UNIVERSITAT DE LLEIDA
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ECOFISIOLOGÍA DE CEPAS DE *FUSARIUM* PRODUCTORAS DE FUMONISINAS

ECOPHYSIOLOGY OF FUMONISIN-PRODUCING ISOLATES OF FUSARIUM

Tesi doctoral
Sònia Marín Sillué
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RESUMEN

Desde 1988, año en que se descubrieron las fumonisinas, toxinas de origen fúngico producidas básicamente por especies del género *Fusarium*, numerosos estudios toxicológicos han puesto de manifiesto su implicación en la problemática para la salud animal que se deriva del consumo de piensos y otros productos agrícolas contaminados por *Fusarium* sección Liseola por parte del ganado. Asimismo, se ha llegado a asociar de alguna manera el consumo directo de maíz contaminado, con el desencadenamiento de cancer esofágico en humanos, sin embargo la relación causa-efecto no ha sido demostrada.

Si bien la contaminación masiva de materias primas por fumonisinas por lo general se asocia a países subdesarrollados del continente africano, asiático y Sudamérica, estudios de incidencia llevados a cabo en otros muchos países han demostrado que en mayor o menor cantidad, las fumonisinas se hallan presentes en la mayoría de muestras de maíz para alimentación animal ensayadas. Este hecho ha dado lugar a la proliferación de estudios de todo tipo, dentro de los cuales se enmarca esta tesis, encaminados a dilucidar en qué momento y bajo qué condiciones se producen estas micotoxinas.

Los objetivos de la presente tesis han sido en primer lugar, determinar el impacto que, la a_w, la temperatura y los conservantes químicos, como factores abióticos, ejercen sobre la germinación, crecimiento y producción de FB_1 por *F. moniliforme* y *F. proliferatum* en maíz. En segundo lugar se ha determinado el efecto de las interacciones fúngicas, es decir, cómo influyen cepas fúngicas propias del maíz sobre el desarrollo y producción de FB_1 por las cepas de *Fusarium*, y su relación con el resto de factores abióticos. Posteriormente, a la vista de estos resultados, se ha pasado a evaluar el potencial que la manipulación de los factores abióticos puede tener como sistema de control del grano almacenado. Por último, se han ensayado la cuantificación de la producción de enzimas hidrolíticos por *Fusarium* sección Liseola, como método alternativo de detección de su presencia en grano, así como la determinación del valor calórico del grano contaminado por estas mismas cepas, como método alternativo de cuantificación de la pérdida de calidad del grano.

Se ha demostrado que a pesar de que las cepas de *Fusarium* son capaces de crecer en diferentes substratos, la producción masiva de fumonisinas únicamente se da en el maíz. Se ha visto que sobre este cereal la germinación de estas cepas es posible entre 5 y 37°C y a $a_w \geq 0,88$, el intervalo se estrecha ligeramente para el crecimiento (7-37°C, $a_w \geq 0,90$), y por último, y lo que es más importante, la producción de FB_1 solo es posible entre 10 y 37°C y a $a_w \geq 0,93$, con las consecuencias que este dato reporta desde el punto de vista del control en

maíz y productos derivados. Se ha demostrado que los conservantes a base de propionatos existentes en el mercado son útiles para el control del crecimiento de *Fusarium* spp. *in situ*, pero para el control de la producción de FB_1 sólo es eficaz a concentraciones $\geq 0{,}07\%$.

La presencia de otras cepas fúngicas en cultivos mixtos, en general, inhibió el desarrollo de *Fusarium*, siendo *A. flavus* y *A. niger* las especies que causaron mayor impacto. Su efecto, sin embargo, dependió de los factores abióticos involucrados. En general, las especies de *Fusarium* compitieron con éxito a a_w altas, y mejor a temperaturas cercanas a los 15°C. Sin embargo, se observó que a a_w altas, la producción de FB_1 podía verse potenciada por determinadas especies, principalmente *A. niger*.

Finalmente, además de la determinación de fumonisinas en materias primas, se ha visto que la monitorización de la actividad enzimática en el grano, y de la pérdida de valor calórico del mismo pueden ser buenos índices de la infección del grano por *Fusarium*.

En conclusión, de la presente tesis se deriva que, en condiciones normales, el control de la contaminación por fumonisinas del grano en post-cosecha puede llevarse a cabo con éxito por el simple control de la a_w; en esta etapa la flora acompañante puede incluso actuar como factor adicional de control. Sin embargo, cabe resaltar que a partir de estos mismos resultados se puede deducir que la presencia de fumonisinas en maíz puede ser ya muy alta en el campo, y que años lluviosos puedan provocar niveles altos de las mismas antes del secado.

* INDICE *

I

INTRODUCCIÓN GENERAL

En el pasado, la investigación en el campo de los cereales almacenados, ha puesto el énfasis en la mejora del estado sanitario y del vigor de dichas semillas, con el fin de mejorar los rendimientos (Magan y Lacey, 1989). Se le ha prestado relativamente poca atención a las pérdidas en post-cosecha; las pérdidas en esta etapa pueden llegar a ser del 50% (National Academy of Sciences, USA, 1978). Los principales causantes de dichas pérdidas son roedores, insectos y hongos.

Mediante la comprensión del efecto que los factores ambientales ejercen sobre las especies fúngicas responsables del deterioro del grano, puede ser posible la manipulación del entorno del grano con el fin de conseguir un grano de calidad, prevenir la contaminación por micotoxinas y conseguir periodos de almacenamiento más largos (Magan y Lacey, 1989).

Fusarium moniliforme Sheldon es uno de los más predominantes mohos asociados con el maíz (*Zea mays L*) destinado al consumo humano y animal en todo el mundo (Marasas *et al.*, 1984a). *F. moniliforme* es un fitopatógeno presente en el suelo que se encuentra en todas las regiones productoras de maíz. Puede infectar las plantas de maíz sin provocar síntomas evidentes, e infectar también el grano. Es común encontrar lotes de maíz con un 100% de granos infectados (Marasas *et al.*, 1984a). Este microorganismo invade los tejidos del grano justo debajo del pericarpio, en la región apical. *F. proliferatum* también infecta mayoritariamente el maíz comercial.

Las fumonisinas son micotoxinas con propiedades carcinogénicas que son vehiculadas a través de la dieta humana y animal. Fueron aisladas (fumonisina B_1 y B_2) por primera vez de cultivos de *F. moniliforme* MRC 826 por Gelderblom *et al.* (1988a), en Sudáfrica. Las estructuras de estas fumonisinas fueron caracterizadas por Bezuidenhout *et al.* en 1988.

Thiel *et al.* (1991a) confirmó que *F. proliferatum* era capaz de producir FB_1 y FB_2, tal y como había apuntado Ross *et al.* (1990), y demostró que *F. nygamai* también era productor. Las fumonisinas son producidas por las siguientes especies fúngicas (Scott, 1993): *F. moniliforme, F. proliferatum, F. anthophilum, F. subglutinans, F. dlamani, F. napiforme, F. nygamai* y *Alternaria alternata* f. sp. *lycopersici.* Se ha visto que *F. moniliforme* y/o *F. proliferatum* presentan una frecuencia del 90% o mayor en maíz y que el 90% de las cepas contaminantes son capaces de producir fumonisinas (Bacon y Nelson, 1994).

Se han analizado un amplio rango de sustratos, sin embargo, solo se han encontrado fumonisinas en derivados de maíz, con la excepción de dos casos: unos derivados de avena para alimentación animal en Brasil (Sydenham *et al.*, 1992b) y un forraje en Nueva Zelanda (Scott, 1993). A pesar de que *F. moniliforme* produce grandes cantidades de fumonisinas en cultivos sobre arroz, no se ha encontrado que éstas se den de forma natural en este cereal. Los granos de maiz partidos parecen presentar niveles de fumonisinas diez veces mayores que aquellos del maíz del que proceden, sin embargo, no hay una relación entre el tamaño de los mismos y la concentración de fumonisina (Mirocha *et al.*, 1992).

Wang *et al.* (1991) observaron la similitud estructural de las fumonisinas y la esfingosina, y su capacidad como inhibidores de la biosíntesis de los esfingolípidos.

LOS CEREALES

La proporción de alimento suministrada por los cereales en la dieta humana varía desde el 25% en los países desarrollados hasta el 80-90% en los países más pobres, que utilizan bajas cantidades de otros alimentos. En estos últimos, los cereales representan un papel primordial en la dieta, cubriendo las necesidades energéticas mínimas, aunque no satisface completamente las necesidades de proteínas, aminoácidos esenciales, vitaminas y sales minerales (López, 1991).

El maíz (*Zea mays*), se define botánicamente como una especie herbácea gigante de la familia de las gramíneas. Presenta una raíz pivotante primaria acompañada de otras más pequeñas que emergen casi directamente de la semilla. El cultivo de esta planta es muy versátil debido a su gran adaptabilidad a distintos suelos y climas, independientemente de las altitudes. Es la única planta tropical cultivada comúnmente fuera de los trópicos.

Se clasifica en seis tipos: dentado (o diente de caballo), caracterizado por una depresión en el grano; duro (o córneo), de aspecto liso; harinero (o amiláceo); dulce (o azucarado); de palomitas y céreo (Hawthorn, 1983). El maíz dentado tiene las semillas grandes y aplastadas. Es, con mucho, la mayor de las semillas de los cereales corrientes con un peso medio de 350 mg. El grano está compuesto por cuatro partes principales: cáscara o salvado (pericarpio y cubierta de la semilla), germen, endospermo y pedículo. El salvado constituye el 5-6% del grano; el germen es relativamente grande: un 10-14% del grano, y el resto corresponde al endospermo (Hoseney, 1994).

La composición química del maíz (Tabla 1) varía en función de las variedades, siendo el porcentaje mayoritario el correspondiente a carbohidratos. El embrión contiene un 30% de aceite con un contenido elevado en ácidos linoleico y linolénico, lo cual lo hace adecuado para la producción del mismo. La principal proteína es la zeína, deficiente en aminoácidos esenciales como son la lisina y el triptófano. Otra de las proteínas más importantes es la

glutenina, ésta de mejor calidad. El grano de maíz es rico en fósforo y pobre en calcio. No es muy rico en vitaminas, aunque contiene algo de provitamina A (Hawthorn, 1983).

El maíz es usado principalmente en la alimentación del ganado. Se incorpora siempre en los piensos compuestos para aves, cerdos y corderos. Debido a su elevado poder energético prima la rapidez del engorde en vez del crecimiento equilibrado, por lo que se recomienda su uso combinado con otros alimentos (Guerrero, 1977).

Tabla 1. *Composición química del grano de maíz (en %) (Hawthorn, 1993)*

Carbohidratos	Humedad	Proteínas	Grasas	Fibra	Cenizas
68,1-72,0	10,8-13,5	9,3-10,0	4,0-4,4	1,7-2,3	1,3-1,5

CONSECUENCIAS DEL CRECIMIENTO FÚNGICO EN EL GRANO

Los mohos pueden actuar como fitopatógenos en el campo, causando lesiones en tallos, hojas, inflorescencias y granos inmaduros, con las pérdidas económicas que ello comporta. El crecimiento fúngico y el deterioro del grano almacenado vienen determinados principalmente por la humedad, el rango de especies contaminantes, y de como éstos interaccionan con la temperatura y la composición gaseosa. La actividad fúngica puede causar el deterioro rápido del grano, a veces con calentamiento espontáneo. También da lugar a pérdidas de materia seca, de valor nutritivo, cambios bioquímicos (como el incremento de ácidos grasos), enmohecimiento visible, decoloración del grano, olores extraños, pérdida de germinabilidad, reducción de su calidad para el procesado y producción de micotoxinas (Christensen y Kaufmann, 1969; Pomeranz, 1982; Magan y Lacey, 1988).

Existe una amplia variedad de cambios bioquímicos que acompañan a la invasión fúngica del grano. Entre ellos el aumento de azúcares reductores y ácidos grasos, modificaciones en proteínas, incluyendo cambios en la actividad enzimática, y la producción de constituyentes fúngicos o metabolitos, como la quitina, el ergosterol, antibióticos y micotoxinas. En muchos casos la mayor preocupación acerca del grano enmohecido es su valor nutritivo, si éste es apropiado para el consumo como alimento o para alimentación animal. Los posibles efectos en la nutrición se detallan a continuación (Sauer, 1988).

a) Una mejora general. No hay problemas de modificación de la palatabilidad; vitaminas y antibióticos pueden estar presentes, y los constituyentes del grano han sido metabolizados a formas más digeribles.

b) Reducción de la palatabilidad como único problema. El consumo del mismo y el coeficiente de engorde pueden verse reducidos, pero la eficiencia puede ser normal o aceptable.

c) Reducción de la palatabilidad y de la digestibilidad. El coeficiente de engorde y la eficiencia se ven reducidos. Se puede compensar mediante complementos a la dieta.

d) Palatabilidad satisfactoria, pero digestibilidad reducida. Consumo normal, pero el ratio consumo:ganancia de peso se ve reducido.

e) Existencia de toxicidad. Se pueden dar varios tipos de efectos tóxicos.

Micotoxinas

Las micotoxinas son metabolitos producidos por hongos que crecen en alimentos y que son tóxicos cuando son ingeridos por el hombre u otros animales. Su presencia en cereales depende de un amplio número de factores, entre los que se encuentran factores físicos, químicos y biológicos (Fig. 1). Hay casos en los que la toxicidad se da por el consumo de una cantidad relativamente grande de estructuras fúngicas que se presenta mezclada con el grano. La aflatoxina es la micotoxina más conocida y probablemente la más importante. Es un problema en zonas cálidas y tropicales, donde es un problema de campo y de almacén. Se ha detectado en una gran variedad de cereales y sus derivados, y en otros productos, aunque donde es más común es en nueces y maíz (Sauer, 1988).

ECOLOGÍA FÚNGICA

El deterioro de alimentos y productos almacenados antes y después de la cosecha comprende un amplio abanico de hongos que difieren en cuanto a sus características ecológicas. Abarcan desde fitopatógenos, patógenos 'débiles' y saprófitos superficiales que dañan las materias primas antes de la cosecha, hasta mohos que causan pérdidas de materia seca y de calidad durante el almacenamiento. Algunos de ellos producen metabolitos tóxicos con más incidencia en el consumidor que cualquier efecto en la apariencia del producto (Lacey, 1989).

La colonización de las partes aéreas de las plantas empieza tan pronto como las hojas e influorescencias se encuentran expuestas al aire. Normalmente, las bacterias son las primeras en colonizar, pero son inmediatamente seguidas por las levaduras, y finalmente por mohos patógenos y saprófitos. Estos mohos continúan su desarrollo a lo largo de la vida de la planta, pero especialmente cuando la planta llega a la senescencia y las semillas maduran. La cosecha marca un profundo cambio en el ecosistema y marca la transición desde el ambiente fluctuante del campo a las condiciones relativamente estables del almacén. Este paso viene acompañado de un profundo cambio en la composición de la microflora (Lacey, 1989).

Los hongos presentes en las plantas antes de la cosecha se han denominado tradicionalmente mohos de campo, e incluyen especies de *Cladosporium, Alternaria, Epicoccum, Verticillium, Fusarium* y mohos fitopatógenos. En cereales almacenados, estos mohos persisten solamente si el grano está lo suficientemente seco como para prevenir el desarrollo de los hongos típicos de almacén. Estos últimos, están presentes en pequeña cantidad antes de la cosecha y son principalmente especies de los géneros *Aspergillus* y *Penicillium.* Si la humedad aumenta, el crecimiento fúngico se hace más vigoroso, dando lugar al calentamiento espontáneo de la masa de grano y al crecimiento de hongos más termotolerantes como *Absidia, Rhizomucor* y *Humicola*, acompañados de actinomicetes termófilos (Lacey, 1989).

La distinción entre mohos de campo y de almacén no es clara, ya que se ha visto que especies de campo pueden crecer en algunos casos en almacén. Esto llevó a la designación de un grupo intermedio; por otra parte también se ha comprobado que especies de almacén, especialmente *A. flavus,* pueden crecer en el campo en zonas tropicales húmedas, e incluso en regiones templadas, proponiéndose un grupo intermedio para especies de campo que a veces lo hacen en el almacén, como *Fusarium* spp. (Pelhate, 1968). Así pues, los

microorganismos deberían de clasificarse en función de sus requerimientos ecológicos, más que en función de su procedencia (Lacey, 1989).

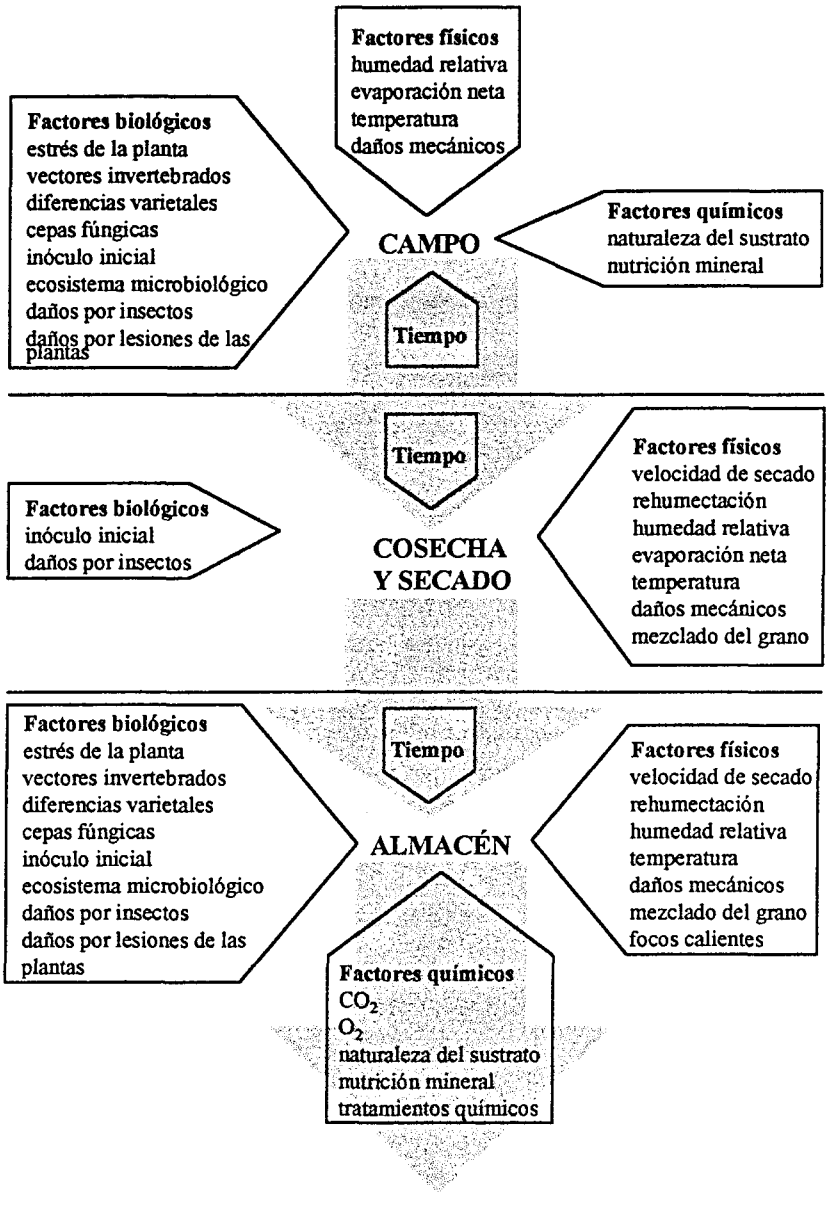


Figura 1. Factores que afectan a la formación de micotoxinas en el grano.

Las especies aisladas en grano almacenado abarcan desde los xerófilos *Eurotium* spp., los moderadamente xerófilos *A.candidus* y *A. flavus,* ligeramente xerófilos *A. fumigatus,* los psicrotolerantes *P. aurantiogriseum* y *P. verrucosum*, los mesófilos *P. corylophilum* y *P. rugulosum* y el termófilo *Talaromyces thermophilus*. Entre los hidrófilos están *Fusarium* spp., mucorales y otros (Lacey y Magan, 1991).

Tanto antes de la cosecha como en el almacén, el crecimiento de los mohos viene determinado por el entorno, especialmente por la disponibilidad de agua, la temperatura y la composición gaseosa; por la interacción con otros microorganismos, y con artrópodos; y por las medidas adoptadas para su control (Lacey, 1989).

Actividad de agua

En microbiología se han usado tres medidas de la disponibilidad de agua: la humedad relativa de equilibrio (ERH), la actividad del agua (a_w) y el potencial hídrico (Φ) (Tabla 2) (Lacey, 1989).

Tradicionalmente se ha utilizado la medida de la humedad de los alimentos como base para determinar la potencialidad de los mismos para alterarse, sin embargo, la actividad de agua da una idea del 'agua disponible' que los microorganismos pueden utilizar para su desarrollo. La relación entre a_w y porcentaje de humedad depende de la composición de cada alimento; la figura 2 ilustra el caso del maíz en grano.

El agua afecta a la colonización de las plantas de varias maneras. El agua de lluvia puede dispersar las esporas fúngicas salpicándolas desde las hojas o desde el suelo, arrastrándolas de las hojas al suelo, y aportando el agua necesaria para la descarga de ascosporas.

La migración de humedad debida al movimiento del vapor de agua desde zonas más calientes a zonas más frías, donde se condensa, es una importante causa de deterioro durante el almacenamiento y transporte del grano. Puede deberse a la radiación solar en un lado del almacén, a la diferencia de temperatura entre el grano y el ambiente o al calentamiento

Tabla 2. Relación entre la actividad del agua (a_w) y el potencial hídrico (-MPa)

a_w	ψ (-MPa)
0,999	0,14
0,995	0,69
0,99	1,38
0,98	2,78
0,95	7,06
0,90	14,5
0,85	22,4
0,80	30,7
0,75	38,6
0,70	49,1
0,65	59,2
0,60	70,3

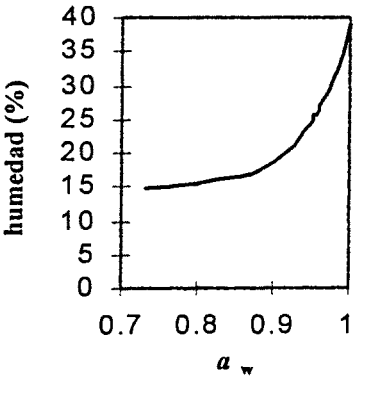


Figura 2. Curva de adsorción del maíz (Marín, datos sin publicar)

provocado por artrópodos y microorganismos. Consecuentemente, granos que han sido secados uniformemente pueden desarrollar áreas con una a_w suficiente para permitir el desarrollo microbiano (Lacey, 1989). Los mohos de almacén son normalmente capaces de germinar y crecer a a_w más bajas que los mohos de campo, tal y como muestran las tablas 3 y 4. La fase de latencia previa a la germinación aumenta conforme disminuye la a_w (Magan y Lacey, 1988). La edad de las esporas y la nutrición también puede afectar al período de latencia (Snow, 1949). La actividad fúngica en cereales almacenados depende estrechamente de la disponibilidad de agua; conforme la actividad de agua aumenta, un mayor número de mohos pueden crecer y la actividad metabólica aumenta hasta causar el calentamiento espontáneo del grano. La mayor tolerancia a las bajas a_w se da a la temperatura óptima de crecimiento. La germinación de esporas a menudo se da a a_w ligeramente menores que a las que se da el crecimiento miceliar. El crecimiento es a menudo anormal cerca de los límites de a_w para la germinación, con hinchamiento de los tubos germinales, contracción de las células de los tubos germinales, e hifas malformadas y con septos cortos, especialmente en *Penicillium* spp., *Fusarium* spp. y *Alternaria alternata* (Schneider, 1954; Armolik y Dickson, 1956; Mislivec y Tuite, 1970; Magan y Lacey, 1984a).

El conocimiento de la influencia de la interacción entre la a_w y la temperatura en el crecimiento fúngico es de particular importancia para entender la ecología de cada especie individual y la interrelación entre ellas. No todos los hongos presentan su crecimiento máximo cerca de 1,00 a_w, por ejemplo, *E. repens, E. amstelodami, E. chevalieri, E. rubrum* y *E. tuber* crecen más rápido entre 0,90-0,95 a las temperaturas óptimas (Fig. 3; Tabla 4). Las cepas de una misma especie difieren poco en sus requerimientos de a_w/temperatura para crecer; Pitt (1979) utilizó las velocidades de crecimiento a diferentes a_w y temperaturas como un criterio taxonómico para discernir entre especies de *Penicillium*.

A menudo las condiciones bajo las cuales un moho se desarrolla en mayor medida, son aquellas bajo las cuales sobrevive o compite mejor, no aquellas a las que crece mejor. Por ejemplo, *Eurotium* spp. predominan en el grano a 0,70 a_w, a pesar de que su óptimo está alrededor de 0,90 a_w. Las especies que predominan son un buen indicador de las condiciones bajo las cuales se ha almacenado el grano.

Los requerimientos de a_w se han usado como método de diferenciación entre mohos. Así Pelhate (1968) definió mohos xerofílicos, mesofílicos e higrofílicos en base a su habilidad para crecer a 0,95, 0,95-1,00 o solamente a 1,00 a_w, respectivamente.

Temperatura

La mayoría de hongos que viven sobre plantas antes de la cosecha, crecen bien entre 0 y 30°C. Por lo que respecta a la etapa en almacén, los hongos difieren ampliamente en las temperaturas que permiten su crecimiento y en las que lo hacen máximo. Sin embargo, la mayoría de hongos crecen en el rango 10-40°C y tienen el óptimo alrededor de 25-35°C (Tabla 5). El descenso de la temperatura provoca una relentización del metabolismo, y es una medida que se usa frecuentemente para frenar el deterioro. La actividad metabólica en productos húmedos almacenados libera calor que si no se evacúa mediante ventilación, puede dar lugar a un calentamiento espontáneo (Lacey, 1989).

Tabla 3. a_w mínimas que permiten la germinación de algunos mohos del grano

Especies	a_w mínima	Temperatura (°C)	Fase lag (días)	Referencia
Acremonium strictum	0,97	24	-	Arbad, 1976
Alternaria alternata	0,85	25	-	Magan y Lacey, 1984a
Alternaria sp.	0,91	24	-	Arbelaez, 1971
	0,94	32	-	Arbelaez, 1971
Aspergillus candidus	0,75	25	15	Snow ., 1944
	0,70	25-35	>95	Ayerst, 1969
	0,76	20	-	Magan y Lacey, 1984b
A. flavus	0,80	-	120	Armolik *et al.*, 1956
	0,75	30-40	>95	Ayerst, 1969
	0,80	37	-	Pitt y Miscamble, 1995
A. fumigatus	0,94	25	-	Magan y Lacey. 1984a
	0,86	35	-	Magan y Lacey. 1984a
	0,80	30-45	>95	Ayerst, 1969
A. nidulans	0,82	25	18	Snow *et al.*, 1944
	0,80	35	-	Magan y Lacey. 1984a
	0,75	10-45	>95	Ayerst, 1969
A. niger	0,84	25	10-11	Pitt, 1968
				Snow *et al.*, 1944
	0,78	30-35	32-95	Ayerst, 1969
A. oryzae	0,80	37	-	Pitt y Miscamble, 1995
A. parasiticus	0,80	37	-	Pitt y Miscamble, 1995
A. restrictus	0,75	22-25	15	Pelhate, 1968
				Snow *et al.*, 1944
	0,72	30	64	Smith y Hill, 1982
A. tamarii	0,75	30-40	>95	Ayerst, 1969
A. terreus	0,78	40	>95	Ayerst, 1969
A. versicolor	0,80	25	14-63	Pitt *et al.*, 1968
	0,78	25	24	Snow *et al.*, 1944
	0,76	25	-	Magan y Lacey. 1984a
	0,75	25	58-64	Smith y Hill, 1982
Botrytis cinerea	0,93	20	-	Snow *et al.*, 1944
Chrysosporium fastidium	0,69	25	48	Pitt *et al.*, 1968
Cladosporium herbarum	0,88	24-25	7	Arbelaez, 1971
				Snow *et al.*, 1944
	0,85	25	-	Magan y Lacey. 1984a
C. cladosporioides	0,86	25	-	Magan y Lacey. 1984a
Epiccocum nigrum	0,86	10	-	Magan y Lacey. 1984a
Eurotium amstelodami	0,74	25	63	Pitt *et al.*, 1968
	0,71	30-35	-	Magan y Lacey. 1984a
	0,70	25-30	32-95	Ayerst, 1969
E. chevalieri	0,71	30	16-32	Ayerst, 1969
E. intermedius	0,75	25	53	Snow *et al.*, 1944
E. repens	0,72	20-25	-	Magan y Lacey. 1984a
	0,70	20-35	>95	Ayerst, 1969
E. ruber	0,70	25	120	Snow *et al.*, 1944
	0,70	20-35	>95	Ayerst, 1969
Fusarium culmorum	0,91	25	56	Schneider, 1954
	0,87	20-25	-	Magan y Lacey. 1984a

Especies	a_w mínima	Temperatura (°C)	Fase lag (días)	Referencia
	0,90	20	28	Magan, 1988
F. moniliforme	0,91	24	-	Arbelaez, 1971
	0,94	32	-	Arbelaez, 1971
Gibberella zeae	0,94	-	-	Arbelaez, 1971
Monascus bisporous	0,61	25	120	Pitt *et al.*, 1968
Mucor spinosus	0,93	25	3	Snow *et al.*, 1944
Paecilomyces variotii	0,84	25	9	Pitt *et al.*, 1968
Penicillium aurantiogriseum	0,84	25	9	Snow *et al.*, 1944
	0,80	20-25	-	Magan y Lacey. 1984a
	0,80	5-35	>95	Ayerst, 1969
P. brevicompactum	0,83	30	28	Mislivec y Tuite, 1970
	0,80	20-25	-	Magan y Lacey. 1984a
P. chrysogenum	0,80	-	14	Galloway, 1935
P. citrinum	0,83	16-23	28, 14	Mislivec y Tuite, 1970
	0,81	30	60	Mislivec y Tuite, 1970
P. expansum	0,83	16-23-30	21, 10, 60	Mislivec y Tuite, 1970
P. fellutanum	0,80	25		Pitt *et al.*, 1968
			12, 28	Snow *et al.*, 1944
P. funiculosum	0,95	16	-	Mislivec y Tuite, 1970
	0,90	23-30	-	Mislivec y Tuite, 1970
P. glabrum	0,81	23	20	Mislivec y Tuite, 1970
P. hordei	0,80	25-30	-	Magan y Lacey. 1984a
	0,85	20	28	Magan, 1988
P. islandicum	0,80	5-40	>95	Ayerst, 1969
P. oxalicum	0,86	23-30	5, 4	Mislivec y Tuite, 1970
P. palitans	0,83	16-23	27, 12	Mislivec y Tuite, 1970
P. piceum	0,79	25-30	-	Magan y Lacey. 1984a
P. puberulum	0,81	23	28	Mislivec y Tuite, 1970
P. roquefortii	0,83	10-25	-	Magan y Lacey. 1984a
P. rugulosum	0,86	26	9	Snow *et al.*, 1944
P. urticae	0,81	23	60	Mislivec y Tuite, 1970
Rhizopus sp.	0,91	32	-	Arbelaez, 1971
	0,94	24	-	Arbelaez, 1971
R. stolonifer	0,93	25	2	Snow, 1949
	0,84	-	16	Snow, 1949
Scopulariopsis brevicaulis	0,90	-	14	Galloway, 1935
Nigrospora oryzae	0,91	24	-	Arbelaez, 1971
	0,94	32	-	Arbelaez, 1971
Trichoderma harzianum	0,92	20	28	Magan, 1988
T. viride	0,94	-	-	Magan, 1988
	0,92	20	28	Magan, 1988
Trichothecium roseum	0,90	20	-	Snow, 1949
Verticillium lecanii	0,90	20-25	-	Griffin, 1963
Wallemia sebi	0,75	-	22	Pelhate, 1968

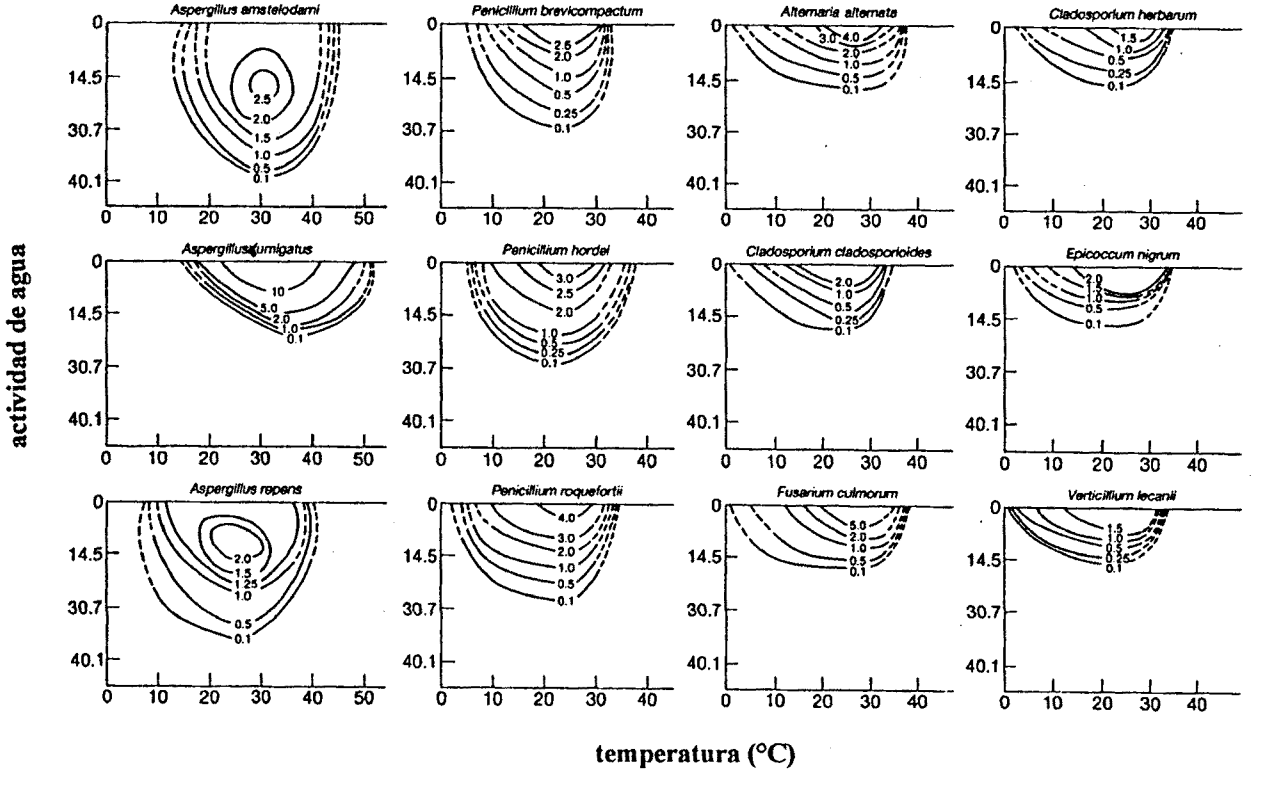


Figura 3. Representación en forma de líneas isopletas de las combinaciones a_w/temperatura que permiten velocidades de crecimiento equivalentes (adaptado de Magan y Lacey, 1984b; Cooke y Whipps, 1993).

Tabla 4. a_w mínimas y óptimas para el crecimiento

Especies	Mínima	Óptima	Referencia
Alternaria alternata	0,88	1,00	Lacey y Magan, 1991
Aspergillus candidus	0,75	0,90-0,98	Lacey y Magan, 1991
	0,75	>0,98	Ayerst, 1969
A. flavus	0,78	0,95	Lacey y Magan, 1991
	0,78	>0,98	Ayerst, 1969
	0,80	0,97-0,98	Pitt y Miscamble, 1995
A. fumigatus	0,85	0,98-0,99	Lacey y Magan, 1991
	0,82	>0,97	Ayerst, 1969
A. nidulans	0,78	0,97	Ayerst, 1969
A. niger	0,78	>0,98	Ayerst, 1969
A. nomius	0,81	0,97-0,98	Pitt y Miscamble, 1995
A. oryzae	0,80	0,97-0,98	Pitt y Miscamble, 1995
A. parasiticus	0,78	0,95	Lacey y Magan, 1991
	0,80	0,96-0,98	Pitt y Miscamble, 1995
A. restrictus	0,71	0,96	Lacey y Magan, 1991
A. tamarii	0,78	>0,98	Ayerst, 1969
A. terreus	0,78	0,97	Ayerst, 1969
A. versicolor	0,78	0,95	Lacey y Magan, 1991
Botrytis cinerea	0,93-0,95	1,00	Lacey y Magan, 1991
Cladosporium cladosporioides	0,88	1,00	Lacey y Magan, 1991
Cladosporium herbarum	0,85	0,95	Lacey y Magan, 1991
Eurotium amstelodami	0,71-0,73	0,93-0,96	Lacey y Magan, 1991
	0,71	0,93	Ayerst, 1969
E. chevalieri	0,71	0,93-0,95	Lacey y Magan, 1991
	0,71	0,93	Ayerst, 1969
E. repens	0,75	0,93-0,95	Lacey y Magan, 1991
	0,71	0,93	Ayerst, 1969
E. tuber	0,71	0,93	Ayerst, 1969
E. rubrum	0,70	0,93	Lacey y Magan, 1991
Fusarium avenaceum	0,89	1,00	Lacey y Magan, 1991
F. culmorum	0,89	0,99	Lacey y Magan, 1991
F. graminearum	0,89	0,98-0,99	Lacey y Magan, 1991
F. moniliforme	0,87		Lacey y Magan, 1991
F. poae	0,89	1,00	Lacey y Magan, 1991
F. sporotrichioides	0,88		Lacey y Magan, 1991
F. subglutinans	0,89	1,00	Lacey y Magan, 1991
F. tricinctum	0,81-0,83	>0,98	Lacey y Magan, 1991
Penicillium aurantiogriseum	0,82	>0,98	Ayerst, 1969
P. brevicompactum	0,78-0,82	1,00	Lacey y Magan, 1991
P. digitatum	0,90	1,00	Lacey y Magan, 1991
P. expansum	0,82-0,83	1,00	Lacey y Magan, 1991
P. islandicum	0,83	>0,97	Ayerst, 1969
P. roquefortii	0,83	1,00	Lacey y Magan, 1991
Rhizopus stolonifer	0,93	1,00	Lacey y Magan, 1991
Wallemia sebi	0,69	0,95	Lacey y Magan, 1991

Tabla 5. Intervalos de temperatura requeridos para el crecimiento de mohos de cereales

Especies	Rango de temperatura	Temperatura óptima	Referencia
Alternaria alternata	0-35	20-25	Lacey, 1989
Aspergillus candidus	3-44	25-32	Lacey, 1989
	10-44	32	Ayerst, 1969
A. flavus	6-45	35-37, 30	Lacey, 1989
			Wheeler *et al.*, 1991
	12-43	33	Ayerst, 1969
A. fumigatus	10-55	40-42	Lacey, 1989
	12-53	40	Ayerst, 1969
A. nidulans	12-47	37	Ayerst, 1969
A. ochraceus		25-30	Wheeler *et al.*, 1991
A. parasiticus	6-45	35-37, 30	Lacey, 1989
			Wheeler *et al.*, 1991
A. restrictus	9-40	30	Lacey, 1989
A. versicolor	4-39	25-30	Lacey, 1989
			Wheeler *et al.*, 1991
Botrytis cinerea	-2-35	22-35	Lacey, 1989
Cladosporium cladosporioides	-5-32	24-25	Lacey, 1989
C. herbarum	-10-32	25	Lacey, 1989
Eurotium amstelodami	5-46	33-35	Lacey, 1989
E. chevalieri	5-43	30-35	Lacey, 1989
	10-42	33	Ayerst, 1969
E. repens	7-40	25-27	Lacey, 1989
	7-38	24	Ayerst, 1969
E. rubrum	5-40	25-27	Lacey, 1989
Fusarium avenaceum	-3-31	25	Lacey, 1989
F. culmorum	<0-31	25	Lacey, 1989
F. equiseti		30	Wheeler *et al.*, 1991
F. graminearum		24-26, 25	Lacey, 1989
			Wheeler *et al.*, 1991
F. moniliforme	2-37	22-28, 25-30	Lacey, 1989
			Wheeler *et al.*, 1991
F. poae	2-39	22-28	Lacey, 1989
F. sporotrichioides	-2-35	22-28	Lacey, 1989
F. subglutinans	2-38	22-28	Lacey, 1989
F. tricinctum	<0-35	25	Lacey, 1989
Penicillium aurantiogriseum	-2-32	23	Lacey, 1989
	<5-32	23	Ayerst, 1969
P. brevicompactum	12-30	23	Lacey, 1989
P. digitatum	6-37	20-25	Lacey, 1989
P. expansum	-6-35	25-26	Lacey, 1989
P. islandicum		30	Wheeler *et al.*, 1991
	10-38	31	Ayerst, 1969
P. roquefortii	<5-35	25	Lacey, 1989
Rhizopus stolonifer	5-34	26-29	Lacey, 1989
Wallemia sebi	>5-35	23-25	Lacey, 1989

Efecto de la a_w y la temperatura sobre la producción de micotoxinas

A menudo se ha investigado la capacidad de determinados hongos para producir micotoxinas bajo las condiciones óptimas de crecimiento y en medios ricos, más que en el rango de condiciones ambientales bajo las cuales suelen producirse las micotoxinas. La capacidad para producir micotoxinas en las condiciones de temperatura y a_w límites para el crecimiento, es de particular interés por el riesgo que comporta (Magan y Lacey, 1989).

Northolt *et al.* (1976, 1979, 1982) pusieron de manifiesto la relación entre disponibilidad de agua, temperatura, crecimiento y producción de micotoxinas. La producción de aflatoxinas, patulina, ácido penicílico y ocratoxinas a diferentes a_w y temperaturas se comparó para diferentes especies. Habitualmente la a_w mínima que permitió el crecimiento fue más baja, así como más amplio el rango de temperaturas, que aquellas condiciones que permitieron la

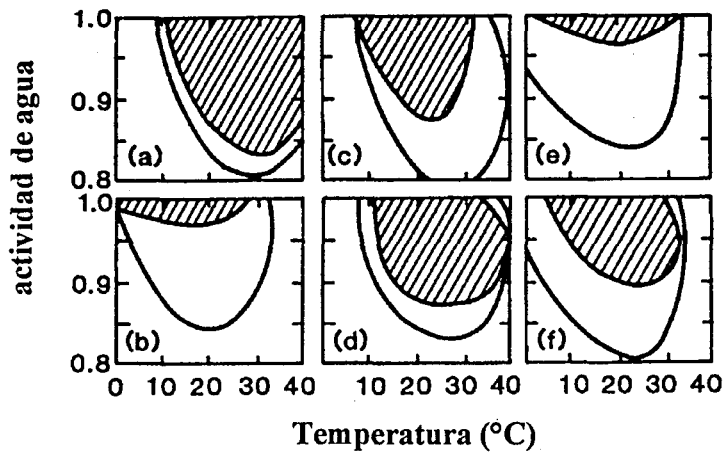


Figura 4. Condiciones a_w/temperatura límites que permiten el crecimiento y la producción de micotoxinas por parte de (a) *Aspergillus flavus* y aflatoxina, (b) *A. ochraceus* y ácido penicílico, (c) *Penicillium aurantiogriseum* y ácido penicílico y (d) *P. aurantiogriseum* y *ocratoxina* A (adaptado de Northolt, 1979; Lacey y Magan, 1991).

producción de micotoxinas (Fig. 4).

La tabla 6 compara las a_w mínimas para crecimiento y producción de micotoxinas para una serie de especies. Los efectos de la a_w y la temperatura sobre la producción de micotoxinas a menudo difieren de los efectos sobre crecimiento y germinación y también pueden diferir para una toxina producida por dos especies diferentes.

Tabla 6. a_w mínimas para el crecimiento y la producción de micotoxinas por algunos mohos del grano

Especies fúngicas	Micotoxina	a_w mínima para		Referencia
		Crecimiento	Producción toxina	
A. flavus	Aflatoxina	<0,80	0,82	Multon, 1988
A. flavus		0,78-0,84	0,84	Diener y Davis, 1970
A. parasiticus		0,84	0,87	Northolt, 1979
A.ochraceus	Ocratoxina	0,77	0,85	Bacon *et al.*, 1973
P. aurantiogriseum		0,82-0,85	0,87-0,90	Northolt, 1979
P. viridicatum		-	0,80	Patterson, 1986
P. viridicatum		0,80-0,81	0,83-0,86	Northolt, 1979
A. ochraceus	Ácido penicílico	0,77	0,88	Northolt, 1979
P. aurantiogriseum		0,82-0,85	0,97	Northolt, 1979
P. patulum	Patulina	0,81	0,95	Northolt, 1979
P. expansum		0,82-0,84	0,99	Northolt, 1979
A. clavatus		-	0,99	Northolt, 1979
A. viridicatum	Citrinina	-	0,85	Patterson, 1986
A. versicolor	Sterigmatocistina	-	0,85	Patterson, 1986
P. commune	Acido ciclopiazónico	-	0,85	Gqaleni *et al.*, 1996

Concentración de iones hidrógeno

La mayoría de hongos crecen bien en el rango de pH entre 3 y 8 y tienen su óptimo cercano al 5. Compiten pobremente contra bacterias a pH 7, pero por debajo de 5, los hongos van predominando gradualmente. El cambio de pH puede alterar la respuesta fúngica al resto de factores, como la a_w (Lacey, 1989).

Las especies de *Aspergillus* y *Fusarium* toleran mal pH muy ácidos (2-3), excepto *F. graminearum*, capaz de crecer a pH 2,1 a 30°C. Las especies de *Aspergillus* estan menos afectadas por pH alcalinos. Las especies de *Penicillium* son capaces de crecer en un amplio rango de pH, y su crecimiento se ve menos afectado por pH bajos que el de los otros géneros (Wheeler *et al.*, 1991).

Composición gaseosa

Los hongos que causan deterioro en productos almacenados se consideran habitualmente aerobios obligados, sin embargo la presión parcial de O_2 necesaria para su crecimiento se ha sobrestimado a menudo. La concentración de O_2 en la atmósfera debe reducirse hasta <0,14% para que el crecimiento lineal se reduzca en un 50%. Un aumento en la concentración de CO_2 hasta el 5-10% puede estimular el crecimiento de algunas especies, especialmente cuando la a_w es alta, y puede ser necesario más de un 15% CO_2 para reducir el crecimiento a la mitad (Lacey, 1989).

Se han utilizado atmósferas modificadas para el control de insectos y hongos en granos almacenados, pero el nitrógeno debe estar libre de oxígeno, mientras que los regímenes CO_2/O_2 útiles para controlar insectos son insuficientes para controlar hongos (Lacey, 1989).

Bajas concentraciones de oxígeno (<1%) y/o concentraciones determinadas de CO_2 o N_2 son altamente efectivas en la prevención del desarrollo de mohos en grano y en la inhibición de determinadas micotoxinas, como aflatoxinas, ocratoxina, patulina, ácido penicílico y toxina T-2. Sin embargo, los niveles de CO_2 necesarios para inhibir el crecimiento fúngico son mucho mayores que los requeridos para la inhibición de la producción de micotoxinas. Los grados de inhibición conseguidos mediante elevadas concentraciones de CO_2 dependen del resto de factores ambientales, como la humedad relativa y la temperatura (Paster y Bullerman, 1988).

Conservantes químicos

La infección del grano durante el almacenamiento puede prevenirse o reducirse mediante el uso de fungicidas precosecha, y mediante el uso de ácidos débiles como el benzoico, sórbico, propiónico, acético, nitroso y sulfuroso, y sus sales. Sin embargo, las pequeñas concentraciones permitidas en alimentos pueden ser efectivas solamente hasta un pH una unidad por encima de su pK_a; a pH más altos, existe demasiado poco ácido no disociado como para afectar el crecimiento fúngico. El ácido debe distribuirse uniformemente entre la masa del grano, sobretodo si se tienen que controlar mohos tolerantes a este tipo de ácidos. Si no es así, estos mohos pueden crecer en zonas tratadas insuficientemente, metabolizando el ácido y permitiendo el crecimiento de especies menos tolerantes (Lacey, 1989).

La utilización de los insecticidas está siendo sometida a un estricto control, que se extiende a los productos fungicidas en ensilado de cereales. A estas restricciones hay que añadir los condicionantes propios de su aplicación. Trabajando con fungicidas a base de propionatos, en maíz con contenidos de humedad entre 18 y 20% y a temperaturas superiores a 20°C, Coll *et al.* (1994) concluyeron que el factor humedad era de los menos influyentes en los niveles de recuentos fúngicos. A medida que se aumentaba la temperatura, la eficacia se condicionaba mucho a la dosis utilizada y a la humedad.

La adición de 1-2% de bicarbonato amónico en granos partidos de maíz provoca una reducción de 5,1-5,9 log ufc g^{-1}; los mismos niveles de bicarbonato sódico provocan reducciones de 1,2-2,0 log ufc g^{-1} (Montville y Shih, 1991).

Irradiación

La irradiación puede ser un método efectivo para el control post-cosecha, sin embargo las diferentes especies difieren en cuanto a susceptibilidad, de manera que podrían ser necesarias dosis demasiado altas. Por ejemplo, se necesitan dosis de 0,3 kGy para eliminar *Penicillium* spp. de semillas de maíz, 1,2 kGy para eliminar *Aspergillus* spp., 6,0 kGy para *Bacillus* spp. y 12 kGy para levaduras y *Fusarium* spp. (Cuero *et al.*, 1986).

Mediante la irradiación se ha comprobado que es posible destruir las esporas de mohos, pero los efectos de la irradiación en la formación de micotoxinas parecen ser contradictorios. Paster y Bullerman (1988) observaron un aumento en la producción de aflatoxinas en trigo

irradiado, mientras que la irradiación del maíz y la cebada antes de la inoculación dio lugar a niveles menores de aflatoxinas.

Ventilación

La ventilación del grano con aire a temperatura ambiente o templado, arrastra el calor y el CO_2, introduce O_2 y arrastra o introduce humedad, dependiendo de la humedad relativa ambiente y de la a_w del grano. De esta manera se rompe cualquier equilibrio que se hubiera podido establecer en el grano, haciendo que la microflora experimente condiciones diferentes a las de la superficie de los granos. El cómo este fenómeno afecta al crecimiento fúngico ha sido poco estudiado (Magan y Lacey, 1989).

Se ha de evitar la adición de agua en el proceso de ventilación cuando las humedades relativas son altas (Lacey y Magan, 1991).

Interacciones microbianas

Los hongos raramente se dan en forma de monocultivo en productos almacenados, sino que lo hacen como un grupo de especies de hongos y bacterias que interactúan entre ellas. Las especies involucradas y la naturaleza de sus interacciones pueden verse modificadas por la temperatura, a_w, composición gaseosa y otros factores, determinando las especies predominantes (Lacey, 1989). Se han descrito toda una serie de posibles relaciones entre las especies fúngicas involucradas: neutralismo (ninguno de los dos se beneficia), comensalismo y mutualismo (respectivamente, uno o ambos microorganismos se benefician), amensalismo (uno se ve perjudicado, mientras que el otro no se beneficia), y competición (ambos sufren dependencia sobre el mismo sustrato).

La interacción entre hifas fúngicas se da inevitablemente y puede dar lugar a interacciones intra e interespecíficas. Las interacciones entre mohos y otra microflora del grano puede también tener un profundo efecto en la habilidad de las cepas componentes para producir micotoxinas (Cuero *et al.*, 1987). Se ha demostrado que las interacciones fúngicas sobre agar pueden abarcar desde el entrecruzamiento entre hifas sin efecto sobre el ratio de crecimiento de ambas colonias, pasando por la inhibición mutua en contacto o a distancia, hasta la inhibición de un microorganismo, mientras que el otro continua creciendo sobre él. Asignando valores a estas reacciones se pueden derivar Índices de Dominancia (I_D) (Magan y Lacey, 1984a). Estos autores encontraron I_D mayores (especies más competitivas) en *P. brevicompactum, P. hordei, P. roquefortii, A. fumigatus* y *A. nidulans*. Los I_D variaron en función de la a_w, siendo *F. culmorum* la única especie de campo capaz de competir con los mohos de almacén. Estos resultados difirieron de los obtenidos posteriormente sobre grano autoclavado, el cual fue analizado mediante diluciones decimales (ufc g^{-1}) y siembra directa (Magan y Lacey, 1985). Así, *A. versicolor,* que había resultado muy poco competitivo en el estudio anterior, mostró gran capacidad para competir en grano. Por otra parte, *F. culmorum, A. alternata* y *P. hordei* mostraron buenas capacidades para competir. Se demostró que dicha habilidad está íntimamente ligada a las condiciones abióticas.

La figura 5 muestra los efectos de la a_w y la temperatura en la colonización del grano almacenado aerobicamente. Las interacciones interespecíficas y otros factores determinan que a menudo los hongos sean más numerosos cerca de las combinaciones a_w / temperatura límites para su crecimiento que en sus óptimos (Magan y Lacey, 1989).

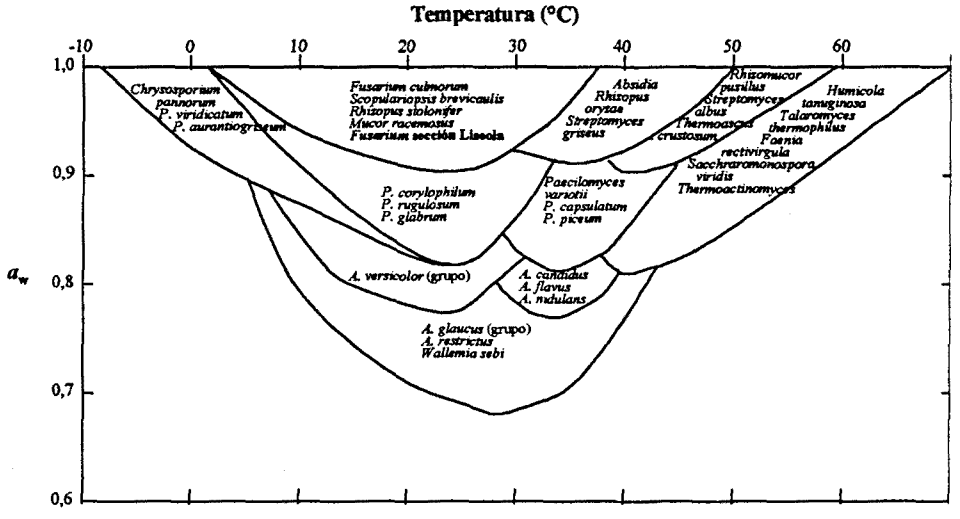


Figura 5. Condidiones límite de a_w y temperatura para el crecimiento de microorganismos de almacén (Magan y Lacey, 1989). *P=Penicillium; A=Aspergillus*

El efecto que las interacciones ejercen sobre la germinación y el crecimiento de *A. flavus, P. verrucosum, F. poae* y *Hyphopichia burtonii* agrupados dos a dos, así como su variación en función de la a_w y la temperatura ha sido estudiado por Ramakrishna *et al.* (1993). La colonización y la producción de *ocratoxina* A en el caso de *Penicillium verrucosum,* y de toxina T-2 en el caso de *Fusarium sporotrichioides,* en cebada irradiada, se ve afectada de diferentes maneras por las presencia de flora acompañante y en función de la a_w y la temperatura: mientras la colonización se ve disminuida en diferentes grados, la producción de micotoxinas puede verse inhibida o favorecida (Ramakrishna *et al.*, 1996a,b).

Así, en el caso de *A. flavus,* las especies que compiten podrían metabolizar las aflatoxinas, alterar el metabolismo de *A. flavus,* competir por el sustrato necesario para la producción de aflatoxinas o dar lugar a condiciones desfavorables para su producción. Por ejemplo, en el campo, la flora acompañante, como *F. moniliforme* inhibe a *A. flavus* impidiéndole contaminar más granos sanos de la mazorca con aflatoxinas (Wicklow *et al.*, 1988). Cepas atoxigénicas de *A. flavus* inoculadas antes de la cosecha han mostrado su efectividad en la inhibición de la producción de aflatoxinas, y han sido sugeridas como agentes de biocontrol en pre- y post-cosecha (Brown *et al.*, 1991).

Interacciones entre hongos y artrópodos

Los insectos son agentes importantes en el deterioro de los productos almacenados, por su propia actuación, y por su relación estrecha con la acción fúngica. Los insectos pueden

potenciar el crecimiento fúngico por los daños que provocan el grano; por su actividad metabólica que provoca aumento de a_w y temperatura; y por su papel en la diseminación de esporas. También pueden inhibir el crecimiento fúngico mediante la secreción de quinonas o alimentándose del propio hongo (Lacey, 1989).

Características del grano

Los granos dañados son mucho más susceptibles a la invasión fúngica y también presentan mayor enmohecimiento visible. El aumento en susceptibilidad depende de la naturaleza del daño, del genotipo del grano y del período de almacenamiento.

El secado del grano se hace con el fin de reducir el contenido de humedad del grano, y que de esta manera no sea posible el crecimiento de mohos en el almacén. Sin embargo, si no se lleva a cabo debidamente, puede tener el efecto contrario. Por ejemplo, un calentamiento demasiado rápido puede causar grietas en los granos, aumentando su susceptibilidad a la invasión fúngica, mientras que un tratamiento demasiado alto puede alterar la relación entre contenido de humedad y a_w, dando lugar posiblemente a una mayor susceptibilidad al enmohecimiento a un porcentaje de humedad dado (Flannigan, 1978).

Tiempo de almacenamiento

El tiempo de almacenamiento interacciona con el resto de factores para determinar el grado de enmohecimiento. Consecuentemente, cuanto más largo es, más seco o más frío debe conservarse. El almacenamiento prolongado del grano puede hacer aumentar su susceptibilidad al crecimiento fúngico.

CONTROL DEL GRANO ALMACENADO

El uso de sistemas para monitorizar la infestación del grano y su calidad, sistemas de ventilación por aire natural, y la respuesta rápida del que gestiona el almacén, permite que el grano sea protegido con pérdidas económicas y riesgos para la salud negligibles. Las variables monitorizadas suelen ser humedad, temperatura, gas intergranular, pérdidas de masa, calidad del grano, insectos, microflora, materiales extraños, y micotoxinas (Fig. 6) (Sinha, 1995). Mientras que las estrategias de prevención y control son la limpieza del grano, preparación de los graneros, mantenimiento preventivo, sistemas de secado y aireación, detección de plagas. y control químico de las mismas con insecticidas de contacto y fumigación (Fig. 7) (Cink y Harin, 1989). Cuando la infección del grano está presente, las estrategias a seguir se basan en ajustar de forma precisa los separadores de grano y realizar una limpieza a conciencia del mismo; de esta manera se eliminan la mayoría de granos afectados. Un secado adecuado, y un almacenamiento en condiciones evitaran posterior crecimiento fúngico. Una infección sin síntomas evidentes es más difícil de tratar; dichos granos no pueden ser separados por ninguno de los métodos estándar. Es necesario realizar investigación en métodos que permitan tratar estos granos (Munkvold y Desjardins, 1997).

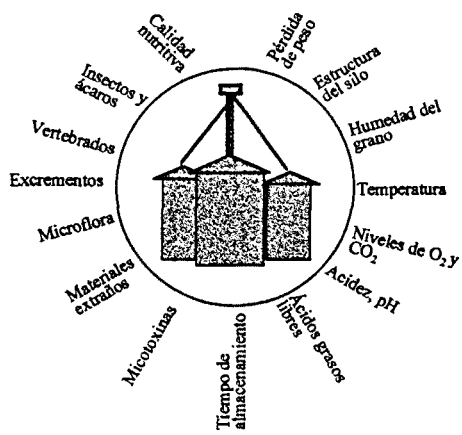


Figura 6. Esquema de las principales variables de seguimiento de la calidad y control aplicadas al grano almacenado correctamente (Sinha, 1995)

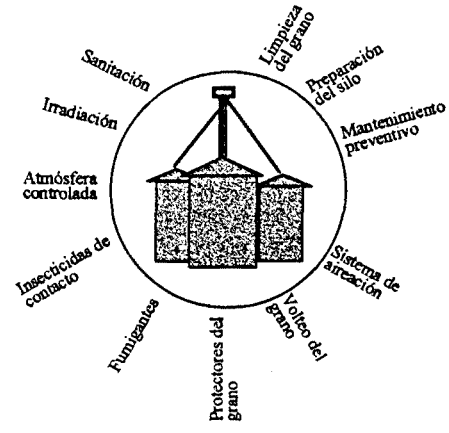


Figura 7. Esquema de las principales herramientas de control usadas en grano almacenado correctamente (Sinha, 1995).

Las nuevas tendencias en almacenamiento de granos, lo hacen en grandes graneros y silos, en los que se aplican los últimos avances tecnológicos. Sin embargo, ocasionalmente se producen problemas, por causas naturales o humanas, como pluviometria por encima de lo normal durante la cosecha, stock grande de grano del año precedente, o almacenamiento prolongado debido a pobres ventas. Cuando esto ocurre los granjeros tienen que enfrentarse a infestaciones, contaminación por micotoxinas, y otros problemas de pérdidas de calidad (Sinha, 1995).

En países en vías de desarrollo la gestión del grano almacenado se hace más difícil, debido básicamente a (Lindblad y Druben, 1980):

- Amplia variedad de estructuras utilizadas.
- Condiciones climáticas favorables al crecimiento y multiplicación de insectos, roedores y hongos.
- Bajos ingresos de los granjeros.
- Falta de conocimientos y formación acerca de técnicas de gestión del grano.

EL GÉNERO *FUSARIUM*

Características del género *Fusarium*

El género *Fusarium* pertenece al orden de los Hifomicetes. Sus hifas forman pequeños agregados, a veces coloreados, sobre los cuales se forman los conidióforos que producen dos tipos de conidios según las condiciones de crecimiento: los macroconidios, arqueados, fusiformes, subdivididos por 3-10 tabiques transversales, y los microconidios, dispuestos en cadenas o en falsas cabezas (Laurent, 1991). Estos criterios, además de la ausencia de

clamidosporas, clasifican a *F. moniliforme* dentro de la sección Liseola, junto a cuatro especies más: *F. proliferatum, F. subglutinans, F. anthophilum* y *F. thapsinum.*

Muchas especies de *Fusarium* han sido descritas como productoras de tricotecenos y zearalenona. Sin embargo, han surgido problemas debido a los diferentes sistemas taxonómicos utilizados. En un intento de remediar esta situación, se estableció en la Universidad del estado de Pennsylvania la "International Toxic *Fusarium* Reference Collection" (Marasas *et al.*, 1984a). Cepas de *Fusarium* de todas las procedencias fueron validadas taxonómicamente y su capacidad productora de micotoxinas fue confirmada. De entre las especies pertenecientes a la sección Liseola, tan solo *F. moniliforme* resultó ser productora de diacetoxiscirpenol y zearalenona. Otros metabolitos secundarios producidos por dicha especie son: deoxinivalenol, ácido fusárico, fusarinas, fusariocinas y toxina T-2 (Marasas *et al.*, 1984)

A pesar de haberse descrito como moho de campo, *Fusarium* spp. puede desarrollarse en almacén cuando la humedad es alta y la temperatura baja. Son importantes como patógenos de cereales, causando lesiones en tallo y raíces, así como infecciones en las espigas. Las diferentes especies son capaces de producir diferentes tricotecenos, moniliformina y fumonisinas. Las especies más habituales en maíz son *F. graminearum, F. moniliforme* y *F. subglutinans.* La infección del maíz es más probable justo antes de la emergencia de la espiga si se dan 70-80 mm de lluvia durante los 6-9 días siguientes, mientras que períodos de 10-16 días después de la emergencia con menos de 60 mm de lluvia harán la infección poco probable. El moho crece bajo la vaina, y después de la cosecha, una vez extraída ésta, continua creciendo hasta que el a_w cae hasta valores de 0,90 (Lacey y Magan, 1991).

Ecología de *Fusarium*

Los efectos de la a_w y la temperatura en el crecimiento de *Fusarium avenaceum, F. culmorum, F. poae* y *F. tricinctum* han sido estudiados *in vitro* en agar extracto de trigo (Magan y Lacey, 1984c). Las a_w óptima y mínima para el crecimiento de todas las especies excepto *F. poae* fueron 0,995-0,99 y 0,89-0,90 a las temperaturas óptimas (Tabla 7). La a_w óptima para el crecimiento de *F. poae* decreció de 0,995 a 0,98 al aumentar la temperatura de 25 a 30°C. A a_w>0,98 *F. culmorum* y *F. poae* crecieron más rápido (6-8 mm d^{-1}) que *F. avenaceum* y *F. tricinctum* (4-5 mm d^{-1}), pero a a_w menores la diferencia fue menor.

La germinación de cepas de *Fusarium roseum* 'Graminearum', 'Culmorum' y 'Avenaceum' en función del tiempo a 25°C ha sido estudiada en un rango de a_w entre 0,94 y 1,00, observándose como ésta disminuye conforme lo hace la a_w, y mostrándose óptima a 1,00 a_w, Las a_w mínimas para germinar en un período de 24 h oscilaron alrededor de 0,94-0,96 a_w (Sung y Cook, 1981).

Los efectos combinados de temperatura, a_w, y pH sobre el crecimiento de una cepa de *F. oxysporum* f. sp. *lycopersici* han sido puestos de manifiesto por Besri (1980). Estableció unas condisiones óptimas de 0,99 a_w, un pH alrededor de la neutralidad y una temperatura de 25-30°C, seguido de 20-35°C, 15°C y 10°C, en función de la a_w. Se ha determinado que el pH más idóneo para la producción de FB_1 para *F. proliferatum* se halla entre 3 y 4 (Keller *et al.*, 1997)

Tabla 7. a_w y temperaturas mínimas y óptimas para el crecimiento de algunas especies de *Fusarium* (Lacey y Magan, 1991).

	Temperatura (°C)		a_w (para crecer)	
	Rango	óptima	mínima	óptima
F. avenaceum	-3-37	25	0,89	1,0
F. culmorum	<0-37	25	0,89	0,99
F. graminearum	Nd	24-26	0,89	0,99-0,98
F. moniliforme	2-37	22-28	0,87	nd
F. poae	2-39	22-28	0,89	1,0
F. sporotrichioides	-2-35	22-28	0,88	nd
F. subglutinans	nd	25	nd	nd
F. tricinctum	<0-35	25	0,89	1,0

Aunque la infección en lotes de maíz suele situarse en el intervalo 9-91%, determinados años el 100% de los granos de maíz pueden estar infectados por *F. moniliforme*; el exceso de lluvia puede ser el responsable (Marasas *et al.*, 1988a).

La a_w afecta significativamente tanto al crecimiento de *F. moniliforme*, como a la biosíntesis de fumonisinas por el mismo sobre maíz autoclavado. Una reducción de un 5% en la a_w reduce a la tercera parte los niveles de fumonisina B_1 producidos, mientras que una reducción del 10% (de 1 a 0,90 a_w) provoca una reducción de 300 veces el nivel de fumonisina y de 20 veces del crecimiento fúngico determinado por el nivel de ergosterol. A 0,85-0,86 a_w no se detecta crecimiento ni producción de fumonisina B_1 (Cahagnier *et al.*, 1995).

La capacidad potencial de 73 cepas de *F. moniliforme* para producir fumonisinas ha sido estudiada sobre maíz humedecido y autoclavado por LeBars *et al.* (1994). Las cepas se dividieron en grupos según su capacidad productora siendo el grupo más numeroso el de las capaces de producir entre 800-3200 $\mu g\ g^{-1}$. La producción fue mayor a 20°C, seguida de 25, 15, 30 y 10°C. A 5, 35 y 40°C la producción fue nula. Se ensayaron contenidos de humedad entre 27 y 39%, siendo la producción máxima a 32%. La cinética de producción de fumonisina B_1 por *F. moniliforme* MRC 826 en función del crecimiento fúngico sobre maíz humedecido y autoclavado ha sido estudiada a diferentes temperaturas. Mientras que el nivel de ergosterol fue significativamente mayor a 25 que a 20°C, no se detectaron diferencias entre ambas temperaturas en cuanto a la máxima producción de fumonisina B_1, aunque sí entre las producciones medias. A 30°C la producción máxima fue muy baja (0,6 $g\ kg^{-1}$) (Alberts *et al.*, 1990).

La infección del maíz por *F. moniliforme* guarda una correlación negativa con la presencia de *F. graminearum* y *F. subglutinans* en el mismo en estudios realizados en Sudáfrica (Rheeder *et al.*, 1990). De la misma manera se ha demostrado una correlación negativa entre la presencia de fumonisinas y aflatoxinas en maíz cultivado en Tailandia (Yoshizawa *et al.*, 1996). Aunque en estudios posteriores Shetty y Bhat (1997) no encontraron correlación de ningún tipo entre la presencia de aflatoxina B_1 y fumonisina B_1 en muestras de sorgo, maíz y piensos procedentes de la India.

La producción de zearalenona en maíz por cepas de *F. graminearum, F. culmorum* y *F. oxysporum* es claramente mayor a 0,97 que a 0,95 a_w; la cantidad de toxina acumulada aumenta hasta los 30 días (Jiménez *et al.*, 1996).

FUSARIUM SECCIÓN LISEOLA

Biología de Fusarium moniliforme

Fusarium moniliforme es el nombre que se ha dado a cualquiera de las seis especies biológicas (o mating populations) que comparten la forma teleomorfa denominada *Gibberella fujikuroi*. Dos de estas seis especies biológicas, identificadas como "A" y "D", producen micotoxinas. Las cepas pertenecientes al grupo A crecen como endofitos en el maíz y a menudo comprenden más del 90% de las cepas de *Fusarium* aisladas de granos de maíz sanos (Leslie, 1996).

Gibberella fujikuroi (Sawada) Ito en Ito y K. Kimura es el teleomorfo para muchas de las especies anamorfas de la sección Liseola de *Fusarium*, tales como *Fusarium moniliforme* Sheldon, *Fusarium subglutinans* (Wollenweber y Reinking) Nelson, Toussoun y Marasas, y *Fusarium proliferatum* (Matsushima) Nirenberg (Nelson *et al.*, 1983). Aunque estos mohos se hallan ampliamente distribuidos en plantas salvajes y cultivadas, se asocian más comúnmente a lesiones en el tallo y raíces del maíz y sorgo (Shurtleff, 1980; Frederiksen, 1986), y las pérdidas económicas debidas a estas especies en estos cultivos se cifran en centenares de millones de dólares al año.

Además de su capacidad fitopatógena, las cepas de este grupo fúngico son capaces de producir una serie de metabolitos secundarios. Miembros de este grupo son productores conocidos de fumonisinas y moniliformina (Marasas *et al.*, 1984a; Thiel *et al.*, 1991a; Leslie *et al.*, 1992b), y han sido utilizados comercialmente para la síntesis de ácido giberélico (Phinney y West, 1960). También se ha demostrado su capacidad para producir deoxinivalenol, diacetoxiscirpenol, ácido fusárico, fusarinas, fusariocinas, toxina T-2 y zearalenona (Marasas *et al.*, 1984a), sin embargo, la dificultad para su identificación, desde el punto de vista taxonómico, ha dado lugar a una situación confusa. Así, la entidad anamorfa descrita por Snyder *et al.* (1945) como *Fusarium moniliforme* ha sido subdividida en cuatro especies morfológicas (*F. moniliforme, F. proliferatum, F. subglutinans, F. thapsinum*), y en seis especies biológicas (A-F) (Tabla 8).

El uso de parentales puede ser una herramienta útil para obviar las dificultades que plantean entidades biológicas diferentes, pero con una morfología muy similar.

Los miembros de la población A son casi universalmente productores de fumonisinas. Los miembros de la población D también son conocidos por producir fumonisinas; la variabilidad en cuanto a los niveles producidos es mucho más amplia que en la población A, asimismo son más comunes las cepas no productoras. Otro carácter útil para discernir entre poblaciones son los huéspedes sobre los que se establecen. Así, la población A es más común en maíz donde puede representar el 90% de la micoflora del grano (Campbell *et al.* 1992; Campbell y Leslie, 1993). Los miembros de las poblaciones D y E se aíslan en altas frecuencias en maíz, sobretodo en los tallos, mientras que los miembros de las poblaciones B y F (*G. thapsina*) son menos frecuentes en este cereal. En sorgo predomina la población F (*G. thapsina*), mientras que las poblaciones D y B presentan frecuencias inferiores.

Tabla 8. Especies biológicas (mating populations) de *Gibberella fujikuroi* y sus correspondientes anamorfos (*Fusarium*)

Mating populations	Anamorfo *Fusarium*
A	*F. moniliforme*
	F. verticillioides
	F. subglutinans
B	*F. sacchari*
	F. neoceras
C	*F. fujikuroi*
	F. proliferatum
D	*F. proliferatum*
E	*F. subglutinans*
	F. sacchari var. subglutinans
F	*
G	*F. nygamai*
Desconocida	*F.anthophilum*
	F. annulatum
	F. beomiforme
	F. dlamini
	F. napiforme
	F. proliferatum var. minus
	F. sacchari var. elongatum
	F. succisae

* La "mating population" F, se había asociado a cepas de *F. moniliforme* con características claramente diferenciadas de las pertenecientes a la "mating population" A. Sin embargo, recientemente dichas cepas han sido descritas como constituyentes de una nueva especie, *F. thapsinum*, perteneciente a la sección Liseola, con su forma teleomorfa *Gibberella thapsina* (Klittich *et al.*, 1997). Dichas cepas no son capaces de producir fumonisinas, razón por la cual pertenecían a un grupo vegetativo compatible diferente, con *F. moniliforme* como forma anamorfa (F) (Leslie, 1993; Leslie *et al.*, 1992a). Por lo tanto la presencia de cepas de *F. moniliforme* identificadas por el método morfológico tradicional en alimentos no supondría un indicio cierto de la posible contaminación por fumonisinas.

El ciclo de *Fusarium* sección Liseola en el maíz

Además del parasitismo como modo de vida, estos hongos pueden existir como saprófitos, y su mejor definición es como parásitos no obligados (Fig. 8). Su naturaleza como parásito no obligado permite al patógeno *Fusarium moniliforme* parasitar un amplio espectro de huéspedes. En el maíz esta especie fúngica existe como un endofito intercelular localizado en hojas, tallos y raíces, que no produce síntomas. Puede existir enteramente como un endofito, colonizando sistémicamente el grano, y permaneciendo en él hasta la germinación, infectando la nueva planta, que más tarde madura y le permite colonizar nuevos granos. Además de este modo de existencia endofito, este hongo también se presenta como saprofito, colonizando tejidos de maíz debilitados o muertos que sirve para la diseminación y supervivencia de la estructura de este hongo; durante la etapa saprofita activa, *F. moniliforme* coloniza los restos de plantas, dando lugar a un inóculo de considerable densidad tanto de micro como macroconidios (Bacon y Hinton, 1996).

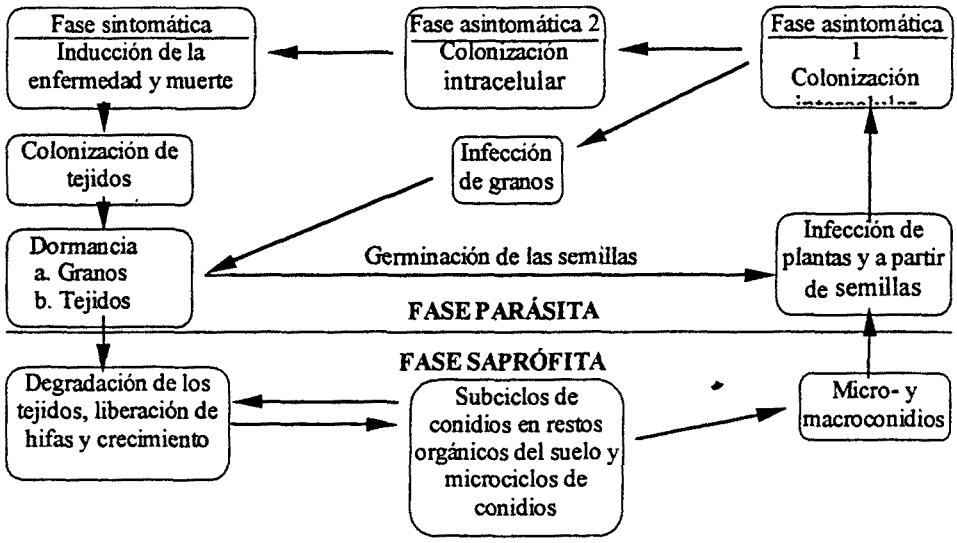


Figura 8. Vida y ciclos de infección de *F. moniliforme* en el maíz (Bacon y Hinton, 1996).

El alto número de huéspedes potenciales de *F. moniliforme* indica que este hongo produce un amplio rango de fitotoxinas o enzimas no específicos que le permiten colonizar estos huéspedes. Se ha demostrado que varias de estas fitotoxinas son micotóxicas, ésta podría ser una segunda función (Bacon y Hinton, 1996).

La producción de bajos niveles de micotoxinas, por ejemplo fumonisinas, por *F. moniliforme* es más probable durante la etapa en que éste no produce síntomas sobre la planta. Si el ciclo de la enfermedad empieza muy pronto, las micotoxinas no entraran en la cadena trófica. Sin embargo, si la enfermedad se desencadena al final del ciclo de vida de la planta (después del desarrollo del grano), las concentraciones de micotoxinas serán probablemente mayores (Bacon y Hinton, 1996).

La infección de los granos de maíz vía sistémica, a partir de las semillas o tallos infectados es un componente o factor que determina el porcentaje de granos infectados (Figura 9). Sin embargo la vía de infección más importante es la "silk infection" (Munkvold *et al.*, 1997).

F. moniliforme se sitúa en el área justo debajo del tejido vascular de cada grano. En granos sanos se encuentran sólo pequeñas porciones de hifas de este moho. No obstante, este pequeño inóculo puede resultar altamente invasivo si las condiciones de almacenamiento son poco adecuadas. (Bacon *et al.*, 1992).

La mayoría de las cepas de *Fusarium proliferatum* son capaces de producir grandes cantidades de fumonisinas, pero se sabe bien poco acerca de la epidemiología y la ecología de esta especie. En la literatura publicada antes de 1976, *F. proliferatum* era universalmente mal identificado como *F. moniliforme*. *F. proliferatum* es casi tan común como *F. moniliforme* en maíz cultivado en zonas templadas, y puede ser aislado de tejidos tanto sintomáticos como asintomáticos, incluyendo las semillas. *F. proliferatum* es un patógeno común en otros cultivos, como el espárrago. Esta especie comparte muchas características

morfológicas con *F. moniliforme*. Parece probable que ambas especies compartan un ciclo patógeno similar, pero todavía no está demostrado (Munkvold y Desjardins, 1997).

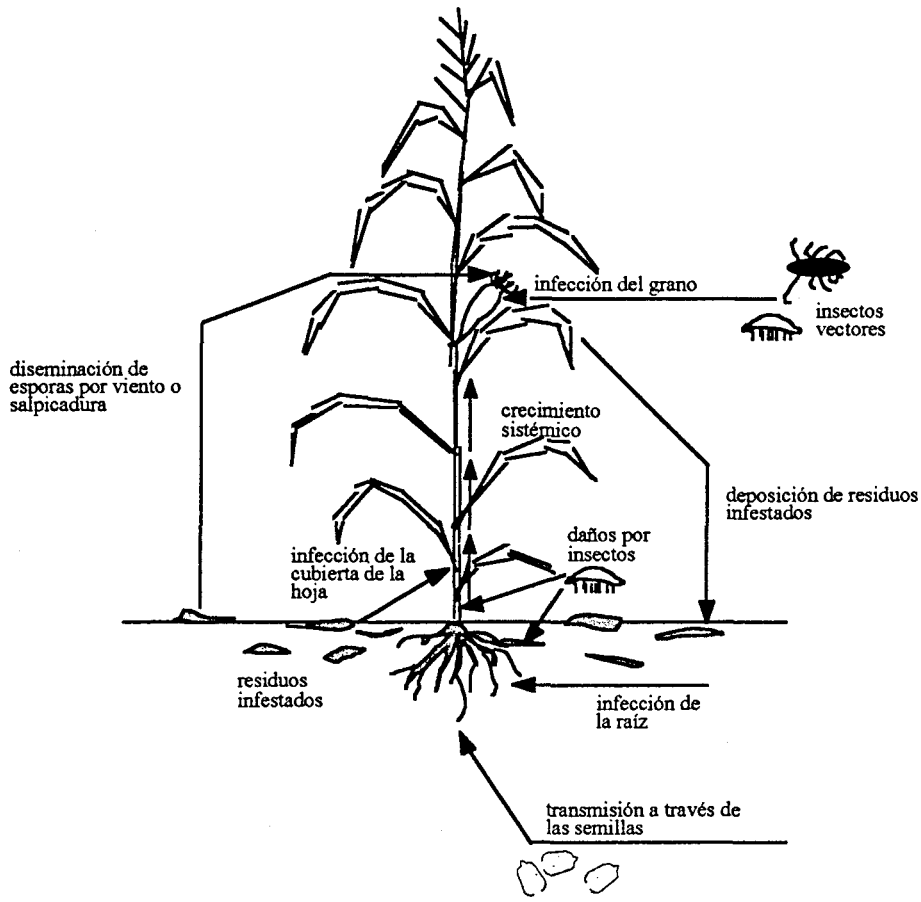


Figura 9. Ciclo de *F. moniliforme* en el maíz. Se ilustran diferentes rutas, pero su importancia relativa no se indica. La ruta más común es a través de los silks o daños por insectos (Munkvold y Desjardins, 1997).

Incidencia de *Fusarium* sección Liseola

Se han realizado numerosos estudios sobre la incidencia de *Fusarium* en maíz y derivados. Se han estudiado básicamente los dos siguientes aspectos: a) la incidencia de *F. moniliforme* y *F. proliferatum* en maíz, fracciones de maíz molturadas, palomitas y maíz dulce; b) la incidencia de las fumonisinas en maíz y derivados de maíz para alimentación humana y animal.

Se han encontrado niveles medios de infección por *Fusarium* de 8,4-36,2 % en maíz dentado, de los cuales *F. moniliforme*, *F. proliferatum*, *F. subglutinans* o *F. graminearum* resultaban ser las especies mayoritarias dependiendo del año de cosecha y de la localización geográfica. Años fríos y húmedos favorecen la proliferación de especies diferentes de *F.*

moniliforme, mientras que este último se vería favorecido por el calor y el estrés hídrico (Bacon y Williamson, 1992). En un muestreo realizado en países de América Central, África y Asia se observó que *Fusarium* spp. era el grupo de mohos mayoritario, y dentro de éste *F. moniliforme* era la especie más frecuentemente aislada (Macdonald y Chapman, 1997).

En palomitas se encontró una infección media de 1,8-10,3 %, de los granos y el 70,6-91,7% de las cepas fueron identificadas como *F. moniliforme*, en el análisis de maíz dulce, tanto en fresco (mazorca), como en grano congelado, se obtuvieron porcentajes de infección de 0,3 y 8%, respectivamente, concluyéndose que este último valor era probablemente debido a contaminación cruzada durante la manipulación del mismo. En este caso las especies de *Fusarium* aisladas fueron principalmente *F. semitectum* (Cagampang, 1994; Katta, 1994).

Cagampang (1994) realizó otro estudio en maíz para alimentación humana, y en las diferentes fracciones procedentes de la molturación del mismo. Así, encontró en el maíz entero porcentajes de infección por *Fusarium* que oscilaron entre el 10 y 28% de los granos. Las fracciones que presentaron recuentos de *Fusarium* mayores fueron el germen y la fibra, seguidas de la harina y por último (<100 ufc g^{-1}), las sémolas.

LAS FUMONISINAS

Las fumonisinas son micotoxinas producidas por diferentes especies del género *Fusarium*, y en menor cuantía, por el género *Alternaria*. De entre las especies de *Fusarium*, aquellas que pertenecen a la sección Liseola son las más productoras de fumonisinas, a excepción de *F. subglutinans*. *F. moniliforme* es el mayor productor de fumonisinas, mientras que *F. anthophilum* y *F. dlamini* son considerados los menores ya que no están asociados al maíz u otros cereales importantes para la alimentación humana. Estas especies solo han sido aisladas en zonas geográficas restringidas. Por contra, *F. napiforme* y *F. nygamai* parasitan sobretodo el sorgo y el mijo (Nelson *et al.*, 1992). La fumonisina B_1 (FB_1) es en general la más sintetizada, seguida de la fumonisina B_2 (FB_2). La producción de fumonisinas varía según la cepa estudiada.

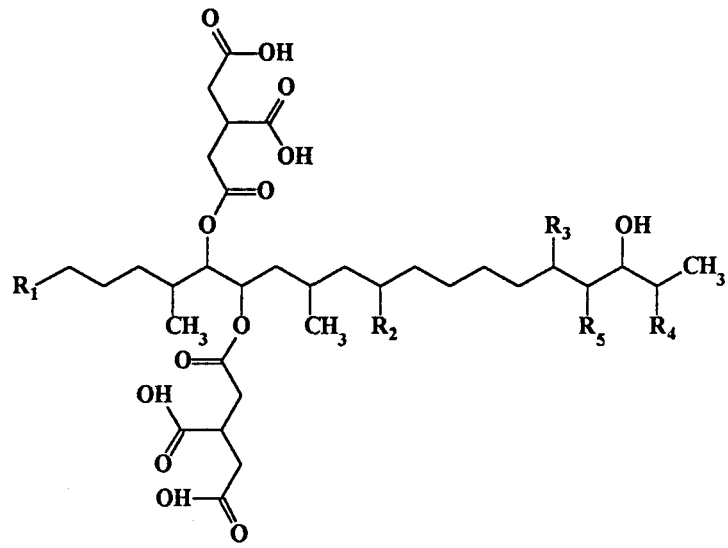
El maíz es, con mucho, el mejor sustrato para la toxicogénesis de *F. moniliforme*, aunque cantidades importantes de FB_1 se producen también en maíz molido y en alimentos preparados para ratas de laboratorio (Holcomb *et al.*, 1993b).

En 1991, Cawood *et al.* aislaron y caracterizaron dos nuevas fumonisinas, FB_3 y FB_4, a partir de cultivos de *F. moniliforme* MRC 826. Se demostró que los derivados N-acetil (FA_1 y FA_2) referenciados previamente por Bezuidenhout *et al.* (1988) eran metabolitos minoritarios de este hongo.

En muestras contaminadas de forma natural, la FB_1 supone un 70% de las fumonisinas totales presentes (Plattner *et al.*, 1992; Poling *et al.*, 1996). La FB_2 y FB_3 son producidas en cantidades similares entre sí, pero siempre menores que la FB_1. La FB_4, FA_1 y FA_2 aparecen en cantidades todavía más pequeñas (Cawood *et al.*, 1991).

Las 16 fumonisinas que han sido aisladas y caracterizadas (Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988a; Cawood *et al.*, 1991; Plattner *et al.* , 1992; Branham *et al.*, 1993; Musser *et al.*, 1996; Poling *et al.*, 1996; Mackenzie *et al.*, 1998) hasta ahora son: fumonisina B_1 (FB_1), fumonisina B_2 (FB_2), fumonisina B_3 (FB_3), fumonisina B_4 (FB_4), fumonisina A_1

(FA_1), fumonisina A_2 (FA_2), fumonisina A_3 (FA_3), fumonisina A_4 (FA_4), fumonisina C_1 (FC_1), fumonisina C_2 (FC_2), fumonisina C_3 (FC_3), fumonisina C_4 (FC_4), fumonisina P_1 (FP_1), fumonisina P_2 (FP_2), fumonisina P_3 (FP_3) y fumonisina iso-B_1 (FB_1) (Fig. 10).



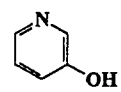
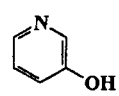
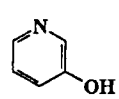
Fum.	R_1	R_2	R_3	R_4	R_5
B_1	CH_3	OH	OH	NH_2	H
B_2	CH_3	H	OH	NH_2	H
B_3	CH_3	OH	H	NH_2	H
B_4	CH_3	H	H	NH_2	H
A_1	CH_3	OH	OH	CH_3CONH	H
A_2	CH_3	H	OH	CH_3CONH	H
A_3	CH_3	OH	H	CH_3CONH	H
A_4	CH_3	H	H	CH_3CONH	H
C_1	H	OH	OH	NH_2	H
C_2	H	H	OH	NH_2	H
C_3	H	OH	H	NH_2	H
C_4	H	H	H	NH_2	H
iso-B_1	CH_3	OH	H	NH_2	OH
P_1	CH_3	OH	OH		H
P_2	CH_3	H	OH		H
P_3	CH_3	OH	H		H

Figura 10. Estructura molecular de las fumonisinas B_1, B_2, B_3, B_4, A_1, A_2, A_3, A_4, C_1, C_2, C_3, C_4, P_1, P_2, P_3 e iso-B_1.

Características de las fumonisinas

Químicamente las fumonisinas presentan como estructura una cadena lineal de 20 carbonos con: dos moléculas de ácido 1,2,3-propanotricarboxílico unidas mediante un enlace de tipo éster a los grupos hidroxilo de los carbonos 14 y 15, dos grupos metilo en los carbonos 12 y 16, un grupo amino en el carbono 2 y de tres a cinco hidroxilos en los carbonos 3, 5, 10, 14 y 15.

Las fumonisinas se presentan bajo la forma de sólidos amorfos (Gelderblom *et al.*, 1988a). Su peso molecular es de 722 g mol^{-1} para la FB_1 y de 706 g mol^{-1} para la FB_2. El punto de fusión de la FB_1 está entre 103-105°C (Dupuy, 1994). Son compuestos polares, solubles en agua, y más todavía en metanol y en la mezcla acetonitrilo:agua. Son insolubles en solventes apolares. Las fumonisinas no absorben la luz ultravioleta ni la visible, y no son fluorescentes. Una derivatización es, pues, necesaria para su detección después de la separación cromatográfica. El espectro infrarrojo de la FB_1 revela absorciones máximas a 3450, 2934, 1729 y 1632 cm^{-1} (Scott, 1993).

Las fumonisinas se consideran, generalmente, termoestables; por ejemplo, la concentración de FB_1 presente en una solución acuosa sometida a ebullición durante 30 minutos y secadas a 60°C durante 24 horas no sufrió apenas cambios. Estas micotoxinas son termoestables con lo que los tratamientos térmicos utilizados en el procesado del maíz no son suficientes para destruirlas totalmente (Dupuy *et al.*, 1993a; Dupuy, 1994). Sin embargo, estudios de estabilidad de la FB_1 realizados en harina de maíz demostraron que cuando esta toxina era sometida a tratamientos térmicos próximos a 100°C, su concentración disminuía ligeramente y que la destrucción era completa a 220°C (Scott, 1993).

Métodos de control. Detoxificación

La eficacia de los tratamientos químicos corrientemente utilizados para detoxificar los alimentos contaminados por micotoxinas es variable en el caso de las fumonisinas. La amonización del maíz contaminado reduce sólo en un 45% la concentración de FB_1, sin modificar su toxicidad en ratas (Norred *et al.*, 1991; Voss *et al.*, 1995). Por el contrario, el agua caliente asociada a un tratamiento térmico reduce en un 95% la concentración de FB_1 en el maíz, y la toxicidad de ésta en ratas (Hendrich *et al.*, 1993; Sydenham *et al.*, 1995). Este efecto estaría asociado con la pérdida del pericarpio de los granos, estructura que contiene la mayor parte de toxina. Durante el proceso de molturación y posterior refinado del maíz, se da una disminución progresiva de la toxina (Viljoen *et al.*, 1993). Cuando el maíz contaminado es molturado por vía húmeda, la mayoría de fumonisina va a parar al gluten, fibra, germen, y agua de remojo, mientras que muy poca cantidad se detecta en el almidón (Bennet *et al.*, 1996).

Por otra parte, se han probado métodos de procesado dirigidos especialmente a la destoxificación, sin embargo una complicación común a estos métodos ha sido que la reducción de la concentración de fumonisinas no siempre conlleva una reducción de su toxicidad. Las moléculas de fumonisina pueden verse alteradas de distintas maneras de modo que no son detectables pero sí tóxicas (Munkvold y Desjardins, 1997).

La fermentación alcohólica va acompañada de una destrucción parcial de la FB_1, mientras que la fracción no degradada se localiza en el orujo y no en la fracción alcohólica (Bothast *et*

al., 1992). Por contra, en cervecería, los rotos del maíz, a menudo utilizados como adjuntos, deben estar libres de fumonisinas, ya que éstas no son destruidas por el proceso de fermentación (Scott y Lawrence, 1995; Scott *et al.*, 1995). De hecho, la cerveza fabricada a partir de maíz contaminado es considerada como una fuente primaria de consumo de fumonisinas por parte del hombre en áreas de África con altos niveles de cáncer esofágico.

El tamizado o la separación mecánica constituye un método eficaz de descontaminación del maíz. En efecto, las partículas finas de maíz (<3 mm) constituyen del 5 a 20% de los cargamentos de maíz y concentran el 26,2-69,4% de la FB_1 (Sydenham *et al.*, 1994).

Los productores de maíz en E.E.U.U. han puesto, hasta la fecha, poca atención en la reducción del nivel de fumonisinas. Debido a la falta de regulación de su presencia y a la naturaleza esporádica de los brotes de PPE y ELEM, los productores no lo han considerado una prioridad. Sin embargo existen diferentes métodos para hacer frente a estas micotoxinas. Existen dos categorías: resistencia genética a *F. moniliforme*, y manejo y procesado del grano para separar los granos infectados y prevenir que el crecimiento fúngico continúe después de la cosecha (Munkvold y Desjardins, 1997).

Métodos estándar de almacenamiento de grano debieran prevenir la acumulación de fumonisinas en el grano almacenado, ya que *F. moniliforme* no crece en grano almacenado por debajo de 18-20% (Kommendahl y Windels, 1981), estando estos valores muy por encima del 13-14% recomendado para el almacenamiento de maíz a largo plazo. El nivel de fumonisinas no debería aumentar en el almacén si el grano se guarda en unas condiciones adecuadas de humedad y temperatura (Munkvold y Desjardins, 1997).

Toxicología

A continuación se exponen una serie de estudios sobre los efectos tóxicos que presentan las fumonisinas sobre los seres vivos.

Butler (1902) reprodujo leucoencefalomalacia (LEM) en caballos mediante la ingestión de maíz contaminado de forma natural en E.E.U.U. Sheldon (1904) describió la presencia de *F. moniliforme* en maíz enmohecido implicado en toxicosis en animales de granja en E.E.U.U. Wilson y Maronpot (1971) reprodujeron LEM en un asno con un cultivo puro de *F. moniliforme* aislado de maíz en Egipto. Kellerman *et al.* (1972) indujo un síndrome hepatotóxico en caballos con un cultivo puro de *F. moniliforme* aislado de maíz en Sudáfrica. Marasas *et al.* (1976) reprodujeron LEM en caballos, con un cultivo puro de *F. moniliforme* aislado de maíz en Sudáfrica. Marasas *et al.* (1981) demostraron que *F. moniliforme* predominaba significativamente más en maíz procedente de áreas con alta incidencia de cáncer esofágico en humanos que en el procedente de zonas de baja incidencia. Kriek *et al.* (1981) indujo un edema pulmonar (PES) en cerdos con un cultivo puro de *F. moniliforme* aislado de maíz en Sudáfrica. Marasas *et al.* (1984b) indujeron cáncer hepático en ratas con un cultivo puro de *F. moniliforme* aislado de maíz en Sudáfrica. Gelderblom *et al.* (1988b) demostraron la capacidad inductora de cultivos de *F. moniliforme* en la iniciación del cáncer hepático en ratas. Marasas *et al.* (1988b) indujo un síndrome similar a la LEM en un caballo por inyección intravenosa de FB_1, dato que confirmaron Laurent *et al.* (1989). También en 1989, Voss *et al.* detectaron FB_1 y FB_2 en muestras de maíz asociadas con brotes de LEM en caballos, aunque no cuantificaron los niveles. A finales de 1989-principios de 1990, la ingestión de granos rotos de maíz de la cosecha de 1989 en E.E.U.U.

produjo un elevado número de brotes de LEM en caballos y de PES en cerdos. Kellerman *et al.* (1990) reprodujeron las lesiones cerebrales propias de la LEM en caballos por ingestión vía oral de FB_1, y consecuentemente el efecto causante de la FB_1 de la LEM en caballos quedó probado. Por otra parte, Harrison *et al.* (1990) reprodujeron el PES en un cerdo por inyección intravenosa de FB_1. En 1991, Gelderblom *et al.* indujeron cáncer hepático en ratas suministrándoles durante 18-26 meses una dieta que contenía 50 mg kg^{-1} FB_1. Wilson *et al.* (1992) indujeron LEM en un caballo alimentándolo con rotos de maíz contaminado naturalmente con un contenido total de 4519 mg FB_1. Los mismos autores produjeron síntomas clínicos suaves y pasajeros, así como lesiones cerebrales histopatológicas suaves en caballos sometidos a una dieta que contenía 8 ppm FB_1. Haschek *et al.* (1992) indujeron PES en cerdos alimentándolos con granos rotos de maíz que contenían 166 mg kg^{-1} FB_1 y 48 mg kg^{-1} FB_2 y por inyección intravenosa de 72 mg FB_1. Por su parte Osweiler *et al.* (1992) obtuvieron el mismo resultado alimentándolos con rotos de maíz con 92 y 28 mg kg^{-1} de FB_1 y FB_2, respectivamente. Ross *et al.* (1993) indujeron LEM en ponies alimentados de granos rotos de maíz naturalmente contaminado (40,56-51,46 g FB_1). Recientemente, se ha observado que la administración de fumonisina B_1 en pavos provoca un aumento de peso en el hígado de los mismos (Bermúdez *et al.*, 1997).

Los efectos toxicológicos de la FB_1, son:

- Causa leucoencefalomalacia (LEM) en caballos
- Causa edema pulmonar (PES) en cerdos
- Es hepatotóxico en ratas
- Es hepatocarcinogénico en ratas
- Promueve e inicia cáncer de hígado en ratas
- No es mutagénica ni genotóxica
- Es citotóxica para cultivos de células de mamíferos
- Es fitotóxica
- Inhibe la biosíntesis de los esfingolípidos
- Ha sido asociada con el riesgo de cáncer esofágico (EC) en humanos

La Agencia Internacional para la Investigación contra el Cáncer (IARC) ha catalogado las micotoxinas derivadas de *Fusarium moniliforme* como 'carcinógenos del Grupo 2B', es decir, posiblemente carcinogénicos para los humanos. Se consideró que había una 'inadecuada evidencia' de la carcinogenicidad de la FB_1 en humanos debido a la exposición por vía oral, aunque al mismo tiempo concluyeron que los cultivos de *F. moniliforme* mostraban 'suficiente evidencia' de carcinogenicidad en animales experimentales (IARC, 1993). Niveles de tolerancia en maíz y derivados deben ser establecidos en base a la ingesta diaria probable (ID) y la ingesta diaria admisible (IDA). Sin embargo, dos vertientes deben ser tenidas en cuenta, por una parte, niveles de tolerancia demasiado altos pueden poner en peligro la salud humana y animal (niveles >8000 ng g^{-1} pueden causar ELEM en caballos)

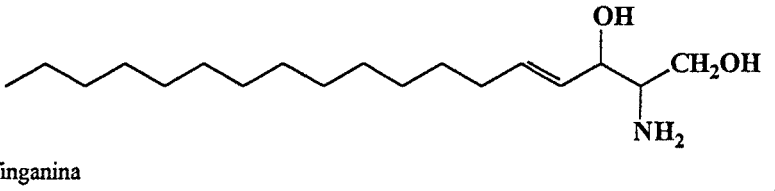
(Marasas *et al.*, 1993a,b), mientras que si los niveles de tolerancia son demasiado bajos, las consecuencias económicas podrían ser desastrosas, por ejemplo, niveles <1000 ng g^{-1} provocarían que el 34,5% de los productos a base de maíz para alimentación humana en E.E.U.U. se declararan ilegales (Thiel *et al.*, 1992).

Mecanismo de acción

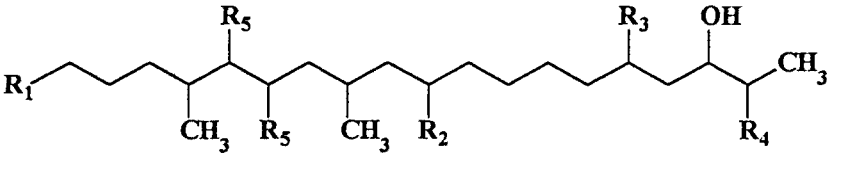
Las fumonisinas son inhibidores de la esfinganina (esfingosina) N-aciltransferasa (ceramidasintasa) *in vitro*, y exhibe una inhibición de tipo competitivo respecto a los dos sustratos de este enzima (esfinganina y acil-CoA), debido a la similitud estructural entre éstas y la esfinganina (Fig. 11). Si se anulan los ácido tricarboxílicos de la fumonisina B_1, esta actividad se reduce 10 veces, mientras que la fumonisina A_1 (que es acetilada en el grupo amino) es inactiva. Estudios con diversos tipos de células (hepatocitos, neuronas, células del riñón, fibroblastos, macrofagos, y células vegetales) han establecido que la fumonisina B_1 no solo bloquea la biosíntesis de los esfingolípidos complejos, sino que también da lugar a la acumulación de esfinganina (Fig. 12). Parte de la esfinganina es metabolizada a 1-fosfato y degradada a hexadecanal y fosfato-etanolamina, que es incorporada en la fosfatidiletanolamina. La esfinganina también se libera de las células, y debido a que aparece en sangre (Wang *et al.*, 1992) y orina, puede ser utilizada como biomarcador de la exposición a fumonisinas. La acumulación de estos compuestos bioactivos, así como la disminución de esfingolípidos complejos, puede ser la causa de la toxicidad, y quizás de la carcinogenicidad, de las fumonisinas (Merrill *et al.*, 1996).

El nivel de esfinganina en el suero del caballo puede usarse para la detección del consumo de fumonisinas, y si se detecta suficientemente pronto, puede permitir una respuesta antes de que la micotoxina haya provocado daño irreversible en el animal. El ratio esfinganina/esfingosina es un indice de la exposición a las fumonisinas (Wang *et al.*, 1992).

También es posible que las fumonisinas inhiban otros enzimas que interaccionen con las bases de cadena larga (esfinganina o esfingosina) o con los acil-CoA. Para comprobar esta posibilidad, la fumonisina B_1 ha sido ensayada con la esfingosina quinasa y la serina palmitoiltransferasa (Wang *et al.*, 1991), y ninguna de estos enzimas fue inhibido por los niveles que sí que dieron una completa inhibición de la ceramida sintetasa.



Esfinganina



Fumonisina

Figura 11. Estructura general de las fumonisinas y de la esfinganina.

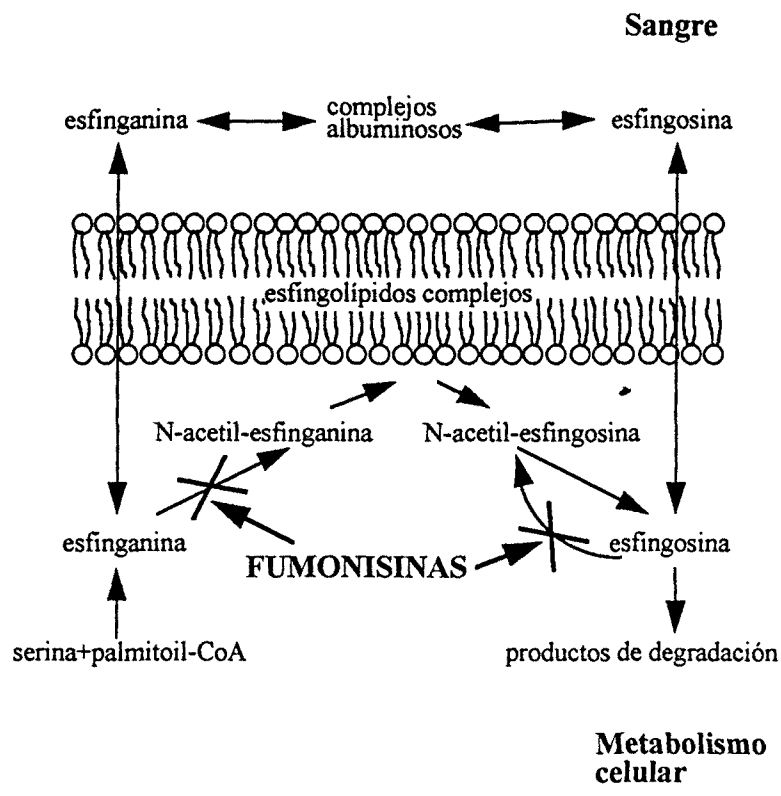


Figura 12. Mecanismo de acción de las fumonisinas (Sydenham *et al.*, 1991).

La exposición a las fumonisinas se traduce en un cambio en el comportamiento de las células, inhibición de su crecimiento y citotoxicidad. Existen dos explicaciones probables para la muerte de las células después de que la biosíntesis de los esfingolípidos haya sido inhibida por las fumonisinas:

- La acumulación de esfingosina libre es citotóxica de las células e inhibe su crecimiento. Se sabe que las bases de cadena larga son capaces de inhibir la proteína quinasa C, activar la fosfolipasa D, inhibir la Na^+/K^+ ATPasa, inducir la defosforilación de la proteína del retinoblastoma, inducir la liberación del Ca^{2+} de las reservas intracelulares, y de afectar a un buen número de sistemas reguladores celulares (Merrill *et al.*, 1993).

- El cese de la biosíntesis de esfingolípidos complejos puede alterar el comportamiento de la célula, así como podría provocar la muerte de la misma, basándose en resultados obtenidos para el enzima serina palmitoiltransferasa con mutantes (Hanada *et al.*, 1990).

Incidencia

En este apartado se procede a dar una serie de datos sobre la incidencia de las fumonisinas en diferentes países. Se ha estructurado en base al destino de las muestras (alimentación animal o humana), y a su relación con la aparición de enfermedades.

Las condiciones ambientales de una área específica de cultivo influye de manera determinante en la acumulación de fumonisinas en el maíz. En base a los datos publicados sobre la incidencia y niveles de fumonisinas en diferentes países/áreas geográficas, se pueden identificar 4 grupos de países: Europa del Este (Polonia, Rumania y Croacia), Unión Europea (Italia y Portugal), África (Benin y Zambia) y Sudamérica (Argentina). En el primer grupo se dieron niveles despreciables o ausencia completa de fumonisinas, mientras que en el grupo africano se encontraron niveles intermedios, con el 20% de muestras con > 1000 ng g^{-1}. En los dos grupos restantes el 100% de las muestras resultaron positivas y el nivel de contaminación fue más alto. La contaminación en muestras procedentes de Argentina fue significativamente más alta que la de cualquier otro país (Visconti, 1996).

Los niveles más altos de fumonisinas se han encontrado en derivados de maíz no asociados con alimentación humana. Algunos estudios han demostrado que el nivel medio de fumonisinas puede multiplicarse por diez en rotos de maíz, respecto al maíz entero (Murphy *et al.*, 1993). Asimismo, Cagampang (1994) obtuvo en maíz destinado a molturación seca, concentraciones de 0,1-3,5 μg g^{-1}, que una vez fraccionado se localizaban principalmente en la fibra y el germen, siendo la concentración menor en las sémolas.

El nivel medio de fumonisinas totales encontradas en 14 muestras de maíz asociado con LEM fue de 10,8 μg g^{-1} según Thiel *et al.* (1991b). Ross *et al.* (1991b) concluyó que sustratos con concentraciones > 10μg g^{-1} de FB_1 no eran seguros para la alimentación de caballos' mientras que Wilson *et al.* (1992) concluyeron que pueden derivarse problemas de la administración de dietas con 8 ppm FB_1 en caballos.

Asimismo, niveles de 8,85 μg g^{-1} FB_1 y 3,00 μg g^{-1} FB_2 (Shephard *et al.*, 1990), 12-130 μg g^{-1} FB_1 y <1-17 μg g^{-1} FB_2 (Plattner *et al.*, 1990) y 37-122 μg g^{-1} FB_1 y 2-23 μg g^{-1} FB_2 (Wilson *et al.*, 1990) se detectaron en muestras de maíz asociadas con brotes de LEM en caballos. Niveles de FB_1 (<1-160 μg g^{-1}), FB_2 (<1-49 μg g^{-1}) y FB_3 (<1-2,6 μg g^{-1}) se han encontrado en maíz, rotos de maíz y piensos a base de maíz asociados con brotes de LEM (Plattner *et al.*, 1990; Shephard *et al.*, 1990; Wilson *et al.*, 1990; Ross *et al.*, 1991a, b; Thiel *et al.*, 1991b; Ross *et al.*, 1992; Sydenham *et al.*, 1992a, b; Wilson *et al.*, 1992; Caramelli *et al.*, 1993; Ross *et al.*, 1993). Los niveles de FB_1 y FB_2 en tres muestras de maíz comercial en Sudáfrica oscilaron entre 0,4-4,4 μg g^{-1} FB_1 y 0,1-1,3 μg g^{-1} FB_2 (Shephard *et al.*, 1990). Sydenham *et al.* (1993) encontraron en muestras procedentes de Argentina concentraciones superiores a 2 μg g^{-1}, con máximos de 6 y 10 μg g^{-1}; los recuentos de *F. moniliforme* oscilaron entre 10^4 y $7 \cdot 10^5$ ufc g^{-1}, ambos muy altos para tratarse de maíz entero y sano. En muestras asociadas con LEM se dieron niveles de FB_1 de 1,3-27 μg g^{-1} y de FB_2 de 0,1-12,6 μg g^{-1} (Marasas *et al.*, 1979; Kellerman *et al.*, 1990).

Por otra parte, se han encontrado niveles de <1-330 μg g^{-1} FB_1 y <1-48 μg g^{-1} FB_2 en rotos de maíz asociados con brotes de PES en E.E.U.U. durante 1989/90 (Harrison *et al.*, 1990; Ross *et al.*, 1991a; Colvin y Harrison, 1992; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Ross *et al.*, 1992; Riley *et al.*, 1993a; Motelin *et al.*, 1994).

En alimentación humana, la mayoría de maíz en grano considerado de buena calidad presenta niveles de fumonisinas de 1 $\mu g\ g^{-1}$ o menores (Sydenham *et al.*, 1991; Pittet y Tornare, 1992; Pittet *et al.*, 1992). Los estudios realizados en derivados de maíz muestran como los productos menos procesados (harina de maíz, rotos...) presentan las mayores concentraciones (< 4$\mu g\ g^{-1}$), mientras que productos altamente procesados como los copos de maíz o las palomitas no presentan o en concentraciones despreciables. Los snacks suelen dar negativos, mientras que las tortillas, fibra de maíz y maíz en lata presentan concentraciones muy bajas (<0,4 $\mu g\ g^{-1}$) (Pittet *et al.*, 1992; Stack y Eppley, 1992; Doko y Visconti, 1994; Pestka *et al.*, 1994; Sanchis *et al.*, 1994, 1995; Zoller *et al.*, 1994).

Se han determinado los niveles de FB_1 y FB_2 en muestras destinadas a alimentación humana en zonas con alta incidencia de cancer esofágico (EC). Así, en una muestra, se obtuvieron niveles para granos sanos e infectados con *Fusarium* de 44 y 83 $\mu g\ g^{-1}$ FB_1, respectivamente (Sydenham *et al.*, 1990a). Consecuentemente, la FB_1 es una sustancia presente normalmente en la dieta de los habitantes de las zonas con riego alto de EC en Transkei. Muestras enmohecidas y no enmohecidas de maíz procedente de zonas de Transkei con alta incidencia de EC presentaron 3,45-46,9 $\mu g\ g^{-1}$ FB_1 y 0,9-16,3 $\mu g\ g^{-1}$ FB_2, concentraciones mucho mayores que las de muestras procedentes de áreas con baja incidencia de EC (Sydenham *et al.*, 1990b). Rheeder *et al.* (1992) determinaron niveles de fumonisinas en maíz procedente de zonas de Sudáfrica con alta incidencia de EC, mostrando que en maíz sano se daban niveles de 6,7-10,2 $\mu g\ g^{-1}$ mientras que en maíz enmohecido ascendía a 63,2-140,5 μg g. Asimismo, Chu y Li (1994) encontraron en muestras de maíz enmohecido procedente de zonas con alta incidencia de EC de China, concentraciones de 18-155 $\mu g\ g^{-1}$ FB_1. Yoshizawa *et al.* (1994) encontraron niveles similares de fumonisinas (0,87 $\mu g\ g^{-1}$ FB_1, 0,45 $\mu g\ g^{-1}$ FB_2) en muestras procedentes de zonas de alto riesgo de EC que en muestras de bajo riesgo, sin embargo el porcentaje de muestras contaminadas sí que resultó ser mayor en zonas con alto riesgo de EC. Niveles relativamente altos de fumonisinas se han encontrado también en derivados de maíz destinados a alimentación humana en otras zonas donde la incidencia del EC es importante, como el Norte de Italia (Doko y Visconti, 1993, 1994) y en Carolina del Sur (E.E.U.U.) (Sydenham *et al.*, 1991). Sin embargo, el EC no ha sido reproducido experimentalmente en animales, con lo cual no hay ninguna prueba de la relación causa-efecto (Marasas, 1994).

Muestras enmohecidas destinadas a la fabricación de cerveza presentaron niveles altos de fumonisinas (117,5 $\mu g\ g^{-1}$ FB_1 y 22,9 $\mu g\ kg^{-1}$ FB_2) (Rheeder *et al.*, 1992). Scott y Lawrence (1995) analizaron 37 marcas de cervezade las cuales sólo dos presentaron una contaminación superior a 5 $ng\ ml^{-1}$ (15 y 49 $ng\ ml^{-1}$).

Se ha determinado la influencia de procesos industriales en los niveles de fumonisinas presentes en las materias primeras. Así se ha comprobado que cuando se hace la fermentación alcohólica del maíz, las fumonisinas son estables bajo las condiciones que se dan durante la fermentación, sin embargo el etanol queda libre de esta toxina, mientras que se concentra en el bagazo restante, normalmente destinado a alimentación animal. Por otra parte la molturación por vía húmeda del maíz da lugar a los siguientes resultados: a partir de maíz naturalmente contaminado con 13,9 $\mu g\ FB_1\ g^{-1}$, el almidón queda libre de fumonisinas, mientras que el resto: gluten (5,1-5,8 $\mu g\ FB_1\ g^{-1}$, 4,7-4,9 $\mu g\ FB_2\ g^{-1}$), fibra (2,7-5,7 $\mu g\ FB_1\ g^{-1}$, 2,1-3,1 $\mu g\ FB_2\ g^{-1}$), germen (1,3-3,1 $\mu g\ FB_1\ g^{-1}$, 0,7-1,6 $\mu g\ FB_2\ g^{-1}$). El agua de remojo y las aguas de proceso contuvieron el 22% de las fumonisinas recuperadas (Bennet *et al.*, 1996).

Análisis de muestras de leche pasterizada no homogeneizada y pasterizada comercial, dieron niveles de fumonisina B_1 por debajo de 5 ng ml^{-1}. El calentamiento de la leche a 62°C durante 60 minutos simulando la paterización, o bien la conserrvación de la leche a 4°C durante 11 días no redujeron la concentación de fumonisinas (Maragos y Richard, 1994).

En la figura 13 se muestras las posibles vías de incidencia de las fuminisinas en maíz, así como las posibles vías de toxiinfección.

Métodos analíticos de determinación de fumonisinas

Las fumonisinas presentan dos grupos ácido tricarboxílico y pueden por tanto adquirir una carga negativa neta. La naturaleza aniónica de éstas es la base del uso extendido de la purificación de muestras mediante columnas de intercambio aniónico (SAX) (Fig. 14). Esta propiedad también permite que las fumonisinas puedan ser separadas por técnicas electroforéticas.

La cuantificación por HPLC con detección por fluorescencia de un derivado obtenido a partir del grupo amino libre (Shephard *et al.*, 1990) es el método analítico más extendido para alimentos destinados a humanos y animales. Este es un buen método para determinar FB_1, FB_2 y FB_3, pero no sus análogos n-acetilados, presentes a veces en algunas muestras en pequeñas concentraciones. Los resultados obtenidos mediante GC/MS (Plattner *et al.*, 1990) muestran buena correlación con los obtenidos por HPLC; sin embargo, esta técnica tampoco permite determinar los análogos n-acetilados.

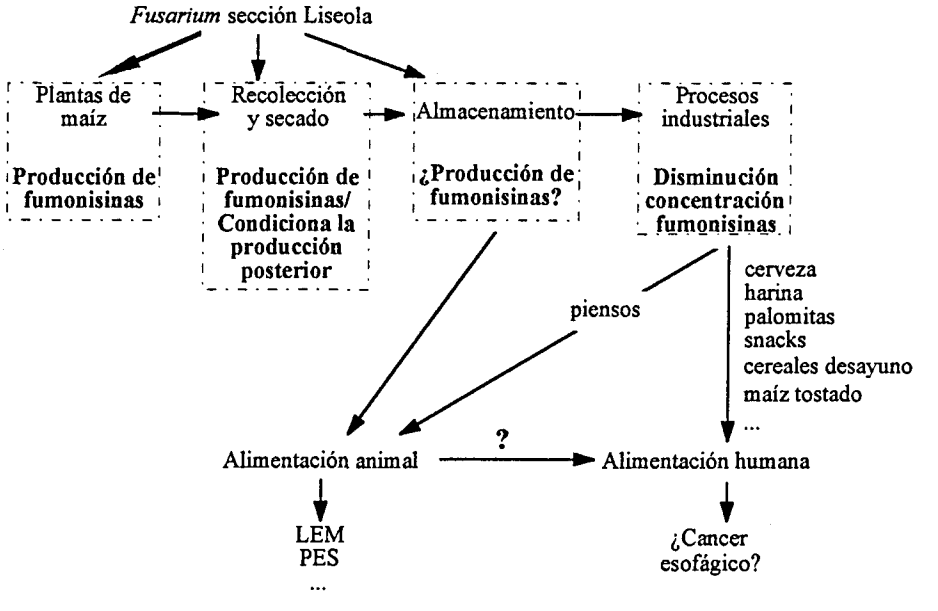


Figura 13. Vías de contaminación del maíz por fumonisinas; posibles vías de toxiinfección.

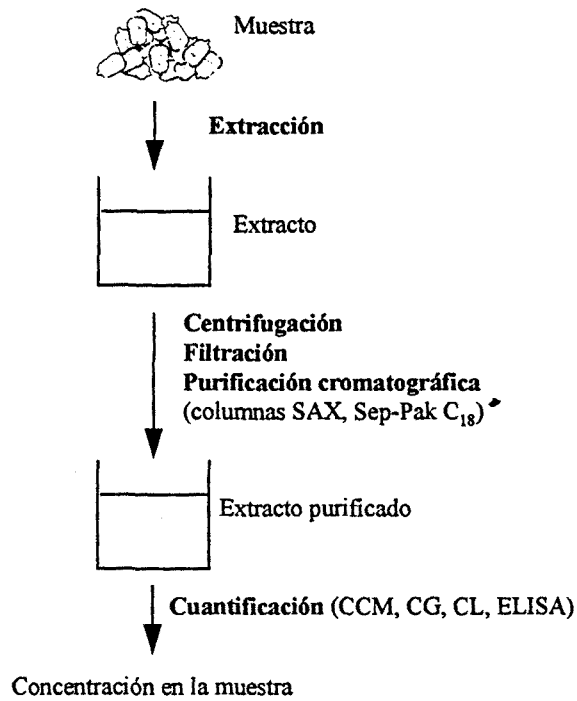


Figura 14. Extracción y detección de fumonisinas

En 1990 se desarrollan los primeros métodos para la detección y cuantificación de fumonisinas FB_1 y FB_2 en maíz y se utilizan por primera vez para determinar los niveles naturales en este cereal:

- Análisis por HPLC del derivado maleico de la FB_1 por detección ultravioleta y del derivado fluorescamina por fluorescencia (Sydenham *et al.*, 1990a).
- Análisis por HPLC con derivatización pre-columna con o-ftaldialdehido (OPA) y detección por fluorescencia y unos límites de detección de 50 ng g^{-1} FB_1 y 100 ng g^{-1} FB_2 (Shephard *et al.*, 1990).
- Análisis por hidrólisis y GC/MS, sensible para bajos niveles de ppm (Plattner *et al.*, 1990).

Dado que las fumonisinas no presentan ningún cromóforo fuerte, la mayoría de métodos analíticos pasan por la derivatización para conseguir compuestos fluorescentes (Tabla 9), así con este fin se ha usado: o-ftaldialdehido (OPA; Shephard *et al.*, 1992; Stack y Eppley, 1992), naftaleno dicarboxaldehido (NDA; Bennet y Richard, 1994), fluorescamina (Sydenham *et al.*, 1990a; Rottinghaus *et al.*, 1992), 4-fluoro-7-nitrobenzofurazan (Scott y Lawrence, 1992), fluoresceina isotiocianato (Maragos, 1995), y (9-fluorenilmetil) cloroformato (Holcomb *et al.*, 1993a). El límite de detección de estos métodos químicos suele estar entre 50 ppb y 1 ppm.

Tabla 9. Diferentes técnicas de cuantificación de la FB_1 (Thibault *et al.*, 1997)

TÉCNICA	LÍMITE DE DETECCIÓN (FB_1)	REFERENCIA
HPLC[1], derivatización con:		
Anhídrido maleico	10 $\mu g\ g^{-1}$	Sydenham *et al.*, 1990a
Fluorescamina	1 - 5 $\mu g\ g^{-1}$*	Holcomb *et al.*, 1993b Ross *et al.*, 1991a
O-ftaldialdehido	0,05 - 0,4 $\mu g\ g^{-1}$*	Shephard *et al.*, 1990, 1994
Naftaleno-2,3-dicarboxialdehido y cianuro de potasio	1 $\mu g\ g^{-1}$	Dupuy, 1994
9-fluorenil-metilcloroformato	0,2 $\mu g\ g^{-1}$	Holcomb *et al.*, 1993a Chen *et al.*, 1995
4-(N,N-dimetilaminosulfonil)-7-fluoro-2,1,3 benzoxadiazol	0,01 $\mu g\ g^{-1}$	Akiyama *et al.*, 1995
Detección con el sistema ELSD[2]	6 - 220 $\mu g\ ml^{-1}$	Wilkes *et al.*, 1995
Otras CL[3], derivatización con:		
4-fluoro-7-nitrobenzofurazan	0,1 $\mu g\ g^{-1}$	Scott y Lawrence, 1992
Naftaleno-2,3-dicarboxialdehido y cianuro de potasio	0.1 $\mu g\ g^{-1}$	Scott y Lawrence, 1992
O-ftaldialdehido y 2-mercaptoetanol	0,004 - 0,05 $\mu g\ g^{-1}$*	Sydenham *et al.*, 1992b Trucksess *et al.*, 1995
TLC[4], revelado con:		
P-anisaldehido	10 - 500 $\mu g\ g^{-1}$	Ross *et al.*, 1991c Sydenham *et al.*, 1990a
P-anisaldehido	estandar FB_1: 0,05 µg	Dupuy *et al.*, 1993b
Ninhidrina	estandar FB_1: 0,05 µg	Dupuy *et al.*, 1993b
Fluorescamina	0,1 $\mu g\ g^{-1}$	Rottinghaus *et al.*, 1992
CG[5], acoplado a la FID[6] o a la MS[7]		
El extracto purificado es hidrolizado, esterificado y acilado: identificación del ácido tricarboxílico	0,5 $\mu g\ g^{-1}$	Sydenham *et al.*, 1990a
ELISA[8]		
Competición entre FB_1 y FB_1-ovoalbúmina	0,05 0,1 $\mu g\ ml^{-1}$	Azcona-Oliveira *et al.*, 1992a
Competición entre FB_1 y FB_1-peroxidasa	0,1 $\mu g\ ml^{-1}$	Azcona-Oliveira *et al.*, 1992b

*según el sustrato; 1, cromatografía líquida de alta resolución; 2, detector por dispersión luminosa; 3, cromatografía líquida; 4, cromatografía de capa fina; 5, cromatografía de gases; 6, detector de ionización de llama; 7, espectrometria de masas; 8, ensayos inmunoenzimáticos.

Recientemente se han desarrollado métodos por electroforesis capilar de un derivado fluoresceina isotiocianato. Es un método altamente sensible que pasa por una purificación mediante columnas de afinidad y derivatización del grupo amino libre (Maragos, 1995). El límite de detección está por debajo de 50 ppb en maíz, y se han obtenido porcentajes medios de recuperación del 89% en muestras de maíz contaminadas con 0,25-5 ppm FB_1. Los resultados se correlacionan bien con los obtenidos con SAX/HPLC y C_{18}/HPLC. Otro método de detección de fumonisinas, pero que no requiere derivatización, es el FAB/MS (Korfmacher *et al.*, 1991). El FAB es sensible a los diferentes tipos de matriz con lo que variaciones en la matriz dan lugar a variaciones en los resultados obtenidos.

Ultimamente se han ensayado la detección y cuantificación de estas toxinas por HPLC utilizando detector por dispersión luminosa o espectrómetro de masas mediante electrospray, sin necesidad de derivatizar. En ambos casos fue necesaria una purificación de las muestras cuando éstas eran contaminadas de forma natural. Los límites de detección se situaron en 10-50 ng FB_1 para el detector por dispersión luminosa y <1 ng para el espectrómetro de masas mediante electrospray (Plattner *et al.*, 1996).

Métodos inmunoquímicos para fumonisinas. En varios laboratorios se han conseguido anticuerpos mono y policlonales contra fumonisinas y sus derivados hidrolizados. Se han utilizado en varios immunoensayos y pueden detectar entre 5 y 5000 ng FB_1 ml^{-1} en una solución estándar limpia. Sin embargo, en el caso de muestras, la matriz puede provocar interferencias. Por lo tanto, sin una purificación previa, la mayoría de immunoensayos pueden utilizarse únicamente como una herramienta para "screenings" en maíz y piensos con concentraciones entre 0,5 y 10 ppm. Los anticuerpos también se están utilizando en las columnas de afinidad, que se utilizan como método de purificación (Chu, 1996).

En general, se han obtenido resultados fiables utilizando tanto ELISA competitivo directo como indirecto, siempre y cuando la concentración de las muestras fuera superior a 1 ppm. Aunque en maíz y derivados con altos niveles de contaminación, no es necesaria una purificación de las muestras previa al immunoensayo, la sensibilidad y precisión del ELISA se mejoró cuando las muestras se sometían a una purificación con columnas rellenas de fase sólida, tales como columnas SAX (Usleber *et al.*, 1994) o columnas Sep-Pak C_{18} de fase reversa (Chu *et al.*, 1995; Maragos y Miklasz, 1995; Maragos *et al.*, 1996). Se ha intentado correlacionar los resultados obtenidos de immunoensayos con los obtenidos por métodos químicos, sin embargo, los resultados han sido inconsistentes; mientras unos autores encontraron buena correlación, otros encontraron una amplia discrepancia. En general, los niveles obtenidos por ELISA fueron mayores que los obtenidos por métodos químicos (Pestka *et al.*, 1994; Shelby *et al.*, 1994; Usleber *et al.*, 1994; Dreher y Usleber, 1995; Trucksess, 1995). La purificación previa mediante columnas SAX dio lugar a niveles más bajos en el test ELISA, pero todavía mayores a los obtenidos por HPLC (Usleber *et al.*, 1994). Estas irregularidades han sido generalmente atribuidas a la posible reacción cruzada de los anticuerpos para FB_1 con otras fumonisinas, con toxinas de este mismo grupo que podrían estar conjugadas con otros compuestos en los sistemas biológicos, o con otros compuestos estructuralmente relacionados, aunque también debe tenerse en cuenta como posible factor el bajo porcentaje de recuperación de los métodos químicos.

Recientemente se ha desarrollado un método ELISA competitivo directo para la detección de fumonisina B_1 hidrolizada (HFB_1), con un límite de detección de 5 ng g^{-1} para muestras entre 5-1000 ng g^{-1} (Maragos *et al.*, 1996).

La selección de un método para ser utilizado universalmente para el análisis de fumonisinas dependerá de la disponibilidad de instrumental, del límite de detección del método, así como de su reproducibilidad y precisión. La determinación de FB_1, FB_2 y FB_3 en maíz por HPLC en fase reversa de los derivados de fumonisinas mediante OPA usando detección por fluorescencia fue estudiada colaborativamente. Los resultados de dos estudios en colaboración a nivel internacional indicó que tanto la precisión como la reproducibilidad de este método lo hacían adecuado para ser considerado oficialmente para el análisis de muestras con concentraciones de hasta 8 ppm FB_1, 3,2 ppm FB_2 y 1,6 ppm FB_3 en maíz (Thiel *et al.*, 1996).

Los estudios realizados sobre muestreos han demostrado que cuando se muestrean lotes contaminados de forma no homogénea, cuanto menor sea el nivel de contaminación de la toxina, mayor se hace el error de muestreo (FAO, 1993).

Legislación

Actualmente en España, no existe ningún límite legal establecido en cuanto a fumonisinas. En materia de micotoxinas solo las aflatoxinas están legisladas. El único país que prevee un límite para fumonisinas es Suiza, que establece que la suma de FB_1 y FB_2 sea inferior a 1 ppm (FAO, 1997).

MÉTODOS DE CUANTIFICACIÓN FÚNGICA

Tradicionalmente, los métodos culturales microbiológicos se han usado para determinar la abundancia relativa de las diferentes especies en una muestra de grano (Lacey *et al.*, 1980; King *et al.*, 1986), tales como la siembra directa de granos en el medio de cultivo, o la siembra mediante diluciones decimales. Sin embargo, estos métodos cuantifican sólo hongos cultivables y a menudo requieren largos periodos de incubación en medios selectivos (Magan, 1993).

Las predicciones acerca del periodo máximo de almacenamiento en la práctica se han venido basando en el trabajo de Kreyger (1972), que usaba la presencia de enmohecimiento visible como criterio para determinar que el grano había alcanzado niveles de deterioro inaceptables. Kreyger (1972) defendía que pérdidas del 2% tenían poca importancia sobre la calidad del grano. Sin embargo, Seitz *et al.* (1982a,b) observaron que se pueden dar niveles inaceptables de deterioro en el maíz, antes de llegar a un 0,5% de pérdida de materia seca, y antes de que el crecimiento fúngico se hiciera visible.

Se han desarrollado una serie de métodos que permiten una detección temprana del deterioro, antes de que el enmohecimiento se haga visible. Estos pueden dividirse en aquellos que cuantifican la biomasa fúngica total tales como la degradación de componentes del grano, como aumento del porcentaje de azúcares reductores (Hummel *et al.*, 1954; Farag *et al.*, 1985a), o como incremento de ácidos grasos libres (Zeleny y Coleman, 1938; Farag *et al.*, 1985b); la detección de enzimas fúngicos (Jain *et al.*, 1991); la determinación bioquímica de quitina, ergosterol (Seitz *et al.*, 1977), y adenosin trifosfato (ATP); y el empleo de métodos electroquímicos basados en la conductividad, impedancia y capacitancia. De los métodos anteriormente mencionados, algunos de ellos tienen su principal inconveniente en el hecho de que las sustancias a determinar son compuestos que se

encuentran de forma natural en el grano, como es el caso de la quitina o el ATP. Entre los métodos que permiten detectar determinadas especies fúngicas se encuentran las sondas de ADN y las técnicas de immunofluorescencia e immunoensayos. Las ventajas de los immunoensayos es la rapidez, precisión y sensibilidad, y el hecho que la presencia previa de mohos puede ser detectada en alimentos que han sido tratados por calor o filtrados durante el procesado (De Ruiter *et al.*, 1993).

El seguimiento de la respiración es otro de los métodos usados para medir la actividad metabólica en cereales almacenados (White *et al.*, 1982a, 1982b). Tanto los mohos contaminantes, como el grano, respiran, pero la contribución de cada uno de ellos al ratio de respiración es motivo de controversia.

La presencia de compuestos volátiles de origen fúngico ha sido propuesto como índice rápido para la determinación del deterioro del grano (Kaminski *et al.*,1973, 1985; Börjesson *et al.*, 1989, 1992; Adamek *et al.*, 1992; Twiddy, 1994).

Estudios realizados con especies de *Fusarium, Penicillium* y *Rhizopus* muestran como la longitud de las hifas de los mismos y el nivel de ergosterol aumentan paralelamente. Para *Fusarium* spp., dada su poca capacidad para esporular, a diferencia de los otros dos géneros, los niveles de ufc aumentaron simultaneamente con los de ergosterol y la longitud de las hifas (Schnürer, 1993).

ESTUDIOS DE ECOLOGÍA FÚNGICA EN CEREALES

Para experimentos *in vitro* sobre medios de cultivo en el campo de la ecología fúngica, las reacciones de especies fúngicas frente a diferentes temperaturas y a_w se han cuantificado en términos de proporción de esporas germinadas, crecimiento lineal y producción de esporas. En el caso de cereal en grano se ha utilizado natural, autoclavado o irradiado; el crecimiento fúngico sobre estos sustratos puede cuantificarse mediante una serie de métodos, pero ninguno es ideal. Los métodos que requieren aislamiento en cultivo permiten contabilizar solamente aquellas especies capaces de crecer en dicho medio y a la temperatura de incubación fijada. La siembra mediante dilución decimal, permite el recuento de diferentes especies, pero favorece a las especies que más esporulan, y a las que crecen más rápido. Este método da una idea vaga de la colonización real que existe en el grano. La siembra directa de los granos, asimismo, favorece a las especies más rápidas en crecer y que compiten mejor. El ensayo del ATP determina la actividad microbiana en el grano, mientras que el análisis de quitina o ergosterol determinan la cantidad de biomasa fúngica. Sin embargo, estos métodos no permiten identificar las diferentes especies presentes (Magan y Lacey, 1988). Parámetros importantes desde el punto de vista ecológico son los siguientes: a_w mínima para germinar, temperaturas mínima y máxima que permiten la germinación, fase de latencia previa a la germinación, velocidad de crecimiento del tubo germinal, a_w mínima para crecer, y velocidad de crecimiento radial de colonia.

Es importante destacar el hecho de que la a_w mínima absoluta se da a los niveles óptimos del resto de factores, como la temperatura. Sin embargo, para cada temperatura, la a_w mínima será diferente. Normalmente la a_w mínima que permite la germinación es la misma que permite el crecimiento, aunque a veces la germinación se da a una a_w más baja sin que se dé luego el crecimiento (Ayerst, 1969).

Lacey *et al.* (1994) utilizaron un respirómetro electrolítico automático para monitorizar la respiración en muestras de grano a diferentes a_w y temperaturas. La monitorización de la producción de CO_2 había sido propuesta como medida del deterioro del maíz por Steele *et al.* (1969). Posteriormente se demostró que en maíz almacenado con un alto contenido de humedad (19-22%) la producción de CO_2 debida a la respiración global guarda una buena correlación con los recuentos de *Aspergillus* y *Penicillium*, con el porcentaje de granos infectados, y con la pérdida de capacidad germinativa del grano. *Aspergillus* y *Penicillium* fueron los géneros predominantes; cuando se llegó al 0,5% de pérdida de materia seca, los recuentos eran de $9 \cdot 10^5$-$5 \cdot 10^6$ ufc g^{-1} (Fernandez *et al.*, 1985). Asimismo, la invasión fúngica y el contenido de aflatoxinas pueden ser inaceptables antes de llegar al 0,5% de pérdidas; el inóculo inicial, particularmente de *A. flavus* afecta significativamente al alcance de la invasión (Seitz *et al.*, 1982a,b).

Para el caso del arroz se ha observado que el porcentaje de materia seca perdida no guarda correlación con el porcentaje de granos infectados. El seguimiento del incremento en la concentración de ergosterol es un mejor indicador de la calidad de granos con alto contenido de humedad (Naewbanij *et al.*, 1986).

Para estudios de la ecología de los mohos del grano se ha utilizado maíz tanto natural, irradiado, como autoclavado. La a_w para los experimentos se ajusta a partir de los datos obtenidos de las curvas de sorción; se ha observado que el maíz autoclavado presenta una relación a_w/contenido de agua diferente, asimismo, el proceso de esterilización por calor modifica las características nutricionales de este cereal. La γ-irradiación esteriliza el grano sin afectar a su germinabilidad y por lo tanto causa menos cambios que el autoclavado (Magan y Lacey, 1988). La producción de micotoxinas en maíz no estéril es menor, probablemente debido a la existencia de flora competitiva. En general, la producción de micotoxinas es mayor en maíz autoclavado, que en maíz irradiado (O'Neill *et al.*, 1996).

Se ha utilizado un diseño factorial para el estudio del efecto de la temperatura, a_w , período de incubación, sustrato y sus interacciones sobre la producción de aflatoxinas (Asevedo *et al.*, 1993) y la coproducción de aflatoxinas y ácido ciclopiazónico por parte de una cepa de *A. flavus* (Gqaleni *et al.*, 1997). El análisis de la varianza reveló que había una compleja interacción entre estos factores, y que esto afectaba a las cantidades de micotoxinas producidas.

MODELIZACIÓN APLICADA A HONGOS

La modelización matemática del crecimiento fúngico, y la habilidad para predecir si un hongo en particular será capaz de crecer en un alimento, y en caso afirmativo hasta qué punto lo hará, no ha recibido en absoluto un interés similar al despertado por la modelización del crecimiento bacteriano. Uno de los problemas principales es la dificultad para recoger datos útiles suficientes y reproducibles para la modelización.

Asumiendo que la cantidad de nutrientes es suficiente (como ocurre normalmente en alimentos), el crecimiento microbiano viene controlado por la temperatura, la a_w y el pH; factores adicionales como la presencia de conservantes, tratamiento por calor, y envasado en atmósferas modificadas, también contribuyen. En principio, si se tiene suficiente información sobre los factores que controlan el crecimiento, las respuestas de los microorganismos pueden predecirse.

En el caso de los hongos, existe una cierta complejidad asociada a la cuantificación del crecimiento fúngico. La medida del ratio de extensión miceliar (velocidad de crecimiento radial) es probablemente el método más simple y más directo, pero no representa necesariamente la verdadera naturaleza del crecimiento fúngico; las hifas pueden penetrar la matriz tri-dimensional de los alimentos, con lo cual los recuentos celulares tampoco son apropiados.

Se han utilizado modelos probabilísticos para predecir el crecimiento de *Zygosaccharomyces bailii* en soluciones modelo (Cole *et al.*, 1987). Los efectos del pH y del ácido sórbico sobre el crecimiento de *Penicillium chrysogenum, Cladosporium cladosporioides* y *Ulocladium atrum* fueron puestos de manifiesto mediante la modelización de las concentraciones mínimas inhibitorias en función del pH (Skirdal y Eklund, 1993); este modelo es útil para explicar el efecto conservante del ácido sórbico a diferentes pH, pero no tiene en cuenta el efecto de otros factores. Pitt (1993) modelizó los efectos de la temperatura, pH, a_w y tamaño de la colonia en el crecimiento fúngico y en la producción de aflatoxinas; dicho modelo tomó en consideración los cambios en velocidad de crecimiento, la masa fúngica, producción de aflatoxinas, la degradación de toxinas, y la disminución de la velocidad de crecimiento con respecto al aumento de la concentración fúngica.

Gibson y Hocking (1994) utilizaron un modelo empírico para los efectos de la a_w sobre el crecimiento en superficie de 4 especies de *Aspergillus*. Estudiaron la conveniencia del uso de modelos previamente usados en bacterias para la interpretación del crecimiento radial de cepas de *A. flavus, A. oryzae, A. parasiticus* y *A. nomius*. En primer lugar llevaron a cabo el ajuste de los diámetros de colonia a una curva de crecimiento; a partir de este ajuste se obtuvo la velocidad de crecimiento máxima (g) para cada a_w. Dichos valores se ajustaron en función de la a_w utilizando un modelo lineal de regresión. La a_w se transformó según: $b_w = \sqrt{(1-a_w)}$ y el modelo utilizado fue: $\ln g = C_0 + C_1 b_w + C_2 b_w^2$, donde los coeficientes C_0, C_1 y C_2 fueron calculados por regresión.

Algunos investigadores han encontrado una correlación significativa entre la inversa de la fase de latencia y la velocidad de crecimiento lineal en *A. restrictus* y *A. versicolor* dentro de los límites de a_w y temperaturas que permiten el crecimiento (Ayerst, 1969; Smith y Hill, 1982).

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II

OBJETIVOS

El objetivo principal de la presente tesis es profundizar en la ecología ligada al desarrollo de las especies fúngicas micotoxigénicas *Fusarium moniliforme* y *F. proliferatum*, en relación con la infección del maíz, mediante estudios detallados del efecto de los factores abióticos, y las interacciones fúngicas, todo ello enfocado desde el punto de vista de la germinación, crecimiento y producción de fumonisinas de dichas especies. Dicho objetivo se subdividió en los siguientes subobjetivos:

- Impacto de la a_w, temperatura y conservantes químicos, y sus interacciones sobre la germinación, crecimiento y producción de fumonisina B_1 por *F. moniliforme* y *F. proliferatum.*

- Importancia de las interacciones fúngicas entre la microflora propia del maíz y las especies de *Fusarium* sección Liseola *in vitro* y *in situ* en maíz en grano, y la interrelación con los factores abióticos.

- Evaluación del potencial de la manipulación de los factores ambientales como sistema de control del deterioro del maíz por *F. moniliforme* y *F. proliferatum.*

- Cuantificación y evaluación de la producción de enzimas hidrolíticos en las etapas tempranas de colonización del maíz por *F. moniliforme* y *F. proliferatum*, y la pérdida de valor calórico del mismo debido al deterioro, como medidas de la calidad del grano, todo ello en función de los factores abióticos.

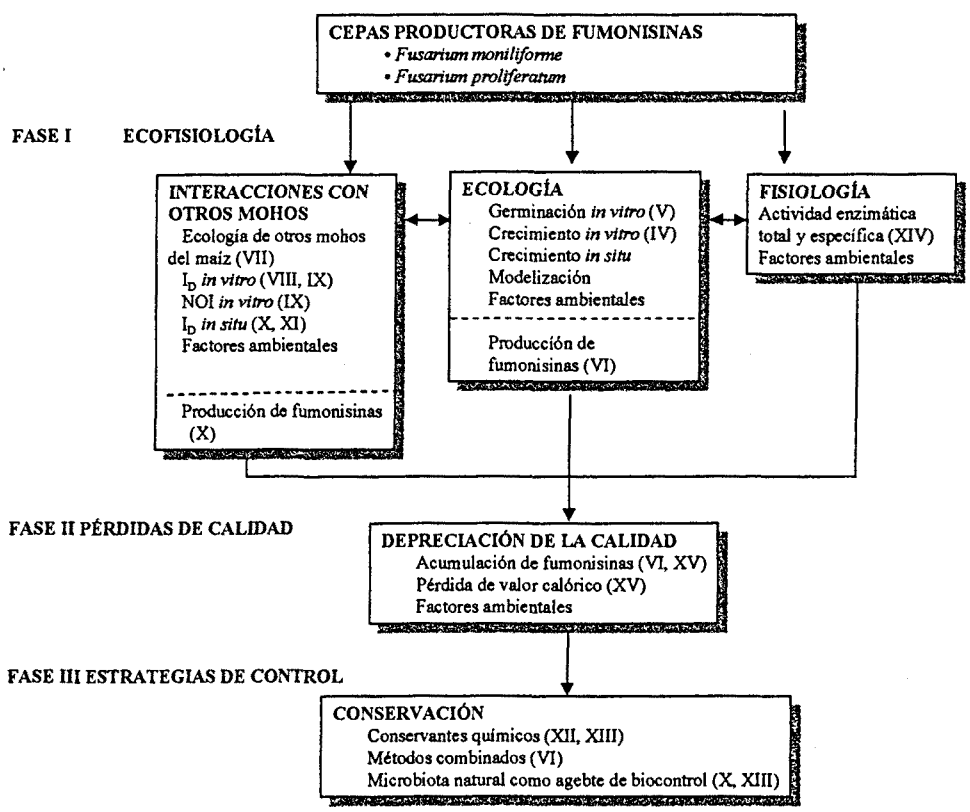
OBJECTIVES

The main objective of the thesis was to improve our understanding and knowledge of the ecology of the fungal toxigenic species F. moniliforme and F. proliferatum in relation to infection of maize by detailed studies on the effect of environmental factors, and interactions with other spoilage fungi on germination, growth and fumonisin production. This has been met by carrying out the following detailed series of studies:

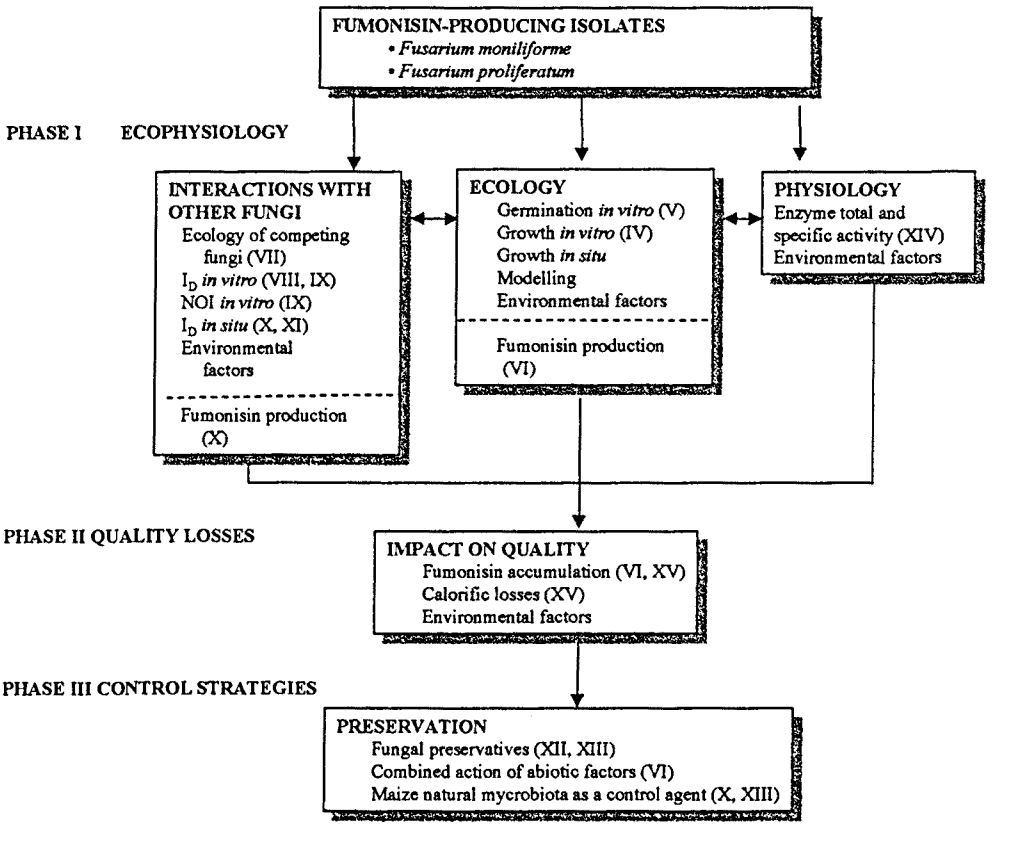
- *The impact of a_w, temperature and chemical preservatives, and their interactions on germination, growth, and fumonisin B_1 production by F. moniliforme and F. proliferatum.*
- *The importance of fungal interactions between contaminant maize fungi and Fusarium Liseola section species in vitro and in situ in maize grain, and the relationship with abiotic factors.*
- *Evaluate the potential of manipulation of environmental conditions as a means of controlling grain spoilage by F. moniliforme and F. proliferatum.*
- *Quantification of hydrolytic enzyme production during early colonisation of maize and calorific losses as a result of spoilage by Fusarium Liseola section species in relation to abiotic factors.*

III

PLAN DE TRABAJO



WORK PLANNING



IV

COMPARISON OF GROWTH AND FUMONISIN B_1 PRODUCTION BY *FUSARIUM MONILIFORME* AND *FUSARIUM PROLIFERATUM* ON MAIZE, WHEAT AND BARLEY GRAIN

S. Marín[1], N. Magan[2], J. Serra[1], A.J. Ramos[1] and V. Sanchis[1]

[1]Food Technology Department, University of Lleida, CeRTA, Rovira Roure, 177, 25198 Lleida, Spain
[2]Cranfield Biotechnology Centre, Cranfield University, Cranfield, Bedford, MK43OAL, U.K.

ABSTRACT

Two isolates each of *F. moniliforme* and *F. proliferatum* isolated from maize were compared for their growth and fumonisin B_1 production capacity on maize, wheat and barley extract agars and on irradiated maize, wheat and barley grain in relation to water availability (water activity, a_w; 0.995-0.88) and temperature (7-37°C for growth; 15 and 25°C for fumonisin B_1). Growth rates of the isolates of both *Fusarium* spp. were similar or higher on wheat and barley than that on maize extract agar. Overall regardless of temperature, both isolates of *Fusarium moniliforme* and *F. proliferatum* only produced fumonisin B_1 on irradiated maize grain at 0.98-0.93 a_w but none on wheat or barley. The implications and possible causes of these findings are discussed.

J. Food Sci. (submitted)

INTRODUCTION

It has been demonstrated that species of *F. moniliforme* and/or *F. proliferatum* are frequenly (>90%) isolated from maize, and that most of the isolates of these species are able to produce fumonisins . In addition to corn, *F. moniliforme* is commonly isolated from several grains including wheat, rice and oats. It also occurs, although infrequently, on other food and non-food commodities (Bacon and Nelson, 1994).

The highest levels of fumonisins have been found in corn-based products not associated with human food; most grain considered of good quality has levels of 1 $\mu g\ g^{-1}$ or less (Sydenham et al., 1991; Pittet and Tornare, 1992; Pittet et al., 1992). Studies in corn-based products revealed that low-processed food, e.g.maize flour or screenings, had higher concentrations ($<4\ \mu g\ g^{-1}$), while highly processed foods such as corn flakes and popcorn contained negligible or no fumonisins.

A wide variety of commodities have been analysed for fumonisins. To date, however, these metabolites have only been reported in corn and corn-based foods and feeds, with the exception of reports of fumonisins in 'black oats' feed from Brazil (Sydenham et al., 1992), contamination of New Zealand forage grass (Scott, 1993) and contamination of Indian sorghum (Shetty and Bhat, 1997). Despite the fact that *F. moniliforme* produces high concentrations of fumonisins in rice cultures, they have not been found to occur naturally in this cereal. However, strains isolated from agricultural products like wheat, barley and sorghum, have been shown to produce fumonisin B_1 in laboratory culture (Bacon and Nelson, 1994; Sala et al., 1994). In the laboratory, maize is by far the best substrate for *F. moniliforme* toxin production, although significant amounts of FB_1 can be produced on milled maize, rice and rat feed (Holcomb et al., 1993).

The objective of this work was to investigate the potential of fumonisin-producing strains to grow at different aw levels in maize wheat and barley based substrates and irradiated grains,and compare the biosynthesis of fumonisin B_1 in these substrate types. This is important in establishing the possible reasons why very few reports of the natural occurrence of fumonisins in cereals such as wheat and barley.

MATERIALS AND METHODS

Fungal isolates

The fungi used in this study were all isolated from maize (Sala et al., 1994) and included two isolates of *F. moniliforme* Sheldon (25N, 85N) and two of *F. proliferatum* (Matsushima) Nirenberg (73N, 131N). All isolates chosen produced fumonisins (Sala et al., 1994).

Medium

The basic media used in this study were 3% maize, barley and wheat extract agar with a pH of 6.31±0.35, 5.93±0.22 and 6.34±0.21, respectively. These were made by boiling 30g dry cereal/L water for 60 min. The resulting mixture was filtered through a double layer of muslin and the volume was made up to 1L. The water activity of these basal media was 0.995 a_w. The a_w of the media was modified by the addition of glycerol to obtain a_w levels of

0.98, 0.96, 0.94, 0.92, 0.90, and 0.88. The water activity of all media was determined with a Novasina Thermoconstanter TH200 (Novasina AG, Zurich, Switzerland).

Irradiated cereals

Spanish maize, barley and wheat were irradiated with 12 kGrays of gamma irradiation and stored at 4°C. The grain contained no fungal infection or contamination but had retained germinative capacity.

For all experiments, irradiated cereals were weighed into sterile flasks and rehydrated to the desired treatment a_w levels (0.93, 0.95 and 0.98) by addition of sterile distilled water. The amount of water added was calculated from a moisture adsorption curve for each cereal. The grain treatments were allowed to equilibrate at 4°C for 48 hours, with periodic shaking. Finally, the a_w values were confirmed by using a Novasina Thermoconstanter TH200.

Inoculation, incubation and growth rates assessment

Molten media (15 mL) were poured into 9-cm-diameter sterile Petri plates. Rehydrated maize was placed in sterile Petri plates (20g/plate, approximately) forming a single layer of grains.

A 5-mm diameter agar disk was taken from the margin of a 7-day-old growing colony of each isolate on malt extract agar and transferred to the centre of each plate. After that, Petri plates containing media of the same a_w were sealed in polyethylene bags, while plates containing grain at the same a_w were placed in closed chambers with beakers containing glycerol-water solutions of the same a_w as the plates in order to create an atmosphere with a same equilibrium relative humidity (E.R.H.). Bags were incubated at 7, 10, 15, 25, 30, and 37°C, while containers were incubated at 15 and 25°C. All treatments were repeated three times.

Every day during the incubation period growing colonies were measured with the aid of a binocular magnifier. Two diameters were obtained from each colony; then, growth rates (mm d^{-1}) were calculated by linear regression of colony radius against time for each strain at each set of conditions tested.

Inoculation and incubation of grain for fumonisin B_1 determination

Beakers containing 60 g of rehydrated grain of each treatment were inoculated with 0.5 ml of a microconidial spore suspension (2.4×10^7 spores ml^{-1}) of each isolate, and shaken vigorously. The inoculated irradiated maize was placed in sterile Petri dishes (20 g per plate), and incubated as described previously for 28 days. The experiment was repeated twice.

Fumonisin B_1 quantification

Samples were incubated for four weeks, and then frozen until extraction and analyses. They were extracted using a modification of the Shephard method (Shephard et al., 1990) as

described by Sanchis et al. (1994). After extraction, purified sample residue was dissolved in 0.5 ml of methanol. Two hundred µl of *o*-phtaldialdehyde (OPA) reagent, prepared according to Shephard et al. (1990) were added to a 50µl sample solution. Fifty µl of this solution were injected into the HPLC system within 2 min of derivatization. The eluent was methanol+0.1 M NaH_2PO_4 (75+25) adjusted to pH 3.35 with *o*-H_3PO_4. The flow rate was 0.8 ml min^{-1}. The recovery rate varied according to the following equation:

Recovery rate (%) = $135.14\, x^{-0.1294}$ where x = spiked FB_1 (ppm) with $0.1<x<100$

Reference standard of FB_1 was purchased from CSIRO, Division of Food Science and Technology, Pretoria, South Africa.

Simultaneously, a portion of each sample (10g) was dried in an oven at 105°C for 17 h (ISTA, 1976), to determine the moisture content, and calculate the fumonisin B_1 concentrations on a dry matter basis.

Statistical analyses of results

Analysis of variance was carried out in order to find significant differences between growth on agar media after 6 days, and on cereal grains after 5 days. The same analysis was applied to fumonisin concentrations. Analysis were made by using the SAS (Statistical Analysis System) version 6.12 (SAS Institute Inc.).

RESULTS

Differences between growth on wheat, barley and maize extract agar

The optimum temperature for growth for *F. proliferatum* and *F. moniliforme* was 30°C regardless of medium used (Fig. 1). Under the conditions tested growth rates were in the range 0-5.5 mm d^{-1} for *F. proliferatum* and 0-6.5 for *F. moniliforme*. *F. proliferatum* strains were able to grow between 7-37°C, while those of *F. moniliforme* only grew between 10-37°C. In general, growth rates increased with water availability. Slow growth was however observed at 0.88 a_w by some of the strains at 25°C.

In general, at 5-15°C growth was favoured on wheat, and sometimes on maize. However, at 25-30°C, growth was maximum on barley extract, and lower on maize and wheat extracts. Finally, at 37°C, growth was maximum on maize extract. Isolates of *F. moniliforme* grew faster than those of *F. proliferatum*; moreover, there were significant differences between growth of isolates belonging to the same species.

Analysis of variance revealed that all single factors (temperature, a_w, different media, different isolates) and their interactions had a significant influence on growth rates ($P<0.01$) (Table 1).

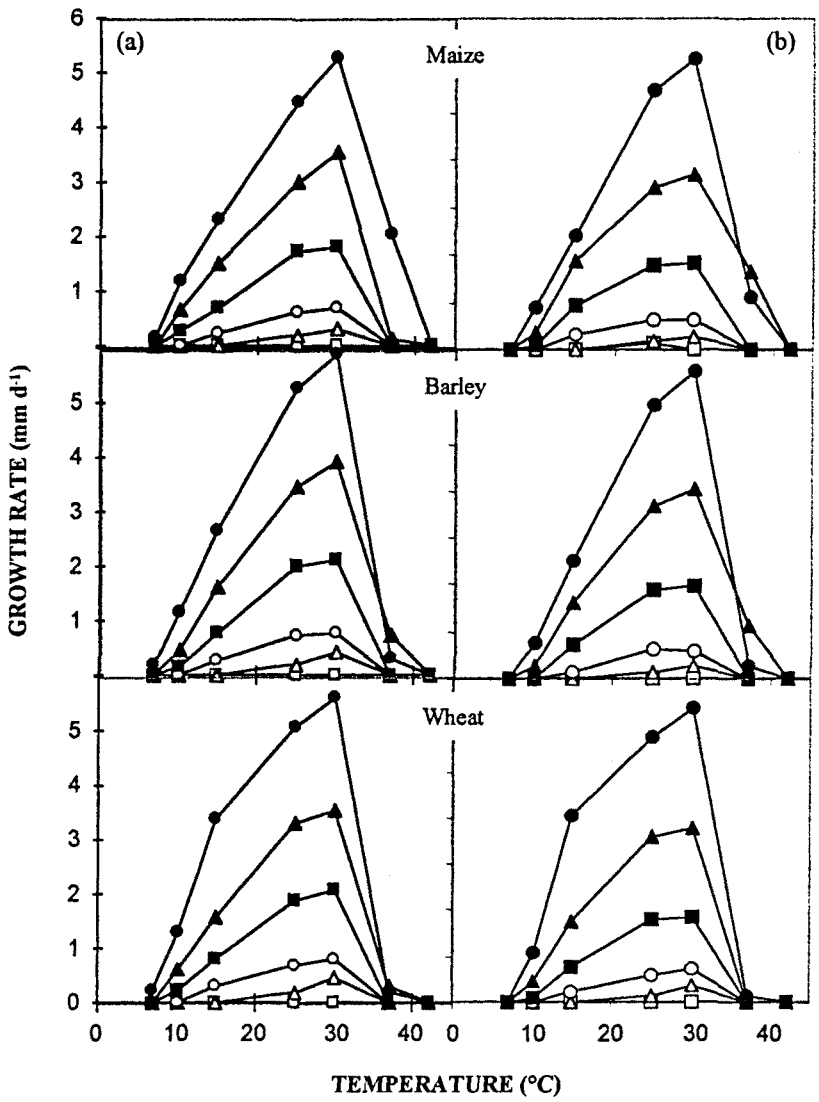


Figure 1. Effect of a_w, 0.88(□), 0.90(△), 0.92(○), 0.94(■), 0.96(▲), 0.98 (●)and temperature on growth rate of (a) *F. proliferatum* (131N), and (b) *F. moniliforme* (85N) on maize, barley and wheat extract agar.

Differences between growth on wheat, barley and maize grains

Maize was the poorest grain substrate supporting growth under the conditions tested. The *Fusarium* species grew best on barley, then on wheat and finally on maize, regardless of a_w, except for isolate 73N of *F. proliferatum* which grew better on wheat, than either barley or maize (Fig. 2).

Table 1. Analysis of variance of growth of *F. moniliforme* and *F. proliferatum* after 6 days, under different conditions. Significance of different media (M), a_w, temperatures (T) and isolates (S).

		F. proliferatum		*F. moniliforme*	
Factor	DF	MS	F	MS	F
a_w	4	4331.8	47518.6**	5717.3	39619.6**
T	4	4084.2	44801.9**	5188.4	35954.3**
S	1	2.5	27.6**	32.0	221.7**
M	2	25.7	282.5**	2.6	18.4**
a_w x T	16	397.3	4357.7**	652.6	4522.3**
a_w x S	4	4.9	54.7**	9.9	68.6**
a_w x M	8	10.1	110.5**	12.0	83.5**
T x S	4	0.2	1.9	1.9	12.89**
T x M	8	12.8	140.0**	11.6	80.2**
S x M	2	0.9	9.9**	0.8	5.7**

**Significant $P<0.01$

There was an important effect of a_w on the growth of the *Fusarium* isolates with rates increasing with available water, regardless of the cereal. However, *F. proliferatum* (131N) grew most rapidly at 0.95 a_w and 0.98 a_w on all the substrates tested. In general, isolate 25N of *F. moniliforme* grew best (2.57-6.36 mm d^{-1}) while isolate 131N of *F. proliferatum* was the slowest (2.54-5.16 mm d^{-1}); however, this depended on a_w levels and media used.

Analysis of variance revealed that all single factors (a_w, different cereals, different isolates) and some of their two-way interactions had a significant influence on the variability of results ($P<0.01$) (Table 2).

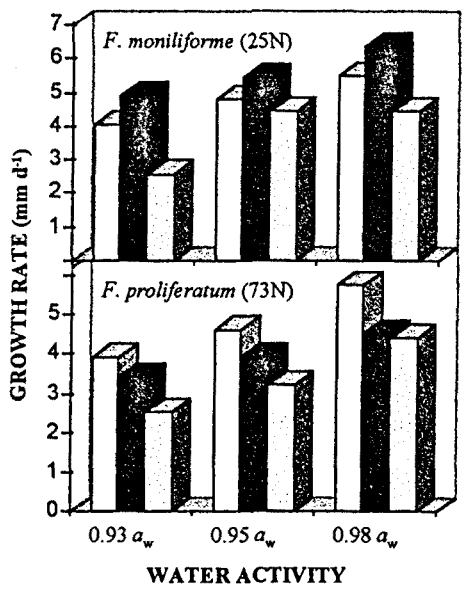


Figure 2. Effect of a_w on growth rate of *Fusarium* isolates at 25°C on wheat (□), barley (■) and maize (□) irradiated grain.

Table 2. Analysis of variance of the growth of *Fusarium* isolates on grain cereals at 25°C. Significance of different cereals (C), isolates (S) and a_w.

FACTOR	DF	MS	F
C	2	223.07	55.52**
S	3	52.87	13.16**
C × S	6	51.83	12.90**
a_w	2	732.80	182.37**
C × a_w	4	18.39	4.57**
S × a_w	6	7.98	1.99
C × S × a_w	12	7.68	1.91

**Significant, $P<0.01$

Differences between fumonisin B_1 production on wheat, barley and maize

Fumonisin B_1 production was always much higher on maize than on any of the other cereals (Fig. 3). The maximum amount produced on maize was 2019.2 μg g^{-1} grain, while for wheat and barley, concentrations were 3.5 and 1.5 ppm, respectively.

Fumonisin B_1 production on maize was significantly higher at 0.98 a_w, lower at 0.95 a_w, and lowest at 0.93 a_w. Fumonisin B_1 production in maize by isolates of *F. proliferatum* was high, but lower for those of *F. moniliforme*. However, there was an important interaction between isolates and a_w, that meant that at 0.93-0.95 a_w, isolates produced equal amounts of fumonisin B_1, while at 0.98 a_w *F. proliferatum* isolates produced a much greater quantity than those of *F. moniliforme*. In wheat and barley, similar concentrations were produced by all of the isolates at the a_w levels tested (0.28-3.55 ppm).

All single factors (isolates, cereals and a_w), as well as two-, three- and four-way interactions had a significant influence on fumonisin B_1 concentration in samples ($P < 0.01$) (Table 3).

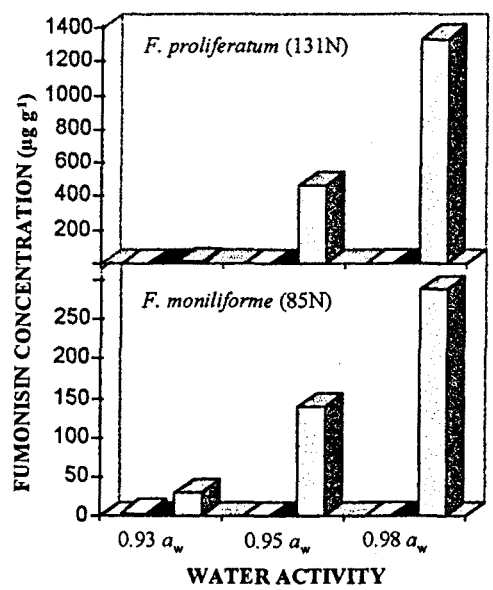


Figure 3. Effect of a_w on fumonisin B_1 production by *Fusarium* isolates at 25°C on wheat (□), barley (■) and maize (□) irradiated grain.

Table 3. Analysis of variance of FB_1 production on grain cereals at 25°C. Significance of different cereals (C), isolates (S) and a_w.

FACTOR	DF	MS	F
C	2	1421202	318.41**
S	3	194821	43.65**
C × S	6	195385	43.77**
a_w	2	682477	152.90**
C × a_w	4	684127	153.27**
S × a_w	6	144879	32.46**
C × S × a_w	12	144649	32.41**

Correlation between growth rate and fumonisin B_1 production on different cereals

There was an inverse correlation between growth and fumonisin B_1 production. This correlation was significant at 0.93 a_w (Pearson correlation coefficient, -0.8146), but not at 0.95 and 0.98 a_w (Pearson correlation coefficients, -0.4420 and -0.5024, respectively).

DISCUSSION

Mean levels of infection by *Fusarium* of 8.4-36.2% have been found in dent maize, with *F. moniliforme, F. proliferatum, F. subglutinans* or *F. graminearum* the most common species depending on the year of harvest and the geographical location (Bacon et al., 1992).

Fusarium is a contaminant of 88-100% of corn-based products for animal consumption as whole corn kernels, screenings and feeds in Spain. The presence of fumonisins has been detected in 12 out of 17 maize samples, 14 out of 15 samples of screenings and 18 out of 18 feed samples, with concentrations ranging from 650 to 920 ng g^{-1} (Sala et al., 1994).

Fusarium Liseola section strains are more common contaminants of maize than other cereals, like wheat and barley. Consequently, the low incidence of *Fusarium* Liseola section in these cereals could be the cause of the absence of fumonisins in such substrates. An extensive study by Sala (1993) on Spanish feed samples revealed that *Fusarium* species contaminated 14 out of 15 samples of maize, 3 out of 8 of wheat, 7 out of 17 of barley, 14 out of 17 of sorghum and 9 out of 24 of feed. Almost 100% of the *Fusarium* strains isolated from maize, wheat and feed samples belonged to *F. moniliforme* and *F. proliferatum*, while in barley the percentage was 60%. More than 60% of the *F. moniliforme* isolates were fumonisin B_1 producers and half of those were *F. proliferatum*. Out of 58 fumonisin producing isolates found, 43 had been isolated from maize, 2 from barley and 1 from wheat. However, the proportion between producers/non producers isolates did not different due to there origin.

Another reason for the possible absence of fumonisins in wheat and barley, could be the competing microflora, which is quite different to that present on maize. They could inhibit in some way the synthesis of fumonisins by *Fusarium* isolates, or on the other hand, could be

able to degrade the mycotoxin as soon as it is produced. It has been shown that the production of fumonisins by *F. moniliforme* and *F. proliferatum* can be altered in different ways by other maize fungi. For example, fumonisin production seems to be enhanced by the presence of A.*niger*, *A. flavus* and *A. ochraceus* under certain environmental conditions, while under most of the conditions of temperature and a_w tested, the production remains variable or diminishes (Marín et al., 1998).

In the present study it has been demonstrated that fumonisin-producing isolates of *Fusarium* are well adapted to grow on barley and wheat as they are for maize. *Fusarium* isolates were able to grow under similar conditions of temperature and a_w on wheat, barley and maize extract agars. At 25°C, the temperature level chosen for further fumonisin experiments, *Fusarium* isolates grew significantly faster on barley extract agar, than on wheat and then on maize extract agar. On whole grains, growth, in general, was faster on wheat; however *F. moniliforme* isolates grew better on barley in most cases. The size of the grains could be important for colonising fungi; small continuous layers of wheat or barley may provide an easier to colonise substrate than maize whose larger size results in greater intergranular air spaces. However the results suggest that perhaps the nutritional status of maize, and its structure may result in slowrer colonisation patterns on both maize extract and whole maize grains. Growth rates on cereal grains were much higher than on agar media, particularly at 0.93-0.95 a_w, but not at 0.98 a_w.

This study has demonstrated that although fumonisin-producers can colonise barley and wheat more rapidly than maize, they were unable to produce fumonisins in these cereals. Negligible amounts of fumonisin B_1 were found, ranging from 0.28 to 3.55 ppm, under a range of conducive water availabilities, which allowed high fumonisin production on maize. It is known that environmental conditions have a great effect on fumonisin production (Marín et al., 1995, 1998; Cahagnier et al., 1995). Our experiments were carried out at 25°C which is a suitable temperature for fumonisin production on maize. It is possible that fumonisins could be produced on the other cereals under different temperature regimes. However, it is worth noting that *F. moniliforme* has also been demonstrated to be unable to produce high concentrations of fumonisins in the laboratory on groundnuts and soybeans (< 5 ppm) (Holcomb et al., 1993).

Different hypotheses might explain these results: i) firstly, the isolates used in this study were originally isolated from maize, and while adaptation to growth on other cereals has been proven, it may be impossible for them to adapt their secondary metabolism to produce fumonisins in different substrates; (ii) one or several nutritional components present in barley and wheat could act as inhibitors of fumonisin biosynthesis, or a component of maize could have the capacity of being used for the initiation of biosynthesis of fumonisins, (iii) moreover, it has been shown that irradiation produces within maize one or more compounds which are inhibitory to aflatoxin biosynthesis or in some way alters the essential precursors to aflatoxin formation. It is not known if this also occurs with fumonisin production. However, if so, the effect of irradiation could lead to different consequences for different cereals; and finally, (iv) it is known that the ratio of protein/carbohydrates is important in favouring the synthesis of some mycotoxins. For example, with patulin or penicillic acid production by *P. roqueforti* where a high rate protein/carbohydrates is not suitable for synthesis (toxigenesis) (LeBars, 1988). These rates are 0.22 for wheat, 0.15 for barley and 0.12 for maize; although the rate for maize was the lowest, it is not significantly different from that for barley.

In conclusion, although section Liseola *Fusarium* species are not the predominant mycoflora of wheat and barley, and consequently they are from this point of view safer than maize, even if these cereals for any reason were heavily contaminated by fumonisin producers, there is the possibility that these strains would not be able to synthesise fumonisins in these cereals.

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V

WATER AND TEMPERATURE RELATIONS AND MICROCONIDIAL GERMINATION OF *FUSARIUM MONILIFORME* AND *F. PROLIFERATUM* FROM MAIZE

S. Marín, V. Sanchis, A. Teixidó, R. Sáenz, A.J. Ramos, I. Viñas, N. Magan

Food Technology Dept., Lleida University, UdL-IRTA, Centre R+D de Lleida, CeRTA, Rovira Roure 177, 25198 Lleida, Spain
Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, U.K.

ABSTRACT

The effects of water activity (a_w, 0.994-0.85 = 0.4-21.0 (-)MPa water potential), temperature (5-42°C), and their interactions on microconidial germination of three isolates each of *Fusarium moniliforme* and *Fusarium proliferatum* were determined in vitro on a maize meal extract medium. Temporal germination rates of microconidia of isolates of both species were significantly influenced by both a_w and temperature. Germination was very rapid at > 0.94 a_w with an almost linear increase with time. Germination rates of microconidia of *F.moniliforme* were slower than those of *F.proliferatum* isolates at marginal a_w levels and 5-25°C, while at higher temperature (30-37°C), the former germinated more rapidly than the latter. The a_w minima for germination of isolates of both species was 0.88, with none occurring at 0.85 a_w over a 40-day incubation period. At 37°C, isolates of *F. moniliforme* had slightly lower a_w minima than those of *F. proliferatum*. The narrowest range of a_w for germination was at 5°C, and none occurred at 42°C. The effect of a_w × temperature interactions on the lag phases (h) prior to germination and the germination rates (h^{-1}) were estimated using the Gompertz model and the Zwietering equation. This showed that lag phases were shorter at 25-30°C and 0.994-0.98 a_w, and were increased to 10-500 h at marginal temperatures (5-10°C) for *F. proliferatum* and longer for *F. moniliforme*. At marginal a_w levels (0.92-0.90), lag times were increased to > 250 h. Germination rates (h^{-1}) were different for the two species. Microconidia of *F. moniliforme* germinated optimally at 25-37°C and 0.96-0.98 a_w, but this changed to 30°C at 0.90-0.94 a_w while germination of microconidia of *F. proliferatum* remained optimum at 30°C regardless of a_w. There were statistically significant ($P < 0.01$) effects of a_w, temperature, isolate, and two- and three-way interactions for *F.proliferatum*, but there were no intraisolate effects for *F. moniliforme*. The ecological significance of these data for understanding colonization patterns of these important fumonisin-producing fungi are discussed.

Key words: water activity, temperature, germination, fumonisin-producing, *Fusarium* spp.

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INTRODUCTION

Fungal spores contaminate the surfaces of ripening crops and, depending on the climatic conditions, will rapidly germinate and colonize the surface or infect the maturing grain. Maize is particularly susceptible to colonization and infection immediately after silk emergence (Sutton 1982; Miller 1994) when conducive environmental conditions exist for 10-20 days, and *Fusarium* spp. can cause severe ear rot. Recently, there has been particular interest in the infection of maize kernels during ripening and postharvest storage by *Fusarium moniliforme* and *Fusarium proliferatum* because of their capacity to produce a group of mycotoxins, the fumonisins (Ross et al. 1990; Thiel et al. 1991).

Two of the most important abiotic parameters determining the potential for spore germination and growth of propagules on the grain surface are water availability (water activity, a_w; water potential (-MPa)) and temperature. In the context of grain, a_w is equivalent to the solute potential component, which predominates in such substrates. Knowledge of the effect of these parameters alone and their interactions on germination of *Fusarium* spp. is limited. The range of a_w and temperatures for germination of *Fusarium culmorum* isolates from wheat grain and wheat straw (Magan and Lacey 1984a; Magan 1988) and of the *F.roseum/culmorum/graminearum* (Gp I & II) complex have been determined (Sung and Cook 1981). Germination of macroconidia, chlamydospores and ascospores of the *F. roseum* complex occurred optimally at between -0.1 to -2.0 MPa water potential (= 0.999-0.985 a_w) with none occurring at -6.0 to -8.0 MPa (= 0.955 to 0.942 a_w). However, their studies were limited to a period of only 24h and one temperature (25°C). More extensive work with a wheat isolate of *F. culmorum* demonstrated a wider range of temperature and a_w for germination, with limits for germination of 0.87 a_w (25°C), than that for growth (0.90 a_w, Magan and Lacey 1984a). However, no information is available on the water and temperature relations of spore germination for isolates of *F. moniliforme* and *F. proliferatum*, especially those that produce fumonisins. Such knowledge is important as it represents the critical first stage in the colonization process resulting in infection of grain and possibly toxin production. Recently, *F. moniliforme* and *F. proliferatum* were shown to grow in vitro at between 0.994 and 0.90 a_w over the temperature range 20-35°C (Marín et al. 1995a) and produce fumonisin B_1 and B_2 down to 0.925 a_w on maize grain (Marín et al. 1995b).

The objectives of this study were to determine the effect of a_w, temperature and their interactions on (a) temporal microconidial germination rates, (b) lag times for germination, and (c) intrastrain differences of three isolates each of *F. moniliforme* and *F. proliferatum* in vitro on a maize extract medium.

MATERIALS AND METHODS

Isolates

The fungi used in this study were all isolated from maize (Sala et al. 1994) and included three isolates of *F. moniliforme* Sheld. (25N, 123N, 85N) and three of *F. proliferatum* (Matshushima) Nirenberg (73N, 131N, 55N). All isolates chosen produced fumonisins (Sala et al. 1994).

Medium

The basic medium used in this study was a 3% maize meal extract agar (MMEA) with a pH of 5.5. This was made by boiling 30 g dry maize/L water for 60 min. The resulting mixture was filtered through a double layer of muslin and the volume made up to 1 L. The water activity of this basal medium was 0.994 (a_w = -0.4 MPa water potential). The a_w of the medium was modified by the addition of glycerol to obtain a_w levels of 0.98, 0.96, 0.94, 0.92, 0.90 and 0.88 (Marín et al. 1995a). The water activity of all media was determined with a Thermoconstanter Novasina TH200 (Novasina AG, Zurich, Switzerland).

A number of solutes can be used to modify a_w, including both ionic (NaCl, KCl, $MgCl_2$) and nonionic (glucose, glucose + fructose, glycerol) ones. Other compounds such as polyethylene glycol (PEG 200-6000), have a increasing matric potential component that is less comparable to grain substrates. In this study glycerol was preferred because of its nonionic nature and stability over a wide temperature range. The a_w of ionic solutes varies with temperature and they have been implicated in increased growth at intermediate a_w levels (Brownwell and Schneider 1984) and being toxic at very high concentrations (Luard 1983; Magan 1988). They are also not representative of the type of solute potentials generated in cereal grains. On moist maize, non-xerotolerant fungi such as *Fusarium* spp. do not accumulate low molecular weight polyols such as glycerol and erythritol to a significant degree. Spores were collected from this medium and placed on the glycerol amended media. Thus, initiation of germination would not be influenced directly by the glycerol in the medium, although later during mycelial growth it might be utilized as a carbon source (Marín 1995a).

Culture, incubation and spore germination measurement

Fungi were grown on moist maize grain (50g maize + 20 ml distilled water) for 7 days at 25°C to obtain heavily sporulating cultures. Spores were suspended in sterile distilled water containing one drop of a wetting agent (Tween 80). Stock spore suspensions (1 ml) were added to 2 ml of sterile water previously modified with glycerol to the required water availability treatment. The final water activity of the treatments were 0.85, 0.88, 0.90, 0.92, 0.94, 0.96 and 0.98 a_w, and the final concentration of spores was in the range 1-5 $\times$ 10^6 spores/ml.

A 0.1-mL aliquot of the spore suspensions was pipetted onto MMEA plates of the same a_w and spread on the surface of the agar medium with a sterile bent glass rod (hockey stick) as quickly and carefully as possible. Petri dishes of the same a_w treatment were enclosed in polyethylene bags and incubated at 5, 10, 15, 25, 30, 37 and 42°C. Initial experiments showed that this method gave consistent and repeatable results. The final experiments were carried out with at least three replicate petri plates per treatment.

Periodically, depending on the treatment, three agar discs (5 mm diameter) were aseptically removed from each replicate plate using a cork borer, placed on a slide, and examined microscopically. Fifty single spores per disc (150/replicate petri plate; 450/treatment) were examined. Spores were considered to have germinated when the germ-tube was equal to or greater than the diameter of the spore. The experiments were carried out for a maximum of 40 days. The minimum condition for germination was categorized as any treatment temperature or a_w at which 10% germination was observed.

Statistical treatment of the results

The variable measured was the percentage germination at different a_w/temperatures against time. The percent germination at each a_w/temperature condition was plotted against time, and nonlinear regression was used to estimate the following two parameters at the 95% confidence level: (a) maximum germination rate (h^{-1}) and (b) lag phase (h). The Gompertz model (1825) was used as the fitting equation (Zwietering et al. 1990).

$$\%\ germination = A \exp\{-\exp[\frac{\mu_m e}{A}(\delta - t) + 1]\}$$

where A = asymptotic value where the germination rate becomes constant (100% in most cases); μ_m = maximum specific germination rate (h^{-1}) given by the slope of the line when the spores germinate exponentially; δ = lag phase (h).

The percent germination after 24 h at different a_w and temperatures was *logit* transformed (log(x/(100-x))) in order to homogenize variance and analyzed by ANOVA for determination of effects of a_w, temperature, isolate, and two- and three-way interactions. Both nonlinear regressions and analysis of variance were made by using SYSTAT (version 5.0, SYSTAT inc.) statistic package.

RESULTS

Temporal effects of water activity on germination

Figure 1 shows that the germination of microconidia of an isolate of *F. moniliforme* (25N) and *F. proliferatum* (73N) was very rapid at > 0.94 a_w, with an almost linear increase with time. However, under more extreme water stress the rate of spore germination, particularly for the isolate of *F. moniliforme*, was slower. The minimum a_w at which germination occurred was 0.88, although under these water availability conditions the lag times prior to germination were 50-100 h and germination was slow. At 0.85 a_w (the lowest level of water activity tested), no germination occurred within 40 days.

The minimum a_w for germination of the isolates of *F. moniliforme* and *F. proliferatum* changed with temperature using a 40-day incubation period (Table 1). At 30 and 37°C, the isolates of *F. moniliforme* had slightly lower a_w minima for germination than that for *F. proliferatum*. No germination of microconidia was observed at 42°C regardless of a_w. The narrowest range of a_w for germination was at the marginal temperature of 5°C.

Analysis of variance of the effect of a_w, temperature, and two- and three-way interactions showed that there were no differences among isolates of *F.moniliforme*, although all other factors and interactions were significant ($P < 0.01$, Table 2). For *F.proliferatum*, one-, two- and three-way interactions were all statistically significant.

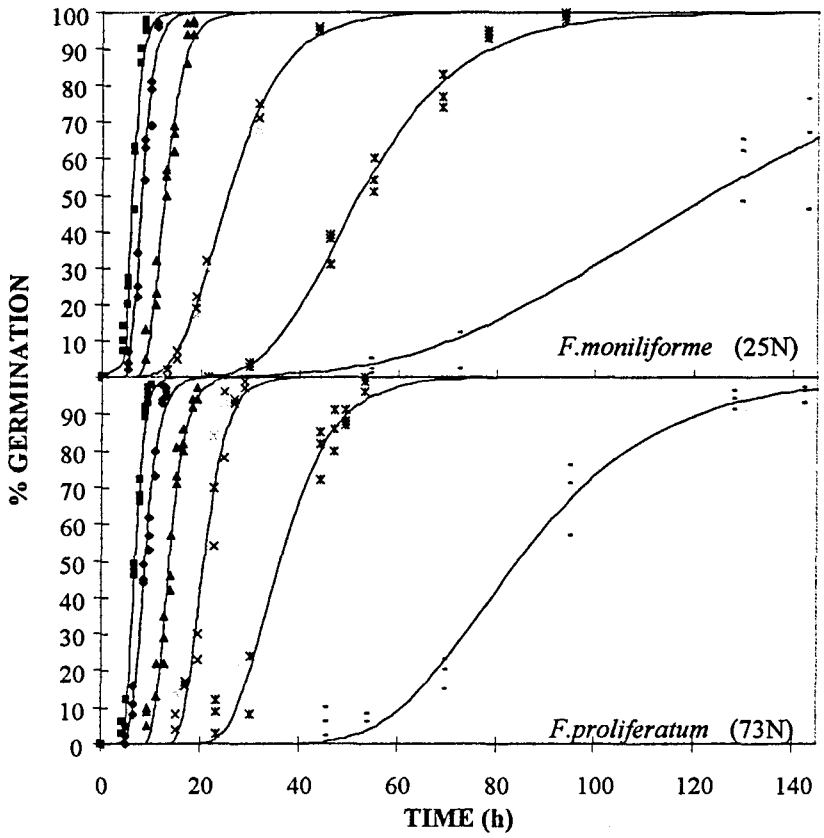

Figure 1. Effect of water activity and time on germination (%) of spores of one isolate of *Fusarium moniliforme* (25N) and one of *F.proliferatum* (73N) on MMEA at 25°C. Water activity levels shown are 0.98 (■), 0.96 (◆), 0.94 (▲), 0.92 (×), 0.90 (✳), and 0.88 (ı).

Water activity × temperature effects on lag phases prior to germination

The lag phases (h) prior to germination increased significantly as temperature was changed from optimal to marginal conditions at each steady-state a_w for both *F. moniliforme* (25N) and *F. proliferatum* (131N, Fig. 2). For *F. moniliforme*, at high a_w (0.98), a reduction in temperature to 5-10°C increased the lag phase to 25-150 h. Under similar conditions, microconidia of *F. proliferatum* had longer lag phases, particularly at 5°C. At marginal temperatures for germination the lag phases at low a_w (0.92-0.90) were increased to > 150 h for isolates of both species. Under the driest conditions (0.88 a_w), germination only occurred at 25-30°C for *F. moniliforme* and 25°C for *F. proliferatum*, with lag phases of about 50-70 and 60-100 h respectively. In general, lag phases were shorter for *F. moniliforme* isolates than for those of *F. proliferatum* at 15-37°C and 0.92-0.98 a_w, while under other environmental conditions tested there was little difference between species. The curves show that, under such dry conditions and more marginal temperature conditions no germination would occur. The profiles generally suggest shorter lag phases at conditions close to the maximum/optimum temperatures and a_w levels than minimum temperatures and a_w levels, for both *F. moniliforme* and *F. proliferatum*.

Table 1. The minimum water activity (a_w) at different temperatures for germination of three isolates of each of *Fusarium moniliforme* and *F. proliferatum* on a maize meal extract agar after 40 days incubation.

		Temperature (°C)						
Species	Isolates	5	10	15	25	30	37	42
F. moniliforme	25N	0.96	0.92	0.90	0.88	0.88	0.92	N.G.
	85N	0.96	0.92	0.90	0.88	0.90	0.92	N.G.
	123N	0.96	0.92	0.90	0.90	0.90	0.92	N.G.
1.1.1.F. proliferatum	55N	0.96	0.94	0.90	0.90	0.92	0.92	N.G.
	73N	0.94	0.92	0.88	0.88	0.90	0.94	N.G.
	131N	0.96	0.92	0.90	0.88	0.90	0.94	N.G.

NG, no germination

The minimum a_w criterion was 10% of spores germinated.

Table 2. Analysis of variance of effect of water activity (a_w), temperature (t), and different isolates (i) on germination of three isolates of *Fusarium moniliforme* (25N, 85N, 123N) and *F.proliferatum* (55N, 73N, 131N) on a maize meal extract agar.

		F. moniliforme		*F. proliferatum*	
Source of variation	Df	Mean Square	F	Mean Square	F
a_w	5	2431.72	937.57*	2534.40	2805.57*
T	5	202.51	779.41*	1697.87	1879.54*
i	2	4.83	1.86	126.31	139.83*
$a_w \times T$	25	259.69	100.13*	223.69	247.62*
$a_w \times i$	10	7.96	3.07*	11.85	13.12*
$T \times i$	10	7.38	3.07*	25.29	27.99*
$a_w \times T \times i$	50	5.90	2.28*	35.28	39.05*

*significant, $P < 0.01$

Germination percentage after 24 h at different a_w and temperature was transformed to *logit* (log(x/(100-x))) for analysis.

Water activity × temperature effects on germination rates

The profiles of germination rates (h^{-1}) show that the optimum tested temperature for germination changed from 37°C and 0.96-0.98 a_w to 30°C at 0.94-0.88 a_w for *F. moniliforme* (25N, Fig. 3). The temperature range for germination was markedly decreased at 0.90-0.88 a_w, the limits for germination. By contrast, the germination rates of an isolate of *F. proliferatum* (131N) at the tested temperatures remained optimum at 30°C, regardless of a_w, except at 0.90 and 0.88 a_w where this changed to 25°C. As with *F. moniliforme* there was a decrease in the temperature range and germination rates as a_w was reduced close to the minimum (0.90-0.88 a_w) for germination. In general, *F. moniliforme* microconidia germinated faster than those of *F. proliferatum* at 30 and 37°C regardless of a_w, and at 25°C and 0.98-0.94 a_w but slower than those of *F. proliferatum* at 5-15°C regardless of a_w.

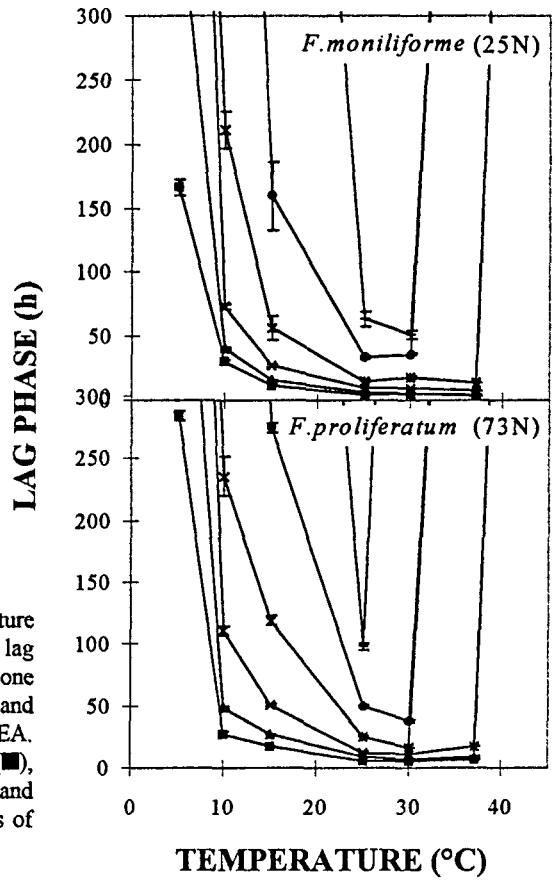

Figure 2. Combined effect of temperature and water activity on the length of the lag phase prior germination of spores of one isolate of *Fusarium moniliforme* (25N) and one of *F.proliferatum* (131N) on MMEA. Water activity levels shown are 0.98 (■), 0.96 (▲), 0.94 (×), 0.92 (✳), 0.90 (●), and 0.88 (+). Error bars show standard errors of the estimated parameters.

DISCUSSION

This study showed that germination of microconidia of fumonisin-producing isolates of *F. moniliforme* and *F. proliferatum* are markedly influenced by a_w, temperature, and their interactions, and complements data recently reported on the effects of these factors and pH on mycelial growth of these same species and isolates (Marín et al. 1995a). The range of a_w conditions over which germination occurred was wider than that for growth. Both species were able to germinate at 0.88 a_w at optimum temperature, while growth was restricted to > 0.90 a_w on a similar MMEA (Marín et al. 1995a).

No information on germination of microconidia of known fumonisin-producing isolates of *F. moniliforme* and *F. proliferatum* has previously been reported. Survival and viability of *F. moniliforme* microconidia on glass cover slips was found to be influenced by equilibrium relative humidity (ERH) and temperature (Liddell and Burgess 1985). For example, microconidia remained viable for up to 70 days at 75-86% ERH and 25-35°C. Interestingly, hyphae and secondary microconidia were often produced. The production of secondary microconidia may have been due to the exhaustion of endogenous reserves on the glass surface. Lacey (1989), in a review of *Fusarium* water relations, reported growth over a wide temperature range (2-37°C) and down to 0.87 a_w. However, in most cases germination has usually been found to occur at a

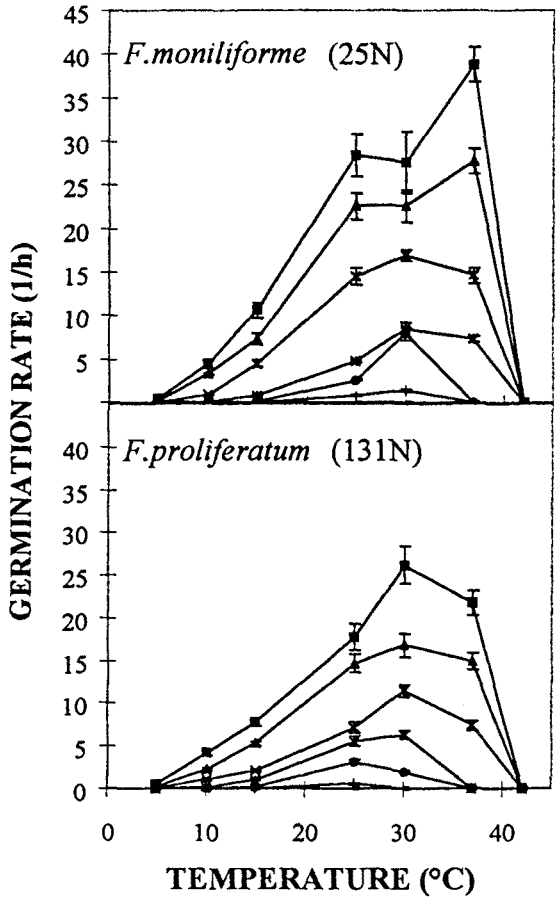

Figure 3. Combined effect of temperature and water activity on the maximum germination rate of spores of one isolate of *Fusarium moniliforme* (25N) and one of *F.proliferatum* (131N) on MMEA. Water activity levels shown are 0.98 (■), 0.96 (▲), 0.94 (×), 0.92 (✳), 0.90 (●), and 0.88 (+). Error bars show standard errors of the estimated parameters.

lower a_w than that for growth (Lacey and Magan 1991). A comparison can, however, be made with other cereal colonizing species such as *F. culmorum* and *F. graminearum*, for which more detailed information is available. For example, for a *F. culmorum* isolate from cereal residue, a_w minima of 0.90-0.925 with glycerol and NaCl as solutes at 20°C were reported, and from wheat grain at 0.89 at 25°C on glycerol-amended medium was reported (Magan 1988; Magan and Lacey 1984a).

In the present study, within 24 h the microconidia of *F. moniliforme* and *F. proliferatum* germinated optimally at 0.994-0.98 a_w, and rapidly over a wide a_w range, with more than 50% of spores of the former and 90% of the latter successfully developing germ tubes at 0.92 a_w and 25°C. Sung and Cook (1981) found that macroconidia of a corn isolate of *F. graminearum* (Gp II) germinated optimally at 0.99-0.985 a_w, and down to 0.955 a_w at 25°C after a similar incubation period on water agar modified with the ionic solute NaCl. This suggests that the germinative capacity of these two species may be more adapted to a wider range of water availability conditions than *F. graminearum* (Gp II) species or indeed macroconidia of isolates of *Fusarium roseum* "*graminearum*" (Gp I), or "*culmorum*" and "*avenaceum*" which had minima of 0.95-0.93 a_w (Sung and Cook 1981). Speed of germination over a wide range of

environmental conditions over relatively short times may be critical in giving a species a competitive advantage in colonizing the maize grain surfaces during ripening. It has been suggested that *F. moniliforme* is adapted to warm, dry climatic conditions, *Fusarium subglutinans* is adapted to cooler temperate conditions, with *F. graminearum* as an intermediate group (Rheeder et al. 1990a). Unfortunately, direct comparisons with *F. subglutinans* cannot be made as no information exists at present on the water relations of germination or growth of this species (Lacey and Magan 1991).

Sutton (1982) found that moisture content is a major factor in determining maize ear rot. A period of 10-20 days exists when the ripening kernels are susceptible and moisture content is reduced from > 50 (= 1.00 a_w) to 20% (0.90 a_w). Persistent wetness periods of 48 h were conducive to infection at this stage by *F. graminearum*. Our study suggests that contaminant spores of *F. moniliforme* and *F. proliferatum* may require shorter time periods for effective germination and establishment over quite a wide range of maize kernel moisture contents (Marín et al. 1995a). Both Blaney et al. (1986) and Rheeder et al. (1990a, 1990b) found negative correlations between isolation of *F. moniliforme* and *F. graminearum*, suggesting that preferential establishment by *F. moniliforme* may prevent other pathogens such as *F. graminearum* from colonizing the same maize kernels. Indeed, Blaney et al. (1986) suggested that competition for substrate, production of antagonistic metabolites, and unspecified environmental factors may be involved. Our study suggests that *F. moniliforme* and *F. proliferatum* may well be adapted for successfully and perhaps preferentially colonizing such niches.

Lag phases prior to germination were found to be profoundly influenced by both temperature and interactions with a_w. The lag phases were longest at minimum temperature and low a_w levels than at maximum temperatures at which lag phases were generally short or no germination occurred. These results for *F. moniliforme* and *F. proliferatum* are similar to those obtained previously with a range of other field and storage grain fungi. For example, lag times, in days, were shown to increase at lowered a_w and with interaction with temperature, pH and gas composition (Ayerst 1969; Magan and Lacey 1984b). A significant correlation also was found between the reciprocal of germination lag times and linear growth for spoilage fungi such as *Aspergillus restrictus* and *A. versicolor* over the a_w and temperature range permitting growth (Smith and Hill 1982). However, such detailed profiles of lag phases and germination rates for these *Fusarium* species have not previously been available and provide useful background information to improve our understanding of the ecology of these fungi.

Germination rates for *F. moniliforme* were optimum at 25-37°C at > 0.96 a_w but changed to 30°C at lower a_w levels. By contrast, for *F. proliferatum*, 30°C was optimum for germination over almost the whole a_w range tested. Germination rates were optimum at 0.994-0.98 a_w and reduced slightly with decreasing a_w. The optimum temperature for germination was different from that found for growth *in vitro* on a similar substrate for which optimum temperature was 25°C for *F. proliferatum* and 30°C for *F. moniliforme* (Marín et al. 1995a). Statistical analyses of data on germination rates showed that there was little difference among three fumonisin-producing isolates of *F. moniliforme*, while there were significant differences among those of *F. proliferatum*. However, the results showed clearly that all other one, two- and three-way interactions were statistically significant. The impact of a_w and temperature on germination (this study), growth (Marín et al. 1995a), and on fumonisin B_1 and B_2 production (Marín et al., 1995b) on maize by isolates of these two species has provided detailed background knowledge on the

ecological competence of these species for colonization and infection of maize and for concomitant mycotoxin production.

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VI

TWO-DIMENSIONAL PROFILES OF FUMONISIN B_1 PRODUCTION BY *FUSARIUM MONILIFORME* AND *FUSARIUM PROLIFERATUM* IN RELATION TO ENVIRONMENTAL FACTORS AND POTENTIAL FOR MODELLING TOXIN FORMATION IN MAIZE GRAIN

S. Marín[1], N. Magan[2], N. Belli[1], A.J. Ramos[1], V. Sanchis[1]

[1]Food Technology Department, University of Lleida, CeRTA, Rovira Roure 177, 25198 Lleida, Spain
[2]Applied Mycology Group, Cranfield Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, UK

ABSTRACT

This study has examined in detail the effect of temperature (7 – 37°C) and water availability (water activity, a_w, 0.89 – 0.97) on fumonisin B_1 production by an isolate of *Fusarium moniliforme* and *F. proliferatum* on irradiated maize grain after incubation for 28 days. The optimum conditions for *F. moniliforme* and *F. proliferatum* were 30°C at 0.97 a_w and 15°C at 0.97 a_w, respectively. The maximum concentrations were 2,861 ppm and 17,628 μg g^{-1} dry wt maize grain, respectively. At marginal a_w/temperature conditions for growth (e.g. 0.89-0.91 a_w) non-detectable concentrations of FB_1 (<0.1 ppm) were produced. A high variability was found between replicates for *F. moniliforme*, but not for *F. proliferatum*. This data was used to construct two-dimensional diagrams of all the a_w × temperature conditions favourable for FB_1 production for the first time. The data was also subjected to a polynomical regression which demonstrated that there was a very good fit for 15-30°C range of temperature and at 0.97 a_w. However, at marginal environmental conditions this was not possible. This suggests that it may be possible to predict within a limited environmental range the potential for significant FB_1 production.

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INTRODUCTION

In recent years, *Fusarium* species belonging Section Liseola have attracted much attention because of their ability to produce fumonisins. Among them, *Fusarium moniliforme* and *Fusarium proliferatum* are the major producers. Since the elucidation of fumonisins in 1988 by Gelderblom *et al.* much work has been carried out on this subject. A number of surveys have shown that a high percentage of samples of corn-based feed are contaminated by fumonisins (Wilson *et al.*, 1990; Ross *et al.*, 1991). They have also been found in samples intended for human consumption (Sydenham *et al.*, 1991; Pittet *et al.*, 1992). From an ecological point of view, the influence of some abiotic factors on development of fumonisin producers has been established (Marín *et al.*, 1995a, 1996). It has also been shown that water availability (water activity, a_w) and temperature are crucial in determining the extent of fumonisin production by these species (Alberts *et al.*, 1990; LeBars *et al.*, 1994; Marín *et al.*, 1995b; Cahagnier *et al.*, 1995). However, results from these previous studies are confusing, possibly due to intraspecific differences, or the different methodologies used.

Well known mycotoxins such as aflatoxins, patulin or cyclopiazonic acid have been studied in detail and the profiles for mycotoxin production by several species determined under different environmental conditions (Northolt *et al.*, 1977, 1978; Gqaleni *et al.*, 1996, 1997). In general, it has been shown that the range of temperatures which allows growth of toxin producers is similar to that which allows mycotoxin formation. However, the a_w range for mycotoxin production is often narrower than that for growth. Consequently, when growth is prevented or controlled, the mycotoxin contamination should be significantly reduced or inhibited.

It must also be remembered that *Fusarium* spp. do not occupy or contaminate maize and other cereals in isolation. The competing mycoflora present in maize may have a significant role in determining niche occupation and fumonisin accumulation (Marín *et al.*, 1998a, b). Thus, a wide range of biotic and abiotic factors in the stored grain ecosystem will determine the final quality of the stored grain.

The objective of the present study was to investigate in detail the profiles of fumonisin B_1 production by one isolate each of *F. moniliforme* and *F. proliferatum* in relation to a_w (0.89-0.97) and temperature (7-37°C), and determine whether the conditions for fumonisin formation are more restricted than those for growth.

MATERIALS AND METHODS

Fungal isolates

Two isolates belonging to the *Fusarium* Liseola section, one of *Fusarium moniliforme* Sheldon (25N) and one of *F. proliferatum* (Matsushima) Nirenberg (73N), were used in these experiments. These isolates were all isolated from maize and have previously been demonstrated to be high fumonisin-producers (Sala, 1993). All the isolates are held in the Food Technology Dept. Culture Collection of the University of Lleida, Spain.

Irradiated maize grain

Spanish dent maize grain was irradiated with 12 kGrays of gamma irradiation and stored aseptically at 4°C. The grain contained no fungal infection or contamination but had retained germinative capacity. The initial water content of the grain was 13.9% (=0.71 a_w).

Inoculation and incubation of maize for fumonisin B_1 studies

40 g of irradiated maize grain were weighed into sterile 250 ml beakers and rehydrated to the required a_w by addition of sterile distilled water using a moisture absorption curve. Experiments were carried out at 0.97, 0.95, 0.93, 0.91 and 0.89 a_w, Beakers of each treatment were inoculated with 0.5 ml of a microconidial spore suspension of each isolate to obtain a final concentration of 2×10^5 spores g^{-1} maize, and shaken vigorously. The inoculated irradiated maize was placed in sterile Petri dishes (20 g per plate). Plates of the same a_w treatment were enclosed in closed plastic containers together with beakers of a glycerol-water solution at the same a_w as the plates, to maintain constant ERH inside the chambers and incubated at 7, 10, 15, 20, 25, 30 and 37°C. The experiment was repeated twice.

Fumonisin B_1 quantification

Samples were incubated for 28 days, and then frozen until extraction and analyses. They were extracted using a modification of the Shephard *et al.* (1990) method as described by Sanchis *et al.* (1994). After extraction, purified sample residue was dissolved in 0.5 ml of methanol. Two hundred µl of *o*-phtaldialdehyde (OPA) reagent, prepared according to Shephard *et al.* (1990) were added to a 50 µl sample solution. Fifty µl of this solution were injected into the HPLC system within 2 min of derivatization. The eluent was methanol+0.1 M NaH_2PO_4 (75+25) adjusted to pH 3.35 with *o*-H_3PO_4. The flow rate was 1 ml min^{-1}. Reference standard of FB_1 was purchased from CSIRO, Division of Food Science and Technology, Pretoria, South Africa. The limit of detection was 0.1 ppm and the recovery rate varied according to the following equation:

Recovery rate (%) = $135.14\,x^{-0.1294}$ where x = spiked FB_1 (ppm) with $0.1<x<100$

Simultaneously, a portion of each sample (10g) was dried in an oven at 105°C for 17 h (ISTA, 1976), to determine the moisture content, and the fumonisin B_1 concentrations calculated on a dry matter basis.

Statistical analyses of the data

First of all, data were transformed by $y=log(x+1)$, were x is FB_1 concentration in µg g^{-1} dry maize, in order to homogenise variance. Analyses of variance of data were carried out by using the PROC GLM procedure in the SAS System (SAS Institute Inc., version 6.12). Water activity and temperature were included in the program in a RANDOM instruction. As the interaction a_w × temperature was significant ($P < 0.01$), further separate analyses were done for each level of temperature or water activity. After that, non-linear regressions were carried out by using EXCEL version 5.0, and the predictive model equation and R-squared obtained in each case.

RESULTS

Interspecific differences

Table 1 summarises the concentrations of fumonisin B_1 produced by the two isolates under the treatment conditions used. In general, the *F. proliferatum* isolate had optimum production at 15°C, while for *F. moniliforme*, FB_1 production was maximum at higher temperatures. *F. proliferatum* produced maximum FB_1 at 0.97 a_w and 15°C (17,628 μg g^{-1} dry wt), while *F. moniliforme* only produced a maximum amount of 2,861 ppm at 0.97 a_w and 30°C. Interestingly, there was a high variability in FB_1 production by replicates of the isolate of *F. moniliforme*, while those of *F. proliferatum* were quite consistent.

Table 2 shows that statistically, both isolates had a similar response to a_w and temperature as neither a_w × isolate nor temperature × isolate interactions were significant. However, the isolates produced significantly different amounts of FB_1 ($P<0.01$).

Table 1. Mean concentrations of FB_1 produced in relation to temperature and a_w levels

Temp. (°C)	Species	0.89 a_w	0.91 a_w	0.93 a_w	0.95 a_w	0.97 a_w
7	*F. moniliforme*	l.d.	l.d.	l.d.	l.d.	l.d.
	F. proliferatum	l.d.	l.d.	l.d.	l.d.	l.d.
10	*F. moniliforme*	l.d.	l.d.	l.d.	5.93	20.55
	F. proliferatum	l.d.	l.d.	28.44	274.21	1762.12
15	*F. moniliforme*	l.d.	l.d.	l.d.	183.99	386.91
	F. proliferatum	l.d.	l.d.	69.34	1495.82	17627.74
20	*F. moniliforme*	l.d.	l.d.	2.73	1198.66	1795.03
	F. proliferatum	l.d.	l.d.	22.58	1334.51	14623.87
25	*F. moniliforme*	l.d.	l.d.	0.18	966.11	1734.71
	F. proliferatum	l.d.	l.d.	32.46	686.75	8708.30
30	*F. moniliforme*	l.d.	l.d.	l.d.	319.76	2861.21
	F. proliferatum	l.d.	l.d.	32.46	125.49	73.67
37	*F. moniliforme*	l.d.	l.d.	l.d.	429.28	1.74
	F. proliferatum	l.d.	l.d.	l.d.	l.d.	l.d.

l.d., below the limit of detection

Table 2. Analysis of variance of FB_1 production on irradiated maize grain inoculated with *Fusarium* species after a 28-day incubation period. Significance of a_w, temperature (T), isolates (I), and their interactions.

FACTOR	DF	MS	F
I	1	4.02	25.61**
a_w	4	29.46	13.81**
a_w × I	4	0.68	1.47
T	6	7.07	2.60
a_w × T	24	1.93	4.18**
I × T	6	1.26	2.74*
a_w × I × T	24	0.46	2.94**

*, significant $P<0.05$

**, significant $P<0.01$

Effect of water activity and temperature on fumonisin B_1 production

Fig. 1 details the two-dimensional effect of a_w and temperature on the production of FB_1 by both *F. moniliforme* and *F. proliferatum*. The numbers on the isopleths joining conditions at which similar levels of FB_1 are produced are shown, with dotted lines used where extrapolation has been made. Overall, FB_1 concentration increased with a_w for both species, with non-significant production at 0.89-0.91 a_w under all the temperature levels tested. Similarly, no FB_1 was produced at 7°C by both isolates, and at 37°C by the *F. proliferatum* one. However, F. *moniliforme* still yielded significant amounts of FB_1 at 37°C at the highest a_w examined (0.95-0.97 a_w). For *F. proliferatum* optimum temperature for production was 15°C, followed by 20, 25, 10 and 30°C. For *F. moniliforme* the optimum temperature varied between 20-25-30°C, then 15 and 37°C. Both a_w and temperature were statistically significant (Table 3). However, a_w was the most important factor affecting FB_1 production.

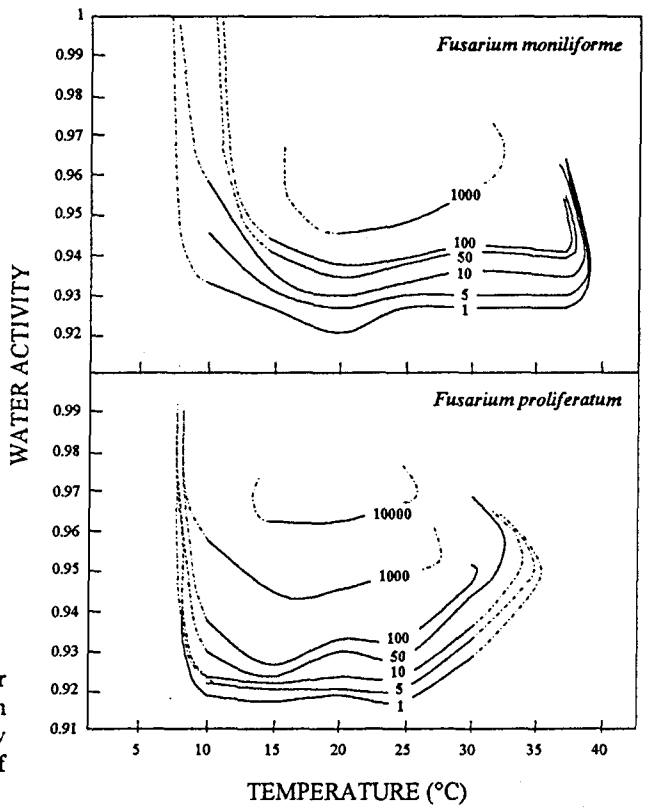

Figure 1. Combined effect of water activity and temperature on fumonisin B_1 production by *Fusarium* species after 28 days of incubation on irradiated maize.

Table 3. Analysis of variance of FB_1 production on irradiated maize grain inoculated with *Fusarium moniliforme* and *F. proliferatum* after a 28-day incubation period. Significance of a_w, temperature (T), and their interaction.

		F. moniliforme		*F. proliferatum*	
FACTOR	DF	MS	F	MS	F
a_w	4	12.98	13.35**	16.97	12.17**
T	6	2.42	2.49	5.90	4.23**
a_w x T	24	0.98	3.33**	1.41	71.75**

**, significant, $P<0.01$
*, significant, $P<0.05$

Table 4 shows the detailed analysis of variance for each separate temperature and a_w levels. It is important to note the close relationship between a_w and temperature. For example, temperature only has a significant effect at ≥0.93 a_w for *F. proliferatum*, and none for *F. moniliforme* except for 0.97 a_w. On the other hand, a_w always has a statistically significant effect at 15-30°C for both isolates, and at 10°C for the isolate of *F. proliferatum*.

Table 4. Analysis of variance of FB_1 production on irradiated maize grain inoculated with *Fusarium* species after a 28-day incubation period under each separate level of a_w and temperature (T).

			F. moniliforme		*F. proliferatum*	
LEVEL	FACTOR	DF	MS	F	MS	F
0.89	T	6	-	-	-	-
0.91	T	6	-	-	0.02	1.00
0.93	T	6	0.11	0.79	1.20	30.13*
0.95	T	6	2.53	2.30	3.72	387.1**
0.97	T	6	3.74	17.93**	4.82	164.24**
7	a_w	4	-	-	-	-
10	a_w	4	0.48	2.16	5.22	262.30**
15	a_w	4	3.43	1453.00**	6.99	1367.00**
20	a_w	4	5.16	38.79**	6.85	388.47**
25	a_w	4	5.12	54.23**	5.90	243.28**
30	a_w	4	4.01	6.38*	2.05	48.63**
37	a_w	4	0.75	0.65	0.02	0.78

**, significant, $P<0.01$
*, significant, $P<0.05$

Modelling of the fumonisin B_1 production

Modelling of the fumonisin B_1 production as a function of a_w was possible over the range 10-30°C for *F. proliferatum* and 15-30°C for *F. moniliforme*, under the other marginal levels of a_w or temperature, the influence was not significant, and consequently fitting models were not used. A third-degree polynomial function was the best fitting equation in most of the cases (Table 5). The temperature factor was significant for fitting to the model under certain a_w conditions only. Fig. 2 and Fig. 3 show the fitting which was achieved using the data in relation to a_w and temperature.

Table 5. Modelling of the fumonisin B_1 production as a function of temperature and a_w.

LEVEL	*F. moniliforme*	*F. proliferatum*
0.93 a_w	-	$log(ppm\ FB_1+1)=0.0004T^3-0.0422T^2+1.1207T-5.5525$; $R^2=0.94$
0.95 a_w	-	$log(ppm\ FB_1+1)=0.0009T^3-0.0769T^2+1.8611T-9.0118$; $R^2=0.91$
0.97 a_w	$log(ppm\ FB_1+1)=5.9104T^3-448.53T^2+9843.3T-50114$; $R^2=0.89$	$log(ppm\ FB_1+1)=0.0006T^3-0.0468T^2+1.0109T-4.8369$; $R^2=0.83$
10°C	-	$log(ppm\ FB_1+1)=-14602a_w^3+41153a_w^2-38585a_w+12037$; $R^2=0.94$
15°C	$log(ppm\ FB_1+1)=-3747a_w^3+1048817a_w^2-97764a_w+3035$; $R^2=0.98$	$log(ppm\ FB_1+1)=-30277a_w^3+84632a_w^2-78863a_w+24453$; $R^2=0.94$
20°C	$log(ppm\ FB_1+1)=-5266.5a_w^3+147085a_w^2-1368135a_w+42385$; $R^2=0.97$	$log(ppm\ FB_1+1)=-33788a_w^3+94622a_w^2-88224a_w+27389$; $R^2=0.96$
25°C	$log(ppm\ FB_1+1)=-48724a_w^3+136186a_w^2-126773a_w+39304$; $R^2=0.98$	$log(ppm\ FB_1+1)=-26337a_w^3+73796a_w^2-68834a_w+21375$; $R^2=0.96$
30°C	$log(ppm\ FB_1+1)=-7708.9a_w^3+22366a_w^2-21544a_w+6892.3$; $R^2=0.83$	$log(ppm\ FB_1+1)=-36828a_w^3+102865a_w^2-95681a_w+29640$; $R^2=0.77$

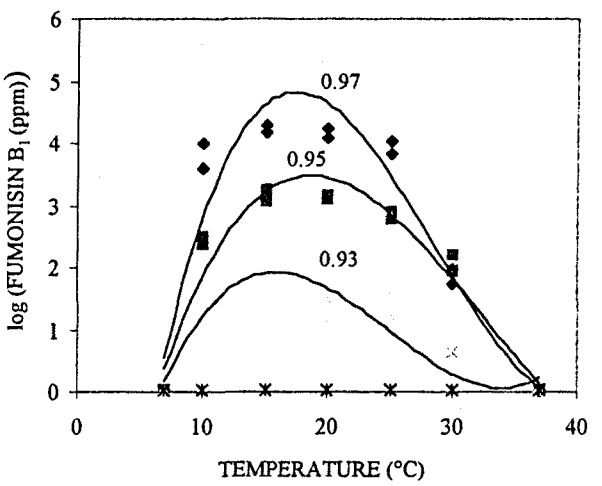

Figure 2. Modelling of the fumonisin accumulation by *F. proliferatum* after 28 days of incubation as a function of temperature. 0.97 a_w (◆), 0.95 a_w (■), 0.93 a_w (▲), 0.91 a_w (✕), 0.89 a_w (✱).

DISCUSSION

This study has developed detailed two-dimensional profiles of conditions which allow the production of FB_1 for the first time. This data also shows clearly that environmental factors have a significant effect on the concentrations of FB_1 produced. This data can be compared with that for growth *in vitro* on a maize meal agar (Marín *et al.*, 1995) where growth minima were observed to be in the a_w and temperature of 0.90 a_w and 5-35°C.

Extensive work has been carried out previously on the combined effect of a_w and temperature on aflatoxin, patulin and cyclopiazonic acid production (Northolt *et al.*, 1977,

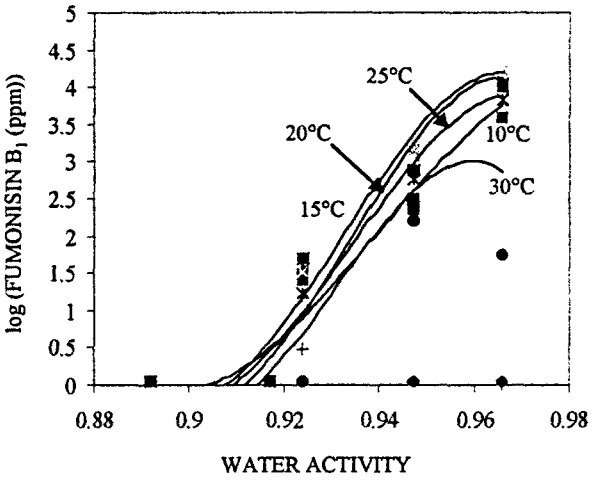

Figure 3. Modelling of the fumonisin accumulation by *F. proliferatum* after 28 days of incubation as a function of a_w. 7°C (◆), 10°C (■), 15°C (▲), 20°C (✕), 25°C (✱), 30°C (●), 37°C (+).

1978; Gqaleni *et al.*, 1996, 1997). However this is the first detailed study on fumonisin B_1 production as affected by a_w and temperature. Although only one isolate of *F. moniliforme* and *F. proliferatum* were used in this study, the data does provide some useful general conclusions on the ecological parameters which influence growth and mycotoxin production.

Previous studies on the effect of a_w and temperature on mycotoxin production have been carried out predominantly on synthetic media (Northolt *et al.*, 1977, 1978; Gqaleni *et al.*, 1996, 1997). However, the direct assay on irradiated maize grain with retained germinative capacity may be a more reliable approach for comparison to those in bulk stored grain. Most other studies have been carried out by setting a_w and temperature to levels approximately optimum for mycotoxin production, e.g. aflatoxin (Asevedo *et al.*, 1993; Gqaleni *et al.*, 1997). However, in this study we have tried to establish those limits which prevent fumonisin formation, similar to what carried out by Northolt *et al.* (1977) for aflatoxins.

A 28-d incubation period was chosen in this experiment as it is a commonly used incubation time by researchers when determining fumonisin producing capacity of *Fusarium* strains: Previously 15 d at 25°C plus 15 d at 15°C (Sala *et al.*, 1994; Sanchis *et al.*, 1995), 21 d at 25°C (Thiel *et al.*, 1991), and 4 weeks at 25°C have been used (LeBars *et al.*, 1994). LeBars *et al.* (1994) suggested that *F. moniliforme* itself degraded the fumonisin after a period of time. This occurred approximately 5 weeks after incubation. Other studies suggest that this degradation begins only 13 weeks after incubation at 20-25°C (Alberts *et al.*, 1990).

Fumonisin B_1 concentration increased with a_w levels and was optimum at 15-30°C, with a maximum FB_1 accumulation as high as nearly 18,000 ppm. No FB_1 was produced at 0.89-0.91 a_w regardless of temperature level. Similarly, no FB_1 was found in samples incubated at 7°C for both isolates, and at 37°C for *F. proliferatum*. Moreover, the latter produced concentrations always < 5 ppm at 0.93 a_w. Concentrations of >3000 ppm, 450 ppm, 10 ppm and 0.1 ppm, were obtained at 1, 0.95, 0.90 and 0.85 a_w, respectively, after a similar incubation period on autoclaved maize inoculated with one isolate of *F. moniliforme* (Cahagnier *et al.*, 1995). Although the incubation temperature was not reported, these

trends parallel those obtained in the present study for *F. moniliforme*. The *F. moniliforme* isolate had optimum production at 30-20°C, followed by 25°, 15° and 10°C. The FB_1 production rate by a *F. moniliforme* isolate on saturated autoclaved maize (1.00 a_w) was maximal at 20°C (1100 ppm); and decreased sharply depending on temperature in the following order: 25 (900 ppm), 15 (400 ppm), 30 (100 ppm), and 10°C. At 35°C, FB_1 was not detected over the 10 weeks of the experiment. The range for growth of this strain was 5->35°C (LeBars *et al.*, 1994). Larger amounts of about 6100 and 9300 ppm at 20 and 25°C, respectively were produced by a *F. moniliforme* isolate after a 4 week incubation period, but again on saturated autoclaved maize (Alberts *et al.*, 1990). It is however important to note that FB_1 production by *F. moniliforme* isolates is subjected to a low repeatibility. In contrast, FB_1 production by the *F. proliferatum* isolate used in this study showed a low error variability. This isolate produced highest concentrations of FB_1 at lower temperatures (15°C).

Experiments to determine the minimum toxic dose of fumonisins to animals have indicated that ponies consuming naturally contaminated feeds containing FB_1 at levels as low as 8 μg g feed are at risk from developing ELEM (Wilson *et al.*, 1992). Recommended maximum fumonisin B_1 concentrations for livestock feed are 5 ppm for horses and other equine species, 10 ppm for porcine species and 50 ppm for beef cattle and poultry (Miller *et al.*, 1996). Consequently, grain maintained below 0.93 a_w, or at higher a_w but at low temperature (<10°C) would not allow FB_1 formation over these levels. However, in Switzerland the legal limit for FB_1+FB_2 in maize products has been provisionally fixed at 1 ppm (FAO, 1995).

Recent studies have demonstrated that these isolates were able to germinate at a minimum a_w of 0.88 and grow at 0.90 a_w in vitro. The temperature range for germination and growth was about 4-35°C. Optimum temperature for growth was 25-30°C and 30-37°C for germination (Marín *et al.*, 1995a, 1996). The same isolates were used in the present study, and their ability to produce FB_1, although in small amounts, in the same temperature range for growth and germination has been demonstrated. However FB_1 production was restricted to >0.91 a_w. Previously, it had been concluded that a reduced a_w inhibits aflatoxin production more than growth, while temperature ranges for growth and aflatoxin production were similar for *A. parasiticus* NRRL 2999 (Northolt *et al.*, 1976). Growth of patulin producers was observed over a relatively wide a_w range (0.85-0.99) but patulin production only over a much narrower range. The lowest a_w permitting production of patulin was 0.95, which was the a_w limit for production by *P. patulum*. On the other hand, the temperature range for production of patulin was wide and almost equalled that for growth (Northolt *et al.*, 1978).

Current methods of reducing fumonisins fall into two general categories: genetic resistance to *F. moniliforme*, and grain handling and processing to remove infected kernels and prevent continued fungal development after harvest (Munkvold *et al.*, 1997). Standard grain storage procedures should prevent the development of fumonisins in stored grain. Generally, fumonisin concentrations are not believed to increase during storage as long as proper conditions of grain moisture and temperature are maintained (Munkvold *et al.*, 1997). The fact that fumonisins can be produced in large amounts at intermediate a_w, and even in small amounts at relatively low a_w, shows that it is critical to avoid any delay before harvested maize is dried. Any delay would enable establishment of *Fusarium* spp. and concomittant

fumonisin production. Subsequent stable storage conditions which prevent initiation and growth of spoilage species are necessary for effective long term grain quality conservation.

There is much debate on the role of fumonisins in the ecology of *Fusarium* species. It has been suggested that production of aflatoxins and cyclopiazonic acid by *A. flavus* might allow it to colonise more substrate and aid its survival in a particular ecological niche. It may thus modify the competitive ability of competing fungi and other microorganisms and inhibit their invasion of already colonised substrate (Gqaleni *et al.*, 1997). Recent studies have certainly suggested that interactions and dominance of *Fusarium* spp. is complex with intra and interspecific interactions occurring and being influenced by the presence of other species and changing environmental conditions (Marín *et al.*, 1998a, b).

Interestingly, significantly higher amounts of FB_1 were obtained by using the same isolates as those used in this study on autoclaved maize (Marín *et al.*, 1995b). However, the general trend of *F. moniliforme* was the same, while production of FB_1 by *F. proliferatum* at 30°C was much lower than at 25°C on irradiated maize than was on autoclaved one. It must be taken into account that autoclaved maize was inoculated at a single point and consequently, after the same incubation period, the fungus had colonised a smaller amount of grain. Mycotoxin production (aflatoxin, deoxynivalenol, acetyl deoxynivalenol, zearalenone) has been demonstrated to be higher on irradiated cereals than on heat-sterilised grain. It has been suggested that this pattern of mycotoxin production is possibly caused by changes in the grain brought about by autoclaving, which favour mycotoxin production and possibly induced changes in irradiation-sterilised grain which inhibit mycotoxin production (O'Neill *et al.*, 1996; Smith *et al.*, 1987).

Gqaleni *et al* (1997) suggested that a full factorial design experiment as carried out in the present experiment was useful as it allows the analysis of interactions between a range of different factors applied at different levels simultaneously and are economical and save time. In mycotoxin studies it demonstrates the complex factors controlling mycotoxin production by fungi and helps to explain their variable concentration in natural substrates. Furthermore a separate analysis of such interactions led to knowledge of the significance of a particular factor at each level, and consequently, as in the present example, enables certain data fitting to be carried out to specific models. We have previously reported on predictions of the lag phase for germination, and growth rates for *Fusarium* spp. in relation to environmental conditions (Marin *et al.*, 1996) using Gompertz's (1825) approach. However, very little effort has been concentrate on predictive modelling of filamentous fungal growth and toxin production as has been carried out for bacteria. This may well be because of the inherent complexities associated with the quantification of fungal growth (Gibson and Hocking, 1997). Moreover, few attempts have been carried out to model mycotoxin production, except those of Pitt (1993, 1995) on aflatoxin modelling in time.

As the effect of a_w on fumonisin accumulation was much more marked, its impact was easily modelled to third-degree polynomial models under most temperature levels (10-30°C), except at 7 and 37°C where concentrations were lower regardless of a_w. In general, the differences between FB_1 production at the different temperatures tested were not high enough to provide general models, except at high a_w. Consequently, this could be an starting point to try to predict the expectable amount of FB_1 found in maize grain stored at a certain temperature and moisture content, however, the initial inoculum and storage time are crucial in determining the final amount produced.

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VII

ECOLOGICAL DETERMINANTS FOR GERMINATION AND GROWTH OF SOME *ASPERGILLUS* AND *PENICILLIUM* SPP. FROM MAIZE GRAIN

S. Marín[1], V. Sanchis[1], R. Sáenz[1], A. J. Ramos,[1] I. Vinas, N. Magan[2]

[1]Food Technology Dept. CeRTA. UdL-IRTA. Lleida University. Rovira Roure 177, 25198 Lleida, Spain.
[2]Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, UK.

SUMMARY

This study compared the effect of temperature (5-45°C), water availability (water activity, a_w; 0.995-0.75) and their interactions on the temporal rates of germination and mycelial growth of three mycotoxigenic strains of *Aspergillus ochraceus*, and one isolate each of *A.flavus*, *A.niger*, *Penicillium aurantiogriseum* and *P.hordei* in vitro on a maize extract medium. Germination was very rapid at > 0.90 a_w with an almost linear increase with time for all species. However, at < 0.90 a_w the germination rates of *A.flavus* and *P.hordei* were slower. The a_w minima for germination were usually lower than for growth and varied with temperature. The effect of a_w x temperature interactions on the lag phases (h), prior to germination, and on the germination rates (h^{-1}) were predicted for the first time for these fungi using the Gompertz model modified by Zwietering. This showed that *A.flavus*, *A.niger* and the two *Penicillium* spp. had very short lag times between 0.995-0.95 a_w over a wide temperature range. At marginal temperatures these were significantly higher, especially at < 10°C for *Aspergillus* spp. and > 30°C for *Penicillium* spp. There were also statistically significant differences between lag phases and germination rates for three different isolates of *A.ochraceus*. The *Aspergillus* spp. also germinated faster than the *Penicillium* spp. The temperature x a_w profiles for mycelial growth varied considerably between species, both in terms of rates (mm d^{-1}) and tolerances. Predictions of the effects of important environmental factors such as temperature, a_w and their interactions on lag times to germination, germination rates and mycelial growth are important in the development ofhurdle technology approaches to predicting fungal spoilage in agricultural and food products.

Key words: water activity, temperature, *Aspergillus*, *Penicillium*, germination, prediction, lag phase, growth, maize.

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INTRODUCTION

A range of xerotolerant/xerophilic *Aspergillus* and *Penicillium* spp. fungi are known to colonise maize kernels in Spain both pre- and post-harvest (Jimenez *et al.* 1985). The dominant species include *Aspergillus flavus*, *A.ochraceus*, *A.niger*, *Penicillium aurantiogriseum* and *P.hordei*. Their interactions and that with pathogens such as *Fusarium* spp. can influence infection levels in maize kernels by *A.flavus* and the production of aflatoxins (Wicklow *et al.* 1980), ochratoxins and perhaps fumonisins (Miller 1994, 1996). Water availability is probably the single most important environmental factor affecting their germination, growth and establishment on such nutrient rich substrates although it will vary both spatially and temporally (Magan 1988; Cooke and Whipps 1993).

There have been some studies of the effect of water availability (water activity, a_w) on germination of toxigenic isolates of *A.flavus, A.ochraceus,* and *A.niger* from a variety of substrates including animal feeds, temperate cereal grains, oilseeds, dried fish and milk powder (Lacey 1989). However, many have only been carried out at one temperature, often close to optima for germination or growth (Pitt and Hocking 1977; Andrews and Pitt 1987). A few studies of such spoilage fungi have included effects of a_w x temperature interactions (Ayerst 1969; Mislivec and Tuite 1970; Magan and Lacey 1984; Pitt and Miscamble 1995). Such interacting abiotic factors are important as they represent the fundamental two-dimensional niche in which these fungi may be able to effectively germinate, grow and actively compete for an allocation of the available resource. Magan and Lacey (1988) suggested that lag times for growth, rates of germination and germ tube extension, were good criteria for comparing the capabilities of different fungal spores for colonising grain surfaces under different a_w conditions. Recently, Pitt and Miscamble (1995) found that a_w minima for germination times of conidia of isolates of *A.flavus* and related species, from sources other than maize, changed with temperature (from 0.82/25°C, to 0.81/30°C and to 0.80/37°C), although predictions of lag phases or rates of germination were not investigated and statistical evaluations were not made. There is also no information at present on comparisons of the germinative and growth capacities of strains of *A.ochraceus.*

Recently, there has been interest in the potential of using a range of ecological traits such as lag phases for germination, germination rates and growth rates in the development of hurdle technology approaches for predicting spoilage (Leistner 1994; 1995). Such information is sparse for spoilage fungi when compared to that available on spoilage bacteria (Wijtzes *et al.* 1992). This study was carried out to compare the effect of a_w, temperature and their interaction on, (a) the germination rate; (b) the lag phase prior to germination by using the Gompertz model; and (c) compare mycelial growth profiles of an isolate of *A.flavus*, three isolates of *A.ochraceus*, and competitors such as *A.niger*, *Penicillium aurantiogriseum* and *P.hordei* in vitro on a maize extract medium.

MATERIALS AND METHODS

Fungal isolates

The fungal species used in this study were one isolate of *Aspergillus flavus* (3.36), one of *A.niger* (3.37), three isolates of *A.ochraceus* [NRRL3174, (3.38), (3.113)], and one each of

Penicillium aurantiogriseum (3.13), and *P.hordei* (3.92). The numbers in brackets are codes of cultures held in the Food Technology Department of the University of Lleida culture collection.

Medium

The basic medium used in this study was a 3% maize meal extract agar (MMEA) with a pH of 5.5. This was made by boiling 30 g dry maize meal l^{-1} in water for 60 min and filtering the resultant mixture through a double layer of muslin. The volume was made up to 1 l. The water activity of this basal medium was 0.994 a_w. This was modified by the addition of known amounts of the non-ionic solute, glycerol (Marín, Sanchis and Magan 1995) to obtain a series of stepwise reductions in a_w between 0.994-0.75 a_w. The a_w of all media was determined with a Novasina Humidat ICII (Novasina AG, Zurich, Switzerland).

Inoculation, incubation and measurement

Germination studies. Fungi were grown on sterile moist maize grain (50 g maize + 20 ml distilled water) for 7 days at 25°C to obtain heavily sporulating cultures. Spores were suspended in sterile destilled water containing a drop of a wetting agent (tween 80). Stock spore suspensions (1 ml) were added to 2 ml sterile glycerol/water solutions to give 3 ml of solution at the required water availability. The final spore concentration was in the range 1-5 x 10^6 spores ml^{-1}.

A 0.1 ml aliquot of the spore suspensions were pipetted onto MMEA plates of the same a_w and spread using a surface - sterilised bent Pasteur pipette. Petri plates of the same a_w treatment were enclosed in polyethylene bags and incubated at 5, 10, 15, 25, 30, 37 and 42°C. Initial experiments with these and other maize grain fungal species (Magan 1988; Marin *et al.* 1996) showed that this method gave consistent and reliable results. All experiments were carried out with at least three separate replicate Petri plates per treatment.

Periodically (hours, and days), depending on the treatment, three agar discs (5 mm diameter) were aseptically removed from each treatment plate using a cork borer, placed on a slide and examined microscopically. A total of 50 single spores per disc (150 per replicate Petri plate; 450 per treatment) were examined. Spores were considered to have germinated when the germ-tube was longer than the diameter of the spore. The experiments were all carried out for up to 40 days.

Growth studies. Actively growing, 5- to 7-day-old colonies of the species and isolates grown on the MMEA were used for all experiments. Agar plugs (5 mm diameter) taken from the growing margins of the colonies were aseptically placed in the centre of each treatment Petri plate. Petri plates of the same a_w and temperature were sealed in polyethylene bags. The a_w x temperature experiments were carried out over the temperature range of 5-45°C. In all cases, the experiments were carried out with at least three replicates per treatment. The Petri plates were examined daily or as necessary and the diameter of the growing colonies measured in two directions at right angles to each other. Measurements were carried out for a maximum of 30 days.

Statistical treatment of the results

The two variables measured were (a) the percentage germination and (b) the radial mycelial extension at different a_w/temperatures against time. For germination, the percentage at each a_w/temperature condition was plotted against time, and the non-linear regression was used to estimate the following two parameters at the 95% confidence level: (a) maximum germination rate (h^{-1}) and (b) lag phase (h). The Gompertz model (1825) was used as the fitting equation (Zwietering *et al.* 1990).

$$(1) Percentage(\%) germination = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t)+1\right]\right\}$$

where A = asymptotic value where the germination rate becomes contant (100% in most cases); μ_m = maximum specific germination rate (h^{-1}) given by the slope of the line when spores germinate exponentially; λ= lag phase (h).

The radial growth rates (mm day^{-1}) at each a_w and temperature treatment were obtained from the slopes of the linear regression of the linear parts of the temporal growth curves (Marín, Sanchis and Magan 1995).

The percentage germination after 24 h at different a_w and temperatures was logit transformed (log(x/(100-x)) in order to homogenise variances and analysed by ANOVA for determination of effects of a_w, temperature, species, and two- and three-way interactions.

Colony radius after 6 days were analysed by a one-way analysis of variance so that effects of single factors (a_w, temperature) and two factors (a_w x temperature) could be assessed for statistically significant differences. Both non-linear regressions and analysis of variance were made by using SYSTAT (version 5.0, SYSTAT Inc.) statistical package.

RESULTS

Temporal effects of water activity on germination

Fig. 1. shows that the germination of conidia of *A.flavus*, *A.niger*, *P.aurantiogriseum* and *P.hordei* at 25°C was very rapid at > 0.90 a_w with an almost linear increase with time. However, under more extreme water stress the germination, particularly for the *A. flavus*, was slower. The minimum a_w at which germination occurred varied from 0.80 to 0.85, for the *Aspergillus* spp., although under these water availability conditions the lag times to germination were 30-100 hours and germination was slow. At 0.75 a_w, the lowest level of water stress tested, none of the isolates tested was able to germinate, and at 0.80 a_w only the isolates of *A. ochraceus* and A.*niger* germinated during the 40 day time period of our experiments (Table 1). *A.niger* was able to germinate at 0.80 a_w whereas *A.flavus* at only 0.85 a_w . There were also some differences between the temporal rates of germination of the three isolates of *A.ochraceus*, particularly at 0.80 a_w (Fig. 2). These data were used to predict a_w x temperature interactions on lag phases prior to germination (h) and germination rates (h^{-1}) for all species and isolates tested.

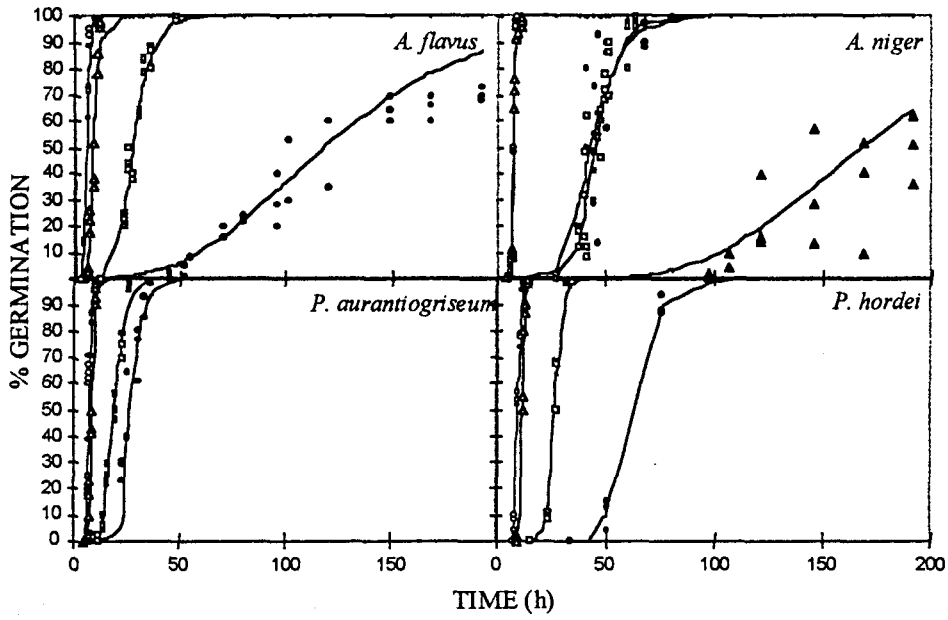

Figure 1. Effect of the water activity on the germination of conidia of *A. flavus, A. niger, P. aurantiogriseum* and *P. hordei* at 25°C on MMEA. Water activity levels are 0.994 (○), 0.95 (△), 0.90 (□), 0.85 (●) and 0.80 (▲).

Table 1. Minimum water activity for germination/growth found at different temperature levels for the isolates of *Aspergillus* and *Penicillium* tested on MMEA. Germination is considered to take place when a 10% germination is achieved, and growth when a growth rate higher than 0.1 mm/day is achieved.

	Temperature (°C)						
	5	10	15	25	30	37	42
A. flavus (3.36)	N.G./N.G	N.G./N.G	0.95/0.95	0.85/0.85	0.85/0.85	0.85/0.90	0.90/0.95
A. niger (3.37)	N.G./N.G	0.95/N.G	0.90/0.90	0.80/0.85	0.80/0.85	0.80/0.85	0.85/0.90
A. ochraceus (3.113)	N.G./N.G	0.85/N.G	0.80/0.85	0.80/0.85	0.80/0.85	0.90/0.90	N.G./N.G
A. ochraceus (3.38)	N.G./N.G	0.85/0.87	0.85/0.85	0.80/0.85	0.85/0.85	0.90/N.G	N.G./N.G
A. ochraceus (NRRL 3174)	N.G./N.G	0.85/0.87	0.80/0.85	0.85/0.85	0.80/0.85	0.90/N.G	N.G./N.G
P. hordei (3.92)	0.90/0.95	0.85/0.90	0.85/0.85	0.85/0.85	0.85/0.90	N.G./N.G	N.G./N.G
P. aurantiogriseum (3.13)	0.90/0.95	0.85/0.90	0.85/0.85	0.85/0.85	0.90/0.90	N.G./N.G	N.G./N.G

N.G.: no germination, no growth

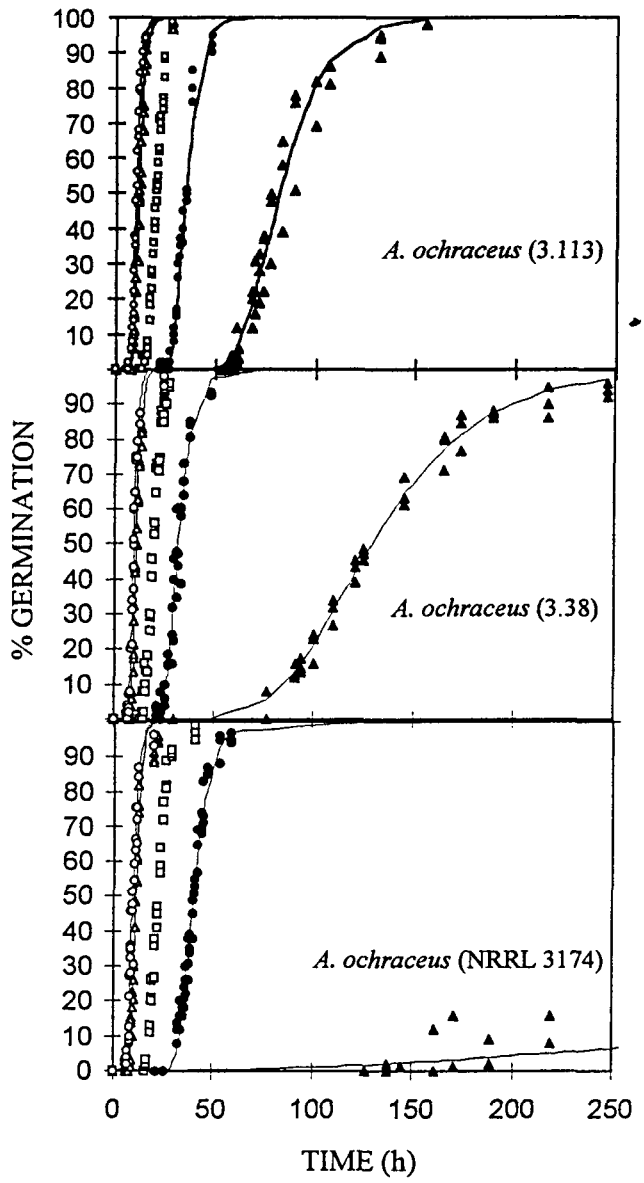

Figure 2. Effect of the water activity on the germination of conidia of three isolates of *A. ochraceus* at 25°C on MMEA. Water activity levels are 0.994 (○), 0.95 (△), 0.90 (□), 0.85 (●) and 0.80 (▲).

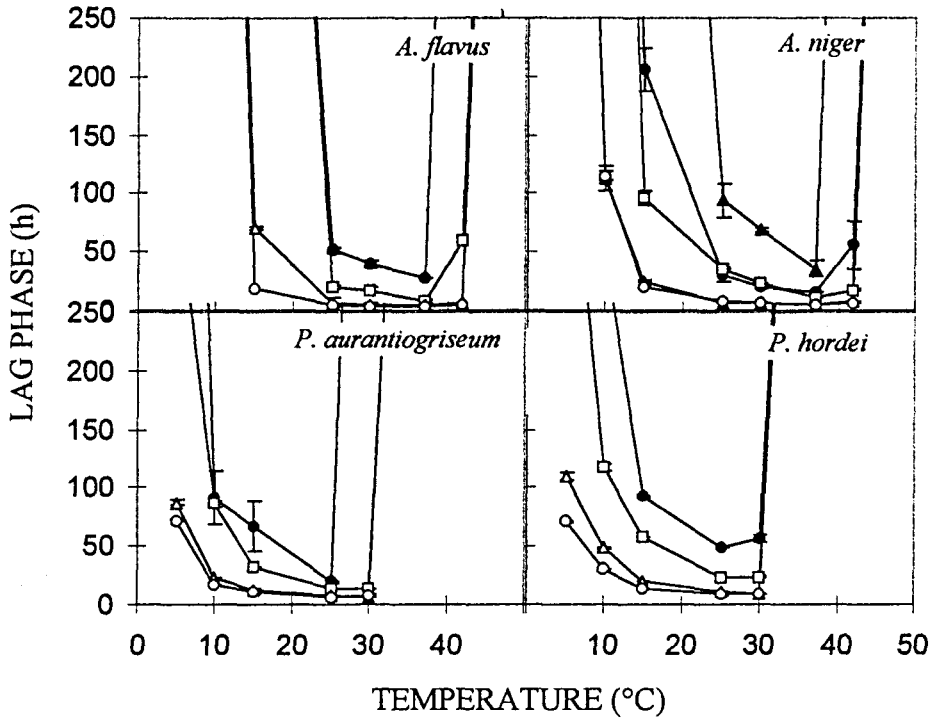

Figure 3. Effect of water activity and temperature on the lag phase before germination of *A. flavus, A. niger, P. aurantiogriseum* and *P. hordei* on MMEA. Water activity levels are 0.994 (○), 0.95 (△), 0.90 (□), 0.85 (●) and 0.80 (▲). Error bars show standard error of estimated parameters.

Predictions of *m* spp. were at 0.994 and 0.95 a_w over a wide range of temperatures (Fig. 3). At marginal temperatures the lag phases were increased, most markedly for the *Penicillium* spp. at > 30°C. This contrasted with that for *A.niger* and *A.flavus* which had very short lag phases, even at 42°C, over a wide a_w range (0.994-0.90 a_w). On the other hand, at low marginal temperatures, the lag phases were increased, more markedly for the *Aspergillus* than for the *Penicillium* spp. (< 10°C).

There were also significant differences in lag phases for the three isolates of *A.ochraceus*. One isolate (NRRL3174) was able to germinate at 0.80 a_w at 15-30°C, with lag phases of between 50 and 250 h, while the other two isolates germinated over a narrower range of temperature at 0.80 a_w. However, the temperature ranges were similar for all three isolates (Fig. 4). 0.994 (○), 0.95 (△), 0.90 (□),

Water activity x temperature effects on germination rates

There were distinct differences between *A.niger* and *A.flavus* with the former having a much broader temperature x a_w range for germination than the latter (Fig. 5). The rates of

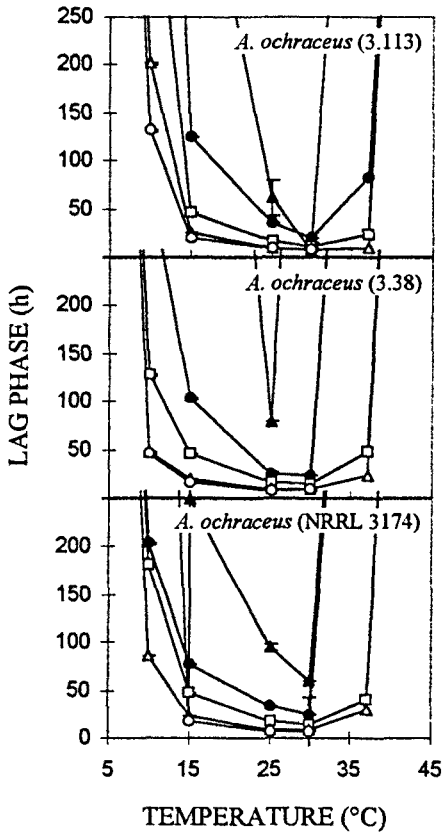

Figure 4. Effect of water activity and temperature on the lag phase before germination of three isolates of *A. ochraceus* on MMEA. Water activity levels are 0.994 (○), 0.95 (△), 0.90 (□), 0.85 (●) and 0.80 (▲). Error bars show standard error of estimated parameters.

germination for the two *Aspergillus* spp. were also more rapid than that of the two *Penicillium* spp. tested. There were also significant differences in a_w x temperature germination rates for the three isolates of *A.ochraceus* (Fig. 6). Both optimum temperature and a_w varied. Statistical analyses showed that there were significant intra-strain differences ($P < 0.001$) due to a_w, temperature, isolate, two- and three-way interactions (Table 2).

Table 2. Analysis of variance of the effect of water activity, temperature, different isolates and their interactions on the percentage of germination of *Aspergillus ochraceus* after 24 hours of incubation on MMEA.

Factor	df	MS	F
Water activity	3	281.88	3645.15**
Temperature	3	332.61	4301.23**
Isolate95 (△	2	5.21	67.32**
Water activity x Isolate	6	5.03	65.01**
Water activity x Temperature	9	46.49	601.23**
Isolate x Temperature	6	7.23	93.47**
Water activity x Isolate x Temperature	18	5.77	74.64**

** Significant $P<0.001$

Note: percentages of germination were transformed as follows: $\sqrt{percentage + 0.5}$

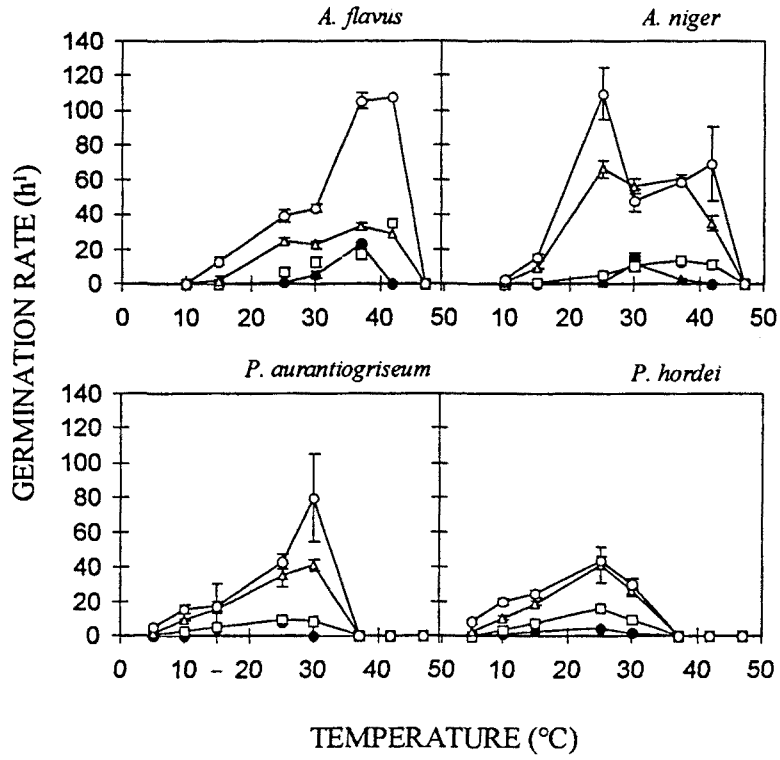

Figure 5. Effect of water activity and temperature on the germination rate of *A. flavus*, *A. niger*, *P. aurantiogriseum* and *P. hordei* on MMEA. Water activity levels are 0.994 (○), 0.95 (△), 0.90 (□), 0.85 (●) and 0.80 (▲). Error bars show standard error of estimated parameters.

Water activity x temperature effects on mycelial growth

Changing a_w at different steady-state temperatures affected growth rates of all the species examined in this study. Fig. 7 compares the a_w x temperature profiles for *A.flavus*, *A.niger* and the two *Penicillium* spp. examined. *A.flavus* and *A.niger* grew almost twice as fast as the *Penicillium* spp. with distinct optimum a_w and temperatures for growth of 0.994 and 30 and 37°C respectively. For *P.aurantiogriseum* and *P.hordei* optimum was at 0.95 a_w (25°C). The *Aspergillus* spp. did not grow at 5°C, and the *Penicillium* spp. not at > 30°C.

Optimum growth of the isolates of *A.ochraceus* over the a_w x temperature range tested showed optima at 0.95 a_w for two isolates over the temperature range 10-30°C and at 0.95-0.995 for the other one (Fig. 8). No growth occurred at > 37°C and at < 0.85 a_w. Maximum growth rates were also 3-4 mm d^{-1}, much less than for *A.flavus* or *A.niger*. Table 3 shows that analyses of the effect of a_w, temperature, isolate and two- and three way interactions were statistically significant.

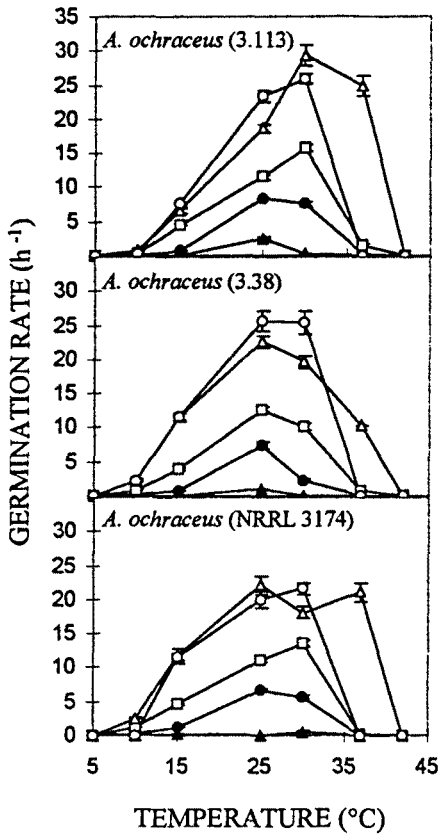

Figure 6. Effect of water activity and temperature on the germination rate of three isolates of *A. ochraceus* on MMEA. Water activity levels are 0.994 (O), 0.95 (△), 0.90 (□), 0.85 (●) and 0.80 (▲). Error bars show standard error of estimated parameters.

Table 3. Analysis of variance of the effect of water activity, temperature, different isolates and their interactions on the colony radius of *Aspergillus ochraceus* after 6 days of incubation on MMEA.

Factor	Df	MS	F
Water activity	4	881.45	4514.15**
Temperature	3	2241.74	11480.56**
Isolate	2	204.76	1048.64**
Water activity x Isolate	8	26.98	138.19**
Water activity x Temperature	12	88.82	454.89**
Isolate x Temperature	6	31.84	163.05**
Water activity x Isolate x Temperature	24	7.62	39.05**

** Significant $P<0.001$

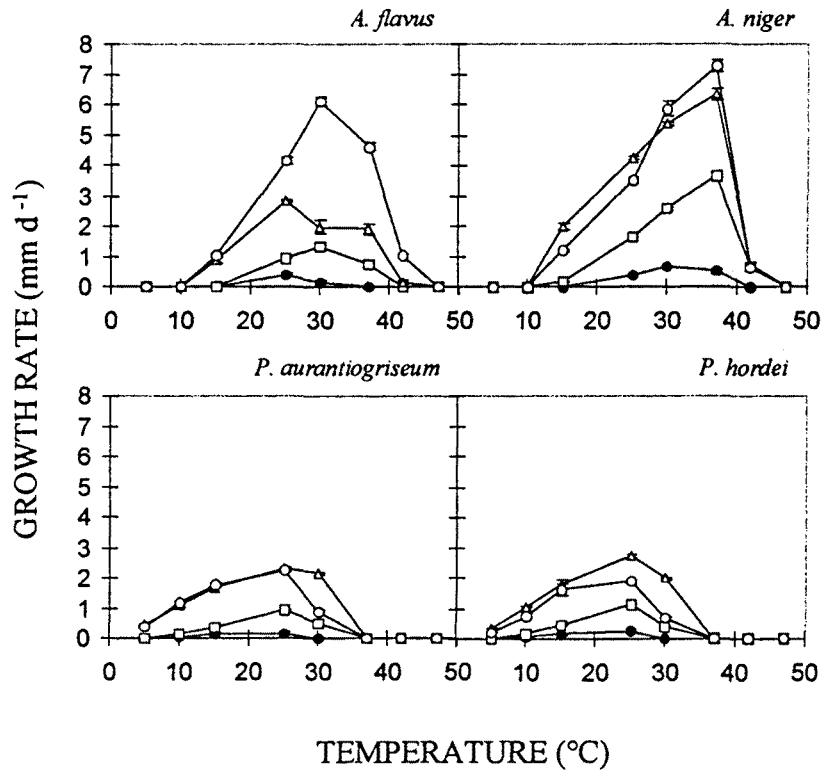

Figure 7. Effect of water activity and temperature on the growth rate of *A. flavus, A. niger, P. aurantiogriseum* and *P. hordei* on MMEA. Water activity levels are 0.994 (○), 0.95 (△), 0.90 (□), and 0.85 (●). Error bars show standard error of estimated parameters.

DISCUSSION

In this study, germination of conidia and mycelial growth of isolates of some *Penicillium* and *Aspergillus* spp. isolated from maize grain were found to be significantly influenced by a_w, temperature and their interactions. The range of a_w conditions for germination at optimum temperatures was generally found to be wider than that for mycelial growth, as recently shown for fumonisin-producing species of *Fusarium* from maize, and for other *Aspergillus* and *Penicillium* spp. (Mislivec and Tuite 1970; Magan and Lacey 1984; Marín *et al.* 1996). In our study, at a_w minima for germination (0.80-0.85) predicted lag times to spore germination of *A.flavus, A.niger, P.aurantiogriseum* and *P.hordei* were in the range 20-100 h on a maize meal extract agar, depending on temperature. In contrast, germination times for isolates of *A.flavus/A.parasiticus* group from sources other than maize on a malt-yeast based medium modifed with glucose:fructose was found to be in the range 30-40 days with minima of 0.81 a_w at 25-37°C (Pitt and Miscamble 1995). Niles *et al.* (1985) demonstrated that on irradiated maize grain, growth of *A.flavus* was optimum over the range 0.84-0.96, with a minimum of 0.80 a_w, and optimum temperature of 25-30°C. There has been much debate

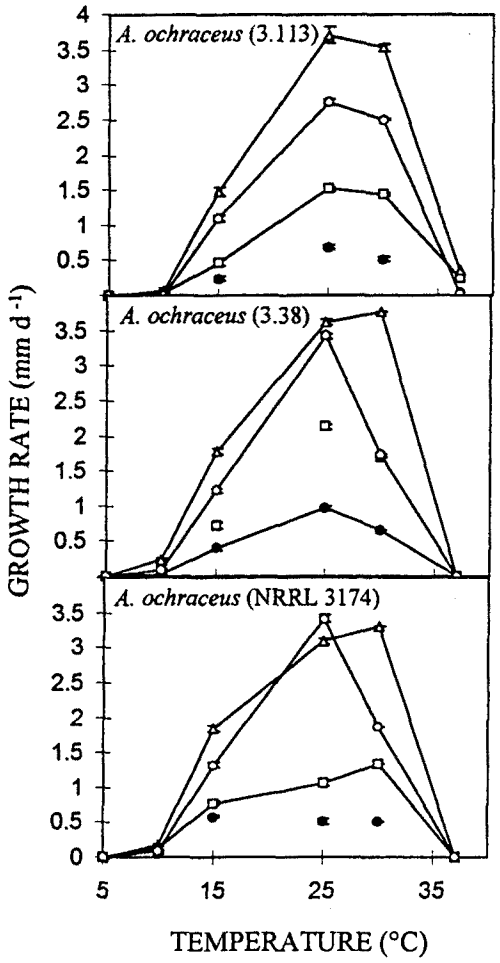

Figure 8. Effect of water activity and temperature on the growth rate of three isolates of *A. ochraceus* on MMEA. Water activity levels are 0.994 (○), 0.95 (△), 0.90 (□) and 0.85 (●). Error bars show standard error of estimated parameters.

about the capability of *A.niger* to inhibit *A.flavus* and aflatoxin production on maize grain (Horn and Wicklow 1983). The predicted germination rates obtained for *A.niger* had two optima for temperature at 25 and 37-42°C at 0.994 and 0.95 a_w, while for *A.flavus* the optimum was found at 37-42°C and 0.995 a_w. The a_w x temperature growth profiles also demonstrated that *A.niger* grew faster than *A.flavus* over a wider temperature range at > 0.90 a_w with temperature optima of 37 and 30°C, respectively. This suggests that from an ecological point of view establishment of spores of *A.niger* may occur more rapidly than for those of *A.flavus*.

Lag times for *P.aurantiogriseum* and *P.hordei* from wheat varied from < 1 at 0.995 a_w to 12-14 days at 0.80 a_w and were increased by changing pH from 6.5 to 4 (Magan and Lacey 1984). Extensive studies of *Penicillium* spp. from maize by Mislivec and Tuite (1970) provided detailed minima and ranges for germination but no detailed information on lag times or germination rates.

Ayerst (1969) determined lag times and growth rates on equilibrated malt extract media, modified over potassium hydroxide solutions for a range of species and isolates. The results

obtained were similar to ours. Under optimum temperature conditions, the lag time at the minimum a_w for germination for *A.flavus*, *A.niger* and *P.cyclopium* (=*P.aurantiogriseum*) varied from 4 to 32 days (0.80 a_w, 30-40°C), 8 to 95 days (0.77-0.80 a_w, 30-35°C) and 2 to 4 days (0.85 a_w, 25°C), respectively. The variability was mainly due to differences between isolates.

Environmental factors such as temperature, pH and nutrition effects on growth and sclerotial production have been extensively studied for ochratoxin producers of *A.ochraceus* (Paster and Chet 1979). However, the effect of a_w and temperature on germination and growth of isolates have not previously been examined in detail. Northolt (1979) examined minima for growth of *A.ochraceus* isolates from poultry feed and found that ochratoxin A and penicillic acid producers grew down to 0.83 a_w at 25-30°C, and 0.80 and 25-30°C respectively, on malt extract sucrose/glycerol media. However, germination times, or predictions of lag phases and germination rates were not examined.

Most of the work dealing with predictive modeling has been done with bacteria. Modeling in bacteria is based on the amount of colony forming units (CFUs) g^{-1} or CFUs ml^{-1} which is a more useful measurement of growth kinetics for bacteria than for fungi. Parameters such as lag time, generation time, maximum growth rate and maximum cell concentration have been estimated for bacteria (Skinner *et al.* 1994). Mycelium dry weight, colony radius, CFU and ergosterol concentration have been used as measurements of fungal growth, but none of these seem to be as reliable as CFUs are for bacteria, for modeling of fungal activity in food substrates (Magan, 1993). The present work is an example of how different environmental conditions can affect the activity of spoilage fungi of feeds and foods, using direct measurements of germination and growth, (percentage germination and colony radius, respectively), and rate of germination, lag phase prior germination, and rate of growth as the estimated parameters.

This study has shown that it is possible to use detailed data on germination of spoilage fungi to develop predictions of both lag phases (h) and germination rates (h^{-1}) in relation to a_w x temperature. It is also possible to distinguish effectively between tolerances of species and isolates of the same species to a_w, temperature and their interactions. These types of data are critical as a base line for any studies involving preservative screening for control of mould spoilage in food and agricultural products. Such data is also essential for the development of predictive modelling of fungal behaviour and for hurdle technology approaches based on single, multiple and interacting factors.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, grant ALI94 0417-C03-01) and to the Catalonian Government (CIRIT, Comissió Interdepartamental de Recerca i Innovació Tecnològica) for their financial support.

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VIII

EFFECT OF WATER ACTIVITY AND TEMPERATURE ON COMPETING ABILITIES OF COMMON MAIZE FUNGI

S. Marín[1], E. Companys[1], V. Sanchis[1], A. J. Ramos[1], and N. Magan[2]

[1]Food Technology Dept., Lleida University, UdL-IRTA, CeRTA, Rovira Roure 177, 25198 Lleida, Spain
[2]Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford, MK43 OAL, U.K.

ABSTRACT

The effect of water activity (a_w, 0.995-0.85) and temperature (15, 25°C) on the *in vitro* inter- and intra-specific interactions between thirteen fungi commonly isolated from maize grain were investigated. The fungi were paired and their interactions given a numerical score to obtain an Index of Dominance (I_D) for each species. *Aspergillus niger* had the highest overall I_D in most of the conditions tested, while the *Aspergillus* species tested were also quite dominant. *Fusarium* species appeared to be dominant only at high water availability (0.995 a_w), while *Eurotium* species dominated at the lower a_ws (0.85-0.90). The relative growth rates of each fungus were also calculated under the same range of environmental conditions. In general, *Aspergillus*, *Fusarium* and *Trichoderma* species grew most rapidly under the combination of a_w and temperature conditions in which they were able to grow, while *Penicillium* species had the slowest growth rates. There was a positive correlation between growth rate and I_D for *Trichoderma viride* and three *Fusarium* spp. but not for *Aspergillus* and *Penicillium* and *Eurotium* spp. The different possible strategies in fungal composition for grain are discussed in relation to these *in vitro* findings.

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INTRODUCTION

Maize is often harvested moist with a moisture content of 18-20% (≡0.90-0.93 a_w) and subsequently dried. During this process, or if inefficiently dried, the environmental conditions are often conducive to rapid spoilage by *Fusarium*, *Aspergillus*, and *Penicillium* spp. which can result in a significant decrease in grain quality and the production of harmful mycotoxins. Such harvested maize grain contains mycelium and spores of a wide range of fungal species (Rheeder, Marasas & van Wyk, 1990). These species will inevitably come into contact. If environmental conditions are conducive they will rapidly grow and compete for the rich maize grain substrate resulting in inter-specific interactions between individual and groups of species with some becoming more dominant than others. The most important factors influencing such interactions are water availability, temperature and intergranular atmosphere (Lacey & Magan, 1991). Information is available on the type of interactions occurring between common fungal colonisers of wheat and barley grain (Magan & Lacey, 1984; Ramakrishna, Lacey & Smith, 1993) but less information is available on maize mycota. Wicklow *et al.* (1980, 1988) examined the associations between fungi on maize kernels and the influence on aflatoxin production by *A. flavus*, but not in relation to abiotic factors.

Several approaches have been considered in order to predict which fungi will become dominant under a given set of environmental conditions. Magan & Lacey (1984, 1985) established five different types of reaction when hyphae from different fungi meet, ranging from mutual intermingling, mutual antagonism to dominance of one species over another with an increasing numerical score of 1 to 5. These were added to give each species an Index of Dominance (I_D) to compare the competitive capabilities of species under different environmental conditions. This approach was slightly modified by others (Ramakrishna *et al*, 1993; Wheeler & Hocking, 1993). These systems were all designed to try and predict the dominance of individual species under different storage regimes.

Understanding the outcome of interactions between these fungi under different environmental conditions may enable more accurate predictions of which individual groups of species may initiate spoilage in stored maize.

The objectives of this study were to examine the effect of a_w and temperature on (a) growth rates, (b) patterns of hyphal interactions, and (c) correlations between dominance indices with growth rates of species of *Fusarium*, *Aspergillus*, *Eurotium*, *Penicillium* and *Trichoderma* spp.

MATERIAL AND METHODS

Isolates

Isolates used in this study were from Spanish maize (Aldea, 1996), and they were considered to be the most common species present in this cereal. They were: *Fusarium graminearum* Schwabe, *F. subglutinans* (Wollenw. & Reinking), *F. proliferatum* (Matsush.) Nirenberg, *Aspergillus niger* van Tiegh., *A. parasiticus* Speare, *A. tumarii* Kita, *A. glaucus* Link:Fr., *Penicillium implicatum* Biourge, *P. aurantiogriseum* Dierckx, *P. canescens* Sopp, *Eurotium chevalieri* L. Magin (2 strains), *E. repens* de Bary and *Trichoderma viride* Pers..

Medium

The basic medium used was PDA (potato dextrose agar), so that growth was not limited by nutrient supply (Bu'lock, 1975). The water activity (a_w) of the basic medium was 0.995. The a_w of this medium was modified to 0.98, 0.95, 0.90, and 0.85 by adding different amounts of glycerol (Dallyn, 1978).

Inoculation, incubation and growth assessment

For the I_D experiments, agar discs (5 mm diam.) were taken from the growing margin of a colony of each of the paired strains and placed 4.4 cm apart in Petri plates of each a_w treatment. Slow growing strains were placed 2.2 cm apart. After inoculation, Petri plates of the same a_w treatment were enclosed in polyethylene bags and incubated at 15 and 25°C.

For comparison of relative growth rates Petri plates of each treatment were centrally inoculated with agar discs as described previously. After inoculation and incubation as described previously, the diameter of the growing colonies were measured daily. The measurements were made in two directions at right angles to each other. At the end of the experiment, the temporal increase in radius was plotted against time and the linear regression was calculated in order to estimate the growth rate in mm d^{-1} under each set of environmental conditions for each species.

Each treatment consisted of three replicates, and the experiments were performed twice. In order to examine the effect of a_w, temperature, and interspecific differences on growth rate, analysis of variance of the radius of the colonies after 5 d was done using SAS Windows, version 6.03 (SAS Institute Inc.).

Types of interaction and I_D calculation

During an 8-week incubation period, the interactions between mycelia of interacting fungi were examined macro- and microscopically, and each fungus given a numerical score using the following scoring system: Mutual intermingling (1), mutual antagonism on contact (2), mutual antagonism at a distance (3), dominance of one species on contact (4, dominant species; 0, inhibited species), dominance at a distance (5, 0) as described previously by Magan & Lacey (1984). Although this system is not quantitative numerical scores were added to enable relative comparison of competitiveness of individual apecies against all others to be made under different environmental regimes.

RESULTS

Effect of water activity and temperature on interspecific interactions

Some patterns were observed when some of the interactions between species were examined (Table 1). For example, interactions between *Fusarium* species were always mutually antagonistic upon contact. At 25°C and high a_w *Fusarium* species often exhibited dominance on contact. At lowered a_w (< 0.96 a_w) mutual antagonism was more common, and sometimes they were dominated by other species.

Trichoderma viride, a fast growing species, similarly dominated other species at high a_w, but was involved in mutual antagonism under even slight water stress, and was dominated at lower a_w, where it grew slowly or was unable to grow.

Significantly, upon contact, *A. niger* was able to dominate almost all the species tested. Only at low a_w and 15°C was it mutually antagonistic to others. Only at 0.995 a_w was *T. viride* able to dominate *A. niger*.

The interactions among *Eurotium*, and *Penicillium* species were always mutually antagonistic either on contact or at a distance (2, 3). At very low a_w levels, however, *E. chevalieri*, *E. repens*, *A. glaucus* and *A. tamarii* often dominated on contact; however, some competing species were unable to grow in such conditions.

Finally, decreasing temperature from 25° to 15°C often led to change from dominance on contact (4) to mutual antagonism on contact or at a distance (2, 3).

Water activity and temperature effects on Index of Dominance (I_D)

Fusarium species had an increasing I_D as a_w, was increased from 0.85 to 0.995 (Fig. 1) although it was 0 at 0.85 a_w; this difference was not temperature dependent. *T. viride* and *A. niger* had the highest I_D score at 0.995 a_w and 25°C. When a_w was slightly reduced, however, *T. viride* became markedly less competitive.

Interestingly, *A. niger* had the highest I_D values of all the species in the range 0.90-0.995 a_w, while *A. tamarii* appeared to be dominant at 0.95-0.85 a_w, regardless of temperature. The total I_D scores for *A. niger* at 15°C were, however, always smaller than those at 25°C. *A. parasiticus*, however, had I_D values which were not dependent on temperature, except at low a_w levels, and it was not markedly dominant at any of the set of conditions tested.

Aspergillus glaucus and the *Eurotium* species followed a very similar pattern (Fig. 1): they had the lowest I_D at 0.995 a_w, increasing at 0.98, 0.95 and 0.90 a_w where their I_D became similar to those of the other species tested, and finally at 0.85 a_w they had the highest I_D except for those of *A. tamarii* which were similar.

Penicillium species showed intermediate levels of I_D, mostly higher at 15 than at 25°C. They were not very dependent on a_w although the optimum was always at 0.90 a_w.

Table 1. Example of the interaction types between species in relation to water activity (a_w) at 25°C.

	vir	*gra*	*Sub*	*pro*	*nig*	*par*	*tam*	*gla*	*Ch*	*rep*	*imp*	*aur*	*can*
	0.98 a_w												
vir	-	1	2	2	0	4	4	4	4	4	4	4	2
gra	1	-	2	2	0	2	4	4	4	4	4	4	4
sub	2	2	-	2	0	2	4	4	4	4	2	4	2
pro	2	2	2	-	0	2	4	4	4	4	2	4	2
nig	4	4	4	4	-	4	4	4	4	4	4	4	4
par	0	2	2	2	0	-	2	4	4	2	4	2	2
tam	0	0	0	0	0	2	-	4	4	4	3	4	2
gla	0	0	0	0	0	0	0	-	2	2	3	0	2
che	0	0	0	0	0	0	0	2	-	2	2	2	2
rep	0	0	0	0	0	2	0	2	2	-	2	2	2
imp	0	0	2	2	0	0	3	3	2	2	-	2	2
aur	0	0	0	0	0	2	0	4	2	2	2	-	2
can	2	0	2	2	0	2	2	2	2	2	2	2	-
	0.90 a_w												
vir	-	0	0	0	0	0	0	0	0	0	0	0	0
gra	0	-	0	0	0	0	0	0	0	0	0	0	0
sub	4	4	-	2	0	2	0	0	2	3	0	0	0
pro	4	4	2	-	0	2	0	2	2	3	0	0	0
nig	4	4	4	4	-	2	4	4	4	4	4	4	4
par	4	4	2	2	2	-	2	2	2	2	2	2	2
tam	4	4	4	4	0	2	-	4	4	2	2	4	2
gla	4	4	4	2	0	2	0	-	2	2	2	2	2
che	4	4	2	2	0	2	0	2	-	2	2	2	2
rep	4	4	3	3	0	2	2	2	2	-	2	2	2
imp	4	4	4	4	0	2	2	2	2	2	-	2	2
aur	4	4	4	4	0	2	0	2	2	2	2	-	2
can	4	4	4	4	0	2	2	2	2	2	2	2	-

Key: vir, T. viride; gra, F. graminearum; sub, F. subglutinans; pro, F. proliferatum; nig, A. niger; par, A. parasiticus; tam, A. tamarii; gla, A. glaucus; che, E. chevalieri; rep, E. repens; imp, P. implicatum; aur, P. aurantiogriseum; can, P. canescens.

Scores are applied to species at left interacting with those listed above.

Water activity and temperature effects on growth rates

Fusarium species had increasing growth rates with a_w, except for *F. proliferatum* and *F. subglutinans* at 25°C where growth rate decreased when a_w was increased from 0.98 to 0.995 a_w (Fig. 2). They were, however, only able to grow in the range 0.95-0.995 a_w. *Fusarium graminearum* had growth rates much higher than those of the other *Fusarium*

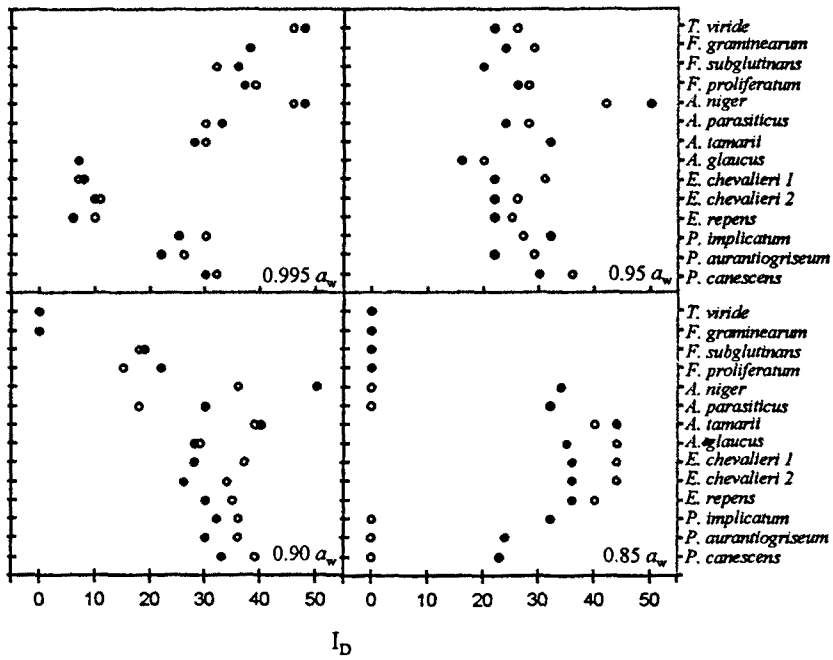

Figure 1. Effect of water activity on the Index of Dominance at 25°C (●) and 15°C (O) of common maize fungi on PDA.

species. This figure represents an example of one species each of *Fusarium*, *Eurotium* and *Penicillium* spp., because in each case all species of the same genus tested behaved in a very similar way.

Aspergillus glaucus and *Eurotium* species were slow-growing (Fig. 2b); neither were able to grow at more than 5 mm d^{-1} at 25°C and 3 mm d^{-1} at 15°C. *A. glaucus* followed a similar pattern to that of the *Eurotium* species: they were able to grow through the whole range of a_w tested, having their optimum a_w at 0.95, and being able to grow at high a_w (0.995) only at a reduced growth rate. The only difference was that *A. glaucus* and *E. repens* had their optimum at 15°C and 0.98 a_w, while *E. chevalieri* strains had optimum growth at 0.95 a_w.

Aspergillus niger and *A. parasiticus* (Fig. 3) were able to grow over the whole range of a_w tested (0.85-0.995), except at 15°C and 0.85 a_w, and the optimum was at 25°C and 0.98 a_w. On the other hand, *A. tamarii* had an optimum between 0.95 and 0.98 a_w, and was able to grow faster than the other *Aspergillus* spp. at 0.85 a_w at both temperatures. *Penicillium* species grew more slowly than all the other fungi tested. They were not able to grow at 0.85 a_w or only at an insignificant growth rate, and their optimum a_w was between 0.95 and 0.98.

Trichoderma viride, grew fastest at 0.995 a_w, attaining almost 35 mm d^{-1} but, as with *Fusarium* species, it was not able to grow at 0.85 and 0.90 a_w.

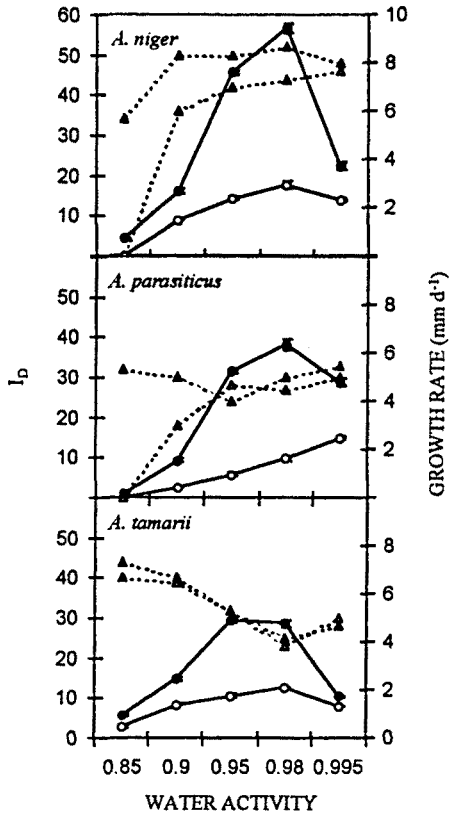

Figure 2. Effect of water activity on Index of Dominance at 15°C (△) and 25°C (▲), and on growth rate at 15°C (O) and 25°C (●) of *Aspergillus* species on PDA. Error bars show standard errors of the estimated growth rates.

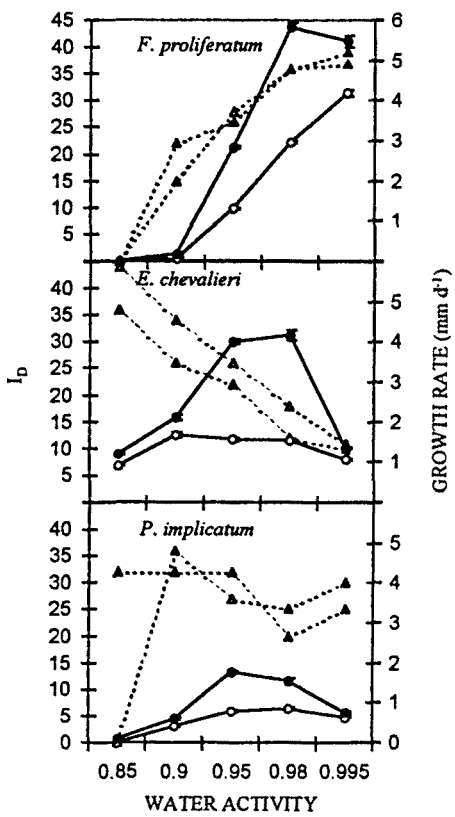

Figure 3. Effect of water activity on Index of Dominance at 15°C (△) and 25°C (▲), and on growth rate at 15°C (O) and 25°C (●) of *Fusarium proliferatum, Eurotium chevalieri* and *Penicillium implicatum* on PDA. Error bars show standard errors of the estimated growth rates.

For the whole experiment, single factors (a_w, temperature, species) and two and three-way interactions, were found to be statistically ($P < 0.01$) significant. The exceptions were for temperature x species interactions for *Eurotium*, *Penicillium* and *A. glaucus*.

Correlation between I_D and growth rat

Some correlation was found between I_D and growth rate for *Fusarium* species (R^2 = 0.713-0.860) and *T. viride* (R^2=0.883); both I_D and growth rate increased with a_w in a similar way, although the effect of temperature was not as strong for both.

Interestingly, *Fusarium, Trichoderma* and *Aspergillus* grew fastest when growing at high a_w, and they had high I_D total scores in these wet conditions; on the other hand, *A. glaucus* and *Eurotium* species had higher growth rates at 0.85-0.90 a_w than the others, and they had the highest I_D in these dry conditions.

DISCUSSION

This study has shown that both interaction type, I_D and growth rates are critically dependent on environmental conditions such as a_w and temperature. Overall, *A. niger* was the most competitive species, with the highest total I_D score. *A. niger* was able to reduce the growth rate of other strains at a distance in agar substrates, suggesting that dominance may involve the production of inhibitory metabolites. In addition, *A. niger* had high growth rates at intermediate a_w levels. Recently, Marín *et al.* (1998), reported that some *Aspergillus* species were able to inhibit the growth of some *Fusarium* species. In contrast, the dominance of *Fusarium* and *Trichoderma* species, particularly at > 0.95 a_w could be predominantly due to their ability to grow rapidly and invasively. The ability of *F. moniliforme* and *F. proliferatum* to germinate and grow under different environmental conditions has already been demonstrated (Marín, Sanchis & Magan, 1995*a*; Marín *et al.* 1995*b*, 1996). Although the *Penicillium* species are known to export antifungal metabolites they did not have high numerical total I_D scores under the conditions tested, and grew significantly more slowly than the *Trichoderma*, *Fusarium* and *Aspergillus* spp. This contrasts with previous *in vitro* studies by Magan & Lacey (1984) on cereal fungi where *Penicillium brevicompactum* grew very slowly but was the dominant species because of the production of metabolites such as mycophenolic acid. Wicklow *et al.* (1988) indicated that common fungal colonists of corn kernels interfere with the ability of *A. flavus* to infect preharvest maize; *F. moniliforme* was particularly effective in inhibiting kernel infection by *A. flavus*. On the other hand, Rheeder *et al.* (1990) found a negative correlation on the isolation of *F. moniliforme* and that of *F. graminearum* and *F. subglutinans* in maize kernels. Wheeler & Hocking (1993), studied interactions among xerophilic species isolated from dry fish and obtained similar results to ours: none of the fungi intermingled freely, while mutual interactions on contact (2:2) were quite common. Species having a faster growth rate than their competitor tended to inhibit on contact, and then continued to grow around the inhibited fungus. At 0.90 and 0.84 a_w the xerophilic fungi exhibited mutual inhibition on contact, or inhibition of one or both species on contact, with inhibited species continuing to grow at a significantly reduced rate.

Previously, total I_D scores were not found to be correlated with growth rate (Magan & Lacey, 1984, 1985; Whipps & Magan, 1987; Wheeler & Hocking, 1993). The reaction derived from contact between hyphae may not depend on growth rate *per se*, especially if one of the species tested produces antifungal metabolites. The difference in growth rates between two interacting fungi may need to be very high to affect hyphal interactions between species, as was found with *Fusarium* and *Trichoderma* species. Interestingly, in this study only good correlations were found between I_D and growth rate for *T. viride* and the *Fusarium* spp.

In the present study, *F. graminearum*, *F. subglutinans* and *F. proliferatum* had higher I_D scores when paired with storage species (*Aspergillus*, *Penicillium* and *Eurotium* spp.). Previously, Magan & Lacey (1984) found that a *Fusarium culmorum* strain isolated from wheat was one of the few field fungi tested which was able to compete successfully in terms of I_D against other field and storage fungi, particularly at 0.95 and 0.98 a_w. The *Eurotium* and *A. glaucus* spp. were uncompetitive at 0.98 and 0.95 a_w. At 0.90 and 0.85 a_w, however, they became markedly more competitive, especially at 25°C. Their xerophilic nature enables growth under conditions in which very few of the other species examined are able to grow

effectively, and thus to a large extent avoiding competition from many other species which grow optimally at > 0.95 a_w.

Stored grain was first considered as a man-made ecosystem by the pioneering work of Sinha (1973) which showed that a range of abiotic and biotic factors impacted on the interactions and dominance of fungal groups and their role in causing deterioration and quality loss. By using this approach and principle component analyses Sinha (1973, 1995) demonstrated that interactions between species and environmental factors were important influences on safe storage life of grain. McLean & Berjak (1987) found that a succession of spoilage fungi colonized stored maize at 0.85 and 1.00 a_w over 4-7 mon storage periods, with *Aspergillus*, *Fusarium* and *Penicillium* spp. being dominant. The water availability range used was, however, limited and no attempts were made to examine the interactions between such fungi and the role of abiotic factors in dominance by individual or groups of fungi.

The present work gives a general impression of how a range of fungal species from maize may interact with each other and their potential competitiveness. Although the *in vitro* I_D study does not reflect directly what may occur in the stored maize ecosystem, it does suggest the possible outcome during hyphal interaction under different environmental conditions. The enhanced competitiveness due to metabolite production in agar media needs to be treated with caution as in grain substrates the production patterns and role may be very different (Magan & Lacey, 1984, 1985). Magan & Lacey (1985), however, only found a good correlation between I_D totals and colonization for *P. brevicompactum*, *P. hordei*, *A. candidus* and *A. nidulans*. It was notable that *Epicoccum nigrum* and *P. brevicompactum*, two of the most competitive fungi *in vitro*, because of their ability to produce inhibitory substances, were both relatively poor competitors in wheat grain. Such substances may only be produced in small amounts and diffuse more slowly in grain substrates.

In terms of growth rates, *Fusarium*, *Trichoderma* and *Aspergillus* species had fastest growth at high water availability (0.98-0.995 a_w), while *Eurotium* and *Aspergillus* species were the fastest in the range 0.90-0.95 a_w, and the *Eurotium* species grew effectively at 0.85 a_w. The colonization of stored grain is a complex process involving more than the antagonistic capabilities of the fungi. If metabolites are unable to diffuse from grain to grain, growth rate may become more important than in agar culture. Also nutritional factors and tolerance of extreme temperatures and a_w levels can also play a part (Magan & Lacey, 1984).

The study of fungal interactions has attracted much interest due to the fact that mycotoxin production by toxigenic species which are present in stored grain can be both enhanced or inhibited by the other species which are colonising the grain. Cuero, Smith & Lacey (1987) showed how aflatoxin production by *A. flavus* was stimulated by *Bacillus amyloliquefaciens* and *Hyphopichia burtonii* when growing together on irradiated maize and rice grains under certain environmental conditions. They suggested that metabolic activity altered the substrate, enhancing growth of *A. flavus* or its ability to produce aflatoxin or both. Similarly, compounds from these micro-organisms could have been released, leading to enhanced growth or enhanced toxin production or both. Wicklow *et al.* (1980) reported detectable levels of aflatoxin production when *A. flavus* was grown with the yeast *Candida guilliermondi* on freshly harvested maize, but none when *A. flavus* was paired with *A. niger* or *T. viride*. Production of aflatoxin by *A. parasiticus* has been shown to be inhibited by *A. oryzae* (Maing, Ayres & Koehler, 1973) and *Rhizopus nigricans* (Weckbach & Marth,

1977). Stimulation of aflatoxin production was found in dual cultures of *A.flavus* with *P. purpurogenum* (Moss & Badii, 1982). Recently, Yoshizawa, Yamashita & Chokethaworn (1996) showed that levels of both fumonisins and aflatoxins in corn samples had a negative relationship to each other, suggesting the interaction of fumonisin- and aflatoxin-producers on kernels during infection and contamination by these mycotoxins.

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IX

ENVIRONMENTAL FACTORS, *IN VITRO* INTERACTIONS, AND NICHE OVERLAP BETWEEN *FUSARIUM MONILIFORME, F. PROLIFERATUM,* AND *F. GRAMINEARUM, ASPERGILLUS* AND *PENICILLIUM* SPECIES FROM MAIZE GRAIN

S. Marín[1], V. Sanchis[1], A. J. Ramos[1], I. Viñas[1], N. Magan[2]

[1]Food Technology Department, University of Lleida, UdL-IRTA, CeRTA, Rovira Roure 177, 25198 Lleida, Spain.
[2]Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, U.K.

SUMMARY

The effects of temperature and water availability on growth and interactions between fumonisin-producing isolates of *Fusarium moniliforme* and *F. proliferatum* and seven other fungi from maize grain were determined *in vitro*. The type of interaction and index of dominance (I_D) between species were markedly influenced by temperature and a_w. Generally, *F. moniliforme* and *F. proliferatum* were very competitive and dominant against the *Penicillium* spp. and *A. flavus*. They were in turn dominated by *A. niger*, but mutually antagonistic when paired with *F. graminearum* and *A. ochraceus*. Under slightly drier conditions (< 0.98 a_w) *A. ochraceus* became more competitive and dominant over the fumonisin-producing species. *A. flavus* was dominant only at 30°C and < 0.96 a_w.. *F. moniliforme* and *F. proliferatum* demonstrated dominance against all species over a range of temperatures and 0.994 to 0.96 a_w. At lower a_w levels they were less competitive. The growth rate of the two fumonisin-producing species was significantly reduced by *F. graminearum*, regardless of a_w. *F. moniliforme* and *F. proliferatum* reduced growth of *Penicillium* and *Aspergillus* spp., especially at > 0.96 a_w. At < 0.96 a_w, growth of these species was unaffected. Using Biolog plates the effect of a_w and temperature on utilisation patterns of carbon sources in maize were evaluated for the first time. The niche overlap indices relative to *F. moniliforme* and *F. proliferatum* were determined and compared with that of each interacting species. NOIs for *F. moniliforme* and *F. proliferatum* were > 0.90 at >0.96 a_w and 25 and 30°, indicative of co-existence with other species. Most of species had NOIs > 0.90, except in some cases when paired with *F. moniliforme*, where NOIs < 0.80 suggested the occupation of different niches. Although there was no significant correlation between the I_D and NOI methods both suggested that the niche overlap between species was in a state of flux and significantly influenced by both temperature and water availability. This suggests that interpretation of I_D, or NOIs carried out under one set of environmental conditions may be misleading when considering interactions between species and also where screening for biocontrol potential is being considered.

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INTRODUCTION

Fusarium moniliforme and *F. proliferatum* are economically important because they cause maize ear rot and produce the fumonisin mycotoxins. During ripening the grain carries a wide range of contaminant field and storage fungi (King & Scot, 1981; Wicklow, 1988). The ability of these *Fusarium* spp. to colonise this ecological niche and produce mycotoxins suggest that they may have strong competitive capabilities. Blaney, Ramsey & Tyler (1986) suggested that the negative correlation between isolation frequencies of *F. graminearum* and *F. moniliforme* on maize grain may have been due to competition for substrate, production of antagonistic substances, and environmental factors, although none of these possibilities have been investigated in detail. *Fusarium moniliforme* also has been reported to suppress the growth of other ear-colonising species (Rheeder *et* al., 1990*b*; Miller, 1994). The primary environmental factors that influence growth and interactions between *Fusarium* spp. and other fungal colonists, particularly *Aspergillus* and *Penicillium* spp., are temperature and water availability (water activity, a_w). Previous work has already been done on the ability of these *Fusarium* species to grow and produce fumonisins under different a_w x temperature conditions (Alberts *et al.*, 1990; Le Bars *et al.*, 1994; Cahagnier, Melcion & Richard-Molard, 1995; Marín, Sanchis & Magan, 1995*a*; Marín *et al.*, 1995*b*).

A range of interspecific interactions can occur between fungi including mutual intermingling (representing overlapping domains), mutual inhibition (antagonism, or combat), and dominance by one species over another (Cooke & Rayner, 1984). These interaction types were given numerical scores and an index of antagonism to investigate the *in vitro* capabilities of different grain spoilage fungi (Wicklow *et al.*, 1980). Using an index of dominance (I_D) it was shown that interactions were profoundly influenced by water activity, temperature and nutrient substrate (Magan & Lacey, 1984). Interactions between species have also been shown to influence the production of mycotoxins in cereals (Wicklow *et al.*, 1980; Cuero, Smith & Lacey, 1987; Ramakrishna, 1990). However, very little information is available on the type of interspecific interactions which might occur between fumonisin-producing strains of *F. moniliforme* and F. *proliferatum*, and *F. graminearum*, *Aspergillus* and *Penicillium* spp. Furthermore, environmental factors may exert some selective pressure influencing the community structure and dominance of individual species. It has been suggested that the coexistence of microorganisms, particularly on plant surfaces, may be mediated via nutritional resource portioning (Wilson & Lindow, 1994*a*, *b*). *In vitro* carbon utilisation profiles were used to determine niche overlap indices (NOI) and thus the level of ecological similarity. It has been proposed that NOI values of > 0.9 represents coexistence between species in an ecological niche, while scores of < 0.9 represent occupation of separate niches (Wilson & Lindow, 1994*a*, *b*). In recent studies, however, the impact that changes in environmental factors might play in determining the level of coexistence or dominance of species in a niche has been neglected. These neglected variables are critical in developing an understanding of the conditions that enable *F. moniliforme* and *F. proliferatum* to become dominant (Marín *et al.*, 1995*a*, *b*).

The objectives of this study were to examine the effect of water activity (0.92-0.994 a_w) and temperature (10-30°C) on *in vitro* interspecific interactions and growth between fumonisin-producing isolates of *Fusarium moniliforme* and *F. proliferatum*, and other grain fungi. The effect of environmental factors on *in vitro* carbon source utilisation patterns and NOIs for the fumonisin-producing strains of *F. moniliforme* and *F. proliferatum* in relation to all other species was determined and compared with the I_D values.

MATERIALS AND METHODS

Fungi

The species used were *Fusarium moniliforme* Sheldon (25N), *F. proliferatum* (Matsushima) Nirenberg (73N), *Aspergillus flavus* Link (3.36), *A. niger* van Tieghem (3.37), *Penicillium aurantiogriseum* Dierckx (3.13), *P. griseofulvum* Dierckx (3.88), *P. citrinum* Thom (3.53) - the numbers in parentheses are the reference numbers for the cultures held at the Food Technology Dept., University of Lleida, Spain-, and *F. graminearum* Schwabe (CECT 2150, ATCC 26557), *A. ochraceus* Wilhelm (NRRL 3174).

Media preparation

The basic medium used was a 3% maize meal extract agar (MMEA) with a pH of 5.5 (Marín *et al*, 1995*a*). The water activity (a_w) of the basic medium was 0.994 and this was modified with glycerol to 0.98, 0.96, 0.94, 0.92. The a_w of all media was confirmed by measurement in a Novasina IC II (Novasina AG, Zurich, Switzerland).

Inoculation and measurement of interactions

Stock cultures of each species on MMEA provided inocula for the interaction experiments. Control growth rates for each species were obtained by inoculating MMEA plates centrally with a 5 mm diam. disc from the growing margin of the stock culture under all test treatment conditions, and measured as previously described (Marín *et al.*, 1995*a*). For interactions, two species were inoculated 4.4 cm apart on 9cm Petri plates. *F. moniliforme* and *F. proliferatum* were tested against each other and each of the other fungi. After inoculation, treatments of the same a_w were sealed in polyethylene bags and incubated at 10, 15, 25, 30, and 37° for up to 8 weeks. All experiments were carried out with at least three separate replicates per treatment.

Four radial measurements were periodically made of each colony, one on a line forming the two inoculation points, the other 3 at 90° intervals around the colony. The equivalent radius (= radius of a circle with the same area of the colony shape measured) obtained for each colony was linear regressed with time to obtain the growth rate. In the case of slow-growing fungi growing towards a faster growing interacting species, and at low a_w, the growth rate of the slower growing species was calculated until it stopped growing because of the dominance of the other. Analysis of variance of growth rates allowed an assessment of whether or not a fungus was affected by the others, or by water activity and temperature (SYSTAT, version 5.0, SYSTAT Inc.).

Classification of interactions between species

The I_D was developed to measure the ability of a species to dominate under a particular set of environmental conditions (Magan & Lacey, 1984). The interaction of each dual culture was examined macroscopically, the type of interaction determined, and numerical scores assigned. These scores were added to obtain the overall Index of Dominance (I_D) for each species in comparison with that of *F. moniliforme* and *F. proliferatum*.

Niche Overlap Index

Experiments were carried out in Biolog (GN MicroPlates, BIOLOG, Inc., Calif.) plates to determine the C-sources utilised by the fungi. The 98 substrates present in a Biolog plate include carbohydrates, carboxylic acids, amino acids, amines and amides, and miscellaneous carbon compounds. We only considered the results which came from carbon sources present in maize: dextrin, D-fructose, D-galactose, α-D-glucose, D-melobiose, D-raffinose, sucrose, D-alanine, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, D-serine, L-serine, and L-threonine. Spores of each species obtained from MMEA were suspended in sterile water and centrifuged three times. Subsequently the spores were washed and centrifuged in a 0.25M 2-(N-morpholino)ethanosulphonic acid (MES, Sigma Chemical Co.) buffered at pH 5.5 solution. The solutions were modified to 0.92, 0.96 and 0.994 a_w levels using the ionic solute NaCl (Lang, 1967). The final concentration of the spore suspension was 10^7 spores ml^{-1} and 100 ml was added to each test wells. An ionic solute was used instead of glycerol because glycerol is one of the C-sources used in the Biolog test plates. The relative effects of non-ionic and ionic solutes on mycelial growth has already been reported by Marín *et al* (1995*a*). The Biolog plates were incubated at 15, 25 and 30° for up to 14 d prior to examination for utilisation of carbon sources.

The Niche Overlap Index (NOI) was determined as follows (Wilson & Lindow, 1994*a*):

$$NOI = \frac{\text{No of C - sources in common between the 2 fungi}}{\text{Total No of C - sources utilized by target 'pathogen'}}$$

The NOI ratios were compared for each set of environmental conditions and comparisons made with the I_D scores.

RESULTS

Water activity x temperature effects on interactions and I_D

The numerical interaction scores and indices of dominance for *F. moniliforme*, *F. proliferatum* and other species are shown in Tables 1 and 2. These data show that over a wide range of temperature and a_w levels both species are very competitive with scores of 4 against *P. aurantiogriseum*, *P. griseofulvum*, *P. citrinum* and *A. flavus*. Both *Fusarium* spp. were, however, consistently dominated by *A. niger*. At high a_w mutualistic antagonism occurred between *F. moniliforme*, *F. proliferatum* and *A. ochraceus*. At reduced a_w levels (0.98-0.94 a_w) *A. ochraceus* was more competitive and dominated the *Fusarium* spp. *A. flavus* was dominant only at 30° and < 0.96 a_w.

The I_D for the *Fusarium* spp. against each interacting species reflects their *in vitro* dominance. Both *F. moniliforme* and *F. proliferatum* are very competitive over a wide range of temperatures and between 0.994-0.96 a_w. However, at 0.94 a_w both are less competitive. Generally, in these experiments, *F. proliferatum* was slightly more competitive than *F. moniliforme*.

Table 1. Effect of water activity (a_w) and temperature on numerical interaction scores and I_D for *Fusarium moniliforme* and paired species.

a_w		10°	15°	25°	30°	I_D
0.994	*F. proliferatum*	0/4*	0/4	0/4	0/4	0/16
	F. graminearum	2/2	2/2	2/2	2/2	8/8
	P. aurantiogriseum	4/0	2/2	4/0	4/0	14/2
	P. griseofulvum	2/2	2/2	4/0	4/0	12/4
	P. citrinum	2/2	4/0	4/0	2/2	12/4
	A. flavus	N.G.	4/0	4/0	4/0	12/0
	A. niger	N.G.	0/4	0/4	0/4	0/12
	A. ochraceus	N.G.	2/2	2/2	2/2	6/6
	I_D	10/10	16/16	20/12	18/14	64/52
0.98	*F. proliferatum*	0/4	0/4	0/4	0/4	0/16
	F. graminearum	2/2	2/2	2/2	2/2	8/8
	P. aurantiogriseum	4/0	4/0	4/0	4/0	16/0
	P. griseofulvum	4/0	2/2	4/0	4/0	14/2
	P. citrinum	4/0	4/0	4/0	2/2	14/2
	A. flavus	N.G.	4/0	4/0	4/0	12/0
	A. niger	N.G.	0/4	0/4	0/4	0/12
	A. ochraceus	2/2	0/4	0/4	0/4	2/14
	I_D	16/8	16/16	18/14	16/16	66/54
0.96	*F. proliferatum*	2/2	2/2	2/2	2/2	8/8
	F. graminearum	2/2	2/2	2/2	2/2	8/8
	P. aurantiogriseum	4/0	4/0	4/0	4/0	16/0
	P. griseofulvum	4/0	4/0	4/0	4/0	16/0
	P. citrinum	4/0	4/0	2/2	2/2	12/4
	A. flavus	N.G.	4/0	4/0	0/4	8/4
	A. niger	N.G.	0/4	0/4	0/4	0/12
	A. ochraceus	2/2	0/4	0/4	0/4	2/14
	I_D	18/6	20/12	18/14	14/18	70/50
0.94	*F. proliferatum*	2/2	2/2	2/2	2/2	8/8
	F. graminearum	2/2	2/2	2/2	2/2	8/8
	P. aurantiogriseum	0/4	4/0	4/0	2/2	10/6
	P. griseofulvum	0/4	4/0	2/2	2/2	8/8
	P. citrinum	2/2	2/2	2/2	2/2	8/8
	A. flavus	N.G.	2/2	4/0	0/4	6/6
	A. niger	2/2	0/4	0/4	0/4	2/14
	A. ochraceus	0/4	0/4	0/4	0/4	0/16
	I_D	8/20	16/16	16/16	10/22	50/74

N.G. : no growth

* *F. moniliforme* score/other species score

Table 2. Effect of water activity (a_w) and temperature on numerical interaction scores and I_D for *Fusarium proliferatum* and paired species.

a_w		10°	15°	25°	30°	I_D
0.994	*F. moniliforme*	4/0*	4/0	4/0	4/0	16/0
	F. graminearum	2/2	2/2	2/2	2/2	8/8
	P. aurantiogriseum	4/0	4/0	4/0	4/0	16/0
	P. griseofulvum	4/0	4/0	4/0	4/0	16/0
	P. citrinum	4/0	4/0	4/0	4/0	16/0
	A. flavus	N.G.	4/0	4/0	4/0	12/0
	A. niger	N.G.	0/4	0/4	0/4	0/12
	A. ochraceus	N.G.	2/2	2/2	2/2	6/6
	I_D	18/2	24/8	24/8	24/8	*90/26
0.98	*F. moniliforme*	4/0	4/0	4/0	4/0	16/0
	F. graminearum	2/2	2/2	2/2	2/2	8/8
	P. aurantiogriseum	4/0	4/0	4/0	4/0	16/0
	P. griseofulvum	4/0	4/0	4/0	4/0	16/0
	P. citrinum	4/0	4/0	4/0	4/0	16/0
	A. flavus	N.G.	4/0	4/0	4/0	12/0
	A. niger	N.G.	0/4	0/4	0/4	0/12
	A. ochraceus	4/0	0/4	2/2	2/2	8/8
	I_D	22/2	22/10	24/8	24/8	92/28
0.96	*F. moniliforme*	2/2	2/2	2/2	2/2	8/8
	F. graminearum	2/2	2/2	2/2	2/2	8/8
	P. aurantiogriseum	4/0	2/2	4/0	4/0	14/2
	P. griseofulvum	4/0	4/0	4/0	4/0	16/0
	P. citrinum	4/0	4/0	4/0	4/0	16/0
	A. flavus	N.G.	4/0	4/0	2/2	10/2
	A. niger	2/2	0/4	0/4	0/4	2/14
	A. ochraceus	4/0	0/4	0/4	0/4	4/12
	I_D	22/6	18/14	20/12	18/14	78/46
0.94	*F. moniliforme*	2/2	2/2	2/2	2/2	8/8
	F. graminearum	2/2	2/2	2/2	2/2	8/8
	P. aurantiogriseum	0/4	4/0	4/0	2/2	10/6
	P. griseofulvum	2/2	2/2	4/0	4/0	12/4
	P. citrinum	2/2	4/0	2/2	2/2	10/6
	A. flavus	N.G.	4/0	4/0	0/4	8/4
	A. niger	N.G.	0/4	0/4	0/4	0/12
	A. ochraceus	2/2	0/4	0/4	0/4	2/14
	I_D	10/14	18/14	18/14	12/20	58/62

N.G. : no growth

* *F. proliferatum* score/other species score

Effect of interspecific interactions on growth of individual species

The growth rates of both *F. moniliforme* and *F. proliferatum* are markedly decreased by the interaction with *F. graminearum* ($P<0.01$), regardless of a_w.(Fig. 1). The growth of *F. graminearum* was not affected by either of the fumonisin-producing species. Their growth was not affected by interactions with the *Penicillium* spp. (Fig. 2a, 3a). Growth rates were, however, reduced during interactions with some *Aspergillus* spp., particularly *A. niger* (Fig. 2b, 3b) ($P<0.01$). The growth rates of the *Aspergillus* and *Penicillium* spp. were, however often significantly affected, by the *Fusarium* spp., particularly at 0.994 and 0.98 a_w (Fig. 2c, d; 3 c, d). The growth of competitive species, such as *A. ochraceus* and *A. niger*, was relatively unaffected by the interactions with *F. moniliforme* and *F. proliferatum*, particularly at lower water availability levels (0.94 and 0.96 a_w). There was no direct relationship between competitiveness, as measured by I_D, and growth rates of the individual species.

Effect of water availability and temperature on carbon source utilisation patterns and niche overlap indices

Changing a_w or temperature altered the number of carbon compounds utilised (niche size, NS) by *F. moniliforme* (Table 3) and *F. proliferatum* (Table 4) and the other species examined. At the lowest a_w and temperature levels examined (0.92 a_w, 15°) *P. aurantiogriseum*, *P. citrinum* and *A. ochraceus* were the only species able to utilise some of the carbon sources tested. All species were able to utilise more carbon sources as the water availability increased to 0.96 and to 0.994 a_w at all temperatures.

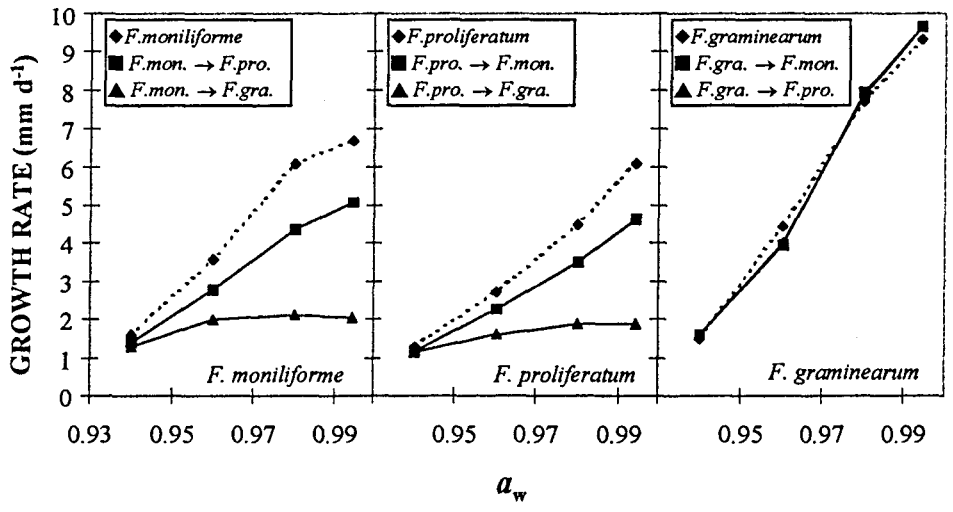

Fig. 1. Growth rate of *Fusarium moniliforme*, *F. proliferatum* and *F. graminearum*, when growing alone and interacting with each other at 25° and different water activity levels on MMEA. Error bars are S.E.M. and are only given where they are bigger than the marker.

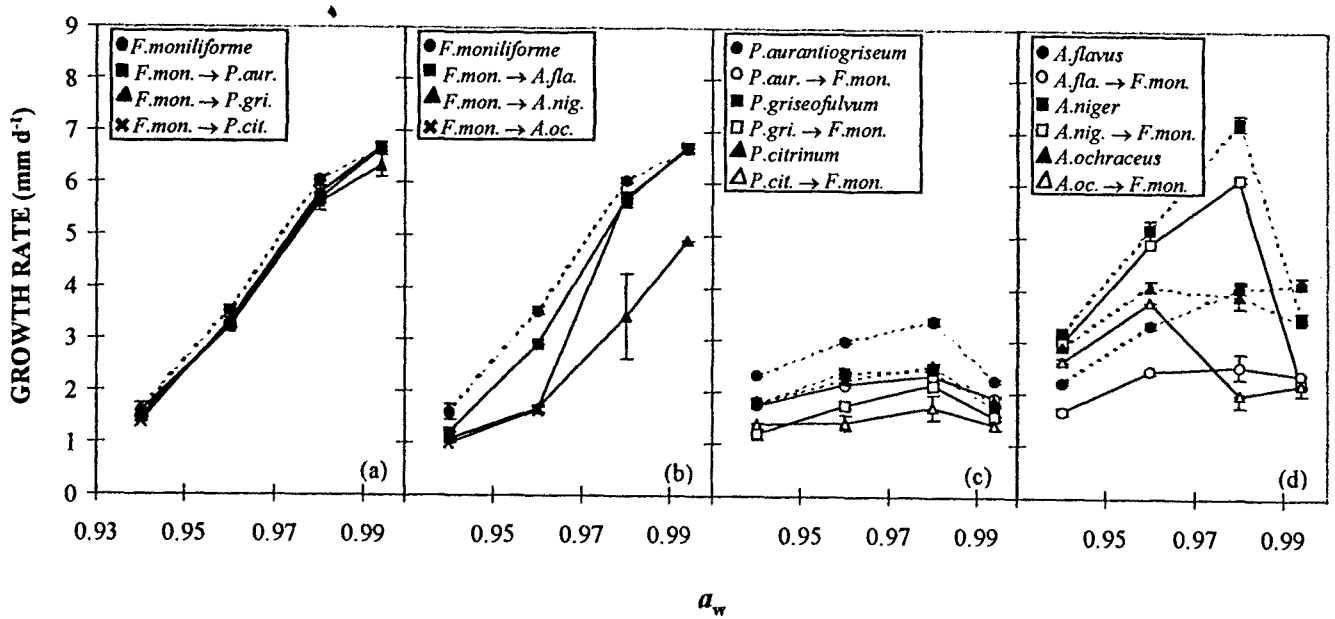

Fig. 2. Growth rate of (a, b) *Fusarium moniliforme* when growing alone and interacting with *Penicillium* and *Aspergillus* spp.; (c) *P. aurantiogriseum, P. griseofulvum* and *P. citrinum* when growing alone and interacting with *F. moniliforme* and; (d) *A. flavus, A. niger* and *A. ochraceus* when growing alone and interacting with *F. moniliforme* at 25° at different a_w levels. Error bars are S.E.M. and are only given where they are bigger than the marker.

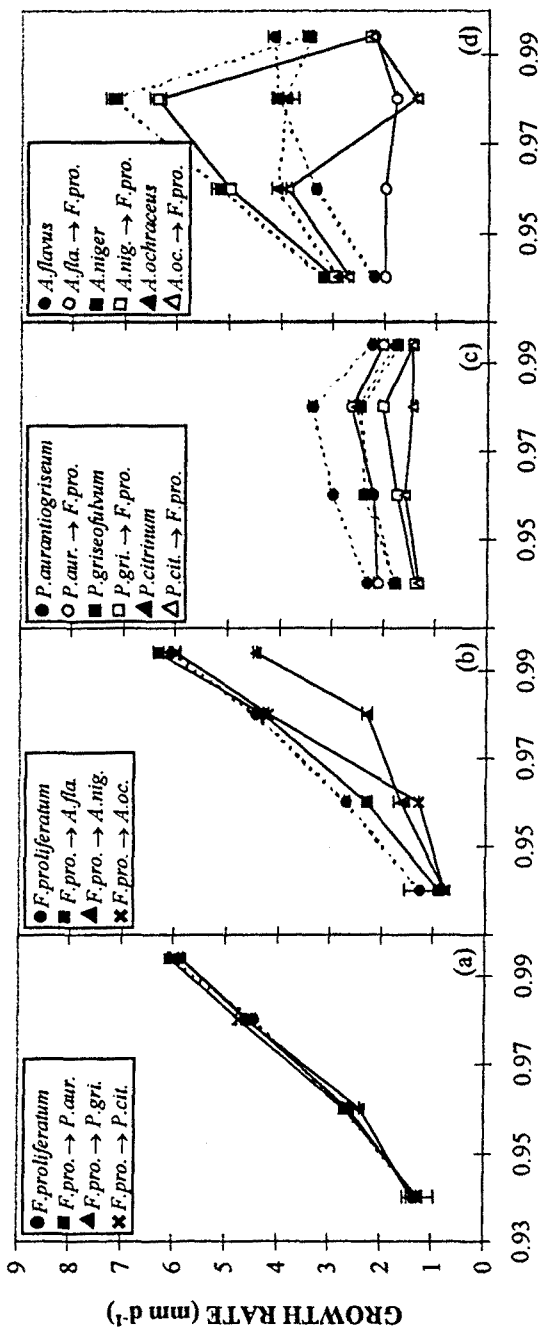

Fig. 3. Growth rate of (a, b) *Fusarium proliferatum* when growing alone and interacting with *Penicillium* and *Aspergillus* spp.; (c) *P. aurantiogriseum*, *P. griseofulvum* and *P. citrinum* when growing alone and interacting with *F. proliferatum* and; (d) *A. flavus*, *A. niger* and *A. ochraceus* when growing alone and interacting with *F. proliferatum* at 25° at different a_w levels. Error bars are S.E.M. and are only given where they are bigger than the marker.

Table 3. NOIs for fungi paired with *F.moniliforme*, derived from carbon source utilization data at different a_w and temperatures.

Water activity		0.92		0.96		0.994
15°	Niche size* (*F.moniliforme*)	0		7		15
	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}
F. proliferatum	0	0/0	12	1 /0.58	18	1 /0.83
F. graminearum	0	0/0	15	1 /0.47	18	1 /0.83
P. aurantiogriseum	11	0/0	15	1 /0.47	16	1 /0.94
P. griseofulvum	0	0/0	6	0.71/0.83	15	0.93/0.93
P. citrinum	7	0/0	14	1 /0.50	18	1 /0.83
A. flavus	0	0/0	12	1 /0.58	15	1 / 1
A. niger	0	0/0	5	0.71/ 1	12	0.80/ 1
A. ochraceus	7	0/0	13	1 /0.54	13	0.87/ 1
25°	Niche size* (*F.moniliforme*)	12		14		18
	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}
F. proliferatum	13	1 /0.92	14	1 / 1	18	1 / 1
F. graminearum	11	0.92/ 1	15	1 /0.93	18	1 / 1
P. aurantiogriseum	9	0.67/0.89	13	0.93/ 1	16	0.89/ 1
P. griseofulvum	12	0.92/0.92	15	0.93/0.87	17	0.94/ 1
P. citrinum	14	1 /0.86	18	1 /0.78	18	1 / 1
A. flavus	12	0.83/0.83	15	1 /0.93	16	0.89/ 1
A. niger	8	0.58/0.87	13	0.93/ 1	14	0.78/ 1
A. ochraceus	13	1 /0.92	15	0.93/0.87	17	0.94/ 1
30°	Niche size* (*F.moniliforme*)	13		12		18
	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}
F. proliferatum	14	1 /0.93	14	1 /0.86	18	0.94/0.94
F. graminearum	13	1 / 1	17	1 /0.71	18	1 / 1
P. aurantiogriseum	8	0.62/ 1	13	1 /0.92	17	0.94/ 1
P. griseofulvum	9	0.69/ 1	15	1 /0.80	18	1 / 1
P. citrinum	13	1 / 1	18	1 /0.67	18	1 / 1
A. flavus	13	1 / 1	17	1 /0.71	17	0.94/ 1
A. niger	10	0.77/ 1	13	1 / 1	14	0.78/ 1
A. ochraceus	13	1 / 1	16	1 /0.75	16	0.89/ 1

*maximum=18

NSS: Niche size of (partner) strain

$NOI_{Fus.}$ represents the proportion of the carbon compounds utilized by *F. moniliforme* that were also utilised by the competing strain

NOI_{strain} represents the proportion of the carbon compounds utilized by the strain that were also utilised by *F. moniliforme*

Table 4. NOIs for fungi paired with *F.proliferatum*, derived from carbon source utilization data at different a_w and temperatures.

Water activity		0.92		0.96		0.994	
15°	Niche size* (*F.proliferatum*)	0		12		18	
		NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}
F. moniliforme		0	0/0	7	0.58/ 1	15	0.83/ 1
F. graminearum		0	0/0	15	1 /0.80	18	1 / 1
P. aurantiogriseum		11	0/0	15	1 /0.80	16	0.89/ 1
P. griseofulvum		0	0/0	6	0.42/0.83	15	0.83/ 1
P. citrinum		7	0/0	14	1 /0.86	18	1 / 1
A. flavus		0	0/0	12	0.92/0.92	15	0.83/ 1
A. niger		0	0/0	5	0.42/ 1	12	0.67/ 1
A. ochraceus		7	0/0	13	1 /0.92	13	0.72/ 1
25°	Niche size* (*F.proliferatum*)	13		14		18	
		NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}
F. moniliforme		12	0.92/ 1	14	1 / 1	18	1 / 1
F. graminearum		11	0.85/ 1	15	1 /0.93	18	1 / 1
P. aurantiogriseum		9	0.69/ 1	13	0.93/ 1	16	0.89/ 1
P. griseofulvum		12	0.92/ 1	15	0.93/0.87	17	0.94/ 1
P. citrinum		14	1 /0.93	18	1 /0.78	18	1 / 1
A. flavus		12	0.62/0.92	15	1 /0.93	16	0.89/ 1
A. niger		8	0.62/ 1	13	0.93/ 1	14	0.78/ 1
A. ochraceus		13	1 / 1	15	0.93/0.87	17	0.94/ 1
30°	Niche size* (*F.proliferatum*)	14		14		18	
		NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}	NSS	$NOI_{Fus.}$/ NOI_{strain}
F. moniliforme		13	0.93/ 1	12	0.86/ 1	18	1 / 1
F. graminearum		13	0.93/ 1	17	1 /0.82	18	1 /0.94
P. aurantiogriseum		8	0.57/ 1	13	0.93/ 1	17	0.94/ 1
P. griseofulvum		9	0.64/ 1	15	0.93/0.87	18	1 / 1
P. citrinum		13	0.93/ 1	18	1 /0.78	18	1 / 1
A. flavus		13	0.93/ 1	17	1 /0.82	17	0.94/ 1
A. niger		10	0.71/ 1	13	0.93/ 1	14	0.78/ 1
A. ochraceus		13	0.93/ 1	16	0.93/0.81	16	0.89/ 1

*maximum=18

NSS: Niche size of (partner) strain

$NOI_{Fus.}$ represents the proportion of the carbon compounds utilized by *F. proliferatum* that were also utilised by the competing strain

NOI_{strain} represents the proportion of the carbon compounds utilized by the strain that were also utilised by *F. proliferatum*

The NOIs varied considerably for interacting species with a_w and temperature. The highest NOIs for *F. moniliforme* and *F. proliferatum* were obtained at 0.96 a_w and 25-30°, while most of the other species tested obtained higher NOIs especially when paired with *F. proliferatum* at 25-30°; these species obtained their highest NOIs at 0.994 a_w and 25-30°. Under high water availability conditions and 25-30° the NOI of *F. moniliforme* and *F. proliferatum* was often > 0.9 indicative of the coexistence of these and other species. However, against other species, particularly *P. aurantiogriseum, P. griseofulvum,* and *A. niger,* these two *Fusarium* species had NOIs < 0.8 indicative of occupation of different niches. It was noteworthy that *F. moniliforme* and *F. proliferatum* had similar NOIs relative to *F. graminearum* at 25-30° and 0.96-0.994 a_w, although at 30° and 0.96 a_w tested *F. graminearum* had much lower NOIs than the fumonisin-producing species.

No direct correlation was found between NOI and I_D. Generally, interactions between *F. moniliforme* and other fungi suggests that against *P. citrinum* and *A. flavus* a high I_D score and a high NOI score were obtained. However, with *A. ochraceus* and *F. proliferatum* there was a low I_D and a high NOI; and with *A. niger* there was a low I_D and NOI. Interactions of *F. proliferatum* with other fungi showed similar results.

DISCUSSION

In interspecific interactions between fungi, ecological factors will exert selection pressure on the mycota, influencing the dominance of species (Magan & Lacey, 1985; Ramakrishna, 1990). No studies have been reported of the impact that changing environmental factors have on such interactions, and the level of niche overlap. Elegant studies of *A. flavus* and other maize fungi *in vitro* and on single grains demonstrated the influence of these interactions on aflatoxin production by *A. flavus* (Wicklow *et al.*, 1980; Wicklow, 1988). Abiotic factors, which are important both pre- and post-harvest, were not examined. For example, between silking and harvest, ripening maize kernels have an initial water content of between 40-50% (a_w = 1.0). That is reduced to between 25-20% (a_w = 0.95-0.90) as the kernels ripen (Inglett, 1970). Thus, 10-20 d exist when water availability conditions would favour colonisation, and perhaps metabolite production, by these *Fusarium* spp. over the other fungi tested. Marín *et al.* (1996) have shown that both *F. moniliforme* and *F. proliferatum* germinate rapidly over a wide range of a_w and temperature. Higher than desirable moisture content at harvest could extend the time available for *Fusarium* growth and colonisation.

This study has shown that *F. moniliforme* and *F. proliferatum* are able to dominate several other common maize contaminating fungi over a wide range of temperature and water availability conditions. Based on I_D values, the isolate of *F. proliferatum* was more dominant than that of *F. moniliforme*, particularly at 0.994 and 0.98 a_w. Direct interactions between the two fumonisin-producing species at 0.96-0.94 a_w resulted in mutual antagonism. Pairings of *F. moniliforme* and *F. proliferatum* with *F. graminearum* resulted in mutually antagonistic interactions upon contact, regardless of a_w level. Studies on pre-harvest inoculated maize have shown that viable counts and infection of kernels by *F. graminearum* declined and *F. moniliforme* increased in mid-season (Miller, Young & Trenholm, 1983; Al-

Heeti, 1987). There are also reports that *F. moniliforme* may repress the growth of other colonisers of ripening maize (Rheeder, Marasas & van Wyk, 1990*a*).

Interspecific interactions between fungi have previously been studied *in vitro* in relation to the micro-ecology of spoilage fungi of wheat, barley and maize (Wicklow *et al.*, 1980; Magan & Lacey, 1984; Ramakrishna, 1990), but the relationship between competitiveness against other spoilage fungi of fumonisin-producing strains of *F. moniliforme* and *F. proliferatum* has not previously been examined. Fumonisins are secondary metabolites; this suggests that where *Fusarium* is present as an endotroph, production of fumonisins may be more important for their function in retaining a niche than they are for acquiring the niche initially but, where contamination comes from leaves or soil, establishment may be helped by mycotoxin production. We found no direct relationship between growth rate of the individual species and their competitiveness. Thus at lowered a_w conditions some *Penicillium* spp. were dominant even though they grew more slowly. Magan & Lacey (1984, 1985, 1988) obtained similar results with the slow growing *P. brevicompactum* the most dominant species both *in vitro* and on sterile wheat grain. This dominance may have been due in part to its ability to produce mycophenolic acid, which may have improved competitiveness and enabled *P. brevicompactum* to exclude other fungi.

Ecological similarity and coexistence between microbial species usually has been examined in relation to the suitability of biological control agents for control of pathogens on plant surfaces (Wilson & Lindow, 1994*a*, *b*). The impact of changing temperature or water availability on the NOI has not been considered. In this study the emphasis was on finding evidence to explain why *F. moniliforme* and *F. proliferatum* are such competitive fungi. We obtained NOIs using the criteria of Wilson & Lindow (1994*a*) for each species in a pair. In our study, *P. citrinum* utilised the widest range of maize-related carbon sources followed by *A. ochraceus*, *P. aurantiogriseum*, *A. flavus* and *Fusarium* spp. The strain of *F. proliferatum* used in this study used more C-sources than *F. moniliforme*, but had a narrower overlap with other species, suggesting that *F. proliferatum* might be more competitive because it can assimilate uncommon C-sources (C-sources which are assimilated by only one of the fungi in a pair). The total and common C-sources utilised by each fungus was markedly influenced by both water availability and temperature. This implies that the amount of niche overlap changes with environmental conditions.

The comparison of NOI data suggests that *F. graminearum* at 15° may be at a competitive advantage over *F. moniliforme* and *F. proliferatum* and that it may utilise resources that they can not. By contrast, at 25-30°, these *Fusarium* spp. appear to coexist in the same niche.

Of the two fumonisin-producing species the isolate of *F. proliferatum* appears to be more competitive than *F. moniliforme* over a wide range of temperature and a_w conditions. Blaney *et al.* (1986) and Rheeder *et al.* (1990*a*, *b*) found negative correlation between the isolation of *F. moniliforme* and *F. graminearum* suggesting that colonisation by *F. moniliforme* may prevent other pathogens such as *F. graminearum* from colonising the same maize kernels. The NOI data also show clearly that *P. citrinum* usually has lower NOI ratios when compared to the *Fusarium* spp., while the latter have high NOI ratios, indicative of occupation of different size niches; *P. citrinum* is able to occupy the same niche as the *Fusarium* spp. in terms of C-sources and in addition has the capability of assimilating some more uncommon sources. This suggests that *P. citrinum* could be at an ecological

advantage. On the other hand, NOI data show how *Fusarium* species have low NOI values while *A. niger* and *P. aurantiogriseum* have high NOIs; compared to I_D values these results suggest that *A. niger* has a different competing strategy, probably metabolite production. Finally *F. graminearum*, *A. flavus* and *A.ochraceus* share their niches with fumonisin-producing *Fusarium* species, and thus compete for the same sources.

Because glycerol is one of the C-sources in the Biolog screen we substituted NaCl for glycerol to modify the water availability conditions of the test. This may not have been ideal but the results suggest that the NOIs are markedly modified by environmental factors. These need to be taken into account for a realistic examination of ecological similarity, niche differentiation and the potential for pre-emptive exclusion which has not been considered before. It may be possible to improve the tests by using C-sources closer to the natural substrates (Wilson & Lindow, 1994*a*). Indeed, Lawlor (1980) has suggested that measures of species similarity that are independent of resource abundance may reflect the evolutionary divergence of resource utilisation patterns due to past competitive pressures. Although consumer-environment interactions have been recognised as important determinants of overlap and similarity between species, only rarely have they been examined in detail.

In this study, we used two approaches to examine interspecific interactions that may occur between maize colonising fungi. There was no general correlation between I_D and NOI. I_D depended more on a_w and temperature than on NOI, suggesting that some factor other than C-source is influencing competitiveness. Competitive ability can probably be attributed to a combination of several factors including growth rate, metabolite production, niche overlap, and interactions with environmental factors. The most important conclusion to be drawn from our data is that the types of interactions observed and the range of C-sources utilised was variable and was dependent upon both temperature and a_w level. This type of information is critical for understanding the competitive abilities of these fungi and their potential for niche overlap and pre-emptive exclusion under economically important environmental conditions.

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Escola Tècnica Superior d'Enginyeria Agrària

ECOFISIOLOGÍA DE CEPAS DE *FUSARIUM* PRODUCTORAS DE FUMONISINAS

ECOPHYSIOLOGY OF FUMONISIN-PRODUCING ISOLATES OF FUSARIUM

Tesi doctoral
Sònia Marín Sillué
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X

COLONIZATION OF MAIZE GRAIN BY *FUSARIUM MONILIFORME* AND *FUSARIUM PROLIFERATUM* IN THE PRESENCE OF COMPETING FUNGI AND THEIR IMPACT ON FUMONISIN PRODUCTION

S. Marín[1], V. Sanchis[1], F. Rull[1], A. J. Ramos[1] And N. Magan[2]

[1]Food Technology Dept., Lleida University, CeRTA, Rovira Roure 177, 25198 Lleida, Spain.
[2]Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford, MK43 OAL, U.K.

ABSTRACT

This study was carried out to determine the effect of water activity (a_w) and temperature on the patterns of colonization of maize grain by isolates of *F. moniliforme* and *F. proliferatum* in the presence of interacting spoilage fungi such as *Aspergillus flavus*, *A. niger*, *A. ochraceus* and *Penicillium implicatum* over four week incubation periods. The impact that such interactions have on *Fusarium* infection of maize grain, populations, and on the production of fumonisins were all evaluated. At 0.93-0.95 a_w, interactions of the two *Fusarium* spp. with other species to a large extent resulted in mutual inhibition on contact or overgrowth by the *Aspergillus* and *Penicillium* spp. regardless of temperature and growth rates of the species. Seed infection by the *Fusarium* spp. at 25°C was influenced mainly by *A. flavus* and *A. niger* with a reduction of infection by up to 45 and 30%, respectively, after two weeks. At 15°C and lowered a_w levels, infection was reduced and sometimes completely inhibited by *A. niger*, *A. ochraceus* and *P. implicatum*. By contrast, populations of the *Fusarium* spp. (CFUs g^{-1} grain) were reduced mainly by *A. flavus* and *P. implicatum*. *A. niger* reduced populations of the *Fusarium* spp. at 0.93 and 0.95 a_w, particularly after 4 weeks incubation. Interaction with *A. niger*, *A. ochraceus* and *A. flavus* at 15 and 25°C and 0.98 a_w resulted in a significant stimulation in fumonisin production by both *Fusarium* spp. Interaction with *P. implicatum* resulted in a decrease in fumonisin production by *F. moniliforme*. However, interaction with *F. proliferatum* resulted in an enhanced production at both temperatures and 0.95 and 0.98 a_w. This study has shown that fungal interactions may act as an additional control factor in *Fusarium* spp. development on grain. However, although interactions lead to a decreased colonization by *Fusarium*, mainly at lower a_w, a decrease in fumonisin production accumulation does not occur at the same time. Knowledge of these interactions is essential for improving effective control procedures for preventing growth and mycotoxin production by such fungi.
Keywords: *Fusarium*, grain, interactions, mycotoxins, fumonisins

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INTRODUCTION

Fusarium moniliforme and *F. proliferatum* are important colonizers of maize with the ability to produce fumonisins and other mycotoxins. Their presence in stored grain can significantly decrease quality and economic value of the harvested grain. Fumonisins have been shown to be involved in leukoencephalomalacia in equine species *(25, 26)*, associated with pulmonary edema syndrome in swine *(30)* and implicated in esophageal cancer in humans *(3, 27)*.

Fusarium species do not however colonize maize grain in isolation. To become established they need to compete effectively against other colonizers including a range of *Aspergillus* and *Penicillium* spp. Previous studies have detailed the important influence of water activity (a_w) and temperature on the ability of these *Fusarium* species to germinate, grow and produce fumonisins *in vitro* and on maize grain *(1, 2, 9, 13, 14, 15)*. To understand the reasons why *Fusarium* spp. are able to sometimes dominate the maize grain ecosystem an understanding is required of the complex interactions which occur between biotic and abiotic factors and their impacts on growth and interactions between the *Fusarium* spp. and other interacting fungi and their influence on mycotoxin production.

The important influence of environmental factors on fungal interactions has been demonstrated in a range of ecosystems *(10, 11, 16, 17, 20, 21, 22, 28)*. Recent work by Ramakrishna *et al. (21, 22)* showed that a_w and temperature had a significant effect on fungal interactions between *F. sporotrichioides* and *P. verrucosum* and competing mycoflora on barley grain, and T-2 toxin and ochratoxin A formation, respectively.

However, studies on fungal interactions involving *Fusarium* Liseola species are very limited. Wicklow *et al. (29)* found a negative correlation between *A. flavus* and *F. moniliforme* in kernel infection in studies carried out before fumonisins had been discovered. More recently, Yoshizawa *et al. (31)* reported a negative relationship between aflatoxin and fumonisin concentration in Thai maize samples. Marín *et al. (16)* in detailed studies demonstrated for the first time that fumonisin producing strains of *F. proliferatum* and *F. moniliforme* were very competitive against a range of other maize colonizers over a range of environmental conditions *in vitro*. Niche overlap indices (NOI) based on utilization patterns of carbon/nitrogen sources found in maize showed that *F. proliferatum* was more competitive than *F. moniliforme*, and that depending on a_w and temperature different niches were occupied by other species such as *A. niger* and *P. aurantiogriseum (16)*. However, the effect of such fungal interactions on growth and fumonisin production on maize has not previously been examined in detail.

The objective of the present work was to determine the effect that a range of common maize colonists (*A. niger, A. flavus, A. ochraceus* and *P. implicatum*) have on *F. moniliforme* and *F. proliferatum* development on irradiated maize grain under different a_w and temperature levels in relation to (i) growth rate, (ii) hyphal interactions, (iii) seed infection, (iv) populations of *Fusarium* spp. based on colony forming units (CFUs), and (v) on temporal accumulations of fumonisins in the maize grain.

MATERIAL AND METHODS

Fungal isolates

Single isolates of six different species were used in this study: *Fusarium moniliforme* Sheldon (25N), *F. proliferatum* (Matsushima) Nirenberg (73N), *Aspergillus niger* van Tieghem (3.37), *A. ochraceus* Wilhem (NRRL 3174), *A. flavus* Link (3.36), and *Penicillium implicatum* Biourge (3.204). The *Fusarium* species were isolated from maize, while the other species are common contaminants of maize in Spain *(24)*, but they were isolated from different substrates. The *Fusarium* isolates used in the present study have previously been shown to be very high fumonisin producers in culture and on maize grain *(14)*. All the strains used in the study are mantained in the Food Technology Department fungus collection of the University of Lleida, Spain.

Grain preparation and a_w

Spanish dent maize grain was irradiated with 12 kGrays of gamma irradiation and stored aseptically at 4°C. The grain contained no fungal infection or contamination but had retained germinative capacity. The initial water content of the grain was 13.9% (=0.71 a_w).

For all experiments, irradiated maize was weighed into sterile flasks and rehydrated to the desired treatment a_w levels (0.93, 0.95 and 0.98) by addition of sterile destilled water. The amount of water added was calculated from a moisture adsorption curve for the grain. The grain treatments were allowed to equilibrate at 4°C for 48 hours, with periodic shaking. Finally, the a_w values were confirmed by using a Novasina Humidat IC I Thermoconstanter.

Inoculation, incubation and growth assessment

Rehydrated maize was placed in sterile Petri plates (20g/plate, approximately) forming a single layer of grains *(14)*. A 5-mm diameter agar disk was taken from the margin of a 5 day-old growing colony of each isolate on malt extract agar and transferred to the center of each plate. After that, plates containing grain at the same a_w were placed in sealed containers along with beakers containing glycerol-water solutions of the same a_w as the plates in order to create an atmosphere with a same equilibrium relative humidity (E.R.H.). Containers were incubated at 15 and 25°C. All treatments were repeated three times.

Every day during the incubation period growing colonies were measured with the aid of a binocular magnifier. Two diameters were obtained from each colony; then, growth rates (GR) expressed as mm d^{-1} were calculated by linear regression of colony radius against time for each strain at each set of conditions tested.

Inoculation, incubation and assessment of indices of dominance

As in the previous section, rehydrated maize was placed in sterile Petri plates (20g/plate, approximately) forming a single layer of grains. A 5-mm diameter agar disk from each isolate of the pair tested for interaction were placed on the grain 4.4 cm apart. After that,

plates containing grain at the same a_w were placed in sealed containers and incubated as described above. All treatments were repeated three times.

Periodically, growing colonies were observed macroscopically and the type of interactions occurring assessed using the categories given by Magan *et al. (10)*, subsequently scores were given to each type of interaction and added to obtain a so-called Index of Dominance. The scores were based on mutual intermingling (1/1), mutual inhibition on contact (2/2), mutual inhibition at a distance (3/3), dominance on contact (4/0), and dominance at a distance (5/0). In the last two cases the former score is for the dominating species and the second for the dominated. These scores were then added for each species individually to obtain a total Index of Dominance under different environmental conditions.

Inoculation, incubation, population changes, and percent kernel infection during interaction under different a_w × temperature conditions

Flasks containing 75 g of rehydrated maize were inoculated with 1 ml of one of the spore suspensions described below; this volume had already been subtracted from the initial amount of water added for the rehydration to maintain the grain a_w. Spore suspensions consisted of (i) a single inoculum of *F. moniliforme* or *F. proliferatum* (2×10^6 spores ml^{-1}), or (ii) a mixture (1+1) of a *Fusarium* species and one of the other isolates paired (4×10^6 spores ml^{-1}), or (iii) a mixture (1+1) of both *Fusarium* species (4×10^6 spores ml^{-1}). Inoculum was homogeneously spread by vigorous shaking of the flasks and then maize was placed in sterile Petri plates (approx. 25g/plate). After this, plates containing grain of the same a_w were placed in sealed containers and incubated as described previously. Incubation periods were for 2 and 4 weeks at 15 and 25°C. All experimental treatments were repeated three times.

After incubation, plates were analyzed for fungal populations (CFU g^{-1}) by serial dilution plating using both MEA (malt extract agar) and MEA-salt (10% NaCl) as enumerating media. *Fusarium* were counted on plates bearing between 5 and 150 colonies.

Furthermore, seed infection was assessed by directly plating 30 grains surface desinfected with 2% sodium hypochlorite for 2 min, onto MEA and MEA-salt. Percentages of grains infected by *Fusarium* species were determined.

The remaining grains of the 4-week-old treatments were frozen at -20°C for later fumonisin analysis.

Fumonisin concentration determination

Extraction of samples. Molded maize samples were ground in a mill. A 2-g subsample was mixed with 30 ml of methanol/distilled water (3+1) for extraction and stirred for 20min on a magnetic stirrer. To separate the solid components, the stirred sample was centrifuged for 15 min at 3000 rpm. The supernatant was filtered through a paper filter and the filtrate was diluted 1:7.5 with PBS (0.55 g $NaH_2PO_4 \cdot H_2O$; 2.85 g $Na_2HPO_4 \cdot 2H_2O$, 8.7 g NaCl; add 1000 ml distilled water). This dilute filtrate was diluted again at least 1:3 with methanol/PBS (1+9) and was employed in the test. Higher contaminated samples had to be diluted more

with methanol/PBS. Extracts were frozen and stored until analyses using an ELISA test kit (Ridascreen® Fumonisin Fast, R-Biopharm GmbH, Darmstadt, Germany).

Enzyme immunoassay procedure. The competitive enzyme immunoassay for the quantitative analysis of fumonisins Ridascreen® Fumonisin Fast was used, and the instructions given by the manufacturer for the development of the test strictly followed. The results of the analysis were obtained photometrically at 450 nm using a Titertek Multiskan PLUS.MK II microtiter spectrophotometer. The specifity of the test has been determined by analysing the cross-reactivities to corresponding substances: Fumonisin B_1 (100%), fumonisin B_2 (40%), fumonisin B_3 (100%). According to the sample preparation procedure, the detection limit for fumonisins is 0.1 $\mu g\ g^{-1}$. The recovery rate in spiked maize meal samples is 60%.

Determination of fumonisin concentration in samples. The values calculated for the standards were entered in a system of co-ordinates on semilogarithmic axis against the fumonisin concentration in ppb. The calibration curve should be virtually linear in the 1-125 ppb range (R^2 = 0.977-0.986, in our case). The fumonisin concentration corresponding to each sample was obtained by interpolating in the calibration curve. After that, concentrations were multiplied by the corresponding factor of dilution, and finally divided by dry matter value corresponding to each sample. Dry matter was determined by drying samples at 105°C for 17 h *(6)*.

Statistical analyses of the data

Analysis of variance were made for colony radius after 8 days, CFUs g^{-1}, seed infection, and fumonisin concentration by using SAS program version 6.11 (SAS Institute, Inc.), and LSD tests performed when needed. CFU data were transformed prior to analysis by [1], while % of infection were transformed by [2] to homogenize variance. Finally, correlation analyses were carried out between the different variables, in order to give a clearer explanation of which aspects of fungal interactions are expressed by each one.

[1] $$y = sin^{-1}\sqrt{\frac{\%infection}{100}}$$

[2] $$y = \log(CFUg^{-1})$$

RESULTS

Growth rates of *Fusarium spp.*

Generally, growth rate was increased from 0.93 to 0.98 a_w at both temperatures. Furthermore, on irradiated maize grain the *Fusarium* species grew faster at 25°C than at 15°C (Table 1). Maximum growth rates of about 3.6 and 3.5 mm d^{-1} were obtained for F. *moniliforme* and *F. proliferatum* at 0.98 a_w and 25°C. *F. proliferatum* grew faster than *F. moniliforme* at lower a_w levels (0.93 and 0.95). All single factors, as well as their two- and three-way interactions, had a significant effect on colony radius ($P < 0.01$).

Table 1. Effect of water activity and temperature on growth rates and Indices of dominance (I_D).

Temp. (°C)	a_w	Growth rate (mm d^{-1})	I_D of *F. moniliforme* paired with					
			A. niger	*A. ochr.*	*A. flavus*	*P. implic.*	*F. prolif.*	**Total**
15	0.93 a_w	0.387	2	0	2	2	2	**8**
	0.95 a_w	1.121	2	2	2	2	2	**10**
	0.98 a_w	1.780	2	4	4	4	0	**14**
25	0.93 a_w	1.411	0	2	0	0	0	**2**
	0.95 a_w	2.165	2	0	2	0	0	**4**
	0.98 a_w	3.589	2	2	2	4	0	**10**
		Total	**10**	**10**	**12**	**12**	**4**	**48**
Temp. (°C)	a_w	Growth rate (mm d^{-1})	I_D of *F. proliferatum* paired with					
			A. niger	*A. ochr.*	*A. flavus*	*P. implic.*	*F. monil.*	**Total**
15	0.93 a_w	0.523	2	0	2	2	2	**8**
	0.95 a_w	1.276	2	2	2	2	2	**10**
	0.98 a_w	1.663	2	4	4	4	4	**18**
25	0.93 a_w	1.666	0	2	2	0	4	**8**
	0.95 a_w	2.531	2	0	0	0	4	**6**
	0.98 a_w	3.488	2	4	2	4	4	**16**
		Total	**10**	**12**	**12**	**12**	**20**	**66**

Effects of fungal interactions with other fungi on colonization of maize grain by *F. moniliforme* and *F. proliferatum*.

Indices of Dominance: Growth of both *F. moniliforme* and *F. proliferatum* was inhibited in a similar way when their hyphae met those of other interacting species (Table 1). At 25°C, mutual inhibition on contact, and inhibition and overgrowth by other species were the most common reactions observed. However, at 15°C mutual inhibition on contact was the most common reaction at 0.93-0.95 a_w. Interestingly, 0.98 a_w/15°C was the only treatment condition at which the two *Fusarium* spp. overgrew the other competing species, with the exception of *A. niger*. Furthermore, *F. proliferatum* was able to dominate and overgrow *F. moniliforme* at 25°C regardless of a_w level, and at 15°C/0.98 a_w, while at 0.95-0.93 a_w they mutually inhibited each other in contact.

Seed infection: The percentage of maize grains infected by *F. moniliforme* and *F. proliferatum* when growing in pure culture or when interacting with each other or other competing species is shown in Fig. 1. Interspecific interaction between *F. moniliforme* and *F. proliferatum* had no influence on the percentage maize infected by either species. In the presence of *Aspergillus* spp. no effect was observed at 15°C on the percentage (%) grain infected by *Fusarium moniliforme*. However, after 4 weeks and lowered a_w levels (0.93 and

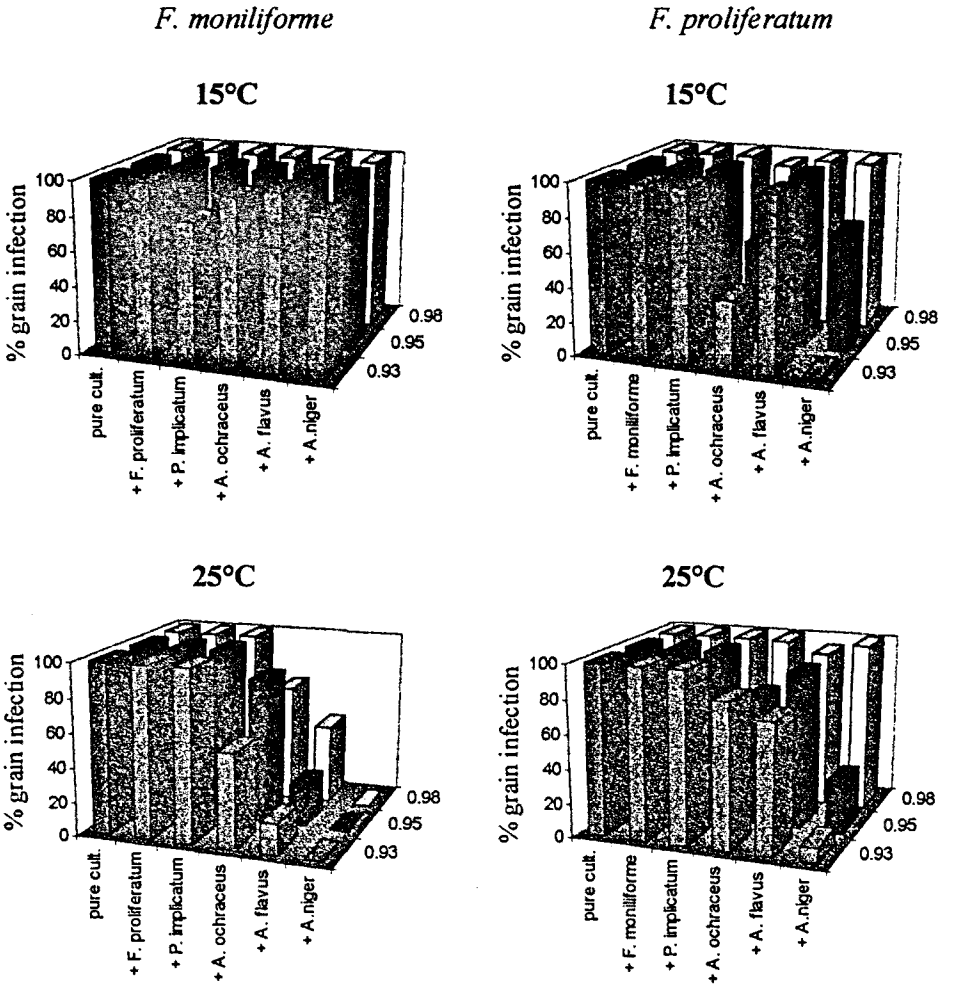

Figure 1. Influence of water activity and temperature on *Fusarium* spp. grain infection (%) in the presence of competing mycoflora after a 4-weeks incubation period.

0.95) lower percentage of maize grains were infected by the *Fusarium* spp. in the presence of *A. niger*, *A. ochraceus* and *P. implicatum*.

At 25°C, colonization by *A. flavus* and *A. niger* markedly decreased the percentage infection of maize grains by both *Fusarium* spp. *A. niger* reduced colonization of *F. moniliforme* in grain by > 30% after 2 weeks, and by 100% after 4 weeks. The effect of interactions between the *Aspergillus* spp. and *F. proliferatum* was smaller. The effect of *A. flavus* also depended on the *Fusarium* species, with greater inhibition of *F. moniliforme* (53-87% reduction) than *F. proliferatum* (7-75% reduction). The activity of *A. ochraceus* only

affected *Fusarium* infection after 4 weeks, with *F. moniliforme* infection of grain being reduced (15-45%) more than that of *F. proliferatum.* In general, colonization by *P. implicatum* did not affect the ability of the two *Fusarium* spp. to colonize maize grain.

No differences were found between 2- and 4-weeks incubation period, while other single factors (a_w, temperature, and species paired), and most of their two-, three- and four-way interactions were significant ($P<0.01$).

Colony forming units: Because the populations of individual species (CFU g^{-1} grain) were significantly higher when using MEA than MEA-salt media, all results are presented on populations isolated on the former medium only. All single factors (a_w, temperature, incubation period, and species paired), as well as their two-, three- and four-way interactions were significant ($P<0.01$). In pure culture, more CFUs of *F. moniliforme* and *F. proliferatum* were present on maize from the 0.98 a_w than at 0.95 and 0.93 treatments, from 25 rather than 15°C and after four rather than two weeks incubation (Fig. 2). Interactions between *F. moniliforme* and *F. proliferatum* resulted in populations of the former species being significantly more reduced than those of *F. moniliforme*, particularly at 15°C.

Both *P. implicatum* and *A. flavus* markedly influenced the isolation of *Fusarium* spp. populations from maize at 25°C. *P. implicatum* markedly decreased the number of CFU g^{-1} grain under almost all the conditions tested, but mainly, at 25°C (16-37% log CFU reduction of *F. moniliforme*) regardless of a_w. *A. flavus* colonization also significantly reduced the population of *F. moniliforme* and *F. proliferatum* isolated from the maize grain, particularly at 25°C.

The influence of *A. niger* on populations of *Fusarium* spp. was dependent on a_w, affecting total numbers particularly at 0.93-0.95 a_w, but less so 0.98 a_w. The impact was also much greater at 25° than at 15°C. Similarly, decrease of *Fusarium* colonization when interacting with *A. ochraceus* was also important at 0.93-0.95 a_w, and more marked after 4 weeks incubation. However, it significantly decreased populations of *F. proliferatum,* but not those of *F. moniliforme.*

Effects of fungal interactions on fumonisin production by *F. moniliforme* and *F. proliferatum*

Fumonisin production by *F. moniliforme* and *F. proliferatum* in maize grain, when growing alone or paired with other species in the different environmental treatments are shown in Fig. 3. All single factors (a_w, temperature and species paired) were significant ($P<0.01$), as well as their two- and three-way interactions.

In the absence of competition: At 15°C *F. moniliforme* produced increasing amounts of fumonisin with increasing a_w. In contrast, at 25°C fumonisin concentration decreased with increasing a_w. *F. proliferatum* produced considerably smaller amounts of fumonisins when compared to *F. moniliforme* with maximum concentrations found at 15°C and 0.95 a_w. For *F. moniliforme*, production at 25°C decreased with increasing a_w.

In the presence of *A. niger*: In general, fumonisin production by *Fusarium* was inhibited by competition with *A. niger*. However, at 15°C and 0.98 a_w, this production by *F.*

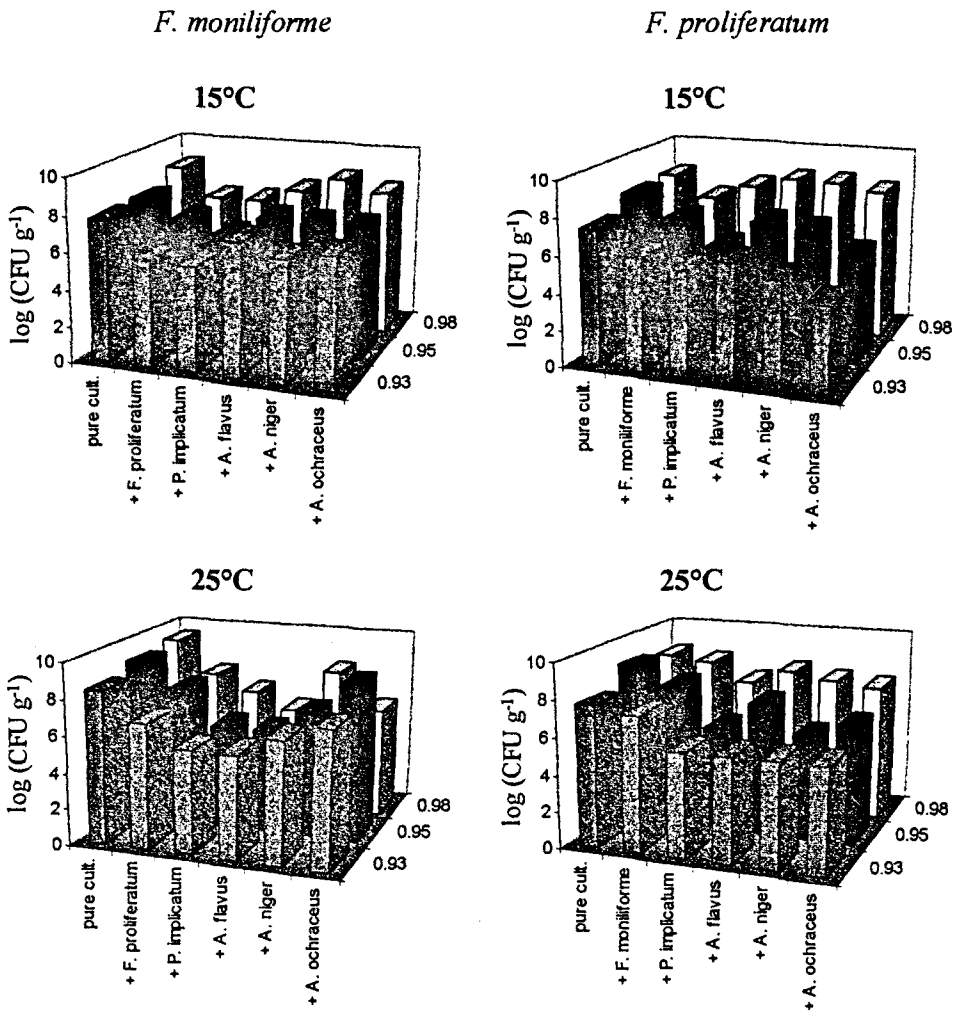

Figure 2. Influence of water activity and temperature on *Fusarium* spp. populations on maize grain (CFU g^{-1}) in the presence of different species after a 4-weeks incubation period.

proliferatum was significantly enhanced. Interaction with *F. moniliforme* resulted in a similar effect at 0.98 a_w and both temperatures.

In the presence of *A. ochraceus*: Production of fumonisins by *F. moniliforme* was stimulated by interaction with *A. ochraceus* at 0.98 a_w and both temperatures, and at 0.95 a_w at 25°C. Fumonisin production by *F. proliferatum* was also enhanced at 0.98 a_w at 15°C. Under all other test conditions fumonisin production was decreased due to fungal competition with *A. ochraceus*.

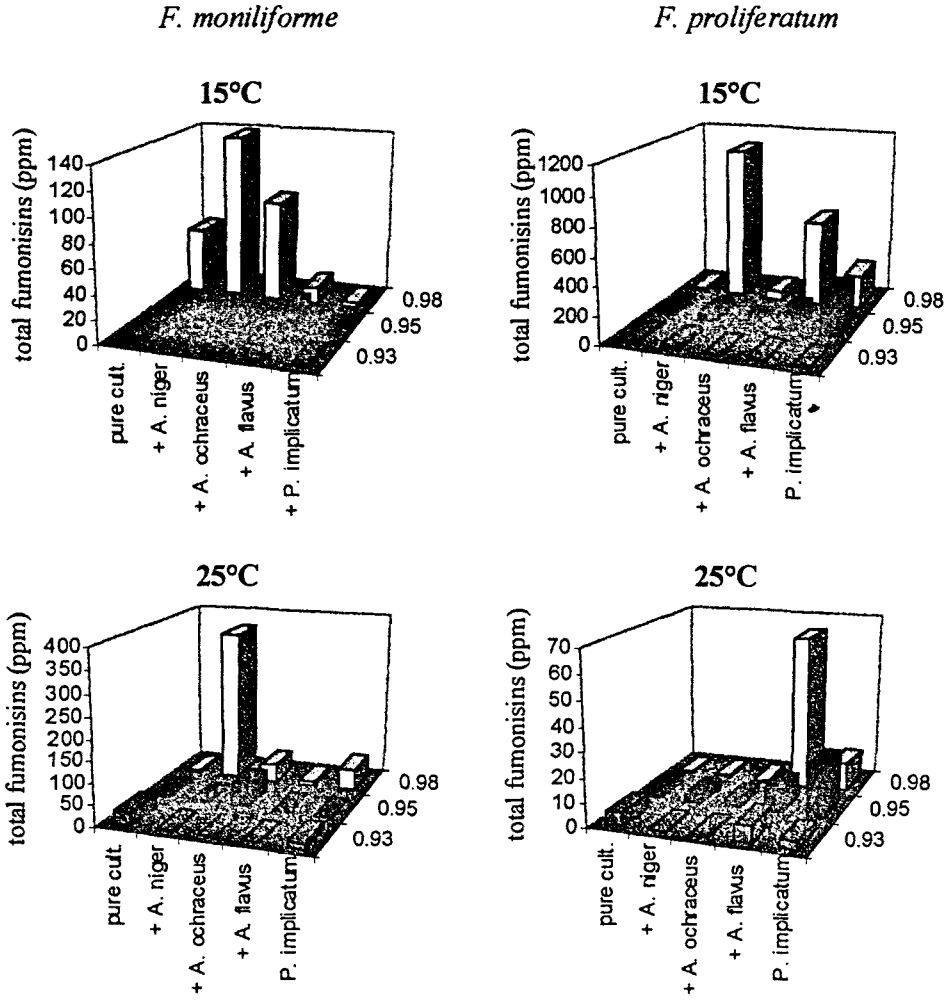

Figure 3. Influence of water activity and temperature on production of fumonisin B_1 by *Fusarium* spp. on maize grain (ppm) in the presence of competing mycoflora after a 4-weeks incubation period.

In the presence of *A. flavus*: With *F. proliferatum* fumonisin production was stimulated by interaction with *A. flavus* under all the conditions tested except for 0.93 a_w/15°C. By contrast, interaction between *F. moniliforme* and *A. flavus* resulted in a higher fumonisin concentration at 15°C and 0.93-0.95 a_w, and 25°C at 0.98 a_w.

In the presence of *P. implicatum*: Fumonisin production by *F. moniliforme* was decreased under all the conditions except for 0.98 a_w at 25°C, while interaction with *F. proliferatum* resulted in fumonisin production being enhanced at 0.95-0.98 a_w and both temperatures.

Effect of interaction between *F. moniliforme* and *F. proliferatum* on fumonisin production: In general, a less than additive fumonisin concentration was found when both *Fusarium* spp. competed for the same maize grain niche when compared to pure cultures of each species (Fig. 4). Interestingly, under specific environmental conditions (0.95, 0.98 a_w and 25°C) the amount of fumonisin produced by the species interacting on maize grain resulted in a synergistic increase in concentrations produced.

Correlation analyses

Table 2 shows an extensive Pearson correlation coefficient analyses carried out for *F. moniliforme* and *F. proliferatum* paired with competing species, between data accumulated in this study and that obtained from a previous study *(16)*. Niche Overlap Indices were based on the common C-sources assimilated by both interacting species paired/total C-sources assimilated by an interacting species *(16)*. The Niche Overlap Indices, growth rates, Index of Dominance, CFU, seed infection and fumonisin production were all analyzed.

The fungal populations (CFU g^{-1} grain) in paired cultures correlated well with fumonisin production in most of the cases. Moreover, correlation between Indices of Dominance and CFUs in mixed cultures was found in some cases, but in general Index of Dominance did not correlate with other factors. Neither Niche Overlap Indices (NOI) nor seed infection levels (SI) correlated with other factors in mixed cultures.

In contrast, correlation with pure cultures showed that growth rates correlated well with fungal populations (CFUs) for both *F. moniliforme* (0.872*) and *F. proliferatum* (0.722*), while there was a negative correlation between growth rate and fumonisin production which was significant for *F. proliferatum* (-0.669*) but not for *F. moniliforme* (-0.017).

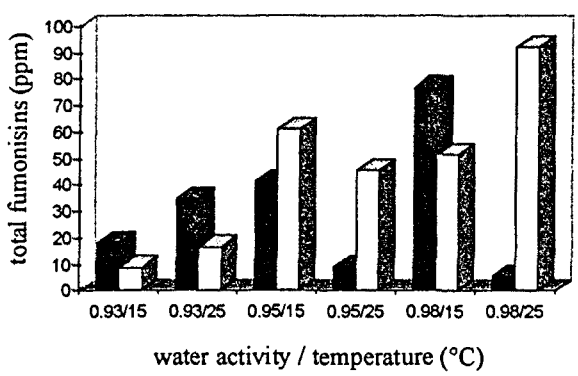

Figure 4. Influence of water activity and temperature on differences between total fumonisin production by *F. moniliforme* and *F. proliferatum* growing separately (■) and when co-cultured (□).

Table 2. Pearson correlation coefficients among growth rate (GR), Index of Dominance (I_D), CFU, seed infection (SI), Niche Overlap Indices (NOI) and fumonisin production (F) for *Fusarium* species growing in paired cultures.

	F. moniliforme paired with					*F. proliferatum* paired with				
	A. niger	*A. ochraceus*	*A. flavus*	*P. implic.*	*F. proliferatum*	*A. niger*	*A. ochraceus*	*A. flavus*	*P. implic.*	*F. moniliforme*
NOI *vs* I_D	0.112	0.523	0.135	-	0.112	-0.125	0.511	0.009	-	0.894*
NOI *vs* CFU	0.675*	0.404	-0.026	-	0.675*	0.377	0.437	0.485	-	0.877*
NOI *vs* SI	-0.431	-0.082	-0.303	-	-0.431		0.252	-0.229	-	-0.287
NOI *vs* GR	0.830*	0.579*	0.600*	-	0.830*	0.899*	0.613*	0.664*	-	0.780*
NOI *vs* F	0.499*	0.305	-0.104	-	-	0.117	0.101	0.306	-	-
I_D *vs* CFU	0.328	0.056	0.777*	0.811*	0.328	0.241	0.655*	0.524	0.783*	0.774*
I_D *vs* SI	0.488	0.141	0.629*	0.328	0.488		0.495	0.519	-	-0.245
I_D *vs* GR	0.149	0.236	0.107	0.368	0.149	0.092	0.453	-0.268	0.206	0.721*
I_D *vs* F	0.280	0.505	0.594*	0.474	-	0.212	0.527	0.802*	0.381	-
CFUm *vs* SI	-0.002	0.215	0.795*	0.315	-0.003		0.549	0.461	-	-0.183
CFUm *vs* GR	0.728*	0.332	-0.267	0.646*	0.728*	0.299	0.559	0.375	0.382	0.779*
CFUm *vs* F	0.789*	0.409	0.737*	0.627*	-	0.718*	0.723*	0.727*	0.288	-
SI *vs* GR	-0.589*	-0.241	-0.556	0.314	-0.589*		0.382	-0.543	-	0.071
SI *vs* F	-0.275	0.134	0.872*	0.086	-		0.229	0.458	-	-
GR *vs* F	0.846*	0.512	-0.179	0.825*	-	-0.112	-0.087	-0.065	-0.274	-

*Significant correlation with a 95% confidence level.

DISCUSSION

This study is the first detailed examination of fungal interactions of species of the *Fusarium* Liseola group with other spoilage fungi on maize and the impact that this may have on fumonisin production under different interacting environmental conditions. In our study, colonization of maize by *Fusarium* spp. was clearly inhibited to varying degrees by *Aspergillus* and *Penicillium* species at 25°C depending on the a_w and the species tested. Recently Ramakrishna *et al. (21, 22)* reported the effects that fungal competition had on *Fusarium sporotrichioides* colonization of barley grain and T-2 toxin formation. Seed infection and populations of *F. sporotrichioides* were inhibited or dominated to a greater or lesser extent by the presence of *A. flavus*, *Penicillium verrucosum* or *Hyphopichia burtonii* at 0.90-0.97 a_w levels and 20-30°C. However, as *F. sporotrichioides* produced very sparse hyphal colonization than the other competing species, it is likely that it competed poorly on the grain surface and was rapidly overgrown.

The present study has demonstrated how important environmental factors are in determining the rates and levels of fungal colonization of grain. This supports previous *in vitro* results and those on temperate cereal grains *(8, 12)*. The profound influence that a_w and temperature have on the dominance of different species in the maize grain niche correlates with previous *in vitro* studies with these same fungi *(16)*. Earlier work has shown that *F. moniliforme* and *F. proliferatum* were dominated by several other common maize contaminating fungi at <0.96 a_w *(16)*. Based on Index of Dominance, *F. proliferatum* was more dominant than *F. moniliforme*, particularly at 0.994 and 0.98 a_w. Wicklow *et al. (29)* found that common fungal colonists of maize kernels interfered with the ability of *A. flavus* to infect maize preharvest. *F. moniliforme* was particularly effective in inhibiting kernel infection of *A. flavus*. However, effects of a_w and of *A. flavus* on *F. moniliforme* infection was not reported.

Previous *in vitro* studies on the Niche Overlap Indices *(16)* reflected that *F. proliferatum* might be more competitive because it was able to assimilate a number of uncommon C-sources. The studies also showed that the *Fusarium* spp. species occupied wider niches than *A. niger* and *P. aurantiogriseum*, while *F. graminearum*, *A. flavus* and *A. ochraceus* shared their niches with fumonisin-producing *Fusarium* species, and thus often competed for the same sources.

Although infection of maize grain by the competing *Fusarium* species was mutually antagonistic at 0.93-0.95 a_w, the results based on the populations isolated from the grain suggested that at 15°C *F. proliferatum* competes better than *F. moniliforme*. Previous studies have demonstrated that *F. proliferatum* is more adapted to low temperatures *(15)*. A significant negative association was demonstrated between *F. moniliforme* and *F. graminearum* in maize grain in South Africa by Rheeder *et al. (23)*. Elegant studies of intra-strain competition between *F. moniliforme* strains within maize plants showed that the percentage of kernels infected with a particular strain was lower in multiple-inoculation treatments than in the single strain inoculation treatments *(18)*. It is common for maize plants to be infected with more than one strain of *F. moniliforme* and other *Fusarium* species; consequently multiple pathways are probably functioning in these plants and the strains are competing for colonization of the kernels and other tissues.

Although fumonisin production was expected to be inhibited by fungal competition, quite often interactions led to a significant increase in fumonisin concentration, mainly at high water availability (0.98 a_w). In previous studies with *F. sporotrichioides* in barley it was found that patterns of T-2 toxin production changed with environmental conditions, species and with time *(21)*. For some of the conditions tested (a_w, temperature, time), T-2 toxin production in the presence of *A. flavus* and *P. verrucosum* was higher than that in pure culture. On maize, zearalenone production by *F. graminearum* was markedly decreased in paired culture with *A. flavus* at 16°C but not at 25°C *(4)*.

Interesting studies by Yoshizawa *et al. (31)* on Thai maize, showed that fumonisins were present in visually healthy maize as well as moldy maize. Surprisingly, there was no significant difference in levels of these toxins between the two sample groups. Samples contaminated with high concentrations of fumonisins (1000 ng g^{-1}) contained low levels of aflatoxins. They demonstrated a negative relationship between these two groups of toxins suggesting that interactions between fumonisin and aflatoxin-producers had occurred. These studies were based on naturally contaminated samples and thus the a_w or temperature regimes of the maize grain were unfortunately not known.

In the present study there was a negative correlation between growth rate and fumonisin production when *F. proliferatum* colonized the maize grain alone. In general, in interactions between the *Fusarium* spp. and other spoilage fungi the populations of mixed of cultures (CFUs g^{-1} grain) and fumonisin concentration correlated well, while relative grain infection levels did not. Colony forming units arise almost entirely from spores. Thus, heavily sporulating species will be overestimated and less abundantly sporulating species underestimated *(7)*. Previous studies by Magan *et al. (11)* in experiments with field and storage fungi of wheat grains found that often when few colonies grew on dilution plates many grains still yielded these fungi. In contrast, Ramakrishna *et al. (21)* found a decrease in colonization by *F. sporotrichioides* in mixed fungal cultures, when compared with growth in pure culture, whether measured as seed infection or populations (CFUs). This generally led to decreased T-2 toxin production. Other studies by Ramakrishna *et al. (22)* also showed that with *P. verrucosum* seed infection or fungal populations gave little indication of ochratoxin production in barley grain in paired cultures.

Cahagnier *et al. (2)* reported on the temporal fumonisin production by *F. moniliforme* on autoclaved maize. The concentrations that they obtained with French isolates were much higher than those obtained in the present study with irradiated maize. The reason could be that autoclaving significantly modifies maize nutritional status and its structure than irradiation, enabling more rapid colonization to occur. Indeed, O'Neill *et al. (19)* reported that different amounts of deoxynivalenol, acetyl deoxynivalenol and zearalenone were produced in fresh, irradiated and autoclaved maize. The lowest mycotoxin production was observed on non-sterile maize, followed by that on irradiated maize, and then on autoclaved maize. This may be an important consideration when extrapolating results to the field situation.

This work has shown that although growth rate and number of CFUs are probably useful measures to assess fungal colonization by *Fusarium* spp. in maize grain, they do not correlate properly in mixed cultures. The ability to grow fast and sporulate abundantly are both important for occupation of an ecological niche *(10, 20)*. Two aspects of competition must be taken into account: primary resource capture and combat *(5)*. Prolific production of

spores, quick germination of these, possession of appropppriate extracellular enzymes, and high growth rates allow species to succeed in primary resource capture. However, indices of dominance reflect the ability of a species for combat and can be important when the density of the initial fungal inoculum is high. However, in naturally contaminated maize the presence of other spoilage fungi and their relative colonization will significantly influence the production of fumonisins by *Fusarium* spp. Thus, if production of fumonisins is a defense reaction by these *Fusarium* spp. to exclude other competing fungi, then understanding these interactions is critical to interpret the patterns of natural contamination of maize grain, pre- and post-harvest. Production of fumonisins and other mycotoxins by spoilage fungi may thus be in a constant state of flux being impacted by a range of interacting biotic and abiotic factors simultaneously.

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In memory of the work of Dr. John Lacey (1937-1998)

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XI

COLONISATION AND COMPETITIVENESS OF *ASPERGILLUS* AND *PENICILLIUM* SPECIES ON MAIZE GRAIN IN THE PRESENCE OF *FUSARIUM MONILIFORME* AND *FUSARIUM PROLIFERATUM*

S. Marín[1], V. Sanchis[1], F. Arnau[1], A.J. Ramos[1] and N. Magan[2]

[1]Food Technology Dept., CeRTA, Universitat de Lleida, Rovira Roure 177, 25198 Lleida, Spain
[2]Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, UK.

ABSTRACT

The effects of different steady-state water activity levels (a_w, 0.93, 0.95 and 0.98) and temperature (15 and 25°C) on colonisation patterns of *Aspergillus* and *Penicillium* spp., when colonising irradiated maize grain in the presence of *Fusarium moniliforme* and *Fusarium proliferatum* were assayed in terms of populations (colony forming units, CFUs g grain^{-1}), seed infection and colonisation rates. The activity of *F. moniliforme* and *F. proliferatum* in grain reduced the presence of *Aspergillus flavus, Aspergillus niger, Aspergillus ochraceus* to some extent, particularly at 15°C and higher water availabilities (0.95-0.98 a_w). In contrast, colonisation patterns of *Penicillium implicatum* on maize grain were unaffected by either *Fusarium* spp. in terms of CFUs or seed infection. Correlations were made between CFUs, seed infection, growth rates and niche overlap indices and hyphal interactions to try and link key indicators of competitiveness and dominance by an individual species.

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INTRODUCTION

Maize grain is colonised by a mixture of spoilage fungi pre- and postharvest. The dominant species depends on a number of abiotic and biotic factors. Of particular importance are the prevailing water availability and temperature conditions which determine the dominance of groups of fungi in the maize grain ecosystem. In recent years *Fusarium moniliforme* and *F. proliferatum* have attracted much interest because of their competitive ability and the production of the fumonisin group of mycotoxins (Cawood et al., 1991). Detailed studies have previously shown that isolates of these two species germinate, grow and produce fumonisins over a wide range of environmental conditions (Marín et al., 1995a; 1995b; 1996). Recently, Marín et al. (1998a) also demonstrated that these *Fusarium* species compete effectively and can exclude other species over a range of water activities and temperatures *in vitro*. However, few studies have examined the reasons why under certain conditions these Fusaria are competitive, while under others they may be dominated by other *Aspergillus*, *Eurotium* and *Penicillium* spp. The competing strategies of each species when co-cultured on maize grain have not been examined in detail previously and may help understand the conditions under which these groups of species successfully dominate, particularly in the stored maize grain ecosystem.

Different approaches to fungal competition have been used before, such as development of an Index of dominance (I_D) values (Magan and Lacey, 1984; 1985; Cuero, et al., 1987; Wheeler and Hocking, 1993; Marín et al., 1998b) and comparison of growth rates (Magan and Lacey, 1984; 1985; Cuero et al., 1987; Whipps and Magan, 1987; Ramakrishna et al., 1993; 1996a; 1996b). More recently, Ramakrishna et al. (1996a; 1996b) assessed the effects of fungal competition in terms of the population structures and levels of seed infection of barley grain to explain interactions between fungi in grain and their impact on mycotoxin production. Percentage of seed infection has also been frequently used as a measure of fungal interference (Cuero et al., 1987; Wicklow et al., 1988; Rheeder et al., 1990).

However, very little work has been done on the impact that the presence of *Fusarium moniliforme* or *F. proliferatum* may have on the occurrence and development of other common maize fungi (Wicklow et al., 1988; Rheeder et al., 1990; Marín et al., 1998a; 1998b). *F. moniliforme* has been demonstrated to act as an inhibitor of *A. flavus* and infection by other *Fusarium* spp. (Wicklow et al., 1988; Rheeder et al., 1990). Indeed, Yoshizawa (1996) reported a negative correlation between levels of fumonisins and aflatoxins in Thai corn. It has been suggested that the production of fumonisins by Fusaria of the Section Liseola might give them an advantage in order to outcompete other fungal colonisers of maize grain (Marín et al., 1995a).

The objective of this study was to investigate the impact of *F. moniliforme* and *F. proliferatum* on the development of common co-colonisers such as *A. niger*, *A. flavus*, *A. ochraceus* and *P. implicatum* under different environmental conditions, by using four different criteria, (a) relative growth rates, (b) types of hyphal reactions, (c) population structure, and (d) percentage maize kernel infection.

MATERIAL AND METHODS

Fungal isolates

Isolates of six different species were used in this study: *Fusarium moniliforme* Sheldon, *F. proliferatum* (Matsushima) Nirenberg, *Aspergillus niger* van Tieghem, *A. ochraceus* Wilhem, *A. flavus* Link, and *Penicillium implicatum* Biourge. All species were isolated from maize and are common contaminants in Spain (Sala, 1993). All the isolates used are held in the Food Technology Department culture collection of the University of Lleida.

Grain

Spanish maize grain was irradiated with 12 kGrays of gamma irradiation and stored at 4°C. The grain contained no fungal infection or contamination but had retained germinative capacity. The initial water content and water activity (a_w) of the grain were 13.9% and 0.71, respectively.

For the experiments, irradiated maize was weighed in sterile flasks and rehydrated to the desired treatment a_w levels (0.93, 0.95 and 0.98) by addition of sterile distilled water. The amount of water added was calculated from a moisture adsorption curve for the grain. The grain treatments were allowed to equilibrate at 4°C for 48 hours, with periodic shaking. Finally, the a_w values were confirmed by using a Novasina Thermoconstanter TH200, Axair Ltd. Systems for Air Treatment, Pfäffikon, Switzerland.

Inoculation, incubation and growth assessment

Rehydrated maize was placed in 9-cm diameter sterile Petri plates (Bibby Sterilin Ltd., Stone, Staffs, U.K.) (20g/plate, approximately) forming a single layer of grains. Then a 5-mm diameter agar disk was taken from the margin of a 5-days old growing colony of each isolate on malt extract agar at 25°C and transferred to the centre of each plate. After that, plates containing grain at the same a_w were placed in sealed containers with beakers of glycerol-water solutions of the same a_w as the treatments in order to maintain the correct equilibrium relative humidity. Containers were incubated at 15 and 25°C. All treatments were repeated three times.

Every day during the incubation period growing colonies were measured with the aid of a binocular magnifier (Leica, Z45E, Leica Inc., Buffalo, U.S.A). Two diameters were obtained from each colony; then, growth rates (mm d^{-1}) were calculated by linear regression of colony radius *versus* time for each strain at each set of conditions tested. Then, relative growth rates were calculated as follows:

$$\text{relative growth rate} = \frac{\text{growth rate isolate}}{\text{growth rate Fusarium spp.}}$$

Interactions between species on maize grain

Under the same treatment conditions detailed above, pairs of species were inoculated as 5-mm diameter agar plugs of each species placed on the grain layer 4.4-cm apart. Treatments were incubated as described previously, and the experiment repeated three times.

Periodically, growing colonies were observed macroscopically and the type of interaction assessed using a modified method of Magan and Lacey (1984). Their scores were based on intermingling (1), mutual inhibition on contact (2/2), mutual inhibition at a distance (3/3), dominance on contact (4/0), and dominance at a distance (5/0). The latter score was for the antagonized species. These scores were then added for each species individually to obtain an overall Index of Dominance (I_D). Instead of 5 categories, we only devised two types of categories as all interactions were either mutual antagonism on contact, or inhibition of one species and the inhibited species being overgrown. Numerical scores were given with both fungi being given 0 in the former category, and 1 or -1 for the dominant, and inhibited species in the latter category.

Fungal populations in mixed inoculation experiments and percentage infection of maize kernels

Flasks containing 75 g of rehydrated maize were inoculated with 1 ml of one of the spore suspensions described below; this volume was initially subtracted from the amount of water added to rehydrate the irradiated maize treatments. Spores were harvested from the surface of a 14-days old colony of each isolate grown on malt extract agar at 25°C, and suspended in 0.01% tween 80 sterile destilled water (1-2 drops in 1000ml water). Spore suspensions consisted of a single inoculum of each strain (2×10^6 spores/ml) or a mixture (1+1) of a *Fusarium* species and one of the other isolates tested (4×10^6 spores/ml). Inoculum was homogeneously spread by vigorous shaking of the flasks and then maize was placed in sterile Petri plates (25g/plate, approximately). After that, plates containing grain at the same a_w were placed in closed containers and incubated as described previously. Incubation periods were 2 and 4 weeks. All treatments were repeated three times.

After incubation, plates were destructively sampled and analysed for CFU g^{-1} by dilution plating using both Malt extract agar (MEA; 20 g malt extract; 20 g glucose; 1 g peptone; 1000 ml destilled water; pH=5.5) and low a_w Malt salt agar (MSA; 20 g malt extract; 20 g glucose; 10 g NaCl; 1 g peptone; 1000 ml destilled water; pH=5.5) as enumerating media. Peptone saline (8.5 g NaCl, 1 g peptone, 1000ml destilled water, pH=5.5) was used as diluent, and homogenization of samples was carried out by using an Stomacher Lab-Blender 400 (BA 6021, Seward Medical UAC House, London, U.K.). After incubation at 25°C, plates bearing between 5 and 150 colony forming units (CFUs) were enumerated for populations of individual *Aspergillus* and *Penicillium* species.

Thirty maize kernels were taken from each sample and assayed by direct plating on both MEA and MSA, after 2-min. surface desinfection with 2% sodium hypochlorite (2 ml NaClO in 100 ml solution). The percentage (%) maize kernels infected by *Aspergillus* and *Penicillium* species were determined.

Statistical analyses of the data

Analysis of variance were made for colony radius after 8 days, CFUs g^{-1} and % of infection, by using SAS program version 6.11 (SAS Institute, Inc., Cary, N.C., U.S.A.). CFU data were transformed prior analysis by [1], while % of infection were transformed by [2]. Same software was used to obtain Pearson correlation coefficients and thus try to correlate the different approaches to fungal competition accumulated in this study and that obtained from a previuo study (Niche Overlap Indices, NOIU) Marín et al., 1998b). Niche Overlap Indices were based on the common C-sources assimilated by both interacting species paired/total C-sources assimilated by an interacting species..

$$[1]\quad y = \log(CFUg^{-1})$$

$$[2]\quad y = sin^{-1}\sqrt{\frac{\%infection}{100}}$$

RESULTS

Differences in relative growth rates among species

All the species grew faster at 25 than 15°C, while in general growth rates increased with a_w, except for *P. implicatum* which showed faster growth at 0.95 a_w, and even a higher growth rate at 0.93 than at 0.98 a_w (data not shown).

At 25°C *Fusarium* species were the slowest, with a maximum growth rate of 3.59 mm d^{-1} at 0.98 a_w, while *P. implicatum* was the fastest with a maximum of 6.32 mm d^{-1} at 0.95 a_w. *A. niger* had the best growth rate of the *Aspergillus* species on irradiated maize grain (data not shown).

Interestingly, *Fusarium* species grew faster than the other species at 15°C and 0.95-0.98 a_w, with a maximum growth rate of 1.78 mm d^{-1} at 0.98 a_w. *A. flavus* had less ability to grow at the lower temperature (data not shown).

Analysis of variance showed that there were statistically significant ($P < 0.01$) differences in relation to a_w, temperature and species. In general, *P. implicatum* grew fastest, followed by *A. niger* and *A. ochraceus*, while there were little differences between the growth rates of *A. flavus* and the *Fusarium* species.

Table 1 shows the relative growth rates (RGR, growth rate of species/growth rate *Fusarium*) obtained by the four species tested under the different environmental conditions. Relative growth rates were in general <1 at 0.95-0.98 a_w at 15°C. *P. implicatum* had a RGR < 1 only at 0.98 a_w and 15°C. Interestingly only *A. ochraceus* had a value <1 at 25°C and 0.98 a_w, showing poor ability of *Fusarium* species to grow quickly when compared to the others. Similar RGR values were obtained in relative to both *F. moniliforme* and *F. proliferatum.*

Table 1. Effect of water activity and temperature on relative growth rates (growth rate species/growth rate *Fusarium*) of the species tested on irradiated maize grain.

Temp. (°C)	Water activity	*Aspergillus niger*	*Aspergillus ochraceus*	*Aspergillus flavus*	*Penicillium implicatum*
		with reference to *Fusarium moniliforme*			
15	0.93 a_w	1.44	1.75	0.44	2.00
	0.95 a_w	0.80	0.88	0.39	1.25
	0.98 a_w	0.83	0.82	0.45	0.56
25	0.93 a_w	2.36	2.07	1.76	3.69
	0.95 a_w	2.05	1.57	1.40	2.92
	0.98 a_w	1.34	0.86	1.42	1.21
		with reference to *Fusarium proliferatum*			
15	0.93 a_w	1.07	1.29	0.32	1.48
	0.95 a_w	0.70	0.77	0.34	1.10
	0.98 a_w	0.89	0.88	0.48	0.60
25	0.93 a_w	2.00	1.75	1.49	3.12
	0.95 a_w	1.75	1.34	1.19	2.49
	0.98 a_w	1.38	0.88	1.46	1.25

Hyphal interactions of *Aspergillus* and *Penicillium* species when paired with *Fusarium* species

Table 2 shows the scores obtained by *Aspergillus* and *Penicillium* species when paired with *F. moniliforme* and *F. proliferatum* under the different environmental treatments tested. In general, both *Aspergillus* spp. and *P. implicatum* were inhibited and often overgrown (-1) by both *Fusarium* species at 0.98 a_w. In contrast, at 0.93-0.95 a_w, they were either mutuallyinhibitory on contact (0) or dominant (1) against the *Fusarium* spp.

Both *Aspergillus* and *Penicillium* species behaved similarly, with *A. niger* and *A. ochraceus* least dominated at 15°C, while at 25°C the scores obtained by each species depended on water availability. There was no difference between the effect of *F. moniliforme* and that of *F. proliferatum* on the competing abilities of the species studied.

Effect of *Fusarium Liseola* section species on populations of *Aspergillus* and *Penicillium* species

The effect of the different media (MEA, MSA) used for enumeration showed that in general there was not significant difference (data not shown). Thus, results are only presented in this section based on MEA results. Table 3 shows how temperature (15 and 25°C), time (2 and 4 weeks), water activity (0.93, 0.95 and 0.98), and presence of *Fusarium* species (control, *F. moniliforme* and *F. proliferatum*) had a significant influence on the results obtained. Analysis of variance revealed that most of two-way interactions were also significant. The CFUs g^{-1} increased with temperature and a_w for all the species, except *A. ochraceus* which had a maximum at 0.93-0.95 a_w (4.2×10^7-9.8×10^8 CFU g^{-1}), and a minimum at 0.98 a_w (2.8×10^6-7.6×10^8 CFU g^{-1}).

Table 2. Effect of water activity and temperature on type of interactions and scores given to *Aspergillus* and *Penicillium* species when growing paired with *Fusarium moniliforme* and *Fusarium proliferatum*.

Temp. (°C)	Water activity	Aspergillus niger	Aspergillus ochraceus	Aspergillus flavus	Penicillium implicatum	Total
		paired with Fusarium moniliforme				
15	0.93 a_w	0	1	0	0	1
	0.95 a_w	0	0	0	0	0
	0.98 a_w	0	-1	-1	-1	-3
25	0.93 a_w	71	0	1	1	3
	0.95 a_w	0	1	0	1	2
	0.98 a_w	0	0	0	-1	-1
	Total	1	1	0	0	2
		Paired with Fusarium proliferatum				
15	0.93 a_w	0	1	0	0	1
	0.95 a_w	0	0	0	0	0
	0.98 a_w	0	-1	-1	-1	-3
25	0.93 a_w	1	0	0	1	2
	0.95 a_w	0	1	1	1	3
	0.98 a_w	0	-1	0	-1	-2
	Total	1	0	0	0	1
	TOTAL	2	1	0	0	3

Figure 1 shows the effect of the presence of *Fusarium* species on colonisation of grain by *Aspergillus* species and *P. implicatum* at different a_w and temperature levels. Although populations of fungi were significantly higher after 4 weeks than after 2 for all species, the trends observed for the level of inhibition were in general quite similar regardless of time of incubation (data not shown).

Colonisation of grain by *A. niger* based on CFUs g^{-1} grain was clearly inhibited by the presence of *Fusarium* species at 15°C at all a_w levels (5-40% reduction on a log basis), while the effect was less clear at 25°C. Similarly, *A. flavus* numbers were markedly inhibited at 15°C over the whole range of a_w (18-44% reduction), but not at 25°C.

In contrast, *F. moniliforme* significantly affected the development of *A. ochraceus* at 15 and 25°C, although the effect was more important at 15°C (24-48% of reduction). *F. proliferatum* also inhibited this species at both temperatures, but the effect was not as pronounced. At 25°C the inhibition was clearer at 0.98 a_w.

Finally, *P. implicatum* populations were least affected at 15°C (0-19% reduction), with similar results at 25°C. The maximum inhibition of this fungus was caused by *F. moniliforme* after 4 weeks incubation.

Overall, the impact of *Fusarium* species on other competing species was often more important at high a_w levels. *F. proliferatum* exerted similar or no inhibition at 0.93-0.95 a_w,

Table 3. Analysis of variance of the effect of temperature (T), time (t), water activity (a_w), and presence of *Fusarium* species (c), and their interactions on *Aspergillus* and *Penicillium* species populations on maize.

	Aspergillus flavus		*Aspergillus niger*		*Aspergillus ochraceus*		*Penicillium implicatum*	
FACTOR	MS	F	MS	F	MS	F	MS	F
C	18.83	266.08**	10.75	107.04**	43.85	699.69**	6.38	277.85**
T	207.11	2926.00**	90.81	903.98**	2.77	203.84**	2.21	96.24**
c × T	14.11	199.41**	15.34	152.74**	8.09	129.06**	0.02	0.89
t	2.44	34.53**	6.62	65.88**	8.11	129.38**	1.22	53.19**
c × t	0.82	11.66**	0.05	0.52	1.11	17.69**	1.38	60.26**
T × t	0.00	0.01	5.69	56.68**	3.77	60.15**	1.39	60.57**
c × T × t	0.16	2.28	0.98	9.81**	0.03	0.41	0.15	6.52**
a_w	2.19	30.89**	4.33	43.14**	3.89	62.10**	1.53	66.50**
c × a_w	3.08	43.48**	2.60	25.90**	4.93	78.69**	0.70	30.56**
T × a_w	0.26	3.68*	0.79	7.84**	9.60	153.18**	0.29	12.62**
c × T × a_w	0.19	2.68*	0.80	7.97**	1.02	16.30**	0.20	8.69**
t × a_w	0.00	0.07	2.68	26.67**	1.83	29.16**	4.45	193.74**
c × t × a_w	0.33	4.65**	0.34	3.36*	0.19	2.97*	0.69	30.18**
T × t × a_w	0.07	1.06	0.28	2.76	0.74	11.86**	0.33	14.60**
c × T xt × a_w	0.24	3.45*	0.32	3.16*	0.34	5.47**	0.06	2.40

*Significant $P<0.05$

**Significant $P<0.01$

but inhibited all the species except for *A. flavus*, at 0.98 a_w. In general, *F. moniliforme* inhibited the other competing species with increasing a_w.

Effect of *Fusarium Liseola* section species on kernel infection by *Aspergillus* and *Penicillium species*

Table 4 shows how temperature (15 and 25°C), time (2 and 4 weeks), water activity (0.93, 0.95 and 0.98), and the presence of *Fusarium* species (control, *F. moniliforme* and *F. proliferatum*) had a significant influence on the percentage isolation of *Aspergillus* and *Penicillium* species from irradiated maize grain. Grain infection was higher at 25°C than at 15°C, and isolation of *Aspergillus* and the *Penicillium* species decreased with increasing water availability. The effect of time was significant for *A. niger* and *A. ochraceus*, where infection was lower initially but increased after 4 weeks incubation (data not shown).

A. niger was not inhibited when grown in combination with the *Fusarium* spp. at 25°C (Figure 2), while it was inhibited at 15°C at 0.93-0.98 a_w, particularly at 0.98 a_w after 2 weeks (77% reduction) (data not shown). Similarly with *A. flavus*, it was not inhibition at 25°C, but at 15°C the *Fusarium* spp. significantly (58-100% reduction) inhibited infection over the whole a_w range tested.

In contrast, *A. ochraceus* was not competitive in the presence of the *Fusarium* spp. at 25°C and 0.98 a_w (76-100% reduction in infection), and at 15°C and 0.98 a_w. The effect of *F. moniliforme* was more marked than that of *F. proliferatum*. However, infection of grain by *P. implicatum*, was not affected by co-inoculation with the *Fusarium* species.

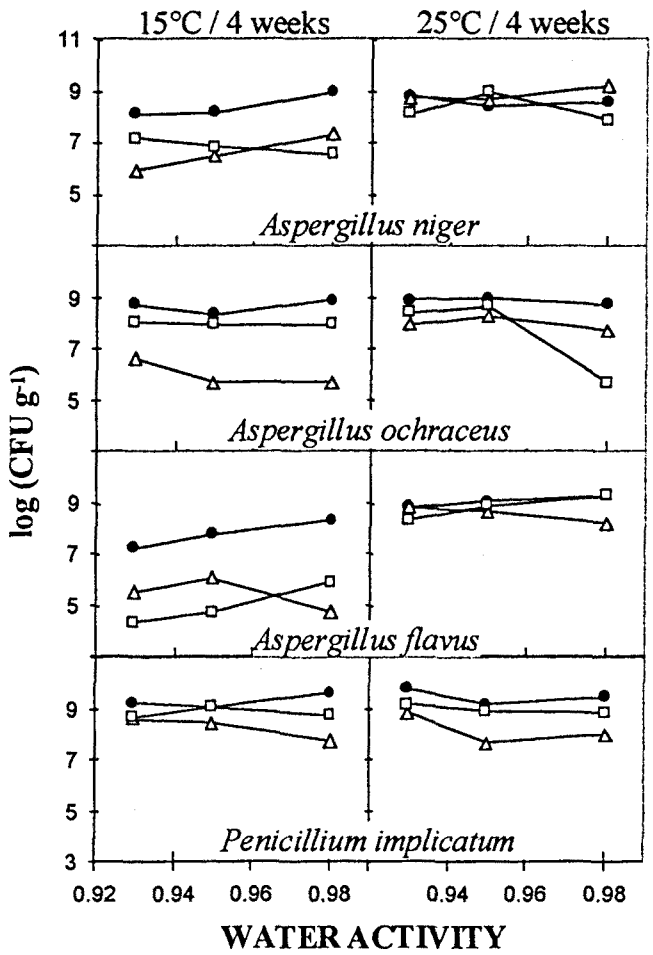

Figure 1. Effect of water activity and temperature on *Aspergillus niger, Aspergillus ochraceus, Aspergillus flavus* and *Penicillium implicatum* population (CFU g^{-1} irradiated maize) in pure culture (●), and in the presence of *Fusarium moniliforme* (△) and *Fusarium proliferatum* (□).

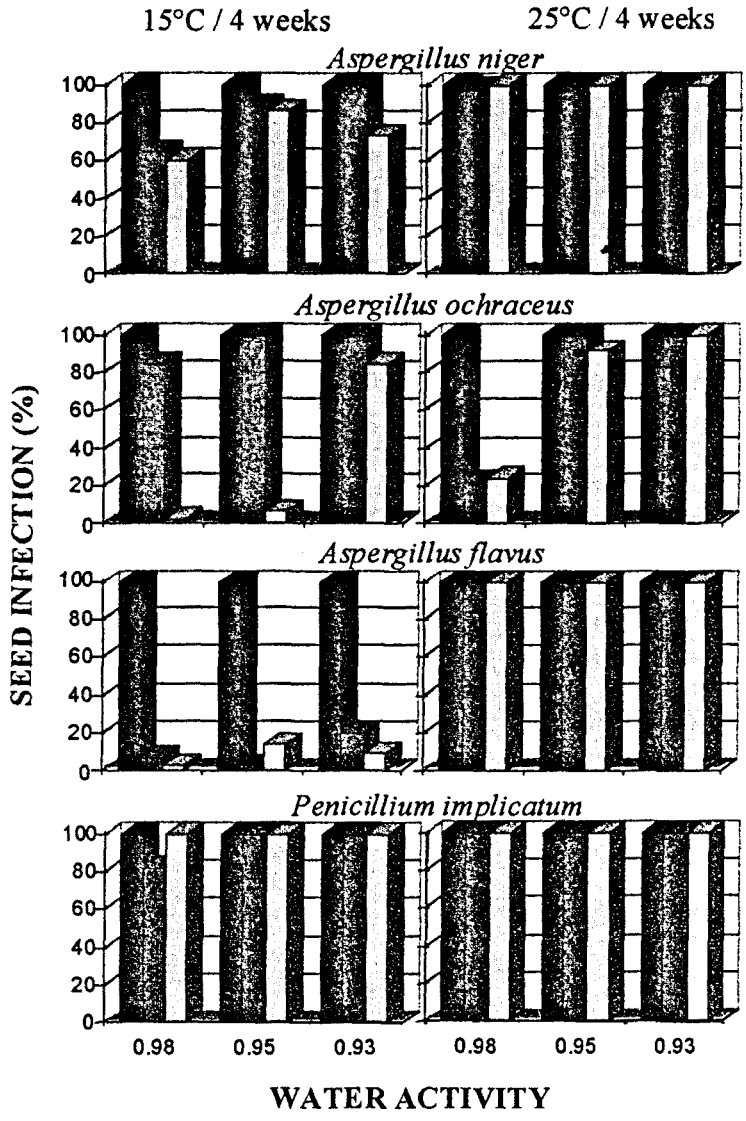

Figure 2. Effect of water activity and temperature on *Aspergillus niger, Aspergillus ochraceus, Aspergillus flavus* and *Penicillium implicatum* maize grain infection (%) in pure culture (■), and in the presence of *Fusarium moniliforme* (□) and *Fusarium proliferatum* (■).

Table 4. Analysis of variance of the effect of temperature (T), time (t), water activity (a_w), and presence of *Fusarium* species (c), and their interactions on *Aspergillus* and *Penicillium* species infection of maize kernels.

	Aspergillus flavus		**Aspergillus niger**		**Aspergillus ochraceus**		**Penicillium implicatum**	
FACTOR	MS	F	MS	F	MS	F	MS	F
c	3.83	582.03**	1.34	153.71**	4.46	274.12**	0.02	49.89**
T	15.30	2324.87**	1.34	153.71**	0.29	17.90**	0.02	49.89**
c × T	3.83	582.03**	1.34	153.71**	1.86	114.27**	0.02	49.89**
t	0.01	2.02	0.11	12.44**	0.12	7.63**	0.00	3.73
c × t	0.01	1.73	0.11	12.44**	0.06	3.53*	0.00	3.73*
T × t	0.01	2.02	0.11	12.44**	0.00	0.26	0.00	3.73
c × T × t	0.01	1.73	0.11	12.44**	0.03	1.74	0.00	3.73*
a_w	0.09	13.37**	0.05	5.24**	2.90	178.57**	0.02	49.89**
c × a_w	0.03	4.48**	0.05	5.24**	0.87	53.78**	0.02	49.89**
T × a_w	0.09	13.37**	0.05	5.24**	0.87	53.39**	0.02	49.89**
c × T × a_w	0.03	4.48**	0.05	5.24**	0.33	20.16**	0.02	49.89**
t × a_w	0.02	2.65	0.01	1.54	0.04	2.46	0.00	3.73*
c × t × a_w	0.02	2.38	0.01	1.54	0.02	1.57	0.00	3.73**
T × t × a_w	0.02	2.65	0.01	1.54	0.08	5.19**	0.00	3.73*
c × T ×t × a_w	0.02	2.38	0.01	1.54	0.04	2.56*	0.00	3.73**

*Significant $P<0.05$

**Significant $P<0.01$

Correlation between different competing criteria for determining competitive strategies

There was good correlation between growth rates of *A. niger* and *A. flavus* with CFUs and seed infection (SI) (Table 5). Correlation was also found between niche overlap indices (NOI) and growth rate (GR) for the *Aspergillus* species. Moreover the RGR often correlated well with hyphal reactions (HR), CFU and SI for *Aspergillus* species but not with the NOI. In general, no correlation was found between HR and CFU or SI; interestingly an inverse slight correlation was also found between HR and NOI. Moreover, good correlation was found between CFU and SI for the *Aspergillus* species. *P. implicatum* did not show much correlation between the parameters studied.

DISCUSION

This study has shown how the outcome of interactions between fungi on maize grain depends on environmental conditions and the types of species present. This study has indicated that a_w × temperature interactions have a profound effect on the potential for fumonisin-producing *Fusarium* spp. to exclude other *Aspergillus* and *Penicillium* spp. from

Table 5. Pearson correlation coefficients among growth rate (GR), relative growth rate (RGR), hyphal reaction scores (HR), CFU, seed infection (SI) and niche overlap index (NOI) for *Aspergillus* and *Penicillium* species.

	Aspergillus niger	*Aspergillus ochraceus*	*Aspergillus flavus*	*Penicillium implicatum*
GR *vs* NOI	0.464	0.646*	0.533*	-
GR *vs* HR	0.195	0.008	0.115	0.574*
GR *vs* CFU	0.911*	0.395	0.910*	0.252
GR *vs* SI	0.851*	0.145	0.953*	0.294
RGR *vs* NOI	0.004	-0.295	0.468	-
RGR *vs* HR	0.689*	0.539*	0.129	0.892*
RGR *vs* CFU	0.498	0.371	0.930*	0.026
RGR *vs* SI	0.752*	0.531*	0.966*	0.364
CFU *vs* NOI	0.619*	0.144	0.494	-
CFU *vs* HR	0.345	0.337	0.100	0.015
CFU *vs* SI	0.830*	0.861*	0.873*	-0.100
SI *vs* NOI	0.266	-0.059	0.282	-
SI *vs* HR	0.416	0.484	0.321	0.363
NOI *vs* HR	0.272	-0.589*	-0.496	-

*Significant correlation with a 95% confidence level.

its niche. At 0.98 a_w the *Fusarium* spp. were particularly competitive. Previously, studies by Blaney et al. (1986) found a negative correlation between the isolation frequencies of *F. graminearum* and *F. moniliforme* and attributed this to competition for substrate, production of antagonistic substances, or environmental conditions that differentially influenced corn ear infection by these two fungi. Furthermore, Rheeder et al. (1990) found negative correlation between the presence of *F. moniliforme* and other *Fusarium* species and also concluded that environmental conditions accounted for these effects. However, few studies have examined the interaction of the Fusaria with other common *Aspergillus* colonisers of maize grain. The experiments were carried out with differents *Aspergillus* species as indicator organisms. However, many *Aspergillus* species is particular *A. niger* and *A. flavus* have an optimum growth al 30-37°C (Marín et al., 1998c). The experiments were performed at 15 and 25°C. The possibility exists that the *Aspergillus* species are more competitive at their optimum temperature.

In our study no correlation was found between hyphal reactions and CFUs, grain infection or growth rate. Previously, Magan and Lacey (1985) found little correlation between Indices of Dominance obtained on agar medium and populations on grain assessed by both direct and dilution plating. Magan and Lacey (1984; 1985), Whipps and Magan (1987), Wheeler et al. (1993) and Marín et al. (1998a) all failed to find a direct relationship between growth rate of the individual species and their competitiveness in terms of Index of Dominance (I_D), both on agar and cereal grain substrata. Thus, for example, Marín et al. (1998a) found that at lowered a_w conditions some *Penicillium* spp. were dominant (I_D) even though they grew more slowly; interestingly the *P. implicatum* isolate had the fastest growth rates on maize grain of those examined in the present study. Whipps and Magan (1987) reported that the interactions (I_D)

changed markedly and were easily predicted from growth rates alone. All approaches, however, may be suitable for the determination of competitiveness. Two aspects of competition must betaken into account: primary resource capture and combat (Cooke and Whipps, 1993). Prolific production of spores, quick germination of these, possession of appropriate extracellular enzymes, and high growth rates allow species to succeed in primary resource capture. However, hyphal reactions reflect the ability of a species for combat and can be important when the density of the initial inoculum is high.

Moreover, in our study good correlation was found for some of the species between relative growth rate and hyphal reactions; this might mean that if one species is able to grow comparatively faster than another, then it will be able to overgrow the competitor when their hyphae meet. Thus the ability to grow faster might not, per se, be a guarantee of dominance, except where a wide difference in growth rates between species occurs. Interestingly, relative growth rates in general correlated well for *Aspergillus* species with CFU and SI which suggests that a species with a fast growth rate under certain environmental conditions is able to colonise a greater proportion of the grain ecosystem, and if competitive exclude other species with lower growth rates. Consequently RGR could in some circumstances be an easy measure to predict which species, e.g. *Aspergillus vs Fusarium* spp., might dominate the ecosystem under different environmental conditions.

In freshly harvested grain contaminant spores of individual species are likely to vary in concentration and spatially. In our studies this was not taken account of because of the complexity of the matrix of treatments required. However, low concentrations of spores of one species may under conducive environmental conditions to be outcompeted by more stress-tolerant and competitive species. Metabolites, perhaps including mycotoxins, produced in grain by either species may then either inhibit a competing species or it could be degraded and utilised. Overall, only two reaction types were observed in this study: Inhibition on contact (combative interaction) and overgrow (combative interaction with secondary resource capture). Growth rates of pure cultures on maize grain are probably a function of primary resource capture prior to any competitive interactions occurring. Thus, fungi in stored grain may form discrete 'mutually inhibited' colonies (Ramakrishna et al., 1993).

Interestingly, in the present study the *Aspergillus* spp. were inhibited by the *Fusarium* species under environmental conditions to which they were better adapted, e.g. 15°C and high a_w, in terms of growth rate, but not in terms of CFUs. This implies that the relative growth rate may be an important measure of the competing ability of a species. Heavy-sporulating species (*Aspergillus* and *Penicillium* spp.) are selected for in the dilution plating method at the expense of *Fusarium* species (especially *F. proliferatum*) producing wet spore droplets in a matrix. However, in our study the heavily sporulating P. *implicatum* was quite competitive not only in terms of CFU, but in infection percentage and growth rate.

Recent work has also examined the use of niche overlap index (NOI) as a measure of fungal competitiveness and niche exclusion under different environmental parameters (Marín et al., 1998b). The total and common C-sources utilised by each fungus was found to be markedly influenced by both a_w and temperature. This implies that the amount of niche overlap changes with environmental conditions. The strain of *F. proliferatum* used in that study used more C-sources than *F. moniliforme*, but had a narrower overlap with other species, suggesting that *F. proliferatum* might be more competitive. Again, there was no general

correlation between I_D and NOI. *A. niger*, *A. flavus*, *A. ochraceus*, *P. aurantiogriseum*, and *F. graminearum* were among the species which shared their niches with fumonisin-producing *Fusarium* species, and thus competed for the same sources, and consequently are likely to inhibit each other. In the present study only correlations between growth rate and NOI for some species was significant. An inverse correlation between hyphal reactions and NOI were obtained and could be explained by the fact that the more an individual species shares its niche with others the more likely it will be dominated by them.

This is the first time that impact of *F. proliferatum* on infection of irradiated maize grain by other fungal species has been shown and demonstrated to be similar to that of *F. moniliforme* depending on interacting *Aspergillus* or *Penicillium* spp. The impact of *F. moniliforme* sometimes resulted in interesting interactions, especially when paired with *A. ochraceus* in terms of CFU and grain infection, while there was no difference in hyphal reactions and RGR values. The study of Marín et al. (1998b) showed that *F. moniliforme* and *F. proliferatum* were able to dominate several other common maize contaminating fungi over a wide range of temperature and water availability conditions on maize extract agar. However, based on I_D values, this isolate of *F. proliferatum* was more dominant than that of *F. moniliforme*, particularly at 0.994 and 0.98 a_w.

Previously, Rheeder et al. (1990) pointed out that *F. moniliforme* may serve as a deterrent to kernel invasion by other seed-infecting fungi, with potential for use as a biocontrol agent. However, stable non-mycotoxigenic strains would have to be examined to try and control infection and fumonisin production by these Fusaria both pre- and post-harvest in maize as has been demonstrated for *A. flavus* and other species (Cole and Cotty, 1990).

Our results suggest that although *A. flavus* and *A. ochraceus* strains produce aflatoxins and ochratoxins, respectively, they were inhibited consistently by *F. moniliforme*. Previous work by Wicklow et al. (1988) indicated that common fungal colonists of maize kernels interfere with the ability of *A. flavus* to infect preharvest maize; *F. moniliforme* was particularly effective in inhibiting kernel infection by *A. flavus*. It has been suggested that kernels initially infected with *F. moniliforme* may be resistant to later infection by *A. flavus* if the *Fusarium* hyphae induce metabolic host resistance (e.g., papillae, cell wall thickening, phytoalexins). The presence of competing fungi may explain why some kernels with high levels of aflatoxin may be often located next to kernels that were toxin-free(Wicklow et al., 1988). *F. moniliforme* is capable of rapidly colonising wounded kernel tissues, thus reducing the resource nutrient pool available to fungi such as *A. flavus*, or may interfere in some other way with its ability to produce aflatoxin (Ullstrup, 1970).

P. implicatum remained unaffected by competition in terms of both seed-infection and number of CFU, while for *Aspergillus* species both CFU and percentage seed infection decreased, demonstrating a clear parallel in the decrease in colonisation by both *A. ochraceus* and *A. flavus*. Ramakrishna et al. (1996b) in a similar study with *P. verrucosum* identified 4 different patterns which depended upon the competing species involved: (i) the percent seed infection and CFU were unaffected by competition, as for *P. implicatum*; (ii) the percent seed infection and number of CFU increased initially more slowly during competition than in pure culture; (iii) seed infection and CFU were markedly decreased by competition, as shown for *Aspergillus* species in this study; and (iv) seed infection was markedly decreased but its sporulation was unaffected during competition. We found significant correlation between CFU and grain infection in *Aspergillus* species while not for

P. implicatum. They found significant correlation between CFU and seed infection for 20 and 30°C, and 0.90-0.95 a_w, but not at 0.97 a_w (Ramakrishna et al., 1996b).

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XII

CONTROL OF GROWTH AND FUMONISIN B_1 PRODUCTION BY *FUSARIUM MONILIFORME* AND *FUSARIUM PROLIFERATUM* ISOLATES IN MOIST MAIZE WITH PROPIONATE PRESERVATIVES

S. Marín[1], V. Sanchis[1], D. Sanz[1], I. Castel[1], A. J. Ramos[1], R. Canela[1], N. Magan[2]

[1]Food Technology Department, University of Lleida, CeRTA, Rovira Roure 177, 25198 Lleida, Spain
[2]Applied Mycology Group, Cranfield Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, UK

SUMMARY

The effect of propionic acid, its sodium salt or a commercial formulation of propionates (0.03, 0.05 and 0.07%), on growth and fumonisin B_1 production by *Fusarium moniliforme* and *F. proliferatum* isolates was evaluated on irradiated maize at different water activities (a_w, 0.93, 0.95, 0.98) and temperatures (15, 25°C). The four isolates grew at all a_w × temperature treatments in the absence of propionates. At the highest propionate concentration tested (0.07%), however, growth was restricted to 0.98 a_w, for *F. proliferatum* isolates but not for those of *F. moniliforme*. Inhibition of growth was maximum when propionates were added in the acid form. In the presence of low propionate concentrations (0.03%), growth was sometimes enhanced probably due to assimilation of these compounds by the fungus. Water activity, temperature, concentration and source of propionate, as well as most two-, three-, four-, and five-way interactions had a significant influence on growth of *Fusarium* isolates. None of the assayed treatments had any effect on fumonisin B_1 production by *F. moniliforme* isolates. For *F. proliferatum*, regardless of treatment, fumonisin concentration was higher when maize was incubed at 15°C than at 25°C. Higher fumonisin B_1 production occurred in the absence of propionates, and in general concentration decreased with increasing doses of preservatives. Single factors (a_w, propionate concentrations and temperature) and temperature × a_w and propionate concentration × temperature interactions had a significant effect on fumonisin production ($P<0.01$). Moreover, propionate concentration was the single most important factor, besides temperature, which affected fumonisin B_1 production.

Int. J. Food Microbiol. (in press)

INTRODUCTION

Maize grain is often harvested at a moisture content which is conducive to growth, colonization and mycotoxin production by a range of fungi, especially *Fusarium* species. If drying to safe moisture contents (14-15% = 0.70-0.73 a_w) is delayed or inefficient such fungi can rapidly colonize the grain depending on the prevailing equilibrium relative humidity and temperature during storage. Sometimes grain is treated with preservatives such as propionic and sorbic acids and their salts for controlling spoilage fungi in stored maize. However, it is critical that the dosage used should always take account of the initial grain moisture content (water availability), storage temperature, and level of initial inoculum, which all interact in the stored maize grain ecosystem.

Fusarium Liseola section species have been demonstrated to be important contaminants of maize in southern European countries and in North and South America (8, 9, 24, 30, 31, 32). The ability of a high percentage of isolates of both *F. moniliforme* and *F. proliferatum* to produce fumonisins, and other mycotoxins has been demonstrated (26). Recent studies have already detailed the environmental factors, including water activity (a_w) and temperature, over which isolates of these species are able to germinate, grow and produce fumonisins (18, 19, 20). However, no information is available on the effect of commonly used fungal preservatives on the development of *F. moniliforme* and *F. proliferatum* on maize and the potential for controlling fumonisin production. It is however important to note that the common aliphatic acid based preservatives are fungistatics only, and not fungicides. There thus needs to be good coverage and contact between the preservative and the grain surface for effective control (15). Thresholds of application are also critical as suboptimal concentrations have previously been demonstrated to be metabolized by spoilage fungi, in some cases stimulating growth and mycotoxin production (1, 15, 16, 17). However, in other cases their use post-harvest has been found to be effective for controlling the development of aflatoxigenic fungi (4, 6, 12) and aflatoxin production (2, 25).

It is important to recognize that there are complex interactions between environmental factors, grain type, oxygen availability and level of contaminant load which will all impact on the efficacy of preservatives for control of spoilage fungi and mycotoxin production. While some studies conducted with potassium sorbate have shown potential for storage of high-moisture corn (13), efficacy varied with corn moisture content, types of molds and storage conditions. However, potential for control of growth of *Fusarium* spp. and prevention of fumonisin production have not previously been evaluated.

The objectives of the present work were to investigate (a) the effectiveness of propionic acid, sodium propionate, and a commercial formulation of propionates for controlling growth of two fumonisin producing isolates each of *F. moniliforme* and *F. proliferatum* on irradiated maize grain as a function of water activity and temperature, (b) to examine ratios of propionates for potential synergistic effects, and (c) the potential for control of fumonisin B_1 production using the commercially available preservative mixture in maize grain.

MATERIALS AND METHODS

Fungal isolates

Four isolates belonging to the *Fusarium* Liseola section: two of *Fusarium moniliforme* Sheldon (25N, 85N) and two of *F. proliferatum* (Matsushima) Nirenberg (73N, 131N), were used in the experiments. These isolates were all isolated from maize and have previously been demonstrated to be high fumonisin-producers (26). All the isolates are held in the Food Technology Dept. Culture Collection of the University of Lleida, Spain.

Irradiated maize grain

Spanish dent maize grain was irradiated with 12 kGrays of gamma irradiation and stored asepticaly at 4°C. The grain contained no fungal infection or contamination but had retained germinative capacity. The initial water content of the grain was 13.9% (=0.71 a_w).

Preservatives

Propionic acid alone, sodium propionate, and a commercial mixture of propionates were used in these studies. The commercial mixture contained propionic acid, ammonium propionate, sodium propionate, and coadjuvants (1,2-propanediol monoesters and fatty acids). The preservatives were diluted to give the required concentration of propionates to the grain (0.03, 0.05 or 0.07%). Stock preservatives were all filtered through a 0.22 μm filter, and stored at 7°C.

Effect of preservatives, water activity and temperature on growth on irradiated maize

Sixty gram of irradiated maize were weighed into sterile 250 ml beakers and rehydrated to the required a_w (0.93, 0.95 and 0.98) by addition of sterile distilled water using a moisture absorption curve. A 0.5 ml aliquot of the water was replaced by a suitable propionate treatment (propionic acid, sodium propionate, or commercial mixture of propionates), to give the grain a final concentration of 0.03, 0.05, and 0.07 g propionate/100 g maize of each preservative. The mean pH of the maize grain treatments were 5.10, 5.33 and 5.49, at 0.93, 0.95 and 0.98 a_w, respectively. At the same time controls containing water only were also prepared. Flasks were subsequently refrigerated at 4°C for 48 h with periodic shaking to allow absorption and equilibration. The rehydrated maize was placed in sterile 9 cm Petri dishes forming thin layers of grains (= 20 g).

Plates were inoculated centrally with a 5-mm diameter agar disk taken from the growing margin of a 5-day old colony of each isolate grown on malt extract agar (MEA). Plates with the same a_w were enclosed in sealed plastic containers together with beakers of a glycerol-water solution at the same a_w as the plates, to maintain constant ERH inside the boxes. Two temperatures of incubation were assayed, 15 and 25°C, and the experiment was repeated three times.

To examine the effect of ratios of two preservatives, mixtures of propionic acid and sodium propionate were used in the ratio 0+100, 25+75, 50+50, 75+25 and 100+0, of each, respectively, using an overall concentration of 0.05 g propionate/100 g maize dose (0.05%). Table 1 shows the change in pH which occurred with treatment. In this study the same three a_w were assayed, but experiments only carried out at 25°C. The experiment was repeated three times.

Assessment of growth was made every day during the incubation period, with maize grain cultures being examined using a binocular magnifier, and two diameters of the growing colonies measured at right angles to each other until the colony reached the edge of the plate. The radius of the colonies were plotted against time, and a linear regression applied in order to obtain the growth rate as the slope of the line.

Inoculation and incubation of maize for fumonisin B_1 studies

Experiments were carried out as previously described at 0.93, 0.95 and 0.98 a_w, at 15 and 25°C but only with the commercial mixture of propionates at three treatment levels: 0, 0.03 and 0.07%. Beakers of each treatment were inoculated with 0.5 ml of a microconidial spore suspension (2.4×10^7 spores ml^{-1}) of each isolate, and shaken vigorously. The inoculated irradiated maize was placed in sterile Petri dishes (20 g per plate), and incubated as described previously. The experiment was repeated twice.

Fumonisin B_1 quantification

Samples were incubated for four weeks, and then frozen until extraction and analyses. They were extracted using a modification of the Shephard *et al.* method (28) as described by Sanchis *et al.* (27). After extraction, purified sample residue was dissolved in 0.5 ml of methanol. Two hundred µl of *o*-phtaldialdehyde (OPA) reagent, prepared according to Shephard et al. (28) were added to a 50 µl sample solution. Fifty µl of this solution were injected into the HPLC system within 2 min of derivatization. The eluent was methanol+0.1 M NaH_2PO_4 (75+25) adjusted to pH 3.35 with *o*-H_3PO_4. The flow rate was 0.8 ml min^{-1}. Reference standard of FB_1 was purchased from CSIRO, Division of Food Science and Technology, Pretoria, South Africa.

Simultaneously, a portion of each sample (10g) was dried in an oven at 105°C for 17 h (11), to determine the moisture content, and present fumonisin B_1 concentrations on a dry matter basis.

Table 1. Effect of different ratios of acid propionic/sodium propionate on pH of maize treated with a 0.05% dose under different water activity levels.

	ratios propionic acid + sodium propionate				
a_w	0+100	25+75	50+50	75+25	100+0
0.93	4.975	5.275	4.860	4.945	4.715
0.95	5.235	5.145	4.965	5.795	5.050
0.98	5.550	5.410	5.480	5.260	5.035

pH determination of treatments

60 g subsamples of maize, rehydrated and equilibrated after addition of preservatives were prepared for each treatment in duplicate as described in the previous sections. After equilibration, samples were ground and pH measured by using a pH electrode (Crison microp H2001).

Statistical analyses of the data

Analysis of variance were made for colony radius after 9 days, and fumonisin B_1 concentration by using SAS program version 6.12 (SAS Institute, Inc.). Finally, correlation analyses were carried out between both variables.

RESULTS

Effect of propionates on growth of isolates of *Fusarium* spp. in relation to a_w and temperature

In general, in the absence of preservative treatment *F. moniliforme* isolates grew slower (0.5 to 4.5 mm d^{-1}) than those of *F. proliferatum* (1 to 5 mm d^{-1}) over the a_w range tested, with isolate 73N of *F. proliferatum* colonizing the irradiated maize most rapidly. Growth was also faster at 25 than 15°C regardless of a_w. Overall, propionic acid alone was more efficient in controlling *Fusarium* growth, with the commercial mixture being more effective than sodium propionate alone over the time scales of our experiments. However, all three reduced growth rates when compared to controls (Fig. 1 and 2). Furthermore, growth was always inhibited more as propionate doses were increased. *F. proliferatum* isolates were much more sensitive to all three propionate treatments, with growth being completely inhibited at 0.93 a_w in the presence of 0.05 and 0.07% concentration. In general, none of the isolates and species were able to grow in the presence of 0.07% at 0.93 a_w with either the commercial mixture or sodium propionate. By using propionic acid alone no growth was found with 0.07% concentration at both 0.93 or 0.95 a_w. Reductions in growth rate of about 1 mm d^{-1} were achieved at 0.98 a_w, while these were approx. 2-3 mm d^{-1} at 0.93 a_w and 15 and 25°C, respectively.

Effects of a_w, temperature, time, preservative type, concentration, and two, three, four and five-way interactions on growth of the *Fusarium* isolates and species were all statistically significant, with a_w and temperature being the most important ones.

Effect of ratios of propionic acid and sodium propionate on growth of Fusarium species

In this experiment an attempt was made to establish the possible existence of a synergistic effect when different ratios of propionic acid and sodium propionate were applied. A 0.05% dose was chosen based on the results presented above. Generally all ratios of the two preservatives tested led to significantly better control than untreated controls for both isolates of *F. moniliforme* and *F. proliferatum*. The highest inhibition was always found

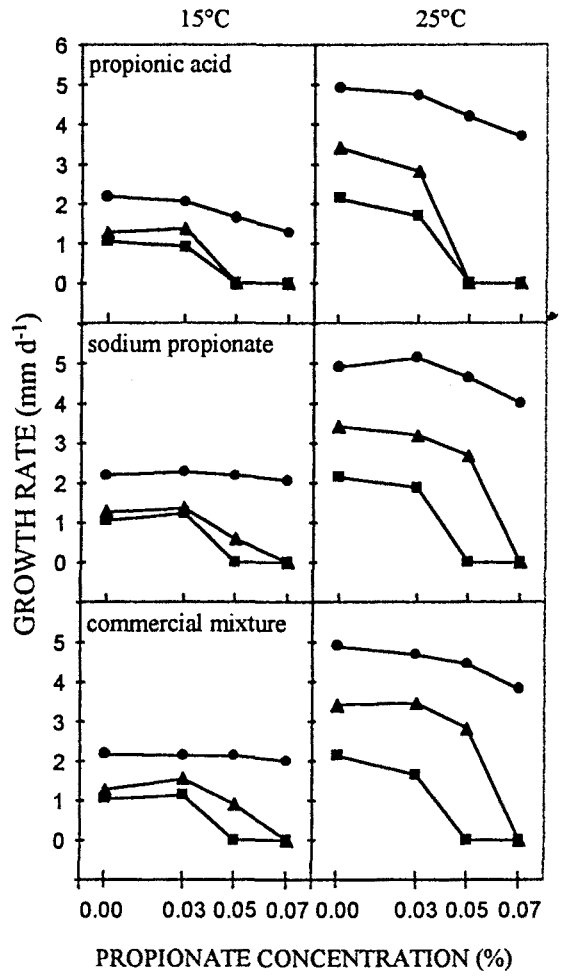

FIG. 1. Effect of different sources and doses of propionates added to irradiated maize on growth rate (mm d^{-1}) of *Fusarium moniliforme* (85N). Combined effect of a_w (●, 0.98; ▲, 0.95; ■, 0.93) and temperature.

when 100% propionic acid was used (Fig. 3). In general, the effects of the other mixtures depended closely on a_w and the isolate tested. There were no significant differences in efficacy of various ratios against the isolates of *F. moniliforme*, while differences found for *F. proliferatum* isolates did not follow any consistent pattern.

In the driest maize a_w treatment (0.93 a_w) 100% propionic acid, and 100% sodium propionate were equally effective, while for other ratios (75+25, 50+50, 25+75 propionic acid+sodium propionate) there was a decreasing effectiveness. However, at 0.95 and 0.98 a_w 100% propionic acid was most effective in controlling growth, with no significant differences between other ratios used.

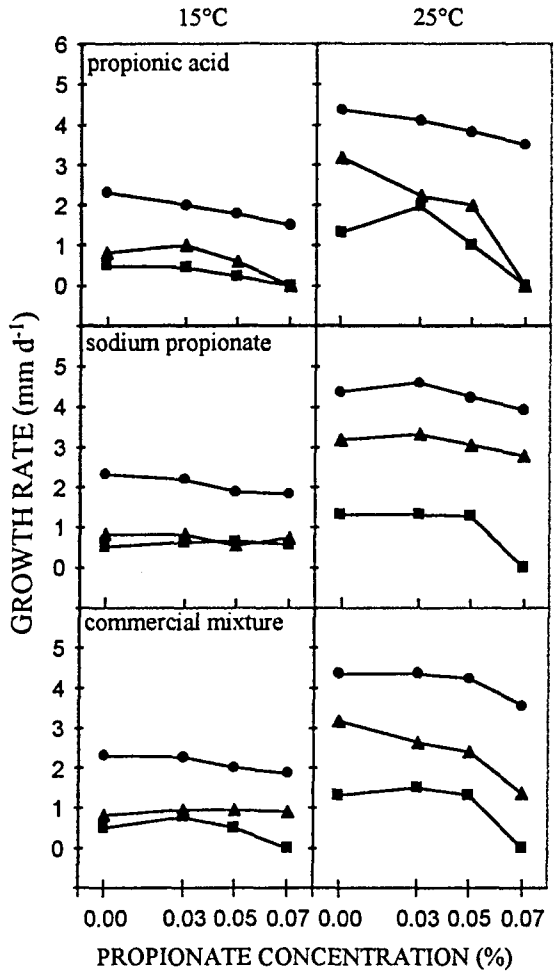

FIG. 2. Effect of different sources and doses of propionates added to irradiated maize on growth rate (mm d^{-1}) of *Fusarium proliferatum* (73N). Combined effect of a_w (●, 0.98; ▲, 0.95; ■, 0.93) and temperature.

Statistical analyses of isolate, a_w, ratio of propionic acid+sodium propionate, and two and three way interactions showed that all factors significantly affected growth of the *Fusarium* isolates and species ($P < 0.01$, Table 2). As before, the major effect was due to a_w.

Effect of propionate preservative on fumonisin B_1 production

In general isolate 73N of *F. proliferatum* produced more FB_1 than isolate 131N, although the maximum concentration present in maize colonized by the latter was 140 ppm (Fig. 4). At 15°C, production of FB_1 by isolate 73N was maximum at 0.95 a_w, and minimum at 0.98 a_w, when the preservative was not used. However, in the presence of the preservative, effect of a_w on fumonisin concentration changed. Similarly, no effect of a_w or dose of propionates was found at 25°C. Increasing the dose of propionate led to a decrease in FB_1 production by

F. proliferatum isolates at 15°C, regardless of a_w. In contrast, isolates of *F. moniliforme* were unaffected by any of the factors tested, and they produced smaller amounts of FB_1, regardless of the treatment (2-12 ppm).

All singled factors assayed (isolate, temperature, a_w, and propionate concentration), and two, three and four way interactions were statistically significant (Table 3). In this case the most important factor was temperature, as higher amounts of FB_1 were produced at 15 than 25°C by the isolates of *F. proliferatum*.

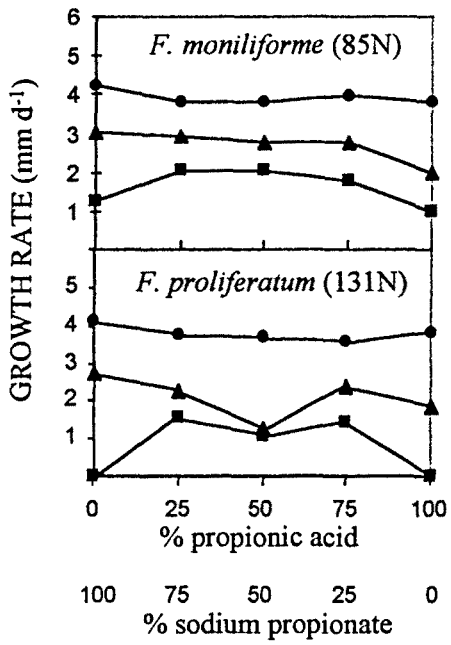

FIG. 3. Effect of different ratios of propionic acid/sodium propionate added to irradiated maize at 25°C on growth rate (mm d^{-1}) of *Fusarium* isolates. Effect of a_w (●, 0.98; ▲, 0.95; ■, 0.93).

Table 2. Significance of fungal isolates (I), water activity (a_w), and percentage of propionic acid (%) in a mixture propionic acid/sodium propionate added to a dose of 0.05% to irradiated maize, and their interactions on growth of *Fusarium* spp. on irradiated maize after 9 days incubation.

FACTOR	DF	MS	F
I	3	205.32	46.30**
a_w	2	11180.89	2521.03**
I × a_w	6	67.99	15.33**
%	5	840.90	189.60**
I × %	15	32.73	7.38**
a_w × %	10	88.64	19.99**
I × a_w × %	30	20.19	4.55**

**significant, $P < 0.01$

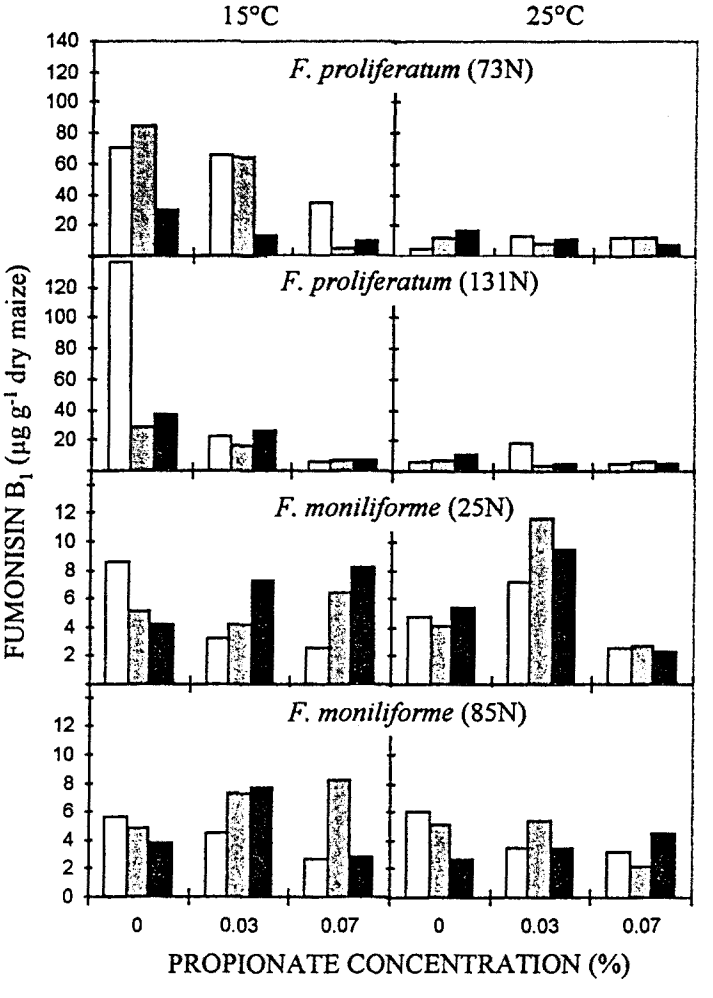

FIG. 4. Effect of propionate concentrations (0.03, 0.07%) on fumonisin B_1 production by two isolates of *Fusarium proliferatum* (73N, 131N) and *F. moniliforme* (25N, 85N) on irradiated maize grain stored at 0.93 (□), 0.95 (▨) and 0. 98 (■) water activity. Results are means of two replicates per treatment.

Correlation analyses

Neither positive nor negative correlation was found between growth rates and fumonisin B_1 production by *Fusarium* Liseola section isolates.

Table 3. Significance of fungal isolates (I), temperature (T), water activity (a_w), and dose (D) of propionates, and their interactions on fumonisin B_1 production by *Fusarium* spp. on irradiated maize after 4 weeks incubation.

FACTOR	DF	MS	F
I	3	5320.9	42.47**
T	1	9590.9	76.55**
I × T	3	2989.9	23.87**
a_w	2	1292.8	10.32**
I × a_w	6	781.7	6.24**
T × a_w	2	1252.3	10.00**
I × T × a_w	6	695.9	5.55**
D	2	3242.1	25.88**
I × D	6	1057.5	8.44**
T × D	2	2517.2	20.09**
I × T × D	6	1063.6	8.49**
a_w × D	4	300.3	2.40
I × a_w × D	12	383.5	3.06**
T × a_w × D	4	605.5	4.83**
I × T × a_w × D	12	519.3	4.15**

**significant, $P < 0.01$

DISCUSSION

This work has shown the complex interactions which take place between the prevailing abiotic factors, fungal colonization and preservatives in the stored grain ecosystem. Propionates have been shown to inhibit the growth of *Fusarium* species on maize grain at very low concentrations. However, efficacy, was closely dependent on the other environmental factors. The highest dose tested was 0.07% propionate because this was recommended for commercial applications. In contrast, in vitro screening on agar media suggested that doses of up to 1% propionate were not adequate to inhibit growth of spoilage fungi of hay such as *Paecilomyces variotii* and *Eurotium* species (14). In their study, species isolated from hay, including *Fusarium culmorum*, were all able to grow in agar containing 0.2% propionic acid. However, other studies have suggested that when using other forms of the preservative (e.g. potassium sorbate) *Fusarium roseum* was more sensitive than *Aspergillus parasiticus* (13).

Propionic acid was found to be the most effective preservative for preventing growth of fumonisin producing *Fusarium* species. However, it is known that the aliphatic acid alone is more volatile than its salts with good short-term efficacy. Thus, perhaps under longer incubation periods the salts may have been more effective. There is an indication that acids with an odd number of carbon atoms are relatively more active as inhibitors than those with an even number (14). However, *Aspergillus* species were also inhibited by smaller concentrations of propionic acid than ammonium propionate on agar media over a range of a_w levels (16). In the present study using the lowest dose (0.03%) sometimes enhanced colonization of maize grain. This may partially be due to poor contact between the preservatives and the fungi, or metabolism of low concentrations as has been demonstrated

previously. For example, *Eurotium* species and spoilage yeasts metabolized 0.2% propionic acid, both in treated hay (37% m.c.) and in liquid media (14, 17).

Although the commercial mixture used is sold as a 'synergistic mixture of propionates' containing both fast acting (e.g. propionic acid, ammonium propionate) and more stable ones (e.g. sodium propionate), we did not obtain better results when a same dose consisting of mixtures of propionic acid and sodium propionate was applied. Indeed, control of fungal growth was worse than that obtained with single propionates. Possibly, doses of the two components separately were too small, enabling the fungus to metabolize one or both components of the formulation. Efficacy of these preservatives is dependent on pH, propionic acid, as well as sorbic and benzoic acids, have best antimicrobial activity when they are 50% undissociated. The efficacy of these acids therefore depends on the dissociation constant, pK_a, which is 4.87 for propionic acid. Thus at pH 4.5, 5.0, 5.5, and 6.0, there is about 70, 43, 19, and 6.9% of undissociated acid, respectively (7). The pH of treatments in this study varied from 4.7 to 5.7, with different patterns depending on a_w levels. Consequently, in most of the cases the percentage of undissociated acid was between 19 and 43%. This pH variation probably did not exert a great influence on preservative efficacy. However, good correlation was found between growth and pH of treatments at 0.93 and 0.98 a_w, which suggests that besides the major effect of a_w, different pH levels led to varying degrees of preservatives efficacy.

Fumonisin B_1 concentrations in samples were low compared with, for example, that obtained by Cahagnier *et al.* (3) at 0.95 a_w after a 28-day at 25°C incubation period with a *F. moniliforme* isolate (>400 ppm). However, they used autoclaved maize in their experiments. It is known that mycotoxin production is higher on autoclaved cereals than on irradiated ones (23). On the other hand, similar low fumonisin concentrations were found in samples of irradiated maize inoculated with *F. moniliforme* and *F. proliferatum*, incubated under similar conditions, and analyzed by using ELISA tests (21).

F. proliferatum isolates produced much higher fumonisin B_1 concentrations than those of *F. moniliforme*. However, significant production by the former species only occurred at 15°C, with production increasing as a_w was decreased, directly opposite to that obtained for growth. However, fumonisin concentrations produced decreased as preservative concentration was increased. By contrast, production of aflatoxins in cultures and growth of isolates of *A. flavus* from molded hay has previously been shown to be stimulated by up to 2% propionic acid (1, 5). Higher acids, such as sorbic acid, have also been found to stimulate growth and aflatoxin production of *A.flavus*, and T-2 toxin by *Fusarium acuminatum* (10, 22). However, Ray & Bullerman (25) found that 1% sorbate completely inhibited aflatoxin production, with 0.5% causing 50% reduction, and 0.1% only 10% inhibition. Mycotoxin production by *Aspergillus parasiticus, Penicillium commune* and *P. patulum* was almost completely inhibited by 0.1-0.15% sorbate (2). Finally, a 0.5% ammonium propionate dose was able to control the growth of fungi in moist groundnuts, including aflatoxigenic species, as well as the production of aflatoxins (4). Complete inhibition of ochratoxin A production by *P. aurantiogriseum* was obtained using 0.1% propionic acid (25°C) or 0.05% (30°C) (29).

In terms of water activity and temperature, the synthesis of fumonisin B_1 by *F. proliferatum* was induced under the environmental stress conditions for growth. A negative correlation was found between growth rate and FB_1 production for these isolates. Interestingly, a

certain amount of fumonisin B_1 was still found in samples where treatments had prevented growth of the *Fusarium* species. Similarly, it has been observed that *Aspergillus parasiticus* appeared to produce much less mycelial mass at the end of the incubation period in corn containing 0.5% sorbate than in corn without sorbate, although aflatoxin levels were similar. Low amounts of aflatoxin, but no visible growth, were also found in samples treated with 1.5% sorbate and inoculated with *A. parasiticus* (13). They concluded that this may have resulted from very limited metabolic activity of the mold or possible carry-over from the inoculum.

ACKNOWLEDGMENTS

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XIII

SELECTIVE EFFECT OF PROPIONATES ON MAIZE MYCOFLORA AND IMPACT ON FUMONISIN B_1 ACCUMULATION

S. Marín[1], N. Magan[2], M. Abellana[1], A. J. Ramos[1], and V. Sanchis[1]

[1]Food Technology Dept., Lleida University, CeRTA, Rovira Roure 177, 25198 Lleida, Spain
[2]Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford, MK43 OAL, U.K.

ABSTRACT

The effect of a commercial mixture of propionates at two different doses (0.05% and 0.1%) on fungal spoilage of natural maize stored at 0.85, 0.90 and 0.95 water activity (a_w) was investigated. Parallel treatments with added inoculum of *Fusarium* Liseola section isolates (*Fusarium moniliforme* and *F. proliferatum*) were carried out in order to determine the effect of fungal interactions on the development of fumonisin-producers on maize in relation to preservative efficacy. Fungal colonisation of grain was measured as fungal counts (CFUs g^{-1} maize). In general, no differences were found between inoculated and uninoculated samples. Besides the selective effect of a_w on maize mycoflora, it was demonstrated that most genera which colonise maize remained unaffected by the preservative concentrations applied. However, *Penicillium* populations (CFUs g^{-1} maize) counts decreased significantly. As they represent a major component of the total fungal counts, an overall control of total mycoflora was observed. Furthermore, there was a significant statistical interaction between preservative and a_w levels, with the preservative activity enhanced at low a_w. The concentrations of fumonin B_1 were unaffected by treatment with no significant differences in concentrations found. This suggests that the natural mycoflora of maize may act as inhibitors of *Fusarium* development, and consequently of fumonisin biosynthesis.

J. Stored Prod. Res. (submitted)

INTRODUCTION

Propionic acid and propionate formulations are highly effective mould inhibitors, commonly used in the food industry (cakes, bakery products, cheese) as calcium and sodium salts. Several reports exist on the efficacy of these fungistats as preservatives of stored high moisture cereals (Skrinjar *et al.*, 1995). However, little information exists on whether such preservatives may be effective in controlling the growth of *Fusarium* spp. or their mycotoxins, either pre-harvest or post-harvest

For maize, environmental factors during ripening and storage can exist which favour rapid invasion of maize and concomitant production of fumonisins by *Fusarium* Liseola species. The most important factors influencing growth and fumonisins production are water availability, and temperature (Marín *et al.* 1995a, b; 1996). Other related factors include mechanical and biological damages and microbial competition (Marín *et al.*, 1998a, b). However, as environmental factors are quite difficult to control in the field, where conditions are exceptionally suitable for fumonisin production, effective post-harvest storage treatment would be needed to ensure that production is inhibited. It may be possible to use a combination of a slightly reduced water activity (a_w) combined with the addition of chemical preservatives for this purpose. However, few studies have investigated this possibility. Previous studies with these organic acids, particularly propionic acid, has shown that a 0.07% dose of propionate may be useful to avoid *Fusarium* Liseola section from growing in moist maize (0.93-0.98 a_w). However, a certain level of fumonisin B_1 was still detected (<40 ppm). Most previous studies deal with *A. flavus* and other aflatoxigenic fungi, and aflatoxin production (Calori-Domingues *et al.*, 1996). Propionic acid was shown to control growth of aflatoxigenic fungi and aflatoxin production in high moisture maize kernels (Smith, 1985; Rusul, 1987).

The objectives of the present work were to examine (a) the efficacy of a propionate-based preservative and interactions with a_w in preventing fungal development in naturally-contaminated maize, and (b) by the addition of *Fusarium moniliforme* and *Fusarium proliferatum* inoculum, to test the ability for control of both *Fusarium* populations and other species, and fumonisin B_1 production in relation to preservative concentration × a_w interaction.

MATERIALS AND METHODS

Culture material

Three isolates each of *F. moniliforme* Sheldon (25N, 85N, 123N) and *F. proliferatum* (Matsushima) Nirenberg (55N, 73N, 131N) were used in this study. These isolates have previously been found to be fumonisin producers (Sala, 1993), and are held in the Food Technology Department Collection of the University of Lleida (Spain).

Preservatives

A commercial antifungal preservative based on a mixture of propionates was purchased from a local supplier. This product consists of sodium propionate, ammonium propionate,

propionic acid, 1,2-propanediol monoesters and fatty acids. Recommended dosages were a 500 g T^{-1} dose for sound, well-dried grain (14% m.c.), doses of 1 kg T^{-1} for initially spoilt grain, with a m.c. of 14-16%, and finally 3 kg T^{-1} for very contaminated grain with a m.c. >16%. Suitable solutions were prepared from the mixture and filter-sterilised through a 0.22 µm filter, and stored at 7°C until used.

Rehydration and inoculation of maize in microporous bags

A factorial experiment was designed consisting of 3 factors, a_w (0.85, 0.90 and 0.95), preservative concentration (0, 500 and 1000 g T^{-1}), and either *Fusarium* inoculation or uninoculated treatments. Each treatment was repeated three times.

150 g subsamples of natural maize were weighted into sterile flasks and adjusted to 0.85, 0.90 and 0.95 a_w) by addition of sterile distilled water and propionate solution, and flasks stored at 4°C for 48 h to modify the grain to the required a_w. They were regularly mixed to obtain a uniform treatment. For treatments which were spiked with inoculum of the *Fusarium* spp. 1 ml of a 3×10^7 spores ml^{-1} suspension containing a mixture of spores of the six *Fusarium* isolates mentioned above in equal parts was added. Additional volumes represented by both preservative and spore suspension had been subtracted from the initial amount of water added to the grain. After shaking flasks, grain was transferred to surface-sterilised microporous bags, and sealed. Bags were then enclosed in sealed environmental chambers containing beakers of glycerol-water solutions of the same a_w as the treatments in order, to maintain the correct equilibrium relative humidity. Chambers were incubated at 25°C for a 28 days period.

Assessment of fungal populations

Colonisation of grain was assessed as colony forming units (CFUs) g^{-1} after 7, 14 and 21 days incubation. 10 g subsamples were destructively analysed by serial dilution using malt extract agar (MEA), and confirmed at random on DG18 medium. Plates bearing between 5 and 150 CFUs were enumerated for total *Aspergillus, Penicillium, Eurotium* and *Fusarium* colonies, as well as *A. flavus.*

Determination of fumonisin B_1 formation

The production of fumonisin B_1 in maize by natural and inoculated *Fusarium* species was determined after 28 days of incubation by HPLC. 10 g subsamples were ground and extracted by blending them in 20 ml methanol-water (3+1). Extracts were filtered and centrifuged. Filtrate (5 g) was loaded on a preconditioned SAX column and eluted with 0.5% acetic acid in methanol. The eluate was evaporated to dryness in a rotavapour, redissolved in methanol, and finally evaporated under a gentle stream of nitrogen and dissolved in methanol for HPLC. Fumonisin was coupled to OPA and assayed by HPLC, by comparison with external standards, using methanol:0.1 M dihydrogen sodium phosphate (3+1) (pH 3.35) as mobile phase at a 0.8 ml min^{-1} flow rate. Reference standard of FB_1 was purchased from CSIRO, Division of Food Science and Technology, Pretoria, South Africa.

Dry matter determination

The percentage dry matter in each sample was determined by drying subsamples of approximately 10 g at 105°C for 17 h (International Seed Testing Association (ISTA), 1976). Thus, all results are presented on dry weight basis.

Statistical treatment of data

Analysis of variance of the effect of a_w, doses of propionates, time and addition of *Fusarium* were performed for each separate genus (*Aspergillus, Eurotium, Penicillium, Fusarium*), for *A. flavus*, and for total counts. Fumonisin B_1 concentration in samples was analysed in a similar way. All statistical analyses were made using SAS version 6.12 (SAS Institute, Inc.).

RESULTS

Relative incidence of species

A. flavus was present in the lowest populations in maize grain at 0.85-0.90 a_w, while *Eurotium* species were isolated in the least amounts at 0.95 a_w. *Penicillium* species were most abundant at 0.95 a_w, while at 0.85-0.90 a_w, *Aspergillus, Penicillium, Eurotium* and *Fusarium* species accounted for a major or minor part depending on propionate concentration. *Penicillium* species were the most abundant at 0.90-0.95 a_w in absence of propionate. However, *Aspergillus* populations reached similar levels at both 500-1000 ml T^{-1} propionate concentration.

Statistically, there was no significant change in either *Aspergillus, Penicillium* or *Fusarium* counts when an additional inoculum of *Fusarium* was used (Table 1). However, the presence of populations of *Eurotium* species increased in *Fusarium*-inoculated maize grain samples.

Effects of water activity and time on fungal populations

Interestingly, total populations and single genera increased significally with time, except for *Fusarium* populations, which remained unchanged throughout the experimental period.

Water activity was the most significant factor ($P < 0.01$) because of its influence on fungal populations. *A. flavus, Fusarium* and *Penicillium* species increased their populations with increasing a_w, although other *Aspergillus* species showed similar populations at 0.90 and 0.95 a_w, while decreased at 0.85 a_w. In contrast, *Eurotium* species showed higher populations at 0.85-0.90 a_w and lower at 0.95 a_w (Fig. 1). Both time and a_w showed a significant interaction ($P<0.01$). Thus, for example, *Eurotium* populations decreased significantly from 0.90 to 0.95 a_w, after 14 and 21 days storage.

Table 1. Analysis of variance of *A. flavus*, *Eurotium* sp., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and total counts (log(CFU g^{-1})) on natural contaminated maize. Significance of additional inoculation of *Fusarium* (I), time (t), preservative dose (P) and water activity (a_w), and their interactions.

		A. flavus		*Eurotium*		*Aspergillus*		*Penicillium*		*Fusarium*		Total	
	DF	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
I	1	0.3	1.3	6.2	15.5**	0.6	1.9	0.0	0.3	0.9	1.8	0.1	0.8
T	2	3.0	15.5**	5.6	14.1**	13.0	40.2**	7.5	51.6**	0.2	0.4	6.3	74.4**
Ixt	2	0.1	0.4	0.6	1.4	0.0	0.1	0.3	2.4	1.0	2.0	0.1	1.8
P	2	1.5	7.6**	2.4	6.0**	0.7	2.1	12.3	83.9**	0.7	1.5	0.8	9.5**
IxP	2	0.1	0.7	0.7	1.9	0.1	0.2	0.3	2.3	0.3	0.6	0.0	0.5
txP	4	0.1	0.8	0.6	1.4	0.8	2.4	0.3	2.1	0.4	0.7	0.4	4.8**
IxtxP	4	0.0	0.2	0.4	1.0	0.0	0.1	0.4	2.6*	0.4	0.8	0.1	1.1
a_w	2	85.1	434.4**	7.7	19.3**	5.2	16.0**	76.4	522.1**	32.0	65.6**	22.8	269.7**
I× a_w	2	0.1	0.3	2.2	5.6**	0.7	2.3	0.3	2.3	0.9	1.8	0.1	0.9
t× a_w	4	2.8	14.5**	4.1	10.2**	2.4	7.3**	0.5	3.3*	0.7	1.4	0.4	5.0**
Ixt× a_w	4	0.1	0.3	0.6	1.6	0.3	0.9	0.1	0.6	0.4	0.8	0.0	0.5
P× a_w	4	1.6	8.0**	3.3	8.2**	2.9	8.9**	1.0	7.0**	0.2	0.4	0.1	1.1
IxP× a_w	4	0.7	3.6**	1.2	2.9*	0.9	2.8*	0.3	1.8	0.0	0.1	0.1	1.3
txP× a_w	8	0.1	0.6	0.6	1.6	0.5	1.7	0.8	5.6**	0.4	0.7	0.1	0.8
IxtxP× a_w	8	0.1	0.5	0.6	1.6	0.3	1.1	0.3	1.8	0.4	0.8	0.1	0.9

* significant, $P< 0,05$
**significant, $P< 0,01$

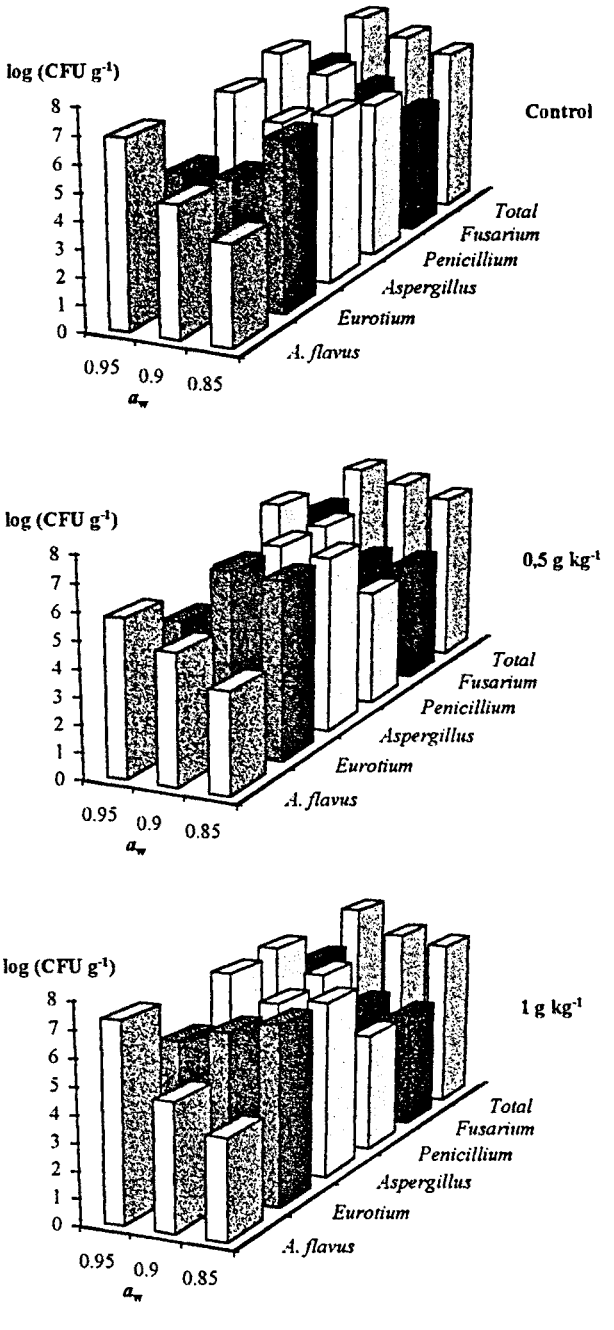

Figure 1. Effect of a_w and concentration of propionates on maize mycoflora after a 21-day incubation period at 25°C

Effects of propionates on fungal populations

Total populations were equally inhibited by 500 and 1000 ml T^{-1} propionate. However, detailed results showed that the *Penicillium* isolates were the only main group inhibited, with mean populations of 6.69, 6.23 and 5.87 (log CFU) for the control, 500 and 1000 ml T^{-1} doses, respectively. The other genera had a low sensitivity to this preservative. As *Penicillium* species are often the major component of grain mycoflora, its sensitivity to propionates accounts for the major controlling effect in the grain. The efficacy of the propionate treatments against the *Penicillium* spp. was significantly dependent on a_w ($P<0.01$), with better control at low a_w. However, for total fungal populations, the effect of propionates was closely related to the incubation time ($P<0.01$), showing high inhibitory effects after 7 days, but lower efficacy after 14-21 days. On the other hand, populations of *Aspergillus* species populations seemed to be stimulated by preservative addition to the maize grain.

In general, the effect of propionates was closely linked to a_w for all of fungal groups. Thus, the statistical interaction a_w × preservative was significant in most cases ($P<0.01$). Figure 2 shows how, in general, at 0.85 a_w the effect of a 0.1% preservative is quite marked, while at 0.90-0.95 a_w there is no effect, with the exception of the *Penicillium* species.

Effects of treatments on fumonisin B_1 production

No effect was exerted by either a_w or propionates on fumonisin concentration of samples (Table 2). There was also no difference between fumonisin levels in maize grain inoculated with *Fusarium* species and uninoculated samples (Table 3). The mean fumonisin level was 6.66 μg g^{-1}. The only interaction which resulted significant was that of additional inoculation of *Fusarium* Liseola × propionate doses ($P<0.05$).

Table 3. Analysis of variance of fumonisin B_1 production by *Fusarium* sp. on naturally contaminated maize. Significance of additional inoculation of *Fusarium* (I), preservative dose (P) and water activity (a_w), and their interactions.

FACTOR	DF	MS	F
I	1	16.49	1.35
P	2	0.81	0.07
IxP	2	44.20	3.62*
a_w	2	11.48	0.94
a_w×I	2	9.51	0.78
a_w×P	4	15.52	1.27
a_w×IxP	4	16.58	1.36

Table 2. Fumonisin B_1 concentration ($\mu g\ g^{-1}$) in naturally contaminated maize incubated at 25°C for 28 days.

Water activity	Preservative concentration	Additional *Fusarium* inoculation	Non-additional *Fusarium* inoculation
0.85	0%	3.71	9.27
	0.05%	11.15	4.96
	0.1%	5.36	9.98
0.90	0%	4.78	5.65
	0.05%	5.72	7.34
	0.1%	3.38	8.04
0.95	0%	8.57	9.46
	0.05%	6.42	3.61
	0.1%	5.97	6.58

DISCUSSION

This study complements previous work in which the inhibitory effect of propionate on growth of *F. moniliforme* and *F. proliferatum* was demonstrated to be effective at low doses (0.07%), but only at high a_w levels (0.93-0.98 a_w) (Marín *et al.*, 1999). However, while these conditions inhibited *Fusarium* growth, a low concentration of fumonisin B_1 was still detected (Marín *et al.*, 1999). As discussed previously (Skrinjar *et al.*, 1995; Marín *et al.*, 1999), the effect of propionates depends closely on the other environmental factors such as a_w and temperature. The present work focused on the biotic factors, i.e., the influence of natural microflora of maize on *Fusarium* development and its relationship with preservatives and a_w. The present study was carried out at 25°C; a regim under which *Fusarium* spp. should be inhibited by other fungal species which colonise maize grain (Marín *et al.*, 1998d) However, at lower temperatures (e.g. 15°C) *Fusarium* has been demonstrated to be more adapted than *Aspergillus* species (Marín *et al.*, 1998b, c), but not as well as *Penicillium* species (Marín *et al.*, 1998a, b, c).

For the first time the selective effect of preservatives on maize microflora has been demonstrated. Furthermore, the tolerance of some fungal species to certain doses of propionates has been shown. For example, populations of *Aspergillus* species increased with concentration of preservative, while *Eurotium* species were unaffected by the preservative. Similarly, the incidence of all predominant storage fungi of stored rice, sorghum and groundnut under tropical conditions were found to decrease due to propionic acid treatment, except for *Eurotium* spp. which increased (Patkar *et al.*, 1995). With inshell groundnuts, 0.3 and 0.5% ammonium propionate was demonstrated to exert a selective effect on the natural micoflora, with *Eurotium* spp. representing more than 50% of the total fungal counts. This effect was evident with 0.3% propionic acid and controlled potentially aflatoxigenic fungi up to day 14 of incubation (Calori-Domingues, 1996).

It has been reported that subinhibitory doses together with inadequate distribution of chemicals could favour fungal growth on the treated material with an initial low level of contamination. A change in the dominance of genera and species can occur, enabling the growth of species tolerant to preservative treatment (Smith, 1985; Lacey, 1989). *In vitro* studies by Mutasa *et al.* (1990a) with potassium sorbate (0.1-0.4%) on growth of tobacco spoilage fungi showed that at both 0.85 and 0.96 a_w and 25°C members of the *Eurotium* group were tolerant to sorbate, while *Aspergillus* and *Penicillium* species were less so, with some species able to metabolise the preservative rapidly (Mutasa *et al.*, 1990b).

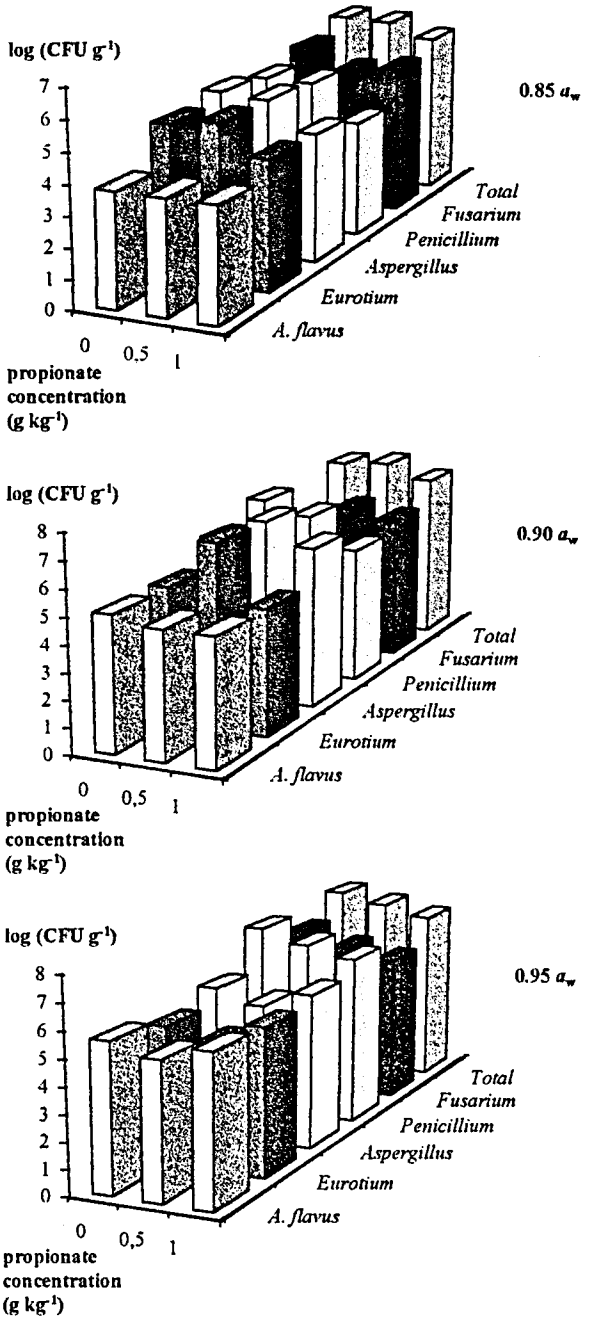

Figure 2. Effect of propionates and a_w on maize mycoflora after a 7-day incubation period at 25°C

In fungi colonizing propionic-acid-treated hay, small additions of propionate to the medium tended to suppress conidial development and to enhance cleistothecium formation, but larger additions inhibited both. Other organisms isolated from hay, including *Fusarium culmorum*, failed to grow when more than 0.4% propionic acid was incorporated in the agar medium (Lord *et al.*, 1981). The ability of certain fungi to metabolize propionic acid can be a problem. Thus *Penicillium variotii* and *Eurotium repens* grew well in 0.2 and 0.4% propionate, taking 1 and 2 weeks to metabolise the chemical completely in agar culture. However, no growth occurred in 0.8 and 1% propionate, even after 30 days, with propionic acid concentrations remaining unchanged. Members of the *Eurotium* group varied in their tolerance to propionate. The inhibitory action of propionic acid has been demonstrated in maize stored with high moisture content. A dose of 0.4% added directly to rehydrated maize controlled contamination for more than 35 weeks (Sauer, 1974). When high moisture maize was heavily inoculated with *Aspergillus flavus/parasiticus* and treated with 1% propionic acid, growth and formation of aflatoxins were inhibited for about 19 weeks (Vandergraft, 1975).

Similarly, production of aflatoxins in cultures by *A. flavus* and by isolates of the same fungus from moulded hay has been stimulated by up to 0.2% propionic acid (All-Hilli *et al.*, 1979; Clevstrom *et al.*, 1981) and by 0.025% sorbic acid. The latter also stimulated production of T-2 toxin by *Fusarium acuminatum* (Gareis *et al.*, 1984) while higher concentrations were shown to inhibit mycotoxin production to varying degrees. .

In the present study, propionate efficacy was improved at low a_w, and was most inhibitory during the first week incubation. High treatment concentrations of propionic acid inhibit growth, but the organisms are not killed and growth may thus only be delayed (Lord *et al.*, 1981). Consequently, *in vitro* screening for the efficacy of preservatives should take into account the combined effect of temperature and a_w on growth of the microorganisms involved. Propionate at concentrations of between 0.01-0.5% were assayed by Skrinjar *et al.* (1995); with up to 0.05% at 25°C markedly decreasing the growth and sporulation of *Penicillium aurantiogriseum*. Growth was completely inhibited at 0.1-0.5% at 30°C. Inhibition of ochratoxin (OA) production by propionate was also demonstrated. Complete inhibition of mycotoxin production was found using 0.1% PA (25°C) or 0.05% (30°C), although some growth could be still observed in media with 0.1% PA at 30°C. They concluded that rather than a single factor affecting OA production, multiple action of various inhibitors would be needed against fungal contaminants in food protection (Skrinjar *et al.*, 1995).

Previously, potassium sorbate has been evaluated as a potential preservative for storage of whole kernel autoclaved yellow dent maize at 18, 24 and 30% moisture content. In general, mycotoxin and CO_2 production were reduced with increasing levels of sorbate. However, samples treated with 0.5% sorbate and inoculated with *A. parasiticus* contained about the same amount of aflatoxin as the control at the end of the incubation period. The sorbate was more effective on maize with lower moisture content and in sealed containers, where high concentration of CO_2 accumulated during incubation (Lee *et al.*, 1986).

In this study a_w was shown to be the most significant factor in selecting maize microflora. Thus, *A. flavus* was the minor group at 0.85 and 0.90 a_w, while *Eurotium* populations were lowest at 0.95 a_w. The populations of *Penicillium* species increased in at 0.90-0.95 a_w. The

profiles of reponse of some of these species to a_w and temperature have been extensively reported elsewhere (Marín *et al.*, 1998a; Abellana, 1999).

It was interesting to note that there was no effect of preservative on fumonisin B_1 production. Similarly, Marín *et al.* (1999) experiments found no significant effect of either propionate concentration or a_w at 25°C. In our experiments smaller amounts of fumonisins were found in the samples. This suggests that the competition between species can perhaps influence the synthesis of such mycotoxins. Probably, the low concnetrations of fumonisin B_1 found in the samples were produced at the beginning of the incubation period. However as soon as the other species were able to colonise the grain, *Fusarium* isolates were unable to compete successfully and then neither growth nor fumonisin B_1 production occurred. This is supported by the fact that *Fusarium* counts were the only ones that did not change with time. Previously, it has been demonstrated the ability of some *Aspergillus* and *Penicillium* to inhibitthe growth of *Fusarium* species in maize grain at 25°C (Marín *et al.*, 1998b).

Herting and Drury (1974) studied the relative antifungal activities of volatile fatty acids on cereals and found propionic acid to be an effective antifungal agent at a level of 0.8% with cereals containing 20% moisture. Propionic acid was more effective than calcium propionate in controlling fungal population density in cereals where *Aspergillus* was dominant (Paster, 1979). De Boer (1988) suggests the application of propionic acid as a preservative in food industry at levels of 0.1-0.3%. Propionic acid concentrations of 0.2-0.3% have been showed as suitable for traditional storage of maize in India for periods up to 12 months, effectively inhibiting the growth of fungi (Kumar *et al.*, 1993).

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XIV

EFFECT OF WATER ACTIVITY ON HYDROLYTIC ENZYME PRODUCTION BY *FUSARIUM MONILIFORME* AND *FUSARIUM PROLIFERATUM* DURING COLONISATION OF MAIZE

S. Marín[1], V. Sanchis[1], A.J. Ramos[1] and N. Magan[2]

[1]Food Technology Dept., CeRTA, UdL-IRTA, Universitat de Lleida, Rovira Roure 177, 25198 Lleida, Spain .
[2]Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, UK.

SUMMARY

The effect of different water availabilities (water activity, a_w; 0.98-0.93) and time (up to 15 days) on the production of seven hydrolytic enzymes by strains of *F. moniliforme* and *F. proliferatum* during early colonisation of gamma-irradiated living maize grain were examined in this study. Both the total activity (μmol 4-nitrophenol min^{-1} g^{-1} maize) and specific activity (nmol 4-nitrophenol min^{-1} μg^{-1} protein) were quantified using chromogenic p-nitrophenyl substrates. The dominant three enzymes produced by the fungi on whole colonised maize kernels were α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase. The other four enzymes were all produced in much lower total amounts and in terms of specific activity (β-D-fucosidase, α-D-mannosidase, β-D-xylosidase and N-acetyl-α-D-glucosaminidase), similar to that in uncolonised control maize grain. There were significant increases in the total production of the three predominant enzymes between 3-15 days colonisation, and between 3-6 days in terms of specific activity when compared to untreated controls. The total and specific activity of the α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase, were maximum at 0.98 a_w with significantly less being produced at 0.95 and 0.93 a_w, with the exception of the total activity of α-D-galactosidase which was similar at both 0.95 and 0.93 a_w. Single factors (time, a_w, and inoculation treatment), two- and three-way interactions were all statistically significant for the three dominant enzymes produced except for specific activity of β-D-glucosidase (two and three-way interactions) and for total activity of α-D-galactosidase in the time × a_w treatment. Thisstudy suggests that these hydrolytic enzymes may play an important role in enabling these important fumonisin-producing *Fusarium* spp. to rapidly infect living maize grain over a wide a_w range.

Key words: hydrolytic enzymes, water activity, maize grain, *Fusarium* spp., total activity, specific activity, infection

Int. J. Food Microbiol. (in press)

INTRODUCTION

Fusarium moniliforme and *Fusarium proliferatum* are commonly present on maize causing severe ear or kernel rot resulting in significant deterioration in quality and production of a number of mycotoxins including fusarins, moniliformin and fumonisins (Nelson, 1992; Nelson et al., 1993). Recent work has shown that *F. moniliforme* can infect maize systemically via seed, but that infection predominantly occurs during silking (Munkvold et al., 1997). It has also been shown that these *Fusarium* spp. are able to germinate and grow over a range of water availabilities (0.90-0.995 water activity, a_w) (Marin et al., 1995; 1996) which parallel moisture contents during silking and during post-harvest drying and storage. However, little information is available on the type of enzymes and the enzymatic capacity of these *Fusarium* spp. under environmental conditions conducive to infection.

Infection of maize grain by *Aspergillus flavus* has received more interest and pectinases, amylases and cutinases have been implicated in this process (Cotty et al., 1990; Woloshuk et al, 1996; Guo et al., 1996). The pericarp is the outermost layer of the maize kernel and provides effective protection from fungal invasion. It consists of several layers of cells which differ in their degree of degradation and cell wall thickness (Wolf et al., 1952). Thus the production of a range of enzymes may be advantageous for infection of maize kernels by *Fusarium* spp. pre- or post-harvest.

It has been suggested that the production of specific hydrolytic enzymes by spoilage fungi on temperate cereals can be a good early indicator of the initiation of moulding in grain post-harvest (Magan, 1993a). For example, both Stevens and Relton (1981) and Jain and Lacey (1991) using chromogenic 4-nitrophenyl substrates found that the largest quantities of enzymes produced by *Aspergillus*, *Penicillium* and *Eurotium* spp. were N-acetyl-β-D-glucosaminidase and α-D-galactosidase. Indeed, Jain and Lacey (1991) found that a range of xerophilic spoilage fungi (*Aspergillus*, *Eurotium* and *Penicillium* spp.) were able to produce β-glucosidase and N-acetyl-β-glucosaminidase, while only some of them showed α-galactosidase, β-galactosidase and α-glucosidase activity during colonisation of barley/wheat grain. However, practically none showed β-glucuronidase, α-mannosidase and α-fucosidase activity. Other semi-quantitative and agar-based enzymatic tests have suggested that *F. moniliforme* and *F. proliferatum* produce large amounts of the hydrolytic enzymes such as N-acetyl-β-D-glucosaminidase and β-glucosidase (Sala, 1993) although effects of environmental parameters were not investigated.

The objectives of this study were to quantify in detail the effect of a_w on the temporal production of seven glycosidase enzymes by strains of *F. moniliforme* and *F. proliferatum* and their potential role in infection of living gamma-irradiated maize grain.

MATERIALS AND METHODS

Fungal isolates

Two strains each of *Fusarium moniliforme* Sheldon (25N, 85N) and *Fusarium proliferatum* (Matsushima) Nirenberg (73N, 131N) were used in all experiments. All isolates were maintained on malt extract agar (MEA -20 g malt extract, 20 g glucose, 1 g peptone, 20 g

agar, 1000 ml destilled water, pH=5.5). All the strains are held in the Food Technology Dept. collection of the University of Lleida, Spain.

Grain

Spanish maize grain was irradiated with 12 kGrays of gamma irradiation and stored at 4°C. Following this treatment the grain contained no fungal infection but had retained germinative capacity (unpublished information). The initial water content and water activity (a_w) of the grain were 13.9% and 0.71, respectively.

Rehydration, inoculation and incubation of grain maize

Irradiated grain, 150 g, were placed in sterile flasks and rehydrated to the desired treatment a_w levels (0.93, 0.95 and 0.98) by addition of sterile destilled water. The amount of water added was calculated from a moisture adsorption curve for the grain. The grain treatments were allowed to equilibrate at 4°C for 48 h, with periodic shaking. Finally, the a_w values were confirmed by using a Novasina Thermoconstanter TH200 (Axaid Ltd., Pfäffikon, Switzerland).

Spore suspensions were obtained by harvesting spores from cultures of each isolate maintained on MEA and suspending them in sterile destilled water with 0.002% Tween 80. The final concentration of the suspensions was assessed by using an haemocitometer and was adjusted to 3×10^7 spores ml^{-1}. A 500 µl of spore suspension was added to each treatment flask and shaken vigorously to obtain an even inoculation of the maize grain. This was taken into account in relation to the initial addition of sterile water to the grain. Uninoculated controls of the same a_w were also prepared. After equilibration, 10 g subsamples of inoculated grain were placed in 9cm sterile Petri plates. Replicates of the same a_w treatment were placed in impermeable to water plastic containers together with two 100 ml beakers containing a glycerol-water solution (Dallyn, 1978) of the same equilibrium relative humidity value as the a_w of the maize grain substrate. These were incubated at 25°C for the duration of the experiment.

Samples were taken after 3, 6, 9, 12 and 15 days incubation and removed for analyses of enzymes. All enzymatic assays were carried out within 8 hours. All treatments were repeated three times.

Extraction of enzymes from grain

Two different methods of sample extraction, similar to those described previously by Jain and Lacey (1991), were applied to all replicates and treatments.

(i) Ground samples: Enzymes were extracted by grinding 4 g of sample with 8 ml of 10mM potassium phosphate extraction buffer (pH 7.2) using a Commercial Waring blender (Waring Ltd., UK). A 2 ml aliquot of this suspension was then centrifuged in eppendorf tubes in a benchtop microfuge (MSE Cenetaur 2, Norwich, UK) for 15 min at 450 x *g* to obtain a clear supernatant.

(ii) Unground samples: Enzymes were extracted by weighing 2 g samples in glass Universal bottles containing 4 ml of 10mM potassium phosphate extraction buffer (pH 7.2). These were shaken on a wrist action shaker (Ikalabortechnik KS250 basic, Janke and Kunkel GmbH and Co., Staufen, Germany) for 1 h at 4°C. The washings were then decanted into 1 ml plastic eppendorf tubes and centrifuged in a benchtop microfuge (MSE Cenetaur 2, Norwich, UK) for 15 minutes at 450 × *g* and supernatant removed with care. Aliquots of all the extracts were frozen and stored at -80°C for subsequent protein analysis.

Total enzyme activity determination

The activity of seven glycosidases was assayed using the 4-nitrophenyl substrates (Sigma, England). The concentrations and buffers used for each enzyme assay are listed in Table 1. Enzyme activity was measured by the increase in optical density at 405 nm, caused by the liberation of 4-nitrophenol upon enzymatic hydrolysis of the substrate, three minutes after stopping the reaction with 1M Na_2CO_3. To allow rapid assay of multiple samples a Multiscan plate reader (Life Sciences International, Cambridge, UK) was used (Jain and Lacey, 1991).

For analyses the reaction mixture consisted of 40 µl of substrate solution in appropriate buffer, 40 µl of enzyme extract (previously diluted, if necessary) and 20 µl of the appropriate buffer. These were placed in the wells of the microtitre plate, along with appropriate controls and incubated for an hour at 37°C. The reaction was stopped by adding 5 µl Na_2CO_3. A calibration curve of 4-nitrophenol concentration vs. absorbency at 405 nm was previously constructed. Total enzyme activity was expressed as µmol 4-nitrophenol released min^{-1} g^{-1} dry maize.

Specific enzyme activity determination

Total protein in the extracellular enzyme extract was measured using a Bicinchoninic acid protein assay (BCA) procedure. In order to carry out rapid analysis of multiple samples, a micro-assay method using a microtitre plate reader was used. A BCA protein assay reagent kit (Pierce, England) consisting of BCA reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.2N sodium hydroxide), BCA reagent B (solution containing 4% cupric sulphate) and Albumin standard (containing bovine serum albumin at a concentration of 2.0 mg ml^{-1} in a solution of 0.9% saline and 0.05% sodium azide) was used.

BCA reagent A, 50 parts, were mixed with 1 part of reagent B to obtain the working reagent, which is stable for at least 1 day when stored in a closed container at room temperature. A 10 µl volume of each standard or unknown sample were pipetted into the appropriate microtitre plate wells, while 10 µl of the diluent were used for the blank wells. A 200 µl volume of the working reagent were added to each well, and after shaking the plates were incubated at 37°C for 30 minutes. After cooling the plates to room temperature, the absorbence at 540 nm was measured in a plate reader. A calibration curve made with albumin standards was used in order to obtain protein concentrations in the enzyme extracts. These values were related to enzyme concentrations to calculate specific activity of the different enzymes tested in nmol 4-nitrophenol released min^{-1} μg^{-1} protein.

Table 1. Summary of the hydrolytic enzymes assayed for in this study, their substrates, concentrations, buffer and pH used.

Enzyme	Substrate	conc. (mM)	Buffer	pH
β-D-fucosidase	4-nitrophenyl-β-D-fucopyranoside	2.0	25mM acetate	5.0
α-D-galactosidase	4-nitrophenyl-α-D-galactopyranoside	4.0	25mM acetate	5.0
β-D-glucosidase	4-nitrophenyl-β-D-glucopyranoside	2.0	25mM acetate	5.0
α-D-mannosidase	4-nitrophenyl-α-D-mannopyranoside	4.0	25mM acetate	5.0
β-D-xylosidase	4-nitrophenyl-β-D-xylopyranoside	2.0	25mM acetate	5.0
N-acetyl-α-D-glucosaminidase	p-nitrophenyl-N-acetyl-α-D-glucosaminide	2.0	25mM acetate	4.2
N-acetyl-β-D-glucosaminidase	p-nitrophenyl-N-acetyl-β-D-glucosaminide	2.0	25mM acetate	4.2

Statistical analyses of the results

Variance of both total and specific enzyme activity results were analysed and effects of the different factors involved in the experiments and their interactions were shown at the 99% level ($P < 0.01$). LSD tests were also made (α=0.05) for the significant factors found. All statistical analysis were made by using SAS 6.11 (SAS Institute Inc.).

RESULTS

Comparison of the total enzyme activity and specific activity in irradiated unground and ground maize grain

Table 2 compares (i) the total activity and (ii) the specific activity of β-D-glucosidase and N-acetyl-β-D-glucosaminidase at 0.95 a_w after 15 days incubation for *F. moniliforme* (25N) and *F. proliferatum* (131N). This shows that although the total enzyme activity was higher in ground samples, the specific activity was often significantly higher in unground maize grain inoculated with strains of *F. moniliforme* and *F. proliferatum* when compared with untreated controls. Because of our interest in determining the role of enzymes and their activity in the infection of maize grain by these fungi all results are presented for total and specific activity in unground samples as we were specifically interested in their involvement in penetration of the grain surface by the mycelia of the *Fusarium* species. Furthermore enzymes extracted from the mycelia growing on the grain surface better represents their potential in the early stages of the infection.

By using both methods of extraction it was shown how total enzyme activity was markedly higher in extracts from ground samples in both inoculated and uninoculated maize due to the higher amount extracted from the maize grain itself. In general, specific activity (Table 2) in ground samples was also higher than that from unground whole kernels, although the differences were not as marked as in the measurement of the total enzyme activity.

Table 2. Effect of the enzyme extraction method in both total activity (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific activity (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein) of *F. moniliforme* (25N) and *F. proliferatum* (131N) found in samples at 0.95 a_w after 15 days.

		N-acetyl-β-D-glucosaminidase				β-D-glucosidase			
		SA	LSD groups	TA	LSD groups	SA	LSD groups	TA	LSD groups
Ground samples	Uninoculated	5.533	A	10.535	A	0.905	A	1.748	A
	25N	5.371	A	45.358	B	11.532	C	108.819	C
	131N	5.154	A	48.992	C	9.897	B	95.862	B
Ungroun d samples	Uninoculated	0.785	A	1.444	A	0.479	A	0.882	A
	25N	12.292	C	8.220	B	9.058	C	4.824	B
	131N	5.590	B	8.225	B	6.033	B	6.128	C

*groups with different letters are significantly different ($P<0.05$).

The total enzyme activity expresses the overall increase or decrease in activity which can be due to both an increase in the enzyme amount or in the specific activity of the ones present. However, the specific activities obtained in relation to the amount of protein present reflects the level of activity of each enzyme. Thus the specific activity measurement represents important complimentary information to the overall total enzymes quantified and reflects the possible enzyme dynamics and behaviour in infection of maize kernels. Because of this, all data are presented for both total and specific activity by the *Fusarium* spp. in unground maize samples.

Comparison of the total and specific activity of the seven glycosidase enzymes produced by *F. moniliforme* and *F. proliferatum* on whole maize grain at different water availabilities

In extracts from uninoculated maize the enzyme β-D-glucosidase was found to be predominantly present with the most activity (1.94 μmol 4-nitrophenol min^{-1} g^{-1} dry grain; 1.05 nmol 4-nitrophenol min^{-1} μg^{-1} protein), followed by N-acetyl-β-D-glucosaminidase (1.62 μmol 4-nitrophenol min^{-1} g^{-1} dry grain; 0.88 nmol 4-nitrophenol min^{-1} μg^{-1} protein) and α-D-galactosidase (0.61 μmol 4-nitrophenol min^{-1} g^{-1} dry grain; 0.33 nmol 4-nitrophenol min^{-1} μg^{-1} protein).

Table 3. Comparison of both total activity (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific activity (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein) of *F. moniliforme* (25N) and *F. proliferatum* (131N) found in samples at 0.95 a_w after 15 days for the seven enzymes tested.

Enzyme	*F. moniliforme* (25N)		*F. proliferatum* (131N)		Uninoculated	
	TA	SA	TA	SA	TA	SA
β-D-fucosidase	0.1326	0.4489	0.1256	0.2942	0.2087	0.1134
α-D-galactosidase	1.5607	5.0122	2.9311	6.8927	0.6085	0.3305
β-D-glucosidase	2.9108	9.4702	5.1953	12.8688	1.9413	1.0544
α-D-mannosidase	0.1508	0.4937	0.0865	0.2019	0.3296	0.2695
β-D-xylosidase	0.3179	1.0380	0.1322	0.3219	0.1857	0.1008
N-acetyl-α-D-glucosaminidase	0.1512	0.5099	0.1438	0.3513	0.2903	0.1577
N-acetyl-β-D-glucosaminidase	1.4549	4.6107	3.8795	8.9933	1.6176	0.8786

Table 3 compares the total and specific activity of the seven enzymes produced by the *Fusarium* spp. on maize after 15 days at 0.95 a_w. The most important enzymes produced by strains of the two *Fusarium* spp. in terms of total and specific activity were α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase. The other four enzymes examined were all present at levels similar to those of the uninoculated control grain, both in terms of concentration and specific activity.

Interactions between assayed factors

Table 4 shows the analyses of variance of results and the effect of single, two and three-way factors on the total and specific activity of the three enzymes of interest. For α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase, single factors (time -t-; water activity -a_w-; and inoculation treatment -s-), and most of two and three way interactions were significant ($P < 0.01$). All two- and three-way interactions were significant except for $a_w \times s$, and $t \times a_w \times s$ for production of β-D-glucosidase and for $t \times a_w$ for total activity of α-D-galactosidase. This wide significance of interactions means that single factors, besides being significant, have different trends depending on the levels of the other single factors, which makes the results more difficult to be interpreted.

Effect of water activity and time on the three predominant glycosidases produced by the *F. moniliforme* and *F. proliferatum* on maize grain

For the total enzyme activity, Fig. 1 compares the effect of a_w and time on the activity of the three main glycosidase enzymes produced by a strain of *F. moniliforme* (25N) and *F. proliferatum* (131N). In general, activity of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase, all increased with increasing a_w and time. However, a_w had an important influence on activity of these three dominant enzymes produced by both *Fusarium* spp. The α-D-galactosidase activity was least affected by a_w, while N-acetyl-β-D-glucosaminidase and β-D-glucosidase activity was higher at 0.98 a_w, but did not show much difference at 0.95 and 0.93 a_w (Table 5).

For the specific activity, a comparison of the three predominant enzymes is shown in Fig. 2. Considering the whole testing period, optimum activity was at 0.95 a_w for α-D-galactosidase and β-D-glucosidase, while activities were similar at 0.93 and 0.98 a_w. The exception was N-acetyl-β-D-glucosaminidase where specific activity was optimum at 0.98 a_w, and much lower and similar at both 0.95 and 0.93 a_w (Table 5). The maximum activity of this enzyme was found after 6 days and 9 days for *F. moniliforme* and *F. proliferatum*, respectively, at 0.98 a_w. However, at 0.95 and 0.93 a_w the enzyme was produced later.

Strain differences

Table 6 shows the statistical comparison of the total and specific activity of the three major hydrolytic enzymes assayed in this study. Significant differences in the specific activity of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase produced by the *F. moniliforme* strains on maize kernels were observed. Furthermore, *F. moniliforme* isolates had higher β-D-glucosidase and N-acetyl-β-D-glucosaminidase total activity than *F. proliferatum*.

Table 4. Analysis of variance of N-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase total (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein) activity at different levels of incubation time (t), water activity (a_w), and inoculation treatments (s).

			TA		SA	
Enzyme	Factor	DF	MS	F	MS	F
N-acetyl-β-D-glucosaminidase	T	4	130.62	19.66**	140.93	14.14**
	a_w	2	681.55	102.57**	171.68	17.22**
	t × a_w	8	42.49	6.40**	37.91	3.80**
	S	4	182.24	27.43**	336.98	33.80**
	t × s	16	32.65	4.91**	52.96	5.31**
	a_w × s	8	81.87	12.32**	28.57	2.87**
	t × a_w × s	29	24.78	3.73**	23.71	2.38**
β-D-glucosidase	T	4	73.58	35.10**	92.80	15.50**
	a_w	2	112.35	53.59**	48.69	8.13**
	t × a_w	8	9.51	4.54**	16.69	2.79**
	S	4	139.61	66.59**	226.90	37.90**
	t × s	16	15.54	7.41**	24.94	4.17**
	a_w × s	8	26.07	12.44**	8.27	1.38
	t × a_w × s	29	7.49	3.57**	6.72	1.12
α-D-galactosidase	T	4	49.16	23.55**	65.56	20.53**
	a_w	2	22.90	10.97**	51.16	16.02**
	t × a_w	8	3.17	1.52	14.96	4.68**
	S	4	88.79	42.53**	202.61	63.44**
	t × s	16	17.33	8.30**	18.95	5.93**
	a_w × s	8	8.60	4.12**	9.49	2.97**
	t × a_w × s	29	3.83	1.84*	5.79	1.82*

**Significant $P < 0.01$
*Significant $P < 0.05$

F. proliferatum isolates had significantly higher α-D-galactosidase specific activity than those of *F. moniliforme*, while for the other two enzymes there was no significant difference between isolates of the same species.

There was no significant difference between either the total or specific activity of the three enzymes at 0.98 a_w. However, both *Fusarium* spp. produced less of these enzymes at 0.95 and 0.93 a_w, with no significant difference in specific activity of the three enzymes at these two a_w levels.

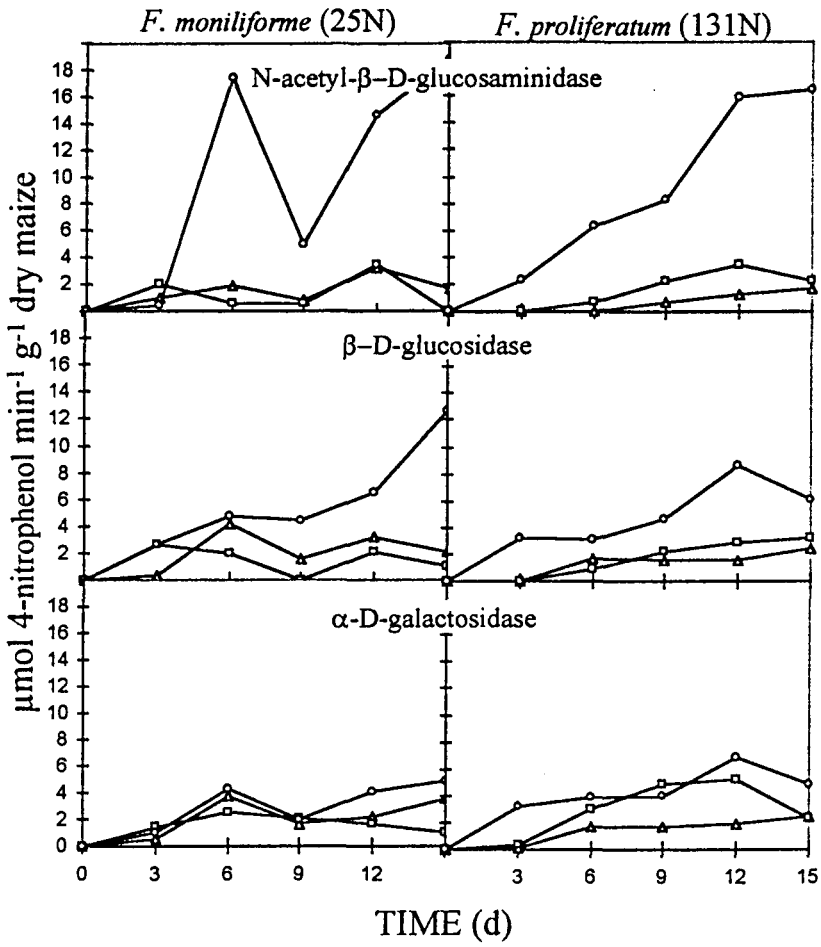

Figure 1. Effect of time and water activity, 0.93 (△), 0.95 (□) and 0.98 (○), in total N-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase activity from *F. moniliforme* (25N) and *F. proliferatum* (131N) inoculated on maize grain. Blanks have been substracted from the results given.

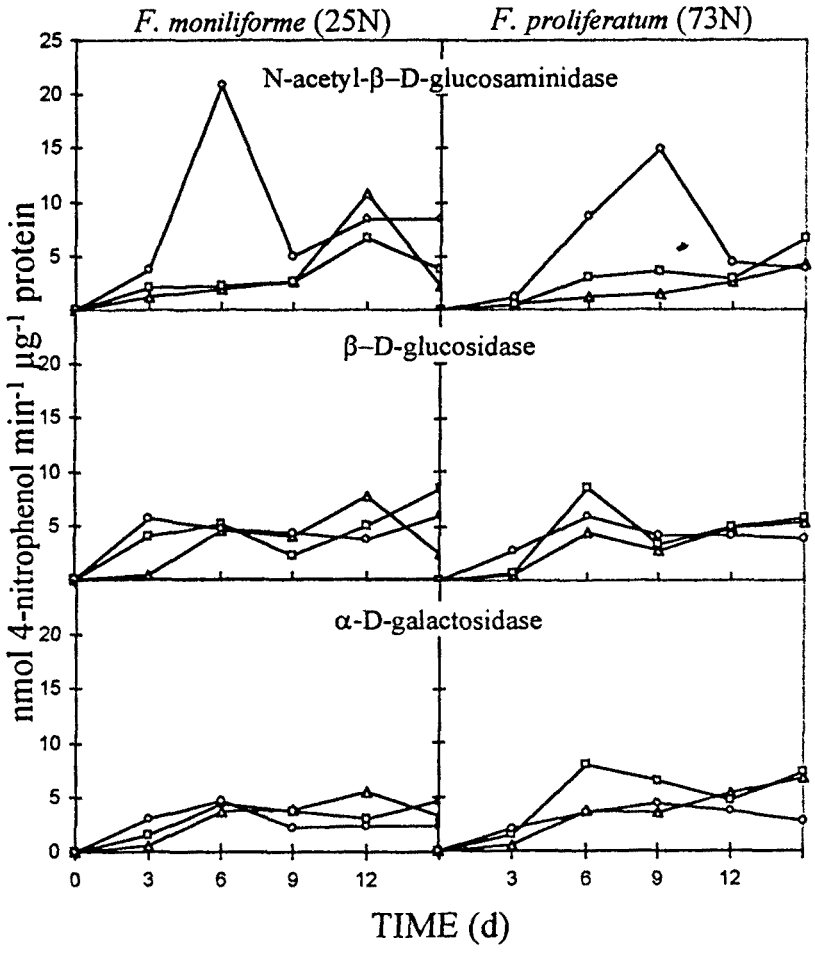

Figure 2. Effect of time and water activity, 0.93 (△), 0.95 (□) and 0.98 (○), in specific N-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase activity from *F. moniliforme* (25N) and *F. proliferatum* (73N) inoculated on maize grain. Blanks have been substracted from the results given.

Table 5. LSD test showing the effects of water activity in N-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase total activity (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific activity (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein).

	N-acetyl-β-D-glucosaminidase			β-D-glucosidase			α-D-galactosidase		
(i) TA	Level	LSD Group	Mean	Level	LSD Group	Mean	Level	LSD Group	Mean
a_w	0.98	A	8.2281	0.98	A	5.0455	0.98	A	3.3387
	0.95	B	3.0213	0.95	B	3.5439	0.95	A	3.0319
	0.93	B	2.2458	0.93	C	1.9651	0.93	B	1.9040
(ii) SA	Level	LSD Group	Mean	Level	LSD Group	Mean	Level	LSD Group	Mean
a_w	0.98	A	6.9092	0.95	A	5.2831	0.95	A	4.3913
	0.95	B	4.1744	0.98	B	3.9898	0.93	B	3.0693
	0.93	B	3.8511	0.93	B	3.3274	0.98	B	2.7670

*Groups with different letters are significantly different ($P<0.05$); UNIN.: uninoculated

Table 6. LSD test showing the differences between species and isolates in N-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase total activity (TA, μmol 4-nitrophenol min^{-1} g^{-1} dry maize) and specific activity (SA, nmol 4-nitrophenol min^{-1} μg^{-1} protein).

	N-acetyl-β-D-glucosaminidase			β-D-glucosidase			α-D-galactosidase		
(i) TA	Level	LSD Group	Mean	Level	LSD Group	Mean	Level	LSD Group	Mean
F. Moniliforme	73N	B	3.6231	73N	C	3.3268	25N	B	2.8157
	85N	A	6.6864	85N	A	6.5070	85N	A	4.1883
F. proliferatun	131N	A	5.4397	131N	C	3.5626	73N	B	3.4306
	25N	A	6.1862	25N	B	4.2209	131N	B	3.4470
	UNIN.	C	1.4446	UNIN.	D	0.8821	UNIN.	C	0.3661
(ii) SA	Level	LSD Group	Mean	Level	LSD Group	Mean	Level	LSD Group	Mean
F. Moniliforme	85N	C	4.9856	85N	B	4.7373	25N	C	3.3931
	73N	C	4.7984	73N	B	4.6026	85N	C	2.9770
F. proliferatun	25N	B	6.3545	25N	B	5.0138	73N	B	4.4931
	131N	A	8.1938	131N	A	6.4496	131N	A	5.9679
	UNIN.	D	0.7857	UNIN.	C	0.4793	UNIN.	D	0.1964

*Groups with different letters are significantly different ($P<0.05$); UNIN.: uninoculated

DISCUSSION

This study has shown that over a wide range of a_w levels *F. moniliforme* and *F. proliferatum* are able to colonise living maize kernels and produce a range of hydrolytic enzymes. The three enzymes with the highest total and specific activity produced during the first 3-9 days were α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase, when compared with uninoculated maize grain. Previous work has shown that both these fumonisin-producing fungi are able to rapidly germinate, and grow over a wide range of temperatures and water availabilities (Marín et al., 1995; 1996) enabling them to compete effectively in this niche.

This study has also shown that more useful information can be obtained by quantifying enzymes produced on the surface of the grain by these pathogens than by grinding samples. This may be particularly so where live irradiated grains are used as opposed to autoclaved dead grain. Irradiated grain may be more realistic as it has retained germinative capacity and some of the natural defences, such as phytoalexins, present in natural harvested grain (Cuero et al., 1986). The high specific activity of the predominant enzymes produced by both *Fusarium* spp., at 0.98-0.93 a_w is indicative of a potential role in the maize infection process.

Fungi have evolved different strategies to invade seeds and other plant tissue. Generally, fungi secrete a cocktail of hydrolytic enzymes including cutinases, cellulases, pectinases and proteases (Knogge, 1996). There are three major types of enzyme required for the hydrolysis of crystalline cellulose to glucose. These are endoglucanases, exoglucanases and β-glucosidases. Penetration of plants may be achieved quite simply by entry through the plant's natural openings or by utilising a route through non-cellulosic areas. It has been shown that *A. flavus* can produce cell-wall degrading pectinases, and may secrete extracellular cutinases to enable effective infection through the pericarp of maize kernels (Guo et al., 1996). There is now evidence that *F. moniliforme* colonises maize grain predominantly during silking but that it is also able to systemically infect seed and be transported within the plant to the next generation (Munkvold et al., 1997). Therefore the production of a range of enzymes is important for effective colonisation of ripening or harvested seeds.

Recently Sala (1993) in complementary work on maize mycoflora investigated *Fusarium* species in a similar way. She found that only 3 *F. proliferatum* isolates out of a total of 13 *F. moniliforme* and *F. proliferatum* strains had α-galactosidase activity, while all of them had β-glucosidase and N-acetyl-β-glucosaminidase activity. The β-glucosidase and N-acetyl-β-glucosaminidase activities were high in general but there were no differences between *F. moniliforme* and *F. proliferatum* isolates. She found no β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase and α-fucosidase in any of the isolates. However, in her studies no detailed quantification of the effects of a_w or time on total or specific activity were carried out. In our experiments all the *F. moniliforme* and *F. proliferatum* isolates used were able to produce α-D-mannosidase on moist maize (0.98 a_w) although in small amounts. In contrast, α-galactosidase activities were higher than suggested by these semi-quantitative studies.

The present study suggests that α-D-galactosidase, β-D-glucosidase, N-acetyl-β-D-glucosaminidase, and, perhaps, β-D-xylosidase may be suitable indicators of *F. moniliforme* and *F. proliferatum* colonisation of maize. In general, expressing total activity in relation to

maize weight may be a more useful and rapid method than measurements of specific activity on a protein basis because the former method is more sensitive in detecting fungal production of enzymes than the latter. Furthermore, the washing and extraction method was more useful for early detection because it enabled quantification of the increase in fungal activity after 3 days. Grinding of samples was less sensitive, but could be useful for examining fungal enzyme production during internal mycelial colonisation of the grain although interference from internal maize enzymes could make interpretation of the data more difficult.

It has been suggested that some of these hydrolytic enzymes may be good indicators of the early colonisation of cereal grain by spoilage fungi, particularly xerotolerant and xerophilic species (Jain and Lacey, 1991; Magan, 1993a, b). Indeed, increases in some of these enzymes were observed prior to any visible moulding had occurred. In the present study on maize, enzyme activity by *Fusarium* spp. from washing extracts showed that N-acetyl-β-D-glucosaminidase, followed by α-D-galactosidase and β-D-glucosidase were important indicators of infection. In experiments with barley and wheat grain, *Penicillium* or *Aspergillus* spp. were found to produce little or no N-acetyl-α-D-glucosaminidase and α-D-glucosidase, while more α-D-mannosidase and β-D-xylosidase activity was generally found, but varied with cereal type. The activity of α-D-galactosidase, N-acetyl-β-D-glucosaminidase, and β-D-glucosidase was always much more greater than that in uninoculated control grain. The latter three enzymes were found to be produced in greatest amounts at 25% moisture content (= 0.98 a_w) after 30 days incubation. They suggested that N-acetyl-β-D-glucosaminidase, α-D-galactosidase, β-D-xylosidase/xylopiranosydase and perhaps β-D-glucosidase would be suitable candidates to detect activity of spoilage by xerophilic fungi, and pointed out that other enzymes (e.g. β-D-xylopiranoside) detected in greater amount in > 25% moisture content grain, might be useful for detecting activity of more mesophilic fungi (Jain and Lacey, 1991). Flannigan and Bana (1980) reported how β-xylosidase and α-glucosidase activities increased with relative humidity (81-92%) of storage atmosphere in extracts from barley inoculated with *A. chevalieri* and stored for 5 months.

This study has shown that some of these hydrolytic enzymes may play an important role in enabling these fumonisin-producing *Fusarium* spp. to rapidly colonise maize grain over a range of water availability conditions. This together with the capability for rapid germination and growth (Marín et. al., 1995; 1996) could partially explain their competitiveness in the maize grain niche and their ability for excluding other fungi once becoming established.

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IMPACT OF *FUSARIUM MONILIFORME* AND *F. PROLIFERATUM* COLONISATION OF MAIZE ON CALORIFIC LOSSES AND FUMONISIN PRODUCTION UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

S. Marín[1], V. Homedes[1], V. Sanchis[1], A. J. Ramos[1] and N. Magan[2]

[1]Food Technology Dept., CeRTA, Universitat de Lleida, Rovira Roure 177, 25198 Lleida, Spain
[2]Applied Mycology Group, Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, U.K.

ABSTRACT

The effect of water activity (a_w, 0.92-0.98), temperature (15-30°C) and incubation period (2-4 weeks) on growth, calorific losses and fumonisin production by one isolate of *Fusarium moniliforme* and two of *F. proliferatum* were studied on maize-based substrates. The calorific losses (kJ) of maize flour due to fungal growth in relation to temperature and a_w were quantified and found to be maximum at 0.98 a_w (17-64 %, after 4 weeks, depending on temperature level) with practically none at 0.92 a_w (0-9%). This suggests that growth of mycotoxigenic Fusarium can contribute to both nutritional losses and toxic contamination of such staple substrates. Total fumonisin concentrations were optimally produced on ground maize at 30°C and 0.98 a_w and were 143.9 μg g^{-1} for *F. moniliforme*, and 51.0-104.3 μg g^{-1} for the *F. proliferatum* isolates, respectively. In general, both fumonisin production and fungal biomass decreased with temperature and a_w. Good correlation was found between fumonisin concentration and fungal biomass, while calorific losses correlated inversely with both fumonisin concentration and fungal biomass. Single, two- and three- and four-way interactions of a_w, temperature, time and isolate were all significant ($P < 0.01$) for calorific losses, and for fumonisin production, with the exception of a_w x temperature x time interaction.

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INTRODUCTION

In many parts of the world, because of poor post-harvest practices, up to 50% of the durable crops may be lost because of mould spoilage and insect damage (Harris and Lindblad, 1978; Sode *et al.*, 1995). Utilisation of nutritionally poor quality staple grains and flour are therefore sometimes a fact of life in many tropical and subtropical regions of the world. However, very little attempt has been made to quantify the nutritional losses in terms of calorific value in such deteriorated staple foodstuffs (Sinha, 1982). Prasad and Prasad (1982) reported changes in calorific value of *Linum usitatissimum* L. due to seed-borne infection by spoilage fungi although the water availability was not determined or controlled. Recently, the contamination and colonisation of maize grain by *Fusarium moniliforme* Sheldon and *F. proliferatum* (Matsushima) Nirenberg has received significant attention because of their pre- and post-harvest colonisation of moist maize and concomitant production of the fumonisin group of mycotoxins (Miller *et al.*, 1995). Indeed, consumption of such spoiled maize has been linked with areas featuring high oesophageal cancer in some parts of the world (Rheeder *et al.*, 1992).

In stored grain ecosystems the most important environmental factors influencing fungal deterioration of grain are the water availability (water activity, a_w) and temperature of storage, as well as the gas composition (Sinha, 1973, 1995; Magan and Lacey, 1988). Recent studies have shown that *F. moniliforme* and *F. proliferatum* isolates can grow over a relatively wide range of a_w levels in moist maize (down to 0.90 a_w = 19% moisture content; Marín *et al.*, 1995a, 1996, 1997). Some work has also shown that both fumonisin B_1 and B_2 production is markedly influenced by a_w and temperature (Cahagnier *et al.*, 1995; Marín *et al.*, 1995b). However, no attempts have previously been made to correlate growth, mycotoxin production and calorific losses in maize or indeed any cereals. Some elegant studies have been made by Sinha and colleagues on the bioenergetics of insects feeding on different grains to quantify losses under different storage conditions (Sinha *et al.*, 1986; White and Sinha, 1987; Demianyk and Sinha, 1988; Sinha, 1995). However, no such considerations have previously been given to calorific losses due to fungal spoilage of staple cereals.

The objectives of this work were to determine in detail the effects of a_w (0.92-0.98), temperature (15-30°C) and time (2-4 weeks) on (a) calorific losses on a coarse maize flour, (b) *in vitro* growth –mycelial dry weight- on a maize extract agar and (c) fumonisin production by *F. moniliforme* and *F. proliferatum* when grown on a coarse maize flour.

MATERIALS AND METHODS

Fungal isolates

One isolate of *Fusarium moniliforme* (25N) and two isolates of *F. proliferatum* (73N, 131N) were used in all experiments. These isolates have previously been shown to be high fumonisin producers in culture and on maize grain (Marín *et al.*, 1995b). The isolates are deposited in the Food Technology Dept. collection of the University of Lleida, Spain.

Maize

Spanish maize grain with an initial water content (wet weight basis) of 13.9% (=0.71 a_w) was ground and sifted to an homogenous particle size (0.45 mm mean diameter).

Ground maize samples were weighed in flasks and rehydrated to the desired a_w treatment levels (0.92, 0.95, 0.98) by the addition of distilled water with reference to a moisture adsorption curve. The maize treatments were allowed to equilibrate at 4°C for 48 h, with periodic shaking. After that, sealed flasks were autoclaved for 20 min at 121°C. Finally, the a_w values were confirmed by using a Novasina Humidat IC I Thermoconstanter (Novasina, Switzerland).

The initial calorific value of ground maize was 19.69±0.13 kJ g^{-1} (4.712 kcal) dry matter of maize flour.

Inoculation and incubation of ground maize

Sterile humidified ground maize was placed in thin layers in sterile Petri plates (12 g/plate). Inoculation of each isolate was made by 17 applications of 8 µl each of a 10^7 spores/ml spore suspension uniformly located all over the plate. Spore suspensions were prepared in glycerol-water solutions at the treatment a_w levels.

The different treatments tested in the experiment were the following: 0.92, 0.95 and 0.98 a_w, incubation temperatures of 15, 20 and 30°C, and incubation periods of 2 and 4 weeks. All treatments were repeated three times.

After inoculation, Petri plates with the same water activity were placed in sealed containers along with 2 beakers containing a 100 ml glycerol-water solution providing the same relative humidity as the enclosed Petri plates (Dallyn, 1978), and incubated. After that, final samples were carefully homogenised, transferred to sealed polyethylene bags, and frozen until analyses were carried out.

Calorific value determination

A Gallenkamp Autobomb calorimeter was used for this purpose. This apparatus measures the heat released by the combustion of a known weight of sample with pressured oxygen. Samples were lyophilised prior to calorific value determination. Samples contained both maize and fungal mycelium, so that results are in practice a slight underestimation of the actual calorific losses in maize. Samples placed in the calorimetric bomb suffer a complete combustion which results in the heating of the water which surrounds the bomb core. From the increase in temperature of the water, the calorific value of the sample can be calculated as follows:

$$[1] Q_{sample} = \{(T_f - T_i) * K\} - Q_c$$

where:

Q_{sample}, kJ released by combustion of sample; T_f, final temperature of the water bath; T_i, initial temperature of the water bath; K, 10.43 kJ/°C (2.494 kcal/°C), constant reference

value previously calculated from combustion of benzoic acid; Q_c, kJ released by the combustion of the cotton threat used as ignition starter.

Fumonisin analyses

Total fumonisin concentration in samples was determined by using a commercial ELISA kit (enzyme-linked immunosorbent assay). RIDASCREEN® Fumonisin Fast is a competitive enzyme immunoassay for the quantitative analysis of fumonisin-residues in corn and corn products. The mean lower detection limit of the RIDASCREEN® Fumonisin Fast test is about 0.2 $\mu g\ kg^{-1}$. According to the test preparation record, the detection limit for fumonisin in corn and corn products is 9 $\mu g\ kg^{-1}$ (Ridascreen, 1995). Extraction and preparation of the samples, as well as test performance were carried out as suggested and described in the kit.

Growth studies

Fungal biomass was quantified as mycelial dry weight. Fungi were grown on maize extract agar modified with glycerol to produce water activities equivalent to those of the ground maize samples (Marín *et al.*, 1995a). The same treatments as for the ground maize experiments were applied with three replicates each. Maize extract agar was inoculated with 136 µl of a 10^7 spores/ml spore suspension and then spread with a bent glass spreader. Petri plates of the same a_w were enclosed in polyethylene bags and incubated. After that, the agar plus growing colonies on its surface was heated and the mycelium was separated from the melted agar. Mycelial mats were lyophilised and weighed.

Statistical treatment of results

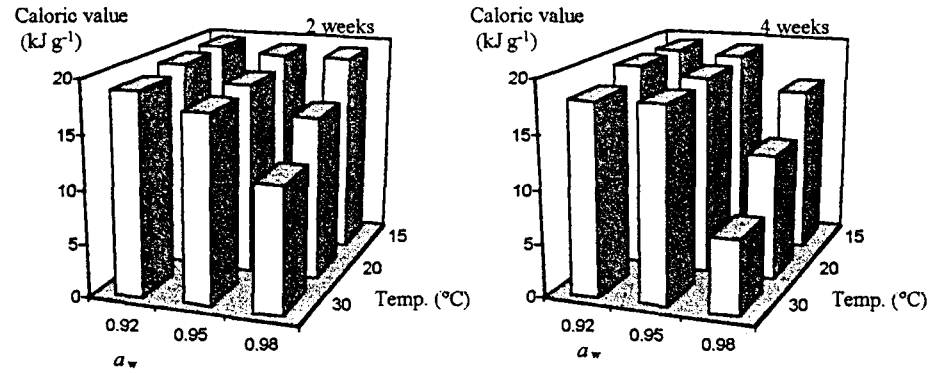
Analysis of variance for the different sets of results were carried out by using the SAS package (version 6.11, SAS Institute Inc.). Ridasoft version 2.0. was used to calculate fumonisin concentration in the samples.

RESULTS

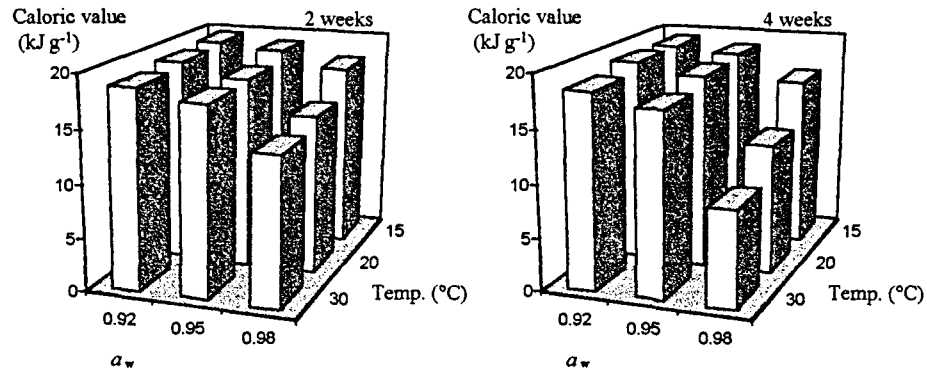
Effect of water activity, temperature and time on calorific losses of maize

Fig. 1 compares the effect of time, a_w and temperature treatments on the calorific losses in $kJ\ g^{-1}$ dry weight maize. This shows that there were significant losses at 0.98 a_w particularly at 20 and 30°C after both 2 and 4 weeks incubation. Losses of 30-65% (4.33-12.74 $kJ\ g^{-1}$) of the initial calorific value were found under these conditions. There were slight losses in calorific value at 0.95 (0.57-2.33 $kJ\ g^{-1}$), but practically none at 0.92 a_w.

Fusarium moniliforme (25N)



Fusarium proliferatum (73N)



Fusarium proliferatum (131N)

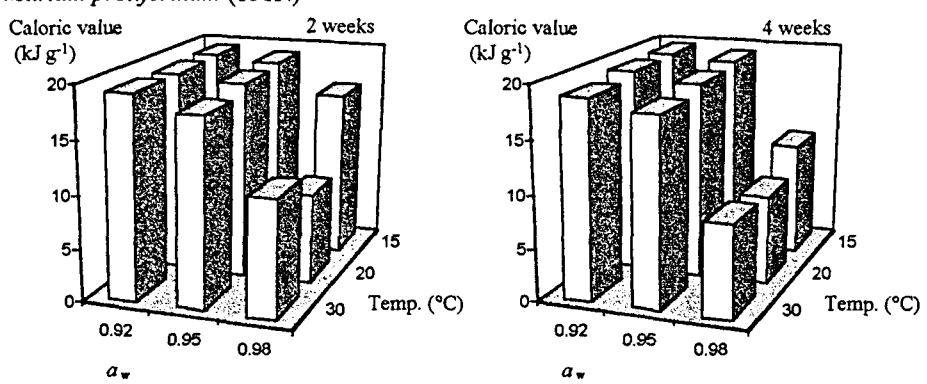


Figure 1. Effect of water activity, temperature and time on calorific losses of maize due to *Fusarium* spp. inoculation.

Table 1 shows that all single-, two-, three- and four-way interactions were statistically significant ($P < 0.01$). Different levels of each factor were significantly different, except for the isolates. Comparison of species and isolates showed that in general there was no difference between the isolate of *F. moniliforme* and 73N of *F. proliferatum*, but that the isolate 131N of *F. proliferatum* caused significantly greater losses in calorific value than the other two when compared to untreated controls.

Effect of water activity, temperature and time on biomass of *Fusarium spp.*

In vitro studies of the growth of these two species and isolates showed that fungal biomass paralleled the results obtained in relation to calorific losses (Fig. 2). Biomass was high in the 0.98 a_w treatment after both two and four weeks growth, and optimum at 30°C (0.478-1.103 g^{-1} dry mycelium). The isolate of *F. moniliforme* and one of the *F. proliferatum* isolates (131N) developed an appreciable biomass at 0.92 a_w at both 20 and 30°C after two and four weeks incubation; indeed isolate 131N was able to generate a measurable amount of mycelium at 0.92 a_w and 15°C. By contrast, isolate 73N of *F. proliferatum* did not develop a significant biomass when compared to the other isolates.

Correlation between increases in biomass and losses in maize calorific value were found to be significant with $P < 0.05$ (Pearson correlation coefficient = -0.7266).

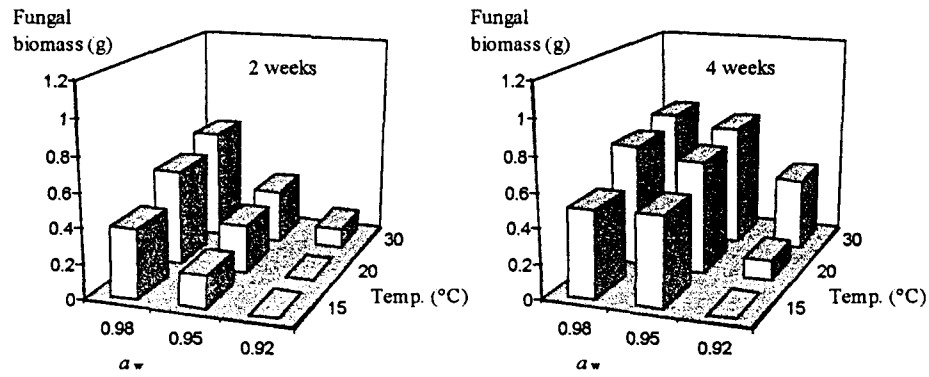
Table 2 shows that all single factors were significant ($P < 0.01$). Different levels of each factor led to results significantly different, even for the isolates. Two-way interactions such as a_w x T, a_w x S and S x t were also significant, where S represents different isolates.

Table 1. Effect of water activity (a_w), temperature (T), incubation period (t) and differences between *Fusarium* isolates (S) on calorific losses of ground maize.

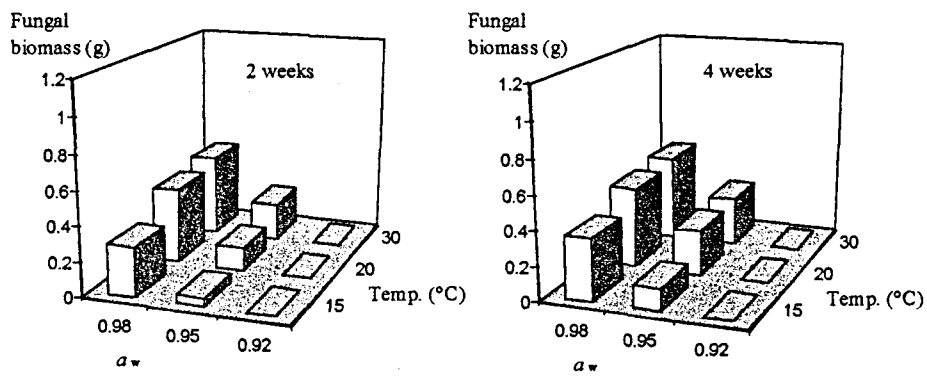
Source	DF	SS	F value
A_w	2	95506411.92	7179.07**
T	2	9158127.47	688.40**
A_w x T	4	7634170.03	286.92**
S	2	1613667.96	121.30**
A_w x S	4	4565064.04	171.57**
T x S	4	1025687.75	38.55**
A_w x T x S	8	1538970.69	28.92**
T	1	2846900.98	427.99**
A_w x t	2	5344649.06	401.75**
T x t	2	437419.70	32.88**
A_w x T x t	4	403417.44	15.16**
S x t	2	86742.56	6.52**
A_w x S x t	4	224082.30	8.42**
A_w x T x S x t	12	1083844.17	13.58**

**Significant P < 0.01

Fusarium moniliforme (25N)



Fusarium proliferatum (73N)



Fusarium proliferatum (131N)

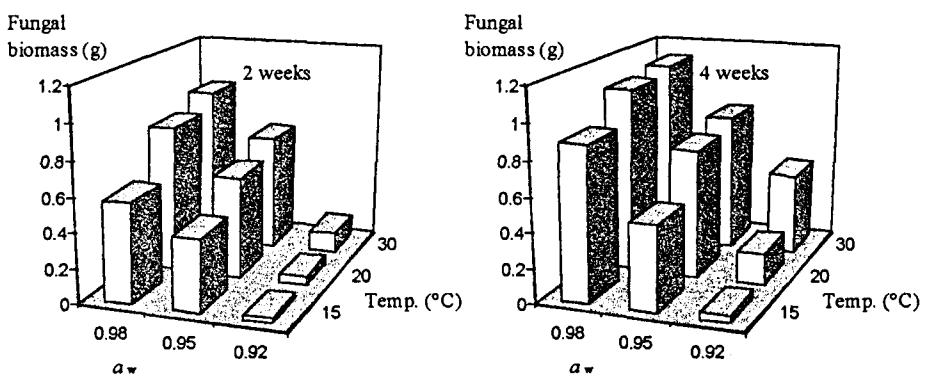


Figure 2. Effect of water activity, temperature and time on biomass of *Fusarium* spp.

Table 2. Effect of water activity (a_w), temperature (T), incubation period (t) and differences between *Fusarium* isolates (S) on fungal biomass generated on maize extract agar.

Source	DF	SS	F value
a_w	2	5.39	386.48**
T	2	0.96	68.80**
a_w x T	4	0.08	2.80*
S	2	3.20	229.48**
a_w x S	3	0.17	8.27**
T x S	4	0.03	1.12
a_w x T x S	5	0.01	0.46
t	1	0.68	98.36**
a_w x t	2	0.04	3.00
T x t	2	0.02	1.38
a_w x T x t	4	0.11	3.98**
S x t	2	0.12	8.56**
a_w x S x t	3	0.13	6.12**
a_w x T x S x t	8	0.03	0.61

*Significant $P < 0.05$

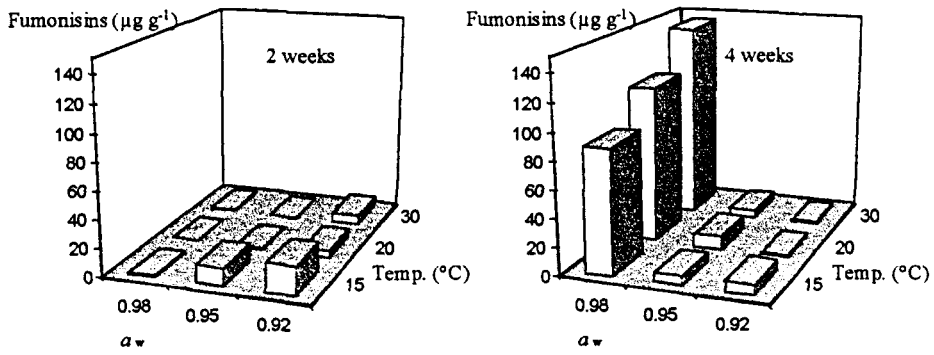
**Significant $P < 0.01$

Effect of water activity, temperature and time on total fumonisin production

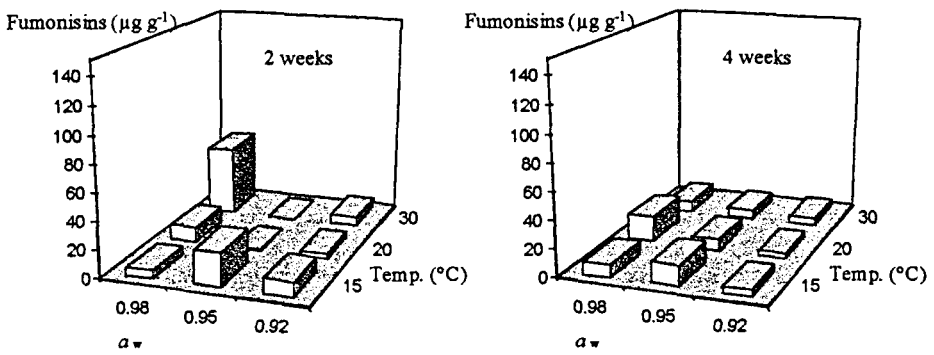
Fig. 3 shows the relationship between treatment factors and total fumonisin production for *F. moniliforme* (25N) and *F. proliferatum* (73N, 131N). Generally, most fumonisins were produced at 30°C and 0.98 a_w for all three isolates examined (0.78-143.95 μg fumonisin g^{-1} dry matter). However, there were interactions between temperature, a_w and storage time. In some cases more fumonisins were produced after two weeks than after four weeks storage, with some breakdown of fumonisins occurring during the latter period especially for *F. proliferatum* (73N). Furthermore, there was an increase in fumonisin production at reduced a_w levels (0.92 and 0.95) at marginal temperatures, particularly 15°C, when compared to those concentrations at higher temperatures and even at higher a_w.

Table 3 shows the statistical effects of single, two, three and four-way interactions. All single factors and most of the interactions were significant ($P < 0.01$). In general, the three water availability levels tested, and times of incubation, led to results significantly different; while for temperatures, there were no differences between means at 15 and 20°C, with 30°C giving the highest fumonisin production for those isolates tested. For the *F. moniliforme* isolate and 131N of *F. proliferatum* there were no significant differences, but they produced much more fumonisins than isolate 73N of *F. proliferatum*. Interestingly, direct correlation was found between fumonisin production and fungal biomass (Pearson correlation coefficient = 0.5023), and inverse correlation between fumonisins and calorific losses (Pearson correlation coefficient = -0.6263), with $P < 0.05$.

Fusarium moniliforme (25N)



Fusarium proliferatum (73N)



Fusarium proliferatum (131N)

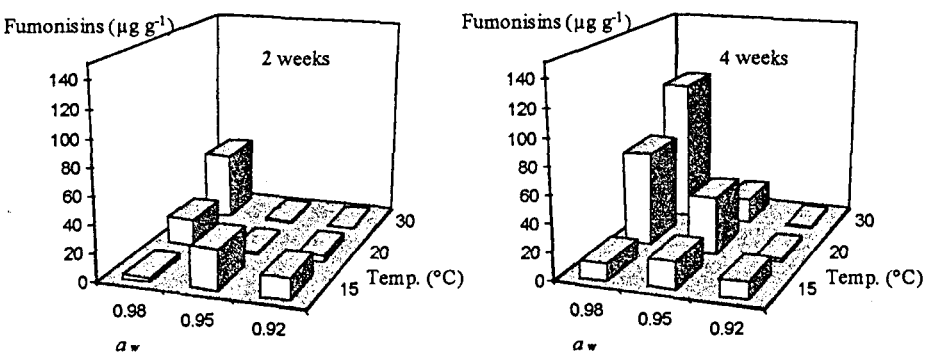


Figure 3. Effect of water activity, temperature and time on total fumonisin production by *Fusarium* spp.

Table 3 Effect of water activity (a_w), temperature (T), incubation period (t) and differences between *Fusarium* isolates (S) on total fumonisin production on ground maize.

Source	DF	SS	F value
a_w	2	2.30 E+10	215.44**
T	2	9.18 E+08	8.58**
a_w x T	4	1.02 E+10	48.03**
S	2	3.54 E+09	33.12**
a_w x S	4	7.24 E+09	33.82**
T x S	4	6.18 E+08	2.89*
a_w x T x S	8	2.38 E+09	5.56**
T	1	7.39 E+09	138.09**
a_w x t	2	1.28 E+10	119.63**
T x t	2	1.72 E+09	16.10**
a_w x T x t	4	4.14 E+08	1.93
S x t	2	7.24 E+09	67.65**
a_w x S x t	4	1.65 E+10	77.15**
a_w x T x S x t	12	3.99 E+09	6.23**

*Significant $P < 0.05$
**Significant $P < 0.01$

DISCUSSION

This study is one of the first of its kind to try to quantify the relationship between storage regimens, fungal colonisation, mycotoxin production and potential calorific losses from a staple cereal such as maize. Stored grain systems can be considered a food-web involving the producers (grain), the primary and secondary consumers (particularly insects) and the spoilage organisms (fungi, bacteria and actinomycetes). Thus, when grain or flour is consumed, part of the energy is assimilated for the production of biomass in the consumer, while part is lost as by-products through respiration and the production of carbon dioxide and water. Sinha (1982) stated that, to understand the bioenergetics of storage agricultural substrates, the energy flow from the grain to consumers needed to be integrated since insects, mites, and fungi all caused nutrient losses (vitamins, lipids, proteins and carbohydrates), functional and aesthetic changes, and produced toxic metabolites.

A study by Prasad and Prasad (1982), showed that the calorific losses caused by six different seed-borne fungi on linseed varied with time at 28°C. There was a significant decrease in the initial calorific value (1.896 kJ; 7.9348 kcal) g^{-1} seed. *Aspergillus niger* Van Tieghem infection resulted in maximum calorific losses (25.2% within 15 days, and 49.28% within 30 days) on surface-sterilised seeds, while losses were higher on autoclaved seeds, especially due to *F. oxysporum* Schlecht. (47.06% and 54.68%, respectively). Unfortunately the water availability was not monitored or controlled in their study making comparisons with our study more difficult. However, the calorific losses reported are comparable to those obtained in the present study for *F. Liseola* species but only at very high a_w levels > 0.98 a_w, which is optimum for growth of these fungi.

Studies of the energy flow and calorific losses from grains to different developmental stages of some insect pests have been elegantly demonstrated by White and Sinha (1981, 1987), Sinha *et al.* (1986) and Demianyck and Sinha (1988). However, such studies with fungi are more difficult because of the inherent difficulty of separating the fungal biomass from the substrate. In this study the data have been presented on a dry weight basis so that the loss in

actual dry matter through respiration as well as the residual losses in nutritional components accounted for of in the calculations. The only factor not included was the actual fungal biomass present in the maize substrate. Thus the actual losses were probably slightly underestimated. However, the marked decrease in total calorific value in the maize substrate, especially at 0.98 and 0.95 a_w and 15-30°C suggests that mycotoxigenic fungi such as *F. moniliforme* and *F. proliferatum* not only contaminate such substrates but also directly affect the nutritional value for human consumption. Losses due to fungal activity in grain have been extensively examined in wheat and maize, by analising dry matter losses but not over a range of a_w levels, particularly in maize. Such bioenergetics data have been obtained and used in a simulation model of the insect pest *Cryptolestes ferrugineus* (Stephens) to predict activity on a species basis which can give detailed and accurate knowledge of the activity of individual species under different environmental conditions (Campbell and Sinha, 1990). Indeed, the present study has demonstrated that there are marked isolate differences in the two fumonisin producing *F. proliferatum* isolates examined. For example, isolate 131N appears to consume a lot of energy, producing a high biomass although it is slow growing (Marín *et al.*, 1995a).

This study found good correlation between calorific losses and total fumonisin production and biomass for the isolates examined. The effect of a_w on concentrations of fumonisins produced was profound. Previously, we demonstrated that on autoclaved sterile maize the ratio of fumonisin B_1 to B_2 was modified with a_w and temperature (Marín *et al.*, 1995b). Results obtained in that and other by (Cahagnier *et al.*, 1995) on whole autoclaved maize showed higher fumonisin concentrations than those obtained on ground autoclaved maize in the present one.

Previously, studies have been carried out on the dry matter losses of different cereals due to fungal activity (Kreyger, 1972; White *et al.*, 1982; Seitz *et al.*, 1982; Lacey *et al*, 1994). Losses result from the utilisation of carbohydrate during fungal metabolism and grain respiration. Estimates differ for the amounts of allowable dry matter losses before grain is rejected for human or animal consumption. In high moisture maize (25% m.c.) a loss of 0.5% dry matter can occur in 7 days, sometimes without any visible moulding. However, this was found to be sufficient to render maize grain unfit for use, and to also contain aflatoxins. Kreyger (1972) considered grain to be fit for animal feed with dry matter losses of up to 2%. However, Hall and Dean (1978) suggested 1% dry matter loss was acceptable in grain for food use and that this could be applied to both wheat and maize. However such studies have not considered implications for deterioration in nutritional value on a calorific basis. Different grain types all have an inherent but different calorific value (Sinha, 1982). A 1% dry matter loss in maize would represent a decrease of 0.112 kJ (0.047 kcal) g^{-1}. This must be an underestimate as we found much larger calorific losses at 0.98 and 0.95 a_w after two weeks storage. However, nutritional losses from a maize meal flour may be greater than that from whole undamaged kernels. Although ergosterol has been used to quantify fungal activity in high moisture maize its relationship varies with fungal species and thus is difficult to use as an overall marker of the activity of fungi in calculations of calorific values and in the development of energy budgets. This work has demonstrated that colonisation of maize by fungi such as *Fusarium* spp. can result in both significant calorific losses at > 0.95 a_w and also significant contamination with fumonisins which would have a marked impact if used for either human or animal consumption. More work is now needed to quantify the calorific losses which can occur when staple grains are colonised by more xerophilic and xerotolerant spoilage fungi, particularly *Aspergillus* and *Penicillium* spp.

ACKNOWLEDGEMENTS

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DISCUSIÓN GENERAL

GENERALIDADES

La base de partida para el desarrollo del presente trabajo son los estudios previos realizados por Sala (1993) en los cuales se determinó la incidencia de *Fusarium* en muestras de cereales y piensos destinadas a la alimentación animal en Catalunya, así como la capacidad toxigénica de las cepas aisladas de las mismas. En dicho estudio se comprobó que 14 de 15 muestras de maíz presentaban contaminación por *Fusarium*, mientras que las proporciones eran 3/8, 7/17, 14/17 y 9/24, para trigo, cebada, sorgo y piensos, respectivamente

Se aislaron un número importante de cepas de *Fusarium* (4 de trigo, 76 de maíz, 5 de cebada, 11 de sorgo y 4 de piensos), a partir de las muestras analizadas, de las cuales, para el caso de trigo, maíz y piensos, porcentajes cercanos al 100% pertenecieron a las especies *F. moniliforme* y *F. proliferatum*, mientras que para cebada, sorgo y colza, los porcentajes de especies de *Fusarium* pertenecientes a la sección Liseola fueron de 60, 63 y 29%, respectivamente.

El 66% de las cepas de *F. moniliforme* resultaron productoras de FB_1, y el 62% de FB_2, mientras que para *F. proliferatum* los porcentajes fueron de 52% y 47%, respectivamente. De las 30 cepas de *F. moniliforme* que resultaron productoras, 21 provenían de maíz, 5 de sorgo, 1 de trigo, 1 de pienso y 2 de colza; las de *F. proliferatum* se distribuyeron en 22 de maíz, 2 de cebada y 1 del resto.

Asimismo se ha comprobado la incidencia de *Fusarium* y fumonisinas en productos para la alimentación animal y humana (Sanchis *et al*, 1994, 1995; Castella, 1997). La incidencia de *Fusarium* en las muestras de maíz, granos rotos y pienso a base de maíz osciló entre el 88-100%. Por lo que respecta a la contaminación por fumonisinas, 12 de 17 muestras de maíz, 14 de 15 de granos rotos de maíz y 18 de 18 muestras de piensos presentaron fumonisinas en concentraciones que oscilaron entre 650 y 920 ng g^{-1}. En las muestras para alimentación humana, 3 de 15 muestras de maíz triturado, 2 de 12 de copos de maíz, 2 de 11 de snacks, 0 de 9 de maíz tostado y 1 de 3 de harina de maíz resultaron contaminadas por fumonisinas, con concentraciones entre 60 y 130 ng g^{-1} (Sala, 1993).

De las 7 especies de *Fusarium* con conocida capacidad para producir fumonisinas, sólo las pertenecientes a la sección Liseola, y especialmente *F. moniliforme* y *F. proliferatum* se han asociado con la contaminación masiva de productos agrícolas por estas micotoxinas (Sala, 1993; Castella, 1997). Esta es la razón por la cual la presente tesis se centra en el estudio de cepas pertenecientes a estas dos especies. Dado que la fumonisina B_1 es la que se encuentra más frecuentemente y en mayores concentraciones, y se ha asociado comunmente a patologías en animales y humanos, los estudios de fumonisinas se han orientado mayormente a su determinación.

Por otra parte los experimentos se realizaron utilizando el maíz como substrato, puesto que el maíz y sus derivados son casi de forma exclusiva los productos en los que se han detectado fumonisinas de forma natural, y por lo tanto conlleva un riesgo para la salud humana y animal, con las consiguientes pérdidas económicas asociadas.

CAPACIDAD TOXIGÉNICA DE CEPAS DE *FUSARIUM* PRODUCTORAS DE FUMONISINAS EN CEREALES DIFERENTES DEL MAÍZ. ESTUDIO COMPARATIVO

El maíz es siempre la base de los productos en los que se ha encontrado fumonisinas de forma natural hasta la fecha. Únicamente, como casos excepcionales, se han hallado en unos derivados de avena en Brasil (Sydenham *et al.*, 1992), un forraje en Nueva Zelanda (Scott, 1993) y en muestras de sorgo en la India (Shetty y Bhat, 1997). También se ha señalado la existencia de pequeñas cantidades en trigo y cebada en España (Castella, 1997).

En principio, se podrían apuntar diferentes causas de la baja incidencia natural de fumonisinas en cereales tan importantes en la alimentación humana como son el trigo y la cebada:

- La baja incidencia de cepas de *Fusarium* en estos cereales, comparada con la del maíz (Sala, 1993)
- La flora acompañante propia de estos cereales impida la proliferación y producción de fumonisinas por estas cepas, o bien sea capaz de degradar estas micotoxinas una vez éstas han sido producidas

Sin embargo, se ha demostrado que cepas altamente toxigénicas sobre maíz son incapaces de sintetizar fumonisinas sobre cebada y trigo, bajo condiciones análogas y en ausencia de flora acompañante. Asimismo, ha quedado demostrada la capacidad de las mismas cepas para crecer sobre estos cereales tal y como lo hacen sobre maíz (C-IV). Por lo tanto, un nuevo factor posiblemente ligado a la diferente composición de estos cereales se añade a las hipótesis expuestas con anterioridad. Posibles explicaciones de este efecto podrían ser:

- Existe algún componente inhibidor en trigo y cebada, o estimulador en maíz de la síntesis de FB_1
- Un bajo ratio proteína/carbohidratos favorece la toxicogénesis de algunas micotoxinas (LeBars, 1988); en este caso el ratio del maíz es ligeramente más bajo que el del trigo y la cebada

Finalmente, hay que tener en cuenta que las cepas utilizadas en el estudio se aislaron de maíz y podrían tener un problema de adaptación a cereales diferentes de éste. Por otra parte, se sabe que la irradiación produce en el maíz uno o varios compuestos inhibitorios de la síntesis de aflatoxinas (Smith *et al.*, 1987); el efecto de la irradiación sobre la posterior síntesis de fumonisinas no ha sido estudiado, sin embargo, de existir, podría tener consecuencias diferentes en los diferentes cereales.

CONDICIONES QUE PERMITEN LA GERMINACIÓN, CRECIMIENTO Y PRODUCCIÓN DE FUMONISINAS DE *FUSARIUM MONILIFORME* Y *FUSARIUM PROLIFERATUM* EN MAÍZ

Germinación

La disponibilidad de agua en el protoplasto es esencial para la germinación, dado un nivel de agua suficiente, el resto de factores pasan a tener un mero efecto regulador.

Fusarium spp. puede estar presente en un substrato durante períodos largos de tiempo, durante los cuales la a_w puede cambiar. Es importante, por lo tanto, conocer las condiciones óptimas de a_w para la germinación y crecimiento, así como el intervalo que permite crecimiento sub-óptimo (Magan y Lacey, 1984c).

La germinación fue posible en el rango 5-37°C. La a_w mínima para germinar se dio a 0,88 a_w a la temperatura óptima de 25°C. El 100% de esporas de dichas especies germinaron antes de 24 horas a 0,98-0,994 a_w y 25°C, mientras que la fase de latencia previa a la germinación se fue alargando conforme la a_w disminuyó o la temperatura se alejó de la óptima (C-V). La capacidad para germinar rápidamente puede suponer una ventaja de cara a la competencia entre especies para colonizar el ecosistema, estableciéndose y tomando recursos de forma rápida.

La duración de nuestros experimentos en germinación fue de 40 días (C-V), sin embargo se ha visto que los microconidios de *F. moniliforme* pueden sobrevivir hasta 900 días a humedades relativas (HR) de 33% y 75% y a temperaturas bajas, de 5-15°C, y 5°C, respectivamente; mientras que a HR de 86-100% y 15-25°C y 100% y 5 y 35°C los microconidios no sólo sobrevivieron, sino que germinaron antes de los 900 días (Liddell y Burgess, 1985). Estos datos dan una idea del potencial de estas cepas para sobrevivir en el grano almacenado y causar problemas posteriores si se da una rehumidificación del mismo por cualquier causa. Si la humedad del grano aumenta puntualmente de 14 a 17% (0,72 a 0,88 a_w aprox.) debido a la fluctuación de la temperatura, sólo son necesarios 2-4 días para que se inicie la germinación de las esporas de *Fusarium* que se hallan presentes de forma abundante por norma general; si el aumento es hasta 0,90 a_w la germinación se da en menos de 2 días. Los recuentos fúngicos (ufc g^{-1}) revelan niveles altos de *Fusarium* en grano almacenado, sin embargo unas condiciones correctas de almacén no son convenientes para su proliferación. Por otra parte la presencia de otras cepas contaminantes pueden modificar dicha cinética (C-XIII).

Se ha propuesto el uso de la duración de la fase de latencia como índice del potencial para desarrollarse de un determinado moho bajo unas determinadas condiciones, dado que en

general una fase de latencia larga implica un crecimiento posterior lento y a la inversa. Sin embargo, para las cepas de *Fusarium* utilizadas en el presente estudio se ha comprobado la existencia de correlación entre la velocidad de crecimiento y el inverso de la fase lag (C-IV, V) a todas las temperaturas y a_w, excepto a 37°C, donde el crecimiento no fue posible pero la fase de latencia previa a la germinación fue corta. Dicha afirmación corrobora la señalado por Ayerst (1969) para una serie de especies fúngicas para las cuales, tras una fase de latencia dada, el crecimiento parecía ser más rápido a temperatura baja que a alta; por otra parte, a temperatura cercana a la máxima, la germinación se daba de manera normal, pero el crecimiento iba siendo cada vez más lento hasta que cesaba (Ayerst, 1969).

Crecimiento

El rango de temperaturas en el que se detectó crecimiento fue entre 4 y 37°C. El rango de a_w que permitió el crecimiento fue más restringido que aquel que permitió la germinación. Ambas especies fueron capaces de germinar a 0,88 a_w a la temperatura óptima, mientras que el crecimiento se vio restringido a a_w iguales o mayores de 0,90, ambos sobre agar extracto de maíz. En este caso, el óptimo también se dio a la máxima a_w de las ensayadas (0,994) (Marín *et al.*, 1995; C-IV).

Antes del descubrimiento de las fumonisinas Joffe *et al.* (1993) habían establecido una temperatura óptima para el crecimiento de cepas de *F. moniliforme* de 22,5-27,5°C, con el máximo entre 32 y 37°C y el mínimo entre 2,5 y 5°C. No se daba crecimiento por debajo de -150 bares (0,90 a_w aprox.) de potencial osmótico y el óptimo estaba en -10 bares (0,993 a_w aprox) (Wilson *et al.*, 1975). Lacey (1989) en una revisión sobre las necesidades hídricas de *Fusarium*, observó crecimiento en el rango de temperaturas entre 2 y 37°C, y a a_w mínima de 0,87 a_w. Magan y Lacey (1984c) encontraron que cepas de *Fusarium* spp. crecían sobre agar extracto de trigo a partir de 0,89-0,90 a_w. La a_w óptima estaba en 0,995 a_w, independientemente de la temperatura. El perfil de respuesta a la a_w /temperatura fue similar en nuestros resultados (C-IV), siendo el rango de velocidades mostrado por *Fusarium* Liseola similar al de *F. culmorum* o *F. poae* (hasta 7 mm d^{-1} de velocidad), aunque superior a los de *F. avenaceum* y *F. tricinctum* (hasta 5 mm d^{-1}). La tendencia fue muy similar en los diferentes cereales ensayados, sin embargo el crecimiento a 5-10°C fue ligeramente más rápido sobre agar extracto de trigo, a 25-30°C más rápido en cebada, y por último a 37°C más rápido en maíz (C-IV).

El crecimiento fue óptimo a 30°C, seguido de 25, 15 y 10°C (C-IV). Alberts *et al.* (1990) determinaron los niveles de ergosterol como medida del crecimiento en cultivos de *F. moniliforme* sobre maíz autoclavado y en condiciones de a_w cercana a 1. Tras 2 semanas de incubación (similar al período para determinar la velocidad de crecimiento en nuestro caso), la concentración de ergosterol fue mayor a 30°C, seguida de 25°C y 20°C; podría ser que en los primeros estadios el crecimiento se viera favorecido a 30°C, y por el contrario, más tarde se situara en 20-25°C. A las 4 semanas la concentración a 20 y 25°C fue similar, y a 30°C, menor. A partir de las 6 semanas, el nivel de ergosterol ya no aumentó a 30°C, mientras que aumentó lentamente a 20 y 25°C. La humedad no se controló en el experimento, con lo cual ésta fue disminuyendo a lo largo del mismo.

Mientras que el descenso de a_w de 1 a 0,90, parece no afectar mucho a los recuentos detectados en muestras de maíz autoclavado inoculado con *F. moniliforme*, la reducción a 0,85 a_w hace que los recuentos se mantengan en el nivel inicial a lo largo del tiempo. A 1 y

0,95 a_w la produccion de esporas es casi idéntica, y un poco inferior a 0,90 a_w. Sin embargo, la producción de ergosterol en el mismo experimento resultó verse muy influida por la a_w. La producción de biomasa (ergosterol) fue idéntica a 1 y 0,95 a_w, pero se vio considerablemente retardada en el tiempo a 0,95 a_w. A 0,90 a_w, la fase estacionaria de crecimiento se alcanzó a los 20 días; la biomasa formada fue 5,5 y 2 veces menor que aquella a 1 y 0,95 a_w, respectivamente. A 0,85 a_w no se detectó ergosterol (Cahagnier *et al.*, 1995)

De estos resultados se deriva que las especies de *Fusarium* pertenecientes a la sección Liseola presentan una diferencia, básicamente con respecto a los requerimientos hídricos, comparadas con otras especies del mismo género. Así, a pesar de que la clasificación en mohos de campo y mohos de almacén de Christensen y Kaufmann (1974), identifica a *Fusarium* como hongo de campo, especies como las aquí tratadas, con requerimientos hídricos menores que otras típicas de cereales de zonas húmedas, como *F. graminearum* o *F.culmorum*, podrían colocarse en un grupo intermedio, tal y como sugirió Pelhate (1968), dado que a pesar de tener el óptimo a la máxima a_w, son capaces de crecer relativamente bien a a_w de 0,92-0,95. *F. moniliforme* ha sido definido como hongo xerófilo, junto con la mayoría de hongos imperfectos, dada su capacidad para crecer alrededor de los –200 bares (=0,87 a_w, aprox.) (Woods y Duniway, 1986).

En el campo, se da un periodo de 10-20 días durante los cuales los granos de maíz en periodo de maduración pasan de >50% (=1,00 a_w) hasta 20% (0,90 a_w) durante el cual son susceptibles a la proliferación de *Fusarium* sobre los mismos (Sutton, 1982). Este es un tiempo más que suficiente para permitir la germinación y establecimiento de *F. moniliforme* y *F. proliferatum* en el maíz antes de la cosecha. En condiciones de sequedad o de bajas temperaturas, las fases de latencia de dichas cepas se alargan, siendo en las condiciones límites mencionadas, incapaces de iniciar la germinación. Por otra parte las temperaturas en el periodo previo a la recolección pueden ser cercanas al óptimo para el desarrollo. El período transcurrido entre la recolección y el secado, que debe ser lo más corto posible, será crucial para la calidad del grano. Una vez en el almacén, ya seco, la temperatura varía a lo largo del tiempo, pudiendo provocar condensaciones de agua sobre el grano, y creando nuevas oportunidades a la proliferación. La acción de otros microorganismos, artrópodos e insectos, y la propia respiración del grano, son otros posibles desencadenantes de la rehumidificación del grano.

Producción de fumonisinas

Las condiciones a_w /temperatura que permiten el crecimiento no son las mismas que aquellas que conllevan la producción de fumonisinas (C-VI), tal y como demostraron Northolt y Bullerman (1982) para la toxigénesis de cepas de *Aspergillus* y *Penicillium* y Magan *et al.* (1984a) para *Alternaria alternata*. El rango de temperaturas en el que se produjo FB_1 fue más reducido que el que permitió germinación y crecimiento; mientras que la reducción del rango de a_w fue todavía más acusada, básicamente para *F. moniliforme* (C-VI). Muchos estudios en ecología de cepas micotoxigénicas se han llevado a cabo sobre medio sintético, sin embargo se ha demostrado que algunas toxinas pueden ser producidas a a_w más bajas en alimentos, de las que se han fijado usando medios sintéticos (Patterson y Damoglou, 1986).

La producción de fumonisina B_1 en maíz irradiado se dio básicamente en el intervalo 10-30°C, mientras que a 7°C no se detectó FB_1 (límite de detección, 0,1 ppm) y a 37°C, únicamente la cepa de *F. moniliforme* fue capaz de producir. La producción óptima fue a

15°C para *F. proliferatum* y a 20-30°C para *F. moniliforme* (C-VI). En general, las cepas de *F. proliferatum* mostraron una mejor adaptación a temperaturas bajas en todas las etapas de desarrollo. La producción de FB_1 por *F. moniliforme* MRC 826 sobre maíz autoclavado a a_w=1 aprox. fue significativamente mayor a 25°C que a 20°C entre las semanas 3 y 7 (Alberts *et al.*, 1990), y en nuestro caso el máximo para *F. moniliforme* (25N) se dió a 30°C a la mayor a_w estudiada (0,97 a_w) (C-VI). La producción a 20 y 25°C comenzó a las 2 semanas y continuó aumentando durante la fase estacionaria confirmando la relación inversa entre el crecimiento miceliar y la producción de metabolitos secundarios (Alberts *et al.*, 1990). Sin embargo, en nuestros experimentos realizados a 2 y 4 semanas, la concentración a 2 semanas no fue siempre menor que aquella a 4 (C-X, XV). La concentración de FB_1 empezó a disminuir a las 13 semanas. La máxima concentración se obtuvo a las 11 o 13 semanas y estuvo alrededor de 17900 ppm (base seca); se concluyó que las condiciones más efectivas para producción de FB_1 en laboratorio eran 7 semanas a 25°C, sin embargo la a_w no fue tenida en cuenta (Alberts *et al.*, 1990). Los períodos de incubación utilizados en laboratorio para la producción de FB_1 están entre 21 y 30 días habitualmente (Bacon y Nelson, 1994), razón por la cual se eligió un período de incubación de 28 días en la mayoría de experimentos (C-IV, VI, XII).

También en maíz autoclavado, y a a_w=1 (aprox.), se ha demostrado que tras 10 semanas la producción de FB_1 por una cepa de *F. moniliforme* es máxima a 20°C (1900 ppm aprox, tras 5 semanas), seguida de 25, 15, 30 y 10°C (LeBars *et al.*, 1990). A 35°C, a pesar de su habilidad para crecer, no se detectó FB_1 (límite de detección, 3-5 ppm). El crecimiento se dio entre 5 y 35°C, mientras que la producción de FB_1 se vio restringida a 13-28°C, aprox. (los autores consideraron que la producción era significativa si era aprox. > 200 ppm). En esta parte del estudio la a_w no fue controlada. Sin embargo, se realizó un estudio paralelo a 20°C, y humedades de 27, 32, 36 y 39%, y se determinó la concentración de FB_1 después de 2 y 3 semanas; la máxima producción se dio a 32% (0,98 a_w, aprox) (300 ppm aprox). A diferencia de lo que ocurre con otras toxinas, (aflatoxinas, ocratoxina A,..) la producción no aumentó con el contenido de humedad hasta el 50%, esto puede ligarse con el hecho de que el estado morfológico y fisiológico del hongo depende de la a_w, y este a su vez influye sobre la capacidad de producción, así el crecimiento miceliar es menos toxigénico que el desarrollo de esporodoquios (Nelson *et al.*, 1991). Como en nuestro caso, vieron como la concentración total transcurridas 3 semanas era menor que aquella al cabo de 2 semanas; ello fue atribuido a la disminución de oxígeno presente durante la incubación. Además sugirieron que el moho degradaba su propia toxina una vez producida (LeBars *et al.* 1994).

La producción aumentó con la a_w hasta el mayor de los valores ensayados: 0,97 a_w. Entre 0,95-0,97 a_w, se produjeron cantidades de FB_1 considerables: entre 2 y 2861 ppm para la cepa de *F. moniliforme* y entre 75 y 17627 ppm para la de *F. proliferatum*. A 0,93 a_w se encontró 3 ppm FB_1 en el caso de *F. moniliforme*, mientras que *F. proliferatum* produjo concentraciones entre 20 y 70 ppm (C-VI). Por debajo de 0,93 a_w no se detectó FB_1 en ningún caso. La biosíntesis de fumonisina por *F. moniliforme* en maíz depende fuertemente de la a_w. Para un mismo grado de crecimiento medido en términos de concentración de ergosterol, la biosíntesis de fumonisina decreció 3 veces cuando la a_w se redujo de 1 a 0,95 a_w (Cahagnier *et al.*, 1995). A 0,90 a_w donde el crecimiento aun fue importante, la producción de fumonisina fue siempre menor de 10 ppm. No se detectó fumonisina a 0,85 a_w. Tras 8 días de incubación a una a_w de 1, se sintetizaron 500 ppm de fumonisina B_1, mientras que eran necesarios 30 días para conseguir este valor a 0,95 a_w. La producción a los 21 días a 1,00 a_w era 3000 ppm aprox, a los 28 d a 0,95 a_w era 400 ppm aprox, a los 30

días y 0,90 a_w era 10 ppm aprox y a los 20 días a 0,85 a_w, 0,1 ppm (Cahagnier *et al.*, 1995). La concentración máxima acumulada fue menor que la obtenida en maíz irradiado, tendencia que coincide con lo que ocurre para el caso de las aflatoxinas, como se discutirá posteriormente.

Se sabe que la presencia de fumonisina B_1 en maíz se ve incrementada drásticamente (hasta 10 veces) en muestras que provienen de zonas afectadas por lluvia, comparadas con muestras de zonas menos lluviosas (Shetty y Bhat, 1997).

Consecuentemente, entre la maduración en el campo y el secado definitivo, se dan toda una serie de oportunidades para la producción de FB_1, dado que ésta es posible en un amplio rango de condiciones. Se ha apuntado que la producción de FB_1 en almacén es poco probable (Munkvold *et al.*, 1997), sin embargo, el hecho que hasta niveles cercanos a 0,90 a_w, un incremento muy pequeño de humedad conlleve un aumento grande del valor de a_w, puede ser un punto a tener en consideración. Finalmente, un factor básico a tener en cuenta es la presencia del resto de microflora propia del grano, aspecto que se tratará más adelante.

Sobre medio de cultivo líquido se ha visto que *F. proliferatum* produce cantidades máximas de FB_1 a pH entre 3,7 y 4,2, mientras que por otra parte por encima de 5 la producción de fumonisina B_1 baja en picado. El efecto del pH sobre el crecimiento (medido como micelio seco), y la producción de fumonisina fue inverso por encima o por debajo de pH=5. El maíz tiene un pH ligeramente superior a 5,6, con lo cual, en principio restringiría la producción, sin embargo en los experimentos con conservantes el pH se redujo hasta 4,7-4,8, sin que hubiera aumento de la concentración (C-XII); sin embargo hay que tener en cuenta el balance entre la acción conservante del propionato y la estimuladora del pH (Keller *et al.*, 1997).

Bajo condiciones óptimas de incubación, los miembros de la 'mating population (MP)' A produjeron en maíz autoclavado entre 0,01-3,99 mg g^{-1} (media 2 mg g^{-1}), los de la D, 0,03-3,15 mg g^{-1} (media 1,69 mg g^{-1}). A la vista de estos resultados se puede afirmar que, no hubo diferencias significativas entre las cantidades de fumonisinas producidas por ambas MP, mientras que cantidades muy pequeñas fueron producidas por los miembros de la MP E (Chulze *et al.*, 1998). De manera similar a lo que ocurre en España, en Argentina las especies responsables de la contaminación por fumonisinas cuando el maíz es recolectado son *F. moniliforme* MP A y *F. proliferatum* MP D (Sala, 1993; Marín, datos sin publicar). En nuestro caso, las cepas de *Fusarium proliferatum* (MP D) utilizadas resultaron generalmente mayores productoras de toxinas que las de *F. moniliforme* (MP A) en las condiciones óptimas.

A lo largo de los experimentos se ha detectado una variabilidad importante en cuanto a la capacidad productora de las cepas, sin embargo, ésta no ha disminuido, como cabría esperar, debido a la sucesiva subcultivacion de las cepas con la consiguiente pérdida de habilidad toxigénica, común en *Fusarium* (Nelson, 1992; Komer *et al.*, 1988). Por lo tanto, se ha atribuido dicho fenómeno a la variabilidad intrínseca del comportamiento de estas cepas, dando lugar a resultados poco repetibles y reproducibles.

La velocidad de crecimiento de las cepas sobre maíz autoclavado fue siempre superior que sobre agar, sobretodo a a_w<0,95 (Marín *et al.*, 1995a,b; C-IV); la mayor concentración de nutrientes y la pérdida de consistencia debida al tratamiento térmico puede facilitar el acceso

de los mohos a los mismos. Por otra parte las velocidades obtenidas sobre maíz irradiado (C-IV, X) son similares a las observadas sobre agar, y muy inferiores a las de maíz autoclavado. Del mismo modo, el crecimiento de *A. flavus*, así como la producción de aflatoxina, son significativamente menores sobre maíz irradiado que sobre maíz autoclavado (Smith *et al.*, 1987). La irradiación puede destruir la flora sin afectar la respiración y composición química del grano. Cuando el grano es autoclavado, éste muere y se modifica fisicoquímicamente, alterando la naturaleza y disponibilidad de nutrientes.

Análogamente, la producción de FB_1 resultó mayor en maíz autoclavado que en irradiado (Marín *et al.*, 1995b; C-VI, X). En general, la producción de otras micotoxinas como deoxinivalenol, acetil deoxinivalenol y zearalenona, es mayor en maíz esterilizado por calor, que en aquel esterilizado por radiación. Se concluyó que ello era debido a cambios sufridos por el maíz en el autoclavado, que favorecían la producción de micotoxinas, o posiblemente a cambios inducidos en el maíz esterilizado por radiación que inhibían la producción de micotoxinas. Como resultado de la irradiación, se pueden producir compuestos radiolíticos en el grano que interfieran en el metabolismo secundario de los hongos presentes (O'Neill *et al.*, 1996). Al aplicar radiación y autoclavado, la producción de aflatoxina es menor que autoclavando solo, con lo cual la irradiación crea algún mecanismo inhibidor de la síntesis de aflatoxina (Smith *et al.*, 1987).

De la misma manera que la repetibilidad propia de los experimentos en los que se determinaron fumonisinas, fue baja, las concentraciones obtenidas en los diferentes apartados reflejan una baja reproducibilidad. La causa se habría de buscar posiblemente en el porcentaje de granos rotos en el maíz utilizado como medio de cultivo en los experimentos, que a la vista de los resultados en global, podría ser determinante. Así es necesario puntualizar que el porcentaje de rotos en el grano utilizado para los experimentos de los C-IV y VI, fue mayor que para el resto, tal y como refleja la alta producción observada en dichos casos. Por otra parte, no se observaron resultados significativamente diferentes cuando se realizaron análisis por ELISA, comparados con los obtenidos por HPLC (C-X, XII).

FACTORES QUE DETERMINAN LA OCUPACIÓN DE UN NICHO ECOLÓGICO

Los hongos dependen para su actividad de fuentes de carbono, como los carbohidratos, de los cuales tienen una gran demanda. Por lo tanto existe una íntima asociación con las fuentes de carbono, y la actividad más importante durante el desarrollo vegetativo es la adquisición y utilización de estas fuentes. Teniendo en cuenta las diferentes habilidades enzimáticas mostradas por los hongos y la naturaleza fácil o difícilmente asimilable de los substratos a colonizar, esto último tiene un profundo efecto sobre las pautas de desarrollo. Las características del substrato determinan no solo qué tipo de mohos van a crecer en un caso determinado, sino también la duración de la vida de los mismos en dicho substrato. Todavía más, el éxito de la captura y asimilación de las fuentes de energía están fuertemente influidas por el impacto adicional de la competencia, estrés y perturbaciones (Cooke y Whipps, 1993).

Un ambiente de estrés es aquel en el cual para la mayoría de hongos, la producción de biomasa está restringida por la continua imposición de un estrés. Esto, puede deberse simplemente a un factor, o a una combinación de factores. En muchas ocasiones el

crecimiento se ve impedido, pero el estrés puede llegar a ser tolerado por hongos que o bien poseen características fisiológicas adecuadas, o que se pueden adaptar mediante una alteración temporal de su comportamiento, e incluso, algunos de ellos han desarrollado mecanismos que refuerzan su dependencia de factores de estrés específicos, que limitan el desarrollo de especies inadaptadas (Cooke y Whipps, 1993). Las especies tolerantes al estrés persisten si las condiciones estresantes se mantienen, sujetas a ser reemplazadas si éste se alivia; no necesariamente crecen rápido, germinan o se reproducen (Magan, 1997). En el caso de la microbiota del maíz, *Aspergillus*, *Penicillium* y *Eurotium* pertenecerían a esta categoría, siendo *Eurotium* el grupo que ha desarrollado una estrategia más especializada para soportar el estrés hídrico.

Una perturbación ocurre cuando toda o parte de la biomasa fúngica es destruida o sujeta a nuevas presiones selectivas debido a cambios en las condiciones ambientales. Tiene dos aspectos diferentes: primero, existen perturbaciones destructivas por una perturbación violenta; sucesos de este tipo se dan en la naturaleza y afectan a hábitats fúngicos pero son relativamente raros. En segundo lugar, existen perturbaciones enriquecedoras, que son bastante frecuentes, naturales y no destructivas y se dan cuando hay un input de material orgánico que contiene una biomasa fúngica propia baja (Cooke y Whipps, 1993). Las especies ruderales son efímeras, sólo capaces de utilizar fuentes fácilmente asimilables, rápidamente y a veces únicamente enfocados a la reproducción (Magan, 1997). *Aspergillus* y *Penicillium* podrían incluirse también en esta categoría.

La competencia se define como la demanda por parte de dos o más individuos de la misma o diferente especie del mismo recurso. La competencia adopta diferentes formas, que comprenden diferentes mecanismos, pero se han de tener en cuenta dos aspectos principales: la captura primaria de recursos y el combate. El primero es el proceso que permite el acceso inicial, influir sobre un determinado recurso, ocupar el territorio. El segundo comprende la defensa del territorio ocupado, captura secundaria de recursos, de otro ocupante establecido (Cooke y Whipps, 1993) (Fig. 1). Las especies combativas son persistentes y de larga vida, capaces de defender los recursos capturados, con o sin crecimiento rápido y germinación de esporas, reproducción lenta o intermitente y buena competencia enzimática (Magan, 1997). *Fusarium* podría pertenecer a este tipo de microorganismos, pero sólo lo haría bajo condiciones no estresantes, de lo contrario, los hongos resistentes al estrés predominan. Entraría en competencia con especies de *Aspergillus* y *Penicillium* básicamente, y a a_w muy altas, con mucorales y bacterias.

Realmente las estrategias de resistencia al estrés de adaptación a un nicho en el cual existen condiciones adversas para la mayoría de microorganismos pueden ser una solución, mientras que aquellos microorganismos que comparten nicho con una gran cantidad de especies microbianas, como es el caso de *Fusarium*, se ven obligados a desarrollar estrategias de competición, y dependerá del resto de especies que entren en juego, que sea o no capaz de ocupar un determinado nicho.

Todas aquellas condiciones a_w/temperatura por debajo de la isopleta que limita la germinación y el crecimiento de *Fusarium* son condiciones bajo las cuales, de mantenerse constantes, no dan lugar a desarrollo de *Fusarium*, pero son condiciones propicias para especies de *Aspergillus*, *Penicillium* y *Eurotium*. A <0,90 a_w, *Penicillium* se establece rápidamente a temperaturas bajas, mientras que *Aspergillus* lo hace a temperaturas altas, mientras que existe una banda de temperaturas que es común a ambos géneros, y en la cual,

jugarán un papel más importante las estrategias de competición que la adaptación a condiciones estresantes. Por otra parte, a a_w <0,85, *Eurotium* es probablemente el género predominante dada su adaptación al estrés hídrico. Sin embargo, el crecimiento de dichas especies puede ser la causa del aumento de a_w, hasta condiciones adecuadas para el género *Fusarium*, aunque, dado que las especies anteriores han sido las primeras en colonizar el sustrato, ello no significa que las nuevas condiciones de humedad permitan el desarrollo de *Fusarium*. La importancia de las interacciones en la colonización del grano por parte de las diferentes especies fúngicas se discuten en el siguiente apartado.

ESTRATEGIAS DE COMPETICIÓN

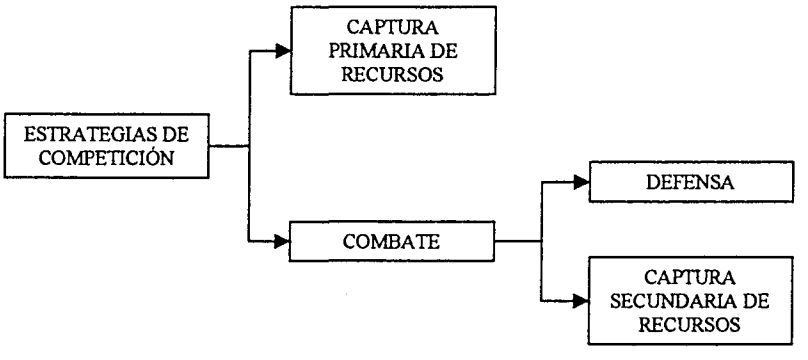

Fig. 1. Estrategias de competición, según Cooke y Whipps (1993)

La mayoría de hongos crecen en substratos naturales en un rango de a_w menor que el observado en los medios de laboratorio. La competencia durante la colonización, y otros muchos factores, están indudablemente involucrados en la reducción del rango de a_w.

Qué hongo será el predominante no es solo una cuestión de llegar en el momento adecuado al sitio adecuado, sino que depende de una rápida consolidación en el nuevo sustrato. La velocidad es esencial en este paso, junto con la capacidad de economizar la energía endógena, y de esta manera maximizar el potencial del inóculo, siendo este último favorecido si los tubos germinativos pueden reaccionar direccionalmente al sustrato colonizado. La disposición de las hifas es aquella que lleva a una toma más eficiente de nutrientes (Cooke y Whipps, 1993).

Se consideran colonizadores primarios aquellos bien dotados para la captura primaria de recursos, es decir aquellos que crecen rápido y cuyas esporas germinan rápido. Es muy importante cuando se trata de extrapolar datos sobre agar a la realidad, que existen especies con velocidades de crecimiento reducidas que tienen un crecimiento miceliar explotativo más que explorativo (Dowson *et al.*, 1989). Un hongo se considera combativo cuando crece a una velocidad similar en ausencia o presencia de otros, o cuando es capaz de frenar la velocidad de los otros (Robinson *et al.*, 1993).

Magan y Lacey (1984b, 1985) concluyeron que la a_w, la temperatura, y los nutrientes del substrato tenían una fuerte influencia sobre el antagonismo, competencia y dominancia de los

mohos. La producción de metabolitos secundarios, como las micotoxinas, también se ve afectada y pueden contribuir al éxito de una especie en particular por exclusión de otras del nicho ecológico.

Las velocidades de germinación, de crecimiento, así como la capacidad para esporular son factores determinantes en la captura primaria de recursos. La mayor o menor importancia de ésta sobre el combate, dependerá lógicamente de las condiciones ambientales, cuando éstas sean estresantes la captura primaria de recursos predominará sobre el combate y a la inversa. Bajo las condiciones ambientales favorables que llevan a capacidades similares de las diferentes especies para crecer y germinar, el combate se hará importante.

Captura primaria de recursos por *F. moniliforme* y *F. proliferatum* en relación con otros mohos propios del maíz. Es *Fusarium* Liseola una sección aventajada?

La flora propia del maíz se compone básicamente de especies de *Aspergillus, Penicillium, Eurotium* y *Fusarium*. Varias cepas de *Eurotium* han sido ensayadas por Abellana *et al.* (1999); los resultados obtenidos reflejan como estas cepas tienen un nicho ecológico mucho más amplio que el de *Fusarium*, sin embargo, en la zona de solapamiento las velocidades de germinación y crecimiento de *Eurotium* son mucho menores que las del resto de especies presentes. A continuación se comparará la habilidad de las cepas de *Fusarium* para desarrollarse bajo diferentes condiciones medioambientales, con las de algunos *Aspergillus* y *Penicillium* ensayados (C-VII).

Germinación. En primer lugar los microconidios de *Fusarium* no fueron capaces de germinar por debajo de 0,88 a_w, con lo cual su nicho presenta un tamaño reducido; dentro de las condiciones que permiten la germinación, el resto de las especies también son capaces de germinar, por lo tanto la preponderancia de una u otra dependerá de la duración de la fase de latencia y de la velocidad con la cual sea capaz de germinar (C-V, C-VII).

Fusarium no presentó una velocidad de germinación mucho menor en general a la presentada por especies de *Aspergillus* spp. y *Penicillium* spp. bajo las mismas condiciones, sin embargo, por encima de 30°C las velocidades de las cepas de *Aspergillus* spp. son mayores que las de *Fusarium*. En el rango 15-30°C, *Aspergillus* spp. y *Penicillium* spp. compiten en igualdad de condiciones; siendo *Fusarium* solamente capaz de germinar a una velocidad similar a 15°C y a_w altas. Por debajo de 15°C predomina *Penicillium* spp. A 7°C y 0,95 a_w *Fusarium* y *Penicillium* poseen velocidades similares (Fig. 2). Por lo que respecta a la fase de latencia, se repite la tendencia de la velocidad. A temperatura >30°C, las especies de *Aspergillus* son las primeras en germinar, mientras que por debajo de 15°C lo hacen las de *Penicilium. Fusarium* es capaz de competir en igualdad de condiciones a a_w alta (0,95-1,00 a_w) y en el intervalo 10-37°C (Fig,. 3). Por lo tanto las cepas de *F. moniliforme* y *F. proliferatum* son capaces de germinar prontamente en un rango amplio, aunque después la velocidad sea lenta comparada con el resto de especies. .En consecuencia, pese a que estas especies de *Fusarium* tienen un nicho más amplio que el del resto de especies de *Fusarium* típicas del maíz, esto no les supone ninguna ventaja competitiva desde el punto de vista de la germinación dado que son condiciones óptimas para el desarrollo fúngico en general y existen otras especies colonizando el grano que tienen velocidades de germinación favorables y fases de latencia más cortas.

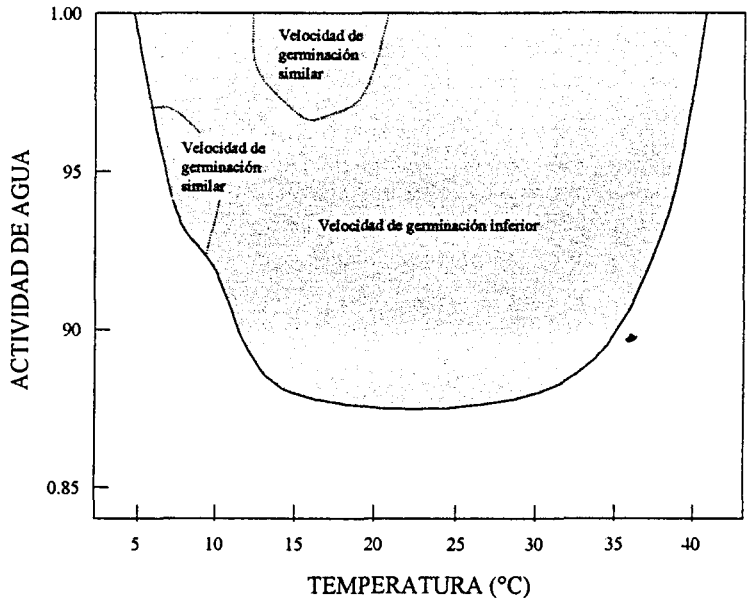

Figura 2. Velocidad de germinación relativa de *Fusarium* spp. respecto a otras especies que ocupan su nicho ecológico (temperatura/a_w)

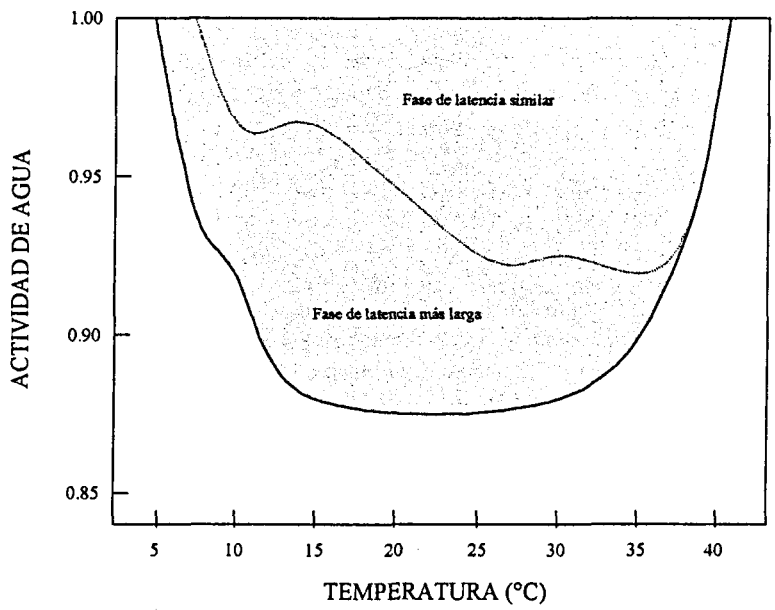

Figura 3. Fase de latencia relativa de *Fusarium* spp. respecto a otras especies que ocupan su nicho ecológico (temperatura/a_w)

Crecimiento. El crecimiento de *F. moniliforme* y *F. proliferatum* fue más rápido que el del resto de especies en un amplio marco de condiciones dentro de las que permitieron su crecimiento. En este caso, el factor biótico hizo que se estrechara el intervalo de crecimiento aproximadamante a 10-30°C y 0,95-1,00 a_w. (Fig. 4). *Fusarium* spp. creció a velocidad similar al resto de especies sobretodo a <25°C. A >25°C las especies de *Aspergillus* crecieron más rápido en algunos casos. Tomando la a_w más baja a la que *Fusarium* spp. fue capaz de crecer (0,90), su velocidad siempre fue <0,5 mm d^{-1}, mientras que las especies de *Penicillium* llegaron a 1 mm d^{-1}, y las de *Aspergillus* a 2 mm d^{-1}, incluso a 3 mm d^{-1} en el caso de *A. niger* (Marín *et al.*, 1995; C-VII) y 2.63 mm d^{-1} para *Eurotium* (Abellana *et al.*, 1999). Por lo tanto, por lo que respecta a crecimiento, *Fusarium* podría competir con éxito a a_w altas (0,95-1,00), dado que tiene un crecimiento invasivo, aunque su micelio es menos denso que el de otras especies fúngicas; sin embargo el retraso en la germinación podría ser determinante para la captura primaria de recursos.

Bajo a_w = 0,93-0,98 y a 15 y 25°C, *in situ* sobre maíz, *Fusarium* spp. creció más rápidamente a 15°C y 0,95-0,98 a_w (C-X, XI). Hay que tener en cuenta que fuera de estas temperaturas posiblemente las aptitudes para competir de estas especies se ven disminuidas en favor de *Aspergillus y Penicillium*, y de *Eurotium* si se usaran a_w menores de las escogidas

Capacidad combativa de *F. moniliforme* y *F. proliferatum*

Se ensayaron los Indices de Dominancia (I_D) de *Fusarium* spp. frente a *A. niger*, *A. ochraceus*, *A. flavus*, *P. aurantiogriseum*, *P. griseofulvum*, *P. citrinum* y *F. graminearum*. El I_D da una idea de la capacidad para combatir, entendiéndose por combatir, o bien la capacidad de mantener el territorio conseguido, o bien la capacidad para capturar territorio

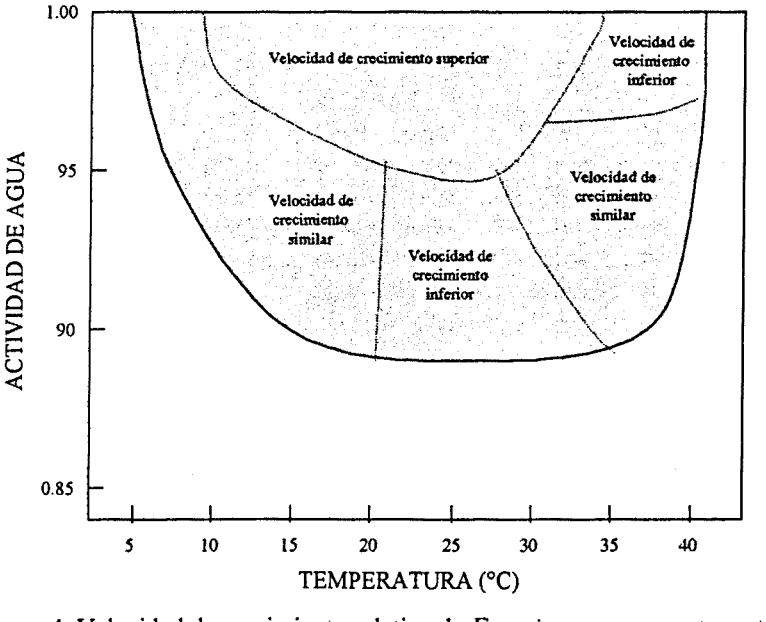

Figura 4. Velocidad de crecimiento relativa de *Fusarium* spp. respecto a otras especies que ocupan su nicho ecológico (temperatura/a_w)

inicialmente ocupado por otro. Hay que tener en cuenta que este es sólo un aspecto de la competencia, y no determina ni mucho menos cual será el hongo dominante; de ahí que no se haya podido relacionar con la velocidad de crecimiento (Magan y Lacey, 1984b; C-VIII, C-IX, C-X, C-XI). La estrategia combativa comprende dos aspectos, el primero, la producción de sustancias difusibles o volátiles con efectos antibióticos sobre las otras especies, y el segundo, el propio contacto físico entre las hifas de las diferentes especies. Es interesante comprobar que estas habilidades se ven afectadas por la temperatura y la a_w (C-VIII, IX); probablemente unas condiciones ambientales desfavorables conllevan una mayor esporulación y un cambio en el metabolismo, y el paso hacia estrategias tolerantes al estrés. Bajo condiciones de humedad alta, *Fusarium* compitió exitosamente debido a la capacidad invasiva de sus hifas capaces de ocupar un amplio espacio a la búsqueda de nutrientes y de descartar otras especies, principalmente *Penicillium*, de micelio peor dotado. *A. niger*, por su parte presentó una estrategia probablemente basada en ambos factores, producción de sustancias volátiles o difusibles, y un micelio invasivo, con lo cual dominó en la mayoría de casos. Por su parte, la competencia con *A.flavus* y *A. ochraceus* se basó en los mismos principios que *Fusarium* y fue más igualada. Por otra parte, a a_w <0,94, las hifas de *Fusarium* pierden su capacidad invasiva y son dominados por el resto de especies en la mayoría de casos. *Fusarium proliferatum* obtuvo I_D mayores que *F. moniliforme*. La misma tendencia a disminuir el I_D conforme baja la a_w se demostró para 2 cepas de *F. proliferatum* y *F. subglutinans* al enfrentarlas a 12 cepas pertenecientes a especies de *Aspergillus, Penicillium, Eurotium* y *Trichoderma* (C-VIII). En general, la reacción que se dio cuando se pusieron en contacto especies de *Fusarium* entre ellas fue antagonismo mutuo. A pesar de que las especies de *Penicillium* en general son conocidas productoras de metabolitos secundarios, no obtuvieron I_D significativamente mayores que el resto de especies ensayadas.

De la misma manera, los I_D hallados sobre maíz reflejaron una mayor capacidad combativa de las especies de *Fusarium* a a_w alta, aunque los I_D fueron menos favorables que en agar. Únicamente dominaron a aquellas temperaturas en que se había demostrado su mayor adaptación comparado con las otras especies (15°C). A 25°C las especies de *Fusarium* fueron inhibidas en contacto y sobrepasadas (0,93-0,95 a_w, principalmente) o experimentaron inhibición mutua en contacto (0,98 a_w). A 15°C *F. proliferatum* mostró más capacidad combativa que *F. moniliforme* (C-X, XI).

El número de fuentes de carbono asimilables por un hongo puede determinar la colonización de un sustrato determinado o no, y la intensidad de la misma. Así, la cantidad de fuentes de carbono asimilables por cada especie varió con la temperatura y a_w (C-IX), aunque *P. citrinum* mostró gran capacidad para asimilar un mayor numero de fuentes en cualquier condición, mientras que *A. niger* fue el que menos asimiló bajo todas las condiciones. Sin embargo, la determinación de los Indices de solapamiento de nicho (NOI) es una medida de la capacidad para combatir; una vez las hifas de dos colonias entran en contacto, la existencia de fuentes asimilables por uno de ellos en el territorio ocupado por el otro, incapaz de asimilarlos, puede llevar a la captura secundaria de recursos. A grandes rasgos, de las especies ensayadas la única especie que presentó NOI favorable frente a *Fusarium* desde este punto de vista fue *P. citrinum*, sin embargo *Fusarium* presento NOI favorable frente a *P. aurantiogriseum, P. griseofulvum* y *A. niger*, existiendo diferencias según a_w y temperatura.

Consecuentemente, I_D y NOI recogen diferentes aspectos combativos, con lo cual no necesariamente debe haber una relación entre ellos. El NOI da una idea de la capacidad de

una especie para adueñarse del territorio de otro, mientras I_D recoge todos los aspectos combativos. Cuando las producción de compuestos volátiles no sea importante, podrá haber más relación entre ambos parámetros. La cepa de *F. proliferatum* utilizada fue capaz de asimilar más fuentes de carbono que la de *F. moniliforme*, y además su nicho presentó un solapamiento menor con el de otras especies, sugiriendo que tendría un mayor potencial para competir. Existe una clara diferencia entre la capacidad combativa de *A. niger* medida como NOI o I_D, ello implica que posiblemente *A. niger* presenta una estrategia diferente, posiblemente basada en la producción de metabolitos, como indica la inhibición de determinadas especies a distancia sobre agar (C-VIII).

Basándose en la incidencia sobre cada grano, se ha observado una correlación negativa entre la presencia de *F. moniliforme* y *F. graminearum*. La infección de los granos por *F. moniliforme* puede servir como disuasorio de la posterior infección por otros hongos (Wicklow *et al.*, 1987) y tener efecto protector contra otros patógenos (Van Wyk *et al.*, 1988). *F. moniliforme* parece ser uno de los primeros en colonizar las mazorcas de maíz en el campo, infectando los granos antes que *Penicillium* y otros mohos (Hesseltine y Bothast, 1977; King., 1981). En estudios realizados en incidencia en granos por siembra directa en muestras procedentes de Carolina del Norte se vio que *F. moniliforme* estaba significativamente correlacionado negativamente con todas las especies comúnmente aisladas (Wicklow *et al.*, 1988). Este caso es diferente del que se reproduce en nuestros experimentos, ya que nuestra inoculación es simultánea, y en el campo *Fusarium* estaría presente en el grano con anterioridad, con lo cual sería más importante la capacidad para defender el territorio ocupado, y a la a_w alta que se da en el campo, se ha demostrado que tanto NOI como I_D son favorables.

Determinación de la capacidad para competir

Ninguna de estas medidas, I_D, NOI o velocidades de crecimiento y germinación es mejor que otra para predecir la microflora predominante bajo unas condiciones determinadas, simplemente dan información sobre diferentes aspectos. Por ejemplo, cuando el maíz almacenado tenga una alta contaminación inicial, las aptitudes combativas serán más relevantes; por el contrario, cuando el inóculo inicial sea pequeño, la captura primaria de recursos será decisiva y por lo tanto la capacidad para germinar, crecer y esporular rápidamente.

Se determinó el efecto de unas cepas sobre las velocidades de crecimiento de las otras, que se puede considerar indicativo de las aptitudes para la captura de recursos, y a su vez, combativas; el crecimiento de *Fusarium* se vio afectado por la presencia de especies de *Aspergillus* y *Fusarium*, pero no por las de *Penicillium*. El efecto fue más acusado a a_w 0,98, cercana al óptimo de todas las especies. Únicamente *A. niger* y *P. citrinum* parecieron mostrar una actividad propiamente combativa provocando halos de inhibición que desaparecerían con el tiempo (C-IX). Para especies con velocidades de crecimiento altas e invasivas como *Trichoderma viride* o las especies de *Fusarium*, si que se encontró una relación entre el I_D y la velocidad, de manera que el carácter invasivo de sus hifas puede darles una ventaja física cuando se encuentran con las hifas de otras especies peor dotadas (C-VIII).

La determinación del número de unidades formadoras de colonias fúngicas en medios de cultivo, pese a ser un método sesgado de cuantificación fúngica, como se discutirá

posteriormente, es uno de los pocos métodos que permiten valorar el resultado de la competencia entre cepas en un substrato. Las especies de *Fusarium* fueron mayormente inhibidas (cuando se realizaron recuentos) a 25°C, en presencia de especies como *A. niger, A. flavus, A. ochraceus* y *P. implicatum*. En general, la cepa de *F. proliferatum* se vio más inhibida que la de *F. moniliforme*, pero ninguna de las especies ensayadas presento una habilidad sobresaliente para inhibir a las especies de *Fusarium* (reducciones de 0-40%, en general) (C-X). Hay que tener en cuenta que los recuentos se hicieron sobre placas conteniendo ambas especies, con lo cual, en el caso de especies con elevada capacidad de esporulación es posible que las colonias de *Fusarium* no pudieran llegar a ser contadas por la masiva presencia de colonias de otro tipo.

La reducción de la infección por especies de *Fusarium* debida a la presencia de otras cepas de *Aspergillus* spp. y *Penicillium* spp., fue más importante a 25 que a 15°C, y más acentuada a las más bajas a_w ensayadas (0,93 a_w). *Aspergillus niger* tuvo un efecto inhibidor muy acentuado llegando a anular la infección de *Fusarium*, las otras especies de *Aspergillus* también la inhibieron en mayor o menor medida, mientras que la otra especie ensayada, *P. implicatum*, no tuvo apenas efecto (C-X).

Es importante la producción de fumonisinas para competir?

La mayoría de estudios realizados en interacciones fúngicas relacionadas con la producción de micotoxinas, como las aflatoxinas, se centran en la influencia de las interacciones fungicas sobre la producción, pero no en la posible ventaja competitiva que la producción tóxica puede suponer.

Las fumonisinas son metabolitos secundarios; esto sugiere que si *Fusarium* está presente como endotrofo, la producción de fumonisinas puede ser más importante para su capacidad para retener un nicho que para ocupar el mismo en un principio, sin embargo, si la contaminación proviene de las hojas o el suelo, el establecimiento puede ser ayudado de la producción de micotoxinas.

Sin embargo, en el presente caso no se observó correlación alguna entre la concentración de FB_1 y las poblaciones de cepas de *Aspergillus* y *Penicillium* cuando éstos crecieron en cultivos mixtos junto a cepas de *Fusarium* sección Liseola (C-X, XI), con lo cual en principio no se puede asociar a la producción de FB_1 el papel de estrategia de competición.

EFECTO DE CEPAS DE *ASPERGILLUS* Y *PENICILLIUM* SOBRE LA PRODUCCIÓN DE FUMONISINAS

El estudio de las interacciones fúngicas ha captado más atención debido al hecho que la producción de micotoxinas por cepas toxigénicas puede verse inhibida o favorecida por la presencia de otras especies. Así se ha visto que la producción de aflatoxinas se ve potenciada por la presencia de determinadas bacterias (Cuero *et al.* 1987). Wicklow *et al.* (1980) observaron que *A. flavus* producía aflatoxinas sobre maíz en presencia de *Candida guilliermondi*, mientras que era incapaz de hacerlo en presencia de *A. niger* o *Trichoderma viride*. Dicha producción también se ha visto inhibida por *A. oryzae* (Maing *et al.*, 1993) y *Rhizopus nigricans* (Weckbach *et al.*, 1977). Por otra parte la presencia de *P. purpurogenum* estimuló la producción de aflatoxina (Moss y Baidii, 1982).

La producción de fumonisinas en presencia de cepas de *A. niger*, *A. flavus*, *A. ochraceus* y *P. implicatum*, se vió en general inhibida, excepto bajo algunas condiciones en las cuales se vio estimulada, dichas condiciones en general coincidieron con las a_w más altas (0,98) y ambas temperaturas (15° y 25°C) (C-X). Las causas de la inhibición podrían ser la propia competencia por los nutrientes, la producción de alguna sustancia inhibidora de la síntesis o la degradación de la fumonisina una vez producida por parte de las cepas ensayadas. Se demostró que bajo algunas condiciones la producción de fumonisinas en co-cultivo de *F. moniliforme* y *F. proliferatum* conduce a una mayor producción que cuando se cultivan por separado (C-X). Las causas de esta estimulación podrían ser la producción, por las cepas acompañantes, de sustancias precursoras de la síntesis, o que la propia competencia hiciera que las cepas de *Fusarium* pasaran prematuramente del ciclo vegetativo a la producción de metabolitos secundarios.

F. culmorum produce cantidades menores de deoxinivalenol, acetil deoxinivalenol y zearalenona en maíz no estéril que en esterilizado, ello fue atribuido a la presencia de flora competitiva en el maíz no estéril. Es posible que la flora acompañante compitiera con *F. culmorum* reduciendo los nutrientes disponibles y el espacio e impidiendo la síntesis de micotoxina (O'Neill *et al.*, 1996). Otros autores han demostrado la reducción de la producción de zearalenona en cultivos mixtos (Eugenio *et al.*, 1970; Cuero *et al.*, 1988). Otras explicaciones de la baja producción en grano no estéril es que la flora natural puede producir sustancias inhibidoras de la producción de micotoxinas (Mislivec *et al.*, 1988), o destruir las micotoxinas tan pronto éstas son producidas, por ejemplo mediante la producción de enzimas que degraden las toxinas. La transformación microbiana y destoxificacion de tricotecenos ha sido ensayada *in vitro* (Kiessling *et al*, 1984; Beeton y Bull, 1989).

Estudios de Yoshizawa *et al.* (1996) mostraron que había fumonisinas en muestras tanto de maíz sano como enmohecido, sin diferencia significativa. Las muestras con altos niveles de fumonisinas (1ppm) contenían bajos niveles de aflatoxinas. Se demostró una relación negativa entre ambas micotoxinas, concluyéndose que se había dado una interacción entre cepas productoras de aflatoxinas y fumonisinas.

CUAL ES LA MEJOR MANERA DE CUANTIFICAR EL CRECIMIENTO FÚNGICO? Y PARA CUANTIFICAR QUÉ ESPECIES PREDOMINAN?

Los análisis microbiológicos de muestras pueden perseguir dos tipos de objetivos: el primero, determinar la actividad fungica presente en un sustrato -para la evaluación de sistemas y condiciones de almacenamiento-, segundo, la identificación de la microflora presente en una muestra -para la identificación de riesgos para la salud- (Kaspersson, 1986). En nuestros experimentos se ha pretendido siempre determinar la presencia de *Fusarium* spp. y otros mohos bajo diferentes condiciones de incubación/almacenamiento, con lo cual se ha recurrido al uso de métodos estándar de enumeración de microorganismos para alimentación animal, es decir el recuento de unidades formadoras de colonias después de la siembra en placa de un banco de diluciones, o bien la siembra directa en placa de los granos.

La determinación de las unidades formadoras de colonia (ufc) es probablemente el método más extendido en micología aplicada a alimentación animal. Sin embargo, este sistema tiene

una serie de inconvenientes derivados de la imposibilidad para discernir si las ufc provienen de fragmentos de micelio, agregados, conidios individuales o grupos, u otras esporas (Rose y Bradley, 1980; Jarvis *et al.*, 1983). Christensen y Meronock (1976) sugirieron que las colonias provenían casi enteramente de esporas. Consecuentemente, las especies con gran capacidad para esporular son sobreestimadas y las que esporulan poco, son subestimadas. Los diferentes niveles de propágulos de las diferentes especies no darán estimaciones fiables de la actividad fúngica (Jarvis *et al.*, 1983). En nuestro caso, las especies de *Fusarium*, que esporulan poco, resultan subestimadas, principalmente *F. proliferatum*, especie especialmente poco esporuladora. Sin embargo, dado que en nuestro caso no se trataba de realizar comparaciones interespecíficas, sinó de cada cepa determinada con su control, se decidió que el método era adecuado (C-VIII, X). Se sabe que el porcentaje de esporas del total de biomasa fungica representa únicamente un 2-3% (Schnurer *et al.*, 1995).

La siembra directa en placa puede utilizarse, o bien para determinar la microflora presente en un substrato, o bien para la enumeración de los granos infectados por un hongo especifico. Mediante esta técnica, el hongo crece a partir de micelio y de esporas, y esto debe tenerse en cuenta en la interpretación de los resultados (Kaspersson, 1986). El principal inconveniente que se encontró en el empleo de dicha técnica fue que, un grano puede estar infectado por varias especies, siendo una la mayoritaria, y sin embargo todas ellas tendrán un mismo resultado positivo. Además, en nuestro caso en la mayoría de experimentos se obtuvieron granos infectados por varias especies, en los cuales se hace difícil discernir entre ellas. Cuando la contaminación es alta, frecuentemente el 100% de granos resultan infectados y el método deja de ser útil (C-VIII, X).

Entre los métodos para determinar la actividad fúngica en muestras, que sin embargo no permiten discernir entre diferentes microorganismos, se encuentran la cuantificacion de ATP, ergosterol, quitina, y en algunos casos los recuentos en placa, y infección fúngica. De hecho, cualquier método de cuantificación de actividad fúngica, debería ir acompañado de una identificación de la microflora complementaria. Sin embargo, para medidas de la actividad fúngica en continuo, sin necesidad de muestrear, la monitorizacion de la evolución del CO_2, o la medida en continuo de la temperatura de almacenamiento, pueden ser métodos muy útiles para almacenamientos a gran escala (Kaspersson, 1986).

Las diferencias en las pautas de crecimiento entre las diferentes especies, los problemas metodológicos, y los efectos ambientales hacen que la búsqueda de un factor de conversión entre ergosterol y biomasa sea infructuosa. Sin embargo para determinados mohos, los cambios en los niveles de ergosterol reflejan exactamente cambios en la longitud miceliar (Schnurer *et al.*, 1995). En la determinación de ufc se ha encontrado una correlación mejor entre éstos y el contenido de ergosterol al utilizar medios con a_w baja (DG18, a_w=0,95) que al usar MEA (Schnurer y Jonsson, 1992). Para especies de *Aspergillus* y *Penicillium*, cuando el ergosterol se multiplica por 2, las ufc lo hacen por 10 (Cahagnier *et al.*, 1993). La a_w baja produce un aumento de esporulacion en algunos casos, con lo que el método de ufc no es reflejo real del efecto de la a_w, mientras que el ergosterol recoge el crecimiento miceliar, que es más real.

A la búsqueda de cual de las medidas empleadas del crecimiento podía considerarse la más representativa, se intentaron establecer correlaciones entre ellas, estudiando tanto cultivos puros como mixtos.

Cultivos puros

Se ha demostrado, para *Fusarium* spp. que existe correlación entre ufc y velocidad de crecimiento. Análogamente, se ha demostrado que para especies con poca capacidad para esporular, como *F. culmorum* existe una buena correlación entre los cambios en la extensión de las hifas, ufc y contenido en ergosterol. Mientras que especies con gran capacidad de esporulación, producen incrementos altos de ufc, sin que aumente la longitud de las hifas y el contenido en ergosterol (Schnurer, 1993).

En muchos casos, pero no siempre, la reproducción se ve favorecida por condiciones que no son óptimas para el crecimiento vegetativo. Se podría esperar que en situaciones favorables para la expansión vegetativa, la captura de recursos fuera la principal misión del micelio, mientras que en condiciones desfavorables la propagación de los propagulos se viera promovida. Para hongos productores de conidios con estrategias ruderales, por ejemplo, *Aspergillus*, *Penicillium* y *Trichoderma*, el micelio joven puede alcanzar la madurez 24 h después de la germinación de las esporas. Diferentes factores interaccionan en provocar el cambio del crecimiento trófico a la etapa reproductiva, incluyendo varios mecanismos endógenos y un rango de reguladores exógenos. Generalmente estos actúan para asegurar que el esfuerzo reproductivo se concentre en un momento en que las condiciones ambientales sean probablemente favorables, o pidan una liberación de esporas, y su subsecuente germinación o la deposición en estado de dormancia. La a_w es un factor mayoritario en el control de la reproducción. Con desecación gradual, las rutas de síntesis de metabolitos secundarios pueden verse activadas, con lo cual hasta que el stress hídrico dañe fatalmente los procesos celulares, la reproducción puede verse potenciada (Magan, 1997).

Entre velocidad de crecimiento y producción de fumonisinas la correlación fue negativa; ello indicaría que condiciones que no favorecen el crecimiento vegetativo, sí que favorecen el paso al metabolismo secundario (C-X).

Cultivos mixtos

Para las especies de *Aspergillus* la velocidad de crecimiento relativa mostró una correlación significativa con I_D, ufc y porcentaje de infección, cuando éstas crecieron junto a especies de *Fusarium* (C-XI), lo cual supondría que cuando la habilidad para la captura primaria de recursos de una cepa es mucho superior que la de aquella con la que compite, la capacidad combativa pasaría a un segundo término. Asimismo, las especies de *Aspergillus* mostraron una relación directa entre los recuentos y los porcentajes de infección de granos (C-XI). Se concluyó que, cuando se trata de especies de *Aspergillus* y *Fusarium*, las velocidades de crecimiento de las colonias pueden ser un índice válido, por comparación, del potencial de una cepa para competir contra otra.

Por el contrario, la cepa de *Penicillium* no presentó ninguna correlación entre dichos parámetros (C-XI). Dicha cepa, y la mayoría de las cepas de *Penicillium* presentes en el grano, tienen una gran capacidad esporuladora, que no necesariamente guarda relación con el incremento de diámetro de la colonia. Por todo esto, un recuento de colonias alto no se traduce en una porción amplia de substrato colonizado.

Fusarium presentó alta capacidad de crecimiento a 15°C dado que a esta temperatura es capaz de crecer más rápido que el resto. Sin embargo, los recuentos fúngicos, dado que

dichas especies esporulan poco, se vieron sensiblemente reducidos por la presencia de otros mohos. Por otra parte, en cultivos mixtos se demostró buena correlación entre ufc y fumonisinas (C-X), lo cual supone que las condiciones que favorecen la esporulación, y por tanto la finalización del ciclo vegetativo, favorecen a su vez la producción de metabolitos secundarios, tal y como apuntó Schnurer *et al.* (1995).

Es importante hacer notar que se utilizaron inóculos equivalentes de las dos cepas en cada caso; la presencia de una cepa con un inóculo inicial escaso, puede hacer que bajo determinadas condiciones sea desplazada por especies ruderales y competitivas.

PÉRDIDA DE CALIDAD DEBIDO A LA CONTAMINACIÓN FÚNGICA. CUAL ES LA MEJOR MANERA DE CUANTIFICAR LA PÉRDIDA DE CALIDAD?

Tradicionalmente se han utilizado el enmohecimiento visible y el porcentaje de pérdida de materia seca (DML) como parámetros indicadores de la calidad del grano almacenado. Sin embargo, las estimaciones de las pérdidas de materia seca permisibles difieren. Una pérdida de materia seca del 0,5%, en la mayoría de casos, sin que haya crecimiento visible, es suficiente para que el maíz no sea apto (Saul y Lind, 1958; Saul y Steele, 1966; Seitz *et al.*, 1982a). Kreyger (1972) consideraba que un grano con un 2% de pérdida de materia seca todavía era adecuado para alimentación animal. Hall y Dean (1978) asumieron que una pérdida del 1% durante un almacenamiento de un año era aceptable para el consumo humano, tanto en trigo como en maíz. Sin embargo, la medida de pérdidas pequeñas (<0,1%) por el sistema de pesada está sujeta a errores porque el peso de micelio fungico habría de ser sustraído de la materia seca total.

Obviamente, en nuestros estudios se observaron pérdidas de materia seca muy superiores, provocadas, por un lado por los niveles altos de humedad suministrados al maíz, y por otro, a la inoculación del cereal a razón de 2×10^5 esporas fúngicas por g de maíz en la mayoría de casos (C-IV, VI, X, XI, XII, XIII). Por otra parte, la presencia de micelio fúngico en el maíz al determinar la materia seca de una muestra hace que, dado el alto contenido de humedad del mismo, el porcentaje de materia seca de la muestra sea pequeño, con lo cual las pérdidas de materia seca contabilizadas son sobrestimadas. Las cepas de *Fusarium* inoculadas sobre el maíz, junto con la respiración del grano provocaron en 28 días pérdidas de entre 0 y 3% de materia seca bajo las diferentes condiciones de temperatura y a_w ensayadas; la presencia de cepas de *A. niger* o *A. flavus* provocó un aumento de dicho porcentaje (C-X). La pérdida de calidad se puede cuantificar en continuo por estimación a partir de la liberación de CO_2 por respiración (Hamer *et al.*, 1991). Por otra parte se ha visto que la DML se debe básicamente a la respiración del grano (Seitz *et al.*, 1982a, b)

Seitz *et al.* (1982a,b) vieron como la contaminación por *A. flavus* y la concentración de aflatoxinas era inaceptable mucho antes de que la pérdida de materia seca llegara al 0,5% establecido por Saul y Steele (1966). Por el contrario, en aquellos casos en que la pérdida de materia seca del maíz fue inferior al 0,5% (a 0,89 a_w), no se encontraron fumonisinas (C-VI). La siembra directa en placa y los análisis de aflatoxinas detectaron la presencia de *A. flavus*, antes de que los niveles de ergosterol empezaran a subir, estos lo hicieron después del 0,5%. El 0,5% se alcanzó en maíz con 24,7-25,6% humedad (=0,97 a_w) al cabo de 9-13

días y al cabo de 4 días con 22,9% humedad (=0,95 a_w), esto sin inocular y almacenado en silos (Seitz *et al.*, 1982b).

Un método paralelo de determinación de la pérdida de calidad puede ser la pérdida de valor calorífico (C-XV), sin embargo, dicha determinación no tiene en cuenta, el valor calorífico correspondiente al micelio fúngico, que de este manera queda incluido dentro del valor obtenido, no obstante, como cuando dicho maíz se utiliza como alimento para ganado, presenta el micelio, la medida es suficientemente adecuada. Utilizando este método, se registraron pérdidas de valor calórico en el maíz inoculado con *Fusarium* de entre 0 hasta 64%. Es interesante destacar que se observó una correlación inversa significativa entre el valor calórico de las muestras de maíz y la biomasa fúngica, y la concentración de fumonisinas presentes (C-XV). Tanto la respirometría, como el seguimiento de la pérdida de materia seca, o del valor calórico, así como la determinación del nivel de ergosterol son parámetros útiles para la cuantificación fúngica en grano, sin embargo, probablemente no son lo suficientemente sensibles para detectar el enmohecimiento en sus principios.

Los recuentos fúngicos se han utilizado habitualmente como una medida de la calidad de diferentes substratos, así se ha considerado maíz normal, alta y muy altamente contaminado aquel que contiene 3×10^4, 7×10^4 y 1×10^5 ufc g^{-1}, respectivamente. En maíz naturalmente contaminado y almacenado a 0,90 o 0,95 a_w los recuentos a partir de la primera semana entran en la categoría de muy altos, únicamente a 0,85 a_w y con la ayuda de conservantes se puede entrar en la categoría de contaminación alta (C-XIII).

Contrariamente a lo que ocurre con *Fusarium* y la producción de fumonisinas, se ha encontrado correlación entre la producción de ocratoxina A por *P. verrucosum* y la infección fungica y ufc en cultivo puro; sin embargo no hay ninguna relación cuando se trata de cultivos mixtos. Por otra parte bajo la mayoría de condiciones hubo una buena relación entre infección y ufc (Ramakrishna *et al.*, 1996), norma que si que siguen las especies productoras de fumonisinas. La determinación del crecimiento visible de *F. sporotrichioides* sobre granos inoculados mantuvo buena relación con ufc y porcentaje de infeccion, durante las primeras etapas de la colonización, más adelante no se pudieron ver diferencias en el crecimiento visible, mientras que las ufc continuaron aumentando. Ninguno de estos métodos cuantifican biomasa fúngica (Ramakrishna *et al.*, 1996).

Otros métodos de medida de pérdida de calidad son: pérdida de la capacidad germinativa del grano y determinación de los cambios en la composición del grano (mucho más costoso).

La concentración de micotoxina en el grano fluctúa constantemente, y viene determinada por la combinación de un cierto número de factores. Podría darse simultáneamente estimulación, inhibicion y degradación en diferentes puntos de la masa del grano y a diferentes tiempos. La concentración final de ocratoxina es el resultado de la integración de todos estos cambios, aunque el grado de colonización es un buen indicador del nivel de contaminación por ocratoxina, en el caso de *P. verrucosum* (Ramakrishna *et al.*, 1996).

Se ha visto que muestras calificadas de maíz 'bueno' a simple vista contenían de 0 a 10,5 ppm fumonisinas, mientras que aquel que se calificó de obviamente enmohecido contenía entre 0,6 y 63,2 ppm (Richard *et al.*, 1993; Sydenham *et al.*, 1991). En el maíz calificado de bueno, puede haber o no correlación entre el nivel total de fumonisinas y el número de unidades formadoras de colonias (Sydenham *et al.*, 1992), aunque si que se ha encontrado

correlación cuando los datos se expresan en porcentaje de infección (Rheeder *et al.*, 1992; Sydenham *et al.*, 1990). Asimismo, muestras altamente enmohecidas procedentes de la China presentaron una concentración de 18-155 ppm, mientras que muestras aparentemente no contaminadas presentaron niveles de 20-60 ppm FB1 (Chu y Li, 1994).

FACTORES ABIÓTICOS COMO ESTRATEGIAS DE CONTROL DE LA PRODUCCIÓN DE FUMONISINAS

El control de las fumonisinas en el grano, puede hacerse desde varias vertientes:

- Mejorando genéticamente las plantas de maíz para conseguir hacerlas resistentes a *F. moniliforme*. El inconveniente es que es difícil de ensayar visualmente, dado que la infección puede no producir síntomas. Existe una técnica de ingeniería genética en desarrollo para detoxificar las fumonisinas en la propia planta (Munkvold *et al.*, 1997)
- Control biológico, dadas las muchas vías de infección de *F. moniliforme* en el campo, parece que la opción más conveniente sería el biocontrol basado en cepas no toxigénicas. Como en el caso de *A. flavus*, con resultados prometedores (Brown 1991)
- Destoxificacion, tiene el inconveniente que a veces no se detecta la fumonisina, pero sigue siendo tóxica
- Procesado del grano para separar los granos infectados. Fácil separar granos rotos, pero no los enteros sin síntomas. Los screenings no deberían usarse para alimentación animal, aunque esto se suele hacer, y es lo que causa más problemas
- Prevenir el desarrollo fúngico después de la cosecha, es el caso que nos ocupa

F. moniliforme no es solamente el patógeno más común encontrado en maíz, sino que además se encuentra entre los hongos más comúnmente aislados de plantas sanas de maíz. Esta especie fúngica es una constante de las plantas y semillas de maíz, de manera que es capaz, sin producir síntomas, de transmitirse vía semilla a las nuevas plantas e infectar los granos (Munkvold *et al.*, 1997). Por los estudios realizados hasta la fecha es razonable concluir que el maíz normalmente contiene fumonisinas. El maíz es uno de los productos agrícolas más importantes en EEUU como ingrediente mayoritario de piensos. También la harina de maíz es básica en la alimentación en regiones de Africa, Asia y América Central y del Sur. En Norteamérica y Europa los derivados del maíz son componentes importantes de muchos alimentos procesados, como los cereales para el desayuno, snacks, refrescos, y cerveza. Es sabido que las fumonisinas no son destruidas por muchos de los procesos industriales. Dado que a menudo se da la infección asintomática de los granos de maíz, no es posible separar los granos infectados mediante los controles físicos tradicionales (Munkvold *et al.*, 1997), con lo cual éstos pasaran al almacén, confiando al secado y al posterior control del almacén el peso de la conservación.

Es importante evitar por todos los métodos el prealmacenamiento del maíz antes del secado, dado que en ese momento las condiciones de humedad son las óptimas para la producción de fumonisinas en el mismo (Cahagnier *et al.*, 1995).

A partir de los diagramas de isopletas (Fig. 5) se puede ver como es posible controlar la producción de fumonisinas por la simple combinación a_w/T, eso si, si las condiciones de

almacenamiento son adecuadamente controladas y mantenidas constantes mediante aireación periódica. Tal y como se observa en la figura 5 el intervalo de condiciones que permiten controlar la producción de FB_1 es mucho más amplio que aquel que se requiere para controlar el crecimiento o la germinación de las cepas productoras en cada caso. Un almacenamiento a <0,93 a_w aseguraría el mantenimiento de un maíz libre de fumonisinas procedentes del campo, a niveles < 1 ppm a cualquier temperatura. El almacenamiento refrigerado también conseguiría la misma finalidad (C-VI). Por otra parte la estimulación de la producción de FB_1 por otras especies fúngicas presentes en el grano se da en general a a_w altas (C-X), valores bajo los cuales, ya no sería factible el almacenamiento, con lo cual a los niveles de a_w a los que designamos como 'seguros' la propia microflora del maíz podría incluso actuar como factor de control adicional.

Interesantes, y a la vez desafortunados resultados se obtuvieron con la aplicación de conservantes a base de propionatos en maíz. La combinación de dosis de 0,07% de propionato en maíz a 0,93 a_w consiguió inhibir por completo el crecimiento de *Fusarium* inoculado en forma de micelio. Sin embargo, cuando se realizó la inoculación en las mismas condiciones en forma de esporas - es conocido que las condiciones requeridas para germinar son menos restrictivas que las requeridas para crecer-, no solo las cepas fueron capaces de germinar sino que incluso produjeron fumonisinas, en cantidades importantes en el caso de *F. proliferatum* (C-XII). Sin embargo, las mismas concentraciones de propionatos en maíz contaminado de forma natural no tuvieron ningun efecto con respecto a los blancos, aunque la concentración de fumonisinas no fue muy alta, debido al efecto controlante de la propia microflora (C-XIII).

Se ha intentado el control de la producción de aflatoxinas mediante conservantes, tipo sorbatos, en maíz almacenado en silos cerrados y con un contenido de humedad relativamente alto. La eficiencia depende de la humedad, y de los microorganismos presentes, de forma que la dosis necesaria para inhibir la mayoría de los presentes e inhibir la presencia de aflatoxina, 1%, fue demasiado alta para considerarse económica (Lee *et al.*, 1986).

Las dosis mínimas recomendadas por fabricantes de conservantes (alrededor del 0,05%), son efectivas en maíz seco y correctamente almacenado, con un 0,7 de a_w; la adición del conservante en este caso previene del deterioro en caso de que se da una rehumidificacion puntual. Sin embargo, el control de la humedad y temperatura del grano sigue siendo básico. Por otra parte, es más común el uso de conservantes para maíz ensilado con alto contenido de humedad (18-20%), en este caso las dosis deben aumentarse por encima de 0,2% (Coll *et al.*, 1994).

Se considera que el consumo por équidos de maíz conteniendo 8 ppm FB_1 puede producir ELEM (Wilson *et al.*, 1992). Asimismo, se han recomendado concentraciones máximas de FB_1 de 5, 10 y 50 ppm en équidos, porcino y, vacuno y aves, respectivamente (Miller *et al.*, 1996). Se ha observado como *Fusarium* a pesar de producir grandes cantidades de fumonisinas dentro de un determinado rango de a_w y temperaturas, dicho rango abarca en sus extremos condiciones que se pueden dar en post-cosecha antes del secado; probablemente es en este punto cuando se da la mayor producción de fumonisinas, sin embargo, después del secado, no deberían producirse en absoluto. Si por una falta de control adecuado en al almacén se da un rehumedecimiento del grano, la presencia de otras especies fungicas en el grano, más adaptadas a a_w más bajas, parecen actuar como factor controlante

de la producción de fumonisinas, pese a que los recuentos de *Fusarium* siguen siendo altos. Sin embargo, en este caso (C-XIII) *A. flavus* aparece como una de las especies mayoritarias, con lo cual, seria interesante conocer si en dichas condiciones *A. flavus* solo está presente o es capaz de sintetizar aflatoxinas. En muestras de maíz naturalmente contaminado e incubado por periodos de 1, 2, 3, 4 semanas, se han encontrado concentraciones de <20 ppm, cuando la concentración a tiempo 0 era < 1ppm, por lo tanto pequeñas cantidades de fumonisinas se producen probablemente en el almacén cuando se da una pequeña condensación de agua en aquellos granos que ya estaban previamente infectados, sin embargo, debido a la presencia de otros hongos, *Fusarium* podría no ser capaz de infectar nuevos granos (C-XIII).

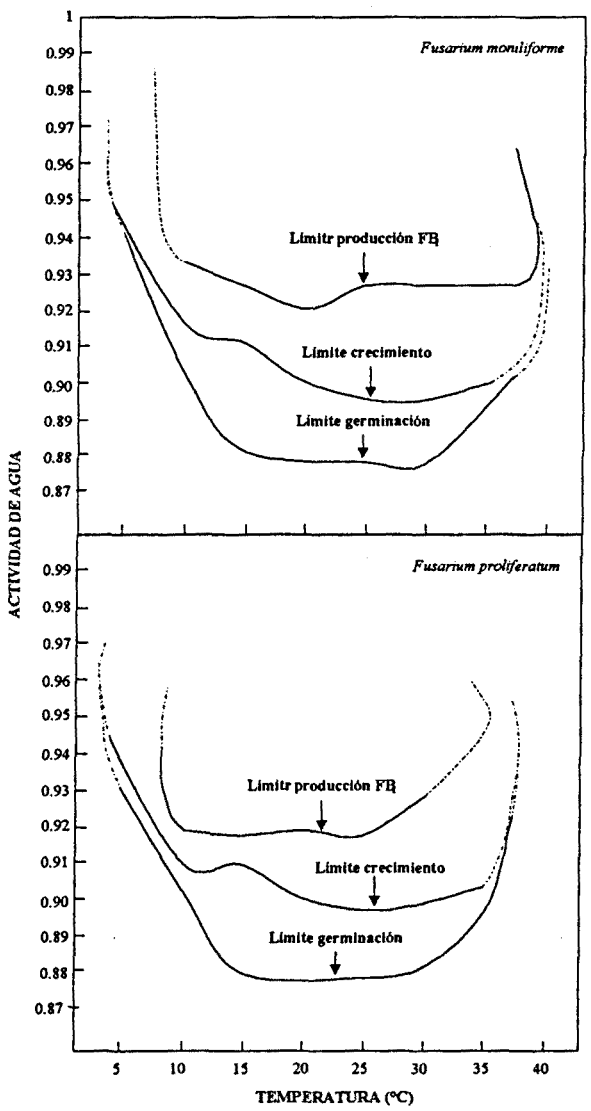

Figura 5. Lineas isopletas indicando las combinaciones a_w/T que limitan la producción de FB_1 (1 ppm), el crecimiento (0,1 mm d^{-1}), y la germinación (10% esporas) de cepas de *F. moniliforme* y *F. proliferatum*.

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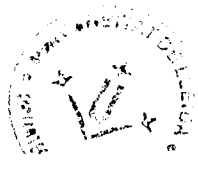
XVII

CONCLUSIONES

- A pesar de que *Fusarium moniliforme* y *F. proliferatum* son capaces de crecer en muchos substratos, el maíz parece ser el substrato idóneo para la producción de fumonisina B_1.
- Se han determinado por primera vez el efecto combinado de la a_w y la temperatura sobre la colonización del maíz por *F. moniliforme* y *F. proliferatum* y las concentraciones de FB_1 acumuladas en el mismo. La germinación es posible entre 5-37°C y ≥ 0,88 a_w, el crecimiento entre 5-35°C y ≥ 0,90 a_w, mientras que la producción de FB_1 solo se da a 10-37°C y ≥ 0,93 a_w.
- Las especies de *Fusarium* tienen un rango de temperatura favorable muy amplio. Consecuentemente, sólo si se mantiene la a_w del grano por debajo de 0,93, se controlará la producción de FB_1 después de la cosecha, sea cual sea la temperatura ambiente. Sin embargo, en el campo se dan niveles mucho más altos de humedad, en especial en años húmedos, con lo cual la presencia de un cierto nivel de fumonisinas en el maíz en el momento de la recolección puede ser muy frecuente.
- Se ha demostrado que los conservantes a base de propionatos existentes en el mercado son útiles para el control del crecimiento de *Fusarium* spp. *in situ* pero el control de la producción de fumonisinas sólo fue eficaz a concentraciones ≥ 0,07%.
- Las interacciones *in vitro* de *Fusarium* spp. con otras especies fúngicas dependen de los factores abióticos. Estos mohos se ven mayormente afectados por la presencia de *A. niger* y *A. flavus*. La microflora natural del maíz afecta al desarrollo de *Fusarium, in situ,* de diferentes maneras, dependiendo de los factores ambientales. Así, en general, las especies de *Fusarium* compiten con éxito a a_w altas, y mejor a temperaturas cercanas a los 15°C. La presencia de flora competitiva contribuye a la reducción del nicho de condiciones abióticas donde *Fusarium* es capaz de crecer. A a_w altas se ha comprobado que la producción de FB_1 puede verse potenciada por la presencia de otras especies fúngicas, como *A. niger*.
- Además de la determinación de fumonisinas en materias primas, se ha demostrado que la monitorización de la actividad enzimática en el grano, y de la pérdida de valor calórico del mismo pueden ser buenos índices de la infección del grano por *Fusarium*.



CONCLUSIONS

- *Although Fusarium moniliforme and F. proliferatum isolates were able to grow on many substrates, maize is the major nutritional grain substrate for FB_1 production.*

- *The two-dimensional profiles of the environmental factors, water activity and temperature, on colonisation by F. moniliforme and F. proliferatum. and FB_1 production in vitro and in maize grain have been determined for the first time.*

- *Overall, the Fusarium species had a wide favourable temperature range. Consequently, provided a aw level of < 0.88 is maintained, post-harvest spoilage and FB_1 production will be prevented. However, higher moisture levels (> 0.88 a_w) pre-harvest in the field, particularly in high humidity conditions would almost certainly result in fumonisin accumulation in the ripening maize grain.*

- *Niche overlap and in vitro dominance of the Fusarium spp. was markedly influenced by the presence of a range of competing fungi, especially A.niger and A.flavus and by environmental factors. In situ, the natural mycoflora of maize also affected Fusarium development in different ways, depending on the prevailing abiotic factors. In general, Fusarium species were able to compete successfully at high water availabilities, and better as temperature approached 15oC. The presence of other fungi narrowed the niche of abiotic conditions over which these Fusarium spp. were able to grow and compete effectively. Under high a_w conditions it was demonstrated that in some cases FB_1 production can be enhanced by natural maize mycoflora.*

- *This study demonstrated that existing propionic acid-based preservatives can control growth of the Fusarium spp. in vitro and in situ but that control of fumonisin production was not complete except at high concentrations (0.07 %).*

- *Besides the quantification of fumonisins in raw materials, other methods for quantifying quality losses in grain, such as enzyme activity, and calorific losses, enumeration of fungal counts, and grain infection, were all found to be useful indicators of Fusarium colonisation and infection of maize.*

XVIII

FUTURAS INVESTIGACIONES

La presente tesis plantea una serie de interrogantes aún por despejar. Así, por ejemplo la causa por la cual las fumonisinas se biosintetizan en grandes cantidades en maíz, mientras que en cereales de composición similar, como el trigo y la cebada, la biosíntesis es casi nula, queda sin resolver.

Los requerimientos hídricos necesarios para la producción de FB_1, son valores que se dan en el campo durante un cierto período de tiempo, con lo cual el control en el campo adquiere una importancia capital, las técnicas de biocontrol pre-cosecha o bien la búsqueda de variedades de maíz resistentes al ataque de *Fusarium* o la mejora genética de las existentes pueden ser la solución.

Dado que, hasta la fecha, el uso de materias primas libres de fumonisinas en los procesos industriales, no está garantizado, sería importante determinar cómo influyen cada uno de los procesos industriales en los que se utiliza el maíz como materia prima, sobre la presencia de fumonisinas en el producto acabado. En la literatura se pueden encontrar algunos estudios de este tipo.

Finalmente, teniendo en cuenta que en un substrato como el maíz, se halla como flora natural un 'cocktail' de especies fúngicas dentro de las cuales se hallan productores de diferentes micotoxinas, se habría de hacer una aproximación a la problemática de todas las micotoxinas a un mismo tiempo, viendo como el cultivo mixto de diferentes especies fúngicas afecta a unas y otras micotoxinas; podría ser que la propia competencia interespecífica sirviera como medida de control de algunas micotoxinas..

XIX

WATER ACTIVITY, TEMPERATURE AND PH EFFECTS ON GROWTH OF *FUSARIUM MONILIFORME* AND *F.PROLIFERATUM* ISOLATES FROM MAIZE

S. Marin[1], V. Sanchis[1] and N. Magan[2]

[1]Food Technology Dept., Lleida University, UdL-IRTA, Centre R+D de Lleida, Rovira Roure 177, 25006 Lleida, Spain
[2]Biotechnology Centre, Cranfield University, Cranfield, Bedford MK43 0AL, U.K.

ABSTRACT

The effects of water activity (a_w, 0.994-0.90 ≡ 0.4 to 14.0 (-)MPa water potential), temperature (4-45°C) and pH (3.6, 5.5, 7.0), and their interactions on growth of isolates of *Fusarium moniliforme* and *F.proliferatum* were determined in vitro on a maize extract agar medium. Growth of two isolates of *F.moniliforme* and four insolates of *F.proliferatum* were significantly influenced by water activity regardless of solute type used (NaCl, glycerol or glucose). However, at steady state a_w levels, growth was optimum at 0.994-0.98 a_w and reduced significantly at 0.92 a_w. Further detailed studies with one isolate of *F.moniliforme* (25N) and two of *F.proliferatum* (73N, 131N) showed that growth occurred over the a_w range 0.994 to 0.90 a_w in the temperature range 20-35°C with slight differences between species. Growth did occur at 4°C and 0.994-0.96 a_w but no growth was recorded at 40 and 45°C regardless of a_w. Profiles of a_w x temperature relations for growth of these two species were constructed from these data for the first time. Optimum pH for growth was 5.5 and 25°C for both isolates of *F.proliferatum*, and pH 7.0 and 30°C for the isolate of *F.moniliforme*. However, for the latter isolate at < 0.98 a_w, optimum pH and temperature for growth changed. The effect of pH, temperature and a_w, alone, two-way and three-way interactions were all found to be statistically significant for these three isolates. The ecological significance of this information for understanding these important fumonisin-producing fungi is discussed.

Key words: water activity, temperature, fumonisin-producing, *Fusarium moniliforme*, *F.proliferatum*, maize

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INTRODUCTION

Fusarium moniliforme and *F.proliferatum* have received much attention in recent years because of their infection and concomittant production of the mycotoxins, fumonisins, in maize kernels (Ross *et al.*, 1990: Thiel *et al.*, 1991). Although environmental factors such as water availability and temperature are known to be important in determining rates of colonization of grain substrates very few studies have been carried out on *F.moniliforme* (Woods and Duniway, 1986) and none on *F.proliferatum*. The water and temperature relations of other *Fusarium* spp. from wheat grain and ears (Magan and Lacey, 1984) and that of *F.roseum/culmorum/graminearum* isolates from soil and crop residue (Cook and Papendick, 1972; Wearing and Burgess, 1979; Magan and Lynch, 1986) have been determined. Knowledge of the effect of such interactions between environmental factors on growth of fumonisin-producing isolates of these two fungi is critical for an understanding of the ecological niche occupied by these important *Fusarium* spp. so that remedial measures to prevent their establishment can be implemented.

The objectives of this study were thus to determine the effect of (a) water activity, (b) temperature, (c) pH and their interactions on growth of isolates of *F.moniliforme* and *F.proliferatum* in vitro on a maize extract medium.

MATERIALS AND METHODS

Isolates

The isolates used in this study were all cultured from maize (Sala *et al.* 1993) and included two isolates of *F.moniliforme* Sheld. (25N, 123N) and four insolates of *F.proliferatum* (Matshushima) Nirenberg (37N, 63N, 73N, 131N). All isolates chosen produced fumonisins (Sala *et al.*, 1993).

Medium

The basic medium used in this study was a 3% maize meal extract agar (MMEA) with a pH of 5.5. This was made by boiling 30 g of dry maize meal/1 L water for 30-60 mins. The resulting mixture was filtered through a double layer of muslin and the volume was made up to 1 L. The water activity (a_w) of this basal medium was 0.994 ($a_w \equiv$ -0.4 MPa water potential). The a_w of this and all media was determined with a Novasina Humidat IC II (Novasina AG, Zurich, Switzerland).

Water activity studies

Initial studies were carried with MMEA modified with either glycerol (Dallyn, 1978), NaCl (Lang, 1967), or glucose (Scott, 1957) to 0.98, 0.96, 94 and 92 a_w. NaCl and glucose were added directly to the medium together with 2 % technical agar (Lab M), while amounts of glycerol necessary to make up required volumes of the MMEA were calculated and added. Subsequent detailed experiments of the interaction of a_w x temperature on growth of three isolates, one of *F.moniliforme* (25N), and two of *F.proliferatum* (73N, 131N), were carried out on unmodified medium (0.994 a_w) and media modified with glycerol to 0.98, 0.96, 0.94,

0.92 and 0.90 a_w. Glycerol was chosen in these studies because of its temperature stability over the a_w range used. Molten media (15 mL) was poured into 9-cm-diameter sterile Petri plates.

Hydrogen ion concentration and water activity studies

Studies were carried out with the same two isolates of *F.proliferatum* and one of *F.moniliforme*. The basic pH of the MMEA was 5.5. This was modified with glycerol to 0.98, 0.96 and 0.92 a_w and subsequently with buffers (0,1M citric acid and 0,2M Na_2HPO_4) to final pH values of 3.6, 5.5 and 7.0, respectively.

Culture, incubation and measurement

Growing 10-14 day old colonies of the *Fusarium* spp. and isolates on potato dextrose agar (PDA) were used for inoculating all experiments. Agar plugs (5 mm diameter) taken from the growing margins of the colonies were aseptically placed in the centre of each treatment Petri plate. Petri plates of the same a_w were sealed in polyethylene bags. The comparison of different solutes to modify a_w was carried out at 25°C; the a_w x temperature experiments were carried out at 5 - 45 °C in increments of 5°C. The a_w x pH experiment was carried out at 20, 25 and 30°C only. In all cases experiments were carried out with three replicates per treatment. The Petri plates were examined daily or as necessary and the diameter of the growing colonies measured in two directions at right angles to each other. Measurements were carried out for a maximum of 30 days.

Statistical treatment of the results

In all cases the linear regression of increase in radius against time (in days) was used to obtain the growth rates (mm/day) under each set of treatment conditions. The growth rates were then analysed by a two way analysis of variance so that effects of single factors (isolate, solute, a_w, temperature, pH), two factors (a_w x temperature; a_w x pH; pH x temperature) and in one case three factor interactions (a_w x temperature x pH) could be assessed for statistically significant differences. Growth rates were evaluated by analysis of variance with SAS software (SAS Institute, version 6.03,Cary, N.C.). Statistical significance was judged at the level $P < 0.05$.

RESULTS

Water activity, solute type and isolate differences

When a_w was decreased to < 0.98 with the ionic solute NaCl, nonionic solute glycerol, or glucose, these was a significant reduction in colony radius of an insolate of *F.moniliforme* and *F.profileratum* over time (Fig.1). This data was used to determine the growth rates (mm/day) of each species in relation a_w and solute type. Analyses of the data demonstrated that there was a statistically significant effect of a_w for insolates of both *F.moniliforme* and *F.proliferatum*, while solute type had little overall effect on growth rate (Table 1). However, interactions between a_w and solute were statistically significant for all isolates.

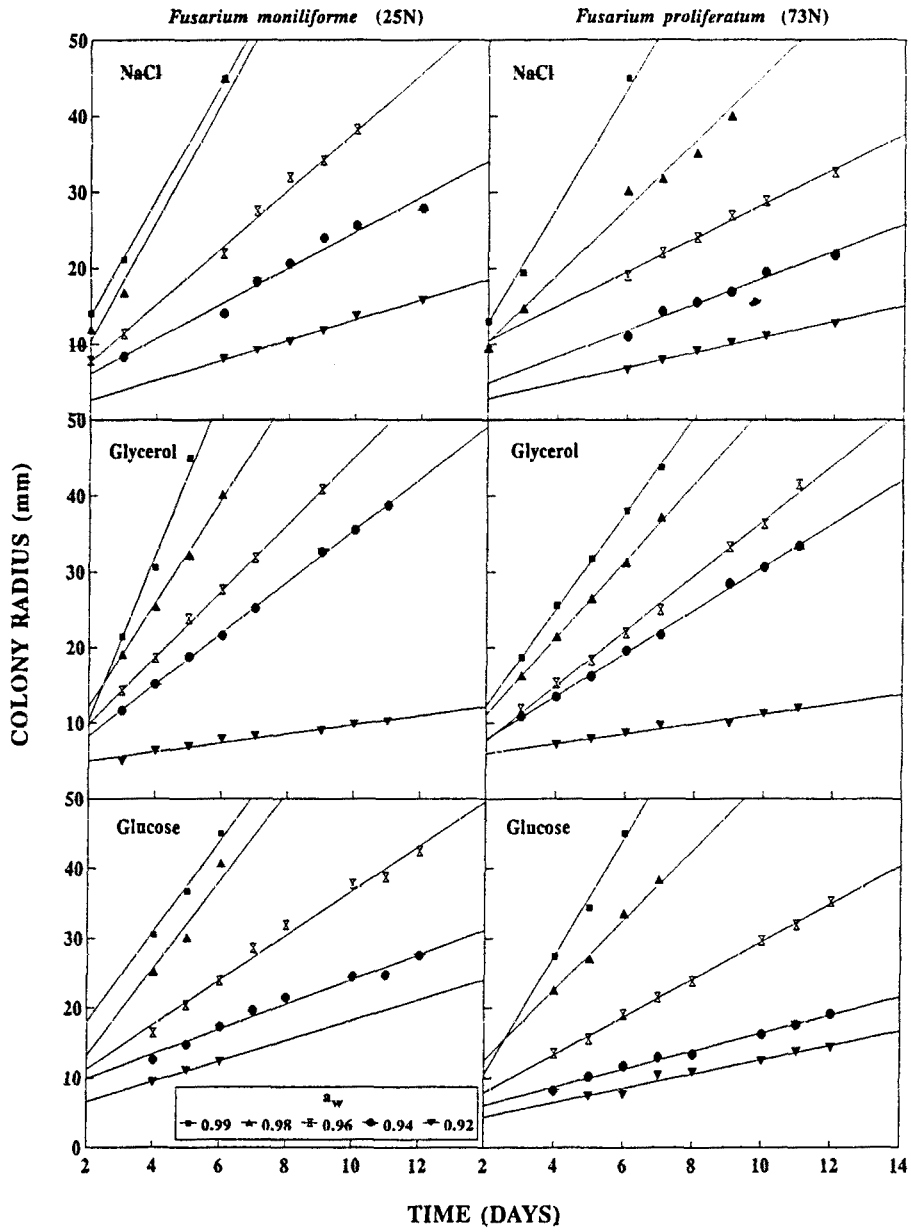

Figure 1. Effect of three different solutes (NaCl, glycerol and glucose) to modify water activity on increase in growth over periods of 28 days of an isolate of *Fusarium moniliforme* and *F.proliferatum* on a maize meal extract agar at 25$_o$C.

Table 1. Effect of water activity (a_w), solute type and two-way interactions on growth of isolates of *Fusarium proliferatum* and *F.moniliforme* in a maize meal extract medium

		Fusarium proliferatum			
		37N		63N	
	df	MS	F	MS	F
a_w	4	53.28	709.46*	59.91	304.89*
Solute	2	0.71	9.48*	0.08	0.43
a_w x solute	8	1.82	24.31*	2.11	10.78*
		Fusarium proliferatum			
		73N		131N	
	df	MS	F	MS	F
a_w	4	68.20	1591.81*	69.21	1120.68*
Solute	2	0.62	14.59*	5.45	88.20*
a_w x solute	8	2.04	47.56*	5.26	85.12*
		Fusarium moniliforme			
		25N		123N	
	df	MS	F	MS	F
a_w	4	103.24	695.84*	41.53	126.62*
Solute	2	4.52	30.49*	0.25	0.75
a_w x solute	8	4.26	28.75*	1.80	5.49*

* Significant $P < 0.001$.

There were, however, quite marked in growth rates of the two isolates of *F.moniliforme* and four of *F.proliferatum* dependent on solute type at each a_w level tested (Fig. 2). At high a_w levels, (e.g. 0.98 a_w), growth rates were higher on the glucose-modified medium than on NaCl or glycerol media. However, at 0.94 a_w growth rates were faster on glycerol-amended medium. Statistical analyses of the data shows there were significant differences between the isolates tested regardless of the solute used to modify a_w (Table 2).

Table 2. Analysis of variance of effect of different solutes (NaCl, glucose, glycerol) used to control water activity (a_w) in relation to growth of four isolates of *Fusarium proliferatum* and two isolates of *F.moniliforme* on maize meal extract agar at 25°C

Solute	Source of variation	DF	MS	F
NaCl	Isolate	5	4.49	27.16*
	a_w	4	150.54	909.42*
	Isolate x a_w	20	2.30	13.87*
Glucose	Isolate	5	5.32	23.97*
	a_w	4	147.56	664.61*
	Isolate x a_w	20	1.06	4.79*
Glycerol	Isolate	5	11.94	224.78*
	a_w	4	96.40	1814.40*
	Isolate x a_w	20	3.14	59.17*

* Significant $P = 0.0001$.

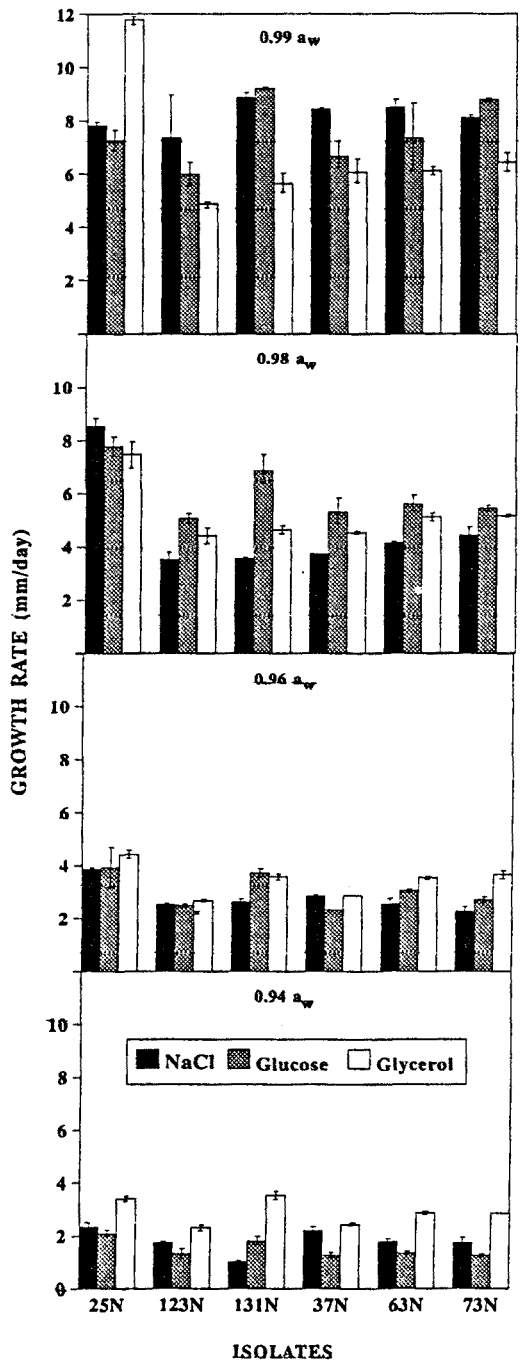

Figure 2. Comparison of growth rates (mm day^{-1}) of two isolates of *Fusarium moniliforme* and four of *F.proliferatum* in relation to water activity modified by different solutes (NaCl, glycerol, glucose) on a maize meal extract agar at 25°C. Bars indicate Least Significant Differences between treatments (P = 0.05).

Water activity x temperature effects on growth

Changing a_w at different steady-state temperatures affected the growth of an insolate of *F.moniliforme* and *F.proliferatum* in different ways (Fig. 3). *Fusarium.moniliforme* had a_w optimum for growth of 0.98-0.994 a_w. and 25-30°C. For *F.proliferatum* the optimum was 0.994 a_w at 24-30°C. However for *F.proliferatum* at 35°C the optimum changed to 0.96 a_w. Generally at 0.94-0.96 a_w, growth rates was reduced by approx. 50% regardless of temperature. Both species grew very slowly at 0.90 a_w, and only at optimum temperatures.

This data was used to construct profiles of growth for the two isolates of *F.proliferatum* and one insolate of *F.moniliforme* at different a_w x temperature levels (Fig. 4). The isopleth lines show similar conditions of a_w and temperature at which specific growth rates occur.

pH, water activity and temperature effects on growth

For two insolates of *F.proliferatum*, optimun pH for growth was 5.5 at 20,25, and 30 °C on a maize extract medium (Fig. 5). For one insolate of *F.moniliforme* (25N), growth was optimum at pH 7.0 at 0.994 and 0.98 a_w. However, at 0.96 a_w, there was no difference in pH optima, and at 0.92 a_w, optimun pH was changed to 5.5 at 30°C. Generally, for all isolates examined growth was slowest at pH 3.6.

Growth of both isolates of *F.profileratum* and one isolate of *F.moniliforme* was significantly affected by a_w, pH, temperature, and two-way and three-way interactions on the maize extract medium (Table 3).

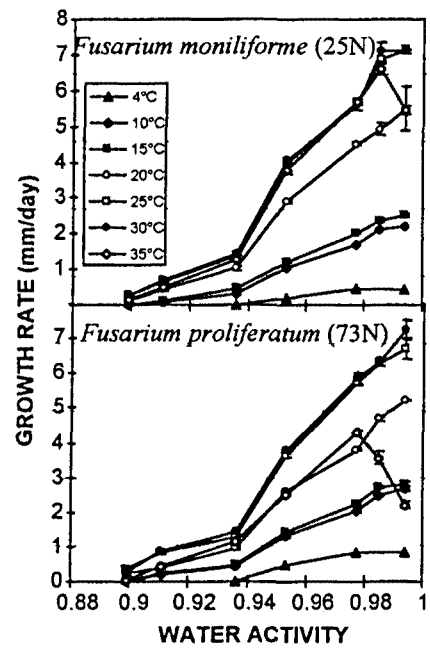

Figure 3. The effect of water activity and temperature on growth rate (mm day^{-1}) of one isolate of *Fusarium moniliforme* and two of *F.proliferatum* on a maize meal extract agar. Bars indicate Least Significant Differences (P = 0.05).

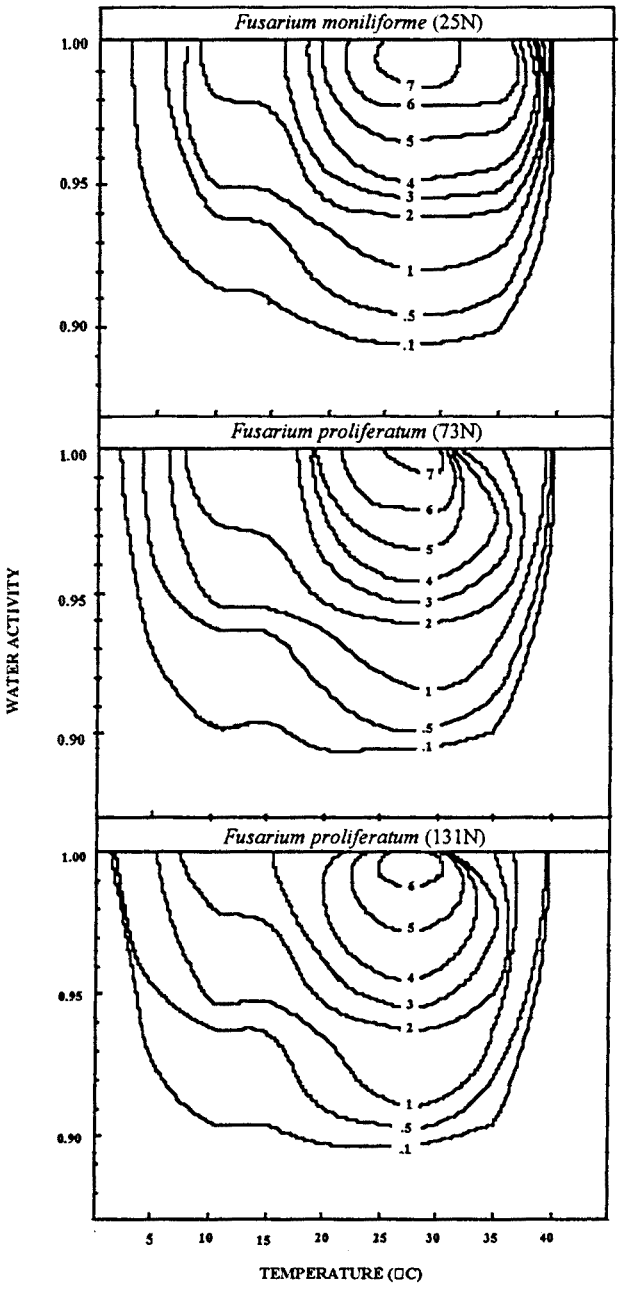

Figure 4. Diagrammatic representation of interaction of temperature and water activity on growth of *Fusarium moniliforme* and *F.proliferatum* isolates. The numbers on the isopleths are growth rates in mm day^{-1} (dotted lines indicate extrapolation).

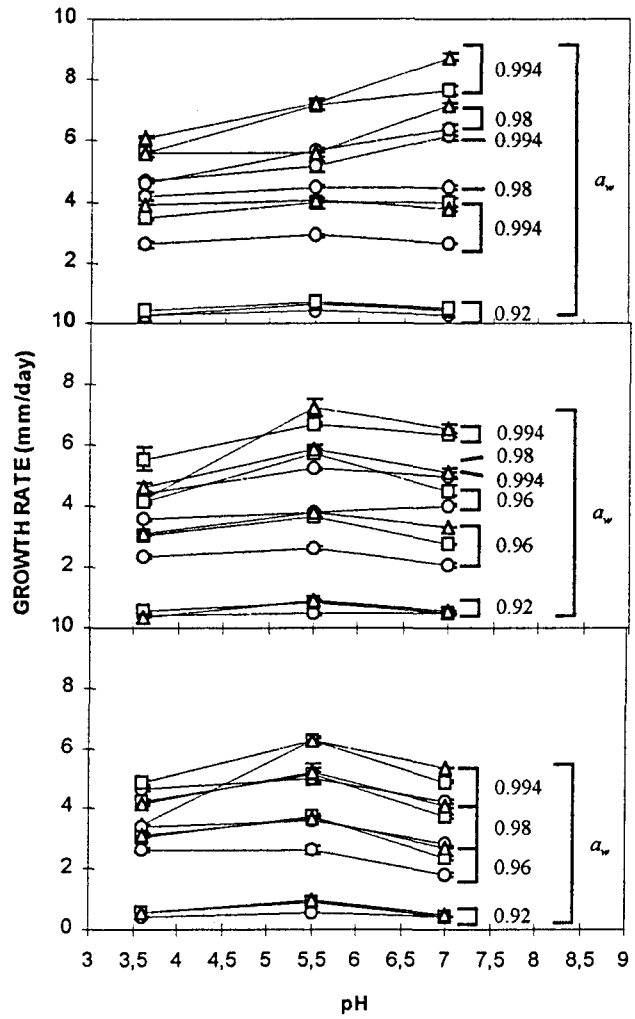

Figure 5. Effects of pH, temperature and water activity on growth rate (mm day^{-1}) of isolates of *Fusarium moniliforme* and *F.proliferatum* on a maize meal extract agar. Bars indicate Least Significant Differences between treatments (P = 0.05).

DISCUSSION

This is the first detailed study of the water, temperature and pH relations of the growth of isolates of *F.moniliforme* and *F.proliferatum* with known fumonisin-producing ability (B1 and B2, Sala *et al.* 1993). This study has demonstrated that there were significant (P=0.05) differences between isolates of *F.moniliforme* and *F.proliferatum* in relation to growth over

a range of water activity (a_w) conditions, regardless of solute used to modify water availability.

Table 3. Analysis of variance of effect of water activity (a_w), temperature (t) and pH on growth of two isolates of *F.proliferatum* (73N, 131N) and one of *F.moniliforme* (25N) on a maize meal extract agar

		F.moniliforme		*F.proliferatum*			
		25N		73N		131N	
Source of variation	DF	MS	F	MS	F	MS	F
a_w	3	189.37	13869.95*	134.81	7932.37*	97.54	8773.81*
t	2	16.32	1195.23*	9.37	551.13*	5.29	475.50*
pH	2	6.69	489.99*	6.80	400.15*	7.91	711.85*
a_w x t	6	1.57	114.77*	0.91	53.60*	0.56	50.39*
a_w x pH	6	2.17	159.21*	1.13	66.60*	0.98	88.02*
t x pH	4	0.61	44.55*	0.95	55.75*	1.03	93.35*
a_w x t x pH	12	0.23	17.20*	0.31	18.50*	0.28	25.11*

* Significant P = 0.0001.

Comparison of solute type to modify the a_w of the maize extract medium showed that there were significant differences in growth rates depending on the solute used. Growth at 0.995-0.96 a_w was often fastest when the easily metabolizeable glucose was used as the solute, while at < 0.96 a_w isolates of both species had higher growth rates on glycerol-amended media. Previously, the use of ionic solutes such as NaCl has resulted in some stimulation of the growth of fungi at > 0.98 a_w while proving to be toxic at < 0.94 a_w when present at higher concentrations (Cook and Christen, 1976; Sung and Cook, 1981; Magan and Lacey, 1984). Besri (1980) compared the effect of NaCl, KCl, glucose and saccharose on growth of *Fusarium oxysporum* f.sp. *lycopersici*. With most solutes mycelial growth was stimulated at 0.99 a_w, with optimum growth occurring at between 0.99-0.997 a_w depending on temperature. The differences in growth response of species to solutes may be due to salt ions (Na^+) being taken up by hyphal cells and enabling maintenance of cell functioning and turgor at low concentrations. However, excess ions can inhibit enzyme activity (Luard, 1983). Glycerol in amended media may also have been utilized by the *Fusarium* spp. test isolates to help overcome water stress. Polyols, particularly glycerol, erythritol and mannitol, have been shown to function as compatible solutes, being accumulated by both xerotolerant yeasts and filamentous fungi (Brown, 1978; Luard, 1982) providing protection for enzymes and their activity at low a_w. However, because of the temperature and a_w stability of glycerol, this nonionic solute was used in all subsequent studies.

Experiments with one isolate of *F.moniliforme* (25N) and two isolates of *F.proliferatum* (73N, 131N) showed that the optimum a_w and temperature for growth were 0.994-0.96 a_w and 25-35°C (25N), 0.994 and 25-30°C (131N), and 0.994 and 25-30°C (73N), respectively. The minimum for growth was found to be at approximately 0.89-0.90 a_w at optimum temperature. The temperature range for mycelial growth of *F.moniliforme* and *F.proliferatum* was found to be in the range 4-35°C. No growth was observed at 40 or 45°C. Previously, Woods and Duniway (1986) found that the optimum and minimum a_w for one isolate of *F.moniliforme* was 0.98, and 0.88-0.87 a_w, respectively. However, their study wwas carried before fumonisins were characterized and at an unspecified steady-state temperature (room temperature). Lacey (1989) suggested a temperature range of 2-37°C. Le Bars *et al.* (1993) showed that growth of a fumonisin-producing isolate of *F.moniliforme*

from France grew optimally at 25°C on PDA, but the effect of water availability and temperature were not examined. Thus, very little information has previously been available on the optimum a_w for growth of *F.moniliforme* (Lacey, 1989) and none on the water and temperature relations of *F.proliferatum*.

Previously, other *Fusarium* spp. such as *F.culmorum* and *F.graminearum* causing root rot of wheat in the Pacific Northwest, USA were found to grow optimally at 0.995-0.99 a_w at 20-30°C, with a minimum in the range 0.92-0.94 a_w at 25°C although experimental periods were very short (7 days). The wilt pathogen *F.oxysporum* f.sp. *lycopersici* did not grow at 35°C, regardless of a_w level (Besri, 1980). However, there may be possible ecological differences between *Fusarium* spp. causing ear blight and those responsible for root rot and stem base diseases. For example, Magan and Lacey (1984) found that isolates of *Fusarium* spp. (*F.avenaceum*, *F.culmorum*, *F.poae* and *F.tricinctum*) from infected wheat ears and grain grew down to 0.89-0.90 a_w. Optimum a_w was at 0.995 a_w regardless of temperature, except for *F.poae* where the optimum changed from 0.995 at 25°C to 0.98 a_w and 30°C.

The data in our study is relevant to colonization of maize kernels by these *Fusarium* species during ripening and post-harvest storage. For example, between silking and harvest ripening maize kernels have an initial water content (w.c.) of about 40-50% ($a_w \equiv 1.0$). Subsequently this is reduced to between 25 and 20% w.c. ($a_w \equiv 0.95$ and 0.90, respectively) as the kernels ripen (Inglett, 1970). These water availability conditions favouring colonization and tissue metabolite production by *F.moniliforme* and *F.proliferarum* exists over a wide time period.

Griffin (1981) suggested that the effect of pH on growth of fungi was generally less marked than that of a_w. However, in this study we found that the effect of pH, a_w x pH, pH x temperature and three-way interactions were all statistically significant influences on growth of the two isolates of *F.proliferatum* and one of *F.moniliforme* examined. There were however some differences between the two isolates of *F.proliferatum* and the isolate of *F.moniliforme*. For example, isolates 73N and 131N of *F.proliferatum* grew optimally at pH 5.5, regardless of a_w or temperature. By contrast, isolate 25N grew optimally at pH 7 at 0.994-0.98 a_w at 20, 25 and 30°C. At low a_w (0.92) this was changed to pH 5.5 and 30°C. Previously no marked difference in growth of *F.oxysporum* f.sp. *lycopersici* at pHs 4.4, 6.07, and 7.05 was obtained over a range of a_w at 25°C. The optimum a_w for growth was found to be 0.985 and the minimum 0.90, regardless of solute used to modify a_w (Besri, 1980).

In our study the pH levels used were specifically chosen in order to evaluate inhibitory effects on growth under different interacting environmental factors. Moist cereals are often treated with organis acids (propionic, benzoic and sorbic acids) to inhibit growth of such spoilage fungi. The pK_a values for maximum efficacy is between 3 and 4. Our study suggests that at such acidic pH levels opportunities may exist for effective inhibition of growth of these fungi.

Griffin (1981) divided fungi into five groups based on their water activity tolerances. *Fusarium* spp. were placed in group 3, with a moderate sensitivity to water stress and optimum a_w of 0.99 and a minimum of 0.89-0.93 a_w. He placed xerotolerant species (e.g. *Aspergillus flavus*, many *Pencillium* spp.) in group 4 and xerophilic fungi (e.g. *Eurotium* spp.) in group 5. Pelhate (1968) suggested that besides the traditional classification of grain

fungi as field and storage fungi (Christensen and Kaufmann, 1974) an important intermediate group of fungi, including *Fusarium* spp. which could be important spoilage fungi should be included. Because *F.moniliforme* and *F.proliferatum* can become established on maize early, their ability to actively grow over a range of intermediater a_w levels and a range of temperatures could be important in determining their competitive ability at intermediate grain moisture contents. For example, at 0.95 a_w isolates of both species grew at up to 50% of their growth rates at optimum a_w and temperature.

This study has determined the profile of a_w x temperature relations for growth of fumonisin producing isolates of *F.moniliforme* and *F.proliferatum* on a maize extract medium for the first time. However, the a_w and temperature range for mycotoxin production may differ from that for growth. Such differences have been previously found for *Penicillium* and *Aspergillus* spp. (Northolt and Bullerman, 1982) and for *Alternaria alternata* (Magan, Cayley, and Lacey, 1984). However, more detailed information is required on the water relations of fumonisin B1 and B2 production by isolates of *F.moniliforme* and *F.proliferatum* (Le Bars *et al.* 1994; Cahagnier *et al.*, 1995; Marín *et al.*, 1995).

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XX

EFFECT OF WATER ACTIVITIES AND TEMPERATURE ON GROWTH AND FUMONISIN B_1 AND B_2 PRODUCTION BY *FUSARIUM PROLIFERATUM* AND *F. MONILIFORME* ON MAIZE GRAIN

S. Marin[1], V. Sanchis[1], I. Vinas[1], R. Canela[1] And N. Magan[2]

[1]Food Technology Dept., Lleida University, UdL-IRTA, Centre R+D de Lleida, Rovira Roure 177, 25006 Lleida, Spain.
[2]Biotechnology Centre, Cranfield University, Cranfield, Bedford, MK43 0AL, U.K.[1]

ABSTRACT

The effect of different intermediate water activities (a_w, 0.968, 0.956, 0.944, 0.925) and temperature (25 and 30°C) on colonisation and production of fumonisin B_1 (FB_1) and B_2 (FB_2) on sterile layers of maize by *Fusarium proliferatum* and *F. moniliforme* isolates was determined over periods of 6 weeks. Generally, both *F.moniliforme* and *F.proliferatum* grew faster with increasing a_w and best at 30°C. All three isolates produced more FB_1 than FB_2 regardless of a_w or temperature. Very little FB_1 and FB_2 were produced at 0.925 a_w, with maximum produced at 0.956 and 0.968 a_w at both temperatures tested. Most FB_1 and FB_2 were produced by *F.moniliforme* (25N), followed by *F.proliferatum* (73N and 131N). At all a_w levels and both temperatures there was an increase in FB_1 and FB_2 concentration with time. Statistical analyses of a_w, temperature, time, two and three-way interactions showed some significant differences between isolates and FB_1 and FB_2 production.

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INTRODUCTION

Fusarium spp. in the section Liseola are known to produce a group of mycotoxins, the fumonisins, with fumonisins B_1 (FB_1) and B_2 (FB_2) being the most important ones found in naturally contaminated maize and in cultures (Cawood *et al.* 1991). These mycotoxins have received particular attention because of their association with cancer-promoting activity and the induction of leucoencephalomalacia (LEM) in horses (Gelderblom *et al.* 1988; Marasas *et al.* 1988). They have been commonly found in a wide range of corn-based foods in most parts of the world (Doko and Visconti 1993; Sanchis *et al.* 1994).

Little attention has been given to the range of environmental factors which might allow growth and accumulation of these mycotoxins in grain substractes. Although, some studies have been carried out on effect of temperature and moisture content on FB_1 (Albers *et al.* 1990, Le Bars *et al.* 1994) by *F.moniliforme*, none have considered colonization ability and produccition of FB_1 and FB_2 at intermediate moisture contents on grain. This is important as it represents the range of conditions under which growth and mycotoxin production may be initiated naturally on inadequately dried grain.

The objectives of this study was to determine the effect of water activity (a_w) and temperatures on colonization and production of FB_1 and FB_2 by Spanish isolates of *F.proliferatum* (73N, 131N) and *F.moniliforme* (25N).

MATERIALS AND METHODS

Organisms and culture system

Two isolates of *F.proliferatum* (73N, 131N) and one of *F.moniliforme* (25N) isolated from Spanish maize and previously found to be significant producers of fumonisins (Sanchis *et al.* 1994) were used in this study.

Sub-samples of 500 g of grain were placed in 2.5 l conical flasks. To each of four flasks,

Spanish maize (1993 harvest) with an initial moisture content of 13.75% (wet weight basis, 0.713 a_w) was used in this experiment. Different amounts of water (148.8, 102.5, 62.35 and 45 ml), calculated from a moisture absorption curve for the maize, was added to sub-samples of 500 g of grain in 2-5 l conical flask. The maize was equilibrated at 4°C for 24 h, weighed, autoclaved for 30 min. (121°C, 120 kPa) and then reweiged. The loss of water (between 2 and 5 ml) was made up with sterile distelled water. The final a_w levels of each treatment were 0.968, 0.956, 0.944 and 0.925 as determined in a Novasina Humidat IC II at 25°C. Single layers of grain (approx. 15 g) were carefully placed in 9 cm sterile Petri plates in a laminar flow cabinet. These were inoculated centrally with a 5 mm diameter agar plug from the growing margin of colonies of each species and isolate on 2% maize meal agar. Petri plates of the same treatment a_w were stacked in plastic chambers or anaerobic jars together with 250 ml of glycerol/water solutions of the same a_w to help maintain the equilibrium relative humidity in the chambers (4). Ttreatments were incubated at 25 and 30°C for up to 6 weeks.

Every 2-3 d, treatments were examined and the colony diameters, in two directions at right angles to each other, were measured in each of four replicates per treatment a_w and

temperature. After 3 and 6 weeks two replicate samples were removed, air dried and used for fumonisin analyses. The increase in radial growth was determined and used to calculate the growth rate (mm d^{-1}) under each set of treatment conditions for each species and isolate.

Fumonisin analyses

The samples were extracted using a modification of the Sherhard method (Sherphard *et al.* 1990) as described by Sanchis *et al.* (1994). After extraction, purified sample residue was dissolved in 0.2 ml of methanol. Two hundred µl of O-Phtaldialdehyde (OPA) reagent, prepared according to Shephard *et al.* (1990) were added to a 50 µl sample solution. Twenty µl of this solution were injected into the HPLC withim 2 min of derivatization. The eluent was methanol 0.1 mol l^{-1} KH_2PO_4 (13:7) adjusted to pH 3.35 with O-H_3PO_4. The flow rate was 1.0 ml min^{-1}. Reference standards of FB_1 and FB_2 were purchased from CSIRO, Division of Food Science and Technology, Pretoria, South Africa.

Growth rates and fumonisins concentrations were evaluated by analysis of variance using SAS software (SAS Institute, version 6.03.). Pairs of results for fumonisin analyses were generally within 5-19% of each other. Statistical significance was determined at the 95% confidence level.

RESULTS AND DISCUSSION

Figure 1 shows that for insolates of both *F.moniliforme* and *F.proliferatum* there was an increase in groth rate as a_w and temperature were increased. However, there was a marked difference between the two isolates of *F.proliferatum* at steady-state temperatures. At the higest a_w tested (0.968), one insolate of *F.proliferatum* (73N) grew faster than the other isolates tested. Colonization of simple layers of maize grain was faster at 30°C than at 25°C, espacially at 0.944, 0.956 and 0.968 a_w. This contrast with recent data on *in vitro* growth of these two species where 25°C, was found to be optimun for growth (Marin *et al.* 1995). Le Bars *et al.*(1994) suggested that 25°C was optimun for groth on a potato dextrose medium but did not examine any a_w or a_w × temperature effects.

Table 1 shows that there was an increase in fumonisin production with time, all three isolates produced more FB_1 than FB_2 regardless of a_w or temperature. Very little FB_1 or FB_2 was produced at 0.925 a_w at both temperatures. Maximum FB_1 and FB_2 was produced by isolates of both *F.moniliforme* and *F.proliferatum* at 0.956 and 0.968 a_w at 25 and 30°C. However, some intraspecific differences between the *F.proliferatum* isolates were observed. Isolate 73N produced significantly more FB_1 and FB_2 than isolate 131N. Calculation of the ratios of FB_1 and FB_2 showed that after 3 and 6 weeks incubation *F.proliferatum* (131N) had higher ratios (3.43-9.08 FB_1:FB_2) than the others. For isolate 73N of *F.proliferatum* and that of *F.moniliforme* there was no marked change in the ratio of FB_1:FB_2 at Z0.956 than at lower a_w levels(4.27-5.41 and 2.89-4.89, respectively).

Statistical analyses showed that there were significant single treatment effects for FB_1 production (Table 2). For FB_2 production any a_w was significant for all isolates examined. There were no consistent patterns with regard to two-way interactions between factors for the species and isolates tested. However, there were significant effects of $t \times a_w$, $a_w \times T$ for FB_1 and FB_2 production. Three-way interactions were not statistically significant.

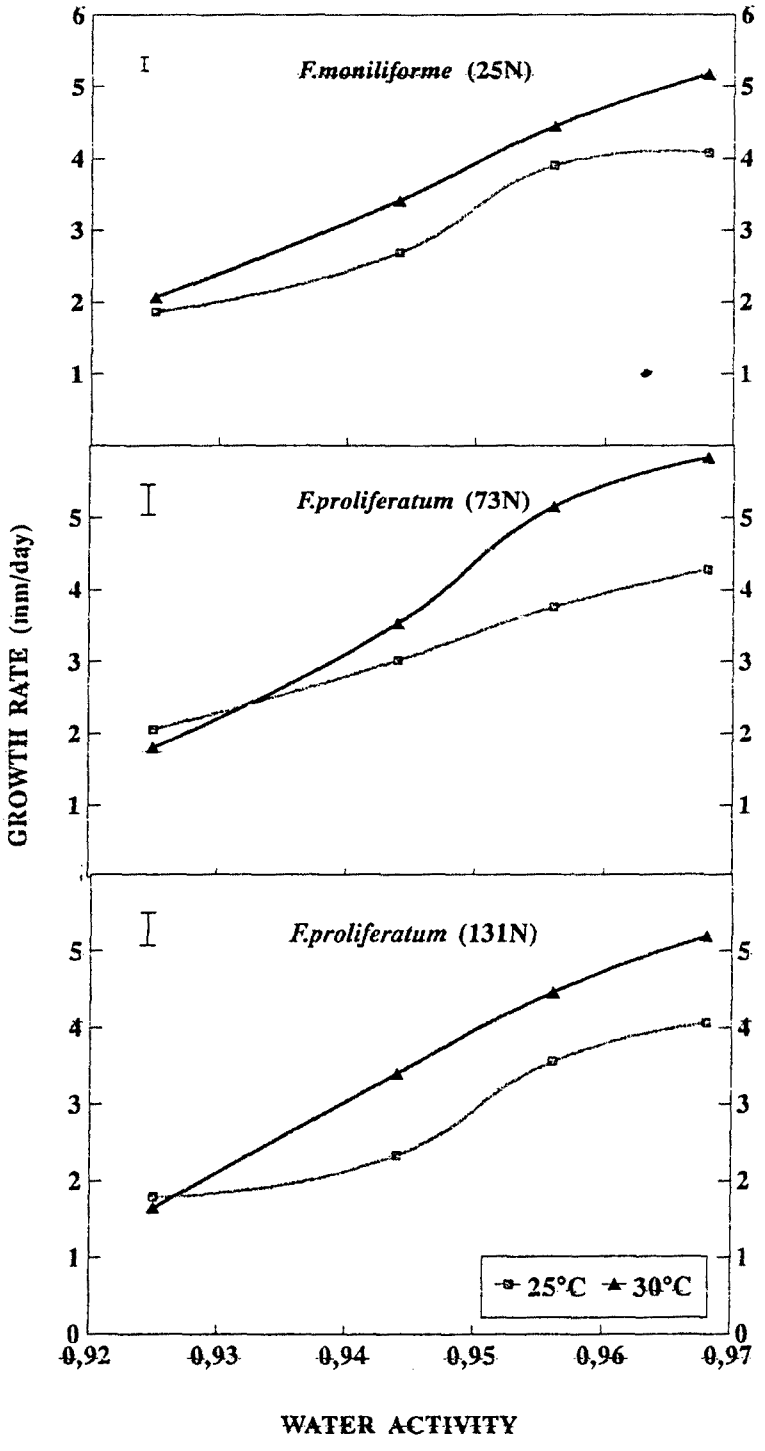

Figure 1. The effect of water activity and temperature on growth rate (mm day-1) of one isolate of *Fusarium moniliforme* and two of *F.proliferatum* on single layers of sterile maize grain. Bars indicate Least Significant Difference (P = 0.05).

Table 1. Effect of water activity (a_w), temperature and time on production of fumonisin B_1 and B_2 ($\mu g\ g^{-1}$ grain) by two isolates of *Fusarium proliferatum* and one of *F.moniliforme* on layers of sterile maize grain.

Temperature °C	25 °C				30 °C			
Time (weeks)	3		6		3		6	
Fumonisins	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2
Species/isolate	*F.proliferatum* (73N)							
0.968	35.8	3.9	68.2	6.0	13.7	16.2	35.9	2.8
0.956	25.8	3.8	74.3	6.3	16.8	4.8	150.1	2.8
0.944	34.6	4.0	43.1	3.8	1.3	6.1	1.9	3.7
0.925	2.7	4.9	4.2	6.1	0.5	4.9	0.8	2.8
Species/isolate	*F.proliferatum* (131N)							
0.968	116.4	2.0	95.1	2.5	21.0	2.2	40.2	5.6
0.956	44.9	2.1	77.9	2.2	76.6	3.1	37.9	4.9
0.944	28.3	2.3	49.9	2.8	2.2	3.3	1.9	5.9
0.925	2.8	3.9	5.6	4.4	0.4	2.6	1.0	3.4
Species/isolate	*F.moniliforme* (25N)							
0.968	57.0	2.6	102.6	5.2	77.9	4.4	466.1	4.3
0.956	571	2.4	93.6	5.7	70.3	4.6	559.7	4.9
0.944	53.2	4.8	14.4	4.6	0.9	3.9	1.1	3.7
0.925	3.0	4.2	6.8	4.9	0.3	4.2	6.0	2.9

The isolates examined in this study are representative of high fumonisins producers in Spain only and those from other parts of the world may deffer in the quantities of fumonisins produced. This study has examined colonization and production of funomisins from a single inoculation point source.

Table 2. Statistical table of the significance of effects of water activity (a_w), temperature (t) and time (T), two- and three-way interactions on fumonisins B_1 and B_2 production by isolates of *Fusarium proliferatum* and *F.moniliforme*

Isolate N°.		*F.proliferatum* 73N		*F.proliferatum* 131N		*F.moniliforme* 25N	
Fumonisin		FB_1	FB_2	FB_1	FB_2	FB_1	FB_2
Factor	d.f.	F	F	F	F	F	F
T	1	3.22	1.13	23.18	13.42	4.98	3.24
a_w	3	20.79	20.70	23.95	26.06	3.65	3.34
T	1	3.63	8.25	36.53	54.70	3.69	2.69
$T \times a_w$	3	2.21	3.12	5.59	2.45	1.56	0.69
$T \times t$	1	0.7	1.37	2.12	0.01	3.23	3.68
$a_w \times t$	3	9.94	8.31	3.89	9.04	1.39	0.96
$T \times a_w \times t$	3	0.76	1.00	0.64	0.67	1.16	1.23

Other studies have inoculated the entire maize substracte with often high concentrations of spore inoculum. In such studies, Alberts *et al.* (1990) found that for *F.moniliforme* (MRC 826), 30°C was optimun for growth and production of FB_1 on moist maize over a 7 week period. Their study was, however, carried out without accurete control or determination of water availity. Surprising, Le Bars *et al.* (1994) found that 20°C was optimun for producction of FB_1 by *F.moniliforme* in French maize grain stored for up to 10 weeks. They

suggested that moisture content was not an important factor in production in maize with a limited moisture content range (>27% m.c.=0.97 a_w are particularly critical in partially dried maize where growth and fumonisins production by *F.moniliforme* and *F.proliferatum* can easy accur. The canges in the ratios of FB_1:FB_2 in relation to a_w and temperature have not previously been examined. Naturally contaminated maize grain and animal feedstuff were found to heve ratios of 6 (Marasas *et al.* 1988), while on milled moist maize culture the original fumonisins isolate *F.moniliforme* (MRC 826) was found to be approximately 1.9-2.1 (Alberts *et al.* 1993), Spanish isolates of *F.moniliforme* and *F.proliferatum* grown on maize cultures had ratios of 1.5-3.6 and 1.1-3.5 respectively (Sanchis *et al.* 1994)

Poorly dried maize with intermediate moisture contents (0.93-0.97 a_w) represent conditions under which colonization and production of FB_1/FB_2 by these *Fusarium* spp. are most likely to be initialed naturally. Under these conditions, matabolite production may provide a competitive adventage to eneble rapid estabvlishment on maize grain. Studies are now in progress to determine the effec of interactions between *Fusarium* spp. and other maize mycoflora to determine the effect on patterns of colonization and production of fumonisins under different environmental conditions.

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EXCLÒS DE PRÉSTEC