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# Ecophysiology of environmental *Aspergillus fumigatus* and comparison with clinical strains on gliotoxin production and elastase activity

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**Significance and Impact of the Study:** *Aspergillus fumigatus* is one of the main opportunist pathogen agents causing invasive aspergillosis. Rural workers present a constant exposition to *A. fumigatus* spores caused by feed-borne manipulation. In this study, environmental *A. fumigatus* strains were able to grow and produce gliotoxin onto the studied conditions including the lung ones. Environmental and clinical strains were physiologically similar and could be an important putative infection source in rural workers.

**Keywords**
*Aspergillus fumigatus*, $a_W$, elastase activity, environment, gliotoxin production, pH.

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## Abstract

The aim of this manuscript was to study the influence of water activity ($a_W$) and pH in the ecophysiological behaviour of *Aspergillus fumigatus* strains at human body temperature. In addition, gliotoxin production and enzymatic ability among environmental ($n = 2$) and clinical ($n = 5$) strains were compared. Ecophysiological study of environmental strains was performed on agar silage incubated at 37°C, studying the interaction at eight $a_W$ levels (0·8, 0·85, 0·9, 0·92, 0·94, 0·96, 0·98 and 0·99) and eight pH levels (3·5, 4, 4·5, 5, 6, 7, 7·5 and 8). Considering the influence of the assumed lung conditions on growth of *A. fumigatus* ($a_W$ 0·98/0·99 and pH of 7/7·5), the optimal condition for the development of *A. fumigatus* RC031 was at $a_W$ 0·99 at pH 7. At $a_W$ 0·98/0·99 and pH of 7/7·5, the highest growth rate and the lowest lag phase was reported, whereas there were no significant differences at $a_W$ 0·98/0·99 and pH 7/7·5 interactions on growth of *A. fumigatus* RC032. Gliotoxin production of *A. fumigatus* strains was evaluated. The gliotoxin production was similar in clinical and environmental strains. Elastin activity was studied in solid medium, highest elastase activity index was found for clinical strain *A. fumigatus* RC0676, followed by the environmental strain *A. fumigatus* RC031. Opportunistic environmental strains can be considered as pathogenic in some cases when rural workers are exposed constantly to handling silage.

## Introduction

*Aspergillus* spp. species are saprophytic fungus widely distributed in nature that produce abundant small spores in soil and, decay organic matter and air. *Aspergillus* genus is recognized as a major cause of infections in immune compromised patients. This expanding population is composed of patients with prolonged neutropenia, advanced HIV infections, hereditary immunodeficiency and patients undergoing allergenic hematopoietic stem cell or pulmonary transplant (Walsh *et al.*2008). Moreover, *Aspergillus fumigatus* is one of the main opportunist pathogen agents causing invasive aspergillosis with high levels of mortality and morbidity (Stanzani *et al.* 2005). It is estimated that a person is able to inhale several hundred of viable spores each day. Rural workers present

a constant exposition to *A. fumigatus* spores caused by feed-borne manipulation (Millner *et al.* 1994; González Pereyra *et al.* 2008a,b, 2009; Alonso *et al.* 2013) making infection highly probable.

*Aspergillus fumigatus* produce a wide number of extrolites, gliotoxin among them. This mycotoxin belongs to the epipolythiodioxopiperazine family and possesses immunosuppressive functions, such as inhibition of macrophage phagocytosis, mitogen-activated T cells proliferation, mast activation, cytotoxic T cells response and monocyte apoptosis, among others (Yamada *et al.* 2000; Stanzani *et al.* 2005). It also inhibits neutrophils NADPH (Tsunawaki *et al.* 2004), suppresses the production of reactive oxygen species deteriorating the neutrophils phagocyte capacity (Orciuolo *et al.* 2007), and reduces the epithelial cell ciliar mobility leading to its damage (Amitani *et al.* 1995). It has been shown that this toxin is produced in animal with induced aspergillosis, and in 80% of invasive aspergillosis patients, the serum concentrations ranged between 166 and 785 ng $ml^{-1}$ (Richard *et al.* 1996; Lewis *et al.* 2005). Other studies suggest that gliotoxin has a direct role in the aspergilosis virulence in immunocompromised patients without neutropenia (Sugui *et al.* 2007; Spikes *et al.* 2008). Because of the importance of gliotoxin as virulence factor, its detection is important to determine the virulence capacity of *A. fumigatus* strains. Many studies agree that the virulence of *A. fumigatus* is multifactorial, being associated with the structure, growth capacity and adaptation to stress conditions, mechanisms of immune system evasion and capacity to cause damage in the host. Some extracellular enzymes such as proteases have the capacity to degrade collagen and elastin, the main components of the pulmonary mucosa.

Several articles have shown the relation between *A. fumigatus* elastin activity and its invasiveness from environmental and clinical origin strains (Blanco *et al.* 2002; García *et al.* 2006). Alvarez-Pérez *et al.* (2010) considered that elastase activity (EA) is among the most important pathogenicity factors of this mould and in particular, there is a strong correlation between EA in *A. fumigatus* and invasive aspergillosis.

Few studies have informed the influence of pH and $a_W$ on *A. fumigatus* development. Pena *et al.* (2014) studied the behaviour of two feed-borne *A. fumigatus* strains isolated from fermented corn silage and brewer's grains and found that the optimum condition for *A. fumigatus* growth was 37°C/0·98 $a_W$. Other studies (Alonso *et al.* 2015) demonstrated that *A. fumigatus* strains were able to grow at 25°C even if the availability of oxygen was reduced at very low pH (4–5). However, no studies have been conducted with *A. fumigatus* strains from silages to which farm workers are exposed. This fact become a great interest and allows providing further details about the pathogenic potential of these strains and the risk of workers in contact with them. The aim of this work was to study *in vitro* the influence of water activity ($a_W$) and pH on growth rate and lag phase of environmental *A. fumigatus* strains at human body temperature. Moreover, gliotoxin production and the enzymatic ability among environmental and clinical strains were compared.

## Results and discussion

In this study, environmental *A. fumigatus* strains were able to grow and produce gliotoxin onto the studied conditions including the physiological conditions present in lung which are the target of *A. fumigatus* infection. The lung conditions are pH and $a_W$ levels present in lung human tissue, which are the target of *A. fumigatus* infection. It has been proposed that *A. fumigatus* is able to adapt to the humid environment of lungs (Pollmächer and Figge 2015) and able to develop at pH 7·38–7·4 (Wood and Schaefer 1978). Based on these reports, in this work the $a_W$ 0·98/0·99 and pH 7/7·5 levels were assumed to be similar to those present in lung.

### Ecophysiological study

The growth of environmental strains *A. fumigatus* RC031 and *A. fumigatus* RC032 (the highest and the least producing strains) was evaluated at different $a_W$ and pH conditions including the lung ones.

The analysis of variance (ANOVA) of the single effect (strain, $a_W$ and pH), two- and three-way interactions, showed that all factors, alone and their interactions, were statistically significant ($P \leq 0{\cdot}0001$) in relation to lag phases and growth rates for both *A. fumigatus* studied strains. These results justify that the study of the factor influence onto each *A. fumigatus* strain should be done separately.

The ANOVA showed that $a_W$ and pH exerted a significant influence ($P < 0{\cdot}0001$) onto the lag phase and growth rate of *A. fumigatus* RC031 strain, as well as their interaction (data not shown). The extreme pH (3·5, 4 and 8) increased the lag phase and decreased de growth rate, whereas intermediate pH showed the lowest lag phase. At pH 7 and 7·5, there were no statistical difference among them in relation to the lag phase. In general, the lag phase at $a_W$ 0·98 and 0·99 was the shortest, and there were no statistical differences among them; but at $a_W$ 0·99 the growth rate was the highest at all studied pH. There was development at pH 5, 6, 7, and 8 (Table 1). Only at $a_W$ 0·8 there was no development of *A. fumigatus*.

The ANOVA for the *A. fumigatus* RC032 strain showed that $a_W$, pH and their interaction had a significant influence ($P < 0{\cdot}0001$) onto lag phase and growth rate

**Table 1** Growth rate ($\mu_{max}$) and lag phase ($\lambda$) of *Aspergillus fumigatus* strains at different pH and water activity levels at 37°C

| | | RC031 | | RC032 | |
|---|---|---|---|---|---|
| $a_W$ | pH | $\mu_{max}$ (mm day$^{-1}$) ± SD | $\lambda$ (day) ± SD | $\mu_{max}$ (mm day$^{-1}$) ± SD | $\lambda$ (day) ± SD |
| 0·80 | 3·5 | – | – | – | – |
| | 4 | – | – | – | – |
| | 4·5 | – | – | – | – |
| | 5 | 0·428 ± 0·04 | 12·5 ± 0·01 | 0·017 ± 0·01 | 30·02 ± 0·03 |
| | 6 | 0·471 ± 0·04 | 11·0 ± 0·04 | 0·020 ± 0·01 | 30·14 ± 0·34 |
| | 7 | 0·503 ± 0·07 | 13·0 ± 0·01 | – | – |
| | 7·5 | – | – | 0·007 ± 0·01 | 29·12 ± 0·15 |
| | 8 | 0·407 ± 0·07 | 14·6 ± 0·04 | – | – |
| 0·85 | 3·5 | – | – | – | – |
| | 4 | 0·361 ± 0·16 | 15·03 ± 0·04 | – | – |
| | 4·5 | 0·974 ± 0·33 | 11·78 ± 0·31 | 0·013 ± 0·01 | 28·43 ± 1·44 |
| | 5 | 0·910 ± 0·06 | 10·89 ± 0·27 | 0·011 ± 0·01 | 26·43 ± 0·20 |
| | 6 | 0·550 ± 0·19 | 6·55 ± 1·02 | 0·016 ± 0·01 | 28·41 ± 0·60 |
| | 7 | 0·331 ± 0·02 | 7·57 ± 0·87 | 0·016 ± 0·01 | 24·44 ± 0·37 |
| | 7·5 | 0·249 ± 0·04 | 7·23 ± 0·35 | 0·016 ± 0·01 | 23·29 ± 0·39 |
| | 8 | 0·436 ± 0·05 | 6·45 ± 0·43 | 0·013 ± 0·01 | 24·33 ± 0·02 |
| 0·90 | 3·5 | – | | – | – |
| | 4 | 0·581 ± 0·01 | 5·51 ± 0·22 | – | – |
| | 4·5 | 0·308 ± 0·05 | 6·09 ± 0·48 | 0·019 ± 0·01 | 16·50 ± 0·54 |
| | 5 | 0·212 ± 0·06 | 7·56 ± 1·45 | 0·029 ± 0·01 | 17·27 ± 0·36 |
| | 6 | 1·067 ± 007 | 7·25 ± 0·15 | 0·019 ± 0·02 | 16·79 ± 0·73 |
| | 7 | 0·176 ± 0·02 | 5·27 ± 0·14 | 0·026 ± 0·01 | 16·79 ± 0·73 |
| | 7·5 | 0·878 ± 0·13 | 6·15 ± 0·4 | 0·005 ± 0·01 | 15·03 ± 0·20 |
| | 8 | 0·245 ± 0·03 | 5·87 ± 0·19 | 0·008 ± 0·01 | 15·56 ± 0·03 |
| 0·92 | 3·5 | – | | – | – |
| | 4 | 0·885 ± 0·02 | 4·17 ± 0·34 | 0·042 ± 0·01 | 16·31 ± 0·45 |
| | 4·5 | 0·802 ± 0·02 | 1·52 ± 0·31 | 0·019 ± 0·04 | 15·26 ± 0·01 |
| | 5 | 0·649 ± 0·02 | 3·25 ± 0·32 | 0·019 ± 0·01 | 14·49 ± 0·51 |
| | 6 | 0·464 ± 0·02 | 1·33 ± 0·40 | 0·017 ± 0·01 | 5·38 ± 0·83 |
| | 7 | 0·518 ± 0·01 | 5·84 ± 0·34 | 0·014 ± 0·01 | 6·7 ± 0·62 |
| | 7·5 | 0·272 ± 0·04 | 6·32 ± 0·04 | 0·012 ± 0·01 | 8·3 ± 0·02 |
| | 8 | 0·682 ± 0·12 | 7·89 ± 0·01 | 0·003 ± 0·01 | 6·5 ± 0·34 |
| 0·94 | 3·5 | 0·695 ± 0·01 | 8·96 ± 0·02 | 0·028 ± 0·01 | 17·65 ± 0·04 |
| | 4 | 1·545 ± 0·04 | 0·02 ± 0·17 | 0·033 ± 0·01 | 16·46 ± 0·01 |
| | 4·5 | 2·275 ± 0·08 | 0·58 ± 0·19 | 0·074 ± 0·01 | 16·55 ± 0·39 |
| | 5 | 1·641 ± 0·13 | 0·14 ± 0·19 | 0·089 ± 0·01 | 14·77 ± 0·24 |
| | 6 | 2·382 ± 0·16 | 1·17 ± 0·17 | 0·066 ± 0·08 | 1·82 ± 0·23 |
| | 7 | 2·393 ± 0·13 | 1·37 ± 0·13 | 0·050 ± 0·08 | 0·73 ± 0·42 |
| | 7·5 | 2·191 ± 0·12 | 0·92 ± 0·18 | 0·046 ± 0·01 | 0·89 ± 0·12 |
| | 8 | 2·053 ± 0·04 | 0·84 ± 0·11 | 0·037 ± 0·01 | 0·88 ± 0·25 |
| 0·96 | 3·5 | 1·834 ± 0·01 | 5·03 ± 0·11 | 0·148 ± 0·01 | 16·7 ± 0·29 |
| | 4 | – | – | – | – |
| | 4·5 | 2·641 ± 0·02 | 2·8 ± 0·22 | 0·164 ± 0·14 | 15·55 ± 0·17 |
| | 5 | 2·605 ± 0·02 | 2·45 ± 0·23 | 0·163 ± 0·13 | 5·34 ± 0·16 |
| | 6 | 2·784 ± 0·02 | 1·32 ± 0·17 | 0·154 ± 0·01 | 0·44 ± 0·14 |
| | 7 | 2·731 ± 0·01 | 1·07 ± 0·21 | 0·153 ± 0·01 | 0·77 ± 0·1 |
| | 7·5 | 3·039 ± 0·01 | 0·64 ± 0·15 | 0·153 ± 0·01 | 0·58 ± 0·13 |
| | 8 | 2·989 ± 0·01 | 0·21 ± 0·20 | 0·161 ± 0·01 | 1·33 ± 0·17 |
| 0·98 | 3·5 | 5·897 ± 0·13 | 0·15 ± 0·17 | 0·306 ± 0·01 | 2·34 ± 0·01 |
| | 4 | 6·601 ± 0·25 | 0·25 ± 0·15 | 0·301 ± 0·01 | 0·26 ± 0·02 |
| | 4·5 | 6·845 ± 1·20 | 0·26 ± 0·14 | 0·302 ± 0·01 | 0·35 ± 0·03 |
| | 5 | 6·714 ± 0·14 | 0·32 ± 0·18 | 0·299 ± 0·01 | 0·56 ± 0·01 |
| | 6 | 6·738 ± 0·37 | 0·36 ± 0·01 | 0·299 ± 0·01 | 0·55 ± 0·01 |
| | 7 | 6·523 ± 0·27 | 0·40 ± 0·01 | 0·305 ± 0·01 | 0·44 ± 0·31 |
| | 7·5 | 5·636 ± 0·67 | 0·45 ± 0·02 | 0·300 ± 0·01 | 0·57 ± 0·02 |
| | 8 | 5·170 ± 0·01 | 0·45 ± 0·03 | 0·302 ± 0·01 | 0·69 ± 0·04 |

**Table 1** (continued)

| | | RC031 | | RC032 | |
|---|---|---|---|---|---|
| $a_W$ | pH | $\mu_{max}$ (mm day$^{-1}$) ± SD | $\lambda$ (day) ± SD | $\mu_{max}$ (mm day$^{-1}$) ± SD | $\lambda$ (day) ± SD |
| 0·99 | 3·5 | 5·698 ± 0·04 | 2·15 ± 0·28 | 0·418 ± 0·01 | 1·26 ± 0·58 |
| | 4 | 6·732 ± 0·04 | 0·15 ± 0·69 | 0·376 ± 0·01 | 0·19 ± 0·11 |
| | 4·5 | 6·851 ± 0·04 | 0·15 ± 0·61 | 0·425 ± 0·01 | 0·25 ± 0·14 |
| | 5 | 6·708 ± 0·13 | 0·15 ± 0·59 | 0·420 ± 0·01 | 0·44 ± 0·39 |
| | 6 | 6·675 ± 0·12 | 0·15 ± 0·58 | 0·423 ± 0·01 | 0·41 ± 0·86 |
| | 7 | 7·154 ± 0·11 | 0·15 ± 0·58 | 0·310 ± 0·01 | 0·40 ± 0·45 |
| | 7·5 | 5·551 ± 0·01 | 0·15 ± 0·15 | 0·313 ± 0·01 | 0·32 ± 0·01 |
| | 8 | 5·650 ± 0·02 | 0·15 ± 0·17 | 0·314 ± 0·01 | 0·21 ± 0·15 |

Media of estimated parameter ± standard deviation.

(data not shown). The Fisher LSD test showed that at $a_W$ 0·9 and higher had the lowest lag phase, while at pH 3·5 and 7 the lag phase was increased. In general, pH did not exert an important influence on growth. The highest growth rate was at pH 4·5, 5 and 6, while at pH 8 the growth rate diminished; the pH 7 and 7·5 showed no statistical differences. The highest $a_W$ showed the lowest lag phase and highest growth rate, at $a_W$ 0·8 and 0·9 the lag phase increased, while at $a_W$ 0·98 and 0·99 the lowest lag phase without significant differences was observed (Table 1).

Considering the influence of the assumed lung conditions on growth of *A. fumigatus* ($a_W$ 0·98/0·99 and pH of 7/7·5), Table 1 shows that the optimal condition for the development of *A. fumigatus* RC031 was at $a_W$ 0·99 at pH 7. At $a_W$ 0·98/0·99 and pH of 7/7·5 the highest growth rate and the lowest lag phase was reported, whereas there were no significant differences at $a_W$ 0·98/0·99 and pH 7/7·5 interactions on growth of *A. fumigatus* RC032.

*Aspergillus fumigatus* produces conidia (asexual spores) that are easily dispersed into the air, ensuring ubiquity in both indoor and outdoor environments (Morris *et al.* 2000). The inhalation of the airborne conidia is followed by conidial deposition in the bronchioles or alveolar spaces. In healthy individuals, conidia that are not removed by mucociliary clearance encounter epithelial cells or alveolar macrophages, the primary resident phagocytes of the lung and responsible for the phagocytosis and killing of *Aspergillus* conidia as well as the initiation of a proinflammatory response into the site of infection. Conidia that evade macrophage killing and germinate become the target of infiltrating neutrophils that are able to destroy hyphae. The risk of developing invasive aspergillosis results primarily from a dysfunction in these host defences in combination with fungal attributes (such as gliotoxin production) that allow *A. fumigatus* survival and growth in this pulmonary environment (Taylor *et al.* 2009). Several authors have reported that rural workers are exposed continuously to greater amounts of *A. fumigatus* conidia because of the fungus prevalence in animal feed including silage (González Pereyra *et al.* 2008a,b, 2009; Alonso *et al.* 2013), so they could have a high risk for invasive aspergillosis. It is important to highlight that recent studies have demonstrated that environmental and clinical *A. fumigatus* strains have no genetic differences among them (Pena *et al.* 2015). In this work, the simulated lung conditions had a significant influence onto the growth parameters increasing the growth rate and decreasing the lag phase of the studied *A. fumigatus* strains suggesting that they could develop and produce invasive aspergillosis in immunocompromised individuals.

### Gliotoxigenic capacity

Gliotoxigenic capacity analyses from the environmental and clinical *A. fumigatus* strains showed that all of them were able to produce gliotoxin (Table 2). For both environmental *A. fumigatus* strains, the gliotoxin production ranged between 2·88 and 657 $\mu$g g$^{-1}$. RC031 gliotoxin production was markedly higher than the other studied strains while *A. fumigatus* RC032 was the least gliotoxin-producing strain.

The gliotoxin role in the pathogenesis of *A. fumigatus* is not totally understood, the gliotoxin presence in environmental isolation suggests that it is involved in the establishment of infection. Indeed, the fact that *A. fumigatus* is the most notorious pathogen of the genus and the greater gliotoxin producer, could indicate the importance in establishing aspergillosis. Several studies have demonstrated that gliotoxin at concentrations among 25–200 ng mg$^{-1}$ has immunosuppressive effects in lymphocytes and macrophages (Murayama *et al.* 1996; Stanzani *et al.* 2005). Moreover, Kupfahl *et al.* (2008) showed that environmental and clinical *A. fumigatus* strains produced similar gliotoxin concentrations among them and showed immunosuppressive effects *in vitro*. Both, environmental strains *A. fumigatus* RC031 and *A. fumiga-*

*tus* RC032 in this work produced gliotoxin under the simulated lung physiological conditions at concentrations that could have produced immunosuppressive effects.

### Elastase activity determination

At 96 h growth, it was observed the maximum elastase activity followed by a gradual decrease in the subsequent days for all the studied strains. The highest elastase activity index was found for the clinical strain *A. fumigatus* RC676, followed by the environmental strain *A. fumigatus* RC031 (Table 3).

Elastin is an essential part of various human tissues that depend on elasticity. These connective tissues include the skin, lung and arteries. Elastin provides these elastic tissues with the ability to stretch and recoil and plays a critical role in supporting and maintaining healthy cells (Daamen *et al.* 2007; Almine *et al.* 2010).

García *et al.* (2006) demonstrated a clear link between elastase activity and pathogenicity using what they have named the elastase activity index (EAI). In our study all the tested strains showed elastase activity with the maximum rate at 96 h followed by a decrease in the following days. Apparently, *A. fumigatus* decreases the production of elastase in the presence of another source of nitrogen different from elastin, such as yeast extract, that when used releases amino acids, which explains the EAI decrease after 120 h (Kothary *et al.* 1984). The studied strains showed a growth rate greater than those from other studies, the experiment lasted 7-days (168 h), while in other studies the EAI lasted 15 days (Blanco *et al.* 2002).

Blanco *et al.* (2002) demonstrate that there is a correlation between EAI and the fungus patogenicity; high EAI levels (>1·17) were related with invasive aspergilomas, while EAI < 1 had a low probability of invasive aspergillosis, they could be related with aspergillomas and colonized patients. In this study, although the tested strains showed an EAI > 1 at 24 h incubation time, only one of the clinical strains showed EAI greater than 1·17. However, the other clinical strains had been isolated from a patient with aspergillosis. García *et al.* (2006) explained this behaviour demonstrating that environmental *A. fumigatus* strains without an initial elastase activity could colonize mice lungs and adapt to produce high elastase levels after successive inoculations.

All studied strains were able to grow in lung physiological conditions. Besides all of them were able to produce gliotoxin, and a significant production of elastin enzyme being both considered as virulence factors. Therefore, the results suggest that opportunistic environmental strains could be considered as pathogen mainly when rural workers are constantly exposed to silage.

**Table 2** Gliotoxin production from environmental and clinical *Aspergillus fumigatus* strains (Media ± standard deviation)

| *A. fumigatus* gliotoxin production | | |
|---|---|---|
| *Origin* | Strains | ng $g^{-1}$ |
| Rural environment | RC031 | 8636·55 ± 0·13 |
| | RC032 | 4·56 ± 0·05 |
| Clinical | RC0391 | 6·68 ± 0·06 |
| | RC0537 | 17·87 ± 0·09 |
| | RC0548 | 4430·85 ± 0·05 |
| | RC0621 | 714·39 ± 0·02 |
| | RC0676 | 35·85 ± 0·02 |

## Materials and methods

### Fungal species

Two *A. fumigatus* strains, *A. fumigatus* RC031 and *A. fumigatus* RC032, isolated from fermented corn silage intended for dairy cattle in Argentina, were used. Moreover, five clinical origin strains, *A. fumigatus* RC0391, *A. fumigatus*, *A. fumigatus* RC0537, *A. fumigatus* RC0548, *A. fumigatus* RC0621, *A. fumigatus* RC0676 were used. These strains were kindly provided by Dr. Clara López of the National University of Rosario These strains were identified by morphological taxonomy proposed by Samson *et al.* (2007) and deposited in the National University of Río Cuarto, Córdoba, Argentina (RC) Collection Centre. These isolates were stored and maintained in glycerol (Sigma-Aldrich Co., St. Louis, MO) at 15% water (w/v) at −20°C.

### Environmental fungal strains isolation

Total fungal counts were performed onto Dichloran Rose Bengal Chloranphenicol agar (DRBC) and Dichloran 18% Glicerol agar (DG18), being the former, a general media used for estimating total culturable fungi (Abarca *et al.* 1994), and the latter has low water activity ($a_W$) and favours xerophilic fungi development (Pitt and Hocking, 1997). Ten grams (10 g) of each sample were homogenized in 90 ml of 0·1% peptone water solution for 30 min in an orbital shaker, obtaining the dilution $10^{-1}$. 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.

**Table 3** Elastase activity index (EAI) for environmental and clinical *Aspergillus fumigatus* strains (media ± standard deviation)

| | *A. fumigatus strains* | | | |
|---|---|---|---|---|
| Incubation time (h) | RC031 | RC032 | RC0548 | RC0676 |
| 0 | 0·00 ± 0·00 | 0·00 ± 0·00 | 0·00 ± 0·00 | 0·00 ± 0·00 |
| 24 | 1·00 ± 0·01 | 1·00 ± 0·01 | 1·00 ± 0·02 | 1·16 ± 0·01 |
| 48 | 1·00 ± 0·02 | 1·03 ± 0·01 | 1·02 ± 0·02 | 1·19 ± 0·02 |
| 72 | 1·02 ± 0·02 | 1·01 ± 0·01 | 1·04 ± 0·02 | 1·19 ± 0·01 |
| 96 | **1·08 ± 0·02** | **1·02 ± 0·01** | **1·04 ± 0·01** | **1·20 ± 0·00** |
| 120 | 1·02 ± 0·02 | 1·02 ± 0·02 | 1·05 ± 0·01 | 1·02 ± 0·02 |
| 144 | 0·87 ± 0·01 | 0·89 ± 0·02 | 0·93 ± 0·02 | 0·93 ± 0·03 |
| 168 | 0·84 ± 0·02 | 0·82 ± 0·01 | 0·81 ± 0·01 | 0·79 ± 0·01 |

Maximum rate of elastase activity index are marked in bold.

### Clinical strains isolation

Sputum samples were collected from patients with pulmonary aspergillosis whose causal agent was identified as *A. fumigatus* using standard techniques (Brightling *et al.* 2000). Isolation of *A. fumigatus* from sputum was performed as described by Pashley *et al.* (2012). Briefly, sputum was separated from saliva and 170 mg (±80 mg) neat plug inoculated onto potato dextrose agar plates supplemented with 16 $\mu$g ml$^{-1}$ chloramphenicol, 4 $\mu$g ml$^{-1}$ gentamicin and 5 $\mu$g ml$^{-1}$ fluconazole. Plates were sealed and incubated at 37°C for up to 7 days, with frequent observations.

### Morphological identification of *Aspergillus* section *Fumigati*

The 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 and 37°C), MEA (25°C), Oatmeal Agar OAT (25°C), Yeast Extract Sacharose Agar YES (25°C), Creatine Saccharose Agar CREA (25°C) and Czapek Agar CZ (25°C). The plates were incubated 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.

### Gliotoxicogenic capacity of *Aspergillus fumigatus* strains

Gliotoxin production of *A. fumigatus* strains was analysed. Until the analysis, the strains were preserved on MEA at 4°C. For the gliotoxin production determination, *A. fumigatus* strains were replicated in 250 ml Erlenmeyer with 100 ml of Yeast Extract Sucrose (YES) broth. A 5 mm diameter plug was taken from a 7 days MEA culture and used to inoculate the YES broth; then, it was incubated at 37°C for 3 days in 150 rev min$^{-1}$ agitation. The broth was filtered through Watman No 1 paper (Schleicher and Schuell Whatman group, London, UK). 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 dissolved with 200 $\mu$l of methanol and conserved at −70°C until HPLC quantification (HPLC Waters e2695; Waters Corp., Milford, MA).

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 (Waters Corp.) with a loop of 20 $\mu$l equipped with Waters 2998 detector arrangements diodes (Waters Corp.). Gliotoxin separation was performed at room temperature on a Phenomenex Luna RP C18 column (150 × 4·6 mm, 5 $\mu$m, Phenomenex 177 Inc, Torrance, CA) fitted with a C18 guard column using an isocratic mode: 75% aqueous 1% acetic acid and 25% acetonitrile. The mobile phase flow was 1·5 ml min$^{-1}$ and the volume injection was 20 $\mu$l. Detection was done at 268 nm and retention time was 8·5–8·8 min. A column washing of 5 min at 95% acetonitrile followed by 2 min of stabilization at running conditions was performed among chromatographic runs. Retention time was properly checked by the co-injection technique. Working solutions were prepared dissolving the pure gliotoxin in 5 ml HPLC quality acetonitrile. The 1 mg ml$^{-1}$ solution was then obtained from this solution (5 mg per 5 ml) and held at −20°C. The gliotoxin standard solutions in mobile phase were prepared from the working solution in methanol, after solvent evaporation.

A calibration curve was obtained injecting 20 $\mu$l of each witness solution (30, 20, 12·5, 7·5, 6 and 0·3 $\mu$g ml$^{-1}$ of mobile phase). The instrumental detection limits (LOD) and quantification limits (LOQ), were determined in fungal extracts and standard solutions of gliotoxin based on the signal/noise (S/N) relation of 3 : 1 for LOD and 190 7 : 1 for LOQ. Organic solvents were high-performance liquid chromatography (HPLC) grade from Sintorgan (Buenos Aires, Argentina). HPLC-grade water was obtained from a Labconco WaterPro Mobile purification system Model 90901-01.

### Ecophysiological study

The effect of the single and interacting factors $a_W$ and pH on both the growth parameters (growth rate and lag phase) and gliotoxin production by *A. fumigatus* strains, including $a_W$ and pH present in human lung ($a_W$ 0·98/0·99-pH 7/7·5), was evaluated.

The culture medium was prepared using dried silage (30 g). Then, distilled water was added and heated at 80°C. The solution was filtrated, the volume to 1000 ml was completed with distilled water, and then agar-agar (3% w/v) was added. The basic medium $a_W$ 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 and Fox 1980). Final $a_W$ values of representative medium samples were checked with a meter $a_W$ (AquaLab Series 3, Labcell Ltd., Basingstoke, Hants, UK). Each medium was autoclaved at 120°C for 20 min. After that the pH was measured and adjusted to 3·5, 4, 4·5, 5, 6, 7, 7·5 and 8 by the addition of adequate volumes of HClorNaOH concentrate solutions, using a pH meter (ATIOrion, Boston, MA).

Petri plates (20 ml) were centrally needle-inoculated from conidial suspensions in semisolid agar (0·2%) for each treatment (Pitt 1979). 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 37°C. Considering eight $a_W$ and eight pH were 64 plates. The assay was performed in triplicate.

### Growth parameters measurement

Two diameters of the growing colonies were measured at right angles to each other until the colony reached the edge of the plate. The radii of the colonies were plotted against time, and a linear regression applied to obtain the growth rate (mm day$^{-1}$) as the slope of the line. Lag phase before growth (h) in each treatment was determined as the abscissa from the growth rate curves.

### Elastase production

Production of the enzyme elastase from *A. fumigatus* RC031 and *A. fumigatus* RC032 strains was determined. The clinical isolates *A. fumigatus* RC548 and *A. fumigatus* RC676 were used as positive control, evaluated from halo elastin production according to Blanco *et al.* (2002) and Alvarez-Pérez *et al.* (2010).

The used methodology was performed by Blanco *et al.* (2002). Elastase activity was studied in a solid medium containing: 0·05% elastin (Sigma), 0·05% yeast carbon base (Difco Laboratories, Detroit, MI), 0·01% rose bengal (Sigma) and 1·5% agar (Merck, Darmstadt, Germany) in 0·05 mol l$^{-1}$ borate buffer, pH 7·6. The medium was sterilized with flowing steam (100°C) for 30 min. Sterile 0·2% agar was used to collect spores from a 7-day-old slant of *A. fumigatus* grown on MEA. Plates containing 20 ml of solid medium were inoculated with a loop ful in a central spot and incubated at 37°C for 15 days. Elastase activity was observed as a zone of clearing around the colony. The diameter of colony growth and the diameter of the halo of elastin lysis (elastase activity) were measured on days 7, 10, and 15 of incubation. The elastase activity index (EAI) was calculated by dividing the elastase diameter by the mycelium diameter.

### 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 INFOSTAT for Windows ver. 2012 (Córdoba University, Córdoba, República Argentina). Statistical significance was judged at $P \leq 0{\cdot}0001$. When the analysis was statistically significant, Fisher's Least Significant Difference (LSD) test was used to determine the significance of each individual parameter and their interactions on lag phases and growth rates at the level $P \leq 0{\cdot}05$.

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## Conflict of Interest

The authors have no conflicts of interest to declare.

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