA) for effects that are judged to have no threshold, i.e. for genotoxic carcinogens such as AFs. These models employ biologically-appropriate mathematical extrapolations from observed animal cancer incidence data (usually derived from tests using high doses) to estimate the expected cancer incidence at the low levels typical of ordinary human exposure. If epidemiological cancer data are available, they also can be used in quantitative risk assessment models (FAO, 2006) Scientific knowledge allows the identification of a practical biological 'threshold' experimentally, or the identification of an exposure level that correlates to an acceptable level of risk, via dose–response modelling and quantitative risk assessment.

AFs exposure has been correlated to human liver cancer. Observations concerning the interaction between hepatitis B infection and AFs suggest two separate AFs potencies; one is apparent in populations in which chronic hepatitis infections are common, the other in populations in which chronic hepatitis infections are rare. Mean potency values for these two groups were chosen, of 0.3 and 0.01 cancers per year per 100000 population per ng AFs ingested per kg body weight per day, respectively (JECFA, 1999).

4. RISK MANAGEMENT

All these toxicological evaluations are the basis for the existing maximum permitted levels of chemical hazards in food. Analysis of the derivation of maximum levels in food differentiates between the duties of the various risk management and risk assessment bodies. The latter evaluate the health effects and intake of a contaminant (including derivation of values for TDI). Risk managers, by setting maximum levels, consider the outcome of the risk assessment process, the concentration of a contaminant in food, socio-economic arguments, the technical feasibility of derived values and other issues. Because setting maximum levels by the Codex Alimentarius Commission (CAC) and European Union (EU) requires the agreement of independent nations, political debate is also an obvious part of the risk management process (Schneider et al., 2007).

Within the EU, the maximum levels legally bind to all member states. By definition, the Codex Alimentarius values only represent recommendations, yet they have attained a prescriptive character due to their acceptance by the WTO as international hygienic standards.

Food safety measures based on risk assessments are generally designed to reduce risks to a target level, and risk managers must determine the degree of health protection they are aiming to achieve. Through good communication with risk managers, risk assessors will likely have examined the relative impacts of different controls on reducing risks, providing the risk managers with objective data that supports decisions on the most appropriate controls. The overriding objective of risk management is to maximize risk reduction while ensuring that the measures employed are efficient and effective and not overly restrictive (FAO, 2006). Where chemicals are not intentionally used in food production settings, more specific risk management options often are evaluated (e.g. imposing conditions on harvesting, providing information to consumers so that they can voluntarily limit exposure).

Exposure guidelines such as PTWIs can then provide a reference point for maximum safe intake, and risk management measures can be put in place that aim to prevent consumers from exceeding that safe upper limit of exposure. When other risk modelling approaches are used, such as linear modelling for carcinogenic effects, different risk management options may be identified and evaluated, such as banning or severely restricting the presence of the chemical.

4.1. The ALOP concept

The concept of ALOP was introduced in the WTO Agreement on the application of the SPS Agreement in 1995 (WTO, 1995). An ALOP is defined in the SPS agreement as: "The level of protection deemed appropriate by the Member establishing a sanitary or phytosanitary measure to protect human, animal or plant life or health within its territory". The purpose of the SPS-Agreement was to increase the transparency of SPS-measures. It is the prerogative of individual Member states to determine what constitutes an ALOP that is appropriate for its population ((de Swarte and Donker, 2005). The acceptable level of risk is the level adopted following consideration of public health impact, technological feasibility, economic implications, and that which a society regards as reasonable in the context of and in comparison with other risks in everyday life (Schothorst, 1998)(van Schothorst, 1998).

An ALOP can be expressed in a range of terms, for instance from broad public health goals to a quantitative expression of the probability of an adverse public health consequence or an incidence of disease (de Swarte and Donker, 2005) . This concept was initially defined for microbiological hazards. With mycotoxins, often there is no proof of causality between the hazard and an individual case of a food-borne disease because impacts of chemical hazards may be more chronic in nature. On the other hand the TDI concept is based on scientific considerations, which certainly can be taken into account in the ALOP/FSO approach.

Two main approaches are applied to setting an ALOP in selecting risk management options in the mycotoxin case:

- *Notional zero risk approach*. Hazards are kept at levels that equate to a pre-determined "negligible" or "notional zero" risk, based on a risk assessment indicating that such low exposure levels are reasonably certain not to cause harm. This is the approach applied to most mycotoxins. For the majority of mycotoxins no acute effects are observed thus the dose-response relationship can not be derived. This approach does not produce precise estimates of risk versus dose and cannot model the impact of various interventions in terms of risk reduction. It thus provides an ALOP that is pre-determined by public policy to be "notional zero risk" (FAO, 2006).

- *"Threshold" approach.* Risks must be kept below a specific numerical level as pre-determined by public policy; this approach may be used for chemical hazards, particularly carcinogens. A level of risk that is judged acceptable can be defined by public policy, and risk management measures can then be chosen to keep risk below that "threshold," sometimes referred to as a "virtually safe dose." The FSO and ALOP are linked by the dose–response relationship which estimates the risk of illness given a specified consumption of a hazard.

The threshold approach is applied to AFs. The 49th Joint FAO/WHO Expert Committee on Food Additive (JECFA) session held in 1999 took as example, an area with low AFs food contamination and with a population having a small prevalence of carriers of hepatitis B: AFs levels based on European monitoring of AFB_1 in peanuts, maize and their products were used, and a population with 1% carriers of hepatitis B was assumed. From the potencies given earlier, this yielded an estimated average population potency of 0.013 cancers per year per 100000 population per ng AFs per kg body weight per day. Based on European monitoring, if all lots with contamination above 20 μg/kg are removed and it is assumed that these foods are ingested according to the "European diet", the mean estimated intake of AFs is 19 ng per person per day. Assuming an adult human weight of 60 kg, the estimated population risk is 0.0041 cancers per year per 100000 people. If a 10 μg/kg hypothetical standard is applied, the average AFs intake is 18 ng per person per day, resulting in an estimated population risk of 0.0039 cancers per year per 100000 people. Thus, reducing the hypothetical standard from 20 μg/kg to 10 μg/kg yielded a drop in the estimated population risk of approximately two additional cancers per year per 10^9 people, well beyond the level of detection. The second example pertained to areas with higher contamination. For these purposes, Chinese data on AFB_1 in peanuts, maize and their products were used and areas with a larger population fraction as carriers of hepatitis B (in this case, a population with 25% hepatitis B carriers was assumed). The estimated potency for this population is 0.083 cancers per year per 100000 people. Using 20 μg/kg and 10 μg/kg hypothetical standards and the "Far Eastern" diet, the average estimated intake was 125 ng AFs per person per day yielding an average population risk of 0.17 and 0.14 cancers per year per 100000 people, respectively. Thus, reducing the hypothetical standard for this population from 20 μg/kg to 10 μg/kg yielded a drop in the estimated population risk of 0.03 cancers per year per 100000 people. This is a greater decrease in risk, but still barely detectable (JECFA, 1999; Pitt, 2004).

The major improvements of the ALOP/FSO methodology relating to risk assessment are i) that current risk assessment focuses mainly on life sciences, while ALOP/FSO methodology must also take into account socio-economic and technological consequences of risk management; consequently, in the future, life sciences, social sciences and engineering need to co-operate more closely to develop integrated scenarios for assessing risk management options, and ii) in order to set meaningful ALOPs and consequent FSOs a better knowledge of the impact of a food safety hazard is needed. Epidemiological data can help gain more insight on the impact and to develop models on major sources of infection and public health impact of food-borne illnesses. Often, epidemiological data are not accumulated in a way that it is directly usable in risk assessment. There is clear room for improvement in that respect (de Swarte and Donker, 2005).

4.2. The FSO concept, a food safety management metric

The FSO is the maximum frequency and/or concentration of the hazard in a food at the time of consumption and is preceded by the PO, which is the maximum frequency and/or concentration of a hazard in a food at a specified step in the food chain before the time of consumption (ICMSF, 2002), that still provides or contributes to the achievement of an FSO or ALOP, as applicable. While Codex considers FSOs only for microbial hazards (the maximum frequency and/or concentration of a microbiological hazard in a food at the time of consumption that provides the appropriate level of protection) (C. A. C. CAC, 2003), in principle, the concept could apply to other types of hazards as well.

In this context, the agro-food industry would use FSOs as means to co-ordinate risk management in the production process throughout the farm-to fork production chain (de Swarte and Donker, 2005).In the particular case of mycotoxins, both mycotoxigenic fungi (which is not a biological hazard per se) and mycotoxins, as chemical hazards, should be controlled.

Once an FSO is set, the food industry is responsible for setting up management systems that deliver a level of food safety in compliance to the FSO. Performance criteria (PC) and other metrics on the operational level can be derived by food industry from FSOs by chain-reversal, in effect articulating appropriate food safety standards for individual links in the chain. Such standards as well as particular control measures that government may choose to

mandate should be enforced and inspected by (private and public) certification and inspection systems (de Swarte and Donker, 2005).

In the case of a chemical hazard such as mycotoxins, the limits set by a country for mycotoxins in foods can be logically considered also to have the status of a FSO.

Full implementation of the FSO concept calls for a quantitative FSO so that PO and PC can be specified, and a HACCP plan developed. Ideally, all the approaches described above would converge on an appropriate FSO for a food.

4.3. Meeting the FSO

Good hygiene practices (GHP) and HACCP are the primary tools available to control chemical hazards in food operations. Thus, FSOs must be based on a realistic assessment of what can be achieved through GHP and HACCP.

POs are linked to the FSO and, when proposed by governments, can be viewed as a kind of milestones that governments provide as guidance in order to help meet the FSO. For example, (European Commission, 2006) sets certain maximum limits for cereals and nuts which still have to undergo physical treatments before direct human consumption. However, POs can also be decided on by operational food safety managers as an integral part of the design of the production of a food in a supply chain.

A PC, is the effect of one or more control measure(s) needed to meet or contribute to meeting a PO, while a Control Measure (CM) is any action and activity that can be used to prevent or eliminate a food safety hazard or to reduce it to an acceptable level (it can be products specifications, guidelines on microbial control, hygiene codes, maximum levels, specific information). There are many different types of CM, instigated by regulation or chosen by the industry, the proper functioning of which needs to be monitored and verified by the industry.

A broad range of CM is used in the food continuum from primary production, processing and manufacturing, transport and distribution, storage and retail to preparation and consumption of the food. CM may include a variety of practices applied at various stages

(e.g., good agricultural and animal production practices, good hygiene practices during manufacture and processing, good consumer handling practices) (JECFA, 2006).

CM in the food industry regarding mycotoxins may fall into these activities:

- Ensuring control of initial levels of hazards (e.g. avoiding nuts and spices from certain origins, avoiding raw materials from primary producers not adhering to good agricultural practices, establishing requirement specifications with suppliers and requiring verifiable documentation e.g., letters of guarantee or certificates of analysis attesting the status of microbiological, chemical and physical hazards in the incoming raw material, using sampling and analyses, as necessary, and using appropriate methods based on established criteria to reject unacceptable ingredients or products).

- Preventing an unacceptable increase of hazards

a) preventing contamination, for example adopting GHPs, that minimise mycotoxin contamination from transport, drying and storage facilities establishments or processing equipment and from the aqueous solutions in fruits and nuts, due to poor renovation. GHPs besides minimize product contamination through cross-contamination between raw and processed product; for the particular case of mycotoxins, it is also important to prevent from contamination by mycotoxigenic fungi, which may further develop and produce mycotoxin in subsequent process stages.

b) preventing fungal growth during transportation, storage and processing, for example, cold storage of apples, adjusting a_W in stored cereals, nuts, coffee or spices, adding preservatives in stored fruits and cereals, controlling temperature and moisture/ a_W in dehydrating fruits, adjusting storage times, use of packaging techniques and materials to protect food from contamination, or implementing effective controls within the food processing environment (e.g., pest control).

- Reducing or eliminating hazards

a) selecting ingredients (e.g. applying electronic sorters to reject nuts that are likely to contain AFs, culling fruits for fruit juice production that are likely to contain patulin, rejecting rotten

grape bunches that are likely to contain OTA, cleaning of cereals will end in separation of mouldy grains which account for most of *Fusarium* toxins and AFs in a lot…).

b) additionally some measures which are not implemented to control mycotoxins or that are intrinsic of the food process, may exert a certain control on mycotoxins:

inactivating mycotoxins, to some extent, because mycotoxins are quite heat stable (e.g. heat treatments, like roasting, frying, baking, commercial sterilisation, fermentation processes).

physical segregation of the most contaminated fractions of raw materials (e.g. milling of cereals, must extraction from grapes or malt, pressure-washing of apples, centrifugation, filtration…)

POs, as milestones, are not intended to be enforced but should provide guidance to designing the correct operational control measures at the step in the chain that the POs govern. Complying with the hazard level tolerated at the moment of consumption (FSO) is a shared responsibility for all parties together and requires an appropriate design of the complete chain which is helped by specifying POs and PCs as food control guidance targets or food safety management measures at relevant points in the production chain (Gorris, 2005).
In practice, FSOs are achieved through the establishment and implementation of performance and process criteria. In every step of the food chain it is necessary to know the effect of every treatment, PC, as well as the process parameters, Process Criterion (PcC) (t, T, pH, a_W) which can be applied in any level neither in the final product, Product Criterion (PdC) (pH, a_W, gaseous atmosphere). PdC assure that the hazard level never overtake safety levels before being cooked or consumed (Stringer, 2005).

When PC are established, a consideration must be given to the initial level of a hazard and changes occurring during production, distribution, storage, preparation and use of a product. PC account for a number of hazard increases and reductions that can be expressed by the following equation:

$$Ho - \sum R + \sum I \leq FSO$$

where

Ho=Initial level of the hazard
∑R=Total (cumulative) reduction of the hazard
∑I=Total (cumulative) increase of the hazard

FSO, Ho, R and I are expressed in μg/kg for mycotoxins.

Thus the PC indicate, the change in hazard level required at a specific step in order to reduce the hazard level at the start of the step (Ho) to a level at the end of the step that complies with the PO or the FSO when it is at the chain end. PCs in general will be decided on by food safety managers as key points in the design of the production of a food in a supply chain. PCs can be achieved by one or more CM and as such are a reflection of the concrete management measures that assure a product is safe and produced to the proper specifications. PCs can be about a required reduction of the hazard, avoiding increase (limit to 0) or assuring a minimal increase.

It should be recognized that the parameters that may be used in the above equation are point estimates, whereas in practice, they will have a distribution of values associated with them. If data exist for the variance associated with the different parameters, then the underlying probability distributions may be established using an approach similar to that in risk assessment (Cole, 2004).

4.3.1. AFs in pistachio nuts. A case study.

Nuts present low a_w, and due to their intrinsic characteristics, fungi are the major microbiological contaminants. Some of these moulds are mycotoxigenic, so high levels of mycotoxins have frequently been reported in nuts. One of the most consumed nuts in the world is pistachio. Presence of mycotoxigenic fungi in pistachio has been reported from the orchards and from the market (Bayman et al., 2003; Fernane et al., 2010a).Fungal infection mainly occurs during the nut developmental phase in the orchard and in post-harvest processing stages (Denizel et al., 1976; Fernane et al., 2010b). In the case of pistachios, the dominant mycobiota are *Aspergillus* section *Nigri*, *A. flavus* and *Penicillium* spp (Denizel et al., 1976; Fernane et al., 2010b).

Several studies have reported that *Aspergillus* spp. causes decay in nuts in different parts of the world, such as California (USA) (M.A. Doster and Michailides, 1994)Iran ((Mojtahedi et al., 1979), and Turkey (Denizel et al., 1976). The most important mycotoxins found are the AFB_1, B_2, G_1 and G_2 and OTA. In the last ten years (2000-2010) the Rapid Alert System for Food and Feed (RASFF) notified 7191 alerts, border rejections and informations regarding mycotoxins, of which 79.60% were for nuts, nuts products and seeds, 37.13% being for pistachio. The most frequent mycotoxins were AFs (2667 notifications), followed by OTA (5 notifications), besides co-occurrence was reported in two cases (RASFF, 2011).

In 2001, FAO published the Manual of the application of the HACCP system in mycotoxin prevention and control, considering two pistachio processing lines after harvest according to the different procedures applied in Asian producing countries. The fast dehulling process line involves fast dehulling (within 24 h after harvest) for preventing staining, floating segregation and quickly drying to 5-6% water content to prevent fungal development. The objective of this line is to reach a good-condition-for-storing product until it is further processed. This process is followed by the major producing countries. Other countries such Turkey or Syria, based on traditional practices, follow slow dehulling process lines, where pistachios are sun dryed and stored for months until they are dehulled, segregated by either flotation and drying or by air gravity separators. Subsequent steps are followed by both lines, including sorting, roasting, packaging and storage/shipping (Figure 1). Pistachios are sorted to remove closed-shell nuts which are sent to other industries for rehydration and mechanical or manual craking (Campbell et al., 2003). If required, hand sorting could complete other electronic processes for removing stained nuts and others with visible insect damage (Pearson and Schatzki, 1998); finally, very small and insect damaged nuts are sorted. It is known that high AFs levels are found in very small and insect damaged nuts, becoming this final process an important step to reduce mycotoxin contamination (Schatzki and Pan, 1996).

CAC proposed a maximum level of 15 μg/kg AFs total in almonds, hazelnuts and pistachios intended for further processing and a level of 10 μg/kg AFs total in 'ready-to-eat' almonds, hazelnuts and pistachios (Codex Stan, 1995). The European Commission (EC) recently amended the Commission Regulation 1881/2006 through Regulation 165/2010, imposing a maximum AFs level in pistachios to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs of 12 μg/kg (AFB_1), 15

μg/kg (total AFs), of 8 μg/kg (AFB_1) and 10 μg/kg (total AFs) for pistachio intended for direct human consumption or use as an ingredient in foodstuffs.

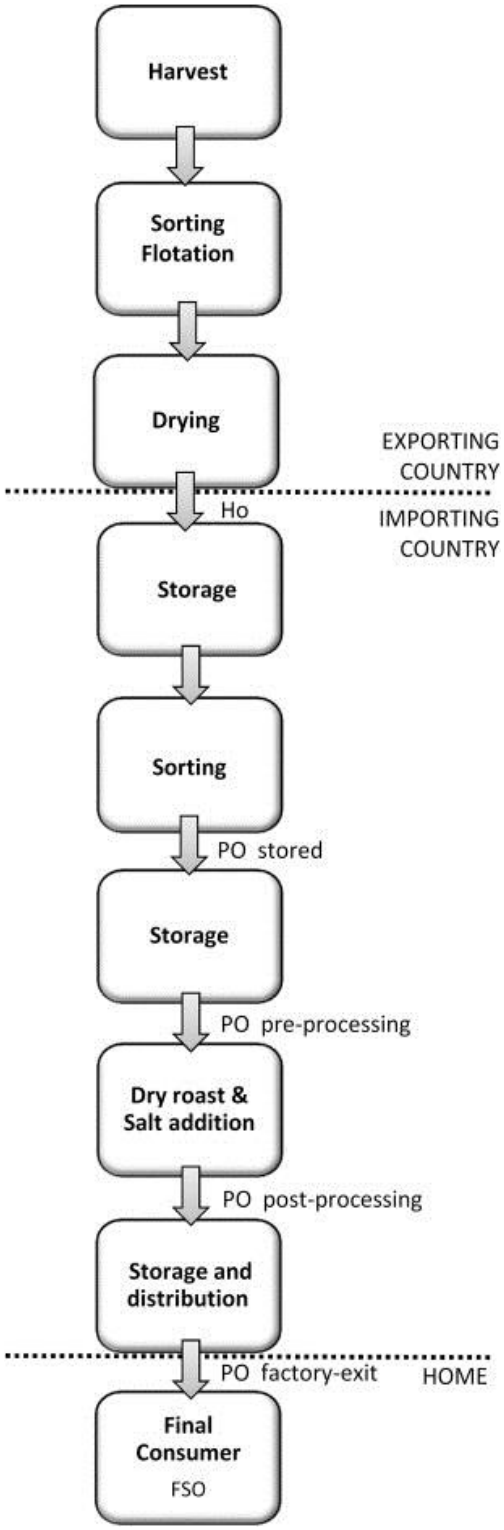


Figure 1 Flow diagram of processing of pistachio.

The aim of the present section is to analyze how the PO, PC, PcC and PdC concepts can be applied in the case of a pistachio importing and processing company to guarantee FSO compliance regarding AFs. As most of the existing literature deals with AFB_1, in some cases extrapolation to total AFs was done. Taking into account the ∑I and ∑R given by the processing steps, food managers must limit the levels of contaminants in the raw materials (Ho) in order to achieve the FSO. In the present case study the value of FSO for AFs is ≤10 μg/kg. The company chosen for the example imports hulled pistachios after flotation separation and drying, and undergoes sorting and roasting as main steps (**Figure 1**).

4.3.1.1 Initial level of AF contamination (Ho)

In our case the maximum value of Ho is that set by European Commision EC Regulation for total AFs in pistachios to be subjected to sorting, or other physical treatment, 15 μg/kg. An established Ho value should led processing companies to accept only those raw material batches which allow compliance with the final PO of the company in the final product. Evidently the Ho established must be achievable from application of Good Agricultural Practice (GAP) and Integrated Phytosanitary Management (IPSM) which seek to reduce the mould spore count in the orchard and reduce the chances of insect attack (Boutrif and Canet, 1998). These practices can assist in limiting mycotoxins formation, but do not guarantee their absence. Therefore if unrealistic low Ho values were required for assuring the FSO, processing steps might need to be redesigned in order to produce a safe product.

4.3.1.2. Increase of AF during storage and processing (∑I)

Increase of AFs concentration is linked to aflatoxigenic moulds present in pistachio. The only opportunities for this to happen are those in which conducive environmental conditions occur together with an extended period of time: initial storage of pistachio nuts and storage prior or after final packaging. Roasting is expected to kill fungi, thus, mycotoxin formation after roasting is unlikely, unless further fungal contamination occurs afterwards.

During storage steps a zero increase of AFs is desirable (Table 2). Provided pistachios are adequately dried and maintained in the dried state during storage, mycotoxin producing fungi cannot grow. Environmental conditions like temperature, moisture and atmosphere must be controlled; also a regular fumigation can be adequate for pest control during storage. Storage

temperature is a main factor on AFs accumulation in pistachio, with a sharp increase at 25-30ºC; maximum AFs levels were found at 20-30% moisture content (m.c.) in pistachios. For AFs prevention, pistachios should be kept under 10% m.c., alternatively they could be stored at a m.c.as high as 25% under cool conditions (<10ºC) (unpublished data).

Moreover, FAO (2001) recommended to reach a m.c.of 5 to 6% after drying and optimum storage conditions of 10ºC or lower and 65 to 70% relative humidity (R.H.). Moreover for postprocessing storage R.H below 70% and temperature between 0-10ºC is recommended depending on expected storage duration. The lower the temperature the longer the storage life.

4.3.1.3. Reduction in AFs levels during sorting and processing (∑R)

As absence of mycotoxins in the raw material cannot be guaranteed, relying on inductial processes for a certain AFs reduction is required.

a) Sorting

It is recognised that sorting and physical segregation significantly reduce the AFs content of consignments of nuts. Mycotoxins are mainly linked to mouldy nuts, damaged by insects, small, deformed and discoloured ones. Removal of pistachio nuts with high the contamination by sorting caused a decrease in contamination of 2 to 4 times in processed pistachios compared to non-processed pistachios (Schatzki, 1995). Park, (2002) quantified physical cleaning, where mold-damaged kernels, seeds or nuts are removed from the intact commodity, may result in 40–80% reduction of AFs. (Schatzki and Pan, 1996) related the AFs reduction from pistachios previously partitioned by water flotation with the elimination of the stained nuts, which include the scalpers, the eye rejects, the hand pick out (HPO) insects, the HPO dye floaters, and the meat sinkers. Considering that the company imports dehulled pistachios after flotation separation and drying, the company sorts by size and only removes the meat and the scalpers. The elimination of this part implies a roughly drop of 26 % on the AFs content and 2 % of the product (**Table 2**).

Table 2 Food safety metrics applied to total AFs in roasted pistachio production.

Step	PC	PcC	PdC	PO (μg/kg)
Receiving of pistachio	–	–	–	≤15 (1881/2006)
Storage	Zero increase	PC achievable by: <10% mc any *T* <10 °C any mc *T* < 20 °C mc < 20%	–	≤15
Sorting	26%	Separation of meats and scalpers	–	≤11.1
Storage	Zero increase	PC achievable by: <10% mc any *T* <10 °C any mc *T* < 20 °C mc < 20%	–	≤11.1
Roasting	About 30%	150 °C 20 min	–	≤7.77
	About 40%	150 °C 30 min		≤6.66
	About 50%	200 °C 20 min		≤5.55
Storage	Zero increase	PC achievable by: <10% mc any *T* <10 °C any mc *T* < 20 °C mc < 20%	–	≤7.77 ≤6.66 ≤5.55
Distribution	Zero increase	–	PC achievable by: <10% mc any T	≤7.77 ≤6.66 ≤5.55
Consumer	–	–	PC achievable by: <10% mc any *T*	FSO ≤ 10 (10-U) (1881/2006)

U = measurement uncertainty.

b) Roasting

AFs have high decomposition temperatures, ranging from 237 to 306ºC and AFB_1 is quite stable to dry heating (Betina, 1989; Rustom, 1997). Although these temperatures are higher

from those actually used by the nuts industry, it is usually accepted that the heat treatment decreases the concentration of AFs to some extent. However conflicting results have been published about the effect of the heat treatments on peanuts and pistachios (Ariño et al., 2009; Farah et al., 1983; Lee et al., 1969; Ozkarsli, 2003; Pluyer et al., 1987; Rustom, 1997; Waltking, 1971; Yazdanpanah et al., 2005). In general the extent of the destruction achieved was very dependent on the initial level of contamination, heating temperature and time. The effects of heat in naturally contaminated peanuts by oven roasting at 150ºC for 30 min caused a 30-45% reduction of AFB_1, while in artificially contaminated peanuts treated under the same conditions, the inactivation was 48-61% (Pluyer et al., 1987). Degradation of aflatoxins in peanuts roasted at 150 º C for 30 min increased with the addition of ionic salts in a range from 38%, 41.5% and 47.6% in unsalted peanuts, and salted with 20 $\mu g \cdot kg^{-1}$ and 50 $\mu g \cdot kg^{-1}$ respectively (Ozkarsli, 2003). In pistachio, the results regarding degradation of AFs due to roasting are also contradictory. (Yazdanpanah et al., 2005) studied the effect of roasting for 30, 60 and 90 minutes at different temperatures (90, 120 and 150ºC). The milder treatment (90ºC-30min) reported slightest effect while the most extreme treatment resulted in the degradation of over 95% of AFB_1 but the pistachio showed a burned appearance. The roasting process at 150ºC for 30 min showed significant reduction of AFB_1 and AFB_2 without any noticeable change in taste of sample. Also the rate of reduction was plotted against the initial amount and linear correlation was not found. On the other hand, (Ariño et al., 2009) studied the effect of roasting on AFs: four commercial batches of raw pistachios in-shell from Iran were salted (1% salt content) and roasted at 120ºC for 20 min in a roasting industry in Spain. This study did not obtain significant differences in relation with AFs reduction after roasting. However the level of contamination of the starting material was low, ranging from 0.12 to 0.18 μg/kg.

Analysing the existing results on the effect of time and temperature in connection with the degradation of AFs, it can be observed that high temperatures (200-400ºC) produce higher mycotoxin reduction (**Figure 2**). Moreover lower temperatures need longer exposition time than higher temperatures for obtaining the same reduction percentages. Thus the percentage of reduction depends on temperature, time as well as the initial mycotoxin contamination (**Table 2**).

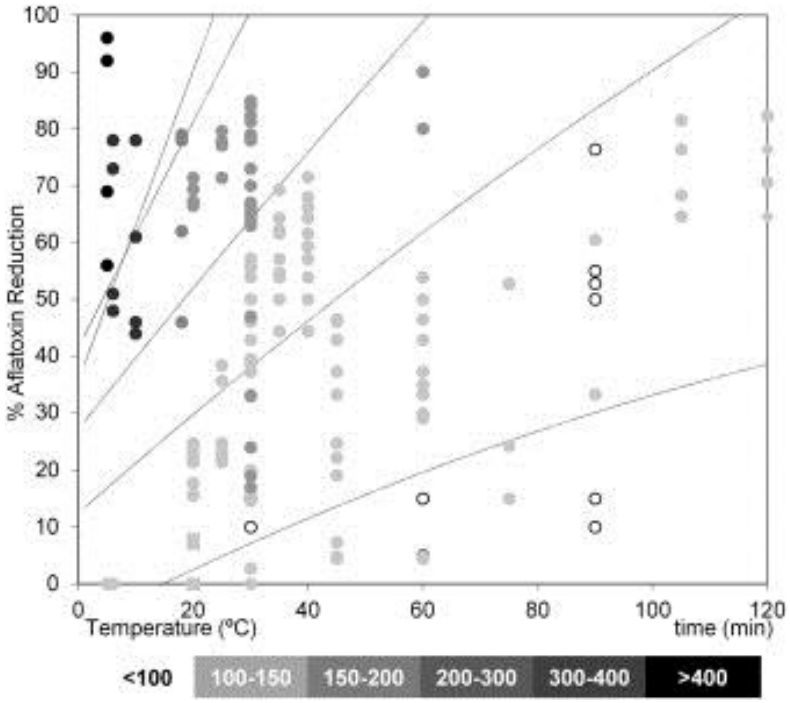


Figure 2 Summary of the existing results regarding aflatoxin reduction due to roasting. Lines represent the temperature levels leading to a given reduction for a certain roasting time with Ho=10μg/kg. (Source of date: Ariño *et al.,* 2009; Lee *et al.,* 1969; Pluyer *et al.,* 1987; Rustom *et al.*, 1997 and Yazdanpanah *et al.,* 2005).

4.3.1.4. FSO, PO and uncertainty

The FSO was taken from the maximum levels in EC Regulation 1881/2006 amending by 105/2010 for total AFs (10 μg/kg). Taking into account the information in the previous subsections, the process steps were individually considered (**Table 2**) and PCs determined when required. Moreover, according to these PCs, possible PcC and PdC were calculated, using previously published results. This process can either be done forward, starting from the PO guideline in the raw material, or backward, starting from the FSO to be accomplished in the final product.

The most common measure of uncertainty is variance. The variance of an estimated parameter statistical dispersion, indicates how far from the expected values are. Hence the results should be reported as "x ± 2*u*" or "x ± *U*", where x is the result; and *u* is the standard measurement of uncertainty. The expanded measurement of uncertainty (2*u* = U) gives a confidence level of approximately 95%, assuming normality of the reported results (EURACHEM, 2000).

For the particular case of mycotoxins, the maximum level as set in the EC Regulation 1881/2006 (FSO) must be over the final product PO to take U into account. EC Regulation (401/2006) states as criterion for acceptance of a lot or sublot that the laboratory sample conforms to the maximum limit, taking into account the correction for recovery and U. According to the performance criteria for AFs analysis (EC 401/2006, recommended RSD_R=21% for a concentration of 10 μg/kg), calculated U would take a value of 4.224 μg/kg. Thus in this case the PO for the final product should take a value of 10-4.224~5.776 μg/kg. Thus, in this example, given the recommended value of U, either the PO at the reception of the pistachio should be lower than 15 μg/kg or the industrial process might need to be redesigned to comply with the maximum level. Considering the current bibliography plus the associated uncertainty, only a final FSO of 10 μg/kg could be reached if a reduction of 50% was achieved during roasting.

An additional point which has not been addressed in this example is sampling uncertainty. According to (Ozay et al., 2007) sampling uncertaintity may account for 99.53% total uncertaintity, while U would just be 0.09%. At the moment, the EU project *Selection and improving of fit-for-purpose sampling procedures for specific foods and risks* is running with the aim of evaluating sampling uncertainty for a range of hazards and sampling plans, including AFs in pistachio nuts. The sampling plans for official control are stated by governments, while the food industry might use different sampling plans for their quality control systems.

As a conclusion, for sampling and determination of AFs concentration there is a need to state the PO for the final product lower than the FSO, taking into account the uncertainty value, U (FSO-U=PO). In Australia, one peanut shelling company sorted peanuts until the mean AFs content of samples from any one lot did not exceed 3 μg/kg (PO): this provided 95% confidence that any lot would meet the 15 μg/kg FSO (Pitt, 2004).-

4.3.2. The need for predictive modelling to reach performance criteria

When seeking for appropriate PC, PcC and PdC the authors found a lack of kinetic models from where to draw data for both AFs production in pistachio nuts as a function of storage conditions and AFs inactivation as a function of time, temperature, moisture… From this example it is clear that models are required to adequatelly adjust PcC, PdC and PC especially during the storage and thermal treatments.

Regarding mycotoxins, (Garcia et al., 2009) described two approaches in the mycotoxin production modelling. One modelling approach is preventing mould growth in all the steps of the production and processing of food and thus indirectly prevent mycotoxin production. The other modelling approach involves directly model mycotoxin production as a function of environmental factors in those steps of the process. However this alternative is associated with several disadvantages as high intraspecific variability in mycotoxin production plus a high variability in the mycotoxin production by a given strain in a given substrate. The application of predictive microbiology in risk management may serve to determine the conditions required to avoid the growth of fungi and therefore the production of mycotoxins in steps such as storage and processing.

Likewise, predictive modelling can be also applied to quantify the mycotoxin reduction through certain processing steps. Ideally, decontamination steps, in addition to assuring an adequate wholesome food suply, should: inactivate, destroy or remove the mycotoxins; not produce or leave toxic residues in the food/feed; retain nutritive value and food/feed acceptability of the product; not alter significantly the properties of the product and destroy fungal spores (Kabak et al., 2006). Moreover, some processing steps may indirecty destroy mycotoxins. In general, factors that may influence the fate of mycotoxins during food processing include the presence of other constituents and enzymes, m.c. of the raw material, processing temperature, pH, pressure, and the mycotoxin concentration (Scott, 1991). All these variables should be explored and their impact on mycotoxins destruction be modeled. This would provide a valuable tool for PC, PcC and PdC calculation.

5. CONCLUSIONS

The present study demonstrates that the emerging risk management metrics, FSO, PO and PC, might be also applied to the mycotoxin hazard. The example here presented underlined the need for better and more structured information on the impact of the storage and processing steps on mycotoxins accumulation. Moreover, the problem of the impact of uncertainty in PO and FSO compliance was brought up.

ACKNOWLEDGEMENTS

The authors are grateful to Spanish government (project AGL2010-22182-C04-04) and EC, KBBE - Food, Agriculture and Fisheries and Biotechnology (project 222738- Selection and

improving of fit-for-purpose sampling procedures for specific foods and risks) and Comissionat per a Universitats i Recerca d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (AGAUR) and European Social Fund for the financial support.

REFERENCES

Abarca, M. L., F. Accensi, J. Cano, F. J., & Cabañes (2004). Taxonomy and significance of black aspergilli. *Antonie Van Leeuwenhoek Int.J.Gen.Mol.Microbiol.,* 86(1), 33-49.

Akkerman, R., Farahani, P. & Grunow, M. (2010). Quality, safety and sustainability in food distribution: A review of quantitative operations management approaches and challenges. *OR Spectrum,* 32(4), 863-904.

Ariño, A., Herrera, M., Estopañan, G., Rota, M. C., Carramiñana, J.J., Juan, T. *et al.,* (2009). Aflatoxins in bulk and pre-packed pistachios sold in Spain and effect of roasting. *Food Control,* 20(9), 811-814.

Aziz, N. H., Attia, E. S., & Farag, S. A. (1997). Effect of gamma-irradiation on the natural occurrence of Fusarium mycotoxins in wheat, flour and bread. *Nahrung,* 41(1), 34-37.

Baert, K., De Meulenaer, B., Kamala, A., Kasase, C., & Devlieghere F. (2006). Occurrence of patulin in organic, conventional, and handcrafted apple juices marketed in Belgium. *Journal of Food Protection,* 69(6), 1371-1378.

Bayman, P., Baker, J. L. & Mahoney, N. E. (2003) Aspergillus on tree nuts: Incidence and associations *Mycopathologia* 155 (3) 161-169.

Betina, V. (1989). Mycotoxins: Chemical, biological and environmental aspects. *Bioactive Molecules,* 9 114-150.

Boonzaaijer, G., Bobeldijk, I., & van Osenbruggen W. A. (2005). Analysis of patulin in dutch food, an evaluation of a SPE based method. *Food Control,* 16(7), 587-591.

Boutrif, E., & Canet C. (1998). Mycotoxin prevention and control: FAO programmes. *Revue de Médicine Vétérinaire.,* 149(6), 681-694.

Butler, T. (1902). Notes on a feeding experiment to produce leukoencephalitis in a horse, with positive results. *American Veterinary Review*, 26, 748- 751.

CAC (Codex Alimentarius Commission). (2003). Report of the Thirty-fifth Session of the Codex Committee on Food Hygiene. ALINORM 03/13A.

CAC (Codex Alimentarius Commission). (2004). Codex General Guidelines on Sampling. CAC/GL 50-2004).

CAC (Codex Alimentarius Commission). (2008). Codex Committee on Methods of Analysis and Sampling. *Guidance On Measurement Uncertainty and Uncertainty of Sampling,* 2008.

CAC(Codex Alimentarius Commission). (2007). Principles and Guidelines for the Conduct of Microbiological Risk Management (MRM). CAC/GL 63-2007.

Cano-Sancho, G., Marin, S., Ramos, A. J., & Sanchis, V (2009a). Survey of patulin occurrence in apple juice and apple products in Catalonia, Spain, and an estimate of dietary intake F*ood Additives and Contaminats: Part B Surveillance* 2 (1), 59-65.

Cano-Sancho, G., Marin, S., Ramos, A. J., & Sanchis, V (2009b). Presence of thichotecenes and co-occurence in cereal based food from Catalonia (Spain). F*ood Control* 22 (3-4), 490-495.

Cano-Sancho, G., Marin, S., Ramos, A.J., Peris-Vicente, J. & Sanchis, V. (2010) Occurrence of aflatoxin M1 and exposure assessment in Catalonia (Spain) *Revista Iberoamericana de Micologia* 27 (3), 130-135.

CAST (Council for Agricultural Science and Technology)(2003) Mycotoxin: risk in plant, animal, and human system, Task Force report, No. 139, Ames, Iowa, USA.

Chen, J., Dagher, S. M., Fisher, C. E., Grunow, W., Hattan, D. G., Kawamura, Y. et al. (2000). Evaluation of certain food additives and contaminants. *WHO Tech. Rep. Ser.,* (896), i-viii+1-127.

Chu, F. S., & Li G. Y. (1994). Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. *Applied and Environmental Microbiology,* 60(3), 847-852.

Cole M., (2004). Food Safety Objectives : concept and current status. Lecture presented at the 36th Symposium of the Swiss Society of Food Hygiene, Zurich, 8th October 2003. Mitteilungen aus Lebensmitteluntersuchung und Hygiene, 95, pp. 13–20.

Commission Regulation (EC) 178/2010 of 2 March 2010, amending Regulation (EC) No 401/2006 as regards groundnuts (peanuts), other oilseeds, tree nuts, apricot kernels, liquorice and vegetable oil (2010). *Official Journal of the European Union,* L 52 32-43.

Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union,* L364 5-24.

Commission Regulation (EU) No 165/2010 of 26 February 2010, amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxin. (2010). *Official Journal of the European Union L,* 50 8-12.

Coronel, M. B., Marin, S., Cano, G., Ramos, A.J. & Sanchis, V. (2011). Ochratoxin A in Spanish retail ground roasted coffee: Ocurrence and assessment of the exposure in Catalonia. *Food Control*, 22 (3-4), 414-419.

Costantini, A. B., Heinrich, W. & Lars I. Q. (1994). Mycotoxins in human health. *Newsletter, WHO Collaborating Center for Mycotoxins in Food,* 1 1.

de Swarte, C. & Donker R. A. (2005). Towards an FSO/ALOP based food safety policy. *Food Control,* 16(9 SPEC. ISS.), 825-830.

Denizel, T., Jarvis, B., & Rolfe E. J. (1976). A field survey of pistachio (Pistacia vera) nut production and storage in Turkey with particular reference to aflatoxin contamination. *Journal Science of Food and Agriculture,* 27(11), 1021-1026.

Doster, M. A., & Michailides T. J. (1994) a. Aspergillus molds and aflatoxins in pistachio nuts in California. *Phytopathology,* 84(6), 583-590.

Doster, M. A., & Michailides T. J. (1994) b. Development of Aspergillus molds in litter from pistachio trees. *Plant Disease,* 78(4), 393-397.

EUROCHEM/CITAG. Guide CG4 (2000). Quantifying uncertainty in analytical measurement. (2nd ed.).

FAO (Food and Agriculture Organization of the United Nations) (2006) Food safety risk analysis. A guide for national food safety authorities. Avaliable from http://www.who.int/foodsafety/publications/micro/riskanalysis06.pdf

FAO/IAEA manual on the application of HACCP system in mycotoxin prevention and control. (2001). *Food Nutrition,* 1-130.

Farah, Z., Martins, M. J. R., & Bachmann M. R. (1983). Removal of aflatoxin in raw unshelled peanuts by a traditional salt boiling process practiced in the north east of Brazil. *Lebensmittel Wissenschaft und Technologie,* 16 122-124.

Fernane, F., Cano-Sancho, G., Sanchis, V., Marin, S., & Ramos A. J. (2010) a. Aflatoxins and ochratoxin A in pistachios sampled in Spain: Occurrence and presence of mycotoxigenic fungi. *Food Additives and Contaminants: Part B Surveillance,* 3(3), 185-192.

Fernane, F., Sanchis, V., Marín, S., & Ramos A. J. (2010) b. First Report on Mould and Mycotoxin Contamination of Pistachios Sampled in Algeria. *Mycopathologia,* 1-7.

Frisvad, J. C., Smedsgaard, J Samson, R. A., Larsen, T. O. & Thrane, U. (2207) Fumonisin B2 production by *Aspergillus niger*. *Journal of. Agriculture and. Food Chemistry, 55,* 9727–9732

Fung, F., & Clark R. F. (2004). Health effects of mycotoxins: A toxicological overview. *Journal of Toxicology Clinical Toxicology,* 42(2), 217-234.

Garcia, D., Ramos, A. J., Sanchis, V., & Marín S. (2009). Predicting mycotoxins in foods: A review. *Food Microbiology,* 26(8), 757-769.

Gil-Serna, J., Vázquez, C., Sardiñas, N., González-Jaén, M. T., & Patiño B. (2011). Revision of ochratoxin a production capacity by the main species of *Aspergillus* section *Circumdati*. *Aspergillus steynii* revealed as the main risk of OTA contamination. *Food Control,* 22(2), 343-345.

Gökmen, V., & Acar J. (1998). Incidence of patulin in apple juice concentrates produced in Turkey. *Journal of Chromatography A,* 815(1), 99-102.

Gorris, L. G. M. (2005). Food safety objective: An integral part of food chain management. *Food Control,* 16(9 SPEC. ISS.), 801-809.

Harrison, M. A. (1989). Presence and stability of patulin in apple products: A review. *Journal of Food Safety,* 9 147-153.

Harwig, J., Kuiper-Goodman, T. & Scott, P.M. (1983). Microbial food toxicants: ochratoxins. In: *Handbook of Foodborne Diseases of Biological Origin.* Reichcigl, M. (ed.). CRC Press, Boca Raton, Florida, 193-238.

Hazel, C. M., & Patel, S. (2004). Influence of processing on trichothecene levels. *Toxicology Letters,* 153(1), 51-59.

Hopkins, J. (1993) The toxicological hazards of patulin. Food and Chemical Toxicology 31(6),454-456.

Hussein, H. S., & Brasel J. M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology,* 167(2), 101-134.

IARC (International Agency for Research on Cancer) (1986). Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans.,40.

IARC (International Agency for Research on Cancer) (1999). Overall evaluations of carcinogenicity to humans. IARC monographs, Vol. 1–73, pp.1–36.

IARC (International Agency for Research on Cancer) (2002). Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, 82.

ICMSF (International Commission on Microbiological Specifications for Foods) (1998). In: *Microorganisms in Foods 6: Microbial Ecology of Food Commodities*, Blackie, London (1998).

ICMSF (International Commission on Microbiological Specifications for Foods). (2002). Microorganisms in Foods 7: Microbiological Testing in Food Safety Management, Kluwer Academic/Plenum Publishers, New York (2002).

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (1995): Evaluations of certain food additives and contaminants.WHO. Technical Report Series, No. 859.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (1999). Evaluation of certain food additives and contaminants (Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 884

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (2001) Safety evaluations of certain mycotoxins in food. *WHO Food Additives Series 47*, World Health Organization, Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). (2006). Summary and Conclusions of the Sixty-fourth Meeting of the JEFCA. Rome, 8-17 February 2005.

JECFA(Joint FAO/WHO Expert Committee on Food Additives) (2007). Summary and Conclusions of the Sixty-eight Meeting of the JEFCA. Geneva, 19-28 June 2007.

Jelinek, C. F., Pohland, A. E., & Wood G. E. (1989). Worldwide occurrence of mycotoxins in foods and feeds--an update. *Journal of the Association of Official Analytical Chemists,* 72(2), 223-230.

Kabak, B., Dobson, A. D. W., & Var I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: A review. *Critical Reviews in Food Science and Nutrition,* 46(8), 593-619.

Kanora, A., & Maes, D. (2009). The role of mycotoxins in pig reproduction: A review. *Veterinary Medicine,* 54(12), 565-576.

Kawashima, L. M., Valente Soares, L. M (2006). Ocurrence of fumonisin B1, aflatoxins B1, B2, G1 and G2, ochratoxin A and zearalenone in corn product. *Ciencia e Tecnologia de Alimentos*, 26 (3), 516-521.

Kellerman, T. S., Marasas, W. F., Pienaar, J. G., & Naudé, T. W. (1972). A mycotoxicosis of equidae caused by Fusarium moniliforme sheldon. A preliminary communication. *Onderstepoort Journal Veterinary Research,* 39(4), 205-208.

Kriek, N. P., Kellerman, T. S., & Marasas, W. F. (1981). A comparative study of the toxicity of Fusarium verticillioides (= F. moniliforme) to horses, primates, pigs, sheep and rats. *Onderstepoort J.Vet.Res.,* 48(2), 129-131.

Krogh, P., Axelsen, N. H., Elling, F., Gyrd-Hansen, N., Hald, B., Hyldgaard-Jensen, J., et al. (1974). Experimental porcine nephropathy. Changes of renal function and structure induced by ochratoxin A- contaminated feed. *Acta Pathology et Microbiologica Scandinavica. Section [A],* 0(Suppl 246), 1-21.

Kuiper-Goodman, T., Scott, P. M., & Watanabe, H. (1987). Risk assessment of the mycotoxin zearalenone. *Regulatory Toxicology and Pharmacology,* 7(3), 253-306.

Lammerding A. M. & Fazil, A (2000). Hazard identification and exposure assessment for microbial food safety risk assessment. *International Journal of Food Microbiology*, 58 (3), 147-157.

Lee, L. S., Cucullu, A. F., Franz Jr., A. O., & Pons Jr. W. A. (1969). Destruction of aflatoxins in peanuts during dry and oil roasting. *Journal of Agriculture and Food Chemistry,* 17(3), 451-453.

López, T. A., Odriozola, E. R., Milano, G. D., Sobredo, J.C., Donadio, L. H. & Rocco, J. A. (1988).Hyperestrogenism in seine dure to natural posioning with zearalenone. *Revista Argentina de Microbiologia,* 20 (3), 119-123.

Manning, R. O., & Wyatt R. D, (1984). Toxicity of *Aspergillus* ochraceus contaminated wheat and different chemical forms of ochratoxin A in broiler chicks. *Poultry Science,* 63(3), 458-465.

Mantle, P. G. (2002). Risk assessment and the importance of ochratoxins. *International Biodeterioration &.Biodegradation,* 50(3-4), 143-146.

McKinley, E. R., & Carlton W. W. (1991). *Mycotoxins and Phytoalexins, Patulin,* Sharma, P., Salunkhe, D.K., Eds.; CRC Press: Boca Raton, FL, USA

Mojtahedi, H., Rabie, C. J., & Lubben A. (1979). Toxic aspergilli from pistachio nuts. *Mycopathologia,* 67(2), 123-127.

Ozay, G., Seyhan, F., Yilmaz, A., Whitaker, T.B., Slate, A.B.,& Giesbrecht, F.G. (2007). Sampling hazelnuts for aflatoxin: Effect of sample size and accept/reject limit on reducing the risk of misclassifying lots 2007 *Journal of AOAC International* 90 (4), 1028-1035.

Ozkarsli, M, (2003).Effect of traditional-roasting and microwave-roasting on aflatoxin B1 in peanuts. MSc Thesis, Cukurova University Institute of Natural and Applied Sciences, Adana, Turkey.

Park, D. L. (2002). Effect of processing on aflatoxin. *Advances in Experimental Medicine and Biology,* 504 173-179.

Pearson, T.C., & Schatzki, T.F. (1998). Machine Vision System for Automated Detection of Aflatoxin-Contaminated Pistachios. *Journal of Agricultural and Food Chemistry*, 46 (6), 2248-2252.

Pfeiffer, E., Groß, K., & Metzler M. (1998). Aneuploidogenic and clastogenic potential of the mycotoxins citrinin and patulin. *Carcinogenesis,* 19(7), 1313-1318.

Pitt. J. I. (2004) Application of food safety objective concept to the problem of aflatoxin in peanuts. *Mitteilungen aus Lebensmitteluntersuchung und Hygiene*, 95, 52-58.

Plattner, R. D., Weisleder, D., Shackelford, D. D., Peterson, R., & Powell R. G. (1992). A new fumonisin from solid cultures of *Fusarium moniliforme*. *Mycopathologia,* 117(1-2), 23-28.

Pluyer, H. R., Ahmed, E. M., & Wei C. I. (1987). Destruction of aflatoxins on peanuts by oven- and microwave-roasting. *Journal of Food Protection.,* 50(6), 504-508.

Prandini, A., Tansini, G., Sigolo, S., Filippi, L., Laporta, M., Piva, G. et al., (2009)_On the occurrence of aflatoxin M1 in milk and dairy products *Food and Chemical Toxicology* 47, 984-991.

Rahimi, E., Bonyadian, M., Rafei, M. & Kazemeini, H.R. (2010)_Occurrence of aflatoxin M1 in raw milk of five dairy species in Ahvaz, Iran. *Food and Chemical Toxicology* 48, 129-131.

RASFF (Rapid Alert System for Food and Feed) available from: https://webgate.ec.europa.eu/rasff-window/portal/index.cfm?event=searchResultList.

Riley, R. T., Kemppainen, B. W. & Norred W. P. (1985). Penetration of aflatoxins through isolated human epidermis. *Journal of Toxicology and Environmental Health,* 15(6), 769-777.

Rustom, I. Y. S. (1997). Aflatoxin in food and feed: Occurrence, legislation and inactivation by physical methods. *Food Chemistry,* 59(1), 57-67.

SCF (Scientific Committee of Food) (1999). Opinion of the Scientific Committee on Food on Fusarium-toxins Part 1: Deoxynivalenol (DON), (expressed on 2 December 1999) http://ec.europa.eu/food/fs/sc/scf/out44_en.pdf

SCF (Scientific Committee of Food) (2000a). Minutes of the 120th Meeting of the Scientific Committee on Food held on 8 and 9 March 2000 in Brussels, Minute statement on patulin. http://ec.europa.eu/food/fs/sc/scf/out55_en.pdf

SCF (Scientific Committee of Food) (2000b). Opinion of the Scientific Committee on Food on Fusarium-toxins Part 2: Zearalenone (ZEA), (expressed on 22 June 2000) http://ec.europa.eu/food/fs/sc/scf/out65_en.pdf.

SCF (Scientific Committee of Food) (2000c). Opinion of the Scientific Committee on Food on Fusarium-toxins Part 4: Nivalenol (expressed on 19 October 2000) http://ec.europa.eu/food/fs/sc/scf/out74_en.pdf

SCF (Scientific Committee of Food) (2002). Opinion of the Scientific Committee on Food on Fusarium-toxins Part 6: Group evaluation of T-2 toxin, HT-2toxin, nivalenol and deoxynivalenol. (adopted on 26 February 2002) http://ec.europa.eu/food/fs/sc/scf/out123_en.pdf

SCF (Scientific Committee of Food) (2003). Updated opinion of the Scientific Committee on Food on Fumonisin B1, B2 and B3 (expressed on 4 April 2003) http://ec.europa.eu/food/fs/sc/scf/out185_en.pdf

Schatzki, T. F. (1995). Distribution of aflatoxin in pistachios. 2. Distribution in freshly harvested pistachios. *Journal of Agriculture and Food Chemistry,* 43(6), 1566-1569.

Schatzki, T. F., & Pan J. L. (1996). Distribution of aflatoxin in pistachios. 3. Distribution in pistachio process streams. *Journal of Agriculture and Food Chemistry,* 44(4), 1076-1084.

Schneider, K., Ollroge, I., Clauberg, M. & Schuhmacher-Wolz U. (2007). Analysis of risk assessment and risk management processes in the derivation of maximum levels for environmental contaminants in food. *Food Additives & Contaminants,* 24(7), 768-776.

Schothorst, R. C., & Van Egmond H. P. (2004). Report from SCOOP task 3.2.10 "collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states" Subtask: Trichothecenes. *Toxicology Letters,* 153(1), 133-143.

Scott, P. M. (1991). Possibilities of reduction or elimination of mycotoxins present in cereal grains. *Cereal Grain.Mycotoxins, Fungi and Quality in Drying and Storage,* 529-572.

Scott, P.M., Lombaert, G.A., Pellaers, P., Bacler, S., Kanhere, S.R., Sun, W.F., et al. (1989). Application of capillary gas chromatography to a survey of wheat for five trichothecenes. *Food Additives and Contaminants,* 6 (4), 489-500.

Soriano M.J. (2007). Micotoxinas en Alimentos. (1st ed.), España. Ediciones Díaz de Santos. (Chapter 12)

Stoloff, L. (1975). Patulin, a contaminant of apple juice. *New York State Agricultural Experiment Station Special Report,* 19, 51–54.

Stringer, M. (2005). Food safety objectives - Role in microbiological food safety management. *Food Control,* 16(9 SPEC. ISS.), 775-794.

Tanaka, T., Hasegawa, A., Yamamoto, S., Lee, U., Sugiura, Y. & Ueno, Y. (1988). Worldwide contamination of cereals by the *Fusarium* mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. Survey of 19 countries. *Journal of Agriculture and Food Chemistry,* 36(9-10), 979-983.

van Schothorst, M. (1998). Principles for the establishment of microbiological food safety objectives and related control measures. *Food Control,* 7 187-193.

van Schothorst, M. (2005). A proposed framework for the use of FSOs. *Food Control,* 16(9 SPEC. ISS.), 811-816.

Viñas, I.; Vela, E. & Sanchis, V. (1993) Capacidad productora de patulina de cepas de *Penicillium expansum* procedentesde centrales hortofrutícolas de Lleida. *Revista Iberoamericana de Micología* 10, 30-32.

Waltking, A. E., Bleffert, G., & Kiernan M. (1968). An improved rapid physicochemical assay method for aflatoxin in peanuts and peanut products. *Journal of the American Oil Chemists´Society,* 45(12), 880-884.

WHO (World Trade Organisation)(2010). Safety and efficacy issues. (2010). Drug Information., 24(1), 25-29.

WHO IARC (1990) Patulin. WHO Food Additives Series. 26: 143-165.

Wichmann, G., Herbarth, O., & Lehmann I. (2002). The mycotoxins citrinin, gliotoxin, and patulin affect interferon-γ rather than interleukin-4 production in human blood cells. *Environmental Toxicolgy,* 17(3), 211-218.

Wild, C. C. P. & Gong, Y. Y. (2010). Mycotoxins and human disease:a largely ignored global health issue. *Carcinogenesis* 31, 71–82.

WTO (World Trade Organisation). (1995). The WTO agreement on the application of sanitary and phytosanitary measures (SPS Agreement). Available from http://www.wto.org/english/tratop_e/sps_e/ spsagr_e.htm.

Yazdanpanah, H., Mohammadi, T., Abouhossain, G., & Cheraghali A. M. (2005). Effect of roasting on degradation of Aflatoxins in contaminated pistachio nuts. *Food and Chemical Toxicology,* 43(7), 1135-1139.

Annex II

First report of field application of atoxigenic *Aspergillus flavus* as candidate biocontrol agents

Antonio Mauro [a], Esther Garcia Cela [b], Peter J Cotty [c], Paola Battilani [a,*]

[a] Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122, Piacenza, Italy; [b] Food Technology Department, Lleida University, XaRTA-UTPV, Rovira Roure 191, E-25198 Lleida, Spain; [c] United States Department of Agriculture, Agricultural Research Service, School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA.

Aflatoxins are secondary metabolites produced by several species of *Aspergillus* on important commodities like maize, cottonseed, peanuts and pistachio nuts. *Aspergillus flavus* is the main responsible for aflatoxins contamination in maize worldwide, almost the only one in Italy. Although several strategies have been applied worldwide to reduce pre-harvest aflatoxins contamination, biological control with atoxigenic strains of *A. flavus* is the most effective. Atoxigenic strains displace aflatoxin producers during crop development resulting in a relevant reduction in aflatoxins contamination in grains. The competitive efficiency of strains is crucial; therefore, native strains must be applied in order to guarantee the best results in term of reduction of aflatoxin contamination. In previous studies, 2 Italian atoxigenic strains were selected as candidate biocontrol agents based on *in vitro* studies. The aim of this research was to confirm their efficacy in field. A field trial was carried out during the 2012 maize growing season (March-September) in 8 fields located in 3 regions of north Italy: Emilia Romagna (ER; 1 field), Lombardia (LO; 2 fields) and Veneto (VN; 5 fields).

Each field consisted of almost 2 ha managed according to the cropping system commonly followed by farmers; 1 ha was treated with the biocontrol agents, a mix of 2 strains belonging to 2 different VCG (IT019 and IT006) and 1 ha represented the untreated control. Sorghum was used as the atoxigenic strain carrier for field trials and it was distributed at V4-V6 maize growing stage using a fertiliser spreader. A randomized design with three replicates was used. At commercial ripening (UR 22-24%), 10 ears from each plot were randomly collected, de-husked, shelled and the kernels dried at 45°C for 3 days prior to be milled. A total of 6

samples (3 replicates for treated and 3 for the control) of maize flour from each field were obtained. *A. flavus* enumeration was carried out on the maize flour through the dilution technique onto a Modified Rose-Bengal Agar.

Members of *Aspergillus* section *Flavi* were initially identified based on colony morphology and the number of colony-forming units (CFU) recorded. Ten discrete *A. flavus* colonies, from 2 independent isolation procedure, were recovered from each sample, transferred on 5/2 agar, incubated for 5-7 days at 31°C and saved in sterile water vials. Single spore colonies were used to estimate the relative abundance of the biocontrol agents released using the vegetative compatibility analysis through the complementation between non-utilizing nitrate auxotrophs (*nit* mutants).

Aflatoxins and fumonisins were analyzed in all the samples according to the method of Stroka et al. (1999) and Visconti et al. (2001), respectively.

Data were subjected to the analysis of variance (ANOVA) using the software IMB SPSS Statistics 21 (IMB, Somers, NY, USA). Mean separations were performed on data using Tukey's Honestly Significant difference test. Data on aflatoxin B_1 and percentage of reduction compared to the untreated test were ln and arcsin transformed, respectively, prior to the analysis. Fields with aflatoxin B_1 contamination lower than 1 μg/kg in both thesis were not considered for the analyses.

A. flavus population ranged between $6.69 \cdot 10^4$ and $6.98 \cdot 10^6$ CFU/g in the control and from $6.72 \cdot 10^5$ to $1.52 \cdot 10^7$ CFU/g in the treated thesis. On average, the population was higher in the thesis treated with the two Italian atoxigenic strains compared to the untreated thesis ($P<0.001$; $5.80 \cdot 10^6$ *vs* $3.30 \cdot 10^6$). Statistical differences were also observed among fields and they were more relevant than those between treated and control areas of the same field where the visual observation never showed differences.

Vegetative compatibility analysis was conducted on 60 isolates for each field, 30 from untreated and 30 from the treated field areas. The percentage of the IT019 was 25.2% and 30.0%, respectively in the untreated and treated area, while it was 53.5% and 61.9% for IT006.

Regarding aflatoxin B_1, in 4 fields the concentration was less than 1.0 μg/kg in both theses; these fields were excluded from statistical analysis. In the other fields, the average aflatoxin B_1 concentration in the treated thesis (4.2 μg/kg) was statistically different from the value of the untreated fields (71.1 μg/kg); this result is confirmed considering single fields.
No differences in fumonisin concentration were observed between the treated and control areas.

These results confirm the ability of the selected atoxigenic strains of *A. flavus* to significantly reduce aflatoxin contamination in maize grain below the limit of 5 μg/kg. The VCG study showed that the recovery of IT006 is significantly higher compared to IT019, both in treated and control plots. The dispersal of atoxigenic strains in untreated areas is surely delayed respect to the planned distribution; due to this reason and because of the lower amount of atoxigenic strains, they cannot reduce aflatoxin contamination, or at least they exert a very light effect.

Based on these field results, the selected strains, in particular IT006 can be confirmed as candidate biocontrol agent to mitigate aflatoxin contamination in maize in Italy, an emerging problem as confirmed in 2012 when very severe contamination was signaled.

This summary briefly reports the activity where Esther Garcia Cela was involved during her period abroad spent in Piacenza, Università Cattolica del Sacro Cuore. In collaboration with PhD Antonio Mauro and with the supervision of Prof. Paola Battilani. A paper is in preparation and it will be submitted to a peer reviewed Journal. Because a related patent is pending, it was not possible to publish data before.