Determination of optimum growth conditions for gliotoxin production by *Aspergillus fumigatus* and development of a novel method for gliotoxin detection

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Gliotoxin is a toxic metabolite of *Aspergillus fumigatus* Fresenius and other fungi. It has been suggested that this toxin may play an important role in the pathogenesis of aspergillosis as gliotoxin has immunosuppressive activity both *in vitro* and *in vivo*. We have determined the optimum growth conditions for the production of gliotoxin by selected isolates of *A. fumigatus* using a number of defined media. Gliotoxin was detected by thin layer chromatography and high performance liquid chromatography. The carbohydrate source, concentration of carbohydrate in the growth medium and incubation temperature were all found to influence gliotoxin production. Optimum growth conditions for gliotoxin production in our study were Czapek-Dox broth containing 30% glucose and incubation at 37 °C. Most of the gliotoxin was produced after 29 h incubation, during the exponential phase of growth. A novel method for screening large numbers of *A. fumigatus* isolates for gliotoxin production, which is both quick and easy, has also been developed, based on the ability of gliotoxin to inhibit the adherence of lung fibroblast (L929) cells to plastic microtitre plates.

**Keywords**  *Aspergillus fumigatus*, gliotoxin, L929 cells

Introduction

*Aspergillus fumigatus* is commonly implicated as the causal agent of both allergic lung disease and invasive aspergillosis, a serious, life-threatening condition in neutropenic patients. Although *A. fumigatus* is ubiquitous in the environment, it has been suggested that this fungus must possess some specific virulence factors that enable it to become a pathogen, since other ubiquitous fungi are unable to cause similar disease.

The ability of *A. fumigatus* to produce the extracellular metabolite gliotoxin has received attention over recent years, as it has been shown that this compound has potent immunosuppressive activity *in vitro* and *in vivo*. Gliotoxin can inhibit the adherence of peritoneal and alveolar macrophages to plastic surfaces [1,2], as well as inducing apoptosis of these cells [3,4] and other cells of haematopoietic origin [5]. This compound is able to inhibit phagocytosis by rodent macrophages and mitotic stimulation of lymphocytes. Additional biological properties of gliotoxin include inhibition of fungal [6] and bacterial [7] growth, and antiviral activity [8]. Gliotoxin has been shown to slow ciliary beating frequency in association with epithelial damage at a concentration above 0.2 µg ml⁻¹ and production of this toxin by *A. fumigatus in vivo* may, therefore, help this fungus to colonize the airways [9].

Gliotoxin production was first demonstrated to occur *in vivo* by Bauer [10] who recovered the toxin from bovine udder tissue naturally infected with *A. fumigatus*. 

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The gliotoxin concentration analysed (9.2 mg kg\(^{-1}\) under) was approximately 100 times higher than that known to produce morphological changes in cells. This toxic has also been shown to be produced in vivo in turkeys with aspergillosis [11], and as an ingested toxin in camels that consumed gliotoxin-containing mouldy hay contaminated with \(A.\) \textit{fumigatus} [12].

Until now, the screening of natural isolates of \(A.\) \textit{fumigatus} for gliotoxin production has involved large-scale batch culture of the fungus in Czapek-Dox broth [13] or rice medium [14], followed by purification of the toxin by chloroform extraction and detection by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) [14].

Relatively little work has been done on the different variables that affect gliotoxin production in vitro in \(A.\) \textit{fumigatus}. Investigation of the conditions and factors that affect gliotoxin production by \(A.\) \textit{fumigatus} will both add to our understanding of the potential role of gliotoxin in pathogenesis and assist in the development of a simpler small scale assay for gliotoxin concentration.

In this study, we have used several different media and incubation temperatures in an attempt to determine the optimum growth conditions for the production of gliotoxin by \(A.\) \textit{fumigatus} in vitro. This study was undertaken so that large numbers of \(A.\) \textit{fumigatus} isolates recovered from patients and from the environment could be screened for gliotoxin production using standardized, optimum conditions for the production of this toxin. Strains and mutants which were unable to produce gliotoxin could then be recovered and their virulence determined in an experimental animal model of aspergillosis.

**Methods**

**Organisms and storage conditions**

The optimum growth conditions for gliotoxin production were investigated using three isolates of \(A.\) \textit{fumigatus} (PHLS Mycology Reference Laboratory, Leeds, UK, Nos 006, 039 and 086). Isolate 006 was recovered post-mortem from a liver transplant patient with invasive aspergillosis, 039 was isolated from the environment and isolate 086 from the sputum of a liver transplant recipient with no sign of aspergillosis.

All other isolates used in this study were recovered following UV mutagenesis of isolate 006. Isolates were stored at \(-70\) °C using a bead cryopreservation system (Protect System, Heywood, UK) [15].

Influence of growth medium on gliotoxin production

The three \(A.\) \textit{fumigatus} isolates were grown on Sabouraud glucose agar (SAB) plates for 2 days at 37 °C and the conidia were then harvested with sterile 0.5% Tween 20 and adjusted to a concentration of 10⁷ conidia ml\(^{-1}\) in distilled water based on haemocytometer counts. One millilitre volumes of this conidial suspension were used to inoculate 100 ml of liquid medium, Czapek-Dox broth (CDB; 30 g carbohydrate (glucose, lactose, maltose or sucrose), 3 g Na₂NO₃, 0.5 g MgSO₄·7H₂O, 0.5 g KCl, 0.01 g FeSO₄ in 11 distilled water), in 250 ml flasks. The cultures were incubated at 37 °C in a shaking incubator at 1400 rpm for 2, 4, 6 or 10 days. The ability of the isolates to produce gliotoxin in brain heart infusion (BHI, Merck, Lutterworth, UK) broth (Oxoid Ltd, Basingstoke, UK) and Sabouraud broth at 37 °C (Oxoid Ltd, Basingstoke, UK) was also determined.

In further experiments, the concentration of glucose in the CDB was varied (20, 30, 40 and 50 g l\(^{-1}\)) and other incubation temperatures (10, 20 and 30 °C) were also used. The incubation time in these experiments was 2 days.

Growth curve for \(A.\) \textit{fumigatus}

Flasks (250 ml), each containing 100 ml of CDB (30 g l\(^{-1}\) glucose; CDG) were inoculated with 1 × 10⁷ \(A.\) \textit{fumigatus} conidia and incubated at 37 °C. After 2, 4, 6, 10, 18, 29, 36 and 48 h, and 4 days, one flask was removed and the contents filtered through a Whatman No. 1 filter paper in a Büchner funnel and dried using negative pressure. The pre-weighed filter papers and fungal growth were dried overnight at 60 °C and allowed to cool for at least 3 h at room temperature before weighing. A growth curve was constructed of dry fungal biomass versus incubation time.

Preparation of extracts for detection of gliotoxin

After the relevant incubation time, fungal growth from cultures of \(A.\) \textit{fumigatus} isolates 006 and 086 was removed from the CDG by filtration through Whatman No. 1 filter paper in separate Büchner funnels and the filtrate shaken three times for 10 min each with 50 ml chloroform (Hyper Solv, BDH) at 25 °C. The chloroform fractions were collected, pooled and evaporated to dryness on a rotary evaporator (Büchi) at reduced pressure and 47 °C. For detection of gliotoxin, dried extracts were dissolved in 200 µl methanol (Hyper Solv, BDH) and stored at -70 °C until assay.
Determination of optimum growth conditions for gliotoxin production by Aspergillus fumigatus

Detection of gliotoxin by TLC

Detection of gliotoxin was performed by TLC. Ten microlitres of each chloroform extract in methanol and of a 1 mg ml\(^{-1}\) gliotoxin standard in methanol, were spotted onto a Silicagel 60 plate with fluorescence indicator (Merck, Damstadt, Germany) approximately 3 cm from the bottom edge. The plates were developed for 10 cm in an inclined tank with a methanol:chloroform (10:90, v/v) solvent system. The plates were air dried and sprayed with 25% sulphuric acid in distilled water and heated at 110 °C for 15 min.

Detection of gliotoxin by HPLC

Gliotoxin was also detected by HPLC (Perkin Elmer) as described by Richard et al. [14]. The mobile phase was 50% methanol (HyperSolv, BDH):50% deionized-distilled water. The gliotoxin extract was diluted 1:50 in mobile phase and 20 μl of each fraction was injected into the HPLC. A standard curve of peak height versus gliotoxin concentration was constructed by injection of 20 μl of gliotoxin standards (Sigma; 50, 100 and 200 ng ml\(^{-1}\) dissolved in mobile phase) into the HPLC and the amount of gliotoxin in the samples was calculated from the standard curve.

The sensitivity of chloroform extraction and HPLC for recovery and detection of gliotoxin was determined by spiking 100 ml of BHI, CDB or Sabouraud broth with 20 μl of gliotoxin solution (original concentration, 1 mg ml\(^{-1}\)) and incubating at 37 °C for 2 days. The broth was then filtered through Whatman No. 1 filter paper in a Büchner funnel and chloroform extraction was performed as described previously. The amount of gliotoxin recovered was then determined from a standard curve.

UV mutagenesis of A. fumigatus

Parent isolate 006 was subjected to UV mutagenesis in an attempt to produce mutants which were deficient in gliotoxin production. Briefly, 5 ml of conidia suspension containing 10\(^{4}\) conidia ml\(^{-1}\) in sterile distilled water were exposed to UV light (265 nm) at a distance of 25 cm from the light source for different periods of time. One-hundred microlitres of this suspension were then spread out on a plate of growth medium and incubated at 37 °C for 2 days. The plating medium chosen allowed only limited growth of A. fumigatus colonies without sporulation and was composed of 0.2% glucose, 0.08% deoxycholate, 0.5% yeast extract, 2% agar [16]. Many discretely separated colonies could easily be scored on a plate. Over 1000 random survivors from UV mutagenesis were obtained and screened for gliotoxin production.

Growth of mutants

A suspension containing 10\(^7\) conidia ml\(^{-1}\) in sterile distilled water was prepared from each of the 1000 surviving irradiated colonies and 10 μl of the conidia suspension was inoculated into 200 μl of CDG in wells of 96-well microtitre plates (Millipore, Watford, UK). The plates were incubated at 37 °C in a moist atmosphere for 48 h. Culture supernatants from all 1000 mutants were filtered using a multiscreen filtration system (Millipore, UK) into a second 96-well plate and then screened for gliotoxin production using the L929 lung fibroblast cell assay described below.

Lung fibroblast (L929) cells

The mouse lung fibroblast cell line (L929) was derived from normal subcutaneous alveolar and adipose tissue of a 100-day-old male C34/An mouse. Dulbecco’s modified eagle’s medium (DMEM) containing 10% foetal calf serum (FCS) was used as the culture medium.

Resurrection of L929 cells from liquid nitrogen

Five millilitres of DMEM (10 × concentrated) was prepared and 9 ml of Hank’s balanced salt solution (HBSS) was warmed separately to 37 °C. A frozen vial containing 1 ml of HBSS with L929 cells was thawed gently at 37 °C and added, with a sterile Pasteur pipette, to the warmed HBSS. The mixture was then centrifuged at 12000 rpm (Denley BS 400) for 5 min and the supernatant discarded. The pellet was resuspended in 1 ml DMEM, transferred into a 50 ml flask that contained freshly prepared culture medium, gassed with 5% CO\(_2\) in air and incubated for 48 h at 37 °C.

Passage of L929 cells

The resurrected cells were washed three times with sterile phosphate-buffered saline (PBS) pH 7.3, and then 1 ml of pre-warmed trypsin-EDTA (0.05% (w/v) trypsin and 0.02% (w/v) EDTA) and 1 ml of PBS were added to a 50 ml culture flask. The enzyme was spread across the cells and incubated at 37 °C for 2 min. When the cells had detached from the plastic, they were pipetted up and down to dissociate clumps and transferred to 1 ml of culture medium.

A small volume of cells was removed to count and viability was assessed with Trypan blue. Cells were transferred into a 200 ml flask containing fresh DMEM (three times the original volume) to a final concentration of 5 × 10\(^6\) L929 cells ml\(^{-1}\) and the cells were then gassed with 5% CO\(_2\) in air and incubated at 37 °C for 2–3 days.
Viability of L929 cells

Viability of the L929 cells was determined with 1% Trypan blue (BDH). Ten microlitres of L929 cells was added to 10 μl of 1% Trypan blue and, after approximately 2 min, the total number of cells and the number of cells that did not exclude Trypan blue (dead cells) were counted using a haemocytometer. The percentage of viable L929 cells was calculated as follows:

\[
\text{Total number of cells} - \text{total number of dead cells} \times 100
\]

L929 lung fibroblast assay

Adherence of L929 mouse fibroblast cells to the plastic surface of 96-well microtitre plates was quantified by a neutral red assay after 2–3 days incubation at 37 °C.

L929 cells were labelled with neutral red in HBSS medium as follows: neutral red (0.04% (w/v) in 10 μl of 1% ethylacetate was added to 90 μl of L929 cells (5 × 10⁵ ml⁻¹) and incubated for 15 min at 37 °C. The cells were centrifuged at 13000 rpm for 5 min, washed twice in DMEM containing 1% FCS and resuspended in the same medium to a concentration of 5 × 10⁵ L929 cells ml⁻¹.

One-hundred microlitres of this cell suspension was pipetted into the wells of a 96-well flat bottomed tissue culture plate (Millipore) and an equal volume of *A. fumigatus* culture supernatant or pure gliotoxin, serially diluted from 0.5–10 μg ml⁻¹ in 1% ethylacetate, was added. The plates were incubated at a 5% CO₂ atmosphere for 2 h at 37 °C. The culture medium was then discarded and the cell monolayers were washed by immersing the microtitre plate once in PBS pH 7.3, at room temperature. The PBS was discarded and the neutral red was released from the remaining adherent cells by addition of 100 μl of 0.05 M acetic acid in 50% ethanol to each well. The absorbance in each well was measured at 570 nm with a microtitre plate reader (Dynatech 600) and was proportional to the number of adherent cells.

To determine the effect of ethylacetate alone on the L929 cells, control ethylacetate dilutions were prepared in DMEM containing 10% FCS (final ethylacetate concentration, 0.01–8%) and these were substituted for gliotoxin in the assay.

The reproducibility of the assay was determined on two separate occasions using three concentrations of gliotoxin (0.5, 0.75 and 1 μg ml⁻¹) in 1% ethylacetate and 5 × 10⁵ L929 cells ml⁻¹.

Results

Effect of medium composition and incubation temperature on gliotoxin production

When gliotoxin production was examined for three isolates of *A. fumigatus* in media containing different carbohydrates, a significant modification of gliotoxin production was achieved for isolates 006 and 086 depending on the carbohydrates used. Most of the gliotoxin was produced in the first 2 days of incubation with glucose or sucrose as the carbohydrate source and after 4 days with lactose or maltose. Isolate 006 produced more gliotoxin than isolate 086 with all of the carbohydrates tested. Isolate 039 did not produce gliotoxin with any of the carbohydrates (data not shown).

As the highest concentration of gliotoxin was produced with glucose during the first 2 days of incubation, the optimum growth conditions were determined further varying two factors, incubation temperature and glucose concentration. A temperature of 37 °C (Table 1) and a glucose concentration of 30 g l⁻¹ (Table 2) appeared to be optimal for gliotoxin production *in vitro*. Increasing or decreasing the amount of carbohydrates or reducing the temperature below 37 °C caused a decrease in gliotoxin production and biomass (Tables 1 and 2).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Temperature (°C)</th>
<th>Biomass (g)</th>
<th>Concentration of gliotoxin (mg ml⁻¹)</th>
<th>Concentration of gliotoxin (mg g⁻¹ biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>006</td>
<td>10</td>
<td>0.06</td>
<td>0.43</td>
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</tr>
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<td></td>
<td>20</td>
<td>0.08</td>
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<td>30</td>
<td>0.10</td>
<td>1.24</td>
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<td>37</td>
<td>0.14</td>
<td>3.75</td>
<td>5.35</td>
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<td>086</td>
<td>10</td>
<td>0.05</td>
<td>0.12</td>
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<td></td>
<td>20</td>
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<td>0.10</td>
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<td>37</td>
<td>0.12</td>
<td>2.70</td>
<td>4.50</td>
</tr>
</tbody>
</table>

Table 1 Effect of temperature on biomass and gliotoxin production after 2 days incubation in CDG (30% glucose) broth

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The sensitivity of HPLC for the detection of gliotoxin was determined using the chloroform extraction procedure on BHI, CDG and SAB broth spiked with a standard gliotoxin concentration. Chloroform extraction was found to result in recovery of 95% of the gliotoxin.

Growth curve and gliotoxin production

Growth curves for *A. fumigatus* isolates 006 and 086 cultured in CDG are shown in Fig. 1. The exponential growth phase began after 4 h and extended over another 40 h. The stationary phase was reached at around 48 h. The production of gliotoxin by isolates 006 and 086 started at 29 h (Fig. 2). Isolate 006 produced more gliotoxin than isolate 086. Maximum gliotoxin production occurred after approximately 48 h incubation at 37 °C.

UV mutagenesis

The effect of exposure of *A. fumigatus* isolate 006 to UV light was quantified by determining the percentage survivors at different exposure times. A survival of 25% was chosen as a suitable measure of mutagenesis [17] and was obtained after 1 min 50 s exposure at a distance of 25 cm from the UV light source. This exposure time was, therefore, used in subsequent experiments to produce *A. fumigatus* mutants defective in gliotoxin production.

Effect of gliotoxin in the lung fibroblast assay

Preliminary studies were performed to look at the effect of the solvent, ethylacetate, on the fibroblast cells. At a concentration of 4% or higher, ethylacetate caused necrosis of the fibroblast cells after 2 h incubation at 37 °C with a drastic decrease in the optical density compared to the control (L929 cells without ethylacetate). However, a concentration of 1% ethylacetate did not have any apparent effect on the lung cells and there was no decrease in the optical density when compared with the control. For subsequent experiments, therefore, neutral red and gliotoxin standards were diluted in 1% ethylacetate.

The incubation time at which gliotoxin affected the

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**Table 2** Effect of glucose concentration on biomass and gliotoxin production after 2 days incubation at 37 °C

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mass of glucose (g l(^{-1}))</th>
<th>Biomass (g)</th>
<th>Concentration of gliotoxin (mg ml(^{-1}))</th>
<th>Concentration of gliotoxin (mg g(^{-1}) biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>006</td>
<td>20</td>
<td>0.090</td>
<td>0.81</td>
<td>1.8000</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.135</td>
<td>2.01</td>
<td>5.3400</td>
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<tr>
<td></td>
<td>40</td>
<td>0.094</td>
<td>0.33</td>
<td>0.7020</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.085</td>
<td>0.18</td>
<td>0.4230</td>
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<td>0.39</td>
<td>0.9620</td>
</tr>
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<td></td>
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<td>0.120</td>
<td>2.68</td>
<td>4.4600</td>
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<td></td>
<td>40</td>
<td>0.080</td>
<td>0.27</td>
<td>0.6750</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.079</td>
<td>0.15</td>
<td>0.3790</td>
</tr>
</tbody>
</table>
viability of the cells was determined using different concentrations of gliotoxin. Gliotoxin, at concentrations of 0·5–1 μg ml⁻¹, did not appear to affect the adherence of L929 mouse lung fibroblast cells to the plastic after 2 h incubation at 37 °C in 5% CO₂, with optical densities close to that of the control. After 2 h exposure to gliotoxin at a concentration of 0·75 μg ml⁻¹, however, some of the cells lost the ability to adhere to the plastic, although they did remain viable and excluded Trypan blue. Viability of the L929 cells was lost completely after exposure to 1 μg ml⁻¹ gliotoxin for 2 h. An incubation time of 2 h was, therefore, chosen for screening A. fumigatus culture supernatants for gliotoxin. This assay was found to be reproducible.

**Screening of A. fumigatus mutants for gliotoxin**

Using the fibroblast assay developed, three UV-irradiated colonies derived from A. fumigatus 006 were found to produce low or undetectable levels of gliotoxin, a result confirmed by TLC. The more sensitive technique, HPLC, showed that two of these isolates were non-producers of gliotoxin while the third produced a low concentration of gliotoxin which was undetectable by the fibroblast assay or TLC.

**Discussion**

Although the airborne pathogen, A. fumigatus, is capable of causing a range of clinical infections in humans and animals, there are many other airborne fungi that have never been implicated in disease. This has lead to the suggestion that A. fumigatus must produce some specific virulence factors that are important in helping the fungus to colonize and invade the airways [9]. Several groups have looked at the role of gliotoxin in the pathogenesis of aspergillosis as this metabolite is produced by over 95% of isolates of A. fumigatus [18] and has associated immunosuppressive activity in vitro and in vivo [10–14].

We have attempted to determine the optimum growth conditions for gliotoxin production in vitro using two A. fumigatus isolates, 006 and 086. When these two isolates were grown in the presence of different carbohydrates, gliotoxin production was higher in the presence of glucose and sucrose, confirming an earlier study by Kurbutskaya & Trostanetsky [19], but was decreased when lactose or maltose were the carbohydrate sources. Therefore, the composition of the medium used to grow A. fumigatus has an important influence on gliotoxin production. Incubation temperature and the concentration of glucose in the broth were also important in determining the amount of gliotoxin produced. The optimum incubation temperature was found to be 37 °C in the presence of 30% glucose.

When we investigated the ability of BHI and SAB to support gliotoxin production, however, no gliotoxin was detected with either isolate, suggesting that these two growth media lack some components which are vital for gliotoxin production.

According to Mullbacher et al. [20], the concentration of gliotoxin produced by A. fumigatus in CDB varies from 20 to 80 μg ml⁻¹ below 50 °C after approximately 4–7 days. This concentration of gliotoxin has been shown to inhibit the growth of several Gram-negative bacteria and fungi, and virus replication in vitro [21]. Gliotoxin at a concentration of 20–50 ng ml⁻¹ has been shown to have an inhibitory effect on phagocytosis of carbon particles by peritoneal exudates [22] and to cause an abrogation of T-cell immune mechanisms [10]. The highest concentration detected in our study was 4·8 μg ml⁻¹ after 2 days incubation at 37 °C in CDG. In this study, gliotoxin production was shown to decrease with time and occurred mainly in the exponential phase of growth. The subsequent decline in activity may have been due to catabolism, to instability of the compound under the culture conditions used, or, perhaps, to the production of an antagonist late in culture [22]. Richard et al. [14] showed that the percentage recovery of gliotoxin from rice medium was 83·8% by HPLC. The average recovery of gliotoxin in our experiments was 95%.

Conventional methods of TLC and HPLC [14] are time consuming to perform for detection of gliotoxin and we, therefore, set out to develop a more rapid method which could be used to screen large numbers of isolates at a time. The lung fibroblast assay although less sensitive than HPLC, was easy to use and had good reproducibility.

One environmental isolate of A. fumigatus (039) tested by HPLC was found to be gliotoxin-negative in this study, confirming earlier observations that a small but significant number of environmental and clinical isolates fail to produce gliotoxin (V. Hopwood, unpublished data).

The fibroblast assay was used to determine the presence or absence of secreted products of A. fumigatus that caused the adhering fibroblasts to detach from the plastic microtitre dish wells. It is possible that components other than gliotoxin might cause the detachment of fibroblasts from the microtitre dish walls and therefore the confirmation of this screening result by HPLC or TLC is important, as was shown in this study. In total, only two gliotoxin-negative isolates were detected with the fibroblast assay amongst UV survivors, and one weak gliotoxin-producing isolate. The results of this study...
show that our L929 lung fibroblast assay is suitable for the rapid screening of large numbers of A. fumigatus isolates for gliotoxin production and avoids the batch culture and purification procedures used previously in other studies.

References