Anti-*Fusarium moniliforme* Activity and Fumonisin Biodegradation by Corn and Silage Microflora

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ABSTRACT

Studies were carried out to isolate microorganisms from corn and silage screened for their ability to inhibit F. moniliforme *growth (strain 113F) in association with fumonisin detoxification. Among 150 isolates four Gram-positive bacilli and one yeast with inhibitory activity were selected. The inhibition zone ranged from 50 to 72.5 mm using cultures, and from 25 to 52.5mm for crude alcoholic extract. The isolates S9, S10, S69 (sporulated bacilli) and SE3071 (yeast) degraded 43, 48, 83 and 57% of the initial FB_1 concentration, respectively. The pH increased gradually in the medium during incubation for biodegradation process.*

Key words: *Fusarium moniliforme*, fumonisin, biodegradation

INTRODUCTION

Fumonisins belong to a mycotoxin group produced by *Fusarium moniliforme* on corn (Marasas, et al., 1984). The compound is propane-1,2,3- tricarboxylic acid of 2-amino 12,16-dimetil polihydroxy eicosane diesters, with C_{14} and C_{15} esterified by hydroxyl of the carboxylic acid (Bezuidenhout et al., 1988). Although the literature describes eight fumonisins, only FB_1, FB_2 and FB_3 have been detected naturally in corn (Gelderblom et al., 1988; Cawood et al., 1991; (Sydenham et al., 1991; Thiel et al., 1992). Characterized as tricarboxylated amino polyalcohol, similar to esfingosins, they cause toxicity, inducing cerebral lesion-LEME in horses, lung edema in swines, imunodepression in poultry and hepatic carcinogenicity in rats (Marasas et al., 1988; Gelderblom et al., 1991; Norred,1993). Recent works have also reported nephrosis, thrombosis, atherosclerosis and toxicosis in other animals (Norred, 1993; Galvano et al.,1997). In humans, the natural occurrence was associated with oesophageal cancer in South Africa and China (Sydenham et al., 1990; Rheder et al., 1992; Wang et al., 1995).

The removal of fine corn particles reduces FB_1 contamination from 26% to 69%, but normal drying or heating treatment is ineffective for total fumonisin removal (Sydenham et al., 1994). The use of $Ca(OH)_2$ and/or H_2O_2 and $NaHCO_3$ reduced respectively, 81% and 100% of FB_1 (Park et al., 1997). A promising procedure is the adsorption method that uses multisequestring agents, like aluminum silicates and sepiolites, with recent inclusion of activated carbon, capable of adsorbing 100% of AFB_1 and FB_1 (Galvano et al., 1997). However, chemical agents affect functional properties of the grain, while adsorbents could affect nutritional components.

Considering the worldwide contamination of corn by *F. moniliforme* in the field, and that fumonisins are produced in the pre-drying step of harvested corn (Hirooka et al., 1996; Ono et al., 1998), one promising option could be the control by using antagonists microorganisms isolated directly from plant microbiota (Motomura et al., 1996). In this work, the effectiveness of microorganisms isolated from the natural habitat of corn and silage, for the control of *F. moniliforme* and fumonisins degradation, without causing drastic alterations in the ecosystem, were analyzed.

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MATERIAL AND METHODS

Microorganisms: The reference microorganism was *F. moniliforme* strain 113F, isolated from feed involved in animal intoxication. The initial productivity reached 54.21 mg/g and 87.31 mg/g of FB_1 and FB_2, respectively (Hirooka et al., 1996). The antagonic microorganisms were isolated from corn and silage obtained from Paraná State (north region), Brazil. The screening was conducted as described by Motomura (1995) and plated in Man, Rogosa & Sharpe (MRS) medium (Oliveira, 1995). The antagonists belonging to the *Bacillus* sp. group were cultivated in brain heart infusion (BHI) broth and the yeasts in malt extract broth. The cultures were maintained at 4ºC.

Screening of antagonic microorganisms: The silage and corn isolates were submitted to antibiogram, using the pour plate technique in potato dextrose agar - PDA inoculated with 10^6 propagules/mL of *F. moniliforme* (Motomura, 1995). After solidification, 0.1 mL of antagonist culture, or crude alcoholic extract previously prepared with ethanol and then concentrated 5 times at 45ºC, was applied into the center of the agar plate (Motomura et al., 1996). The plates were then incubated at 25ºC for 7 days and the inhibition zone were measured.

Fumonisin production in corn culture: The corn culture was obtained by inoculating 2 mL (10^6 spores/mL) of *F. moniliforme* 113F on the surface of 100g of ground corn, previously humidified with 100mL of distilled water and autoclaved for 30min. After incubation at 25ºC for 30 days, the culture was solvent treated as described below and FB_1 analyzed by HPLC (Weibking et al., 1993).

Preparation of material for fumonisins degradation assay: Cleaning of the crude culture was conducted according to Weibking et al. (1993), using 400mL of acetone:chloroform 75:25 (V/V), by overnight agitation of the sample at 180 rpm at 25°C. The extracted material was filtered through Whatman n° 1 filter paper, and the culture residue cleaned again with acetone:chloroform 75:25. The solid residue was evaporated in a air circulation chamber at 40ºC for 48 hours, ground and stored at -7°C.

For the fumonisin degradation assay, 0.015M phosphate saline buffer - PBS at pH 7.0 was added to the dried corn culture in a 10:1 (V/V) ratio. The suspended material was extracted at 180 rpm, 25° C for 30min, centrifuged at 10,000xg and filtered through Whatman n°1 filter paper.

Fumonisins degradation by selected microorganisms: The five microorganisms that showed higher anti-*F.moniliforme* activity were cultivated in 200mL of BHI broth (*Bacillus* spp.) at 35°C, or in malt extract broth (yeasts) at 25ºC, respectively. One mL of standardized culture of antagonists (absorbance 0.3 at 600nm) was transferred to tubes containing 4mL of BHI or malt extract broth amended with 4mL of *F. moniliforme* culture suspension in PBS with known concentration of FB_1. The assay for each microorganism consisted of five determinations made in triplicate, incubated at 25°C (yeast) or 35 °C (*Bacillus* sp.). Every two days, during 15 days, the culture was interrupted for fumonisin analysis, centrifuged at 10,000xg, and filtered through a Whatman n°1 filter paper.

The analyses consisted of pH determination, along with fumonisin quantification by HPLC. The remaining FB_1 was calculated as μg/g, considering that the treated corn culture contained 230 μg/g of FB_1. The control consisted in the culture medium added with treated corn culture, but without antagonist.

Determination of fumonisins by HPLC: The fumonisins were determined using the method of Shephard et al. (1990), modified by Ueno et al. (1993). One mL of the culture filtrate was clarified in Sep Pak Accell Plus QMA Cartridges, previously conditioned with 6mL methanol: water at 3:1(V/V) ratio, followed by elution with 3mL methanol. The toxin was eluted with 10mL ethanol with 0.5% acetic acid, evaporated at 40°C and re-suspended in 1mL of methanol. After drying, 2mL of methanol-water (3:1) was added and evaporated under nitrogen at 50ºC. For analysis, it was suspended in 800 μL of methanol-water (3:1) and a 200μL aliquot was dried under nitrogen. After derivatization with 200μL of ortho-phtaldehyde (40mg orthophtaldehyde, 1mL methanol, 5mL 0.1M sodium borate and 50μL 2-mercaptoethanol), the analysis was carried out in isocratic HPLC (Shimadzu LC-10AD) using reverse phase C_{18} column (250x4.6mm), with 5μm of Supelco's nucleosil. The mobile phase consisted of methanol-sodium phosphate 0.1M (80:20) adjusted to pH 3.3. The equipment was conditioned to a flow of 1mL/min. with

wavelength of excitation and emission of 335nm and 450nm (Shimadzu F 535), respectively.

RESULTS AND DISCUSSION

A total of 150 microorganisms were isolated from corn and silage using MRS culture medium. Submitting the same isolates to antibiogram test (Motomura, 1995) against *F. moniliforme* 113F, inhibitory activity was detected in 28 isolates. The antagonists were identified as 13 yeasts and 15 bacteria, with predominance of bacilli over cocci and lactic bacteria. Similar data were obtained in a previous work with corn and soil samples (Motomura et al., 1996). This predominance could be explained by the use of surface samples of silage, which favors development of aerobic groups, to the detriment of lactic bacteria. The antimicrobial activity on the silage surface may be important in natural biocontrol of mycotoxigenic fungal proliferation, which is favored by aerobiosis.

Four *Bacillus* sp. and one yeast, designated as S1, S9, S10, S69 and SE3071, respectively, showed highest anti-*F.moniliforme* activity (Table 1). The inhibition zone using whole culture ranged from 50 to 72.5mm, compared to the crude extract of the same cultures which ranged from 25 to 52.5mm, indicating loss of inhibitory activity during the concentration of the alcoholic extract by heating at 45ºC. Higher losses occurred with strains S1, S9, SE3071, S69 and S10 in a decreasing order. The instability of yeast product may be due to the expression of antagonistic activities as a product of the "Killer" factor, constituted by toxic peptic compounds (Walker et al., 1995; Kashiwagi et al., 1997).

Besides the anti-*F.moniliforme* effect, an ideal condition would be if the same microorganism was able to degrade the toxic fungal metabolites. Tables 2 and 3 contain data on the activity regarding FB_1 degradation, along with the effect of antagonist growth on the pH variation and the decrease in toxin concentration.

Of the five microorganisms that inhibited the growth of *F. moniliforme*, only the strain S_1 *Bacillus* did not degrade the toxin, as the FB_1 decreased21.4% compared to the control. without inoculum that was degraded 31% (Table 2).

The high polarity of fumonisins probably induces spontaneous reaction of decomposition (Hirooka et al., 1993). This was observed during the maintenance of dried corn culture (Weibking et al., 1993), whose initial level of 298 µg/g was reduced to 230µg/g, during a storage period of six months at -7°C.

Table 1 - Inhibition of *F.moniliforme* 113F by antagonic microorganisms isolated from corn and silage.

Microorganisms	Gram/Wirtz	Inhibition zone (mm)		Origin
		Whole culture	Crude extract	
Bacillus S1	Sporulated G+	72	52.5	Silage
Bacillus S9	Sporulated G+	72.5	47.5	Silage
Bacillus S10	Sporulated G+	70	39.5	Silage
Bacillus S69	Sporulated G+	65	35	Silage
Yeast SE3071	-	50	25	Corn

Table 2 - Degradation of FB_1 (µg/g) by corn and silage microorganisms.

Time (days)	Microorganisms					
	S1	S9	S10	S69	SE3071	CONTROL
0	230	230	230	230	230	230
2	*	197.64	136.15	135.22	166.27	-
4	*	155.91	122.93	127.66	162.46	-
6	180.67	131.3	119.19	38.72	131.80	156.66

- Interferents in the extract.

Table 3 - pH Variation in a period of six days.

Microorganisms	pH

	Day 0	Day 2	Day 4	Day 6
S1	7.5	7.2	7.9	8.3
S9	6.7	7.2	8.3	8.6
S10	6.9	7.2	7.7	8.1
S69	6.9	7.3	8.0	8.0
SE3071	5.9	6.5	7.4	7.7
CONTROL	6.8	6.8	6.8	6.8

The strains S9, S10, S69 and SE3071 showed promising results for FB_1 degradation, causing decreases of 43, 48, 83 and 57%, respectively. In Table 2, data up to the 6^{th} day of the experiment are shown, since problems occurred with chromatogram interpretation, possibly caused by interference of microbial metabolism. Regarding the strain S1, problems with microbial contaminant interference were observed, which hindered the chromatogram interpretation after two days.

In the control treatment, the pH was maintained at 6.8, while in the assays inoculated with antagonistic microorganisms it increased gradually, suggesting an alkalization process as a result of microbial growth (Table 3). After six days, the pH values varied from 7.7 to 8.6. Probably, the release of C_2 amino group from the backbone of fumonisin molecule played significant role in the detoxification process, being the pH determination, a suitable parameter for monitoring biological degradation. (Jackson et al., 1996).

Although the literature reports increasing number of chemical detoxification processes, with emphasis on the adsorption using multifunctional chelating agents (Galvano et al., 1997; Park et al., 1997), there are still doubts regarding the negative effect on nutritional and commercial value of a chelate adsorbed products. Emphasis should be given to the promising potential of strain S69, which showed an antagonistic activity associated with detoxification twice more effective than the other microorganisms. The strain S_1 was not very effective in grain detoxification, but it could still be used in the field, with the intention to control diseases caused by *F. moniliforme*, since it showed the largest inhibition zone, in terms of stability of the compound secreted (Table 1).

The fumonisins degradation by the selected microorganisms is an interesting method, since nowadays there is no effective procedure for the control of fumonisins produced at the final stage of the maturation process in corn.

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RESUMO

A aplicação de microrganismos visando controle de fungos micotoxigênicos ou detoxificação em armazenagem consiste de uma área promissora, já que reduz contaminação de ecossistema por resíduos agrotóxicos. Visando controle biológico, microrganismos isolados de milho e silagem foram analisados perante efeito anti-*F. moniliforme* (linhagem 113F) em associação com a detoxificação de fumonisinas. Após análise de 150 isolados, selecionou-se quatro bacilos Gram-positivos e uma levedura com melhor atividade inibitória. O halo de inibição variou de 50 a 72.5 mm usando culturas íntegras e 25 a 52.5mm, para extrato bruto de cultivo. Os isolados S9, S10, S69 (bacilos esporulados) e SE3071 (levedura) degradaram 43%, 48%, 83% e 57% de FB_1 respectivamente, em relação à concentração inicial. O pH aumentou gradativamente com o tempo de incubação.

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