Departament de Sanitat i d'Anatomia Animals
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Aspergillus SECCIÓN *Nigri*: ESTUDIO FISIOLÓGICO Y MOLECULAR DE ESPECIES OCRATOXÍGENAS.

Memoria presentada para optar
al grado de doctor

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CERTIFICAN:

que Don Alexandre Esteban Franco ha realizado el presente trabajo sobre "*Aspergillus* sección *Nigri*: estudio fisiológico y molecular de especies ocratoxígenas" bajo nuestra dirección en el Departament de Sanitat i d'Anatomia Animals de la Universitat Autònoma de Barcelona.

Y para que conste, a efectos de ser presentada como Memoria de Tesis para optar al grado de Doctor en Medicina y Sanidad Animales, firmamos el presente certificado en Bellaterra a 19 de Diciembre de 2005.

M.L. Abarca Salat F.J. Cabañes Saenz

Esta memoria de Tesis Doctoral se presenta como compendio de publicaciones. La relación de los artículos es la siguiente:

- **Effects of temperature and incubation time on production of ochratoxin A by black aspergilli.** Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***Research in Microbiology*****, 155 (2004) 861-866.**

- **Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species.** Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***International Journal of Food Microbiology*** **(aceptado para publicación).**

- **Study of the effect of water activity and temperature on ochratoxin A production by *Aspergillus carbonarius*.** Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***Food Microbiology*** **(aceptado para publicación).**

- **Effect of pH on ochratoxin A production by *Aspergillus niger* aggregate species.** Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***Food Additives and Contaminants*** **(aceptado para publicación).**

- **Influence of pH and incubation time on ochratoxin A production by *Aspergillus carbonarius* in culture media.** Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***Journal of Food Protection*****, 68 (2005) 1435-1440.**

- **Isolation and characterization of six polymorphic microsatellite loci in *Aspergillus niger*.** Esteban, A., Leong, S.L., Tran-Dinh, N. ***Molecular Ecology Notes*****, 5 (2005) 375-377.**

- **Comparison of ERIC, AFLP and microsatellite markers in the study of *Aspergillus niger* aggregate and *Aspergillus carbonarius*.** Esteban, A., Leong, S.L., Hocking, A., Abarca, M.L., Cabañes, F.J., Tran-Dinh, N. **(enviado para su publicación).**

ÍNDICE

1.-INTRODUCCIÓN

1.1.-Importancia de *Aspergillus* sección *Nigri* (Gams *et al.*)

Los componentes de la sección *Nigri* son unos de los de mayor importancia dentro del género *Aspergillus*. Se hallan distribuidos de forma ubicua a nivel mundial y crecen en una gran variedad de sustratos: semillas, granos, forrajes, piensos, frutas, verduras y cuero entre otros, siendo considerados como hongos responsables del deterioro de alimentos (70, 189, 197).

Algunas especies de *Aspergillus* sección *Nigri* se utilizan a nivel industrial como fuente de enzimas extracelulares y ácidos orgánicos para ser utilizados en la industria alimentaria. Los productos obtenidos de *Aspergillus niger* poseen la categoría GRAS ('Generally Recognised As Safe') de la FDA (Food and Drug Administration) y se utilizan en cantidades significativas como aditivos alimentarios (4). Éstos incluyen, entre otros, α-amilasas, catalasas, celulasas, glucoamilasas, lipasas, pectinasas y proteasas, así como ácido cítrico y ácido glucónico (35, 177). Inicialmente las cepas utilizadas en la producción industrial de estos productos se seleccionaban tras mutagénesis. Con posterioridad, el desarrollo de cepas recombinantes permitió utilizar algunas de las especies de la sección *Nigri* para la obtención de diferentes enzimas a nivel industrial mediante la expresión de genes específicamente seleccionados (203, 212). Todos los preparados de enzimas y las cepas utilizadas para su producción son sometidos a controles para detectar la posible producción de metabolitos tóxicos que pudieran afectar la seguridad de estos productos en el mercado (39, 177).

En Asia se encuentra muy difundida su utilización junto a otras especies fúngicas, sobre todo en la elaboración de alimentos y bebidas fermentadas (277). *Aspergillus awamori* y otros microorganismos se utilizan como fermentos en la producción del koji, una masa elaborada a partir de cereales y leguminosas fermentados que constituye la base del miso, la salsa de soja o el sake (33, 60).

No obstante, algunas especies incluidas en esta sección son capaces también de participar en procesos patológicos en el hombre y los animales. *Aspergillus niger* es una de las especies asociadas a la aspergilosis invasiva pulmonar y es a menudo agente causal de aspergiloma (118). Esta especie se aísla clínica o subclínicamente del oído externo en el hombre y suele aparecer implicada en micosis en diferentes localizaciones, aunque en alguna ocasión también en infecciones diseminadas (65, 118). En animales se considera también un patógeno oportunista habiéndose descrito casos de aspergilosis en algunas especies de mamíferos y aves (229). También

se halla involucrado en algunas enfermedades profesionales por el riesgo de hipersensibilidad que comporta la inhalación de sus esporas (212). La inhalación de enzimas en polvo también puede desencadenar reacciones alérgicas. En este sentido, se han descrito casos de reacciones asmáticas causadas por la inhalación de enzimas provenientes de *A. niger* presentes en aditivos de panadería (136, 194, 209).

Aspergillus niger es la especie de *Aspergillus* más comúnmente responsable del deterioro de la fruta fresca recolectada (189). Asimismo, es el agente causal de la antracnosis del algodonero y el carbón o añublo en los higos (114, 172, 225).

Algunas especies de esta sección pueden también producir diversos metabolitos secundarios tóxicos. Estas micotoxinas no se habían detectado en cereales y por ello, se consideraba que no suponían un riesgo para el hombre o los animales. Sin embargo, desde la primera descripción de la producción de ocratoxina A (OTA) por *A. niger* var. *niger* (1) y *A. carbonarius* (105) los miembros de esta sección están adquiriendo una mayor importancia como hongos productores de micotoxinas.

En la Tabla 1 se detallan los metabolitos secundarios y toxinas producidas por algunas especies de *Aspergillus* sección *Nigri*.

Tabla 1.-Metabolitos secundarios y toxinas producidas por algunas especies de *Aspergillus* sección *Nigri* (67, 81, 83, 163, 176, 208).

Especie	Metabolitos secundarios y toxinas
A. aculeatus	Ácido secalónico B, D y F Aculeasinas Eumodina Endocrocina Okaraminas Neoxalina
A. carbonarius	Aurasperona Naftopironas Ocratoxina A Piranonigrina Piranopirrol
A. japonicus	E-64 Festuclavina
A. niger	Ácido glutacónico Ácido 4-hiroximandélico Ácido Kójico Ácido monoglucosil oxi octadecanoico Asnipirona Asperenonas Aspergillinas Aspereillonas Asperrubrol Aurasperonas Dehidroflavininas Flaviolina Flavininas Genisterinas Kotaninas Malforminas A, B, C Naftopironas Nigragillina Neoequinulina A Nigerazinas Ocratoxina A Orlandina Oroboles Piranonigrina Piranopirrol Pirofen Tubingensina A y B

1.2.-Taxonomía

La primera descripción del género *Aspergillus*, llevada a cabo por Micheli en 1729, distinguía nueve especies considerando el color de la colonia. Una de esas especies era *Aspergillus capitatus capitulo pullo* que se caracterizaba por ser de color negro. Posteriormente algunos miembros actuales de la sección *Nigri* fueron incluidos en otros géneros como por ejemplo *Ustilago phoenicis* o *U. ficuum*, que más tarde se denominarían *A. phoenicis* o *A. ficuum* respectivamente. En el año 1859 Cramer describió el género *Sterigmatocystis* para incorporar las especies biseriadas de *Aspergillus*. La especie tipo del género, *S. antacustica*, se considera actualmente un probable sinónimo de *A. niger*. Van Tieghem en 1867 describió *A. niger* a partir de aislamientos procedentes de diversos sustratos y situó esta especie en el género *Sterigmatocystis* basándose en la estructura biseriada de las cabezas conidiales. Saccardo en 1886 aceptó el concepto de *Sterigmatocystis* e hizo nuevas combinaciones para las especies conocidas como *Aspergillus* 'negros'. Wehmer en 1901 reunió en un solo grupo a las especies negras de *Sterigmatocystis* y *Aspergillus*, aunque algunos autores continuaron describiendo especies de *Aspergillus* negros dentro del género creado por Cramer (11).

Ya en el año 1926, Thom y Church (249) agruparon las especies en secciones caracterizadas por el color de la colonia y sus características morfológicas. Dentro de los *Aspergillus* negros, consideraron 13 especies (tanto uniseriadas como biseriadas). Mosseray en 1934 describió hasta 35 especies de *Aspergillus* negros, pero la clave de identificación que elaboró era poco práctica por la gran cantidad de características contempladas (197). Posteriormente Thom y Raper (250) subdividieron este grupo en tres series dependiendo de diversos criterios morfológicos y propusieron 15 especies.

Raper y Fennell en 1965 (197) elaboraron la monografía más completa del género *Aspergillus* y redujeron a 12 el número de especies dentro del que ellos denominaron grupo *A. niger*. Las especies dentro de este grupo descritas con posterioridad no fueron aceptadas en la revisión realizada en 1979 por Samson (206). Posteriormente, Al-Musallam (11) y Kozakiewicz (114) realizaron nuevas aportaciones a la taxonomía de las especies del grupo *A. niger*. Gams *et al.* (86) reclasificaron el género *Aspergillus* siguiendo las normas del código de nomenclatura botánica y crearon la sección *Nigri*, incluida dentro del subgénero *Circumdati*. *Aspergillus niger* fue considerada la especie tipo de la sección.

1.2.1.-Criterios morfológicos

En la monografía elaborada por Raper y Fennell, todas las especies de *Aspergillus* con cabeza conidial oscura se incluyeron en el grupo *A. niger*. En este grupo se incluyeron 12 especies y dos variedades que se distinguían por las estructuras uni o biseriadas de los conidióforos, el color de la colonia y de las cabezas conidiales, la forma, tamaño y ornamentación de los conidios, y la velocidad de crecimiento en agar Czapek (197).

Al-Musallam revisó la taxonomía de este grupo considerando características morfológicas y de cultivo después de 7 y 14 días de incubación. Teniendo en cuenta estos caracteres, propuso su división en 5 especies claramente distinguibles entre sí mediante criterios morfológicos (*A. carbonarius, A. ellipticus, A. heteromorphus, A. helicotrix* y *A. japonicus*), y el agregado *A. niger*. La especie *A. carbonarius* se reconocía fácilmente por el gran tamaño de sus estructuras microscópicas, en especial sus conidios de más de 6 μm de tamaño. Las especies *A. ellipticus, A. heteromorphus* y *A. helicotrix* presentaban respectivamente conidios, métulas o esclerocios con formas muy características. *Aspergillus japonicus*, la única especie uniseriada, incluía dos variedades: *A. japonicus* var. *japonicus* y *A. japonicus* var. *aculeatus* que se distinguían por el tamaño de las vesículas y la forma de los conidios. El agregado *A. niger* se caracterizaba por presentar colonias con tonalidades negruzcas y conidios más o menos globosos, de diámetros inferiores a 6 μm y una ornamentación más o menos marcada. Las dos especies que integraban el agregado (*A. niger* y *A. foetidus*) se diferenciaban por el diámetro de las colonias y *A. niger*, a su vez, se dividía en seis variedades y dos formas. Éstas se diferenciaban básicamente según el grado de rugosidad y ornamentación de los conidios (11).

Kozakiewicz (114) constató que la maduración de los conidios de estas especies es muy lenta, siendo de hasta 5 semanas en el caso de las cepas pertenecientes al agregado *A. niger*. Desestimó por tanto la clasificación de Al-Musallam (11) e hizo una nueva propuesta basada en la ornamentación de los conidios utilizando técnicas de microscopía electrónica de barrido (SEM). Distinguió dos patrones básicos de ornamentación conidial: conidios verrugosos o conidios espinosos y dividió la sección en diez especies y ocho variedades. El agregado quedó dividido en tres especies (*A. acidus, A. citricus* y *A. niger*).

Recientemente Abarca *et al.* (4) realizaron una revisión de la sección *Nigri* con el fin de ofrecer una visión general de la problemática taxonómica y la importancia de los miembros de esta

sección. Estos autores propusieron una sencilla clave taxonómica para identificar las especies más comunes de la sección utilizando criterios morfológicos.

Samson *et al.* (208) propusieron 4 nuevas especies en la sección *Nigri*: *A. costaricaensis*, *A. piperis*, *A. sclerotioniger* y *A. lacticoffeatus*. También incluyeron *A. vadensis*, que había sido propuesta como una nueva especie en la sección (67), y validaron la especie *A. homomorphus*, propuesta anteriormente (237). La diferenciación de los 15 taxones aceptados en la sección se basaba en características morfológicas, el perfil de 9 metabolitos secundarios y la secuenciación del gen de la β-tubulina. No obstante, el número de cepas ensayadas correspondientes a las cuatro nuevas especies fue muy bajo: 1 cepa en el caso de *A. costaricaensis*, *A. piperis* y *A. sclerotioniger*, y 3 cepas en el caso de *A. lacticoffeatus*.

En la Tabla 2 se resumen las especies de *Aspergillus* sección *Nigri* propuestas por Raper y Fennell (197), Al Musallam (11), Kozakiewicz (114) y Samson *et al.* (208).

Tabla 2.-Especies propuestas en *Aspergillus* sección *Nigri* según diferentes autores.

Raper y Fennell (197)	Al-Musallam (11)	Kozakiewicz (114)	Samson *et al.* (208)
A. japonicus Saito	*A. japonicus* var. *japonicus* Saito	*A. japonicus* Saito	*A. japonicus* Saito
A. aculeatus Iizuka	*A. japonicus* var. *aculeatus* (Iizuka) Al-Musallam	*A. atroviolaceus* Moss.	*A. aculeatus* Iizuka
A. carbonarius (Bainier) Thom	*A. carbonarius* (Bainier) Thom	*A. carbonarius* (Bainier) Thom	*A. carbonarius* (Bainier) Thom
A. heteromorphus Batista y Maia	*A. heteromorphus* Batista y Maia	*A. fonsecaeus* Thom y Rapper	*A. sclerotioniger* Samson y Frisvad
A. ellipticus Raper y Fennell	*A. ellipticus* (Raper y Fennell) Al-Musallam	*A. heteromorphus* Batista y Maia	*A. heteromorphus* Batista y Maia
A. niger van Tieghem	*A. helicothrix* Al-Musallam	*A. ellipticus* Raper y Fennell	*A. ellipticus* Raper y Fennell
A. ficuum (Reichard) Hennings	Agregado *A. niger*:	*A. helicothrix* Al-Musallam	*A. niger* van Tieghem
A. tubingensis (Schöber) Mosseray	*A. niger* var. *niger* van Tieghem	*A. niger* var. *niger* van Tieghem	*A. tubingensis* (Schöber) Mosseray
A. phoenicis (Corda) Thom	*A. niger* var. *niger* f. *hennebergii* (Blochwitz) Al-Musallam	*A. niger* var. *ficuum* (Reich.) Kozakiewicz	*A. vadensis* de Vries *et al.*
A. pulverulentus (McAlp) Thom	*A. niger* var. *phoenicis* (Corda) Al-Musallam	*A. niger* var. *tubingensis* (Moss.) Kozakiewicz	*A. costaricaensis* Samson y Frisvad
A. awamori Nakazawa	*A. niger* var. *phoenicis* f. *pulverulentus* (McAlp) Al-Musallam	*A. niger* var. *phoenicis* (Corda) Al-Musallam	*A. piperis* Samson y Frisvad
A. foetidus (Naka.) Thom y Raper	*A. niger* var. *awamori* (Nakazawa) Al-Musallam	*A. niger* var. *pulverulentus* (McAlp) Kozakiewicz	*A. lacticoffeatus* Samson y Frisvad
A. foetidus var. *acidus* Naka, Simo y Watanabe	*A. niger* var. *nanus* (Mont.) Al-Musallam	*A. niger* var. *awamori* (Nakazawa) Al-Musallam	"*A. brasiliensis*"
A. foetidus var. *pallidus* Naka, Simo y Watanabe	*A. niger* var. *usamii* (Sakaguchi *et al.*) Al-Musallam	*A. acidus* Kozakiewicz	*A. foetidus* Thom y Rapper
	A. niger var. *intermedius* (Speg.) Al-Musallam	*A. citricus* var. *citricus* (Wehmer) Moss.	*A. homomorphus* Steiman, Guiraud, Sage y Seigle-Mur. ex Samson y Frisvad
	A. foetidus Thom y Raper	*A. citricus* var. *pallidus* (Naka, Simo y Watanabe) Kozakiewicz	

1.2.2.-Criterios fisiológicos y bioquímicos

Se han utilizado diversos criterios fisiológicos y bioquímicos en el estudio de las especies pertenecientes a *Aspergillus* sección *Nigri* con el fin de paliar las dificultades que surgen al utilizar exclusivamente criterios morfológicos, sobre todo con los componentes del agregado *A. niger*.

Al estudiar la utilización como fuentes de carbono de más de cien compuestos, incluyendo azúcares, alcoholes, oligo y polisacáridos, ácidos orgánicos, aminoácidos y nucleótidos, sólo 7 compuestos (melicitosa, xilitol, galactitol, ácido vanílico, ácido cis-aconítico, L-serina, L-tirosina) revelaron patrones variables de utilización entre las especies de la sección *Nigri*. Más del 95% de las 400 cepas ensayadas pertenecientes al agregado *A. niger* utilizaron los 7 compuestos como única fuente de carbono a excepción de L-tirosina. Con respecto las cepas de *A. carbonarius,* éstas utilizaron melicitosa y xilitol como única fuente mientras que la asimilación de galactitol fue variable. Ninguna de estas cepas utilizó ácido vanílico, ácido cis-aconítico, L-serina o L-tirosina. De las especies uniseriadas, sólo la L-tirosina fue utilizada como única fuente de carbono (96, 112, 266).

El análisis de isozimas ha sido también aplicado a las especies de *Aspergillus* sección *Nigri* (96, 112, 153). Al analizar la 6 fosfogluconato deshidrogenasa (6PGDH), la aspartato transaminasa (GOT), la dihidrofolato reductasa (DHFR) y la glucosa 6 fosfato deshidrogenasa (G6PDH) se observó una gran variabilidad entre las diferentes especies de la sección y también entre las cepas pertenecientes al agregado *A. niger* (153). Kevei *et al.* (112) obtuvieron unos patrones homogéneos con la malato deshidrogenasa NADP dependiente y con la glutamato deshidrogenasa NADP dependiente al analizar diversas cepas de *A. carbonarius*. De éstas, una cepa identificada como IN7 mostró un patrón distinto, presentó además unos patrones de RFLP (Restriction Fragment Length Polymorphisms) y RAPD (Random Amplification of Polymorphic DNA) característicos y unos conidios más pequeños, por lo que se indicó que podría tratarse de una nueva subespecie de *A. carbonarius* (112). Al analizar la arilesterasa y las glutamato y malato deshidrogenasas en cepas de *A. japonicus* y *A. aculeatus*, se observó poca variabilidad (96).

En un trabajo en el que se valoraba la capacidad de hidroxilar progesterona por diferentes especies de la sección, todas las cepas fueron capaces de transformar la progesterona a 21-hidroxiprogesterona. A excepción de *A. foetidus* var. *pallidus* y *A. foetidus* var. *acidus* que transformaron la progesterona sólo a este producto, el resto de especies estudiadas produjeron

además otros compuestos hidroxilados diferentes. La producción de 11α,15α-dihidroxiprogesterona se consideró una característica fisiológica propia de las cepas pertenecientes a *A. aculeatus* (157).

Parenicová *et al.* (176) estudiaron los perfiles de metabolitos secundarios de especies uniseriadas dentro de la sección *Nigri*. Las cepas clasificadas como *A. japonicus* producían alcaloides de indol y un metabolito polar, mientras que los aislamientos de *A. aculeatus* producían neoxalina, okaraminas, compuestos de parahercuamidelica y ácido secalónico.

Samson *et al.* (208) utilizan además de algunos criterios morfológicos y moleculares, la producción de 9 metabolitos secundarios (ácido secalónico, aflavininas, antafumicina, asperazina, kotaninas, lactonas corimbíferas, naftopironas, OTA y piranonigrina) para obtener diferentes perfiles taxonómicos de las cepas y justificar la propuesta de las nuevas especies en la sección *Nigri*.

1.2.3.-Criterios basados en el análisis de DNA

Para contrarrestar las limitaciones que se presentan al utilizar los criterios morfológicos y bioquímicos, en el estudio de las especies de esta sección se han considerado también criterios basados en el análisis de DNA que pueden proporcionar una información más objetiva. Las técnicas moleculares empleadas en la sección *Nigri* se han dirigido principalmente a esclarecer tanto la taxonomía de las especies uniseriadas (*A. japonicus* y *A. aculetus*), como a profundizar en el conocimiento de las especies biseriadas, especialmente las especies incluidas en el agregado *A. niger*.

1.2.3.1.-Especies uniseriadas: *Aspergillus japonicus* / *A. aculeatus*.

Las especies uniseriadas de esta sección son *A. japonicus* y *A. aculeatus*, aunque siguiendo ciertos criterios morfológicos se ha propuesto una sola especie (*A. japonicus*) y dos variedades (*A. japonicus* var. *japonicus* y *A. japonicus* var. *aculeatus*) (11). Es por ello que junto a estos criterios se han utilizado diversas técnicas moleculares para lograr clarificar su situación taxonómica (4, 96, 117, 153, 175, 176, 208, 278).

Kusters-van Someren *et al.* (117) analizaron los patrones de RFLP del rDNA obtenidos mediante digestión del DNA total con *Sma*I y observaron que *A. aculeatus* y *A. japonicus* aparecían idénticos. Otros autores diferenciaron *A. japonicus* y *A. aculeatus* tras el análisis de los patrones de RFLP del rDNA obtenidos con *Eco*RI y con *Pst*I-*Sal*I (153, 275). Visser *et al.* (275) observaron que la cepa *A. aculeatus* CBS 114.80 mostraba un patrón distinto al resto de cepas de *A. aculeatus*.

Hamari *et al.* (96) no observaron diferencias entre los patrones del rDNA de las dos especies. No obstante, la aplicación de los enzimas de restricción *Bgl*II, *Eco*RI y *Pvu*II en mtDNA permitió la obtención de hasta 7 patrones distintos. Estos resultados, juntamente con los criterios fisiológicos estudiados confirmaron la distinción de las dos especies como dos grupos diferenciados (96). Parenicová *et al.* (175, 176) distinguieron *A. aculeatus* de *A. japonicus* mediante RFLP y la utilización de diferentes combinaciones de enzimas de restricción. No obstante, la secuenciación de las regiones ITS-5,8S del rDNA no permitió diferenciarlas como especies distintas (175, 176). Basándose en la secuenciación de esta región, los patrones de RFLP ensayados y los perfiles de metabolitos secundarios consideraron que la cepa *A. aculeatus* CBS 114.80 podría representar un tercer taxón uniseriado (176).

Peterson (185), en el árbol filogenético obtenido tras secuenciar las regiones D1 y D2 de la unidad 28S del rDNA, observó que las especies uniseriadas se agrupaban separados del resto de especies biseriadas de la sección.

La secuencia de un fragmento del gen mitocondrial del citocromo *b* mostró que, tanto *A. japonicus* como *A. aculeatus* presentaban la misma secuencia de aminoácidos por lo que se consideró que pertenecían a una misma especie (278). Sin embargo, Hamari *et al.* (98) describió la incapacidad de transmisión de los genomas mitocondriales entre ambas especies.

Abarca *et al.* (4) observaron que los dos taxones uniseriados compartían una secuencia de la región ITS-5,8S del rDNA idéntica, y que por tanto podrían representar una sola especie según este criterio.

Samson *et al.* (208) analizaron la secuencia del gen de la β-tubulina y junto con el perfil de producción de metabolitos secundarios y diferentes criterios morfológicos, incluyeron en la lista provisional de miembros de la sección a *A. japonicus* y *A. aculeatus* como dos especies distintas.

1.2.3.2.-Especies biseriadas.

1.2.3.2.1.-*Aspergillus carbonarius*.

Las características morfológicas de *A. carbonarius* hacen que sea una especie dentro de la sección *Nigri* claramente diferenciada del resto (4). De igual modo, el análisis de RFLP del rDNA y del mtDNA y de RAPD permiten igualmente esta diferenciación (117, 153, 174, 175, 176, 266).

Al analizar la variabilidad intraespecífica de diversas cepas de *A. carbonarius* mediante RFLP del rDNA y mtDNA y mediante RAPD se observaron patrones muy similares entre las diferentes cepas, aunque con ligeras variaciones (112). Sólo una cepa (IN7) presentó patrones de restricción de rDNA y mtDNA y de RAPD diferentes al resto, al igual que diversos caracteres morfológicos y fenotípicos distintivos. Los autores propusieron que este aislamiento representaría una nueva subespecie de *A. carbonarius* denominada "*A. carbonarius* var. *indicus*" (112, 266). Los mapas físicos del mtDNA de la cepa IN7 mostraron diferencias en el tamaño de este mtDNA en comparación con otras cepas de *A. carbonarius*, aunque el contenido genético era casi idéntico (97).

El análisis filogenético de la región ITS-5,8S de las especies de esta sección reveló que *A. carbonarius* se diferenciaba claramente del resto de especies ensayadas. *Aspergillus carbonarius* formaba un grupo separado dentro de las especies biseriadas (4, 175, 176). Resultados similares se observaron al analizar las regiones D1 y D2 de la unidad 28S del rDNA (185).

Recientemente, Bau *et al.* (26) y Serra *et al.* (219) detectaron cepas inicialmente identificadas como *A. carbonarius*, aisladas en la Península Ibérica, que no producían OTA y presentaban conidios de un diámetro inferior al característico de esta especie. Éstas además se diferenciaron del resto de cepas de *A. carbonarius* mediante RAPD y secuenciación de la región ITS-5,8S del rDNA (26). Estas diferencias se han confirmado mediante AFLP y secuenciación del gen de la calmodulina, por lo que se ha realizado la propuesta de una nueva especie dentro de la sección *Nigri*, denominada "*A. ibericus*" (220).

1.2.3.2.2.-Agregado *Aspergillus niger*.

Se han utilizado diversas técnicas moleculares con el fin de clarificar la taxonomía de los miembros de este agregado ya que morfológicamente las especies que lo componen son indistinguibles (4).

Kusters-van Someren *et al.* (117) observaron dos patrones de RFLP del rDNA al realizar la digestión con *Sma*I en cepas del agregado *A. niger* (rDNAs tipos I y II). Como el cultivo neotipo de *A. niger* (CBS 554.65) pertenecía al grupo I y el cultivo tipo de *A. tubingensis* (CBS 134.48) al grupo II, propusieron denominar a las cepas del grupo I como *A. niger* y a las del grupo II como *A. tubingensis*. Estos dos grupos eran morfológicamente idénticos. Posteriormente, Mégnégneau *et al.* (153) confirmaron estos resultados mediante el análisis de RFLP del DNA total con *Sma*I, *Eco*RI y *Pst*I en cepas de colección. En este trabajo obtuvieron cuatro patrones de restricción del rDNA que se podían agrupar en dos grupos principales que coincidían con los propuestos por Kusters-van Someren *et al.* (117).

Los patrones de restricción del rDNA obtenidos por Varga *et al.* (263) con *Sma*I coincidieron con trabajos anteriores (153) y, mediante RFLP del mtDNA con diferentes enzimas de restricción, agruparon 47 cepas de colección en cinco patrones distintos. Estos patrones se podían agrupar en dos grupos principales que se correspondían con los obtenidos por Kusters-van Someren *et al.* (117). En otro trabajo posterior, detectaron hasta 12 patrones de restricción del mtDNA al utilizar la combinación *Hae*III-*Bgl*II en cepas aisladas de suelo (264). Estos patrones se agrupaban también en dos grupos principales que correspondían con los propuestos por Kusters-van Someren *et al.* (117) con la excepción de un tercer patrón. Este tercer patrón de RFLP se observó solamente en 6 cepas aisladas de suelo de Brasil que no se distinguían morfológicamente de las cepas agrupadas como *A. niger* o *A. tubingensis*. Estos autores propusieron que las cepas pertenecientes a este tercer patrón representarían una subespecie de *A. niger* o una nueva especie del agregado *A. niger*, provisionalmente denominada *"A. brasiliensis"* (264). Kevei *et al.* (113) demostraron que era posible transferir mitocondrias entre cepas del agregado *A. niger* que presentaban los diferentes patrones de RFLP del mtDNA detectados por Varga *et al.* (263, 264). Tal transferencia no fue posible entre cepas del agregado y cepas de *A. carbonarius* o *A. japonicus*.

La división del agregado *A. niger* en los grupos *A. niger* y *A. tubingensis* fue confirmado de nuevo por Visser *et al.* mediante la utilización de diferentes enzimas de restricción y la hibridación con diferentes sondas (275).

Parenicová *et al.* (174, 175) en un estudio con 23 cepas de colección del agregado *A. niger*, describieron un nuevo patrón de RFLP del rDNA obtenido con *Pst*I-*Sal*I. Este patrón estaba representado por las cepas tipo de variedades de *A. foetidus*. Como consecuencia de ello propusieron la división del agregado en tres taxones morfológicamente idénticos: *A. niger*, *A. tubingensis* y *A. foetidus*. Las cepas representando el nuevo grupo no habían sido estudiadas previamente, pero se clasificarían como *A. tubingensis* al digerir el DNA con *Sma*I siguiendo el método descrito por Kusters-van Someren *et al.* (117). En un trabajo posterior (176), se propuso la división del agregado en 4 especies morfológicamente idénticas: *A. niger*, *A. tubingensis*, *A. foetidus* y "*A. brasiliensis*". Esta división se propuso tras considerar los patrones de restricción obtenidos con *Kpn*I-*Xho*I y *Pst*I-*Sal*I. No obstante, al realizar la digestión del DNA con *Pst*I-*Sal*I y la hibridación con el gen 28S del rDNA, que anteriormente había permitido separar cepas de *A. foetidus* del resto de especies del agregado (174), *A. niger* y "*A. brasiliensis*" compartían el mismo patrón de rDNA (176).

El análisis de las regiones ITS-5,8S del rDNA de las cepas de la sección *Nigri* incluidas en otro estudio (175) permitió determinar que las diferencias entre las 4 especies anteriormente comentadas (*A. niger*, *A. tubingensis*, *A. foetidus* y "*A. brasiliensis*") eran mínimas. Las secuencias de *A. niger* y *A. tubingensis* se diferenciaban en sólo 3 nucleótidos y las de *A. foetidus* y *A. tubingensis* en 2 nucleótidos. Entre las secuencias de *A. niger* y *A. foetidus* habían 5 diferencias (175). Los árboles filogenéticos obtenidos del análisis de estas regiones del DNA realizados por diferentes autores (4, 176, 269) muestran las cepas del agregado *A. niger* claramente diferenciadas del resto de cepas de la sección *Nigri*. En el agregado se agrupan conjuntamente los cuatro grupos propuestos: *A. niger*, *A. tubingensis*, *A. foetidus* y "*A. brasiliensis*". No obstante, las cepas representativas de *Aspergillus tubingensis* y *A. foetidus* se agrupan juntas en un subgrupo y la secuencia de "*A. brasiliensis*" es la más distinta dentro del agregado. Al realizar la secuenciación de las regiones D1 y D2 de la unidad 28S del rDNA las especies pertenecientes al agregado se diferencian del resto de especies de la sección aunque dentro del agregado no se observa una variabilidad remarcable (185).

Yokoyama *et al.* (278) dividieron el agregado *A. niger* en dos grupos representados por *A. niger* y *A. awamori* tras secuenciar un fragmento del gen mitocondrial del citocromo *b*, añadiendo así más confusión a la taxonomía de este grupo.

Accensi *et al.* (9) secuenciaron la región ITS-5,8S de las cepas tipo de *A. niger* (CBS 554.65) y de *A. tubingensis* (CBS 134.48). Al comparar estas secuencias se localizó una diana para la enzima *Rsa*I en la secuencia de *A. niger*. Tras realizar la digestión, se obtenían dos patrones de rDNA dentro del agregado *A. niger*: el patrón denominado N (2 fragmentos, 519 y 76 pares de bases (pb)) y el patrón T (un fragmento de 595 pb). Ambos grupos correspondían con los dos propuestos por Kusters-van Someren *et al.* (117). En este estudio se ensayó una cepa de "*A. brasiliensis*" utilizando esta técnica y se clasificó como tipo N. La cepa tipo de *A. foetidus* var. *acidus* (CBS 564.65), incluida en el grupo *A. foetidus* por Parenicová *et al.* (174, 176), se clasificaría como tipo T. La simplicidad de esta técnica permite fácilmente la detección de los patrones N y T, presentando menos variabilidad que otros patrones de RFLP descritos anteriormente por otros autores (117, 153, 174, 176, 263, 264). En un estudio posterior, Accensi *et al.* (10) pusieron de manifiesto que todas las cepas productoras de OTA estudiadas tienen un patrón de RFLP tipo N. Este hecho ha sido corroborado en estudios posteriores en los que se han estudiado los patrones de RFLP de alrededor de 200 cepas del agregado *A. niger* de diferentes orígenes, incluyendo también cepas de colecciones internacionales (3, 27, 51, 126). No obstante, recientemente se ha descrito la producción de OTA por 3 cepas aisladas de uva que han presentado un patrón T (152).

En la Tabla 3 pueden observarse algunos patrones obtenidos con los estudios moleculares llevados a cabo con las especies del agregado *A. niger* y *A. carbonarius*.

Tabla 3.-Diferentes patrones moleculares de cepas del agregado *A. niger* y *A. carbonarius* según distintos autores.

	RFLP rDNA (*Sma*I) (117)	RFLP rDNA (*Sma*I, *Eco*RI, *Pst*I) (153)	RFLP mtDNA (263)	RFLP mtDNA (*Hae*III-*Bgl*II) (264)	RFLP rDNA (*Sma*I) (264)	RFLP mtDNA (*Hae*III) (112)	RFLP (*Pst*I-*Sal*I) + Southern *pel*A (174)	RFLP (*Pst*I-*Sal*I) + Southern 28S (174)	RFLP rDNA (*Sma*I) (174)	RFLP ITS-5.8S rDNA (9)	RFLP mtDNA (*Hae*III-*Bgl*II, *Hae*III) (266)	RFLP rDNA (*Sma*I) (266)	RFLP (*Pst*I-*Sal*I) + Southern *pel*A (176)	RFLP (*Pst*I-*Sal*I) + Southern 28S (176)	RFLP (*Kpn*I-*Xho*I) + Southern 28S (176)	RAPD y secuenc. ITS 5.8S rDNA (26) AFLP y secuenc. gen calmodulina (220)
A. niger	I	I I’	1a 1b 1c	1a 1b 1c 1d 1e	I I’		E / F / D	C	B	N	1a 1b 1c 1d 1e	I	B	B	B	
A. tubingensis	II	II II’	2a 2b	2a 2b 2c 2d 2e 2f	II II’		A / B	B / D	C	T	2a 2b 2c 2d 2e 2f	II-II’	D	C	A	
“*A. brasiliensis*”				3	III					N	3a 3b	III	G	B	D	
A. foetidus							C	D	C	T			C	D	A	
A. carbonarius						1a 1b	J		A		C1a C1b	C1	A	A	C	*A. carbonarius* “*A. ibericus*”
“*A. carbonarius* var. *indicus*” (IN7)						2					C2	C2				

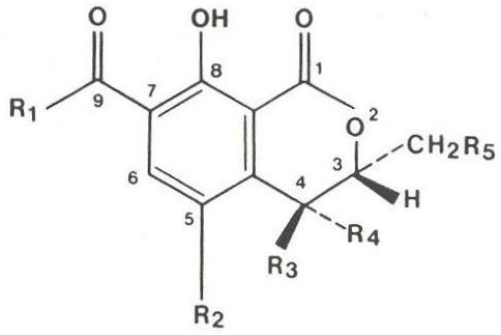
1.3.-Las ocratoxinas

Las ocratoxinas son un grupo de micotoxinas producidas por diversas especies de los géneros *Aspergillus* y *Penicillium*. Estos metabolitos fúngicos son ácidos orgánicos débiles y, entre los diferentes tipos, la ocratoxina A (OTA) es la que presenta una mayor toxicidad. Ésta presenta efectos nefrotóxicos, carcinógenos, teratógenos e inmunosupresores. La OTA consiste en una dihidroisocumarina unida por el grupo 7-carboxilo a una molécula de L-β-fenilalanina mediante un enlace amida. Su estructura química es: (R)-N-((5-cloro-3,4-dihidro-8-hidroxi-3-metil-1-oxo-1H-2-benzopirano-7-il)carbonil)-L-fenilalanina. La ocratoxina B (OTB) es el derivado no clorado de la OTA y es significativamente menos tóxica tanto *in vivo* como *in vitro*. La ocratoxina C (OTC) es el éster de la OTA y su posible potencial tóxico, aunque se considera prácticamente nulo, se ha descrito específicamente sobre algunas líneas celulares de monocitos en el hombre (166).

La ocratoxina α (OTα) y la ocratoxina β (OTβ) son productos de la hidrólisis de la OTA y OTB respectivamente, no poseen la molécula de la fenilalanina y no se consideran tóxicos (115, 166). La OTα se ha detectado en la orina de animales de experimentación a los que se les ha administrado OTA (233, 238). En animales rumiantes se ha observado un proceso de detoxificación de la OTA ingerida a OTα llevada a cabo en el rumen (233). En la Figura 1 se muestra la estructura química de las diferentes ocratoxinas

La OTA está estructuralmente relacionada con la citrinina (115). Esta micotoxina puede aparecer como co-metabolito de la OTA en *Penicillium verrucosum* (189).

Fig.1 Estructura química de las diferentes ocratoxinas (115).



R1	R2	R3	R4	R5	
Fenilalanina	Cl	H	H	H	Ocratoxina A
Fenilalanina	H	H	H	H	Ocratoxina B
Fenilalanina, etil éster	Cl	H	H	H	Ocratoxina C
OH	Cl	H	H	H	Ocratoxina α
OH	H	H	H	H	Ocratoxina β

1.3.1.-Hongos productores de ocratoxina A

El primer aislamiento de OTA data del año 1965 y se asoció al género *Aspergillus,* concretamente a *A. ochraceus* (258). Desde su descubrimiento, la producción de esa micotoxina se ha asociado a distintas especies pertenecientes también a la sección *Circumdati*: *A. auricomus, A. elegans, A. insulicola, A. melleus, A. ostianus, A. petrakii, A. sclerotiorum, A. sulphureus, Neopetromyces muricatus, Petromyces albertensis* y *Petromyces alliaceus* (21, 82, 103, 265). Las especies ocratoxígenas *Petromyces albertensis* y *P. alliaceus* se consideran ahora sinónimos y se han reclasificado en la sección *Flavi* (185, 267). Recientemente Frisvad *et al.* (84) propusieron nuevas especies en la sección *Circumdati,* la mayoría de ellas productoras de OTA: *A. cretensis, A. flocculosus, A. pseudoelegans, A. roseoglobulosus, A. steynii* y *A. westerdijkiae*. De manera esporádica, también se ha descrito la producción de OTA en especies incluidas en otras secciones (*Aspergillus*, *Fumigati*, *Terrei*, *Usti*, *Versicolores* y *Wentii*) (2).

La sección *Nigri* por el contrario ha adquirido una gran importancia como productora de OTA desde la primera descripción de la producción de esta micotoxina por *A. niger* var. *niger* (1) y por *A. carbonarius* (105). Los porcentajes descritos de aislamientos ocratoxígenos en el caso del agregado *A. niger* varían entre el 0,6 y el 50%, mientras que este porcentaje es cercano al 100% en

A. carbonarius (4, 25, 91, 126, 146, 200, 205, 219, 251, 257). Aunque algunos autores han señalado la capacidad de las especies uniseriadas de la sección para producir OTA (24, 62, 146), este hecho requiere confirmación ya que no se consideran especies productoras (25, 26, 91, 126, 176, 200, 208, 246).

Dentro del género *Penicillium*, la producción de OTA se detectó inicialmente en *Penicillium viridicatum* (262) y posteriormente en otras especies, en algunos casos no siempre correctamente identificadas debido a la complejidad taxonómica del género. En la actualidad, se ha confirmado que los aislamientos que fueron identificados como *P. viridicatum* productores de OTA y citrinina son en realidad *P. verrucosum* (59, 188) aunque todavía hoy aún se cita en diversas revisiones a la especie *P. viridicatum* como productora de OTA. Actualmente *P. verrucosum* se ha dividido en 2 especies ocratoxígenas: *P. verrucosum* y *P. nordicum* (54, 121, 207).

1.3.2.-Presencia y legislación de ocratoxina A

La presencia de OTA ha sido descrita en múltiples sustratos. Se ha detectado OTA en cereales, incluyendo maíz, cebada, trigo, sorgo, centeno, avena y arroz (191); leguminosas (69, 191, 261) y en productos cárnicos de cerdo y aves (53, 61, 87, 88, 104, 108, 110, 115, 138, 186, 193, 216, 228, 256, 261) y leche de vaca (226). La presencia de OTA en estos animales de producción se produce tras el consumo de piensos que contienen la micotoxina (7, 85, 88, 104, 193). No obstante, algunas especies ocratoxígenas también han sido aisladas de productos con un alto contenido en proteínas y grasa como la carne y el queso (106, 116, 121, 137). La contaminación por OTA también se ha detectado en multitud de otros alimentos, tal y como muestra la Tabla 4.

Tabla 4.-Presencia de OTA en diferentes alimentos.

Sustrato	País	Referencia
Alimentos infantiles a base de cereales	Italia	32, 34
Café	Alemania	41, 101, 171
	Bélgica	224
	Brasil	66, 127, 210, 243, 255
	Canadá	133
	Dinamarca	110
	España	48
	Europa[a]	259
	Holanda	260
	Hungría	74, 247
	Italia	155, 199
	Japón	159, 253, 254
	Portugal	151
	Qatar	5
	Reino Unido	181, 228
	Suiza	36, 43, 44, 109, 190, 192, 239, 271, 279
Cerveza	Alemania	41, 68, 107, 154
	Bélgica	242
	Canadá	217, 231
	Dinamarca	110
	España	124
	Italia	274
	Japón	160, 253
	Sudáfrica	168
	Suiza	279
	Turquía	93
Chocolate y cacao	Alemania	72
	España	50, 221
	Reino Unido	228
Especias	Bélgica	248
	Qatar	5
	Reino Unido	180
Galletas, bizcochos y cereales de desayuno	Alemania	72
	España	37
Pan	España[b]	125
	Italia	272
Pasas	Alemania	72
	Argentina	146, 200
	Canadá	134
	Qatar	5
	Reino Unido	139, 140, 228
Queso	Alemania	72
Regaliz	Alemania	42
Salsa de soja	Japón	253
Salsas[c]	Alemania	148
Té	Alemania	41
Uva	Italia	23
Vinagres	Alemania	148
	Francia	150

(sigue)

(Tabla 4, continua)

Vino, mosto y zumo de uva	Alemania	147, 148, 170
	Brasil	202
	Canadá	162, 231
	España	28, 38, 49, 135
	Francia	150, 204, 205
	Grecia	232, 236
	Italia	23, 55, 56, 120, 130, 187, 244, 245, 273, 274
	Japón	253
	Marruecos	76
	Portugal	198
	Sudáfrica	222, 234
	Suiza	279, 280
	Reino Unido	228
	Estados Unidos	223
Zumos[d]	Alemania	148

[a]: Estudio en colaboración.
[b]: OTA también detectada en muestras de pan de Francia, Holanda, Bélgica, Italia, Alemania, Irlanda, Austria, Suiza, Hungría, Estados Unidos, Túnez y Brasil.
[c]: Ketchup, mostaza, salsa de pimienta y salsa barbacoa.
[d]: Zumos de grosella, tomate y zanahoria.

En Europa se estima que al menos el 50% de la ingesta de OTA en la dieta proviene de los cereales y sus derivados (22). Después de los cereales, el vino se considera la segunda fuente de OTA (15% de la ingesta) en la dieta europea (12). Estudios recientes han mostrado que miembros de *Aspergillus* sección *Nigri*, y principalmente *A. carbonarius*, son la fuente principal de contaminación de OTA en uva y como consecuencia en el vino (24, 25, 51, 91, 126, 145, 201, 204, 205, 218, 219, 251, 257) y en uvas pasas (3, 100, 146, 200, 257). También podrían ser una fuente de contaminación en café (45, 109, 243, 247).

En 1991, el Comité Conjunto de Expertos sobre Aditivos Alimentarios de la FAO/OMS (Joint Expert Committee on Food Additives) estableció un valor de ingesta tolerable semanal para OTA de 112 ng OTA/kg (16 ng/kg/día). En 1998, debido a las propiedades tóxicas de la OTA, el Comité Científico sobre Alimentos de la UE (European Union Scientific Committee for Food) estimó que el nivel aceptable de seguridad en la ingesta diaria de OTA debía estar por debajo de 5 ng/kg. Desde el año 2002 la Unión Europea (UE) dispone de una normativa por la que se establecen los niveles máximos de OTA en distintos alimentos. Aunque la última actualización data del año 2005, estos límites serán revisados en un futuro próximo y los límites de OTA en café verde, frutos secos distintos de las uvas pasas, cerveza, cacao y productos del cacao, vinos de licor, productos cárnicos, especias y regaliz están pendientes de ser incluidos (13, 14, 16). Algunos países de la UE tales como Italia o Dinamarca presentan una normativa propia más restrictiva y, fuera de la UE, diversos países también han incluido límites para la OTA en algunos productos alimentarios (15). En la Tabla 5 se muestra el contenido máximo de OTA tolerado en alimentos para los estados miembros de la UE.

Tabla 5.-Contenido máximo de OTA tolerado en alimentos para los estados miembros de la UE (16).

Sustrato	Límite OTA (μg/kg)
Cereales (incluido el arroz y el alforfón) y productos derivados de los mismos:	
-cereales en grano sin transformar (incluido arroz sin transformar y alforfón)	5
-productos derivados de los cereales (incluidos productos transformados a base de cereales y los cereales en grano destinados al consumo humano directo)	3
Uvas pasas (pasas de Corinto, sultanas y otras variedades de pasas)	10
Café:	
-café tostado en grano y café tostado molido, con excepción del café soluble	5
-café soluble (café instantáneo)	10
Vino (tinto, blanco y rosado)[a] y otras bebidas a base de vino y/o mosto de uva[b]	2[c]
Zumo de uva, ingredientes de zumo de uva en otras bebidas, incluido el néctar de fruta y el zumo de uva concentrado reconstituido[d]	2[c]
Mosto de uva y mosto de uva concentrado reconstituido, destinados a consumo humano directo[d]	2[c]
Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad[e]	0,5
Alimentos dietéticos destinados a usos médicos especiales dirigidos específicamente a lactantes[f]	0,5
Café verde, frutos secos distintos de las uvas pasas, cerveza, cacao y productos del cacao, vinos de licor, productos cárnicos, especias y regaliz (a determinar antes del 30-06-06)	---

[a]: Vinos, incluidos los vinos espumosos, pero excluidos los vinos de licor y los vinos con un grado alcohólico volumétrico no inferior al 15% vol., tal como se definen en el Reglamento (CE) nº 1493/1999 (DO L 179 de 14.7.1999, p. 1), y los vinos de fruta.

[b]: Vinos aromatizados, bebidas aromatizadas a base de vino y cócteles aromatizados de productos vitivinícolas, tal como se definen en el Reglamento (CEE) nº 1601/91 del Consejo (DO L 149 de 14.6.1991, p. 1). El contenido máximo de ocratoxina A aplicable a estas bebidas está en función de la proporción de vino y/o mosto de uva presente en el producto acabado.

[c]: El contenido máximo se aplica a los productos procedentes de la cosecha de 2005 en adelante.

[d]: Zumos de frutas, incluidos los zumos de frutas a base de concentrado, los zumos de frutas concentrados y el néctar de frutas, tal como se definen en los anexos 1 y 2 de la Directiva 2001/112/CE del Consejo, de 20 de diciembre de 2001, relativa a los zumos de frutas y otros productos similares destinados a la alimentación humana (DO L 10 de 12.1.2002, p. 58), y los productos derivados de la uva.

[e]: Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad, tal como se definen en el artículo 1 de la Directiva 96/5/CE de la Comisión, de 16 de febrero de 1996, relativa a los alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad (DO L 49 de 28.2.1996, p. 17). Directiva cuya última modificación la constituye la Directiva 2003/13/CE (DO L 41 de 14.2.2003, p. 33).

El contenido máximo relativo a los alimentos elaborados a base de cereales y los alimentos infantiles para lactantes y niños de corta edad se refiere a la materia seca, que se determina con arreglo a lo dispuesto en la Directiva 2002/26/CE.

[f]: Alimentos dietéticos destinados a usos médicos especiales, tal como se definen en el apartado 2 del artículo 1 de la Directiva 1999/21/CE de la Comisión, de 25 de marzo de 1999, sobre alimentos dietéticos destinados a usos médicos especiales (DO L 91 de 7.4.1999, p. 29).

El contenido máximo relativo a los alimentos dietéticos para usos médicos especiales dirigidos específicamente a los lactantes se refiere:

- en el caso de la leche y los productos lácteos, a los productos listos para el consumo (comercializados como tales o reconstituidos de acuerdo con las instrucciones del fabricante).
- en el caso de los productos distintos de la leche y los productos lácteos, a la materia seca, que se determina con arreglo a lo dispuesto en la Directiva 2002/26/CE.

1.3.3.-Métodos de detección de hongos productores de ocratoxina A

La extracción de OTA se realiza con disolventes orgánicos y en condiciones ácidas para que la molécula no se halle disociada. Tras un proceso de purificación, los métodos más comúnmente utilizados para detectar la presencia de OTA en el laboratorio son las técnicas de cromatografía en capa fina (TLC) y la cromatografía líquida de alta eficacia (HPLC) (213, 256).

Los primeros ensayos laboratoriales para detectar micotoxinas en cultivos fúngicos requerían mucho material y eran procesos lentos y tediosos, sobre todo cuando se necesitaba estudiar un gran número de cepas. Habitualmente el hongo se desarrollaba en sustratos naturales o en medios de cultivo sólidos o líquidos y a continuación se realizaba la extracción, purificación y concentración de la toxina elaborada. La técnica más utilizada para detectar la micotoxina era la TLC (46, 214, 215).

Filtenborg y Frisvad (77) propusieron un sistema más simple basado en la detección de la toxina presente en un bocado de agar (agar technique plug). Este método resultó eficaz para la detección de algunas micotoxinas extracelulares entre las que se incluye la OTA. Se recortaban bocados de forma cilíndrica conteniendo micelio fúngico y medio de cultivo y éstos se transferían directamente a una placa de TLC. Posteriormente, propusieron alguna modificación para la detección de micotoxinas intracelulares que consistía en añadir unas gotas de líquido de extracción al bocado de agar para liberar las toxinas del micelio (78). El medio agar extracto de levadura sacarosa (YES, 189) se recomendó para la detección de micotoxinas extracelulares y el medio agar Czapek extracto de levadura (CYA, 189) resultó muy eficaz para la detección de toxinas intracelulares en diferentes estudios (78-80).

A partir del método del bocado de agar descrito anteriormente, Bragulat *et al.* (40) desarrollaron un método más sencillo y limpio basado en la detección y cuantificación de OTA mediante HPLC. Se introducían tres bocados extraídos de la colonia fúngica en un vial con 1 ml de metanol durante una hora, se filtraba el extracto y se cuantificaba con HPLC. En este trabajo se recomendaba los medios CYA y YES, dependiendo de la especie, para estudios de producción de OTA en medio de cultivo.

Aprovechando la fluorescencia emitida por algunas micotoxinas, diversos autores (71, 129) desarrollaron un medio de cultivo que permitía la detección cualitativa de aflatoxinas. Tras incubar las cepas aflatoxígenas en el medio agar crema de coco (CCA), las colonias se observaban fluorescentes a la luz ultravioleta. Este medio de cultivo fue igualmente eficaz para la detección de cepas productoras de OTA y presentó resultados comparables a los obtenidos con la técnica de TLC (100).

Otros autores han propuesto métodos inmunológicos con anticuerpos monoclonales específicos contra OTA (ELISA) para la detección de cepas ocratoxígenas, pero no se utilizan tan habitualmente en cultivos fúngicos como las técnicas cromatográficas (246, 265).

Recientemente se han descrito diferentes técnicas basadas en PCR para la detección de DNA de hongos ocratoxígenos. Los polimorfismos obtenidos mediante AFLP (Amplified Fragment Length Polymorphism) y RAPD han permitido diseñar cebadores específicos para la detección de *A. ochraceus* y *A. carbonarius* en café verde. Estos cebadores, si bien presentan una elevada especificidad para estas especies fúngicas, no permiten la distinción entre las cepas productoras y no productoras de OTA (164). Otros cebadores se han diseñado a partir de la secuenciación de regiones concretas ya conocidas del DNA. De este modo, la presencia de zonas variables en el gen de la calmodulina ha permitido desarrollar cebadores específicos para la identificación de cepas de *A. japonicus* y *A. carbonarius* (184). Las diferencias inter-específicas detectadas en las regiones ITS-5,8S del rDNA también han permitido la detección y diferenciación de algunas especies dentro de *Aspergillus* sección *Nigri* mediante cebadores específicos (92). Asimismo, en un trabajo donde se diseñaron cebadores a partir de esta región de DNA, se detectó específicamente *A. ochraceus* o *A. carbonarius* cuando se ensayaron en diferentes especies de *Aspergillus* (182). Con la reciente identificación y caracterización de genes de la policétido sintasa, muy posiblemente involucrados en la biosíntesis de OTA (167), Geisen *et al.* (90) detectaron específicamente *P. nordicum* mediante el uso de cebadores dirigidos a esta región de DNA. Sin embargo, estos cebadores no fueron útiles para detectar otras especies ocratoxígenas como *P. verrucosum* o *A. ochraceus*. Posteriormente Dao *et al.* (63) diseñaron 2 pares de cebadores a partir de la secuencia de un gen de la policétido sintasa de una cepa de *A. ochraceus*. Al ensayar estos cebadores con diferentes especies, se observó que un par permitía la detección de especies de *Penicillium* y *Aspergillus* productoras de OTA o citrinina y el otro par de cebadores detectaba exclusivamente *A. ochraceus*.

En este momento se están desarrollando técnicas basadas en microsistemas que permitan detectar la producción de micotoxinas de forma rápida y eficaz en un futuro próximo. Estas técnicas incluyen la detección específica de micotoxinas mediante biosensores y sensores químicos basados en electrodos, anticuerpos o receptores sintéticos. También se están desarrollando sistemas para la detección de hongos toxígenos mediante chips de DNA (DNA arrays) que utilizados en sondas se unirían de forma complementaria a secuencias de DNA específicas del hongo estudiado. También se han hecho algunos ensayos con nariz electrónica para la detección de compuestos volátiles asociados a la producción de algunas micotoxinas o bien liberados durante el crecimiento fúngico y lengua electrónica para la detección de estos compuestos en sustratos líquidos (132, 144). Mediante nariz electrónica se han podido diferenciar cepas de *P. verrucosum* productoras y no productoras de OTA en pan (161) y ha sido posible detectar trigo contaminado con micotoxinas utilizando este sistema (252). Recientemente se ha decrito la diferenciación de cuatro especies del género *Aspergillus* (*A. flavus*, *A. ochraceus*, *A. oryzae* y *A. versicolor*) mediante la utilización de lengua electrónica (230).

1.3.4. Factores que influyen en la producción de ocratoxina A

Existen múltiples factores que intervienen en el desarrollo fúngico y también en la biosíntesis de metabolitos secundarios. Algunos de éstos son intrínsecos, es decir, dependen exclusivamente de la propia cepa fúngica, de su base genética. Recientemente, varios trabajos han permitido la detección de algunos genes que podrían estar involucrados en la biosíntesis de OTA. Inicialmente se caracterizaron genes que codificaban una policétido sintasa expresada exclusivamente cuando el hongo se desarrollaba en condiciones favorables para la producción de OTA. La identificación se realizó en *A. ochraceus* (167) y en *P. nordicum* (90, 111) pero entre ambas especies estos genes presentaban poca homología. Se han podido identificar además en *P. nordicum* otros genes de enzimas posiblemente involucradas igualmente en el proceso de biosíntesis: una péptido sintasa no ribosómica, una halogenasa, una fenilalanina tRNA sintetasa, una metilasa y un fragmento homólogo a genes ABC transportadores (73, 111).

Por otro lado, existen una serie de factores extrínsecos que afectan al comportamiento de la cepa y hacen que la invasión fúngica, la infección y la elaboración de micotoxinas en diferentes sustratos

naturales dependan en gran medida de ellos. Estos factores se pueden dividir en bióticos y abióticos.

1.3.4.1.-Factores bióticos

El crecimiento fúngico con otros organismos presentes en el sustrato afecta tanto el desarrollo del hongo como la producción de micotoxinas. Los primeros colonizadores de muchos sustratos naturales son las bacterias, y después son seguidas por levaduras y hongos miceliares. Este hecho puede afectar el comportamiento de las especies micotoxígenas. Así, al estudiar la incubación de *A. parasiticus* en un sustrato donde se había desarrollado previamente *Lactobacillus casei*, se observó que el crecimiento del hongo se producía más lentamente y la producción de aflatoxina era más baja. Resultados similares se detectaron con *Streptococcus lactis* como organismo competitivo (165).

El crecimiento conjunto en un sustrato de determinadas especies fúngicas puede influir de forma significativa en la producción de OTA. Se ha descrito la inhibición de la producción de OTA por *P. verrucosum* en cebada en presencia de *A. flavus* y *Hyphopichia burtonii* (195). En estudios llevados a cabo con *A. ochraceus* en maíz, se observó que la producción de OTA puede verse inhibida o estimulada al crecer en contacto con diversas especies de *Alternaria*, *Aspergillus* y *Eurotium*. Este efecto se producía cuando el sustrato presentaba valores de actividad de agua (a_w) superiores a 0,95 a_w, por lo que los factores abióticos pueden influir significativamente en el efecto de la micobiota competidora sobre la producción de OTA (122, 123).

En sustratos naturales la presencia de insectos puede facilitar la colonización fúngica e influir así en el contenido de micotoxinas. Además del daño físico que pueden generar en el sustrato, también actúan como vectores diseminando los conidios de especies fúngicas, algunas de ellas productoras de micotoxinas (102, 169, 211, 270).

1.3.4.2.-Factores abióticos

Las condiciones ambientales ejercen una importante influencia en el crecimiento fúngico así como sobre la producción de micotoxinas. Tanto en el campo como en los productos recolectados y almacenados, el crecimiento y la esporulación de los posibles hongos contaminantes depende en

gran medida de estos factores ambientales (47, 128, 143, 165). Esto ha propiciado el desarrollo de diversos modelos de producción de micotoxinas para estudiar el efecto de estos factores abióticos. La composición del sustrato, incluyendo la a_w y el pH de éste, y la temperatura son algunos de los factores estudiados. En relación a la OTA, la mayoría de estudios se han realizado con cepas pertenecientes a *A. ochraceus* y *P. verrucosum* que son las especies clásicamente consideradas productoras de OTA.

1.3.4.2.1.-Sustrato

La producción de micotoxinas está muy ligada a la composición del sustrato. Las diferencias en las características físicas y químicas del sustrato, la disponibilidad de agua y oxígeno, el contenido en azúcares, proteínas o aminoácidos o las trazas de minerales presentes puede influir de forma significativa en la producción de OTA y otras micotoxinas (75, 95, 119, 169).

La OTA es un contaminante natural que se halla en gran variedad de sustratos vegetales, incluyendo cereales y leguminosas (69, 191, 261). Madhyastha *et al.* (141) observaron que para *A. ochraceus* algunas leguminosas son mejores sustratos para la producción de OTA que cereales como el maíz o el trigo. Sin embargo el trigo resultó ser mejor sustrato que las oleaginosas para *P. verrucosum*. En otros trabajos la producción de OTA en cereales por cepas de *P. verrucosum* de la propia micoflora fue variable dependiendo del cereal (6, 8). Hay que tener en cuenta que la composición de un mismo cereal puede ser diferente entre sus variedades y aquéllas con un mayor contenido en amilosa y agua son las que generalmente reúnen condiciones más favorables para un mayor acúmulo de OTA (18, 19, 57). El desarrollo fúngico podría alterar esta composición al cabo del tiempo y producir diferentes niveles de OTA relacionados con la composición final del sustrato tras la incubación (142).

Un alto nivel de proteína, y en concreto de los aminoácidos prolina y ácido glutámico, estimula la producción de OTA por *A. ochraceus* y *P. verrucosum* (*P. viridicatum*) tanto en cebada como en medios sintéticos. Este hecho podría sugerir que la contaminación por OTA de diversos sustratos naturales estaría condicionada por la presencia de estos dos aminoácidos (75, 95). Las fuentes de carbono del sustrato también ejercen un efecto sobre la producción de OTA. Al incubar varias especies de *Aspergillus* (*A. melleus, A. ochraceus* y *A. sulphureus*) en un medio líquido con diferentes fuentes de carbono se observó una mayor producción de OTA al utilizar glucosa o

sacarosa que otros azúcares como la maltosa, manosa, galactosa, xilosa o arabinosa (119). En otro trabajo, los niveles máximos de OTA se observaron también al utilizar glucosa o sacarosa en vez de lactosa o fructosa como única fuente de carbono (158). La concentración de azúcar presente también influye en los niveles de OTA producidos. Se detectó la máxima producción de OTA por *A. ochraceus* cuando el medio contenía un 4% de sacarosa y un 2% de extracto de levadura (64). En otro estudio, la máxima producción de OTA por *A. ochraceus* se obtuvo al utilizar un medio con un 0,625% de glucosa en comparación con niveles superiores de hasta 10% de este azúcar (158).

La concentración de sales minerales en el medio también influye en la producción de OTA ya que algunas especies fúngicas resultan particularmente sensibles a la disponibilidad de ciertos elementos traza que intervienen en reacciones metabólicas muy específicas (57, 119, 128, 235). La adición de zinc al sustrato potenció la producción de OTA y también el crecimiento fúngico (57, 158). La concentración óptima de sales de zinc para la producción de OTA por *A. ochraceus* se estableció en un rango entre 0,055 y 2,2 mg/l. Los rangos óptimos para las sales de cobre y hierro se establecieron en 0,004-0,04 mg/l y 1,2-24 mg/l respectivamente para esta especie (235).

Los diferentes estudios centrados en el efecto de los factores abióticos sobre la producción de OTA se han llevado a cabo utilizando un gran número de medios naturales y sintéticos. Entre éstos cabe destacar ocasionalmente la utilización de análogo de pan (183), de medio sintético similar a la uva (30, 31, 156), agar extracto de malta (MEA) (128, 158), medio sintético Adye-Mateles (AM) (158) o de medio Raulin-Thom (RT) (58). También se ha utilizado el medio CYA en diversas especies ocratoxígenas de *Aspergillus* (40), aunque es el medio YES el que se ha incluido más frecuentemente en los diferentes trabajos (40, 47, 58, 89, 227, 246, 268). El medio YES resultó ser un buen sustrato para la producción de OTA en cepas de *Aspergillus* sección *Circumdati* y para cepas del agregado *A. niger* de la sección *Nigri*. Para las cepas de *A. carbonarius* se obtuvieron los máximos niveles de OTA en el medio CYA (40).

El tiempo durante el que se realiza la incubación también condiciona la cantidad de micotoxina que se puede encontrar en el sustrato. Aunque su efecto sobre el contenido de OTA es diferente dependiendo de la cepa, se pueden detectar niveles altos de OTA después de 7-10 días de incubación. En un estudio realizado en el medio líquido YES, la concentración máxima de OTA se alcanzó a los 7 días de incubación en algunas especies (*A. melleus* y *A. albertensis*) y a los 10 días

para las cepas del agregado *A. niger*, *A. carbonarius* y *Penicillium verrucosum*. En el caso de *A. ochraceus*, dependiendo de la cepa, este máximo se observó entre los 7 y 10 días de incubación (58, 246, 268).

1.3.4.2.2.-Actividad de agua

La actividad de agua (a_w) del sustrato es uno de los factores que más influye en el crecimiento fúngico y la producción de micotoxinas en los alimentos almacenados (128). En *P. verrucosum* (*P. viridicatum*) se ha descrito un mínimo de a_w de 0,80 para el crecimiento (52, 183, 189) y un mínimo de 0,83-0,86 a_w para la producción de OTA (128, 165) con una producción máxima a una a_w de 0,95-0,99 en medio sintético (128) y de 0,90-0,93 en medio natural y análogo de pan (17, 99, 183).

En el caso de *A. ochraceus* el mínimo de crecimiento se ha observado a 0,77-0,80 a_w (189, 196, 240) mientras que la producción de OTA se ha detectado a partir de 0,83-0,90 a_w y el óptimo a 0,95-0,99 a_w con pequeñas variaciones dependiendo del estudio y el medio de cultivo empleado (128, 240).

En *Aspergillus* sección *Nigri* el mínimo valor de a_w para el crecimiento de *A. carbonarius* se ha descrito a 0,88-0,90 a_w (29, 109, 156). En el caso del agregado *A. niger*, se ha descrito la germinación de algunas cepas a valores de 0,77-0,80 a_w (149, 189). Vujanovic *et al.* (276) detectaron crecimiento lento de *A. niger* a 0,76 a_w, mientras que otros autores determinaron que el mínimo valor de a_w para el crecimiento era 0,82 a_w (178). En relación a la producción de OTA, existen pocos estudios que evalúen la influencia de la a_w en especies ocratoxígenas de la sección *Nigri*. Los estudios llevados a cabo hasta el momento incluyen cepas de orígenes muy restringidos y utilizando sustratos naturales o análogos para la incubación de éstas. La producción máxima de OTA por cepas de *A. carbonarius* incubadas en medio sintético similar a la uva se observó en el rango 0,95-0,99 a_w (30, 31, 156). Al incubar una cepa desarrollada sobre granos de café, el máximo de producción se observó a 0,99 a_w (109). En un estudio que incluyó dos cepas del agregado *Aspergillus niger* aisladas de uva e incubadas en medio sintético similar a la uva, se detectó producción de OTA de una de esas cepas a partir de 0,90 a_w que fue el mínimo valor ensayado. La máxima producción de micotoxina fue diferente dependiendo del tiempo de

incubación, aunque el máximo total de OTA de esas dos cepas se observó en un rango de a_w de 0,98-0,995 (30).

1.3.4.2.3.-Temperatura

Además del agua disponible en el sustrato, la temperatura es una de las variables con un papel más significativo en el control del desarrollo microbiano y la producción de toxinas (128). Este factor tiene una considerable influencia en los requerimientos de agua por parte del hongo por lo que en muchos estudios se ha valorado conjuntamente con la a_w (20, 31, 99, 156, 196, 240).

Los diversos márgenes de temperatura en los que se observa OTA exhiben claras diferencias en la capacidad de crecimiento y producción de micotoxina según la especie. En general, *Penicillium* spp. presentan una producción de OTA a unos rangos de temperatura inferiores a los de las especies de *Aspergillus* estudiadas. Este hecho permite relacionar la producción de OTA en climas fríos a *Penicillum* spp, concretamente *P. verrucosum*, mientras que en zonas de clima cálido y templado la producción de OTA está más relacionada con especies ocratoxígenas del género *Aspergillus* (2, 100, 128, 131, 189, 191, 241).

El rango de temperatura de crecimiento de *P. verrucosum* (*P. viridicatum*) se ha descrito entre 0 y 31ºC (128, 189) mientras que la producción de OTA por esta especie se ha detectado entre 4 y 31ºC (128, 165), con un óptimo de producción alrededor de los 20-25ºC en diferentes medios sintéticos y naturales (89, 94, 99, 128, 227). Bullerman (47) sin embargo, no detectó producción de OTA a una temperatura de 5ºC y obtuvo mayor producción a 12ºC que a 25ºC en medio YES. Por otro lado, cuando se utilizó queso como sustrato la producción de toxina se observó solamente a 20 y 24ºC (165).

En el caso de *A. ochraceus*, mientras que el crecimiento se ha descrito entre 8 y 37ºC (128, 189), la producción de OTA se observó desde una temperatura de 10-12ºC hasta 37ºC (58, 128, 240), con una producción máxima entre 30 y 37ºC (20, 128, 240). En otros trabajos el máximo de producción de OTA se detectó a temperaturas inferiores, entre 25 y 30ºC, cuando la incubación se realizó en diferentes sustratos naturales (maíz, trigo y cebada) (58, 196). Para *Petromyces albertensis* (*Aspergillus albertensis*) se detectaron los niveles máximos de OTA en YES a 30ºC y 7

días de incubación. Después de 10 días, los niveles de OTA producidos a diferentes temperaturas (16, 15, 30 y 35ºC) eran muy similares (268).

En las cepas pertenecientes a *Aspergillus* sección *Nigri* se han descrito rangos de crecimiento entre 8 y 41ºC para *A. carbonarius* (45, 156) y entre 6 y 47ºC para el agregado *A. niger* (173). Sólo existen dos estudios sobre la influencia de esta variable en la producción de OTA, llevados a cabo muy recientemente e incluyendo exclusivamente cepas aisladas de uva. Al incubar cepas de *A. carbonarius* en medio sintético similar a la uva se observó producción OTA a partir de 15ºC y se detectaron los máximos de producción de OTA a una temperatura de 15-20ºC (31, 156). Hasta el momento, no se han publicado estudios sobre la influencia de la temperatura en la producción de OTA por cepas del agregado *A. niger*.

1.3.4.2.4.-pH

Este factor puede influir también en el crecimiento del hongo y la producción de micotoxinas. Sin embargo, su efecto no parece ser tan limitante como el de otros factores abióticos, por lo que se suele estudiar en modelos que incluyen otras variables (47, 143, 183).

El rango de pH descrito para el crecimiento de *P. verrucosum* es 2,1-10 (189). En un estudio en el que se incubó una cepa de *Penicillium* no identificada en medio YES, la producción de OTA se detectó por encima de pH 3,5 que fue el mínimo valor ensayado (47). Patterson y Damoglou (183) incubaron una cepa de *P. verrucosum* (*P. viridicatum*) en análogo de pan y detectaron OTA entre pH 4 y pH 8. En todos los casos, la máxima producción de OTA se ha detectado a un pH cercano a 6 (47, 89, 183).

En el intervalo de pH entre 3 y 7,8 los niveles máximos de OTA producido por un cepa de *A. sulphureus* se detectaron a pH 6-6,3. En ese estudio se observó que la producción de OTA a pH 4 disminuía más de tres veces en comparación con los valores observados a pH 6 (119). En el caso de *A. ochraceus* el crecimiento ha sido descrito en un rango entre pH 2,2 y 10 (158, 189) mientras que la producción de OTA ha sido descrita en un intervalo inferior, entre 5,5 y 8,5, obteniéndose la producción máxima a pH 5,5-6 (158). El rango de crecimiento para *A. niger* se ha descrito entre

1,4 y 9,8 (173). Hasta el momento, no existen estudios realizados con cepas de *Aspergillus* sección *Nigri* productoras de OTA valorando el efecto de esta variable sobre la producción de toxina.

1.3.4.2.5.-Otros factores abióticos

La composición de la atmósfera donde se desarrolla el hongo puede afectar el crecimiento de las especies productoras de OTA y también la posible presencia de la micotoxina en el sustrato (165, 179). En un trabajo que incluía una cepa de *A. ochraceus* se observó que la producción de OTA se inhibía completamente con un porcentaje de CO_2 del 30% independientemente de los niveles de O_2 presentes. Este efecto inhibitorio no estaba relacionado con un efecto directo sobre el crecimiento fúngico ya que el crecimiento sólo se veía afectado a partir de niveles del 60% de CO_2 y superiores (179). Posteriormente, en un trabajo que incluía tres cepas de *P. verrucosum* incubadas en trigo se observó que la presencia de un 25% de CO_2 inhibía el crecimiento aproximadamente un 40%. La producción de OTA se vio inhibida más de un 75% cuando la presencia de CO_2 era del 50% (52).

Por último, es necesario considerar que existe una interacción entre los diferentes factores abióticos descritos anteriormente. De esta forma, los requerimientos de agua por parte del hongo dependen de otras variables ambientales como la temperatura y el sustrato (169). También se ha descrito una interacción significativa entre la temperatura de incubación y el pH del sustrato donde se desarrolla el hongo (47, 227). El pH puede afectar también las interacciones entre otros factores abióticos como la a_w y la temperatura debido a sus efectos sobre procesos metabólicos implicados en el desarrollo fúngico (143).

En la Tabla 6 se muestran los rangos de a_w, temperatura y pH en los que se ha descrito el crecimiento y producción de OTA de diferentes especies ocratoxígenas.

Tabla 6.-Rangos de a_w, temperatura y pH en los que se ha descrito el crecimiento y producción de OTA de diferentes especies ocratoxígenas.

Especie	a_w		Temperatura (ºC)		pH	
	Crecimiento	OTA	Crecimiento	OTA	Crecimiento	OTA
P. verrucosum	≥0,80 (52, 183, 189)	≥0,83-0,86 (128, 165)	0-31 (128, 189)	4-31 (128, 165) >5 (47) 20, 24 (165)	2,1-10 (189)	>3,5 (47) 4-8 (183)
A. ochraceus	≥0,77 (189) ≥0,80 (196, 240)	≥0,83-0,87 (128) ≥0,90 (240)	8-37 (128, 189)	10-37 (58, 128, 240)	2,2-10 (158, 189)	5,5-8,5 (158)
A. carbonarius	≥0,88 (156) ≥0,90 (29, 109)	>0,90 (31, 109)	8-41 (45) >10 (156)	≥15 (31, 156)	---	---
Agregado *A. niger*	≥0,76 (276) ≥0,77 (189) ≥0,80 (149) ≥0,82 (178)	≥0,90 (30)	6-47 (173)	---	1,4-9,8 (173)	---

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2.-OBJETO DEL ESTUDIO

La ocratoxina A (OTA) es una micotoxina que está recibiendo una especial atención en todo el mundo por su marcado carácter nefrotóxico y por su amplia distribución. Estudios recientes han demostrado que posee además propiedades carcinógenas, teratógenas e inmunotóxicas.

Desde su descubrimiento en 1965, la producción de esta micotoxina se asocia clásicamente a *Aspergillus ochraceus* y *Penicillium verrucosum*. No obstante, la incidencia y distribución habitual de estas especies no permiten explicar la elevada presencia de la ocratoxina A en una gran variedad de alimentos destinados al consumo humano y animal, por lo que cabe pensar que otras especies fúngicas pueden estar también implicadas.

Desde la primera descripción de la capacidad productora de OTA por *Aspergillus niger* var. *niger* en 1994, se han publicado un importante número de trabajos de investigación sobre la capacidad ocratoxígena de las especies incluidas en la sección *Nigri*. La capacidad de *A. niger* de elaborar OTA supone un riesgo inesperado para la salud humana y animal, ya que es una especie ampliamente utilizada en la industria alimentaria y posee el estatus GRAS (generally recognized as safe) de la FDA.

En la actualidad, las especies que se aceptan como ocratoxigénicas en esta sección son *A. carbonarius* y las incluidas en el denominado "agregado *A. niger*". En los estudios realizados hasta el momento, el porcentaje de cepas del agregado *A. niger* productoras se encuentra entre el 0,6 y el 50% mientras que en el caso de *A. carbonarius* este número es prácticamente del 100%. La producción de una micotoxina depende no sólo del genotipo de la cepa sino también de toda una serie de factores ambientales que van a ejercer su influencia en el crecimiento y metabolismo de la cepa. La mayoría de investigaciones sobre el efecto de diferentes factores en la producción de OTA se han centrado en las especies clásicamente consideradas como productoras, *Aspergillus ochraceus* y *Penicillium verrucosum*. En el caso de las especies ocratoxígenas de la sección *Nigri*, se desconocen las condiciones que favorecen la producción de esta micotoxina.

Por otro lado, la taxonomía de esta sección es compleja y está en contínua revisión. La dificultad que presenta la clasificación basada únicamente en características morfológicas ha propiciado la aplicación de otros criterios de clasificación basados en la biología molecular. En un intento de clarificar la taxonomía del agregado *A. niger* algunos autores han propuesto dividirlo en dos o más especies considerando los resultados obtenidos mediante diferentes técnicas moleculares.

Por tanto, atendiendo a la gran importancia que está adquiriendo la ocratoxina A, consideramos de interés el estudio de las condiciones que influyen en la producción de esta micotoxina, así como la caracterización molecular de las cepas ocratoxígenas dentro de *Aspergillus* sección *Nigri*.

Por todo ello el objeto del presente trabajo ha sido:

- Estudiar cepas de diferentes orígenes de *A. carbonarius* y del agregado *A. niger* productoras y no productoras de ocratoxina A con el fin de determinar las diferentes condiciones que influyen en la producción de micotoxina.

- Estudiar la influencia de la temperatura de incubación en la producción de ocratoxina A.

- Estudiar la influencia de la actividad de agua (aw) del medio de cultivo en la producción de ocratoxina A.

- Estudiar la influencia del pH del medio de cultivo en la producción de ocratoxina A.

- Realizar la caracterización de las cepas mediante técnicas de biología molecular (RFLP, secuenciación y RAPD).

- Evaluar la utilidad de algunos marcadores moleculares (AFLP, ERIC-PCR y microsatélites) para la caracterización de cepas de *A. carbonarius* y el agregado *A. niger*.

3.-ARTÍCULOS

3.1 Effects of temperature and incubation time on production of ochratoxin A by black aspergilli. Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***Research in Microbiology*, 155 (2004) 861-866.**

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Effects of temperature and incubation time on production of ochratoxin A by black aspergilli

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Abstract

The effects of temperature (5–45 °C) on the growth and production of ochratoxin A (OTA) by eighteen strains of *Aspergillus* section *Nigri*, cultured on Czapek yeast autolysate agar (CYA) and on yeast extract sucrose agar (YES), were studied for an incubation period of 30 days. Isolates were selected to include different sources and different reported abilities to produce OTA. Temperature ranges for OTA production were more restrictive than those for growth and each strain tested differed in its optimum conditions for OTA production. *Aspergillus niger* aggregate strains achieved maximum OTA levels in YES medium mainly at 20–25 °C. The *A. carbonarius* strains produced the highest OTA levels in CYA medium at 15 or 20 °C. Significant amounts of OTA were produced after only five days of incubation. Due to their ability to produce OTA at a wide range of temperatures, OTA can be continuously produced in the field. This fact has to be taken into account in commodities such as grapes, raisins and wine, where *A. carbonarius* and members of the *A. niger* aggregate are considered to be the main sources of the OTA contamination.

Keywords: *Aspergillus niger*; *Aspergillus carbonarius*; Grapes; Ochratoxin A; Temperature; Wine

1. Introduction

Ochratoxin A (OTA) is a nephrotoxic mycotoxin naturally found in a wide range of food commodities throughout the world. Among other toxic effects, OTA is carcinogenic, teratogenic, genotoxic and immunosuppressive [16,26]. It has been classified as "possibly carcinogenic to humans" (group 2B) by the International Agency for Research in Cancer. Since March 2002 maximum OTA levels in cereals and dried vine fruits are regulated by the EU and in the near future, the Commission will possibly include maximum limits for OTA in other food products (wine, grape juice, coffee, beer, cocoa and spices) [8].

Aspergillus ochraceus and *Penicillium verrucosum* are the typical OTA-producing species, but their incidence and natural occurrence do not explain the widespread OTA contamination reported in some food products. Since the first description of OTA production by two species belonging to *Aspergillus* section *Nigri*, *Aspergillus niger* var. *niger* [1] and *Aspergillus carbonarius* [19], the significance of black aspergilli as toxin-producing fungi has changed. It is now considered that in substrates such as grapes, raisins and wine the source of OTA contamination detected is due mainly to *A. carbonarius* and the species included in the *A. niger* aggregate [2]. Currently, other *Aspergillus* species included in section *Nigri* are not considered to be OTA producers.

The *A. niger* aggregate is formed by two species, *A. foetidus* and *A. niger*, with the latter species consisting of six varieties and two formae [7], but these taxa are very difficult to distinguish from each other by morphological means. At the molecular level many attempts have been made to divide the taxa included in the *A. niger* aggregate into two or more species [4]. On the basis of their ITS-5.8S rDNA restriction

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fragment length polymorphism (RFLP) patterns, they can be grouped into two patterns, designated N and T [5]. To date, all of the OTA-positive isolates belonging to the *A. niger* aggregate, whose RFLP patterns are known, are of type N [3,6,14].

The reported percentages of ochratoxigenic isolates belonging to the *A. niger* aggregate are much lower than for *A. carbonarius* species [2] and very little is known about the optimal conditions for OTA biosynthesis. Current scientific literature on the influence of environmental factors on OTA production has been focused mainly on *A. ochraceus* and *P. verrucosum*. To date, only a few studies have reported the effect of environmental conditions such as water activity [12,21] and incubation time [31,33] on the amount of OTA produced by some isolates of *Aspergillus* section *Nigri*. As nothing is known about the optimum temperature for OTA production by these species, the aim of this study was to elucidate the effects of temperature and incubation time on OTA production by isolates belonging to the *A. niger* aggregate and *A. carbonarius.*

2. Material and methods

2.1. Fungal *isolates*

Twelve isolates of the *A. niger* aggregate (ten of pattern N and two of pattern T) and six isolates of *A. carbonarius* were used in this study. Isolates were selected to include different sources and different reported abilities to produce OTA. The origin and OTA properties of the eighteen strains studied are shown in Table 1. The RFLP pattern type of the twelve isolates belonging to the *A. niger* aggregate following Accensi et al. [5] are also included.

2.2. Culture conditions

OTA production was determined on two culture media: yeast extract sucrose (YES) agar, which contained, per liter, 20 g of yeast extract, 150 g of sucrose, 0.5 g of magnesium sulfate and 20 g of agar and Czapek yeast extract (CYA) agar, which contained, per liter, 1 g of K_2 HPO_4, 10 ml of Czapek concentrate, 1 ml of trace metal solution, 5 g of yeast extract, 30 g of sucrose and 15 g of agar [28].

Inocula were prepared by growing the strains on malt extract agar at 25 °C for 7 days. Conidia suspensions of each isolate were prepared in an aqueous solution of 0.05% Tween 80. After filtering through sterile cheesecloth they were adjusted to approximately 10^6–10^7 conidia per ml as determined by a counting chamber. Each medium was point inoculated with 1 µl of the adjusted suspension. Plates were incubated at nine different temperatures from 5 to 45 °C (at 5 °C intervals). Each assay was performed in duplicate.

2.3. OTA production and quantification

OTA production was analyzed after 5, 10, 15, 20 and 30 days of incubation at each temperature assayed using a previously described high-pressure liquid chromatography (HPLC) screening method [13]. On each sampling occasion, three agar plugs were removed from different points of the colony and extracted with 0.5 ml of methanol. The extracts

Table 1
Aspergillus strains used in this study

Species	Strain (source)[a]	OTA production reported [Ref.][b]	RFLP pattern [Ref.][b]
A. niger aggregate:			
A. niger var. *niger*	A-75 (feedstuffs, CCFVB)	+ [1]	N [6]
A. niger var. *niger*	A-136 (soy, CCFVB)	+ [1]	N [6]
A. niger var. *niger*	A-942 (raisins, CCFVB)	+ [3]	N [3]
A. niger var. *niger*	A-943 (grapes, CCFVB)	+	N
A. awamori	CBS 139.52	+ [24]	N [6]
A. foetidus	CBS 618.78	+ [31]	N [6]
A. niger	CECT 2088	– [32]	N
A. niger	CBS 554.65	– [6]	N [6]
A. niger var. *niger*	A-946 (coffee, CCFVB)	–	N
A. niger	CBS 121.55	–	N
A. tubingensis	CBS 134.48	– [6]	T [6]
A. niger var. *niger*	A-947 (grapes, CCFVB)	– [14]	T [14]
A. carbonarius	NRRL 67	+ [31]	
A. carbonarius	CBS 127.49	+ [21]	
A. carbonarius	A-941 (grapes, CCFVB)	+ [14]	
A. carbonarius	M325 (apples, supplied by HMLJ Joosten)	– [21]	
A. carbonarius	CBS 110.49	+ [21]	
A. carbonarius	A-1082 (raisins,CCFVB)	– [3]	

[a] Abbreviations: CCFVB, Culture Collection of Veterinary Faculty of Barcelona, Bellaterra (Barcelona), Spain; CBS, Centralbalbureau voor Schimmelcultures, Utrecht, The Netherlands; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; NRRL, Northern Agricultural Research Service Culture Collection, Peoria, IL, USA.

[b] No reference in OTA production ability or in RFLP pattern means that the data were obtained in our laboratory and have not been published.

were filtered and injected into the HPLC. OTA detection and quantification was made by a Waters LCM1 chromatograph with a fluorescence detector Waters 2475 (excitation wavelength: 330 nm/emission wavelength: 460 nm), and with a column C18 Spherisorb S5 ODS2, 250 × 4.6 mm. Twenty µl of each extract were applied. The mobile phase, with a flow rate of 1 ml/min, consisted of the following linear gradient: acetonitrile, 57%; water, 41% and acetic acid, 2% [10]. The extracts with the same retention time as OTA (around 6.8 min), were considered positive. Confirmation was made through derivatization of OTA in its methyl-ester [20]. The detection limit of the extraction procedure and the HPLC technique was 0.02 ng OTA and the quantification limit of HPLC technique with the extraction procedure was 0.05 µg/g for this mycotoxin.

2.4. Data analysis

Data obtained were analyzed statistically by means of the one-way analysis of variance test and Student's test. All statistical analyses were performed using SPSS software (Version 10.0).

3. Results and discussion

The isolates belonging to the *A. niger* aggregate grew in YES medium from 10 to 45 °C with the exception of strain A-946 that did not grow at 10 °C. In CYA medium, four strains (A-942, CBS 554.65, CBS 121.55 and A-947) grew from 10 to 45 °C and the eight remaining strains had a minimum growth temperature of 15 °C. All the *A. carbonarius* strains grew in YES medium between 10 and 40 °C. In CYA medium, the growth temperature range was narrower and they grew from 15 to 30 °C (A-941), to 35 °C (NRRL 67, CBS 127.49, M325, CBS 110.49) or to 40 °C (A-1082). The reported growth temperatures for *A. niger* are minimum, 6–8 °C, maximum, 45–47 °C, and optimum 35–37 °C [25] and for *A. carbonarius* the growth temperature range is 10–40 °C [27], but nothing is known about temperature range for OTA production.

Six out of the 12 isolates of *A. niger* aggregate and four out of the 6 *A. carbonarius* strains tested produced OTA. *A. carbonarius* strains produced significantly more OTA than that produced by strains belonging to the *A. niger* aggregate ($P < 0.01$). Culture media played an important role in the amount of mycotoxin produced. The mean OTA concentration produced by the *A. niger* aggregate strains in YES medium (3.33 µg/g) was significantly higher ($P < 0.01$) than in CYA medium (1.35 µg/g). On the contrary, *A. carbonarius* strains produced significantly ($P < 0.01$) higher amounts of OTA in CYA (52.14 µg/g) than in YES medium (13.52 µg/g). These results were in accordance with previous studies [13].

OTA concentrations produced in the optimal culture medium at each temperature and incubation time are summarized in Tables 2 (*A. niger* aggregate strains in YES medium) and 3 (*A. carbonarius* strains in CYA medium). Temperature ranges for OTA production were more restrictive than those for growth and each strain tested differed in its optimum conditions for OTA production. The OTA production kinetics of representative strains at all temperatures tested are shown in Figs. 1 and 2. As observed in Table 2, *A. foetidus* CBS 618.78 only produced OTA at 25 and 30 °C, whereas in the remaining positive strains OTA was detected from 10 or 15 to 25, 30 or 35 °C depending on the strain. Although the temperature range for OTA production varied between strains, the six positive isolates were able to produce OTA at 25 °C. It is important to note that 25 °C is the temperature selected for the screening of OTA-producing ability by *A. niger* aggregate isolates [13]. Except for strains A-942 and A-943, the optimum temperature range for OTA production was 20–25 °C and in most cases the highest OTA level was achieved after only 5 days of incubation. At 15 °C, maximum OTA concentration was achieved after 10–20 days of incubation. With increasing incubation temperature, the maximum OTA level was obtained earlier, after 5 or 10 days (Table 2 and Fig. 1).

Three *A. carbonarius* strains (NRRL 67, CBS 127.49, M325) produced OTA between 15 and 35 °C. *A. carbonarius* A-941 did not grow at 35 °C (Table 2). The highest amounts of OTA were obtained in CYA medium at 15 or 20 °C. OTA was detected after 5 days of incubation from 20 to 30–35 °C. When the temperature was 15 °C, all the positive strains produced OTA after 10 days of incubation, achieving the maximum level after 30 days of incubation. At the other temperatures tested the highest OTA concentration was recorded after different incubation times, depending on the strain (Table 3 and Fig. 2).

In spite of OTA being a stable metabolite, in some cases the OTA content decreased considerably with increasing incubation time or just at the final incubation time. Some authors have suggested that this may be due to the fact that the strains remove and assimilate the phenylalanine moiety from the OTA molecule as other nitrogen sources in the culture media become exhausted [31,33].

It is not known why the percentage of OTA-positive isolates in *A. carbonarius* may reach 100%, while the percentage is much lower for isolates belonging to the *A. niger* aggregate [4], nor what are the optimal conditions for the expression of OTA biosynthesis. In this study, the six *A. niger* aggregate strains initially considered as OTA-negative did not produce this mycotoxin at any of the temperatures tested for a period of 30 days. In the broad range of temperatures assayed, strains with the RFLP pattern of type T did not produce OTA. To date OTA production has never been demonstrated in type T strains [6].

A. carbonarius strains CBS 110.49 and A-1082 did not produce detectable levels of OTA at any of the temperatures tested. Joosten et al. [21], using coffee cherries as a substrate, reported that *A. carbonarius* CBS 110.49 was a very low producer strain, and *A. carbonarius* M325 was

Table 2
OTA concentration (mean and one standard error) produced by the six OTA-producing strains of *A. niger* aggregate in YES medium at each temperature and incubation time tested

Strain	Days	OTA concentration (μg/g)					
		10 °C	15 °C	20 °C	25 °C	30 °C	35 °C
A. niger	5	NG	nd	25.61 ± 0.11^{c}	28.31 ± 3.92^{c}	7.80 ± 3.49^{b}	0.18 ± 0.01^{a}
var. *niger*	10	NG	6.62 ± 0.39^{c}	30.76 ± 0.61^{e}	10.47 ± 0.97^{d}	2.44 ± 0.73^{b}	0.06 ± 0^{a}
A-75	15	nd	2.5 ± 0.95^{a}	16.99 ± 4.94^{c}	10.18 ± 1.44^{b}	1.57 ± 0.06^{a}	0.02 ± 0.03^{a}
	20	nd	3.83 ± 0.80^{ab}	23.92 ± 5.66^{c}	8.53 ± 1.06^{b}	0.57 ± 0.07^{a}	0.03 ± 0.05^{a}
	30	nd	2.66 ± 0.34^{b}	10.08 ± 1.14^{d}	6.50 ± 0.57^{c}	0.48 ± 0.07^{a}	nd
A. niger	5	NG	nd	17.44 ± 11.05^{b}	49.40 ± 0.78^{c}	10.24 ± 0.47^{ab}	0.12 ± 0.04^{a}
var. *niger*	10	NG	1.73 ± 0.04^{a}	6.11 ± 6.12^{ab}	11.93 ± 2.74^{b}	4.11 ± 0.75^{a}	0.03 ± 0.01^{a}
A-136	15	nd	0.83 ± 0.07^{a}	3.80 ± 3.34^{a}	11.17 ± 2.07^{b}	3.27 ± 0.44^{a}	0.03 ± 0.01^{a}
	20	nd	1.44 ± 0.42^{b}	1.14 ± 20^{b}	10.55 ± 0.59^{d}	2.74 ± 0.44^{c}	0.02 ± 0.02^{a}
	30	nd	1.48 ± 0.02^{a}	6.66 ± 7.68^{a}	9.40 ± 1.54^{a}	2.55 ± 0.23^{a}	nd
A. niger	5	NG	0.65 ± 0.74^{a}	18.06 ± 8.44^{b}	12.59 ± 3.31^{ab}	1.27 ± 0.47^{a}	nd
var. *niger*	10	nd	24.98 ± 9.41^{b}	20.84 ± 11.34^{b}	2.54 ± 0.02^{a}	0.23 ± 0.13^{a}	nd
A-942	15	nd	16.09 ± 1.57^{d}	8.66 ± 0.31^{c}	2.13 ± 0.14^{b}	0.15 ± 0.03^{a}	nd
	20	0.99 ± 0.49^{a}	33.58 ± 8.24^{b}	8.93 ± 4.82^{a}	1.60 ± 0.01^{a}	0.11 ± 0.01^{a}	nd
	30	3.24 ± 0.81^{a}	21.37 ± 6.67^{b}	6.05 ± 0.81^{a}	1.46 ± 0.33^{a}	0.12 ± 0.07^{a}	nd
A. niger	5	NG	nd	2.42 ± 0.47^{b}	0.35 ± 0.13^{a}	nd	nd
var. *niger*	10	NG	1.06 ± 0.47^{a}	2.19 ± 1.61^{a}	0.15 ± 0.01^{a}	nd	nd
A-943	15	nd	1.09 ± 0.06^{b}	2.50 ± 0.39^{c}	0.38 ± 0.23^{a}	nd	nd
	20	nd	2.87 ± 1.99^{a}	1.14 ± 0.63^{a}	0.21 ± 0.03^{a}	nd	nd
	30	0.08 ± 0.11^{a}	2.41 ± 1.22^{b}	1.12 ± 0.05^{a}	0.22 ± 0.02^{a}	nd	nd
A. awamori	5	NG	nd	6.07 ± 2.77^{ab}	10.62 ± 4.95^{b}	nd	nd
CBS 139.52	10	NG	0.40 ± 0.11^{a}	6.86 ± 2.86^{a}	6.06 ± 6.24^{a}	0.08 ± 0^{a}	nd
	15	nd[a]	0.81 ± 0.42^{a}	3.25 ± 0.28^{ab}	5.66 ± 3.16^{b}	0.04 ± 0.06^{a}	nd
	20	nd	2.55 ± 1.04^{b}	2.13 ± 0.71^{ab}	7.71 ± 1.16^{c}	0.77 ± 0.03^{a}	nd
	30	nd	1.84 ± 0.74^{b}	2.60 ± 0.70^{b}	8.66 ± 0.90^{c}	0.23 ± 0.24^{a}	nd
A. foetidus	5	NG	nd	nd	97.68 ± 42.38^{b}	3.90 ± 1.10^{a}	nd
CBS 618.78	10	NG	nd	nd	5.30 ± 0.42^{c}	1.12 ± 0.52^{b}	nd
	15	nd	nd	nd	11.50 ± 8.62^{a}	0.90 ± 0.50^{a}	nd
	20	nd	nd	nd	10.30 ± 1.20^{b}	0.46 ± 0.12^{a}	nd
	30	nd	nd	nd	3.91 ± 4.02^{a}	0.29 ± 0.04^{a}	nd
Mean	5	NG	0.11 ± 0.27^{a}	11.60 ± 10.21^{a}	33.16 ± 35.94^{b}	3.87 ± 4.31^{a}	0.05 ± 0.08^{a}
	10	nd	5.80 ± 9.70^{ab}	11.13 ± 12.06^{a}	6.07 ± 4.51^{ab}	1.33 ± 1.65^{a}	0.01 ± 0.02^{a}
	15	nd	3.55 ± 6.20^{ab}	5.86 ± 6.14^{b}	6.83 ± 4.84^{b}	0.99 ± 1.27^{a}	0.01 ± 0.01^{a}
	20	0.16 ± 0.40^{a}	7.37 ± 12.90^{b}	6.21 ± 9.25^{ab}	6.48 ± 4.47^{ab}	0.77 ± 1.0^{a}	0.01 ± 0.01^{a}
	30	0.55 ± 1.32^{a}	4.96 ± 8.09^{b}	4.42 ± 3.83^{b}	5.02 ± 3.78^{b}	0.61 ± 0.96^{a}	nd

Values with the same superscript within each strain and incubation time are not significantly different ($P < 0.05$). NG: not growth; nd: not detected (limit of detection 0.05 μg/g).

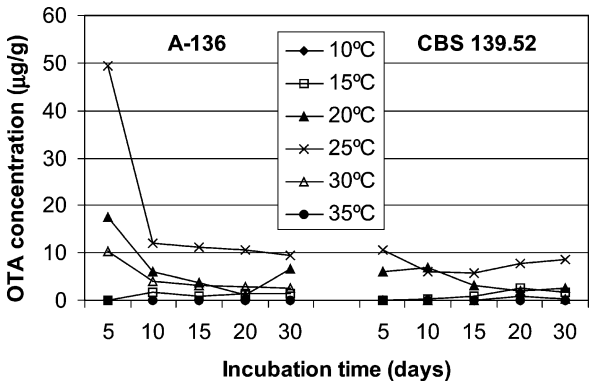

Fig. 1. Kinetics of OTA production in YES medium by the *A. niger* aggregate strains A-136 and CBS 139.52 at each temperature tested.

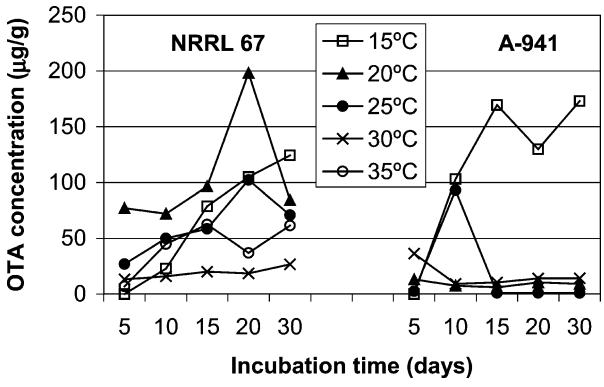

Fig. 2. Kinetics of OTA production in CYA medium by the *A. carbonarius* strains NRRL 67 and A-941 at each temperature tested.

Table 3
OTA concentration (mean and one standard error) produced by the four OTA-producing strains of *A. carbonarius* in CYA medium at each temperature and incubation time tested

Strain	Days	OTA concentration (μg/g)				
		15 °C	20 °C	25 °C	30 °C	35 °C
A. carbonarius	5	nd	77.15 ± 11.77^{c}	26.92 ± 5.10^{b}	13.12 ± 0.99^{ab}	6.88 ± 0.05^{a}
NRRL 67	10	22.76 ± 7.06^{a}	72.09 ± 8.71^{c}	49.95 ± 3.92^{b}	15.82 ± 3.53^{a}	44.95 ± 0^{b}
	15	78.76 ± 13.03^{b}	96.54 ± 8.67^{b}	58.55 ± 1.97^{ab}	20.10 ± 2.35^{a}	62.44 ± 27.08^{ab}
	20	105.03 ± 40.15^{ab}	198.27 ± 55.30^{b}	102.40 ± 16.88^{ab}	18.58 ± 1.22^{a}	36.91 ± 7.46^{a}
	30	124.43 ± 7.14^{b}	84.42 ± 29.75^{ab}	70.76 ± 1.17^{ab}	26.64 ± 6.28^{a}	61.35 ± 29.47^{ab}
A. carbonarius	5	nd	205.35 ± 3.92^{d}	49.12 ± 7.46^{c}	16.38 ± 0.39^{b}	13.44 ± 2.96^{b}
CBS127.49	10	60.22 ± 12.16^{a}	484.52 ± 99.68^{b}	33.86 ± 2.35^{a}	37.48 ± 1.94^{a}	5.01 ± 1.10^{a}
	15	204.52 ± 57.69^{b}	221.72 ± 59.26^{b}	43.35 ± 0.39^{a}	37.74 ± 2.35^{a}	8.77 ± 2.86^{a}
	20	127.93 ± 43.56^{b}	245.31 ± 21.19^{c}	37.94 ± 23.43^{a}	46.62 ± 3.14^{a}	2.57 ± 0.43^{a}
	30	220.06 ± 10.60^{c}	147.08 ± 32.18^{b}	51.92 ± 16.21^{a}	54.84 ± 8.94^{a}	2.68 ± 1.41^{a}
A. carbonarius	5	nd	13.16 ± 2.56^{a}	2.48 ± 0.61^{a}	36.35 ± 9.81^{b}	NG
A-941	10	103.23 ± 29.83^{b}	7.43 ± 1.48^{a}	93.24 ± 22.76^{b}	9.16 ± 1.18^{a}	NG
	15	169.56 ± 49.84^{b}	6.14 ± 0.12^{a}	1.09 ± 0.42^{a}	10.38 ± 1.16^{a}	NG
	20	129.87 ± 9.42^{b}	10.41 ± 4.90^{a}	1.02 ± 0.33^{a}	14.01 ± 3.49^{a}	NG
	30	173.16 ± 118.52^{a}	9.16 ± 0.31^{a}	0.81 ± 0.16^{a}	14.18 ± 1.30^{a}	NG
A. carbonarius	5	nd	12.30 ± 1.93^{b}	0.37 ± 0.10^{a}	1.74 ± 1.92^{a}	nd
M325	10	26.36 ± 15.30^{a}	8.86 ± 1.29^{a}	0.54 ± 0.03^{a}	3.68 ± 0.95^{a}	1.08 ± 1.20^{a}
	15	97.40 ± 16.87^{b}	4.62 ± 0.81^{a}	0.48 ± 0.10^{a}	4.71 ± 1.11^{a}	1.30 ± 1.14^{a}
	20	43.29 ± 3.14^{b}	6.91 ± 0.23^{a}	0.43 ± 0.10^{a}	6.35 ± 2.39^{a}	0.75 ± 0.04^{a}
	30	82.70 ± 27.47^{b}	5.80 ± 0.09^{a}	0.52 ± 0^{a}	4.71 ± 1.69^{a}	0.72 ± 0.23^{a}
Mean	5	nd	76.99 ± 90.80^{b}	19.72 ± 23.0^{a}	16.90 ± 14.41^{a}	6.77 ± 6.72^{a}
	10	53.14 ± 37.41^{a}	143.22 ± 229.52^{a}	44.39 ± 38.52^{a}	16.53 ± 14.82^{a}	17.01 ± 24.28^{a}
	15	137.56 ± 59.38^{b}	82.25 ± 102.43^{ab}	25.87 ± 29.62^{a}	18.23 ± 14.47^{a}	24.17 ± 33.35^{a}
	20	101.53 ± 40.43^{b}	115.22 ± 124.55^{b}	35.45 ± 47.96^{a}	21.39 ± 17.56^{a}	13.41 ± 20.37^{a}
	30	150.08 ± 59.52^{b}	61.61 ± 67.55^{a}	31.00 ± 35.87^{a}	25.09 ± 21.77^{a}	21.58 ± 34.46^{a}

Values with the same superscript within each strain and incubation time are not significantly different ($P < 0.05$). NG: not growth; nd: not detected (limit of detection 0.05 μg/g).

OTA-negative. In our study, *A. carbonarius* M325 produced detectable levels of OTA from 15 to 35 °C, with a maximum level after 15 days of incubation at 15 °C. The OTA-negative *A. carbonarius* A-1082 is now under study because its morphological and genetic characteristics differs from the remaining *A. carbonarius* strains studied [15].

Our study shows that OTA-positive strains of *Aspergillus* section *Nigri* are able to produce OTA at a wide range of temperatures, with the optimal temperature usually being lower than those reported for growth. Although this feature is strain-related, knowledge of the influence of this parameter will provide us with potential tools to minimize OTA production in foods, especially in those where OTA contamination has been related to black aspergilli.

Among the fungi most commonly reported from foods, *A. niger* is more prevalent in warmer climates, both in field situations and in stored foods [28]. *A. carbonarius* has not been reported as frequently, probably because all of the black aspergilli are commonly regarded as "*A. niger*". Black aspergilli have been reported as being the predominant mycobiota of grapes [9,11,14,17,22,23,29,30] and raisins [3,18]. In grapes, they can be isolated at all developmental stages of the grapes, but the highest levels of isolation are achieved at harvesting [9,11,30]. Black spores give protection from sunlight and UV light, providing a competitive advantage in such habitats [28].

It is worth noting that according to our results significant amounts of OTA can be produced in only five days. Due to the ability to produce OTA at a wide range of temperatures, OTA can be continuously produced in the field, mainly at harvest time although there are large day–night temperature variations. Other climatic condition such as rain may also have a great influence on OTA contamination of wine because it favors overgrowth of OTA-producing black aspergilli. The use of different practices in grape cultivation in order to control black aspergilli contamination (e.g. fungicide treatments) will be of great importance in order to minimize the final OTA content in grapes and consequently in wine.

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3.2 Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species. Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***International Journal of Food Microbiology*** **(aceptado para publicación).**

Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species

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Abstract

The effect of water activity (a_w) (0.82-0.99) on growth and ochratoxin A (OTA) production by twelve *Aspergillus niger* aggregate strains, cultured in Czapek Yeast Autolysate agar (CYA) and Yeast Extract Sucrose agar (YES), was studied for an incubation period of 30 days. The strains were selected to include diverses sources, different reported abilities to produce OTA and different ITS-5.8S rDNA Restriction Fragment Length Polymorphism (RFLP) pattern. They were characterized by Random Amplification of Polymorphic DNA (RAPD) and ITS-5.8S rDNA and 28S rDNA (D1/D2) sequencing. Regardless of the a_w value tested, YES was a better culture medium than CYA for OTA production. The a_w range for OTA production was narrower than that for growth. OTA was produced from 0.90 a_w, 0.92 a_w, 0.94 a_w or 0.96 a_w to 0.99 a_w depending on the strain and the culture medium. The molecular study differentiated strains into two groups which corresponded to the RFLP types N and T although it did not distinguish them by their source of isolation or OTA producing abilities. Our results show that *A. niger* aggregate strains are able to grow and produce OTA over a wide a_w range. These results will lead to a better understanding of the contribution of *A. niger* aggregate in OTA contamination of food and feed.

1. Introduction

Mycotoxin contamination of food and feed represents a high risk for human and animal health. Ochratoxin A (OTA) is a mycotoxin which contaminates foods such as cereals, coffee, grapes, cocoa, wine, beer and spices. It commands attention as it has been implicated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity and immunotoxicity in both animals and humans (O'Brien and Dietrich, 2005). It has been classified as a possible human carcinogen (group 2B) by the IARC (IARC, 1993). The European Commission has established maximum limits for OTA in cereals, dried vine fruits, wine, coffee and infant foods (Commission of the European Communities, 2002, 2004, 2005).

OTA has been mainly associated with *Aspergillus ochraceus* and other species included in section *Circumdati* and to *Penicillium verrucosum*. Nevertheless, since the first description of OTA production by *Aspergillus niger* var. *niger* (Abarca et al., 1994) and by *A. carbonarius* (Horie, 1995) members of *Aspergillus* section *Nigri* are achieving a greater significance regarding OTA content in some food commodities. The source of OTA contamination in grapes, raisins and wine is due mainly to *A. carbonarius* and the species included in the *A. niger* aggregate (Abarca et al., 2004). It is worth noting that *Aspergillus niger* products hold the GRAS (Generally Regarded as Safe) status from the Food and Drug Administration and is a widely applied industrial species for large-scale biotechnological production of organic acids and enzymes (Bigelis and Lausure, 1987) in the food industry.

The taxa included in the *A. niger* aggregate are very difficult to distinguish by morphological means. Hence, many attempts have been made to divide these taxa into two or more species (Abarca et al., 2004). On the basis of their ITS-5.8S rDNA Restriction Fragment Length Polymorphism (RFLP) patterns, two groups can be observed, designated as N and T (Accensi et al., 1999). To date, all OTA-positive isolates belonging to the *A. niger* aggregate were classified in type N, whereas type T strains were not able to produce OTA (Accensi et al., 2001; Cabañes et al., 2002; Abarca et al., 2003; Leong et al., 2004).

Knowledge of the influence of environmental parameters on OTA production may contribute to prevention of OTA contamination in food commodities. Nevertheless, very little is known about optimal conditions for OTA production by *A. niger* aggregate strains. In a previous study focused on the influence of temperature, maximum OTA levels were achieved at 20-25ºC in YES medium (Esteban et al., 2004). In synthetic grape juice medium, maximum OTA production of two *A. niger* aggregate strains has been recently reported at 0.98-0.995 a_w (Bellí et al., 2004).

The aim of the current work is to study the influence of a_w on growth and OTA production by different species belonging to the *A. niger* aggregate. The strains were selected to include different sources, different reported ochratoxigenic ability and different ITS-5.8S rDNA RFLP patterns. Analysis of genetic relationships between the strains included in the study was performed using several molecular techniques.

2. Materials and methods

2.1 Fungal strains and culture conditions

The reported OTA production, RFLP pattern and source of isolation of the strains used in this study are shown in Table 1. The strains were grown on malt extract agar at 25 ºC for 7 days. Conidia suspensions of each isolate were prepared in an aqueous solution of 0.05% Tween 80. The inocula were adjusted to approximately 10^6-10^7 conidia per ml, determined by a counting chamber. The physiological study was carried out on two culture media: Czapek Yeast Extract (CYA) agar and Yeast Extract Sucrose (YES) agar (Pitt and Hocking, 1997) with a final pH of 6.9-7. Both media were adjusted to different water activity values (0.82, 0.86, 0.90, 0.92, 0.94, 0.96, 0.98, 0.99) by addition of appropiate quantities of glycerol determined by the construction of a calibration curve. The final a_w of the culture media was determined with Novasina Thermoconstanter TH 200 (Novasina, Zurich, Switzerland). Plates were centrally point inoculated with 1 µl of the adjusted suspension and incubated at 25 ºC. Each assay was performed in duplicate. For each experiment, the a_w values of control plates were determined after 30 days of incubation and not significant variation was detected.

2.2 Determination and quantification of OTA

OTA production was determined after 5, 10, 15, 20 and 30 days of incubation at each a_w value assayed following a previously described high-pressure liquid chromatography (HPLC) screening method (Bragulat et al., 2001). Three agar plugs were removed from different points of the colony and these were extracted with 0.5 ml of methanol. The extracts were filtered and 20 µl was injected into the HPLC. OTA detection and quantification was made by a Waters LCM1 chromatograph with a fluorescence detector Waters 2475 (excitation wavelength: 330 nm / emission wavelength: 460 nm), and with a column C18 Spherisorb S5 ODS2, 250 x 4.6 mm. The mobile phase, with a flow rate of 1 ml/minute, consisted of the following isocratic program:

acetonitrile, 57%; water, 41% and acetic acid, 2% (Bauer and Gareis, 1987). The extracts with a peak at the same retention time as OTA (around 6.8 minutes) were considered positive. Confirmation was made through derivatization of OTA to its methylester (Hunt et al., 1980). The detection limit of the extraction procedure and the HPLC technique was 0.02 ng OTA and the quantification limit of HPLC technique with the extraction procedure was 0.05 μg g^{-1} for this mycotoxin.

Data obtained from the different a_w conditions tested were statistically analysed by means of one-way analysis of variance test and Student's test. All statistical analyses were performed using SPSS software (Version 12.0).

2.3 DNA extraction

Fungal DNA was extracted as described by Accensi et al. (1999). The strains were inoculated in 1.5 ml Eppendorf tubes containing 500 μl of Sabouraud broth (2% glucose, w/v; 1% peptone w/v) supplemented with chloramphenicol (1 mg l^{-1}) and incubated overnight in an orbital shaker at 300 rpm at 30 ºC. Mycelium was recovered after centrifugation and washed with NaCl 0.9% (w/v), frozen in liquid nitrogen and ground to a fine power with a pipette tip. The powder was incubated for 1h at 65 ºC in 500 μl extraction buffer (Tris-HCl 50 mM, EDTA 50 mM, SDS 3% and 2-mercaptoethanol 1%). The lysate was extracted with phenol:chloroform (1:1, v/v), 3 M NaOAc and 1 M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8). DNA was cleaned with 'Geneclean kit II' (BIO 101, La Jolla, CA, USA), according to the manufacturer's instructions.

2.4 Sequencing of the ITS1-5.8S rDNA-ITS2 and D1/D2 of 28S rDNA regions

The sequencing reaction was carried out using the sequencing commercial system 'ABI Prism Big Dye Terminator v 3.0 Ready Reaction Cycle Sequencing Kit' (Applied Biosystems, Gouda, The Netherlands). The sequence of both chains was obtained with the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), described by White et al. (1990) for the ITS1-5.8S-ITS2 region and NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (O'Donnell, 1993) for the D1/D2 region of 28S rDNA, following the indications of the

manufacturer. An Applied Biosystems 'ABI Prism 310 Genetic Analyzer' sequencer was used to obtain the DNA sequences. The sequences were aligned by using the software program Clustal X (1.8) (Thompson et al., 1997). Once aligned, they were analysed using the Neighbor-Joining method (Saitou and Nei, 1987) with the program 'Mega' (2.1) (Kumar et al., 2001).

2.5 RAPD analysis

PCR reaction was carried out in a 20 μl reaction volume using a Perkin-Elmer 2400 thermocycler. The mixture for the reaction contained: 10x PCR buffer (10 mM); 3mM of $MgCl_2$; 280 μM of each dNTP (dATP, dCTP, dTTP and dGTP); 0.55 μM of primer ari1 (5'-TGCTTGGCACAGTTGGCTTC-3') (Castellá et al., 2002); 1 U of Tag DNA polymerase and between 0.1 and 10 ng of DNA. The reaction started with an initial denaturing step of 5 minutes at 95 ºC, 44 cycles of 1 minute at 95 ºC, 1 minute at 36 ºC and 4 minutes at 72 ºC, followed by a final elongation step of 7 minutes at 72 ºC.

Electrophoresis was conducted on a 2% (w/v) agarose gel in Tris-boric acid-EDTA (TBE). The molecular weights of the amplified products were obtained in comparison to the '100 bp molecular ruler' (Bio-Rad Laboratories S.A., Barcelona). The profiles of bands obtained were analysed using the UPGMA method (Page and Holmes, 1998) of the 'Diversity Database' 2.2.0. software. The reactions were performed in triplicate.

3. Results and discussion

3.1 Water activity effect on growth and OTA production

All the strains grew in CYA and YES media from 0.86 a_w to 0.99 a_w and three of them (CBS 554.65, A-946, CBS 121.55) also grew in YES medium adjusted at 0.82 a_w from 20 days of incubation. The germination of *A. niger* strains has been reported at 0.77 a_w (Pitt and Hocking, 1997) and 0.80 a_w (Marín et al., 1998). Slow growth of *A. niger* isolates was reported at 0.76 a_w on malt extract agar (MEA) (Vujanovic et al., 2001) whereas in another study the minimum water activity limit for growth was 0.82 using the same culture medium (Parra and Magan, 2004).

Five of the six reported ochratoxigenic strains produced quantifiable levels of OTA. The strain CBS 618.78 produced only trace amounts of OTA in this study. Tables 2 and 3 show the concentration detected at each a_w value and incubation time in CYA and YES medium respectively. Mean OTA concentration produced by all the positive strains at all sampling times is

shown in Fig. 1. The a_w range for OTA production was narrower than that for growth and each strain differed in its optimum conditions for OTA production. The a_w range in which OTA production occurred was wider for most of the strains in CYA than in YES, but the concentration achieved was higher in YES medium mainly at the 0.96-0.99 a_w range (Fig. 1). The mean OTA concentration produced in YES medium (4.38 μg g^{-1}) was significantly higher ($p<0.01$) than in CYA medium (1.88 μg g^{-1}). Regardless the initial a_w value, YES was more suitable for OTA production than CYA medium for the *A. niger* aggregate strains. These results were in accordance with two previous studies of *A. niger* aggregate strains (Bragulat et al., 2001; Esteban et al., 2004), however CYA medium was reported to be more suitable than YES medium for OTA production by *A. carbonarius* strains (Bragulat et al., 2001; Esteban et al., 2004; Esteban et al., 2005).

All isolates produced OTA on both media after only 5-10 days of incubation. In CYA medium (Table 2) the maximum levels of OTA were detected within the range 0.94-0.98 a_w after 5-10 days of incubation. In YES medium the optimal conditions for OTA production were observed after 5-10 days of incubation at 0.96-0.99 a_w. Apart from CBS 618.78 which produced only trace amounts of OTA, A-943 produced in both media the lowest amounts of OTA in comparison with the rest of strains studied.

The amounts of OTA detected decreased when increasing incubation time. Some authors suggested that strains could remove and assimilate the phenylalanine moiety from the OTA molecule, as other nitrogen sources of the culture medium become exhausted (Téren et al., 1996).

The percentage of OTA producing strains in the *A. niger* aggregate is low (Abarca et al., 2004) although it is still unknown if this is due to a genetic based feature. It is necessary to test different environmental conditions to find out the optimal conditions for OTA production which may clarify this fact. In our study, the six strains initially considered as OTA-negative (Table 1) did not produce the mycotoxin at any of the a_w values tested for a period of 30 days. In a previous study, these six strains did not produce OTA at any of the temperatures tested (Esteban et al., 2004). Four of these strains showed N RFLP pattern whilst two of them showed T RFLP pattern. To date OTA production has never been demonstrated in type T strains (Accensi et al., 2001; Cabañes et al., 2002; Abarca et al., 2003; Leong et al. 2004).

In synthetic grape juice medium the optimum a_w value for OTA production by two *A. niger* aggregate strains was recently reported to be 0.98-0.995 a_w, after 5 to 10 days of incubation (Bellí et al., 2004). Studies focused on *A. carbonarius* established the optimum a_w range for OTA production between 0.95-0.99 a_w in synthetic grape juice medium (Bellí et al., 2004, 2005; Mitchell et al., 2004), whereas maximum OTA accumulation was observed at 0.99 a_w when one isolate from green coffee was incubated on coffee cherries (Joosten et al., 2001).

3.2 Analysis of genetic relationships between the strains

Many attempts have been carried out to clarify the taxonomy within *A. niger* aggregate by using different molecular techniques and several authors have proposed division of the *A. niger* aggregate into two or more species (Abarca et al., 2004). The ITS1-5.8S-ITS2 rDNA sequencing analysis of these strains showed very similar sequences which included 602 base pairs (bp). The strain CBS 554.65 showed a G insertion in the 5.8S region. The two strains with T RFLP pattern (CBS 134.48 and A-947) differed from the type N strains at three substitutions in ITS1 and ITS2 regions. These differences were reflected in the phylogenetic tree assessed (Fig. 2A). Analysis of D1 and D2 regions has shown to be a suitable tool to differentiate species within *Aspergillus* section *Nigri* (Peterson, 2000). In our study, the sequences obtained by the analysis of 28S rDNA (D1/D2 regions) included 586 bp. Strains with T RFLP pattern differed from N RFLP strains at two positions. Two different clusters were observed in the phylogenetic tree assessed (Fig. 2B). In both phylogenetic trees, one cluster contained the two strains with RFLP type T and the other one grouped the type N strains. This latter group included both OTA-positive and OTA-negative strains.

The differentiation between the two RFLP types was also reflected when observing the dendrogram obtained with the Random Amplification of Polymorphic DNA (RAPD) technique (Fig. 3). The two type T strains (CBS 134.48 and A-947) were also clustered separately from type N strains. Type N strains cluster showed a great variability and was divided in two main groups, both containing minor branches including producing and non-producing strains. On the basis of RAPD analysis, Megnegneau et al. (1993) proved the existence of a high level of intraspecific variability among strains of the *A. niger* aggregate, consistent with the RFLP analysis of rDNA which grouped the strains in two main groups.

In our study, no single molecular technique used could distinguish the strains by their source of isolation or their OTA producing abilities. For example, in the RAPD analysis, three OTA negative strains (CBS 554.65, A-946, CECT 2088) were clustered together with the producer strain CBS 618.78. The OTA negative strain CBS 121.55 was clustered jointly with the two OTA positive strains A-942 and A-943.

3.3. Implications on food sources

The strains studied have been able to grow at a wide range of a_w in both CYA and YES media. This would explain why *A. niger* is considered the most common *Aspergillus* species responsible for post-harvest decay of fresh fruit and is also among the most frequently fungi isolated from nuts and sun dried products, such as vine fruits (Pitt and Hocking, 1997; Heenan et al., 1998; Abarca et al., 2003). The black spores of this species apparently provide protection from sunlight and UV light, providing a competitive advantage in such habitats. *A. niger* aggregate species are isolated from grapes in the field, achieving the highest levels in late grape growth stages (Battilani et al., 2003; Serra et al. 2003; Bau et al., 2005). They are also isolated from coffee raw materials. *Aspergillus niger* has been frequently detected on OTA contaminated coffee cherries and green coffee (Bucheli and Taniwaki, 2002; Taniwaki et al., 2003). The isolation of these species from such products may represent one source of OTA contamination in the field.

Moreover, according to the results obtained, significant amounts of OTA can be achieved at only 5 days of incubation at 25ºC. This feature can also lead to the contribution of OTA contamination in those products, stored or manufactured after harvesting, where *A. niger* aggregate species are frequently isolated together with other ochratoxigenic black aspergilli. The drying process performed in some products such as coffee or raisins has not prevented OTA contamination (Bucheli and Taniwaki, 2002; Taniwaki et al., 2003). Leong et al. (2004) reported that black aspergilli can grow in the fruit during the initial stages of drying until the water activity falls below around 0.85 a_w. Coffee cherries contain still 25-50% of water after 5 days of drying, enough to support growth of black aspergilli (Bucheli and Taniwaki, 2002).

Our results show the ability of *A. niger* aggregate strains to grow and produce OTA in a wide a_w range. Moreover significants amounts of OTA can be produced after only five days of incubation since the a_w achieved by some substrates after the drying process could still allow the development of this species and the production of mycotoxin.

Further studies on the effect of physiological variables on growth and OTA production deserve a significant concern. It may lead us to better understanding of the role of *A. niger* aggregate in OTA contamination in food and feed where black aspergilli members are usually isolated.

Acknowledgements

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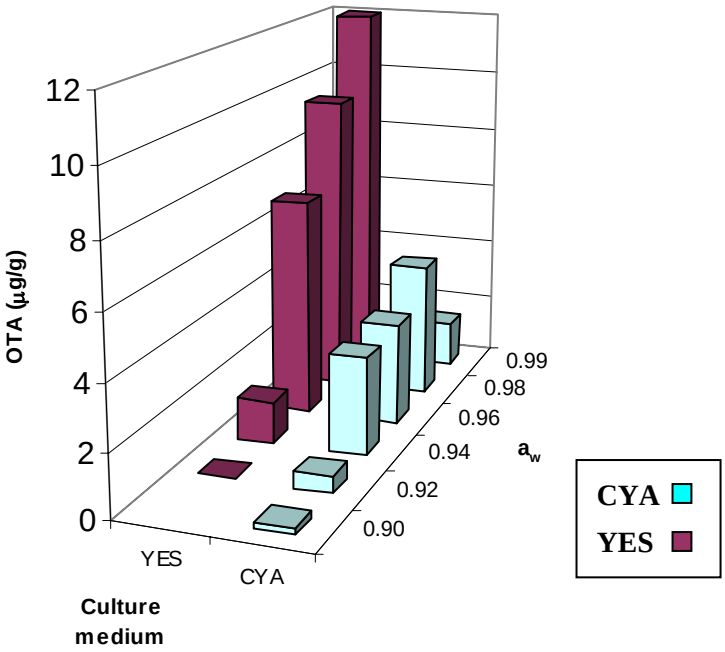
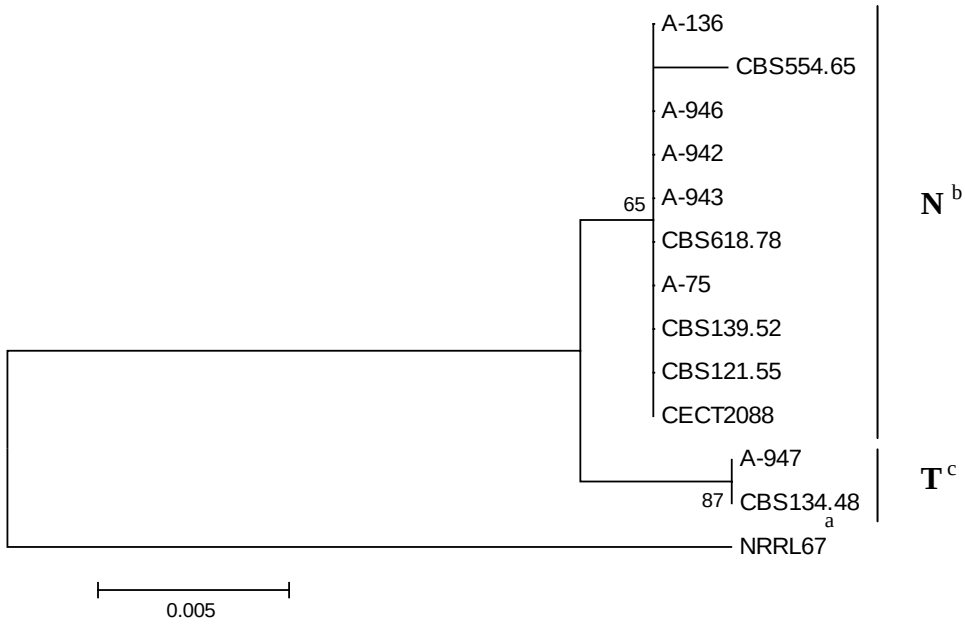


Fig. 1. Mean OTA concentration produced at all sampling times by all the OTA-producing strains at each a_w value and culture media tested.

A



B

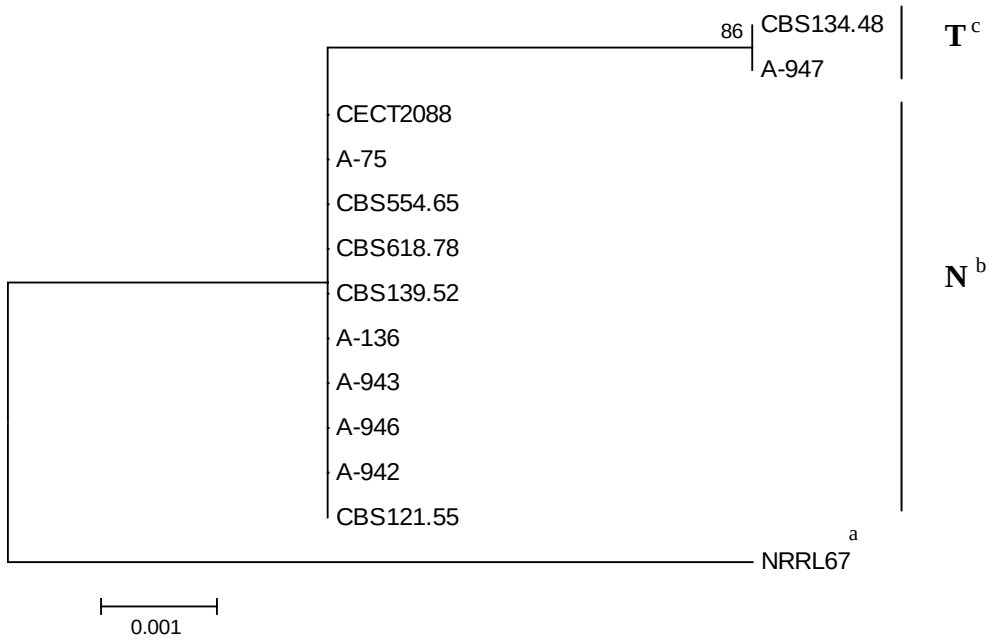


Fig. 2. Phylogenetic trees constructed from ITS-5.8S rDNA-ITS2 (A) and D1/D2 (B) sequences of the *A. niger* aggregate strains included in this study using neighbour-joining method. Bootstrap percentages calculated from 1,000 resamplings are indicated at nodes.
[a]: *A. carbonarius* strain (NRRL67) was included as outgroup in the analysis.[b]: N RFLP type; [c]: T RFLP type (Accensi et al., 1999).

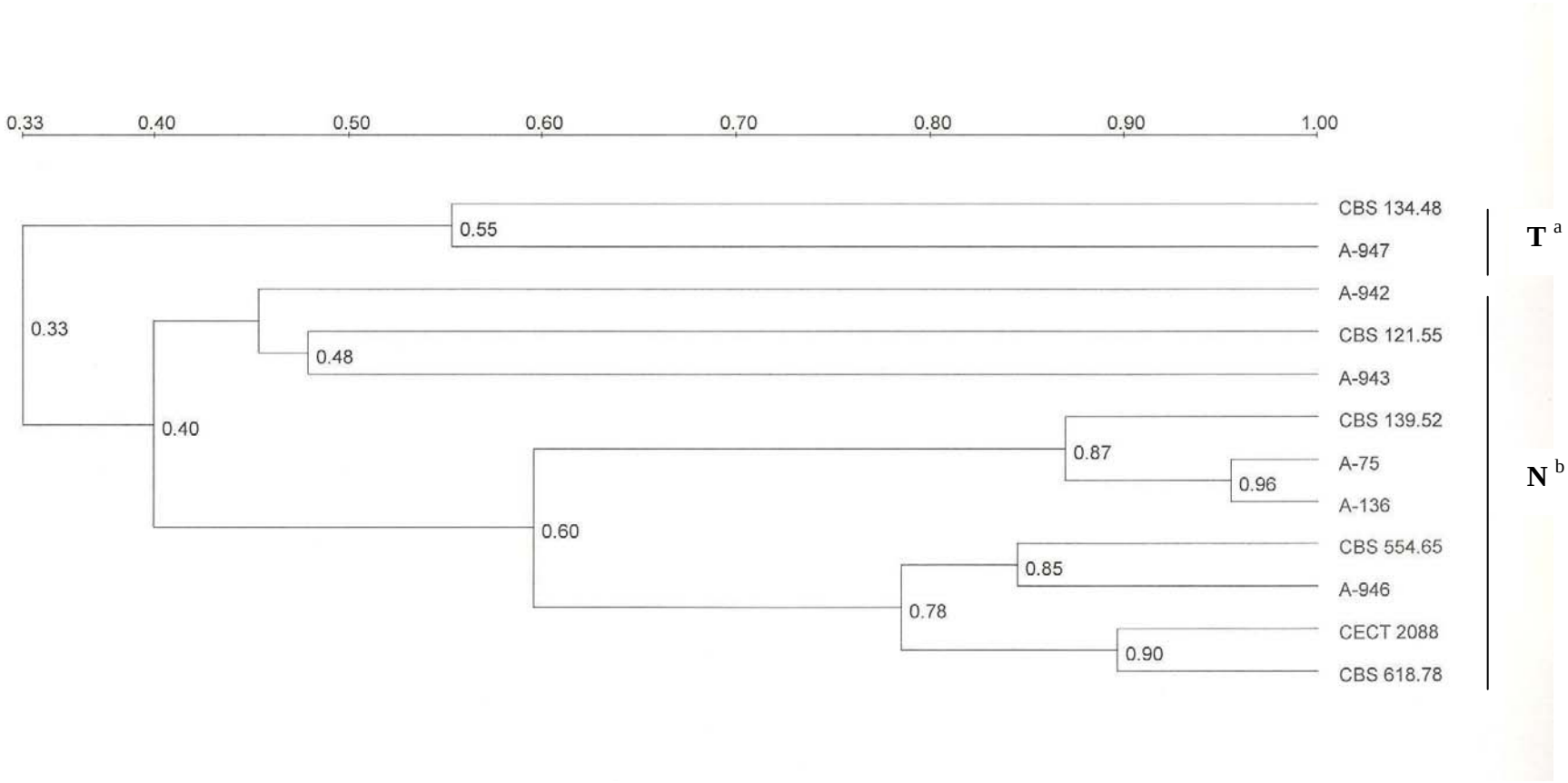


Fig. 3. UPGMA dendrogram of the *A. niger* aggregate strains used in this study assessed from the comparison of RAPD fingerprintings generated with primer ari1. The scale represents taxonomic distance. [a]: T RFLP type; [b]: N RFLP type (Accensi et al., 1999).

Table 1.
Aspergillus niger aggregate strains used in this study.

Species	Strain (source)[a]	OTA production reported (Ref.)	RFLP pattern (Ref.)
A. niger var. *niger*	A-75 (feedstuffs, CCFVB)	+ (Abarca et al., 1994; Esteban et al., 2004)	N(Accensi et al., 2001)
A. niger var. *niger*	A-136 (soy, CCFVB)	+ (Abarca et al., 1994; Esteban et al., 2004)	N(Accensi et al., 2001)
A. niger var. *niger*	A-942 (raisins, CCFVB)	+ (Abarca et al., 2003; Esteban et al., 2004)	N(Abarca et al., 2003)
A. niger var. *niger*	A-943 (grapes, CCFVB)	+ (Esteban et al., 2004)	N(Esteban et al., 2004)
A. awamori	CBS 139.52 (kuro-koji)	+ (Ono et al., 1995; Esteban et al., 2004)	N(Accensi et al., 2001)
A. foetidus	CBS 618.78 (unknown)	+ (Téren et al., 1996, Esteban et al., 2004)	N(Accensi et al., 2001)
A. niger	CECT 2088 (unknown)	- (Varga et al., 2000; Esteban et al., 2004)	N(Esteban et al., 2004)
A. niger	CBS 554.65 (tannin-gallic acid fermentation)	- (Accensi et al., 2001; Esteban et al., 2004)	N(Accensi et al., 2001)
A. niger var. *niger*	A-946 (coffee, CCFVB)	- (Esteban et al., 2004)	N(Esteban et al., 2004)
A. niger	CBS 121.55 (otomycosis)	- (Esteban et al., 2004)	N(Esteban et al., 2004)
A. tubingensis	CBS 134.48 (unknown)	- (Accensi et al., 2001; Esteban et al., 2004)	T(Accensi et al., 2001)
A. niger var. *niger*	A-947 (grapes, CCFVB)	- (Cabañes et al., 2002; Esteban et al., 2004)	T(Cabañes et al., 2002)

[a] Abbreviations: CCFVB, Culture Collection of Veterinary Faculty of Barcelona, Bellaterra (Barcelona), Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CECT, Colección Española de Cultivos Tipo, Valencia, Spain.

Table 2
OTA concentration (mean and mean standard error) produced by the five OTA-producing strains of *A. niger* aggregate in CYA medium at 25ºC at each a_w value tested.

		OTA concentration(μg/g)						
Strain	days	0.86	0.90	0.92	0.94	0.96	0.98	0.99
A-75	5	NG	nd	nd	5.86±1.87 ^{a,b}	10.17±3.53 ^{b}	4.87±2.49 ^{a,b}	3.98±0.63 ^{a,b}
	10	nd	nd	0.40±0.25 ^{a}	6.74±2.37 ^{b}	6.63±3.42 ^{b}	2.46±0.43 ^{a,b}	1.47±0.20 ^{a}
	15	nd	nd	0.04±0.05 ^{a}	4.59±1.26 ^{b}	3.85±0.95 ^{b}	1.67±1.00 ^{a}	1.82±0.10 ^{a}
	20	nd	nd	0.10±0.06 ^{a}	3.72±0.95 ^{a}	2.55±0.92 ^{a}	6.91±8.02 ^{a}	1.72±0.62 ^{a}
	30	nd	nd	nd	1.91±0.91 ^{b}	1.72±0.08 ^{b}	1.78±0.03 ^{b}	1.03±0.21 ^{a,b}
A-136	5	NG	nd	nd	12.95±4.82 ^{b}	12.7±1.56 ^{b}	13.36±0.90 ^{b}	4.08±0 ^{a}
	10	nd	nd	nd	2.06±0.28 ^{b}	3.65±0.12 ^{c}	3.05±1.17 ^{b,c}	1.89±0.39 ^{b}
	15	nd	nd	nd	1.38±0.25 ^{a,b}	2.55±0.34 ^{b}	1.96±1.20 ^{b}	1.73±0.45 ^{a,b}
	20	nd	nd	nd	1.50±0.52 ^{b}	2.84±0.49 ^{c}	4.75±0.30a ^{d}	2.54±0.28 ^{c}
	30	nd	nd	nd	1.00±0.09 ^{a}	1.92±0.45 ^{a}	6.76±1.56 ^{b}	1.63±0.43 ^{a}
A-942	5	NG	nd	nd	1.50±0.66 ^{a,b}	3.35±1.51 ^{b}	10.28±0.01 ^{c}	1.37±0.04 ^{a,b}
	10	nd	1.27±1.51 ^{a}	6.47±0.18 ^{b}	11.15±1.29 ^{c}	4.17±1.32 ^{a,b}	2.50±1.96 ^{a}	0.75±0.20 ^{a}
	15	nd	1.86±0.66 ^{a,b}	2.22±0.51 ^{a,b}	9.31±0.21 ^{c}	3.42±1.27	3.92±1.14 ^{b}	0.94±0.04 ^{a}
	20	nd	1.47±0.76 ^{a}	1.88±0.13 ^{a}	8.21±2.74 ^{b}	2.54±0.86	3.94±0.26 ^{a}	0.98±0.23 ^{a}
	30	nd	0.45±0.42 ^{a}	1.48±0.37 ^{b}	4.91±0.19 ^{c}	3.44±0.47	4.89±0.04 ^{c}	0.66±0.13 ^{a}
A-943	5	NG	nd	nd	nd	0.06±0 ^{b}	0.07±0.01 ^{b}	0.12±0.03 ^{c}
	10	nd	nd	0.34±0.08 ^{a}	0.24±0.21 ^{a}	0.22±0.08 ^{a}	nd	nd
	15	nd	nd	0.12±0.04 ^{a}	0.19±0.11 ^{a}	0.31±0.20 ^{a}	nd	0.16±0.04 ^{a}
	20	nd	nd	nd	0.29±0.03 ^{b}	0.73±0.13 ^{c}	nd	0.07±0.01 ^{a}
	30	nd	nd	nd	0.25±0.08 ^{b}	0.51±0.14 ^{c}	nd	nd
CBS 139.52	5	NG	nd	nd	nd	0.49±0.11 ^{a}	12.6±7.86 ^{a}	3.05±1.22 ^{a}
	10	nd	nd	nd	nd	8.79±2.47 ^{b}	5.49±1.33 ^{c}	2.22±0.17 ^{a}
	15	nd	nd	nd	nd	1.94±1.22 ^{b}	5.09±0.41 ^{c}	2.35±0.28 ^{b}
	20	nd	nd	nd	nd	0.83±0.35 ^{a}	4.23±1.18 ^{b}	2.18±0.35 ^{c}
	30	nd	nd	nd	nd	1.05±0.28 ^{b}	6.04±0.09 ^{c}	0.29±0.04 ^{a}

^{a,b,c,d} Values with the same superscript within each strain and incubation time are not significantly different ($p < 0.05$); nd: not detected (limit of detection 0.05 μg g^{-1}).; NG: not growth

Table 3
OTA concentration (mean and mean standard error) produced by the five OTA-producing strains of *A. niger* aggregate in YES medium at 25ºC at each a_w value tested.

		OTA concentration(μg/g)						
Strain	days	0.86	0.90	0.92	0.94	0.96	0.98	0.99
A-75	5	NG	nd	nd	3.31±0.57 [a]	9.99±14.13 [a]	23.86±4.70 [a]	54.12±16.88 [b]
	10	nd	nd	nd	6.99±3.08 [a,b]	13.04±1.97 [b,c]	18.50±6.36 [c]	20.54±1.57 [c]
	15	nd	nd	nd	1.19±0.13 [a]	3.06±1.85 [a]	2.92±1.61 [a]	9.91±2.79 [b]
	20	nd	nd	nd	0.45±0.04 [a]	3.82±4.50 [a]	1.39±0.86 [a]	15.32±2.28 [b]
	30	nd	nd	nd	0.96±0.27 [a]	0.65±0.72 [a]	2.91±2.32 [a,b]	5.02±0.86 [b]
A-136	5	NG	nd	nd	3.81±2.46 [a]	32.96±0.97 [b]	59.66±10.59 [c]	49.40±14.92 [b,c]
	10	nd	nd	nd	0.99±0.42 [a]	15.61±3.71 [b]	18.04±0.39 [b]	8.88±3.14 [c]
	15	nd	nd	nd	0.81±0.10 [a]	7.35±2.55 [a]	3.89±3.92 [a]	3.40±1.82 [a]
	20	nd	nd	nd	0.28±0.13 [a]	5.49±0.04 [b]	6.72±0.81 [b]	6.01±3.59 [b]
	30	nd	nd	nd	0.23±0.06 [a]	2.96±1.15 [a]	8.07±7.21 [a]	0.56±0.27 [a]
A-942	5	NG	nd	nd	2.30±1.36 [a]	16.78±3.54 [b]	16.32±2.14 [b]	16.50±3.79 [b]
	10	nd	nd	0.56±0.52 [a]	7.54±0.98 [a]	18.23±4.82 [b]	4.72±1.96 [a]	1.08±0.04 [a]
	15	nd	nd	0.19±0.10 [a]	1.87±1.97 [a]	9.69±4.99 [b]	4.18±1.39 [a]	1.58±0.16 [a]
	20	nd	nd	0.09±0.01 [a]	0.71±0.30 [a]	6.39±4.22 [a]	3.54±2.41 [a]	1.66±0.04 [a]
	30	nd	nd	nd	1.14±1.08 [a,b]	3.22±0.17 [c]	1.89±0.06 [b]	0.64±0.27 [a,b]
A-943	5	NG	nd	nd	nd	0.17±0.02 [b]	0.21±0.11 [b]	0.21±0.01 [b]
	10	nd	nd	nd	nd	1.06±0.92 [a]	0.06±0.08 [a]	nd
	15	nd	nd	nd	nd	0.30±0.23 [a]	nd	0.05±0 [a]
	20	nd	nd	nd	nd	nd	nd	nd
	30	nd	nd	nd	nd	nd	nd	nd
CBS 139.52	5	NG	nd	nd	0.11±0.03 [a]	8.90±2.79 [a]	25.53±4.71 [b]	41.07±3.14 [c]
	10	nd	nd	nd	0.47±0.44 [a]	11.46±2.09 [a]	22.48±22.37 [a]	31.69±5.26 [a]
	15	nd	nd	nd	nd	0.37±0.04 [a]	7.80±4.51 [b]	17.99±0.51 [c]
	20	nd	nd	nd	nd	0.55±0.55 [a]	1.53±0.54 [a]	8.88±2.19 [b]
	30	nd	nd	nd	nd	0.35±0.22 [a]	4,10±2.64 [a,b]	4.83±1.12 [b]

[a] [a,b,c] Values with the same superscript within each strain and incubation time are not significantly different ($p < 0.05$); nd: not detected (limit of detection 0.05 μg g^{-1}).; NG: not growth

3.3 Study of the effect of water activity and temperature on ochratoxin A production by *Aspergillus carbonarius*. Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***Food Microbiology*** **(aceptado para publicación).**

Study of the effect of water activity and temperature on ochratoxin A production by *Aspergillus carbonarius*

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Summary

The effect of water activity (a_w) (0.78-0.99) and temperature (15 ºC and 30 ºC) on growth and production of ochratoxin A (OTA) of six *Aspergillus carbonarius* strains was studied in two culture media: Czapek Yeast Autolysate agar (CYA) and Yeast Extract Sucrose agar (YES), during a period of 30 days. The strains were selected to include different sources and different reported abilities to produce OTA and were characterized by RAPD and ITS-5.8S rDNA sequencing. CYA showed to be better culture medium than YES for OTA production in the isolates tested. OTA concentration was higher at 15 ºC than at 30 ºC. At 30 ºC, ranges for OTA production were more restrictive than those for growth. OTA was produced from 0.86 a_w, 0.90 a_w or 0.94 a_w depending on the strain. At 15 ºC, growth and OTA production were detected only in the 0.94-0.99 a_w range. The molecular study performed showed that five of the strains were conspecific and no correlation was found between molecular data and the OTA production level or origin. The remaining strain had never been able to produce OTA and will probably represent a new species in the *Aspergillus* section *Nigri*. Our results show that *A. carbonarius* is able to grow and produce OTA in a wide range of water activities at both high and low temperatures.

1. Introduction

The mycotoxin contamination of agricultural products is a serious health hazard with an increasing attention worldwide. Within the mycotoxins which regard more concern it is worthy to mention ochratoxin A (OTA). It is classified as a possible human renal carcinogen (group 2B) and it has been associated to the development of urinary tract tumours and renal diseases in different parts of the world. It is also teratogenic, carcinogenic and is clearly an immunosuppressive agent (O'Brien and Dietrich 2005). To date the European Commission has established limits for OTA in cereals, dried vine fruits, wine, coffee and infant foods (Anonymous 2002, 2004, 2005).

OTA was until recently believed to be produced only by *Aspergillus ochraceus* and related species belonging to section *Circumdati* and by *Penicillium verrucosum*, but these species are not considered the main OTA source in some commodities. Since the first description of OTA production by *Aspergillus niger* var. *niger* (Abarca et al. 1994), the significance of *Aspergillus* section *Nigri* is achieving a higher attention when studying OTA production. *Aspergillus carbonarius* has been also reported as an OTA-producing species in this section (Horie 1995). Recent surveys have clearly shown that *A. carbonarius* is the main source of OTA in food commodities such as wine, grapes, dried vine fruits, and probably in coffee (Abarca et al. 2004). Nevertheless, little information is available about the ecology of *A. carbonarius.* Although it was originally reported to cause grape rot (Raper and Fennell 1965) it has not been commonly reported, probably because all the black aspergilli are very frequently identified as "*A. niger*". In that sense, the taxonomy of this section is under revision and many attempts have been made in order to clarify the number of species included using morphologic, molecular and metabolic criteria (Abarca et al. 2004).

Due to the growing awareness of the potential hazards of *A. carbonarius* as contaminant of food and feed it is very important to know the effect of environmental conditions on growth and OTA production by this species. The aim of this work is to analyze the influence of water activity (a_w) on growth and OTA production by six *A. carbonarius* strains at two incubation temperatures (15 and 30 ºC) over a period of 30 days. The strains were selected to include different sources and different reported ochratoxigenic ability. Analysis of genetic relationships within the studied strains has been also performed through different molecular techniques.

2. Material and methods

2.1. Fungal isolates and culture conditions

Six isolates of *A. carbonarius* were included in the study. The reported OTA production and source of isolation is shown in Table 1. The strains were grown on malt extract agar (Pitt and Hocking 1997) at 25 ºC for 7 days. Conidia suspensions of each isolate were prepared in an aqueous solution of 0.05% Tween 80. The inocula were adjusted to approximately 10^6-10^7 conidia per ml as determined by a counting chamber. The physiological study was carried out on two culture media: Czapeck yeast extract (CYA) agar and Yeast extract sucrose (YES) agar (Pitt and Hocking 1997). These media were adjusted to eight water activity values (0.78, 0.82, 0.86, 0.88, 0.90, 0.94, 0.98, 0.99) by addition of different amounts of glycerol. The final a_w of the culture media was determined with Novasina Thermoconstanter TH 200 (Novasina, Zurich, Switzerland). Plates were centrally point inoculated with 1 µl of the adjusted suspension and incubated at 15 and 30 ºC. Each assay was performed in duplicate.

2.2. Determination and quantification of OTA

OTA production was determined after 5, 10, 15, 20 and 30 days of incubation at each a_w value and temperature following a previously described high-pressure liquid chromatography (HPLC) screening method (Bragulat et al. 2001). Three agar plugs were removed from different points of the colony and were extracted with 0.5 ml of methanol. The extracts were filtered and 20 µl was injected into the HPLC. OTA detection and quantification was made by a Waters LCM1 chromatograph with a fluorescence detector Waters 2475 (excitation wavelength: 330 nm / emission wavelength: 460 nm), and with a column C18 Spherisorb S5 ODS2, 250 x 4.6 mm. The mobile phase, with a flow rate of 1 ml/minute, consisted of the following isocratic program: acetonitrile, 57%; water, 41% and acetic acid, 2% (Bauer and Gareis 1987). The extracts with the same retention time as OTA (around 6.8 minutes) were considered positive. Confirmation was made through derivatization of OTA in its methyl-ester (Hunt et al. 1980). The detection limit of the extraction procedure and the HPLC technique was 0.02 ng OTA and the quantification limit of HPLC technique with the extraction procedure was 0.05 µg/g for this mycotoxin.

Data obtained from the different a_w conditions tested were statistically analysed by means of one-way analysis of variance test and Student's test. All statistical analyses were performed using SPSS software (Version 10.0).

2.3. DNA extraction

Fungal DNA was extracted as described by Accensi et al. (1999). The strains were inoculated in 1.5 ml Eppendorf tubes containing 500 µl of Sabouraud broth (2% glucose, w/v; 1% peptone w/v) supplemented with chloramphenicol (1 mg/l) and incubated overnight in an orbital shaker at 300 rpm at 30 ºC. Mycelium was recovered after centrifugation and washed with NaCl 0.9% (w/v), frozen in liquid nitrogen and ground to a fine power with a pipette tip. The powder was incubated for 1h at 65 ºC in 500 µl extraction buffer (Tris-HCl 50 mM, EDTA 50 mM, SDS 3% and 2-mercaptoethanol 1%). The lysate was extracted with phenol: chloroform (1:1, v/v), 3 M NaOAc and 1 M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8). DNA was cleaned with 'Geneclean kit II' (BIO 101, La Jolla, CA, USA), according to the manufacturer's instructions.

2.4. Sequencing of the ITS1-5.8S-ITS2 rDNA region

The sequencing reaction was carried out using the sequencing commercial system 'ABI Prism Big Dye Terminator v 3.1 Cycle Sequencing Kit' (Applied Biosystems, Gouda, The Netherlands) following the indications of the manufacturer. The sequence of both strands was obtained with the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), described by White et al. (1990). An Applied Biosystems 'ABI Prism 3100 Genetic Analyzer' sequencer was used to obtain the DNA sequences. The sequences were aligned by using the software program Clustal X (1.8) (Thompson et al. 1997). Once aligned, they were analysed using the Neighbor-Joining method (Saitou and Nei 1987) with the program 'Mega' (2.1) (Kumar et al. 2001).

2.5. RAPD analysis

PCR reactions for the RAPD analysis were performed using three primers: ari1, M13 and P8. It was carried out with 0.1 to 10 ng of DNA in a 20 µl reaction volume and using a Perkin-Elmer 2400 thermocycler (Perkin Elmer Cetus corporation, Emeryville, CA, USA).

The mixture for the reaction with primer ari1 contained: 10x PCR buffer (10 mM); 3 mM of $MgCl_2$; 280 µM of each dNTP (dATP, dCTP, dTTP and dGTP); 0.55 µM of primer (5'-TGCTTGGCACAGTTGGCTTC-3') (Castellá et al. 2002); 1 U of Taq DNA polymerase. The reaction

started with an initial denaturing step of 5 minutes at 95 ºC, 44 cycles of 1 minute at 95 ºC, 1 minute at 36 ºC and 4 minutes at 72 ºC, followed by a final elongation step of 7 minutes at 72 ºC.

For primer M13 (5'-GAGGGTGGCGGTTCT-3') (Vassart et al. 1987), the mixture reaction contained: 10x PCR buffer (10 mM); 3 mM of $MgCl_2$; 250 μM of each dNTP (dATP, dCTP, dTTP and dGTP); 0.59 μM of primer; 1 U of Taq DNA polymerase. The reaction started with an initial denaturing step of 1 minute at 95 ºC, 30 cycles of 20 seconds at 95 ºC, 1 minute at 50 ºC and 20 seconds at 72 ºC, followed by a final elongation step of 6 minutes at 72 ºC.

For primer P8 (5'-GGAGCCCAC-3') (Voigt et al. 1995), the mixture reaction contained: 10x PCR buffer (10 mM); 3 mM of $MgCl_2$; 250 μM of each dNTP (dATP, dCTP, dTTP and dGTP); 0.51 μM of primer; 1 U of Taq DNA polymerase. The reaction started with an initial denaturing step of 5 minutes at 95 ºC, 30 cycles of 30 seconds at 95 ºC, 1 minute at 32 ºC and 30 seconds at 72 ºC, followed by a final elongation step of 6 minutes at 72 ºC.

Electrophoresis was conducted on a 2% (w/v) agarose gel in Tris-boric acid-EDTA (TBE). The molecular weights of the amplified products were obtained in comparison to the '100 bp molecular ruler' (Bio-Rad Laboratories S.A., Barcelona, Spain). The profiles of bands obtained were analysed using the UPGMA method (Page and Holmes 1998) of the 'Diversity Database' 2.2.0 software (Bio-Rad Laboratories S.A., Barcelona, Spain). The reactions were performed in triplicate for each of the RAPD primers studied.

3. Results and discussion

3.1. Water activity effect on growth and OTA production

When the temperature of incubation was 30 ºC all the strains grew in YES medium over the range of 0.82-0.99 a_w. In CYA medium the a_w range was narrower: two strains started their growth at 0.86 a_w (NRRL 67, CBS 127.49), one strain at 0.88 a_w (A-941) and three strains at 0.90 a_w (M325, CBS 110.49, A-1082). When incubation was performed at 15 ºC, all the isolates grew from 0.94 to 0.99 a_w at either CYA or YES medium (Figure 1A). Although relatively little is known about physiology of *A. carbonarius*, recent studies reported the minimum growth of this species on synthetic grape juice at 0.88-0.90 a_w (Bellí et al. 2004a, Mitchell et al. 2004) and on coffee berries at 0.90 a_w (Joosten et al. 2001).

Four out of the six *A. carbonarius* strains studied produced OTA. The a_w range for OTA production in each strain was very similar in both media (Figure 1B). Nevertheless, the mean OTA

concentration produced in CYA medium (11.92 μg/g at 30 ºC and 29.99 μg/g at 15 ºC) was higher than in YES medium (0.94 μg/g at 30 ºC and 20.27 μg/g at 15 ºC) being this difference statistically significant ($p<0.01$) at 30 ºC. The fact that CYA is better than YES medium for OTA production by *A. carbonarius* is in agreement with previous studies (Bragulat et al. 2001, Esteban et al. 2004, 2005).

OTA concentration produced in CYA medium at each a_w value tested is shown in Table 2 (30 ºC) and Table 3 (15 ºC). At 30 ºC, a_w ranges for mycotoxin production were more restrictive than those for growth and each strain differed in its optimum conditions for OTA production. With the strains A-941 and M325, OTA was detected from 0.94 to 0.99 a_w. The strains CBS 127.49 and NRRL 67 started OTA production at 0.86 and 0.90 a_w respectively.

At 15 ºC the four strains produced OTA over the range 0.94-0.99 a_w. It is worth to highlight that OTA concentration was statistically higher ($p<0.01$) at 15 ºC than at 30 ºC with the strains NRRL 67, A-941 and M325. The strain M325 had been described as OTA negative when coffee cherries were used as a substrate (Joosten et al. 2001). On previous studies, this strain was also able to produce OTA in CYA and YES medium incubated at different temperature and pH values (Esteban et al. 2004, 2005).

The optimum temperature range for OTA production in CYA medium by *A. carbonarius* has been established in a previous study at 15-20ºC (Esteban et al. 2004). In contrast, optimum growth has been reported at 30ºC (Bellí et al. 2004a, Leong et al. 2004, Mitchell et al. 2004). According with our results, Mitchell et al. (2004) studying eight strains isolated from grapes, identified 0.95-0.98 a_w and 15-20 ºC as optimum conditions for OTA production on synthetic grape juice medium. In the same medium, maximum OTA accumulation by two *A. carbonarius* strains isolated from grapes was obtained at 0.96 a_w , but the only tested temperature was 25 ºC (Bellí et al. 2004b). Optimal conditions for OTA production on coffee cherries was 25 ºC and 0.99 a_w using one strain isolated from green coffee (Joosten et al. 2001).

The strains CBS 110.49 and A-1082 did not produce detectable levels of OTA over the a_w range studied. In previous studies these two strains were not able to produce OTA at any of the temperatures and pH values tested (Esteban et al. 2004, 2005). The reported percentage of OTA producing strains in *A. carbonarius* is quite variable (Abarca et al. 2001, 2004), probably depending on the detection method used, but reaches the 100% in many studies (Cabañes et al. 2002, Leong et al. 2004, Sage et al. 2004, Bau et al. 2005a). The strain CBS 110.49 was reported as a very weak OTA-producing strain using coffee cherries as a substrate (Joosten et al. 2001). So, possibly we have not found yet its optimal nutritional requirements to produce OTA, or it has probably lost this ability. The other OTA-negative strain A-1082 was isolated from dried vine fruits (Abarca et al. 2003) and

was initially identified as *A. carbonarius*. Further examinations showed some morphological and genetic characteristics different from the remaining *A. carbonarius* strains studied. The strain A-1082 in addition to other strains isolated from grapes are now under study and will probably represent a new species in section *Nigri* tentatively named as "*A. ibericus*" (Cabañes et al. 2004, Bau et al. 2005b).

3.2. Molecular characterization

The phylogenetic tree obtained after sequencing the ITS1-5.8S-ITS2 rDNA region determined that five *A. carbonarius* strains (M325, CBS 110.49, NRRL67, A-941 and CBS 127.49) were conspecific (Fig. 2). Sequence analysis of the ITS regions was proved to be a suitable tool to separate *A. carbonarius* isolates from other black Aspergilli (Parenicova et al. 2000). In contrast, strain A-1082 was clearly differentiated. As has been mentioned above, this strain may represent a new species. This fact was confirmed with the dendrograms obtained with the three RAPD primers used (Fig. 3). Strain A-1082 always showed a different RAPD pattern and was located separately from the rest of strains in the three dendrograms assessed. The five *A. carbonarius* strains were grouped in a separate clade containing different subdivisions. No correlation was observed between the obtained clusters and the OTA production level or origin of the strains.

RAPD has been used in black Aspergilli to assess the genomic variability among different strains of the group, including *A. carbonarius* (Megnegneau et al. 1993). This molecular technique was also used to study the intraspecific variation of this species comparing the molecular polymorphism obtained with several phenotypic characters (Kevei et al. 1996). Fungaro et al. (2004) applied RAPD assays to identify amplification products specific for *A. carbonarius* and designed specific oligonucleotides which were successfully used as primers to detect *A. carbonarius* strains in coffee beans. In the same study they tried to distinguish between toxigenic (n=23) and non-toxigenic (n=6) strains of *A. carbonarius* isolated from coffee. Despite the large number of random primers used (471), no association was found between RAPD genotype and the ability to produce OTA. As we have mentioned above the percentage of OTA-producing strains reaches 100%, so the main question is if non-toxigenic *A. carbonarius* isolates do really exist in the field. In fact, the five *A. carbonarius* strains used in our study had been previously reported as OTA-producing strains (see Table 1). Maybe for that reason no correlation was found between molecular data and toxigenic properties of the studied strains.

3.3. Agricultural implications

Although isolates may have a slightly different response, our results show that *A. carbonarius* is able to grow and produce OTA in a wide range of water activities at both high and low temperatures. These results contribute to a better understanding of the ecophysiology of this species. As we have mentioned above, *A. carbonarius* appears to be less common than *A. niger,* but many earlyer surveys have probably not differentiated both species. Since *A. carbonarius* has been identified as an important source of OTA, some reports dealing with the origin of this mycotoxin in some substrates have reported their occurrence separately.

In grapes, *A. carbonarius* are recovered in all development stages of berries, reaching the highest level of isolation at harvest (Serra et al. 2002, Battilani et al. 2003, Bau et al. 2005a). In dried vine fruits, the occurrence of this species is very high (Abarca et al. 2003, Heenan et al. 1988, Magnoli et al. 2004). Leong et al (2004) reported that black aspergilli can grow in the fruit during initial stages of drying until the water activity drops below around 0.85 $a_{w.}$ Furthermore, black spores provide protection from sunlight and UV light, providing competitive advantage in such habitats (Pitt and Hocking 1997). All these facts would explain the association of *A. carbonarius* with dried vine fruits and probably with other dried fruit products.

In coffee beans, it seems that *A. carbonarius* infection mostly occurs after harvest (Taniwaki et al. 2003). Drying of fruits appears to be a risky step for natural occurrence of black aspergilli and OTA production. Indepently of the method of drying used, cherries contain sufficient water (still 25-50% after 5 days of drying) to support growth of *A. carbonarius* (Bucheli et al. 2002).

So, in accordance with our results, since significant amounts of OTA can be produced after only five days of incubation, the length of the drying process could provide sufficient time for growth and OTA production in the above mentioned substrates.

Further studies on the effect of different environmental factors on growth and OTA production by *A. carbonarius* will increase our knowledge of the ecology of this important species.

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Table 1. *Aspergillus carbonarius* strains used in this study.

Strain (source)[a]	OTA production reported (Ref.)
A. carbonarius NRRL 67 (Brazil)	+ (Téren et al. 1996, Esteban et al. 2004, 2005)
A. carbonarius CBS 127.49 (coffee)	+ (Joosten et al. 2001, Esteban et al. 2004, 2005)
A. carbonarius A-941 (grapes, CCFVB)	+ (Cabañes et al. 2002, Esteban et al. 2004, 2005)
A. carbonarius M325 (apples, supplied by HMLJ Joosten)	- (Joosten 2001); + (Esteban et al. 2004, 2005)
A. carbonarius CBS 110.49 (air)	+ (Joosten 2001); - (Esteban et al. 2004, 2005)
A. carbonarius A-1082 (raisins, CCFVB)	- (Abarca et al. 2003, Esteban et al. 2004, 2005)

[a] Abbreviations: CCFVB, Culture Collection of Veterinary Faculty of Barcelona, Bellaterra (Barcelona), Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; NRRL, Northern Agricultural Research Service Culture Collection, Peoria, IL, USA.

Table 2. OTA concentration (mean and mean standard error) produced by the four OTA-producing strains of *A. carbonarius* in CYA medium at 30 ºC at each a_w and incubation time tested.

		OTA concentration(μg/g)					
Strain	days	0.86	0.88	0.90	0.94	0.98	0.99
NRRL 67	5	NG	NG	0.64±0.35^{a}	8±1.88^{b}	9.62± 3.20^{b}	3.33±1.02^{a,b}
	10	NG	nd	11.48±2.71^{a}	1.93±0.20^{b}	2.24± 0.09^{b}	7.05±0.61^{c}
	15	nd	nd	1.66±0.69^{a}	1.62±0.60^{a}	1.78± 0.01^{a}	6.52±1.21^{b}
	20	nd	nd	0.67±0^{a}	1.41±0.72^{a}	3.31± 1.66^{b}	7.71±0.31^{c}
	30	nd	nd	1.43±0.18^{a}	2.87±1.61^{a}	1.48± 1.05^{a}	7.53±0.18^{b}
CBS127.49	5	NG	nd	64.65±6.72^{a,b}	84.08±4.31^{b}	39.96±20.41^{a,c}	18.60±2.75^{a,d}
	10	nd	12.49±1.07^{a}	178.43±130.69^{a}	62.16±7.06^{a}	181.54±3.54^{a}	42.46±14.52^{a}
	15	nd	18.84±3.02^{a,b}	58.85±18.02^{b,c}	67.44±25.51^{c}	16.18±1.14^{a,b}	25.48±1.10^{a,b}
	20	0.22±0.31^{a}	17.00±2.32^{a}	56.61±21.18^{b}	23.04±12.95^{a}	19.24±0.19^{a}	18.35±0.12^{a}
	30	nd	13.23±0.35^{a,b}	13.05±8.24^{a,b}	30.53±9.41^{b}	19.26±1.10^{b}	21.21±1.85^{b}
A-941	5	NG	NG	nd	0.31±0.09^{a}	1.15±0.21^{b}	0.22±0^{a}
	10	NG	nd	nd	nd	0.27±0.01	0.21±0.02^{b}
	15	NG	nd	nd	nd	0.34±0.21	0.22±0.07^{a}
	20	NG	nd	nd	nd	0.82±0.52	0.11±0.04^{a}
	30	NG	nd	nd	0.15±0.13^{a}	0.75±0.18^{b}	0.22±0.10^{a}
M325	5	NG	NG	nd	nd	0.13±0.01^{a}	0.18±0.26^{a}
	10	NG	NG	nd	nd	nd	0.14±0.01
	15	NG	NG	nd	0.16±0.12^{a}	nd	0.13±0.02^{a}
	20	NG	NG	nd	0.03±0.04^{a}	0.38±0.05^{b}	0.08±0.02^{a}
	30	NG	NG	nd	0.29±0.10^{a}	0.77±0.15^{b}	0.13±0.06^{a}

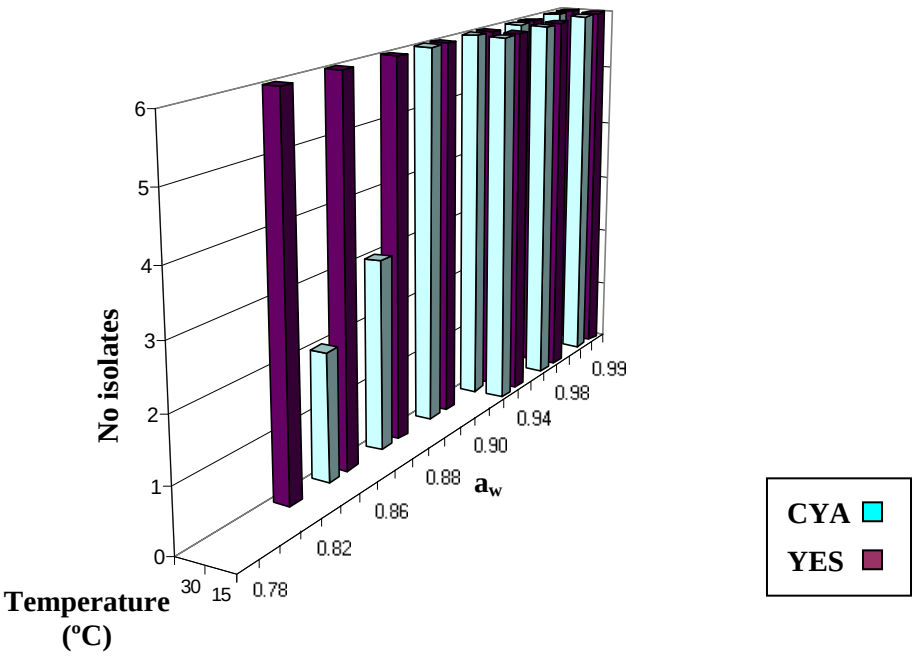
^{a,b,c} Values with the same superscript within each strain and incubation time are not significantly different ($p < 0.05$); nd: not detected (limit of detection 0.05 μg/g).; NG: not growth

Table 3. OTA concentration (mean and mean standard error) produced by the four OTA-producing strains of *A. carbonarius* in CYA medium at 15 ºC at each a_w and incubation time tested.

		OTA concentration(μg/g)		
Strain	days	0.94	0.98	0.99
NRRL 67	5	NG	nd	0.64±0.91
	10	NG	31.52±7.23	48.84±7.06
	15	7.14±0.42[a]	79.09±1.96[b]	48.57±8.24[c]
	20	25.87±12.64[a]	378.51±52.58[b]	89.64±22.37[a]
	30	30.17±0.19[a]	50.23±19.23[a]	46.90±9.02[a]
CBS127.49	5	NG	nd	nd
	10	NG	42.13±3.21	48.29±3.14
	15	nd	74.37±22.75[a]	63.83±5.49[a]
	20	9.69±0.09[a]	38.85±2.35[b]	55.78±3.53[c]
	30	73.18±15.65[a]	46.34±0.40[a]	64.66±9.81[a]
A-941	5	NG	nd	nd
	10	0.08±0.02[a]	14.27±1.18[b]	28.58±4.31[c]
	15	nd	4.44±0.16[a]	16.93±3.53[b]
	20	2.94±0.16[a]	3.83±2.11[a]	12.21±6.28[a]
	30	9.53±0.66[a]	5.31±1.29[b]	16.40±0.27[c]
M325	5	NG	nd	0.14±0.19
	10	NG	16.56±3.54	23.25±1.03
	15	nd	10.55±1.56[a]	14.76±9.73[a]
	20	1.31±1.03[a]	8.35±1.06[a]	14.43±11.77[a]
	30	3.21±0.06[a]	8.32±2.59[a,b]	19.70±5.88[b]

[a,b,c] Values with the same superscript within each strain and incubation time are not significantly different ($p < 0.05$); nd: not detected (limit of detection 0.05 μg/g).; NG: not growth

A



B

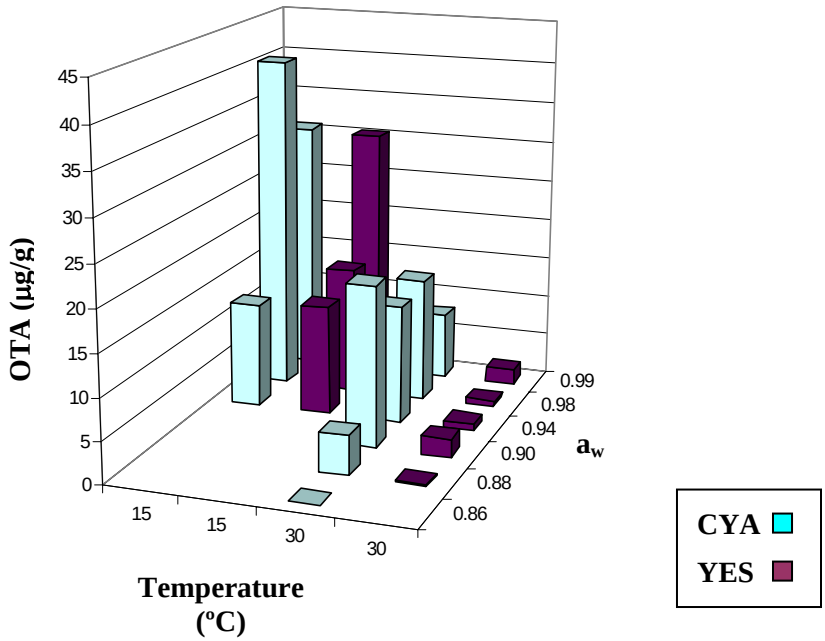


Fig. 1. A: Number of *A. carbonarius* strains developed at each a_w value and medium tested at 15 and 30 ºC. B: Mean OTA concentration produced by the OTA-producing strains of *A. carbonarius* at each a_w value and medium tested at 15 and 30 ºC.

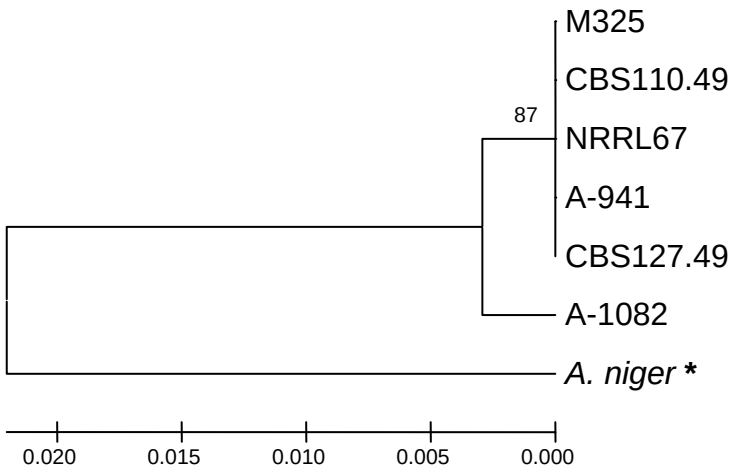
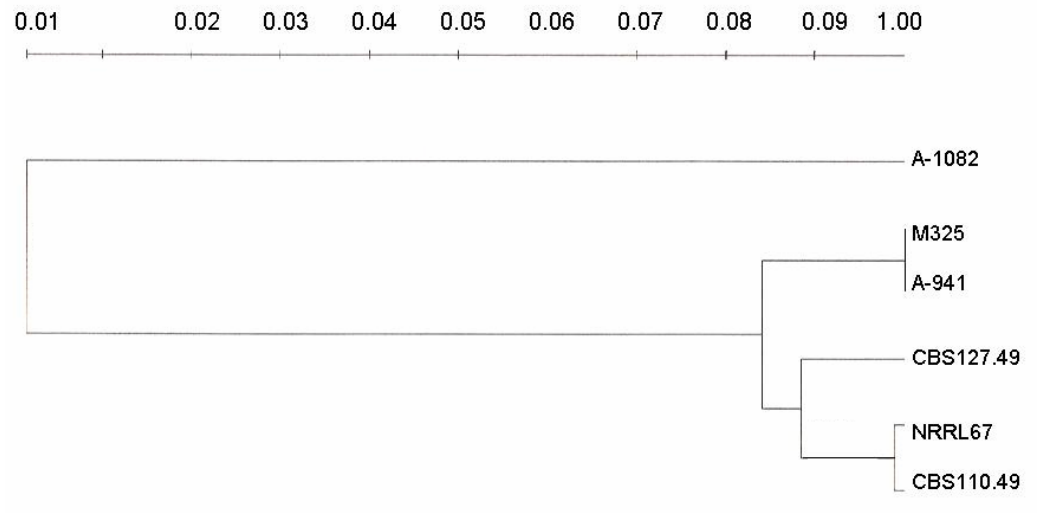
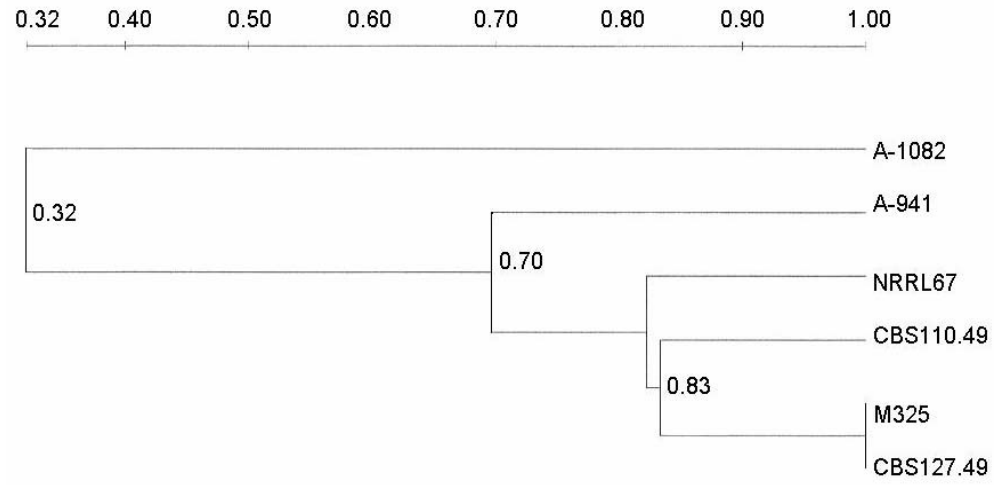


Fig. 2. Phylogenetic tree constructed from ITS1-5.8S-ITS2 rDNA sequences of the *A. carbonarius* strains included in this study using neighbour-joining method. Bootstrap percentages calculated from 1,000 resamplings are indicated at nodes.
*****: *A. niger* aggregate strain (A-75, CCFVB) was included as outgroup in the analysis.

A



B



C

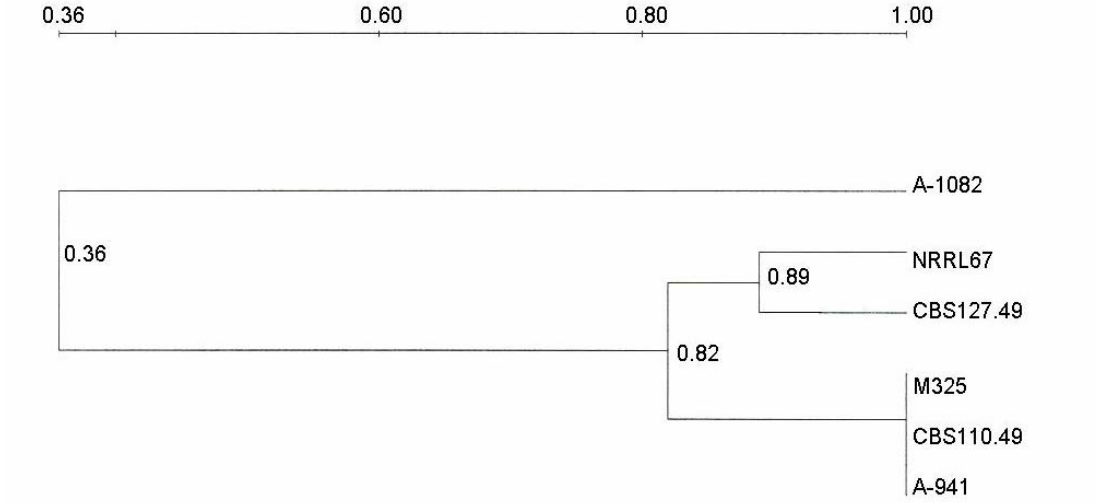


Figure 3. UPGMA dendrograms of the *A. carbonarius* strains used in this study assessed from the comparison of RAPD fingerprintings generated with (A) primer ari1, (B) primer M13, (C) primer P8. The scale represents taxonomic distance.

3.4 Effect of pH on ochratoxin A production by *Aspergillus niger* aggregate species. Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***Food Additives and Contaminants*** **(aceptado para publicación).**

Effect of pH on ochratoxin A production by *A. niger* aggregate species

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Keywords: *Aspergillus niger*, coffee, grapes, raisins, ochratoxin A, pH.

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Abstract

The effect of pH (2-10) on growth and ochratoxin A (OTA) production by twelve *Aspergillus niger* aggregate strains was studied in two culture media: Czapek Yeast Autolysate agar (CYA) and Yeast Extract Sucrose agar (YES), during a period of 30 days. The strains were selected to include different sources, different reported abilities to produce OTA and different ITS-5.8S rDNA RFLP patterns. YES showed to be better culture medium than CYA for OTA production. In this medium, OTA was produced from pH 2 or pH 3 to pH 10 depending on the strain. Our results show the ability of *A. niger* aggregate strains not only to grow but also to produce OTA over a wide pH range. These results will lead to a better understanding of the role of *A. niger* aggregate strains in the OTA contamination of several food commodities.

Introduction

Among the different mycotoxins which merit special concern for the hazard they represent in food commodities, ochratoxin A (OTA) deserves particular attention. OTA is a nephrotoxic mycotoxin which possesses as well carcinogenic, teratogenic, genotoxic and immunosuppressive effects (O'Brien and Dietrich 2005). The International Agency for Research on Cancer (IARC 1993) has rated OTA as a possible human carcinogen (group 2B). The European Commission has established limits for OTA in cereals, dried vine fruits, wine, coffee and infant foods. These limits will be reviewed in the short term and maximum limits for more products such as cocoa, beer, meat products, spices or liquorice will be included (Comission Regulation 2002, 2004, 2005).

OTA was until recently believed to be produced only by *Aspergillus ochraceus* and by *Penicillium* verrucosum. The description of OTA production by two species belonging to *Aspergillus* section *Nigri*, *Aspergillus niger* var. *niger* (Abarca et al. 1994) and *Aspergillus carbonarius* (Horie 1995), may contribute to explain the widespread OTA contamination reported in some food and feed products. This distribution was unclear only taking into account the incidence and natural occurrence of the previously described OTA-producing species.

Within *Aspergillus* section *Nigri* it is known that isolates included in the *A. niger* aggregate can produce a range of different toxic metabolites (Frisvad and Thrane 2000) but it is the ability of some of their members to produce OTA what deserves special concern. It is considered that in substrates such as grapes, raisins and wine the source of OTA contamination detected is due to *A. carbonarius* and some species included in the *A. niger* aggregate (Abarca et al. 2004). Furthermore, it is worth to highlight that *A. niger* products hold the GRAS (Generally Regarded as Safe) status from the Food and Drug Administration and it is used as source of enzymes and organic acids for food processing, despite the reported ability to produce OTA (Bigelis and Lausure 1987).

The taxa included in the *A. niger* aggregate are extremely difficult to distinguish one from each other by morphological means. In an attempt to solve the classification of these fungi, several

molecular techniques have been carried out in order to clarify the taxonomy and divide these taxa into two or more species (Abarca et al. 2004). On the basis of their ITS-5.8S rDNA restriction fragment length polymorphism (RFLP), the strains were grouped in two patterns, designated N and T (Accensi et al. 1999). To date the OTA-producing isolates with RFLP pattern known are classified as type N (Accensi et al. 2001; Cabañes et al. 2002; Abarca et al. 2003; Leong et al. 2004; Esteban et al. 2004).

Little is known about the environmental factors which can affect the OTA production by species of the *A. niger* aggregate. In a previous study on the effect of temperature, the *A. niger* aggregate strains showed the highest levels of OTA in Yeast Extract Sucrose (YES) agar at 20-25ºC (Esteban et al. 2004).

As nothing is known about the influence of pH, the aim of this study is to know its effects on growth and OTA production by different species belonging to the *A. niger* aggregate at 25ºC over a period of 30 days. The strains were selected to include different sources, different reported ochratoxigenic ability and different ITS-5.8S rDNA RFLP patterns.

Material and methods

Fungal strains and inocula preparation

Twelve isolates of the *A. niger* aggregate were used in this study (ten of N RFLP pattern and two of T pattern). The reported OTA production, source of isolation and RFLP pattern is shown in Table I. The strains were grown on malt extract agar (Pitt and Hocking 1997) for 7 days at 25ºC. Conidia suspensions of each isolate were prepared in an aqueous solution of 0.05% Tween 80. After filtering through sterile cheesecloth they were adjusted to approximately 10^6-10^7 conidia per ml as determined by a counting chamber.

Culture conditions

OTA production was determined on two culture media: yeast extract sucrose (YES) agar and Czapek yeast extract (CYA) agar (Pitt and Hocking 1997). The pH of the culture media was varied from 2 to 10. Adjustment of pH was performed before autoclaving by adding HCl (1 and 10 N) and NaOH (1 N). The pH value was measured using a pHmeter GLP21 (Crison Strumenti S.p.A., Carpi, Italy). The sterilization of the media adjusted at pH 2, 3 and 4 was carried out by autoclaving the agar separately from the rest of components in order to avoid solidification problems. The pH of each media was checked after sterilization. Each plate was point inoculated with 1 μl of the adjusted suspension. Plates were incubated at 25ºC. Each assay was performed in duplicate.

OTA production and quantification

OTA production was analysed after 5, 10, 15, 20 and 30 days of incubation at each pH value following a previously described high-pressure liquid chromatography (HPLC) screening method (Bragulat et al. 2001), being efficient at the range of pH tested. On each sampling occasion, three agar plugs were removed from different points of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. OTA detection and quantification was made by a Waters LCM1 chromatograph with a fluorescence detector Waters 2475 (excitation wavelength: 330 nm/emission wavelength: 460 nm). Twenty μl of each extract were applied. The mobile phase, with a flow rate of 1 ml/min, consisted of the following isocratic program: acetonitrile, 57%; water, 41% and acetic acid, 2% (Bauer and Gareis 1987). The extracts with the same retention time as OTA (around 6.8 min), were considered positive. Confirmation was made through derivatization of OTA in its methyl-ester (Hunt et al. 1980). The detection limit of the extraction procedure and the HPLC

technique was 0.02 ng OTA and the quantification limit of HPLC technique with the extraction procedure was 0.01 μg g^{-1} for this mycotoxin.

Data analysis

Data obtained were analyzed statistically by means of the one-way analysis of variance test and Student's test. All statistical analyses were performed using SPSS software (Version 12.0).

Results and discussion

The twelve strains grew in CYA and YES media adjusted from pH 2 to10. According to our results, the pH range for growth in *Aspergillus niger* was previously reported to be from 1.4 to 9.8 (Panasenko 1967).

Six out of the 12 strains tested produced detectable levels of OTA in both media. Tables II and III show the concentration detected at each pH value and incubation time in CYA and YES medium respectively. In CYA medium most of the strains produced OTA at all the pH values tested. The strain CBS 618.78 produced OTA from pH 4 to 8 and A-943 was a weak OTA-producing strain with detected values very close to the detection limit of the technique at some pH values. In YES medium, the strains produced OTA from pH 2 (strains A-942, A-943 and CBS 139.52) or pH 3 (strains A-75 and A-136) to pH 10. However, this range was lower for the strain CBS 618.78 which produced OTA from pH 5 to 8.

Mean OTA concentration produced at each pH value by the six positive strains is shown in Figure 1. The concentration achieved by all the strains in YES was higher than in CYA medium except at pH 3 (Figure 1). At this pH value the strains showed a slight higher OTA production in CYA medium. The total mean OTA concentration produced in YES medium (4.42 μg g^{-1}) was significantly higher (Student's t $P<0.01$) than in CYA medium (1.38 μg g^{-1}) which supports that YES

is better than CYA medium for OTA production. This result was in accordance with previous studies which reported the suitability of YES medium to test OTA production by *A. niger* aggregate strains (Bragulat et al. 2001; Esteban et al. 2004). On the contrary, CYA medium has been reported to be more suitable than YES medium in *A. carbonarius* (Bragulat et al. 2001; Esteban et al. 2004; Esteban et al. 2005).

In both media most of the strains produced OTA after only 5-10 days of incubation and showed different optimal values for OTA production. In CYA medium (Table 2) the strains showed the optimal OTA production within the range pH 3-8. In YES medium (Table 3) the optimal conditions for OTA production were reached within the range pH 5-10 and always after 5-10 days of incubation. In both media, the strain A-943, isolated from grapes, showed the lowest OTA production in comparison with the rest of strains studied.

In accordance with previous studies (Esteban et al. 2004) the OTA content decreased when increasing incubation time. It has been suggested that this may be due to the fact that the strains remove and assimilate the phenylalanine moiety from the OTA molecule as other nitrogen sources become exhausted (Téren et al. 1996).

It is not known yet why the percentage of OTA-producing strains in the *A. niger* aggregate is low (Abarca et al. 2004), nor if this is due to a genetic based feature. To clarify this fact it is necessary to study the effect of different environmental conditions on OTA production. In this study, the six strains initially considered as OTA negative did not produce the mycotoxin at any of the pH values tested for the period of 30 days. In a previous study, these six strains did not produce OTA at any of the temperatures tested (Esteban et al. 2004). The strains with the RFLP pattern of type T did not produce OTA in the range of pH values assayed. To date OTA production has never been demonstrated in type T strains (Accensi et al. 2001; Cabañes et al. 2002; Abarca et al. 2003; Leong et al. 2004; Esteban et al. 2004).

There are not previous works regarding the effect of pH on OTA production by *A. niger* aggregate strains. Nevertheless, in the case of *A. carbonarius* it has been recently reported that the strains are

able to produce OTA in a broad pH range (pH 2 to 10) (Esteban et al. 2005). The results obtained here with the *A. niger* aggregate strains state that they are also able to grow at a wide range of pH values (pH 2-10). This would explain why *A. niger* is so commonly isolated from a great variety of food commodities and is considered the most common *Aspergillus* species responsible for the postharvest decay of fresh fruit (Pitt and Hocking 1997). The black aspergilli belonging to the *A. niger* aggregate are the predominant *Aspergillus* species isolated from grapes. They are isolated along the different developmental stages of berries although it is at harvesting when the highest levels are observed (Battilani et al. 2003; Serra et al. 2003; Bau et al. 2005). The black spores provide protection from sunlight and UV light, providing a competitive advantage in such habitats (Pitt and Hocking 1997). It is worth to mention that the pH of grapes increases during ripening, achieving a final pH range of 3-4 (Splittstoesser 1987; FDA 2003). *Aspergillus niger* has been also reported to be the predominant fungi in dried vine fruits (Heenan et al. 1998; Abarca et al. 2003) which show pH ranges of 3.8 to 4.1 (FDA 2003). *Aspergillus niger* aggregate strains have been also isolated from different stages of coffee beans, before and after harvesting (Taniwaki et al. 2003). The pH of unfermented coffee beans can range from 5.4 to 6.4 (Thompson et al. 2001) and therefore, the time before the arrival of the cherries at the factory may represent a high risk for OTA production (Bucheli and Taniwaki 2002). The remarked conditions could support growth and also the production of OTA on these substrates.

It is worth noting that according to our results *A. niger* aggregate strains can grow and produce OTA in a wide pH range. Moreover significant amounts of OTA can be produced at only 5 days. This would lead to confirm their contribution in the OTA contamination of substrates, either in the field or after harvesting, due to the presence of the proper environmental conditions for the production of the mycotoxin.

Further studies on the effect of environmental factors on growth and OTA production are necessary. A better knowledge of the ecology of these species will provide us with management strategies to reduce the OTA contamination of the substrates where they are usually isolated.

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Table I. *Aspergillus niger* aggregate strains used in this study.

Species	Strain (source)[a]	OTA production reported (Ref.)	RFLP pattern (Ref.)
A. niger var. *niger*	A-75 (feedstuffs, CCFVB)	+ (Abarca et al. 1994; Esteban et al. 2004)	N (Accensi et al. 2001)
A. niger var. *niger*	A-136 (soy, CCFVB)	+ (Abarca et al. 1994; Esteban et al. 2004)	N (Accensi et al. 2001)
A. niger var. *niger*	A-942 (raisins, CCFVB)	+ (Abarca et al. 2003; Esteban et al. 2004)	N (Abarca et al. 2003)
A. niger var. *niger*	A-943 (grapes, CCFVB)	+ (Esteban et al. 2004)	N (Esteban et al. 2004)
A. awamori	CBS 139.52 (kuro-koji)	+ (Ono et al. 1995; Esteban et al. 2004)	N (Accensi et al. 2001)
A. foetidus	CBS 618.78 (unknown)	+ (Téren et al. 1996; Esteban et al. 2004)	N (Accensi et al. 2001)
A. niger	CECT 2088 (unknown)	- (Varga et al. 2000; Esteban et al. 2004)	N (Esteban et al. 2004)
A. niger	CBS 554.65 (tannin-gallic acid fermentation)	- (Accensi et al. 2001; Esteban et al. 2004)	N (Accensi et al. 2001)
A. niger var. *niger*	A-946 (coffee, CCFVB)	- (Esteban et al. 2004)	N (Esteban et al. 2004)
A. niger	CBS 121.55 (otomycosis)	- (Esteban et al. 2004)	N (Esteban et al. 2004)
A. tubingensis	CBS 134.48 (unknown)	- (Accensi et al. 2001; Esteban et al. 2004)	T (Accensi et al. 2001)
A. niger var. *niger*	A-947 (grapes, CCFVB)	- (Cabañes et al. 2002; Esteban et al. 2004)	T (Cabañes et al. 2002)

[a] Abbreviations: CFFVB, Culture Collection of Veterinary Faculty of Barcelona, Bellaterra (Barcelona), Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CECT, Colección Española de Cultivos Tipo, Valencia, Spain.

Table II. OTA concentration (mean and mean standard error) produced by the six OTA-producing strains of *A. niger* aggregate in CYA medium at 25ºC at each pH value tested.

		OTA concentration(μg/g)								
	days	2	3	4	5	6	7	8	9	10
A-75	5	nd [a]	1.78±1.02 [a]	1.92±1.12 [a]	7.82±0.55 [b]	1.47±0.27 [a]	0.74±0.07 [a]	1.33±0.77 [a]	0.93±0.42 [a]	0.64±0.16 [a]
	10	1.70±0.49 [a]	1.57±0.66 [a]	1.59±0.49 [a]	1.19±0.03 [a]	0.82±0.13 [a]	0.69±0.08 [a]	0.79±0.23 [a]	0.39±0.03 [a]	0.41±0.06 [a]
	15	0.30±0.06 [a]	3.09±0.89 [b]	1.38±0.08 [a]	1.10±0.31 [a]	0.89±0.01 [a]	0.81±0.01 [a]	0.89±0.06 [a]	0.35±0.05 [a]	0.21±0 [a]
	20	0.57±0 [a]	1.09±1.01 [a]	1.44±0.07 [a]	0.41±0.06 [a]	0.92±0.02 [a]	0.62±0.27 [a]	1.00±0.30 [a]	0.32±0.04 [a]	0.31±0.01 [a]
	30	0.18±0.05 [a]	1.22±1.20 [a]	1.55±0.04 [a]	0.95±0.60 [a]	1.21±0.91 [a]	0.61±0.13 [a]	0.87±0.13 [a]	0.45±0.08 [a]	0.21±0.06 [a]
A-136	5	0.03±0.04 [a]	4.00±1.13 [b]	2.79±0.35 [ab]	9.31±2.12 [c]	2.44±0.47 [ab]	1.92±0.82 [ab]	1.54±0.20 [ab]	1.50±0.13 [ab]	1.67±0.12 [ab]
	10	3.68±0.71 [a]	2.93±0.20 [a]	1.98±0.03 [b]	2.01±0.57 [b]	0.77±0.23 [c]	1.17±0.03 [bc]	0.90±0.21 [c]	0.43±0.19 [c]	0.58±0.06 [c]
	15	1.14±0.28 [a]	4.16±0.54 [b]	2.00±0.05 [c]	1.83±0.28 [c]	1.06±0.12 [a]	0.67±0 [a]	0.69±0.27 [a]	0.54±0.26 [a]	0.51±0.11 [a]
	20	1.50±1.08 [ab]	2.86±0.19 [c]	1.98±0.09 [bc]	0.71±0.06 [ab]	1.09±0.01 [ab]	1.02±0.01 [ab]	0.82±0.25 [ab]	0.50±0.27 [a]	0.46±0.17 [a]
	30	1.24±0.71 [ab]	2.80±0.05 [c]	1.80±0.20 [b]	1.67±0.31 [ab]	1.41±0.78 [ab]	0.81±0.18 [ab]	0.59±0.13 [ab]	0.33±0.09 [a]	0.30±0.06 [a]
A-942	5	0.42±0.18 [a]	7.24±1.66 [b]	4.23±0.43 [c]	3.86±2.28 [ac]	1.21±0.04 [ac]	0.88±0.09 [ac]	1.32±0.34 [ac]	0.99±0.31 [ac]	0.44±0.14 [a]
	10	3.51±1.84 [ab]	4.90±0.66 [b]	2.91±0.64 [ac]	1.04±0.02 [c]	0.86±0.05 [c]	1.00±0.17 [c]	1.10±0.37 [c]	0.84±0.11 [c]	0.52±0.10 [c]
	15	0.22±0.01 [a]	8.53±0.29 [b]	1.92±0.21 [c]	0.89±0.06 [de]	1.06±0.13 [e]	0.92±0.36 [de]	1.07±0.07 [e]	0.91±0.15 [de]	0.37±0.04 [ad]
	20	0.16±0.01 [a]	7.00±0.38 [b]	2.62±0.61 [c]	1.28±0.21 [d]	1.07±0.01 [de]	0.87±0.07 [ade]	1.22±0.20 [d]	0.65±0.04 [ade]	0.31±0.06 [ae]
	30	0.36±0.01 [ab]	4.59±0.31 [c]	1.37±0.10 [d]	0.49±0.06 [ab]	0.76±0.21 [b]	0.81±0.11 [b]	1.28±0.05 [d]	0.52±0.16 [ab]	0.25±0.04 [a]
A-943	5	nd [a]	0.14±0.01 [b]	nd [a]	nd [a]	0.05±0 [c]	nd [a]	nd [a]	nd [a]	nd [a]
	10	0.10±0.01 [a]	0.26±0.06 [b]	nd [c]	nd [c]	0.03±0.04 [c]	nd [c]	nd [c]	nd [c]	nd [c]
	15	0.19±0.04 [a]	0.22±0 [a]	nd [b]	nd [b]	nd [b]	nd [b]	nd [b]	0.09±0.05 [c]	nd [b]
	20	nd [a]	0.07±0.09 [a]	nd [a]	nd [a]	0.03±0.04 [a]	nd [a]	0.06±0.01 [a]	0.08±0.04 [a]	nd [a]
	30	nd [a]	0.08±0.11 [a]	nd [a]	nd [a]	0.05±0.06 [a]	nd [a]	nd [a]	nd [a]	nd [a]
CBS 139.52	5	4.87±1.99 [ab]	10.50±0.21 [c]	1.21±1.03 [a]	3.85±0.62 [ab]	2.90±1.02 [ab]	2.06±0.41 [ab]	2.68±1.47 [ab]	5.69±1.4 [b]	3.81±0.42 [ab]
	10	14.58±2.02 [a]	7.77±2.90 [b]	0.65±0.91 [c]	2.35±0.90 [c]	1.87±0.48 [c]	1.87±0.33 [c]	2.02±0.52 [c]	3.24±1.34 [c]	3.06±0.57 [c]
	15	2.07±0.54 [ab]	8.63±1.22 [c]	0.42±0.28 [a]	1.55±0.22 [ab]	2.30±0.86 [ab]	1.95±0.25 [ab]	3.14±0.53 [b]	2.79±0.78 [b]	1.95±0.12 [ab]
	20	1.80±1.37 [a]	15.35±4.59 [b]	0.63±0.80 [a]	1.41±0.43 [a]	2.04±0.37 [a]	2.02±0.42 [a]	2.57±1.22 [a]	3.64±1.71 [a]	3.78±1.58 [a]
	30	2.14±0.59 [a]	7.69±2.23 [b]	0.39±0.55 [a]	3.01±0.37 [a]	1.76±0.02 [a]	1.56±0.18 [a]	2.56±0.50 [a]	4.16±2.79 [a]	2.82±0.15 [a]
CBS 618.78	5	nd [a]	nd [a]	nd [a]	0.12±0.01 [ab]	0.17±0.01 [b]	0.28±0.12 [c]	0.08±0.01 [ab]	nd [a]	nd [a]
	10	nd [a]	nd [a]	nd [a]	1.04±0.50 [a]	0.25±0.09 [a]	0.52±0.39 [a]	1.84±2.47 [a]	nd [a]	nd [a]
	15	nd [a]	nd [a]	nd [a]	0.68±0.15 [b]	0.73±0.14 [b]	0.78±0.34 [b]	0.46±0.35 [ab]	nd [a]	nd [a]
	20	nd [a]	nd [a]	nd [a]	0.07±0.02 [a]	0.49±0.11 [b]	0.55±0.03 [b]	0.25±0.20 [a]	nd [a]	nd [a]
	30	nd [a]	nd [a]	0.05±0.06 [a]	0.96±0.56 [b]	0.27±0.06 [a]	0.31±0.09 [a]	0.04±0.06 [a]	nd [a]	nd [a]

[a,b,c,d,e] Values with the same superscript within each strain and incubation time are not significantly different ($p < 0.05$); nd: not detected (limit of detection 0.01 μg g^{-1}).

Table III. OTA concentration (mean and mean standard error) produced by the six OTA-producing strains of *A. carbonarius* in YES medium at 25ºC at each pH value tested.

		OTA concentration(μg/g)								
	days	2	3	4	5	6	7	8	9	10
A-75	5	nd [a]	0.67±0.11 [a]	7.27±0.24 [ab]	16.82±0.39 [b]	18.22±3.01 [b]	14.82±7.45 [b]	12.89±2.45 [b]	15.46±3.79 [b]	8.46±0.52 [ab]
	10	nd [a]	0.69±0.01 [a]	4.43±0.04 [abc]	4.80±1.84 [abc]	9.72±1.34 [c]	9.01±1.18 [c]	15.02±4.14 [d]	7.24±0.02 [bc]	2.54±0.35 [ab]
	15	nd [a]	0.27±0.12 [a]	2.20±0.87 [ab]	4.19±1.03 [bc]	4.75±0.45 [bc]	8.15±2.36 [d]	6.49±0.55 [cd]	3.72±1.25 [bc]	0.50±0.11 [a]
	20	nd [a]	0.52±0.15 [a]	1.81±0.37 [ab]	0.09±0.12 [a]	3.53±0.34 [b]	5.91±0.46 [c]	3.53±1.33 [b]	2.93±0.86 [b]	0.91±0.23 [a]
	30	nd [a]	0.15±0.02 [a]	0.96±0.13 [ab]	1.57±0.54 [bc]	2.24±0.62 [c]	2.65±0.26 [c]	3.56±0.78 [d]	1.60±0.21 [bc]	0.44±0.15 [ab]
A-136	5	nd [a]	5.91±0.71 [a]	26.78±4.51 [bc]	37.93±1.22 [d]	34.69±1.18 [cd]	34.66±2.08 [cd]	20.27±0.90 [be]	19.90±1.06 [be]	15.94±6.93 [e]
	10	nd [a]	0.91±0.43 [ab]	6.19±0.43 [ab]	11.55±3.92 [ab]	6.39±0.39 [ab]	6.55±0.23 [ab]	7.08±0.76 [ab]	13.98±9.57 [b]	2.62±0.21 [ab]
	15	nd [a]	1.04±0.20 [a]	2.34±0.40 [a]	1.75±1.05 [a]	5.33±1.10 [a]	3.55±2.27 [a]	6.37±5.10 [a]	6.15±0.26 [a]	0.29±0.01 [a]
	20	nd [a]	0.39±0.13 [a]	1.56±0.32 [ab]	0.18±0.10 [a]	1.54±0.49 [ab]	3.84±1.77 [b]	1.83±0.91 [ab]	3.43±0.16 [b]	0.69±0.37 [a]
	30	nd [a]	0.22±0.03 [ab]	0.73±0 [ab]	0.69±0.27 [ab]	2.44±0.71[c]	1.52±0.35 [bc]	1.02±0.28 [ab]	2.45±0.69 [c]	0.28±0.06 [ab]
A-942	5	nd [a]	2.06±0.47 [ab]	10.89±1.87 [bcd]	5.38±1.09 [abc]	12.90±6.56 [cd]	11.66±2.36 [bcd]	5.49±0.90 [abc]	18.23±1.44 [d]	11.38±3.59 [bcd]
	10	3.25± 3.00 [a]	0.15±0.03 [a]	2.29±1.39 [a]	1.32±0.38 [a]	2.34±0.08 [a]	2.85±0.73 [a]	1.26±0.07 [a]	4.97±1.54 [a]	5.05±2.62 [a]
	15	1.18±0 .44 [abc]	0.33±0.18 [a]	0.89±0.38 [ab]	0.58±0.02 [a]	1.91±0.42 [c]	1.54±0.13 [bc]	2.86±0.05 [d]	0.39±0.02 [a]	1.70±0.19 [bc]
	20	1.48±1.57 [a]	nd [a]	0.61±0.04 [a]	0.35±0.18 [a]	1.29±0.02 [a]	1.19±0.50 [a]	1.00±0.18 [a]	1.13±0.39 [a]	0.87±0.19 [a]
	30	0.39±0.26 [a]	0.14±0.01 [a]	0.33±0.02 [a]	0.36±0.11 [a]	1.25±0.54 [a]	0.76±0.08 [a]	1.01±0.04 [a]	0.84±0.20 [a]	1.27±0.64 [a]
A-943	5	nd [a]	nd [a]	0.23±0.09 [a]	0.12±0.01 [a]	0.16±0.05 [a]	0.16±0.01 [a]	nd [a]	0.57±0 [a]	1.39±0.48 [b]
	10	0.11±0.15 [a]	0.18± 0.26 [a]	nd [a]	nd [a]	0.15±0.02 [a]	nd [a]	nd [a]	0.20±0.06 [a]	0.13±0 [a]
	15	0.12±0.02 [a]	0.17±0.24 [a]	nd [a]	nd [a]	nd [a]	nd [a]	nd [a]	0.07±0.03 [a]	nd [a]
	20	nd [a]	0.12±0.16 [a]	nd [a]	nd [a]	nd [a]	nd [a]	0.08± 0.01 [a]	0.09±0 [a]	nd [a]
	30	nd [a]	0.09±0.12 [a]	nd [a]	nd [a]	nd [a]	nd [a]	nd [a]	nd [a]	nd [a]
CBS 139.52	5	0.03±0.04 [a]	16.82±3.14 [bc]	17.37±7.70 [bc]	58.00±1.34 [d]	10.25±7.95 [abc]	14.74±0.28 [bc]	19.65±1.80 [c]	19.03±7.65 [c]	1.88±0.23 [ab]
	10	0.12±0.16 [a]	3.58±0.75 [a]	1.11±0.79 [a]	17.12±5.13 [a]	9.54±10.88 [a]	11.97±9.62 [a]	16.96±4.77 [a]	42.27±4.59 [b]	18.74±3.28 [a]
	15	nd [a]	3.43±3.29 [a]	9.60±5.33 [a]	16.38±4.24 [a]	13.69±2.76 [a]	9.05±4.79 [a]	8.92±5.58 [a]	7.87±9.38 [a]	5.73±0.86 [a]
	20	nd [a]	4.61±0.70 [a]	4.14±2.49 [a]	4.88±2.61 [a]	10.01±0.40 [a]	12.56±1.92 [a]	12.43±4.50 [a]	17.98±17.78 [a]	5.88±1.21 [a]
	30	nd [a]	2.70±0.08 [a]	3.29±0.02 [a]	11.66±1.34 [a]	6.46±1.87 [a]	7.66±0.70 [a]	6.03±4.05 [a]	11.59±12.43 [a]	2.32±0.11 [a]
CBS 618.78	5	nd [a]	nd [a]	nd [a]	24.56±15.90 [b]	5.49±7.50 [a]	0.25±035 [a]	4.12±2.01 [a]	nd [a]	nd [a]
	10	nd [a]	nd [a]	nd [a]	1.17±0.55 [a]	0.95±0.59 [a]	0.21±0.29 [a]	30.56±18.72 [b]	nd [a]	nd [a]
	15	nd [a]	nd [a]	nd [a]	nd [a]	1.63±2.22 [a]	0.40±0.02 [a]	0.95±0.08 [a]	nd [a]	nd [a]
	20	nd [a]	nd [a]	nd [a]	nd [a]	0.12±0 [a]	0.99±0.059 [b]	1.21±0.04 [b]	nd [a]	nd [a]
	30	nd [a]	nd [a]	nd [a]	nd [a]	0.51±0.06 [b]	0.63±0.03 [c]	0.10±0.07 [a]	nd [a]	nd [a]

[a,b,c,d,e] Values with the same superscript within each strain and incubation time are not significantly different ($p < 0.05$); nd: not detected (limit of detection 0.01 μg g^{-1}).

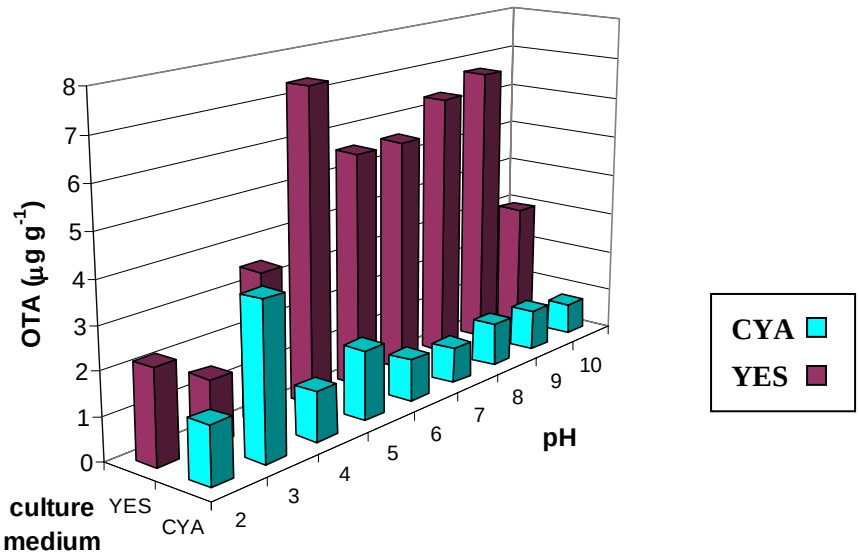

Fig.1. Mean OTA concentration produced by the OTA-producing strains at each pH value and culture media tested.

3.5 Influence of pH and incubation time on ochratoxin A production by *Aspergillus carbonarius* in culture media. Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J. ***Journal of Food Protection,* 68 (2005) 1435-1440.**

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Influence of pH and Incubation Time on Ochratoxin A Production by *Aspergillus carbonarius* in Culture Media

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ABSTRACT

The effect of pH (2 to 10) and temperature (15 and 30°C) on growth and production of ochratoxin A (OTA) of six strains of *Aspergillus carbonarius* was studied in two culture media: Czapek yeast autolysate agar and yeast extract sucrose agar. Isolates were selected by their different source and different reported ability to produce OTA. Regardless of the initial pH or the temperature tested, Czapek yeast autolysate agar has been shown to be the best culture medium for OTA production by *A. carbonarius.* In this medium, OTA was produced from pH 2 to 10 at the two incubation temperatures tested. The results obtained show the ability of *A. carbonarius* to not only grow but also produce OTA over a wide pH range at high or low temperatures. This may help explain why this species is considered the main OTA source in some substrata.

Ochratoxin A (OTA) is a mycotoxin of growing importance in the last decade. The International Agency for Research on Cancer has rated OTA as a possible human carcinogen (category 2B). Although the role of OTA in human disease is still speculative, its acute nephrotoxicity, immunosuppressive actions, and teratogenic effects in animal models, coupled with its ability to be carried through the food chain, merit concern *(10, 18).* The European Commission has established limits for OTA of 10 μg/kg in dried vine fruit, 5 μg/kg in raw cereals, 3 μg/kg in cereal-derived products, 5 μg/kg for roasted coffee, 10 μg/kg for instant coffee, 2 μg/kg for wine, grape juice, and grape must, and 0.5 μg/kg for foods for infants and young children. By 30 June 2006, the European Commission will review these limits and consider the inclusion of maximum levels for OTA in green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices, and liquorice *(15–17).*

Although currently *Aspergillus ochraceus* and *Penicillium verrucosum* are considered typical OTA-producing species, they are unlikely to be significant sources of OTA in some substrates. Since the first description of OTA production by two species belonging to *Aspergillus* section *Nigri* (*Aspergillus niger* var. *niger (4)* and *Aspergillus carbonarius (20)*), the significance of these species as mycotoxin-producing fungi has increased *(1).* Recent surveys have shown that *A. carbonarius* is the main OTA source in wine, grapes, dried vine fruits, and probably coffee *(3).*

To prevent OTA contamination, it is necessary to determine the influence of environmental parameters on mycotoxin production. The effect of water activity *(9, 22),* temperature *(19, 24),* and incubation time *(32, 35)* on OTA production by some isolates of *A. carbonarius* have been recently reported. In one such study *(19),* we stated that *A. carbonarius* strains produced OTA from 15 to 35°C, achieving the highest levels in Czapek yeast extract agar (CYA) at 15 or 20°C. Similar results have been reported in synthetic grape juice medium *(24).* Because nothing is known about the effect of pH, the aim of this study is to determine the influence of pH on OTA production by *A. carbonarius* at two incubation temperatures (15 and 30°C) during a period of 30 days.

MATERIALS AND METHODS

Strains and preparation of inoculum. Six isolates of *A. carbonarius* were used in this study. Isolates were selected by their different source and different reported ability to produce OTA. The origin and OTA properties of the six strains studied are given in Table 1.

The strains were grown on malt extract agar *(28)* for 7 days at 25°C. Conidia suspensions of each isolate were prepared in an aqueous solution of 0.05% Tween 80. After filtering through sterile cheesecloth, they were adjusted to approximately 10^6 to 10^7/ml of conidia as determined by a counting chamber.

Growth media and incubation conditions. OTA production was determined in two basal culture media: yeast extract sucrose (YES) agar and CYA *(28).* The pH of the culture media was varied from 2 to 10. Adjustment of pH was performed before autoclaving by adding HCl (1 and 10 N) and NaOH (1 N). The pH value was measured using a pHmeter GLP21 (Crison Strumenti S.p.A., Carpi, Italy). Each plate was point inoculated with 1 μl of the adjusted suspension. Plates were incubated at two different temperatures: 15 and 30°C. Each assay was performed in duplicate.

OTA production and quantification. OTA production was analyzed after 5, 10, 15, 20, and 30 days of incubation at each pH value and temperature assayed following a previously described high-pressure liquid chromatography (HPLC) screening method *(11).* On each sampling occasion, three agar plugs were

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TABLE 1. Aspergillus carbonarius *strains used in this study*

Strain (source)[a]	OTA production reported (reference)[b]
A. carbonarius NRRL 67 (Brazil)	+ *(32)*
A. carbonarius CBS 127.49 (coffee)	+ *(22)*
A. carbonarius A-941 (grapes, CCFVB)	+ *(13)*
A. carbonarius M325 (apples, supplied by HMLJ Joosten)	− *(22)*
A. carbonarius CBS 110.49 (air)	+ *(22)*
A. carbonarius A-1082 (raisins, CCFVB)	− *(2)*

[a] CCFVB, Culture Collection of Veterinary Faculty of Barcelona, Bellaterra (Barcelona), Spain; CBS, Centralbalbureau voor Schimmelcultures, Utrecht, The Netherlands; and NRRL, Northern Agricultural Research Service Culture Collection, Peoria, Ill.
[b] +, positive; −, negative.

removed from different points of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. OTA detection and quantification were performed with a Waters LCM1 chromatograph (Waters Cromatografia, Barcelona, Spain) with a Waters 2475 fluorescence detector (excitation wavelength, 330 nm; emission wavelength, 460 nm) and with a C18 Spherisorb S5 ODS2 column (250 by 4.6 mm). Twenty microliters of each extract was applied. The mobile phase, with a flow rate of 1 ml/min, consisted of the following linear gradient: acetonitrile, 57%; water, 41%; and acetic acid, 2% *(8)*. The extracts with the same retention time as OTA (approximately 6.8 min) were considered positive. Confirmation was made through derivatization of OTA in its methyl-ester *(21)*. The detection limit of the extraction procedure and the HPLC technique was 0.02 ng of OTA, and the quantification limit of the HPLC technique with the extraction procedure was 0.05 μg/g for this mycotoxin.

Data analysis. Data obtained were analyzed statistically by means of one-way analysis of variance test and Student's *t* test. All statistical analyses were performed using SPSS software (version 10.0; SPSS Inc, Chicago, Ill.).

RESULTS AND DISCUSSION

All the studied strains grew in CYA and YES media adjusted from pH 2 to 10 and incubated at 15 and 30°C with only one exception: the strain CBS 110.49 did not grow in CYA medium adjusted at pH 10 when the incubation was performed at 15°C. The growth pH range for *A. carbonarius* has not been previously reported. Nevertheless, as expected, our results show a broad similarity to *A. niger* with established pH limits of 1.5 to 9.8 *(26)*.

Two of the six strains tested (CBS 110.49 and A-1082) did not produce detectable levels of OTA at any of the experimental conditions assayed. The strain CBS 110.49 was reported as a very weak OTA-producing strain, using coffee cherries as substrate *(22)*. The OTA-negative strain A-1082, isolated from dried vine fruits in a previous study *(2)*, is now under study, because its morphological and genetic characteristics differ from the remaining *A. carbonarius* strains studied *(7, 14)*.

In CYA medium, OTA was produced from pH 2 to 10 at the two incubation temperatures tested. In YES medium, the pH range for OTA production was sometimes narrower. Thus, at 15°C OTA production began at pH 3. At 30°C, the pH ranges were 2 to 10 (strains A-941 and M325), 3 to 9 (strain NRRL 67), and 3 to 10 (strain CBS 127.49).

Although the pH range for OTA production was similar in both media, the concentration produced in each medium was statistically different. The mean OTA concentration produced by the positive isolates in CYA medium (16.0 μg/g at 30°C and 19.2 μg/g at 15°C) was significantly higher ($P < 0.01$) than in YES medium (1.63 μg/g at 30°C and 2.69 μg/g at 15°C). So, regardless of the initial pH or the temperature tested, CYA has been shown to be the best culture medium for OTA production by *A. carbonarius* in agreement with previous reports *(11, 19)*.

OTA concentrations produced in CYA medium at each pH level tested and incubation time are given in Tables 2 (incubation temperature of 30°C) and 3 (incubation temperature of 15°C). Table 4 summarizes the maximum OTA level produced under the different assay conditions studied.

When CYA plates were incubated at 30°C, all the strains produced detectable levels of OTA after only five days of incubation, except for the strain NRRL 67 at pH 5 (Table 2). The optimum pH range for OTA production proved different for each strain. The strain A-941 isolated from grapes achieved a significantly higher OTA level at pH 2. In the same way, the strain M325 isolated from apples, which is a weak OTA-producing strain, produced the highest level after 5 days of incubation at pH 2. This strain was reported as OTA negative when coffee cherries were used as substrate *(22)*. The strain CBS 127.49 produced maximum OTA concentration in the pH range 4 to 7, and the strain NRRL 67 produced similar OTA levels at all the pH ranges tested.

When the plates were incubated at 15°C, OTA was detected after 10, 15, or 20 days of incubation, depending on the pH level tested (Table 3). The strains showed a similar trend to that observed at 30°C, but the highest OTA levels were obtained generally at a higher pH range (5 to 7). Nevertheless, the strain A-941 achieved the highest OTA level at pH 2 but after 30 days of incubation.

Incubation temperature also played an important role in the OTA concentration produced at the different pH levels tested. Although 30°C has been reported as an optimal growth temperature for *A. carbonarius (9, 23, 24)*, the optimum temperature range for OTA production in CYA medium has been established at 15 to 20°C *(19)*. When the plates were incubated at 15°C, OTA concentrations produced by the strains NRRL 67 and M-325 were significantly higher ($P < 0.01$). The strain A-941 also produced higher amounts of OTA at 15°C in the pH range of 3 to 10, but at pH 2 OTA concentration produced at 30°C was higher. The strain CBS 127.49 produced more OTA when incubated at 30°C ($P < 0.05$).

Our results show that *A. carbonarius* isolates are able to produce OTA at a wide range of pH values (2 to 10) on CYA medium, whereas other OTA-producing species such as *A. ochraceus* did not produce OTA outside the range of pH 5.5 to 8.5 on modified Adye-Mateles synthetic medium *(25)*. *P. verrucosum* was reported to produce OTA from pH

TABLE 2. *OTA concentration produced by the four OTA-producing strains of* A. carbonarius *in CYA medium at 30°C at each pH (2 to 10) and incubation time tested*

		OTA concentration (μg/g)[a]								
Strain	Days	2	3	4	5	6	7	8	9	10
NRRL 67	5	7.79 ± 2.23 AB	11.49 ± 3.80 B	1.66 ± 0 CD	ND	7.24 ± 0.41 ABC	8.43 ± 2.27 AB	5.63 ± 0.47 ACD	4.36 ± 0.28 ACD	2.66 ± 1.16 ACD
	10	11.21 ± 1.02 A	7.47 ± 0.32 BC	7.63 ± 0.20 BC	8.77 ± 2.19 AB	9.19 ± 1.03 AB	5.79 ± 0.63 BC	7.20 ± 0.88 BC	4.20 ± 0.59 C	4.34 ± 0.84 C
	15	4.16 ± 2.75 A	8.47 ± 1.78 A	10.54 ± 0.95 A	6.62 ± 0.10 A	8.29 ± 0.66 A	7.20 ± 2.50 A	5.97 ± 0.38 A	6.72 ± 5.16A	3.66 ± 1.17 A
	20	2.31 ± 0.22 A	8.11 ± 0.10 C	9.34 ± 3.68 C	16.52 ± 0.46 D	7.72 ± 0.07 BC	7.27 ± 1.69 BC	5.76 ± 1.40 ABC	7.29 ± 0.42 BC	2.92 ± 0.23 AB
	30	3.21 ± 2.55 A	6.16 ± 0.91 A	13.62 ± 2.93 B	20.45 ± 0.51 C	7.70 ± 1.18 A	9.34 ± 3.27 AB	6.64 ± 0.79 A	7.63 ± 0.88 A	5.08 ± 1.65 A
CBS 127.49	5	20.34 ± 2.06 A	47.73 ± 15.70 B	121.22 ± 11.22 C	18.29 ± 2.63 A	17.32 ± 2.98 A	10.08 ± 1.53 A	8.14 ± 0.43 A	8.51 ± 3.27 A	14.18 ± 3.25 A
	10	21.32 ± 1.48 A	36.63 ± 11.78 C	28.78 ± 5.94 BC	17.32 ± 0.94 AB	25.23 ± 6.87 ABC	15.21 ± 0.47 AB	12.02 ± 0.37 AB	6.68 ± 0.58 A	14.12 ± 3.46 AB
	15	14.85 ± 5.54 A	34.80 ± 8.56 AB	42.74 ± 8.87 B	42.99 ± 16.60 B	18.34 ± 2.94 A	16.57 ± 4.04 A	14.68 ± 1.25 A	10.72 ± 2.35 A	8.75 ± 0.67 A
	20	7.45 ± 3.04 A	27.50 ± 10.24 A	78.95 ± 31.90 B	29.72 ± 5.61 A	26.89 ± 0.59 A	35.60 ± 10.48 A	12.00 ± 1.06 A	19.02 ± 9.07 A	9.02 ± 0.52 A
	30	2.61 ± 2.51 A	22.42 ± 20.72 AB	44.62 ± 13.02 BC	61.13 ± 0.20 C	24.34 ± 2.79 AB	204.52 ± 13.19 D	17.01 ± 2.33 AB	25.22 ± 3.51 AB	12.68 ± 1.66 AB
A-941	5	158.74 ± 7.06 A	7.13 ± 1.23 B	1.85 ± 0.30 B	3.04 ± 1.61 B	1.21 ± 0.10 B	1.59 ± 0.33 B	3.11 ± 0.96 B	3.00 ± 0.98 B	0.63 ± 0.01 B
	10	230.33 ± 2.35 A	6.55 ± 2.35 B	3.51 ± 0.18 C	1.60 ± 0.16 C	1.18 ± 0.23 C	1.12 ± 0.33 C	1.96 ± 0.44 C	1.15 ± 0.02 C	0.74 ± 0.06 C
	15	289.99 ± 65.53 A	4.86 ± 1.14 B	2.62 ± 0.23 B	1.72 ± 0.11 B	1.62 ± 0.18 B	1.07 ± 0.06 B	2.84 ± 1.58 B	0.93 ± 0.27B	0.41 ± 0.03 B
	20	157.62 ± 32.17 A	4.83 ± 1.47 B	2.13 ± 0.98 B	4.87 ± 1.10 B	1.38 ± 0.22 B	1.79 ± 0.91 B	2.40 ± 0.83 B	1.55 ± 0.10 B	0.52 ± 0.04 B
	30	151.24 ± 2.75 A	5.20 ± 0.47 BC	2.74 ± 0 BCD	5.55 ± 0.22 B	1.46 ± 0.54 D	2.09 ± 0.39 CD	2.85 ± 0.95 CD	1.10 ± 0.12 D	0.78 ± 0.27 D
M325	5	25.35 ± 6.54 A	3.18 ± 0.10 B	4.32 ± 1.17 B	4.16 ± 0.01 B	1.09 ± 0.10 B	1.43 ± 0.33 B	2.53 ± 1.04 B	1.37 ± 0.57 B	0.58 ± 0.22 B
	10	7.25 ± 0.48 A	4.89 ± 1.44 AB	7.22 ± 3.18 A	2.74 ± 0.01 B	1.39 ± 0.43 B	1.02 ± 0.34 B	1.19 ± 0.08 B	1.04 ± 0.23 B	1.12 ± 0 B
	15	4.23 ± 0.61 A	6.00 ± 1.00 B	5.20 ± 0.12 AB	4.84 ± 0.13 AB	1.83 ± 0.76 C	1.07 ± 0.03 C	1.69 ± 0.15 C	0.82 ± 0.23 C	0.85 ± 0.28 C
	20	2.69 ± 1.22 AB	4.10 ± 0.30 B	7.37 ± 0.42 C	3.45 ± 0.47 B	1.54 ± 0.50 AD	1.50 ± 0.16 AD	2.81 ± 0.36 AB	1.04 ± 0.11 D	0.64 ± 0.20 D
	30	1.67 ± 1.07 A	6.59 ± 2.64 AB	8.42 ± 1.94 B	14.92 ± 4.04 C	1.70 ± 0.54 A	3.13 ± 0.80 A	2.13 ± 0.49 A	0.98 ± 0.37 A	1.36 ± 0.18 A

[a] Values are mean ± 1 standard error. Values with the same letter within each strain and incubation time are not significantly different ($P < 0.05$). ND, not detected (limit of detection, 0.05 μg/g).

TABLE 3. *OTA concentration produced by the four OTA-producing strains of* A. carbonarius *in CYA medium at 15°C at each pH (2 to 10) and incubation time tested*

Strain	Days	OTA concentration (μg/g)[a]								
		2	3	4	5	6	7	8	9	10
NRRL 67	5	ND	ND	ND	ND	ND	ND	NG	NG	NG
	10	ND	7.44 ± 0.36 A	14.38 ± 10.08 AB	21.40 ± 2.55 B	23.59 ± 4.31 B	5.38 ± 1.36 A	ND	ND	NG
	15	0.63 ± 0.69 A	22.85 ± 0.42 AB	21.52 ± 4.73 AB	45.23 ± 9.97 B	29.50 ± 3.34 B	24.51 ± 18.41 AB	24.12 ± 5.42 AB	ND	ND
	20	7.97 ± 2.07 A	23.31 ± 9.02 B	39.63 ± 2.98 B	35.30 ± 11.45 B	36.86 ± 0.23 B	41.85 ± 6.98 B	35.69 ± 1.18 B	4.30 ± 0.86 A	0.25 ± 0.12 A
	30	12.27 ± 0.30 A	19.65 ± 2.83 A	23.87 ± 3.13 AB	29.97 ± 3.92 AB	43.02 ± 9.88 AB	51.62 ± 21.19 B	30.25 ± 0.39 AB	28.65 ± 0.95 AB	26.51 ± 0.08 AB
CBS 127.49	5	ND	ND	ND	ND	ND	ND	ND	ND	NG
	10	ND	11.58 ± 6.87 A	12.58 ± 0.16 A	25.46 ± 1.77 BC	28.03 ± 5.10 C	16.65 ± 0.78 AB	18.81 ± 4.71 ABC	ND	ND
	15	0.22 ± 0.09 A	14.19 ± 2.82 AB	15.71 ± 4.86 AB	21.03 ± 6.59 AB	59.39 ± 30.61 AB	68.88 ± 28.96 B	43.57 ± 28.41 AB	7.99 ± 6.87 AB	1.76 ± 1.11 A
	20	2.51 ± 0.01 A	13.07 ± 4.20 A	17.10 ± 3.92 A	8.05 ± 5.49 A	38.57 ± 4.31 C	32.47 ± 9.81 BC	21.56 ± 2.47 AB	14.12 ± 4.12 A	13.12 ± 7.13 A
	30	9.42 ± 1.80 AB	10.57 ± 1.92 AB	18.79 ± 4.94 ABC	4.25 ± 4.36 A	32.19 ± 6.28 BC	39.13 ± 16.87C	20.76 ± 1.41 ABC	15.29 ± 1.22 AB	27.14 ± 0.94 ABC
A-941	5	ND	ND	ND	ND	ND	ND	ND	ND	NG
	10	ND	37.63 ± 2.90 A	17.65 ± 0.55 AB	30.53 ± 10.20 A	20.71 ± 4.08 A	39.60 ± 13.61 A	25.00 ± 0.35 A	ND	NG
	15	23.67 ± 3.28 A	51.14 ± 10.63 B	45.29 ± 0.31 B	53.84 ± 5.49 B	22.59 ± 0.94 A	45.90 ± 0.15 B	21.42 ± 1.57 A	2.30 ± 0.27 C	0.25 ± 0.17 C
	20	41.4 ± 3.14 A	39.69 ± 1.96 AB	36.91 ± 9.02 AB	32.47 ± 2.75 AB	21.37 ± 2.59 CD	28.64 ± 1.34 BC	14.85 ± 2.24 D	15.46 ± 0.04 D	15.07 ± 1.39 D
	30	101.57 ± 5.49 A	35.25 ± 16.09 B	34.69 ± 7.45 B	28.31 ± 2.35 B	23.14 ± 2.28 B	39.30 ± 6.60 B	21.07 ± 0.59 B	13.76 ± 0.16 B	20.54 ± 2.11 B
M325	5	ND	ND	ND	ND	ND	ND	ND	ND	NG
	10	ND	13.06 ± 4.28 AB	22.84 ± 0.04 BC	41.07 ± 6.28 C	34.97 ± 0.78 C	33.02 ± 16.87 C	ND	ND	NG
	15	31.24 ± 9.01 AB	38.02 ± 5.10 BC	13.32 ± 1.81 AB	46.90 ± 11.38 C	47.46 ± 9.8 C	33.30 ± 7.06 AB	25.82 ± 4.78 AB	10.65 ± 2.16 A	NG
	20	31.16 ± 1.22 A	14.43 ± 3.14 B	24.89 ± 5.06 A	31.64 ± 2.35 A	24.42 ± 0.78 A	29.89 ± 2.63 A	27.78 ± 1.37 A	12.60 ± 0.86 B	NG
	30	18.18 ± 0.67 AB	17.90 ± 4.43 AB	16.79 ± 1.53 AB	31.08 ± 7.85 BC	28.31 ± 3.13 BC	34.69 ± 10.59 C	16.10 ± 0.78 AB	14.10 ± 0.78 AB	5.39 ± 0.42 A

[a] Values are mean ± 1 standard error. Values with the same letter within each strain and incubation time are not significantly different ($P < 0.05$). ND, not detected (limit of detection, 0.05 μg/g); NG, no growth.

TABLE 4. *Maximum OTA concentration produced by each strain under the different assay conditions studied*

Strain	Maximum OTA production at 15°C				Maximum OTA production at 30°C			
	Culture media	pH	Days	Concentration (μg/g)	Culture media	pH	Days	Concentration (μg/g)
NRRL 67	CYA	7	30	51.6	CYA	5	30	20.5
	YES	6	20	9.4	YES	7	5	2.6
CBS 127.49	CYA	7	15	68.9	CYA	7	30	204.5
	YES	6	15	19.2	YES	9	5	16.2
A-941	CYA	2	30	101.6	CYA	2	15	290.0
	YES	4	30	5.4	YES	8	30	5.9
M-325	CYA	6	15	47.5	CYA	2	5	25.3
	YES	6	30	26.8	YES	4	30	7.5

4 to 8, with the greatest level at pH 5.6 *(27)* when growing on a bread analogue.

Strong evidence of the contribution of *A. carbonarius* to the OTA contamination of wine during a microvinification trial has been recently reported *(13)*. In the field, this species was recovered along the different developmental stages of berries, but the highest level of isolation was achieved at harvesting *(5, 6, 29)*. The pH of grapes increases during ripening, achieving a final pH range of 3.0 to 4.0 *(30)*, and dried vine fruits reported pH ranges of 3.8 to 4.1 *(34)*.

A. carbonarius has been isolated from coffee beans by several authors, but the time of invasion is not known. In Brazil, this species was isolated in samples from drying yard and storage on farm but not from the fruits obtained from trees *(31)*. The available data indicate that OTA is likely to be formed between the time of coffee cherry harvest and its arrival in the factory *(12)*. At that time, the pH of unfermented coffee beans can range from 5.4 to 6.4 *(33)*.

Our results show the ability of *A. carbonarius* to not only grow but also produce OTA over a wide pH range at high or low temperatures. This may help explain why this species is considered the main OTA source in some substrata. Further studies on the influence of different environmental conditions on growth and OTA production by *A. carbonarius* will lead to better knowledge of its ecology and will provide us with potential tools to reduce the risk of OTA contamination in foods.

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PRIMER NOTE

Isolation and characterization of six polymorphic microsatellite loci in *Aspergillus niger*

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Abstract

Certain strains of *Aspergillus niger* produce ochratoxin A in food and in animal feeds. Six polymorphic microsatellite markers suitable for population analysis were developed for *A. niger* through screening published sequences for microsatellite repeats. Polymorphism was evaluated for 28 isolates of *A. niger*, including toxigenic strains. Loci displayed six to 13 alleles. Investigation of cross-species amplifications with *Aspergillus carbonarius* and *Aspergillus japonicus* showed limited success.

Keywords: *Aspergillus niger*, microsatellites, ochratoxin A

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Fungi belonging to *Aspergillus* section *Nigri* are distributed worldwide and are considered common food spoilage fungi (Pitt & Hocking 1997). *Aspergillus niger* has GRAS (Generally Recognized As Safe) status (Bigelis & Lasure 1987) and is a source of extracellular enzymes and organic acids in the food industry. However, strains of *A. niger* and the related species, *Aspergillus carbonarius*, have been reported to produce ochratoxin A (OTA) and are associated with OTA contamination in grapes and grape products, and coffee (Abarca *et al*. 2004). OTA is nephrotoxic, immunosuppressive, teratogenic, and carcinogenic (JECFA 1991), and has received increasing attention worldwide because of the potential health hazard it poses to humans and animals.

Species within *Aspergillus* section *Nigri* have been studied by means of morphological and molecular criteria, however, the number and delineation of species in the *A. niger* aggregate are unclear (Abarca *et al*. 2004). A rapid method to differentiate the *A. niger* aggregate into two types: N and T, on the basis of their ITS-5.8S rDNA restriction fragment length polymorphism (RFLP) patterns is available (Accensi *et al*. 1999). Some type N strains produce OTA, but toxigenicity has not been reported for type T strains (Accensi *et al*. 2001). The development of microsatellite markers may clarify taxonomy within the *A. niger* aggregate, showing relationships among strains and possible correlations with mycotoxigenic potential, substrate, and geography.

A total of 3390 sequences of *A. niger* and *A. carbonarius* from GenBank was searched for microsatellites. The FASTA algorithm was used for searches and microsatellite motifs were used as queries. Ten loci were identified among *A. niger* sequences. Primers for polymerase chain reaction (PCR) amplification were designed from sequences flanking the microsatellites using PRIMER 3 (Whitehead Institute for Biomedical Research) and NET PRIMER (Biosoft International) software.

Twenty-eight *A. niger* isolates were grown for 3 days at 25 °C on SNA liquid medium (Nirenberg 1976) and DNA extracted using a modified FastPrep protocol (BIO101, Smith-White *et al*. 2001).

Polymorphic loci were detected by screening a subset of seven *A. niger* strains. Six of the 10 loci were found to be polymorphic (Table 1). The reverse primer from each polymorphic locus was pig-tailed (Brownstein *et al*. 1996) and 5′-labelled with a fluorophor (Biosystems) for sizing with an automated sequencer.

PCR amplifications were carried out in 20-μL volumes containing 1 × PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin), 5% glycerol,

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Table 1 PCR primer sequences, number of alleles, and size range observed for microsatellite loci in *Aspergillus niger*

Locus	Repeat motif	Primer sequence (5′–3′)	Size range (bp)	No. of alleles	*D*	H_O	GenBank Accession no.
ACNM1	$(CA)_{15}$	TCTCGACTCTGGCTCCTACC *FGTTTGCTTACTCACCGACTGGAAAA	464–509	13	0.93	0.90	AY081845
ACNM2	$(CT)_{10}$	TGCCCTTACTCTGCCTCTCT HGTTTCCATTATTCACCCTCCCTTCT	409–446	6	0.80	0.77	AX952973
ACNM3	$(CCA)_{15}$	TAACTTGCCTCCGTGGTTGT RGTTTGAGACCGGAAACATTGGAGTAG	177–215	12	0.91	0.88	BE759201
ACNM5	$(GTT)_{12}$	CGTTTTCTCGGAAGGTTTGA RGTTTGTGCGTGTTGGGGACTATCT	163–204	8	0.73	0.71	ANAJ5117
ACNM6	$(ATC)_{12}$	CGACAGCCGCATCATAGTT FGTTTCCTGCTCTTTTTGCCTTCTTT	429–458	9	0.84	0.81	AY081847
ACNM7	$(GTA)_{10}$	TGAGGGAAGGGGGTTTTATT HGTTTGATCTACGGGGGTGTTTGTC	378–468	10	0.89	0.86	ANI278532

*superscript letters indicate fluorescent labels: F, 6FAM; H, HEX, and R, ROX; *D*, numerical index of discriminatory power; and H_O, observed heterozygosity.

125 μM each dNTP, 5 pmol of each primer, and 0.5 U of *Taq* DNA polymerase (QIAGEN). Approximately 20–40 ng of DNA was used for each reaction. Amplifications were performed in a Hybaid PCR Express thermal cycler with the cycling parameters: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 54 °C (*ACNM2*, *5*, *6*, *7*) or 61 °C (*ACNM1*, *3*), and 1 min at 72 °C, followed by another 10 min at 72 °C.

Five of the six polymorphic loci produced amplicons from a majority of 28 *A. niger* strains, and displayed six to 13 alleles (Table 1). *ACNM6* produced amplicons from only type N *A. niger* strains, however, this included both toxigenic and nontoxigenic strains. The relatively high numerical indices of discriminatory power (Hunter 1991) and observed heterozygosities (Nei 1978) demonstrated diversity within the *A. niger* aggregate.

All polymorphic loci were tested for cross-species amplification in *Aspergillus japonicus* and the highly ochratoxigenic species *A. carbonarius*. *ACNM1* and *ACNM3* produced amplicons from *A. japonicus*, however, double bands were present in *ACNM3*. *ACNM1*, *2*, *3*, *5* and *7* produced amplicons from certain *A. carbonarius* strains, but consistent results could not be achieved, and *ACNM3* again produced double bands. Adjustments to primer design and PCR optimization would be required for cross-species utility.

The high degree of polymorphism in this set of microsatellite markers can be used to clarify taxonomy by delineating species within the *A. niger* aggregate and to elucidate relationships between toxigenic and nontoxigenic strains. Studies of population structure and strain distribution in association with particular commodities and locations can add to the fundamental understanding of OTA production by *Aspergillus* section *Nigri* in food and in animal feeds. Such information can be integrated into strategies to minimize OTA contamination.

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3.7 Comparison of ERIC, AFLP and microsatellite markers in the study of *Aspergillus niger* aggregate and *Aspergillus carbonarius.* Esteban, A., Leong, S.L., Hocking, A., Abarca, M.L., Cabañes, F.J., Tran-Dinh, N. **(enviado para su publicación).**

Comparison of ERIC, AFLP and microsatellite markers in the study of *Aspergillus niger* aggregate and *Aspergillus carbonarius*

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Keywords: *Aspergillus niger, Aspergillus carbonarius, microsatellites, AFLP, ERIC-PCR, ochratoxin A.*

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Abstract

Genetic variation among *Aspergillus carbonarius* and *Aspergillus niger* aggregate strains of differing ochratoxin-producing ability and origin was examined using Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, Amplified Fragment Length Polymorphisms (AFLP) and microsatellite markers. ERIC and AFLP clearly differentiated the *A. niger* aggregate from *A. carbonarius* strains. AFLP was more informative than ERIC, and was able to divide *A. niger* aggregate strains into the two recognised ITS-5.8S rDNA RFLP types, N and T. Microsatellite markers were only applicable in *A. niger* aggregate strains and distributed them according to their RFLP types. No association was observed between the genotypic distribution and ochratoxigenicity, substrate or geography.

1. Introduction

Members of *Aspergillus* section *Nigri* are distributed worldwide and considered common food spoilage fungi (Pitt & Hocking, 1997). They are regarded as benign fungi and within this group, *Aspergillus niger* is commonly used in food processing; products made with *A. niger* hold the GRAS (Generally Recognised As Safe) status from the FDA (Bigelis & Lausure, 1987). Nevertheless, since the first descriptions of ochratoxin A (OTA) production by *A. niger* var. *niger* (Abarca et al., 1994) and *A. carbonarius* (Horie, 1995), the significance of black aspergilli as toxin-producing fungi has increased.

OTA is a nephrotoxic mycotoxin naturally found in a wide range of foods. It is classified as a possible human renal carcinogen (group 2B) (IARC, 1993), and is also teratogenic and immunosuppressive. The European Union has regulated the maximum limits for OTA in an increasing number of foods, most recently encompassing cereals, dried vine fruits, wine, coffee and infant foods (Commission of the European Communities, 2005).

Until the 1990s, the primary producers of OTA were thought to be *A. ochraceus* and related species of *Aspergillus* section *Circumdati* (Hesseltine et al., 1972; Varga et al., 1996) and *Penicillium verrucosum* (Pitt, 1987). However, the incidence and natural occurrence of these species did not explain the widespread OTA contamination in some food products. Ochratoxigenic isolates of *A. niger* and *A. carbonarius* are now considered the primary source of OTA contamination in grapes, wine and dried vine fruits (Abarca et al., 2004).

Within the black aspergilli, taxa included in the *A. niger* aggregate are difficult to distinguish by morphological means (e.g. *A. awamori*, *A. foetidus*, *A. niger*, *A. tubingensis*). Molecular techniques, including RFLP analysis of ribosomal, mitochondrial and chromosomal DNA, RAPD, isoenzymes and sequencing, have been used to clarify the taxonomy of this group, and various authors divided the group into two or more species (Abarca et al., 2004). The *A. niger* aggregate has been divided into two groups, designated types N and T, based on ITS-5.8S rDNA RFLP (Accensi et al., 1999). OTA-positive isolates of the *A. niger* aggregate characterised by this method belong to the type N group (Accensi et al., 2001), however, recently toxigenicity was reported in three type T strains (Medina et al., 2005).

A number of techniques have been applied to the molecular differentiation of fungal strains. Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR is a technique originally based on PCR amplification of regions between neighbouring repetitive elements found interspersed in eubacterial genomes (Hulton et al., 1991; Versalovic et al., 1991).

Fungal species and strains may also be differentiated by this technique (Smith-White et al., 2001), and this paper reports the first application of ERIC-PCR to *Aspergillus* spp.

Amplified fragment length polymorphism (AFLP) is a useful molecular tool able to detect polymorphisms at a large number of independent loci. AFLP fingerprints are reproducible and reliable, and have previously been used to study fungal species (Majer et al., 1996; Castellá et al., 2002). AFLP characterisation of *A. carbonarius* and *A. niger* aggregate strains isolated from coffee revealed no clear link between molecular relatedness of strains and toxigenicity (Schmidt et al., 2004).

Microsatellite markers are another PCR-based means of typing fungi. They are especially useful for the study of closely related organisms (Carter et al., 2004), and have been applied to various genera, including toxigenic fungi such as *Aspergillus flavus* and *A. parasiticus* (Tran-Dinh & Carter, 2000). Recently, six polymorphic microsatellite markers have been isolated and characterized for population analysis in *A. niger* (Esteban et al., 2005).

The aim of this study was to evaluate the suitability of ERIC-PCR, AFLP and microsatellite markers in typing *A. carbonarius* and *A. niger* aggregate strains isolated from a variety of substrates and locations. The toxigenicity of the strains, substrate of isolation and geography, as well as the ITS-5.8S rDNA RFLP pattern of the *A. niger* aggregate strains, were examined for association with the genetic relationships indicated by these methods.

2. Materials and methods

2.1 Fungal strains

The isolates used in this study are listed in Table 1. Forty-two isolates of *A. carbonarius* were included in the analysis, as well as 26 isolates of *A. niger* aggregate strains. Four strains of *A. aculeatus* were also added as an outgroup. The strains were selected to include different sources of isolation and geographical origin, primarily Spain and Australia. Several strains provided from international culture collections were also included in the study.

2.2 OTA-producing ability

Australian *A. carbonarius* isolates were screened for OTA production on coconut cream agar with visualisation under long-wave UV light (Heenan et al., 1998). The remaining strains were screened by HPLC as previously described (Bragulat et al., 2001).

2.3 Genomic DNA extraction

As previously described (Smith-White et al., 2001), strains were grown in liquid medium and DNA extracted using a FastPrep protocol (BIO101, Inc., California, U.S.A.) with three modifications as follows: after cell lysis, proteins in the supernatant were precipitated with 150 µl 3 M potassium acetate adjusted to pH 5.5 with glacial acetic acid; mixing time in the presence of the DNA binding matrix was extended to 30 min; and the matrix was washed with 100 mM sodium acetate in 70 % ethanol and mixed for a further 30 min before centrifugation. DNA was eluted as previously described.

2.4 ITS-5.8S rDNA RFLP of Aspergillus niger *aggregate strains*

Type N and T patterns in *A. niger* aggregate strains were determined as previously described (Accensi et al., 1999). The presence or absence of a target for the restriction enzyme *Rsa*I in the ITS1-rDNA region of *A. niger* aggregate strains enabled the differentiation between the two patterns.

2.5 ERIC-PCR analysis

The primers used in this study were ERIC2F/ERIC1R (5'-AAGTAAGTGACTGGGGTGAGCG-3' / 5'-ATGTAAGCTCCTGGGGATTCAC-3') (Gillings & Holley, 1997). Reaction mixtures (20 µl) containing 1x PCR buffer (Qiagen, Germany), 5 % glycerol, 250 µM each dNTP, 20 pmol of each primer (Proligo®, USA), 1 U of *Taq* DNA polymerase (Qiagen, Germany) and 20-40 ng of genomic DNA as template were amplified in a Hybaid PCR Express Thermal Cycler (Integrated Sciences, Australia; also used for all subsequent amplifications reported in this paper). The reaction commenced with an initial denaturing step of 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 8 min at 68 °C, followed by a final extension step of 8 min at 68 °C. Electrophoresis was conducted on a 2 % (w/v) agarose gel in Tris-boric acid-EDTA (1.1 M Tris, 900 mM borate,

25 mM EDTA, pH 8.3) and images captured using Kodak 1D image analysis software version 3.5.4 (New Haven, USA).

ERIC results were scored by visual inspection. Bands were scored as present (1) or absent (0) for each isolate and comigrating bands were considered homologous characters. The RAPDistance program version 1.04 (Armstrong et al., 1994; http://www.anu.edu.au/BoZo/software/) was used to produce a distance matrix from pairwise comparisons of ERIC profiles using the Dice coefficient (Dice, 1945).

2.6 AFLP analysis

This method was based on a protocol developed for black *Aspergillus* spp. (Perrone et al., 2005). Approximately 400-800 ng of genomic DNA was digested for 2.5 h at 37 ºC with the endonucleases *Eco*RI (13 U) and *Mse*I (7 U) and 10x One-Phor-All buffer (Amersham Biosciences UK Ltd, UK) in a total volume of 40 µl. The ligation reaction was performed with *Eco*RI adapter (4 pmol) and *Mse*I adapter (38 pmol) (Proligo, Australia) using T4-ligase (0.75 Weiss units) and 0.2x T4 ligase buffer (Promega, U.S.A.) in a total volume of 7.5 µl. Pre-amplification reactions were performed with two primer combinations: *Eco*RI-A (5'-GACTGCGTACCAATTCA-3') + *Mse*I-C (5'-GATGAGTCCTGAGTAAC-3'), or *Eco*RI (5'-GACTGCGTACCAATTC-3') + *Mse*I-C (Proligo, Australia). The reaction mixtures (25 µl) containing 1x PCR buffer (Qiagen, Germany), 200 µM each dNTP, 25 pmol of each primer, 0.5 U of *Taq* DNA Polymerase (Qiagen, Germany) and 20-40 ng digested DNA as template were amplified with cycling parameters: 20 cycles of 30 s at 94 °C, 1 min at 56 °C and 1 min at 72 °C. Products from the pre-amplification were diluted 1:10 in Milli Q water for use as template in the selective PCR. Two different primer combinations were used for selective PCR: *Eco*RI-AT (5'-GACTGCGTACCAATTCAT-3') + *Mse*I-CG (5'-GATGAGTCCTGAGTAACG-3') with template from the *Eco*RI-A + *Mse*I-C pre-amplification, and *Eco*RI-G (5'-GACTGCGTACCAATTCG-3') + *Mse*I-CT (5'-GATGAGTCCTGAGTAACT-3') with template from the *Eco*RI + *Mse*I-C pre-amplification. Reaction mixtures for the selective amplification (25 µl) contained 5 µL diluted template DNA from the corresponding pre-amplification in 1x PCR buffer (Qiagen, Germany), 200 µM each dNTP, 20 pmol of each primer (Proligo, Australia) and 2.5 U of *Taq* DNA Polymerase (Qiagen, Germany), and were amplified with cycling parameters: 10 cycles of 1 min at 94 °C, 1 min at 65 °C to 56 °C (decreasing 1 Celsius degree every cycle from 65 °C to 56 °C) and 1 min 30 s at 72 °C, followed by 20 additional cycles of 30 s at 94 °C, 30 s at 56

°C and 1 min at 72 °C. The products were sized with an automated sequencer (ABI Prism® 3700 DNA Analyzer, Applied Biosystems, USA) at SUPAMAC (Camperdown, Australia).

The entire procedure was replicated from DNA digestion stage with both selective primer sets. For each isolate, only amplicon sizes which were detected in both replicates were included in the phylogenetic analysis. Each amplicon within the size range 50-500 bp and above a threshold height was defined as an allele. Data from both sets of selective primers were combined and a distant matrix was produced by pairwise comparison using the Dice coefficient (Dice, 1945) and ADE-4 software version 2001 (Align2, LecPCR and DistAFLP, http://pbil.univ-lyon1.fr/ADE-4/microb/).

2.7 Microsatellite marker analysis

The microsatellite markers and amplification conditions used in this study have been previously described (Esteban et al., 2005). ACNM1, 2, 3, 5 and 7 were used to investigate all *A. niger* aggregate strains, while ACNM6 was used only in type N strains. Each amplicon size (bp) was defined as an allele and the absence of an amplicon was defined as a null allele. Pairwise population distances were calculated using the Microsat2 program (http://hpgl.stanford.edu/projects/microsat/) based on the proportion of shared alleles. For bootstrap analysis, 200 resampled data sets were used.

2.8 Construction of dendrograms

From distance matrices, the neighbour-joining algorithm in Phylip (Felsenstein, 1989; http://evolution.genetics.washington.edu/phylip.html) was used to construct dendrograms, which were drawn using TreeExplorer 2.12 (K. Tamura; http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html). Bootstrap analysis was performed with PAUP* program (D.L. Swofford; Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4, Sinauer Associates, Sunderland, USA), using 1000 resampled data sets.

3. Results and discussion

3.1 OTA-producing ability

The results from the assays for OTA-production by individual strains are stated in Table 1. All *A. carbonarius* strains, except CBS 110.49, and ten strains belonging to the *A. niger* aggregate were able to produce OTA. None of the *A. aculeatus* strains were able to produce OTA.

3.2 *ITS-5.8S rDNA RFLP analysis of* Aspergillus niger *aggregate strains*

The RFLP patterns obtained in the *A. niger* aggregate are listed in Table 1. Within this group, 19 strains showed N RFLP pattern whilst seven of them showed T pattern. The type N strains included both toxigenic and non-toxigenic strains. None of these type T strains were able to produce OTA.

3.3 ERIC-PCR analysis

Considerable variation was observed among the *A. niger* aggregate and *A. carbonarius* strains studied using ERIC-PCR. Sixteen distinct bands were scored and the resulting dendrogram revealed three major clusters corresponding to *A. carbonarius, A. niger* aggregate and *A. aculeatus* (Fig. 1). Analysis of ERIC-PCR suggested greater genetic variation among *A. niger* aggregate strains than among *A. carbonarius* strains. However, the technique did not discriminate the *A. niger* aggregate strains based on their ITS-5.8S rDNA RFLP pattern (Accensi et al., 1999) and one toxigenic strain, A-136, was described as identical with a non-toxigenic strain, A-946. Three toxigenic *A. niger* aggregate isolates (FRR 5695, A-1241 and A-220) were also found outside the main *A. niger* aggregate cluster. Bootstrap analysis provided little support for the ERIC-PCR dendrogram. Perhaps ERIC-PCR surveyed only a small proportion of the genome through the use of a single set of primers. Hence, the 16 bands scored may not be representative of genetic variation within the black *Aspergillus* spp. studied. ERIC-PCR was considered the least informative of the techniques tested.

3.4 AFLP analysis

AFLP analysis showed three major groups, corresponding to *A. carbonarius, A. niger* aggregate and *A. aculeatus* (Fig. 2). Higher genetic variation was seen in the *A. niger* aggregate than in *A. carbonarius*. The *A. niger* aggregate cluster was divided into two

groups, containing type N and type T strains, respectively, as reported from AFLP analysis of isolates primarily from grapes in Southern Europe (Perrone et al., 2005). Furthermore, the type N group was divided in two clusters, with a bootstrap value of 92 %. Relationships between major clusters and substrate or geography were not observed for any species, with isolates from various sources clustered together. Within two clusters of the type N group, toxigenic and non-toxigenic isolates were interspersed.

3.5 Microsatellite markers

The six polymorphic microsatellite markers tested produced suitable amplicons for analysis of *A. niger* aggregate strains. These loci had been unsuccessfully tested for cross-species amplification in *A. carbonarius* and *A. aculeatus* (Esteban et al., 2005) and hence, the data assessed in the molecular analysis incorporated only strains of the *A. niger* aggregate.

The microsatellite analysis divided the *A. niger* aggregate strains into two groups corresponding to types N and T (Fig. 3). The microsatellite dendrogram showed similar topology to the AFLP dendrogram (Fig. 2). Within the type N group, strains were further divided into two clusters, each containing a mixture of toxigenic and non-toxigenic strains, which corresponded to those observed in the AFLP analysis. Despite low support from bootstrap analysis of the microsatellite data, the similarity between the microsatellite and AFLP dendrograms confirms the validity of these techniques, as the sources of polymorphism in AFLP and microsatellite analyses are independent. Low bootstrap support values for the microsatellite dendrogram likely arose due to the relatively small number of loci examined, and the frequency of null alleles for certain loci. As suggested by AFLP analysis, microsatellite data indicated that strains from different sources and geographical origins clustered together in the type T group, as well as in the type N group.

3.6 Molecular relationships indicated by AFLP and microsatellite analyses

The non-toxigenic type T *A. niger* aggregate strains were clearly separated from the type N strains by AFLP and microsatellite analyses. The type N strains were subdivided in two clusters, each of which included both toxigenic and non-toxigenic strains. The genotypic distribution of these strains was not related to their toxigenicity. Lack of association between genotype and toxigenicity was also reported for black aspergilli isolated from coffee; analysis of AFLP with selective primers *Eco*RI-AT + *Mse*I-CT (Schmidt et al., 2004) and RAPD

(Fungaro et al., 2004) distinguished between species and strains of black aspergilli, however, specific genotypical differences between toxigenic and non-toxigenic isolates of the *A. niger* aggregate or *A. carbonarius* were not found. On the other hand, a link between toxigenicity and molecular relatedness was shown in *A. ochraceus* using mitochondrial DNA restriction profiles (Varga et al., 2000). Similarly, AFLP analysis of ochratoxigenic *Penicillium* strains showed a relationship between genotype, capacity to produce OTA and origin (Castellá et al., 2002): strains formed two major clusters, one containing mainly high producing strains isolated from foods, such as cheeses and meat products, and the other one containing moderate to non-producing strains isolated from plants. Those data confirmed the separation of ochratoxigenic *Penicillium* strains into *P. nordicum* and *P. verrucosum*, respectively. Regarding the origin of the black *Aspergillus* strains, our study did not reveal any association between genotypic distribution and substrate or geography. Several isolates from vineyards were closely related to isolates from various other sources. Similarly, isolates from Europe and Australia were interspersed in the AFLP dendrogram. This lack of correlation has also been observed for *A. ochraceus* (Varga et al., 2000).

ERIC, AFLP and microsatellite markers have all shown varying utility for inter- and/or intra-specific differentiation of the *A. niger* aggregate and *A. carbonarius*. AFLP and microsatellite markers generated the most meaningful results and were able to divide the *A. niger* aggregate strains into the two previously defined ITS-5.8S rDNA RFLP patterns (Accensi et al., 1999), as well as suggesting a further subdivision within the N type group. However no association between the distribution of the strains and toxigenicity, geography or substrate of isolation was observed. Although further studies are still necessary to clarify the relationships within the black aspergilli, the availability of reliable molecular markers will increase understanding of the nature of toxin production and the occurrence of ochratoxigenic strains. This may provide useful information to minimise OTA contamination of food and feed in the future.

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Table 1. List of strains studied and their properties.

Reference	Species	Source	Geography[a]	OTA production	RFLP pattern
[b]A-1122	*A. aculeatus*	grapes	Spain	-	n/a[c]
A-1325	*A. aculeatus*	grapes	Spain	-	n/a
A-1355	*A. aculeatus*	grapes	Spain	-	n/a
A-1356	*A. aculeatus*	grapes	Spain	-	n/a
A-642	*A. carbonarius*	soil	Portugal	+	n/a
A-941	*A. carbonarius*	grapes	Spain	+	n/a
A-1040	*A. carbonarius*	raisins	Spain	+	n/a
A-1070	*A. carbonarius*	raisins	Spain	+	n/a
A-1477	*A. carbonarius*	grapes	Spain	+	n/a
A-1500	*A. carbonarius*	grapes	Spain	+	n/a
[d]FRR 5573	*A. carbonarius*	grapes	Australia (Vic)	+	n/a
FRR 5374	*A. carbonarius*	grapes	Australia (Vic)	+	n/a
FRR 5574	*A. carbonarius*	grapes	Australia (Vic)	+	n/a
FRR 5682	*A. carbonarius*	vineyard soil	Australia (NSW)	+	n/a
FRR 5683	*A. carbonarius*	vineyard soil	Australia (NSW)	+	n/a
FRR 5690	*A. carbonarius*	grapes	Australia (NSW)	+	n/a
FRR 5691	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5692	*A. carbonarius*	vineyard soil	Australia (Qld)	+	n/a
FRR 5693	*A. carbonarius*	grapes	Australia (Vic)	+	n/a
FRR 5696	*A. carbonarius*	grapes	Australia (Vic)	+	n/a
FRR 5697	*A. carbonarius*	grapes	Australia (Vic)	+	n/a
FRR 5698	*A. carbonarius*	grapes	Australia (Vic)	+	n/a
FRR 5699	*A. carbonarius*	grapes	Australia (NSW)	+	n/a
FRR 5700	*A. carbonarius*	grapes	Australia (NSW)	+	n/a
FRR 5702	*A. carbonarius*	grapes	Australia (NSW)	+	n/a
FRR 5703	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5704	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5705	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5706	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5707	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5708	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5709	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5710	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5711	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5712	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5713	*A. carbonarius*	vineyard soil	Australia (SA)	+	n/a
FRR 5714	*A. carbonarius*	dried grape stems	Australia (SA)	+	n/a
FRR 5715	*A. carbonarius*	vineyard soil	Australia (Qld)	+	n/a
FRR 5716	*A. carbonarius*	vineyard soil	Australia (Qld)	+	n/a
FRR 5717	*A. carbonarius*	vineyard soil	Australia (Qld)	+	n/a
FRR 5718	*A. carbonarius*	vineyard soil	Australia (Qld)	+	n/a
FRR 5719	*A. carbonarius*	grapes	Australia (Vic)	+	n/a
[e]CBS 127.49	*A. carbonarius*	coffee	unknown	+	n/a
CBS 110.49	*A. carbonarius*	air	Indonesia	-	n/a
[f]M325	*A. carbonarius*	apples	Switzerland	+	n/a
[g]NRRL67	*A. carbonarius*	unknown	Brazil	+	n/a

A-75	*A. niger* aggregate	feedstuff	Spain	+	N
A-136	*A. niger* aggregate	soy	Spain	+	N
A-220	*A. niger* aggregate	feedstuff	Spain	+	N
A-942	*A. niger* aggregate	raisins	Spain	+	N
A-1241	*A. niger* aggregate	grapes	Spain	+	N
FRR 5694	*A. niger* aggregate	vineyard soil	Australia (WA)	+	N
FRR 5695	*A. niger* aggregate	vineyard soil	Australia (SA)	+	N
CBS 139.52	*A. awamori*	Kuro-koji	Japan	+	N
CBS 618.78	*A. foetidus*	unknown	Germany	+	N
[h]MUM 01.05	*A. niger* aggregate	grapes	Portugal	+	N
A-655	*A. niger* aggregate	wheat	Spain	-	N
A-946	*A. niger* aggregate	coffee	Portugal	-	N
A-1743	*A. niger* aggregate	grapes	Spain	-	N
FRR 333	*A. niger* aggregate	rice	Australia (NSW)	-	N
FRR 2522	*A. niger* aggregate	peanuts	Australia (Qld)	-	N
FRR 5722	*A. niger* aggregate	dried grape stems	Australia (Tas)	-	N
CBS 554.65	*A. niger*	tannin-gallic acid ferm	USA	-	N
CBS 121.55	*A. niger*	otomycosis	Switzerland	-	N
[i]CECT 2088	*A. niger*	unknown	USA	-	N
A-487	*A. niger* aggregate	wheat	Spain	-	T
A-615	*A. niger* aggregate	feedstuff	Spain	-	T
A-656	*A. niger* aggregate	soil	Spain	-	T
A-947	*A. niger* aggregate	grapes	Spain	-	T
FRR 5720	*A. niger* aggregate	vineyard soil	Australia (NSW)	-	T
FRR 5721	*A. niger* aggregate	vineyard soil	Australia (Vic)	-	T
CBS 134.48	*A. tubingensis*	unknown	unknown	-	T

[a] Abbreviations for Australian states as follows. Vic: Victoria; NSW: New South Wales; SA: South Australia; Qld: Queensland; WA: Western Australia; Tas: Tasmania.

[b] Culture Collection of Veterinary Faculty of Barcelona, Bellaterra (Barcelona), Spain.

[c] Not applicable

[d] Culture Collection of Food Science Australia, North Ryde (NSW), Australia.

[e] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

[f] Strain provided by Dr. H Joosten.

[g] Northern Agricultural Research Service Culture Collection, Peoria, IL, USA.

[h] Micoteca da Universidade do Minho, Braga, Portugal.

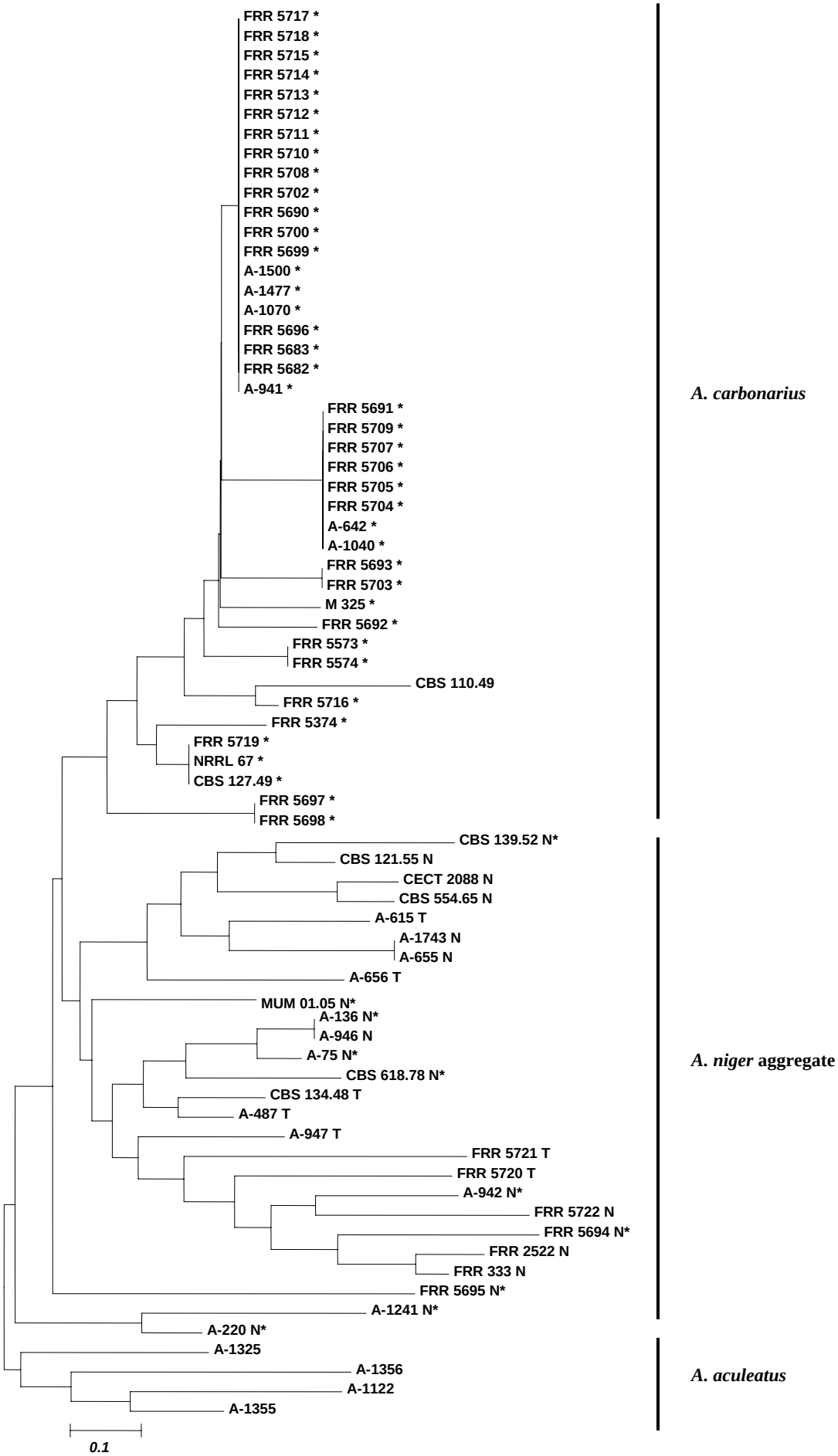
[i] Colección Española de Cultivos Tipo, Valencia, Spain.

Fig. 1. Relationships between *Aspergillus niger* aggregate and *A. carbonarius* strains analysed by ERIC-PCR. N,T: indicates *A. niger* aggregate strains with N or T RFLP pattern (Accensi et al., 1999).*: denotes toxigenic isolates.

Fig. 2. Relationships between *Aspergillus niger* aggregate and *A. carbonarius* strains analysed by AFLP. N,T: indicates *A. niger* aggregate strains with N or T RFLP pattern (Accensi et al., 1999).* : denotes toxigenic isolates.

Fig. 3. Analysis of polymorphisms at microsatellite loci to deduce relationships between strains of the *Aspergillus niger* aggregate. N,T: indicates strains with N or T RFLP pattern (Accensi et al., 1999). * : denotes toxigenic isolates.

Fig. 1


FRR 5717 *
FRR 5718 *
FRR 5715 *
FRR 5714 *
FRR 5713 *
FRR 5712 *
FRR 5711 *
FRR 5710 *
FRR 5708 *
FRR 5702 *
FRR 5690 *
FRR 5700 *
FRR 5699 *
A-1500 *
A-1477 *
A-1070 *
FRR 5696 *
FRR 5683 *
FRR 5682 *
A-941 *
FRR 5691 *
FRR 5709 *
FRR 5707 *
FRR 5706 *
FRR 5705 *
FRR 5704 *
A-642 *
A-1040 *
FRR 5693 *
FRR 5703 *
M 325 *
FRR 5692 *
FRR 5573 *
FRR 5574 *
CBS 110.49
FRR 5716 *
FRR 5374 *
FRR 5719 *
NRRL 67 *
CBS 127.49 *
FRR 5697 *
FRR 5698 *
CBS 139.52 N*
CBS 121.55 N
CECT 2088 N
CBS 554.65 N
A-615 T
A-1743 N
A-655 N
A-656 T
MUM 01.05 N*
A-136 N*
A-946 N
A-75 N*
CBS 618.78 N*
CBS 134.48 T
A-487 T
A-947 T
FRR 5721 T
FRR 5720 T
A-942 N*
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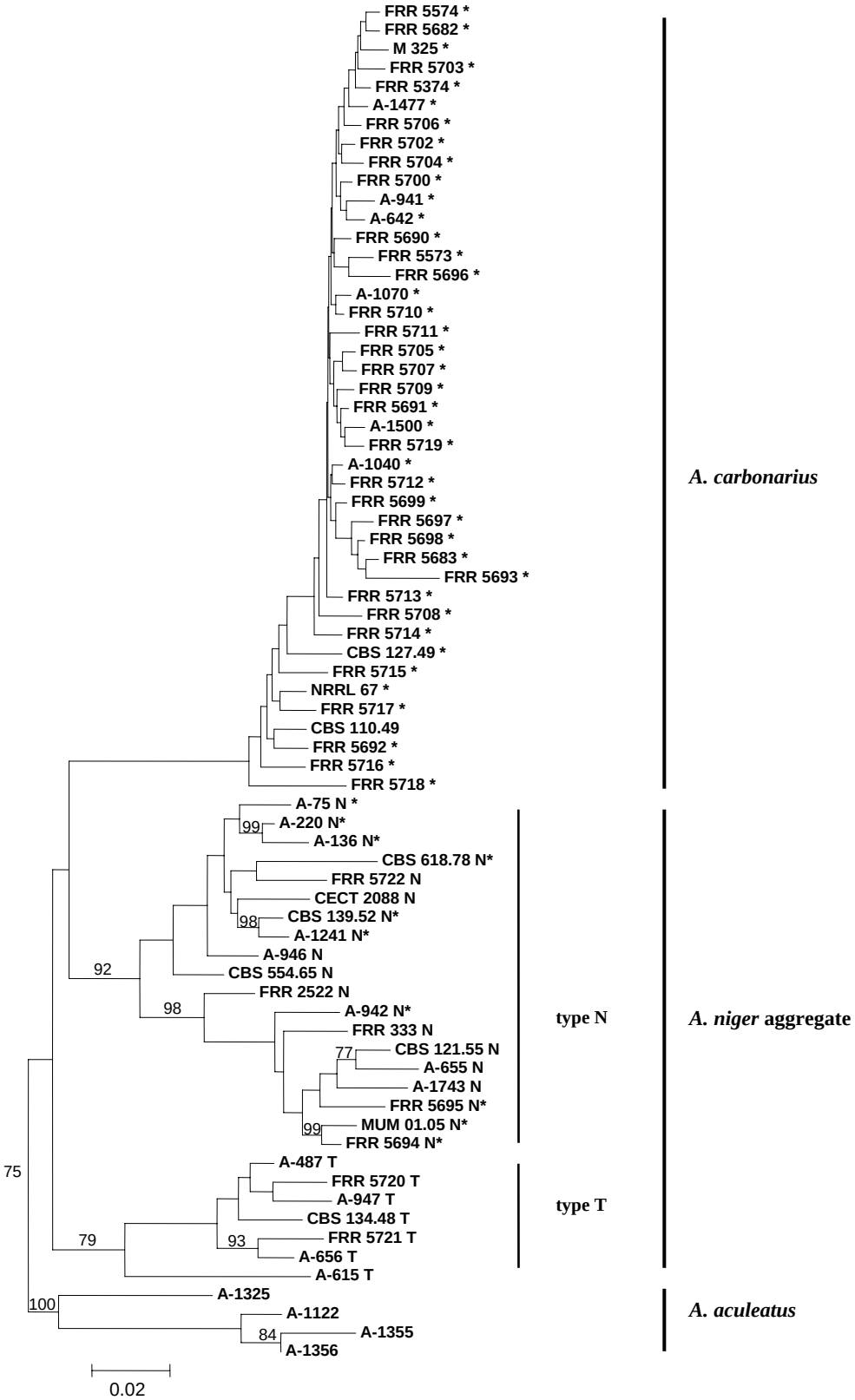
FRR 2522 N
FRR 333 N
FRR 5695 N*
A-1241 N*
A-220 N*
A-1325
A-1356
A-1122
A-1355
0.1
A. carbonarius
A. niger aggregate
A. aculeatus

Fig. 2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FRR 5705 *
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FRR 5712 *
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FRR 5715 *
NRRL 67 *
FRR 5717 *
CBS 110.49
FRR 5692 *
FRR 5716 *
FRR 5718 *
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A-220 N*
A-136 N*
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CECT 2088 N
CBS 139.52 N*
A-1241 N*
A-946 N
CBS 554.65 N
FRR 2522 N
A-942 N*
FRR 333 N
CBS 121.55 N
A-655 N
A-1743 N
FRR 5695 N*
MUM 01.05 N*
FRR 5694 N*
A-487 T
FRR 5720 T
A-947 T
CBS 134.48 T
FRR 5721 T
A-656 T
A-615 T
A-1325
A-1122
A-1355
A-1356
99
98
92
98
77
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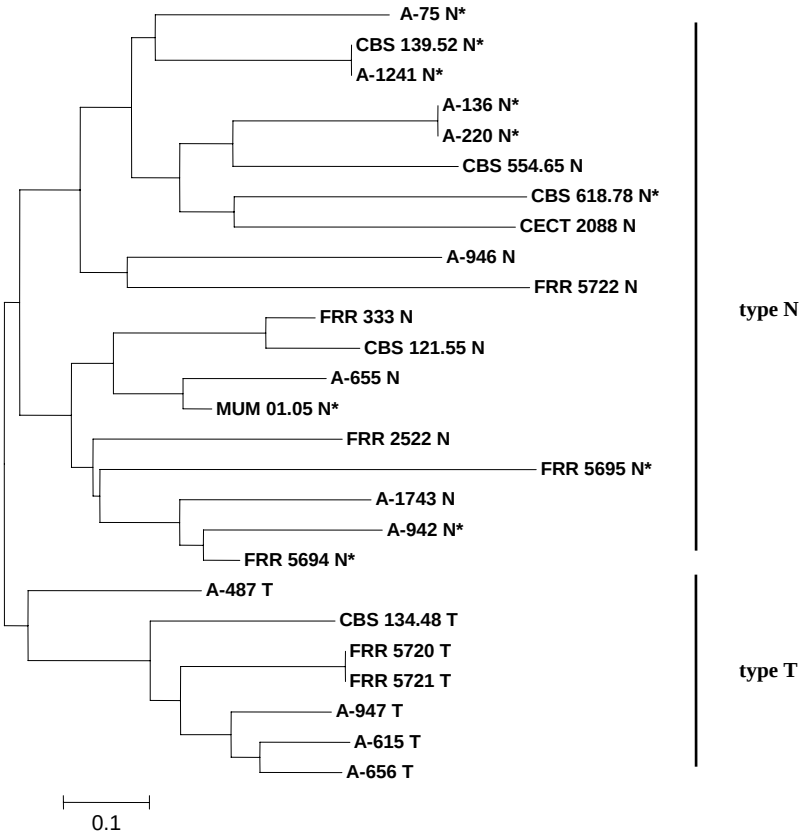
93
100
84
0.02
type N
type T
A. carbonarius
A. niger aggregate
A. aculeatus

Fig. 3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FRR 5722 N
FRR 333 N
CBS 121.55 N
A-655 N
MUM 01.05 N*
FRR 2522 N
FRR 5695 N*
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A-942 N*
FRR 5694 N*
A-487 T
CBS 134.48 T
FRR 5720 T
FRR 5721 T
A-947 T
A-615 T
A-656 T
0.1
type N
type T

4.-RESUMEN GLOBAL DE LOS RESULTADOS Y DISCUSIÓN

En la presente Memoria de Tesis Doctoral se ha realizado un estudio fisiológico y molecular de las especies del género *Aspergillus* sección *Nigri* productoras de ocratoxina A (OTA).

Para realizar el estudio fisiológico se han seleccionado un total de 18 cepas con diferente capacidad de producción de OTA y diferentes orígenes. De éstas, 12 pertenecen al agregado *A. niger* y 6 pertenercen a *A. carbonarius*. El estudio se ha centrado en el efecto de la temperatura de incubación, la actividad de agua (a_w) y el pH sobre el crecimiento y la producción de OTA en los medios de cultivo agar Czapek extracto de levadura (CYA) y agar extracto de levadura sacarosa (YES). Las lecturas se han efectuado a los 5, 10, 15, 20 y 30 días de incubación y se ha determinado, en cada caso, la capacidad de producir OTA mediante HPLC. Todos los ensayos se han realizado por duplicado.

La caracterización molecular de las cepas se ha llevado a cabo mediante RFLP de ITS-5,8S del rDNA, secuenciación de las regiones ITS-5,8S del rDNA y D1/D2 de 28S del rDNA y mediante RAPD. Además, se han evaluado y comparado tres marcadores moleculares (ERIC-PCR, AFLP y microsatélites) con el fin de valorar su idoneidad para la caracterización de cepas pertenecientes a la sección *Nigri*. En este último ensayo se incluyeron las cepas del estudio fisiológico junto con otras 14 cepas del agregado *A. niger*, 36 cepas de *A. carbonarius* y 4 cepas de *A. aculeatus* de diferentes orígenes geográficos y diferentes fuentes de aislamiento.

En el estudio de la influencia de la temperatura de incubación (5-45ºC) sobre el crecimiento y la producción de OTA (artículo 3.1), las cepas del agregado *A. niger* fueron capaces de desarrollarse entre 10 y 45ºC y las de *A. carbonarius* entre 10 y 40ºC. La producción de OTA se observó en un margen más estrecho de temperatura, 10-35ºC para las cepas del agregado *A. niger* y 15-35ºC para las de *A. carbonarius*, detectándose niveles significativos de OTA a los 5 días de incubación. Las cepas del agregado *A. niger* alcanzaron los máximos niveles de OTA en el medio YES incubado a 20-25ºC y las de *A. carbonarius* en el medio CYA a 15-20ºC. Teniendo en cuenta estos resultados, en los restantes estudios fisiológicos, las cepas del agregado *A. niger* se incubaron a 25ºC y las de *A. carbonarius* a 15 y 30ºC.

En los ensayos realizados para conocer la influencia de la a_w (0,78-0,99 a_w) sobre el crecimiento y la producción de OTA (artículos 3.2 y 3.3) todas las cepas del agregado *A. niger* se desarrollaron en los medios CYA y YES con valores de a_w entre 0,86 y 0,99. La producción de OTA se detectó en un margen más estrecho (0,90-0,99 a_w) y las concentraciones más elevadas se observaron tras 5-

10 días de incubación en el medio YES con valores de a_w entre 0,96 y 0,99 (artículo 3.2). Las cepas de *A. carbonarius* se desarrollaron en el medio YES incubado a 30ºC en el margen de a_w de 0,82 a 0,99. A esta temperatura el crecimiento se inició en el medio CYA a valores de a_w más elevados que en el medio YES. La producción de OTA se detectó en un margen más estrecho de a_w que el de crecimiento (0,86-0,99). Cuando la incubación se realizó a 15ºC todas las cepas crecieron y elaboraron OTA en los dos medios de cultivo con valores de a_w desde 0,94 a 0,99. Aunque la temperatura óptima de crecimiento de *A. carbonarius* se ha descrito a 30ºC, en nuestro estudio la concentración de OTA elaborada fue superior a 15ºC que a 30ºC. Los niveles máximos de OTA se alcanzaron en el medio CYA a 0,98-0,99 a_w incubado a 15ºC (artículo 3.3).

Al estudiar el efecto del pH (pH 2-10) sobre el crecimiento y producción de OTA (artículos 3.4 y 3.5) se observó que las cepas del agregado *A. niger* y de *A. carbonarius* fueron capaces de desarrollarse y producir OTA desde pH 2 a pH 10. En el caso de las cepas del agregado *A. niger*, la producción máxima de OTA se detectó en el medio YES en el intervalo de pH 5-10, después de 5-10 días de incubación a 25ºC (artículo 3.4). En el caso de *A. carbonarius*, los valores máximos estaban en función de la cepa en estudio y la temperatura ensayada (15ºC y 30ºC). A 30ºC las concentraciones más elevadas de OTA se obtuvieron en el medio CYA a pH 2, 5 o 7 dependiendo de la cepa. Cuando la incubación se realizó a 15ºC las concentraciones más elevadas se detectaron en general a valores de pH más elevados (pH 5-7) que a 30ºC (artículo 3.5).

En todos los estudios fisiológicos realizados, el medio de cultivo ejerció un importante papel en la concentración de OTA producida. Las cepas de *A. carbonarius* elaboraron niveles de OTA más elevados en el medio CYA y las cepas del agregado *A. niger* en el medio YES, independientemente de la temperatura de incubación, a_w y pH estudiado.

En relación a la capacidad ocratoxígena de las cepas, el porcentaje de aislamientos del agregado *A. niger* productores es bajo, aunque todavía se desconoce si ello es debido al genotipo de las cepas o depende de factores externos. Mediante RFLP de la región ITS-5,8S del rDNA, las cepas del agregado *A. niger* se agrupan en dos patrones denominados N y T. De las 12 cepas del agregado utilizadas en el estudio, 6 habían sido descritas anteriormente como productoras de OTA y tenían un patrón de RFLP tipo N. De las 6 cepas del agregado *A. niger* inicialmente consideradas como no ocratoxígenas, 4 tenían un patrón de RFLP tipo N y 2 tipo T. Ninguna de estas cepas ha sido capaz de producir OTA en los dos medios estudiados ajustados a diferentes valores de a_w y pH a ninguna de las temperaturas de incubación ensayadas durante un período de 30 días. Mediante

análisis de la secuenciación de las regiones ITS-5,8S del rDNA y D1/D2 del 28S del rDNA y mediante RAPD (artículo 3.2) las 12 cepas del agregado se diferenciaron en dos grupos que se correspondían con los dos tipos de patrón de RFLP establecidos previamente (N y T). El grupo que incluía las cepas de tipo N presentaba una gran variabilidad pero no se pudieron diferenciar las cepas productoras y no productoras de OTA.

En el caso de las cepas de *A. carbonarius* el porcentaje de cepas descritas como ocratoxígenas es variable, pero alcanza valores del 100% en muchos estudios. Dos de las cepas incluidas en nuestro estudio habían sido previamente citadas como no ocratoxígenas. Una de ellas (M325), se había descrito como no productora al utilizar granos de café como sustrato pero fue capaz de elaborar OTA en las condiciones analizadas en nuestro estudio. En cambio, la cepa CBS 110.49 considerada como débilmente ocratoxígena al utilizar también granos de café como sustrato, no elaboró OTA en ninguno de los estudios realizados. Cabe pensar por tanto que no se ha utilizado el sustrato idóneo o que probablemente ha perdido la capacidad de producir OTA. Por último, la cepa A-1082, inicialmente identificada como *A. carbonarius*, tampoco fue capaz de producir OTA en ninguna de las condiciones estudiadas. Mediante el análisis de la región ITS-5,8S del rDNA (artículo 3.3) se pudo determinar que las cepas de *A. carbonarius* estudiadas eran coespecíficas excepto la cepa A-1082 que se diferenció claramente de las 5 cepas restantes, hecho también confirmado mediante RAPD. Esta cepa pertenecería a la nueva especie propuesta dentro de la sección *Nigri*, denominada "*A. ibericus*". No se observó ninguna correlación entre las subdivisiones observadas mediante RAPD y el nivel de producción de OTA de las 5 cepas de *A. carbonarius*. Un aspecto a considerar en este punto es si realmente existen en la naturaleza cepas de *A. carbonarius* no ocratoxígenas. Las cepas ensayadas han demostrado esta capacidad como mínimo en alguno de los estudios realizados. Esto podría explicar por qué mediante las técnicas moleculares estudiadas no se pueden diferenciar las cepas en función de sus propiedades ocratoxígenas.

La comparación de los diferentes marcadores moleculares se llevó a cabo utilizando AFLP, ERIC-PCR y los microsatélites previamente caracterizados en el agregado *A. niger* (artículo 3.6). Los tres marcadores evaluados (artículo 3.7) resultaron útiles para valorar la variabilidad inter e intraespecífica en las cepas del agregado *A. niger* y *A. carbonarius*. No obstante, los resultados más significativos se obtuvieron mediante AFLP y el análisis de microsatélites, permitiendo ambos la diferenciación de las cepas del agregado 2 grupos que se corresponden con los dos patrones de RFLP del ITS-5,8S del rDNA previamente establecidos (N y T). En este estudio no pudo

establecerse ninguna relación entre la distribución genotípica de las cepas y su capacidad ocratoxígena, origen geográfico o sustrato de aislamiento.

Los resultados obtenidos en la presente Memoria ponen de manifiesto que las cepas del agregado *A. niger* y de *A. carbonarius* pueden crecer y producir OTA en un amplio rango de temperatura, a_w y pH. Esta capacidad les permitiría la elaboración de la micotoxina en diferentes sustratos en el campo a pesar de que existan importantes variaciones de temperatura entre el día y la noche y explicaría por qué se encuentran entre las especies de *Aspergillus* comunmente responsables de alteraciones en la fruta fresca y desecada. Los procesos de desecación y los cambios de pH no evitarían el crecimiento de las cepas ni la producción de OTA. Estos resultados contribuyen a explicar el papel que desempeñan estas especies como principal fuente de contaminación de OTA en algunos sustratos.

5.- CONCLUSIONES

1. Las cepas del agregado *A. niger* y *A. carbonarius* pueden crecer y producir ocratoxina A en un amplio rango de temperatura, actividad de agua y pH, por lo que se confirma la contribución de estas especies en la contaminación de ocratoxina A de diferentes sustratos en los que se dan las condiciones ambientales óptimas para la producción de la micotoxina.

2. Las especies ocratoxígenas de la sección *Nigri* son capaces de elaborar niveles significativos de ocratoxina A desde los 5 días de incubación.

3. Las cepas del agregado *A. niger* alcanzaron los máximos niveles de ocratoxina A a 20-25ºC en el medio YES, en el intervalo de a_w 0,96-0,99 y en el rango de pH 5-10.

4. Las cepas de *A. carbonarius* alcanzaron los máximos niveles de ocratoxina A a 15-20ºC en el medio CYA, en el intervalo de a_w 0,98-0,99 y en el rango de pH 5-7.

5. Las técnicas de AFLP, microsatélites, RAPD y secuenciación confirman la separación del agregado *A. niger* en dos grupos que se corresponden con los patrones de RFLP previamente establecidos N y T.

6. Las cepas del agregado *A. niger* con un patrón de RFLP tipo T no han producido ocratoxina A en ninguna de las condiciones ensayadas.

7. La cepa A-1082, perteneciente a la nueva especie propuesta de la sección *Nigri* "*A. ibericus*" se diferenció molecularmente del resto de cepas de *A. carbonarius* mediante secuenciación y RAPD y no elaboró ocratoxina A en ninguna de las condiciones estudiadas.

8. Ninguna de las técnicas moleculares estudiadas permitió establecer una correlación entre la distribución genotípica de las cepas y su capacidad ocratoxígena, origen geográfico o sustrato de aislamiento.