Production and purification of fumonisins from a highly toxigenic *Fusarium verticilloides* strain

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SUMMARY

Fumonisins are the major mycotoxins produced by *Fusarium verticilloides* and *F. proliferatum* fungi which are widely found as contaminants in corn and corn screenings. These molecules are hepatotoxic and nephrotoxic for several species and carcinogenic in rodents. Moreover their consumption was linked to high prevalence of human oesophageal cancer in certain geographic areas. The aim of this work was to improve FB1 production and purification procedures in laboratory conditions in order to produce large quantities of semi-purified toxin that may be used in experimental intoxications of farm animals. We used a highly toxigenic strain of *Fusarium verticilloides* (NRRL-3428) isolated from feeds. Influence of substrate, temperature, water content, culture recipient size and screen analysis of the substrate on fumonisin production was tested. Optimal production was obtained when strain was grown on coarsely cracked corn with 50% water content at 21°C during 5 weeks. This allowed the production of 3 to 4 g of fumonisin B1 per kg of culture material. The composition of the extracts was found to be as follow : 54% FB1, 8% FB2, 9% FB3 and 29% of pigments coming from corn. The ratio observed between FB1 and FB2 is comparable to the one reported in naturally contaminated corn. Further purification of these extracts on SAX columns led to the removal of pigments and to obtain of fumonisins extracts pure enough to be used for intra-venous or intra-peritoneal injection.

Keywords : *Fusarium verticilloides* - Fumonisins - production - purification.

RÉSUMÉ

Production et purification des fumonisines issues d'une souche de *Fusarium verticilloides* fortement toxinogène. Par J.D. BAILLY, A. QUERIN, D. TARDIEU et P. GUERRE.

Les fumonisines sont les principales mycotoxines produites par les *Fusarium verticilloides* et *Fusarium proliferatum,* moisissures largement répandues comme contaminants du maïs. Ces molécules sont hépatotoxiques et néphrotoxiques dans plusieurs espèces et carcinogènes chez les rongeurs de laboratoire. De plus leur consommation a pu être reliée à une forte prévalence de cancers de l'œsophage chez les habitants de certaines régions du monde. L'objectif de ce travail était d'améliorer les conditions de production et de purification de la FB1 au laboratoire afin de produire des quantités importantes de toxines pouvant être utilisées dans le cadre d'intoxications expérimentales d'animaux de rente. Nous avons utilisé une souche fortement toxinogène de *Fusarium verticilloides* (NRRL 3428), isolée d'aliments pour animaux. L'influence sur la production de fumonisines du substrat, de la teneur en eau, du matériel utilisé pour la culture et de la granulométrie du substrat a été testée. Les conditions optimales de production correspondent à une culture réalisée sur du maïs grossièrement broyé, à 50% de teneur en eau, à 21°C pendant 5 semaines. Dans ces conditions, la production atteint 3 à 4 g de fumonisine B1 par kg de matériel de culture. La composition des extraits obtenus est la suivante : 54% de FB1, 8% de FB2, 9% de FB3 et 29% de pigments provenant du maïs. Le rapport entre FB1 et FB2 est comparable à celui qui est observé dans du maïs naturellement contaminé. La purification des extraits par passage sur colonne SAX a permis l'élimination des pigments et l'obtention d'extraits suffisamment purs pour pouvoir être utilisés par voie intraveineuse ou intrapéritonéale.

Mots-clés : *Fusarium verticilloides* - Fumonisines - production - purification.

Introduction

Fumonisins are the major mycotoxins produced by *Fusarium verticilloides* and *F. proliferatum* fungi which are widely found as contaminants in corn and corn screenings [17]. This family of mycotoxins involves 15 different toxins but mainly three (FB1, FB2 and FB3) are found in naturally contaminated feeds [21]. A survey of corn in France revealed that 67% of samples were contaminated by *F. verticilloides,* and that more than 80% of these strains were able to produce high levels of FB1, the most abundant and the most toxic molecule of the family [24]. Very large quantities of fumonisin B1 (up to 100 to 200 mg/kg) could be observed in feeds involved in farm animal intoxication cases [5].

Since its isolation in 1988, hepatic and renal toxicity of FB1 were observed in several species, including horses, pigs, lambs, rats, broilers, turkeys and ducks, whereas encephalomalacia and pulmonary oedema were reported in horses and pigs [46]. In rodents, FB1 was reported to be carcinogenic [16, 18] whereas its consumption was linked to high prevalence of human oesophageal cancer in certain geographic areas [20, 26, 47, 51]. Finally all animal species appear sensitive to the toxin, but its toxicity is different from one to another.

At the molecular level, fumonisins are known to disrupt the metabolism of sphingolipids by inhibiting shinganine and shingosine N-acyltransferase. This leads to the accumulation of free sphinganine and to the modification of the sphinganine to sphingosine ratio within tissues and serum of exposed animals [41]. Both sphinganine and sphingosine are active second messengers that may lead to cell death or proliferation, depending on the model used [22, 25]. The sensitivity of cells to free sphinganine may be involved in carcinogenesis since the development of tumours could be related to anarchic cell multiplication after exposure to the toxin [19].

If the toxicity of fumonisins is now well characterised in rodents, little is known on deleterious effects in farm animals. Moreover, the kinetics of the toxins is not often investigated as well as its persistence at the residual level. One of

the main reasons for this is the cost of purified FB1. Thus, in order to clarify consequences of animal exposure during breeding it is necessary to produce large quantities of semi-purified toxin.

The aim of this work was to improve FB1 production and purification procedures in laboratory conditions in order to produce large quantities of semi-purified toxin that may be used in experimental intoxications of farm animals. For this we isolated strains of *Fusarium verticilloides* from feed involved in LEM cases and characterized their toxigenic potential. Fumonisins production was then improved by evaluating influence of substrate, temperature, water content, culture recipient size and screen analysis of the substrate.

Material and methods

SOLVENTS AND REAGENTS

All solvents and reagents used were analytical grade and purchased from Prolabo (Paris, France). FB1, FB2, Fusaric acid, Moniliformin and Fusarin C standards were purchased from Sigma (Saint Louis, MO).

FUNGAL STRAINS

The strain of *Fusarium verticilloides* was isolated from corn by-products feed which had led to two cases of equine leucoencephalomalacia in the region of Toulouse (France) [5]. This feed was shown to contain 125 ppm FB1. Ten grams of this feed were dispersed in 190 ml of 0,5% tween solution using a Waring blendor mixer. One ml of each decimal dilution was plated on both malt agar (2% agar, 2% malt, 50 ppm chloramphenicol) and Potato Dextrose Agar (0,4% potato extract, 2% dextrose, 1.5% agar) media. Pure strain of *Fusarium verticilloides* was isolated after several planting out. This strain identity has been confirmed by the ARS (Agricultural Research Service Culture Collection, Peoria, Illinois), and referenced as strain NRRL-3428. Two referenced strains M-3036 and M-2552, purchased from the *Fusarium* Research Centre (Penn State University, PA 16802, USA) and whose toxigenic potential had already been described [34] were used to compare the toxinegic potential of NRRL 3428 strain.

FUNGAL CULTURE

Fusarium strains were cultivated on potatoes dextrose agar (PDA) medium at 20°C. Each week, cultures were planted out on new Petri dishes to perform mycotoxin production. Long period storage of the strains is done at 4°C in tubes containing PDA medium. Each six months the strains are planted on Petri dishes to control viability and purity.

To assess FB1 production, three pieces of 0.5 cm^2 of 1-week subculture of these strains on PDA were deposed on rice or corn media.

Solid culture media used were prepared as follows : 50 g of rice or corn and various amount of sterile water were added to 15 cm glass Petri dishes and autoclaved 30 min at 121°C before contamination. Water content of the medium was assessed before moulds inoculation, using the French official method [1].

Cultures were then incubated at different temperatures for several times. Each week, cultures were checked for the absence of fungal contamination by macroscopic examination. In case of development of any suspect fungal colony on the substrate, microscopic examination was done. Identification was performed according to Nelson et al [33], Pitt [38], Raper and Fennell [39] and Botton [8]. When contamination was confirmed, culture material was not used for fumonisin production.

FUMONISINS EXTRACTION AND PURIFICATION

FB1 extraction and purification were performed as originally described by Le Bars et al [24]. *Fusarium verticilloides* cultures were extracted with 100 ml of methanol-water (3:1) or 100ml of acetonitrile-water (1:1) by grounding 3 min in a Waring blendor. Extraction yield assessed by spiking blank corn sample with 20 and 200 mg/kg of pure FB1 was 90 ± 3 % whatever the solvent system used. Further purification can be done on methanol-water extract. After filtration on flutted filter n°3 (Fioroni, VWR, Fontenay sous bois, France), 10 ml of the extract were applied to Bond-Elut SAX cartridges (500mg, 2,8 ml) (VWR, Fontenay sous bois, France) and eluted with 14 ml of acidified methanol (0,5% acetic acid). These extracts were evaporated to dryness under a gentle stream of nitrogen and dissolved in methanol. Each extract was diluted or concentrated to be within the range of the calibration curve. Yield of the purification procedure has been calculated by using extracts containing known amounts of pure FB1 (20 and 200 mg/kg) and was found to be 96 ± 2%.

FUMONISINS QUANTIFICATION

Fumonisins were quantified by both thin layer chromatography [14] and HPLC [3].

Filtered extracts before or after purification (2µl) and standards were spotted on thin-layer chromatographic plates (Merck n°5553, VWR, Fontenay sous bois, France). Separation was carried out in 1-butanol-acetic acid-water (20+10+10, v/v/v). After a final drying step, plates were sprayed with a mixture containing methanol, p-anisaldehyde, acetic acid and sulfuric acid (85-0.5-10.5 vol) and heated 10 min at 110°C.

Quantification was performed by using a spectrophotodensitometer (Shimadzu CS930, Shimadzu Corp., Kyoto, Japan) set at 600 nm. The system was coupled to a personal computer data processing system. Linear response was obtained over a range of 50-800 ng FB1. For each plate, 9 extracts were spotted, after dilution if necessary, along with 4 different standard amounts of FB1 (50, 100, 200 and 400 ng). The detection limits in culture material were 30-50 µg/g of initial dry corn when crude extracts were spotted, and 3-5 µg/g if extracts were purified.

For HPLC quantification, 25 µl of the extracts or 25 µl of standard were derivatized with a mixture of 25 µl of borate buffer (pH 8.3), 25 µl of water and 25 µl of O-phtaldialde-

hyde (15 mM) and separated by HPLC using an M2200 (ICS, Toulouse, France) pump, a Prontosil C18, 5 µm, 250x4mm column equipped with a pre-column (ICS, Toulouse, France), and a 8450 fluorescence HPLC-monitor (Shimadzu, Kyoto, Japan). The operating conditions were: liquid phase : NaH_2PO_4 (0.1M pH 3.3)/ Methanol 25/75 (v/v); flow rate : 1 ml/min; injection volume : 10 µl ; Ex 335 nm and Em 440 nm. Quantification was done by measuring peak area with Pic3 data system from ICS (Toulouse, France) and comparing with standard calibration curve. The mean retention time was of 7,5 min and 17,5 min for FB1 and FB2 respectively. Limits of quantification are 0.1 and 0.2 µg/g for FB1 and FB2 respectively [40].

OTHER FUSARIOTOXINS QUANTIFICATION

The extract was tested for the absence of other fusariotoxins.

Fusaric acid was tested as described by Burmeister et al [9]. 5 µl of extract were spotted on TLC plates (Merck n°5553, VWR, Fontenay sous bois, France) that were developed in isopropyl alcohol-ethyl acetate-water-acetic acid (4 :3.8 :2 :0.2). Compounds were then identified by spraying 50% sulphuric-acid followed by charring at 130°C. The detection limit of the method is about 20 µg/g

Moniliformin was tested as described by Desjardin et al [12]. 3 µl of purified extracts were spotted on TLC plates with fluorescence indicator (Merck F_{254} n°5554, VWR, Fontenay sous bois, France). These plates were developed in acetonitrile-water (58 :15) and moniliformin was visualised by quenching of the indicator when observed under short-wave UV light (254 nm). The detection limit of the method is about 10 µg/g.

Fusarin C was tested as described by Cantalejo et al [10]. 3 µl of extracts and standards were spotted on TLC plates (Merck n°5553, VWR, Fontenay sous bois, France). They were developped in diethyl ether-ethyl acetate-methanol (5 :5 :2). Fusarin C was visible as yellow spots under light at 366nm. The detection limit of the method is about 10 µg/g.

Beauvericin was not tested because this metabolite is mainly produced by *Fusarium proliferatum* and *F. subglutinans* and not by *F. verticilloides* and, in all cases, the levels of beauvericin obtained are extremely low compared to FB1 and not able to induce any effect in animals [7, 15].

ERGOSTEROL DETERMINATION

Extraction of ergosterol was performed using the method of Schwardorf and Müller [43] with slight modifications according to AFNOR norm NFV 18-112 [2]. 45 ml methanol, 15 ml ethanol, 6 g KOH and 60 µl pyrogallol (10% in methanol) were added to 15 grams of samples. The mixture was refluxed for 30 min at 80°C, cooled down to 20°C and filtered through fluted paper. Fifteen ml of the saponified mixture was then extracted twice with 30 ml of petroleum ether. This extract was washed twice with acid water (adjusted to pH 2.5 with sulphuric acid), and 5 ml were evaporated to dryness using water bath (60°C) and gentle stream of nitrogen. The residue was then dissolved in appropriate volume of the same solvent as for ergosterol standard solutions (250-500 µl).

Quantification was then performed by fluorodensitometry as previously described [6].

Results and discussion

ISOLATION AND IDENTIFICATION OF THE FUNGAL STRAIN

A single pure strain of *Fusarium* was isolated from feed involved in LEM case [5]. It showed both macroscopic and microscopic characteristics of a *Fusarium verticilloides* strain, mainly abundant microconidia forming long conidial chains and a white aerial mycelium, growing rapidly and becoming tinged with purple after few days of culture on PDA medium [33]. This isolates was addressed to the ARS culture collection for further identification. Sequencing part of the EF-1 alpha gene allowed the confirmation of the identity of this isolate as *Fusarium verticilloides* [36]. This strain is now referenced by the ARS culture collection under the number NRRL 3428

PRODUCTION OF FUMONISINS

Purity of the culture

After 5 weeks of cultures, Petri dishes were macroscopically checked for the absence of fungal contamination and in case of any doubt, microscopic examination was performed. All dishes in which another fungal strain developed were thrown away (figure 3). Indeed, the development of *Penicillium* or *Aspergillus* species on culture medium may lead to synthesis of other mycotoxins that could contaminate the extracts. When contamination was due to the development of species not known to have any toxigenic potential, culture material was also thrown away. Indeed, it has been well established that the development of several fungal species on a substrate lead to the diminution of the production of mycotoxins by toxigenic species [30].

Influence of time

Figure 1 shows the time-dependence production of fumonisins by Fusarium NRRL 3428 strain. Whatever the culture conditions tested, production curve was biphasic with an increase in the amount of produced fumonisins during the former 4 to 5 weeks and then a decrease in the amount of the produced toxins. This production curve is in agreement with previously reported ones [4, 24].

The decrease in fumonisins contents after several weeks of culture may be linked to both the diminution of metabolic precursors and enzymatic cleavage of the toxins within the culture medium [24].

Influence of the substrate

Both rice and corn were tested for fumonisins production. Figure 1A shows that higher FB1 levels were obtained after incubation on maize whereas no significant differences on mycelium growth were observed according to ergosterol quantification. This result is in agreement with a previous

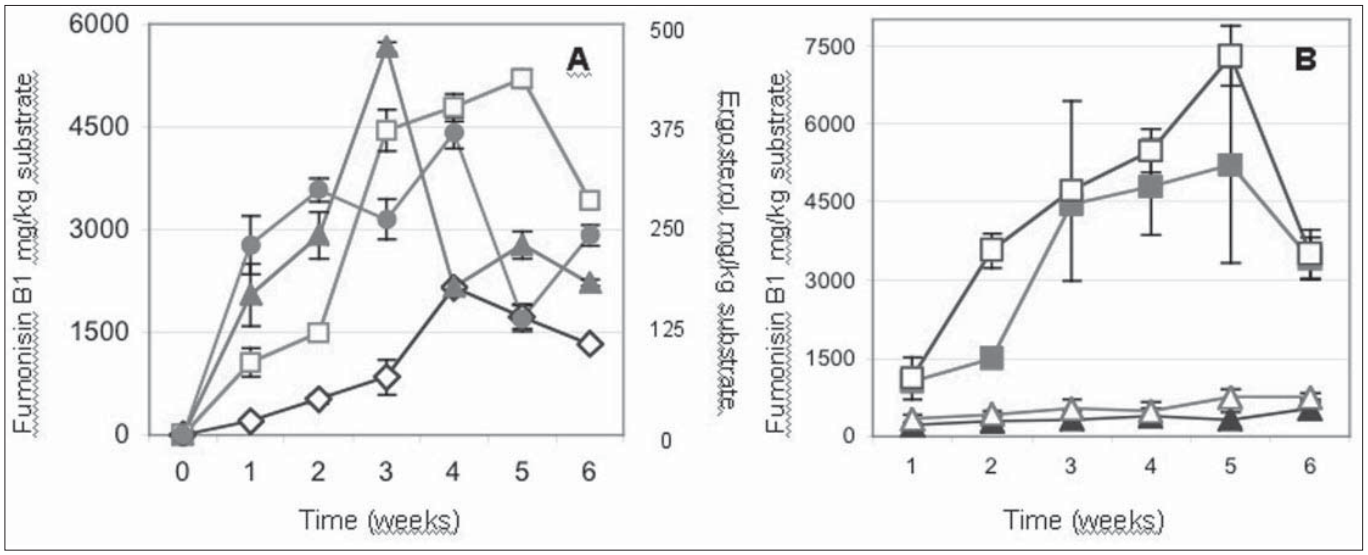


FIGURE 1. — **A :** comparison of growth measured by ergosterol quantification (bold symbols) and FB1 production (light symbols) by NRRL 3428 strain cultivated on rice (◆) or intact corn grains (■). Results are given as the mean (+/- se) of three experiments. **B :** Fumonisin B1 production by NRRL 3428 strain grown on intact grains (■), coarsely cracked corn (□), corn flour (▲) or polenta (△). Results are given as the mean (+/- se) of three experiments.

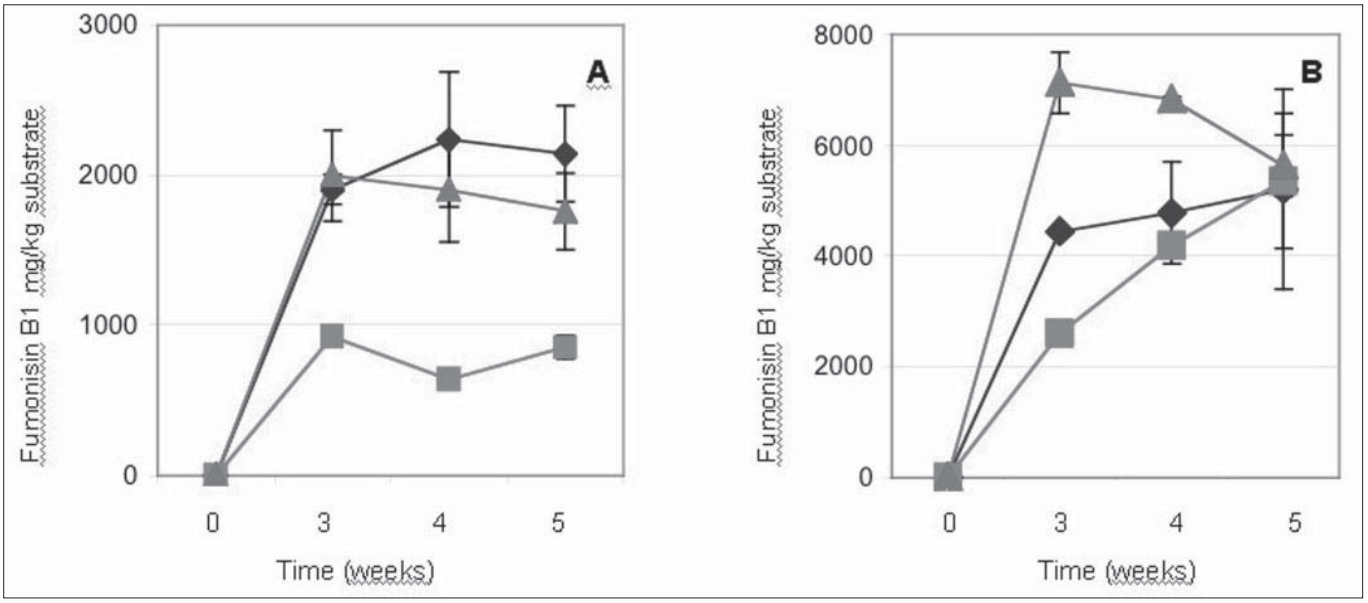


FIGURE 2. — comparison of FB1 production by NRRL 3428 strain and reference strains M-2552 and M-3036 in various culture conditions : **A :** 50g of intact corn grains, 40% water content, 300 ml Erlenmeyer flasks, 25°C, darkness. **B :** 50 coarsely cracked maize, 50% water content, 15 cm Petri dishes, 21°C, darkness. The results are expressed as mean +/- sd of three experiments. -◆-: NRRL 3428; -■-: M 2552; -▲-: M3036.

study that compared several culture substrates for fumonisins production [29] even if rice is often use as culture medium for mycotoxin production at the laboratory scale [44, 48, 50]. For further experiment maize was used as substrate for *Fusarium* growth.

To test substrate screen analysis influence on fumonisin B1 production, corn was left in intact grains, coarsely cracked or reduced into flour. Figure 1B shows that FB1 production was higher when *Fusarium* was cultured on cracked corn than on intact grains whereas corn flour allowed only low level of toxin production. This result can be explained by the fact that the cracking of corn grains made easier the access of the fungus to nutriments. However, when corn flour is used, production is dramatically reduced. In this case, another limiting parameter may be involved. Indeed, with corn flour or polenta, the culture media become too dense to allow an adequate breathing of the fungal strains. It has been well demonstrated that the presence of oxygen is an important parameter for mycotoxins production [23, 49].

Influence of physico-chemical parameters

Both temperature and water content of the substrate are factors that are of particular importance for the growth and the mycotoxinogenesis of *Fusarium* species that are mainly mesophilic and hygrophilic fungal strains usually developing on living plants, acting as parasites [27]. Table I shows influence of temperature and water content respectively on

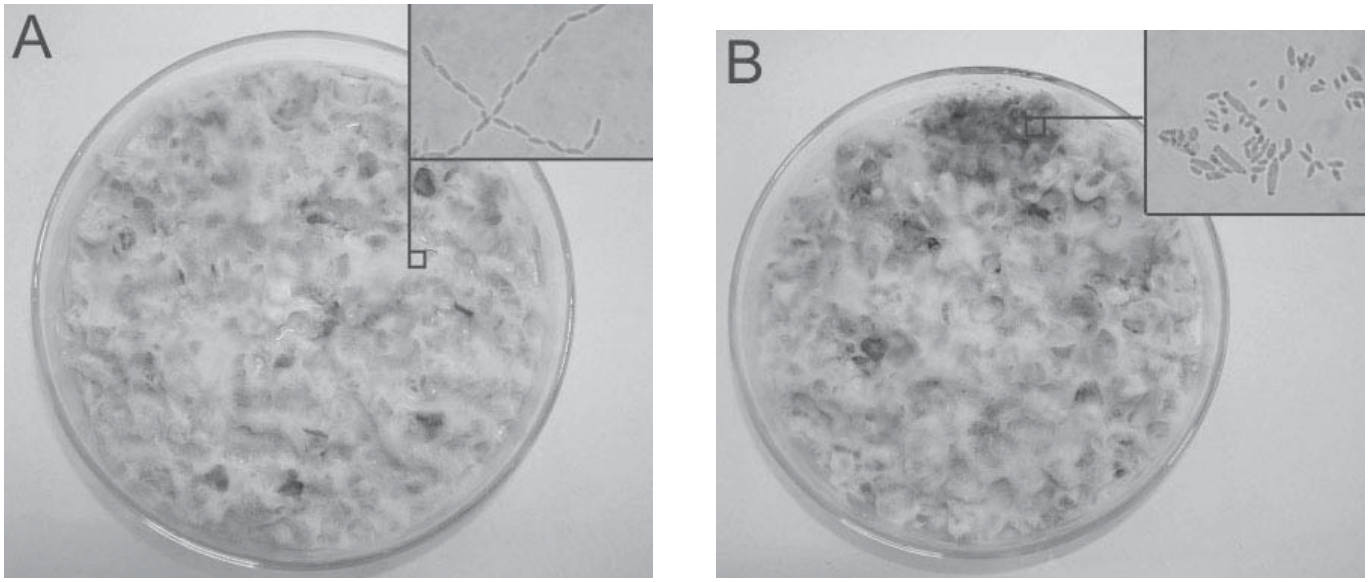
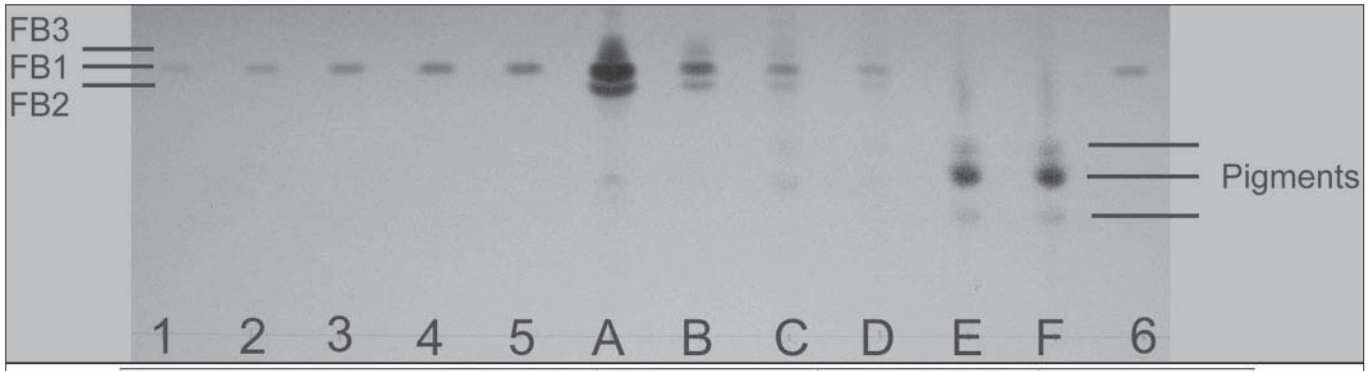


FIGURE 3. — macroscopic and microscopic aspect of cultures after 5 weeks of incubation at 21°C. **A :** pure *Fusarium verticilloides* culture. **B :** Petri dishes contaminated with *Cladosporium herbarum.*



	FB1 (µg/ml)	FB2 (µg/ml)	FB3 (µg/ml)
Crude extract before purification	270	61	28
Purified extract	150	26	15

FIGURE 4. — thin layer chromatography plate after separation with butanol-acetic acid-water (20+10+10, v/v/v) and revelation by spraying 0.5% p-anisaldehyde and heating 10 min at 110°C. lanes 1 to 5 and 6 correspond to FB1 standards ranging from 50 (line 1) to 800 ng (line 5) spotted on the plate. Lane A : crude extract before purification, lane B : extract after purification, lane C : crude extract diluted 10 folds, lane D : purified extract diluted 10 folds, lanes E and F : corn extracts without fumonisins. Results of the quantification by fluorodensitometry are given in the table.

Water content	**34%**	**40%**	**50%**
Corn flour	<1	<1	6
Coarsely cracked corn	85	89	**100**
Intact grains	11	64	82

Temperature	**17°C**	**21°C**	**25°C**
Week 2	25	49	15
Week 3	63	64,5	33
Week 4	70	75	50
Week 5	83	**100**	59
Week 6	88	48	48

TABLE I. — influence of water content and temperature on FB1 production by NRRL 3428 strain. Results are expressed as the percentage of the value obtained in the best culture conditions (7306 mg/kg of culture material obtained by using coarsely cracked corn, 50% water content and incubated at 21°C during 5 weeks).

FB1 production by NRRL 3428 strain. Toxin production was higher when fungal strain was incubated at 21°C than at 17 or 25°C. A water content of 50% allowed higher levels of toxin accumulation in all tested substrates. These results are in agreement with data obtained in other studies [29, 42].

FB1 production optimal conditions taken off for the end of the study were : 5 weeks at 21°C on sterile coarsely cracked maize with water content of 50%.

Comparison with other strains under various culture conditions

To determine the toxigenic potential of the strain we isolated, we tested by comparison with reference strains in two culture conditions.

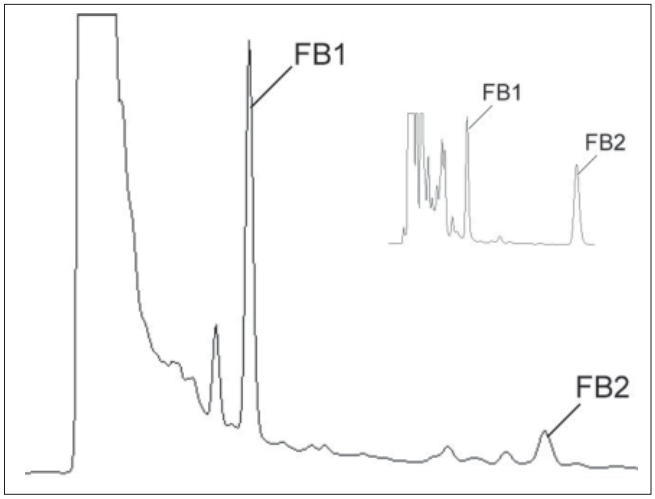


FIGURE 5. — quantification of FB1 and FB2 in purified extracts by HPLC according to AFNOR norm NF EN 13585. One representative chromatogram is shown.

Results are reported on figure 2. In the two culture conditions that were tested, after 5 weeks, levels of fumonisin B1 production by the three used strains were comparable, even if the kinetics of FB1 production was different depending on the strain. However, for the three isolates, levels of production were approximately 2.5 folds higher when strains were grown on coarsely cracked corn at 50% humidity in Petri dishes rather than on intact grains at 40% humidity in 300 ml Erlenmeyer flasks. Moreover, the two tested reference strains produced higher toxin level when growth in our condition rather than those used for their characterization [34].

EXTRACTION AND PURIFICATION OF FUMONISINS

Extraction procedure

Extraction of fumonisins can be performed with both acetonitrile-water (1:1) or methanol-water (3:1) [40, 45]. The major interest of using acetonitrile-water for extraction is the lower toxicity of these solvents compared to methanol. This extraction mixture was used when the extracts were used for oral administration to animals without further purification of fumonisins. When the extracts were devoted to intra-peritoneal administration and therefore needed purification, methanol-water was used for fumonisins extraction.

In order to evaluate the extraction yield of the procedure, four successive extractions were done for each extract. The quantification of the fumonisins content of each filtrate gave the following results : 5475 mg/kg for the first extraction, 2322 mg/kg for the second one, 414 mg/kg for the third one and fumonisins became undetectable by TLC in the fourth extraction filtrate. This assay allowed the determination of an extraction yield about 66% for the first extraction. That is lower that the yield determined with blank corn samples spiked with 20 and 200 mg/kg of pure FB1 that was found to be around 90%. This could be explained by the large quantities of toxins present in the culture material. The extraction yield could have been improved by increasing the solvent volume but this would have led to a diminution of fumonisins concentration in the final extract.

After filtration, the extracts presented a dark brown colour corresponding to pigments that were co-extracted with toxins. Separation of these compounds by thin layer chromatography and quantification by fluorodensitometry showed that composition of these extracts was the following : 54% of FB1 ; 8% of FB2 and 9% of a molecule with a Rf probably corresponding to FB3 (figure 4). The concentration of FB1 and FB2 was confirmed by HPLC. The absence of commercial standard available for FB3 did not allow the exact quantification of this fumonisin. The ratio observed between FB1 and FB2 synthesis by our strain is comparable with those already reported with isolated strains on culture medium [11, 31, 32] and in naturally contaminated corn from various geographic origin [13, 21, 35, 37]. Twenty nine % of the extracts were constituted with pigments mainly coming from corn and that are also found in control culture prepared without *Fusarium* development (figure 4).

Other known fusariotoxins contents (fusarin C, moniliformin and fusaric acid) were below detection limits of the TLC method used (data not shown).

Purification of the extracts

Purification of the extracts by application Bond-Elut SAX cartridges allowed the removal of pigments that became no more detectable (figure 4 and figure 5). The ratio between the three produced fumonisins was unchanged by purification. However, the yield of the purification procedure was only 55% (figure 4). This can probably be linked to the overload of the column by toxins and pigments. Indeed this purification material is usually devoted to purification of naturally contaminated samples with low amounts of toxins. The improvement of the purification procedure may lead to the development of more adequate columns.

Conclusion

The characterization of the impact of chronic exposure to fumonisins in farm animals is usually made difficult by the cost of the purified toxins. The improvement of culture conditions of a highly toxigenic strain of *Fusarium verticilloides* allowed the production of an average of 3 to 4 g of fumonisins per kg of culture material. The extracts contained 71% of fumonisins and corn pigments that may be used for oral administration to farm animals. The further purification of these extracts on SAX columns allowed removal of co-extracted pigments and extracts with a sufficient purity to be used by intra-peritoneal or intra venous routes. In all cases, the toxicity of these extracts may be closely related to the one of naturally contaminated corn since the FB1/FB2 ratio is comparable.

References

1. — AFNOR NF-V-04-401 : determination de l'humidité des aliments. AFNOR éditeur, Paris, 1968.
2. — AFNOR NF-V-18-112 : Aliment des animaux : determination de la teneur en ergostérol, AFNOR éditeur, Paris,1983.
3. — AFNOR NF EN 13585 : dosage des fumonisines B1 et B2 dans le maïs. AFNOR éditeur, Paris, 2002.
4. — ALBERTS J.F., GELDERBLOM W.C.A., THIEL P.G., MARASAS W.F.O., VAN SCHALKWYK D.J., BEHREND Y. : Effects of tem-

perature and incubation period on production of fumonisin B1 by *Fusarium moniliforme. Appl. Environ. Microbiol.,* 1990, **56,** 1729-1733.

5. — BAILLY J.D., RAYMOND I., LE BARS P., GUYOMARD Y., ABADIE J., LE BARS J., GUERRE P., DELVERDIER M., BURGAT V. : Leucoencephalomalacie des équidés : cas rapportés au CNITV, *Rev. Med. Vet.,* 1996, **147,** 787-796.
6. — BAILLY J.D., LE BARS P., PIETRI A., BENARD G., LE BARS J. : Evaluation of a fluorodensitometric method for analysis of ergosterol as a fungal marker in compound feeds. *J. Food Protect.,* 1999, **62,** 686-690.
7. — BOTTALICO A., LOGRIECO A., RITIENI A., MORETTI A., RANDAZZO G., CORDA P. : Beauvericin and fumonisin B1 in preharvest Fusarium moniliforme maize ear rot in Sardinia. *Food Addit. Contam.,* 1995, **12,** 599-607.
8. — BOTTON B. : Moisissures utiles et nuisibles : importance industrielle, 2ème ed., Masson editeur, Paris, 1990.
9. — BURMEISTER H.R., GROVE M.D., PETERSON R.E., WEISLEDER D., PLATTNER R.D. : Isolation and characterization of two new fusaric acid analogs from *Fusarium moniliforme* NRRL 13 163. *Appl. Environ. Microbiol.,* 1985, **50,** 311-314.
10. — CANTALEJO M.J., TORONDEL P., AMATE L., CARRASCO J.M., HERNANDEZ E. : Detection of fusarin C and trichothecenes in *Fusarium* strains from spain. *J. Basic Microbiol.,* 1999, **39,** 143-153.
11. — CASTELLA G., BRAGULAT M.R., CABANES F.J. : Fumonisin production by Fusarium species isolated from cereals and feeds in spain. *J. Food Protect.,* 1999, **62,** 811-813.
12. — DESJARDINS A.E., PLATTNER R.D., NELSON P.E. : Production of fumonisin B1 and moniliformin by *Gibberella fujikoroi* from rice from various geographic areas. *Appl. Environ. Microbiol.,* 1997, **63,** 1838-1842.
13. — DOMIJAN A.M., PERAICA M., JURJEVIC Z., IVIC D., CVJETKOVIC B. : Fumonisin B1, fumonisin B2, zearalenone ad ochratoxin A contamination of maize in Croatia. *Food Addit. Contam.,* 2005, **22,** 677-680.
14. — DUPUY J., LE BARS P., BOUDRA H., LE BARS J. : Thermostability of fumonisin B1, a mycotoxin from *Fusarium moniliforme,* in corn. *Appl. Environ. Microbiol.,* 1993, **59,** 2864-2867.
15. — FOTSO J., LESLIE J.F., SMITH J.S. : Production of beauvericin, moniliformin, fusaproliferin and fumonisins B1, B2, and B3 by fifteen ex-type strains of *Fusarium* species. *Appl. Environ. Microbiol.,* 2002, **68,** 5195-5197.
16. — GELDERBLOM W.C., KRIEK N.P., MARASAS W.F., THIEL P.G. : Toxicity and carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin B1, in rats. *Carcinogenesis.* 1991, **12,** 1247-51.
17. — GELDERBLOM W.C., MARASAS W.F., VLEGGAAR R., THIEL P.G., CAWOOD M.E. : Fumonisins: isolation, chemical characterization and biological effects. *Mycopathologia.* 1992, **117,** 11-6.
18. — GELDERBLOM W.C., ABEL S., SMUTS C.M., MARNEWICK J., MARASAS W.F., LEMMER E.R., RAMLJAK D. : Fumonisin-induced hepatocarcinogenesis : mechanisms related to cancer initiation and promotion. *Environ. Health Perspect.,* 2001, **109** Suppl 2, 291-300.
19. — GELDERBLOM W.C., GALENDO D., ABEL S., SWANEVELDER S., MARASAS W.F., WILD C.P. : Cancer initiation by fumonisin B1 in rat liver : role of cell proliferation. *Cancer Lett.,* 2001, **169,** 127-137.
20. — GROVES F.D., ZHANG L., CHANG Y.S., ROSS P.F., CASPER H., NORRED W.P., YOU W.C., FRAUMENI J.F. Jr : *Fusarium* mycotoxins in corn and corn products in a high-risk area for gastric cancer in Shandong Province, China. *J. Assoc. Off. Anal. Chem. Int.,* 1999, **82,** 657-62.
21. — JECFA : Fumonisins, 47th report of the joint expert committee on food additives, World Health Organization editor, Geneva, 2002.
22. — JONES C., CIACCI-ZANELLA J.R., ZHANG Y., HENDERSON G., DICKMAN M. : Analysis of fumonisin B1 induced apoptosis. *Environ. Health Perspec.,* 2001, **109,** 315-320.
23. — KELLER S.E., SULLIVAN T.M., CHIRTEL S. : Factors affecting the growth of *Fusarium proliferatum* and the production of fumonisin B1 : oxygen and pH. *J. Indus. Microbiol & Biotech.,* 1997, **19,** 305-309.
24. — LE BARS J., LE BARS P., DUPUY J., BOUDRA H. : Biotic and abiotic factors in fumonisin B1 production and stability. *J. Assoc. Off. Anal. Chem. Int.,* 1994, **77,** 517-521.
25. — LI W., RILEY R.T., VOSS K.A., NORRED W.P. : Role of the proliferation in the toxicity of fumonisin B1 : enhanced hepatotoxic response in the partially hepatectomized rat. *J. Toxicol. Environ. Health A.,* 2000, **60,** 441-457.
26. — MARASAS W.F. : Fumonisins : their implications for human and animal health. *Nat. Toxins,* 1995, **3,** 193-8.
27. — MARIN S, MAGAN N., RAMOS A.J., SANCHIS V. : Fumonisin producing strains of *Fusarium* : a review of their ecophysiology. *J. Food Protect.,* 2004, **67,** 1792-1805.
28. — MARIN S., MAGAN N., SERRA J., RAMOS A.J., CANELA R., SANCHIS V. : Fumonisin B1 production and growth of *Fusarium moniliforme* and *Fusarium proliferatum* on maize, wheat and barley grain. *J. Food Sci.,* 1999, **64,** 921-924.
29. — MARIN S., MAGAN N., BELLI N., RAMOS A.J., CANELA R., SANCHIS V. : Two dimentional profiles of fumonisin B1 production by *Fusarium moniliforme* and *Fusarium proliferatum* in relation to environmental factors and potential for modelling toxin formation in maize. *Int. J. Food Microbiol.,* 1999, **51,** 159-167.
30. — MARIN S., SANCHIS V., RULL F., RAMOS A.J., MAGAN N. : Colonisation of maize grain by *Fusarium moniliforme* and *Fusarium proliferatum* in the presence of competing fungi and their impact on fumonisin production. *J. Food. Protect.,* 1998, **61,** 1489-1496.
31. — MARIN S., SANCHIS V., VINAS I., CANELA R., MAGAN N. : Effect of water activity and temperature on growth and fumonisin B1 and B2 production by *Fusarium proliferatum* and *Fusarium moniliforme* on maize grain. *Lett. Appl. Microbiol.,* 1995, **21,** 298-301.
32. — MELCION D., CAHAGNIER B., RICHARD-MOLARD D. : Study of the biosynthesis of fumonisins B1, B2 and B3 by different strains of *Fusarium moniliforme. Lett. Appl. Microbiol.,* 1997, **24,** 301-305.
33. — NELSON P.E., TOUSSOUN T.A., MARASAS W.F.O. : *Fusarium* species : an illustrated manual for identification. Pennsylvania State Univ. editor, 1983.
34. — NELSON P.E., PLATTNER R.D., SHACKELFORD D.D., DESJARDINS A.E. : Production of fumonisins by *Fusarium moniliforme* strains from various substrates and geographic areas. *Appl. Environ. Microbiol.,* 1991, **57,** 2410-2412.
35. — NIKIEMA P.N., WORRILLOW L., TRAORE A.S., WILD C.P., TURNER P.C. : Fumonisin contamination of maize in Burkina Faso, West Africa. *Food Addit. Contam.,* 2004, **21,** 865-870.
36. — O'DONNELL K., KISTLER H.C., CIGELNIK E., PLOETZ R.C. : Multiple evolutionary origins of the fungus causing Panama disease of Banana : concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad Sci USA.,* 1998, **95,** 2044-2049.
37. — ONO E.Y., FUNGARO M.H., SOFIA S.H., FIGUEIRA E.L., GERAGE A.C., ICHINOE M., SUGIURA Y., UENO Y., HIROOKA E.Y. : Trends of fumonisin contamination and animal intoxication through monitoring 1991 to 1997 corn crop in the state of Parana, Brazil. *Mycopathologia,* 2004, **158,** 451-455.
38. — PITT J.L. : Laboratory guide to common *Penicillium* species. Academis Press editor, London, 1988.
39. — RAPER K., FENNELL D.I. : The genus *Aspergillus.* Williams and Wilkins editors, Baltimore, 1965.
40. — RICE L.G., ROSS P.F., DEJONG J. : evaluation of a liquid chromatographic method for the determination of fumonisins in corn, poultry feed, and *Fusarium* culture material. *J. Assoc. Off. Anal. Chem. Int.,* 1995, **78,** 1002-1009.
41. — RILEY R.T., ENONGENE E., VOSS K.A., NORRED W.P., MEREDITH F.I., SHARMA R.P., SPITSBERGEN J., CARLSON D.B., MERRILL A.H. jr. : Sphingolipid perturbation as mechanisms for fumonisin carcinogenesis. *Environ. Health Perspect.,* 2001, **109,** 301-308.
42. — SAMAPUNDO S., DEVLIEHGERE F., DE MEULENAER B., DEBEVERE J. : Effect of water activity and temperature on growth and the relationship between fumonisin production and the radial growth of *Fusarium verticilloides* and *Fusarium proliferatum* on corn. *J. Food Protect.,* 2005, **68,** 1054-1059.
43. — SCHWARDORF K., MULLER H.M. : Determination of ergosterol in cereal, mixed feed components and mixed feeds by liquid chromatography. *J. Assoc. Off. Anal. Chem. Int.,* 1983, **72,** 457-462.
44. — SEWRAM V., MSHICILELI N., SHEPHARD G.S., VISMER H.F., RHEEDER J.P., LEE Y.W., LESLIE J.F., MARASAS W.F. : Production of fumonisin B and C analogues by several *Fusarium* species. *J. Agric. Food Chem.,* 2005, **53,** 4861-4866.
45. — SYDENHAM E.W., SHEPARD G.S., THIEL P.G. : Liquid chromatographic determination of fumonisins B1, B2 and B3 in foods and feeds. *J. Assoc. Off. Anal. Chem. Int.,* 1992, **75,** 313-318.
46. — THIBAULT N., BURGAT V., GUERRE P. : Les fumonisines : nature, origine et toxicité. *Rev. Med. Vet.,* 1997, **148,** 369-388.
47. — UENO Y., IIJIMA K., WANG S.D., SUGIURA Y., SEKIJIMA M., TANAKA T., CHEN C., YU S.Z. : Fumonisins as a possible contributory risk factor for primary liver cancer : a 3-year study of corn harvested in Haimen, China, by HPLC and ELISA. *Food Chem. Toxicol.,* 1997, **35,** 1143-50.

48. — WANG J.J, LEE C.L., PAN T.M. : Modified mutation method for screening low citrinin producing strains of *Monascus purpureus* on rice culture. *J. Agric. Food Chem.,* 2004, **52,** 6977-6982.
49. — WATANABE A., KAMEI K., SEKINE T., HIGURASHI H., OCHIAI E., HASHIMOTO Y., NISHIMURA K. : Cytotoxic substances from Aspergillus fumigatus in oxygenated or poorly oxygenated environment. *Mycopathologia,* 2004, **158,** 1-7.
50. — WILBERT F.M., KEMMELMEIER C. : Identification of deoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone in galactose oxidase-producing isolates of *Fusarium graminearum. J. Basic Microbiol.,* 2003, **43,** 148-157.
51. — YOSHIZAWA T., YAMASHITA A., LUO Y. : Fumonisin occurrence in corn from high- and low-risk areas for human esophageal cancer in China. *Appl. Environ. Microbiol.,* 1994, **60,** 1626-9.