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Mycotoxin risk evaluation in feeds contaminated by *Aspergillus fumigatus*

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Abstract

Aspergillus fumigatus, a common feed contaminant particularly ubiquitous in conserved forages, produces several mycotoxins that can affect the health of animals. The aim of this work was to assess the ability of *A. fumigatus* to produce toxins, particularly gliotoxin, on laboratory media and natural feed substrates. The ability of fourteen *A. fumigatus* strains to produce gliotoxin, verruculogen, fumagillin, and helvolic acid was evaluated on defined medium. Mycotoxin production was widespread; gliotoxin and verruculogen were produced by 70 and 85% of isolates at concentrations up to 62.2 and 3.5 $\mu g\,mL^{-1}$, respectively. In addition, all strains were positive for fumagillin (up to 25.9 $\mu g\,mL^{-1}$) and helvolic acid (up to 3.5 $\mu g\,mL^{-1}$). Growth and gliotoxin production of a gliotoxin-positive isolate was evaluated on several common animal feeds. Growth was extensive on cereals—wheat, corn, barley, and triticale, less pronounced on forage grasses—rye grass, orchard grass, and tall fescue, and negative or scarce growth was observed on leguminous forages—alfalfa and red clover. Gliotoxin production was in general correlated to growth, except for corn and tall fescue. In these two substrates, as well as in leguminous forages the toxin was not detected. Low pH culture conditions, similar to those present in silages, reduced gliotoxin production as compared to controls although growth was unaffected. Concentrations found in cereals and grasses were up to 17.5 and 1.5 $\mu g\,g^{-1}$, respectively. The stability of *A. fumigatus* mycotoxins during storage was evaluated in orchard grass and ryegrass. Toxins were stable in both forages, except for fumagillin whose concentration decreased rapidly. After eight weeks of storage, the amount of gliotoxin, verruculogen, helvolic acid, and fumagillin was 63, 76, 89, and 10% of the initial concentration, respectively. The high concentrations found

Abbreviations: Afu 9, *Aspergillus fumigatus* strain 9; DCM, dichloromethane; MEM, minimum eagle medium; MEM + G, minimum eagle medium supplemented with glucose; YES, yeast extract sucrose

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in experimentally contaminated feed and the relative stability of *A. fumigatus* toxins could pose a potential health risk for animals consuming contaminated feeds.

Keywords: *Aspergillus fumigatus*; Mycotoxin production; Mycotoxin stability; Forage; Cereal

1. Introduction

Production of hay and silage during the growing season is a widespread agricultural practice that allows the conservation of forages for later use throughout the year. The process of drying and ensiling help preserve the quality of forages during extended periods. However, adverse climate conditions or inadequate techniques during harvesting, preparation, or storage of the forage may favour mould development, which is one of the most common problems encountered in stored feeds. Unfavourable weather and storage conditions are also the main causes triggering fungal contamination in cereals. Fungal contamination reduces the nutritive value of feeds and may affect animal and human health. A common contaminant in silage and hay is the saprophyte mould *Aspergillus fumigatus* (Cole et al., 1977; Pelhate, 1987; Schneweis et al., 2001; Smith and Lynch, 1973). This fungus is the main causal agent of invasive aspergillosis in humans and animals (Latge, 2001) and produces several toxic metabolites.

Gliotoxin, one of the major metabolites produced by *A. fumigatus*, has received particular attention because it has potent immunosuppressive, genotoxic, cytotoxic, and apoptotic effects (Nieminen et al., 2002; Upperman et al., 2003; Waring et al., 1988). In addition, this toxin has been associated with cases of clinical aspergillosis (Bauer et al., 1989; Richard et al., 1996; Sutton et al., 1996). Feeds contaminated with this toxin are potentially toxic for animals. Gliotoxin has been linked to a case of intoxication and death in camels consuming contaminated hay (Gareis and Wernery, 1994). Other common *A. fumigatus* toxins are: verruculogen, which has tremorgenic (Land et al., 1987) and genotoxic (Sabater-Vilar et al., 2003) activities, fumagillin, an angiogenesis inhibitor not used therapeutically because of its high cytotoxicity and genotoxicity (Amitani et al., 1995; Ingber et al., 1990), and helvolic acid, a steroidal antibiotic that also has cytotoxic activities (Amitani et al., 1995). Despite their recognized toxicity there is a lack of information describing the production and stability of *A. fumigatus* toxins on feeds.

The purpose of the present work was to assess the ability of *A. fumigatus* strains to produce toxins, particularly gliotoxin, on laboratory media and natural feed substrates. The stability of gliotoxin, verrucologen, fumagillin, and helvolic acid on forage substrates was also examined.

2. Materials and methods

2.1. Mycotoxin production on liquid media

Fourteen strains of *A. fumigatus* (Afu) were screened for their capacity to produce gliotoxin, verruculogen, fumagillin, and helvolic acid in broth media. Table 1 lists the

Table 1
A. fumigatus strains tested for mycotoxin production

Strain	Source	Reference	Isolated from
Afu 01	MNHN[a]	91.2760	Soil
Afu 02	MNHN	88.2521	Soil, oil contaminated
Afu 03	MNHN	96.3921	Cork wine
Afu 04	MNHN	90.2642	Cereal
Afu 05	MNHN	72.2115	Forage
Afu 07	MNHN	65.516	Fruit compote
Afu 08	MNHN	88.2541	Air-conditioning filter
Afu 09	MNHN	97.4053	Sea sediments
Afu 10	ATTC[b]	46645	Human
Afu 11	NRRL[c]	1986	Not specified
Afu 12	INRA-Theix		Wheat straw
Afu 13	INRA-Theix		Skin lesions, cattle
Afu 14	INRA-Theix		Skin lesions, cattle
Afu 15	INRA-Theix		Lung, cattle

[a] National Museum of Natural History (Paris, France).
[b] American Type Culture Collection (ATCC, Marassas, USA).
[c] Agriculture Research Service Culture Collection (Peoria, USA).

origin and isolation source of the strains used. The four strains (Afu 12–15) that were isolated in our laboratory came from a dairy farm in Normandy (France) with cases of clinical aspergillosis. Isolates were maintained on 20 g/L malt agar slants at 4 °C.

The optimal conditions for mycotoxin production, based on the production of gliotoxin by a known *A. fumigatus* (Afu 09) producer strain were determined in various synthetic media previous to the screening test. The effects of incubation time, agitation, low pH, and inoculum size on toxin production were also tested on the same strain. The broth media used were yeast extract sucrose (YES, 20 g yeast extract and 150 g saccharose L^{-1}) (Davis et al., 1966) and Eagle's minimum essential medium (Sigma, France) supplemented with 50 g/L foetal calf serum with or without the addition of 30 g glucose L^{-1} (MEM + G and MEM, respectively). Inocula were prepared from spores harvested from 3-day-old malt extract agar cultures and suspended in sterile 5 mL/L of Tween 80 solution. The media were aseptically inoculated to obtain a final concentration of 2×10^5 spores mL^{-1} and incubated without agitation for 6 days at 37 °C. At the end of the incubation period, mycelia were filtered through pre-weighed filter papers, washed with distilled water, dried overnight at 60 °C, and weighed for determination of fungal biomass. The total filtrate was used for mycotoxin extraction by mixing with an equal volume of dichloromethane (DCM). The mixture was vortexed for 15 s, the phases allowed to separate and the organic layer was filtered through filter paper (Whatman, PS1). The extract was evaporated to dryness under a flow of N_2 gas at 45 °C, the residue was redissolved in 200 μL of methanol–distilled water mixture (1:1) and used for mycotoxin analysis.

2.2. Mycotoxin production on feed substrates

The growth and mycotoxin production of *A. fumigatus* Afu 09 was assayed on various common animal feeds. Isolate Afu 09 was selected from the fourteen strains initially

screened because it was the best gliotoxin producer in liquid media. Five species of forages, three grasses (orchard grass (*Dactylis glomerata*), tall fescue (*Festuca arundinacea*), and ryegrass (*Lolium perenne* L.)) and two leguminous (alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense* L.)) were used as substrates. All forage substrates were dried at 48 °C for 72 h. Five cereals were also tested for their ability to induce mycotoxin production by growing *A. fumigatus* isolate Afu 09. They were: wheat (*Triticum aestivum*), triticale (*Triticosecale*), corn (*Zea mays*), barley (*Hordeum vulgare*), and rice (*Oryza sativa*). The cereals were obtained from a local provider and they had no preservatives or additives that might interfere with fungal growth. Forages were cut in pieces of about 2 cm long and cereals were coarsely ground. Feeds prepared in this way were weighed into 100 mL Erlenmeyer flasks, 5 g for forages and 10 g for cereals, followed by addition of equivalent amount of distilled water, and sterilized by autoclave (120 °C, 10 min). Feeds were aseptically inoculated with Afu 09 spores, obtained as described above, to obtain a final concentration of 2×10^5 spores g^{-1}. Flasks were incubated in static culture at 37 °C for up to 2 weeks and examined daily for fungal growth under binocular loupe. At day 6 and 14 of the incubation period 10 mL distilled water was added to triplicate flasks followed by addition of 40 mL DCM. The feed–solvent mixture was soaked at room temperature for 2 h, followed by 15 min of mechanical agitation, and filtered through filter paper (Whatman, PS1). Three millilitres of the filtrate extract was evaporated to dryness under a flow of N_2 gas at 45 °C, the residue redissolved in 500 μL of methanol–distilled water mixture (1:1) and used for mycotoxin analysis.

2.3. Mycotoxin stability on forages

The stability of different mycotoxins in feeds was studied using two grasses, orchard grass and ryegrass, which were favourable substrates for the production of gliotoxin. Forages were ground to pass a 1 mm screen, 0.5 g dispensed into 15 mL polypropylene Falcon tubes and used 'as is' or humidified with distilled water (1:1) to simulate silage's wet conditions. These forage matrices were spiked with 70 μL of an ethanol solution containing the four mycotoxins followed by evaporation of the solvent under a flow of N_2 gas at 45 °C. Final concentration of gliotoxin, verruculogen, fumagillin, and helvolic acid was 30, 15, 10 and 15 μg g^{-1} of feed, respectively. Triplicate tubes were capped and incubated at room temperature in the dark for 1, 4, and 8 weeks. Stability was also checked under the same conditions of incubation on mycotoxin solutions in 10 mL/L acetic acid solution–acetonitrile (1:1) in the absence of the forage matrix. The toxin extraction was performed in the same incubation tubes following the addition of 0.5 mL of distilled water and 6.5 mL of methanol–chloroform (3:7). Tubes were agitated mechanically for 15 min and filtered through filter paper (Whatman no. 4). Filtrates (3.5 mL each) were transferred to a glass tube and evaporated to dryness under a flow of N_2 gas at 45 °C. The residue was dissolved in 500 μL of methanol–distilled water mixture (1:1), and 50 μL of the extract was analysed by reversed-phase-HPLC. Mycotoxin concentration at time zero was considered as 100 and stability was expressed as percentage decrease from the initial concentration.

2.4. Mycotoxin analysis

Mycotoxins were analysed by HPLC by the method of Frisvad (Frisvad, 1987) with some modifications. Briefly, separation of the four mycotoxins were performed at room temperature on a Nucleosil C_{18} reversed-phase column (150 mm × 4.6 mm, 5 μm, Macherey Nagel, France) fitted with a Nucleosil C_{18} guard-column using a gradient solvent system (solvent A = 10 mL/L of acetic acid, and solvent B = acetonitrile). The solvent program was as follows: the initial percentage of solvent B was 10%, which was raised to 50% in 30 min, then to 90% in 4 min, lowered to 10% in 2 min, and held at 10% at a flow rate of 2 mL min^{-1}.

The four mycotoxins were well separated, their retention times were typically 14.3, 29.9, 32.6 and 33.4 min for gliotoxin, verruculogen, fumagillin and helvolic acid, respectively. Detection was done at each toxin specific wavelength instead of the single 254 nm used in the original method (Frisvad, 1987) because sensitivity was improved and interferences decreased. A diode array detector was set at 242, 268, 271 and 322 nm for the quantification of helvolic acid, gliotoxin, verruculogen, and fumagillin, respectively. Peak areas for gliotoxin, verruculogen, and fumagillin obtained with these specific wavelengths were, respectively, 14, 33, and 86% higher than peak areas obtained at 254 nm. Fumagillin, which is not detected at 254 nm, was well detected at 322 nm at the concentrations used in our assay.

2.5. Statistical analysis

Data was statistically analysed by one-way analysis of variance using the general linear models procedure of SAS (SAS Institute Inc., Cary, NC). Significance was declared at the 5% probability level and differences among means were tested using the Tukey option (Tukey–Kramer method). For the stability experiment the model included toxin, time, and toxin × time after it was established that matrix forage and humidity and their two- and three-way interactions with toxin and time were not statistically significant.

3. Results and discussion

3.1. A. fumigatus growth and toxinogenesis on liquid media

The influence of media and culture conditions were determined previous to the screening test using a gliotoxin-positive strain of *A. fumigatus* (Afu 9). YES medium is widely used to test toxin production in fungi belonging to *Penicillium* and *Aspergillus* species (Bayman et al., 2002; Boudra, 1994; Cvetnic and Pepeljnjak, 1990; Jimenez et al., 1991) and was considered as the reference medium. Compared to MEM, mycelial biomass was almost 8 times higher in this medium, 52.55 ± 1.97 mg mL^{-1} versus 6.88 ± 0.38 mg mL^{-1} at 10 days of incubation ($P < 0.05$). However, YES did not induce the production of gliotoxin at any time throughout the incubation period. In contrast, the toxin was detected in MEMs culture extracts from day 3 of incubation. The addition of glucose to MEM (MEM + G, 30 g L^{-1}) resulted in a two-fold increase in fungal biomass (10.7 ± 0.38 mg mL^{-1}, $P < 0.05$), which

Table 2
Effect of culture conditions on biomass and gliotoxin production by an *A. fumigatus* strain

Treatment	Biomass (mg mL^{-1})	Gliotoxin (μg mL^{-1})
Control[a]	10.7	29.6 ac
Filter-sterilized medium	12.0	26.7 ac
Non-sterilized medium	11.1	40.0 a
Reduced inoculum size[b]	12.6	13.6 bc
Effect of agitation	13.0	16.4 bc
Effect of acid medium (pH 4)	11.6	2.6 b
S.E.M.	0.45	3.58

Within a column, means ($n = 3$) followed by different letters differ ($P < 0.05$); S.E.M.: standard error of the means.
[a] Minimum eagle medium (MEM) broth supplemented with 30 g glucose L^{-1}, pH 6.0, inoculated with 10^5 spores mL^{-1} and incubated at 37 °C without agitation.
[b] Inoculum 10^3 spores mL^{-1}.

was accompanied by a similar increase in gliotoxin production. The parallel increase in biomass and gliotoxin concentration in glucose supplemented MEM suggests that toxin synthesis in this medium was not triggered by the lack of a readily available energy source, but it was stimulated by another constituent(s) present in this medium and absent in YES.

In order to optimise the production of gliotoxin and increase the sensitivity of the screening test the effect of medium sterilization, inoculum size, agitation, and low pH was further assayed on MEM + G. Biomass production was not affected ($P > 0.05$) by any of the parameters tested (Table 2). However, low pH of the medium, reduced size of the inoculum, and agitation had a negative effect on gliotoxin production ($P < 0.05$). The reduced synthesis of gliotoxin at low pH suggests that acid-stored feeds, such as silage, are less likely to have deleterious amounts of this toxin, even in the presence of *A. fumigatus* contamination.

3.2. Screening of *A. fumigatus* strains

The capacity of fourteen *A. fumigatus* strains to produce mycotoxins was examined in MEM + G medium (Table 3). After 3 days of incubation, gliotoxin was found in 10 isolates at concentrations ranging from 0.2 to 23.2 μg mL^{-1}. At 6 days of incubation, however, gliotoxin was found in the culture extract of 5 isolates and the concentration tended to decrease, except for 2 isolates (Afu 09 and 13) that showed increased concentration. Unlike gliotoxin, the concentration of verruculogen, fumagillin, and helvolic acid in positive cultures increased throughout the incubation period. Verruculogen was found in 9 isolates, while fumagillin and helvolic acid were produced by all 14 strains tested after 6 days of incubation.

Of the four secondary metabolites monitored, gliotoxin was the least frequent with nearly 30% of isolates being negative. However, the concentration found in positive strains was usually larger than for the other three metabolites. Gliotoxin is reportedly the most toxic metabolite produced by *A. fumigatus* (Sutton et al., 1996) and the amount produced by some strains is enough to pose a potential animal health problem if found in feeds (Morgavi et al., 2004; Upperman et al., 2003; Watanabe et al., 2003). In general, gliotoxin production

Table 3
Mycotoxin production by *A. fumigatus* strains on minimum eagle medium broth-glucose[a]

Strain	Gliotoxin		Verruculogen		Fumagillin		Helvolic acid	
	3[b]	6[b]	3[b]	6[b]	3[b]	6[b]	3[b]	6[b]
Afu 01	–[c]	–	0.1	–	1.2	1.9	–	0.5
Afu 02	–	–	0.1	–	0.8	4.2	–	0.2
Afu 03	–	–	0.1	2.1	0.5	5.7	–	2.9
Afu 04	23.2	5.8	0.1	–	–	0.2	0.1	2.1
Afu 05	16.9	7.3	–	–	1.5	11.7	–	0.1
Afu 07	5.0	0.8	0.2	0.9	0.4	5.7	–	0.4
Afu 08	4.5	–	–	2.0	0.6	13.7	–	1.1
Afu 09	15.6	62.2	0.1	0.6	0.4	13.8	–	0.6
Afu 10	7.0	–	0.4	1.8	2.9	25.9	0.1	1.0
Afu 11	5.7	–	0.4	3.5	1.4	15.2	0.3	2.2
Afu 12	–	–	0.1	0.3	1.0	6.0	0.5	2.4
Afu 13	11.2	25.1	0.2	–	1.2	14.3	–	0.4
Afu 14	0.6	–	0.4	1.4	3.8	19.0	0.7	2.3
Afu 15	0.2	–	0.5	2.0	2.5	7.1	0.5	2.7

[a] Toxin concentration in $\mu g\,mL^{-1}$.
[b] Incubation time (days).
[c] Not detected.

peaked early during the incubation period and decreased thereafter suggesting that the toxin was unstable in the medium and/or was degraded by the fungus.

The Afu 09 strain was the best gliotoxin producer, and was used for further toxinogenesis studies on feeds.

3.3. *A. fumigatus* growth and toxinogenesis on feed substrates

Extensive fungal growth was observed in all cereal substrates (Table 4). Wheat, corn, barley, and triticale became visibly mouldy after 2 days of incubation, and were covered entirely by Afu 09 after 3 days of incubation. In contrast, *A. fumigatus* grew poorly in forage substrates. Mycelial development was observed macroscopically after 2–3 weeks of incubation on orchard grass, tall fescue, and rye grass. Leguminous were less favourable fungal substrates than grasses. There was not growth on alfalfa and on red clover, fungal growth was poor and detected under loupe magnification only. Cereals were favourable for gliotoxin production with the exception of corn, although corn has been reported to be a suitable substrate for gliotoxin production by an *A. fumigatus* strain (Wenehed et al., 2003). In a similar way as for MEM – G media, gliotoxin concentration was higher in actively growing cultures and decreased in older cultures. Concentration on triticale, the most favourable substrate, was 17.5 and $3.6\,\mu g\,g^{-1}$ at 6 and 14 days of incubation, respectively. Gliotoxin production on forages was lower than on cereals, which is probably a reflection of the limited growth observed on these substrates. Orchard grass and rye grass were the only two forages in which gliotoxin was produced. In contrast, the toxin was not detected in tall fescue, red clover, and alfalfa. For other toxinogenic *Aspergillus*, such as *A. flavus* and *A. versicolor*, forages were also not favourable for

Table 4
A. fumigatus growth and gliotoxin production on feed substrates

Feed	Growth[a]	Gliotoxin ($\mu g\ g^{-1}$)	
		6[b]	14[b]
Cereals			
Wheat	+++	8.9	3.9
Corn	+++	–[c]	–
Barley	+++	5.6	1.4
Triticale	+++	17.5	3.6
Forages			
Orchard grass	+	na[d]	1.5
Tall fescue	+	na	–
Rye grass	++	na	0.9
Alfalfa	–	na	–
Red clover	+	na	–

[a] Growth recorded after 3 and 14 days of incubation for cereals and forages, respectively. (–) Negative, (+) mycelial development observed under loupe magnification, (++) macroscopically visible mycelia, (+++) extensive growth, substrate entirely covered.
[b] Incubation time (days).
[c] Not detected.
[d] Not analysed.

fungal development and mycotoxin production (Le Bars, 1976; Le Bars and Labouche, 1979).

3.4. Stability of *A. fumigatus* toxins on forages

Orchard grass and rye grass, the two forages that were favourable for *A. fumigatus* growth and gliotoxin synthesis were used to test mycotoxins stability during storage at room temperature. The type of forage had no effect ($P > 0.05$) on toxins stability. In addition, two different rates of humidity to simulate hay or silage conditions also had no effect on stability ($P > 0.05$). Table 5 shows for each mycotoxin the pooled data for both forages under dry and wet conditions. *A. fumigatus* toxins were moderately stable throughout the incubation

Table 5
Stability of *A. fumigatus* toxins on feed forages stored at ambient temperature[a]

Toxin	Storage period (weeks)				S.E.M.
	0	1	4	8	
Gliotoxin	100 b	91.7 b	74.0 c	62.7 c	2.81
Fumagillin	100 b	65.9 c	23.6 d	9.2 e	4.11
Helvolic acid	100	98.0	89.8	89.2	2.36
Verruculogen	100 b	87.7 bc	77.8 c	75.9 c	1.66

[a] Toxins were spiked on orchard grass and rye grass and kept under dry or wet storage conditions. No differences ($P > 0.05$) were observed due to forage type or storage conditions (see text). Values shown are pooled means for each toxin ($n = 12$). Concentration at the start of the incubation (week 0) was considered as 100. Within a row, means followed by different letters differ ($P < 0.05$).

period with the exception of fumagillin. Fumagillin concentrations decreased by 35% after one week of incubation and at the end of the 8-week incubation period only 10% was recovered. Concentrations of the other toxins at the end of the 8-week incubation ranged from 63% for gliotoxin up to 90% for helvolic acid. In pure solution, the four mycotoxins were stable with just a 10% concentration decline at 7 weeks of incubation. The decrease in the concentration of fumagillin could be attributed to an interaction with the forage matrix. The role of the epiphytic microbial and fungal populations in fumagillin degradation was likely not important as similar results were observed under wet and dry conditions. Under natural conditions and especially for silages, the natural microbial population could react with or bind to these molecules as was shown for other mycotoxins (El-Nezami et al., 2002; Oatley et al., 2000; Peltonen et al., 2001) and increase toxins' disappearance. In addition, fungi with the ability to produce secondary metabolites can alternatively degrade them, depending on the stage of growth development. This is probably the reason why gliotoxin concentration decreased rapidly in fungal cultures.

In conclusion, *A. fumigatus* was highly toxinogenic. All strains tested produced two or more of the four mycotoxins monitored. Fumagillin and helvolic acid were present in all isolates whereas just a few isolates did not produce gliotoxin and/or verruculogen under the conditions tested. The production varied widely among isolates. Gliotoxin in particular was synthesized at important concentrations by some strains. Although *A. fumigatus* is a major contaminant in conserved forages our results show that fungal growth and gliotoxin production in several common forages is poor. Leguminous, like alfalfa and red clover, as well as the acid conditions present in silages did not favour gliotoxin production and hence these feeds seem not to be a potential source of intoxication. However, contaminated cereals can contain significant concentrations of this toxin. Extracts from *A. fumigatus* grown on barley grain had a negative effect on rumen fermentations (Morgavi et al., 2004). Also, the immunosuppressive and apoptotic effect of gliotoxin are observed at concentrations as low as 0.01 $\mu g\,mL^{-1}$ (Upperman et al., 2003; Watanabe et al., 2003). The concentration of gliotoxin found in experimentally contaminated feeds (17.5–0.9 $\mu g\,g^{-1}$) could, therefore, affect productivity and present a health risk for animals.

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