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PRODUCTION OF FUMONISINS BY STRAINS OF *FUSARIUM MONILIFORME* ACCORDING TO TEMPERATURE, MOISTURE AND GROWTH PERIOD

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ABSTRACT

Production of fumonisins B_1 (FB_1) and B_2 (FB_2) by two Brasilian strains (LAMIC 2999/96 and 113F) and one American strain (NRRL 13616) of *Fusarium moniliforme* were evaluated in laboratory cultures subjected to different temperatures (20, 25, and 30ºC), and moisture contents (25, 34, and 42%) on corn substrate. The cultures were grown during 10, 20, 30, 45, and 60 days, totalizing 135 treatments with two repetitions for each one. The fumonisins were extracted with acetonitrile/water. The clean-up with end-capped C_{18} silica (C_{18ec}) cartridges and fumonisin derivatization with *o*-phtaldialdeyde were carried out through an automated sample processor system (ASPEC), followed by quantification of the toxins through HPLC. Fumonisin production varied widely, reaching average yields from 0.25 to 5515.45 µg/g of FB_1 and from 0.15 to 3032.10 µg/g of FB_2. In the present work, the factors strain, temperature, moisture and days of fungal culture were evaluated, and all of them had a bearing on the amounts of fumonisins produced. The highest FB_1 average yields were obtained by the strain 113F, under the following conditions: 34% moisture content, 60 culture days, and temperature of 25ºC. The highest FB_2 average yield was obtained by the same strain with cultures over 45 days, 42% moisture content, at the temperature of 25ºC. Via regression analysis, the ideal temperature for fumonisins production was, calculated as 24.5 and 24.3ºC (± 2ºC) for FB_1 and FB_2, respectively.

Key words: Fungi*, Fusarium moniliforme,* mycotoxins, fumonisins, abiotic factors.

INTRODUCTION

Fumonisins belong to a large group of toxic metabolites produced by fungi of the genera *Fusarium* (19,26) and *Alternaria* (4). These are natural contaminants of cereals worldwide and are mostly found in corn and corn-based products (9,30,32). The occurrence of fumonisin B_1 (FB_1) in Brazilian feeds was demonstrated by several investigations (6,9,15,21,32).

The FB_1 is the most abundant and toxic metabolite of this group of mycotoxins, representing *ca*. 70% of the total concentration in naturally contaminated foods and feeds, followed by fumonisins B_2 (FB_2) and B_3 (FB_3) (18,20,23). Their action is characterized by inhibition of *de novo* sphingolipid biosynthesis and consequent elevation in the ratio of sphinganine and sphingosine in serum of exposed animals (40). Fumonisins are known to be toxic to domestic animals and to induce leukoencephalomalacia in horses (12) and porcine pulmonary edema (22). Liver hyperplastic nodules and lesions in the distal esophageal mucosa of weaning pigs fed with fumonisin-contaminated feed have also been reported (3). These micotoxins also caused reduced body weight gain in chicks and turkey polts (14,39) and pigs (3). Additionally, epidemiological studies have shown a positive association between exposure to dietary fumonisin and increased risk of human esophageal cancer (5,24,35).

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The production of fumonisins in agricultural commodities depends on such factors as geographical region, season, and the environmental conditions under which the particular grain grows, is harvested and stored. Tropical and subtropical regions are the most favorable for fungi development on cereals and production of these toxins (36). Although cereals are important as substrates, moisture level and temperature are the critical abiotic factors regulating the growth of *Fusarium moniliforme* and the production of fumonisins (2). Low kernel moisture content < 22% should reduce or prevent toxin production in storage (13). Information on the minimal, optimal, and maximum temperature for fumonisin production is uncertain; however, the best temperature range for fumonisin production is 20-28ºC (1). Ryu *et al*. (28) investigated the influence of the temperature and its variations on the FB_1 production. They also tested the effect of cyclic temperatures at intervals of 12 hours on cultures kept at 5 and 25ºC, 10 and 25ºC, and 15 and 30ºC. In addition, they carried out tests on cultures at constant temperature (25ºC) for two weeks followed by four more weeks at 15ºC. In all cultures FB_1 production was observed, however, the highest average was obtained from samples cultured at cyclic temperatures of 10 and 25ºC.

Marín *et al*. (16) surveyed the effects of different temperatures (25 and 30ºC) and water activities (0.968, 0.956, 0.944, 0.925) on fungal growth and fumonisin production by *Fusarium moniliforme* and *Fusarium proliferatum* strains, observing that both increased as the moisture and temperature increased. By evaluating the effect of pH (3.6, 5.5, and 7.0) and temperature (4-45ºC) on the fungal growth, Marín *et al*. (17) concluded that *Fusarium monoliforme* grew better at pH 7.0 and 30ºC, whereas *Fusarium proliferatum* grew better at pH 5.5 and 25ºC.

On harvested samples, the concentration of fumonisins is usually lower than 10 μg/g. Yet, more than two thirds of these samples are positive for such toxins (8,9,30,34). However, very high FB_1 concentrations such as 126 and 330 μg/g have already been found in foods (27).

Under laboratory conditions, high concentrations of fumonisins can be obtained inoculating *Fusarium moniliforne* onto sterile corn with high moisture content. The amount of fumonisin is also dependent upon the strain of *Fusarium moniliforme* employed in the culture (19,37,38). Hence, fumonisin concentrations can be above 10000 μg/g in 13-week culture medium (1,10).

The present work describes the influence of abiotic factors such as moisture, culture time and temperature on production of fumonisins, using three strains of *Fusarium moniliforme*, 1) LAMIC 2999/96, isolated from a corn sample naturally contaminated with FB_1 and FB_2, responsible for an outbreak of horse leukoencephalomalacia in Catuípe's municipal district-RS (15); 2) NRRL 13616, from USA; and 3) 113F, from the Universidade Estadual de Londrina-PR. The choice of such strains was done because they are admittedly high fumonisin producers, according to tests carried out previously.

MATERIALS AND METHODS

Fumonisin production

The fungus isolation and maintainance were carried out according to Mallmann *et al.* (15).

The methodology employed for fumonisin production was adapted from several authors (1,10,19). Corn grains with 11.9% moisture content were utilized to prepare the fungal cultures.

The experiment was outlined with two repetitions of 135 treatments consisting of three strains of *Fusarium moniliforme*, which were evaluated at different culture periods: 10, 20, 30, 45 and 60 days, with 25, 34 and 42% moisture content on the substrate, and cultured at temperatures of 20, 25, and 30ºC as shown in Table 1.

For fumonisin production, the following procedures were adopted for each treatment: 100g of corn with 11.9% moisture content was weighed in triplicate, in 500 ml Erlenmeyer flasks. To each weighed amount deionized water was added (10.2, 18.1 and 25.2 ml, respectively) in order to obtain corn samples with 25, 34 and 42% moisture content, respectively. The flasks were closed with cotton tops. The samples were allowed to stand on shelves for two hours at room conditions, and ten autoclaved for 20 minutes. Thirty minutes later, 0.5 ml of potato dextrose agar containing *Fusarium moniliforme* culture was added to each sample. The flasks were closed again, and shaken manually to homogenize the culture material. They were then covered with aluminium foil and placed in BOD incubator. On the third incubation day, the cultures were vigorously shaken in order to get a better fungus distribution on the samples. After the culture period, the material was autoclaved for 5 minutes, dried in an electrical oven at 45ºC during 15 hours, and ground and stored in freezer at -18ºC until quantification of fumonisins.

Table 1. Experimental outline of culture of 3 strains of *Fusarium moniliforme.*

Culture days	10			20			30			45			60		
Moisture (%)	25	34	42	25	34	42	25	34	42	25	34	42	25	34	42
	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Temperature (ºC)	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25
	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30

Standards

Standards of fumonisins B_1 (FB_1) and fumonisina B_2 (FB_2) were from Sigma Chemical (St. Louis, USA). Stock standard solutions were prepared in acetonitrile/Milli-Q water (50:50, v/v) at 1-5 mg/ml. Working standard solutions were prepared in acetonitrile/Milli-Q water (50:50, v/v) at 5 μg/ml. All standard solutions were stored at -18ºC until use.

Extraction, clean-up and derivatization

The procedures of extraction and preparation of mycotoxins took place according to previously described methodologies (25,31,33), yet with some slight modifications. Clean-up, derivatization and injection procedures were carried out through an automated sample preparation system (ASPEC) (Gilson-Vivier le Bel, France). Samples (10g) were suspended in 50 ml of acetonitrile/water (50:50, v/v) and extracted in a blender (Walita – São Paulo, Brazil) at high speed, for 5 min. Each mixture was filtered through Whatman IV filter paper. Two ml of acetonitrile/water extract were mixed with 6 ml of destiled water for clean-up with 300 mg C_{18ec} silica disposable extraction cartridges (DEC). Before clean-up, the sample pH was adjusted to 5.8-6.5 with 1 M sodium hydroxide when necessary.

The solid phase clean-up, derivatization and injection procedures were performed using ASPEC. The sequence of operations for the automated clean-up of samples was as follows: 1) Condition DEC with 2 ml of acetonitrile. 2) Condition DEC with 2 ml of water. 3) Push 8 ml of sample through DEC (2 ml sample and 6 ml distilled water). 4) Rinse needle. 5) Wash DEC with 5 ml of water. 6) Elute mycotoxins with 2 ml of acetonitrile/water (70:30, v/v), pH 5.8-6.5. 7). Flow rates through DEC were set at 2 ml/min; however, pushing of sample and eluting procedures were performed with flow rate at 1 ml/min.

The ASPEC was programmed to advance to derivatization and injection of samples after each elution, according to the following procedure: 1) Rinse needle. 2) Dispense 200 µl of OPA solution (Dissolve 40 mg of *o*-phthaldialdehyde in 1 ml of methanol and dilute with 5 ml 0.1 M sodium tetraborate. Add 50 µl of 2-mercaptoethanol) into a clean sample tube conditioned in a temperature-controlled rack at 30ºC. 3) Rinse needle. 4) Add 50 µl of test solution. 5) Mix derivative solution (by aspersion and dispense). 6) Rinse needle. 7) Wait for a period of 2 min. 8) Inject 100 µl in the chromatography system. 9) Rinse needle. 10) Rinse injection port. 11) End.

A second derivatization program was used to set up the standard injection and construct a calibration curve. The delivery of OPA and test solutions were conducted at 3 ml/min.

Fumonisin determination by HPLC

The HPLC consist of a GBC Scientific Equipment pump Model LC 1150 (ICI Instruments - Dingley, Australia) on-line with ASPEC. The mobile phases were composed of acetonitrile/water/acetic acid (50:50:1, v/v), (solution A) with a linear gradient of acetonitrile (solution B) according to Chu and Li (5) and Stack and Eppley, (31), with some modifications. For the first 8 minutes of the chromatographic run, the mobile phase consisted of 100% solution A, at which time a step change was made to 85% solution A and 15% solution B. The mobile phase then returned to 100% solution A by means of a linear gradient over a 4-minute period. These mobile phases were filtered through a 0.45 µm Waters HV membrane and pumped at 1 ml/min flow rate over the entire chromatogram. The chromatographic column, 150 x 4.6 mm, ODS, 5 µm, Macherey-Naguel (Düren, Germany) was maintained at a constant temperature of 35ºC in a column oven. Fumonisins were detected by a fluorescence detector (F100, Merck – Schuchardt) with wavelength set at 335 nm for excitation and 440 nm for emission. Calculation of fumonisin concentrations in test samples was based on peak areas compared with those of the standards. The limits of quantification of the method were of 20 and 30 µg/kg for FB_1 and FB_2, respectively.

Statistical analysis

The experimental outline utilized for fumonisin production was entirely random in a factorial experiment based on 3 x 3 x 3 x 5 (3 strains, 3 temperatures, 3 moisture contents, and 5 culture periods), totalizing 135 treatments with two repetitions for each one. The global statistical analysis on fumonisin production data was performed after transformation of their numerical values into decimal logarithm scale. The fumonisin production was then analyzed through analysis of variance (ANOVA) method so that the effects of the interaction of a single factor such as temperature (T) culture days (C), strain (L),and moisture content (U), and of two-factor (T x C, T x L, T x U, C x U, C x L, L x U), and three factors (T x C x L, T x C x U, T x L x U, C x L x U) on fumonisin production might be evaluated. In a unique case, the four-factor interaction (T x C x L x U) was also evaluated. Tukey test ($p<0.05$) was applied for comparison of the averages. Polynominal regression studies were effected analyzing models of first and second degrees (linear and quadratic, respectively). The analyses were done using the statistical pack program SAS Version 6 (1990) (SAS/STAT-SAS Institute Inc. Cary, NC-USA) in an IBM 9276 computer.

RESULTS AND DISCUSSION

All the factors – temperature (T), culture days (C), strains (L), and moisture content (U) – herein evaluated showed relevant importance to the production of the toxins. The majority of the interactions between factors also influenced the production of toxins, except for the interactions of culture days with strains (CS) and culture days with different moisture contents (CU). The interaction between different utilized temperatures, culture days and strains (TCL) exhibited meaningful influence on FB_1 production, however this did not occur in FB_2 production. Table 2

Table 2. Global analysis of variance on FB_1 and FB_2 production data suitably transformed into decimal log scale.

Variation causes	Freedom degrees	Mean square		F		Significance level	
		FB_1	FB_2	FB_1	FB_2	FB_1	FB_2
T*	2	28.68	31.04	306.39	292.37	0.0001	0.0001
C**	4	22.91	24.50	244.81	230.82	0.0001	0.0001
L***	2	0.90	0.93	9.58	8.75	0.0001	0.0003
U****	2	3.91	4.50	41.81	42.36	0.0001	0.0001
TC	8	1.36	1.08	14.57	10.22	0.0001	0.0001
TL	4	1.04	0.80	11.10	7.50	0.0001	0.0001
TU	4	4.16	3.80	44.40	35.82	0.0001	0.0001
CU	8	1.52	1.57	16.19	14.75	0.0001	0.0001
CL	8	0.19	0.17	1.99	1.57	0.0520	0.1399
LU	4	0.14	0.11	1.47	0.99	0.2142	0.4157
TCL	16	0.24	0.14	2.53	1.99	0.0020	0.2127
TCU	16	0.72	0.59	7.67	5.54	0.0001	0.0001
TLU	8	0.22	0.23	2.36	2.19	0.0209	0.0322
CLU	16	0.17	0.19	1.78	1.76	0.0392	0.0432
TCLU	32	0.18	0.23	1.95	2.13	0.0046	0.0015
***** $\overline{X}$		1345.38	606.37				
VC (%)		11.99	15.01				
R^2		0.95	0.94				

* T-Temperature; ** C-Culture days; *** L-Strain; **** U-Moisture content; ***** $\overline{X}$-Mean of yielded fumonisins (µg/g). Values without logarithmic transformation.

also shows that the average yield of FB_1 produced was quite superior to that of FB_2, this latter representing, approximately, 31.1% of the total fumonisins.

In relation to the averages of two repetitions of the 135 treatments of FB_1 and FB_2 production, a great variation in the amount of fumonisins produced was noted. The minimal and maximal productions of FB_1 were 0.25 µg/g and 5515.45 µg/g, respectively.

Concerning FB_2, the minimal and maximal concentrations were of 0.15 µg/g and 3032.10 µg/g, respectively. So, toxin production was obtained under all the conditions evaluated in the present experiment.

The concentration of fumonisins yielded under laboratory conditions is usually higher than toxins produced naturally. Accordingly, the average amounts of the two toxins produced during the experiment, 1345.38 µg/g and 606.37 µg/g of FB_1 and FB_2, respectively, were quite superior to those produced naturally, as reported by several authors (9,23,30,32), who verified maximal concentrations up to 330 µg/g of FB_1. This fact may be attributed to the culture conditions maintained in laboratory, where one has worked with the samples grow under controlled parameters and in previously autoclaved culture medium, facilitating therefore the growth of the inoculated fungi, corroborated by absence of concurrent fungal growth.

Similar concentrations were achieved by Schumacher *et al*. (29), 4360 µg/g of FB_1; and Roos *et al*. (26), who obtained up to 2350 and 320 µg/g of FB_1 and FB_2, respectively. On the other side, less toxins were produced than that reported by Holcomb *et al*. (10), who determined more than 10000 µg/g of FB_1. It is noteworthy to point out here that after the culture period, the fungal cultures were autoclaved for 5 min and dried at 45ºC for 15 hours. According to researches done by Dupuy *et al.* (7) and Jackson *et al*. (11), fumonisins are degraded at high temperatures. When exposed to 75ºC for 8 uninterrupted hours, for example, they loss *ca*. 50% of activity. Consequently, one may corelate here the possible degradation to autoclaving and drying of the cultures.

Fumonisin production as a function of the moisture of the fungal culture

In the study analysis of the effect of the moisture content in corn on fumonisin production (Fig. 1), it was observed that the toxin yield increased of concomitantly to the moisture content increase, at 20ºC and 25ºC. At 30ºC, an inverse effect was noted for both toxins, i.e. the increase of the moisture of the cultures led to a decrease in the amount of yielded toxins. This trend was observed for all strains, despite lack of statistical significance. The highest mean concentrations of FB_1 and FB_2 were yielded less than 42% moisture content, at 25ºC, especially for strain 113F.

According to several authors (2,13), moisture content has a fundamental importance for fumonisin production, once low

concentrations occur in cereals stored at moisture content lower than 22%. In this work, the moisture content significantly in the amounts of produced fumonisins. The moisture content presented significant interaction with other factors, fact that did not take place with different strains which exhibited the same response to the same moisture content in the substrate.

Fumonisin production as a function of the temperature of the fungal culture

The influence of the temperature on fumonisin production may be observed in Fig. 2. The highest toxin prodution was obtained in cultures at 25ºC. At 20ºC, the production decreased and the lowest yields were observed in cultures at 30ºC. The ideal temperatures for FB_1 production, calculated by regression, were of 24.3, 24.7 and 24.5ºC for the strains LAMIC 2999/96, NRRL 13616 and 113F, respectively. From these data, the estimated optimal mean temperature for the FB_1 production was 24.5ºC (±2ºC). The same evaluations were done regarding FB_2 production, and the ideal temperatures were of 24.2, 24.1 and 24.3ºC for the strains LAMIC 2999/96, NRRL 13616 and 113F, respectively. The optimal mean temperature for the production of this toxin was 24.2ºC (±2ºC).

In the interaction between temperature and moisture, the amount of the yielded toxins increased with the increase of the culture days, as evidenced by a greater average yield under different moisture contents in 60-day-old cultures.

Alberts *et al*. (1) studied the adaptation of *Fusarium moniliforme* to different temperatures. They concluded that this fungus grows better at 25ºC than at 20ºC. Its lowest growth was ascertained at 30ºC. In our research, the production of fumonisins followed the same temperature parameters adopted by the above cited researchers. Low concentrations of these toxins were yielded at 30ºC, probably due to the slow growth of the fungus. Measurements of its growth was not emphasized in our experiments. At 20ºC, there were proportioned high yields of fumonisins, yet highest one was obtained at 25ºC.

In our work, the ideal temperatures found for fumonisins production (24.5ºC and 24.2ºC for FB_1 and FB_2, respectively), diverge from those reported by other authors, (27ºC and 28ºC) (37,39). However, our results are similar to those described by other authors (10,14) utilizing the temperature of 25ºC.

Fumonisin production as a function of the fungal strain utilized in cultures

In Fig. 3, it may be observed that the strains show a very similar behavior at same temperature. The interaction of these two factors with the fungal culture time presented a larger output of toxins in 60-day-old cultures at 25ºC. The largest total output was obtained by strain LAMIC 2999/96 cultured at 25ºC for 60 days. However, the best mean production occurred with strain 113F, at 25ºC. A similar behavior was noted for FB_2, when a larger mean production was observed at 25ºC by strain 113F, which also yielded the largest amount of FB_2 at 60 days of culture at the same temperature.

The strains exhibited total fumonisin production in similar amounts but, when the production of each strain was assessed without the interaction with the effects of temperature, moisture and culture periods, their differences were highly significant, as shown in Table 2.

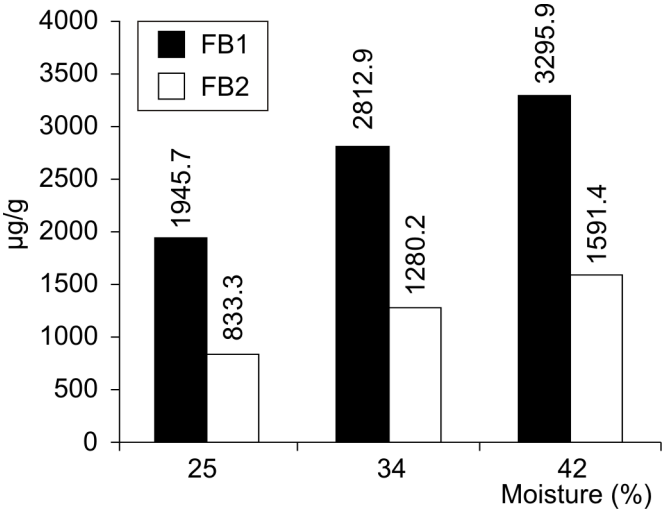

Figure 1. Mean fumonisin output as a function of the culture moisture content.

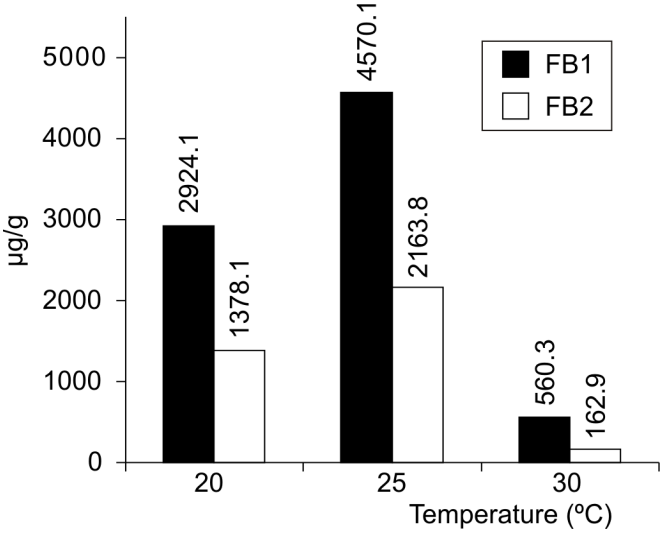

Figure 2. Mean fumonisin output as a function of culture temperature.

Fumonisin production as a function of fungal culture time

In Fig. 4, the effect of the fungal culture period on fumonisin prodution may be observed. The toxin production increased with the increase of the culture time. The largest production was obtained in strain 113F cultured for 60 days under 42% moisture content. For the three strains, FB_1 production reached the maximum of 44.3 µg/g/day in a period of 60 days. In relation to FB_2 production, the maximal mean output occurred on 45 days of culture, especially for strain 113F. The toxin prodution was linear, deriving a theoretical mean increment of 32.1 µg/g/day in the three strains, up to the 45 days of culture at the conditions employed in this work.

The fungal culture period has great importance for production of FB_1 and FB_2 as well. In 10-day-old cultures, the production was lowest, yet in sufficient amount to induce the characteristic pathologies in all species susceptible to these mycotoxins. With the increase of culture periods, the mean production of the toxins raised continually.

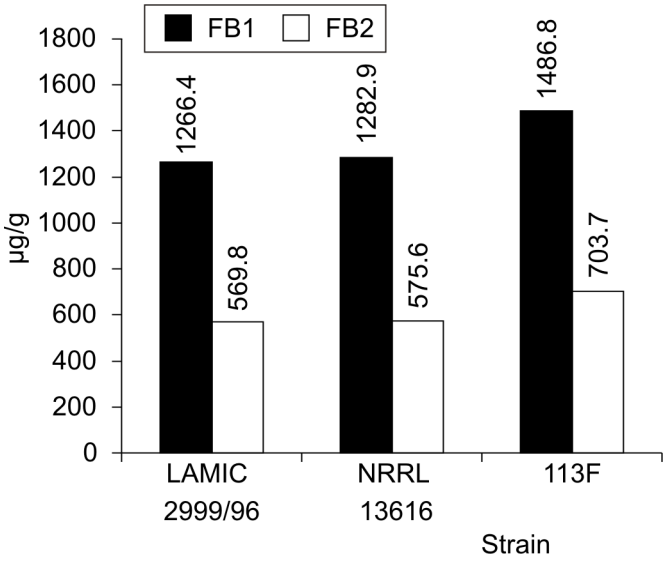

Figure 3. Mean fumonisin output as a function of the strain.

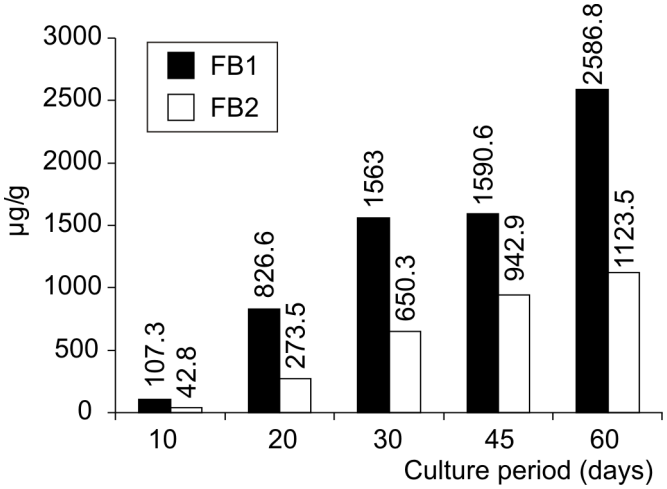

Figure 4. Mean fumonisin output as a function of the fungal culture time.

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RESUMO

Produção de fumonisinas por cepas de *Fusarium moniliforme* de acordo com a temperatura, umidade e tempo de cultura

Produção de fumonisinas B_1 (FB_1) e B_2 (FB_2) a partir de duas cepas brasileiras (LAMIC 2999/96 e 113F) e uma cepa americana (NRRL 13616) de *Fusarium moniliforme* foi avaliada em culturas de laboratório submetidas a diferentes temperaturas (20, 25 e 30ºC) e a diferentes teores de umidade (25, 34 e 42%) em substrato de milho. As culturas foram realizadas em períodos de 10, 20, 30, 45 e 60 dias, totalizando135 tratamentos com duas repetições para cada um. As fumonisinas foram extraídas com acetonitrila/água. A limpeza foi realizada empregando cartuchos de sílica C_{18} encapada (C_{18ec}) e a derivação com *o*-ftalodialdeído foram realizadas por um sistema processador automático de amostras (ASPEC), seguidas por quantificação das toxinas por CLAE. A produção de fumonisinas variou muito, atingindo rendimentos médios de 0,25 a 5515,45 µg/g de FB_1 e de 0,15 a 3032,10 µg/g de FB_2. Neste trabalho, os fatores como cepa, temperatura, umidade e dias de cultura fúngica foram avaliados, e todos estes influenciaram nas quantidades de fumonisinas produzidas. As mais altas produções de FB_1 foram obtidas pela cepa 113F nas seguintes condições: teor de umidade de 34%, 60 dias de cultura, temperatura de 25ºC. A maior produção média de FB_2 foi obtida pela mesma cepa com culturas durante 45 dias, a um teor de umidade de 42%, à temperatura de 25ºC. A temperatura ideal para produção de fumonisinas foi calculada por meio de análise de regressão, sendo 24,5ºC e 24,3ºC (±2ºC) para FB_1 e FB_2, respectivamente.

Palavras-chave: Fungos, *Fusarium moniliforme*, micotoxinas, fumonisinas, fatores abióticos.

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