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Growth and gliotoxin production by feed-borne *Aspergillus fumigatus sensu stricto* strains under different interacting environmental conditions

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RESEARCH ARTICLE

Abstract

In this study the effects of temperature, oxygen tension, water activity (a_W), pH, incubation time and their interactions on (1) the lag phase prior to growth, (2) growth rate and (3) gliotoxin production of two feed-borne *Aspergillus fumigatus sensu stricto* strains, isolated from fermented maize silage and brewer's grains, were evaluated on an agar medium based on these substrates. Regardless of oxygen tension, the growth rate of the two strains decreased significantly as temperature and a_W decreased ($P<0.05$). The optimum conditions for *A. fumigatus* growth were 37 °C, 0.98 a_W for both strains at reduced oxygen tension, regardless of pH level ($P<0.05$). The studied *A. fumigatus* strains were able to grow under several incubation conditions, some of them prevalent in stored animal feeds. Some specific interactions that allowed accumulation of gliotoxin at high levels were found. This study showed that gliotoxin production occurred at more restricted conditions than fungal growth. This fact is important, as by maintaining the appropriate conditions in animal feeds, *A. fumigatus* growth and gliotoxin production can be prevented. In this study, growth rates, lag phases prior to growth and gliotoxin production over a range of environmental conditions provide useful information that can help in predicting the possible fungal contamination of fermented animal feeds. Furthermore, the information is relevant since *A. fumigatus* is an opportunistic pathogen found in cereals and fermented animal feeds and represents a high risk of contamination to animals and farm workers who handle them improperly.

Keywords: growth rate, lag phase, opportunistic pathogen, stored animal feeds, animals

1. Introduction

Aspergillus fumigatus is the most common cause of invasive aspergillosis (IA) in immunocompromised individuals. The IA incidence has increased recently, mainly due to an increasing population of immunocompromised patients (Daly and Kavanagh, 2001; Denning, 1998; Stanzani *et al.*, 2005). Despite advances in early diagnosis and antifungal therapy, IA is still the main cause of death in these patients, with mortality rates between 80 and 95% (Denning *et al.*, 1998; Woo Bok *et al.*, 2006). This thermophilic fungus is frequently found in fermented cereal based-feeds and able to produce tremorgenic mycotoxins and induce neurological syndromes in farm workers who have manipulated mouldy feed (Gordon *et al.*, 1993). Its spores are easily spread through the air and pose a high risk of exposure for both animals and humans, as all *A. fumigatus sensu stricto* isolates are opportunistic and can become pathogenic if they meet the appropriate host (Hong *et al.*, 2010; Land *et al.*, 1987; Latgé *et al.*, 2000).

Gliotoxin (GLI) an epipolythiodioxopiperazine metabolite is a toxin with potent immunosuppressive, genotoxic, cytotoxic and apoptotic effects produced by *A. fumigatus*

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(Nieminen *et al.*, 2002; Upperman *et al.*, 2003; Waring *et al.*, 1988). The toxic and immunosuppressive characteristics of this toxin imply a substantial role of this compound in fungal pathogenicity (Scharf *et al.*, 2012). GLI is produced during the infection process and has been detected in lung and sera of mice, as well as humans infected with *A. fumigatus* (Kupfahl *et al.*, 2006; Lewis *et al.*, 2005). Moreover, this toxin has been linked to intoxication and death in camels who had consumed contaminated hay, and it was produced at high levels *in vivo* by *A. fumigatus* strains recovered from bovine udder (Bauer *et al.*, 1989; Gareis and Wernery, 1994). Furthermore, a recent study demonstrated its presence in cattle feedstuff (Pereyra *et al.*, 2008).

Because of the fact that *A. fumigatus* is an ubiquitous filamentous fungus in the environment, it can be isolated practically from any kind of substrate, especially soil and decaying organic materials. In addition, it is one of the toxicogenic fungi that most frequently contaminates silages (Boysen *et al.*, 2000; Cavaglieri *et al.*, 2005; González Pereyra *et al.*, 2008, 2011). The fungal growth and mycotoxin production is markedly affected by many environmental factors, but the main factors influencing germination, growth and sporulation in fungi are water activity (a_W) and temperature (T) (Bellí *et al.*, 2004; Magan and Lacey, 1984). Few articles have reported the effects of environmental parameters on growth and GLI production by *A. fumigatus*, and all of them assayed clinical strains. Some of these have proposed that a highly aerated condition is responsible for the rapid production of GLI by *A. fumigatus* and that this fungus is able to grow at low oxygen tension (minimum 0.1%) (Hall and Denning, 1994; Watanabe *et al.*, 2004; Willger *et al.*, 2009). Recently, some articles described the influence of pH, T and metal ions on the amylase activity and detoxification of heavy metals by environmental *A. fumigatus* strains (Faryal *et al.*, 2006; Nwagu and Okolo, 2011). Fungal growth results from the complex interaction of several environmental factors; therefore, the comprehension of each factor involved is essential to understand the fungal spoilage in agricultural and food products. Also, the human exposure risk to the farm raw materials could be predicted. No prior information about the effect of different levels of T, oxygen tension, a_W, pH and their interactions on lag phase, growth rate and GLI production by feed-borne *A. fumigatus* strains is available. The aims of this work were to evaluate the effect of T, oxygen tension, a_W, pH and their interactions on (1) the lag phase prior to growth, (2) growth rate and (3) GLI production by feed-borne *A. fumigatus sensu stricto* strains.

2. Materials and methods

Fungal species

Two GLI producing *A. fumigatus sensu stricto* strains were used. *A. fumigatus* RC2063 was isolated from fermented maize silage intended for dairy cattle in Argentina. This strain was identified by morphological taxonomy proposed by Samson *et al.* (2007) and deposited in the National University of Río Cuarto, Córdoba, Argentina (RC) Collection Centre. *A. fumigatus* RC2108 isolated from fermented brewer's grains destined for swine feed in Argentina was obtained from this Collection Centre. These *A. fumigatus* strains were able to produce GLI at 722.77 ng/g and 923.58 ng/g levels, respectively in yeast extract sucrose agar (YES) during 7 days of incubation at 25 °C (G.A. Pena, unpublished data). These isolates were stored in 15% glycerol (w/v) (Sigma-Aldrich, St. Louis, MA, USA) at -20 °C.

Molecular identification to confirm the taxonomic state at species level of these strains was done by sequencing the partial β-tubulin gene (*ben*A) according to Samson *et al.* (2004a) using primers Bt2a and Bt2b (Glass and Donaldson, 1995). For amplification of β-tubulin gene, PCR was performed in a 50 µl reaction mixture containing 2.5 µl of genomic DNA (5 ng/µl), 10 µl of colourless Reaction Buffer 5× with $MgCl_2$ (1.5 mM), 28.25 µl of ultra-pure sterile water, 5 µl dNTPs (0.2 mM), 2 µl of each primer (10 pmol/µl) and 0.25 µl GoTaq DNA polymerase (5 U/µl, Promega Corporation, Madison, WI, USA). Amplification was performed in a MJ Research PTC-200 thermo cycler (GMI Inc., Ramsey, MN, USA), programmed for 5 min at 94 °C followed by 35 cycles of 1 min denaturation at 94 °C followed by primer annealing for 1 min at 58 °C and primer extension for 1 min at 72 °C and a final 7 min elongation step at 72 °C. The obtained *benA* sequences of both *A. fumigatus sensu stricto* strains have been deposited in GenBank database (*A. fumigatus* RC2063: accession number JX277549, *A. fumigatus* RC2108: accession number JX277548).

Medium

The basic medium used in this study was a fermented substrate extract agar (FSEA) at 3% (w/v). This medium was made by weighting 30 g of dried brewer's grain per litre in water. After that, the volume was made up to 1 litre and agar at 2% (w/v) was added. The a_W of the basic medium was modified by the addition of glycerol to 0.937, 0.955 and 0.982 (Dallyn and Fox, 1980). Each medium was autoclaved at 120 °C for 20 min. After that, the pH level of medium was measured and adjusted to 5, 7 and 9 by the addition of variable volumes of HCl or NaOH concentrate solutions, using a pH meter (ATI Orion 529 Main Street Boston, MA, USA). The final FSEA media were poured into 90-mm diameter sterile plastic Petri dishes (20 ml per

plate). Final a_W values of representative medium samples were checked with an a_W meter (AquaLab Series 3, Labcell Ltd., Basingstoke, UK).

Inoculation and incubation

Petri dishes (20 ml) were needle-inoculated centrally by a single point from conidial suspensions in semisolid agar at 0.2% (Samson *et al.*, 2004b). The conidial suspensions were prepared by harvesting spores from heavy sporulating cultures, grown for 7-days at 25 °C on malt extract agar (MEA), and suspending them in the semisolid agar to a concentration of 10^6 spores/ml. The final a_W of these suspensions were set to 0.937, 0.955 and 0.982 using sterile water with glycerol. Inoculated plates of the same a_W were enclosed in permeable polyethylene bags (20 plates per bag) and incubated in darkness for 14 days at 18 and 25 °C under normal oxygen tension, and at 37 °C under normal and reduced (0.4% O_2, 5% CO_2) oxygen tension. A CO_2 incubator (Innova CO-48; New Brunswick Scientific, Edison, NJ, USA) was used to create the modified atmosphere. The temperature of 37 °C was selected based on the range of both animal and human body temperature levels. The experiment was done in triplicate and carried out in two parts. First, the interaction assay of T, a_W and pH at normal oxygen tension was studied. Then, the interaction assay of a_W × pH at 37 °C and reduced oxygen tension was carried out.

Growth measurement

Growing colonies of each replicate plate were measured daily at two perpendicular diameters set at right angles to each other, until the colony reached the edge of the plate. Radial growth rates (mm/h) were subsequently calculated as the slope of the linear regression obtained from plotting the colony radius against time under the linear phase for growth. Lag phase prior to growth in each treatment was defined as the time (h) to reach 4 mm of diameter and determined as the abscissa from the growth rate curves.

Gliotoxin measurement

Gliotoxin extraction

After 3, 7 and 14 days of incubation, GLI was determined following the methodology proposed by Geisen (1996) with some modifications. From each Petri dish, three agar plugs were removed at different points of the colony and extracted with 1 ml of chloroform. The sample-solvent mixture was centrifuged for 20 min at 7,500×*g* and the chloroform phase was recovered. The solutions were filtered, evaporated to dryness and re-dissolved in 300 µl of mobile phase. The extract was analysed by high performance liquid chromatography (HPLC).

Gliotoxin detection and quantification by HLPC

GLI was determined following the methodology proposed by Frisvad (1987), with some modifications. A Perkin Elmer 200 Series HPLC System (Perkin Elmer, Shelton, CT, USA) equipped with an autosampler and UV detection was used for GLI determination. GLI separation was performed at room temperature on a Phenomenex Luna RP C18 (2) column (150×4.6 mm, 5 µm; Phenomenex Inc., Torrance, CA, USA) fitted with a C18 guard column using an isocratic mode: 75% aqueous 1% acetic acid and 25% acetonitrile. A column washing of 5 min with 95% acetonitrile, followed by 2 min of stabilisation at the running conditions was performed between the chromatographic runs. The retention time was properly checked by co-injection technique, as shown in Figure 1. Detection was done at 268 nm. The standard solutions in the mobile phase were prepared from a 1 mg/ml solution of pure GLI (Sigma-Aldrich) in methanol, after solvent evaporation. The calculated instrumental limit of detection (LOD), determined as S/N=3, was 0.23 µg/g and the limit of quantification (LOQ), as S/N=7, was 0.55 µg/g, where S is the signal (intensity of the toxin peak) and N is the signal noise. These limits were determined in the fungal extracts and standard GLI solutions.

Gliotoxin detection and quantification by HPLC-MS/MS

Highly specific and sensitive HPLC-MS/MS was used to confirm the HPLC analysis for fungal extracts with non-detectable (nd) or doubtful concentrations of GLI, following the methodology by Sulyok *et al.* (2007), with some modifications. All HPLC-MS/MS analyses were performed using a Waters 2695 Alliance HPLC (Waters Corporation, Milford, MA, USA) equipped with a Waters Alliance 2685 pump, a Waters Alliance 2695 autosampler, a Waters 2996 PDA diode array detector interfaced to a Quattro Ultima Platinum tandem quadrupole mass spectrometer (Waters) with electrospray ionisation (ESI) source. The column used was a XBridge C18 (2.1×150 mm, i.d. 3.5 µm; Waters) equipped with a guard column (2.1 mm, 10 mm i.d.) of the same material. The interfaces were operated in a positive ion mode. Nebulizer and desolvation gases were nitrogen heated to 150 and 200 °C, respectively. The capillary voltage was 3.00 kV. The nitrogen flows were adjusted to 104 and 678 l/h for cone and desolvation gases, respectively. Multiple-reaction monitoring (MRM) was used for toxin determination. The precursor peak $[M+H]^+$ of GLI (*m/z* 327) and two products peaks (*m/z* 263 and *m/z* 245) were monitored to accomplish both quantitation and qualification criteria. Data acquisition and processing were performed using Mass Lynx V.4.1 (Waters). MRM trace *m/z* 327>263 was used for quantification. Mobile phase A consisted of aqueous 1% acetic acid:5 mM ammonium acetate (99.96%:0.04%, v/w) and mobile phase B of methanol:1% acetic acid:5 mM ammonium acetate

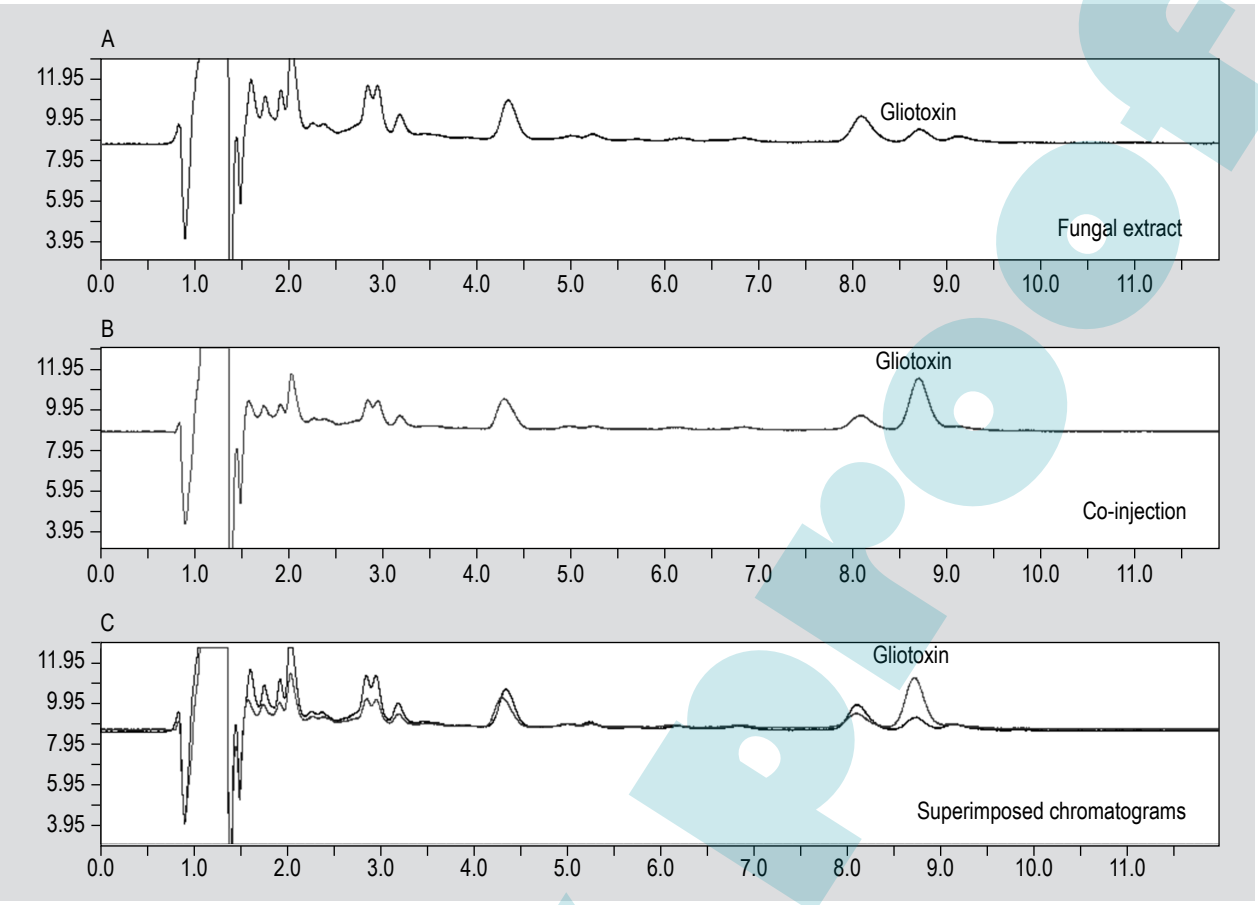

Figure 1. (A) HPLC chromatogram and (B) retention time confirmation by co-injection technique of a positive fungal extract for gliotoxin. (C) superimposed chromatograms (A+B).

(80%:19.96%:0.04%, v/v/w). At the initial time, the eluant was 89.5% mobile phase A and 10.5% of phase B. This composition was kept over 2 min, after which a linear gradient to 97.5% of solvent B was performed for 12 min, and then the eluant was kept at this composition for 3 min in order to clean the column. The initial conditions were stabilised over 5 min before the next injection. The flow rate was 0.2 ml/min. The column temperature was maintained at 22 °C. Aliquots of 20 µl of extracts were injected into the HPLC unit. Four points of identification were used to identify GLI, i.e. retention time of 14.56 min, the precursor $[M+H]^+$ of *m/z* 327 and both product ions (*m/z* 263 and 245). A calibration curve was obtained injecting increasing volumes (5-20 µl) of two working solutions of GLI, 150 and 50 ng/ml in mobile phase, in triplicate. Good linearity with a correlation coefficient higher than 0.997 was obtained for the calibration range. The calculated instrumental LOD (S/N=3) for GLI was 4.3 ng/g and LOQ (S/N=7) was 9.9 ng/g, and were determined in fungal extracts and standard solutions of GLI. The relative within-day and between-day standard deviations (% RSD) were 5.34.

Statistical analysis

In all cases, the linear regression of increase in radius against time was used to obtain the growth rates under each set of growth conditions. The lag phase and growth rate data of each strain were then evaluated by analysis of variance (ANOVA) using InfoStat for Windows 2012 Version 2.03 (SPSS Inc., Chicago, IL, USA). Statistical significance was judged at the level $P \leq 0.0001$. When the analysis was statistically significant, the Fisher's Least Significant Difference test (LSD) was used for determination of the significance of each individual parameter and their interactions on lag phases and growth rates. In the last analysis, the statistical significance was judged at the level $P<0.05$.

For statistical analysis of GLI, data were analysed by the general linear and mixed model (GLMM) using InfoStat, considering treatments, strain, temperature, oxygen tension, a_W, pH and incubation time as fixed effects. Independent errors were considered and the model was corrected to obtain the homogeneity of the variance. The variation between means of each treatment was analysed by Fischer's LSD test ($P<0.05$). The normality and variance homogeneity

were checked for the growth rate and lag phase of the obtained results. Regarding GLI analysis, datasets were transformed as $\log_{10}(x + 1)$ to obtain a normal distribution.

3. Results

The study was designed to simulate the storage conditions of animal feeds, and those related to clinical cases, using the interaction of the T, a_W and pH parameters as a function of oxygen tension.

Effect of temperature, water activity and pH at normal oxygen tension

The variance analysis of the effect of single (T, a_W and pH), two- and three-way interactions, showed that most of the single factors and their interactions were statistically significant ($P \leq 0.0001$) in relation to the lag phases of both *A. fumigatus sensu stricto* strains. All single factors and their interactions were statistically significant respect to growth rates (Table 1). At normal oxygen tension, T was the most influencing factor on lag phase and growth rate for *A. fumigatus sensu stricto* strains. The shortest lag phases (9.50 to 13.35 h for *A. fumigatus* RC2063 strain and 5.25 to 7.57 h for *A. fumigatus* RC2108) were obtained at 37 °C and 0.98 a_W for all pH levels ($P<0.05$). In general, there was an increase in the lag phase as T and a_W decreased. For *A. fumigatus* RC2108, a decrease in T from 37 °C to 25 °C at 0.98 a_W, regardless pH levels, produced an average increase in the lag phase of 6.19 to 48.52 h. Whereas, the same change in T at 0.93 a_W showed a more marked increase in the lag phase of this strain (59.93 to 153.65 h). A similar behaviour was observed for *A. fumigatus* RC2063. Moreover, at 18 °C, 0.93 a_W and pH 5, 7 and 9, both strains showed the most extended lag phases that lasted during the complete incubation period (>336 h) (Figure 2).

On the other hand, the growth rates of the two strains decreased significantly as T and a_W decreased ($P<0.05$). The optimum conditions for growth of *A. fumigatus* RC2063 and RC2108 were 37 °C, 0.98 a_W and pH 7. At this T and at 0.98 and 0.95 a_W, all pH levels showed a statistical significant difference, resulting in an optimum growth rate of both strains at pH 7 for both a_W levels. At 25 °C and 0.93 a_W, regardless of the pH, the growth rate of the strains was significantly lower with respect to the other tested conditions. Finally, for 18 °C and 0.93 a_W at all pH levels growth of both strains was completely inhibited ($P<0.05$) (Figure 2).

Influence of water activity, oxygen tension, pH at 37 °C

The analysis of the influence of a_W and pH on growth of *A. fumigatus* RC2063 and RC2108 at 37 °C and reduced oxygen tension incubation conditions is shown in Table 2. Data from the analysis of growth parameters under normal oxygen tension at 37 °C from the previous analysis were compared with the data under the reduced oxygen tension. The analysis of variance of single (a_W, oxygen tension and pH), two- and three-way interactions, showed that most factors were statistically significant ($P \leq 0.0001$) in relation to lag phases and growth rates at 37 °C for both strains.

Table 3 shows the effects of a_W, pH and oxygen tension on lag phases and growth rates of strains at 37 °C. At 0.98 a_W, all interactions of a_W × pH at reduced oxygen tension were, in general, significantly different from the same interactions at normal oxygen tension on lag phase and growth of *A. fumigatus* RC2108 ($P<0.05$). A similar behaviour was observed for *A. fumigatus* RC2063. At 37 °C and reduced oxygen tension conditions, a_W was the most influencing factor on lag phases and growth rates of the assayed strains. There was a significant increase in the lag

Table 1. Analysis of temperature (T), water activity (a_W), pH and their interactions on lag phase and growth rate of *Aspergillus fumigatus sensu stricto* strains at normal oxygen tension.

Source of variation[1]	df[1]	*A. fumigatus* RC2063				*A. fumigatus* RC2108			
		Lag phase		Growth rate		Lag phase		Growth rate	
		MS[1]	F[1]	MS	F	MS	F	MS	F
T	2	360,438.29	2221.2*	0.12	10,525.07*	109,555.3	2,234.48*	0.13	5,393.1*
a_W	2	142,446.58	877.83*	0.06	5,460.31*	3,5026.7	714.4*	0.04	1,737.21*
pH	2	1,273.73	7.85	0.00023	19.98*	446.1	9.1	0.00038	15.3*
T × a_W	4	65,622.34	404.4*	0.01	805.63*	102,138.77	2,083.21*	0.00049	195.57*
T × pH	4	6,222.04	38.34*	0.00047	41.07*	510.67	10.42*	0.00052	20.77*
a_W × pH	4	819.13	5.05	0.0006	52.82*	261.85	5.34	0.00077	30.8*
T × a_W × pH	8	1054.94	6.5*	0.00027	23.72*	328.18	6.69*	0.0003	11.87*

[1] df = degrees of freedom; MS = mean square; F = F-Snedecor; * = significant at $P \leq 0.0001$.

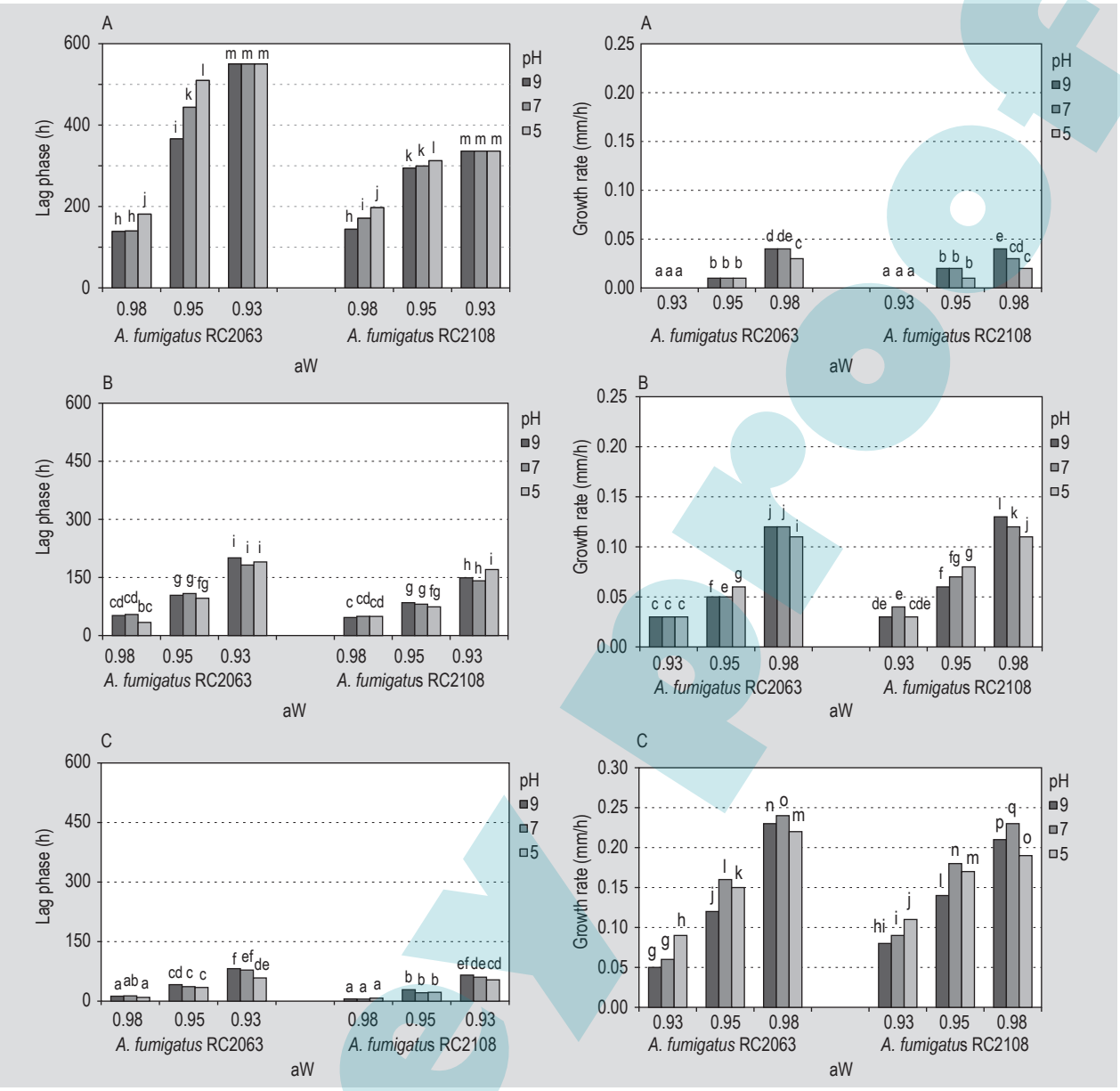

Figure 2. Effect of water activity (a_W) and pH on lag phase (h) and growth rate (mm/h) of *Aspergillus fumigatus* strains at normal oxygen tension and temperatures of (A) 18 °C, (B) 25 °C and (C) 37 °C. Mean values based on triplicate data. Means with a common letter are not significantly different according to LSD test (P<0.05).

phases and a decrease on the growth rates as a_W decreased. Maximum growth rates and the shortest lag phases were observed at the highest a_W (0.98 a_W) regardless of oxygen tension. However, lag phases at reduced oxygen tension were longer than at normal oxygen tension at 37 °C and 0.98 a_W for both strains, whereas the average growth rate was higher (P<0.05) (Table 3). At 0.95 and 0.93 a_W for all pH levels, lag phases were shorter than at normal oxygen tension for both strains. Moreover, when the strains grew at reduced oxygen tension the growth rates were, in general, higher than at normal oxygen conditions, regardless of a_W levels considered. The optimum growth condition at reduced oxygen tension (37 °C) for *A. fumigatus* was 0.98 a_W for both strains, regardless of the pH level (P<0.05).

Effect of interacting environmental conditions on gliotoxin production

The influence of the interacting factors T × a_W × pH × incubation time at normal oxygen tension and a_W × pH × incubation time × oxygen tension at 37 °C on GLI production for both *A. fumigatus sensu stricto* strains was investigated. The analysis of variance of the effect of single (strain, T, a_W, pH and incubation time), two-, three-, four- and five-way interactions, showed that all single factors,

Table 2. Analysis of variance of water activity (a_W), pH, oxygen tension (OT) and their interactions on lag phase and growth rate of *Aspergillus fumigatus sensu stricto* strains at 37 °C.

Source of variation	df[1]	*A. fumigatus* RC2063				*A. fumigatus* RC2108			
		Lag phase		Growth rate		Lag phase		Growth rate	
		MS[1]	F[1]	MS	F	MS	F	MS	F
a_W	2	14,813.1	3,168.07*	0.24	21,111.74*	11,516.46	3,840.93*	0.19	1,041.14*
pH	2	431.38	92.26*	0.0011	99.89*	123.3	41.12*	0.00067	3.7
OT	1	236.86	50.66*	0.03	2,676.85*	3.52	1.17	0.09	496.5*
a_W × pH	4	174.77	37.38*	0.00078	69.17*	107.16	35.74*	0.00089	4.91
a_W × OT	2	183.23	39.19*	0.02	2,007.2*	150	50.03*	0.03	180.83*
pH × OT	2	20.09	4.3	0.00017	15.05*	25.36	8.46	0.0008	4.41
a_W × pH × OT	4	18.53	3.96	0.00031	27.67*	18.86	6.29	0.00075	4.14

[1] df = degrees of freedom; MS = mean square; F = F-Snedecor; * = significant at $P \leq 0.0001$.

Table 3. Growth rates and lag phases of *Aspergillus fumigatus* RC2063 and RC2108 as a function of water activity (a_W), pH and oxygen tension at 37 °C.[1]

a_w	pH	Oxygen tension	*A. fumigatus* RC2063		*A. fumigatus* RC2108	
			Lag phase (h)	Growth rate (mm/h)	Lag phase (h)	Growth rate (mm/h)
0.98	9	reduced	14.46±0.34 ^{b}	0.3563±0.00172^{l}	13.27±0.42^{b}	0.389±0.00079^{i}
		normal	12.45±0.75 ^{ab}	0.2293±0.00378^{jk}	5.75±1.69^{a}	0.214± 0.00916^{gh}
	7	reduced	15.34±0.11^{b}	0.3648±0.0019^{m}	13.66±0.42^{b}	0.3897±0.00078^{i}
		normal	13.35±0.56 ^{b}	0.243±0.001^{k}	5.25±1.14^{a}	0.2333±0.00451^{h}
	5	reduced	14.39±0.29^{b}	0.3541±0.0032^{l}	13.15±0.19^{b}	0.3895±0.00051^{i}
		normal	9.50±2.48 ^{a}	0.218±0.001^{j}	7.57±2.96^{a}	0.194±0.00435^{fg}
0.95	9	reduced	29.77±0.88^{d}	0.1591±0.0024^{g}	20.27±4.43^{c}	0.2341±0.0532^{h}
		normal	41.63±2.16^{f}	0.121±0.002^{e}	28.77±0.73^{d}	0.136 ±0.0131^{d}
	7	reduced	29.39±0.72^{d}	0.1655±0.0008^{h}	21.68±0.71^{c}	0.2188±0.0006^{h}
		normal	36.58±0.77^{e}	0.1603±0.0025^{gh}	21.95±1.18^{c}	0.1846 ±0.0061^{ef}
	5	reduced	25.65±0.72^{c}	0.1649±0.0013^{h}	21.95±0.72^{c}	0.2175 ±0.00089^{h}
		normal	34.49±1.20^{e}	0.1527±0.0075^{f}	22.27±1.07^{c}	0.1686 ±0.0050^{e}
0.93	9	reduced	76.77±5.97^{i}	0.0528±0.0016^{a}	63.11±0.72^{gh}	0.0861±0.0011^{ab}
		normal	81.90±3.05^{j}	0.0546±0.0011^{a}	65.65±3.65^{h}	0.0826±0.0051^{a}
	7	reduced	66.11±2.45^{h}	0.0649±0.0007^{b}	61.90±0.83^{g}	0.0968±0.00045^{ab}
		normal	78.23±2.45^{i}	0.058±0.0026^{a}	60.36±0.76^{g}	0.0873±0.0030^{ab}
	5	reduced	57.04±1.90^{g}	0.0728±0.0021^{c}	46.95±0.58^{e}	0.1212±0.0024^{cd}
		normal	58.47±2.79^{g}	0.092±0.0088^{d}	53.79±1.23^{f}	0.1073±0.0049^{bc}

[1] Values are mean ± standard deviation. Values corresponding to the same letter do not differ significantly according to Fisher's protected LSD test ($P<0.05$).

as well as their interactions were statistically significant ($P \leq 0.0001$) in relation to GLI accumulation at normal oxygen tension. The same behaviour was observed when the analysis of variance of the effect of single (strain, a_W, pH, incubation time and oxygen tension), two-, three-, four- and five-way interaction at 37 °C was carry out.

In general, GLI production did not show a similar pattern to that found in the growth assays. The effect of all interacting environmental factors was variable. GLI was detected at only few growth conditions, but in some of them, GLI was produced at higher levels than on YES medium (G.A. Pena, unpublished data). At normal oxygen tension and 3 days incubation, GLI was not detected at any interaction.

This behaviour was also observed at 18 °C in all a_W × pH × incubation time interactions for both *A. fumigatus sensu stricto* strains. However, at 7 and 14 days, GLI was mainly detected at 37 and 25 °C and at 0.93 a_W conditions for both strains ($P<0.05$). The toxin was also detected at some other conditions, but, in general, at lower levels (Table 4). GLI production for *A. fumigatus* RC2063 and RC2108 was highest at pH 7, 37 °C and 0.93 a_W. Similarly, at reduced oxygen tension and 37 °C, GLI was mainly detected at 0.93 a_W in fungal extracts of *A. fumigatus* RC2108. The toxin was also detected in fungal extracts of this strain at 3 days incubation at levels of 39.6 and 45.9 ng/g, at pH 7 and 9, respectively. The highest GLI concentration at reduced oxygen tension was detected at 7 days incubation, 0.93 a_W and pH 7 for *A. fumigatus* RC2108 (data not shown).

Generally at 37 °C, when the strains produced GLI at normal oxygen tension at 7 days incubation, the toxin concentration was significantly reduced at 14 days ($P<0.05$) (Table 4). The same behaviour was observed at 25 °C and at 0.98 and 0.95 a_W for both studied strains, whereas at this T and the lowest a_W evaluated (0.93 a_W) the strains produced GLI after 14 days incubation, without any detectable toxin during the previous 7 days. This effect in GLI production was also observed at reduced oxygen tension (data not shown). The highest GLI concentration found in the whole assay was detected at normal oxygen tension in *A. fumigatus* RC2063 extracts at 14 days incubation at 25 °C, 0.93 a_W and pH 9 ($P<0.05$).

4. Discussion

In the present study, the lag phase prior to growth and mycelial growth of two *A. fumigatus sensu stricto* strains, isolated from animal feeds, were found to be mainly influenced by T, a_W and oxygen tension and their interactions. Regardless of oxygen tension during incubation, lag phases increased and growth rates decreased as T and a_W decreased. High T and a_W, and microaerophilic conditions are prevalent in fermented feeds, such as silages and brewer´s grains. The studied *A. fumigatus sensu stricto* strains were able to grow under these conditions, with longer lag phases than at normal oxygen tension, but with higher growth rates, at the highest T and a_W tested. Moreover, results of all interactions were found to be diverse and complex. Therefore, each one could be considered in relation to the ecosystem that needs to be studied. The growth rate of *A. fumigatus* appears to be an important parameter influencing the pathogenesis, since it is one of the most prevalent airborne fungal pathogens. Paisley *et al.* (2005) proposed a kinetic microbroth method for spectrophotometrically measuring the growth of clinical

Table 4. Gliotoxin production (ng/g) by *Aspergillus fumigatus sensu stricto* under different temperature, water activity (a_W), pH and incubation time (days) tested at normal oxygen condition.[1,2]

Temperature	a_W	pH	*A. fumigatus* RC2063		*A. fumigatus* RC2108	
			7 days	14 days	7 days	14 days
37 °C	0.98	9	ND^{s}	ND^{s}	ND^{s}	ND^{s}
		7	ND^{s}	ND^{s}	ND^{s}	ND^{s}
		5	ND^{s}	ND^{s}	ND^{s}	ND^{s}
	0.95	9	ND^{s}	ND^{s}	ND^{s}	ND^{s}
		7	ND^{s}	ND^{s}	ND^{s}	ND^{s}
		5	ND^{s}	ND^{s}	ND^{s}	ND^{s}
	0.93	9	420.9^{i}	140.2^{k}	1,404.9^{d}	217.8^{j}
		7	1,820.5^{c}	958.4^{e}	2,377.7^{b}	944.2^{e}
		5	ND^{s}	ND^{s}	ND^{s}	ND^{s}
25 °C	0.98	9	513.5^{g}	45.1^{l}	ND^{s}	ND^{s}
		7	ND^{s}	13.7^{p}	ND^{s}	ND^{s}
		5	ND^{s}	ND^{s}	677.8^{f}	ND^{s}
	0.95	9	692.2^{f}	ND^{s}	40.9^{m}	ND^{s}
		7	23.6^{o}	ND^{s}	ND^{s}	ND^{s}
		5	ND^{s}	12^{q}	ND^{s}	ND^{s}
	0.93	9	NG^{s}	4,583.4^{a}	NG^{s}	688.1^{f}
		7	NG^{s}	450.6^{h}	NG^{s}	ND^{s}
		5	NG^{s}	ND^{s}	NG^{s}	10.1^{r}

[1] ND = not detected after confirmation by HPLC-MS/MS; NG = no growth. All negative samples obtained from HPLC-UV were confirmed by HPLC-MS/MS.
[2] Values with the same letter do not differ significantly ($P<0.05$).

A. fumigatus. They compared the growth rates and LD90 values of these isolates and suggested a correlation between both parameters. However, this link between growth rate and virulence does need further investigation.

In this study, at minimum levels of T and a_w under normal oxygen tension, the *A. fumigatus sensu stricto* strains were not able to grow during any of the evaluated incubation periods. Northolt *et al.* (1995) reported a minimal a_w of 0.85 to 0.95 for growth of *A. fumigatus*. In another study, some *A. fumigatus* strains isolated from marine salterns were able to grow at 0.828 a_W, after 15 days incubation at 30 °C (Tepsic *et al.*, 1997). The source of isolation of these adapted strains could explain this behaviour.

At the present time there are few data available on growth parameters of *A. fumigatus sensu stricto*, especially from strains isolated from feedstuffs. Hall and Denning (1994) reported the influence of some environmental factors on growth of clinical *A. fumigatus* strains. They investigated the growth of 24 *Aspergillus* isolates, of which 10 *A. fumigatus* strains, at low oxygen concentrations (0, 0.025, 0.1, 0.5 and 2.5%). The pathogenic *A. fumigatus* strains were able to grow at low oxygen tensions, but not without oxygen. Also, the influence of interacting environmental parameters, such as T, a_W, oxygen tension, pH and incubation time on GLI production was studied. Some of the specific interactions allowed higher levels of the toxin accumulation than those observed in synthetic YES medium. In general, GLI could be detected at the lowest a_W level assayed. This study showed that GLI production occurred at more restricted conditions than fungal growth (Hall and Denning, 1994). This fact is important because maintaining the appropriate conditions in cereal based-feeds could prevent *A. fumigatus* growth and GLI production. Growth of a fungal colony is not synonymous with mycotoxin production, even for mycotoxigenic fungi, as environmental conditions may allow growth but not the production of mycotoxins. Mycotoxin production may be triggered under stress conditions for growth. Total prevention of fungal growth effectively prevents mycotoxin accumulation (García *et al.*, 2009).

In this study, after GLI had been produced within 7 days incubation (at 37 °C), the toxin concentration was significantly reduced at 14 days, whereas at suboptimal conditions (at 25 °C and the lowest a_W evaluated) the strains produced GLI after 14 days incubation, without any toxin detected during the previous days. Probably, a more active fungal metabolism (high T or a_W) enables a faster GLI production, though not always at the highest levels. The decrease in GLI concentration detected between 7 and 14 days of incubation could be explained by their consumption by the fungus, which can use GLI as a carbon source. Abrunhosa *et al.* (2002), Leong *et al.* (2006) and Varga *et al.* (2000) have reported similar results in other *Aspergillus* species.

The influence of environmental factors exerted significant effects on growth and GLI production of the studied strains. Moreover, it shows for the first time the effects of interacting environmental conditions on lag phase, growth rate and GLI production by feed-borne *A. fumigatus sensu stricto* strains. The ability of this fungus to adapt to diverse environmental conditions could explain why *A. fumigatus* is the most frequently occurring opportunistic pathogenic fungus. The studied *A. fumigatus* strains were able to grow under several incubation conditions, some of them prevalent in stored animal feeds such as fermented feeds. Willger *et al.* (2009) identified primary metabolites in the mammalian lung typically associated with fungal growth under hypoxic environments. However, Watanabe *et al.* (2004) proposed that a highly aerated condition was responsible for the rapid production of GLI by clinical *A. fumigatus* strains.

The fungal growth rates and lag phases prior to growth over a range of environmental conditions provide useful information about the ecology of these rarely studied strains. This information would help in predicting the possible fungal contamination of *A. fumigatus* in fermented animal feeds. This opportunistic pathogen, when found in cereals, represents a high risk to contamination to animals and farm workers who handle them improperly. Other studies including more strains and experimental levels per factor could be useful to develop predictive models expressing the kinetic parameters as a function of the environmental factors tested. These mathematical models could be used to predict the growth boundaries and behaviour of *A. fumigatus* strains in other ecosystems.

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References

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

Bauer, J., Gareis, M., Bott, A. and Gedek, B., 1989. Isolation of a mycotoxin (gliotoxin) from a bovine udder infected with *Aspergillus fumigatus*. Journal of Medical and Veterinary Mycology 27: 45-50.

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

Boysen, M.E., Jacobsson, K.G. and Schnürer, J., 2000. Molecular identification of species from the *Penicillium roqueforti* group associated with spoiled animal feed. Applied and Environmental Microbiology 66: 1523-1526.

Cavaglieri, L.R., González Pereyra, M.L., Pereyra, C.M., Magnoli, C.E., Chulze, S.N. and Dalcero, A.M., 2005. Fungal and mycotoxin contamination of cow feeding stuffs in Argentina. In: Conference on reducing impact of mycotoxins in tropical agriculture. Ghana, Africa, abstract.

Dallyn, H. and Fox, A., 1980. Spoilage of material of reduced water activity by xerophilic fungi. In: Gould, G.H. and Corry, E.L. (eds.) Microbial growth and survival in extreme environments. Academic Press, London, UK, pp. 129-139.

Daly, P. and Kavanagh K, 2001. Pulmonary aspergillosis: clinical presentation, diagnosis and therapy. British Journal of Biomedical Science 58: 197-205.

Denning, D.W., 1998. Invasive aspergillosis. Clinical Infectious Diseases 26: 781-803.

Faryal, R., Lodhi, A. and Hameed, A., 2006. Isolation, characterization and biosorption of zinc by indigenous fungal strains *Aspergillus fumigatus* RH05 and *Aspergillus flavus* RH07. Pakistan Journal of Botany 38: 817-832.

Frisvad, J.C., 1987. High-performance liquid chromatographic determination of profiles of mycotoxins and other secondary metabolites. Journal of Chromatography A 392: 333-347.

García, D., Ramos, A.J., Sanchís, V. and Marín, S., 2009. Predicting mycotoxins in foods: a review. Food Microbiology 26: 757-769.

Gareis, M. and Wernery, U., 1994. Determination of gliotoxin in samples associated with cases of intoxication in camels. Mycotoxin Research 10: 2-8.

Geisen, R., 1996. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. Journal of Applied Microbiology 19: 388-392.

Glass, N.L. and Donaldson, G., 1995. Development of primer 406 sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61: 1323-1330.

González Pereyra, M.L., Alonso, V.A., Sager, R., Morlaco, M.B., Magnoli, C.E., Astoreca, A.L., Rosa, C.A.R., Chiacchiera, S.M., Dalcero, A.M. and Cavaglieri, L.R., 2008. Fungi and selected mycotoxins from pre- and post-fermented corn silage. Journal of Applied Microbiology 104: 1034-1041.

González Pereyra, M.L., Chiacchiera, S.M., Rosa, C.A.R., Sager, R., Dalcero, A.M. and Cavaglieri, L., 2011. Comparative analysis of the mycobiota and mycotoxins contaminating corn trench silos and silo bags. Journal of the Science of Food and Agriculture 91: 1474-1481.

Gordon, K.E., Masotti, R.E. and Waddell, W.R., 1993. Tremorgenic encephalopathy: a role of mycotoxins in the production of CNS disease in humans. Canadian Journal of Neurological Sciences 20: 237-239.

Hall, L.A. and Denning, D.W., 1994. Oxygen requirements of *Aspergillus* species. Journal of Medical Microbiology 41: 311-315.

Hong, S.B., Kim, D.H., Park, I.C., Choi, Y.J., Shin, H.D. and Samson, R.A., 2010. Re-identification of *Aspergillus fumigatus sensu lato* based on a new concept of species delimitation. Journal of Microbiology 48: 607-615.

Kupfahl, C., Heinekamp, T., Geginat, G., Ruppert, T., Hartl, A., Hof, H. and Brakhage, A.A., 2006. Deletion of the *gli*P gene of *Aspergillus fumigatus* results in loss of gliotoxin production but has no effect on virulence of the fungus in a low-dose mouse infection model. Molecular Microbiology 62: 292-302.

Land, C., Hult, K., Fuchs, R., Hagelberg, S. and Lundstrom, H., 1987. Tremorgenic mycotoxins from *Aspergillus fumigatus* as a possible occupational health problem in sawmills. Applied and Environmental Microbiology 193: 262-275.

Latgé, J.P., Verweij, P.E. and Bretagne, S., 2000. Molecular typing of *Aspergillus fumigatus*. In: Samson, R.A. and Pitt, J.I. (eds.) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood Academic Publishers, Amsterdam, the Netherlands, pp. 471-482.

Leong, S.L., Hocking, A.D. and Scott, E.S., 2006. Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium. International Journal of Food Microbiology 110: 209-216.

Lewis, R.E., Wiederhold, N.P., Chi, J., Han, X.Y., Komanduri, K.V., Kontoyiannis, D.P. and Prince, R.A., 2005. Detection of gliotoxin in experimental and human aspergillosis. Infection and Immunity 73: 635-637.

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

Nieminen, S.M., Maki-Paakkanen, J., Hirvonen, M.R., Roponen, M. and Von Wright, A., 2002. Genotoxicity of gliotoxin, a secondary metabolite of *Aspergillus fumigatus*, in a battery of short-term test systems. Mutation Research 520: 161-170.

Northolt, M.D., Frisvad, J.C. and Samson, R.A., 1995. Occurrence of food-borne fungi and factors for growth. In: Samson, R.A., Hoekstra, E.S., Frisvad, J.C. and Filtenborg, O. (eds.) Introduction to food-borne fungi. Centraalbureau voor Schimmelcultures, Baarn, the Netherlands, pp. 243-250.

Nwagu, T.N. and Okolo, B.N., 2011. Growth profile and amylolytic activity of a thermophilic fungus *Aspergillus fumigatus* isolated from soil. Asian Journal of Biotechnology 3: 46-57.

Paisley, D., Robson, G.D. and Denning, D.W., 2005. Correlation between *in vitro* growth rate and *in vivo* virulence in *Aspergillus fumigatus*. Medical Mycology 43: 397-401.

Pereyra, C.M., Alonso, V.A., Rosa, C.A.R., Chiacchiera, S.M., Dalcero, A.M. and Cavaglieri, L.R., 2008. Gliotoxin natural incidence of *Aspergillus fumigatus* isolated from corn silage and ready dairy cattle feed. World Mycotoxin Journal 1: 457-462.

Samson, R.A., Hoekstra, E.S. and Frisvad, J.C., 2004b. Introduction to food and airborne fungi, 7th edition. Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

Samson, R.A., Hong, S., Peterson, S.W., Frisvad, J.C. and Varga, J., 2007. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. Studies in Mycology 59: 147-203.

Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F.A., Frank, J.M. and Frisvad, J.C., 2004a. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. Studies in Mycology 50: 45-61.

Scharf, D.H., Heinekamp, T., Remme, N., Hortschansky, P., Brakhage, A.A. and Hertweck, C., 2012. Biosynthesis and function of gliotoxin in *Aspergillus fumigatus*. Applied Microbiology and Biotechnology 93: 467-472.

Stanzani, M., Orciuolo, E., Lewis, R., Kontoyiannis, D., Martins, S., St. John, L. and Komanduri, K., 2005. *Aspergillus fumigatus* suppresses the human cellular immune response via gliotoxin-mediated apoptosis of monocytes. Blood 105: 2258-2265.

Sulyok, M., Krska, R. and Schuhmacher, R., 2007. A liquid chromatography/tandem mass spectrometric multimycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples. Analytical and Bioanalytical Chemistry 389: 1505-1523.

Tepsic, K., Gunde-Cimerman, N. and Frisvad, J.C., 1997. Growth and mycotoxin production by *Aspergillus fumigatus* strains isolated from a saltern. FEMS Microbiology Letters 157: 9-12.

Upperman, J.S., Potoka, D.A., Zhang, X.R., Wong, K., Zamora, R. and Ford, H.R., 2003. Mechanism of intestinalderived fungal sepsis by gliotoxin, a fungal metabolite. Journal of Pediatric Surgery 38: 966-970.

Varga, J., Kevei, F., Hamari, Z., Tóth, B. and Téren, J., 2000. Genotypic and phenotypic variability among black *Aspergilli*. In: Samson, R.A. and Pitt, J.I. (eds.) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood Academic Publishers, Amsterdam, the Netherlands, pp. 397-411.

Waring, P., Eichner, R., Mullbacher, A. and Sjaarda, A., 1988. Gliotoxin induces apoptosis in macrophages unrelated to its antiphagocytic properties. Journal of Biological Chemistry 263: 18493-18499.

Watanabe, A., Kamei, K., Sekine, T., Waku, M., Nishimura, K. and Miyaji, M., 2004. Effect of aeration on gliotoxin production by *Aspergillus fumigatus* in its culture filtrate. Mycopathologia 157: 245-254.

Willger, S.D., Grahl, N. and Cramer, Jr., R.A., 2009. *Aspergillus fumigatus* metabolism: clues to mechanisms of *in vivo* fungal growth and virulence. Medical Mycology 47: S72-S79.

Woo Bok, J., Woon Chung, D., Balajee, S.A., Marr, K.A., Andes, D., Nielsen, K.F. and Frisvad, J.C., 2006. GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to *Aspergillus fumigatus* virulence. Infection and Immunity 74: 6761-6768.