Predictive mycology and use of natural antifungals to prevent the mycotoxin food hazard

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Predictive mycology and use of natural antifungals to prevent the mycotoxin food hazard

Thesis submitted by

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To fulfil the requerimets of the degree of Doctor

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October 2012

Dissertation presented by Daiana García to obtain the PhD degree from the University of Lleida. This work was carried out under the supervision of Sonia Marín Sillüe and Antonio J. Ramos. The present work has been carried out in the Mycology Research Group in the Food Technology Department and is included in the "Ciéncia i Tecnologia Agraria i Alimentaria" doctorate program. The work is part of the following projects:

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"La verdadera locura quizá no sea otra cosa que la sabiduría misma que, cansada de descubrir las vergüenzas del mundo, ha tomado la inteligente resolución de volverse loca"

Heinrich Heine

AGRADECIMIENTOS

Un 4 de septiembre de 2008 llegué a Lleida sin saber realmente dónde estaba ubicada la ciudad en el mapa, decidida a experimentar las vivencias que me iban a traer los siguientes cuatro años de doctorado. Y a día de hoy, pasado ese tiempo, me doy cuenta que hay mucha gente a la que tengo que agradecer:

… y en primer lugar estás vos que fuiste el que me dio las fuerzas necesarias para enfrentar mi último año de doctorado, para mí, el más difícil. Gracias Fran por tu apoyo y compañía diaria; gracias por ponerte en mi lugar y hacerme sentir tan bien cada día y levantarme el ánimo cuando decaía. Gracias mi amor por todo lo que me diste desde Argentina durante este tiempo. ¡Te amo!

Gracias a los tres jefes del laboratorio por su total confianza puesta en mí. Gracias al Dr. Vicente Sanchis, Dr. Antonio J. Ramos y en especial a la Dra. Sonia Marín. Gracias Sonia por brindarme tus conocimientos, por tu buena predisposición, y sobre todo por tu paciencia al momento de la escritura de la tesis. Los espero en a los tres Argentina!!!

Quiero agradecer también a todo el grupo de Microbiología del Departamento de Tecnología de los Alimentos por los buenos momentos compartidos tanto en el ámbito laboral como las juntadas fueras del laboratorio. Gracias Bernarda, Esther, Amani, Cyndia, Ana, Nuria, Jordi, Arnau, Germán y Montse por la buena onda y por estar presente en todo momento.

Montse, te nombro nuevamente porque te merecés un agradecimiento aparte. Gracias por toda la ayuda que me diste en éstos cuatro años. Gracias por acompañarme en las cosas buenas y en las no tan buenas que sólo vos y yo sabemos. Gracias a vos y a toda tu familia por haberme "adoptado" como una más. Te voy a extrañar mucho, pero te espero en mí país así te llevo de recorrida.

Al personal del grupo de antioxidantes Alba, María, Xenia, Laura, Aida y Manuel que hicieron que cada día sea un buen día, poniendo pilas para poder vivirlo y trabajarlo.

Agradecer en particular a Aida y Germán, mis compañeros de trabajo con los que emprendimos juntos este camino del doctorado y compartimos horas y horas en la sala de análisis, que entre charlas y risas de por medio, hicieron que el trabajo sea más ameno.

Nuevamente agradecer a Aida por su amistar y por abrirme las puertas de su casa y familia, ya sea para compartir una tarde normal, en familia o unas Navidades. Fueron 4 años trabajando en el mismo lugar y me llevo muchos recuerdos… sobre todo los viajecitos ;-) ¡Gracias amiga!. Ahora ya sé a donde van a ser mis próximas vacaciones!!!

Al grupo de investigación de Micología del departamento de Microbiología e Inmunología de la Universidad Nacional de Río Cuarto, especialmente a Sofía Chulze y Germán Barros que desde que empecé la carrera de microbióloga estuvieron presentes. Gracias además por permitirme realizar la estancia de investigación durante marzo-abril de 2010. Gracias Germán por toda tu ayuda y por la buena onda que me tiraste desde mis comienzos del doctorado.

Summary

Filamentous moulds may cause spoilage in raw materials, foods and feeds. Some of them synthesize mycotoxins which are a risk for human and animal health. From the food safety point of view, regarding foodborne moulds only mycotoxins, as chemical hazards, are relevant. Nevertheless, despite the absence of a direct correlation between mould growth and mycotoxins production, the prevention of fungal growth in raw materials and foods leads invariably to the prevention of mycotoxins presence.

Due to the fact that moulds can contaminate foods from raw materials till end products, different strategies could be used at the different steps in the food chain. Preharvest strategies include the use of resistant varieties, crop rotation, soil preparation, optimal irrigation, fertilizer, herbicides, insecticides and chemical and biological agents application. Post-harvest strategies include improved drying and storage conditions, together with the use of natural and chemical agents. Predictive models may be used as a strategy to predict and prevent mycotoxigenjc fungal growth and mycotoxins accumulation.

The present PhD work focused in two main strategies:

a) The use of antifungals of natural origin to prevent mycotoxigenic fungi and mycotoxins.

Equisetum arvense and *Stevia rebaudiana* extracts were analized as possible natural agents to inhibit growth and mycotoxin accumulation in *in vitro* and *in vivo* experiments. Both extracts were effective against mycotoxigenic moulds and the mycotoxigenic *Aspergillus* and *Fusarium* isolates studied were completely inhibited by *E. arvense* extract at 3%. However, the effect decreased in the *in vivo* test. In the last case, *Equisetum* was effective against *Aspergillus* section *Flavi* and *Fusarium* section *Liseola* growth at high water activity levels and with high infection levels, but mycotoxins levels were not significantly affected.

b) The assessment of the usefulness of predictive models to manage the mycotoxin problem.

In an initial experiment, four particular points which deserved in depth study to assess the viability of predictive microbiology in the moulds field were identified: 1) models should be developed for longer time periods; 2) food and raw materials prone to mycotoxin contamination are usually stored under marginal conditions for mould growth, thus performance of models should be checked under such conditions; 3) the impact of the inoculum size in the performance of the models; and 4) the impact of the potential intraspecies variability among isolates in prediction performance.

Prediction of time to growth by kinetic models was clearly linked to inoculum size. On the other hand, the performance of predictive models may be compromised under marginal conditions for fungal growth, the higher variability of results under these conditions results in the need for a higher number of replicates required, specifically for kinetic models. Finally, a high intraspecific variability on growth and mycotoxin levels has proven to be wider for the both isolates studied: *A. carbonarius* and *P. expansum*. For this reason, a greater number of strains should be included to develop models under non optimal conditions for both, growth and for mycotoxin production. A matrix was built from which the number of strains and replicates to be planned for new experiments can be assessed for a reliable estimation of growth

parameters and we concluded that increasing the number of strains in an experiment increases the explained variability much more than including further replicates.

Finally, a first attempt was done to model aflatoxins production as a function of growth parameters and time. Aflatoxins accumulation was shown to be better correlated to colony area than either colony diameter or fungal biomass. Luedeking-Piret model was used for this purpose, and reasonable percentages of variability were explained.

To conclude, probability models applied either to mould growth or mycotoxin production might be a valuable tool in food safety management through the food chain.

Resum

Els fongs filamentosos poden causar deteriorament en les matèries primeres, pinsos i diferents aliments, però a més a més, alguns sintetitzen toxines anomenes micotoxines, que són un risc per la salut humana i animal. Des del punt de vista de la seguretat alimentària, només les micotoxines, com perill químic, són rellevants. No obstant això, i tot i l'absència d'una relació directa entre el creixement fúngic i la producció de micotoxines, la prevenció del creixement de fongs als aliments condueix a la prevenció de la presència de micotoxines.

Degut a que els fongs filamentosos poden contaminar els aliments des de la matèria primera fins al producte final, s'hauria d'utilitzar diferents estratègies de prevenció en els diferents pasos de la cadena alimentària. Les estratègies de pre-collita inclouen la utilització de varietats resistents, rotació de cultius, la preparació del sòl, una irrigació òptima, l'ús de fertilitzants, herbicides, insecticides i l'aplicació d'agents químics i biològics. Per altra banda, les estratègies de post-collita inclouen el millorament de les condicions d'assecat i emmagatzematge junt amb la utilització d'agents químics i biològics. Conjuntament, els models predictius podrien utilitzar-se com una estratègia per la predicció i la prevenció del creixement de fongs i l'acumulació de micotoxines.

Aquesta tesi ha estat enfocada en dos principals estratègies pel control del desenvolupament fúngic:

a) L'ús d'antifúngics d'origen natural per la prevenció de fongs i micotoxines.

Dos extractes vegetals, d'*Equisetum arvense* i *Stevia rebaudiana,* van ser estudiats com a possibles agents inhibidors del creixement de fongs toxigènics i les seves corresponents micotoxines. Les soques estudiades de *Aspergillus* i *Fusarium* van ser completament inhibides i*n vitro* per l'extracte de *E. arvense* al 3 %. No obstant això, aquest efecte es va veure disminuït en l'estudi *in vivo* en llavors de blat de moro, on *E. arvense* va ser efectiu en la inhibició del creixement d'*Aspegillus* secció *Flavi* i *Fusarium* secció *Liseola* amb els majors nivells d'activitat d'aigua provats i pels majors nivells d'infecció. Tot i això, els nivells de micotoxines no van ser significativament afectats.

b) L'avaluació de la utilitat dels models predictius per al maneig del problema de les micotoxines

En un treball inicial van ser identificats quatre punts específics que mereixen un estudi en profunditat per avaluar la viabilitat de la microbiologia predictiva en el camp dels fongs, i concretament en l'emmagatzematge de matèries primeres i aliments: 1) els models predictius haurien de ser útils per a predir durant llargs períodes de temps; 2) els aliments i les matèries primeres propensos a la contaminació per micotoxines, en general, estan emmagatzemats sota condicions marginals per al creixement fúngic; d'aquesta manera el desenvolupament dels models s'haurien de verificar sota aquestes condicions; 3) l'impacte del tamany de l'inòcul podria ser tingut en compte en el desenvolupament dels models; i 4) l'impacte de la potencial variabilitat intra-espècies en la predicció entre aïllats de la mateixa soca.

La predicció del temps fins a l'inici del creixement fúnigic per models cinètics va ser clarament influïda pel tamany d'inòcul. D'altra banda, les prediccions van mostrar ser menys acurades sota les condicions marginals estudiades per al creixement fúngic; la gran variabilitat dels resultats sota aquestes condicions

porta com a conseqüència la necessitat de la utilització d'un gran nombre de repeticions, especialment per als models cinètics. Per últim, es va demostrar una gran variabilitat intra-especifíca en els paràmetres cinètics del creixement i els nivells de micotoxines per les soques estudiades: *A. carbonarius* i *P. expansum*. Per aquest motiu, un gran nombre de soques haurien de ser incloses per al desenvolupament de models tant com pel creixement fúngic com per la producció de micotoxines; l'augment del nombre de soques en un experiment augmenta la variabilitat explicada molt més que incloent-hi més repeticions.

Finalment, es va fer un primer intent de modelització per la producció d'aflatoxines en funció dels paràmetres de creixement i el temps. En aquest experiment es va demostrar que l'acumulació d'aflatoxines es correlaciona millor amb l'àrea de la colònia, que amb el diàmetre o la biomassa. Per al model de la producció d'aflatoxines es va utilitzar el model de Luedeking-Piret, obtenint raonables percentatges de variabilitat explicada.

Per concloure, els models de probabilitat aplicats en aquesta tesi, ja sigui per al creixement de fongs o per la producció de micotoxines, pot ser una eina valuosa en la gestió de la seguretat alimentària al llarg de la cadena alimentària.

Resumen

Los hongos filamentosos pueden causar deterioro en las materias primas, piensos y alimentos varios pero además, algunos sintetizan toxinas llamadas micotoxinas las cuales son un riesgo para la salud humana y animal. Desde el punto de vista de la seguridad alimentaria, sólo las micotoxinas, como peligro químico, son relevantes. Sin embargo, pese a la ausencia de una relación directa entre el crecimiento fúngico y la producción de micotoxinas, la prevención del crecimiento de mohos en los alimentos conduce a la prevención de la presencia de micotoxinas.

Debido a que los hongos filamentosos pueden contaminar los alimentos desde las materias primas hasta su producto final, se deberían emplear diferentes estrategias de prevención en los distintos pasos de la cadena alimentaria. Las estrategias de pre-cosecha incluyen el uso de variedades de resistencia, rotación del cultivo, la preparación del suelo, una irrigación óptima, el uso de fertilizantes, herbicidas, insecticidas y la aplicación de agentes químicos y biológicos. Por otro lado, las estrategias de pos-cosecha incluyen el mejoramiento de las condiciones de secado y almacenamiento junto con el uso de agentes químicos y naturales. Conjuntamente, los modelos predictivos podrían utilizarse como una estrategia para la predicción y prevención del crecimiento de mohos y la acumulación de micotoxinas.

Esta tesis fue enfocada en dos principales estrategias de control del desarrollo fúngico:

a) El uso de antifúngicos de origen natural para la prevención de mohos y micotoxinas.

Dos extractos vegetales, de *Equisetum arvense* y *Stevia rebaudiana*, fueron estudiados como posibles agentes inhibidores del crecimiento de hongos toxigénicos y sus correspondientes micotoxinas. Las cepas estudiadas de *Aspergillus* y *Fusarium* fueron completamente inhibidas *in vitro* por el extracto de *E. arvense* al 3%. Sin embargo, éste efecto se vio disminuido en el estudio *in vivo* en semillas de maíz, donde *E. arvense* fue efectivo en la inhibición del crecimiento de *Aspergillus* sección *Flavi* y *Fusarium* sección *Liseola* a los mayores niveles de actividad de agua ensayados y para los mayores niveles de infección. A pesar de esto, los niveles de micotoxinas no fueron significativamente afectados.

b) La evaluación de la utilidad de los modelos predictivos para el manejo del problema de las micotoxinas.

En un trabajo inicial fueron identificados cuatro puntos específicos que merecen un estudio en profundidad para evaluar la viabilidad de la microbiología predictiva en el campo de los mohos, y en concreto en el almacenamiento de materias primas y alimentos: 1) los modelos predictivos deberían ser útiles para predecir en largos períodos de tiempo; 2) los alimentos y las materias primas propensos a la contaminación por micotoxinas generalmente, están almacenadas bajo condiciones marginales para el crecimiento fúngico; de esta forma el desarrollo de los modelos debería chequearse bajo dichas condiciones; 3) el impacto del tamaño del inóculo podría tenerse en cuenta en el desarrollo de los modelos; y 4) el impacto de la potencial variabilidad intra-especie en las predicciones entre aislados de una misma cepa.

La predicción del tiempo hasta el inicio del crecimiento mediante modelos cinéticos estuvo claramente influenciado por el tamaño del inóculo. Por otro lado, los modelos de predicción estuvieron

comprometidos bajo las condiciones marginales estudiadas para el crecimiento fúngico; la gran variabilidad de los resultados bajo dichas condiciones trae como consecuencia la necesidad de la utilización de un gran número repeticiones, especialmente para los modelos cinéticos. Por último, se demostró una gran variabilidad intra-específica en el crecimiento y los niveles de micotoxinas para las cepas estudiadas: *A. carbonarius* y *P. expansum*. Por éste motivo, un gran número de cepas deberían incluirse para el desarrollo de modelos tanto como para el crecimiento fúngico como para la producción de micotoxinas; el aumento del número de cepas en un experimento aumenta la variabilidad explicada mucho más que incluyendo más repeticiones.

Finalmente, se hizo un primer intento de modelización para la producción de aflatoxinas en función de los parámetros de crecimiento y el tiempo. En dicho experimento se demostró que la acumulación de aflatoxinas se correlacionada mejor con el área de la colonia, que con el diámetro o la biomasa. Para el modelo de la producción aflatoxinas se utilizó el modelo de Luedeking-Piret, obteniéndose razonables porcentajes de variabilidad explicada.

Para concluir, los modelos de probabilidad aplicados en ésta tesis, ya sea para el crecimiento de moho o para la producción de micotoxinas, pueden ser una herramienta valiosa en la gestión de la seguridad alimentaria a través de la cadena alimentaria.

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Nomenclature section

a_w, water activity.

$a_{w\ min}$, a_w below which no growth occurs.

$a_{w\ max}$, a_w over which no growth occurs.

$a_{w\ opt}$, a_w at which μ_{max} is optimal.

μ_{max}, maximum specific growth rate (it is defined as the slope of the growth curve at the point of inflexion) (mm/d, μm/h).

μ_{opt}, maximum growth rate at optimal conditions (mm/d, μm/h).

λ, lag phase or time to growth (defined as the intersection of the line defining the maximum specific growth rate with the x axis) (d, h).

pH_{min}, pH below which no growth occurs.

pH_{max}, pH over which no growth occurs.

pH_{opt}, pH at which the μ_{max} is optimal.

t, time (d, h).

T, temperature (ºC)

T_{min}, temperature below which no growth occurs (ºC)

T_{max}, temperature over which no growth occurs (ºC)

T_{opt}, temperature at which μ_{max} is optimal (ºC)

y_0 or D_o initial colony diameter (or radius), usually zero (μm, mm)

y or D colony diameter (or radius) (μm, mm)

y_{max} or D_{max} maximum colony diameter (or radius) attained, asymptotic value (μm, mm)

1. Introduction

Introduction

Mycotoxins are defined as "fungal metabolites that when ingested, inhaled or absorbed through the skin cause illness to human and animal death" (Pitt, 1996). According to mycotoxins and intake, they can cause autoimmune illnesses, metabolic and biochemical deficiencies; have allergenic properties, reduce reproductive efficiency, and some of them are teratogenic, carcinogenic, mutagenic, and can cause dead. About 400 different mycotoxins have been identified, but only around 20 synthesized by different fungal species, are important as natural contaminants in agricultural products. A high number of filamentous moulds have the capability to produce mycotoxins; however the most important producer genera are *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* (Sidhu, 2002). These moulds have worldwide distribution and they are found in a wide range of environments due to their capacity to utilize a variety of substrates and their relative tolerance to low pH, low water activity (a_w) and low temperature (Atanda et al., 2011; Huis in't Veld, 1996). FAO estimates that around a 25% of world's crops are annually affected by mycotoxins, especially those produced by *Fusarium*, *Aspergillus* and *Penicillum* (FAO, 2004).

Mycotoxins can be produced in pre-harvest and post-harvest, during food and feeds production. Many mycotoxins can contaminate different products and have been detected in a wide variety of vegetable products (rice, wheat, rye, barley, corn, soybeans, sorghum, nuts, spices), in foods and beverages (fruit juices and puree, beer and wine), feed and animal products (dairy, meat, eggs, etc.) being involved in human and animal diseases (Table 1) (FAO, 2004).

Table 1. The most relevant mycotoxins in human and animal health.

Fungal genera	Mycotoxins	Substrate
Aspergillus	Aflatoxins	Peanut, corn, cotton, nuts, soya beans, dairy products, spices, other foods
	Cyclopiazonic acid	Peanut, sunflower
	Sterigmatocystin	Coffee, wheat, cheeses
	Ochratoxin A	Wheat, barley, corn, beans, peanuts, wine, grapes, spices
Penicillium	Patulin	Apples and their derivates
	Ochratoxin A	Cheeses, nuts
	Citrinin	Wheat, barley, corn, rice
	Cyclopiazonic acid	Peanuts, corn
	Penicílic acid	Stored corn, dried beans
Fusarium	T-2 y HT-2 toxins	Wheat, corn, soya beans, animal feeds
	Deoxynivalenol	Wheat, corn, soya beans, animal feeds
	Nivalenol	Wheat, corn, soya beans, animal feeds
	Zearalenone	Corn, animal feeds, soya beans, wheat
	Diacetoxyscirpenol	Wheat, corn, animal feeds
	Fumonisins	Corn, soya beans
Alternaria	Tenuazonic acid	Sunflower, apples, tomatoes
	Alternariol	Sorghum, barley, apples, tomatoes
	Alternariol monomethyl ether	Sunflower, apples, tomatoes
Claviceps	Ergot alkaloids	Rye, wheat

Based on Barros et al. (2008; 2011); CAST (2003); Geisen (1998); Hasan (1996); Viñas et al. (1992).

Besides health problems, presence of mycotoxins in raw materials and food causes important economic losses to both exporting and importing countries, because tolerances allowed in international trade have declined in the last few years.

On the other hand, under the agreement of the World Trade Organization (WTO) about Application of Sanitary and Phytosanitary (agreement MSF), health and safety requirements for food should be based on scientific risk assessment (FAO, 2004). From the food safety point of view, only mycotoxins (as chemical hazards) are important, while moulds and yeast themselves may cause spoilage but have no safety implications. However, presence of a mycotoxigenic mould in foods and feeds may indicate a risk to found mycotoxins (Pitt and Hocking, 1997).

Maximum tolerable levels of dangerous mycotoxins are being set, and an effective management of food security must achieve a secure food supply, therefore should be arise important challenges for responsible authorities of safe food in each nation (FAO, 2006). European legislation aims to harmonize the maximum tolerance levels of mycotoxins in food and feed for all European Union countries, and the requirements of the sampling and analytical techniques, committing all countries to follow the common rules. Besides, worldwide efforts are also underway to establish common guidelines for determining levels of tolerance and methods of control of mycotoxins (Santos et al., 2011).

In general, mycotoxigenic moulds are not aggressive pathogens, but some species can invade and colonize plant tissues synthesizing mycotoxins during cultivation, harvesting, drying, transport, processing and storage (Moss, 1992; Heathcote and Hibbert, 1978). Nevetherless, there are two main reasons whereby control of mycotoxins should be exerted early:

1.- Mycotoxins are stable metabolites and are difficult to remove once formed. Generally are resistent to many processes in the food industry and during the processes, the levels may decrease but they are not completely eliminated.

2.- Mycotoxin analyses are usually complex, expensive and time-consuming, so quality control for mycotoxins is costly.

Moreover, there is no a direct correlation between moulds and their mycotoxins levels in food and feeds; therefore, analysis of fungal levels is not a good indicator of toxin contamination. However, prevention of fungal growth in raw materials and foods leads invariably to prevention of mycotoxins presence.

1.1 Prevention of development of mycotoxigenic fungi

Filamentous moulds are found around the world and they can colonize different ecological niches such soil or decaying materials or can live in symbiosis with both, plants and animals. When a mould grows in

a substrate, according to the toxicological potential of the strain, there is a risk to found mycotoxin contamination (Morales-Valle, 2011). Due to the fact that moulds can contaminate foods and raw materials till end products, any prevention strategy should integrate all the steps in food production (Jouany, 2007), including pre and post-harvest, storage of raw materials and their later processing, storage and transport.

1.1.a Prevention of fungal and mycotoxin contamination in primary production
Mycotoxin accumulation in crops and food products is increasing worldwide due to different factors that affect cereal crops, as climate change, use of plant varieties of high yield but which are susceptible to mycotoxin accumulation, and agricultural practices. Codex Alimentarius has developed some codes for prevention and reduction of mycotoxin in cereals, nuts, fresh fruit, and other raw materials. The elaboration and acceptance of a General Code of Practice by Codex will provide uniform guidance for all countries to consider in attempting to control and manage contamination by various mycotoxins (Kabak et al., 2006). Recommendations for the reduction of various mycotoxins in cereals are practices based on Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP). Control strategies before harvest include:

a) A very common strategy in cereal crops is the use of **resistant varieties** to fungal infections and pests, although it should be noted that varieties which are resistant in a geographical area may not be resistant in other parts of the world (Codex Alimentarius Commission, 2002). This is probably due to differences in the genetic pool within each country's, agronomic techniques and different environmental conditions (Edwards, 2004). Resistant wheat varieties to *Fusarium* Head Blight (FHB) showed a decreased or no production of deoxynivalenol (DON) (Bai et al., 2001; Lehoczki-Krsjak et al., 2010; Tekauz, 2002).
Actually there is interest in obtaining genetically modified seeds in order to improve their resistance to fungal contamination. These resistant varieties prevent mycotoxins accumulation and also avoid contamination by other microorganisms such as bacteria and viruses, thus reducing economic losses for the producer. However, problems in this regard may be the lack of resistant control genotypes and lack of involvement of single major genes (Cary et al., 2011; Munkvold, 2003).

b) **Crop rotation** is also important to prevent fungal contamination in cereals, because it focuses on the idea of breaking the chain of infectious material. Incidence of FHB and DON contamination on wheat grain was higher when corn was the preceeding crop; however, decreased in crops following soya bean (Caron, 1993; Cassini, 1970, 1973; Dill-Macky and Jones; 2000; Edwards, 2004; Rapilly et al., 1973; Teich and Hamilton. 1985; Teich and Nelson, 1984; Vogelgsang et al., 2011). In addition, other crops such linen are not host plants of *Fusarium*, thus can be used as "cleaning crop" (Caron, 1993; Manuca, 1969). Codex recommends that crops such as potatoes, alfalfa, among others, which are not hosts to

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Fusarium species, should be used in crop rotation to reduce inoculum levels of this fungus in the field (Codex Alimentarius Commission, 2002).

Continuos cultivation of peanuts can produce a high population of *Aspergillus flavus/A. parasiticus* in the soil which increases the probability of infection in plants and the consequent production of aflatoxins (Kabak et al., 2006). However, in some environments *Aspergillus* levels can be very high, so rotation crop can become ineffective, and other actions may be required (Codex Alimentarius Commission, 2004).

c) **Soil preparation** before seeding time is also important for prevention of fungal contamination, as any crop management that results in removal, destruction or burial of previous crop residues (infected or not), probably conduces to a decrease in the level of fungal inoculum (Kabak et al., 2006). In ploughing soil, the top of soil is inverted so crop debris of the before culture is mixed with the first 10-30 cm of the superficial land. Some authors concluded that a decrease in crop debris on the soil surface (i.e. after till) helps to decrese inoculum production and mould spores dispersal (Bateman et al., 1998; Cassini, 1970; Jalaluddin and Jenkyn, 1996; McMullen et al., 1997; Miller et al., 1998; Teich and Hamilton, 1985). Dill-Macky and Jones (2000) reported that no till (direct drilling) after wheat or maize cultivation, significantly increase DON contamination of the following wheat crop compared to ploughing. Besides, in Mediterranean climates it is a good practice to leave ploughed land exposed to autumn sunshine as a means of destroying fungal material that could otherwise infect the following crop (Nicholson et al. 2003). In nuts such as peanuts, the type of soil affects fungal infection. For example, sandy soils favour rapid water drainage and mould growth, especially under dry conditions. As clay soils have a higher water holding capability, probability of plant water stress decreases, which may result on reduction of aflatoxin levels in peanuts grown in these soils.

d) An optimal **irrigation** of plants avoids water stress, being less susceptible to fungal development (Codex Alimentarius Commission, 2004; Kabak et al., 2006). Jones et al. (1981) found the lowest levels of *A. flavus* and aflatoxin in irrigated plants of maize. These differences were also observed by Jones et al. (1981) comparing years with lower rainfall with years where rainfall was abundant. However, this difference was not observed for the levels fumonisins produced by *F. verticillioides* (Bruns, 2003). On the other hand, high rainfalls during flowering period provide good conditions for dissemination and infection of plants with *Aspergillus* and *Fusarium* species. Therefore, it is important to avoid irrigation during flowering and ripening period in wheat, barley and rye crops (Codex Alimentarius Commission, 2002; Cotty and Jaime-García; 2007).

e) Soil must be tested to determine if **fertilizer application** is required, because some reports showed that increasing nitrogen level significantly increased the incidence of pathogenic fungi in cereal crops (Martin et al., 1991). Severity and incidence of FHB can be affected by the different fertilization regimes

used (Lemmens et al., 2004; Martin et al., 1991; Suproniene et al., 2011). Lemmens et al. (2004) and Martin et al. (1991) observed a significant increase in the incidence of *Fusarium*, FHB and DON levels in wheat and barley with the increase of nitrogen (70 to 170 and 0 to 80 kg/ha, respectively). However, studies in maize showed higher levels of aflatoxin in grains grown in soil with lower levels of nitrogen (80 kg/ha compared with 120 kg/ha) (Anderson et al., 1975); but there are no differences between aflatoxin and fumonisin levels when nitrogen levels are over 120 kg/ha in maize (Bruns and Abbas, 2005ab). Finally, other studies concluded that nitrogen fertilization decreases the contamination by strains of *Botrytis cinerea* in tomatoes (Lecompte et al., 2010). Besides, lack of nutrients in soil could stress cereal and nut crops, becoming susceptible to future contaminations (Codex Alimentarius Commission, 2004).

f) **Herbicides** use for weed control is important in preventing of various contaminations, because they are a source of toxigenic fungal inoculum (Chulze et al., 2006; Ponsone et al., 2007). A reduction of *Sclerotinia sclerotiorum* and increasing in yield of various cereal seeds was observed due to elimination of weeds (Pynenburg et al., 2011). Ponsone et al. (2007) observed many fungal species in weeds, including ochratoxigenic species in vineyards from Argentina. For this, it is important to remove these weeds for prevention of future contamination of raw materials with mycotoxin-producing moulds.

g) Elimination of **insects** in the field is also important, because insects can carry fungal spores, or wound seeds and fruits making them susceptible to future contaminations. Insect damage and aflatoxin content are positively correlated in maize, peanuts and nuts, increasing their levels before harvest (Catangui and Berg, 2006; Garman and Jewett 1914; Gradziel et al., 1995; Hesseltine et al., 1976; Lillehoj et al., 1975, 1976, 1980; Lynch and Wilson, 1991; Riley, 1882; Wilson et al., 1981). On the other side, patulin production in apples or pears occurs due to mechanical damage by insects and pests, so fruits are susceptible to be contaminated (Kabak et al., 2006).

h) Use of **chemical and biological agents** is other strategy to prevent fungal contamination in the field. There are a variety of fungicides in the market, but evidence of their efficacy against mycotoxigenic fungi in the field is limited. This may be due to their effect on other species, saprophytic or pathogenic, that could act as biological control agents through competition for nutrients (Dalcero et al., 2011).
A great number of chemical compounds have been described as chemical inhibitors of aflatoxigenic moulds and, hence, of aflatoxin levels (Moss, 1992; Ozkaya et al., 1999). While many researches suggested that fungicides may be useful for control of moulds and their mycotoxins in field (Delage et al., 2003; D'Mello et al., 1998; Leyronas et al., 2006), others claim that they are ineffective in the control of mycotoxins of *Fusarium* and *Aspergillus* species, and even stimulate the production (Dalcero et al., 2011; Kabak et al., 2006; Simpson et al., 2001; Wheeler, 1991). No-toxigenic biological agents are applied to compete with the mycotoxin-producing mycobiota, preventing crops contamination. It has been shown

that introduction of non-aflatoxigenic *A. flavus*/*A. parasiticus* strains into the field reduce aflatoxin contamination in maize, peanuts, rice and cotton seeds. This is due to substrate competition and production of some metabolites able to inhibit toxigenic microorganisms (Bhatnagar et al., 2004; Brown et al., 1991; Cleveland et al., 2003; Cotty and Bhatnagar, 1994; Dorner et al., 1992, 1999; Horn et al., 2000; JECFA, 2001). There is a limited number of microorganisms biocompetitive for *Fusarium* contamination in agricultural products (Kabak et al., 2006). Some works have shown a decrease of some *Fusarium* mycotoxins using some bacteria and yeast. For example, *Bacillus subtilis* and *Cryptococcus nodaensis* reduce growth and mycotoxin contamination of this genus (Cleveland et al., 2003).

Finally, environmental conditions as relative humidity, rainfall and temperature have an important effect in fungal development and mycotoxin production in field, but wheather is a not controllable factor in crops. High temperatures are probably the most important factor which influence in *A. flavus* infection and aflatoxin contamination in maize before harvest (Fortnum, 1986; Jones et al., 1980); this was confirmed by Manwiller and Fortnum (1979) and Williams et al. (2003). In non-irrigated peanuts, moisture control is important because drought periods during plant development induce water stress to plants, and if occurs during ripening, increases the susceptibility of invasion and mycotoxin production by *A. flavus* and *A. parasíticus* (Cotty and Bhatnagar, 1994; Dorner et al., 2003; Horn et al., 2000; Moss, 1992). On the other hand, high moisture levels occurring at flowering favour contamination by *Fusarium* species in wheat (Aldred and Magan, 2004; Lacey et al., 1999). For this reason, for the prevention of mycotoxin accumulation a key point in grains production is a good planning of seeding and harvest, to achieve low moisture content and optimum degree of maturity (Kabak et al., 2006; Sweeney and Dobson, 1998).

Due to climate change, a gradual increase in temperature and changes in atmosphere gases concentrations and humidity in different parts of the world is observed. Consequently, the pattern of mycotoxins occurrence in crops in different parts of the world may change (Magan et al., 2011; Paterson and Lima, 2010; 2011; Tirado et al., 2010). Effects of climate change on mycotoxins produced preharvest depend on mould, host and their interaction (Rusell et al., 2010). Nevertheless, environmental changes may affect in different ways mycotoxigenic fungi; in addition, temperature, humidity and rainfall and the temporal pattern of these parameters are known to have an effect on how toxigenic moulds interact with their plant hosts in producing mycotoxins and the broader biological communities of our agricultural ecosystems (Tirado et al., 2010). Temperature and raifall are probably the most affected climatic factors in the future, and these changes may have a very strong impact on plants and their pathogens (Ingram, 1999), including mycotoxins concentratios (Miraglia et al., 2009). In general, if temperatures rise in temperate and cold areas, they could be susceptible to aflatoxin contamination, while in tropical countries such contamination would not appear (Rusell et al., 2010).

1.1.b Prevention of fungal and mycotoxin contamination during storage and transport of raw materials

Post-harvest strategies are important to prevent mycotoxin contamination and include improved drying and storage conditions, together with the use of natural and chemical agents (Kabak et al., 2006). Cereal seeds and nuts can be stored in bags or silos, constituting the ecosystem of raw materials together with various factors involved in storage (temperature, humidity, atmosphere, oxygen levels). The most common recommendation for decreasing mould contamination after harvest is a rapid drying of seeds, which implies a reduction of a_w and thus a reduced risk of moulds and insect development. Besides, physical separation of infected or damaged grains which present morphological visible defects, also conduce to decreased mould and mycotoxin contamination (Hell et al., 2000; Udoh et al., 1997). Another factor influencing grain storage is temperature (Guerzoni, 2003; Heathcote and Hibbert, 1978). In 2002, Codex Alimentarius Commission established that a temperature rise of 2-3 °C can cause fungal growth and infestation by insects; drying of seeds, a correct storage structure protected from rain, soil moisture and rodents, and with minimum temperature fluctuation, was recommended.

For fresh fruits storage at low temperatures is necessary and cooling should be applied quickly to avoid growth of decay-causing agents (Kaback et al., 2006). Besides, different studies have shown that controlled atmospheres reduce fungal growth and patulin accumulation by *Penicillium expansum* on apples and pears (Codex Alimentarius Commission, 2002; Dock et al., 1998; Hoogerwerf et al., 2002; Jackson et al., 2003; Moodley et al., 2002).

Control of fungal growth in raw materials is also possible with the use of fungicides and preservatives or with natural inhibitors. There are a large number of fungicides in the market based on organic acids such as sorbic or propionic acid (Dalcero et al., 2011). But if their application is not performed at the correct dose, moulds can grow, especially mycotoxin producers which metabolize these aliphatic acids (Magan and Aldred, 2007). Besides, as chemical products, they should be used with caution to prevent damage to human and animal health and environment. Moreover, continuous and indiscriminate use of chemical preservatives in foods and feeds could lead to toxic effects for consumers and to development of resistances in microorganisms (López-Malo et al., 2002). Therefore, alternative compounds are searched to replace or enhance current fungicides.

1.1.b.1 Control of fungal contamination through the application of natural products

Many studies have suggested that some essential oils and antioxidant compounds possess antifungal activity (Burow et al., 1997; Cairns and Magan, 2003; Fanelli et al, 2003; Hope et al., 2003, 2005, Hua et al., 1999; Huang et al., 1997; Norton, 1997; Romero et al., 2010). Various works have evaluated the antimicrobial effect of hydro-alcoholic plant extracts on mycotoxigenic moulds (Kukic et al, 2008;

Milanovic et al. 2007) and essential oils (Benkeblia, 2004; López et al., 2004; Rasooli and Owlia, 2005). Many extracts also were studied as inhibitors of mycotoxin production (Nguefack et al., 2004; Soliman and Badeaa et al., 2002). Besides, some phenolic compounds have been detected as inhibitors of aflatoxin production. For example, a decrease of 96%, 74% and 32% on aflatoxin biosynthesis in presence of acetosyringone, syringaldehide and sinapinic acid respectively (4 mM) has been described. Furthermore, natural plant compounds such as the anthraquinones, coumarins and flavonoids can be highly inhibitory in the biotransformation process from aflatoxin B_1 to aflatoxin B_1-8-9-epoxide (Hua et al., 1999). Thus, plant extracts could be an alternative to chemical products for fungal prevention, because they are biodegradable products, leave no residue contaminants in the environment and, their natural origin stimulates their use. Some plant extracts contain antioxidant compounds as poliphenols (flavonoids and phenolic acids, etc.) and others such as terpenes, currently very popular for their effect in human health. Some authors concluded that these compounds could be the basis for the antimicrobial effects of plant extracts (Ebana and Madunagu, 1993). Inhibitory mechanism of action of some vegetable essential oils againt moulds is due to modification in cytoplasm, inhibiting some of its functions, cytoplasmic membrane rupture and inactivation and/or inhibition of intracellular synthesis of enzymes (Srivastava et al., 2008). These effects can occur alone or simultaneously causing the inhibition of spore germination. For this, plant extracts with antimicrobial properties could be used to control mycotoxigenic moulds in raw materials and foods, replacing the use of synthetic chemicals.

For many years, herbs have been used in medicine and nutrition as insect repellents, food preservatives or fragrances; this previous knowledge can be applied for the selection of plant materials to investigate possible antimicrobials. There is no unanimity among the authors consulted regarding to the part of plant to be used for obtaining the extract for use as antifungal. Some of them selected only aerial parts without floral organs (Radulovic et al., 2006), others used the entire plant including the bracts (Kukic et al., 2008), and some of them only bulbs (Benkeblia, 2004), and others did not list which tissue or organ was collected to obtain the extract.

Stevia spp. and *Equisetum* spp. have been described as herbs with antioxidant properties by different studies (Canadanovic- Brunet et al., 2009; Milanovic et al., 2007; Nagai et al., 2005; Radulovic et al., 2006). Species of these plant genera are consumed in different countries as "healthy ingredients" but they could also be useful for the prevention of mould growth and mycotoxin production due to their antioxidant compounds. Currently there are several publications describing the effect produced by several plant extracts on fungal growth (Table 2). However, there are few works in the literature regarding the antifungal effect of extracts of *Equisetum arvense* (Canadanovic-Brunet et al., 2009; Milanovic et al., 2007; Nagai et al., 2005) and there are no researches with *Stevia rebaudiana*.

E. arvense L. (Equisetaceae, subgenus *Equisetum*) is a pteridophyte plant widely distributed in the northern hemisphere, popularly known as "horsetail." Infusions of *E. arvense* have diuretic (Graefe and Veit, 1999), antioxidant (Trouillas et al., 2003), vasorelaxant (Sakurai et al., 2003) and antiinflammatory effects (Martins et al., 2004). Radulovic et al. (2006) characterized composition of *E. arvense* essential oil by chromatographic techniques, and they identified 25 compounds. The main constituents were hexahidrofarnesil acetone (18.34%), cisgeraniol acetone (13.74%), thymol (12.09%) and trans-phytol (10.06%). Milanovic et al. (2007) quantified and characterized phenolic content in 5 hydro-alcoholic extracts of different species of *Equisetum* obtaining differences in flavonoids, in total phenol concentration and antioxidant capability of each species; although there was no correlation between phenol concentration and antioxidant capacity. In both studies, *E. arvense* showed antimicrobial activity similar to that obtained with conventional antibiotics and also inhibited spore germination (Hiraga et al., 1997).

Furthermore, *S. rebaudiana* also known as "sweet herb"; is a semiperennial shrub native to northeastern Paraguay which propagates naturally. Economical importance of this herb is due to a mix of eight diterpene glycosides (which are mainly stevioside and rebaudioside) located in the leaves. It is from 100 to 400 times sweeter than sucrose and for its physico-chemical and toxicological features it can be included in the human diet as a natural dietary sweetener. Some authors have determined the concentration of phenolic compounds and antioxidant activity in alcoholic extracts of this *Stevia* species (Ghanta et al., 2007; Shukla et al., 2009).

Many studies analyzed antioxidant compounds on *E. arvense* and *S. rebaudiana* hydro-alcoholic extracts or essential oils (Table 3). In fact, some phenolic acids and flavonoids present in the extracts have been studied as antimicrobials and they showed antifungal and antibacterial activity (Cushnie and Lamb, 2005; Romero et al., 2010; Merkl et al, 2010). On the other hand, Curry et al. (2008) and Tago et al. (2010) studied *S. rebaudiana* and *E. arvense* toxicity and they were not associated with any sign of clinical toxicity or adverse effects. Thus, both herbs could be considered in the future as possible natural antifungals.

Table 2. Common eseential oils and hydro-alcoholic extracts used against mycotoxigenic moulds

Essential oil of	Commonly from (plant species)	Majoritary components	References where used
Thyme	*Thymus vulgaris*	Thymol, carvacrol, linalool	Montes-Belmont and Carvajal, 1998; Soliman and Badeaa, 2002.
Origano	*Origanum vulgare*	Carvacrol, thymol	López et al., 2004; Marín et al., 2003; 2004; Souza et al., 2007; Velluti et al., 2003.
Cinnamon	*Cinnamomum zeylanicum*	Eugenol, cinnamaldehyde, caryophyllene	Marín et al., 2003; 2004; Montes-Belmont e al., 1998; Soliman y Badeaa, 2002; Velluti et al., 2003.
Mustard	*Brassica hirta, B. juncea, B. nigra, B rapa*	Allyl isothiocyanate	Dhingra et al., 2009; Mari et al., 2002; Nielsen and Rios, 2000.
Clove	*Syzygium aromaticum*	Eugenol, caryophyllene	Awuah and Ellis, 2002; Bluma and Etcheverry, 2008; Marín et al., 2003; 2004; Matan et al., 2006; Montes-Belmont y Carvajal, 1998; Nielsen y Rios, 2000; Reddy et al., 2009; Velluti et al., 2003.
Lemongrass	*Cymbopongon citratus*	Geranial, neral	Adegoke y Odesola, 1996; Dubey et al., 2000; Fandohan et al., 2004; Marín et al., 2003; 2004; Somda et al., 2007; Souza et al., 2007; Velluti et al., 2003; 2004.
Basil	*Ocimum basilicum*	Thymol	Atanda et al., 2007; Fandohan et al., 2004; Montes-Belmont and Carvajal, 1998; Soliman and Badeaa, 2002;
Neem	*Azadirachta indica*	Hexadecanoic acid, oleic acid	Fandohan et al., 2004; Montes-Belmont y Carvajal, 1998; Owolade et al., 2000; Reddy et al., 2009; Somda et al., 2007;
Shiraz thyme	*Zataria multiflora*	Carvacrol	Gandomi et al., 2009
Onion	*Allium cepa*	Organosulfur	Benkeblia et al., 2004
Garlic	*Allium sativum*	Organosulfur	Benkeblia et al., 2004
Mexican tea	*Chenopodium ambrosoides*	Ascaridole, p-cymene, α-terpirene, isoascaridole, limonene	Kumar et al., 2007
Rosemary	*Rosmarinus officinalis*	Piperitone α-terpirene, limonene cineole	Rasooli et al., 2008
Ajowan bishop´s Weeds	*Trachyspermum copticum*	Thymol, p-cimene, α-terpirene,	Rasooli et al., 2008
Horsetail	*Equisetum arvense*	Thymol, linanol 1.8-cineol	Radulovic et al., 2006
Wild artichoke	*Cynara cardunculus*	Saponins, sterols sesquiterpene lactones, flavones,	Kukic et al., 2008
Agave	*Agave. asperrima A. striata*		Sánchez et al., 2005
Horsetail Family	*Equisetum. arvense, E. sylvaticum E. fluviatile, E. palustre, E. temalteia*	Silicic acid, saponins	Canadonovic-Brunet et al., 2009, Milovanovic et al., 2007

Table 3. Antioxidant compounds of *E. arvense* and *S. rebaudiana* as reported in the existing literature.

	General compound	Specific compound *
E. arvense	Phenolic acids	Apigenin 5-*O*-glucoside
		Methyl esthers of protocatechuic
		5-*O*-caffeoyl shikimic acid
		Monocaffeoyl meso-tartaric acid
		Dicaffeoyl *meso*-tartaric acid
	Flavonoids	Quercetin
		Isoquercetin
		Quercetin 3-*O*-glucoside
		Quercetin 3-*O*-(6''-*O*-malonylglucoside)
		Kaempferol 3-*O*-glycoside
	Terpenes	1,8 cineol
		Linalool
		Thymol
		Camphor
S. rebaudiana	Phenolic acids	Pyrogallol
		4-methoxybenzoic acid p-coumaric acid
		4-methylcatechol
		Sinapic acid
		Cinnamic acid
		Salicylic acid
		Gallic acid
		Protocatechuic acid
		Catechin
		Epicatechin
		Caffeic acid
		Chlorogenic acid
		p-coumaric acid
		Rutin
	Flavonoids	Quercetin
		Quercetin dihydrate
		Quercetin-3-*O*-glucoside
		Quercetin-3-*O*-arabinoside
		Apigenin-4'-*O*-glucoside
		Luteolin-7-*O*-glucoside
		Kaempferol-3-*O*-rhamnoside
		Carvacrol
	Terpenes	α-pinene
		Caryophylle
		Limonene

Compiled from Kim (2011), Milovanovic et al. (2007), Mimica-Dukic (2008), Muanda et al. (2011), Radulovic et al. (2006) and Sandhu et al. (2010)

1.2 Use of predictive models in prevention of fungal contamination

1.2.1 Use of predictive models in prevention of fungal contamination in primary production

There are several predictive models for *Fusarium* contamination in cereal grains based on existing information on weather conditions, kind of cereal, pathogens susceptibility, insect damage, regions or seasons, and other factors which could influence in mould growth and mycotoxins production in field (Battilani et al., 2008; De la Campa et al., 2005; Del Ponte et al., 2005; De Wolf, 2003; Franz et al., 2009; Hooker et al., 2002; Molineros et al., 2005; Moschini, 1996; Rossi et al., 2003). In many models the incidence of diseases caused by toxigenic moulds is predicted but they are not adapted for prediction of mycotoxin levels. For this reason, many researchers have tried to relate severity of disease with existing levels of toxin demonstrating an unreliable prediction of mycotoxin using models from symptoms (Paul et al., 2006). Actually, only a predictive model for DON, built for wheat ("DONcast") has been validated and commercialized (Hooker et al., 2002; Schaafsma and Hooker, 2007).

This kind of models may be useful for the prevention of economic losses and reduction of contamination risks, predicting date of planting, optimal time for antifungal application, final level of contamination and a good organization to the next step: storage of grain. However, these models present some limitations: for a good model a lot of information is required and their results depend on the variety of the crop rotation with other crops, type of soil management (plow or not), geographical area, planting date and climatic conditions. Models developed in a given country reveal a strong dependence for the toxin with respect to working region (Franz et al., 2009).

1.2.2. Use of predictive models for prevention of fungal contamination in storage and transport of raw materials

The need to ensure the microbiological quality and safety of food products has stimulated the interest in the use of mathematical models for quantifying and predicting microbial behaviour (Lahlali et al., 2005). For 20 years, predictive microbiology has been developed for predicting the occurrence of food-borne pathogens, although these tools are dedicated to bacteria (Dantigny et al., 2005). Predictive modelling of filamentous fungal growth has not received the same attention as that of bacterial development. Gibson and Hocking (1997) suggested that this may be because of the inherent complexities associated with the quantification of fungal growth. While bacteria reproduce by fission, and normally grow homogenously through a liquid medium or take place only at surfaces, fungal hyphae can penetrate the physical three-dimensional matrix of food (Dantigny et al., 2005). However, the situation has changed and a growing number of studies are available in the literature dealing with predictive modelling of fungi (Baggerman and Samson, 1988; Bayne and Michener,1979; Belli et al., 2004ab; Casella et al., 1990; Char et al., 2005; Dantigny et al., 2002, 2005, 2006, 2007; Fujikawa and Itoh, 1996; Judet et al., 2008; Lopez et al. 2004;

Marín et al., 1996, 1998, 2006ab; Pardo et al., 2004, 2005a,b,c,d, 2006ab; Parra and Magan et al., 2004; Patriarca et al., 2001; Plaza et al., 2003; Samapundo et al., 2005, 2007; Scott and Bernard, 1987). The employment of models may be useful for decision-making purposes to prevent risks for human and animal health (Prandini et al., 2008). With these models, final levels of mold or mycotoxins could be predicted, constituting a useful tool for the food industry.

Membré and Lambert (2008) summarize some applications of predictive microbiology in three groups related to food safety:

Product innovation:
Assessing rate of microbial proliferation, growth limits, or inactivation rate associated with particular food formulations and/or process conditions in order to develop new products and processes, reformulate existing products, and determine storage conditions and shelf-life.

Operational support:
Supporting food safety decisions that need to be made when implementing or running a food manufacturing operation, such as designing in-factory heating regimes, setting critical control points (CCPs) in HACCP, assessing impact of process deviations on microbiological safety and quality of food products.

Incident support:
Estimation of impact on consumer safety or product quality, in case of problems with products on the market.

Thus predictive models development for growth of mycotoxigenic moulds and environmental condition in which they can grow could give information about storage conditions to prevent the occurrence of mycotoxins (Magan and Aldred, 2007). Generally, these models are developed under constant environmental conditions and therefore cannot be used under field conditions. According to their objectives, predictive models in microbiology can be divided into two main categories: kinetic and probabilistic models

a) Kinetic models
These models determine microbial responses in relation to time and environmental conditions, and provide estimates for parameters of growth. Kinetic models can be classified into:

a.1 Primary models

Primary models describe how the number of microorganisms in a population changes with time under specific conditions (Marks et al., 2007). These models form the basis for developing strategies in the field of food safety. The main primary models used by mycologists are:

<u>Gompertz modify model</u>

This is an exponential function that describes an asymmetrical sigmoidal curve. In the last years some researchers have used Gompertz modified equation by Zwietering et al. (1990), because the parameters have a biological interpretation:

$$y = y_{max} * \exp\left\{-\exp\left[\left(\frac{\mu_{max} * \exp(1)}{y_{max}}\right)(\lambda - t) + 1\right]\right\} \qquad [1]$$

y_{max}, μ_{max} and λ are the estimated growth parameters through the non-linear regression algorithm.

<u>Baranyi model (Baranyi and Roberts, 1994)</u>

This is a widely used model for bacteria. The model assumes that, after a certain adjusting period, the growth rate is constant but an upper asymptote after the exponential phase occurs.

$$y = y_0 + \mu_{max}A - \ln\left\{1 + \frac{[\exp(\mu_{max}A) - 1]}{\exp(y_{max} - y_0)}\right\} \qquad [2]$$

$$A = t + \left(\frac{1}{\mu_{max}}\right)\ln\left[\exp(-\mu_{max}t) + \exp(-\mu_{max}\lambda) - \exp(-\mu_{max}t - \mu_{max}\lambda)\right] \qquad [3]$$

y_0, y_{max}, μ_{max} and λ are the estimated growth parameters

If this triphasic model wants to be converted to a biphasic (lag-linear) one, the last logarithmic term in Eq. [2] may be omitted, and then a growth function without upper asymptote can be obtained (Gibson et al., 1994). In most cases, mycelium is developing on an ever "new" medium (i.e. the peripheral zone of the colony); there is no limitation to the growth of the fungal colony due to substrate (Pirt, 1967). In the absence of lag phase (conditions near to the optimum for growth) it would result in the linear model that has been used for many years by mycologists.

a.2 Secondary models

Secondary models describe the influence of environmental factors on key parameters of the primary model; growth parameters (e.g. maximum growth rate), estimated from primary models are then modelled as a function of intrinsic and extrinsic factors of foods. Two of the most important environmental parameters that determine the ability of moulds for growing in foods are a_w and temperature. Mycotoxin production is also affected by these factors; however, the effect on mycotoxins is different from the effect on growth. Table 4 shows published works with secondary models in moulds.

Ratkowsky model (Ratkowsky et al., 1983)

Originally developed for bacteria:

$$\sqrt{\mu_{max}} = b(T - T_{min})\{1 - \exp[c(T - T_{max})]\} \qquad [4]$$

b, c are estimated constants, as well as T_{min} and T_{max}

Initially this model included only temperature, but it has been applied to other factors such as a_w (Tassou et al., 2007):

$$\sqrt{\mu_{max}} = b(a_w - a_{w min})\{1 - \exp[c(a_w - a_{w max})]\} \qquad [5]$$

Linear Arrhenius-Davey equation (Davey, 1989)

Initially applied to model temperature effect on bacterial growth:

$$\ln \mu_{max} = a_0 + a_1/T + a_2/T^2 \qquad [6]$$

where T is absolute temperature (K). It may be extended to a_w and pH (Panagou et al., 2003):

$$\ln \mu_{max} = a_0 + a_1 a_w + a_2 a_w^2 + a_3 pH + a_4 pH^2 + a_5/T + a_6/T^2 \qquad [7]$$

$a0$, $a1$, $a2$, $a3$, $a4$, $a5$, $a6$, are constants to be estimated

Rosso cardinal model

This model was proposed by Rosso et al. (1993), and takes into account cardinal temperatures (T_{min}, T_{max}, T_{opt}). A great advantage of this model is that all parameters have a physiological meaning which clearly facilitate initial parameter estimations and may also aid in future incorporation into the model of underlying cell biological mechanisms (Brul and Klis, 1999).

$$\mu_{max} = \frac{\mu_{opt}(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})\{(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)\}} \qquad [8]$$

The model was some years latter specifically tested on a data set of existing fungal growth data by Rosso and Robinson (2001), taking into account a_w as factor:

$$\mu_{\max} = \frac{\mu_{opt}(a_w - a_{w(\max)})(a_w - a_{w(\min)})^2}{(a_{w(opt)} - a_{w(\min)})\{(a_{w(opt)} - a_{w(\min)})(a_w - a_{w(opt)}) - (a_{w(opt)} - a_{w(\max)})(a_{w(opt)} + a_{w(\min)} - 2a_w)\}}$$

[9]

Gibson model (Gibson et al., 1994)

This model deserves a special mention because it was the first one developed specifically for moulds. It was applied to *A. flavus* for the first time:

$$\ln \mu_{\max} = a_0 + a_1\sqrt{1 - a_w} + a_2(1 - a_w)$$

[10]

$a0$, $a1$, $a2$, are constants to be estimated.

It has also been extended to take into account the combined effect of temperature (Tassou et al., 2008):

$$\ln \mu_{\max} = a_0 + a_1\sqrt{1 - a_w} + a_2(1 - a_w) + a_3 T + a_4 T^2 + a_5 T(1 - a_w)$$

[11]

$a0$, $a1$, $a2$, $a3$, $a4$, $a5$, are constants to be estimated.

Polynomial models

The general expression of the polynomial model is:

$$Y = a_0 + \sum_{i=1}^{I} a_i X_i + \sum_{i=1}^{I} \sum_{j=1}^{I} a_{ij} X_i X_j$$

[12]

ai are the contants to be estimated

X_i are the independent or input variables (a_w, temperature…)

Y is the response variable ($\mu_{\max}$, λ…)

Models based on the Gamma concept (Zwietering et al., 1996)

Gamma concept was proposed by Zwietering et al. (1996) and is based on the assumption that the effect of various factors affecting the growth rate of microorganisms can be combined by multiplying the separate effects. This model has the general form:

$$\gamma = \frac{\mu_{\max}}{\mu_{opt}} = \gamma(T) * \gamma(pH) * \gamma(a_w)$$

[13]

The relative effect of a given variable can be described by the gamma-factor of that variable, for example:

$$\gamma(T) = \left(\frac{T - T_{\min}}{T_{opt} - T_{\min}}\right)^2$$

[14]

$$\gamma(pH) = \frac{(pH - pH_{\min})(pH_{\max} - pH)}{(pH_{opt} - pH_{\min})(pH_{\max} - pH_{opt})}$$

[15]

$$\gamma(a_w) = \frac{(a_w - a_{w\min})}{(1 - a_{w\min})}$$

[16]

The advantage of using this approach is that for every variable determining growth rate, the relative effect can be calculated, by separating the effect of the various variables (Zwietering et al., 1996). However, it needs to be noted that these models may not be applicable to a situation in which the cardinal values of one environmental factor depend on the other factors (Panagou et al, 2003).

Table 4. Examples of secondary kinetic growth models in solid substrates used in the past in Food Mycology

Model	Studied parameters	Fungal species	References
Ratkowsky	Temperature and salts	*Penicillium roqueforti, Trichoderma harzianum, Paecilomyces variotii, Aspergillus niger, Emericella nidulans*	Cuppers et al. (1997)
	Temperature and a_w	*A. niger*	Parra and Magan. (2004)
	Temperature	*Penicillium expansum*	Baert et al. (2007)
Arrhenius-Davey	Temperature and a_w	*Fusarium verticillioides, Fusarium proliferatum*	Samapundo et al. (2005)
	Temperature	*P. expansum*	Baert et al. (2007)
	Temperature and a_w	*Aspergillus carbonarius*	Tassou et al. (2007)
	Temperature and a_w	*Aspergillus flavus, Aspergillus parasiticus*	Samapundo et al. (2007)
	Temperature and pH	*Aspergillus* sección *Nigri*	Silva et al., (2010)
Rosso	Temperature and salts	*P. roqueforti, T. harzianum, P. variotii, A. niger, E. nidulans*	Cuppers et al. (1997)
	Temperature, pH and a_w	*Penicillium chrysogenum, A. flavus, Cladosporium cladosporioides, Alternaria alternata.*	Sautour et al. (2001)
	a_w	*A. flavus, A nomius, A. oryzae, A. parasiticus, A. candidus, A. sydowii, Eurotium amstelodami, E. chevalieri, Xeromyces bisporus*	Rosso and Robinson (2001)
	pH and preservatives	*Penicillium brevicompactum*	Membré et al. (2001)
	Temperature, pH and a_w	*Monascus ruber*	Panagou et al. (2003)
	Temperature and a_w	*A. carbonarius*	Tassou et al. (2007)
	Temperature and a_w	*A. flavus*	Marín et al., (2008)
Polynomial	a_w	*A. flavus*	Gibson et al. (1994)
	Temperature and salts	*P. variotii, A. niger, E. nidulans*	Cuppers et al. (1997)
	a_w	*P. roqueforti*	Valík et al. (1999)
	Temperature, pH and a_w	*P. chrysogenum*	Sautour et al. (2001)
	Temperature and a_w	*Wallemia sebi*	Patriarca et al. (2001)
	Temperature, pH and a_w	*M. ruber*	Panagou et al. (2003)
	Temperature and a_w	*A. niger*	Parra and Magan (2004)
	Temperature and a_w	*A.* sección *Nigri*	Belli et al., (2004ab, 2005b)
	Temperature and a_w	*Aspergillus ochraceus, Penicillium verrucosum*	Pardo et al. (2004, 2005abcd, 2006ab)
	Temperature and a_w	*F.verticilloides, F. proliferatum*	Samapundo et al. (2005)
	Temperatura, salts and a_w	*P. expansum*	Lahlali et al. (2005)
	Temperature and a_w	*A. carbonarius, P. expansum*	Marín et al. (2006ab)
	Temperature and a_w	*A. carbonarius*	Tassou et al., (2007)
	Temperature	*P. expansum*	Baert et al. (2007)
	Temperature and a_w	*Aspergillus* sección *Nigri*	Samapundo et al. (2007)
	Temperature and pH	*Aspergillus* sección *Nigri*	Silva et al., (2010)
	Temperature and a_w	*A. parasiticus*	Galati et al., (2011)
Gamma Concept	Temperature, pH and a_w	*M. ruber*	Panagou et al. (2003)
	Temperature, pH and a_w	*Botrytis. cinerea, P. expansum*	Judet-Correia et al (2010)

a.3 Tertiary models

Tertiary models could be defined as the integration of primary and secondary models in a software using databases.

b) Probability models

These models may be used to predict the probability of contamination by molds and mycotoxins in a particular food. Probabilistic models are useful where the objective is to determine whether or not microbial growth can occur under specific conditions or time. Consequently, probability modelling is particularly useful when pathogenic or mycotoxin-producing species are involved. Probability models allow the prediction of whether a particular event, such as growth or toxin production, might occur, under various conditions (Gibson and Hocking, 1997). Probabilistic growth models are built from the proportion of "growth/no growth" responses throughout the experimental design space at a defined point in time (Brul et al., 2007).

Logistic regression is a useful tool for modelling the boundary between growth and no growth. A logistic regression model relates the probability of occurrence of an event, Y, conditional on a vector, X, of explanatory variables (Hosmer and Lemeshow, 1989). The specific model of the logistic regression is as follows:

$$P(x) = \frac{\exp\left(\sum b_i x_i\right)}{1 + \exp\left(\sum b_i x_i\right)} \qquad [17]$$

where x_i are independent variables (T, a_w, pH, …) and b_i are constants to be estimated.

The logit transformation of P(x) is defined as:

$$\log it\,(P) = \ln \frac{P(x)}{1 - P(x)} = \sum b_i x_i \qquad [18]$$

These models have been used by the following authors for fungi: Battey et al., (2001); (Char et al. 2005); Lindblad et al., (2004); Marín et al., (2008; 2009); Tassou et al., (2008).

c) Model validation

Predictive models are often built on data obtained in laboratory media, thus extrapolation to food products is not straightforward (Dalgaard and Jorgensen, 1998; McClure et al., 1993) because of the complexity of these products. Predictive models are often built under laboratory conditions with synthetic media and take a limited number of factors into account compared to the numerous factors influencing growth moulds in food products (Pinon et al., 2004). Therefore, a good way of validating a model is to compare its prediction to data obtained for food products. Models cannot be used with confidence until such a

comparison is made and hence validation is an essential step enabling researchers to understand the applicable range of models and also the limits of their performance (Jagannath and Tsuchido, 2003).

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2. Objectives and work schedule

Filamentous moulds are found in a wide range of foods and feeds as contaminants. From the food safety point of view, only mycotoxins, as chemical hazards, are relevant, while filamentous moulds themselves may cause spoilage but have no safety implications. A good way to ensure the absence of mycotoxins in food is preventing fungal growth. Prevention of mould contamination could be made in all steps of food chain (pre and postharvest, manufacturing, and storage steps). The prevention of fungal and mycotoxin contamination in primary production is generally dependent of climatic factors. However, during postharvest and storage of food, conditions are more controlled and the implementation of control strategies is easier. For these reasons the following points were addressed in this thesis:

I. To assess the usefulness of the aplication of extracts of *Equisetum arvense* and *Stevia rebaudiana* in order to control mycotoxigenic moulds in postharvest.

II. To study of the usefulness and possible drawbacks of the application of predictive kinetic models to predict mould contamination in pre and postharvest. In particular their performance was tested in some representative mycotoxigenic moulds as affected by:
    a. Inoculum size.
    b. Intraspecies variability.
    c. Marginal conditions for growth.
    d. Constant and cycling temperature.

III. To test the possible use of predictive probability models in prevention of mycotoxigenic mould growth and toxin presence in postharvest.

IV. To apply a predictive kinetic model for mycotoxin accumulation.

V. Validation of predictive models in food system

Aim I

In vitro → Mycotoxigenic moulds

↓ Inoculated

Maize agar medium (2%) (0.85-0.95 a_w)
+
Plant extracts (*Equisetum arvense* and *Stevia rebaudiana*) at different concentrations

→ Incubation → 15-30 ºC 21 days

→ Growth inhibition

→ Mycotoxin production inhibition

Natural antifungals

In vivo → *Aspergillus flavus* and *Fusarium verticillioides*

↓ Inoculated

Maize seeds (0.85-0.95 a_w)
+
with *E. arvense*, *S. rebaudiana* and mix of both (3%)

→ Incubation → 25 ºC 30 days

→ CFUs

→ Aflatoxins and fumonisins level

Aims II, III, IV and V

Prediction of growth → *Aspergillus parasiticus* and *Aspergillus ochraceus* → Incubation on MEA → $0.80\text{-}0.93\ a_w$ and $10\text{-}42\ ^\circ C$

→ Kinetic primary and secondary models

→ Probabilistic models

Validation on food substrates

Predictive models

From these results we studied the effect of the next points in predictive models

1- Inoculum level (1-5; 1-5×10^{-2}; 1-5×10^{-3} spores)

Aspergillus carbonarius and *Penicillium expansum*

Incubation

0.90; 0.92 and 0.98a_w and 15 and 25 ºC Culture medium: MEA

Kinetic and probabilistic models

2- Intraspecific variability on growth and toxin level

Aspergillus carbonarius (30 isolates) and *Penicillium expansum* (79 isolates)

Incubation

0.98 a_w /25ºC;
0.98 a_w /25ºC;
0.98 a_w /25ºC
(*A. carbonarius;* SNM)
0.98 a_w /20ºC
0.90 a_w /1ºC
(*P. expansum;* ACAM)

Obtain mathematically the number of isolates and replicates needed to design an experiment involving mycotoxigenic moulds.

3- Cycling temperatures

Fusarium graminearum and *Fusarium verticillioides*

Incubation

0.99a_w at 15, 20, 25 and 30 ºC; and cycling (12h) of 15/20 ºC; 15/25 ºC and 20/30 ºC. Culture medium: Soybean agar (2%) and in soybeans seeds.

Kinetic models

4- Marginal conditions

Experiments 1, 2 and 3 were made both under marginal and optimal conditions for growth

Prediction of mycotoxin production → *Aspergillus flavus* → Incubation → 0.90 and 0.99 a_w at 25 ºC On MEA and maize grain → Kinetic models for aflatoxins B_1 and B_2

3. Results

3.1 Mould growth and mycotoxin production as affected by *Equisetum arvense* and *Stevia rebaudiana* extracts

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Abstract

Cereals are very important for human and animal diet. However, agricultural products can be contaminated by moulds and their mycotoxins. On the other hand, natural plant products with antimicrobial properties could be a possibility to control mycotoxigenic fungi in foods and feeds. In this study, *Equisetum arvense* and *S. rebaudiana*. extracts were tested for their efficacy against a range of mycotoxigenic fungi. Maize agar medium (MAM 2%) was used for this study and *Equisetum arvense* and *S. rebaudiana*. extracts were added at different concentrations (1-3%) under different water activity (a_w) levels (0.85-0.95). Six mycotoxigenic moulds were inoculated and incubated at different temperatures (15-30 ºC) during 21 days. In general, no growth was observed with *Equisetum* sp. extract at 3% in all studied conditions for all isolates. However, with *S. rebaudiana* extract at 2-3%, growth was not significantly decreased ($p<0.05$) in most of the cases. Finally, in terms of toxin production, results were not conclusive. The use of *Equisetum arvense* extracts as antifungals and antimycotoxigenics and their application should be further explored.

Keywords:

Plant extracts, mould, mycotoxin, *Equisetum arvense, Stevia rebaudiana*.

1. Introduction

Safety of cereals and by-products depends on contamination by microscopic fungi (Barros, Garcia, Oviedo, Ramírez, Torres & Chulze 2008). Moulds are responsible for off-flavour formation, production of allergenic compounds and mycotoxins contamination which are a risk for human and animal health. Mycotoxins are chemical hazards in food products of increasing concern due to the wide range of food types where they can be found (Garcia, Ramos, Sanchis, & Marín, 2010). Approximately 25-40% of cereals world-wide are contaminated with mycotoxins produced by different fungi (Pittet, 1998). Among the fungal genera that are known to produce mycotoxins *Penicillium*, *Fusarium*, *Aspergillus* and *Alternaria* are the most important (Sidhu, 2002). Mycotoxins are associated to the presence of fungal inoculum on predisposed substrates; therefore prevention of fungal growth effectively conduces to prevention of mycotoxin accumulation (Garcia, Ramos, Sanchis, & Marín 2009).

Mould growth is commonly controlled using synthetic fungicides; however, natural antimicrobials have also shown important antifungal properties (López-Malo, Alzadora, & Guerrero, 2000). Antifungal chemicals, mainly low molecular weight organic acids, have been generally used for the preservation of stored grains (López, Theumer, Zygadlo, & Rubinstein 2004). Nowadays, consumers prefer low levels of preservatives in foods or preservative-free products. Moreover, continuous and indiscriminate use of chemical preservatives in foods and feeds, could lead to toxic effects for consumers and to the development of resistances in microorganisms López-Malo, Alzadora, & Guerrero, 2000). Antifungal chemicals, mainly low molecular weight organic acids, have been generally used for the preservation of stored grains (López, Theumer, Zygadlo, & Rubinstein 2004). Besides this compounds have an inhibitory effect on *Aspergillus* spp. and *Fusarium* spp. growth and toxin production (Mahoney & Molyneux 2004; Palumbo, Keeff, & Mahoney, 2007; Radulovic, Stojanovic, & Palic, 2006; Romero, Alberto, & Vaamonde, 2010).

Stevia spp. and *Equisetum* spp. have been described as herbs with antioxidant properties by different studies (Canadanovic-Brunet, Cetkovic, Djilas, Tumbas, Savatovic, Mandic, Markov & Cvetkovic, 2009; Milanovic, Radulovic, Todorovic, Stankovic & Stojanovic, 2007; Nagai, Myoda, & Nagashima, 2005; Radulovic, Stojanovic, & Palic, 2006). Species of these plant genera are consumed in different countries as "healthy ingredients" but they could also be useful in preventing mould growth and mycotoxin production due to their antioxidant compounds. There are few studies that show antifungal activity of *Stevia* spp. and *Equisetum* spp. extracts (Canadanovic-Brunet, Cetkovic, Djilas, Tumbas, Savatovic, Mandic, Markov & Cvetkovic, 2009; Milanovic, Radulovic, Todorovic, Stankovic & Stojanovic, 2007; Nagai, Myoda, & Nagashima, 2005). For this reason the aim of this work was to assess the antifungal and antimycotoxigenic effect of hydro-alcoholic extracts of *Equisetum arvense* and *Stevia rebaudiana*. on different mycotoxigenic moulds.

2. Materials and methods

2.1 Fungal isolates and preparation of inoculum

Six mycotoxigenic isolates were included in this study, *Aspergillus flavus* (UdL-TA 3.215), *A. parasiticus* (UdL-TA 3.18) (both producers of aflatoxins B_1, B_2, G_1 and G_2), *A. carbonarius* (UdL-TA 3.81), *A. westerdijkiae* (UdL-TA 3.183) (producers of Ochratoxin A), *Fusarium verticillioides* (UdL-TA 3.232; producer of Fumonisis B_1 and B_2) and *F. graminearum* (UdL-TA 3.234; producer of deoxinivalenol and zearalenone). The references in brackets are the codes of cultures held in the Food Technology Department Culture Collection of the University of Lleida. The isolates were sub-cultured on potato dextrose agar (PDA) plates and incubated at 25ºC for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from PDA plates and they were suspended in 5 ml of distilled H_2O/glycerol solutions with different water activity (a_w) levels: 0.85, 0.90 and 0.93 for *Aspergillus* species and 0.93 and 0.95 for *Fusarium* species. After homogenizing, the suspensions were adjusted using a Thoma counting chamber to a final concentration of 1-5×10^5 spores/ml.

2.2 Plant extract and medium preparation

Commercial dried *S. rebaudiana.* (PAMIES HORTÏCOLES S.L., Spain) was used in this study. The aerial parts of *E. arvense* (recollected in fields from Catalonia, Spain, 2009-2010) were dried at 40ºC. Dried vegetal matter was extracted with 70% ethanol at room temperature during 5 days. After that, ethanol was evaporated by rota-evaporation and the extracts were stored at 4ºC until their use. A maize agar medium (MAM) was used, composed by a 2% maize meal with water activity properly modified with glycerol to the range of a_w conditions studied (Marín et al., 1995). Plant extracts were aseptically incorporated into the agar after sterilization of the medium by autoclave to give final concentrations of 1, 2, and 3% (w/v) for *E. arvense* and 2 and 3% (w/v) for *S. rebaudiana* extracts. MAM without preservatives and MAM with addition of propionic acid at 0.1% (v/v) were used as controls. The a_w of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of ±0.003, before, during and at the end of the experiment.

2.3 Inoculation and incubation

Petri dishes were inoculated centrally with a needlepoint load. Previous repeated experiments showed that the number of spores inoculated through this technique was 10-100 spores. Plates with the same a_w level were enclosed in polyethylene bags in order to maintain a constant water activity. The incubation temperatures for all moulds were 15, 25 and 30 ºC and a_w 0.85, 0.90 and 0.93 a_w (*Aspergillus* species) and 0.93 and 0.95 a_w (*Fusarium* species). For each condition, 6 Petri dishes were inoculated. Two perpendicular diameters of the growing colonies were measured daily until the colony reached the edge of the Petri dish. The diameters of the colonies were plotted against time.

2.4 **Mycotoxins extraction from culture and quantification**

2.4.a Mycotoxins extraction

Three agar plugs (diameter 4 mm) of each colony were removed from the colonies after 7 and 21 days of incubation and placed in a vial. Aflatoxins (AFs), ochratoxin A (OTA) and fumonisins (FBs) were extracted by adding 1 ml of methanol into the vials, while deoxinivalenol (DON) and zearalenona (ZEA) were extracted with 1 ml of acetonitrile; the vials were shaken for 5 s and allowed to rest. After 60 min, the vials were shaken again and extract filtered (Millex-HV 0.45 μm 25 mm, Millipore Corporation. Bedford, U.S.A.) into another vial and stored at 4 ºC until analysis by HPLC (Waters, Mildford, MA, SA). Plug extraction was performed in triplicate and mycotoxins were expressed per mm^2 of colony.

2.4.b Mycotoxins detection and quantification

General description of the equipment

All mycotoxins were detected and quantified separately by using a HPLC system (Waters 2695, separations module, Waters, Milford, USA) and a C_{18} column (5 μm Waters Spherisorb, 4.6 × 250 mm ODS2). Mobile phase was always pumped at 1 ml min^{-1} and injection volume was always 100 μl. For fluorescence detection a Waters 2475 module (Waters, Milford, USA) was used and for absorbance detection of DON a Waters 2487 module (Waters, Milford, USA) was employed. Quantification was always achieved with a software integrator (Empower, Milford, MA, USA). Mycotoxins were quantified on the basis of the HPLC fluorimetric or absorbance response compared with that of a range of mycotoxins standards.

Aflatoxins: A post column photochemical derivatization system (LC Tech detector, UVC 254 nm, Germany) was used and toxins were detected by fluorescence (λ_{exc} 365 nm; λ_{em} 455 nm). The mobile phase was water: methanol: acetonitrile (70:17:17) and the detection limit of the analysis was about 0.0005 ng/mm^2 for B_2 and G_2, and 0.001 ng/mm^2 for B_1 and G_1, based on a signal-to-noise ratio of 3:1. The range of aflatoxins standards used for quantification was 0.09-0.94 ng/ml.

Ochratoxin A: Detection was achieved by fluorescence (λ_{exc} 330 nm; λ_{em} 460 nm). The mobile phase was acetonitrile: water: acetic acid (57:41:2) and the detection limit of the analysis was about 0.004 ng OTA/mm^2, based on a signal-to-noise ratio of 3:1. The range of OTA standards used for quantification was 0.05-250 ng/ml.

Fumonisins B_1 and B_2: Fumonisins were detected by fluorescence (λ_{exc} 335 nm; λ_{em} 440 nm). The mobile phase was methanol: 0.1M sodium dihydrogen phosphate (77:23), solution adjusted to pH: 3.35 with orthophosphoric acid. Dried extracts were dissolved in methanol and derivatized with OPA (Sydenham et

al., 1996). Detection limit of the analysis was about 0.54 ng/mm^2 for FB$_1$ and 1.68 ng/mm^2 for FB$_2$, based on a signal-to-noise ratio of 3:1. Quantification was achieved with Empower. The range of FBs standards used for quantification was 15-5000 ng/ml.

Zearalenone: Detection was achieved by fluorescence (λ_{exc} 274nm; λ_{em} 445 nm). The mobile phase was acetonitrile: water (60:40) and the detection limit of the analysis was about 0.0085 ng/mm^2, based on a signal-to-noise ratio of 3:1. The range of ZEA standards used for quantification was 5-25 ng/ml.

Deoxynivalenol: DON was detected by absorbance (λ 220nm). The mobile phase was water: acetonitrile: methanol (90:5:5) and the detection limit of the analysis was about 0.25 ng/mm^2, based on a signal-to-noise ratio of 3:1. The range of DON standards used for quantification was 500-15000 ng/ml.

2.5 Statistical analyses

Diameters of growing colonies were plotted against time, and the Baranyi & Roberts (1994) model was used to estimate growth rate and lag phase for each growth condition and isolate. Analysis of variance of growth rates and lag phases was used in order to assess significant differences due to growth conditions and assayed antifungal. Mycotoxins were expressed as ng or µg per mm^2 of colony. LSD test was used to establish the differences among mean values of the variables under the different levels of factors at p<0.05.

3. Results

3.1 Plant extracts effect on mould growth

In general, no growth was observed by using an *E. arvense* extract at 3% in either of the isolates or studied conditions. Analysis of variance (ANOVA) of mould growth showed that the effect of the water activity (a_w), temperature, antifungals, and their interaction were statistically significant (p<0.05). At 0.85 a_w no growth was observed for any of the assayed isolates.

3.1.a. Plant extracts effect on growth of A. flavus and A. parasiticus

In general, when adding *E. arvense* extract at 2-3% to the medium, *A. flavus* and *A. parasiticus* growth was significantly retarded (p<0.05) (Figure 1A and 1B). For *A. flavus*, *E. arvense* extract at 3% completely inhibited growth in all studied conditions, except at 0.93a_w/30 ºC where growth only decreased a 86%. On the other hand, at 0.93 a_w/15 ºC growth was faster in presence of *S. rebaudiana* extract at 2-3% and *E. arvense* extract at 1% compared to the control, and at the other temperatures (25 ºC and 30 ºC) no significant inhibition was recorded. Otherwise, total inhibition was recorded by extracts both at 0.85a_w at all temperature and 0.90a_w/15ºC for *A. parasíticus*. *E. arvense* 3% inhibited growth in a 100% in all studied conditions, except at 0.90a_w/30 ºC where growth was decreased a 95%. On the other hand, *E.*

arvense 1% inhibited growth only at 0.93a_w/15 ºC (42%) and at 0.90a_w/25 ºC compared to control. In general, in *S. rebaudiana* 3% medium, growth decreased significantly (p<0.05) except at 0.90-0.93 a_w/30 ºC and also at 0.93a_w/25ºC where growth was similar to control, while *S. rebaudiana* 2% had little or no effect.
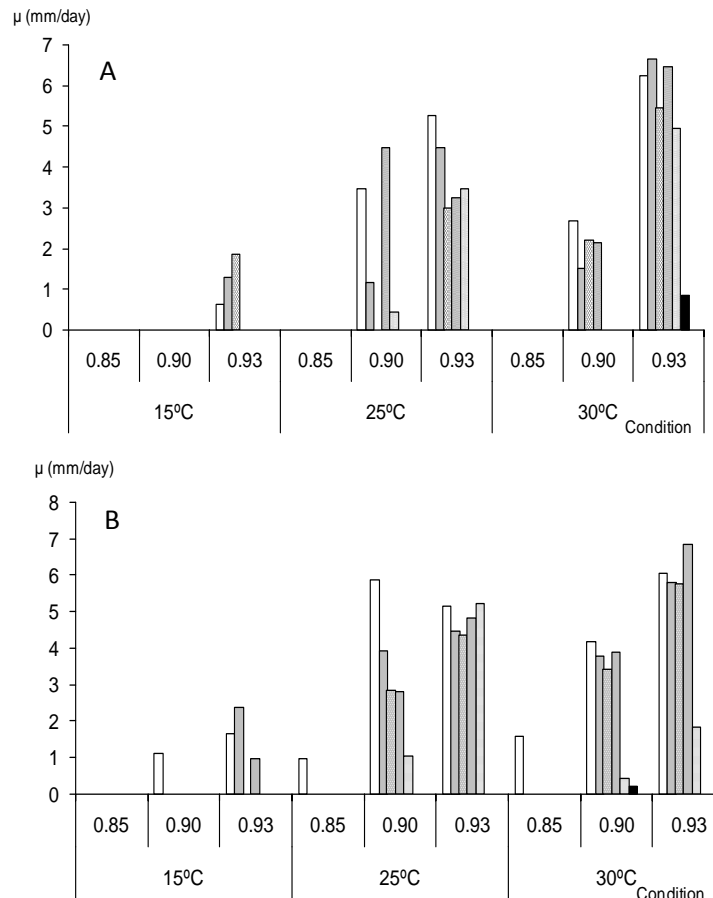
Figure 1: Growth of *A. flavus* (A) and *A. parasiticus* (B) in MAM with plant extracts at all conditions studied. ☐ Control; ▨ Stevia 2%; ▧Stevia 3%; ▨ *E. arvense* 1%; ▨ *E. arvense* 2%; ■ *E. arvense* 3% and ▨ Propionic acid 0.1%

3.1.b Plant extracts effect on growth of A. carbonarius and A. westerdijkae

When *A. carbonarius* and *A. westerdijkae* were inoculated in *E. arvense* extract at 3% medium, growth was significantly decreased (p<0.05) under all studied conditions (Figure 2A and 2B). However, for lower concentrations of this extract *A. carbonarius* grew similar to control, especially under good growth conditions. With *S. rebaudiana* extract, growth was promoted for all conditions assayed, except for 0.85 a_w at all temperatures assayed. Moreover, *A. westerdijkiae,* in presence of *E. arvense* extract at 2% also

decreased growth between 37-100%. In general, *S. rebaudiana* extracts at 2-3% and *E. arvense* extract at 1% increased growth, especially in the latter case.
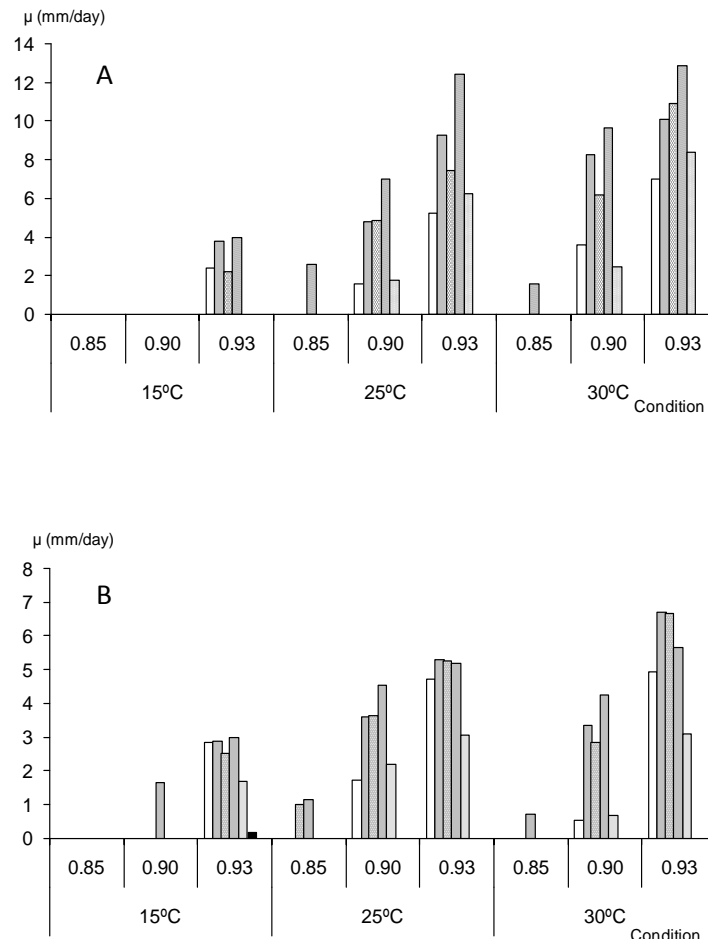
Figure 2: Growth of *A. carbonarius* (A) and *A. westerdijkiae* (B) in MAM with plant extracts at all conditions studied. ☐ Control; ▓ Stevia 2%; ⊠ Stevia 3%; ⊠ *E. arvense* 1%; ⊡ *E. arvense* 2%; ■ *E. arvense* 3% and ▨ Propionic acid 0.1%.

3.1.c Plant extracts effect on growth of F. verticillioides and F. graminearum

In general, extracts of *E. arvense* at 2-3% and *S. rebaudiana* at 3% significantly decreased growth (p<0.05) for *F. verticillioides*, except at 0.95 a_w/25 ºC and 0.95 a_w/30 ºC (Figure 3). *E. arvense* extract at 3% completely inhibited growth under all studied conditions, except at 0.95 a_w/30 ºC, where growth decreased a 86% compared with control. However, *F. graminearum* only significantly ceased growth (p<0.05) in presence of *E. arvense* extract at 3%, for all conditions studied (Figure 4). *E. arvense* extract at 2% at 0.95 a_w/25 ºC decreased growth only a 36% and the rest of the extracts did not decrease the growth or was even higher compared to control. *S. rebaudiana* extract promoted growth for *F.*

graminearum in all studied conditions, except at 0.93 a_w/15 ºC where growth decreased in a 100%. *F. graminearum* was the only assayed mould that could grow in presence of propionic acid at 0.1%, but only at optimum conditions for growth.
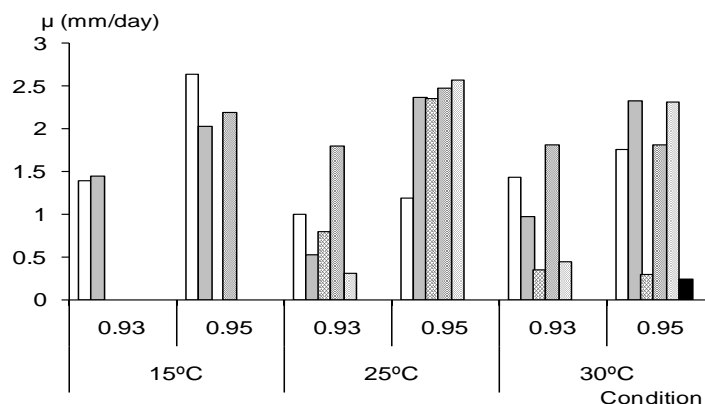
Figure 3: Growth of *F. verticillioides* in MAM with plant extracts at all conditions studied.
□ Control; ▨ Stevia 2%; ▨ Stevia 3%;▨ *E. arvense* 1%;▨ *E. arvense* 2%;■ *E. arvense* 3% and ▨ Propionic acid 0.1%.
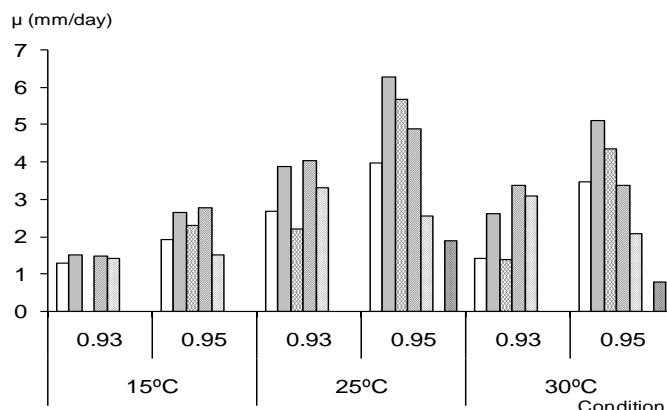
Figure 4: Growth of *F. graminearum* in MAM with plant extracts at all condition studied.
□ Control; ▨ Stevia 2%; ▨ Stevia 3%;▨ *E. arvense* 1%;▨ *E. arvense* 2%;■ *E. arvense* 3% and ▨ Propionic acid 0.1%.

3.2 Plant extracts effect on mycotoxin production

No aflatoxins were detected in the control medium for both *A. flavus* and *A. parasiticus*, possibly due to the growth conditions used. However, stimulation of aflatoxigenesis was recorded in some cases. *A. flavus* produced aflatoxins (AFB$_1$, AFB$_2$ and AFG$_1$) with *S. rebaudiana* at 0.90a_w/25 ºC, 0.93a_w/15 ºC and 0.93a_w/30 ºC after 21 days of incubation (data no shown).The maximum concentration of AFB$_1$ (11.41

ng/mm^2) was recorded at 0.93 a_w/15 ºC with *S. rebaudiana* extract at 2%. In 1% *Equisetum* sp. extract medium, *A. flavus* also produced aflatoxins (AFB$_1$ and AFG$_1$) at 0.93 a_w/15 ºC, 0.93 a_w/25 ºC and 0.93 a_w/30 ºC, although levels of toxins were lower than in *S. rebaudiana* extract medium. *A. parasiticus* synthesized aflatoxins (AFB$_1$, AFG$_1$ and AFG$_2$) at 0.90 a_w/30 ºC in *Equisetum* sp. extract at 1% medium and produced AFG$_2$ at 0.93 a_w/30 ºC with *S. rebaudiana* extract at 3%.

OTA production by *A. carbonarius,* was detected in the control at 0.90 a_w/25-30 ºC and 0.93 a_w/25 ºC (Table 1). In presence of both plant extracts, OTA production was higher than in the control for both 7 and 21 incubation days. With *S. rebaudiana* extract medium, levels of toxins were higher than in the *E. arvense* medium*.* The maximum OTA level (1.415 ng/mm^2) was detected with *S. rebaudiana* extract at 3% at 0.93 a_w/25 ºC at 7 days of incubation. *A. westerdijkiae*, however, only produced OTA in the control at 0.93 a_w/25 ºC at 7 and 21 days of incubation but the levels were below the detection limit (Table 1) With *E. arvense* extract at 2% medium at 0.90 a_w/30 ºC after 21 days of incubation production was 0.033 ng/mm^2, the maximum level produced. Levels of OTA produced by A. *westerdijkiae* were lower than those obtained with *A. carbonarius*.

FBs were detected only in media with plant extracts. With *S. rebaudiana* extract at 2-3% *F. verticillioides* only produced toxin at 0.95 a_w/15 ºC at 0.882 and 0.868 ng/mm^2 levels, respectively, after 21 days of incubation. In *E. arvense* extract medium FB$_1$ was only produced at 0.93 a_w/25 ºC (1.176 ng/mm^2), after 7 days of incubation.

F. graminearum produced DON in *S. rebaudiana* extract at 2-3% and *E. arvense* extract at 2% medium at 21 days of incubation. With *S. rebaudiana* extract at 2 and 3% toxin was produced at 0.93 a_w/15 ºC at a level of 8.652 ng/mm^2 and at 0.95 a_w/25 ºC at a level of 3.424 ng/mm^2, respectively. With *E. arvense* extract at 2% medium, 2.445 ng DON/mm^2 were produced at 0.93 a_w/25 ºC. Table 2 shows the values of *F. graminearum* ZEA production. Higher production of ZEA was found compared with DON. At 15 ºC ZEA was synthesized in *S. rebaudiana* extract at 2% and *E. arvense* extract at 2% medium at 21 days of incubation for both a_w studied, and the amounts produced were higher compared with control. ZEA production only decreased at 0.93 a_w/25 ºC, compared with the control, except with *E. arvense* 2% extract at 21 days of incubation, where the production was similar to control. For other conditions, production was stimulated or was similar to control. The higher amount of this toxin (35.294 ng/mm^2) was obtained with *S. rebaudiana* extract at 3%, at 0.95 a_w/25 ºC and 21 days of incubation.

Table 1. OTA production (ng/mm^2) by *A. carbonarius* and *A. westerdijkiae* in MAM with plant extracts at different conditions

			15 ºC		25 ºC		30 ºC	
		Days	0.90	0.93	0.90	0.93	0.90	0.93
A. carbonarius	C	7	nd	nd	nd	0.012	0.004	<ld
		21	nd	nd	0.037	0.005	nd	nd
	S 2%	7	nd	nd	0.005	0.777	0.018	0.267
		21	nd	1.293	0.310	0.777	0.052	0.302
	S 3%	7	nd	nd	0.006	1.415	0.028	0.700
		21	nd	0.835	0.602	0.875	<ld	0.377
	E 1%	7	nd	nd	0.031	0.785	0.014	0.069
		21	nd	0.530	0.076	0.769	0.202	0.221
	E 2%	7	nd	nd	nd	0.003	nd	nd
		21	nd	nd	nd	0.146	nd	0.264
	E 3%	7	ng	nd	nd	ng	ng	ng
		21	ng	nd	nd	ng	ng	ng
A. westerdijkiae	C	7	nd	nd	nd	nd	nd	nd
		21	nd	nd	nd	<ld	nd	<ld
	S 2%	7	nd	nd	nd	nd	nd	nd
		21	nd	0.012	<ld	nd	nd	nd
	S 3%	7	nd	nd	nd	nd	nd	nd
		21	nd	nd	0.004	nd	nd	nd
	E 1%	7	nd	nd	nd	nd	nd	nd
		21	0.007	0.004	nd	<ld	nd	<ld
	E 2%	7	nd	nd	nd	nd	nd	0.016
		21	nd	nd	nd	0.015	0.033	0.051
	E 3%	7	ng	nd	ng	ng	ng	ng
		21	ng	nd	ng	ng	ng	ng

C: control; S: *Stevia* sp.; **E**: *E. arvense*; **P**: propionic acid; **nd**: no detected; **ld**: limit detection: **ng**: no growth

Table 2. ZEA production (ng/mm^2) by *F. graminearum* in MAM with plant extracts at different conditions.

			15 ºC		25 ºC		30 ºC	
		Days	**0.93**	**0.95**	**0.93**	**0.95**	**0.93**	**0.95**
F. graminearum	C	7	nd	nd	3.333	0.079	nd	0.074
		21	0.226	nd	3.367	1.483	nd	3.135
	S 2%	7	nd	nd	0.209	0.645	nd	0.481
		21	0.871	0.532	2.489	3.956	11.759	10.814
	S 3%	7	ng	nd	nd	1.947	nd	1.160
		21	ng	nd	nd	35.294	nd	nd
	E 1%	7	nd	nd	nd	0.764	0.017	nd
		21	nd	nd	0.899	nd	5.863	4.340
	E 2%	7	nd	nd	0.656	nd	nd	nd
		21	0.747	0.232	8.981	0.357	5.998	nd
	E 3%	7	ng	ng	ng	ng	ng	ng
		21	ng	ng	ng	ng	ng	ng
	P 0.1%	7	ng	ng	ng	0.255	ng	nd
		21	ng	ng	ng	1.652	ng	1.487

C: control; **S**: *Stevia* sp.; **E**: *E. arvense*; **P**: propionic acid; **nd**: no detected; **ng**: no growth.

4. Discussion

Interest in natural therapies and increasing consumers demand for natural, safe and effective products, has resulted in the need to obtain quantitative data on plant extracts (Hammer et al., 1999). Mitscher, Drake, Gollapundi, & Okwute (1987) suggested that it is very important to scientifically study traditional medicinal plants because they may contain new sources of antimicrobial compounds.

E. arvense is used, currently, for biological control of fungi and pests, whereas *S. rebaudiana* is consumed as a natural sweetener. In this research we evaluated the antifungal capability of *E. arvense* and *S. rebaudiana* extracts, at different levels and environmental conditions (temperature and a_w), on six mycotoxigenic moulds: *A. flavus*, *A. parasiticus*, *A. carbonarius*, *A. westerdijkiae*, *F. verticilloides* and *F. graminearum*. Temperature and a_w conditions studied are near to conditions which may occur in cereals in pre and post-harverst, but do not include the optimum growth conditions for these moulds.

Propionic acid is commonly used as an antifungal; in this study it was used as a control to compare the efficacy obtained with the different plant extracts. Our results showed that this acid is a potent antimicrobial for all isolates studied and therefore, avoids mycotoxin production. Only *F. graminearum*

could grow in the presence of propionic acid at 0.1%, but only at suitable conditions for growth (0.95 a_w/25 ºC and 0.95a_w/30 ºC). Besides, *E. arvense* extract at 3% reduced growth of all moulds studied and these results were comparable to those obtained with 0.1% propionic acid. Mechanisms of action of plant extracts are unknown. However, Rasooli & Owlia (2005) and Kale, Cary, Bhatnagar & Bennett (1996) attributed the action to morphological damage caused to mould. Other authors proposed that the inhibitory effect is due to granulation of the cytoplasm, the cytoplasmic membrane rupture and inactivation and/or the inhibition of the intracellular synthesis of enzymes (Srivastava, Singh, Shukla, & Dubey 2008). Our results showed that antifungal capability for *Stevia* and *Equisetum* extracts was dose-dependent. Low levels of extract could result in an increase of growth in some conditions (eg. *A. flavus* in presence of 1% *E. arvense*, 0.93 a_w/15 ºC). In general, high inhibition of growth was observed at marginal conditions for growth, some of them food storage conditions.

In our study, *E. arvense* was the most effective extract for mould inhibition. The use of a 3% of this extract was the most effective in all conditions tested, reaching 100% inhibition for *A. carbonarius* and *F. graminearum* growth. Possibly, antimicrobial effect could be attributed to the presence of various substances, mainly phenols such as thymol or monoterpernes (Pattnaik, Subramanyan, Bapaji & Kole, 1997; Radulovic, Stojanovic, & Palic, 2006). A decreased mould growth, could lead to a decrease of total mycotoxin accumulation. To our knowledge, there are no published works with regard to mycotoxin production in presence of *E. arvense* extracts.

S. rebaudiana showed lower antifungal activity compared with *E. arvense* extract for all isolates tested and it had no effect against *A. carbonarius* and *A. westerdijkae*. Besides, it was dose-dependent for *A. flavus*, where growth increased at low levels of extract. No published works were found regarding mould growth inhibition with *S. rebaudiana* extracts. However, inhibition of *A. flavus* and *A. parasiticus* growth may be a possible alternative in future researches.

Several studies have demonstrated the effectiveness of various essential oils from different plants. Nguefack, Leth, Amvam Zollo, & Mathur (2004) studied the effect of essential oils of *Ocimum gratissimum*, *Thymus vulgaris*, *Cymbopogon citrates*, *Zingiber officinalis* and *Monodora myristia* on *A. flavus*, *A. fumigatus* and *F. verticillioides* growth and mycotoxin production. All essential oils inhibited both mycelial growth and conidial germination. *O. gratissimum* essential oil was more effective on *F. verticillioides* inhibition (86%), *T. vulgaris* for *A. flavus* (81%) and *C. citrates* for *A. fumigatus* (70%) at the same concentration of essential oil assayed (200 ppm). However, *M. myristia* was not effective on growth inhibition, as a dose of 500 ppm only decreased growth of *F. verticillioides* by 22%, and *A. fumigatus* and *A. flavus* development in a 9.8% and 12% respectively.

On the other hand, Sánchez, Heredia & García (2005) worked with *Agave asperrima* and *A. striata* extracts. They found that these extracts decreased growth of *A. flavus* and *A. parasiticus* at 125-500 µg/ml, respectively. However, at 50% of the MIC, AFs production was reduced between 65-97% for all isolates studied. This inhibition could be attributed to the lysis of the mycelium and spores due to the

presence of fungicidal compounds in plant extracts (Shelef, 1994; Namazi, Allameh, Aminshahidi, Nohee & Malekzadeh, 2002). In our study, there was no production of AFs in the control, probably because the a_w tested were below the optimal range for AF production (0.95-0.99 a_w) (International Commission on Microbiological Specifications for Food, 1996). Faraj, Smith, & Harran (1991) examined the effect of a_w (0.90, 0.95 and 0.98) and temperature (25, 30 and 35 ºC) in the production of AFs by *A. flavus* and *A. parasiticus* in irradiated maize and they found highest levels of AF in *A. parasiticus* at 0.98 a_w/25 °C and *A. flavus* at 0.95-0.98 a_w/30 °C.

There are few works on growth of *A. carbonarius* and *A. westerdijkae* in presence of plant extracts. Romero, Alberto, & Vaamonde, (2010) tested the effect of gallic acid (majoritary phenolic compound in grapes) on *A. carbonarius* in Czapek Yeast Extract agar (CYA). They found an increase in the lag phase and decreased growth rate in the presence of the extract and 500 mg/l was totally inhibitory for *A. carbonarius* growth. OTA production was also reduced with gallic acid. On the other hand, Palumbo, Keeff, & Mahoney, 2007 studied the effect of phenolic compounds, including gallic acid, in OTA production and growth of several *Aspergillus* species in synthetic medium. In the case of *A. carbonarius*, gallic acid did not affect growth but tended to inhibit OTA production. However, in our work, growth was only clearly reduced with a 3% of *E. arvense* extract while *E. arvense* 1% and *S. rebaudiana* 2-3% stimulated OTA production. Regarding *A. westerdijkae*, resveratrol (a grape skin component) was effective in controlling this mould in wheat stored for 28 days at different a_w (0.80 to 0.995) and different incubation temperatures (15-25 ºC). Under these conditions it was also reduced OTA production in a 60% (Aldred, Cairns-Fuller & Magan, 2008). In our study, growth of *A. westerdijkae* was inhibited by *E. arvense* extract 2-3%, while toxin production showed considerable variation, production being stimulated at low level of extracts and high temperatures at 21 days of incubation.

With regard to *Fusarium* sp., previous works proved the effectiveness of different essential oils against species of this genus (Rai, Qureshi & Pandey, 1999; Taiga, Suleian, Sule & Olufolaj, 2008). In a maize synthetic medium with 1000 mg/l level of cinnamon and clove essential oils, growth of *F. verticillioides* decreased between 62-80%, respectively, under all conditions tested: 20-30 °C and 0.95 to 0.995 a_w (Velluti, Marín, Gonzalez, Ramos, & Sanchis, 2004). This work agrees with our results where temperature did not affect the results obtained.

Marín, Velluti, Muñoz, Ramos, & Sanchis, (2003) studied the effect of lemongrass essential oil on maize inoculated with *F. verticilloides* and *F. proliferatum*. This study suggested that microbial competition plays an important role in the accumulation of FB_1 and the effectiveness of essential oils in cereals could be much lower than observed in *in vitro* experiments in synthetic medium. The essential oil of lemongrass (500 mg/kg maize) completely inhibited the growth of *F. verticilloides* in maize seeds for 21 days of incubation; however the applied dose affected the germination of grain; at the same conditions both inhibition of growth and ZEA production by *F. gramineaurm* occurred only at low a_w, while production of DON was inhibited at the same conditions as FB_1 (Marín, Velluti, Ramos, & Sanchis, 2004).

Results presented in this study confirm that *E. arvense* extracts were effective for the control of toxigenic moulds growth, while *S. rebaudiana* extract was effective only for *A. flavus*, *A. parasiticus* and *F. verticillioides*. In terms of toxin production, results were not conclusive. However, in the event of total inhibition of growth, mycotoxin production would not take place. Due to the high presence of fungal contamination and the risks of mycotoxins in foods and feeds, it could be interesting to find plant extracts with antifungal effects against the most common fungal species in grain microbiota. The results obtained with extracts of *E. arvense* and *S. rebaudiana* on culture media cannot necessarily be extrapolated to natural ecosystems and should be corroborated in cereal grain. Our data confirm the *in vitro* fungal inhibiting effect of 3% *E. arvense* extract for all isolates studied.

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3.2 Effect of *Equisetum arvense* and *Stevia rebaudiana* extracts on growth and mycotoxin production by *Aspergillus flavus* and *Fusarium verticillioides* in maize seeds as affected by water activity

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Abstract

Cereals are very important for human and animal diet. However, agricultural products can be contaminated by moulds and their mycotoxins. Plant extracts, particularly those of *Equisetum arvense* and *Stevia rebaudiana* have been previously reported to contain antioxidant compounds which might confer them antifungal properties. In this study, *Equisetum arvense* and *Stevia rebaudiana* extracts, were tested for the control of mycotoxigenic fungi in maize. The extracts and a mixture of both were tested for their efficacy on *Aspergillus flavus* and *Fusarium verticillioides* growth. Unsterilised inoculated maize was used and extracts were added under different water activity (a_w) levels (0.85-0.95); a_w is an environmental factor determinant for mould growth. Moulds were inoculated and incubated during 30 days. Results confirmed that the extract of *Equisetum* and a mixture 1:1 of *Equisetum-Stevia* may be effective for the inhibition of both growth of *A. flavus* and aflatoxin production at high water activity levels (pre-harvest conditions). In general, growth of the fumonisin producer was reduced by the use of plant extracts, especially at 0.95 a_w, however, fumonisin presence was not significantly affected. Then, *E. arvense* and *S. rebaudiana* extracts could be an alternative to control aflatoxigenic mycobiota in moist maize.

1. Introduction

Maize (*Zea mays* L.) is one of the main cereals as a source of food, forage and processed products for industry (Chulze, 2010). It is a very important cereal for human and animal diet; however, it can be contaminated by moulds and their mycotoxins. Moulds are responsible for off-flavour formation, production of allergenic compounds and mycotoxins contamination which are a risk for human and animal health. Besides, mycotoxins can be produced in field, in grains of cereals, in raw materials, in transports, in stored food, and in different situations in which conditions are suitable for their production (Garcia et al, 2009). A number of fungi able to produce toxic metabolites, e.g. *Fusarium, Aspergillus* and *Penicillium*, are known to colonize maize (Chulze et al, 1996). On the other hand, the growth of moulds depends on the effects of multiple variables like pH, water activity (a_w), solute concentrations, temperature, atmosphere, time, etc. However, generally a_w and temperature are regarded as the main controlling factors determining the potential for growth (Panagou et al., 2003; Plaza et al., 2003; Dantigny et al., 2005). Critical water content for safe storage of maize corresponds to a water activity (a_w) of about 0.7. Problems in maintaining an adequately low a_w often occur in the tropics where high ambient humidity makes the control of commodity moisture difficult (Chulze, 2010).

Fusarium verticillioides and *Aspergillus flavus* are two important mycotoxigenic moulds and they can colonize maize and their by-products. *A. flavus* is an important aflatoxin producer and can infect maize pre- and post-harvest; an increased presence of aflatoxins can occur if the phases of drying and storage are poorly managed (Chulze, 2010). Within aflatoxins group, aflatoxin B_1 (AFB_1) is considered as a carcinogen type 1 for the International Agency for Research on Cancer (IARC, 1993a). On the other hand, *Fusarium* species are predominantly considered as field fungi. Fumonisin production can however occur post-harvest when storage conditions are inadequate (Marin et al. 2004). *F. verticillioides* is an important fumonisin producer. Fumonisins are considered as "possible human carcinogen" (IARC, 1993). Both aflatoxins and fumonisins are relevant in maize and maize-based foods and feeds due to their widespread occurrence and co-occurrence (Chulze, 2010).

In order to prevent mycotoxin contamination, control of mycotoxigenic mould growth is required in maize and its derivatives. Mould growth is commonly controlled using synthetic fungicides (López-Malo et al. 2000; López et al., 2004); however, natural antimicrobials have also shown important antifungal properties (López-Malo et al. 2000). Continuous and indiscriminate use of chemical preservatives in foods and feeds, can lead to toxic effects for consumers and to the development of resistances in microorganisms (López et al. 2004).

Some researches showed that antioxidant compounds have an inhibitory effect on *Aspergillus* spp. and *Fusarium* spp. growth and toxin production (Mahoney and Molyneux., 2004; Palumbo et al., 2007; Radulovic et al., 2006; Romero et al., 2010; Samapundo et al., 2007). Plant extracts contain different antioxidant compounds such as polyphenols, phenols, flavonoids, etc. which could be the bioactive basis responsible for their antimicrobial property (Ebana and Madunagu., 1993). Extracts with antimicrobial

properties obtained from plants, could be a possibility to control mycotoxigenic fungi in foods and feeds, avoiding the use of chemicals.

Many studies in the case of *Equisetum arvense* and some others in the case of *Stevia rebaudiana* reported their antioxidant constituents (flavonoids and phenolic acids in their aqueous extracts), which might confer them antifungal properties (Table 1). In fact, some phenolic acids, i.e. gallic acid, caffeic acid and protocatechuic acid, all present in *S. rebaudiana* extracts and some flavonoids, i.e kaempherol, quercetin, isoquercetin, apigenin and luteolin, some present in *S. rebaudiana* and *E. arvense* extracts have been shown to have proven either antifungal or antimicrobial activity (Romero et al.. 2010; Merkl et al., 2010; Cushnie and Lamb, 2005). Besides, Curry et al. (2008) and Tago et al. (2010) studied *S. rebaudiana* and *E. arvense* toxicity and they were not associated with any signs of clinical toxicity or adverse effects. There are few studies that show antifungal activity of *Stevia* spp. and *Equisetum* spp. extracts (Canadanovic-Brunet, 2009; Milanovic et al., 2007). For this reason the aim of this work was to assess the antifungal and antimycotoxigenic effect of hydro-alcoholic extracts of *E. arvense* and *S. rebaudiana* on unsterilised maize, inoculated with different levels of *A. flavus* and *F. verticillioides* at different water activity (a_w) conditions.

Table 1. Antioxidant compounds of *E. arvense* and *S. rebaudiana* as reported in the existing in literature.

	General compound	Specific compound*
E. arvense	**Phenolic acids**	Apigenin 5-*O*-glucoside
		Methyl esthers of protocatechuic
		5-*O*-caffeoyl shikimic acid
		Monocaffeoyl meso-tartaric acid
		Dicaffeoyl *meso*-tartaric acid
	Flavonoids	Quercetin
		Isoquercetin
		Quercetin 3-*O*-glucoside
		Quercetin 3-*O*-(6''-*O*-malonylglucoside)
		Kaempferol 3-*O*-glycoside
	Terpenes	1,8 Cineol
		Linalool
		Thymol
		Camphor
S. rebaudiana	**Phenolic acids**	Pyrogallol
		4-Methoxybenzoic acid p-Coumaric acid
		4-Methylcatechol
		Sinapic acid
		Cinnamic acid
		Salicylic acid
		Gallic acid
		Protocatechuic acid
		Catechin
		Epicatechin
		Caffeic acid
		Chlorogenic acid
		p-coumaric acid
		Rutin
	Flavonoids	Quercetin
		Quercetin dihydrate
		Quercetin-3-*O*-glucoside
		Quercetin-3-*O*-arabinoside
		Apigenin-4'-*O*-glucoside
		Luteolin-7-*O*-glucoside
		Kaempferol-3-*O*-rhamnoside
		Carvacrol
	Terpenes	α-pinene
		Caryophylle
		Limonene

Compiled from Radulovic et al., 2006; Mimica-Dukic, 2008; Sandhu et al., 2010; Kim, 2011; Milovanovic et al., 2007 and Muanda et al., 2011

2. Materials and Methods

2.1 Fungal isolates and preparation of inoculum

Two mycotoxigenic isolates were included in this study, *Aspergillus flavus* (UdL-TA 3.215, producer of aflatoxins B_1, B_2) and *Fusarium verticillioides* (UdL-TA 3.232; producer of fumonisis B_1 (FB_1) and B_2 (FB_2)). The references in brackets are the codes of cultures held in the Food Technology Department Culture Collection of the University of Lleida. The isolates were sub-cultured on potato dextrose agar (PDA) plates and incubated at 25ºC for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from PDA plates and they were suspended in 5 mL of distilled H_2O/glycerol solutions with different water activity (a_w) levels: 0.85, 0.90 and 0.95 for *A. flavus* and 0.93 and 0.95 for *F. verticillioides*. After homogenizing, the suspensions were adjusted using a Thoma counting chamber to final concentrations of $1-5\times10^2$; $1-5\times10^4$ and $1-5\times10^6$ spores/mL.

2.2 Plant extract and maize preparation

Commercial dried *S. rebaudiana* (PAMIES HORTÍCOLES S.L., Spain) was used in this study. The aerial parts of *E. arvense* (collected in fields from Catalonia, Spain, 2009-2010) were dried at 40ºC. 50g of dried vegetal material was extracted with 1000 mL of ethanol 70% at room temperature during 5 days. After that, ethanol and part of water were evaporated by rota-evaporation to obtain a 50% (w/v) concentration of plant, and the extracts were stored at 4ºC until their use.

Unsterilised maize was used. Water activity was adjusted by aseptically adding amounts of sterile distilled water to maize in sterile bottles. The bottles were cooled down to 4ºC for 48 hours with periodic hand-shaking during this time. The amount of water necessary to reach the different water activity levels was determined by calibration curves (Garcia et al. 2011), subtracting the volume of extracts to be added. Both plant extracts, separately and the 1:1 mixture of them, were aseptically incorporated into the maize seeds in a rate of 6 ml of 50% extract per 100 g of maize. Final a_w values of seeds were checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of ± 0.003, before, during and at the end of the experiment.

2.3 Inoculation and incubation

Petri dishes were inoculated centrally with 5 µl of each spore suspension. Plates with the same a_w were enclosed in sealed containers along with beakers containing water glycerol solutions of the same a_w as the plates in order to maintain the a_w (Dallyn, 1978). Petri dishes were incubated at 25 ºC and a_w 0.85, 0.90 and 0.93 a_w (*A. flavus*) and 0.93 and 0.95 a_w (*F. verticillioides*). For each a_w condition, 3 Petri dishes were inoculated. Colony forming units (CFUs) were counted after 10, 20 and 30 days by serial dilution plating technique on Dichloran Rose-Bengal Chloramphenicol (DRBC; glucose 10 g; peptone 5 g; potassium dihydrogen phosphate (KH_2PO_4) 1 g; magnesium sulphate $MgSO_4H2O_7$ 0.5 g;

chloramphenicol 2 mL; dichloran 0.002g; Rose Bengal 0.025 g; agar-agar 15 g; destilled water 1000 mL, was used for assessment of CFUs of *A. flavus*. Green Malachite Medium (GMM), a selective medium for *Fusarium* spp. (Peptone 5 g; potassium dihydrogen phosphate (KH_2PO_4) 1 g; magnesium sulphate $MgSO_4H2O_7$ 0.5 g; green malaquite 2.5 µg/mL; agar-agar 20 g; destilled water 1000 mL) was used for counting this mould.

2.4 Mycotoxins extraction from maize

Mycotoxins were extracted after 10, 20 and 30 days of incubation in triplicate by immunoaffinity chromatography and stored at 4 ºC until analysis by HPLC.

2.4 a. Aflatoxins extraction and clean-up

5 g of milled sample were extracted with 15 mL of acetonitril+water (60+40 v/v) and shaken for 10 minutes. The extract was filtered through number 1 filter paper and 2 mL of filltrate were mixed with 14 mL of PBS. Then, the diluted extract was cleaned up by passage through an immunoaffinity column (EASI-EXTRACT AFLATOXIN*.* R-Biopharm Rhone Ltd., Glasgow, Scotland) at a flow rate of 2–3 mL/min. The column was then washed with 20 mL of PBS and left to dry. Aflatoxins were finally eluted from the column with 3 mL methanol+water (50+50, v/v).

2.4 b. Fumonisins extraction and clean-up

10 g of milled sample were extracted with 15 mL of methanol+acetonitril+water (25+25+50, v/v) and 1 g of NaCl and shaken for 20 minutes. The extract was filtered through number 1 filter paper and 10 mL of filtrate were mixed with 40 mL of PBS. Then, the diluted extract was cleaned up through passage through an immunoaffinity column (FUMONIPREP, R-Biopharm Rhone Ltd., Glasgow, Scotland) at a flow rate of 2–3 mL/min. The column was then washed with 20 mL of PBS and left to dry. Fumonisins were finally eluted from the column with 3 mL methanol+water (50+50, v/v).

2.5. Mycotoxins detection and quantification

2.5.a. General description of the equipment

Mycotoxins were detected and quantified separately by using a HPLC system (Waters 2695, separations module, Waters, Milford, USA) and a C_{18} column (5 µm Waters Spherisorb, 4.6 × 250 mm ODS2). Mobile phase was always pumped at 1 mL/min and injection volume was always 100 µL. Waters 2475 module (Waters, Milford, USA) was used for fluorescence detection. Quantification was always achieved with a software integrator (Empower, Milford, MA, USA). Mycotoxins were quantified on the basis of the HPLC fluorimetric response compared with that of a range of mycotoxins standards.

2.5.b. <u>Aflatoxins B_1 and B_2 (AFB$_1$ and AFB$_2$)</u>: A post column photochemical derivatization system (LCTech detector, UVC 254 nm, Germany) was used and toxins were detected by fluorescence (λ_{exc} 365 nm; λ_{em} 455 nm). The mobile phase was water+methanol+acetonitrile (70+17+17) and the detection limit of the analysis was about 0.03 ng/g for B_2 and 0.06 ng/g for B_1, based on a signal-to-noise ratio of 3:1. The range of aflatoxins standards used for quantification was 0.09-2.5 µg/mL. The mean recovery rate was 79% and 85% for AFB$_1$ and AFB$_2$, respectively, when samples were spiked at 0.83-3.33 ng/g and 0.17-0.83 ng/g, respectively.

2.5.c. <u>Fumonisins B_1 and B_2 (FB$_1$ and FB$_2$)</u>: Fumonisins were detected by fluorescence (λ_{exc} 335 nm; λ_{em} 440 nm). The mobile phase was methanol+0.1M sodium dihydrogen phosphate (77+23), solution adjusted to pH 3.35 with orthophosphoric acid. Dried extracts were dissolved in methanol and derivatized with OPA (Sydenham, Shephard, Thiel, Stockenström, Snijman, and Van Schalkwyk, 1996). Detection limit of the analysis was about 0.54 ng/g for FB$_1$ and 1.68 ng/g for FB$_2$, based on a signal-to-noise ratio of 3:1. Quantification was achieved with Empower. The range of FBs standards used for quantification was 0.015-7.82 µg/mL. The mean recovery rate was 93% and 84% for FB$_1$ and FB$_2$, respectively, when samples were spiked at 0.2-2 µg/g and 0.1-1µg/g, respectively.

2.6 Statistical analyses

Analysis of variance of CFUs and mycotoxins was used in order to assess significant differences due to growth conditions (water activity, inoculum concentration, incubation time) and assayed antifungals. . LSD test was used to establish the differences among mean values of the variables under the different levels of factors (extracts, water activity, inoculum size, incubation time) at *p*<0.05.

3. Results

3.1 Plant extract effects on mould growth in maize

Analysis of variance (ANOVA) of the effect of water activity (a_w), inoculum level, different extracts, and their interaction showed that all factors alone and their interaction were statistically significant (p<0.05) in relation with mould growth. Plant extracts effect on growth for both moulds was highly dependent on a_w (p<0.05), while the inoculum size was less important. Extracts were effective at 0.95 a_w in mould growth inhibition; however, for low a_w, results were not so clear.

Figure 1 shows mean values of CFUs for *A. flavus* in plant extract treated maize at different a_w, times of incubation and inoculum level. In general, at 0.95 a_w, CFUs decreased significantly (p<0.05) with the mixture of extracts compared with the control, at all times assayed (10, 20 and 30 days) (>99% inhibition, regardless of the sampling time and inoculum size); at this a_w the extract of *E. arvense* and the mixture of

both extracts had similar results, with higher reductions than *S. rebaudiana.* At low a_w (0.85, 0.90), no concluding statistically significant effect of the extracts was observed.
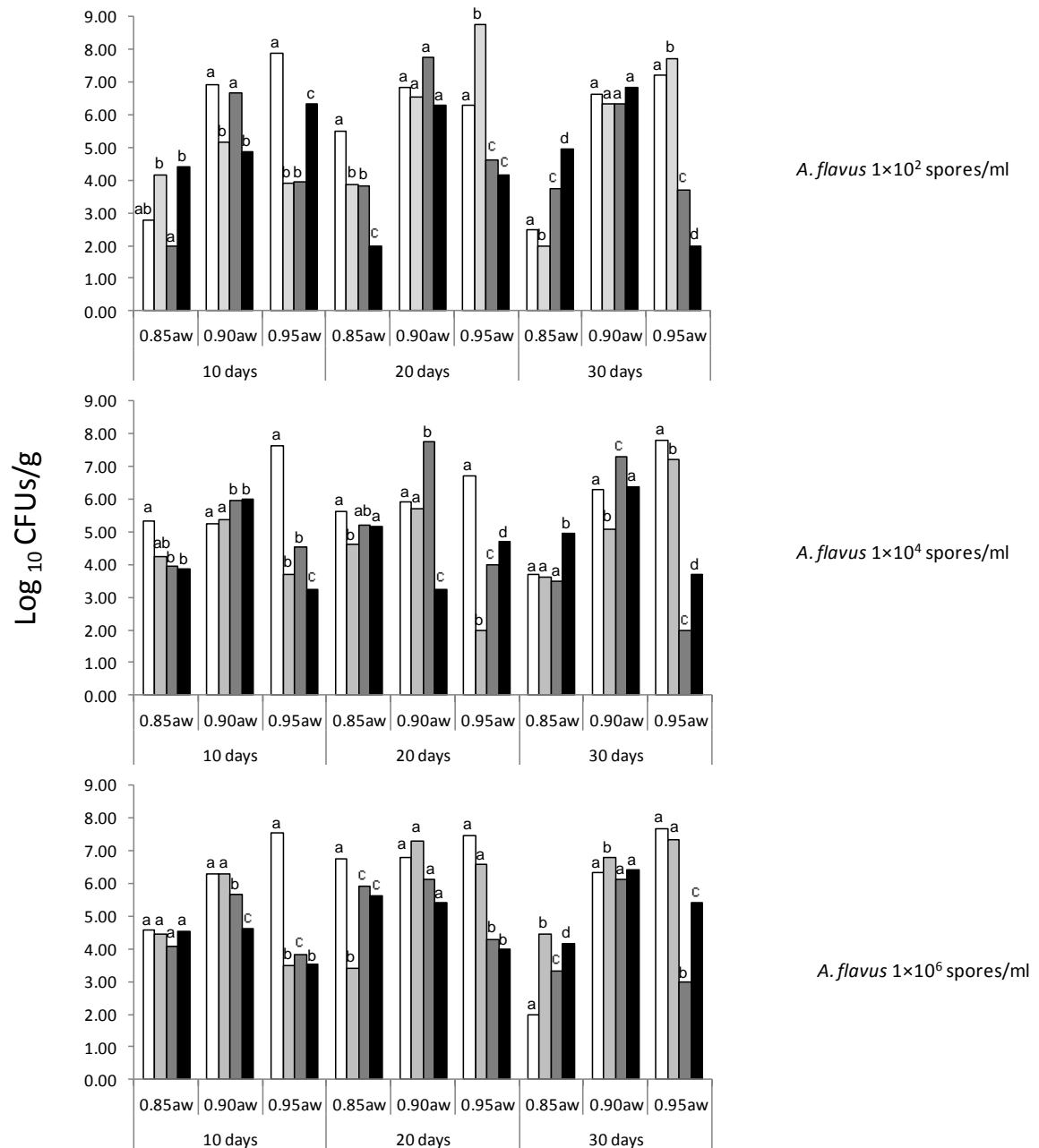
Figure 1: Growth of *A. flavus* in maize seeds with plant extract at different a_w conditions at 25 °C in DRBC medium. Control; ☐ *S. rebaudiana*; ☐ *E. arvense*; ■ Mixture ■ . Bars with different letters are significantly different according to LSD test.
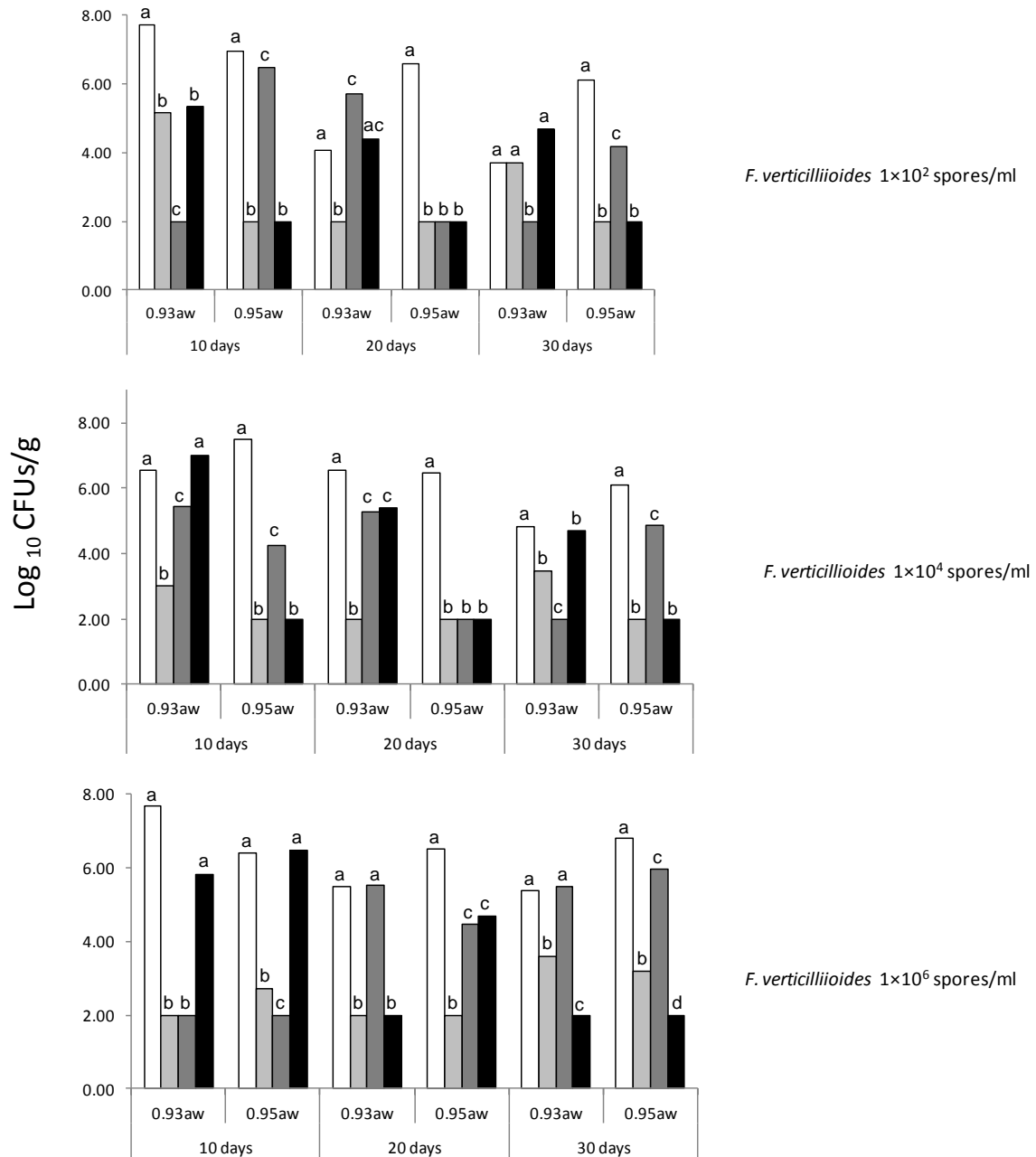
Figure 2: Growth of *F. verticillioides* in maize seeds with plant extract at different a_w conditions at 25 °C in GMM. Control; □ *S. rebaudiana*; □ *E. arvense*; ▨ Mixture ■ . Bars with different letters are significantly different according to LSD test.

Figure 2 shows mean values of CFUs for *F. verticillioides* in plant extracts treated maize grain at different a_w, times of incubation and inoculum level. In general, significantly lower CFU levels were observed in presence of extracts, especially with *S. rebaudiana* extract, compared with the control (>99% of inhibition under all conditions, except at $0.93a_w$/30 days where no change was observed). The extract of *E. arvense* and the mixture of both also reduced growth significantly. In this case, all the extracts worked similarly at both 0.93 and 0.95 a_w.

It must be highlighted that significant stimulation of growth by the extracts was observed in a few cases.

3.2 Plant extracts effects on mycotoxins production in maize

AFB_1 was detected in maize either treated or not (Table 2). No significant differences ($p<0.05$) among inoculum levels were found. At 0.85 a_w, AFB_1 levels were, in general < 5 ng/g, and no differences could be observed between control and treatments. At 0.90 a_w, AFB_1 levels were higher (up to 675 ng/g), however, due to the variability among replicates, significant reductions were rarely observed. Finally, at 0.95 a_w, all 3 extracts led to a decreased aflatoxin presence, *E. arvense* extract being the most effective. *E. arvense* and the plants extract mixture caused reductions up to 100%, while *S. rebaudiana* extract did not have such a marked effect.

Table 2. Aflatoxin B_1 level (ng/g maize) in presence of plant extracts at different a_w conditions at 25 °C.

Inoculum conc. (spores/mL)	Treatment	10 days			20 days			30 days		
		$0.85a_w$	$0.90a_w$	$0.95a_w$	$0.85a_w$	$0.90a_w$	$0.95a_w$	$0.85a_w$	$0.90a_w$	$0.95a_w$
1×10^2	Control	0.13 a	121.15 a	1533.74 a	0.26 a	586.07 a	1077.37 a	2.03 a	713.69 a	1196.97 a
	Stevia sp.	<ld	0.22 b	0.09 b	0.17 a	74.22 ab	1135.92 a	1.40 a	24.58 c	821.37 b
	E. arvense	0.17 a	47.34 ab	0.11 b	5.38 a	24.28 ab	0.06 b	0.43 a	304.76 b	2.03 c
	Mixture	<ld	1.05 b	0.07 b	0.49 a	18.27 b	0.45 b	0.98 a	662.92 a	0.72 c
1×10^4	Control	57.80a	7.78 a	1750.91 a	<ld	10.48 ab	1315.81 a	1.23 a	55.62 ab	1535.13 a
	Stevia sp.	12.09 b	0.34 a	2.49 b	459.16 a	0.95 a	4.39 b	0.42 a	1.71 a	700.13 b
	E. arvense	0.89 b	0.33 a	<ld	<ld	110.52 ab	<ld	<ld	675.39 b	<ld
	Mixture	<ld	1.97 a	0.12 b	0.22 b	165.41 b	0.45 b	0.44 a	624.38b	<ld
1×10^6	Control	<ld	95.07 ab	1537.60 a	<ld	643.73 a	969.23 a	0.09 a	361.90 a	1204.69 a
	Stevia sp.	0.23 a	380.13 a	14.99 b	<ld	145.91 b	207.34 ab	1.01 a	358.01 a	874.73 b
	E. arvense	0.65 a	41.74 b	3.03 b	1.53 c	42.74 b	0.79 b	1.98 a	37.64. ab	<ld
	Mixture	3.46 a	0.97 b	14.87 b	0.56 c	135.23 b	<ld	1.74 a	2.10 b	1.09 c

<ld: below limit of detection

* In each column, for each inoculum concentration, values with the same letter are not significantly different($p<0.05$)

No significant inhibitory effect of extracts was observed in AFB$_2$ production (p<0.05) (Table 3). And regarding FB$_1$, it was detected in maize either treated or not (Table 4). No significant differences (p<0.05) among inoculum levels and different a$_w$ levels assayed were found. However, significant differences (p<0.05) between plant extract treatments and time of incubation were found. The inhibition of production was, however, low, and sometimes stimulation occurred. A slight inhibition of FB1 production occurred with *E. arvense* extract after 20-30 days of incubation. Similar results were found for FB$_2$ (Table 4). *S. rebaudiana* and *E. arvense* extracts led to a decrease in FB2 level after 10 and 20 days, with a decrease of 46 and 51% for 10 days and 71% and 73% for 20 days of incubation, respectively.

It must be highlighted that significant stimulation of mycotoxin production by the extracts was observed in a few cases.

Table 3. Aflatoxin B_2 level (ng/g maize) in presence of plant extracts at different a_w conditions at 25 °C.

Inoculum conc. (spores/mL)	Treatment	10 days			20 days			30 days		
		$0.85a_w$	$0.90a_w$	$0.95a_w$	$0.85a_w$	$0.90a_w$	$0.95a_w$	$0.85a_w$	$0.90a_w$	$0.95a_w$
1×10^2	Control	<ld	7.32 a	0.13 a	<ld	1.70 a	3.44 a	<ld	14.21 a	8.78 a
	Stevia sp.	<ld	<ld	<ld	<ld	0.41 b	7.53 b	<ld	0.19 b	10.17 a
	E. arvense .	<ld	1.87 b	<ld	0.48	0.38 ab	<ld	<ld	1.78 ab	0.03 b
	Mixture	<ld	0.03 c	<ld	<ld	0.09 b	<ld	<ld	3.18 ab	<ld
1×10^4	Control	10.80 a	0.25 a	0.80 a	<ld	2.00 ab	4.24 a	<ld	3.11 ab	15.84 a
	Stevia sp.	0.45 a	<ld	<ld	2.63	<ld	0.60 b	<ld	0.07 a	9.34 a
	E. arvense	<ld	<ld	<ld	<ld	1.99 ab	<ld	<ld	6.52 b	<ld
	Mixture	<ld	<ld	<ld	<ld	8.98 b	<ld	<ld	1.88 ab	<ld
1×10^6	Control	<ld	3.68 a	3.25 a	<ld	2.57 a	8.07 a	<ld	6.47 a	17.13 a
	Stevia sp.	0.03 a	11.64 b	0.18 b	<ld	5.64 a	0.48 b	<ld	2.54 b	9.13 b
	E. arvense	<ld	1.59 a	2.03 b	<ld	2.73 a	<ld	0.04	2.53 b	<ld
	Mixture	0.30 a	0.27 a	0.16 b	<ld	1.86 a	<ld	<ld	<ld	<ld

<ld: below limit of detection

* In each column, for each inoculum concentration, values with the same letter are not significantly different ($p<0.05$)

Table 4. Fumonisin B_1 and B_2 level (μg/g maize) in presence of plant extracts at different a_w conditions at 25 °C.

	10 days		20 days		30 days	
	FB$_1$	FB$_2$	FB$_1$	FB$_2$	FB$_1$	FB$_2$
Control	2.49 a	0.68 a	3.23 c	0.56 a	2.59 ab	0.36 a
Stevia sp.	9.14 b	0.37 b	0.95 a	0.16 b	4.34 c	0.43 a
E. arvense	3.19 a	0.33 b	1.14 ab	0.15 b	1.55 a	0.20 a
Mixture	6.36 ab	0.62 ab	2.2 bc	0.41 a	4.00 c	0.25 a

* In each column, values with the same letter are not significantly different ($p<0.05$)

4. Discussion

Accumulation of mycotoxins in crops is increasing worldwide due to climatic changes, use of different plant varieties of high yield but which are susceptible to mycotoxins accumulation, and agricultural practices (Chulze, 2010). Mycotoxins can be produced before harvest and can increase their level during post-harvest and storage of grain. Therefore, it is necessary an early fungal growth and mycotoxin production control, in field through good agricultural practices. The increasing knowledge of the pollutive, residual, carcinogenic and toxic effects of some synthetic fungicides has resulted in the need to obtain alternatives to control mycotoxigenic strains growth and mycotoxin production. Currently, interest in more natural, safe and effective products, has resulted in the need to obtain quantitative data on plant extracts (Hammer et al., 1999). Mitscher et al. (1987) suggested that it is very important to scientifically study traditional medicinal plants because they may contain new sources of antimicrobial compounds. *E. arvense* is used, currently, for biological control of fungi and pests, whereas *S. rebaudiana* is consumed as a natural sweetener.

In this research we evaluated the antifungal and antimycotoxigenic capability of *E. arvense* and *S. rebaudiana* extracts, and a mixture of them, on growth and mycotoxin production of *A. flavus* and *F. verticillioides* on naturally contaminated maize grain at different a_w and inoculum levels. Conditions studied are near to conditions which may occur in cereals in pre and post-harverst, but do not include the optimum growth conditions for these moulds. Extracts showed certain inhibition of mould growth, especially at the high a_w assayed where *A. flavus* growth was highly inhibited particularly with the mixture of extracts, while for *F. verticillioides*, *S. rebaudiana* extract was more effective. In general, low numbers of CFUs where recorded at lower a_w for both moulds, thus the possible effects of extracts were hindered.

Researches have shown that plant extracts contain antioxidant compounds, mainly phenols such as thymol or monoterpernes which could influence on mould develoment (Radulovic et al. 2006). Some

authors worked with different antioxidants on mould growth and mycotoxin production. Nesci et al. (2007) worked with synthetic antioxidants on *Aspergillus* section *Flavi* populations in maize grain. They found that a mixture of butylated hydroxyanisole (BHA) and propyl paraben (PP) (1:1) affected natural mycobiota and *Aspergillus* section *Flavi* growth on stored maize. On the other side, Etcheverry et al. (2002) and Torres et al. (2003) showed a good control of *F. verticillioides* and *F. proliferatum* and fumonisin production on culture medium and irradiated maize with these antioxidants, under different conditions of a_w and temperature

Mechanisms of action of plant extracts are unknown. However, some authors attributed antimicrobial action to:

i) Presence of OH groups able to form hydrogen bonds that have effects on enzymes (Farag et al. 1989).

ii) Action on mould morphology (Kale et al.,1996; Rasooli and Owlia, 2005).

iii) Inhibitory effect due to granulation of the cytoplasm, cytoplasmic membrane rupture and inactivation and/or the inhibition of the intracellular synthesis of enzymes (Srivastava et al. 2008).

A decreased mould growth could lead to a decrease of mycotoxin production. To our knowledge, there are no published works with regard to mycotoxin production in presence of *E. arvense* and *S. rebaudiana* hydro-alcoholic extracts in maize. In our study, at 0.85 a_w no AFB_1 was detected or level was low, probably due to the reduced a_w plus the biotic interactions. In general, extracts were more effective at the higher a_w level with great inhibition of AFB_1 production. Nesci and Etcheverrry (2006) worked with natural maize phytochemicals on growth and AFB_1 production for *A. flavus* and *A. parasíticus* on maize medium and they found completely inhibition on AFB_1 production at a_w=0.937-0.999. These results agree with ours where phytochemicals inhibition was a_w level dependent. Bluma and Etcheverry (2008) also found that a_w level is an important factor for mycotoxin inhibition. They worked with plant essential oils on *A. flavus* and *A. parasiticus* growth and mycotoxin production. In this work boldus, poleo and mountain thyme essential oils completely inhibited AFB_1 at 2000 and 3000 μg/g of essential oils at 11 incubation days.

Regarding fumonisins, time of incubation was an important factor for production of these mycotoxins. *E. arvense* was more effective compared with *S. rebaudiana* and the mixture. Some studies with essential oils and different antioxidants on *F. verticillioides* have shown inhibition of fumonisins production. López et al. (2004) worked with aromatic plant essential oils, finding that *Origanum vulgare* decreased FB_1 production while *Aloysia triphyla* increased the level. Farnochi, et al. (2005) showed a reduction of fumonisin accumulation between 32-77% with 1000 μg/g of BHA after 28 days. However, the reduction in fumonisin levels could be influenced by the competing mycobiota.

Our results show, in some cases, stimulation of growth and mycotoxins production due to the plant extracts. Inhibition of competing mycoflora might be the reason for an increased population of mycotoxigenic fungi.

Results presented in this study confirm that extract of *Equisetum* and a mixture 1:1 of *Equisetum-Stevia* may be effective for the inhibition of both growth of aflatoxin producers and aflatoxin production at high water activity levels (preharvest conditions). Although growth of the fumonisin producer was reduced by the use of plant extracts at 0.93-0.95 a_w, fumonisin presence was not affected. Due to the widespread fungal contamination and the risks of mycotoxins in foods and feeds, it could be interesting to find plant extracts with antifungal effects against the most common fungal species in grain microbiota. *E. arvense* and *S. rebaudiana* extracts could be an alternative to control aflatoxigenic maize mycobiota in moist grain.

Acknowledgement

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3.3 *Equisetum arvense* hydro-alcoholic extract: phenolic composition and antifungal and antimycotoxigenic effect against *Aspergillus flavus* and *Fusarium verticillioides* in stored maize

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Abstract

Maize is a very important cereal for human and animal diet, but it can be contaminated by moulds and their mycotoxins. On the other hand, natural plant products with antimicrobial properties could be a possibility to control mycotoxigenic fungi in foods and feeds. In this study, *Equisetum arvense* extract was tested for the efficacy on *Aspergillus* section *Flavi* and *Fusarium* section *Liseola* growth. Natural contaminated maize was used in this study and extract was added under different water activities (a_w), 0.90 and 0.95 for *Aspergillus* section *Flavi* and *Fusarium* section *Liseola*, respectively. Moulds were inoculated in maize and incubated during 30 days. Results showed in this study confirm that *E. arvense* extract may be effective for the inhibition of *Aspergillus* section *Flavi* in maize with high level of this mould. Moreover, this extract showed a good inhibition of growth on *Fusarium* section *Liseola* levels. Aflatoxin and fumonisin production was not affected by the extract. Therefore, *E. arvense* extract could be an alternative to control maize mycobiota level in moist grain.

1. Introduction

Mycotoxins are secondary metabolites synthesized by some mould species in foods and feeds which are a risk for human and animal health. When conditions are suitable for mould growth, mycotoxins can be produced and contaminate raw materials. On the other hand, maize is a very important cereal for human and animal diet; however, it can be contaminated by moulds. *Aspergillus* and *Fusarium* species can infect maize pre-harvest, and mycotoxin contamination can increase if storage conditions are poorly managed[1]. *Aspergillus flavus* is the main producer of aflatoxins, the most important mycotoxins in the world's food supplies[2]. Aflatoxins are carcinogenic, mutagenic, teratogenic and hepatotoxic compounds formed as secondary metabolites during the growth of *A. flavus* and *A. parasiticus*[3]. Aflatoxin B_1 (AFB_1) is considered the most important mycotoxin because is considered as a carcinogen type 1 by the International Agency for Research on Cancer[4]. On the other hand, *Fusarium* species are predominantly considered as field fungi and plant pathogens. *F. verticillioides* is an important fumonisin producer and fumonisin B_1 is considered as "possible human carcinogen"[4].

Mould growth is commonly controlled using synthetic fungicides; however, natural antimicrobials have also shown important antifungal properties[5,6]. Extracts obtained from plants with antimicrobial properties could be a possibility to control mycotoxigenic fungi in foods and feeds, avoiding the use of chemicals[7]. Possibly, the antimicrobial effect could be attributed to the presence of various antioxidant substances, mainly phenolic compounds such as polyphenols, flavonoids, and monoterpernes such as thymol, carvacrol[8,9,10]. Besides, antioxidant compounds, reducing mould growth may lead to a decrease of total mycotoxin accumulation, or interfere in the mycotoxin synthesis[6].

Equisetum spp. has been described as an herb with antioxidant properties by different studies[10,11,12,13]. Besides, Tago *et al.*[14] studied *E. arvense* toxicity and it was not associated with any signs of clinical toxicity or adverse effects. Some researches have shown that antioxidant compounds have an inhibitory effect on *Aspergillus* spp. and *Fusarium* spp. growth and toxin production[10,15,16,17,18]. To our knowledge, there is little published information with regard to mycotoxin production in presence of *E. arvense* extracts. Garcia *et al.*[19] used *E. arvense* hydro-alcoholic extract for the control of *A. flavus* and *F. verticillioides* in maize in Petri dishes, and their results confirmed that the extract may be effective for the inhibition of growth of both fungi, specially at high water activity levels (pre-harvest conditions). In general, growth of *F. verticillioides* was reduced by the use of plant extracts; however, fumonisin presence was not significantly affected. Our aim in this work was to assess the antifungal and antimycotoxigenic effect of a hydro-alcoholic extract of *E. arvense* on bulk unsterilised and *A. flavus* or *F. verticillioides* inoculated maize under storage conditions, and to study the phenolic composition of the extract.

2. Materials and Methods

2.1 Fungal isolates and preparation of inoculum

Two mycotoxigenic isolates were included in this study, *Aspergillus flavus* (UdL-TA 3.215), producer of aflatoxins B_1 (AFB_1) and B_2 (AFB_2) and *Fusarium verticillioides* (UdL-TA 3.232), producer of fumonisis B_1 (FB_1) and B_2 (FB_2). The references in brackets are the codes of cultures held in the Food Technology Department Culture Collection of the University of Lleida. The isolates were sub-cultured on potato dextrose agar (PDA) plates and incubated at 25 ºC for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from PDA plates and they were suspended in 5 mL of distilled H_2O/glycerol solutions with different water activity (a_w) levels: 0.90 for *A. flavus* and 0.95 for *F. verticillioides*. After homogenizing, the suspensions were adjusted using a Thoma counting chamber to final concentrations of $1\text{-}5{\times}10^6$ spores mL^{-1}.

2.2 Plant extract preparation and phenolic compound analysis

Aerial parts of *E. arvense* (collected in fields from Catalonia, Spain, 2009-2010) were dried at 40 ºC. 50g of dried vegetal material was extracted with 1000 mL of ethanol:water (1:1) at room temperature during 5 days. After that, ethanol and part of water were evaporated by rota-evaporation to obtain an 80% concentrated plant extract that was stored at 4 º C until use.

Extract was analyzed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C8, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% B over 5 min, 15-20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% B over 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. Spectra were recorded in negative ion mode between *m/z* 100 and 1000. EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) of the previous

experiment using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V.

2.3 Maize preparation

Unsterilised maize was used for this experiment. Initial contamination (colony forming units, CFUs/g) was 1.5×10^5 CFU g^{-1} and 5×10^6 CFU g^{-1} for *A.* section *Flavi* and *F.* section *Liseola*, respectively. Only FB_1 was detected initially with a level of 0.57 µg g^{-1}.

Water activity was adjusted by aseptically adding sterile distilled water to maize in sealed containers with 3 kg of grain. The containers were cooled down to 4 ºC for 48 hours with periodic hand-shaking during this time. The grain a_w tested levels were 0.90 for *A. flavus* and 0.95 for *F. verticillioides*. The amount of water necessary to reach the different a_w levels was determined by calibration curves[20], subtracting the volume of extracts to be added. *E. arvense* extract was aseptically incorporated into the maize seeds to a final concentration of 0.3g kg^{-1}(w/w). An extract of *E. arvense* of 1.15 g mL^{-1} was aseptically incorporated into the maize seeds to a final concentration of 30 mg g^{-1} of maize. Final a_w values of seeds were checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of ± 0.003, before, during and at the end of the experiment.

2.4 Inoculation and incubation

A full factorial design with three replicates per treatment was followed. Overall, 8 treatments were tested: treated/untreated maize, *A. flavus* inoculated/uninoculated maize, *F. verticillioides* inoculated/uninoculated maize. Containers were inoculated with an additional concentration of 1×10^3 spores g^{-1} of grain, thoroughly mixed and incubated at 25 ºC. CFUs were counted after 15 and 30 days by serial dilution plating technique on Dichloran Rose-Bengal Chloramphenicol (DRBC; glucose 10 g; peptone 5 g; potassium dihydrogen phosphate KH_2PO_4 1 g; magnesium sulphate $MgSO_4 \cdot 7H_2O$ 0.5 g; chloramphenicol 2 mL; dichloran 0.002 g; Rose Bengal 0.025 g; agar-agar 15 g; distilled water 1000 mL, was used for assessment of CFUs of *Aspergillus* section *Flavi*. Green Malachite Medium (GMM), a selective medium for *Fusarium* spp. (peptone 5 g; potassium dihydrogen phosphate KH_2PO_4 1 g; magnesium sulphate $MgSO_4 \cdot 7H_2O$ 0.5 g; green malaquite 2.5 mg; agar-agar 20 g; distilled water 1000 mL) was used for counting *Fusarium* section *Liseola*.

2.5 Mycotoxins extraction

Mycotoxins were extracted after 15 and 30 days of incubation in triplicate and were extracted and analyzed as EASI-EXTRACT AFLATOXIN inmunoaffinity column protocol.

2.5.a Aflatoxins extraction and clean-up

5 g of milled sample were extracted with 15 mL of acetonitrile+water (60+40 v/v) and shaken for 10 minutes. The extract was filtered through number 1 filter paper and 2 mL of filltrate were mixed with 14 mL of PBS. Then, the diluted extract was cleaned up by passage through an immunoaffinity column (EASI-EXTRACT AFLATOXIN. R-Biopharm Rhone Ltd., Glasgow, Scotland) at a flow rate of 2–3 mL min^{-1}. The column was then washed with 20 mL of PBS and left to dry. Aflatoxins were finally eluted from the column with 3 mL methanol+water (50+50, v/v).

2.5.b Fumonisins extraction and clean-up

10 g of milled sample were extracted with 15 mL of methanol+acetonitrile+water (25+25+50, v/v) and 1 g of NaCl and shaken for 20 minutes. The extract was filtered through number 1 filter paper and 10 mL of filtrate were mixed with 40 mL of Phosphate Buffered Saline (PBS). Then, the diluted extract was cleaned up through passage through an immunoaffinity column (FUMONIPREP, R-Biopharm Rhone Ltd., Glasgow, Scotland) at a flow rate of 2–3 mL min^{-1}. The column was then washed with 20 mL of PBS and left to dry. Fumonisins were finally eluted from the column with 3 mL methanol+water (50+50, v/v).

2.6 Mycotoxins detection and quantification

2.6.a General description of the equipment

Mycotoxins were detected and quantified separately by using a HPLC system (Waters 2695, separations module, Waters, Milford, MA, USA) and a C_{18} column (5 μm Waters Spherisorb, 4.6 × 250 mm ODS2, Dublin, Ireland). Mobile phase was always pumped at 1 mL min^{-1} and injection volume was always 100 μl. Waters 2475 module (Waters, Milford, USA) was used for fluorescence detection. Quantification was always achieved with a software integrator (Empower, Milford, MA, USA). Mycotoxins were quantified on the basis of the HPLC fluorimetric response compared with that of a range of mycotoxins standards.

Aflatoxins B_1 and B_2 (AFB$_1$ and AFB$_2$): A post column photochemical derivatization system (LCTech detector, UVC 254 nm, Germany) was used and toxins were detected by fluorescence (λ_{exc} 365 nm; λ_{em} 455 nm). The mobile phase was water+methanol+acetonitrile (70+17+17) and the detection limit of the analysis was 0.03 ng g^{-1} for AFB$_2$ and 0.06 ng g^{-1} for AFB$_1$, based on a signal-to-noise ratio of 3:1. The range of aflatoxins standards used for quantification was 0.09-2.5 μg mL^{-1}.

Fumonisins B_1 and B_2 (FB$_1$ and FB$_2$): Fumonisins were detected by fluorescence (λ_{exc} 335 nm; λ_{em} 440 nm). The mobile phase was methanol+0.1M sodium dihydrogen phosphate (77+23), solution adjusted to pH 3.35 with orthophosphoric acid. Dried extracts were dissolved in methanol and derivatized with OPA reagent (o-phthalaldehyde)[21]. Detection limit of the analysis was 0.54 ng g^{-1} for FB$_1$ and 1.68 ng g^{-1} for

FB_2, based on a signal-to-noise ratio of 3:1. Quantification was achieved with Empower. The range of FBs standards used for quantification was 0.015-7.82 µg mL^{-1}.

2.7 Statistical analyses

Analysis of variance of CFUs and mycotoxins results was used in order to assess significant differences due to the assayed antifungal and time. LSD test was used to establish the differences among mean values of the variables under the different levels of factors at *p<0.05*.

3. Results

3.1 Chemical composition of E. arvense hydroalcoholic extract

Table 1 shows the general compounds of *E. arvense* hydro-alcoholic extract obtained by mass analyses. The chromatogram obtained (Figure 1) revealed 13 principal compounds from which kaempferol-3-*O*-rutinoside-7-*O*-glucoside and kaempferol dihexoside were the most predominant in the extract with 10.84 and 7.48 µg mg^{-1} lyophilised extract, respectively.
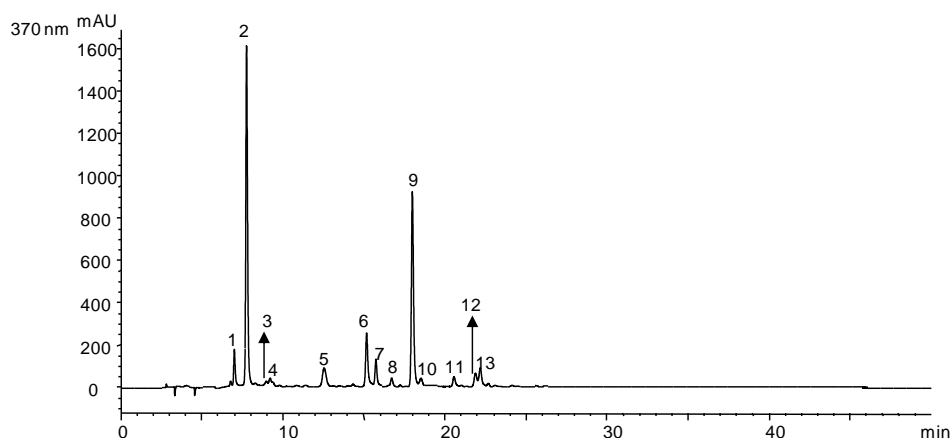
Figure 1: Chromatogram of *E. arvense* analyses at 370 nm. Identification of peak numbers is listed in Table 1.

Table 1. Retention time, maximum wavelength in the visible region (λ max), spectral mass data and identification and concentration of phenolic compounds in *E. arvense* hydro-alcoholic extract.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]⁻ (*m/z*)	MS^2 (*m/z*)	Tentative identification	µg mg^{-1} lyophilised extract
1	7.0	354	787	625, 463, 301	Quercetin 3-*O*-rutinoside-7-*O*-rhamnoside	0.77 ± 0.01
2	7.7	344	771	609, 447, 285	Kaempferol-3-*O*-rutinoside-7-*O*-glucoside	10.84 ± 0.21
3	8.9	358	787	625, 463, 301	Quercetin trihexoside	0.14 ± 0.01
4	9.2	328	355	193	Ferulic hexoside acid	0.14
5	12.5	330	179	135	Caffeic acid	1.82 ± 0.09
6	15.1	372	423	261	Equisetumpyrone	-
7	15.7	352	625	463,301	Quercetin 3-*O*-sophoroside	0.85 ± 0.01
8	16.7	328	309	193,178,149,135	Ferulic acid derivative	0.26 ± 0.04
9	17.9	348	609	447, 285	Kaempferol dihexoside	7.48 ± 0.12
10	18.5	328	193	178, 149, 135	*trans*-Ferulic acid	1.19 ± 0.03
11	20.5	356	463	301	Quercetin 3-*O*-glucoside	0.46 ± 0.02
12	21.8	354	505	463,301	Quercetin acetyl hexoside	0.55 ± 0.01
13	22.6	352	447	285	Kaempferol 3-*O*-glucoside	0.15 ± 0.01

3.2 Effect of E. arvense extract on mould growth in maize

Analysis of variance showed that *E. arvense* extract had a significant effect on *Fusarium* section *Liseola* growth, regardless of incubation time and additional inoculation. Levels of CFUs/g decreased in the presence of *E. arvense* extract (Figure 2). CFU counts were not significantly different after 15 or 30 days. Moreover, within treated and untreated samples, the levels were similar in uninoculated and inoculated treatments ($p<0.05$), which is logical taking into account the initial high level of *Fusarium Liseola* section. For *Aspergillus* section *Flavi* the inhibitory effect of the extract was only observed in the inoculated treatments, regardless of the incubation time ($p<0.05$), with a 45% population reduction. In this case, inoculated control treatments reached higher populations than uninoculated ones ($p<0.05$).
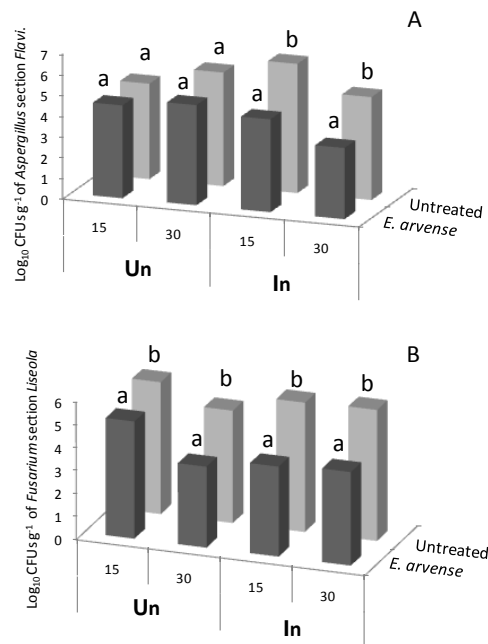
Figure 2: Growth of *Aspergillus* section *Flavi* (A) and *Fusarium* section *Liseola* (B) either *E. arvense*-treated or not, either additionally inoculated or not at 25 °C. Un: uninoculated maize. In: Inoculated maize with $1{\times}10^{3}$ spores per gram of grain.

3.3 Effect of E. arvense extract on mycotoxin production in maize

Analysis of variance showed that, in general, *E. arvense* extract had no significant effect on FB_1 and FB_2 production, for all studied conditions ($p<0.05$) (Table 2). On the other hand, no aflatoxins were detected in any of the treatments, neither in the controls.

Table 2. Fumonisin levels (µg g^{-1}) in maize grain treated with *E. arvense* hydro-alcoholic extract.

Maize condition	Time	Extract	Fumonisin B$_1$		Fumonisin B$_2$	
			Mean	SD	Mean	SD
Uninoculated	15	Untreated	0.34 **a**	0.38	<ld	0
		E. arvense	0.44 **a**	0.49	<ld	0
	30	Untreated	0.80 **a**	0.73	0.18 **a**	0.17
		E. arvense	1.42 **a**	1.39	0.20 **a**	0.22
Inoculated (1×10^3 spores g^{-1})	15	Untreated	0.76 **a**	0.51	<ld	0
		E. arvense	0.25 **a**	0.10	<ld	0
	30	Untreated	<ld **a**	0.00	0.69 **a**	0.26
		E. arvense	0.23 **a**	0.33	0.33 **b**	0.22

Values with the same letter are not significantly different (*p*<0,05)

4. Discussion

Maize (*Zea mays* L.) is one of the main cereals as a source of food, forage and processed products for industry. Stored maize is a man-made ecosystem in which quality and nutritive changes occur because of interactions between physical, chemical and biological factors where fungal spoilage and mycotoxin contamination are of major concern[1].

On the other hand, interest in natural therapies and increasing consumers demand for natural, safe and effective products, has resulted in the need to obtain quantitative data on plant extracts[22]. Mitscher *et al.*[23] suggested that it is very important to scientifically study traditional medicinal plants because they may contain new sources of antimicrobial compounds. *E. arvense* is used, currently, for biological control of fungi and pests. In this research we evaluated the antifungal capability of *E. arvense* hydro-alcoholic extract in stored maize. The extract was effective in reducing *Fusarium* section *Liseola* levels regardless of incubation time and inoculum levels. While in the untreated maize *Fusarium* section *Liseola* population was 4.92 log CFU g^{-1}, in treated maize the population was 3.13 log CFU g^{-1} (36% reduction). Nevertheless, for *Aspergillus* section *Flavi,* extract was only effective in inoculated maize highly contaminated (45% population reduction). This may be due to the lower *Aspergillus* section *Flavi* initial level in the maize.

Garcia *et al.*[7] studied the effect of *E. arvense* on growth and mycotoxin production of six moulds, including *A. flavus* and *F. verticillioides*, in maize agar medium (MAM). They found that the extract completely inhibited growth of both moulds. In a later work Garcia *et al.*[19] studied *E. arvense* extract effect on *A. flavus* and *F. verticillioides* in sterile maize grain in Petri plates, at different conditions of temperature, a_w, and inoculum size. Their results confirmed that the extract was effective for the inhibition of *A. flavus* at high water activity level. However, at 0.90 a_w no statistically significant effect was observed. Moreover, growth of *F. verticillioides* was inhibited at both a_w assayed, 0.93 and 0.95 a_w.

Some researches have shown that plant extracts which contain antioxidant compounds, mainly phenolics and volatile monoterpenes such as thymol, carvacrol etc., can influence on mould development[8,9,10,24,25]. Analysis of our extract showed that it was rich in flavonoids and phenolic acids which could be responsible for the growth inhibition. Our results agree with previous reports that also found flavonoids and phenolic acids in *E. arvense* extracts of other origins[10,12,26,27,28]. Principal compounds of our extract were kaempferol-3-*O*-rutinoside-7-*O*-glucoside and kaempferol dihexoside, which are flavonoid compounds with known antioxidant properties[24,29]. Compounds similar to those found in our extract were detected by Veit *et al.*[30] by HPLC analysis. They analyse phenolics compound in some species of *Equisetum* genus and the main compound in *E. pratense* was kaempferol 3-*O*-rutinoside-7-*O*-glucoside in all samples of this specie. However, the main compound of *E. arvense* was different depending of geographic origin, but quercetin 3-0-(6"-*O*-malonylglucoside) was found to be abundant in all samples examined. In European plants it was always the major flavonoid and comprised between 28 and 50% of the total flavonoid content. In this last work, *E. arvense* extract also presents large amounts of dicaffeoyl-meso-tartaric acid. Singh *et al.*[24] studied the effect of kaempferol-7-*O*-glucoside against bacterial and fungal growth, being effective against *A. flavus*, *Aspergillus niger*, *Fusarium moniliformae* and *Rhizoctonia bataticola*.

Other important phenolic compounds found in our extract were quercetin and caffeic acid; these compounds have shown to completely inhibited growth and aflatoxin production by both *A. flavus* and *A. parasiticus*[31], growth and ochratoxin A production by *Aspergillus carbonarius*[32], and was found effective against *A. flavus*, *A. niger*, *F. moniliformae* and *R. bataticola*[24]. Besides, quercetin was found to reduce patulin accumulation by acting on the transcription level of certain tested genes[33]. On the other hand, Quiroga *et al.*[34] found a fungal growth inhibition that ranged between 80-90 % for *Trichoderma* spp., 60-80 % for *Fusarium oxysporum* and *Penicillium notatum* and >90% for *A. niger* in presence of *Equisetum giganteum* extract, but as they did not analyse the extract the compostion is unknown. Nevertheless, all previously described works were made on culture medium in laboratory conditions, which makes comparison with real systems difficult.

With respect to mycotoxin production, no aflatoxins were found and fumonisin presence was not significantly affected. Garcia *et al.*[19] also found a low relevance of *E. arvense* extract in fumonisins production. As both growth and mycotoxin content were referred to maize weight, lower fungal counts

occurred along with similar levels of mycotoxins, suggesting a higher rate of mycotoxin per fungal biomass in the treated samples; this would confirm the hypothesis that toxin production might be triggered under growth limiting conditions, either nutrient shortage or other adverse conditions. Besides in general results obtained in agar experiments have rarely been fully confirmed in food and feed substrates, some works showed aflatoxin and fumonisins production inhibition on maize by the use of plant essential oils (EOs). Mallozzi *et al.*[35] also studied the effect of some flavonoids on AFB_1 formation, founding a 99% of inhibition due to the addition of the flavonoid kaempheritrin at 100 ppm on YES medium. With respect to FB_1, production, it was reduced by plant phenolics compounds, as chlorophorin (94% of reduction) followed by caffeic acid, ferulic acid, vanillic acid and iroko, which reduced FB_1 levels by 90–91%[36].

Although in general results obtained in agar experiments have rarely been fully confirmed in food and feed substrates, some works showed aflatoxin and fumonisins production inhibition on maize by the use of plant essential oils (EOs). Bluma and Etcheverry[35] worked with boldus, poleo and mountain thyme EOs on *A. flavus* and *A. parasiticus* and they found complete inhibition of AFB_1 at 2000 and 3000 μg/g at 11 incubation days. López *et al.*[6] worked with aromatic plant EOs on the inhibition of *F. verticillioides* on culture medium and their effects on the FB_1 production in maize grain by a toxicogenic strain of *F. verticillioides*. They found that with the EO of *Origanum vulgare* fungal growth was inhibited on semisolid brain-heart infusion broth; with respect to mycotoxin production, no effect on FB_1 was found after 15 days of maize incubation, however the EO exhibited a significant inhibitory effect on the mycotoxin production at 20-30 days, respect to control. They also analysed EO components and characterized a high content of alcoholic and phenolic compounds (terpineol+thymol, 42.3% of the total oil), that may be responsible for the antifungal effect. Mallozzi *et al.*[36] also studied the effect of some flavonoids on AFB_1 formation, founding a 99% of inhibition due to the addition of the flavonoid kaempheritrin at 100 ppm on YES medium. With respect to FB_1, production, it was reduced by plant phenolics compounds, as chlorophorin (94% of reduction) followed by caffeic acid, ferulic acid, vanillic acid and iroko, which reduced FB_1 levels by 90–91%[37].

Most researches used either agar or broth dilution series to assess antimicrobial activity of spices, herbs and their extracts and EOs, and in some cases both assays for comparative purposes, as antimicrobial performance in the two systems can vary[37]. However, results obtained on culture media cannot necessarily be extrapolated to natural ecosystems where there are other factors that influence on the mould growth[20]. Nevertheless, some authors have already used fruit and vegetable model media to investigate plant extracts and/or EO efficacy [38,39,40,41,42]. In most of these cases plant extracts and/or EO efficacy decreased in the food model media, by comparison with the *in vitro* control media. Holley and Patel[37] concluded that the presence of fat, carbohydrate, protein, salt and pH reaction influence the effectiveness of these agents in foods and antimicrobial potency of EOs is also reduced in foods with lower water activity[37].

Interest in natural therapies and increasing consumers demand for natural, safe and effective products, has resulted in the need to obtain quantitative data on plant extracts[22]. The results presented in this study confirm that the extract of *E. arvense* may be effective for the inhibition of *Aspergillus* section *Flavi* and *Fusarium* section *Liseola* growth, especially when seeds are highly contaminated. However, mycotoxins presence was not affected accordingly.

Acknowledgement

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3.4 Modelling the effect of temperature and water activity in the growth boundaries of *Aspergillus ochraceus* and *Aspergillus parasiticus*

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Abstract

The aim of this work was to model the growth of *Aspergillus parasiticus* and *Aspergillus ochraceus*, both mycotoxin producers, near to the growth/no-growth boundaries and validate those models in sterile maize grain, peanuts and coffee beans. Malt extract agar was adjusted to six different water activities: 0.93, 0.91, 0.89, 0.87, 0.85 and 0.80. Plates were incubated at 10, 15, 20, 25, 30, 37 and 42ºC. For each of the 35 conditions 10 Petri dishes were inoculated. Both kinetic and probability models were applied to colony growth data. The results of the present study indicate that the developed probability modelling approach could be satisfactorily employed to quantify the combined effect of temperature and water activity on the growth responses of *A. ochraceus* and *A. parasiticus*. However, validation of kinetic results led to poor goodness of predictions. In this study, the validation samples were placed near to the expected boundaries of the models in order to test them under the worst situation. Probability of growth prediction under extreme growth conditions was somewhat compromised, but it can be considered acceptable.

1. Introduction

Mycotoxins are a chemical risk in food products of increasing concern due to the wide range of food commodities where they can be found. Consumption of mycotoxin contaminated food has been associated with several cases of human poisoning or mycotoxicoses, sometimes resulting in death (Molina and Giannuzzi 1999). *Aspergillus* species have the ability to grow in various climates and, in food products, are among the most important moulds causing both spoilage and mycotoxin production. *A. ochraceus* and *A. parasiticus* are responsible for synthesis of ochratoxin A (OTA) and aflatoxins (B1, B2, G1 and G2), respectively. *A. ochraceus* was the first OTA-producing species described (Van der Mérwe et al. 1965) and is capable of producing OTA when grows on foods during storage (Leistner 1984; Gareis and Scheuer 2000; Spotti et al. 2001, 2002; Castella et al. 2002; Lund and Frisvad 2003; Comi et al. 2004; Matrella et al. 2006; Pietri et al. 2006; Cantoni et al. 1982a,b, 2007). *A. parasiticus* grows rapidly on a wide variety of natural substrates under favourable conditions of temperature and humidity (Bagheri-Gavkosh et al. 2009).This mould can produce aflatoxins in food and feeds and can pose serious health hazards to human and animal.

Mycotoxins are associated to the presence of fungal inoculum on susceptible substrates. Despite the absence of direct correlation between the extent of mould growth and mycotoxin production, prevention of fungal growth effectively conduces to prevention of mycotoxin accumulation (Garcia et al. 2009). In addition, growth is a variable which presents less intraspecific variability, and the kinetics of growth are more known, thus a good alternative to prevent mycotoxin accumulation might be prediction and prevention of growth (Marín et al. 2008a). The growth of moulds in foods and feeds depends on the effects of multiple variables like pH, water activity (a_w), solute concentrations, temperature, atmosphere composition, time, etc. But generally a_w and temperature are regarded as the principal controlling factors determining the potential for growth (Panagou et al. 2003; Plaza et al. 2003; Dantigny et al. 2005).

Mathematical modelling can be a tool to predict and, consequently, to prevent the growth of mycotoxigenic moulds. Secondary kinetic models describe microbial response in relation to time and environmental factors providing estimates for parameters of growth: lag phase (λ) and maximum growth rate (μ_{max}). Probabilistic models study the probability that mould growth or mycotoxin production occur. An integrated description of the microbial response could be given by first establishing the likelihood of growth through a probability model and then predicting the growth parameters, such as specific growth rate and lag time, provided that growth is expected (Masana and Baranyi 2000). Logistic regression is a useful approach to model the growth boundaries (growth/no growth interface) of microorganisms in different areas of food science, such as product development, formulation and food processing (Sosa-Morales et al. 2009). Most published models take into account several factors (water activity, temperature, incubation time) in most cases close to their optimal levels, nevertheless foods and feeds are stored at marginal environmental conditions for mould growth. These researches have been frequently made on solid media adjusted to different a_w and incubated to different temperatures. However,

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results obtained on culture media cannot necessarily be extrapolated to natural ecosystems where there are other factors that influence on the mould growth. For this, validation is an essential step after modelling, enabling researchers to understand the applicable range of models and also the limits of their performance (Jagannath and Tsuchido 2003).

The objectives of the present work were (i) to develop kinetic models for *A. ochraceus* and *A. parasiticus* on a synthetic growth medium as a function of temperature and water activity (reducing fungal growth may also reduce mycotoxin production); ii) to develop growth/no growth interface models (with the objective of preventing from mycotoxin production), and (ii) to validate the developed models with data from independent experiments on selected food. Few previous studies have reported on validated models for moulds. As far as we know this is the first study specifically dedicated to model and validate mould growth at the limits of growth. The objective was to test whether the performance of the predictive models may fail at the boundaries

1.1 Nomenclature section

a_w, water activity

$a_{w\ min}$, minimum a_w for growth

$a_{w\ max}$, maximum a_w for growth

$a_{w\ opt}$, a_w at which μ_{max} is optimal

D, colony diameter (mm)

D_{max}, final diameter of mycelium attained in Petri dishes

μ_{max}, maximum growth rate (mm/d)

μ_{opt}, maximum growth rate at optimal conditions (mm/d, μm/h)

λ, lag phase or time to growth (days)

P, probability of growth (in the range of 0 to 1)

T, temperature (ºC)

T_{min}, minimum temperature for growth (ºC)

T_{max}, maximum temperature for growth (ºC)

T_{opt}, T at which μ_{max} is optimal (ºC)

2. Materials and methods

2.1 Fungal isolates and preparation of inoculum

Two mycotoxigenic isolates were included in this research, one of *A. parasiticus* (UdL-TA 3.18) isolated from peanuts and one of *A. ochraceus* (Udl-TA 3.53) isolated from coffee. The references in brackets are the codes of cultures held in the Food Technology Department Culture Collection of University of Lleida, Spain. The isolates were sub-cultured on malt extract agar (MEA) plates and incubated at 25ºC for 7 days to obtain heavily sporulating cultures. After incubation, a sterile inoculation loop was used to remove the

conidia of each mould from MEA plates and they were suspended in 5 ml of H_2O/glycerol solutions with different water activity levels: 0.93, 0.91, 0.89, 0.87, 0.85 and 0.80.

The suspensions were then filtered through glass wool into sterile 10 ml tubes to remove mycelial fragments. Then the spore suspension was centrifuged at 4000 rpm, at 4ºC for 15 min. The pellet was resuspended with the required H_2O/glycerol solution. The number of spores per ml was then determined using a Thoma counting chamber and the final concentration was adjusted to 10^5 spores/ml.

2.2 Medium

The basic medium used in this study was malt extract agar (MEA) with six different water activities. The a_w of the medium was modified to 0.93, 0.91, 0.89, 0.87, 0.85 and 0.80 by the addition of 304.3 g/l, 376 g/l, 483.4 g/l, 565 g/l and 675 g/l of glycerol respectively. The medium was autoclaved and poured into 9 cm sterile Petri dishes. The pH of the media was in the range of 6.0-6.2. The a_w of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy ± 0.003.

2.3 Inoculation and incubation

MEA plates were inoculated centrally with a needlepoint load. Previous repeated experiments showed that the number of spores inoculated through this technique was 10-100 spores. In particular, 26.0 CFU ± 15.8 have been obtained through this protocol. Inoculation load has been shown to affect lag phase duration, although not growth rates (Sautour et al. 2003). Increased lag phases (up to 23%) were in general observed for *Aspergillus carbonarius* and *Penicillium expansum* when inoculum increased from 0-1 to 10-100 spores (Garcia et al. 2010).

Petri dishes with the same a_w level were enclosed in polyethylene bags in order to maintain a constant water activity and incubated at 10, 15, 20, 25, 30, 37 and 42ºC. Bags do not affect growth by diminishing oxygen availability neither increasing CO_2 concentration as confirmed before comparing growth with plates incubated without bags. The effect of temperature and a_w on the growth response of both fungi was investigated by means of a full factorial design. For each treatment 10 Petri dishes were inoculated (n=10).

2.4 Growth assessment

Fungal growth was observed on a daily basis for an overall period of 90 days by diameter measurements at right angles with the aid of a ruler and a binocular magnifier. The diameter of the colonies was plotted against time.

2.5 Validation

Validation was carried out directly in sterile green coffee beans, peanuts and maize with modified a_w. Validation of models was carried out in peanuts and maize for *A. parasiticus* and in coffee and maize for

A. ochraceus. Table 1 shows incubation conditions chosen for validation of each mould after inoculation. Except for a suitable condition, the combinations were chosen near the growth/no growth boundaries in order to validate the models under those conditions in which prediction may be a key point in food safety. Resolution and accuracy of the measurements in the validation experiments might be compromised by the heterogeneity of the matrices.

Sterile nuts, beans and grains were adjusted to the required a_w levels and poured in Petri plates forming a single layer. Inoculation was carried out as in agar plates. Plates with the same a_w were enclosed in sealed containers along with beakers containing water glycerol solution of the same a_w as the plates which were renewed periodically in order to maintain constant a_w (Dallyn 1978). For each condition 10 Petri dishes were inoculated. Growth assessment was carried out as for MEA experiments.

Table 1. Validation set of conditions for *A. ochraceus* and *A.parasiticus* models.

A. ochraceus		A. parasiticus	
Water activity	Temperature	Water activity	Temperature
0.85	25	0.85	25
0.85	30	0.85	30
0.87	20	0.87	20
0.87	37	0.87	30
0.89	15	0.87	37
0.89	20	0.89	15
0.89	37	0.89	20
0.91	15	0.89	37
0.93	10	0.91	15
0.93	25	0.93	25

2.5.1 Water activity adjustment of the substrates

Seeds were sterilized by autoclave at 120ºC for 20 minutes. Water activity was adjusted by aseptically adding amounts of sterile distilled water to the substrates in sterile bottles. The bottles were cooled down to 4ºC for 48 hours with periodic hand-shaking during this time. The amount of water necessary to reach the different water activity levels were determined by calibration curves (water activity-ml water to be added/g substrate) previously made (Table 2). Final a_w values of each substrate were confirmed with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy ± 0.003. The pH values of maize, peanuts and coffee beans were 6.0, 6.0 and 5.7$\pm$0.1, respectively.

Table 2. Amount of water to be added to each substrate for different a_w levels

a_w	Water (ml/100g)		
	Maize	Peanut	Green coffee
0.85	2.0	4.5	10.5
0.87	4.0	5.0	12.3
0.89	5.0	6.0	15.5
0.91	7.5	9.5	21.5
0.93	11.0	12.0	24.5

2.6 Model development

2.6.1 Kinetic model

A typical two-step modelling approach, including primary and secondary modelling, was employed to quantify the effect of temperature and a_w on the kinetic parameters of *A. ochraceus* and *A. parasiticus.* Initially, estimates of the growth rates of the fungi were obtained by plotting colony diameter changes against time. For each treatment, a non linear regression was applied to estimate the maximum growth rate (μ_{max}, mm d $^{-1}$), lag phase before growth (λ, days), and maximum colony diameter, if applicable, by fitting the experimental data to the primary model of Baranyi and Roberts (1994) [1] by using Statgraphics Plus 5.1 with the non linear regression option.

$$D = \mu_{max} A - \ln\left[1 + \frac{\exp(\mu_{max}A) - 1}{\exp(D_{max})}\right] \quad [1]$$

$$A = t + \left(\frac{1}{\mu_{max}}\right)\ln\left[\exp\left(-\mu_{max}t\right) + \exp\left(-\mu_{max}\lambda\right) - \exp\left(-\mu_{max}t - \mu_{max}\lambda\right)\right] \quad [2]$$

The average estimates of μ_{max} and λ were further fitted to secondary models to describe the effect of temperature and a_w on fungal growth rate. The models are described by the following equations:

a) The combined effects of temperature and water activity were determined according to the gamma concept (Zwietering et al. 1992).

$$\sqrt{\mu_{max}(T, a_w)} = CTPM_2(T, a_w) = \sqrt{\mu_{opt} \cdot \tau(T) \cdot \rho(a_w)} \quad [3]$$

where

$$\tau(T) = \left(\frac{(T - T_{\min})^2 \cdot (T - T_{\max})}{(T_{opt} - T_{\min}) \cdot \left[(T_{opt} - T_{\min})(T - T_{opt}) - (T_{opt} - T_{\max})(T_{opt} + T_{\min} - 2T) \right]} \right) \qquad [4]$$

and

$$\rho(a_w) = \left(\frac{(a_w - a_{w\min})^2 \cdot (a_w - 1)}{(a_{w opt} - a_{w\min}) \cdot \left[(a_{w opt} - a_{w\min})(a_w - a_{w opt}) - (a_{w opt} - 1)(a_{w opt} + a_{w\min} - 2a_w) \right]} \right)$$

$$[5]$$

where $a_{w\max}=1$ according to Sautour et al. (2001).

b) Extended Davey model (Davey 1989)

$$\sqrt{\mu_{\max}} \ (or \ \ln \lambda) = a_0 + a_1 a_w + a_2 a_w^2 + a_3 / T + a_4 / T^2 \qquad [6]$$

Where T is absolute temperature (ºK), and $a_0 \ldots a_4$ are coefficients to be estimated.

c) General polynomial model

$$\sqrt{\mu_{\max}} \ (or \ \ln \lambda) = a_0 + a_1 a_w + a_2 a_w^2 + a_3 T + a_4 T^2 + a_5 T a_w \qquad [7]$$

Where T is temperature (ºC) and $a_0 \ldots a_5$ are coefficients to be estimated.

Similar results should be expected from b) and c) quadratic models, both of them polynomial however, it was decided to include both of them as did Samapundo et al. (2005, 2007) in their studies.

The goodness of fit of the modelling approach was evaluated by the root mean square error (RMSE), which measures the average deviation between observed and predicted values. The smaller the value of this index the better the fit of the model to the experimental data:

$$RMSE = \sqrt{\frac{\sum (predicted - observed)^2}{n - p}} \qquad [8]$$

Where *n* is the number of observations and *p* is the number of parameters to be estimated.

For validation, the indices proposed by Ross (1996) were used:

2.6.1.1 Bias-factor (BF). The BF answers the question whether, on average, the observed values lie above or below the line of equivalence and, if so, by how much. .

$$bias\ factor = 10^{\left(\Sigma \log\left(\mu_{predicted}/\ \mu_{observed}\right)/\ n\right)}$$ [9]

μ is maximum growth rate or other modelled variable

n is the number of observations used in the calculation

Perfect agreement between predictions and observations will lead to a bias factor of 1.

2.6.1.2 Accuracy factor (AF). The AF averages the minimum 'distance' between each point and the line of equivalence as a measure of how close, on average, predictions are to observations. The AF is, thus, a measure of average deviation and may be used as a simple measure of the level of confidence one may have in the model's predictions.

$$accuracy\ factor = 10^{\left(\Sigma |\log\left(\mu_{predicted}/\ \mu_{observed}\right)|/ n\right)}$$ [10]

The larger the value, the less accurate is the average estimate.

2.6.2 Modelling of the growth/no growth interface

For each treatment of the two fungal species, growth data were converted into probabilities of growth by assigning the value of 1 in the case where visible fungal growth was evident, and 0 in the case of absence of growth during the overall period of the experiment. The resulting data were fitted to a logistic regression model as described previously (Ratkowsky and Ross 1995) to determine the growth/no growth boundaries of the fungi under the different a_w and temperature levels assayed. The model employed was a full second order logistic regression model (Battey et al. 2002) that includes also the linear term for time:

$$\text{Logit P} = \ln\left(\frac{P}{1-P}\right) = b_0 + b_1 a_w + b_2 T + b_{11} a_w^2 + b_{22} T^2 + b_{12} a_w T + time$$ [11]

Where b_i are the coefficients to be estimated. The equation was fitted by using Statgraphics® Plus version 5.1 (Manugistics, Inc, Maryland,USA) linear logistic regression procedure. The automatic variable selection option with a backward stepwise factor selection method was used to choose the significant effects ($P<0.05$). The predicted growth/no growth interfaces for $P = 0.1, 0.5$ and 0.9 were calculated and plotted using Microsoft Excel 2003 Solver.

3. Results

3.1 Kinetic primary model

Growth of *A. ochraceus* in solid medium followed, in general, biphasic Baranyi's function (with no upper asymptote) except at 37ºC / 0.85-0.89 a_w and at 10ºC with 0.91-0.93 a_w, where growth followed a sigmoidal curve. For *A. parasiticus* the growth also followed biphasic Baranyi's function; only at 0.85a_w/20ºC a sigmoidal growth curve was observed.

Maximum growth rate (μ_{max}) and lag phase (λ) were estimated through Baranyi's primary model (Table 3). For both moulds under marginal conditions, μ_{max} decreased and λ increased. No growth was observed under extreme conditions (eg. >37ºC, <10ºC, <0,85 a_w for *A. ochraceus* and >42ºC, <15ºC, <0,85 a_w for *A. parasiticus*). In the case of *A. ochraceus*, the higher μ_{max} was observed at 0.93a_w/30ºC with the lowest λ. For *A. parasiticus* the higher μ_{max} was found at 0.93a_w/37ºC but the minor λ was at 0.93a_w/30ºC.

Table 3. Estimated maximum growth rates (μ_{max}) and lag times (λ, starting from 10-100 inoculated spores) for the growth of *Aspergillus ochraceus* and *Aspergillus parasiticus* on malt extract agar at various temperatures and water activity levels.

Temperature (ºC)	Water activity	*Aspergillus ochraceus*		*Aspergillus parasiticus*	
		μ_{max} (mm d^{-1}) $\pm$SE	λ (days) $\pm$SE	μ_{max} (mm d^{-1}) $\pm$SE	λ (days) $\pm$SE
10	0.80	-	-	-	-
	0.85	-	-	-	-
	0.87	-	-	-	-
	0.89	-	-	-	-
	0.91	0.14±0.02	48.3±5.0	-	-
	0.93	0.06±0.01	35.3±9.2	-	-
15	0.80	-	-	-	-
	0.85	-	-	-	-
	0.87	-	-	-	-
	0.89	0.18±0.01	14.8±2.4	0.10±0.02	39.3±9.7
	0.91	0.30±0.03	15.5±4.4	0.24±0.02	23.7±3.7
	0.93	0.30±0.03	11.0±3.2	0.28±0.02	18.0±3.3
20	0.80	-	-	-	-
	0.85	0.22±0.01	10.8±0.7	0.18±0.08	30.6±3.6
	0.87	0.34±0.01	10.0±0.6	0.22±0.00	12.2±1.5
	0.89	0.39±0.03	5.3±0.5	0.28±0.03	9.2±0.9
	0.91	0.56±0.01	4.6±0.4	0.40±0.01	6.2±0.7
	0.93	0.68±0.01	3.9±0.2	0.49±0.02	4.9±0.2
25	0.80	-	-	-	-
	0.85	0.30±0.03	4.2±1.0	0.38±0.03	5.9±0.4
	0.87	0.43±0.03	3.4±0.8	0.61±0.03	3.6±0.4
	0.89	0.58±0.04	2.9±0.8	0.78±0.05	2.9±0.3
	0.91	0.94±0.03	2.9±0.4	1.06±0.02	2.5±0.3
	0.93	1.04±0.06	2.4±0.3	1.08±0.05	2.1±0.2
30	0.80	-	-	-	-
	0.85	0.32±0.01	4.6±0.6	0.41±0.03	4.2±0.3
	0.87	0.45±0.02	3.2±0.6	0.65±0.04	3.2±0.3
	0.89	0.63±0.03	3.5±1.0	0.47±0.05	1.1±0.6
	0.91	0.94±0.03	2.6±0.5	1.17±0.03	1.9±0.1
	0.93	1.15±0.08	2.3±0.2	1.40±0.10	1.5±0.2
37	0.80	-	-	-	-
	0.85	0.10±0.20	22.5±1.3	-	-
	0.87	0.29±0.10	15.1±1.1	0.57±0.05	6.2±1.0
	0.89	0.20±0.03	12.0±1.5	0.88±0.06	4.6±0.2
	0.91	0.23±0.02	9.3±1.7	1.18±0.05	2.3±0.2
	0.93	0.27±0.02	7.5±1.5	1.50±0.07	1.7±0.3
42	0.80	-	-	-	-
	0.85	-	-	-	-
	0.87	-	-	-	-
	0.89	-	-	-	-
	0.91	-	-	-	-
	0.93	-	-	0.48±0.10	4.8±1.7

SE, standard error, n=10. -, no growth observed for 90 days

3.2 Secondary models for the effects of a_w and temperature on the growth rate and lag phase

Response of the moulds to the environmental conditions studied was examined with several existing models. Based on analysis of residues, the square root transformation was introduced to stabilize the variance of the fitted values for the growth rate ($\sqrt{\mu}$), confirming the suitability of this transformation as suggested by Dantigny and Bensoussan (2008).

The cardinal values of environmental factors (minimum, maximum and optimum value) of the secondary model of Rosso and Robinson (2001) are shown in Table 4, whereas the fitted models are presented in Fig. 1. In particular, optimum a_w values were poorly estimated as they were outside the experimental domain, and models should not be applied outside this domain. Taking into account that the experimental design included only suboptimal water activity levels, the model exhibited reasonably good fit to experimental data in terms of calculated RMSE. *Aspergillus parasiticus* was rather fast growing under the best conditions of temperature and a_w tested, based on the estimates of μ_{opt} values of 1.8 mm d^{-1} compared to 1.0 mm d^{-1} for *A. ochraceus*. While $a_{w\,min}$ estimates for growth were similar for both fungi, the optimum estimated temperatures for growth varied from 27.4ºC for *A. ochraceus* to 31.1ºC for *A. parasiticus*, explaining the better adaptation of the later to higher temperatures. Oppositely to what expected, the estimated T$_{min}$, however, was lower for *A. parasiticus* than for *A. ochraceus*.

Table 4. Estimated values and statistics of the coefficients of the Rosso and Robinson (2001) model for *Aspergillus ochraceus* and *Aspergillus parasiticus* at different conditions of temperature and a_w.

Species	Parameter	Estimated value ±SE	RMSE
Aspergillus ochraceus	μ_{opt} (square root)	1.00±0.02	0.128
	T_{min}	6.48±0.71	
	T_{max}	41.83±0.15	
	T_{opt}	27.36±0.27	
	$a_{w\,min}$	0.78±0.01	
	$a_{w\,opt}$	0.93±0.00	
Aspergillus parasiticus	μ_{opt} (square root)	1.33±0.06	0.175
	T_{min}	5.66±0.89	
	T_{max}	42.47±0.16	
	T_{opt}	31.15±0.31	
	$a_{w\,min}$	0.77±0.01	
	$a_{w\,opt}$	0.94±0.01	

SE, standard error derived from non linear regression
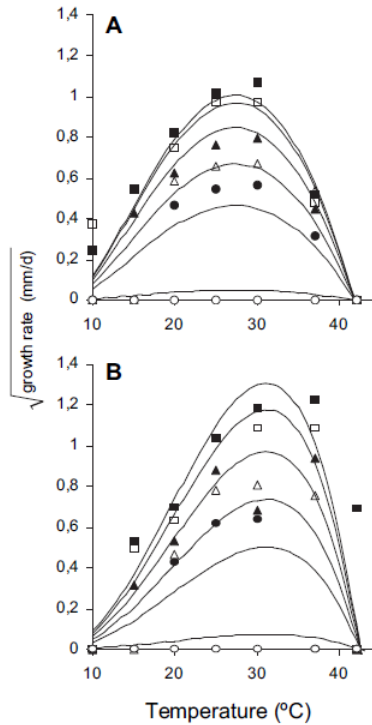
Figure 1: Square root of growth rates (mm d^{-1}) of A) *A. ochraceus* and B) *A. parasiticus* as a function of temperature at different a_w levels (■ 0.93 a_w; □ 0.91 a_w; ▲ 0.89 a_w; △ 0.87 a_w; ● 0.85 a_w; ○ 0.80 a_w). Regression lines are fitted to cardinal model.

For subsequent models, based on analysis of residues, natural logarithm transformation was used (ln λ) for lag phases. In particular, the general polynomial model showed better performance than extended Davey model for both growth rate (RMSE= 0.125-0.189) and lag time (RMSE=0.493-0.696) (Table 5). Most coefficients resulted significant. As far as statistical evaluation is concerned, the models presented better performance for *A. ochraceus* than for *A. parasiticus.* For *A. ochraceus*, lag phases were predicted under 7 days in the range 20-34ºC at 0.87 a_w, and in the range 16-35ºC at 0.93 a_w, whereas lag phases over 30 days were predicted at 0.80 regardless of temperature level, outside the range 18-38ºC at 0.85 a_w, and outside the range 12-40ºC at 0.89 a_w (Figure 2). For *A. parasiticus*, lag phases were predicted under 7 days in the range 22-32ºC at 0.87 a_w, and in the range 18-38ºC at 0.93 a_w, whereas lag phases over 30 days were predicted at 0.80 regardless of temperature level, outside the range 18-36ºC at 0.85 a_w, and outside the range 15-40ºC at 0.89 a_w.

Table 5. Model coefficients for the modelled μ_{max} and λ of *A. ochraceus* and *A. parasiticus*

	Coef.	μ_{max} (mm d^{-1})		λ (days)	
		A. ochraceus	*A. parasiticus*	*A. ochraceus*	*A. parasiticus*
		Estimated value$\pm$SE	Estimated value$\pm$SE	Estimated value$\pm$SE	Estimated value$\pm$SE
Davey	a_0	-264.6$\pm$9.4	-352.5$\pm$15.8	1.21 10^3$\pm$37.3	1.42 10^4$\pm$58.6
	a_1	4.7$\pm$10.6 ns	68.8$\pm$16.8	-180.6$\pm$42.1	-319.7$\pm$62.4
	a_2	-35.5$\pm$4.2	-21.8$\pm$6.5	210.4$\pm$16.8	146.8$\pm$24.3
	a_3	1.56 10^5$\pm$4.16 10^3	1.92 10^5$\pm$6.95 10^3	-6.72 10^5$\pm$1.65 10^4	-7.62 10^5$\pm$2.58 10^4
	a_4	-2.59 10^7$\pm$6.11 10^5	-2.78 10^7$\pm$9.71 10^5	1.09 10^8$\pm$2.42 10^6	1.13 10^8$\pm$3.61 10^6
	RMSE	0.128	0.198	0.509	0.736
Polynomial	a_0	-38.7$\pm$3.1	-23.9$\pm$4.8	206.6$\pm$12.4	146.7$\pm$17.5
	a_1	73.7$\pm$7.2	45.3$\pm$10.9	-408.8$\pm$28.3	-285.8$\pm$40.0
	a_2	-35.7$\pm$4.1	-22.9$\pm$6.2	211.9$\pm$16.3	151.q$\pm$23.0
	a_3	0.3$\pm$0.0	0.1$\pm$0.0	-1.3$\pm$0.1	-0.6$\pm$0.1
	a_4	-0.0$\pm$0.0	-0.0$\pm$0.0	0.0$\pm$0.0	0.0$\pm$0.0
	a_5	-0.2$\pm$0.0	0.1$\pm$0.0	0.6$\pm$0.1	-0.2$\pm$0.1 ns
	RMSE	0.125	0.189	0.493	0.696

SE, standard error derived from non linear regression

ns, not significant
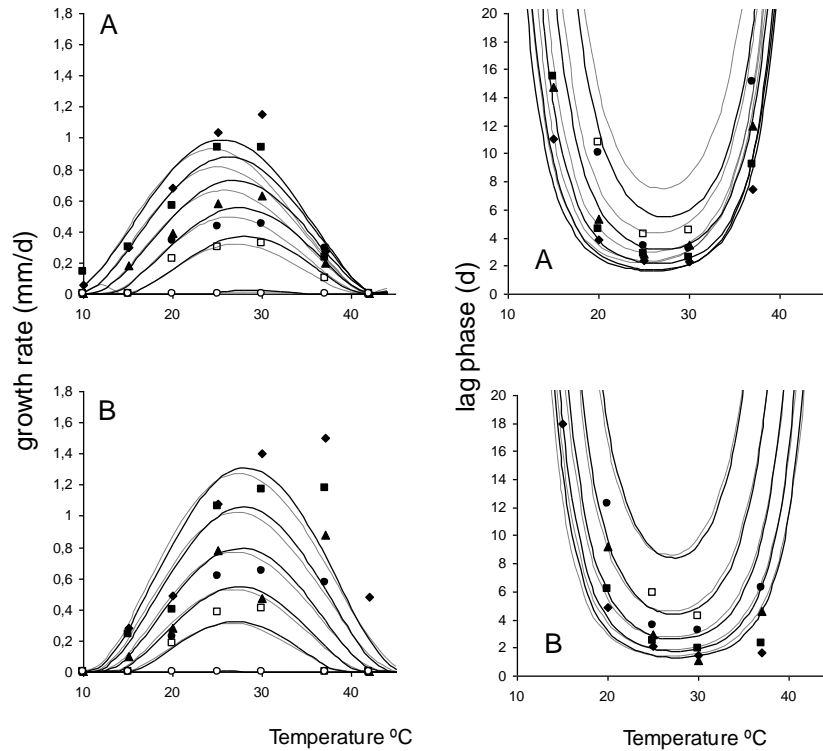
Figure 2: Growth rates (mm d^{-1}) and lag phases (d, starting from 10-100 inoculated spores) of A) *A. ochraceus* and B) *A. parasiticus* as a function of temperature at different a_w levels (◆, 0.93 a_w; ■, 0.91 a_w; ▲, 0.89 a_w; ●, 0.87 a_w; □, 0.85 a_w; ○, 0.80 a_w). Regression lines are fitted to polynomial (—) and extended Davey (---) models. Error bars show standard deviation (n=10).

3.3 Validation of kinetic models on food matrices

Plotting observed versus predicted growth rate values for the four food matrices (figures not shown); it could be visually concluded that the best predictions were done for *A. parasiticus* in maize, and that in general there was a poor accordance between predictions and observed values. Moreover, small differences were observed in the predictions by the three models. Bias and accuracy factors (Bf and Af) were calculated for both species and models (Table 6).

Table 6. Bias and accuracy factors obtained for the validation set.

Species	Food matrix	Model	Validation set	Growth rate (mm d^{-1})		Lag phase (d)	
				Bias factor	Accuracy factor	Bias factor	Accuracy factor
A. ochraceus	Maize grain	Rosso	0.93a_w/25ºC	0.12	1.72	-	-
			Extreme (9)	0.11	26.86	-	-
		Davey	0.93a_w/25ºC	0.13	1.65	7.69	1.82
			Extreme (9)	0.13	29.93	5.18	15.30
		Polynomial	0.93a_w/25ºC	0.12	1.76	10.02	2.37
			Extreme (9)	0.11	33.60	6.82	20.16
	Coffe beans	Rosso	0.93a_w/25ºC	0.64	1.25	-	-
			Extreme (9)	0.48	1.91	-	-
		Davey	0.93a_w/25ºC	0.67	1.19	1.81	1.97
			Extreme (9)	0.56	2.13	1.51	1.68
		Polynomial	0.93a_w/25ºC	0.63	1.28	2.36	2.57
			Extreme (9)	0.48	2.39	1.99	2.21
A. parasiticus	Maize grain	Rosso	0.93a_w/25ºC	1.09	1.07	-	-
			Extreme (9)	0.09	24.06	-	-
		Davey	0.93a_w/25ºC	0.99	1.03	2.53	2.34
			Extreme (9)	0.10	30.13	5.23	13.40
		Polynomial	0.93a_w/25ºC	0.98	1.04	2.49	2.31
			Extreme (9)	0.10	30.97	5.11	13.33
	Peanuts	Rosso	0.93a_w/25ºC	1.01	1.07	-	-
			Extreme (9)	0.29	1.02	-	-
		Davey	0.93a_w/25ºC	0.92	1.18	0.52	1.07
			Extreme (9)	0.33	1.22	0.65	2.23
		Polynomial	0.93a_w/25ºC	0.90	1.20	0.53	1.08
			Extreme (9)	0.33	1.26	0.66	2.21

Extreme (9), the 9 validation experiments listed in table 1, except condition 0.93a_w/25ºC

Validation of *A. ochraceus* growth model in coffee beans led to acceptable Bf and Af at $0.93a_w/25^\circ C$ (0.63-0.67 and 1.19-1.28, respectively) suggesting that the model was conservative as higher values were predicted than the observed ones. Worse indices were obtained for the 9 extreme growth conditions assayed. Bf were unacceptably low for validation in maize grain due to the absence of growth in maize under many conditions; acceptable Af were obtained at $0.93a_w/25^\circ C$, but they revealed a poor predictive power under marginal conditions. No differences were observed among the applied models. Similarly, better results were observed for the validation in coffee beans for lag phases predictions compared to results in maize.

Good validation results were obtained in maize for *A. parasiticus* at $0.93a_w/25^\circ C$ (Bf=0.98-1.10; Af=1.03-1.08); however the models performed poorly in maize under the remaining conditions included in the validation set. The same trend was observed in peanuts at $0.93a_w/25^\circ C$ (Bf=0.90-1.02; Af=1.06-1.20), while bias factors under extreme conditions revealed too fast predicted growth compared to the observed one. No differences were observed among the applied models. Similarly, better results were observed for the validation in peanuts of lag phases predictions compared to results in maize.

3.4 Probability models

The mould growth responses for each of the 10-fold repeated experiments are shown in Table 7. *A. ochraceus* grew under 25 of the assayed conditions, and *A. parasiticus* under 21. The replicated samples did not always show the same response, more markedly when conditions were far from the optimum ones (e.g., at 15 and 37°C). No growth was observed at 0.80 a_w, regardless of the temperature level.

Table 7. **(part 1 of 1)** Experimental variables with logistic growth responses after 1 month for the growth of *A. ochraceus* and *A. parasiticus* in MEA.

a_w	Temperature (ºC)	*A. ochraceus**	*A. parasiticus**
0.80	10	0	0
0.80	15	0	0
0.80	20	0	0
0.80	25	0	0
0.80	30	0	0
0.80	37	0	0
0.80	42	0	0
0.85	10	0	0
0.85	15	0	0
0.85	20	10	9
0.85	25	10	10
0.85	30	10	10
0.85	37	3	0
0.85	42	0	0
0.87	10	0	0
0.87	15	0	0
0.87	20	10	10
0.87	25	10	10
0.87	30	10	10
0.87	37	10	10
0.87	42	0	0

* Observed outcome: number of positive MEA plates at the given a_w out of the 10 inoculated MEA plates after a 90 days incubation at the given temperature

T**able 7 (part 2 of 2).** Experimental variables with logistic growth responses after 1 month for the growth of *A. ochraceus* and *A. parasiticus* in MEA.

a_w	Temperature (ºC)	*A. ochraceus**	*A. parasiticus**
0.89	10	0	0
0.89	15	10	7
0.89	20	10	10
0.89	25	10	10
0.89	30	10	10
0.89	37	10	0
0.89	42	0	0
0.91	10	1	0
0.91	15	9	9
0.91	20	10	10
0.91	25	10	10
0.91	30	10	10
0.91	37	9	10
0.91	42	0	0
0.93	10	8	0
0.93	15	10	10
0.93	20	10	10
0.93	25	10	10
0.93	30	10	10
0.93	37	9	10
0.93	42	0	0

* Observed outcome: number of positive MEA plates at the given a_w out of the 10 inoculated MEA plates after a 90 days incubation at the given temperature

A full second-order logistic regression model including all the linear, quadratic and interaction terms along with the time term was generated. Predicted probabilities with a value of > 0.50 were considered positive responses (growth). With this criterion, the degree of agreement between observations and predictions was 93.0% concordant and 7.0% discordant for *A. ochraceus* and 94% concordant and 6% discordant for *A. parasiticus*. Maximum rescaled R^2 were of 0.684 and 0.738, respectively. Backward stepwise regression did not eliminate any of the linear or quadratic terms of the logistic model, as all of them were statistically significant ($P < 0.05$), thus the models consisted of 7 terms:

For *A. ochraceus*:

Logit (P) = -704.005 + 1373.68 a_w + 5.3746 T + 0.0329 time − 681.468 a_w^2 − 3.7892 a_w T − 0.0384 T^2

For *A. parasiticus*:

Logit (P) = -607.505 + 1220.57 a_w + 2.8928 T + 0.0387 time − 642.99 a_w^2 − 0.3006 a_w T − 0.0481 T^2

where P is the probability of growth and T is the incubation temperature in ºC.

The interaction of a_w with temperature was statistically significant. Plots of probability of growth for temperature and a_w at 7 and 30 days of incubation are presented in Fig. 3. It is graphically depicted that the probability plot shifted to lower water activities for the same temperature level as time advanced, for both fungal isolates. Probability data observed after 1 month were almost equal to those observed after 3 months. In addition, the probability of growth for *A. parasiticus* was lower at the lowest temperatures assayed (10-20ºC) indicating delay in fungal growth of this species. For instance, after 7 days at 0.91 a_w/15°C no growth was observed for *A. parasiticus* as the estimated probability was < 0.50, whereas for *A. ochraceus* growth was evident ($P > 0.90$) at the same conditions.
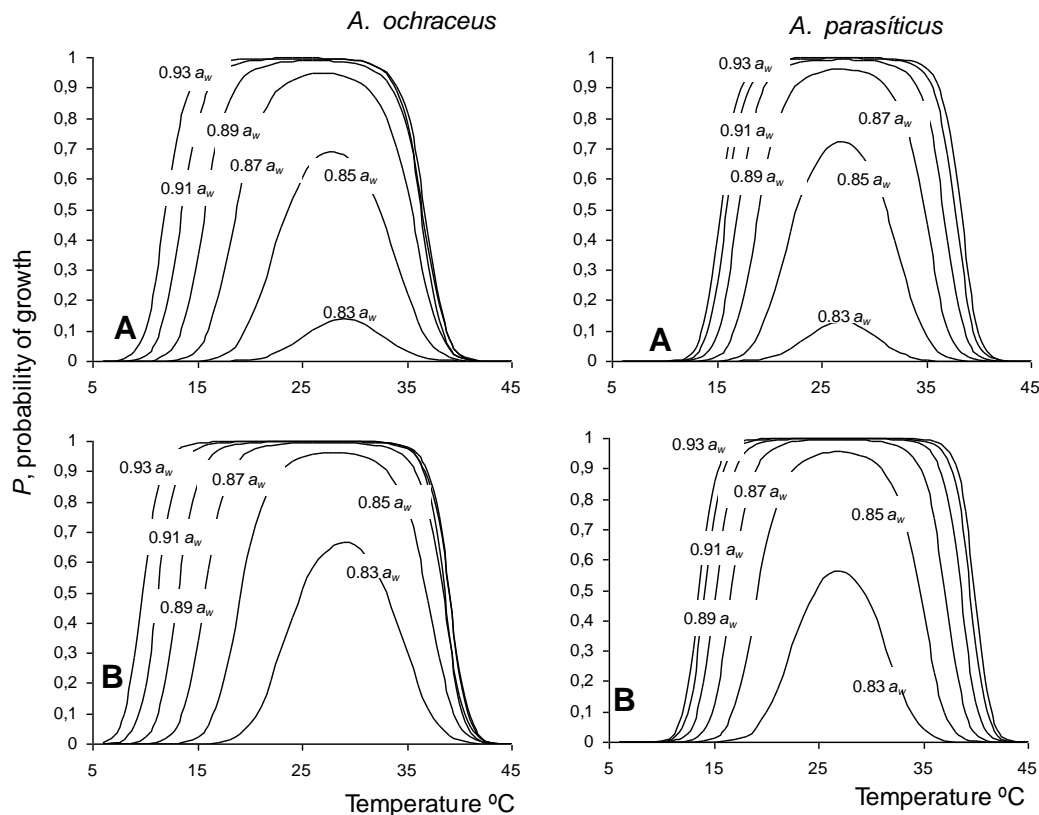
Figure 3: The predicted effect of temperature and water activity on probability of *Aspergillus ochraceus* and *Aspergillus parasiticus* growth in MEA incubated for A) 7 and B) 30 days.

As observed in the figures, probabilities of growth for *A. ochraceus* over 0.80 were predicted in the range 0.87-0.93 a_w at 19-35ºC. Thus for safe storage of food products, a a_w <0.85 should be maintained and temperatures in the range 21-35ºC should be avoided (P<0.5); alternatively, cool storage (<10ºC) could be applied. Probabilities of growth for *A. parasiticus* over 80% were predicted in the range 0.87-0.93 a_w at 19-35ºC. Thus for safe storage of food products, a a_w <0.85 should be maintained and temperatures in the range 20-34ºC should be avoided (P<0.5); alternatively, cool storage (<14ºC) could be applied.

The predicted growth/no growth boundaries at probabilities of 0.1, 0.5 and 0.9, together with the observed growth/no growth cases from which the predictions were derived are presented in Fig. 4 for both *Aspergillus* species. This figure confirms graphically the high percentage of logistic model agreement with experimental data.
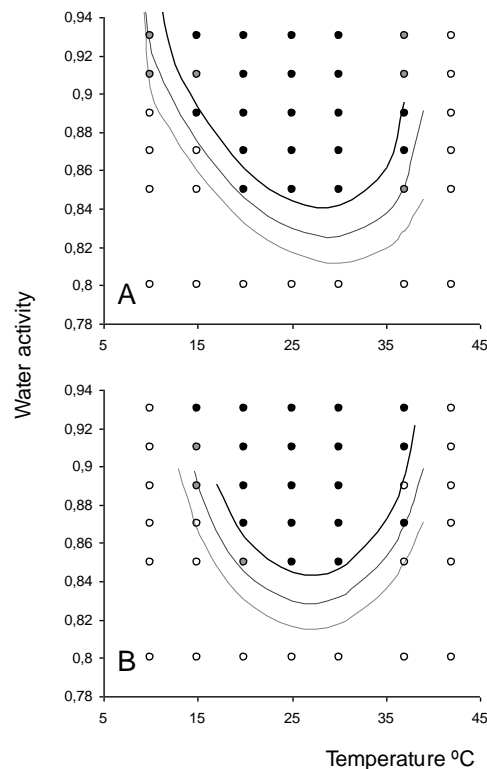
Figure 4: The predicted growth/no growth boundaries for one month with respect to a_w and temperature at probabilities of 0.1, 0.5 and 0.9 for A) *Aspergillus ochraceus* and B) *Aspergillus parasiticus*. ●, 100% observed growth; ◐ 10-90% observed growth; O, no-growth observation.

3.5 Validation of probability models on food matrices

Predicted mould growth responses after one month were compared to those observed in the food matrices for the same period (Table 8). The models predicted growth reasonably successfully in peanuts and coffee (70 and 80% concordance, respectively) considering that the validation samples were located at the boundaries of the domain of the model. For maize, however, there was an agreement of 30 and 40%, for *A. ochraceus* and *A. parasiticus*, respectively, between predicted and observed probabilities. The only condition tested under suitable conditions for growth (0.93a_w/ 25ºC) led to predicted P=1 for both *A. ochraceus* and *A. parasiticus*, which agreed with the observed probability of growth of *A. parasiticus* in maize and peanuts and *A. ochraceus* in coffee, while the observed probability for *A. ochraceus* in maize was 0.60.

Table 8. Comparison of predicted and observed mould growth responses (one month) of *A. ochraceus* and *A. parasiticus* in raw food matrices.

a_w/Temperature		*A. parasiticus*			*A. ochraceus*		
		Predicted probability of growth	Observed outcomes in maize*	Observed outcomes in peanut*	Predicted probability of growth	Observed outcomes in maize*	Observed outcomes in coffee*
0.85	25	0.91	0.0	0.4	0.87	0.0	1.0
0.85	30	0.89	0.0	1.0	0.90	0.0	1.0
0.87	20	0.91	0.0	0.0	0.92	0.2	1.0
0.87	30	0.98	0.6	1.0	-	-	-
0.87	37	0.41	0.9	0.8	0.63	0.0	0.1
0.89	15	0.36	0.1	0.0	0.76	0.0	1.0
0.89	20	0.98	0.3	1.0	0.99	0.0	1.0
0.89	37	0.77	0.5	1.0	0.77	0.8	1.0
0.91	15	0.65	0.4	1.0	0.95	1.0	0.9
0.93	10	-	-	-	0.44	0.6	1.0
0.93	25	1.00	1.0	1.0	1.00	0.6	1.0

* number of positive maize, peanuts or coffee plates at the given a_w out of the 10 inoculated plates after a 90 days incubation at the given temperature.

4. Discussion

Most work in predictive microbiology, dealing with bacterial pathogens and spoilage bacteria, has been carried out under a wide range of temperatures, but mainly under high water availabilities which are common in fresh products prone to be colonized by bacteria. Moulds, however, colonize and spoil mainly those foods (e.g. stored cereals and nuts) in which the reduced water activity prevents bacterial growth. Thus in food mycology it is important the use of predictive models developed under suboptimal water activities, and in the whole range of storage temperatures.

 Two main modelling approaches may be applied for prevention of mycotoxin accumulation in food commodities. The first one consists of predicting and preventing any growth of mycotoxigenic species; the second modelling approach involves direct mycotoxin analyses. In this work, the first approach has been considered; from our experience, although there is a correlation between growth and mycotoxin production, it is not possible to predict mycotoxin accumulation from kinetic growth data: i) Mycotoxin accumulation does not occur at its best under the same conditions as growth; ii) moreover, not all fungal growth results in mycotoxin formation (Marín et al. 2006). The second approach involving modelling of

mycotoxin concentration, may encounter the problem that given the different abilities to synthesize mycotoxins by the different strains of a given species, extrapolation from the models obtained with one or several strains might not be representative for the majority of the strains (Marín et al. 2008a). In addition, growth is a variable which presents less intraspecific variability, and the kinetics of growth are more known, thus we considered prediction and prevention of growth a good alternative to prevent mycotoxin accumulation (Marín et al. 2008b).

The results of this work showed that the examined mycotoxigenic *Aspergillus* species were rather slow growing fungi, with maximum growth rates under the conditions assayed of 1.15 and 1.50 mm d^{-1}, for *A. ochraceus* and *A. parasiticus*, respectively. Previous published works dealing with radial growth rate of *A. ochraceus* showed maximum values of approx. 2-4 mm d^{-1} around 0.93 a_w in different agar media and coffee beans (Marín et al. 1998; Ramos et al. 1998; Pardo et al. 2004a, 2005a,b; Suarez-Quiroz et al. 2004), except that of Pardo et al. (2004b) in barley grains, which showed maximum growth rates of 1mm d^{-1} at such reduced a_w levels. Although many studies deal with growth of *Aspergillus* section *Flavi*, few give details on growth requirements specific for *A. parasiticus*.

Several secondary models have been developed to quantify the effect of these factors on fungal growth (Garcia et al. 2009). Rosso et al. (1993) proposed a model that included three cardinal parameters for temperature (T_{min}, T_{max}, T_{opt}) and the specific growth rate at optimum temperature. This model was later extended to include also water activity (Rosso and Robinson 2001; Sautour et al. 2001). While estimated $a_{w\ min}$ was similar for both fungal species (0.77 and 0.78), the derived cardinal values for temperature (T_{min}, T_{max}, T_{opt}) for *A. ochraceus* were 6, 42 and 27, and 6, 42 and 31 for *A. parasiticus*. Minimum a_w for growth has been reported at 0.80 (Pardo et al. 2004b; Suarez-Quiroz et al. 2004), however, no a_w levels were tested within the range 0.75-0.80, thus our estimation of $a_{w\ min}$ is probably accurate. Regarding cardinal temperatures for *A. ochraceus*, minimum and maximum temperatures have been reported at 10 and 37ºC, respectively (Marin et al. 1998; Ramos et al. 1998; Suarez-Quiroz et al. 2004), while the optimum one was 25-30ºC (experiments done in 5ºC intervals), in agreement with the estimated values by Rosso and Robinson (2001) model. A great advantage of this model is that all parameters have a physiological meaning which clearly facilitates initial parameter estimations and may also aid in future incorporation into the model of underlying cell biological mechanisms (Brul and Klis 1999). In contrast, the estimated coefficients of the polynomial models have no meaning. In addition, similar or slightly worse goodness of fit were observed for the polynomial models in terms of RMSE compared to those obtained for the Rosso and Robinson (2001) model (RMSE=0.128 for *A. ochraceus*; RMSE=0.175 for *A. parasiticus*). Consequently, from our results cardinal model should be favoured over the polynomial ones.

Modelling of lag phase data is of interest because it allows direct prediction of the safe storage/transport periods for a given food commodity. Observed lag phases when growth occurred ranged from 2 to 48 days for *A. ochraceus*, and from 1 to 39 days for *A. parasiticus* (Table 3). Short lag times were observed for *A. ochraceus* at 25-30ºC, whith a sharp increase at 37ºC, while they decreased gradually at lower

temperatures. In the case of *A. parasiticus*, similar values were observed at 25-37ºC, while there was a sharp increase at 20-15ºC. However, modelling of lag phase data obtained under marginal growth conditions becomes difficult, because of no growth results. In our case, the general polynomial model performed better than that proposed by Davey (1989) for both species (RMSE=0.493-0.696).

For practical purposes, like safe storage prediction, an alternative to lag time calculation may be the use of probability of growth estimation through linear logistic regression. While kinetic models allow for calculation of lag time ± 95% confidence interval, from probability models we can infer the time interval in which initiation of growth may be more probable (e.g. $0.50 < p < 0.90$). For example, at 20ºC and 0.87 a_w, lag time confidence intervals were [8.7,11.3] and [9.3,15.2] for *A. ochraceus* and *A. parasiticus*, respectively; those intervals corresponded to 0.85-0.86 and 0.82-0.85 probability of growth, respectively. Probabilistic models using logistic regression have been extensively employed to define the growth boundaries of important foodborne pathogens such as *Listeria monocytogenes* (Koutsoumanis and Sofos 2005; Gysemans et al. 2007; Vermeulen et al. 2007) and *Escherichia coli* (Presser et al. 1998; Skandamis et al. 2007), but their application is rather limited in fungi, where the majority of developed models are kinetic, providing information in terms of growth rate and lag phase or produce response surface and contour plots.

The developed logistic regression models were successfully fitted to the experimental data as the agreement between observed and predicted probabilities was > 93% concordant for both fungal species and R^2 were >0.68. High values for both performance indices have been reported previously for the growth of *A. carbonarius* in pistachio nuts (Marín et al. 2008b) and in synthetic grape juice medium (Tassou et al. 2009). It must be noted that polynomial type logistic regression models are empirical in nature and do not contribute to the understanding of the mechanism involved in microbial growth inhibition. However, this type of models offer the possibility to include the no-growth responses, which are particularly important when efforts are conducted to prevent an event, such as fungal growth, providing thus a significant degree of quality and safety from spoilage (López-Malo et al. 2000). Nevertheless, Ratkowsky and Ross (1995) modeled growth/no growth of *Shigella flexneri* through logistic regression with equations which contained biological meaningful terms (pH, temperature, a_w, and gas concentration).

Probability of growth of *A. ochraceus* higher than 0.9 was estimated after 8 days over 0.91 a_w in the range 15-35ºC. However growth could be delayed by cooling to 10ºC; in such case *P* was <0.1 for 20 days at 0.91a_w, for 51 days at 0.90a_w and indefinitely under this a_w value. Similarly an extension of the unaltered period could be achieved through a_w control, for example *P* did not reach 0.1 in one month at 0.86/15ºC, and over 90 days at 0.82a_w outside the interval 25-34ºC or at 0.80a_w outside the interval 30-31ºC. Probability of growth of *A. parasiticus* higher than 0.9 was estimated after 3 weeks over 0.91 a_w in the range 17-37ºC. However growth could be delayed by cooling to 15ºC; in such case *P* was <0.1 for 27 days at 0.87 or indefinitely under 0.86 a_w. Growth was delayed for 2 months at 0.90/12ºC and for > 90 days under decreasing values of a_w and temperature. Similarly an extension of the unaltered period could

be achieved through a_w control, for example, *P* did not reach 0.1 in 40 days at 0.85a_w/15ºC, over 90 days at 0.82a_w outside the interval 23-31ºC or at 0.80a_w. Taking into account the sorption curves of maize, peanuts (Chen 2000) and coffee (Pittia et al. 2007) the safe moisture contents would be approx. 18, 11 and 18% (dry weight basis), respectively, as suggested by the models developed in this work. Thus in terms of its use in the implementation of HACCP plans the probabilistic approach shown in this study may be of more interest than kinetics modelling.

Very few studies in scientific literature have externally validated the models developed to describe fungal growth, and they rarely present accuracy and bias factors. For example, Battey et al. (2001) constructed a probability model for *A. niger* and *Penicillium spinulosum* on a beverage analogue and validated it in an external set of beverage analogues, too. Samapundo et al. (2007) modelled *A. flavus* and *A. parasiticus* growth on irradiated maize grain, and performed the numerical validation on an external independent set of experiments on irradiated maize grain, too. Baert et al. (2007) developed a series of models for *P.expansum* growth in apple puree agar medium, which were validated in apples. Marín et al. (2008b) validated the probability of growth and OTA models developed for *A. carbonarius* on pistachio nuts in an independent set of experiments prepared on pistachio nuts. However developing models on real food substrates requires much effort, and models developed in a food substrate can not be extrapolated to other food products. A better approach would be to develop the models in general synthetic media and validate results on food substrates, as done in the present work, however the deviations shown confirm that models developed on synthetic media do not present a realistic picture of microbial responses on real food systems, as they do not take into account one of the most important parameters, i.e. that of food matrix.

Validation of kinetic models was carried out in peanuts and maize for *A. parasiticus* and in coffee and maize for *A. ochraceus*. The domain of the models included suboptimal growth conditions (always under 0.93 a_w); within this framework, validation set was constructed including 9 extreme conditions plus a centered condition in the domain. The objective was to test whether the performance of the predictive models may fail at the boundaries. Validation of *A. ochraceus* growth models on coffee beans led to acceptable results under most conditions, while the models for *A. parasiticus* predicted too fast growth under the extreme conditions. Growth of the fungi in maize was in general much slower than predicted by the models. The optimum growth rate is very much dependant on the substrate thus probably explaining the bias and accuracy factors obtained for maize grains. No significant differences were found among the performance of the three models used. The location of grains, beans and nuts in a single layer in Petri plates, which was the method used for validation is not a realistic situation, because the free spaces among particles may compromise fungal growth, making it slower. Is it likely that the resolution and accuracy of measurements in validation studies is lower than on agar plates due to the lack of homogeneity in the matrices tested.

Validation with independent data showed that the developed logistic model could satisfactorily predict the responses of both fungal species at probability level of 0.5. Specifically, the agreement with growth data in food matrices was 70% in coffee, 80% in peanuts and 30-40% for maize.

In conclusion, the results of the present study indicate that the probability developed modelling approach could be satisfactorily employed to quantify the combined effect of temperature and water activity on the growth responses of *A. ochraceus* and *A. parasiticus*. However, validation of kinetic results led to poor goodness of predictions. Boundary models may be important in predicting the most suitable combinations of environmental factors to prevent fungal growth, providing thus a significant degree of quality and safety. In this study, the validation samples were placed near the expected boundaries of the model in order to test it under the worst situation. Probability of growth prediction under extreme growth conditions was somewhat compromised, but it can be considered acceptable. The risk in using the models presented here in real situations may be the differences in the initial inoculum size and the unrealistic constant conditions of temperature and moisture content.

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3.5 Modelling mould growth under suboptimal environmental conditions and inoculum size.

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Abstract

Predictive models can be a tool to develop strategies to prevent mould development and consequently prevent mycotoxins production. The aims of this work were to assess the impact of a) high/low levels of inoculum and b) optimal/suboptimal environmental conditions on either the kinetic or probability. Different levels of spores suspensions of *Aspergillus carbonarius* and *Penicillium expansum* were prepared and inoculated centrally with a needlepoint load on malt extract agar (MEA) with 50 replicates. While optimum conditions led to a colony diameter increase which followed Baranyi's function, suboptimal conditions led to different grow functions. In general, growth rate (μ) and lag phase (λ) were normally distributed. μ showed similar distributions under optimal growth conditions, regardless of the inoculum level, while suboptimal a_w and temperature conditions led to higher kurtosis distributions, mainly when the inoculum levels were low. Regarding λ, more skewed distributions were observed, mainly when the inoculum levels were low. Probability models were not much affected by the inoculum size used to build them. Lower probabilities of growth were in general predicted under marginal conditions at a given time for both strains. The slopes of the probability curves were smaller under suboptimal growth conditions due to wider distributions. Results show that a low inoculum sizes and suboptimal conditions lead to high variability of the estimated growth parameters and growth probability.

1. Introduction

There is a need to ensure the microbiological quality and safety of food products. This has stimulated interest in the use of mathematical models for quantifying and predicting microbial behaviour. Moulds can grow in all kind of foods and feeds and they can produce mycotoxins which are a risk for human and animal health. Mould growth and mycotoxin production are associated to the presence of fungal inoculum on predisposed foods and feeds. Despite the absence of direct correlation between mould growth and mycotoxins production, prevention of fungal growth effectively conduces to prevention of mycotoxin accumulation.

Aspergillus carbonarius and *Penicillium expansum* are two mycotoxigenic moulds. They produce ochratoxin A (OTA) and patulin, respectively. *A. carbonarius* has been found in a great variety of substrates for human and animal consumption in regions with warm and tropical climate. Strains of *A. carbonarius* have been isolated and identified in several Mediterranean wine producing countries, such as Spain, Italy, Israel, Portugal and Greece (Serra et al., 2003; Battilani et al., 2004; Belli et al., 2004a; Mitchell et al., 2004; Tjamos et al., 2004, 2006). Besides, it is the main fungal species responsible for OTA accumulation in dried vine fruits (Abarca et al., 2003). Some researches have proven that high percentages (75-100%) of its isolates are able to produce the toxin (Cabañes et al., 2002; Belli et al., 2005; Marín et al., 2008). As a result, many works have been published during the last few years describing its growth as a function of environmental factors. Some of them included predictive models to be applied in foods manufacturing and storage (Belli et al., 2004; Marín et al., 2006).

P. expansum is the most important species related to blue mould of apples and pears, an important post-harvest disease of these fruits worldwide. This mould grows at low temperatures, thus cold storage does not prevent spoilage but only retards it (Baert, et al., 2008). Moreover *P. expansum* is the main responsible for the occurrence of patulin in apple derivatives (Weidenbörner et al., 2001).

Kinetic models determine microbial responses in relation to time and environmental conditions, and provide estimates for parameters of growth: lag phase (λ) and growth rate (μ). Probabilistic models study the probability that mould growth or mycotoxin production occurs. While kinetic models take into account the amount of growth or mycotoxin, probabilistic models only study presence/absence of them. Most published models take into account the complex interaction of several factors (water activity, temperature, incubation time) in most cases close to their optimal levels, however, existing models rarely take into account the effect of inoculum size. Sautour et al. (2003) concluded that there is a need for standardizing spore preparation in predictive mycology. The inoculum size influences the estimated growth parameters and should be considered in qualitative risk assessment for the design of challenge tests and experiments to gather data for predictive growth models (Baert et al., 2008).

The aim of this work was to assess the impact of a) using high/low levels of inoculum and b) optimal/suboptimal environmental conditions on either the kinetic or probability obtained models and the distribution of estimated parameters. .

1.1 Nomenclature section

D: colony diameter.

Dmax: is the maximal colony diameter (mm).

t: time

λ: is the lag phase (d).

μ: is the growth rate (mm/day).

P: probability of growth

bi: are the coefficients to be estimated

Pmax: maximum probability level attained

k: the slope of the probability function when P=Pmax/2

r: time when P=Pmax/2

2. Materials and methods

2.1 Fungal isolates and preparation of inoculum

Two isolates were included in this study, one of *A. carbonarius* (UdL-TA 3.27) and one of *P. expansum* (UdL-TA 3.80). The references in brackets are the codes of cultures held in the Food Technology Department Culture Collection of University of Lleida. The isolates were sub-cultured on malt extract agar (MEA, pH 5.5) plates and incubated at 25ºC for 7 days to enable significant sporulation. After incubation a sterile inoculation loop was used to remove the conidia from MEA plates and they were suspended in 5 ml of H_2O/glycerol solutions with different water activity levels: 0.98 and 0.90 for *A. carbonarius* and 0.98 and 0.92 for *P. expansum*. The suspensions were then filtered through glass wool into sterile 10 ml tubes to remove mycelial fragments. Then the spore suspension was centrifuged at 1730*g*, 4ºC for 15 min. The pellet was resuspended with the required H_2O/glycerol solution. The number of spores per ml was then determined using a Thoma counting chamber. The suspension was then serially diluted to obtain 1-5 x 10^3, 1-5 x 10^4 and 1-5 x 10^5 spores/ml suspensions.

2.2 Medium

The basic medium used in this study was MEA with three different water activities. The a_w of the medium was modified to 0.98, 0.92 and 0.90 a_w by the addition of 105 g/l, 290 g/l and 321.7g/l of glycerol respectively. The medium was autoclaved and poured into 9 cm sterile Petri dishes. The a_w of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of $\pm$ 0.003.

2.3 Inoculation and incubation

MEA plates were inoculated centrally with a needlepoint load. Previous repeated experiments showed that the number of spores inoculated through this technique were 0-1, 1-10 and 10-100 spores when using spores suspensions of 1-5×10^3, 1-5×10^4 and 1-5×10^5 spores/ml, respectively. In particular, the following numbers of colonies were counted when plated (mean ± standard deviation): 23.8 ± 14.5, 5.8 ± 11.7 and 0.5 ± 0.6 for *A. carbonarius* suspensions and 28.2 ± 17.2, 3.7 ± 6.8 and 0.5 ± 0.4 for *P. expansum* suspensions. The needle inoculation was preferred over a known volume inoculation to avoid the liquid interference, even if the solution is adjusted to the same a_w as the media. For *A. carbonarius* the suboptimal conditions studied were 0.98a_w/15ºC and 0.90a_w/25ºC and for *P. expansum* the conditions were 0.98a_w/15ºC and 0.92a_w/25ºC. In both cases the optimal condition chosen was 0.98a_w/25ºC (table 1). Plates with the same a_w level were incubated in sealed polyethylene bags in order to maintain a constant water activity. For each condition 50 Petri dishes were inoculated.

Table 1. Incubation conditions for each mould.

	A. carbonarius			*P. expansum*		
	10-10^2	1-10	0-1	10-10^2	1-10	0-1
0.98 a_w 25ºC	x	x	x	x	x	x
0.98 a_w 15ºC	x	x	x	x	x	x
0.90 a_w 25ºC	x	x	x			
0.92 a_w 25ºC				x	x	x

2.4 Growth assessment

Two perpendicular diameters of the growing colonies were measured daily until the colony reached the edge of the Petri dish. Diameter was chosen as growth measure; although it may have some shortcomings because it does not account for volume and density of colonies, it is the more suitable technique to assess fungal biomass in solid substrates (Marín et al., 2005). The diameters of the colonies were plotted against time and a non linear regression was applied to estimate the growth rate, μ (mm day^{-1}), lag phase before growth (days), and maximum colony diameter, if applicable.

2.5 Statistical analyses

Kinetic model

For each treatment, diameters were adjusted to Baranyi's function (Eq. 1) (Baranyi et al., 1994) by using Statgraphics Plus 5.1 with the non linear regression option.

$$D = \mu A - \ln\left[1 + \frac{[\exp(\mu A) - 1]}{\exp(D_{max})}\right]$$ (Eq. 1)

$$A = t + \left(\frac{1}{\mu}\right)\ln\left[\exp(-\mu t) + \exp(-\mu \lambda) - \exp(-\mu t - \mu \lambda)\right]$$ (Eq. 2)

D: colony diameter.

D_{max}: is the maximal colony diameter (mm).

t: is time.

λ: lag phase or time to growth (d).

μ: is the growth rate (mm/day).

Omitting no growth results, distribution of μ and λ followed normal distributions (Kolmogorov-Smirnov and Anderson-Darling tests), thus an ANOVA test was applied to assess significant differences among the estimated levels of μ and λ under different initial inoculum levels and environmental conditions.

Probability model

For probability model we used a linear logistic regression analysis (Statgraphics Plus 5.1) to determine the growth/no growth boundaries, assigning 1 and 0 to plates with visible growth and with no visible growth respectively.

$$\log it\ (P) = \ln\frac{P(t)}{1 - P(t)} = b_i + b_i t$$ (Eq. 3)

P: probability of growth

b_i: are the coefficients to be estimated

t: time

Additionally, mean probabilities of growth along time were averaged for the 50 observations, and a logistic regression was carried out as follows:

$$P = \frac{P_{max}}{1 + \exp[k(r - t)]}$$ (Eq. 4)

P_{max}: maximum probability level attained

k: slope of the tangent line through the inflection point

r: inflection point where P equals half of P_{max}

3. Results

3.1. Growth curve modelling

For *A. carbonarius*, under both optimum (0.98 a_w/25 °C) and limiting a_w (0.90 a_w/25 °C) conditions, growth kinetic followed Baranyi's function (with no upper asymptote). However, limiting temperature conditions (0.98 a_w/15 °C) led to full sigmoidal Baranyi's function, and even at limiting inoculum conditions (0-1 spores) a second nearly straight line could be observed after a near-sigmoidal function (fig. 1a).
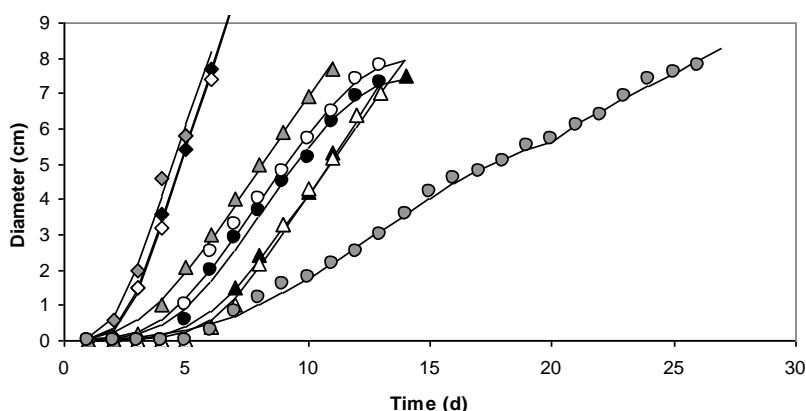
Figure 1a: Growth kinetics of *A. carbonarius.* Observed values (symbols) and Baranyi's fitting curves (lines). (◆) 0.98a_w/25ºC; (▲), 0.9oa_w/25ºC; (●), 0.98a_w/15ºC. Black symbols, 10-100 spores; white symbols, 1-10 spores; grey symbols, 0-1 spores.

For *P. expansum*, sigmoidal Baranyi's growth function could be fitted under optimum conditions (0.98a_w/25ºC). Under limiting conditions of temperature (0.98a_w/15ºC) or water activity (0.92a_w/25ºC) observed diameters followed a lineal trend after a near-sigmoidal one (fig. 1b). This last trend was modelled through a sigmoidal function followed by a linear function, as follows:

with

$$D = \begin{cases} if \ t < \dfrac{D_{max}-b}{\mu_2} & \mu_1 A - \ln 1 + \dfrac{[\exp(\mu_1 A)-1]}{\exp(D_{max})} \\ if \ t > \dfrac{D_{max}-b}{\mu_2} & b + \mu_2 t \end{cases}$$

(Eq.3)

$$A = t + \left(\frac{1}{\mu_1}\right)\ln[\exp(-\mu_1 t) + \exp(-\mu_1 \lambda) - \exp(-\mu_1 t - \mu_1 \lambda)]$$

(Eq.4)

where μ_1 is the maximum slope of the sigmoidal part, μ_2 is the slope of the straight line, and b is the Y-intercept of this line.

The estimated slopes of the straight line were in general smaller than the maximum growth rate estimated through the related sigmoidal curve, suggesting an adaptation to limiting conditions, but at a reduced rate. Besides, for both strains, this second line showed a narrower distribution (higher kurtosis) than the maximum growth rates estimated from the sigmoidal function.
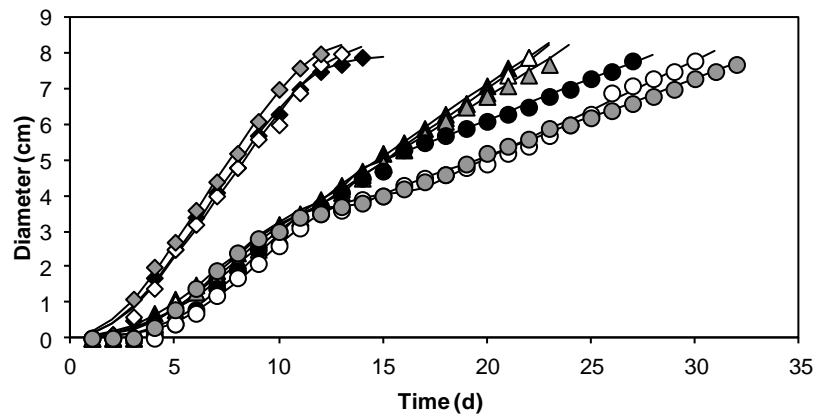
Figure 1b: Growth kinetics of *P. expansum.* Observed values (symbols) and Baranyi's fitting curves (lines). (◆) 0.98a_w/25ºC; (▲), 0.92a_w/25ºC; (●), 0.98a_w/15ºC. Black symbols, 10-100 spores; white symbols, 1-10 spores; grey symbols, 0-1 spores.

3.2. Inoculum size effects on estimated maximum growth rates (μ_1) and lag phases (λ)

Table 2 shows the number of replicates (out of 50) in which there was growth of *A. carbonarius*. At 0.98a_w/25ºC with the highest inoculum level, there was growth in all plates, while only 18 and 7 plates presented growth when 1-10 and 0-1 spores were inoculated. Taking out these no-growth plates, under limiting environmental conditions, growth occurred in 66-96% of the replicates at the higher inoculum level, while at the lower ones growth occurred in 50-100% and 30-70% of the plates, when 1-10 and 0-1 spores were used, respectively (the percentages are on the basis of growing plates under optimal conditions). Table 2 also shows mean and median values of μ_1. In general, no significant differences ($P>0.05$) among estimated μ_1 levels due to inoculum size were found, although the maximum μ_1 values were generally observed at the higher inoculum levels. Regarding λ, no significant differences were observed due to inoculum size under optimum growth conditions. However, there were significant differences among the inoculum levels under limiting growth conditions with the higher value of λ with 0-1 spores at 0.98a_w/15ºC and with 1-10 spores at 0.90a_w/25ºC.

Table 2. Growth parameters values of *A. carbonarius*

			n	n growth	μ$_1$ (mm/day)			λ (d)		
					Mean +	median	SD	Mean +	median	SD
0.98 25	0-1		50	7	2.16a*	2.18	0.14	2.46a	2.48	0.30
	1-10		50	18	2.16a	2.18	0.14	2.49a	2.50	0.32
	10-100		50	50	2.19a	2.18	0.26	2.15a	2.17	0.28
0.90 25	0-1		50	5	1.05b	1.05	0.03	6.11d	3.22	0.18
	1-10		50	19	1.06b	1.06	0.05	6.29d	6.35	1.26
	10-100		50	48	0.97b	0.97	0.09	5.50c	5.41	0.66
0.98 15	0-1		50	2	0.57c	0.57	0.06	6.19d	6.13	0.44
	1-10		50	9	1.06b	1.10	0.13	5.00b	4.46	1.34
	10-100		50	33	1.03b	1.02	0.17	4.77b	4.78	0.83

*Means followed by different letters are significantly different according to Duncan test

For *P. expansum* the number of plates in which growth was observed decreased with decreasing inoculum level too (table 3). At the higher inoculum level 100% of the plates showed growth, with 54-64% and 12-14% growing colonies in plates inoculated with 1-10 and 0-1 spores, respectively. No significant differences among maximum growth rates were found due to inoculum level under optimal environmental conditions ($P>0.05$). However, under suboptimal conditions, small differences were observed. There were significant differences among λ obtained under the different inoculum levels, however no general trend could be established; increasing lag phases were observed with decreasing inoculum size from 10-100 to 1-10, but 1-10 values did not follow the same trend.

Table 3. Growth parameters values of *P. expansum.*

			n	n growth	μ$_1$ (mm/day)			λ (d)		
					Mean +	median	SD	Mean +	median	SD
0.98 25	0-1		50	7	0.92a*	0.91	0.01	2.22a	2.24	0.12
	1-10		50	27	0.87a	0.87	0.02	2.60b	2.26	0.18
	10-100		50	50	0.90a	0.90	0.02	2.53b	2.54	0.14
0.92 25	0-1		50	6	0.60d	0.60	0.04	4.63d	4.71	0.58
	1-10		50	32	0.65c	0.65	0.04	4.68d	4.63	0.32
	10-100		50	50	0.62d	0.61	0.03	4.05c	4.05	0.27
0.98 15	0-1		50	7	0.77b	0.72	0.10	4.56d	4.52	0.13
	1-10		50	28	0.64c	0.64	0.06	5.72f	5.55	0.60
	10-100		50	50	0.67c	0.65	0.10	5.24e	5.21	0.44

*Means followed by different letters are significantly different according to Duncan test

3.3 Inoculum size effects on distributions of μ_1 and λ.

Taking into account only the growing replicates, the range of estimated μ_1 for *A. carbonarius,* was always wider with 10-100 than 1-10 and 0-1 inoculated spores under all conditions studied, due to the higher number of observations (table 4). μ_1 was normally distributed and showed similar distributions under optimal growth conditions, regardless of the inoculum level, while 0-1 and 10-100 inoculum levels led to positives excess kurtosis distributions under suboptimal a_w and temperature conditions, revealing the presence of infrequent extreme deviations. Similarly for λ, the range obtained was wider with 10-100 than 1-10 and 0-1 inoculated spores, but only under good environmental conditions (table 5). Higher kurtosis values were observed when inoculating 1-10 spores, under suboptimal conditions. In general, no over skewed distributions were observed.

Table 4. μ_1 distribution of *A. carbonarius*

		A. carbonarius μ_1 (mm/day)				
		Min*	Max*	Range*	Kurtosis*	Asymmetry*
0.98 25	0-1	1.85	2.37	0.52	-0.29	-0.49
	1-10	1.85	2.37	0.52	-0.10	-0.53
	10-100	1.63	2.80	1.16	-0.02	0.16
0.90 25	0-1	1.00	1.13	0.14	0.34	0.70
	1-10	0.97	1.16	0.18	-0.65	0.09
	10-100	0.69	1.17	0.48	2.29	-1.02
0.98 15	0-1	0.49	0.74	0.25	2.97	1.32
	1-10	0.91	1.26	0.35	-1.08	0.18
	10-100	0.73	1.65	0.92	3.66	1.25

*Statistics calculated for growing observations

Table 5. λ distribution of *A. carbonarius*

		A. carbonarius λ (day)				
		Min*	Max*	Range*	Kurtosis*	Asymmetry*
0.98 25	0-1	1.95	3.00	1.05	-0.65	-0.01
	1-10	1.95	3.00	1.05	-0.82	-0.04
	10-100	1.42	2.68	1.26	-0.48	-0.25
0.90 25	0-1	5.69	6.33	0.64	0.28	0.94
	1-10	2.80	8.11	5.32	2.53	-1.16
	10-100	4.50	7.41	2.91	1.33	1.05
0.98 15	0-1	5.36	7.07	1.71	-0.14	0.41
	1-10	3.61	7.65	4.04	0.48	1.16
	10-100	3.37	6.57	3.20	-0.60	0.10

*Statistics calculated for growing observations

A similar trend was observed for *P. expansum*, with wider μ_1 ranges at higher inoculum levels, due to the higher amount of observations taken under these conditions. Nevertheless, under non optimal environmental conditions the widest range was obtained in 1-10 for suboptimal a_w and in 10-100 for suboptimal temperature (Table 6). Positive excess kurtosis, suggesting extreme observations, was reported at $0.92a_w$ and 25ºC with inoculum levels of 1-10. With respect λ, positive excess kurtosis distributions were observed at 10-100 inoculum level, while with 0-1 spores, distributions showed negative excess kurtosis (Table 7). In general, no over skewed distributions were observed.

Table 6. μ_1 distribution of *P. expansum*

		P. expansum μ_1 (mm/day)				
		Min*	Max*	Range*	Kurtosis*	Asymmetry*
0.98 25	0-1	0.9	0.93	0.02	-2.04	0.35
	1-10	0.83	0.93	0.1	0.34	0.60
	10-100	0.85	0.94	0.1	-0.53	-0.06
0.92 25	0-1	0.54	0.67	0.12	2.10	0.78
	1-10	0.58	0.81	0.23	6.51	1.66
	10-100	0.54	0.68	0.14	0.44	-0.32
0.98 15	0-1	0.67	0.93	0.25	-1.19	0.77
	1-10	0.53	0.77	0.24	-0.20	0.38
	10-100	0.54	0.9	0.36	-0.80	0.56

*Statistics calculated for growing observations

Table 7. λ distribution of *P. expansum*

		P. expansum λ (day)				
		Min*	Max*	Range*	Kurtosis*	Asymmetry*
0.98 25	0-1	2.03	2.36	0.33	-0.60	-0.66
	1-10	2.19	3	0.86	1.56	0.57
	10-100	2.28	3	0.76	2.65	0.68
0.92 25	0-1	3.85	5.3	1.42	-1.63	-0.36
	1-10	4.12	5.4	1.3	-0.43	0.33
	10-100	3.25	4.88	1.63	2.31	0.43
0.98 15	0-1	4.4	4.74	0.35	-1.43	0.47
	1-10	4.94	7.13	2.19	-0.06	0.97
	10-100	4.21	6.8	2.6	2.51	0.64

*Statistics calculated for growing observations

3.4. Suboptimal growth conditions effects on distributions of μ_1 and λ

As already known, μ_1 values were significantly lower under marginal growth conditions, while λ values were significantly higher for both species.

Suboptimal growth temperature conditions led to 34-71% no-growth observations for *A. carbonarius*, while all inoculated plates showed growth of *P. expansum*. Suboptimal growth water activity conditions led to 0-29% no-growth observations for *A. carbonarius* while 0-14% inoculated plates showed no-growth of *P. expansum* (the percentages are on the basis of growing plates observed under optimal conditions of tables 2 and 3). Thus distribution of μ_1 and λ was clearly affected by growth conditions for *A. carbonarius.*

Excluding no-growth observations, *A. carbonarius* showed more near to normal distributions for both μ_1 and λ, with nearer to 0 values for both kurtosis and skew under optimal growth conditions than under marginal ones (Tables 4 and 5). In the case of *P. expansum* this was not so clear, with positive excess kurtosis for μ_1 values at 0.92 a_w and 25ºC compared to control, but not at 0.98a_w/15ºC. For λ, there were not clear variations in the distributions at different growth conditions.

3.5. Inoculum size effects on probability models

Taking into account only the growing plates, the models obtained were affected by the inoculum size, but no general trend was observed at the different growth conditions for *A. carbonarius* and *P. expansum*. Sometimes the probability of growth increased sharply with 0-1 spores, while in other cases this was with 10-100 spores, thus no conclusions were obtained from this point.

3.6. Suboptimal growth conditions effects on probability models

Fig. 2 shows the probability models obtained under the different environmental conditions when no growth observations were not included, representing thus a worst case scenario. For *A. carbonarius* for example, when 10-100 spores were inoculated, 0.9 probability of growth was observed before 2 days under optimum conditions, while more than 5 days were required at 0.98a_w/15ºC and 0.90a_w/25ºC. On the other hand, a probability of 0.05 was reached before 2, 3 and 4 days at 0.98a_w/25ºC, 0.98a_w/15ºC and 0.90a_w/25ºC, respectively. The slopes of the probability curves were smaller under suboptimal growth conditions suggesting wider distributions. For *P. expansum* for example, when 1-10 spores were inoculated, 0.9 probability of growth was observed before 3 days under optimum conditions, while more than 4 and more than 5 days were required at 0.92a_w/25ºC and 0.98a_w/15ºC, respectively. On the other hand, a probability of 0.05 was reached before 1, 2 and 2 days at 0.98a_w/25ºC, 0.92a_w/25ºC and 0.98a_w/15ºC, respectively. The slopes of the probability curves were smaller under suboptimal growth conditions suggesting wider distributions.
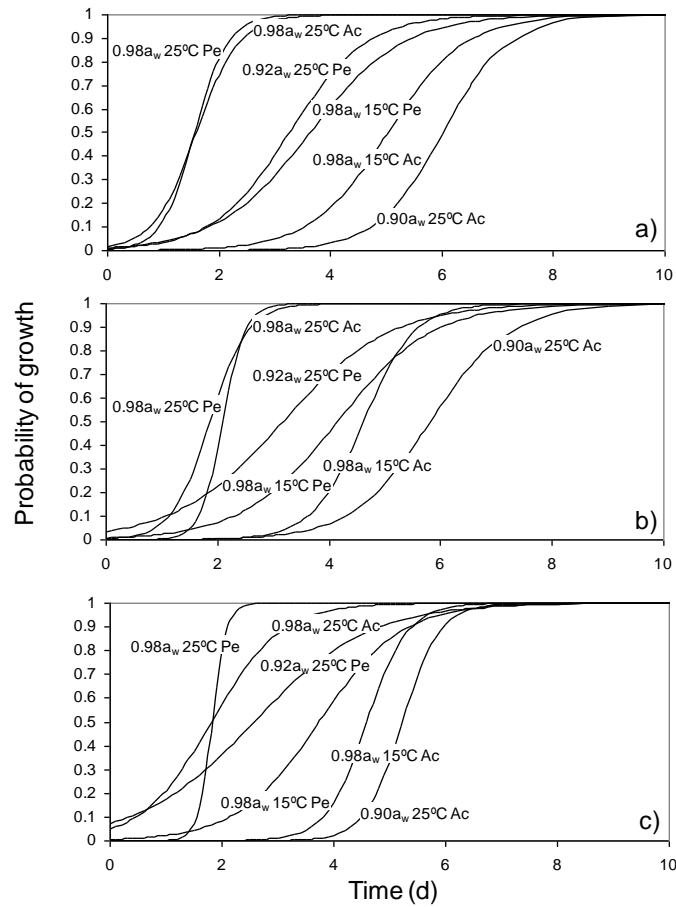
Figure 2: Probability models obtained through linear logistic regression under different environmental conditions for *A. carbonarius* (Ac) and *P. expansum* (Pe). **a)** 0-1 inoculated spores; **b)** 1-10 inoculated spores and **c)** 10-100 inoculated spores.

However, for a better performance of the predictive models no growth observations due to marginal conditions (once rejected those no growth observations due to the low level of inoculum) should be included, although in such cases a worse fitting of such models was observed. Table 8 shows the difference in the predictions obtained for both kinds of models for *A. carbonarius*.

Table 8. Predictions for probability of growth (P) of *A. carbonarius* (inoculation level: 10-100 spores)

Time (d)	P excluding no-growth observations			P including no-growth observations (linear logistic)			P including no-growth observations (logistic)		
	$0.98a_w$ 25ºC	$0.90a_w$ 25ºC	$0.98a_w$ 15ºC	$0.98a_w$ 25ºC	$0.90a_w$ 25ºC	$0.98a_w$ 15ºC	$0.98a_w$ 25ºC	$0.90a_w$ 25ºC	$0.98a_w$ 15ºC
2	0.80	0.00	0.00	0.80	0.07	0.21	0.50	0.00	0.00
4	1.00	0.02	0.16	1.00	0.23	0.28	1.00	0.04	0.12
6	1.00	0.91	0.98	1.00	0.55	0.36	1.00	0.86	0.65
8	1.00	1.00	1.00	1.00	0.84	0.45	1.00	0.96	0.66
10	1.00	1.00	1.00	1.00	0.96	0.55	1.00	0.96	0.66
R^2	0.823	0.903	0.874	0.823	0.614	0.131	1	0.999	0.999

An alternative to include no-growth data due to marginal conditions would be to calculate the mean probability of growth for each time observation and once probabilities plotted against time, to use logistic regression to obtain the probability functions. This approach was used in Figure 3 and the resulting predictions and R^2 are showed as an example in table 8. Sharper slopes were observed in this approach, while maximum probabilities were <1 due to the inclusion of no-growth data. This last approach results in the most realistic predictions.
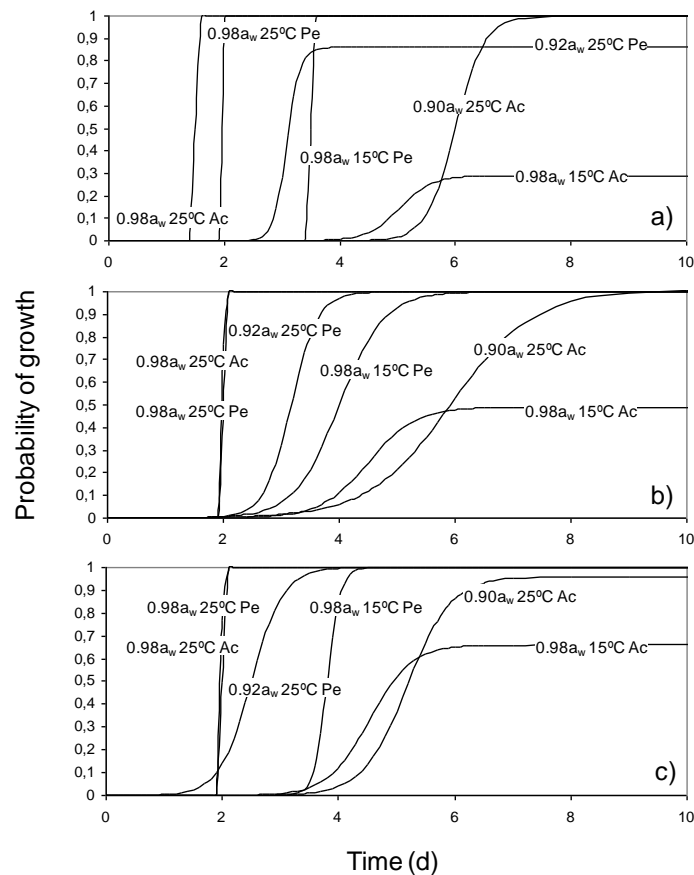
Figure 3: Probability models obtained through logistic regression of the mean probabilities under different environmental conditions for *A. carbonarius* (Ac) and *P. expansum* (Pe). **a)** 0-1 inoculated spores; **b)** 1-10 inoculated spores and **c)** 10-100 inoculated spores.

4. Discussion

Traditionally the growth and ecophysiological characterization of fungal contaminants of foods has been determined using very high inoculum levels in the form of spore suspensions or circular disks cut from the margins of growing colonies (Samapundo et al., 2007). Studies on mould growth use mainly high inoculum levels, while in reality infection of a food occurs with a low number of spores. It may actually be that contamination at low inoculum levels or by individual spores plays a more important role in fungal infection, spoilage of foods and subsequent production of mycotoxins of public and animal health significance than previously thought (Samapundo et al., 2007). Sautour et al. (2003) investigated the influence of inoculum preparation on fungal growth kinetics. The age of the sporulating culture had an effect on the growth rate, while the nature of the diluting solution had no effect on fungal growth kinetics.

In this study, although inoculum was not completely standardised, it has been shown that the inoculum levels tested significantly affected the mean estimations of the kinetic parameters. Excluding no-growth observations, inoculum levels did not affect significantly the rate of growth, while increasing lag phases were in general observed when inoculum decreased from 10-100 to 0-1 spores. The results with 0-1

spores were not representative as only a few plates presented growth. Similarly, at lower inoculum levels of *P. expansum* longer lag phases were observed compared to the higher inoculum levels (Baert et al., 2008). In their case, with the same number of growing replicates under each inoculum level, the standard deviation was also higher for lower inoculum levels, indicating that the variability between replicates is higher when fewer spores are inoculated. This indicates that more replications are necessary to estimate the lag phase of the mould under the specified circumstances. For the growth rate no clear effect of the inoculum size was observed, neither. A reduction of the inoculum size did not result systematically in an increase of the variability (Baert et al., 2008). Sautour et al. (2003) also reported growth at low inoculation levels. Growth of *P. chrysogenum* was also observed when 1 or 10 spores were inoculated under optimum growth conditions, with a decrease in the lag phase duration as the size of the inoculum increased. In their case growth was significantly faster with about 1 spore, while no significant difference in the growth rates were observed when the inoculum size was 10-1000 spores. They concluded that the lag time is strongly dependent on the inoculum size, thus inoculum size should be standardised in order to assess the shelf-life of food products.

Thus for modelling purposes, low inoculum sizes should be used in order to predict the lag phase before growth, while this point is not so important for growth rate prediction. Therefore, using high inoculum levels to estimate the growth parameters will result in estimating a worst case scenario, with the fastest growth and consequently the shortest shelf life. The size of the spore inoculum has been already found to have direct relationship to fumonisin production on corn (Chulze et al., 1999), with an increase in the inoculum size resulting in an increase in the amount of fumonisins produced.

The number of spores that are present (inoculum size) together with the number of spores that will germinate (depending on physiological stage and surrounding conditions) will determine whether growth will occur. The present study shows that the growth of *A. carbonarius* occurred in 100% of the cases under optimal a_w/T conditions when the inoculum was equal or higher than 10-100 spores, and only more than 66% under marginal a_w/T conditions. Decreasing percentages were observed when inoculum sizes were between 0-10 spores. For *P.expansum* the limiting growth conditions tested did not limit the number of growing colonies.

Regarding kinetic parameters, for *A. carbonarius* higher variability (wider distributions) of both growth rates and lag phases was observed under both water activity and temperature suboptimal conditions, while for *P. expansum* this only occurred under limiting conditions of water activity. The existence of no-growth observations results in a wide distribution of kinetic parameters; these observations should be included in the models for a better goodness of prediction of such models, although their inclusion compromises the goodness of fit. These results indicate that more replicates should be inoculated to reduce the variability between replicates when the growth conditions become limiting. For improving the prediction of microbial growth, the distribution of kinetic parameters should be determined (Dantigny et al., 2007). Distributions of biological parameters should be assessed for single spores, while the

distributions presented in this work come from bigger inocula; this was done in order to assess the distribution of growth parameters from realistic inoculum sizes which may be found in food products. The effects of water activity and temperature on the distribution of radial growth rate and lag time for growth of single spores of *Aspergillus flavus* and *Fusarium verticillioides* were assessed (Samapundo et al., 2006) under suitable growth conditions. As in our case, all the distributions were normal. The standard deviations increased with decreasing a_w, but the effect of T on the distribution was less clear. However, no limiting conditions of growth were included in the study and no no-growth cases were recorded.

Fungal growth has been usually modelled using Baranyi's model; in general, worse fittings were reported in the past when growing conditions were far from optimal ones (Marín et al., 2008b). However, less attention has been paid to suboptimal conditions than to optimal ones. From the food safety point of view, however, these conditions are of great importance, and predictions near the boundaries for growth should be much accurate. In this study, it was observed that under most of suboptimal conditions although colony diameter increase followed initially a sigmoidal function, when growth seemed about to cease, a second growth phase was observed at a near to constant rate, although slower. A secondary growth rate was then defined, although most of the conclusions were drawn from the usual growth rate, as it has been the most commonly used in the past. In addition, in those published experiments in which short times for observations were used this secondary phase was probably never observed.

Prevention of fungal growth effectively conduces to prevention of mycotoxin accumulation. Due to the poor correlation observed between growth and mycotoxin production under different environmental conditions (Bellí et al., 2005a; Marín et al., 2004; Morales et al., 2007), kinetic growth models should allow to predict the conditions under which no growth occurs. In such approach, the prediction of the growth rates through kinetic models is only relevant to determine the growth/no-growth boundary (Garcia et al., 2009). Probability modelling is particularly useful when pathogenic or mycotoxin-producing species are involved. Probability models allow the prediction of whether a particular event, such as growth or toxin production, might occur, under various conditions (Gibson and Hocking, 1997). Probabilistic growth models are built from the proportion of "growth/no growth" responses throughout the experimental design space at a defined point in time (Brul et al., 2007).

Probability models shown in this study are of particular interest to account for no-growth situations encountered under marginal growth conditions, because if they are disregarded the resulting secondary models would too much overestimate the risk of these mycotoxigenic fungi to cause safety problems. These models were not much affected by the inoculum size used to build them. Lower probabilities of growth were in general predicted under marginal conditions at a given time for both strains. The slopes of the probability curves were smaller under suboptimal growth conditions due to wider distributions. No previous studies have dealt with the effects of inoculum size and marginal growth conditions on probability models applied to moulds.

As conclusions, it can be stated that for a suitable secondary kinetics model development it is required i) to use low inoculum levels, in particular for lag phase predictions, ii) to use higher number of replicates as no-growth replicates resulting from the use of low inoculum levels should be discarded, iii) to use higher number of replicates for the treatments involving marginal growth conditions to diminish the error associated to higher variability of both μ_1 and λ. Finally, probability models may be a good alternative to model probability of growth near to the growth/no-growth boundaries.

Acknowledgement

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3.6 Is intraspecific variability of growth and mycotoxin production dependent on environmental conditions? A study with *Aspergillus carbonarius* isolates.

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Abstract

Mycotoxin contamination of food products is an important health hazard of a growing interest around the world. *Aspergillus carbonarius* has been considered the main species in ochratoxin A (OTA) accumulation in grapes, wine, dried vine fruits and probably in coffee. Generally, foods and feeds are stored at marginal conditions for mould growth. Different environmental factors could influence on the variability intra-species in their growth capability and mycotoxin production, and this might be increased when marginal conditions exist. This point should be clarified for further development of predictive mycology. The aim of this study was to assess the impact of suboptimal environmental conditions on the intraspecific variability of *A. carbonarius* growth and OTA production using thirty isolates of *A. carbonarius*. SNM plates were inoculated centrally with a needlepoint load. Three a_w/temperature conditions were tested, an optimal one (0.98a_w/25ºC) and two suboptimal ones: 0.90a_w/25ºC and 0.98a_w/37ºC as suboptimal water activity and temperature, respectively, which might take place through over ripening and dehydration of grapes. For each condition, 12 Petri dishes were inoculated, and colony growth and OTA production was measured along time. ANOVA revealed significant differences among μ and λ within the 30 assayed isolates. Coefficients of variation (CV%) revealed a wider dispersion of growth rates at 0.90a_w/25ºC compared to 0.98a_w/25ºC, and a more than 4-fold higher CV at 0.98a_w/37ºC compared to 0.98a_w/25ºC. However, regarding lag phases dispersion they were similar at 0.98a_w/25ºC and 0.90a_w/25ºC and wider at 0.98a_w/37ºC. There were significant differences ($p<0.05$) among OTA levels (ng/mm^2) for the different conditions, values being lower under marginal conditions, and particularly at 0.98a_w/37ºC. Coefficients of variation (CV%) revealed a wider dispersion of OTA production at 0.90a_w/25ºC compared to 0.98a_w/25ºC, while CV at 0.98a_w/37ºC was similar to that at 0.98a_w/25ºC. In order to address the strain variability in growth initiation and prove the well-established notion of reducing OTA in foods by preventing fungal growth, a greater number of strains should be included when developing models under both, suboptimal a_w for OTA and temperature levels for growth.

Keywords:
Intraspecific variability, *Aspergillus carbonarius*, Growth mould, Mycotoxin production

1. Introduction

Mycotoxin contamination of food products is an important health hazard of a growing interest around the world. *Aspergillus carbonarius* has been considered the main species in ochratoxin A (OTA) accumulation in grapes, wine, dried vine fruits and probably in coffee (Cabañes et al., 2002, Abarca et al., 2003). OTA is a toxic fungal secondary metabolite which poses a risk for the human and animal health. The toxicological profile includes teratogenesis, nephrotoxicity and immunotoxicity (Patel et al., 1997; Krogh et al., 1987; Kanisawa et al., 1984; Bendele et al., 1985) and it is classified as a possible human renal carcinogen (group 2B) (International Agency for Research on Cancer, 1993).

A. carbonarius has been found in a great variety of substrates for human and animal consumption in regions with warm and tropical climate. Strains of this mould have been isolated and identified in several Mediterranean wine producing countries, such as Spain, Italy, Israel, Portugal and Greece (Serra et al., 2003; Battilani et al., 2004; Belli et al., 2004a; Mitchell et al., 2004; Tjamos et al., 2004, 2006).

There are many factors that influence mould growth and mycotoxin contamination like temperature, substrate aeration, water activity, inoculum concentration, microbial interactions, physiological state of mould, etc. Many works have been published on the effects of some of these factors on growth of mycotoxigenic strains including one to eleven strains. Some authors reported a high intraspecific variability on the mould growth and mycotoxin production when several strains were included in the studies (Bellí et al., 2004b; Parra and Magan, 2004; Pardo et al., 2004; 2005a; Arroyo et al., 2005; Astoreca et al., 2007, 2010; Romero et al., 2007; Tassou et al., 2009). Some others did not find differences among isolates of the same species (Pardo et al., 2005b; Bellí et al., 2004b).

Moreover the use of cocktail inocula of different isolates to minimize the variation that might be expected among different isolates of the same species has been proposed (eg. Hocking and Miscamble, 1995; Patriarca et al., 2001; Pose et al., 2009; Romero et al., 2007, 2010). This concept was introduced for physiological studies on foodborne bacterial pathogens, particularly in acquisition of data for predictive modelling studies, as a way of determining the extremes of growth limits for particular species (Gibson et al., 1987; Buchanan et al., 1993).

Generally, foods and feeds are stored at marginal conditions for mould growth. Different environmental factors could influence on the variability intra-species in their growth capability and mycotoxin production, and this might be increased when marginal conditions exist. This point should be clarified for further development of predictive mycology. The aim of this study was to assess the impact of suboptimal environmental conditions for growth on the intraspecific variability of *A. carbonarius* growth and OTA production using thirty isolates of *A. carbonarius*.

2. Materials and methods

2.1 Fungal isolates and preparation of inoculum

This work was carried out on thirty isolates of *A. carbonarius* previously isolated from grapes of Spain (Catalonia, Andalucía, La Rioja, Valencia). All of them are maintained in the culture collection of Food Technology Department of Lleida University. The isolates were sub-cultured on malt extract agar (MEA) plates and incubated at 25 ºC for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from MEA plates and they were suspended in 5 ml of H_2O/glycerol solutions with two different water activity levels: 0.98 and 0.90 (optimal and suboptimal condition, respectively). After homogenizing, the suspensions were ajusted using a Thoma counting chamber and the final concentration was adjusted to $1\text{-}5\times10^5$ spores/ml.

2.2 Medium and water activity modification

Growth was determined on synthetic nutrient medium (SNM). This medium simulates grapes composition between veraison and ripeness (Delfini et al., 1982). The initial a_w was 0.99 and was modified to $0.98a_w$ and $0.90a_w$ by adding differents amounts of glycerol (10 g/l and 510 g/l, respectively) and varying its sugars content: (90 g/l of D(+) glucose and 95 g/l of fructose for 0.98 a_w and 70 g/l of D(+) glucose and 30 g/l of fructose for $0.90a_w$ (Valero et al., 2005). Medium was autoclaved and poured into 9 cm sterile Petri dishes. The a_w of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of $\pm$ 0.003, before, during and the end of the experiment.

2.3 Inoculation, incubation and growth assessment

SNM plates were inoculated centrally with a needlepoint load. Previous repeated experiments showed that the number of spores inoculated through this technique was 10-100 spores. In particular, 23.8 CFU $\pm$ 14.5 colonies were counted when plated (mean $\pm$ standard deviation; n=20). Petri dishes with the same a_w level were enclosed in polyethylene bags in order to maintain a constant water activity and were incubated at 25 ºC for both a_w and also at 37 ºC for 0.98 a_w condition. Thus three a_w/temperature conditions were tested, an optimal one (0.98a_w/25ºC), which is an optimal condition for the growth of this mould, and two suboptimal ones: 0.90a_w/25º and 0.98a_w/37ºC as suboptimal water activity and temperature, respectively, which might take place through over ripening and dehydration of grapes. For each condition, 12 Petri dishes were inoculated. Two perpendicular diameters of the growing colonies were measured daily until the colony reached the edge of the Petri dish. The diameters of the colonies were plotted against time.

2.4 OTA extraction from culture and quantification

OTA was extracted by a variation of Bragulat et al. (2001) method. Three agar plugs (diameter 5 mm) were removed along a diameter of the colonies when they reached 20 mm and from the inner, middle and

outer part of the colonies when they reached 40, 60 and 80 mm. When colony diameter was 10 mm only one plug was removed. 1 ml of methanol was added, and the vials were shaken for 5 s. After 60 min, the extracts were shaken and filtered (Millex-HV 0.45 μm 25 mm, Millipore Corporation. Bedford, U.S.A.) into another vial and stored at 4 ºC until the analysis by HPLC instrument (Waters, Mildford, MA, SA). Plug extraction was performed in duplicate.

OTA production was detected and quantified by HPLC with fluorescence detection (λ_{exc} 330 nm; λ_{em} 460 nm) (Waters 2475), using a C_{18} column (5 μm Waters Spherisorb, 4.6 × 250 mm ODS2). The mobile phase (acetonitrile–water–acetic acid, 57:41:2) was pumped at 1 ml min^{-1}. The injection volume was 100 μl and the retention time was around 7 min. The detection limit of the analysis was about 0.21 ng OTA/g SNM or 0.0005 ng OTA/mm^2, based on a signal-to-noise ratio of 3:1. Quantification was achieved with a software integrator (Empower, Milford, MA, USA). OTA was quantified on the basis of the HPLC fluorimetric response compared with that of a range of OTA standards. OTA values were expressed per mm^2 of colony.

2.5 Statistical analyses

Diameters of growing colonies were plotted against time, and Baranyi and Roberts (1994) model was used to estimate growth rate and lag phase for each growth condition (0.90a_w/25ºC, 0.98a_w/25ºC, 0.98a_w/37ºC) and isolate. Analysis of variance of growth rates and lag phases was used in order to assess significant differences due to isolates and growth conditions. OTA was expressed as ng per mm^2 of colony. For OTA results, analysis of variance was applied to assess the significance of growth conditions, colony diameter and intraspecific differences. In both cases, Tukey test was used to establish the differences among mean values of the variables under the different levels of factors at $p < 0.05$. Moreover, analysis of variance was carried out to test of significance of time in OTA accumulation ($p < 0.05$)

3. Results

3.1 Marginal conditions effects on intraspecific growth differences

All isolates of *A. carbonarius* grew under the three conditions tested. Analysis of variance revealed significant differences in growth among isolates and among incubation conditions; moreover, differences among isolates depended on environmental conditions ($p < 0.05$). Table 1 shows mean values of growth rate (μ, mm/day) and lag phase (λ, day) of all isolates for the conditions studied and their dispersion. As expected, there were significant differences ($p < 0.05$) among estimated μ and λ levels for the different conditions, μ values being lower under marginal growth conditions; at 0.90a_w/25ºC growth was inhibited to a higher extent than at 0.98a_w/37ºC, in terms of μ. Regarding λ, it was longer at 0.90a_w/25ºC, but it

decreased at 0.98a_w/37ºC compared to 0.98a_w/25ºC. As a result the high temperature condition was not as suboptimal as expected, at least in terms of λ.

Table 1. Descriptive statistics of growth parameters for the three conditions assayed

Condition	μ (mm/day)						λ (day)					
	mean	Min	Max	Range	SD	CV%	Mean	Min	Max	Range	SD	CV%
0.98a_w/25ºC	1,04a	0.66	1.25	0.58	0,14	13,22	3,69a	2.28	5.14	2.86	0,68	18,38
0.98a_w/37ºC	0,73b	0.45	2.8	2.34	0,44	60,77	3,38b	0.97	4.42	3.45	0,75	22,18
0.90a_w/25ºC	0,33c	0.27	0.69	0.43	0,08	23,06	4,70c	2.71	6.19	3.48	0,83	17,76

*Means followed by different letters are significantly different according to Duncan test

Moreover, ANOVA revealed significant differences among μ and λ within the 30 assayed isolates. Coefficients of variation (CV%) shown in table 1, reveal a wider dispersion of growth rates at 0.90a_w/25ºC compared to 0.98a_w/25ºC, and a more than 4-fold higher CV at 0.98a_w/37ºC compared to 0.98a_w/25ºC. However, regarding lag phases dispersion they were similar at 0.98a_w/25ºC and 0.90a_w/25ºC and wider at 0.98a_w/37ºC. In particular, strain 8 showed a better adaptation to low a_w than the others, and strains 8, 9, 10, 14 and 29 grew better than average at 37ºC (Fig1a). In general, these isolates grew fast under suitable growth conditions, but not significantly faster than the rest. Isolates 3, 8, 11 and 14 initiated their growth significantly earlier than the others at 37ºC, while strains 3, 23 and 29 started growing early at 0.90 a_w; in this case isolates 3 and 8 showed also short lag phases at 0.98a_w/25ºC (Fig1b).
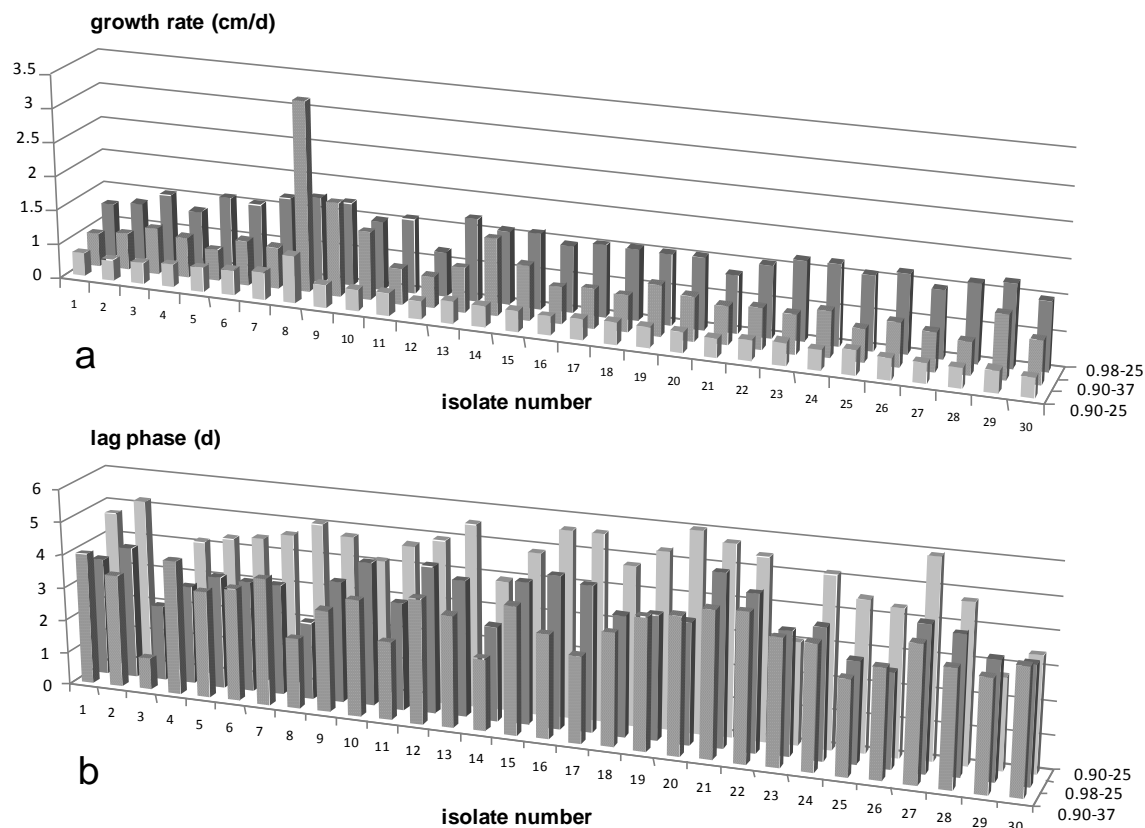
Figure 1: Growth rate (mm/day; 1a) and lag phase (days; 1b) of thirty isolates of *Aspergillus carbonarius* tested at 0.98a_w/25ºC, 0.90a_w/25ºC and 0.98a_w/37ºC in SNM medium.

3.2 Marginal conditions effects on the growth variability within isolates

Twelve Petri dishes were inoculated per isolate. The minimum variability for μ was observed at 0.90a_w/25ºC and the highest was at 0.98a_w/37ºC (table 2). However, the variability of lag phase values increased under marginal environmental conditions with the major value under marginal temperature, but in general the variation was not much wide.

Table 2. Mean (n=30) coefficient of variation of growth parameters

	μ (mm/day)		λ (day)	
Condition	Mean CV%	Range CV%	Mean CV%	Range CV%
0.98a_w/25ºC	10.63	2.93-33.87	11.55	3.57-33.50
0.98a_w/37ºC	24.62	2.05-93.72	17.66	4.15-48.30
0.90a_w/25ºC	5.78	1.86-17.15	13.64	4.89-30.53

Figure 2a shows the variability of growth rate data for each isolate. When isolates grew under marginal water activity level, the major variability was observed for the isolates 14, 22 and 23 (although lower than 18%); under suitable conditions isolates 1, 2, 4 and 6 had the higher variation values (lower than 34%), while under high temperature the isolates with more variable growth rate results were 9, 10, 14, 15 and 29 (up to 94%). It must be highlighted that the more consistent results were obtained for isolate number 8.

Figure 2b shows the variability of lag phase data for each isolate. When isolates grew under suitable conditions, isolates 1, 4, 6, 12 and 30 had the higher variation values (lower than 34%). Under marginal water activity level, the major variability was observed for the isolates 11, 14, 15, 23, 24, 28 and 29 (up to 24%) while under high temperature the isolates with more variable lag phase results were 2, 3, 8, 16 and 23 (lower than 48%).

As a conclusion, some of the isolates which lacked repeatability in their growth rate results, had the same problem for lag phases, except under high temperature.
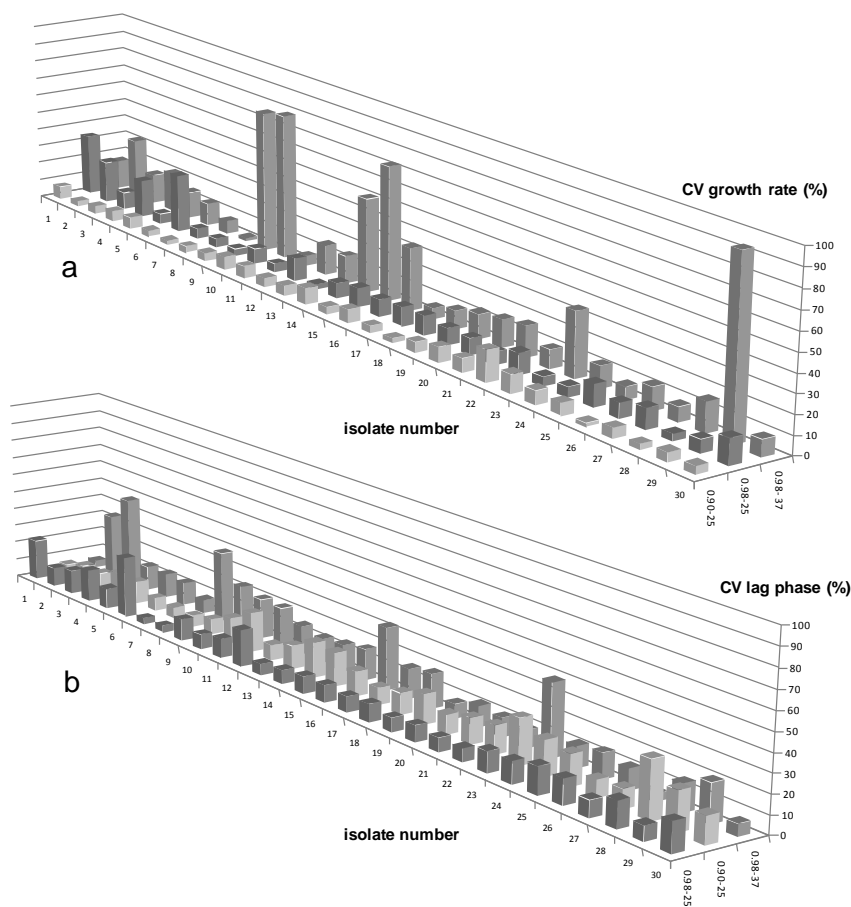

Figure 2: Coefficients of variation (%) of growth rate (a) and lag phase (b) for each isolate of *Aspergillus carbonarius* tested at 0.98a$_w$/25ºC, 0.90a$_w$/25ºC and 0.98a$_w$/37ºC in SNM medium.

3.3 Marginal conditions effects on intraspecific OTA production difference

The analysis of variance of the whole data matrix showed that intraspecific differences, growth conditions and colony diameter, as well as their interactions, had a significant impact on OTA production.

Table 3 shows mean values of OTA production (ng/mm^2) of all isolates for the conditions studied and their dispersion. As expected, there were significant differences (p<0.05) among OTA levels for the different conditions, values being lower under marginal conditions, and particularly at 0.98a_w/37ºC. Coefficients of variation (CV%) shown in table 3, reveal a wider dispersion of OTA production at 0.90a_w/25ºC compared to 0.98a_w/25ºC, while CV at 0.98a_w/37ºC was similar to that at 0.98a_w/25ºC.

Table 3. Descriptive statistics of mean OTA production (ng/mm^2) for the three conditions assayed.

Condition	Mean	Min	Max	Range	SD	CV%
0.98a_w/25ºC	3.48a	0.02	18.06	18.04	4.92	141
0.98a_w/37ºC	0.13c	<ld	0.71	0.71	0.19	146
0.90a_w/25ºC	0.24b	<ld	3.30	3.30	0.76	315

*Means followed by different letters are significantly different according to Duncan test.

The analysis was repeated separately for each growth condition:

At 0.98a_w/25ºC, both colony diameter and intraspecific differences and their interaction affected significantly OTA production. OTA production by the 30 isolates was not significantly different when colonies were 20-40 mm wide (Fig 3). However, when colonies were 10 mm, colonies of isolates 1, 2, 3, 4, 9 and 14 showed high OTA concentrations. Similarly, when colonies attained 60-80 mm, isolates 4, 14, 19 and 30 reached higher OTA concentrations than the remaining isolates. Isolates which produced less than 1 ng/mm^2 regardless of the incubation time were omitted from Figure 3 for clearness.

OTA levels up to 2.1 ng/mm^2 were recorded at 0.98a_w/37ºC, moreover, isolates 1, 12, 19 and 29 did not show detectable levels of OTA. Under these conditions only intraspecific differences affected significantly OTA production, regardless of the colony diameters. Tukey test, revealed, however, few clear differences among isolates, only isolates 5, 17 and 27 produced significantly higher levels of OTA (0.54, 0.71 and 0.58 ng/mm^2, respectively) than the others. The remaining isolates produced between <LD and 0.40 ng/mm^2.
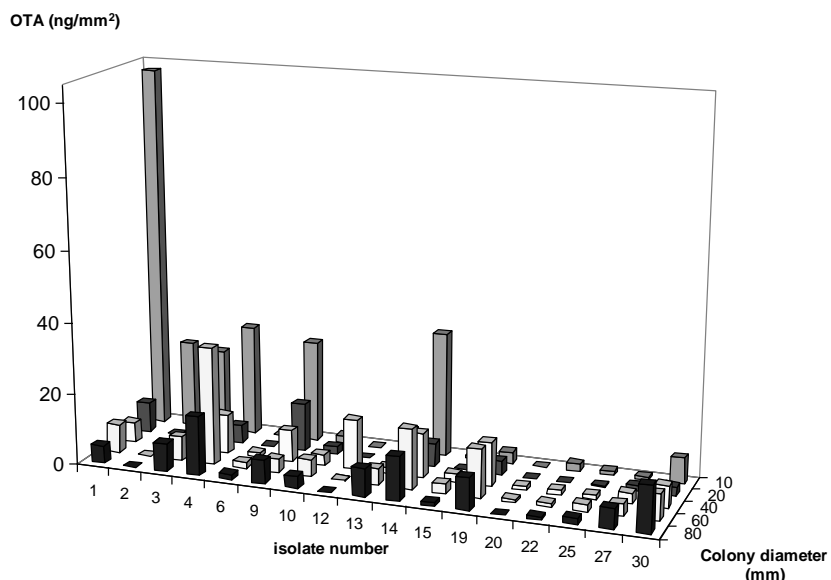
Figure 3: OTA production (ng/mm^2) by some isolates of *Aspergillus carbonarius* tested at 0.98a$_w$/25ºC in SNM medium.

Finally, at 0.90a$_w$/25ºC, both colony diameter and intraspecific differences and their interaction affected significantly in OTA production. The significant impact of colony diameter on OTA production was only observed for isolates 1, 4, 9, 10, 11, 13 and 20 (Fig. 4). Isolates 1 and 4 showed peak values when colonies achieved 40 mm diameter, while isolates 10, 11, 13 and 20 had higher productions in the smaller colonies (10-20mm). For the remaining isolates, OTA production, although variable, was not linked to increasing colony diameters, and mean OTA levels were between <LD and 0.20 ng/mm^2. Isolates 1 and 4 showed significantly higher OTA levels (P<0.05) for most colony diameters, while isolates 9, 11 and 13 showed significantly higher production than 10 and 20, when colonies showed diameters of 10-20mm.
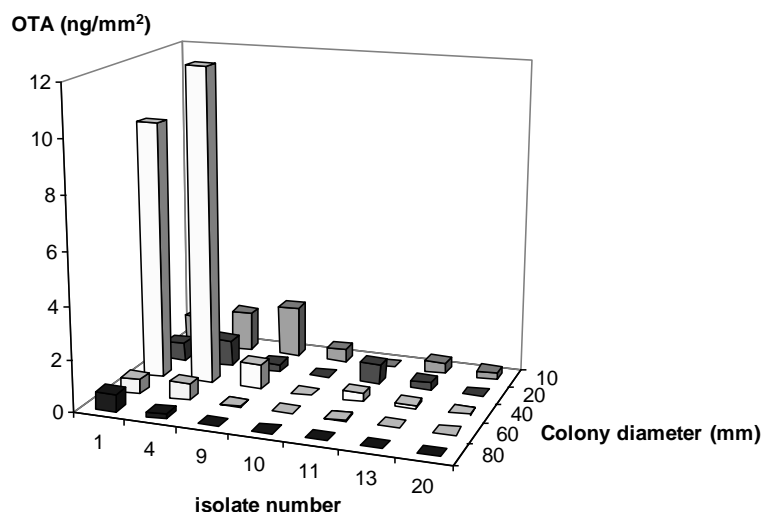
Figure 4: OTA production (ng/mm^2) by some isolates of *Aspergillus carbonarius* tested at 0.90a$_w$/25ºC in SNM medium.

Temporal production of OTA

Analysis of variance showed that time had no effect on the amount of OTA produced (ng/mm^2) at 0.98a_w/37ºC and 0.90a_w/25ºC, that is to say that the mean OTA present across the colony could be considered nearly constant along time. Such constant rates varied from 0.005 to 0.71 ng/mm^2 and from 0.0003 to 3.30 ng/mm^2, respectively.

The effect of time was only significant for certain isolates at 0.98/25ºC (2, 6, 7, 17, 18, 20 and 23) (Fig. 5). Isolates 6, 17, 18 and 20 showed peak production after 7-10 days, while for isolates 2, 7 and 23 the maximum production was in day 5 and decreased afterwards. Additionally, figure 5 presents the increasing size of the colonies and the total accumulated OTA in the colonies. Total OTA followed in general, the same trend as OTA rate in ng/mm^2, thus the size of the colonies was less determinant.
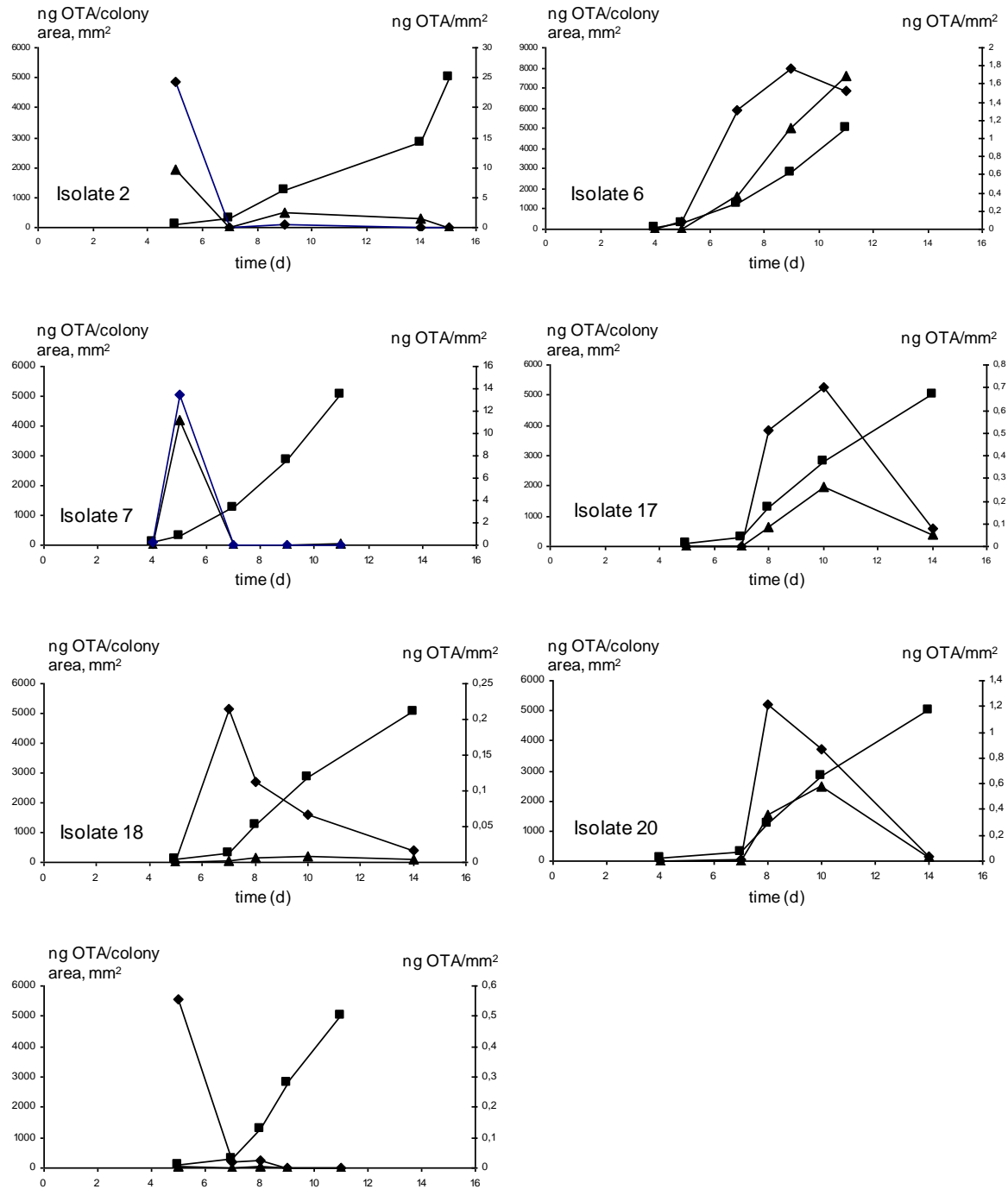
Figure 5: Effect of time in OTA production (◆, ng/mm² of colony; ▲, ng in the total colony) and colony area (■, mm²) of *Aspergillus carbonarius* isolates at 0.98aw/25ºC.

4. Discussion

Individual strains exhibit differences in their growth and in the amount of mycotoxin produced (Romero et al., 2010). These differences can be attributed to isolate geographical origin, substrate, environmental condition, molecular characteristics, etc.

In this research we compared growth parameters and OTA production of thirty isolates of *A. carbonarius* incubated at three different conditions. All isolates grew under all conditions studied. However, some isolates had an exceptionally behavior and showed a good adaptation for both, optimal and suboptimal conditions, compared to the rest of isolates. As we said before, this could be attributed to isolate origin and molecular character. Studies on ecophysiologycal adaptation to different environmental conditions have been carried out with a few isolates, but these results might not be representative for the majority of the strains (eg. Bellí et al., 2004ab, 2005; Esteban et al., 2006; Astoreca et al., 2007; Tassou et al., 2007; Kapetanakou et al., 2009).

When *A. carbonarius* was incubated at $0.90a_w$/25ºC growth was inhibited to a higher extent than at $0.98a_w$/37ºC compared with $0.98a_w$/25ºC. Some published works are in agreement with our results, where mould growth is more affected by marginal a_w conditions than at high temperature. However, the dispersion of the values for both, growth rate and lag phase, was wider at 37ºC. This indicates that under limiting temperature more isolates may be necessary to estimate growth parameters of a given species with a minor variability of data. Most works are made with a few isolates, between two to eleven but, no specific study in intraspecific variability was made. Those works showed differences among growth parameters of the different isolates (Bellí et al., 2004a; Parra and Magan, 2004; Pardo et al., 2004; 2005a; Arroyo et al., 2005; Astoreca et al., 2007, 2010; Romero et al., 2007; Tassou et al., 2009), with the highest variability under marginal conditions. However, Pardo et al. (2005b) studied growth and OTA production of three *A. ochraceus* isolates at different conditions and they did not find differences among the isolates.

Some authors used a cocktail inoculum to minimize the variation that might be expected between different isolates of the same species (eg. Hocking and Miscamble 1995; Patriarca et al., 2001; Pose et al., 2009; Romero et al., 2007; 2010). Although this methodology can be criticized because of the loss of information regarding the responses of individual strains of a species, it is accepted as a legitimate method of achieving a "worst case" scenario (Hocking and Miscamble 1995). Romero et al. (2010) compared growth rate, lag phase and OTA production of a mixed inoculum of four *A. carbonarius* strains with the single strains. They found significant differences on the growth rate and lag phase among the four isolates for the condition studied (30ºC); however, no significant differences between growth rates of each isolate and the mixed inoculum were detected. They concluded that the use of a cocktail inoculum could be a useful approach to obtain data for prediction of the final level of contamination. However, this work was made under suitable growth conditions and, from the food safety point of view, marginal

conditions are of great importance and predictive models should be built near the boundaries for growth (Garcia et al., 2010).

Moreover, in our study, replications within isolates were evaluated. The major variability for growth rate and lag phase was also observed under high temperature with values of CV up to 94% and 48%, respectively. However, isolates with the highest variability for growth rate were different from those with major variability for lag phase. Under the remaining conditions CV of growth parameters were relatively low with a minor variability under suboptimal water activity. This indicates that high temperature promoted intrinsic biological variation.

Garcia et al. (2010) worked with one isolate of *A. carbonarius* and one of *Penicillium expansum* under suboptimal conditions. Their results showed a higher variability between replicates of *A. carbonarius* for both growth parameters under marginal water activity and temperature conditions, while for *P. expansum* this only occurred under limiting conditions of water activity.

Regarding OTA production, in this study, the amounts of OTA produced by isolates varied with marginal conditions. There were significant differences ($p < 0.05$) among OTA levels for the different conditions, values being lower under marginal conditions, and particularly at high temperature. Other works had similar results, where high water activities seemed ideal for maximum OTA production, while OTA yield decreased with water activity reduction (Bellí et al., 2004b, 2005; Cairns-Fuller et al., 2005; Esteban et al., 2006; Romero et al., 2010). Esteban et al. (2006) studied the effect of water activity and temperature on ochratoxin A production by four *A. carbonarius* isolates. They found that OTA concentration was higher at low temperature (15 ºC) than at high temperature (30ºC), but there were differences in optimal condition for mycotoxin production between the isolates studied. Also, Bellí et al. (2005) found the minimum OTA concentration at 30, 35 and 37 ºC and also had differences between isolates in optimal condition for mycotoxin production.

Given the different abilities to biosynthesise OTA by the different strains of the same species, extrapolation of the results obtained with one individual strain might not be representative for the majority of the strains (Marín et al., 2008a; Romero et al., 2010). In our research, OTA production by the 30 isolates was affected significantly by both, colony diameter and intraspecific differences and their interaction. Baert et al. (2007) showed that patulin production of the isolates increased as a function of time and also as a function of the colony surface area. However, there were differences in the amounts of toxin production among isolates. Arroyo et al. (2005) observed similar results, where the three *P. verrucosum* studied had similar profiles of OTA production but the amounts differed among the strains. Also, McCallum et al. (2002) studied patulin production by *P. expansum* and in their results there was a high variability among isolates too. However, Bellí et al. (2005) did not find differences in the amount of toxin produced between two isolates of *A. carbonarius* and two of *A. niger* aggregate. All of these works studied isolates from different geographical origin, and the difference in mycotoxin production between isolates can be attributed to this factor.

The major dispersion of data for OTA production was however, at $0.90a_w/25^oC$. In the other hand, Baert et al. (2007), studied patulin production by eight strains of *P. expansum,* where patulin production was highly reduced when temperature changed from 4 ^oC to 1 ^oC. However, they found that patulin production for high temperature was strain dependent. Besides, their results showed that isolates with slow growth produced less patulin at lower temperatures than isolates with faster growth, indicating differences between the isolates studied.

In our work analysis of variance showed that time had no effect on the rate of OTA produced (ng/mm^2) when marginal conditions were assayed, and OTA concentration across the colony could be considered nearly constant along time. In these conditions of growth, the increase in colony diameters would lead to a near to linear increase in the total accumulated OTA. Bellí et al. (2004b) analyzed OTA production of two *A. carbonarius* isolates where mycotoxin concentration decreased with time, being minimum after 20 days of incubation for both isolates. Probably, toxin was degraded by fungus itself as suggested by Varga et al. (2000) or may be the production of OTA by the fungi could have stopped due to lack of nutrients.

In our case, at 0.98 $a_w/25^oC$, those isolates which showed a significant effect of time on OTA production across the colonies, showed peak values at 5 to 10 days. Few mathematical models have been developed on mycotoxins due to high variability on toxin production by the strains and the lack of a clear trend of mycotoxin production along time.

For this it is difficult to build a kinetic model for mycotoxin production on mould. Molina and Giannuzzi (2002) modeled aflatoxin accumulation in a solid media using a non-linear model. They had a good correlation between model and experimental data. Alternatively, Marín et al. (2008b) applied a probabilistic model to determine OTA presence by *A. carbonarius* in pistachio nuts which was a 94.4% concordant with experimental data. Tassou et al. (2009) employed a similar model for OTA production on a simulated grape juice medium. Their results showed a high concordance rate (98.9-99.1%) between predicted and observed data.

One of the most important aspects of predictive model development is ensuring that predictions made by the model are applicable to real situations (Romero et al., 2010). For this, in this work we took into account suboptimal conditions because they have a great importance from the food safety point of view. Besides, we also worked with many isolates because in natural ecosystems different strains can occupy the same niche and they may have intraspecific differences in their growth and mycotoxin production. To our knowledge, this is the first study on a great number of isolates studied at marginal conditions for mould growth and mycotoxin production. In order to address the strain variability in growth initiation and prove the well-established notion of reducing OTA in foods by preventing fungal growth, a greater number of strains should be included when developing models under both, suboptimal a_w for OTA and temperature levels for growth.

Acknowledgement

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3.7 Intraspecific variability of growth and patulin production of 79 *Penicillium expansum* isolates at two temperature conditions

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Abstract

Penicillium expansum has been considered the main species in patulin production in apples and pears. Generally, fruits are stored at suboptimal conditions for mould growth and this situation could influence on the intra-species variability in their growth capability and mycotoxin production. The aim of this research was to assess the impact of suboptimal environmental conditions on the intra-specific variability of *P. expansum* and patulin production using seventy nine isolates of this mould. Petri dishes with Apple Concentrate Agar Medium (ACAM) medium were inoculated centrally and two temperature conditions were tested, a near to optimal one (20 ºC) and the suboptimal cold storage one (1 ºC). For each condition, 10 Petri dishes were inoculated, and colony growth and patulin production was measured along time. Kruskal-Wallis test revealed significant differences among growth rate (μ) and lag phase (λ) within the seventy nine assayed isolates. Coefficients of variation revealed a wider dispersion of μ (mm/day) and λ (days) at 1 ºC compared to 20 ºC. There were significant differences ($p<0.05$) among patulin levels (ng/mm^2) for the different conditions, values being lower under the lower temperature. Coefficients of variation revealed the wider dispersion of mycotoxin production at 1 ºC. In order to address the strain variability in growth initiation and prove the well-established notion of reducing patulin production in foods by preventing fungal growth, a greater number of strains should be included.

1. Introduction

Growth of moulds in foods is an important quality problem and may lead to significant economic losses for the food industry (Andersen and Thrane, 2006). Mycotoxin contamination of food products is an important health hazard with a growing interest around the world. *Penicillium expansum* is the most important fungal species causing spoilage in cold stored fruit and is the responsible of "blue mould" spoilage in pears and apples. This mould is a psychrophile and can grow at very low temperatures (-2 to -6 ⁰C) (Brooks and Hansford, 1923; Pansenko, 1967). However, the optimum temperature is near 25 ⁰C and the maximum near 35 ⁰C (Pansenko, 1967). Besides, this mould can produce patulin, a mycotoxin with carcinogenic and teratogenic effects which can cause acute and chronic effects if ingested (McKinley et al., 1982; Lee and Röschenthalen, 1987; Pteiffer et al., 1998; Liu et al., 2003). Some authors have reported that 100% isolates of *P. expansum* are patulin producers (Andersen et al., 2004). Moreover, this toxin is both stable in acidic conditions and resistant to thermal inactivation (Lovett and Peeler, 1973), making it difficult to be removed from food products.

Both fungal growth and mycotoxin production are influenced by different factors such as temperature, substrate aeration, water activity, inoculum concentration, microbial interactions, physiological state of mould, genetic information, isolate age, etc., however the effects of these factors on mycotoxin production might be different from those on growth (Garcia et al., 2009). Many works have been published on the effects of some of these factors on growth of mycotoxigenic strains. Some of these works showed a high variability on growth rates and mycotoxin production when several strains were included in the studies (Bellí et al., 2004; Parra and Magan, 2004; Pardo et al., 2004; 2005a; Arroyo et al., 2005; Astoreca et al., 2007, 2010; Romero et al., 2007; Tassou et al., 2009). By contrast, some others did not find differences among isolates of the same species (Pardo et al., 2005b; Bellí et al., 2004). However, a recent work on *Aspergillus carbonarius* growth and ochratoxin A (OTA) production was made with thirty isolates; in this work a wide dispersion was observed in both growth rate and mycotoxin production, especially under marginal growth conditions (Garcia et al., 2011).

The use of cocktail inocula of different isolates in ecophysiological studies to minimize the variation that might be expected among different isolates of the same species has been proposed by different authors (e.g. Hocking and Miscamble, 1995; Patriarca et al., 2001; Pose et al., 2009; Romero et al., 2007, 2010). This concept was introduced for physiological studies on foodborne bacterial pathogens, particularly in acquisition of data for predictive modelling studies, as a way of determining the extremes of growth limits for particular species (Gibson et al., 1987; Buchanan et al., 1993).

Generally, foods and feeds are stored under marginal conditions for mould growth. Different environmental factors could influence the variability of responses of different isolates of the same species in their growth patterns and mycotoxin production, and this might be increased when marginal conditions exist. This point requires clarification for further development of predictive mycology. The aim of this study

was to assess the impact of a non-optimal temperature (1 ⁰C apple storage condition) and a near to optimal one (20 ⁰C), on the intraspecific variability of *P. expansum* growth and patulin production.

2. Materials and methods

2.1 Fungal isolates and preparation of inoculum

This work was carried out on seventy-nine isolates of *P. expansum* previously isolated from apples in Lleida (Spain) during 2004 and 2005 seasons. All of them are maintained in the culture collection of the Food Technology Department of Lleida University. The isolates were sub-cultured on malt extract agar (MEA) plates and incubated at 25 ⁰C for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from MEA plates and they were suspended in 5 ml of H_2O/glycerol solution with 0.98 water activity level. After homogenizing, the suspensions were adjusted using a Thoma counting chamber and the final concentration was adjusted to $1-5\times10^5$ spores/mL.

2.2 Medium preparation and water activity modification

Growth was determined on Apple Concentrate Agar Medium (ACAM, apple concentrate: water 1:7 and agar 15 g/L). The initial a_w was 0.99 and was modified to 0.98 (similar to apple water activity) by addition of glycerol. Medium was autoclaved and poured into 9 cm sterile Petri dishes. The a_w was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of $\pm$ 0.003.

2.3 Inoculation, incubation and growth assessment

Plates were inoculated centrally with a needlepoint load. Previous repeated experiments showed that the number of spores inoculated through this technique were 10-100 spores when using spore suspensions of $1-5\times10^5$ spores/ml. In particular, the following numbers of colonies were counted when plated (mean $\pm$ standard deviation): 28.2 CFU $\pm$ 17.2 for *P. expansum* suspensions. However, the needle inoculation was still preferred over a known volume inoculation to avoid the liquid interference, even if the solution is adjusted to the same a_w as the media. Petri dishes were enclosed in polyethylene bags in order to maintain a constant water activity and were incubated either at 1 ⁰C or at 20 ⁰C. Thus two temperature conditions were tested, a near to optimal one (20 ⁰C) and the suboptimal cold storage one (1 ⁰C). For each condition, 10 Petri dishes were inoculated. Fungal growth was observed on a daily basis for an maximum period of 120 days or until the colony reached the edge of the Petri dish by diameter measurements at right angles with the aid of a ruler. The diameters of the colonies were plotted against time.

2.4 Patulin extraction from culture and quantification

Patulin was extracted from colonies when they were 20, 40 and 60 mm of diameter. Three plugs (diameter 5 mm) were removed along the colony radius from the inner, middle and outer part of colonies. Plugs were weighed and introduced into 3-ml vials. 1 mL of ethyl acetate was added, and the vials were shaken for 5 s. After 60 min, the extracts were shaken and filtered with a 0.45 μm nylon filter and then were evaporated and stored at 4 ⁰C until the analysis by an HPLC system (Waters, Mildford, MA, SA). Plug extraction was performed in duplicate.

Patulin production was detected and quantified by HPLC with UV detection (λ 276 nm) (Waters 2487), using a C_{18} column (5 μm Waters Spherisorb, 4.6 × 250 mm ODS2). The mobile phase (acetonitrile–water–acetic acid, 57:41:2) was pumped at 1 ml min^{-1}. The injection volume was 100 μl and the retention time was around 6.5 min. The detection limit of the analysis was about 0.33 ng patulin/g ACAM or 0.017 ng patulin/mm^2 of colony, based on a signal-to-noise ratio of 3:1. Quantification was achieved with a software integrator (Empower, Milford, MA, USA). Patulin was quantified on the basis of the HPLC absorbance response compared with that of a range of patulin standards (3 to 500 ng/mL).

2.5 Statistical analyses

For growth experiments, the experimental design consisted of 2 categorical factors: intraspecific differences (79 levels) and storage temperature (2 levels) plus time as a covariate while the response variable was colony diameter. For patulin experiments, the experimental design consisted of 2 categorical factors: intraspecific differences (79 levels) and storage temperature (2 levels) plus a quantitative factor: colony diameter (3 levels), while the response variable was patulin concentration. The timing for patulin analysis was set at certain colony diameters instead of at fixed time periods, due to the fact that the temperature levels were so different that at equivalent time periods no patulin would have probably been detected at the lower temperature.

Diameters of growing colonies were plotted against time, and the Baranyi and Roberts (1994) model was used to estimate the growth rate and lag phase for each growth condition (20 ⁰C and 1 ⁰C) and isolate. Analysis of variance of growth rates and lag phases medians was used in order to assess significant differences due to isolates and growth conditions. Patulin was expressed as ng per mm^2 of colony. For patulin results, analysis of variance of medians was applied to assess the significance of growth conditions, colony diameter and intraspecific differences. In both cases, the Kruskal-Wallis test was used to establish the differences among median values of the variables under the different levels of factors at $p<0.05$. Moreover, analysis of variance of medians was carried out to test significance of time in patulin accumulation ($p< 0.05$). Statistical analysis were carried out with Statgraphics® Plus version 5.1 (Manugistics, Inc, Maryland,USA).

3. Results

3.1 Effect of marginal conditions on intraspecific growth differences

All isolates of *P. expansum* grew under both conditions tested. Analysis of variance revealed significant differences in growth among incubation conditions; moreover, significant differences among isolates were found and these differences were dependent on environmental conditions ($p<0.05$). Table 1 shows median values of growth rate (μ, mm/day) and lag phase (λ, day) of all isolates for the conditions studied and their dispersion. Both parameters calculated for the 79 isolates were not normally distributed, besides although under some conditions they followed a gamma distribution, under other conditions they could not be fitted to any known distribution (data not shown). There were significant differences ($p<0.05$) among estimated μ and λ levels for the two conditions, μ values being lower when *P. expansum* grew at 1 ºC compared to 20 ºC. Regarding λ, it was longer at the lower temperature assayed. Regarding dispersion parameters, for μ the major range was at 20 ºC, but with a 13.5 % of CV. At 1 ºC, with μ<2.48 mm/day, the range was narrower (1.63 mm/day) but the CV equaled 17.8%. Similarly, the lag phase CV was 14.3% at 1 ºC and 12.7% at 20 ºC.

According to Kruskal-Wallis test, there were significant differences ($p<0.05$) on growth among isolates within the same condition. However, some isolates were either fast growers (13 and 49) or slow growers (41, 45, 51, 52 and 54), regardless of the temperature level (figure 1). Similarly, some isolates started to grow earlier (3, 42 and 56) or later (43, 49 and 79) than the others at both temperatures. There was no relationship between high growth rate and short lag phase or vice versa ($p<0.05$). On the other hand, there were some isolates (for example, isolate 57) which showed bad adaptation to 20 ºC compared to the others, but when incubated at 1 ºC it showed remarkable ability to grow at this low temperature.

Table 1. Descriptive statistics of growth parameters for the conditions assayed

	μ (mm/day)							λ (days)						
Condition	Median*	Mean	Min	Max	Range	SD	CV%	Median*	Mean	Min	Max	Range	SD	CV%
20 ºC	9.40a	9.09	5.51	11.01	5.50	1.23	13.53	2.44a	2.52	1.73	4.24	2.51	0.32	12.70
1 ºC	1.98b	1.91	0.85	2.48	1.63	0.34	17.80	13.10b	13.61	0.98	25.75	24.77	1.95	14.33

*Medians followed by different letters are significantly different according to Kruskal-Wallis test
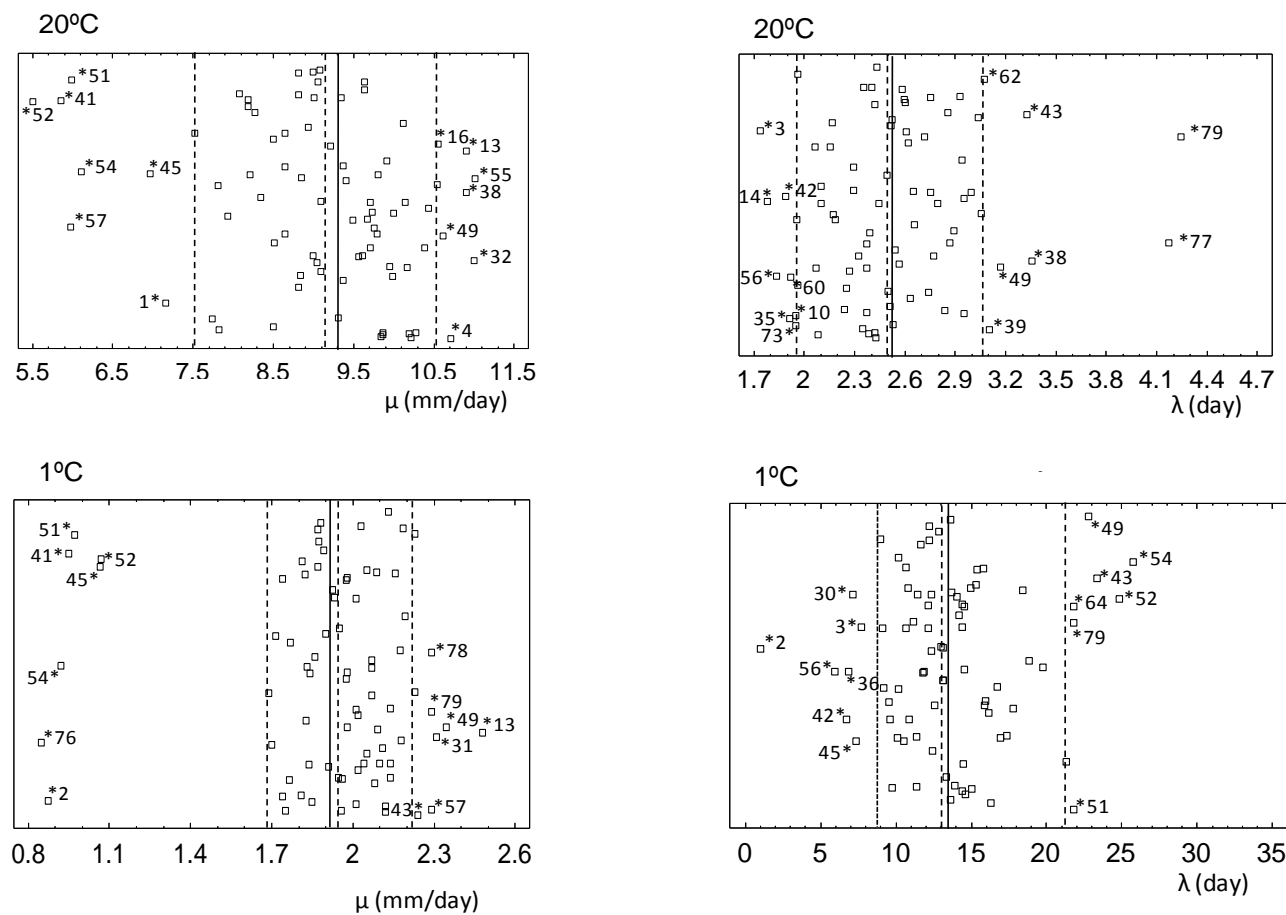
Figure 1: Dispersions of growth rate (mm/day) and lag phase (day) for each isolate of P. expansum tested at 1ºC and 20ºC in ACAM medium. --- Percentiles (10, 50 and 90%) and ___ Media

Regarding growth variability within replicates of isolates, the minimum mean dispersion for μ and λ was observed at 20 ºC; however, for μ no significant differences were found between the CV % at both temperatures. For λ, CV % was higher at 1 ºC than at 20 ºC (table 2).

Table 2. Mean (n=79) coefficient of variation of growth parameters

	μ (mm/day)		λ (day)	
Condition	Mean CV%	Range CV%	Mean CV%	Range CV%
20ºC	4.41	1.01-30.34	11.77	0.62-61.06
1ºC	5.07	0.00-12.23	15.26	0.00-42.57

3.2 Effect of marginal conditions on intraspecific patulin production differences

The analysis of variance of the total data matrix showed that intraspecific differences, growth conditions and colony diameter, as well as their interactions, had a significant impact on patulin production.

Table 3 shows median values of patulin production (ng/mm^2) of all isolates for the two conditions studied and their dispersion. As expected, there were significant differences ($p<0.05$) between patulin levels for the different temperatures, values became lower under marginal temperature. Coefficients of variation (CV%) shown in Table 3 reveal, however, a wider dispersion of the toxin production at 1 ºC. Kruskal-Wallis test was used for the analysis because values were not normally distributed.

Table 3. Descriptive statistics of patulin production (ng/mm^2) for the conditions assayed

Condition	Median	Mean	Min	Max	SD	CV%
20ºC	2.22a	2.14	<ld	5.01	0.96	44.85
1ºC	0.31b	0.37	<ld	1.96	0.35	96.48

*Medians followed by different letters are significantly different according to Kruskal-Wallis test. <ld: under detection limit.

The analysis was repeated separately for each temperature. Production increased significantly with colony size for both temperatures for most of the isolates (table 4). The major variability of patulin production was observed at 1 ºC, regardless of colony size. In general, those isolates which produced more patulin at 20 ºC were different from those that produced the higher amount at 1 ºC, suggesting a strain specific adaptation to stress conditions.

Table 4. Descriptive statistics of median patulin production (ng/mm^2) for the different colony sizes at the two temperature conditions assayed.

	Condition	20 ºC	1 ºC
20 mm	Median	**0.32 a***	**0.01 a**
	Mean	0.55	0.06
	SD	0.83	0.13
	Min	<ld	<ld
	Max	4.72	0.095
	%CV	151	217
40 mm	Median	**2.22 b**	**0.21 b**
	Mean	2.36	0.26
	SD	1.59	0.25
	Min	<ld	<ld
	Max	6.71	0.82
	%CV	67.37	96.15
60 mm	Median	**3.82 c**	**0.57 c**
	Mean	3.51	0.79
	SD	2.08	0.91
	Min	<ld	<ld
	Max	7.98	5.54
	%CV	56.29	115.19

* For a given temperature, different letters next to the means median that significant differences exist between patulin levels produced at different colony sizes according to Kruskal-Wallis test

3.3 Temporal production of patulin

Analysis of variance showed that, in general, time had a significant effect on the amount of patulin produced (ng/mm^2) at both temperature conditions. However, the effect of time was not significant for twenty isolates at 20 ºC and twenty nine for the low temperature, while twelve isolates showed constant production with time at both temperatures assayed. This means that for all these isolates, patulin present across the colony could be considered nearly constant over time. Constant rates varied from 0.33 to 4.79 ng/mm^2 at 20 ºC and for the lower temperature from 0.33 to 0.64 ng/mm^2.

The effect of time was significant for the majority of the isolates. In general for these isolates, patulin production increased with time for both conditions but not linearly. However, some isolates showed a peak production. At 20 ºC there were 13 isolates with a peak production; however, at 1 ºC only 4 isolates showed the peak. Figure 2 (A) shows an example of two isolates at each condition (the increasing size of the colonies and the total accumulated patulin in the colonies is also presented). At 20 ºC isolate 65

showed a peak production after the 7 days of growth, while at 1ºC the maximum production was at day 35 and decreased afterwards for isolate 20. Only isolate 49 showed this trend for both temperatures. Also, figure 2 (B) shows an example of one isolate in which time had no effect on the amount of patulin production at both temperatures assayed.
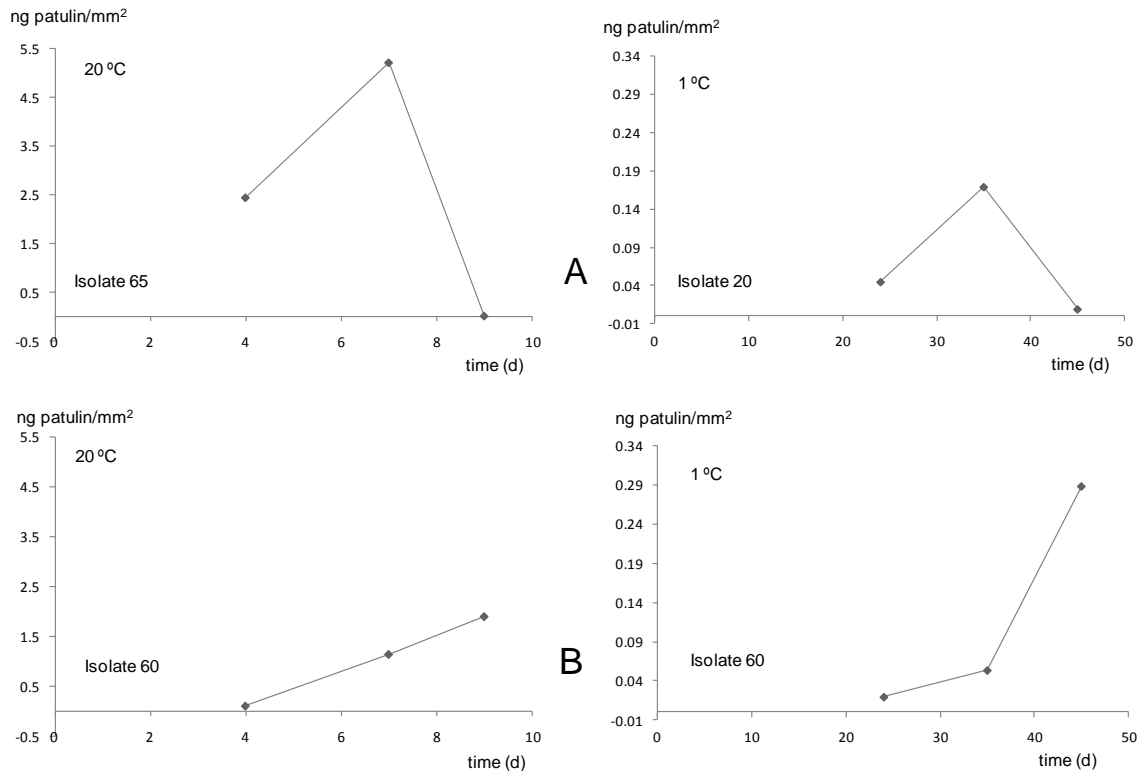
Figure 2: Example of effect of time in patulin production (ng/mm^2 of colony) of some *P. expansum* isolates at each temperature condition.

4. Discussion

Several isolates of *P. expansum* could contaminate the same food. However, individual strains exhibit differences in their growth and in the amount of mycotoxin produced (Romero et al., 2010). In our work, we compared growth parameters (µ and λ) and patulin production of seventy nine isolates of *P. expansum* incubated at two different temperatures. All isolates grew under 1 and 20 ºC. Nevertheless, some isolates showed an exceptional response with a minor adaptation compared to the rest of the isolates and they were not necessarily the same for both temperatures. Isolates 41, 45, 51, 52 and 54 showed the lower growth rate for the two temperatures assayed and 13 and 49 the best adaptation; this could be attributed to an intrinsic character of the isolates. On the other hand, some isolates obtained no

remarkably different growth parameters at 20 ºC, but they showed good adaptation at 1 ºC (2, 31, 57, 78 and 79). Isolate 57 had worse adaptation at 20 ºC, but better than the others at 1 ºC.

Several ecophysiological studies have been developed under different environmental conditions with a few isolates, but these results may not be representative for the majority of the strains due to the ability of each one to adaptation at different conditions (eg. Bellí et al., 2004, 2005; Esteban et al., 2006; Astoreca et al., 2007; Tassou et al., 2007; Kapetanakou et al., 2009). In our work, when isolates were incubated at 1 ºC growth was inhibited to a higher extent than at 20 ºC. Some published studies are in agreement with our results, where mould growth is affected by low temperatures. Besides, at this condition, the dispersion of the values for growth rates and lag phase, was largest compared with 20 ºC. Particularly, the wide dispersion of lag phases is interesting because the estimation of this parameter is crucial to determine the extent of cold storage in apples and other fruits. This indicates that under low temperature more isolates may be necessary to estimate growth parameters for *P. expansum* with higher accuracy. Similar results were obtained by Garcia et al. (2011) with *Aspergillus carbonarius* isolates. They worked with thirty isolates, and they observed that under marginal temperatures for growth, *A. carbonarius* had the major dispersion for both growth parameters.

To our knowledge, no specific study in intraspecific variability of growth and mycotoxin production has been done. However, some ecophysiological researches were made and showed differences among growth parameters of the different isolates studied (Bellí et al., 2004; Parra and Magan, 2004; Pardo et al., 2004; 2005b; Arroyo et al., 2005; Astoreca et al., 2007, 2010; Romero et al., 2007; Tassou et al., 2009), with the highest variability under marginal conditions. Nevertheless, Pardo et al. (2005) studied growth and OTA production of three *A. ochraceus* isolates at different conditions and they did not find differences among the isolates. To minimize these variations, some authors used a cocktail inoculum that might be expected between different isolates of the same species (eg. Hocking and Miscamble 1995; Patriarca et al., 2001; Pose et al., 2009; Romero et al., 2007; 2010). Although this methodology can be criticized because of the loss of information regarding the responses of individual strains of a species, it is accepted as a legitimate method of achieving a "worst case" scenario (Hocking and Miscamble 1995). However, Romero et al. (2010) concluded that the use of cocktail inoculums could be a useful approach to obtain data for prediction of the final level of contamination.

In addition, in this research, replications within isolates were evaluated. The major variability for growth rate and lag phase for the same isolate was observed at low temperature. However, isolates with high variability for growth rate were different from those with major variability for lag phase. Garcia et al. (2010) worked with one isolate of *A. carbonarius* and one of *P. expansum* under suboptimal conditions. Their results showed a higher variability between replicates of *A. carbonarius* for both growth parameters under marginal water activity and temperature conditions, while for *P. expansum* this only occurred under limiting conditions of water activity.

As regards patulin production, in this work, the amount of toxin produced by isolates varied with temperature with values being lower at 1 ºC. Baert et al. (2007) worked with eight isolates of *P. expansum*. They found that patulin production also decreased at 1 ºC compared with the other temperatures assayed (4, 10 and 20 ºC). Other authors also found the higher production of this mycotoxin at high temperatures compared with low temperatures (Morales et al., 2008; Reddy et al., 2010).

As for growth, studies on mycotoxin production by one individual strain might not be representative for the majority of the strains (Marín et al., 2008a; Romero et al., 2010). In our research, patulin production by the majority of isolates was affected significantly by both colony diameter and intraspecific differences and their interaction. Patulin accumulation was major in 60 mm-diameter colonies, than in those of 40 and 20 mm. There are some studies of patulin production for *P. expansum* and the results show variability of patulin production between the isolates assayed (Abramson et al., 2009; McCallum et al. 2002; Menniti et al., 2009; Reddy et al., 2010; Watanabe, 2008). In our work the major dispersion of data for patulin production was at 1 ºC. Reddy et al., (2010) worked with ten isolates of *P. expansum*, and they found variability in patulin production among isolates, however, variability was similar at the two temperatures assayed (25 and 4 ºC) for a given colony diameter in different substrates. Baert et al. (2007) obtained the major variability of patulin values at low temperatures, but at 4 ºC variability was higher than at 1 ºC.

In this research, analysis of variance showed that in general, time had a significant effect on the rate of patulin produced (ng/mm^2) for the majority of the isolates. Maximum, patulin production was reached at different times depending of temperature condition of incubation and a decline of production was observed in the next analysis. Probably, toxin was degraded by fungus itself as suggested by Varga et al. (2000) for OTA accumulation or possibly production of patulin by the fungi may have ceased due to lack of nutrients. On the other hand, the number of isolates which were not affected by time was bigger at 1 ºC than at 20 ºC. Patulin production for these isolates could be considered constant through the time. Garcia et al. (2011) studied OTA production by thirty isolates of *A. carbonarius* and they found that time had no effect on the rate of the mycotoxin production at marginal conditions for growth ($0.90a_w$/25 ºC and $0.98a_w$/37 ºC), so it could be considered nearly constant over time.

One of the most important aspects of predictive model development is ensuring that predictions made by the model are applicable to real situations (Romero et al., 2010). Therefore, in this work we took into account the most important storage temperature conditions because it is of great importance for the fruit-derivates safety point of view. We also worked with many isolates because in natural ecosystems different strains can occupy the same niche and they may have intraspecific differences in their growth and mycotoxin production. Most published models took into account the complex interaction of several factors (water activity, temperature, incubation time) in most cases close to their optimal levels; however, existing models rarely take into account the effect of marginal conditions and microorganisms interaction. To our knowledge, this is one of the first studies on a great number of isolates of *P. expansum* studied at non optimal conditions for mould growth and mycotoxin production. In order to address the strain

variability in growth initiation and prove the well-established notion of reducing patulin in foods by preventing fungal growth, a greater number of strains should be included when developing models for low temperatures, especially when patulin production is the main aim of the work.

Acknowledgement

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3.8 Optimising the number of isolates to be used to estimate growth parameters of mycotoxigenic species

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Abstract

The aim of the present work was to mathematically assess the minimum number of isolates that would lead to equivalent growth parameters estimates to those obtained with a high number of strains. The datasets from two previous works on 30 *A. carbonarius* isolates and 62 *P. expansum* isolates were used for this purpose. First, the datasets were used to produce a global estimation of growth parameters μ (growth rate, mm/d) and λ (time to visible growth, d) under the different experimental conditions, providing also a 95 % confidence interval. Second, a computational algorithm was developed in order to obtain an estimation of the growth parameters that one would obtain using a lower number of isolates and /or replicates, using a bootstrap procedure with 5000 simulations. The result of this algorithm was the probability that the obtained estimation falls in the 95% confidence interval previously produced using all sample isolates. Third, the algorithm was intensively applied to obtain these probabilities for all possible combinations of isolates and replicates. Finally, these results were used to determine the minimum number of isolates and replicates needed to obtain a reasonable estimation, i.e. inside the confidence interval, with a probability of 0.8, 0.9 and 0.95. The results revealed that increasing the number of isolates may be more effective than increasing the number of replicates, in terms of increasing the probability. In particular, 12-17 isolates of *A. carbonarius* led to the same growth parameters as the total 30 (p=0.05) or 9 isolates with p=0.20; by contrast, 25-30 isolates of *P. expansum* led to the same growth parameters as the total 62 (p=0.05) or 18-21 isolates with p=0.20. As far as we know, this is the first study that provides a systematic evaluation of the number of isolates and replicates needed when designing an experiment involving mycotoxigenic moulds responses to environmental factors, and may serve to support decision making in this kind of studies or other similar ones.

Keywords:

Aspergillus carbonarius, Penicillium expansum, number of isolates, predictive models.

1. Introduction

Moulds can grow in many foods and feeds and can produce mycotoxins which are a risk for human and animal health. Mycotoxins, chemical hazards of microbiological origin, are formed by some fungal species that readily colonise crops in the field or after harvest and thus pose a potential threat to human and animal health through the ingestion of food products prepared from these commodities. Mycotoxins cause a diverse range of toxic effects because their chemical structures are very different from each other. Acute effects require that high amounts of mycotoxins are consumed so that such incidents are usually restricted to the less developed parts of the world, where resources for control are limited. Chronic effects are of concern for the long-term health of the human population and are important when these toxins are frequently present in lower amounts. Some of the most common mycotoxins are carcinogenic, genotoxic, or may target the kidney, liver or immune system (Bankole and Adebanjo, 2003).

In the last two decades a huge number of researches have been carried out in order to assess the effects of environmental factors such as water activity (a_w), temperature, pH, antifungals, etc, on growth of known mycotoxigenic fungal species. A more reduced number of works have addressed the mycotoxin production as affected by environmental variables. Most of those works tested a reduced number of levels of factors, and analysis of variance plus multiple range tests were applied to assess the significance of the different levels of factors. For this purpose, one to eleven isolates of the species of interest were usually used (information browsed in Scopus database). Table 1 summarises the details of such studies published in the past decade.

Phenotype strain variability, including micro and macromorphological characters and toxin production has been studied; however, ecophysiological traits, as in this study, have rarely been evaluated in a high number of mycotoxigenic isolates (Morales et al., 2008). By contrast, some studies exist on genotype variability in mycotoxigenic species. Restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA-PCR (RADP-PCR) and other phylogenetic studies on mycotoxigenic species such as *Fusarium culmorum*, *Fusarium poae*, *Aspergillus flavus* and *Aspergillus parasiticus* have shown wide strain variability. Moreover, no correlation has usually been found between geographic origin or host and genotype, either. High genetic diversity can be indicative of a population that has been present in a region over sufficient evolutionary time to acquire variation or it may be due to a single introduction of highly diverse strains or multiple, independent introductions. High genetic diversity can also be due to sexual recombination (Gargouri et al., 2003; Mohankumar et al., 2010; Tran-Dinh et al., 2009). The existence of this genetic variability could provide a wide genetic pool that strains of a species can use to face better both new and different environmental conditions (Somma et al., 2010). PCR analysis showed that RAPDs are effective tools for clustering strains of *F. verticillioides* species, indicating an association between endophytic strains, sites of isolation, seed inbred lines and maize populations (Pamphile and Azevedo, 2002).

Recently, emerging predictive mycology has been pointedly applied to mycotoxigenic fungi. The usefulness of such models may be closely linked to the number of strains used to build them, depending on their genetic diversity as described above. However, in most of the cases, only one strain was used representing the species of interest, as the cost of the studies is obviously a factor to be taken into account. Most of the previous researches showed that the different strains in a species behave differently in their response to factors such as temperature, a_w, pH and antifungals (Table 1). Thus, it is important to establish the minimum number of strains that should be involved in predictive models development. Alternatively, the cocktail inoculum technique could be applied, although the number of strains in the cocktail should also be standardised. Some authors have usually used a cocktail of strains from a single species in their studies (Pose et al., 2009, 2010; Romero et al., 2007). With respect to growth parameters, the mixed inoculum of 4 strains of *A. carbonarius* yielded an average growth rate, which represents the tendency of the individual strains behaviour in optimal growth conditions. As one of the most important aspects of model development is ensuring that predictions made by the model are applicable to real situations, the use of a mixed inoculum could be used to approach this objective.

Lately, some authors pointed out that variability among strains might be increased under marginal conditions for growth and mycotoxin production (Baert et al., 2007; García et al., 2011a). The aim of the present work was to use the datasets from two previous works on 30 *A. carbonarius* isolates (García et al., 2011a) and 79 *P. expansum* isolates (García et al., 2011b) in order to mathematically assess the minimum number of isolates in each case that would lead to equivalent growth parameters estimates to those obtained with all 30 or 62 (taken from the 79) strains. These two mycotoxigenic species were chosen based on the availability of a relatively high number of strains, previously isolated as part of several projects involving them, plus the fact that *P. expansum* contains strains markedly adapted to low temperatures, while *A. carbonarius* contains strains markedly adapted to high temperatures, and some may grow at low water activity levels.

Table 1. Ecophysiological studies involving mycotoxigenic *Penicillium*, *Aspergillus*, *Fusarium* and *Alternaria* species published in the last decade. Data on the number of strains used per species. Data browsed on Scopus.

Year	Number of published studies[a]	Number of strains (range)	Mean number of strains	Works claiming no intraspecies differences in growth (%)[b]	Works claiming no intraspecies differences in mycotoxin production (%)[c]
2000	2	1	1	-	-
2001	6	1-2	1.8	100	-
2002	7	1-4	2.3	100	0
2003	4	1-3	1.5	0	0
2004	19	1-10	4.1	33	11
2005	15	1-8	2.7	66	25
2006	13	1-6	2.8	30	11
2007	22	1-8	2.2	33	33
2008	5	1-11	3.4	0	-
2009	18	1-6	2.3	0	0
2010	16	1-8	3.6	25	0

[a]Only food mycology works were included, industrial mycology and bioremediation works were excluded. One publication can include more than one species of study. Studies on fungal sections were not included.

[b]Those studies with several strains per species, which evaluated the differences among strains in growth jointly were not included in the calculated percentage.

[c]Intraspecies differences evaluated only in proven mycotoxigenic strains.

2. Materials and methods

2.1. Data sets

Two sets of data were used. In the first one, 30 isolates of *A. carbonarius* isolated in previous years from vineyards in north-eastern and southern Spain were included. Spore suspensions of the isolates were point-inoculated on synthetic nutrient medium (SNM, simulating grape composition) and incubated at three a_w/temperature conditions, one optimal (0.98a_w/25ºC) and two suboptimal: 0.90a_w/25ºC and 0.98a_w/37ºC, as suboptimal a_w and temperature, respectively, with 12 replicates per treatment. In each case the growth rate (μ, mm/d) and lag phase (λ, d) were estimated through Baranyi and Roberts model (1994). Thus, a data matrix with 2160 values (30×3×12) couples of parameters (μ (mm/d) and λ (d)) was available (for more details, see García et al. 2011a). A summary of the previously published results is shown in table 2.

In the second data set, 62 isolates of *P. expansum*, isolated from apples from cool storage rooms in the north-eastern part of Spain were included. Isolates had been grown as described above for *A. carbonarius* on apple concentrate agar medium at two different temperature levels: a near to optimal for growth one (20ºC) and the suboptimal cold storage one (1ºC). For each condition, 10 Petri dishes were inoculated. As for *A. carbonarius*, growth parameters were estimated through Baranyi and Roberts models (1994). Thus, a data matrix with 2480 values (62×2×10) couples of parameters (*µ* (mm/d) and *λ* (d)) was available (for more details, see García et al. 2011b). A summary of the results previously published is shown in table 2.

Table 2. Summary of results previously published in García et al. (2011a,b)

	Median μ (mm/d)	CV of μ (%)	Median λ (d)	CV of λ (%)
	Aspergillus carbonarius (n=30)			
0.98a_w/25 ºC	10.4	13.2	3.6	18.4
0.98a_w/37 ºC	5.9	60.8	3.5	22.2
0.90a_w/25 ºC	3.1	23.1	4.7	17.8
	Penicillium expansum (n=79)			
20 ºC	9.1	13.5	2.5	12.7
1 ºC	1.9	17.8	13.6	14.3

The number of missing data values was quite low (12 data points). A random imputation based on a Gaussian distribution was applied.

2.2 Methods

First, linear mixed models were used to assess the variability of the parameters, specifically to ascertain the amount of variability due to replicates respect to that attributable to isolates, using for this purpose the whole dataset. Second, to reduce the variability due to replicates, the mean for each isolate was computed. The resulting datasets where then used to produce a global estimation of growth parameters *µ* and *λ* under the different experimental conditions, providing also a 95 % confidence interval (CI). Third, a computational algorithm was developed in order to obtain an estimation of the growth parameters that one would obtain using a lower number of isolates and/or replicates, using for this purpose a bootstrap procedure with 5000 random samples. The result of this algorithm was the probability that the obtained estimation falls in the CI previously produced using all samples. Fourth, the algorithm was intensively applied to obtain these probabilities for all possible combinations of isolates and replicates. Finally, these results were used to determine the minimum number of isolates and replicates needed to obtain a reasonable estimation, i.e. inside the CI, with a probability of 0.8, 0.9 and 0.95.

2.2.1 Algorithm

Consider n and r the number of isolates and replicates respectively. Assume that N and R are the total number of isolates and replicates, i.e. n ∈ {1,2,…,N} and r ∈ {1,2,…,R}, so that a global estimation (with a CI) can be obtained using all N×R data values. Consider now the problem of computing the same estimation but with a lower number of isolates. In this case, there are $\binom{N}{n} = \dfrac{N!}{n!(N-n)!}$ possible ways of selecting n different isolates from all N. One first approach would be to systematically obtain these estimations for all possible combinations, and therefore report the number of times that the estimation falls inside the global CI, computing a proportion that might be interpreted as the probability of producing an estimation similar to the one with all observations. However, the number of combinations can exponentially grow, which makes this analysis unfeasible at practice for computing reasons. For instance, $\binom{30}{15} = 155,117,520$ possible combinations could be considered when estimating the parameters selecting 15 isolates from a set of 30. In addition to this, if we consider all possible situations and R=12 different replicates, this would lead to $12 \times \sum_{i=1}^{30}\binom{30}{i} = 112,884,901,516$ different estimations of the parameters, with a required computational time of 10.4 years (estimated with a 3 GHz personal computer). To overcome this, the final approach used here is based on a bootstrap algorithm, so that for a given number of replicates (r) and isolates (n) a random selection of 5000 different combinations, from all possible ones, is used to estimate the probability that the estimation falls in the CI. Each bootstrap sample is the result of randomly sampling with replacement from the general N×R data matrix, so that a n×r sub-data matrix is obtained where the mean values for μ and λ can be computed. This procedure is then repeated 5000 times and, thus, the probability of the estimation to fall in the true values for μ and λ (by using the global CI) is computed. With this approach computational time is reduced down to 10.93 hours and quality of the estimation is reasonably acceptable, since the standard error associated to the bootstrap procedure is low, being (0.5*(1-0.5)/5000)^0.5=0.007, so that probability estimations may deviate in ±1.96*0.007=0.014 units with a 95% of statistical confidence.

2.2.2 Implementation

All statistical analyses and algorithms were implemented in R code (©2009) The R Foundation for Statistical Computing). Scripts with the analysis are available as Supplementary Material.

3. Results

Table 3 shows the calculated 95 % CIs under the different environmental conditions. In general the intervals were wider for λ than for μ, and λ intervals were wider under marginal conditions for growth.

Table 4 depicts the percentages of variability due to isolates or due to replicates, obtained from linear mixed models. These results indicate that variability is quite higher among isolates than within replicates. However there is a non negligible variability due to replicates (ranging from 1.98% up to 44.43%), which underlines the importance of taking into account this source of variation for experimental design planning. For μ, the highest standard deviation due to intraspecies differences was observed at $0.98a_w/37^{\circ}C$ (*A. carbonarius*) and at 1 $^{\circ}C$ for λ (*P. expansum)* suggesting higher intraspecies variability under marginal conditions of growth. Repeatibility of the experiments was also worse at $0.98a_w/37^{\circ}C$ for μ and at 1 $^{\circ}C$ for λ (Table 4).

Table 3. Calculated confidence intervals (95%) for μ (mm/d) and λ (d) for *A. carbonarius* (30 isolates) and *P. expansum* (62 isolates)

	Parameter	Condition	CI 95%
A. carbonarius	μ	$0.98a_w/25\ ^{\circ}C$	0.990-1.094
	λ	$0.98a_w/25\ ^{\circ}C$	3.445-3.949
	μ	$0.98a_w/37\ ^{\circ}C$	0.576-0.838
	λ	$0.98a_w/37\ ^{\circ}C$	3.056-3.678
	μ	$0.90a_w/25\ ^{\circ}C$	0.298-0.354
	λ	$0.90a_w/25\ ^{\circ}C$	4.386-5.010
P. expansum	μ	$20\ ^{\circ}C$	0.884-0.951
	λ	$20\ ^{\circ}C$	2.340-2.526
	μ	$1\ ^{\circ}C$	0.179-0.223
	λ	$1\ ^{\circ}C$	11.886-14.040

Table 4: Percentages of variability due to isolates or due to replicates.

			Standard Deviation		Variability (%)	
	Parameter	Condition	Due to isolates	Due to replicates	Due to isolates	Due to replicates
A. carbonarius	μ	$0.98a_w/25\ ^{\circ}C$	0.13	0.11	61.49	38.51
	λ	$0.98a_w/25\ ^{\circ}C$	0.66	0.43	70.70	29.30
	μ	$0.98a_w/37\ ^{\circ}C$	0.34	0.30	55.57	44.43
	λ	$0.98a_w/37\ ^{\circ}C$	0.82	0.51	72.40	27.60
	μ	$0.90a_w/25\ ^{\circ}C$	0.07	0.02	92.97	7.03
	λ	$0.90a_w/25\ ^{\circ}C$	0.81	0.68	58.80	41.20
P. expansum	μ	$20\ ^{\circ}C$	0.13	0.04	91.90	8.10
	λ	$20\ ^{\circ}C$	0.36	0.28	61.95	38.05
	μ	$1\ ^{\circ}C$	0.09	0.01	98.02	1.98
	λ	$1\ ^{\circ}C$	4.19	1.96	82.13	17.87

Figure 1 and 2 show the probability of achieving an acceptable estimation of the parameters (i.e. falling in the CI that one would obtain using all data values) for different number of isolates and replicates (tables S1-S10 in supplementary material contain the exact values). These results reveal that increasing the number of isolates is much more effective than increasing the number of replicates, in terms of increasing the probability. In addition to this, three lines are represented to visualize those combinations of numbers of isolates and replicates that would be needed to reach a probability of 0.8, 0.9 and 0.95, respectively. For *A. carbonarius*, for example, at $0.98a_w$/25 ºC, at least 16 isolates (with 5 replicates) would be required or, for example, a higher number of isolates (17) but with 3 replicates to reach a probability of 0.95 to obtain a μ included in the CI; at $0.98a_w$/37ºC, similar results were observed (minimum of 13 isolates with 8 replicates), while at $0.90a_w$/25ºC at least 12 isolates with 4 replicates were required, in this later case the number of replicates was much less determinant than the number of isolates used. The CI would be reached with p=0.90, with a minimum number of isolates of 11-13, with 3 to 9 replicates, depending on the condition assayed. Finally, at least 9 isolates should be used with 3-6 replicates to reach a 0.80 probability. Regarding λ estimated values, 14 isolates (9-12 replicates) would be required to reach the CI with 0.95 probability, regardless of the condition tested. At least 9 isolates with 5-7 replicates should be used to reach the CI with a probability of 0.80. In the case of *P. expansum* at least 29 isolates (with 6 replicates) would be required to reach a μ included in the CI at 20 ºC with probability of 0.95. At the lower temperature, a minimum of 25 isolates with 2 replicates were required. As for *A. carbonarius* at low a_w, for *P. expansum* at 1 ºC, the number of replicates was much less determinant than the number of isolates used for μ assessment. The minimum acceptable number of isolates would be 23-27, with 1 to 4 replicates, depending the temperature studied for a probability of 0.90, and 21 isolates with 1-4 replicates for a probability of 0.80. Concerning λ estimated values, a minimum of 30 isolates and 10 and 6 replicates for 20 ºC and 1 ºC respectively were needed to reach the CI with 0.95 probability, while 18 isolates with 6-9 replicates should be used, regardless of the temperature tested, to get a 0.80 probability.

Figure 1: Heatmaps representing the probability of obtaining a correct estimation of *μ* and *λ* for different combinations of number of isolates and replicates for *A. carbonarius*. Heatmaps cover three different experimental conditions: 0.98*a*~w~/25ºC, 0.98*a*~w~/37ºC and 0.90*a*~w~/25ºC. Combinations bellow each of the lines are those where a probability higher than 0.95, 0.90 or 0.85 is expected.
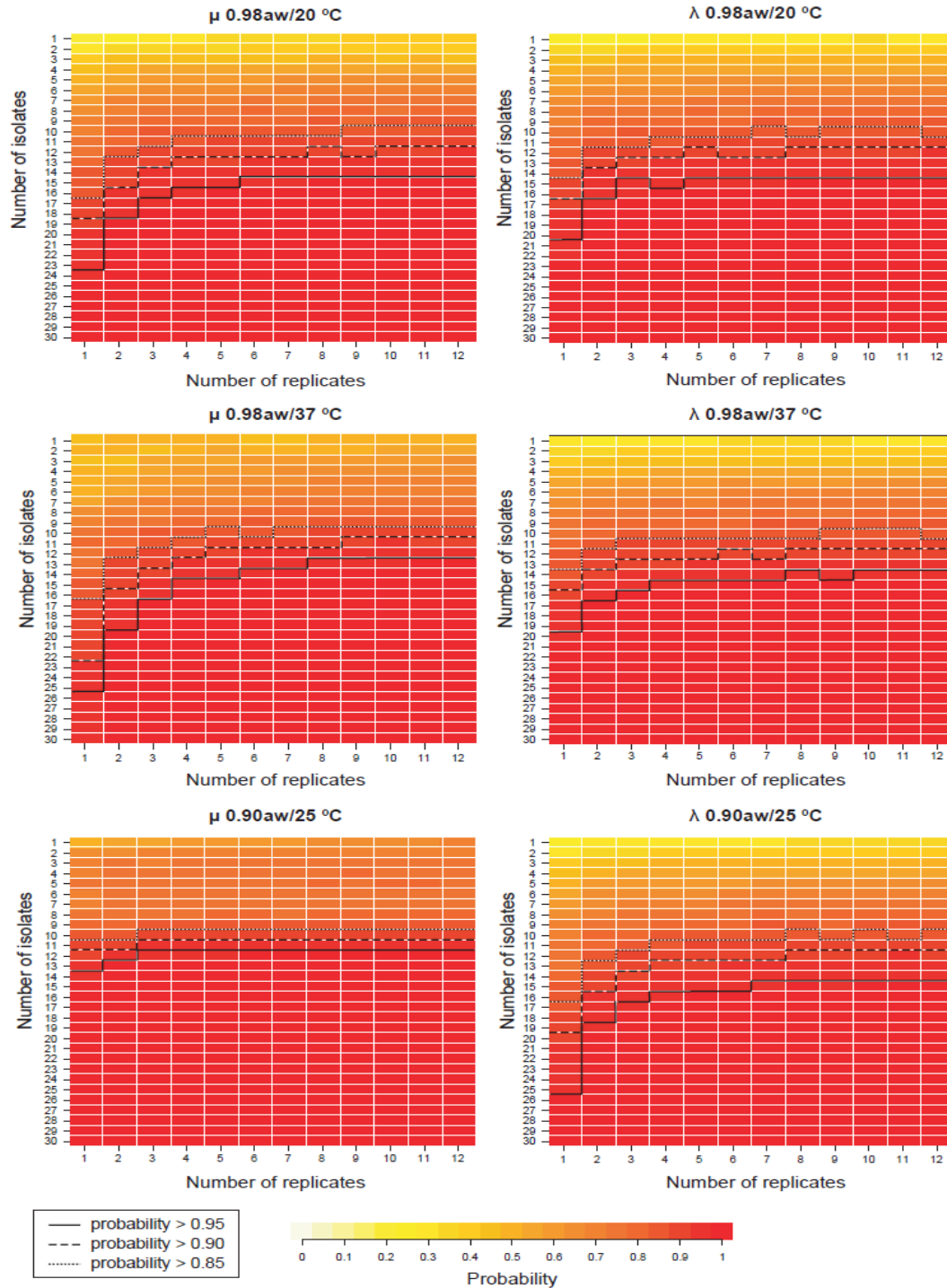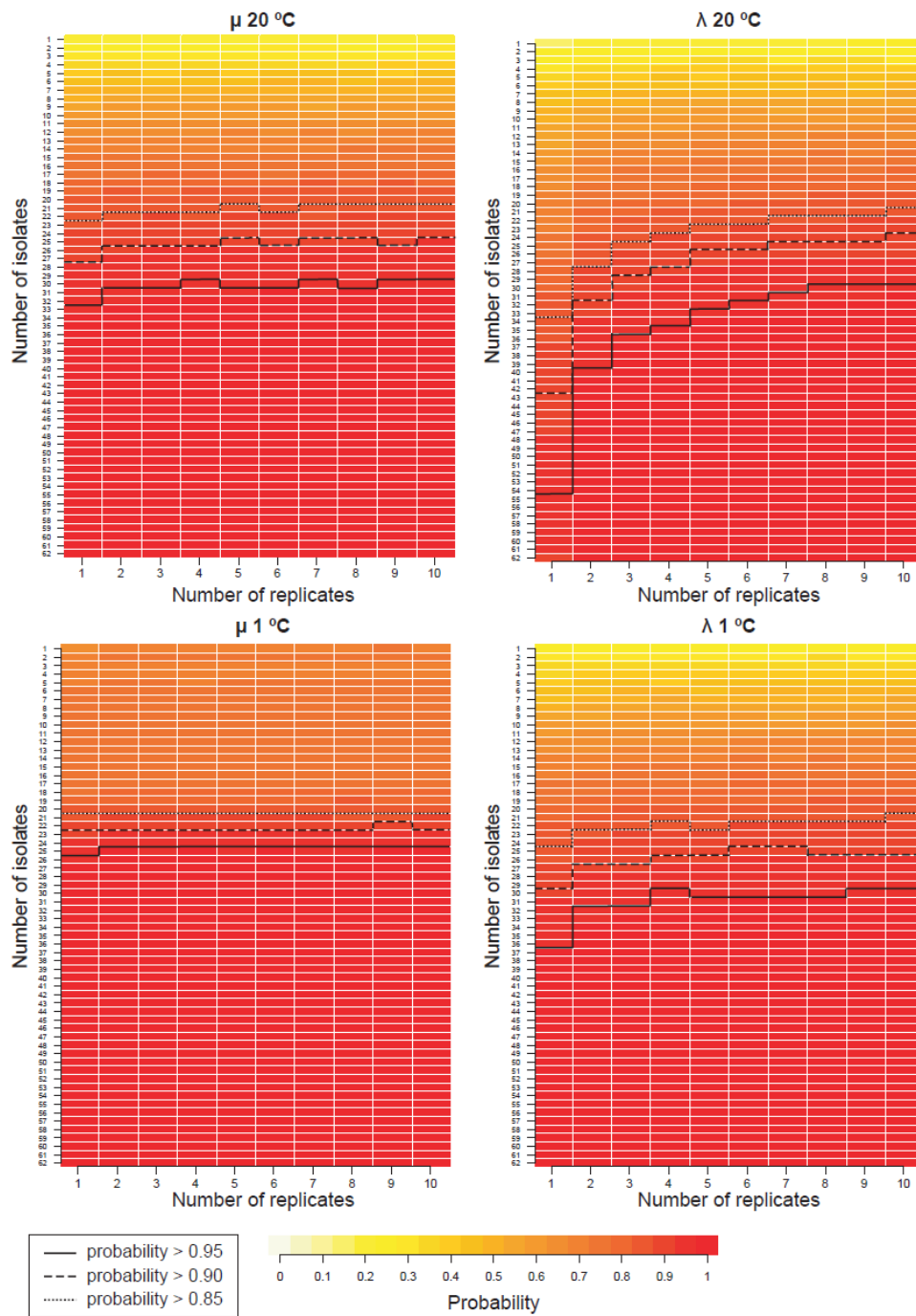
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Figure 2: Heatmaps representing the probability of obtaining a correct estimation of *µ* and *λ* for different combinations of number of isolates and replicates for *P. expansum*. Heatmaps cover two different experimental conditions: 20 ºC and 1 ºC. Combinations bellow each of the lines are those where a probability higher than 0.95, 0.90 or 0.85 is expected.

Ecophysiological studies of moulds have been usually carried out in the past with usually no more than 3 isolates and 3 replicates. Table 5 shows the probability to get a result included in the CI at the different conditions assayed, taking into account results of 3 isolates with 3 replicates. The probabilities ranged from 0.32 to 0.68 for μ, and from 0.34 to 0.49 for λ, both ranges quite low.

Table 5: Probabilities to obtain mean values in the CI built from 30 and 62 isolates of *A. carbonarius* and *P. expansum*, with 3 isolates and 3 replicates.

	Condition	μ	λ
	$0.98a_w$/25 ºC	0.45	0.46
A. carbonarius	$0.98a_w$/37 ºC	0.52	0.46
	$0.90a_w$/25 ºC	0.72	0.47
P. expansum	20 ºC	0.32	0.33
	1 ºC	0.70	0.36

4. Discussion

This work presents an initial approximation to the standardisation of the number of isolates to be used in predictive mycology studies. The final outcome of the applied algorithms are the heatmaps which provide the potential user with a decision support for experimental designs concerning the number of isolates and replicates to be used. In one hand, users may use the results presented here in future works dealing with *A. carbonarius* and *P. expansum*. In the other hand, the algorithms presented may be applied by other researchers to other fungal species if data are available.

From these results, a potential user may determine whether or not including one more replicate in the experimental design will certainly contribute to a better estimation of growth parameters.

The 'cost function' would be obtaining the best confidence of estimation with the minimum number of experiments; the same weight was given to replicates and isolates. From the figures, a researcher may choose using *x* isolates with *y* replicates for a given confidence of the estimation, or alternatively increasing the number of replicates if a smaller number of strains is available. One should be aware, however, that increasing the number of replicates do not compensate a limited amount of strains. A scenario where more replicates do not lead to better estimations might be quite common according to our results.

The sources of variation evaluated in this work were intraspecies differences and within replicates variability. Replicates variability accounted for 2 to 44% of the total variability; this latter value is quite high, but reflects the intrinsic biological variability of fungi in their responses to the environment. Nevertheless, this result supports the need of using replicates to obtain a better estimation of the studied

outcome, reducing the error attributable to the measuring procedure, given that this seems only to be negligible in few specific and quite extreme conditions. Previous studies on the modelling the growth of moulds as a function of environmental variables show that at optimal conditions of growth the variability of μ increases. The square root transformation is some times used. Table 4 of the present work confirms that a higher percentage of the variability is due to replicates at optimal conditions, while under marginal conditions (1ºC for *P. expansum* and 0.90 a_w for *A. carbonarius*) the contribution to variability of intraspecies difference is more important than that of replicates. As a consequence, the number of replicates/isolates required for an accurate estimation of the growth parameters was not lower under optimal conditions of growth. Previous researches pointed out that intraspecies variability could be widened under marginal conditions of growth; the wider calculated CI interval at 37 ºC for *A. carbonarius* reinforced this hypothesis. However, as the confidence interval size was affected by both intraspecies differences and within replicates variability this was not observed for *P. expansum* at 1 ºC. Giorni et al. (2007) worked with 40 strains of *Aspergillus* section *Flavi* inoculated on Czapek medium and incubated at 3 temperatures (15, 25 and 30 °C) and a_w levels (0.83, 0.94, 0.99) for 14 days in the dark. At 15 °C the growth was slow, while at 25 ºC and 30 °C it was very similar and significantly higher; the distribution of strain's growth rates around the median was more dispersed for the conditions leading to faster growth, although individual outliers were more commonly reported under 15-25 ºC. A similar pattern was observed for a_w.

Regarding to the use of linear mixed models to discriminate variability from isolates respect to that from replicates, non linear mixed model could have been used to directly fit kinetic data. However, here the goal was to assess critical values for the number of replicates and isolates to obtain a good estimation of the parameters associated to those kinetic curves and the goodness of the fit of linear mixed models seemed reasonably acceptable and more parsimonius than non-linear mixed models. In addition to this, we have considered not only the estimation for μ and λ, but also their 95% confidence intervals estimated when the standard error from all 30 or 62 isolates are used. Our assumption is that these intervals may contain the true values for μ and λ, which seems quite sensible. Therefore, bootstrap samples are used to assess the error in estimating μ and λ when reducing sample size, using for this assessment 5000 bootstrap samples. This final estimation is also subjected to some uncertainty, but given that the number of samples is quite high (5000 samples) the variability associated to the final estimation is reasonably low, with a standard error lower than 0.007, as reported above. Thus, the final estimated probability of obtaining a correct estimation provided in our work might be subjected to an error, at most, of ±0.014 units, which seems quite reasonable.

One drawback of our study may be that it is assumed that the 'true' values for the estimated parameters are those obtained with either 30 or 62 isolates. Although the number of isolates is quite high, it is uncertain what would have happened if, for example 200 isolates had been included instead. Moreover, *A. carbonarius* strains had quite a wide geographical origin, while *P. expansum* isolates came from a

more limited area; it is uncertain if the inclusion of isolates from a wider geographical area would have lead to a higher number of isolates required for a given probability. Hypothetically, if strains from distant geographical areas have more different growth patterns, their inclusion in the algorithm would have led to wider CI, but the resulting probabilities would not necessarily change.

Studies on *A. carbonarius* populations isolated from grape demonstrated that the grape variety and the culture conditions (year, production zone and treatment) applied have an important effect on the phenotypic (morphological) biodiversity of strains. Strains belonging to different morphological groups, produce different levels of ochratoxin A (OTA); on the other hand, strains belonging to the same morphological groups and coming from different grape varieties, production zones and treatments are able to produce similar levels of OTA. The RAPD-PCR dendrograms of *A. carbonarius* strains showed high genetic diversity among the isolates, however, the clusters were not related to different grape varieties, production zones and treatments neither to OTA production. Therefore, strains belonging to the same phenotypic group did not show genotypic homology (Dachoupakan et al., 2009). By contrast, Martinez-Culebras et al. (2009) found using arbitrary primer-PCR (ap-PCR) that their grape-isolated *A. carbonarius* constituted a homogeneous group with respect to intraspecific genotypic variability. Correlation between cluster analysis and the toxigenic ability of *A. carbonarius* could be established in all the OTA-producing *A. carbonarius* isolates grouped within the same cluster. Phylogenic analysis revealed that all the *A. carbonarius* isolates analysed had identical ITS and IGS sequences. This result agrees with the high degree of similarity (77.8%) obtained by cluster analysis based on ap-PCR profiles, confirming the lack of important molecular variability within *A. carbonarius*.

Regarding *P. expansum*, RAPD results by Elhariry et al. (2011) revealed that the tested strains showed high percentage of similarity and no correlation was observed between cluster analysis and the sources of isolation. On the other hand, some ecophysiological traits were shown to vary in *P. expansum* population, including growth in different media, resistance to fungicides and amount of patulin accumulated (Morales et al., 2008).

As far as we know, this is the first study that has provided a systematic evaluation of the number of isolates and replicates needed when designing an experiment involving mycotoxigenic mould responses to environmental factors and may serve to support decision making in this kind of studies or other similar ones. We think that the fact that scripts from these analyses are available as R code, may help other researchers to undertake similar studies but with different assumptions such as other laboratory conditions or sample features.

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3.9 Impact of cycling temperatures on *Fusarium verticillioides* and *F. graminearum* growth and mycotoxins production in soybean

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Abstract

BACKGROUND: *Fusarium graminearum* and *F. verticillioides* are two very important mycotoxigenic species as they cause diverse diseases in crops. The effect of constant and cycling temperatures on growth and mycotoxin production of these species were studied on soybean based medium and on irradiated soya beans.

RESULTS: *F. graminearum* grew better when was incubated at 15, 20 and 15-20 ºC (isothermal or cycling temperature) during 21 days of incubation. Maximum levels of zearalenone (ZEA) and deoxynivalenol (DON) (39.25 and 1040.4 µg/g, respectively) were detected on soya beans after 15 days of incubation and the optimal temperature for mycotoxin production was: 15 ºC for ZEA and 20 ºC for DON. *F. verticillioides* grew better at 25 ºC in culture medium and at 15-20 ºC, 15-25 ºC on soybean seeds. Fumonisins B_1 (FB_1) was produced only in culture medium, and the maximum level (7.38 µg/g) was found at 15 ºC after 7 days of incubation.

CONCLUSION: When growth and mycotoxin production under cycling temperatures were predicted from the results under constant conditions, observed values were different from calculated for both species and substrate medium. Therefore, care should be taken if data at constant temperature conditions are to be extrapolated to real field conditions.

Keywords:

Fusarium graminearum, F. verticillioides, Mycotoxins, Cycling temperatures.

1. Introduction

Soybean (*Glycine max* L.) is a species of leguminous original from Asia especially used for oil and flour production.[1] This commodity is the main source of protein used for food and feedstuffs throughout the world.[2] Argentina is the third largest producer of soybean; however, it is the main exporter of the by-products, where 57% of flour exports are destined to the European Union.[3] About 34% of soybean oil is exported to China, 20% to India and the rest to countries such as Venezuela, Egypt, Peru and South Africa.[4]

Soybean is often infected by fungi during cultivation or postharvest (in transit or in storage), significantly affecting its productivity.[5] Fungal contamination can cause damage in cereal grains and oilseeds, such as low germination, discoloration, heat, wilt, rot and mycotoxin occurrence. Fungal growth and mycotoxin production are influenced by different factors such as temperature, substrate aeration, water activity (a_w), inoculum concentration, microbial interactions, physiological state of mould, etc.[6] *Alternaria* and *Fusarium* species are the most commonly isolated fungi from soybean in Argentina and in other regions of the world.[7,8,9,10] *Fusarium* species have a large degree of morphological, physiological and ecological diversity and are considered the most important plant pathogens worldwide.[11] Among the *Fusarium* species a few of them are responsible of mycotoxin production, *F. graminearum* is the main deoxynivalenol (DON) and zearalenone (ZEA) producer and *F. verticillioides* is one of the main responsible for fumonisins (FBs) production.[11]

Many studies have been published during the last few years describing the growth of mycotoxigenic fungi as a function of environmental factors. Some of them included predictive models for fungal growth under constant conditions of either temperature or a_w.[12,13,14,15,16,17,18] However, variation of temperature occurs during the development of soybean plant at field stage when *Fusarium* species can colonize this leguminous. Besides, field temperature fluctuation will result in modification of the pattern of growth and mycotoxins production. For this reason, the objectives of the present study were: i) to determine the impact of different constant temperature regimes and cycling temperatures on growth and mycotoxin production on soybean based medium and on irradiated soya beans by two strains of *F. verticillioides* and *F. graminearum* isolated from soybean in Argentina and ii) to asses if growth and toxin production at cycling temperatures could be predicted from data at constant temperature.

2. Material and methods

2.1 Fungal isolates

One strain of *F. verticilliodes* (F5017) and one of *F. graminearum* (F5050) (*F. graminearum sensu stricto* (lineage 7)) isolated from soybean in Argentina were evaluated. The strains are kept in the culture collection at Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto Culture Collection. The isolate of *F. verticillioides* had proven ability to produce fumonisins, while *F. graminearum*

had been proven to produce ZEA and DON[10] The isolates were sub-cultured on carnation leaves agar (CLA) at 25 ⁰C for 7 days to enable significant sporulation.

2.2 Medium

A 2% (w/v) milled soybean agar was used in this study (0.99 a_w). The medium was autoclaved at 121 ⁰C 1 atm for 20 min and poured into 9 cm sterile Petri dishes. a_w of the medium was checked with an AquaLab Series 3 (Labcell Ltd.,Pullman, USA) with an accuracy of ±0.003.

2.3 Soya beans preparation

Soya beans (14.5% moisture content, 1 kg batches) were gamma irradiated (7 kGy) using a Cobalt radiation source and stored aseptically at 4 °C. Beans were adjusted at 0.99 a_w by aseptically adding sterile distilled water simulating a_w at the filling step (1.47 ml/g to the beans in sterile bottles). The bottles were cooled down to 4 ⁰C for 48 hours with periodically hand-shaking during this time. Final a_w values of each bottle were checked with an AquaLab Series 3 (Labcell Ltd.,Pullman, USA) with an accuracy ±0.003. Then, approx. 25 g of seeds were placed in single layers into Petri dishes.

2.4 Inoculation and incubation conditions

Petri dishes were inoculated centrally with a 4 mm diameter agar disc taken from the margin of a 7-day-old colony of each isolate on synthetic nutrient agar[19] at 25 °C and transferred face down to the centre of each plate. Inoculated plates of the same a_w were sealed in containers along with beakers containing a water glycerol solution of the same a_w as the plates in order to maintain the $a_w.$[20] All plates were incubated at 15, 20, 25, and 30 ⁰C and at 12-h cycling intervals at temperatures 15⁰C/20⁰C; 15⁰C/25⁰C and 20⁰C/30⁰C. Temperatures were selected based on mean temperature at R6 soybean growth stage (full seed) where 15 ⁰C was the minimum and 30 ⁰C maximum average temperatures registered in the last years in the Río Cuarto, Córdoba region. The experiments were repeated 3 times, for both culture medium and soya beans. Moreover, separated sets of treatments were prepared for each sampling period: 7, 15 and 21 days.

2.5 Growth assessment

For both, soybean seeds and agar medium, fungal growth was observed on a daily basis for an overall period of 21 days and diameter measurements earned out at right angles with the aid of a ruler and a binocular magnifier.[21]

2.6 Mycotoxins analysis from soybean based medium

2.6.a Extraction

Three agar plugs (diameter 5 mm) of each colony were removed from the inner, middle and outer part of the colonies after 7, 15 and 21 days of incubation and placed in a vial for each repeated experiment. One ml of methanol for FBs and 1 ml of acetonitrile for DON and ZEA were added, and the vials were shaken for 5 sec and allowed to rest. After 60 min, the vials were shaken again and extracts filtered (Whatman Nº 4) into another vial and stored at 4 ºC until analysis by HPLC (Waters, Mildford, MA, SA). Plug extraction was performed in duplicate.

2.6.b Detection and quantification

All mycotoxins were detected and quantified separately by using a HPLC system (Waters 2695, separations module, Waters, Milford, USA). Chromatographic separations were performed on a stainless steel C_{18} reversed-phase column (250 mm x 4.6 mm i.d., 5 µm particle size; Waters Spherisorb, Ireland) connected to a security guard cartridge (10 mm × 4 mm i.d., 5 µm particle size; Waters Spherisorb, Ireland). Injection volume was 100 µl by automatic injector.

For fluorescence detection of fumonisins and zearalenone a Waters 2475 module (Waters, Milford, USA) was used and for absorbance detection of DON a Waters 2487 module (Waters, Milford, USA) was employed. Quantification was always achieved using a software integrator (Empower, Milford, MA, USA). The mycotoxin levels were calculated by comparing the area of the chromatographic peak of the sample with those of the standard calibration curve.

<u>Fumonisins B_1 and B_2</u>: FBs were detected by fluorescence (λ_{exc} 335 nm; λ_{em} 440 nm). The mobile phase was methanol: 0.1M sodium dihydrogen phosphate (77:23) solution adjusted to pH 3.35 with orthophosphoric acid. Dried extracts were dissolved in methanol and derivatized with *o*-phthaldialdehyde (OPA).[22] Detection limit of the analysis was about 0.01 ng/g of culture medium for FB_1 and 0.002 ng/g of culture medium for FB_2, based on a signal-to-noise ratio of 3:1. The range of FBs standards used for quantification was 0.015-5 µg/ml.

<u>Zearalenone</u>: ZEA detection was achieved by fluorescence (λ_{exc} 274nm; λ_{em} 445 nm). The mobile phase was acetonitrile: water (60:40). Samples were dissolved in mobile phase. Detection limit of the analysis was about 0.017 ng/g of culture medium, based on a signal-to-noise ratio of 3:1. The range of ZEA standards used for quantification was 5-25 ng/ml.

<u>Deoxynivalenol</u>: DON was detected by absorbance (λ 220nm). The mobile phase was water: acetonitrile: methanol (90:5:5). Samples were dissolved in mobile phase. Detection limit of the analysis was about

0.31 ng/g of culture medium, based on a signal-to-noise ratio of 3:1. The range of DON standards used for quantification was 0.5-15 µg/ml.

2.7 Mycotoxins analysis from soya beans

Fumonisins B_1 and B_2: Fumonisins were analyzed by using the method of Shephard et al. (1990)[23] as modified by Doko et al. (1995)[24]. The detection limit of the analytical method was 20 µg/kg for both mycotoxins. The HPLC system consisted of a Hewlett Packard 1050 pump (Palo Alto, CA, USA) connected to a Hewlett Packard 1046A programmable fluorescence detector and a Hewlett Packard 3395 integrator. Chromatographic separations were performed on a stainless steel, C_{18} reversed-phase column (150 × 4.6 mm i.d., 5 µm particle size; Luna-Phenomenex, Torrance, CA, USA) connected to a security guard cartridge (4 × 3 mm i.d., 5 µm particle size; Phenomenex).

Deoxynivalenol and zearalenone: DON detection and clean-up was done by using the method described in Barros et al. (2008),[25] while ZEA was analyzed using the methodology proposed by Silva and Vargas (2001).[26]

For DON, mobile phase was water/methanol (88:12) at a flow 1.5 ml/min. Mycotoxin was detected by UV absorbance (λ: 220nm; Hewlett Packard 1100, programmable UV detector, Palo Alto, CA, USA) and quantified by a data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01, Palo Alto, CA, USA). The detection limit of the analytical method was 50 µg/kg. HPLC system consisted of a Hewlett Packard 1100 pump (Palo Alto, CA, USA; Rheodyne manual injector with 50 ul loop, Rheodyne, Cotati, CA, USA). Chromatographic separations were performed on a stainless steel, C18 reverse-phase column (150×4.6 mm i.d., 5 µm particle size; Luna-Phenomenex, Torrance, CA, USA).

For ZEA, mobile phase was methanol/water (80:20, v/v) at a flow rate of 0.5 ml/min and detection was achieved by fluorescence ($λ_{exc}$ 280nm; $λ_{em}$ 460 nm). The detection limit of the analytical method was 10 µg/kg. HPLC system was the same as described above for FBs.

2.8 Statistical analyses

Diameters of growing colonies were plotted against time, and the Baranyi and Roberts (1994)[27] model was used to estimate growth rate and time to growth for each growth condition and isolate (Equation 1 and 2) by using Statgraphics Plus 5.1 with the non linear regression option. Analysis of variance of growth rates and time to growth was used in order to assess significant differences due to growth conditions. Mycotoxins were expressed as µg/g of agar medium/soybean seeds. LSD test was used to establish the differences among mean values of the variables under the different levels of factors at $p < 0.05$.

$$D = \mu A - \ln 1 + \frac{[\exp(\mu A) - 1]}{\exp(D_{\max})} \quad \text{(Eq. 1)}$$

(Eq. 2)

$$A = t + \left(\frac{1}{\mu}\right) \ln[\exp(-\mu t) + \exp(-\mu \lambda) - \exp(-\mu t - \mu \lambda)]$$

D: colony diameter.

$D_{\max}$: is the maximal colony diameter (mm)

t: is time (d)

λ is the time to growth (d)

μ: is the growth rate (mm/day)

3. Results

3.1 Effects of constant and fluctuating temperatures conditions on mould growth

There were significant differences on mould growth due to constant temperatures assayed (p<0.05). In general, 20 ⁰C was the best temperature for the growth of *F. graminearum* and 25 ⁰C for *F. verticillioides*. Figure 1 shows growth rate values (μ, cm/day) for *F. graminearum*, growing on soya beans and soybean based medium. Significant differences in the growth rate among the different temperatures and temperature cycles studied were found (p <0.05) on both soya beans and soybean based medium. On culture medium, the higher growth rate was obtained at 20 ⁰C and at 15/20 ⁰C cycling temperature and on seeds the major growth rate was observed at 15/20 °C. Besides, on soya beans the lower growth rate was observed at 25 °C and 15/25 °C. No growth was found at 30 °C and 25/30 °C in any of the substrates. Regarding time to visible growth, it increased with decreasing growth rates, except at 15/20 °C in soya beans where the higher growth rate was reported together with a relatively long lag time.

Figure 2 shows growth rate (μ, cm/day) for *F. verticillioides* growing on soya beans and soybean based medium. Significant differences on growth rate for the different temperatures and temperature cycles studied were found (p <0.05). On culture medium the higher growth rate was observed at 25 °C and in temperature cycles, the fastest growth was observed at 15/25 ⁰C. The slowest growth was observed at 30 °C. On the other hand, there was a decrease in growth when *F. verticillioides* grew in soya beans compared to the growth obtained on culture medium. On soybeans, higher growth rates were observed at 15 °C, 15/20 °C and 15/25 °C. As for *F. graminearum*, no growth was observed at 30 °C and 25/30 °C on soya beans; however, at these temperatures growth occurred on soybean based medium. Regarding time to visible growth, in general, it increased with decreasing growth rates, except at 15/25 °C in soya

beans where delayed growth was observed with a subsequent relatively high growth rate, similarly growth rate and lag time were not negatively correlated at 30ºC in culture medium.
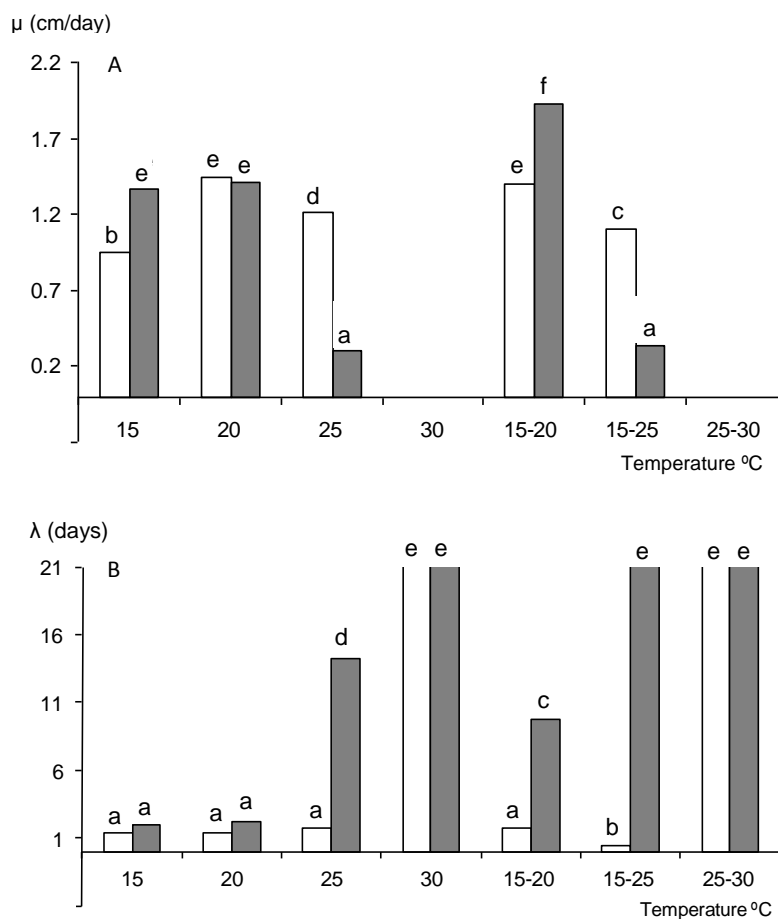
Figure 1: growth rate (μ, cm/day; 1A) and time to growth (λ, days; 1B); of *Fusarium graminearum* on culture medium □ and on soybean seeds ■. Bars with different letter denote significant differences according to LSD test (p<0.05).
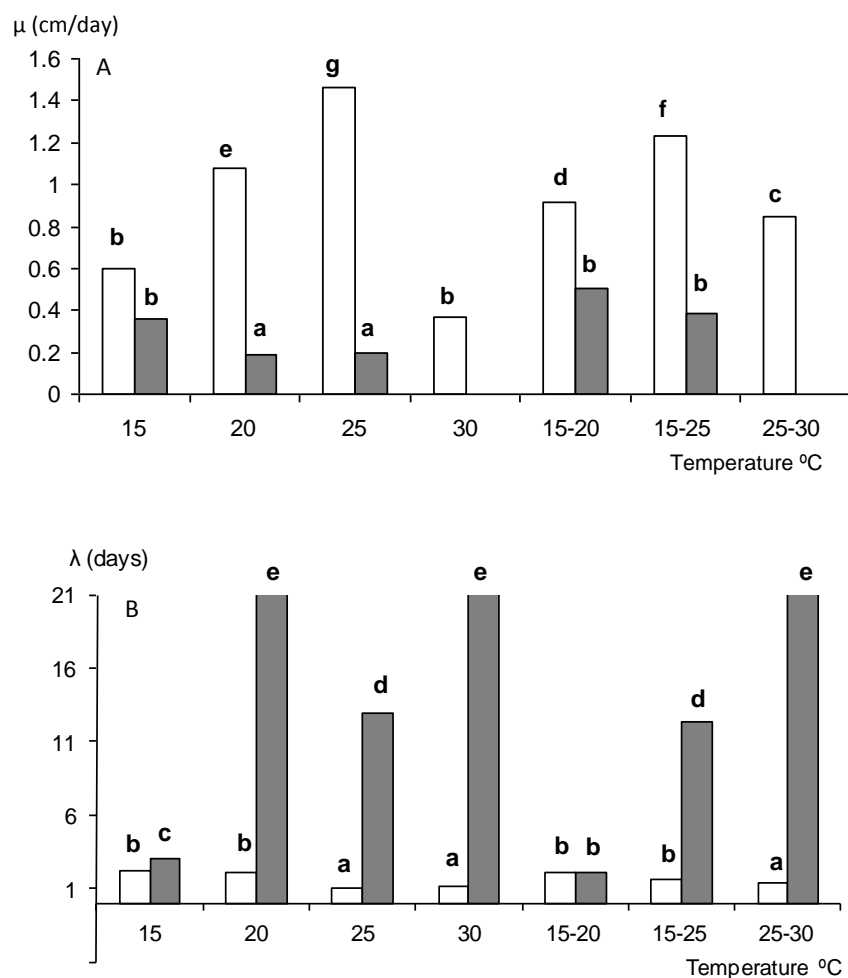
Figure 2: growth rate (μ, cm/day; 1A) and time to growth (λ, days; 1B) of *Fusarium verticillioides* on culture medium □ and on soybean seeds ■. Bars with different letter denote significant differences according to LSD test (p<0.05).

For both species, longer times to visible growth were reported on soy beans compared to agar medium, suggesting an easier initial nutrient uptake in agar medium.

Results obtained under fluctuating temperatures were compared with the sum of 12 hours growth under each constant temperature, for both soya beans and soybean based medium (Table 1 and Table 2, respectively). On culture medium, *F. graminearum* showed significant differences (p<0.05) between observed and calculated growth at cycling 25/30ºC and 15/20 ºC (the difference was small in this later case) (Table 1), while for soya beans this difference occurred at 15/25 ºC and 25/30ºC (Table 2). *F. verticillioides* showed remarkable differences at 15/20 ºC and at 15/25 ºC between observed and calculated growth on culture medium, as well as at 15/20 ºC in soya beans.

As a conclusion, growth at cycling temperatures cannot be predicted from data at constant temperature.

Table 1. Calculated and observed and growth rate (cm/day) of isolates at fluctuating temperatures on culture medium

	μ (cm/day)					
	F. graminearum			*F. verticillioides*		
Condition	15-20 ºC	15-25 ºC	25-30 ºC	15-20 ºC	15-25 ºC	25-30 ºC
Calculated	1.20 b	1.08 a	0.61	3.02 b	0.76 b	0.92 a
Observed	1.40 a	1.10 a	NG	0.91 a	1.23 a	0.85 a

*Different letters on values denote significant differences according to LSD test (p<0.05).
NG: no growth

Table 2. Calculated and observed growth rate (cm/day) of isolates at fluctuating temperatures on soybean seeds

	μ (cm/day)					
	F. graminearum			*F. verticillioides*		
Condition	15-20 ºC	15-25 ºC	25-30 ºC	15-20 ºC	15-25 ºC	25-30 ºC
Calculated	1.39 a	0.84 b	0.15	0.27 b	0.27 a	0.10
Observed	1.93 a	0.34 a	NG	0.50 a	0.39 a	NG

*Different letters on values denote significant differences according to LSD test (p<0.05).
NG: no growth

3.2.a ZEA and DON production on culture medium and soybean seeds at the different temperature conditions

Table 3 shows ZEA production by *F. graminearum* at the different conditions studied. When this mould grew in culture medium, it produced the mycotoxin under all temperatures, although the production was low. The major production was observed at 15/25 °C, but the levels did not exceed 0.35 μg/g agar. Mycotoxin production at 15/20 °C was lower than expected taking into account prediction at constant 15 and 20 ºC, while production at 15/25 °C was higher than the expected, in contrast to growth results. Respect to ZEA production on soya beans, this mycotoxin was only detected under the lowest temperatures studied (15 °C and 15/20 °C); however production levels on seeds were higher compared with production on soybean based medium. The highest level (39.25 μg/g) was observed at 15 ºC after 15 days of incubation.

Table 3. ZEA production on culture medium and soybean seeds at different temperatures at 7, 15 and 21 days.

	ZEA production					
	Culture medium (µg/g agar)			Soybean seeds (µg/g)		
Temperature	7 days	15 days	21 days	7 days	15 days	21 days
15 ºC	<ld	0.23 ± 0.23	0.11 ± 0.03	10.7 ± 0.14	39.25 ± 7.85	31.15 ± 9.83
20 ºC	0.03 ± 0.04	0.29 ± 0.28	0.06 ± 0.08	<ld	<ld	<ld
25 ºC	0.08 ± 0.11	<ld	<ld	<ld	<ld	<ld
30 ºC	NG	NG	NG	NG	NG	NG
15-20 ºC	0.09 ± 0.12	0.04 ± 0.62	0.08 ± 0.11	<ld	19.95 ± 10.39	21.9 ± 1.23
15-25 ºC	0.34 ± 0.45	<ld	0.33 ± 0.35	NG	NG	NG
25-30 ºC	NG	NG	NG	<ld	<ld	<ld

NG: No growth; ld: limit of detection.

Respect to DON production, the toxin was detected in culture medium under all temperatures at which growth occurred, except at 15 °C (Table 4). The higher production was observed at the temperature cycles, where the highest level was recorded at 15/25 °C at 21 days of incubation. When *F. graminearum* grew on soya beans, the DON levels were higher than those observed on culture medium. Production was detected at all temperatures where growth occurred. High levels of production were observed at 15 days of growth, decreasing at 21 days, except at 25 °C, where the highest concentration occurred after 7 days of incubation. The highest level of DON was found at 20 °C (1040.4 ug/g).

Table 4. DON production on culture medium and soybean seeds at different temperatures at 7, 15 and 21 days.

	DON production					
	Culture medium (µg/g agar)			Soybean seeds (µg/g)		
Temperature	7 days	15 days	21 days	7 days	15 days	21 days
15 ºC	<ld	<ld	<ld	158.2 ± 223.73	244.04 ± 192.74	<ld
20 ºC	<ld	<ld	0.35 ± 4.98	<ld	1040.4 ± 11.31	0.25 ± 0.07
25 ºC	0.11 ± 0.16	0.41 ± 0.58	<ld	708.08 ± 993.1	32.09 ± 37.41	<ld
30 ºC	NG	NG	NG	NG	NG	NG
15-20 ºC	<ld	<ld	1.03 ± 1.46	<ld	223.48 ± 259.40	1.18 ± 0.67
15-25 ºC	<ld	<ld	2.48 ± 3.5	<ld	424.62 ± 600.50	0.18 ± 0.25
25-30 ºC	NG	NG	NG	NG	NG	NG

NG: No growth; ld: limit of detection.

3.2.b Fumonisins production on culture medium and soybean seeds at the different temperature conditions

On culture medium *F. verticillioides* produced fumonisin B$_1$ at all temperatures tested at 7 days of incubation (Table 5). The toxin was mainly detected after 7-15 days of growth. The highest production was observed at 15 °C after 7 days of incubation. When *F. verticillioides* grew directly on soya beans, no FBs were detected.

Table 5. FBs production on culture medium and soybean seeds at different temperatures at 7, 15 and 21 days.

	Fumonisin B$_1$ production					
	Culture medium (µg/g agar)			Soybean seeds (µg/g)		
Temperature	7 days	15 days	21 days	7 days	15 days	21 days
15 ºC	7.38 ± 4.56	<ld	<ld	<ld	<ld	<ld
20 ºC	1.77 ± 0.27	1.75 ± 0.50	<ld	<ld	<ld	<ld
25 ºC	0.80 ± 1.14	<ld	0.60 ± 0.86	<ld	<ld	<ld
30 ºC	1.77 ± 2.50	2.55 ± 1.22	<ld	NG	NG	NG
15-20 ºC	0.62 ± 0.87	0.79 ± 1.12	<ld	<ld	<ld	<ld
15-25 ºC	1.27 ± 0.06	0.84 ± 1.19	0.96 ± 0.08	<ld	<ld	<ld
25-30 ºC	1.24 ± 0.29	<ld	0.66 ± 0.004	NG	NG	NG

NG: No growth; ld: limit of detection.

4. Discussion

Great potential exists for infection of different grains with multiple *Fusarium* species, probably resulting in contamination of feed with multiple *Fusarium* mycotoxins. Soybean matrix has been poorly studied compared to other commodities in relation to the mycobiota and their mycotoxin production. However, some researches showed natural contamination with *Fusarium* mycotoxins in soybean and by-products.[7,9,10,28]

There are many published works regarding growth and mycotoxin production by different *Fusarium* species under different conditions.[17,18,29,30,31,32,33,34] However, the majority of these studies were made under isothermal conditions. Nevertheless, these results cannot necessarily be extrapolated to natural ecosystems where temperature conditions fluctuate during the day.

In the present work, growth and mycotoxin production by *F. graminearum* and *F. verticillioides*, isolated from Argentinean soybean, were evaluated at different constant and cycling temperatures on culture

media and soybean seeds. Temperatures assayed in this study simulated field conditions for growing soybean during February, March and April in Argentina. *F. graminearum*, grew better when was incubated at 15 ºC, 20 ºC and 15-20 ºC (isothermal or cycling temperature). Respect to *F. verticillioides,* it grew better at 25 ºC in culture medium and at 15-20 ºC, 15-25 ºC on soybean seeds. Our results agree with other works which found that optimal temperature for growth of different *Fusarium* species were between 20-25 ºC. [17,218,29,30,32,33,34,35] However, Samapundo et al. (2005)[17] and Marín et al., (1995)[31] found that optimal temperature for *F. proliferatum* and *F. verticillioides* growth on corn was 30 ºC; both authors worked with the same strains isolated from Spanish maize. The different optimum temperature reported may be attributed to either the geographical and host origin of the strains which may lead to genetic differences or to the different growth substrate

On the other hand, in general the isolates grew better in culture medium compared with soya beans (except in some conditions for *F. graminearum*); for this reason, care should be taken when using only culture medium for fungal growth studies, because in some cases this may lead to overestimated growth values.

F. graminearum is the most important DON and ZEA producer species isolated from soybean growth stages in Argentina[10] and co-occurrence of ZEA and DON has been well documented. [36,37,38,39,40,41] In our work, *F. graminearum* synthesized both ZEA and DON, and production was affected by temperature, time and substrate. In general, the higher levels of both toxins were recorded in soybean seeds at 15 days of incubation. Besides, levels of DON were higher compared with level of ZEA, and temperatures for higher DON production (15, 15/20 ºC) were similar to those for growth, while temperature for optimal ZEA accumulation varied (15 ºC, 20 ºC; 15/20 ºC and 15/25 ºC). In some cases DON and ZEA production did not occur at cycling temperature but did so at constant one. Eugenio et al. (1970)[42] and Mirocha et al. (1967)[43] concluded that incubation at low temperatures (12 to 14 ºC) following initiation of fungal growth at room temperature resulted in higher amounts of ZEA and DON by some strains of *F. graminearum*. However, Eugenio et al. (1970)[42] and Vaamonde et al (1987)[44] concluded that soybean is not favorable for the production of this mycotoxin. They hypothesized that soybean possess some factors that limits production of ZEA by *Fusarium* isolates.

Contrary, *F. verticillioides* only produced fumonisin B_1 on culture medium and the maximum level was at 30 ºC at 15 days of growth. However, at this temperature the species was unable to grow in soybean seeds. As for *F. graminearum*, under isothermal temperatures the level of FBs was higher compared to cycling temperatures. The optimum condition for FB_1 production on corn was reported to be 20 ºC at a_w between 0.956-0.968.[31,45]. Besides, Ryu et al. (1999b)[46] studied FB_1 production by *F. moniliforme* and *F. proliferatum* in milling rice, with cycling temperatures and they found that the optimal condition for fumonisin B_1 production was different for each species: cycling temperature among 10 and 25 ºC for *F. moniliforme* and among 5 and 25 ºC for *F. proliferatum*. Thus, toxin production depends, among other factors, of temperature condition, isolate and matrix.

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In summary, when growth obtained under fluctuating temperatures was compared to predicted growth by pooling growth at constant temperature, in general, observed values were different from calculated (p<0.05) for both species and incubation medium with some exceptions. Obviously, the real temperature gradient was not reproduced in this work. Therefore, temperature fluctuation influences on the colonization of *Fusarium* species in the field. Taking into account the results in soybean seeds, predicted growth was faster than the observed for *F. graminearum* and vice versa for *F. verticillioides*, while predicted toxin production was in general higher than the observed. Although both of them are fail-safe prediction cases, this highlights the fact that extrapolation from constant condition to real field conditions must be done with extremely care.

Currently, predictive models on growth contamination and mycotoxin production in cereal crops trend to be on field situations to simulate real conditions.[47,48] To our knowledge, this is the first study on *F. graminearum* and *F. verticillioides* on soybean based medium and soya beans where growth parameters and mycotoxin production are studied at isothermal and cycling temperatures. Although incubation of fungal cultures at alternating temperature is impractical, it is very important to study ecophysiological behavior under these conditions, because growth and subsequent mycotoxin production at isothermal conditions seem to be different compared to cycling temperatures.

Acknowledgement

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3.10 Kinetics modeling of aflatoxin production by Aspergillus flavus in maize-based media and maize grain

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Abstract

Predictive mycology has mainly dealt with germination, growth and inactivation of fungi while the mycotoxins production issue remains nearly unexplored. Very few studies provide biomass/colony size data along with mycotoxins data for the same sample times, thus the ratio mycotoxin accumulation per fungal biomass weight / colony size has rarely been reported. For this, the objective of the present study was to model the kinetics of mycotoxin production under the assumption of existing both no-growth-associated and growth-associated production. *A. flavus* growth, and aflatoxins production, was chosen as a model mycotoxigenic microorganism, and it was grown in maize agar medium and maize grain at 0.90 and 0.99 a_w at 25 ºC. A significant positive correlation ($p < 0.05$) was observed among the growth responses (colony radius and biomass weight) in agar medium and colony radius in maize at both a_w levels assayed. Aflatoxins accumulation was shown to be better correlated to colony area than either colony radius or fungal biomass. It was observed that aflatoxin production may not present a clear delay compared to growth, as expected for a secondary metabolite, thus a mixed growth associated production situation can be considered. Luedeking-Piret model was used to model aflatoxins production and reasonable percentages of variability were explained. This is a first attempt to model aflatoxins production as a function of growth parameters and time.

1. Introduction

Predictive mycology has mainly dealt with germination, growth and inactivation of fungi (Dantigny et al. 2005), while the mycotoxins production issue remains nearly unexplored (Garcia et al., 2009). Despite the fact that mycotoxins are produced by fungi, very few studies provide biomass/colony size data along with mycotoxins data for the same sample times. Exceptions are the studies from Magan et al. (1984), Samapundo et al. (2005), Marín et al. (2006) and Baert et al. (2007), that provided the radius/diameter/surface of the colonies along with the concentration of mycotoxins. It is clear from these studies that mycotoxins, as secondary metabolites, are still produced in the stationary phase (i.e., when growth of the colony has already stopped).

During the past decade several publications have reported on different mycotoxins production over time either in synthetic media or food substrates. These studies rarely took into account the possibility to model such production. In such studies, mycotoxin production was assessed at 3-4 time points, incubation periods lasting from some days to some weeks. The highest mycotoxin levels were sometimes reported at the beginning of the incubation period, while in some other cases the concentration increased with time, and in some others the concentration increased since a peak was reached, suggesting either ulterior degradation, or in the case of experiments in agar media with the plug technique, the decrease in average mycotoxin concentration in larger size colonies (Hope and Magan, 2003; Belli et al., 2004; Hope et al., 2005; Esteban et al., 2006a,b; Marín et al., 2006; Ramirez et al., 2006; Astoreca et al., 2007; Morales et al., 2007). This kind of studies quantified the total amount of mycotoxin per weight of sample, but it was not related to the amount of mould biomass or other growth measurement. Thus the increasing mycotoxin concentration over time was the result of no-growth-associated production, plus growth-associated production, if any, but their contribution was not elucidated. Even if toxin concentrations are compared for the same incubation time, the use of these kind of units prevent from taking into account the effect of the size of the colony, as it is expected that bigger colonies will present higher accumulation of toxins (Marín et al., 2006). For this reason, Marín et al. (2006) expressed ochratoxin A (OTA) accumulation in µg OTA colony^{-1} by multiplying the area of each colony at each time of extraction by the µg of OTA per mm^2. Similarly, Northolt (1979) calculated the specific rate of mycotoxin production as r_P/X (µg mycotoxin mg biomass h^{-1}) for the production of aflatoxin B$_1$ by *Aspergillus flavus*.

In one of the few models dedicated to describe the production of mycotoxins as a function of the biomass, Pitt (1993) assumed that toxins were produced simultaneously with growth, and then the rate of production may be proportional to growth rate. The author also mentioned that this assumption would be inappropriate in cases where toxin levels increased after growth ceased.

The objective of the present study was modelling the kinetics of mycotoxin production under the assumption of existing both no-growth-associated (proportional to biomass) and growth-associated production (proportional to growth rate). *A. flavus* growth, and aflatoxins production, was chosen as a model mycotoxigenic microorganism, and it was grown in maize agar medium and maize grain.

2. Material and methods

2.1 Fungal isolates and preparation of inoculum

One mycotoxigenic isolate of *A. flavus* (UdL-TA 3.185) was included in this research. The reference in brackets is the code of this culture held in the Food Technology Department Culture Collection of University of Lleida, Spain. The isolate was sub-cultured on malt extract agar (PDA) plates and incubated at 25 ºC for 7 days to obtain heavily sporulating cultures. After incubation, a sterile inoculation loop was used to remove the conidia of the mould from PDA plates and they were suspended in 5 mL of H_2O/glycerol solutions with two different water activity (a_w) levels: 0.99 and 0.90. After homogenizing, the number of spores per ml was then determined using a Thoma counting chamber and the final concentration was adjusted to 10^4 spores mL^{-1}.

2.2 Maize agar medium (MAM) and maize grain preparation

In this research MAM and maize grain with modified a_w were used. MAM was composed by a 2% maize meal with water activity convenient modified with glycerol to the range of a_w conditions studied (Marín et al., 1995). Maize grain was sterilized by autoclave at 120 ºC during 20 minutes. Water activity was adjusted by aseptically adding amounts of sterile distilled water to maize in sterile bottles. The bottles were cooled down to 4 ºC for 48 hours with periodic hand-shaking during this time. The amount of water necessary to reach the different water activity levels was determined by calibration curves (Garcia et al., 2011). Final a_w values of medium and grain were checked with an Aqua Lab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of ± 0.003, before, during and at the end of the experiment.

2.3 Experimental design

In this work, a series of MAM and maize Petri plates were prepared at 0.90 and 0.99 a_w, and inoculated. During incubation, Petri plates were sampled along time (3 replicates per analysis) for determination of biomass weight, colony radius and aflatoxin content in agar experiments and for colony radius and aflatoxin content in maize experiments.

2.4 Inoculation and incubation

Petri dishes were inoculated centrally with 5 µL of a 1-5 10^5 spores mL^{-1} suspension. Plates with the same a_w were enclosed in sealed containers along with beakers containing water glycerol solution of the same a_w as the plates in order to maintain the a_w (Dallyn, 1978). Ninety MAM Petri dishes and 30 maize grain Petri dishes were inoculated and incubated at 25 ºC.

2.5 Growth assessment

Colony radius (mm) and biomass (mg dry weight) were measured at different time periods. Colony radius was observed on a daily basis or as required for an overall period of 30 days by measurements at right angles with the aid of a ruler and a binocular magnifier, on MAM and maize Petri dishes. Mycelium dry weight was measured as in Taniwaki et al. (2006) on culture medium. Briefly, colonies were cut from the medium, transferred to a beaker containing distilled water (100 mL approximately), then heated in a streamer for 30 min to melt agar. The mycelium remained intact and was collected and transferred to a dried, weighed filter paper and dried at 80 ºC for 18 hs. Then the filter paper was weighed and the dry weight of biomass was calculated by difference.

2.6 Aflatoxins extraction from MAM and maize seeds

2.6.a Aflatoxins extraction from MAM

Three agar plugs (diameter 4 mm) of each colony were removed from inner, middle and outer part of the colonies at different incubation times and placed in a vial. 1 mL of methanol was added, and the vials were shaken for 5 s. After 60 min, the extracts were shaken and filtered (Millex-HV 0.45 µm 25 mm, Millipore Corporation. Bedford, U.S.A.) into another vial and stored at 4 ºC until the analysis by HPLC instrument (Waters, Mildford, MA, SA).

2.6.b Aflatoxins extraction from maize seeds

5 g of milled sample were extracted with 15 mL of acetonitrile+water (60+40 v/v) and shaken for 10 minutes. The extract was filtered through number 1 filter paper and 2 mL of filtrate were mixed with 14 mL of PBS. Then, the diluted extract was cleaned up by passage through an immunoaffinity chromatography column (EASI-EXTRACT AFLATOXIN. R-Biopharm Rhone Ltd., Glasgow, Scotland) at a flow rate of 2–3 mL/min. The column was then washed with 20 ml of PBS and left to dry. Aflatoxins were finally eluted from the column with 3 mL methanol+water (50+50, v/v) and stored at 4 ºC until analysis by HPLC.

2.7 Aflatoxins detection and quantification

Aflatoxins B_1 and B_2 (AFB_1 and AFB_2) were detected and quantified separately by using a HPLC system (Waters 2695, separations module, Waters, Milford, USA) and a C_{18} column (5 µm Waters Spherisorb, 4.6 × 250 mm ODS2). Mobile phase was pumped at 1 mL min^{-1} and injection volume was 100 µL. For fluorescence detection a Waters 2475 module (Waters, Milford, USA) was used. Quantification was achieved with a software integrator (Empower, Milford, MA, USA). Mycotoxins were quantified on the basis of the HPLC fluorimetric response compared with that of a range of mycotoxins standards. A post column photochemical derivatization system (LC Tech detector, UVC 254 nm, Germany) was used and toxins were detected by fluorescence (λ_{exc} 365 nm; λ_{em} 455 nm). The mobile phase was water: methanol: acetonitrile (70:17:17) and the detection limit of the analysis was about 0.5 pg/mm2 for AFB2 and 1

pg/mm2 for AFB1, based on a signal-to-noise ratio of 3:1. The range of aflatoxins standards used for quantification was 0.09-0.94 ng mL-1.

2.8 Statistical analysis

In general, the rate of microbial exponential growth is calculated as

$$\frac{dX}{dt} = \mu X \qquad [1]$$

And after integration microbial concentration is

$$X = X_0 e^{\mu t} \qquad [2]$$

In this work, the general mixed-growth associated Leudeking-Piret model for product formation was used (Shuler and Kargi, 2007), the rate of product formation is as follows

$$\frac{dP}{dt} = (\alpha\mu + \beta)X \qquad [3]$$

To calculate the product formation, equation [3] should be integrated

$$\int_0^p dP = \int_0^t (\alpha\frac{dX}{dt} + \beta X)\,dt \qquad [4]$$

Substitution of equation [2] in equation [4] and integration results in

$$P = X_0 e^{\mu t}\left(\alpha + \frac{\beta}{\mu}\right) \qquad [5]$$

Where:

μ, specific growth rate (mm d^{-1}, cm d^{-1}, etc)

P, product concentration (gP L^{-1})

t, time (h, d)

dP/dt, product accumulation in the culture medium (gP/L h)

X, biomass concentration (gX L^{-1})

β, growth-independent coefficient for P production (gP/gX h)

α, growth-associated coefficient for P production (gP/gX)

However, these calculations are based on the assumption of an exponential microbial growth, which is mostly applicable to unicellular microorganisms. Growth of filamentous fungi in solid substrates may not follow such behavior, as will be discussed in the following section.

3. Results and discussion

3.1. Correlation among growth responses

A significant positive correlation ($p < 0.05$) was observed among the growth responses (colony radius and biomass weight) in agar medium at both a_w levels assayed (Table 1). Positive correlation among these

growth responses has been confirmed elsewhere (Marín et al., 2005), although one variable cannot be fully predicted from the other as they contribute complementary information (Taniwaki et al., 2005). This may be caused by fungi producing thicker more dense colonial morphologies (Wyatt et al., 1995). Biomass measured as mycelium dry weight actually represents growth. However, this method lacks sensitivity and is destructive (Deploey and Fergus, 1975). On the other hand, colony diameter as a measure of fungal biomass takes no account of colony density (Wells and Uota, 1970). Colony diameter is not a good measure of fungal biomass production in aging colonies (Taniwaki et al., 2005). Finally, fungal hyphae can penetrate solid substrates, such as foods, making their extraction difficult (Pitt, 1984). Thus mycelium dry weight cannot be measured in foods, while colony diameter, although easily assessed, is difficult to be applied to real food substrates, and can just be measured in flat, bigger surface foods, such as certain fruits, bakery products and so on.

'Colony density' was calculated by dividing biomass weight by colony area at each time period; the average values were 0.01 and 0.1 mg/mm^2 at 0.99 and 0.90 a_w, respectively. A value of 0.023 mg/mm^2 was reported by Marín et al. (1995) for an *A. flavus* strain in DG18 (0.955 a_w) at 25 ºC.

Colony radius obtained in maize grain showed also a significant correlation ($p < 0.05$) with growth variables measured in agar medium. At 0.99 a_w growth was faster in agar medium than in maize, however, at 0.90 a_w growth in both substrates was similar.

Table 1. Correlation among the recorded growth responses (Pearson coefficients and P-values)

	Radius en agar	Radius in maize	Biomass in agar
0.99 a_w			
Radius in agar	1.00	0.99	0.88
	(0.00)	(0.00)	(0.01)
Radius in maize	-	1.00	0.89
		(0.00)	(0.01)
Biomass in agar	-	-	1.00
			(0.00)
0.90 a_w			
Radius in agar	1.00	0.97	0.98
	(0.00)	(0.00)	(0.00)
Radius in maize	-	1.00	0.98
		(0.00)	(0.00)
Biomass in agar	-	-	1.00
			(0.00)

3.2. Modelling growth data

Only macroscopic models were considered at this point. Macroscopic models simply describe the changes in total biomass with time, and do not rely directly on considerations of hyphal elongation and branching. Parameters are typically determined directly by fitting the model to the biomass profile.

At 0.99 a_w, colony radius increased following a sigmoidal growth curve both in agar and maize (Fig. 1); while growth was visible earlier in maize, the subsequent growth was slower in maize than in agar; in both cases growth rate decreased from day 7. When plotting diameters (or radiuses) of a mould colony growing in an agar Petri plate against time, a lag phase is observed, followed by a linear phase, but in most of the cases no decrease in growth rate is usually observed before the edge of the Petri plate is reached (Marín et al., 2008). This was confirmed in the present study, as growth rate in the agar only slowered when radius reached 40 mm, close to the edge of the dish. On the other hand, an asymptotic phase was observed in maize. This trend was also observed by Valik et al. (1999), who described for *P. roqueforti* diameter a growth curve typical of microbial growth with lag-phase, linear phase and upper asymptote when using 17 cm Petri plates with asymptotic values between 2 and 12 cm, well before the edge was reached. Regarding our results on biomass accumulation, it showed a near to exponential increase with time. As a consequence, kinetics of biomass increase was modeled as proposed in equation [2]. The results of biomass weight at 0.99 a_w, although higher, paralleled those reported by Marín et al. (2005) using an *A. flavus* strain in DG18. Thus while colony radius increased linearly, the increase in biomass was exponential. Hypothetically, mould colonies, while growing at a constant growth rate in diameter, should yield by branching an exponential amount of biomass (Bull and Trinci, 1977).
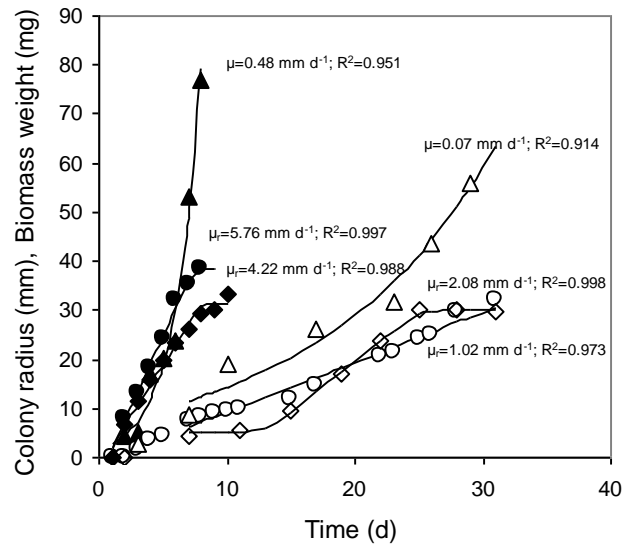

Figure 1: Growth responses of *A. flavus* over time at 0.99 a_w (▲, biomass in MAM; ●, colony radius in MAM; ◆, colony radius in maize) and 0.90 a_w (△, biomass in MAM; ○, colony radius in MAM; ◇, colony radius in maize).

For colony radius, growth was modeled using Baranyi and Roberts (1994) model:

$$r = \mu_r A - \ln\left\{1 + \frac{[\exp(\mu_r A) - 1]}{\exp(r_{\max})}\right\}$$ [6]

$$A = t + \left(\frac{1}{\mu_r}\right)\ln\left[\exp(-\mu_r t) + \exp(-\mu_r \lambda) - \exp(-\mu_r t - \mu_r \lambda)\right]$$ [7]

Where μ_r is radial growth rate (non specific) and λ is time to growth

At 0.90 a_w, colony radius both in MAM and maize seemed to follow a diauxic growth curve, with an initial radial growth rate until they reached a plateau, and finally a linear radius increase at a lower rate than the initial one (Fig. 1).

Fungal growth (colony radiuses or diameters) has been usually modelled using Baranyi's model; in general, worse fittings were reported in the past when growing conditions were far from optimal ones (Marín et al., 2008). However, less attention has been paid to suboptimal conditions than to optimal ones. From the food safety point of view, however, these conditions are of great importance, and predictions near the boundaries for growth should be much accurate (Marín et al., 2012). For *P. expansum*, under limiting conditions of temperature (0.98 a_w/15 ºC) or water activity (0.92 a_w/25 ºC), and for *A. carbonarius* at marginal temperature (0.98 a_w/15 ºC) observed diameters followed a lineal trend after a near-sigmoidal one. (Garcia et al., 2010). This last trend was modelled through a sigmoidal function followed by a linear function. The estimated slopes of the straight line were in general smaller than the maximum growth rate estimated through the related sigmoidal curve, suggesting an adaptation to limiting conditions, but at a reduced rate. Besides, for both strains, this second line showed a narrower distribution (higher kurtosis) than the maximum growth rates estimated from the sigmoidal function.

Probably, in those published experiments in which short times for observations were used this second phase was probably never observed (Garcia et al., 2010).

Initially growth was slower in maize than in agar, although latter they overlapped. Similarly, biomass showed a near to exponential increase only after a lag period. Following these observations, the exponential and Baranyi and Roberts models were applied to biomass and radius data, respectively, from day 7-10.

3.3 Correlation among growth and mycotoxin data

Table 2 shows Pearson correlation coefficients; in agar experiments, significant correlation was obtained for all growth variables and aflatoxins concentration except for AFB$_2$ at 0.90 a_w where production results

followed an erratic trend with time and thus they could not be correlated with growth. For the other 3 remaining cases, aflatoxins production was best correlated to colony area.

Regarding maize experiments, significant positive correlation was found in all cases between AFB_1 and AFB_2 production and colony radius and area; in this case slightly better correlation was found for radius than for area.

Mellon et al. (2002) reported that AFB_1 production paralleled fungal biomass increase during the exponential growth phase in a liquid corn kernel simulation medium. The maximum rate of AFB_1 production coincided with the maximum rate of biomass production, with a peak in both cases at day 4, however, biomass production continued to increase after 4 days, albeit at a lower rate, whereas aflatoxin levels decreased from the peak at 4 days.

Table 2. Correlation among aflatoxins and growth responses (Pearson coefficients and P-values)

			Radius (mm)	Area (mm^2)	Biomass (mg)
Agar experiments	0.99	AFB_1 (ng)	0.9278 (0.00)	0.9802 (0.00)	0.9145 (0.00)
		AFB_2 (ng)	0.8037 (0.02)	0.8973 (0.00)	0.8620 (0.01)
	0.90	AFB_1 (ng)	0.9566 (0.00)	0.9703 (0.00)	0.9218 (0.01)
		AFB_2 (ng)	0.4614 (0.36)	0.4384 (0.38)	0.3172 (0.54)
Maize experiments	0.99	AFB_1 (ng g^{-1})	0.8152 (0.01)	0.7935 (0.01)	-
		AFB_2 (ng g^{-1})	0.7720 (0.01)	0.7123 (0.03)	-
	0.90	AFB_1 (ng g^{-1})	0.8537 (0.01)	0.8293 (0.02)	-
		AFB_2 (ng g^{-1})	0.9042 (0.00)	0.8638 (0.01)	-

Although as secondary metabolites one may expect mycotoxin production to follow a curve paralleling that of growth but slightly delayed, regulation of secondary metabolism is poorly understood (Le Bars, 1988) and the relationship between the rates of primary and secondary metabolism is not clear (Pitt, 1993). In our study, a delay was observed at 0.90 a_w (delay in the initiation of AFB_1 production was about 4-8 days compared to growth), and also at 0.99 a_w, but only in maize (delay in the initiation of AFB_1 production was 2 days compared to growth), while in agar both growth parameters and AFB_1 concentration had detectable values at day 2, when the first observation was carried out, suggesting a behaviour similar to that of primary metabolites.

In our study AFB$_1$ accumulation paralleled colony radius increase, with decreasing rates of production with decreasing rates of radius increase; no decrease in the rate of biomass accumulation was observed, thus toxin accumulation slowed down before any sign of decrease in biomass accumulation was observed. By contrast, Shih and Marth (1974) observed that aflatoxin production generally rose during the logarithmic and deceleration phases of fungal growth, suggesting that the toxin is either a metabolite produced by growing cells or is converted biosynthetically from some other compound by growing cells. However, broader generalizations about mycotoxin formation mechanisms are difficult and the formulation of mechanistic mathematical models is therefore challenging.

3.4. Modelling aflatoxin data under conditions suitable for growth

Aflatoxin content (ng) in agar plates increased with time reaching a plateau at the end of the incubation period, while aflatoxin concentration (ng g^{-1}) in maize reached a maximum at a certain time period and decreased afterwards (Fig. 2 and 4). The analysis for aflatoxins was destructive thus new plates were analyzed at each time period, contributing to an increase in the already known intrinsic variability in mycotoxin production. In a liquid corn kernel simulation medium, AFB$_1$ production increased after 36 h, with a peak at day 4, with the greatest rate of production occurring between days 2 and 4; after aflatoxin production peaked, concentrations decreased over the next 3 days (Mellon et al., 2002).

At the moment there is no widespread primary model associated to mycotoxin production. An interesting review about a descriptive model for growth and aflatoxin formation affected by environmental conditions was presented by Pitt (1993), who developed some equations for the rates of production and degradation of aflatoxin by *A. flavus* and *A. parasiticus* as related to mould growth and environmental conditions. He assumed that the rate of toxin formation is proportional to growth rate and cell mass; in this situation each increment in new cell mass is accompanied by an increment in new toxin mass. This work is the first one and the only existing mechanistic model published for mycotoxin production.

Figure 2: Fitting of AFB$_1$ (■) and AFB$_2$ (■) accumulation in MAM at 0.99 a_w to Leudeking-Piret model [5], [8], [9] based on colony radius/area or biomass (●) as growth variables. Colony radius was fitted to [6] and biomass to [2]

Although Luedeking-Piret model has been often applied in the past to model fungal metabolites formation (mainly primary metabolites) (Marín and Dantigny, 2012), it has only recently been applied to beauvericin production in summerged fermentation (Xu et al., 2011) and aflatoxins production in agar media (Abdel-Hadi et al. 2011).

Regarding AF accumulation in agar experiments, to apply equation [4] linked to colony radius, as X (radius) followed a sigmoidal function; growth was assimilated to a linear function after the time to growth was reached:

If t<λ $\qquad$ X=0; P=0 $\qquad$ [8]

If λ<t<t$_{Xmax}$ $\qquad$ $X = at + b; P = (a\alpha + b\beta)t + a\beta\dfrac{t^2}{2}$

where t$_{Xmax}$ is the time point where the linear model reaches the Xmax value as predicted by the Baranyi and Roberts model, and a~μ$_r$.

If colony area is taken as growth parameter instead of radius:

If t<λ $\qquad$ X=0; P=0 $\qquad$ [9]

If λ<t<t$_{Xmax}$ $\qquad$ $X = \pi(at + b)^2; P = \pi\beta a^2\dfrac{t^3}{3} + \left(\pi\alpha\,a^2 + \pi\beta\,ba\right)t^2 + \left(2\pi\alpha ba + \pi\beta b^2\right)t$

Finally, as biomass increased exponentially, equation [5] was used for prediction.

Figure 2 and table 3 show the fitting results. An observed vs predicted plot was built for AFB$_1$ and AFB$_2$ as predicted through the radius, area or biomass values, from day 2 to day 7 (Fig 3, AFB$_1$). Overall, predicting aflatoxin content through colony area data led to more accurate and less biased predictions than those from colony radius or biomass weight. Still the slope of the line was <1, suggesting fail-dangerous predictions.
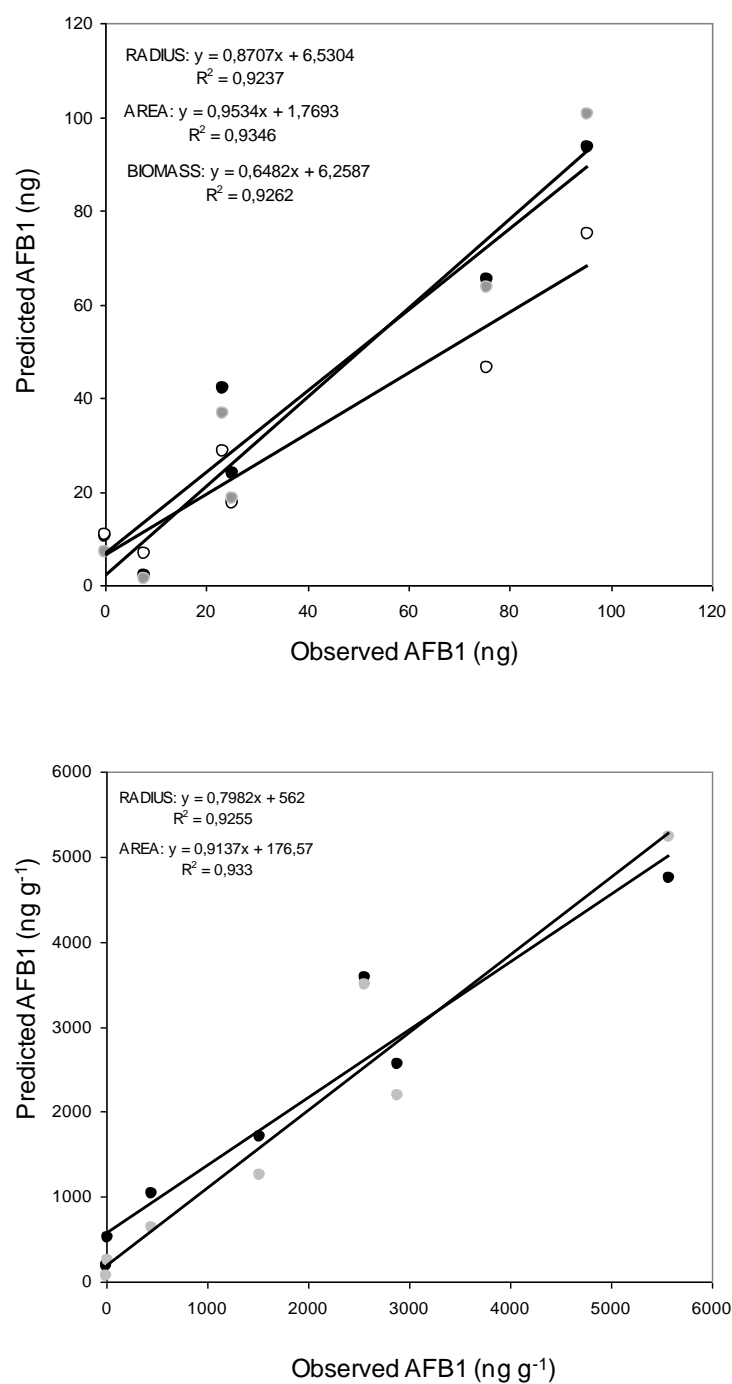
Figure 3: Observed AFB_1 values versus predicted values through equations [5], ○; [8], ●; and [9], ●, in MAM (a) and maize grain (b) at 0.99 a_w.
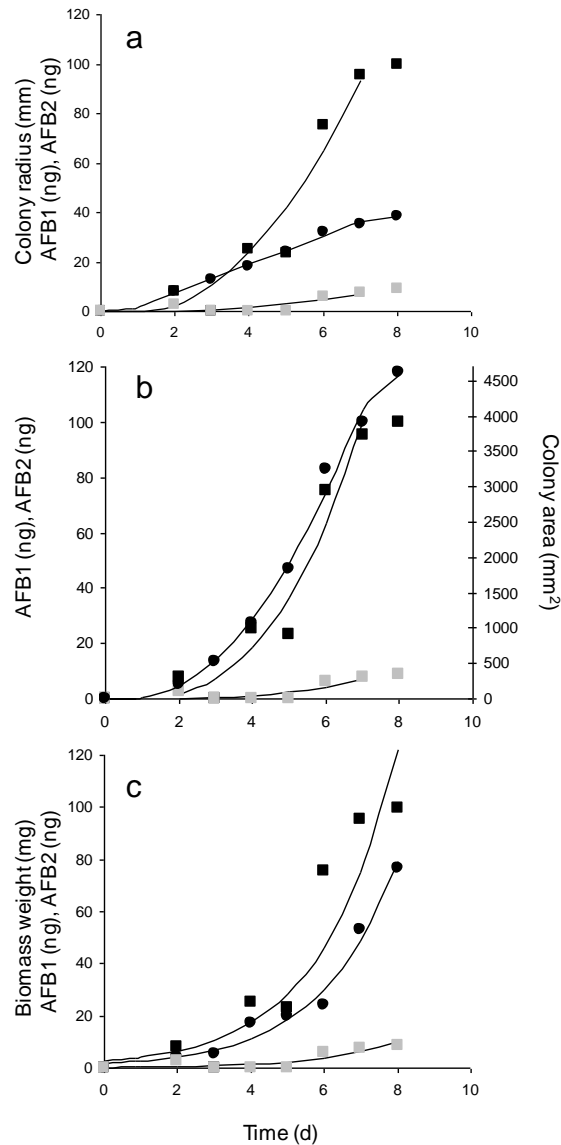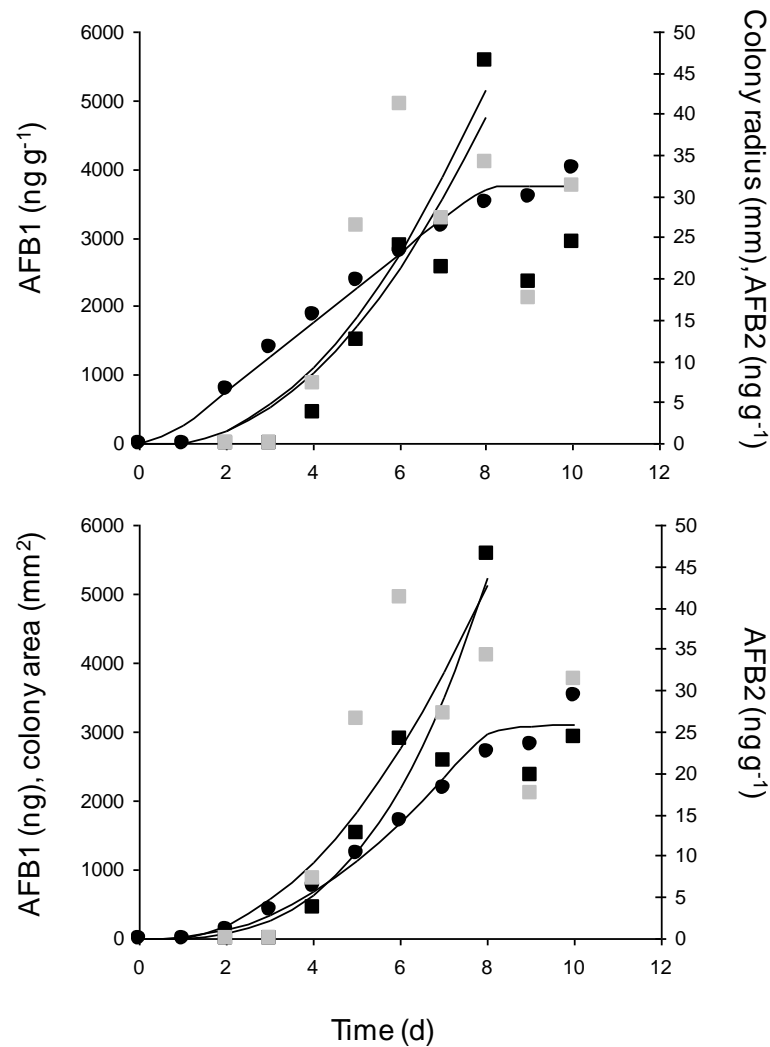
Figure 4: Fitting of AFB$_1$ (■) and AFB$_2$ (■) accumulation in maize grain at 0.99 a_w to Leudeking-Piret model [8], [9] based on colony radius/area (●) as growth variables. Colony radius was fitted to [6].

Regarding AF accumulation in maize experiments, as X followed a sigmoidal function, thus growth was assimilated to a linear function after the time to growth was reached, and equation [8] was applied; whereas for colony area equation [9] was applied (Fig. 4). An observed vs predicted plot was built for AFB$_1$ and AFB$_2$ as predicted through the radius and area values, from day 2 to day 8; overall, predicting AFB$_1$ content through colony area data led to more accurate and less biased predictions than those from colony radius, while no difference was observed for AFB$_2$ (Fig 3, AFB$_1$).

Table 3. Estimated parameters for AFB_1 accumulation through the Leudeking-Piret model [4]

			0.99 a_w	0.90 a_w
Agar experiments	Radius	α (ng mm^{-1})	ns	ns
		β (ng mm d^{-1})	0.829	0.14
		R^2	0.92	0.839
	Area	α (ng mm$^{2-1}$)	0.009	ns
		β (ng mm^2 d^{-1})	0.007	0.003
		R^2	0.93	0.843
	Biomass	α (ng mg^{-1})	0.71	ns
		β (ng mg d^{-1})	0.40	0.067
		R^2	0.816	0.776
Maize experiments	Radius	α (ng mm^{-1})	ns	39.147
		β (ng mm d^{-1})	40.44	8.869
		R^2	0.898	0.869
	Area	α (ng mm$^{2-1}$)	0.25	0.592
		β (ng mm^2 d^{-1})	0.58	0.157
		R^2	0.933	0.958

ns: not significant

Looking at Table 3, a not growth-associated model was predicted for colony radius, while a mixed growth-associated toxin formation was predicted in the remaining cases; α and β values cannot be compared as they are calculated in different units, but for example, predicting AF accumulation through area results would imply that 9 pg AFB_1 (0.25 ng g^{-1} maize) are accumulated per mm^2 of newly generated colony per day simultaneously to growth (similar to what would happen for primary metabolites), while 7 pg of AFB_1 (0.58 ng g^{-1} maize) are accumulated per mm^2 of existing colony.

3.4.1 Toxin accumulation = Toxin formation- Toxin degradation

A decrease in the rate of toxin accumulation and even a negative rate of toxin accumulation was observed from day 7 to 8 in agar experiments, and from day 8 to 10 in maize experiments. In both cases this occurred once a decreased growth rate had been observed in terms of colony radius. That decrease in the rate might be due to i) a decrease in the growth-associated toxin accumulation due to the decrease in the growth rates, ii) a decrease in the rate of toxin production by the existing biomass, or iii) a degradation of toxin by the existing biomass. The last two phenomena could be merged in the β value that could then take either positive or negative values. For figure 2a and 2b, as at time 7.2 d the radius reached its maximum according to Baranyi and Roberts equation, for time over 7.2 the growth-associated terms could be deleted from the model and β kept at the same value, without considering any degradation. As only one observed point was available at this stage, the kinetics of degradation were not studied.

For experiments in maize (figure 4) toxin was modelled until growth reached a null growth rate according to Baranyi and Roberts equation, afterwards growth dependent toxin production must be omitted.

However, growth independent produced toxin would increase at a constant rate as a result of a constant X (and a constant β, if true), unless degradation occurs, in which case β could have a positive or negative value. As a rough estimation, if the three available AFB_1 values from day 8 to 10 (31.38 mm is the constant estimated radius, and 3093.5 mm^2 is the constant estimated area) were taken and the linear trend obtained, the negative slope of that line (-1324), would mean that AFB1 accumulation rate (β) is -42.5 ng mm^{-1} or -0.42 ng mm^{2-1}, which is the balanced result of both production and degradation, with a higher level of the second.

3.5. Modelling aflatoxin data under suboptimal conditions for growth

In this case only AFB_1 data were used as the correlation between AFB_2 and growth data was shown to be weak in agar experiments (section 3.3). As shown in figure 1 growth data showed early growth and a kind of an adaptation time till day 7, at that time mean AFB_1 concentration was 1.08 ng in agar and 1.57 ng g^{-1} in maize. It was decided to model toxin accumulation from day 7 linked to the previous presented growth kinetics (section 3.2).

Similarly to what happened at 0.99 a_w, AFB_1 (ng) in agar increased with time reaching a plateau at the end of the incubation period (31 days), while AFB_1 (ng g^{-1}) in maize reached a maximum after 23 days and decreased afterwards (Fig. 5 and 7).
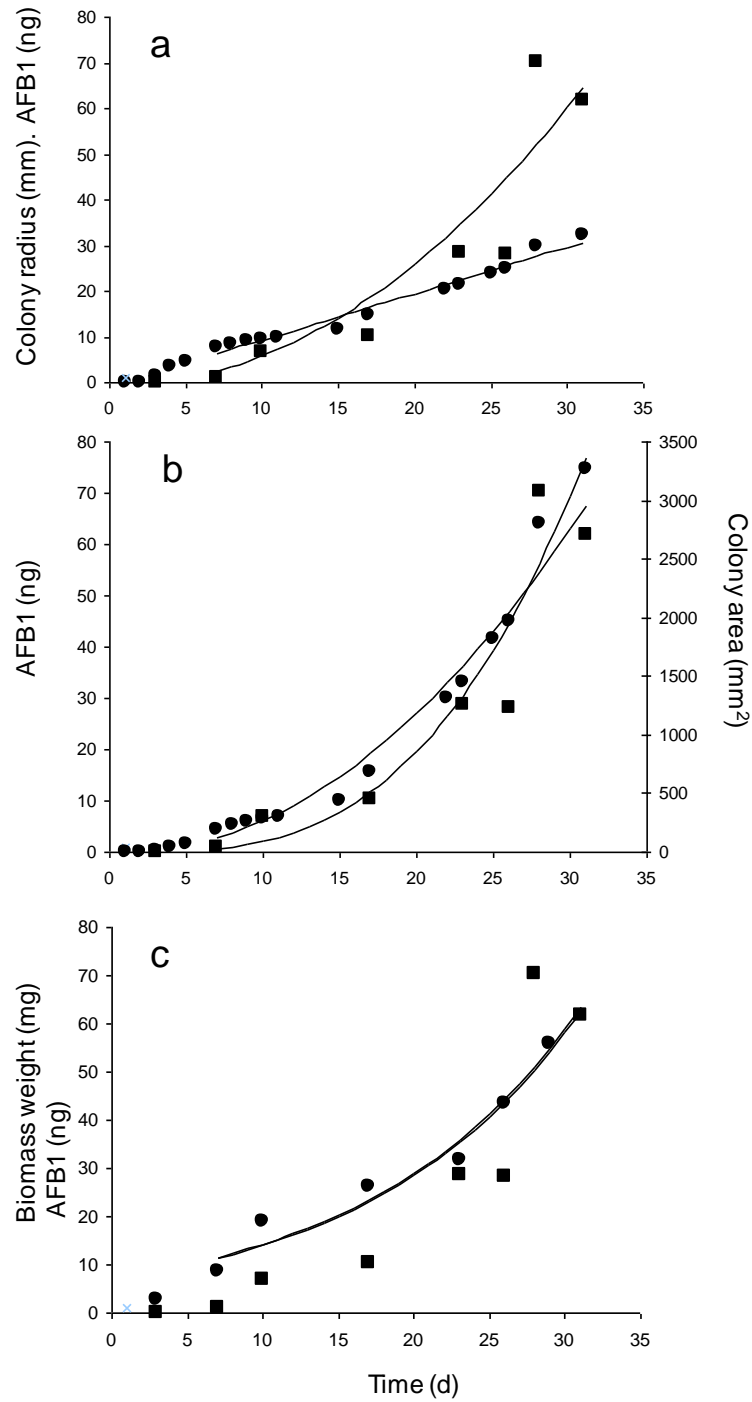
Figure 5: Fitting of AFB$_1$ (■) and AFB$_2$ (■) accumulation in MAM at 0.90 a_w to Leudeking-Piret model [5], [8], [9] based on colony radius/area or biomass (●) as growth variables. Colony radius was fitted to [6] and biomass to [2].

Regarding AF accumulation in agar experiments, as colony radius followed a lag-linear function, growth was assimilated to a linear function after the time to growth was reached and equation [8] was used.

If colony area is taken as growth parameter instead of radius, equation [9] is used.

Finally, as biomass increased exponentially, equation [5] was used for prediction.

Figure 5 and table 3 show the fitting results. An observed vs predicted plot was built for AFB_1 as predicted through the radius, area or biomass values, from day 7 to day 28 (Fig 6). Overall, predicting AFB_1 content through colony area data led to more accurate and less biased predictions than those from colony radius or biomass weight. The slope of the line was nearly 1, suggesting not biased predictions. Looking at Table 3, not growth-associated models were predicted in agar experiments. It should also be taken into account that data till day 7 were not included; in those days growth occurred at a significant rate, but only about 1 ng AFB_1 was accumulated, suggesting that toxin production was delayed respect to growth (as expected for a secondary metabolite). Predicting AF accumulation through area results would imply that 2.6 pg of AFB_1 are accumulated per mm^2 of existing colony.
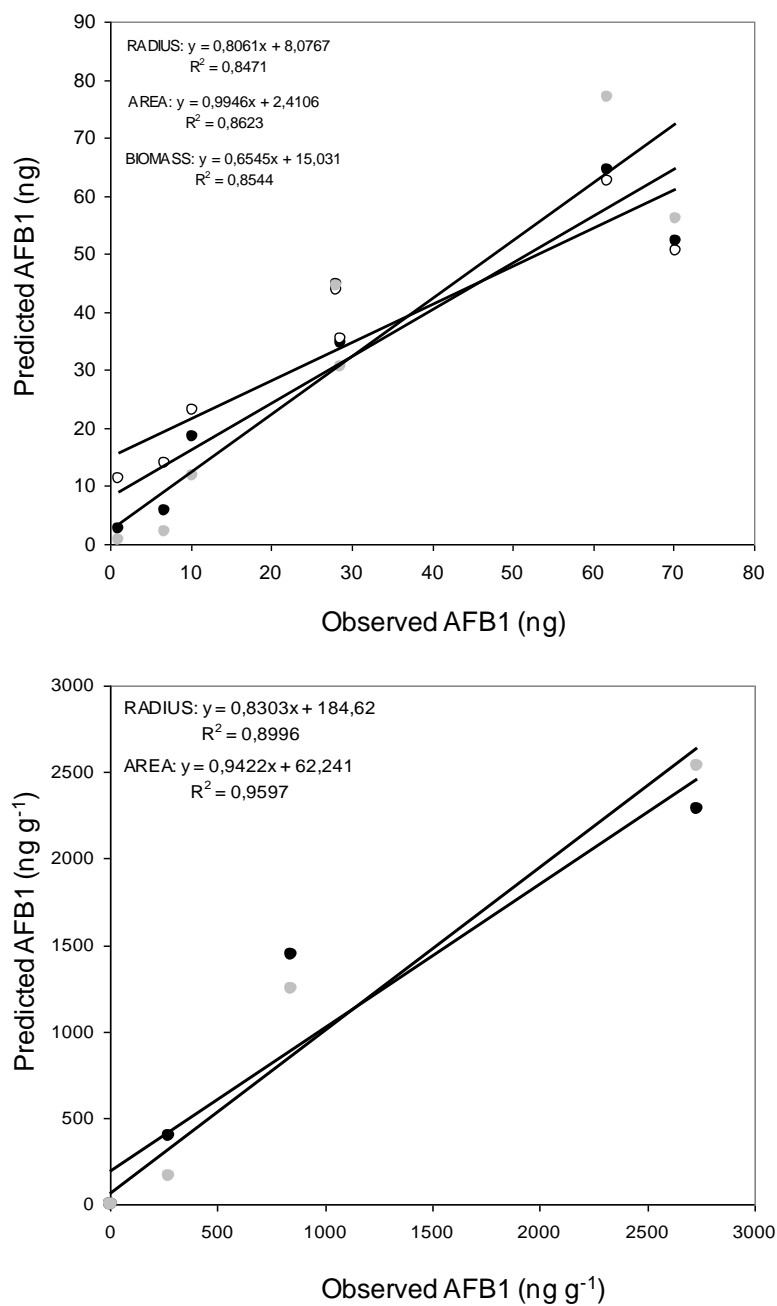
Figure 6: Observed AFB$_1$ values versus predicted values through equations [5], ○; [8], ●; and [9], ●, in MAM (a) and maize grain (b) at 0.90 a_w.

Regarding AF accumulation in maize experiments, as X followed a sigmoidal function, thus growth was assimilated to a linear function after the time to growth was reached, and equations [8] and [9] were applied between day 7 to day 23, because from that day a null growth rate was predicted by Baranyi and Roberts model (Fig. 7). An observed vs. predicted plot was built for AFB_1 as predicted through the radius and area values, from day 7 to day 23 (Fig. 6); overall, predicting AFB_1 content through colony area data led to more accurate and less biased predictions than those from colony radius.

Figure 7: Fitting of AFB_1 (■) and AFB_2 (■) accumulation in maize grain at 0.90 a_w to Leudeking-Piret model [8], [9] based on colony radius/area (●) as growth variables. Colony radius was fitted to [6].

In this case, although from day 1 to 7 growth occurred at a significant rate, with a limited AFB_1 accumulation, a mixed growth-associated toxin formation was predicted from day 7. For example, predicting AFB_1 accumulation through area results would imply that 0.59 n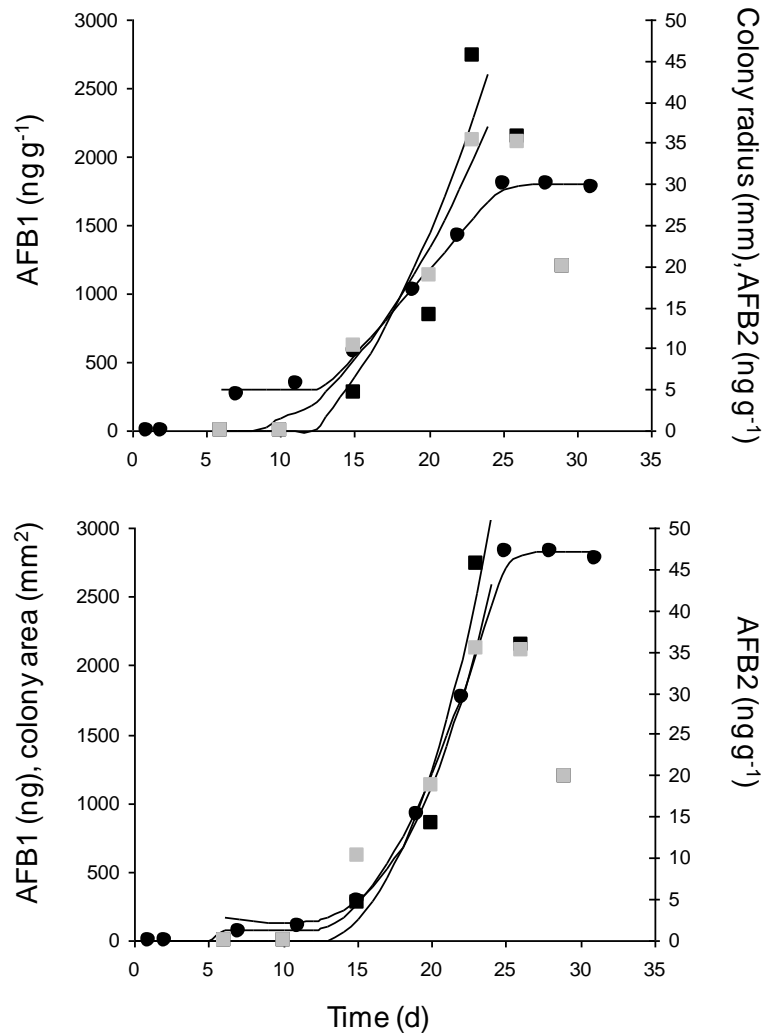g AFB_1 g^{-1} maize are accumulated per mm^2 of newly generated colony per day simultaneously to growth (similar to what would happen for primary metabolites), while 0.96 ng of AFB_1 g^{-1} maize are accumulated per mm^2 of existing colony (Table 3).

As conclusion, aflatoxin production may not present a clear delay in relation to growth. As a result, food safety strategies similar to those for *Staphylococcus aureus*, for which the risk of enterotoxin presence is linked to a certain level (10^4 cel g^{-1}) of microbial concentration in the foodstuff (Stewart et al., 2003), may not be applied here. Moreover, colony area showed better correlation with aflatoxin accumulation than the other parameters, including biomass, thus looking at the modelled results the next step would be producing alpha and beta values under different growth conditions, in order to generate secondary models which allow predicting alpha and beta as a function of i.e. water activity and temperature. This is a first approximation to modelling of mycotoxin production; however, alpha and beta values should be regarded with care as a reduced number of experimental points in the linear growth phase were included.

Acknowledgements

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4. Discussion

Filamentous fungi and their mycotoxins are often found as contaminants in agricultural products, before or after harvest as well as during transportation and storage. Different strategies to prevent mould and mycotoxin contamination have been developed and generally are divided into pre-harvest and post-harvest strategies. The most common approach in pre-harvest is the use of resistant varieties, crop rotation, herbicides, insecticides and biological and chemical antifungals. However, the efficacy of these techniques depends on weather conditions in field. Regarding post-harvest, control of a_w, temperature, use of controlled atmospheres, and different chemical or biological products are used to control moulds development. In this case, environmental conditions are more controlled and implementation of control strategies is easier.

4.1 Hydroalcoholic plant extracts effect on mycotoxigenic moulds development

4.1.1 *Equisetum arvense* and *Stevia rebaudiana* extracts effects on mycotoxigenic moulds growth and toxin production in post-harvest maize

In chapters 3.1, 3.2 and 3.3 we studied antifungal and antimycotoxigenic effect of extracts from *E. arvense* and *S. rebaudiana*, both in *in vitro* and *in vivo* assays, at different concentrations and environmental conditions (temperature and a_w). In both kinds of experiments, conditions studied were near to conditions which may occur in cereals in late ripening and post-harvest, but did not include optimum growth conditions for these moulds.

The antifungal activity of *S. rebaudiana* and *E. arvense* extracts was dose-dependent (chapter 3.1). *E. arvense* extract was the most effective for mould inhibition, and the use of an extract of 115% w/v (115g of plant per 100 mL of water) applied to a final concentration of 3% w/v (3 gram of plant per 100 g of media) was the most effective under all conditions tested, reaching 100% growth inhibition for *A. carbonarius* and *F. graminearum*. *S. rebaudiana* extract showed lower antifungal activity compared to *E. arvense* extract for all isolates tested and it had no effect against *A. carbonarius* and *A. westerdijkae*.

In our study, propionic acid was used as a control to compare the efficacy obtained with the different plant extracts because this acid is commonly used as an antifungal in stored cereals; only *E. arvense* extract applied at 3% led to results comparable to those obtained with this acid. Moreover, high inhibition of growth due to the application of both extracts was observed in maize agar medium at marginal conditions for growth, mainly storage conditions. Results obtained in maize grain (chapter 3.2) confirmed that the extract of *E. arvense* at 115% w/v applied to a final concentration of 3% w/w and a mixture 1:1 of *E. arvense*-*S. rebaudiana* (3%) extracts may be effective for the inhibition of growth of *A. flavus* and *F. verticillioides*. However, oppositely to what was observed in agar medium, the maximum growth inhibition was observed at the higher water activity levels assayed (closer to preharvest conditions). Finally, *E. arvense* extract reduced 36% and 45% the populations of *Fusarium* section *Liseola* and *Aspergillus*

section *Flavi,* respectively; when the initial spore load in maize grain was high, while the effect could sometimes not be observed when the initial contamination was lower. Overall, a decreased antifungal activity was observed from the *in vitro* experiments to the *in vivo* ones. Quiroga et al. (2001) found an inhibition of fungal growth between 80-90 % for *Trichoderm*a spp., 60-80 % for *Fusarium oxysporum* and *Penicillium notatum* and >90% for *A. niger* in presence of *Equisetum giganteum* extract, however it was made only in *in vitro* test.

For both, *in vitro* and *in vivo* assays, results of the effect of the extracts on mycotoxins production were not conclusive. While for agar experiments, a decreased mould growth could lead indirectly to a decrease of total mycotoxin accumulation; in the experiments with maize the quantification of mycotoxin concentration in the grain revealed that the effect of the extracts was not significant. Most researchers used either agar or broth dilution series to assess antimicrobial activity of spices, herbs and their extracts and/or essential oils (EOs), and in some cases both assays for comparative purposes, because antimicrobial performance in the two systems can vary (Holley and Patel, 2005). However, results obtained on culture media cannot necessarily be extrapolated to natural ecosystems where there are other factors that influence on the mould growth and toxin production. Nevertheless, some authors have already used fruit and vegetable model media to investigate extract and/or EO efficacy (Cerrutti and Alzamora, 1996; Del Campo et al., 2000; Hsieh et al., 2001; Ultee and Smid, 2001; Valero and Salmeron, 2003) or applied their extract and/or EOs directly in food material as lemon, grape, orange, nectarine and apricot (Gatto et al., 2011; Sayago et al., 2012). In most of these cases the efficacy of extract and/or EO decreased in the food model media or food, compared with the *in vitro* control media. Holley and Patel (2005) concluded that the presence of fat, carbohydrate, protein, salt and pH reaction influence the effectiveness of these agents in foods and antimicrobial potency of these compounds is also reduced in foods with lower water activity.

4.1.2 *Phenolic composition of E. arvense and S. rebaudiana hydro-alcoholic extracts*

Plant extracts contain different antioxidant compounds such as polyphenols, phenols, flavonoids, etc. which could be the bioactive basis responsible for the antimicrobial property (Ebana and Madunagu 1993; Lemus-Mondaca et al., 2012; Pattnaik et al., 1997; Radulovic et al., 2006; Singh et al., 2011; Weidenbörner et al., 1989). For this reason we analyzed our plant extracts.

E. arvense and *S. rebaudiana* hydro-alcoholic extracts obtained in our laboratory were analyzed by HPLC-DAD and HPLC-MS/MS for flavonoids and phenolic acids detection and quantification (Table 1). Principal compounds of *E. arvense* extract were kaempferol-3-*O*-rutinoside-7-*O*-glucoside and kaempferol dihexoside, which are flavonoid compounds with known antioxidant properties (Le Gall et al., 2003; Singh et al., 2011). Other authors reported a similar range of flavonoids and phenolic acids in *E. arvense*

extracts (Milanovic et al., 2007; Mimica-Dukic et al., 2008; Oh et al., 2004; Radulovic et al., 2006; Sandhu et al., 2010). Similar compounds were detected by Veit et al. (1995) by HPLC analysis in some species of *Equisetum*; kaempferol 3-O-rutinoside-7-O-glucoside being the main compound in *Equisetum pratense* extracts. The main compound of *E. arvense* extract, however, was different depending of geographic origin of the plant material, but quercetin 3-0-(6"-O-malonylglucoside) was found to be abundant in all samples examined. Singh et al. (2011) studied the effect of kaempferol-7-O-glucoside against bacterial and fungal growth and was effective against *A. flavus*, *Aspergillus niger*, *Fusarium moniliforme* and *Rhizoctonia bataticola*. In our case, *E. arvense* extract also contained a high concentration of dicaffeoyl-meso-tartaric acid and to our knowledge there is no publication about this compound against mycotoxigenic moulds.

Regarding phenolic compounds found in *S. rebaudiana* extract, isomers of caffeoylquinic acid were the major constituents. To our knowledge, there is no publication regarding antimicrobial properties of this compound. Abou-Arab and Abu-Salem (2010) studied the total amount of phenolic acids and flavonoids in *S. rebaudiana* (24.01 and 18.93 mg/g dry weight basis of leaves and 33.99 and 30.03 mg/g dry weight basis of callus, respectively). They also studied antifungal activities of *S. rebaudiana* leaves and callus extracted by six types of solvents (acetone, chloroform, hexane, methanol, ethyl acetate and water) and they found a good inhibition for *Aspergillus ochraceus*, *Aspergillus parasiticus*, *A. flavus* and *Fusarium*. However, antifungal study was made only in culture medium (potato dextrose agar).

Other important phenolic compounds (and their derivatives) found in both extracts were quercetin and caffeic acid; these compounds have shown to completely inhibit growth and aflatoxin production by both *A. flavus* and *A. parasiticus* (Aziz et al., 1998), growth and ochratoxin A production by *A. carbonarius* (Romero et al., 2009), and were effective against *A. flavus*, *A. niger*, *F. moniliforme* and *R. bataticola* (Singh et al., 2011).

Table 1. Retention time, maximum wavelength in the visible region (λ max), spectral mass data and identification and concentration of phenolic compounds in *E. arvense* and *S. rebaudiana* liophylised hydro-alcoholic extracts.

Rt (min)	λ_{max} (nm)	Molecular ion [M-H]⁻ (*m/z*)	MS² (*m/z*)	Tentative identification	µg/mg
*Equisetum arvense*
7.0	354	787	625, 463, 301	Quercetin 3-*O*-rutinoside-7-*O*-rhamnoside	0.77 ± 0.01
7.7	344	771	609, 447, 285	Kaempferol-3-*O*-rutinoside-7-*O*-glucoside	10.84 ± 0.21
8.9	358	787	625, 463, 301	Quercetin trihexoside	0.14 ± 0.01
9.2	328	355	193	Ferulic hexoside acid	0.14
12.5	330	179	135	Caffeic acid	1.82 ± 0.09
15.1	372	423	261	Equisetumpyrone	-
15.7	352	625	463,301	Quercetin 3-*O*-sophoroside	0.85 ± 0.01
16.7	328	309	193,178,149,135	Ferulic acid derivative	0.26 ± 0.04
17.9	348	609	447, 285	Kaempferol dihexoside	7.48 ± 0.12
18.5	328	193	178, 149, 135	*trans*-Ferulic acid	1.19 ± 0.03
20.5	356	463	301	Quercetin 3-*O*-glucoside	0.46 ± 0.02
21.8	354	505	463,301	Quercetin acetyl hexoside	0.55 ± 0.01
22.6	352	447	285	Kaempferol 3-*O*-glucoside	0.15 ± 0.01
*Stevia rebaudiana*
7.6	326	391	179, 173, 135	Caffeic acid derivative	0.83 ± 0.12
9.9	326	515	353, 191, 179, 173, 135	Dicaffeoylquinic acid isomer	4.47 ± 0.29
10.7	326	353	191, 179, 173, 135	5-*O*-Caffeoylquinic acid	2.08 ± 0.24
11.4	326	353	191, 179, 161, 135	Caffeoylquinic acid isomer	9.48 ± 0.70
12.9	324	179	135	Caffeic acid	0.29 ± 0.01
16.0	356	609	301	Quercetin-3-*O*-rutinoside	5.42 ± 0.04
16.9	330	515	353, 191, 179, 173, 135	Dicaffeoylquinic acid isomer	3.84 ± 0.08
17.8	328	353	191, 179, 173,161, 135	Caffeoylquinic acid isomer	36.67 ± 1.49
18.1	362	433	301	Quercetin pentoside	2.08 ± 0.04
18.6	354	447	301	Quercetin 3-*O*-rhamnoside	9.56 ± 0.21
18.8	326	515	353, 191, 179, 173,161, 135	Dicaffeoylquinic acid isomer	18.92 ± 1.40
19.6	350	417	285	Kaempferol pentoside	0.34 ± 0.02
20.2	334	515	353, 191, 179, 173, 135	Dicaffeoylquinic acid isomer	1.36 ± 0.01
20.7	334	515	353, 191, 179, 173, 135	Dicaffeoylquinic acid isomer	0.57 ± 0.03
21.0	346	431	285	Kaempferol rhamnoside	0.67 ± 0.02
24	328	515	353, 191, 179, 173, 135	Dicaffeoylquinic acid isomer	1.62 ± 0.05

Results presented in these chapters confirm that *E. arvense* extract was effective for the control of toxigenic moulds growth. *E. arvense* extract could be applied as part of an integrated strategy for alternative control of aflatoxigenic and fumonisin-producing mycobiota in intermediate moisture maize grain.

4.2 Predictive models as a tool to prevent mycotoxigenic moulds growth

Two main modelling approaches may be applied for prevention of mycotoxin accumulation in food commodities. The first approach involves obtaining experimental mycotoxin data over time and modelling of the kinetics of mycotoxin accumulation; the second one consists of predicting and preventing any growth of mycotoxigenic species, as an indirect way of preventing mycotoxin accumulation. This section deals with this second alternative, but we will come back to the first one later.

4.2.1 Kinetic predictive models for growth

Growth is a variable which presents less intraspecific variability than mycotoxin production, and kinetics of growth are more known, thus the best alternative to prevent mycotoxin accumulation might be prediction and prevention of growth (Marín et al., 2008b).

In the last years, many published models have been built based on growth data produced on solid media adjusted to different a_w and incubated at different temperatures (Table 2). From chapter number 3.4 we concluded that predictive models performance is very affected by marginal conditions, including storage conditions of foods and feeds. The risk of using the models presented in this chapter in real situations may be the differences in the initial inoculum size and the unrealistic constant conditions of temperature and moisture content. These points, together with the use of a high number of isolates, were taken into account to plan the following experiments (chapters 3.5, 3.6, 3.7, 3.8 and 3.9).

However, generally, existing works in predictive mycology were carried out under good conditions for growth, with a high inoculum level and with a few isolates. In the present work the following questions were tried to be answered to some extent:

a) Food and feeds are usually contaminated by a limited number of fungal spores, thus are kinetic models dependent on the initial inoculum size?

b) Are kinetic models obtained dependent on the isolates or strains used to develop them?

c) How do kinetic models perform under marginal conditions for growth?

d) Could it be possible to apply kinetic models developed at constant temperature to predict growth under fluctuating temperatures?

Table 2. (part 1 of 2) Kinetic primary and secondary models applied to fungal growth present in the literature.

Model	Fungal species (number of isolates)	a_w range	Temperature range	Inoculum size (spores)	References
Baranyi Polynomial	*A. flavus* (3), *A. oryzae* (3), *A. parasiticus* (3), *A. nomius* (3)	0.82-0.99	30 ºC	No details	Gibson et al. (1994)
Linear	*F. moniliforme* (2); *F. proliferatum* (4)	0.90-0.994	4-45 ºC	$1\text{-}5 \times 10^5$	Marin et al. (1996)
Linear	*A. ochraceus* (3), *A. flavus* (1), *A. niger* (1), *P. aurantiogriseum* (1), *P. hordei* (1),	0.75-0.994	5-45 ºC	$1\text{-}5 \times 10^5$ and agar plug	Marin et al. (1998)
Baranyi Polynomial	*P. roqueforti* (1)	0.11-0.98	25 ºC	No details	Valik et al. (1999)
Baranyi	*Byssochlamys fulva* (1), *Neosartorya fischeri* (1), *Talaromyces avellaneus* (1),	0.85-0.95	25 ºC	A single cleistothecium or ascus	Valik and Piecková (2001)
Linear Polynomial	*P. chrysogenum* (1)	0.75-0.85	15-25 ºC	$1\text{-}5 \times 10^4$	Sautour et al. (2001)
Linear Polynomial	*Wallemia sebi* (cocktail of 4)	0.77-0.96	25 and 30 ºC	$1.7\text{–}2 \times 10^4$	Patriarca et al. (2001)
Baranyi Polynomial Rosso cardinal Gamma concept	*Monascus ruber* (1)	0.93-0.97	20-40 ºC	1×10^4	Panagou et al. (2003,2007)
Linear	*E. rubrum* (1), *E. repens* (1), *W. sebi* (1), *A. penicillioides* (1), *P. roqueforti* (1), *Chrysosporium xerophilum* (1), *Xeromyces bisporus* (1)	0.70-0.92	25, 30 and 37 ºC	No details	Gock et al. (2003)
Linear Polynomial	*A. ochraceus* (3)	0.8-0.99	10, 20, and 30 ºC	$1\text{–}5 \times 10^4$	Pardo et al. (2004)
Linear Ratkowsky square-root Polynomial	*A. niger* (2)	0.90-0.99	25, 30 and 35 ºC	9×10^4	Parra and Magan (2004)
Linear Polynomial	*A. carbonarius* (2), *A. niger* aggregate (2)	0.90-0.98	25 ºC	1×10^3	Bellí et al. (2004a)
Linear Polynomial	*A. carbonarius* (9), *A. niger* aggregate (8), *Aspergillus* section *Nigri* uniseriate (5)	0.90-0.995	10-37 ºC	1×10^3	Bellí et al. (2004b)

Table 2 (part 2 of 2). Kinetic primary and secondary models applied to fungal growth present in the literature.

Model	Fungal species (number of isolates)	a_w range	Temperature range	Inoculum size (spores)	References
Linear Polynomial	*A. carbonarius* (8)	0.90-0.99	15-37 ºC	1×10^3	Bellí et al. (2005)
Baranyi Arrhenius-Davey	*F. verticillioides* (1), *F proliferatum* (1)	0.810 and 0.985	15, 22, 25, and 30 °C	agar plug	Samapundo et al. (2005)
Linear Polynomial	*A. ochraceus* (3)	0.88, 0.90, 0.94, 0.96, 0.98 and 0.99	10, 20, and 30 °C	$1\text{-}5 \times 10^3$	Pardo et al. (2005a)
Linear Polynomial	*A. ochraceus* (3), *P. verrucosum* (3)	0.75-0.99	10, 20 and 30 °C	1×10^2 and 1×10^3	Pardo et al. (2005b, 2006)
Linear	*P. verrucosum* (3)	0.75-0.995	10-25 ºC	5×10^3	Cairns-Fuller et al (2005)
Linear Polynomial	*P. expansum* (1)	0.89–0.98	5–25 °C	5×10^4	Lahlali et al (2005)
Linear	*P. italicum* (1); *P. digitatum* (1)	0.89-0.98	5–25 °C	1×10^4	Lahlali et al (2006)
Gompertz Polynomial	*A. carbonarius* (4)	0.96	7-42 ºC	5×10^3	Marín et al. (2006a)
Gompertz Polynomial	*P. expansum* (2)	0.83-0.99	3-29 ºC	5×10^2	Marín et al. (2006b)
Linear Polynomial Ratkowsky square-root Arrhenius-Davey	*P. expansum* (6)	0.991	2-30 ºC	2×10^3 and 2×10^6	Baert et al. (2007)
Baranyi	*R. oryzae* (1)	0.895 and 0.99	20 and 30 ºC	No details	Carrillo et al. (2007)
Arrhenius-Davey	*A. flavus* (1) *A. parasiticus* (1),	0.801-0.982	16-37 ºC	agar plug	Samapundo et al. (2007)
Baranyi Arrhenius-Davey Rosso cardinal Polynomial	*A. carbonarius* (2)	0.85-0.98	10-40 ºC	5×10^3	Tassou et al. (2007b)
Linear	*P. expansum* (2)	-	4, 12, 25 ºC	$2 \times 10^{1\text{-}6}$	Baert et al. (2008)
Baranyi Rosso and Robinson	*A. flavus* (2)	0.85-0.97	25 ºC	5×10^3	Marín et al. (2009)
Rosso et al. (1993) Sautour et al. (2001) Gamma concept	*B. cinerea* (1) *P. expansum* (1)	0.85-0.99	2-30 ºC	1×10^4	Judet-Correia et al., (2010)
Baranyi Polynomial Arrhenius-Davey	*A.* section *Nigri* (1)	0.97-0.99	17-22 ºC	1×10^3 spores ml^{-1} of liquid culture	Silva et al. (2010)

4.2.1.a Kinetic predictive models as affected by inoculum level

Traditionally growth and ecophysiological characterization of fungal contaminants of foods has been determined using high inoculum levels in the form of spore suspensions or circular disks cut from the margins of growing colonies (Samapundo et al., 2007). It may actually be that contamination at low inoculum levels or by individual spores plays a more important role in fungal infection, spoilage of foods and subsequent production of mycotoxins of public and animal health significance than previously thought (Samapundo et al., 2007).

Excluding no-growth observations, different inoculum levels from 1 to 100 spores did not significantly affect growth rate of *A. carbonarius* and *P. expansum* in chapter 3.5. However, predicted time before growth was in general longer with smaller inoculum size. This is an important point because lag time is a parameter much more useful than growth rate in food safety and food spoilage. A higher number of spores would increase the probability of including individual spores with higher readability for germination and growth. Sautour et al. (2003) concluded that the lag time is strongly dependent on the inoculum size, thus inoculum size should be standardized in order to assess the shelf-life of food products. Thus for modelling purposes, low inoculum sizes should be used in order to predict the lag time before growth, while this point is not so important for growth rate prediction. Therefore, using high inoculum levels to estimate the growth parameters will result in estimating a worst case scenario, with faster growth and consequently shorter safe time.

4.2.1.b Kinetic predictive models as affected by intraspecies differences

Individual strains exhibit differences in their growth and in the amount of mycotoxin produced (Romero et al., 2010). These differences can be attributed to geographical origin, substrate, environmental conditions, molecular characteristics, etc. Thus, results obtained with a little number of isolates might not be representative for the majority of the strains. However, studies on ecophysiological adaptation to different environmental conditions have been frequently carried out with a few isolates (eg; Astoreca et al., 2007. Bellí et al., 2004 ab, 2005; Esteban et al., 2006ab; Kapetanakou et al., 2009; Tassou et al., 2007a).

In chapters 3.6 and 3.7 we compared growth parameters [μ (mm/day) and λ (d)] of thirty isolates of *A. carbonarius* and seventy nine of *P. expansum* to study intraspecific differences within isolates. In both cases, significant differences among isolates were found and these differences were dependent on environmental conditions ($p < 0.05$). Besides, coefficients of variation (CV%) revealed a high variability among growth parameters between isolates and replicates of the same strain specially under non optimal temperature for growth. Mainly, for *P. expansum*, the wide dispersion of λ (d) is worrying because the estimation of this parameter is crucial to determine the extent of cold storage in apples and other fruits

which this mould can contaminate. These results indicate that under limiting temperature for growth more isolates may be necessary to estimate growth parameters of a given species with a minor variability of data.

Some authors used a cocktail inoculum to minimize the variation that might be expected between different isolates of the same species (eg. Hocking and Miscamble 1995; Patriarca et al., 2001; Pose et al., 2009; Romero et al., 2007; 2010). Although this methodology can be criticized because of the loss of information regarding the responses of individual strains of a species, it is accepted as a legitimate method of achieving a "worst case" scenario (Hocking and Miscamble 1995). However, the use of cocktail inoculum could be important to decrease the size of experiments. Nevertheless, this point should be clarified and much more effort is requested for the building of predictive models simulating real situations.

As a consequence of the existing variability within isolates and replicates, there is a need to standardize the number or isolates and replicates to be used in predictive mycology to obtain reliable predictions. Data sets obtained for 30 isolates of *A. carbonarius* (10 replicates each) and 62 of *P. expansum* (12 replicates each) were included in a computational algorithm in order to obtain an estimation of the growth parameters that one would obtain using a lower number of isolates and /or replicates. Results in this study confirmed that increasing the number of isolates is much more effective than increasing the number of replicates, in terms of increasing the probability to obtain the same results of growth parameters. In particular, 12-17 isolates of *A. carbonarius* led to the same growth parameters as the total 30 (p=0.05) or 9 isolates with p=0.20; similarly, 25-30 isolates of *P. expansum* led to the same growth parameters as the total 62 (p=0.05) or 18-21 isolates with p=0.20 (chapter 3.8).

From the results in this chapter, a potential user may determine whether or not including one more replicate in the experimental design will certainly contribute to a better estimation of growth parameters; an scenario where more replicates do not lead to better estimations might be quite common according to our results. Replicates variability accounted for 2 to 44% of the total variability; this latter value is quite high but reflects the intrinsic biological variability of fungi in their responses to the environment.

4.2.1.c Kinetic predictive models as affected by marginal conditions

One of the most important aspects of predictive model development is ensuring that predictions are applicable to real situations. However, most predictive models have been developed to quantify the effect of these factors on fungal growth including optimal conditions (Garcia et al. 2009) (Table 2). By contrast, in food mycology it is important the use of predictive models developed under suboptimal water activities, and under cool to room temperature.

Regarding primary models, fitting growth data (colony diameters) to Baranyi and Roberts (1994) model resulted in poor goodness of fit under suboptimal conditions (chapter 3.5) due, in part, to high differences in growth among replicates under these conditions. The existence of no-growth observations results in a wide distribution of kinetic parameters; these observations should be included in the models for a better goodness of prediction of such models, although their inclusion compromises the goodness of fit. *A. carbonarius* showed the higher variability of both growth rate and lag phase under suboptimal conditions of both a_w and temperature, while for *P. expansum* this only occurred under limiting conditions of water activity (chapter 3.5). The effects of a_w and temperature on the distribution of radial growth rate and lag time for growth of single spores of *A. flavus* and *F. verticillioides* were assessed by Samapundo et al. (2006). As in our case, all the distributions were normal and the standard deviations increased with decreasing a_w, but the effect of temperature on the distribution was less clear. However, no limiting conditions of growth were included in the study and no no-growth cases were recorded.

Moreover, under some suboptimal conditions of growth colony diameter increase followed initially a sigmoidal trend, however, when growth seemed about to cease a second growth phase was observed at a near to constant rate, although slower than the initial one. This trend was also observed in chapter 3.10 when *A. flavus* grew at 0.90 a_w. Thus, Baranyi and Roberts (1994) model was not fully applicable and a secondary growth rate was then defined (chapter 3.5). The reason why this fact was not reported in the literature before may well be that growth data were recorded for short time periods.

Regarding secondary models, the cardinal model proposed by Rosso and Robinson (2001) seemed the more appropriate to model growth rate as a function of a_w and temperature. The model, as applied in chapter 3.4, is based on the gamma concept, that is, independence among factors is assumed, thus a unique T_{min} is estimated, regardless of the a_w level, and a unique a_{wmin} is estimated, regardless of the temperature level. As a consequence, good predictions are expected in the growth area (as long as about 8 levels of a_w and temperature are tested in the 'growth' domain), while predictions are more biased when approaching the growth/no-growth boundary.

4.2.1.d Predicting growth under fluctuating temperature from growth data collected at constant temperature

Fluctuating temperature occurs to a limited extent during transport and storage of raw materials; however this fluctuation of temperature can be recorded through time by sensors and data loggers and such data might be used to predict fungal growth occurrence. Temperature fluctuation acquires a capital importance in the field situation where it is determinant for the extent of fungal attack in crops.

Predictive models, as presented in this thesis, are developed under constant temperature values, and they may be directly applied to storage and transport operations under the assumption of non-fluctuating temperature.

In chapter 3.9, the hypothesis was that growth under fluctuating temperature could be predicted by addition of the diameter increases occurring under the temperature level recorded at each time interval. A field situation for soybean crop in Argentina was simulated, and growth rate of *F. graminearum* and *F. verticillioides* in soya beans were predicted. However, when growth obtained under fluctuating temperatures was compared to predicted growth by pooling growth at constant temperature, in general, the observed values were different from calculated ($p<0.05$) for both species. Therefore, temperature fluctuation influences on the colonization of *Fusarium* species in the field, and extrapolation from constant condition to real field conditions must be done with extremely care.

4.2.2 Predictive probability models applied to mould development

Although those secondary models pointed out in section *4.2.1.c* have been used to describe growth of mycotoxigenic fungi, their drawbacks when used under limiting conditions of growth, commonly encountered in the food safety issues, make us search for alternatives such as probability models. In many situations it is important to ensure that microorganisms do not grow in foods. Probability models are particularly useful when pathogenic or mycotoxin-producing species are involved. Probabilistic growth models are built from the proportion of "growth/no growth" responses throughout the experimental design space at a defined point in time (Brul et al., 2007). If predictions need to be done in the long term, long experiments have to be designed for building appropriate models.

Few studies report on the use of probability models for prediction of mould growth or mycotoxins production. Battey et al. (2001) worked with logistic regression to predict the probability of growth of spoilage moulds (*A. niger* and *Penicillium spinulosum*) in response to various preservative systems in ready to drink beverages at different levels of pH, titratable acidity and sugar content. The boundary between growth and non growth of *E. chevalieri* in milk jam as a function of a_w, pH, potassium sorbate and storage time was predicted by means of the probabilistic model using logistic regression, which provided a wide range of formulation possibilities depending on the targeted shelf life (Char et al. 2005). The same methodology has been applied by Marín et al. (2008b) to develop suitable validated models to predict the growth and OTA production boundaries by an *A. carbonarius* isolated from pistachios as a function of moisture content and storage temperature of pistachios. Probability model was applied for the first time in this work to mycotoxin accumulation. Also, Tassou et al. (2009) developed and validated a probabilistic model to predict OTA production boundaries for two *A.carbonarius* isolates on a synthetic grape juice medium as a function of a_w and temperature. It must be noted that polynomial type logistic regression models are empirical in nature and do not contribute to the understanding of the mechanism involved in microbial growth inhibition. However, this type of models offer the possibility to include the no-growth responses, which are particularly important when efforts are conducted to prevent an event, such as fungal growth, providing thus a significant degree of quality and safety from spoilage (López-Malo et

al. 2000). Nevertheless, Ratkowsky and Ross (1995) modeled growth/no growth of *Shigella flexneri* through logistic regression with equations which contained biological meaningful terms (pH, temperature, a_w, and gas concentration). This approach may be of interest in predictive mycology, too.

Probability models were not much affected by inoculum size used to build them in chapter 3.5. However, lower probabilities of growth were in general predicted under marginal conditions at a given time for both strains studied in this chapter (*A. carbonarius* and *P. expansum*). The slopes of the probability curves were smaller under suboptimal growth conditions due to wider distributions. Results obtained in chapter 3.4 indicate that the developed probability models could be satisfactorily employed to quantify the combined effect of temperature and a_w on the growth responses of *A. ochraceus* and *A. parasiticus*. Prediction of growth probability under extreme growth conditions was somewhat compromised, but it could be considered acceptable. Results obtained in both chapters showed that probabilistic models are less sensitive to marginal conditions and inoculum size than kinetics models. Thus, they could be used in real situations including marginal conditions and low inoculum level.

4.3 Predictive models as a tool to prevent mycotoxins accumulation

4.3.1. Mycotoxins production as affected by intraspecifics variability and marginal environment conditions

As pointed out in section *4.2.1.b*, given the different abilities to synthesize mycotoxins by the different strains of a given species, extrapolation from the models obtained with one or several strains might not be representative of the majority of the strains (Marín et al., 2008a). For this reason, in chapter 3.6 and 3.7 we studied OTA and patulin production of several isolates of *A. carbonarius* and *P. expansum*, at different conditions including marginal ones. In both chapters, we observed a high variability in mycotoxin level among isolates, and variability increased under marginal conditions. In general, coefficient of variation (CV%) for OTA production by *A. carbonarius* was very high and in contrast to growth, the highest variation was at low a_w level. However, variability under near to optimal condition was similar to marginal temperature for growth. CV% of patulin production by *P. expansum* was smaller than of OTA production and also the highest CV% was found under suboptimal condition. This means that limiting environmental conditions increase variability in mycotoxin levels between isolates of the same species and this factor should be considered for further building of predictive models.

4.3.2 Kinetic predictive models for mycotoxin production: a first approximation

From the food safety point of view, mycotoxins are the target to be modeled because moulds and yeast themselves may cause spoilage but have no safety implications. But prediction of a certain mycotoxin level may be particularly difficult because mycotoxin level is mostly a function of the contaminating fungal

strain apart from the environmental conditions which may be included in a model. Due to high variability in toxin production by the strains and the lack of a clear trend of mycotoxin production along time. few mathematical models have been developed on mycotoxins. Molina and Giannuzzi (2002) modeled aflatoxin accumulation in a solid media using a non-linear model. They had a good correlation between model predictions and experimental data.

Several other drawbacks are associated to model directly mycotoxin levels:

- To develop such models a huge number of mycotoxin analyses are required, involving work and economic costs.

- There is a high variability in the mycotoxin production by a given strain in a given substrate (Marín et al., 2008b). Thus, mathematical modeling of mycotoxin formation may be particularly difficult.

- Although as secondary metabolites one may expect mycotoxin production to follow a curve paralleling that of growth but slightly delayed, regulation of secondary metabolism is poorly understood (Le Bars, 1988) and the relationship between the rates of primary and secondary metabolism is not clear (Pitt, 1993).

A. flavus and aflatoxin B_1 and B_2 were chosen to try to build a primary kinetic predictive model in chapter 3.10. This work confirms that aflatoxins B_1 and B_2 are positively correlated with *A. flavus* growth at all conditions studied. In this chapter aflatoxins were modeled by Luedeking-Piret equation. Moreover, colony area showed better correlation with aflatoxin accumulation than the other parameters, including biomass, and it was the preferred option as growth indicator in the Luedeking-Piret equation. A mixed-growth associated model may be required as a result of concomitant aflatoxins production with early growth, contrary to what can be expected from a secondary metabolite. Thus, looking at the modelled results the next step would be producing α and β values under different growth conditions, in order to generate secondary models which allow predicting α and β as a function of i.e. water activity and temperature. This is a first approximation to modelling of mycotoxin production,

A recent work by Abdel-Hadi et al. (2011) building a model for the prediction of aflatoxin B_1 production by *A. flavus* under different a_w and temperatures combining growth rate, aflatoxin B_1 and the expression of 10 genes involving in aflatoxin B_1 production. They also used Luedeking-Piret model and their results showed a good linear regression fit between observed aflatoxin B_1 production and predicted production based in the model.

4.4 Validation of predictive models

Validation is an essential step in predictive modelling. The first stage of validation, when proposing a new type of model is often internal validation (te Giffel and Zwietering, 1999) which means validation is performed on the same data used for building the model (Ratkowsky and Ross, 1995). However, further

external validation, using new data not used for fitting the model, would appear to be essential to confirm the robustness of the model (Delignette-Muller et al., 1994). Besides, predictive models are often built on data obtained in laboratory medium. Extrapolation to predictions in food products is not straightforward (Dalgaard and Jorgensen, 1998; McClure et al., 1993) due to the complexity of these media. Predictive models are often built under laboratory conditions with synthetic media and take a limited number of factors into account compared to the numerous factors influencing growth moulds in food products (Pinon et al., 2004). Therefore, a good way of validating a model is to compare its prediction to data obtained for food products. Models cannot be used with confidence until such a comparison is made and hence validation is an essential step enabling researchers to understand the applicable range of models and also limits of their performance (Jagannath and Tsuchido, 2003).

Very few studies in scientific literature have externally validated the models developed to describe fungal growth (Table 3).

Table 3. Validated kinetic secondary models and probability models.

Model	Substrate	Mould/Mycotoxin	Reference
Probability	Drink beverages	*A. niger* *P. spinulosum*	Battey et al. (2001)
Polynomial Arrhenius-Davey	Corn	*F. verticillioides F. proliferatum*	Samapundo et al. (2005)
Polynomial Arrhenius–Davey	Corn	*A. flavus* *A. parasiticus*	Samapundo et al. (2007)
Probability	Pistachio nuts	*A. carbonarius* and OTA	Marín et al. (2008b)
Probability	Simulated grape juice medium	*A. carbonarius* and OTA	Tassou et al. (2009)
Cardinal	Grape berries	*B.cinerea* *P. expansum*	Judet-Correia et al. (2010)
Polynomial Davey Cardinal Probability	Green coffee Peanut Corn	*A. ochraceus* *A. parasiticus*	Chapter 3.4

Kinetic secondary models developed for *A. ochraceus* and *A. parasiticus* were validated in chapter 3.4. In general, validation of kinetic results led to poor goodness of predictions. In the study, validation samples were placed near to the expected boundaries of the models in order to test them under the worst situation.

Validation of *A. ochraceus* model in coffee beans led to acceptable results under most conditions, while the models for *A. parasiticus* predicted too fast growth under the extreme conditions. Growth of both fungi in maize was in general much slower than predicted by the models. The optimum growth rate is very much dependant on the substrate thus probably explaining the bias and accuracy factors obtained for maize grains. The location of grains, beans and nuts in a single layer in Petri plates, which was the

method used for validation, is not a realistic situation, because the free spaces among particles may compromise fungal growth, making it slower. It is likely that resolution and accuracy of measurements in validation studies is lower than on agar plates due to the lack of homogeneity in the matrices tested.

On the other hand, validation of the probability model with independent data showed that the developed logistic model could satisfactorily predict the responses of both fungal species at probability level of 0.5. Specifically for validation samples placed near the expected boundaries of the model, the agreement with growth data in food matrices was 70% in coffee, 80% in peanuts and 30-40% for maize. Probability of growth prediction under extreme growth conditions was somewhat compromised, but it can be considered acceptable.

As a conclusion, kinetic models applied to predict mycotoxigenic mould growth as a combined function of a_w and temperature, may present some drawbacks under marginal conditions for growth, apart from being dependent on inoculum size and species. Alternatively, probability models are suitable to predict the growth-no growth boundary, although the effects of intraspecific variability, in this case need to be further tested.

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5. Conclusions

5.1 Control of fungal contamination through the application of natural products

- *Equisetum arvense* and *Stevia rebaudiana* extracts contain a series of phenolic compounds that could be responsible of their antimicrobial effect. In *in vitro* assays the extracts were effective against mycotoxigenic moulds and the *Aspergillus* and *Fusarium* isolates studied were completely inhibited by *E. arvense* extract at 3%.

- However, the effect of both extracts decreased on maize, especially at the lower a_w. In *in vivo* assays, the *Equisetum* extract was effective for the inhibition of growth of both *Aspergillus* section *Flavi* and *Fusarium* section *Liseola* at high water activity levels and with high infection levels. The levels of mycotoxins however, were not significantly affected.

5.2 Predictive models as a tool for prevention of fungal contamination

- Prediction of time to growth by kinetic models was clearly linked to inoculum size; low inoculum size would be desirable to better simulate real food conditions. For this reason, inoculum should be standardized for the modelling of time to growth and a higher number of replicates should be taken into account when low inoculum size is used.

- The performance of predictive models may be compromised under marginal conditions for fungal growth. The higher variability of results under some of these conditions results in the need for a higher number of replicates required, specifically for kinetic models.

- A high intraspecific variability on growth and mycotoxin levels has been proven for the isolates of *A. carbonarius* and *P. expansum*. For this reason, a great number of strains should be included to develop models representing the species. For both isolates, intraspecific variability among mycotoxin data was higher than among growth data. A decision support matrix was built from which the number of strains and replicates to be planned for new experiments can be assessed for a reliable estimation of growth parameters. It was concluded that increasing the number of strains in an experiment decreases the explained variability much more than including further replicates.

- Mould growth, and their subsequent mycotoxin production, under isothermal conditions seem to be different compared to cycling temperatures. As consequence, when growth and mycotoxin production under cycling temperatures were predicted from the results under constant conditions, observed values were different from calculated for mycotoxigenic species and substrate medium. Therefore, care should be taken if data at constant temperature conditions are to be extrapolated to real field conditions.

- Probabilistic models were affected by the inoculum size, but no general trend was observed; these models seemed to be less affected by low inoculum compared with kinetic models.

- Lower probabilities of growth were in general predicted under marginal conditions at a given time. The slopes of the probability curves were smaller under suboptimal growth conditions due to wider distributions.

- Validation of kinetic models led to good bias and accuracy factors under conducive conditions for growth, however poor goodness of prediction was observed under the marginal conditions. Nevertheless, probability of growth prediction under extreme growth conditions was somewhat compromised, but it could be considered as acceptable. Boundary models may be important in predicting the most suitable combinations of environmental factors to prevent fungal growth, providing thus a significant degree of quality and safety.

- A first attempt was done to model aflatoxins production as a function of growth parameters and time. Aflatoxins accumulation was shown to be better correlated to colony area than either colony diameter or fungal biomass. Luedeking-Piret model was used for this purpose, and reasonable percentages of variability were explained.

5.1 Control de la contaminación fúngica mediante la aplicación de productos naturales

- Los extractos de *Equisetum arvense* y *Stevia rebaudiana* contienen una serie de compuestos fenólicos que podrían ser los responsables del efecto antimicrobiano. En los ensayos *in vitro* los extractos fueron efectivos contra hongos micotoxigénicos y, las cepas estudiadas de *Aspergillus* y *Fusarium* se inhibieron completamente en presencia de *E. arvense* al 3%.

- Sin embargo, el efecto de ambos extractos disminuyó en maíz especialmente a bajos niveles de a_w. En los ensayos *in vivo* el extracto de *Equisetum* fue efectivo para la inhibición del crecimiento de *Aspergillus* sección *Flavi* y *Fusarium* sección *Liseola* en presencia de altos niveles de a_w e infección. No obstante, los niveles de micotoxinas no se vieron afectados significativamente.

5.2 Modelos predictivos como herramienta para la prevención de la contaminación fúngica y por micotoxinas

- La predicción del tiempo hasta el inicio del crecimiento mediante la utilización de modelos cinéticos fue claramente afectada por el tamaño del inóculo. Bajos niveles de inóculo serían ideales para simular de la mejor manera las condiciones reales de los alimentos, sin embargo, se debería trabajar con un alto número de réplicas a la hora de diseñar experimentos para la construcción de modelos predictivos.

- Los modelos predictivos pueden verse comprometidos bajo condiciones marginales del crecimiento fúngico. La gran variabilidad de resultados obtenidos en algunas de estas condiciones trae como consecuencia la necesidad de la utilización de un alto número de réplicas, especialmente para los modelos cinéticos de predicción.

- El crecimiento de los mohos y su consecuente producción de micotoxinas son diferentes bajo condiciones isotérmicas comparadas con los ciclos de temperaturas. Como consecuencia, cuando se predijo el crecimiento y la producción de toxinas en ciclos de temperatura a partir de los resultados obtenidos en condiciones constantes, los valores observados fueron diferentes de los calculados para las especies micotoxigénicas estudiadas y el medio de crecimiento. Por lo tanto, se debe tener cuidado cuando los datos a temperaturas constantes son extrapolados a condiciones reales de campo.

- Los modelos de probabilidad fueron afectados por el nivel de inóculo, pero no se observó una tendencia general del efecto del inóculo; estos modelos se vieron menos afectados por bajos niveles de inóculo fúngico comparados con los modelos cinéticos.

- Las probabilidades más bajas de crecimiento en general fueron predichas bajo condiciones marginales a un tiempo dado. Las pendientes de las curvas de probabilidad fueron más bajas en condiciones subóptimas de crecimiento debido a la amplia distribución de los datos.

- Con respecto a la validación de los modelos cinéticos se obtuvieron buenos índices de precisión y exactitud bajo las condiciones favorables de crecimiento, sin embargo se obtuvo una mala

validación en las predicciones realizadas bajo condiciones no óptimas de crecimiento. Del mismo modo, se vieron comprometidas las predicciones de probabilidad de crecimiento bajo condiciones extremas. Los modelos de crecimiento / no crecimiento pueden ser importantes para la predicción de las combinaciones más adecuadas de los factores ambientales para prevenir el crecimiento de los mohos, dando así un grado significativo de la calidad y seguridad de los alimentos.

• Se observó una gran variabilidad intra-específica en el crecimiento y los niveles de micotoxinas de *A. carbonarius* y *P. expansum*. Por esto, se debería incluir un gran número de cepas en representación de la especie para el desarrollo de modelos de predicción. En ambas cepas, la variabilidad intra-específica de los niveles de micotoxinas fue mayor que la variabilidad entre los datos de crecimiento. De éste último estudio, se concluyó que en un experimento el aumento en el número de cepas aumenta mucho más la variabilidad explicada que incluyendo más réplicas.

• Por último, se realizó un primer intento para modelar la producción de aflatoxinas en función de los parámetros de crecimiento y el tiempo. La acumulación de aflatoxinas mostró la mejor correlación con el área de la colonia que con el diámetro o la biomasa del moho. El modelo de Luedeking-Piret fue utilizado para la obtención del modelo de producción de toxina, obteniéndose razonables porcentajes de variabilidad explicada.

6. Future research

Taking into account the results obtained in this PhD thesis we suggest different objectives to undertake in the future.

There is a need for in depth study of probability models, in particular on the effect of intraspecies differences in their performance; studies including a large number of strains, both as single or cocktail inocula would be of interest. Moreover, the results on the effects of inoculum size on the performance of the probability models are not considered to be enough, thus they should be extended.

Finally, the accompanying microbiota was in no case taken into account in the development of models in this work, and this can be a point of interest.

Moreover, in parallel, the development of predictive models based on field weather and agronomic variables, is a tool with a growing interest and their application may be crucial for prevention of those mycotoxins which accumulate mainly in the field.

With respect to the application of plant extracts for the control of filamentous fungi and their mycotoxins, the identification of the active principle which led to good results in agar medium could be considered, and try to optimize the conditions for its application in food and raw materials.