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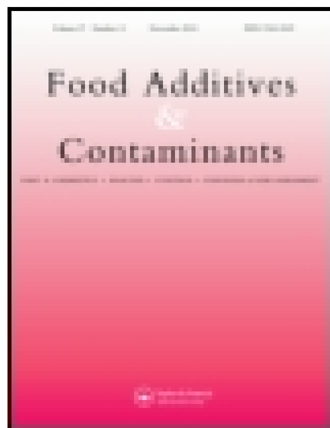

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Physiological behaviour of gliotoxigenic Aspergillus fumigatus sensu stricto isolated from maize silage under simulated environmental conditions

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Physiological behaviour of gliotoxigenic *Aspergillus fumigatus sensu stricto* isolated from maize silage under simulated environmental conditions

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Environmental conditions play a key role in fungal development. During the silage production process, humidity, oxygen availability and pH vary among lactic-fermentation phases and among different silage sections. The aim of this work was to study the physiological behaviour of gliotoxicogenic *Aspergillus fumigatus* strains isolated from maize silage under simulated natural physicochemical conditions – different water activities (a_W), temperatures (T^o), pH and oxygen pressure – on the growth parameters (growth rate and lag phase) and gliotoxin production. The silage was made with the harvested whole maize plant that was chopped and used for trench-type silo fabrication. Water activity and pH of the silage samples were determined. Total fungal counts were performed on Dichloran Rose Bengal Chloramphenicol agar and Dichloran 18% Glycerol agar. The morphological identification of *A. fumigatus* was performed with different culture media and at different growth temperature to observe microscopic and macroscopic characteristics. Gliotoxin production by *A. fumigatus* was determined by HPLC. All strains isolated were morphologically identified as *A. fumigatus*. Two *A. fumigatus* strains isolated from the silage samples were selected for the ecophysiological study (*A. fumigatus sensu stricto* RC031 and RC032). The results of this investigation showed that the fungus grows in the simulated natural physicochemical conditions of corn silage and produces gliotoxin. The study of the physiological behaviour of gliotoxigenic *A. fumigatus* under simulated environmental conditions allowed its behaviour to be predicted in silage and this will in future enable appropriate control strategies to be developed to prevent the spread of this fungus and toxin production that leads to impairment and reduced quality of silage.

Keywords: *Aspergillus fumigatus sensu stricto*; glitoxin; physiological behaviour

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Introduction

Fungal contamination of cattle feedingstuffs is a worldwide problem; the spoilage and reduction of the nutritional quality caused by this contamination can lead to important economic losses for the agricultural/breeder sector. The main genera that produce mycotoxins – *Fusarium, Penicillium, Alternaria* and *Aspergillus* – are widely distributed in nature (Lacey 1989). The production of mycotoxins by some fungal species represents a risk to animal and human health.

Silage-making conserves the nutritive value of green crops by spontaneous lactic fermentation produced by lactic acid bacteria under anaerobic conditions. The quality of the final product depends on the nutritive value of the raw materials and the conditions under which the fermentation occurs. Low sugar levels and the presence of oxygen affects lactic fermentation. If this process does not occur in the proper conditions it could lead to the growth of yeasts that increase the pH followed by an increase in temperature. If the pH increases above the critical limits, the development of other microorganisms that spoil the silage such as *Aspergillus* species, one of the most common genera isolated in silage, can occur (Weissbach 1996; El-Shanawany et al. 2005; Garon et al. 2006; González Pereyra et al. 2008; Alonso et al. 2013). Aspergillosis is defined as the tissue damage caused by fungi of the genera *Aspergillus* (Vanden Bossche 1988). Among 250 species within *Aspergillus* genera, infections in humans and animals are mainly caused by only eight species, and *A. fumigatus* is the main opportunist pathogen (Kradin & Mark 2008). This fungus has been frequently isolated from invasive aspergillosis, a disease that affects immunocompromised patients. The symptoms of aspergillosis can be presented at least in four forms: mycotoxicosis, allergic diseases, saprophytic colonisation, or chronic and invasive or systemic disease (Denning 2006). The severity of the aspergillosis depends on several factors, but the most important is the immune system state of the patient (Vonberg & Gastmeier 2006). Thus, they behave as opportunist pathogens in immunocompromised patients (patients with human immunodeficiency virus (HIV), patients who have had transplants, cancer patients, etc.). *Aspergillus fumigatus* is a widely distributed saprophytic fungus that can be found in the soil or decaying organic

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matter where it performs an essential role in the carbon and nitrogen cycle. Previous studies have shown the prevalence of *Aspergillus* species in silage and finished feed for cattle in feedstuffs (González Pereyra et al. 2008, 2009, 2011; Pereyra et al. 2008; Keller et al. 2012; Alonso et al. 2013).

Gliotoxin, the main mycotoxin produced by *A. fumigatus*, a secondary metabolite from the epipolythiodioxopiperazine family, was described as an antibiotic due to its antibacterial, antiviral and antifungal properties. This toxin exhibits diverse biological activities against the immune system. In murine models, the inhibition of macrophages and polymorphonuclear cells, including phagocytosis and bactericide activity, has been shown. It presents the capacity to inhibit the nuclear transcription factor NF-kB (central regulator of the immune response), and hence prevents the transcription activation of several inflammatory cytokines, haematopoietic growth factors and their receptors, and cellular adhesion molecules (Pahl et al. 1996). Gliotoxin production by *A. fumigatus* can establish an important immune-evasive factor that is mediated by direct effects onto the antigen presenting cells and by direct and indirect effects onto T-cells (Stanzani et al. 2004). Studies by Morgavi et al. (2004) demonstrated that gliotoxin associated with other toxic metabolites present in culture extracts of *A. fumigatus* had negative effects on *in vitro* ruminal fermentation.

Temperature and humidity levels are key factors in fungal growth and mycotoxin production, therefore the environmental conditions play a key role in fungal development. During the silage production process, humidity, oxygen availability and pH can change among lactic-fermentation phases and among different silage sections, therefore a knowledge of *A. fumigatus* growth and gliotoxin production conditions could allow one to predict its behaviour and could help to prevent occurrence in silage. The aim of this work was to study the physiological behaviour of gliotoxicogenic *A. fumigatus* strains isolated from maize silage under simulated natural physicochemical conditions – different water activities (a_W), temperatures (T^o), pH and oxygen pressure – on the growth parameters (growth rate and lag phase) and gliotoxin production.

Materials and methods

Sampling and sample processing

The samples were collected from two dairy farms located in the south dairy area of Córdoba province, Argentina. The silage was made with the harvested whole maize plant that was chopped and used for trench-type silo fabrication which was 25 m long and covered about 125 m^2. Silage samples were collected manually from both kinds of silos in transects at three levels. Three points from the upper section, three points from the middle and three points from the lower section of the silo were sampled at 15 cm depth (Figure 1), making 2 kg samples for each level. A 2 kg sample of silage material was taken from each section. It was homogenised and quartered manually so that a 500 g sample from each sampling site was obtained. All samples were immediately taken to the laboratory, ground in a mill (particle size of 1 mm) and tested for pH and a_W.

Determination of water activity in samples

The measure of the samples a_W was made with an AQUALAB CX2 equipment (Decagon, Devices, Pullman, WA, USA) and calibrated before each measure. Previous to the calibration, the equipment was located in an area with stable temperature and a solution of potassium chloride (CLK) 0.760 a_W was used as reference standard. When readings of the humidity standard were within the stated ranges, duplicate readings of distilled water were made.

Determination of pH in samples

The pH determinations were made in a 150 ml beaker filled halfway with fresh sample. Then enough water

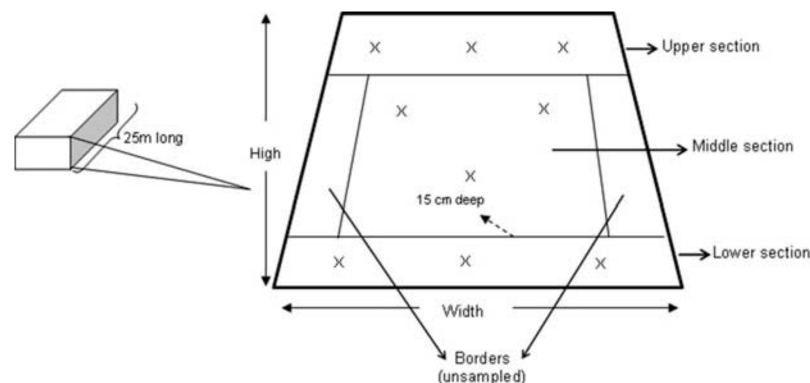

Figure 1. Sampling scheme designed for trench-type silos in different sections. Sampling points are represents by symbol ‘×’.

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(boiled and cooled previously) was to set sample leaving about 1.25 cm of free water over the top. The samples were stirred and left 30 min to rest. The water was removed and the pH lectures were made immediately with a pH meter (ORION Model 210A) and standard pH 4.0 and 7.0 solutions were used.

Isolation of fungus strains

Total fungal counts were performed onto Dichloran Rose Bengal Chloramphenicol agar (DRBC) and Dichloran 18% Glycerol agar (DG18), the former being a general media used for estimating total culturable fungi (Abarca et al. 1994), and the latter having low water activity (a_W) and favouring xerophilic fungi development (Pitt & Hocking 1997). Samples (10 g) were homogenised in 90 ml of 0.1% peptone water solution for 30 min in an orbital shaker, obtaining a dilution of 10^{-1}. Serial dilutions (10^{-2} to 10^{-5}) were made and 0.1 ml aliquots were inoculated in duplicates onto the media described above. The plates were incubated at 25°C for 7–10 days; only plates containing 10–100 colony-forming units (CFU) were used for counting, with results expressed as CFU g^{-1}. Representative colonies of *Aspergillus* section *Fumigati* were transferred for subculturing to tubes containing malt extract agar (MEA) and incubated at 28°C for 7 days for later identification.

Morphological identification of Aspergillus *section* Fumigati

Morphological identification was made according to Samson et al. (2007). Identification for each species was performed with different culture media and growth temperature for the observation of microscopic and macroscopic characteristics. Identification of each species of *Aspergillus* section *Fumigati* was performed from cultures of MEA. Suspensions of conidia in semisolid agar of each strains were used to inoculate three equidistant points in the plates with Czapek yeast extract agar (CYA) (25°C and 37°C), MEA (25°C), oatmeal agar (OAT) (25°C), yeast extract saccharose agar (YES) (25°C), creatine saccharose agar (CREA) (25°C) and Czapek agar (CZ) (25°C). The plates were incubated for 5–7 days at the indicated temperature.

The *A. fumigatus* strains were deposited in the National University of Río Cuarto, Córdoba, Argentina (RC) Collection Centre.

Gliotoxin production by Aspergillus fumigatus *strains*

Gliotoxin production of *A. fumigatus* strains was determined. Until the analysis the strains were preserved on MEA at 4°C. For the determination of gliotoxin production, the *A. fumigatus* strains were replicated in duplicate in 250 ml Erlenmeyer with 100 ml of YES broth. A 5 mm diameter plug was taken from a 7-day MEA culture and used to inoculate the YES broth; it was then incubated at 37°C for 3 days at 1400 rpm agitation. The broth was filtered with Whatman No. 1 paper. Toxin extraction from the filtered liquid was done twice with 50 ml chloroform at 25°C under 10 min agitation. The chloroform fractions were collected and evaporated with a rotator evaporator until dryness. Dry extracts were conserved at −70°C until the HPLC quantification.

Detection and quantification of gliotoxin

Samples were analysed in triplicate. Dry extracts were redissolved in the mobile phase and filtered (Titan filtration system, 17 mm, 0.45 µm; Rockwood, TN, USA) for gliotoxin analysis by HPLC. Gliotoxin detection and quantification were determined following the methodology proposed by Frisvad (1987), with some modifications. The HPLC apparatus used for gliotoxin determination was a Waters e2695 with a loop of 20 µl equipped with a Waters 2998 diode array detector. Gliotoxin separation was performed at RT on a Phenomenex Luna RP C18 column (150 × 4.6 mm, 5 µm; Phenomenex Inc., Torrance, CA, USA) fitted with a C18 guard column using an isocratic mode of 75% aqueous 1% acetic acid and 25% acetonitrile. The mobile phase flow was 1.5 ml min^{-1} and the volume injection was 20 µl. Detection was done at 268 nm and the retention time was 8.5–8.8 min. A column washing of 5 min at 95% acetonitrile followed by 2 min of stabilisation at the running conditions was performed between chromatographic runs. The retention time was checked by co-injection. The working solution (1 mg ml^{-1}) was obtained by dissolving pure gliotoxin in HPLC quality acetonitrile; from this solution (5 mg/5 ml) were obtained the 1 mg ml^{-1} solution and held in −20°C. The gliotoxin standard solutions in mobile phase were prepared from a working solution (1 mg ml^{-1}) in methanol, after solvent evaporation. A calibration curve was obtained by injecting 20 µl of each witness solution (30, 20, 12.5, 7.5, 6, 0.3 and 0.1 µg ml^{-1} of mobile phase). A linear relationship was observed in the calibration curve in the range of toxin used. Quantification of gliotoxin was performed by measuring the areas followed by extrapolation to obtain a calibration curve using standard solutions of gliotoxin. For recovery, assay tubes with YES medium were fortified at two levels and incubated at 25°C for 24 h. The relative standard deviations (RSDr) of the measurements registered on the same day (repeatability) and on different days (reproducibility) were calculated. The average percentages recovered from media spiked with 5 and 7 µg g^{-1} were 100.3% and 92.4% with RSD_r's of 6.6% and 3.8% respectively. Repeatability and reproducibility of the results determined at different concentrations of gliotoxin (0.5–1000 ng injected) ranged from 0.3% to 5.4% and from 3.9% to 12.7%, respectively. The instrumental LODs and LOQs were determined in fungal extracts and standard solutions of gliotoxin based on the

signal/noise (*S/N*) ratio of 3:1 for LOD and of 7:1 for LOQ. The LOD of the method was 0.12 ng g^{-1}.

Ecophysiological study

Two *A. fumigatus* strains isolated from the silage samples were selected for the ecophysiological study: *A. fumigatus sensu stricto* RC031 and *A. fumigatus sensu stricto* RC032. (Strain RC031 was one of the highest producers of gliotoxin, while strain RC032 was one that showed less ability to produce gliotoxin.) These strains were stored in glycerol 15% (Sigma-Aldrich, Buenos Aires, Argentina) at −80°C. The effect of the single and interacting factors a_W, pH, oxygen tension and incubation time on the growth parameters (growth rate and lag phase) and gliotoxin production was evaluated.

The culture medium was prepared using the previously dried silage (30 g). Then distilled water was added and heated to 80°C. The solution was filtrated and the volume completed to 1000 ml with distilled water; then agar–agar (3% w/v) was added. The a_W of the basic medium was modified by the addition of known amounts of glycerol to 0.8, 0.85, 0.9, 0.92, 0.94, 0.96, 0.98 and 0.99 (Dallyn & Fox 1980). Final a_W values of representative medium samples were checked with an a_W meter (AquaLab Series 3, Labcell Ltd, Basingstoke, UK). Each medium was autoclaved at 120°C for 20 min. After that the pH of medium was measured and adjusted to 3.5, 4, 4.5, 5, 6, 7, 7.5 and 8 by the addition of adequate volumes of HCl or NaOH concentrate solutions, using a pH meter (ATI Orion, Boston, MA, USA).

Inoculation and incubation under normal and reduced oxygen tension

Petri plates (20 ml) were centrally needle-inoculated from conidial suspensions in semisolid agar (0.2%). The conidial suspensions were prepared by harvesting spores from heavy sporulating cultures (7-day-old growing colony at 25°C) of each isolate on MEA and suspending them in the semisolid agar. Inoculated plates of the same a_W were enclosed in permeable polyethylene bags (20 plates per bag) and incubated in darkness for 25 days at 25°C under normal oxygen tension. To create a modified atmosphere, a CO_2 incubator (New Brunswick Scientific Innova CO-48, Edison, NJ, USA) was used and plates were incubated under reduced oxygen tension (0.4% O_2, 5% CO_2). Each experiment was performed in triplicate.

Measurement of growth parameters

The growing colonies of each replicate plate were measured daily in two perpendicular diameters at right angles to each other until the colony reached the edge of the plate; for each treatment the measure was held in triplicate. Radial growth rates (mm h^{-1}) were subsequently calculated as the slope of the linear regression obtained from plotting the colony radius of the replicates 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 extraction, detection and quantification

Gliotoxin production of both *Aspergillus* section *Fumigati* strains was evaluated at different growth conditions and incubation days. The toxin extraction was carried out following the methodology proposed by Geisen (1996), with some modifications. Three agar plugs were removed from each treatment plates at different points of the colony and extracted with 1 ml chloroform. The sample-solvent mixture was centrifuged for 20 min at 9500 rpm and the chloroform phase was then recovered. The solutions were filtered, evaporated to dryness, redissolved in 1000 µl of acetonitrile and the extract analysed by HPLC. Gliotoxin determination was performed following the previous methodology.

Statistical analyses

The linear regression of increase in radius against time was used to obtain the growth rates under each set of treatment conditions. The lag phase and growth rate data of each strain were then evaluated by analysis of variance (ANOVA) using Info Stat for Windows Version 2.03 (SPSS Inc., Cary, NC, USA). Statistical significance was judged at $p \leq 0.0001$. When the analysis was statistically significant, LSD Fischer's test was used to determine the significance of each individual parameter and their interactions on lag phases and growth rates at the level $p \leq 0.05$.

Results

Physicochemical properties of silage

At the beginning of sampling, the silage was well compacted and showed colour and odour characteristics of good lactic acid fermentation. The silage upper levels that were in contact with air (without the polystyrene cover) looked dry and some sectors showed a slight fungal contamination. The physicochemical results obtained from the silage samples are shown in Table 1. The pH of middle section silage samples oscillated from 3.5 to 5, while the pH from the lower and upper sections samples oscillated from 3.5 to 7 and from 4 to 7, respectively, with a high number of samples with pHs higher than 4.5. Silage samples a_W oscillated from 0.881 to 0.995.

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Table 1. Silage physicochemical properties.

Maize silage sections	Water activity		pH	
	Mean	Range	Mean	Range
Upper	0.945 ± 0.057	0.881–0.987	5.6 ± 1.3	4–7
Central	0.965 ± 0.006	0.965–0.995	3.9 ± 0.5	3.5–5
Lower	0.976 ± 0.021	0.905–0.988	5.3 ± 1.3	3.5–7

Note: Silage samples, $n = 50$.

Morphological identification of Aspergillus *section* Fumigati *strains*

The studied strains were identified as *A. fumigatus* showing the macroscopic differential characteristics of greyish to turquoise reproductive mycelium, dark green to dull green in the CYA 25°C and CYA 37°C media and inconspicuous white mycelia. In MEA and CYA 25°C the colony diameter oscillated from 30 to 35 mm and in CYA 37°C they showed a 57 mm mean diameter; the reverse showed colourless, yellowish, greyish, green or reddish brown. The microscopic characteristic observed were uniseriate conidiophores, phialides located in the half or second third portion of the vesicle, conidia head columnar, colourless or grey stipe with a smooth wall, and vesicle pyriform to subclavate. The conidia were globose to ellipsoidal, smooth to finely rough with a diameter from 2 to 3 μm.

Toxigenic capacity

Toxigenic capacity analyses from of 54 *A. fumigatus* strains showed a wide variation among them (Table 2). *Aspergillus fumigatus* RC031 gliotoxin production was markedly superior to the other *A. fumigatus* strains and *A. fumigatus* RC032 was the less gliotoxin-producing strain. These strains were selected for the subsequent analyses.

Aspergillus fumigatus *RC031 growth under ecophysiological study*

The analysis of variance of the effect of single (strain, oxygen tension, a_W and pH) two- and three-way interactions, showed that all the factors alone and their interactions were statistically significant ($p \leq 0.0001$) in relation to lag phases and growth rates for both *A. fumigatus* strains. These results justify the separated study of the factors influence onto each *A. fumigatus* strain.

The O_2 tension, a_W and pH, and their interactions, have a highly significant influence ($p < 0.0001$) on the *A. fumigatus* RC031 lag phase and growth rate. The extreme acid and alkaline pH increased the lag phase and reduced the growth rate, while at pHs 5, 6 and 7.5 the lower lag phase was shown (Table 3). Generally, the pH rise increased the growth rate. It is important to

Table 2. Gliotoxin production of *Aspergillus fumigatus* strains.

A. fumigatus strains	ng g^{-1}	*A. fumigatus* strains	ng g^{-1}
RC001	78.58 ± 0.9	RC028	279.65 ± 0.8
RC002	1.50 ± 0.6	RC029	290.50 ± 0.9
RC003	2.03 ± 0.5	RC030	100.74 ± 0.9
RC004	16.73 ± 0.5	RC031	8636.55 ± 0.9
RC005	1.47 ± 0.7	RC032	4.56 ± 0.9
RC006	16767.60 ± 0.8	RC034	n.d.
RC007	31876.16 ± 0.9	RC035	n.d.
RC008	19678.10 ± 0.6	RC036	31.32 ± 0.9
RC009	31907.54 ± 0.7	RC037	4987.86 ± 0.7
RC010	0.986 ± 0.5	RC038	n.d.
RC011	13446.75 ± 1.4	RC039	n.d.
RC012	34987.67 ± 0.9	RC040	6540.48 ± 0.8
RC013	8623.14 ± 0.8	RC041	n.d.
RC014	36222.91 ± 1.5	RC042	n.d.
RC015	24366.96 ± 1.6	RC043	522.17 ± 0.9
RC016	3.04 ± 1.7	RC044	n.d.
RC017	397.35 ± 0.8	RC045	n.d.
RC018	1506.29 ± 0.7	RC046	n.d.
RC019	82.32 ± 0.5	RC048	225.87 ± 0.8
RC020	109.59 ± 0.9	RC049	1621.71 ± 0.9
RC021	24.29 ± 1.1	RC0391	6.68 ± 0.6
RC022	17.42 ± 1.4	RC0537	17.87 ± 1.1
RC023	27.23 ± 1	RC0548	4430.85 ± 1.2
RC025	25.89 ± 1.1	RC0621	714.39 ± 1.3
RC026	1212.16 ± 0.9	RC0676	35.85 ± 0.8
RC027	68.53 ± 0.8		

Note: n.d., Not detected by the used methodology, $n = 162$.

Table 3. Influence of pH and a_W on *Aspergillus fumigatus* RC31 growth.

Media ± SE†	Lag phase (h)	Growth rate (mm h^{-1})
pH		
3.5	1.87 ± 0.02 c	0.02 ± 0.00013 a
4.0	1.83 ± 0.02 bc	0.02 ± 0.00013 a
4.5	1.80 ± 0.02 b	0.03 ± 0.00013 b
5.0	1.73 ± 0.02 a	0.03 ± 0.00013 b
6.0	1.75 ± 0.02 a	0.03 ± 0.00013 b
7.0	1.80 ± 0.02 b	0.03 ± 0.00013 b
7.5	1.74 ± 0.02 a	0.03 ± 0.00013 b
8.0	1.80 ± 0.02 b	0.03 ± 0.00013 b
a_W		
0.80	22.39 ± 1.05 b	n.g.‡ a
0.85	165.96 ± 1.05 e	0.0018 ± 0.00013 b
0.90	169.82 ± 1.05 e	0.0043 ± 0.00013 c
0.92	104.71 ± 1.05 c	0.0100 ± 0.00013 d
0.94	97.72 ± 1.05 bc	0.0200 ± 0.00013 e
0.96	18.19 ± 1.05 d	0.0300 ± 0.00013 f
0.98	29.89 ± 1.05 a	0.0600 ± 0.00013 g
0.99	31.68 ± 1.05 b	0.0800 ± 0.00013 h

Notes: †Standard error
‡n.g., No growth.
Letters in common are not significantly different according to the LSD test ($p < 0.05$), $n = 128$.

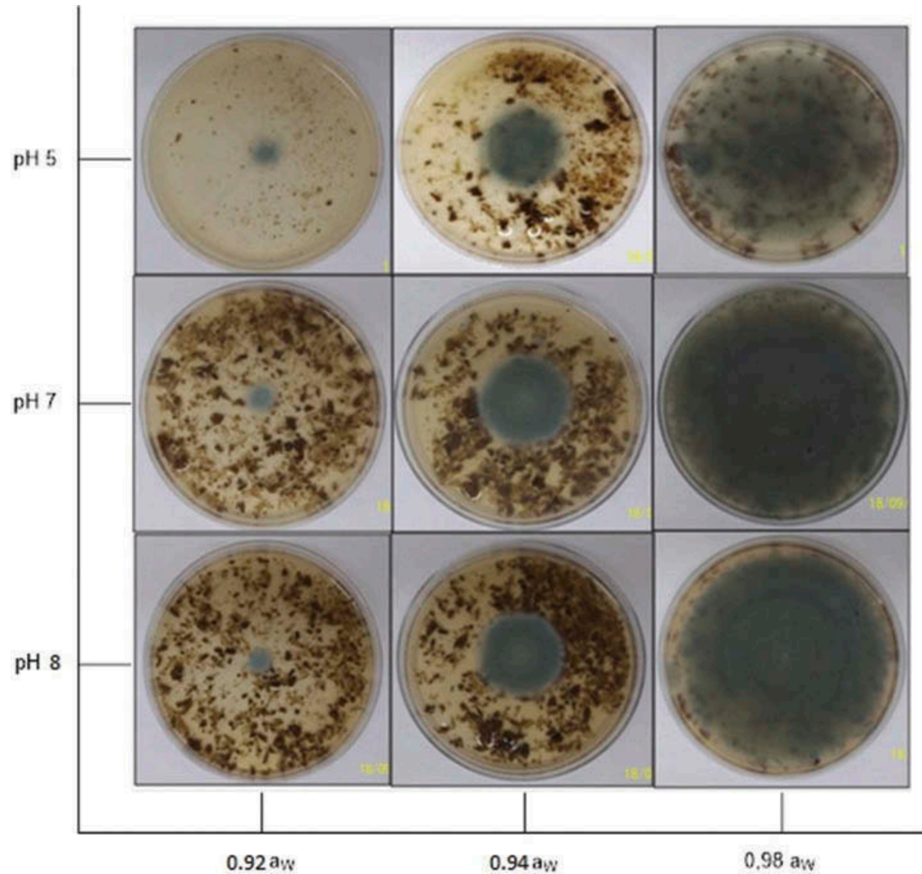

Figure 2. (colour online) Influence of interacting levels of pH, a_W and oxygen tension (reduced) on *A. fumigatus* growth after five incubation days.

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highlight that the highest growth rate was obtained at pH 7.5. The results on the influence of a_w on the lag phase and growth rate showed that the minor lag phase was observed at 0.98 a_W, followed by 0.99 a_w, while the growth rate was increased at increasing a_W levels (Table 3). *Aspergillus fumigatus* RC031 did not grow at 0.8 a_W. Figure 2 shows the influence of interacting levels of pH, a_W and oxygen tension (reduced) on *A. fumigatus* growth after five incubation days.

Aspergillus fumigatus *RC032 growth under ecophysiological study*

The O_2 tension, a_W and pH factors, and their two- and three-way interactions, exert a highly significant influence ($p < 0.0001$) on the *A. fumigatus* RC032 lag phase and growth rate. The minor lag phase was observed at pHs 7.5 and 7, while the extreme pHs (3.5, 4, 4.5 and 8) raised this parameter. Also, the growth rate was affected by the pH; the highest growth rates were at pHs 5 and 7, while it was reduced at pH 3.5 (Table 4). Regarding a_W, the highest a_W showed a minor lag phase and higher growth rate. The lag phase time was reduced at a_W's 0.85 and 0.9, while it was considerably increased at 0.98 (Table 4).

Interaction variables analysis

The analysis of the interaction of oxygen tension, a_W and pH on the growth of *A. fumigatus* showed a relation between the conditions that allowed the growth of the fungus. At normal oxygen tension, the growth was stimulated when pH increased and a_W decreased. Although the same behaviour was observed at reduced oxygen tension, the conditions that influenced the growth required higher pH and a_W than those at normal oxygen tension (Table 5). The results were also analysed to determine the non-permissive growth conditions of *A. fumigatus* and it was observed that with increasing pH and decreasing a_W the *A. fumigatus* growth was inhibited at both oxygen tensions tested. However, more exigent conditions at reduced oxygen tension were necessary to reduce the growth (Table 6).

Table 4. Influence of pH and a_W on *Aspergillus fumigatus* RC32 growth.

Media ± SE[†]	Lag phase (h)	Growth rate (mm h^{-1})
pH		
3.5	144.54 ± 1.05 e	0.02 ± 0.0002 a
4.0	83.18 ± 1.05 d	0.03 ± 0.0002 b
4.5	81.28 ± 1.05 d	0.03 ± 0.0002 b
5.0	70.79 ± 1.05 bc	0.03 ± 0.0002 b
6.0	69.18 ± 1.05 bc	0.02 ± 0.0002 a
7.0	60.25 ± 1.05 a	0.03 ± 0.0002 b
7.5	64.56 ± 1.05 ab	0.03 ± 0.0002 b
8.0	77.62 ± 1.05 cd	0.03 ± 0.0002 b
a_W		
0.80	223.87 ± 1.05 f	0.0010 ± 0.0002 a
0.85	295.12 ± 1.05 g	0.0025 ± 0.0002 b
0.90	199.52 ± 1.05 e	0.0030 ± 0.0002 b
0.92	107.15 ± 1.05 d	0.0100 ± 0.0002 c
0.94	32.36 ± 1.05 b	0.0020 ± 0.0002 d
0.96	45.71 ± 1.05 c	0.0040 ± 0.0002 e
0.98	21.88 ± 1.05 a	0.0060 ± 0.0002 fg
0.99	32.36 ± 1.05 b	0.0070 ± 0.0002

Notes: †Standard error
Letters in common are not significantly different according to the LSD test ($p < 0.05$), $n = 128$

Table 5. Critical limits of interacting environmental factors for *Aspergillus fumigatus* growth for both studied strains.

Oxygen tension	a_W	pH
Normal	0.80	6.00
	0.85	4.50
	0.90	4.00
	0.94	3.50
	0.96	
	0.98	
	0.99	
Reduced	0.85	5.00
	0.90	4.00
	0.92	
	0.94	
	0.96	3.50
	0.98	
	0.99	

Note: $n = 256$.

Aspergillus fumigatus *gliotoxin production under ecophysiological study*

Table 7 shows the production of gliotoxin by *A. fumigates* RC032 strain under different environmental conditions. This production was informed only when it was positive. All other interactions were negative for the gliotoxin production with the technique assayed. In general, gliotoxin was produced at normal oxygen tension; it is important to highlight that at the same a_W level the gliotoxin increased at decreasing pH levels.

Table 6. Critical limits of interacting environmental factors for *Aspergillus fumigatus* growth inhibition for both studied strains.

Oxygen tension	a_W	pH
Normal	0.80	5.00
	0.85	4.00
	0.90	3.50
	0.92	
Reduced	0.80	8.00
	0.85	4.50
	0.90	3.50
	0.92	
	0.94	

Note: $n = 256$.

Table 7. Gliotoxin production RC032 under different ecophysiological conditions.

Oxygen tension	a_W	pH	Gliotoxin (ng g^{-1})
Normal	0.90	4.0	8.10
	0.94	4.0	9.03
	0.94	5.0	76.80
	0.94	6.0	46.61
	0.94	7.5	5.98
	0.96	7.0	10.05
	0.98	3.5	8.05
Reduced	0.90	4.5	6.02

Note: $n = 128$.

Under reduced oxygen tension, the gliotoxin was only produced at similar a_W and pH conditions than those present in natural ensilage.

Discussion

In this study, the influence of interacting simulated natural physicochemical conditions on *A. fumigatus* growth parameters and gliotoxin production were studied. The studied strains were capable of growth under reduced oxygen tension generally with a higher growth rate than at normal oxygen tension. A similar behaviour was observed by Hall and Denning (1994) and Wilger et al. (2009), who studied the effect of low oxygen tension on 24 *Aspergillus* spp. (10 *A. fumigatus* strains). They tested different oxygen tensions (0%, 0.025%, 0.1%, 0.5% and 2.5%) and observed that all *A. fumigatus* strains were capable of growing under low tensions (a minimum of 0.1%), but not in the absence of oxygen. In this work, which analysed the interaction between oxygen tension and pH, it was observed that in both strains the lag phase was reduced at normal oxygen tension and the growth rate increased when the pH rises. These results were related to the aerobic phase of the silage process, where the oxygen is still present and pH varies between 6.0 and

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6.5. Although these conditions prevail naturally between 24 and 72 h, it is enough time for fungal development and both studied strains show the highest growth rate under these conditions. Weissbach (1996) proposed the limit conditions of a_W and pH necessary to ensure silage conservation in which *Clostridium* spp. growth was inhibited, which is one of the principal spoilage bacteria in silage. Studying *A. fumigatus* behaviour under the proposed conditions, it was observed that the lowest pH affected the lag phase raising, however this effect was offset by the highest a_w, thus resulting in lack of fungal growth inhibition by descending pH. Because of this, from the results of this work we propose new limits for silage conservation by adding oxygen tension as another parameter to those previously proposed by Weissbach (1996).

This work shows the conditions under which fungal growth is inhibited. With the highest a_w it is necessary to have the lowest pH. The same happens with oxygen tension. Reduced oxygen tension does not need such extreme conditions to inhibit fungal growth compared with normal oxygen tension. These are more extreme critical conditions than those proposed by Weissbach (1996) which ensure fungal growth inhibition and, by extension, gliotoxin production.

With silage in a good state, the conditions are kept stable when pH varies between 3.8 and 5.0 and there is anaerobiosis. The compaction loss of the silage created by water and/or air infiltration can alter the characteristics, generating changes in pH and a_W. This paper shows the minimal conditions that allow *A. fumigatus* growth; these conditions can be found in the different silage sections. Comparative analysis of the influence of the interactions of oxygen tension, a_w and pH under *in vitro* conditions for *A. fumigatus* with silage's natural environmental conditions allows one to predict the capacity of the fungus to grow. In the silage middle section, the low pH (3.5–5) and reduced oxygen tension are not enough to inhibit growth because of the compensation produced by the high a_W (0.96–0.99). *A. fumigatus* development is also possible in the silage lower section; wherever the reduced oxygen tension should inhibit growth, the higher pH and a_W stimulate fungal growth. Finally the silage upper section conditions are the most permissive for fungal development because of normal oxygen tension and higher pH (4–7) and a_W range. Under these conditions both strains had one of the lowest lag phases and highest growth rate.

The study of the physiological behaviour of gliotoxigenic *A. fumigatus* under simulated environmental conditions allowed one to predict its behaviour in silage. Given that the presence of this fungus in the environment cannot be avoided, it is important based on the knowledge provided by this work to use prevention strategies (physical, biological and/or chemical) to prevent the development and production of toxin by *A. fumigatus*. Measures such as the use of cutting tools that maintain compaction as much as possible while the silo is used are of great importance. Another recommended measure could be the use of biological additives that accelerate the process of oxygen reduction with optimal production of lactic acid, which in turn could provide a probiotic effect in livestock.

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Conflict of interest

No potential conflict of interest was reported by the author(s).

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