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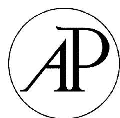

ORIGINAL ARTICLE

Trichothecenes and fumonisins produced in autoclaved tiger nuts by strains of *Fusarium sporotrichioides* and *Fusarium moniliforme*

J. J. Mateo* and M. Jiménez

This work describes for the first time the production of trichothecenes and fumonisins in autoclaved tiger nuts by strains of Fusarium sporotrichioides *and* F. moniliforme *respectively. The study was carried out using a high performance liquid chromatograph with fluorescence detector. The results obtained show that the strains studied are capable of producing these mycotoxins in tiger nuts and that the concentrations of toxin found depend on the variety of tiger nut used as substrate. The amount of type A trichothecenes produced in tiger nuts by the different strains of* F. sporotrichioides *is smaller than that produced by the same strains in other substrates. However, the strains of* F. moniliforme *have shown that their ability to produce fumonisin B1 is similar to that which they have in rice or wheat, although we have not found any detectable amounts of fumonisin B2 in tiger nuts.*

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Introduction

Tiger nut is a tuber of the species *Cyperus esculentus* L. which is used both in animal feed and for human consumption where is used to make Valencian horchata (a nut milk drink). This plant grows in areas with a temperate, humid climate (Pascual 1984). Tiger nut requires soils with special characteristics for quality production. The types of soil suitable for the tiger nut are those which are loose as harvesting requires sieving down to a depth of 15–20 cm of soil where the tuber grows. If it is sieved in compact soils, the tubers come out covered in soil making the washing process much more difficult. Furthermore, in loose, open, sandy soils the tiger nut grows best and has a sweet, strong flavour, a thinner skin and lacks the roots which reduce its value (the hairy tiger nut). It is also bigger and more uniform in size (Vaya 1981, Schwartz 1983). Chemical composition of fresh tiger nuts is shown in Table 1.

After harvesting, the crop must be washed to separate it from the earth, stones, snails, ash, vegetable matter, etc., which must be removed. After washing, the tiger nuts losses humidity in the drying process. During this process, humidity drops from 50% (tender nut) to 11% (dry nut). Drying should be slow and carefully controlled to ensure that the tubers acquires its full organoleptic characteristics (Vaya 1981, Pascual 1984, Pascual 1997).

These are the critical phases in the processing of the nut where the species of fungi present in the tuber, which are potential

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Table 1. Chemical composition of fresh tiger nuts

	% (W/w)
Water	8–10
Lipids	23–30
Starch	30–35
Sucrose	10–15
Cellulose	9–10
Other sugars	0·5–2
Proteins	5–6
Ashes	1·5–2

producers of mycotoxins, could synthesize these toxic metabolites. The washing process does not completely eliminate the remains of soils from the tuber surface and during drying the conditions are suitable for the growth of fungi and the production of secondary metabolites (Vila 1966, Pascual 1997). Some studies have shown the presence of toxigenic fungus in tiger nuts (Adebajo and Oyesiku 1994) and the presence of aflatoxins has even been detected in horchata (Bankole and Eseigbe 1996).

Species of the genus *Fusarium* are widely distributed in nature and often appear associated with vegetable diseases causing serious economic damage to producers (Marasas and Nelson 1987, Marasas et al. 1986, Jelinek et al. 1988, Tanaka et al. 1988, Beasley 1989, Miller and Trenholm 1994). Furthermore, the presence of *Fusarium* fungi should be taken into consideration for health reasons especially, because of its well known ability to produce mycotoxins which have a significant effect on the health of livestock and humans (Ueno 1983, Joffe 1986, Beasley 1989).

The species *F. sporotrichioides* has been described as a producer of type A trichothecenes, which are metabolites with a sesquiterpenoid structure and a double bond at C9–C10 and an epoxide ring at C12–C13, although it does not have a chromophore group; among these toxins, T-2 toxin stands out because of its high toxicity (Cole and Cox 1981), but also significant are HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS) (Yagen and Joffe 1976, Ishii and Ueno 1981, Visconti 1985).

The species *F. moniliforme* is one of the fungi which is most regularly isolated form cereals (Marasas et al. 1984, Sydenham et al. 1995); this species is significant from the toxicological point of view because it has strains which are capable of producing a wide variety of mycotoxins such as moniliformin, fusarin-C, fusaric acid and especially fumonisins (Gelderblom et al. 1988), substances which can provoke illnesses in animals and cause cancer (Ross et al. 1992, Thiel et al. 1992).

This work is a study of the potential of the tiger nut to serve as substrate for the production of type A trichothecenes (T-2 toxin, HT-2 toxin, NEO, DAS) by five producer strains of *F. sporotrichioides* and fumonisins B1 (FB1) and B2 (FB2) by three strains of *F. moniliforme.* Two varieties of tiger nut are analysed, one which is traditionally cultivated in the area of Alboraya, Spain (amella variety) and the other imported from Africa (nigerian variety) and which is characterized by higher yields for the production of the horchata. This work analyses for the first time the risk factor in the consumption of this tuber both directly with no prior treatment and in the form of a refreshing drink (horchata). This paper also presents a comparative study of the tiger nut as substrate for the production of mycotoxins considered previously and other substrates.

Materials and Methods

Fungus strains

Fusarium sporotrichioides strain FSp11 was isolated for *Zea mays*, *F. sporotrichioides* strains FSp12 and Fsp13 were isolated from *Triticum aestivum* and *F. sporotrichioides* strain FSp14 from *Panicum miliaceum*. All these strains were reported to produce type A trichothecenes. *Fusarium sporotrichioides* strain FSp9 was isolated in our laboratory from banana and previous results have shown their ability to produce type A trichothecenes.

Fusarium moniliforme strains FM37, FM25 and CECT 2982 were isolated from *Z. mays.* All these strains have shown their ability to produce fumonisins.

The strain numbers are the reference numbers for the cultures held at the Microbiology Dept., University of Valencia, Spain.

Culture preparation

Fusarium strains were cultivated in potato dextrose agar (PDA) for 7 days at 26°C. Substrates were purchased commercially and used without grinding. One hundred grams of each substrate were placed in a 500 ml Erlenmeyer flask and adjusted to A_w 0·995 with deionized water. The flasks were plugged with cotton, covered with aluminium foil and autoclaved for 30 min at 115°C. The substrate was inoculated with pieces of PDA single-spore cultures and maintained at 26°C. During the first 3 days culture flasks were shaken periodically to disperse inoculum uniformly. After 3 weeks, the cultures were dried at 45°C for 48 h and then finally ground.

Chemicals and reagents

Organic solvents were of HPLC grade from Merck (Darmstadt, Germany). Water was double-distilled in glass. DMAP, standards of T-2 toxin, HT-2 toxin, neosolaniol (NEO), diacetoxyscirpenol (DAS) and FB2 were purchased from Sigma Chemical Co (St Louis, Missouri, USA). FB1 standard (CSIR, Pretoria, South Africa) was used as received. Toxins were transferred quantitatively to volumetric flasks with acetonitrile/water (1/1), resulting in stock standards with concentrations of 1 mg ml^{-1}. Working standards were made up in acetonitrile/water from this stock standard. *o*-Phthaldialdehyde (OPA) was from Fulka (Fluka Chemie, Buchs, Switzerland). Coumarin-carboxylic acid and thionyl chloride were purchased from Aldrich Chemical Co. (Milwaukee, USA).

Type A trichothecenes were analysed by the method of Cohen and Boutin-Mouma (1992) and fumonisins by the methods of Thiel et al. (1993), both with modifications.

Extraction

a) *Trichothecenes.* Samples were finely ground in a laboratory mill and thoroughly mixed. Aliquots (10 g) of the samples were blended with a high speed blender (Ultraturrax T25) at 14 000 rpm with 50 ml of acetonitrile/water (21/4) for 5 min and filtered through Whatman no. 4. The filtrate was defatted with hexane and then concentrated to dryness under vacuum at 40°C. The residue was redissolved in 3·0 ml in chloroform/methanol (9/1) prior to cleanup procedure.

b) *Fumonisins.* Samples were finely ground in a laboratory mill and thoroughly mixed. Aliquots (10 g) of the samples were blended with a high speed blender (Ultraturrax T25) at 14 000 rpm with 50 ml of acetonitrile/water (1/1) for 5 min and filtered through Whatman no. 4.

Cleanup

a) *Trichothecenes.* A 3-ml capacity Florisil Sep-Pak column (Waters Chromatography Division, Millipore Corp., Milford, Massachusetts, USA) was preconditioned with 5 ml of methanol followed by 5 ml of chloroform/methanol (9/1). One and half milliliters of the sample extract were passed through the column. The trichothecenes were eluted from the column with 20 ml chloroform/methanol (9/1) and then concentrated to dryness under vacuum at 40°C. The residue was transferred to a 2 ml screw cap ambar vial with 2 × 0·5 ml of dichloromethane and then sample was evaporated to dryness under slow stream of nitrogen.

b) *Fumonisins.* A 3-ml capacity C_{18} Sep-Pak column (Waters Chromatography Division) was preconditioned with 5 ml of methanol followed by 5 ml of 1% aqueous KCl. Two milliliters of the sample filtrate was added to 5 ml of 1% aqueous KCl and passed through the column. The column was washed with 3 ml of 1% aqueous KCl, followed by 2 ml of acetonitrile/1% aqueous KCl (1/4). Fumonisins were eluted from the column with 2 ml of methanol/water (7/3).

Derivatization conditions

a) *Trichothecenes.* All glassware were rinsed with toluene before use. To each vial containing dry samples, 10 µl of DMAP solution was added, followed by addition of 10 µl of coumarin-3-carbonyl chloride. The vial was closed and the

mixture incubated at 80°C for 20 min. The mixture was then cooled in ice water and then purification procedure followed. The cooled reaction mixture was redissolved in 0·4 ml of toluene and 0·4 ml of 0·05 M dihydrogen phosphate buffer pH 5·0 were added. After vigorous mixing, phases were allowed to separate. Three hundred microlitres of the upper organic phase were transferred to another vial and evaporated to dryness under slow stream of nitrogen. To the residue, 50 μl of HPLC mobile phase acetonitrile/water (65/35) containing 0·75% acetic acid was added, the solution filtered through a 0·20 μm filter and 20 μl injected into the HPLC system.

b) *Fumonisins.* An aliquot of the sample (50 μl) was derivatizated with 200 μl of OPA reagent and injected into HPLC.

HPLC analysis

The trichothecene derivates (20 μl of solution) were analysed using a reverse phase HPLC/fluorescence detector system. The HPLC system consisted of a Waters 600 pump connected to a Waters 474 Scanning Fluorescence detector and a Digital Celebris 590 PC (Millenium Software was used). Chromatographic separations were performed on a stainless steel LiChrospher® 100, C-18 reverse phase column (250 mm × 4 mm, 5 μm particle size) connected to a LiChrospher® 100 C-18 reverse phase precolumn cartridge (4 × 4 mm, 5 μm particle size). The LC column was kept at room temperature with a flow rate of 1·0 ml min^{-1}. For trichotecene determination, the mobile phase was acetonitrile/water (65/35) containing 0·75% acetic acid. The fluorescence detector had the excitation and emission wavelengths set at 292 nm and 425 nm, respectively. For fumonisins analysis mobile phases were (A) methanol/0·05 M sodium dihydrogen phosphate adjusted to pH 5·0 with 2 M NaOH (1/1) and (B) acetonitrile/water (4/1). The gradient program was 100% A during 5 min followed by a rapid increase to 50% A + 50% B with a final hold of 15 min. The fluorescence detector had the excitation and emission wavelengths set at 335 nm and 440 nm, respectively, with a slit width of 18 nm.

Statistical analysis

Statistical analysis of data was performed with the computer program Statgraphics Plus for Windows 2.1. Cluster analysis was performed using the nearest method and the euclidean distance.

Results

a) *Trichothecenes*

Figure 1 shows a typical chromatogram obtained as the result of analysing type A trichothecenes (T-2 toxin, HT-2 toxin, NEO, DAS) produced by *F. sporotrichioides* strain FSp9 in tiger nut of the amella variety. As can be seen there is a good separation among four peaks corresponding to the above mycotoxins.

Table 2 shows the production of the type A trichothecenes mentioned above by the strains of *F. sporotrichioides* used in this work in the two varieties of tiger nut under consideration. As can be seen, the production of the HT-2 toxin is, in all the cases, very small. The strain FSp11 produces a small amount of type A trichothecenes in either of the two varieties of tiger nut, with no difference observed between them; in the remaining cases there is a greater production of trichothecenes in the nigerian variety than in the amella. For the strain FSp12, this higher production is only observed in the case of DAS but for the strain FSp14, the production of this toxin is similar in both varieties of tiger nut.

Comparison of the biosynthesis of the above toxins in tiger nut and in corn, rice or wheat (Table 2) shows that in most cases, the formation of T-2 toxin and HT-2 toxin is much lower in tiger nut than in the other substrates tested; particularly noteworthy is the quantity of T-2 toxin produced in rice when it is inoculated with the strain FSp11, whereas the biosynthesis of HT-2 toxin is more homogeneous in rice, corn and wheat. However, observation of the production of NEO or DAS shows that the biosynthesis of both in tiger nut is comparable to that which occurs in the other substrates studied; nevertheless, there are samples where high quantities of DAS (specially in the samples inoculated with the strains FSp12 and

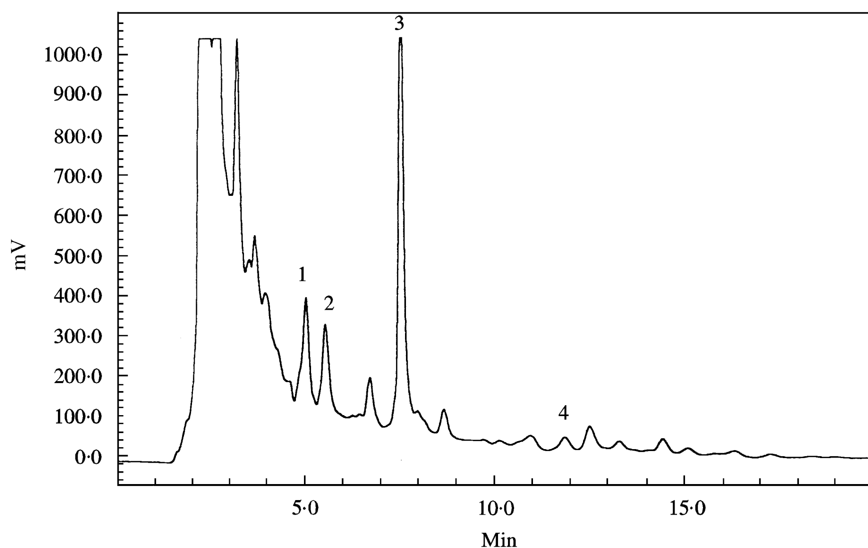

Figure 1. Typical chromatogram obtained as the result of analysing type A trichothecenes produced by *F. sporotrichioides* strain FSp9 in tiger nut of the amella variety. (1 = NEO, 2 = DAS, 3 = T-2 toxin, 4 = HT-2 toxin).

FSp13) or NEO (in rice inoculated with strain FSp13) are produced.

b) *Fumonisins*

Figure 3 shows a representative chromatogram obtained as a result of the analysis of fumonisins produced by *F. moniliforme* strain FM25 in the nigerian variety of tiger nut. FB2 was not detected in any of the samples of tiger nut studied, but was present in the extracts obtained from other substrates, suggesting that the composition of the substrate may affect the transformation of FB1 into FB2.

Table 2 shows the quantities of FB1 produced by the strains of *F. moniliforme* in the two varieties of tiger nut. It can be seen that, as happened with the type A trichothecenes, the formation of FB1 is greater in the variety of tiger nut imported from Africa, reaching maximum amounts of around 40 mg kg^{-1}. It should also be noted that in the amella variety, the production of FB1 is independent of the strain with which it is inoculated and is around 10 mg kg^{-1}. Thus, it appears that in the case of this last variety of tiger nut, its chemical composition determines the formation of fumonisins but in the nigerian variety, the strain of *F. moniliforme* plays an important role in determining the amount of FB1 which is produced.

Comparison of the biosynthesis of FB1 in the tiger nut with that which occurs in corn, rice or wheat shows that in all cases, the greatest synthesis of FB1 occurs in corn. However, in contrast to what happens with type A trichothecenes, the amounts of FB1 produced in tiger nut are comparable to those obtained for the other substrates and were similar to the amount determined for corn when the African tiger nut was inoculated with strain CECT 2982. It should be noted that for the three strains of *F. moniliforme* used, the greatest synthesis of FB1 occurs in corn, although smaller amounts are obtained by inoculation with strain CECT 2982. In the samples of rice inoculated with this strain, very small amounts of this toxin are obtained, but for the other strains, intermediate amounts of FB1 are obtained in this substrate. The same graduation for the production of fumonisins occurs in the tiger nut which suggests that the capacity to produce these mycotoxins is genetically regulated in the different strains tested.

Table 2. Production of type A trichothecenes and fumonisins (mg kg^{-1} dry and ground substrate) by the different *Fusarium* strains and substrates used

		Trichothecenes				Fumonisins	
Strain	Substrate	T-2	HT-2	DAS	NEO	FB1	FB2
FSp9	Rice	0·290	0·106	tr	0·030	–	–
	Corn	0·671	0·089	0·059	0·063	–	–
	Wheat	0·491	0·067	tr	0·040	–	–
	Tiger nut 'amella'	0·041	ND	0·019	0·019	–	–
	Tiger nut 'nigerian'	0·059	0·044	0·029	0·028	–	–
FSp11	Rice	2·780	0·096	0·033	tr	–	–
	Corn	0·035	0·043	ND	tr	–	–
	Wheat	0·282	0·040	tr	0·040	–	–
	Tiger nut 'amella'	0·027	0·02	0·024	0·024	–	–
	Tiger nut 'nigerian'	0·025	ND	0·023	0·021	–	–
FSp12	Rice	0·062	0·089	0·220	tr	–	–
	Corn	0·246	0·064	tr	tr	–	–
	Wheat	0·242	0·050	0·118	0·173	–	–
	Tiger nut 'amella'	0·021	ND	0·019	0·022	–	–
	Tiger nut 'nigerian'	0·022	ND	0·113	0·024	–	–
FSp13	Rice	0·062	0·094	0·857	0·264	–	–
	Corn	tr	0·125	0·175	0·045	–	–
	Wheat	0·099	0·039	0·143	0·050	–	–
	Tiger nut 'amella'	0·03	ND	0·079	0·029	–	–
	Tiger nut 'nigerian'	0·087	0·022	0·108	0·087	–	–
FSp14	Rice	0·067	ND	tr	tr	–	–
	Corn	0·039	0·058	0·030	tr	–	–
	Wheat	tr	0·037	0·041	tr	–	–
	Tiger nut 'amella'	0·057	ND	0·025	0·016	–	–
	Tiger nut 'nigerian'	0·071	ND	0·024	0·066	–	–
CECT 2982	Rice	–	–	–	–	0·421	tr
	Corn	–	–	–	–	37·18	4·532
	Wheat	–	–	–	–	4·516	2·496
	Tiger nut 'amella'	–	–	–	–	11·75	ND
	Tiger nut 'nigerian'	–	–	–	–	21·958	ND
FM25	Rice	–	–	–	–	28·01	11·82
	Corn	–	–	–	–	83·7	13·63
	Wheat	–	–	–	–	8·85	6·17
	Tiger nut 'amella'	–	–	–	–	10·8	ND
	Tiger nut 'nigerian'	–	–	–	–	39·24	ND
FM37	Rice	–	–	–	–	39·78	8·42
	Corn	–	–	–	–	89·19	7·61
	Wheat	–	–	–	–	0·691	0·211
	Tiger nut 'amella'	–	–	–	–	8·36	ND
	Tiger nut 'nigerian'	–	–	–	–	29·19	ND

ND: Not detected.
tr: Trace.
–: Experiment not carried out.

In the cluster analysis of the data obtained (Fig. 4), it can be seen that the samples of tiger nut associate at a small distance, forming two groups according to the variety used; however, these groups are not observed for the other substrates. Thus, it seems clear that the chemical composition of the tiger nut makes the synthesis of fumonisins characteristic of the substrate and different from that which occurs in other substrates tested. Furthermore, the composition of the two varieties of tiger nut is sufficiently different to make the fumonisin production characteristic of each.

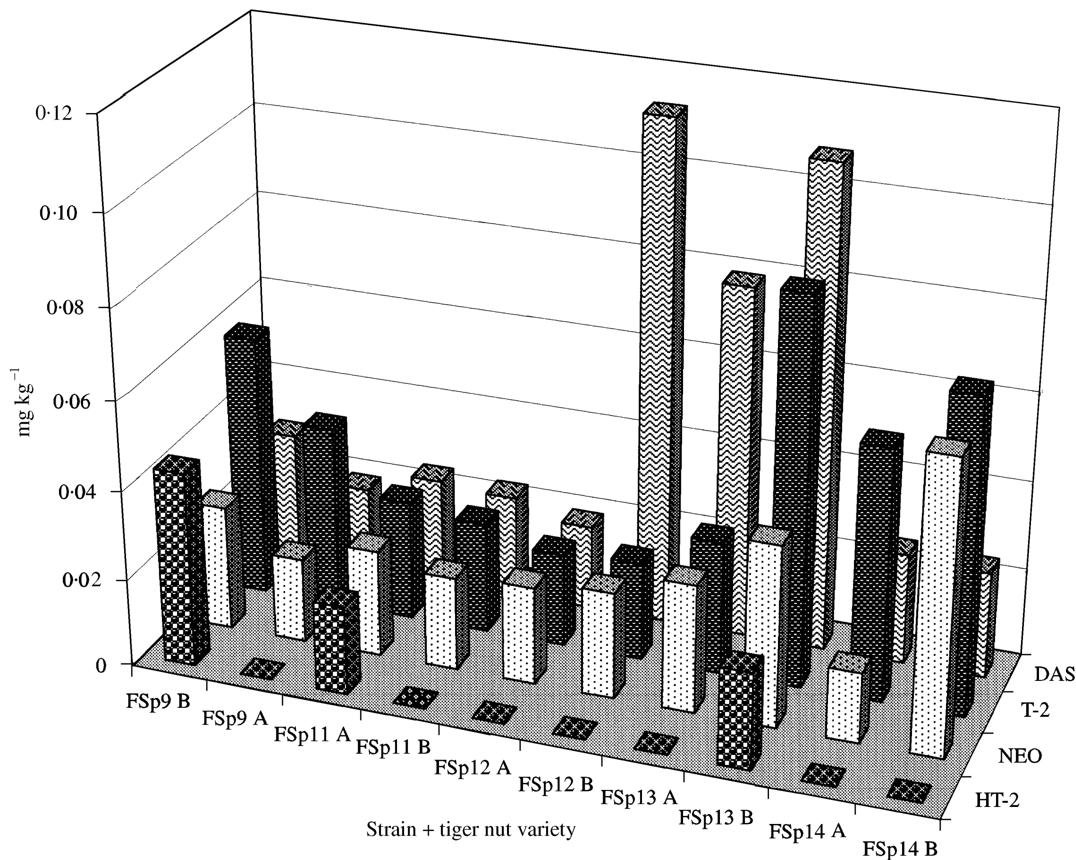

Figure 2. Production of the type A trichothecenes in tiger nut variety by the strains of *F. sporotrichioides*. (A = amella variety, B = nigerian variety).

Discussion

Fusarium sporotrichioides strains have been found to occur naturally in infected grains and feed implicates in human and animal toxicoses (Joffe, 1986). These strains have shown their ability to produce type A trichothecenes in various substrates such as barley, corn, strawberry, wheat or rice (Logrieco et al. 1990, Jimenez and Mateo, 1997).

Many strains of *F. moniliforme*, isolated not only from corn and feeds, but also from other substrates such as sorghum and millet, have been shown to produce fumonisins (Gelderblom et al. 1988, Nelson et al. 1991, Thiel et al. 1991, Sydenham et al. 1992). The commodities in which fumonisins have been mainly detected are corn and corn-based products (Plattner et al. 1990, Bacon and Nelson 1994, Julian et al. 1995, Chulze et al. 1996), but these mycotoxins have been also detected in other substrates, such as rice (Meredith et al. 1996), sorghum (Shetty and Bhat, 1997), milk (Maragos and Richard 1994), wheat (Luo et al. 1990) or forage grass (Mirocha et al. 1992).

In conclusion, this study is to our knowledge, the first work to show the capacity of fungi from the *Fusarium* species to produce both type A trichothecenes and fumonisins in tiger nuts. Bearing in mind the ambient conditions surrounding this foodstuff both before and after harvest and the processing it undergoes before direct consumption or in the form of refreshing drink (horchata), there is no doubt that there is a significant risk factor in the possible presence of the toxins studied in the substrate. New studies are being carried out in our laboratory to evaluate the presence of these

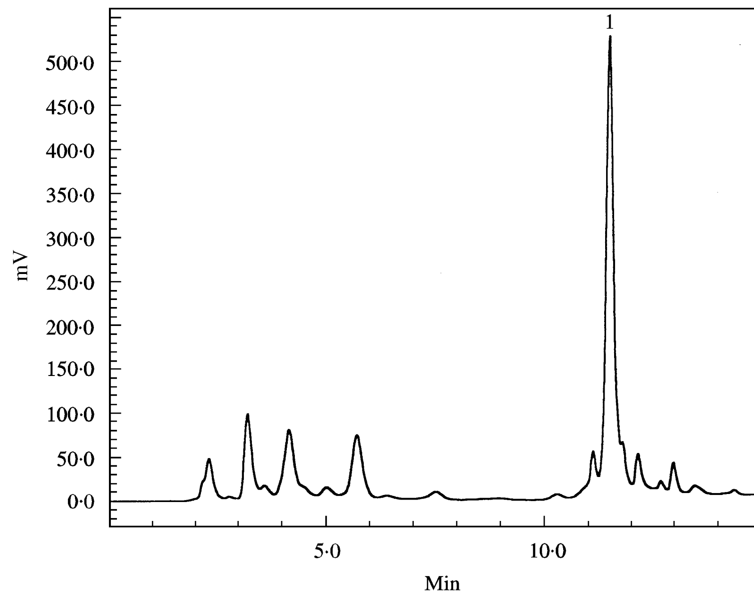

Figure 3. Chromatogram obtained as a result of the analysis of fumonisins produced by *F. moniliforme* strain FM25 in the nigerian variety of tiger nut. (1 = FB1).

toxins in samples naturally contaminated with productive species. We have found that these species of *Fusarium* are frequent in agricultural land and in *Cyperus esculentus*.

It has been shown that the variety imported from Africa is more susceptible to the synthesis of the mycotoxins studied, a point which should be considered, not just yield when deciding to cultivate it. At present only the economic performance of the crop is taken into account due to the lack of research into the mycotoxins in this substrate.

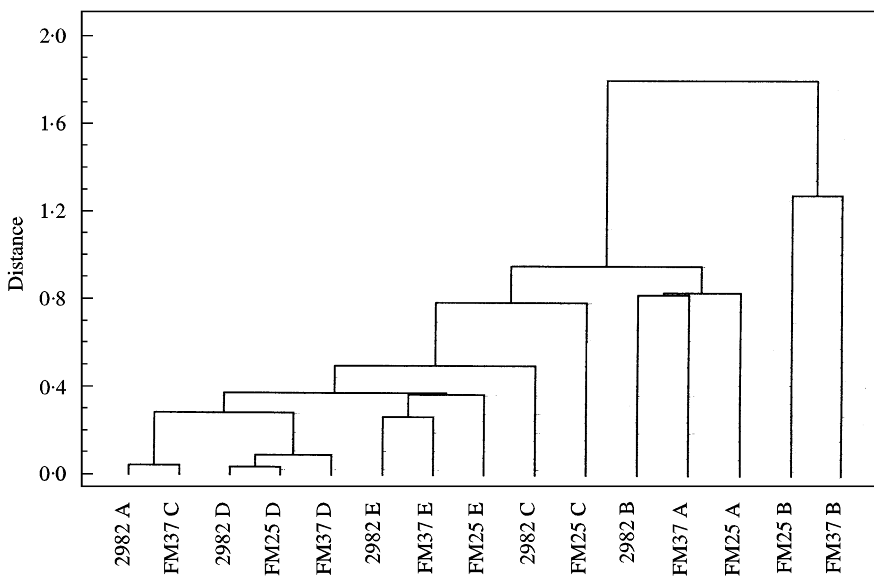

Figure 4. Dendogram obtained as a result of the cluster analysis of the amounts of FB1 produced by the *F. moniliforme* strains in the substrates assaied. (A = Rice, B = Corn, C = Wheat, D = Tiger nut 'amella', E = Tiger nut 'nigerian').

Analysis of the results obtained in this study highlight the need to continue to study other fungal species present in this food and their capacity to biosynthesize toxic metabolites in this substrate. For the present, this work suggests that the capacity of the strains studied here to biosynthesize type A trichothecenes is less in tiger nut than in other substrates, thus reducing the risk factor for consumer health. However, this cannot be generalized to include the other toxins, because in the case of fumonisins, their synthesis is not radically affected by the substrate used.

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