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SHORT COMMUNICATION

CYTOTOXIC EFFECTS OF *FUSARIUM MONILIFORME* EXTRACTS AND FUMONISIN ON A BABY HAMSTER KIDNEY CELL LINE

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Abstract: Four *Fusarium moniliforme* culture extracts prepared from LEM associated corn (extracts 23 and 33) and non-LEM corn (extracts 1 and 2), and fumonisin standards were tested for cytotoxic effects against a baby hamster kidney cell line (BHK-21), using neutral red bioassay. The fungal culture extracts from all four isolates (containing various amounts of FB_1 and FB_2) were toxic to BHK-21 cells. Culture extracts from isolates 1,2 and 23 showed comparable LV_{50} i.e. 56-60 µl/ml. A slightly higher volume (71 µl/ml) of isolate 33 extract was needed to cause LV_{50} to the same cell line. Both FB_1 and FB_2 standards were toxic to BHK-21 cells, however, higher amount of FB_2 was needed to cause NR_{50} to cells compared to FB_1. Toxicity of BHK-21 cells produced by *Fusarium moniliforme* isolate culture extracts from both types of corn samples could be due to FB_1, FB_2 as well as other fumonisins present in the culture extracts. Neutral red bioassay could be adapted for preliminary screening of corn, or other agricultural commodities, prior to chemical analysis, for the presence of fumonisins.

Key words: Cytotoxicity, *Fusarium moniliforme*, fumonisins.

INTRODUCTION

Fusarium moniliforme J. Sheldon, a common fungal contaminant of corn throughout the world, in addition to causing plant diseases, has been implicated in animal and human diseases.[1] *Fusarium moniliforme* infection of corn has been correlated with human oesophageal cancer risk in Transkei, South Africa, where corn is the staple diet.[2] The same fungus present in feed corn, is thought to be responsible for leukoencephalomalacia (LEM) disease in horses, pulmonary edema and hydrothorax in swine, and carcinogenicity in rats.[2] Carcinogenic compounds known as fumonisins produced by *F. moniliforme* isolates are thought to be responsible for LEM disease in equids, and other related diseases.[2]

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Abbreviations: BHK - Baby hamster kidney; LEM -Leukoencephalomalacia; LV_{50} - midpoint lethality volume; NR_{50}-midpoint neutral red value; NR - Neutral red dye.

The type and quantity of mycotoxin(s) present in an agricultural product is dependent on factors including crop species, management practices and the climatic conditions for growth and storage.[3] However, because of the erratic occurrence and distribution of toxins, in a field or in a sample, it is not always economically feasible or reliable to analyze plant material accurately for the presence of mycotoxins, even though chemical analyses are available and sensitive enough to measure toxin levels at microgram or sub microgram levels.[4] Toxic substances may also be present for which chemical analyses are not available. Because of this, bioassays have been developed to screen fungal and plant extracts for the presence of toxic compounds.[1,3,5]

Objectives of this research were to evaluate the cytotoxicity of culture extracts of *F. moniliforme* isolated from corn associated with an outbreak of LEM in horses in Maryland, to a mammalian cell line in comparison to the cytotoxicity of extracts of *F. moniliforme* isolated from corn not associated with an outbreak of LEM, using Neutral red bioassay.[4] For comparison, two fumonisins, FB_1 and FB_2 were included. It is eventually hoped to develop a cell line bioassay as an initial screen for the presence of toxic substances such as the fumonisins which could then be confirmed by appropriate chemical analysis.

METHODS AND MATERIALS

Chemicals: Fumonisin standards were purchased from CSIR, Pretoria, South Africa. Minimum essential medium (MEM), trypsin, and supplements to the culture medium were purchased from Life Technologies Laboratories, Grand Islands, New York. Neutral red dye (NR) was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin.

Growth of mammalian cells: Baby hamster kidney (BHK-21) cells obtained from the American Type Culture Collection (Rockville, Maryland) were maintained in monolayer culture in 25 cm^3 triangular canted-neck tissue culture flasks (Corning Glass Works, Corning, New York). MEM supplemented with 11% heat inactivated fetal calf serum, 11% tryptose phosphate, 2% glutamine was used as the growth medium. A solution of Pen-Strep-Amphotericin B (2%) was also added to the medium. Cells were grown for 3-4 d at 37^0 C in a 5% CO_2 incubator and then cells (10 ml) were subcultured at a density of $1x10^5$ per ml in 25 cm^3 flasks for 3 d. The cells were separated from each other using 0.25% trypsin and a 0.2 ml sample of the cell suspension (2×10^4 cells) was dispensed into microtiter plates (Falcon 96, Kamstrup, Denmark) and incubated in a CO_2 incubator at 37^0 C for 2 d to stimulate monolayer cell formation.[3,5,7]

Neutral red bioassay: *Fusarium moniliforme* culture extracts from 2 isolates (from feed corn associated with LEM), and extracts from 2 other isolates (from non-LEM associated corn) were used for the present investigation. *Fusarium*

moniliforme culture extracts dissolved in 1% methanol (25-200 µl/ml) were added to microtiter plate wells containing cells. Fumonisin standards were also included (10-200 µg/ml) for comparison. Each treatment was replicated six times and included a 1% methanol control. .Treated-cells were incubated for 2 d as previously described. Then the medium over the cells was removed using a Pasteur pipette and replaced with a fresh medium containing 40 mg/ml of neutral red dye (NR), and the plates were incubated for 3 h in a 5% CO_2 incubator to allow for uptake of the NR dye into the lysosomes of viable cells. The medium was then removed using a Pasteur pipette, and the cells were washed with 0.5% formaldehyde-1% calcium chloride. NR dye was extracted from viable cells by adding 0.8 ml of 1% acetic acid in 50% ethanol and pipetting the solution up and down several times. The dye containing solution was collected in 3 ml vials. After 10 min at room temperature, absorbance of the extracted dye was measured at 540 nm using a Spectrophotometer (Diode Array, Hewlett-Packard,).[6,7]

The level of absorbance was calculated by subtracting the absorbance value of each dye sample (culture extract or standard) from the control. A graph of absorbance difference vs. volume of culture extract added per ml or concentration of fumonisin added per ml was plotted. Regression analysis was carried out using Lotus 123 computer program. Fifty percent cell death i.e., mid-point lethality volumes (LV_{50}) for *F. moniliforme* culture extracts and mid-point neutral red values (NR_{50}) for fumonisin standards, were found using the regression line.[6,7] Two bar graphs were drawn for LV_{50} and NR_{50} data obtained using the regression line. Coefficients of determination (r^2) were also calculated for cytotoxicity data for BHK-21 cells.

RESULTS AND DISCUSSION

Regression output obtained for BHK-21 cells treated with *F. moniliforme* culture extracts and fumonisins is presented in Table 1. Coefficient of determination (r^2) for *F. moniliforme* culture extracts and fumonisin standards indicate that only 90-95% of the variability of difference (between the control and each culture extract/standard fumonisin) is explained by volume of culture extract or concentration of standard fumonisin added. The closer the r^2 value to 100%, the more precise the cytotoxicity values obtained using the neutral red bioassay. Since r^2 values are close to 100%, we conclude that the absorbance difference values corresponding to various volumes of culture extracts or concentrations of fumonisin standards added are precise, and therefore the mid-point lethality volumes (LV_{50}) or mid-point neutral red values (NR_{50}) calculated are also precise.

A summary of the results of NR analysis of *F. moniliforme* extracts/ standard fumonisin treated cells as indicated by their LV_{50} and NR_{50} are presented in Table 2, Figure 1 and Figure 2. All *F. moniliforme* culture extracts were toxic to BHK-21 cells and had LV_{50} volumes of 56-71 µl/ml of medium to

same cells. Culture extracts from isolates 1 and 2 (from non-LEM corn) and 23 (from LEM-associated corn) showed comparable LV_{50} volumes i.e., 60 ± 4.0, 60 ± 3.0, and 56 ± 3.4 µl/ml respectively. A slightly higher volume (71.5 ± 3.2 µl/ml) of isolate 33 culture extract was needed to cause LV_{50} to the same cells (Table 1 and Figure 1).

Table 1: Regression output for optical density difference vs. volume of *F. moniliforme* culture extract or fumonisin added (per ml).

Extract/Fumonisin	n	df	a	b	r^2
1	8	6	-14.90	86.62	90.82%
2	8	6	-11.99	102.60	94.90%
23	8	6	-13.25	88.12	93.56%
33	8	6	-11.66	102.90	94.07%
FB_1	7	5	-7.00	65.50	94.44%
FB_2	9	7	-21.41	128.17	92.28%

n = number of observations; df = degrees of freedom (n-2); a = constant (Intercept); b = regression coefficient; r^2 = coefficient of determination.

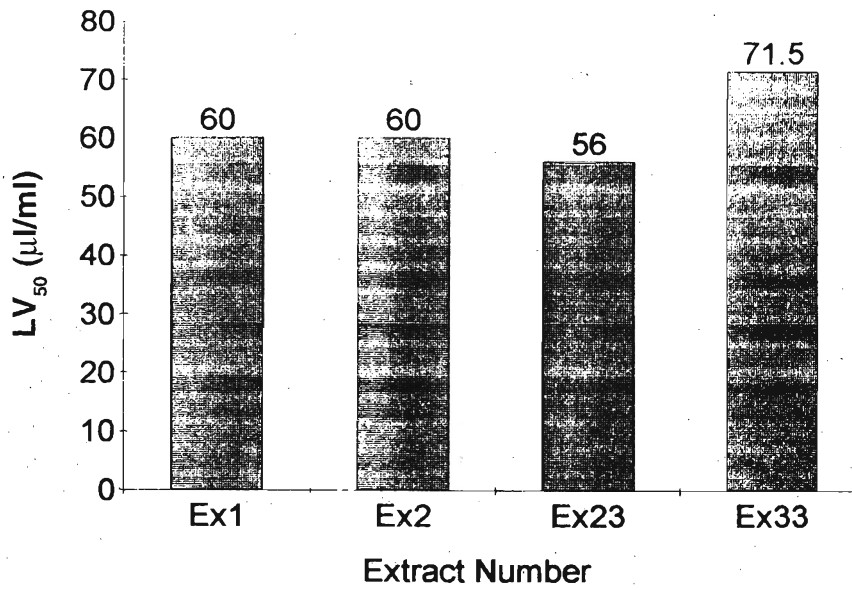

Figure 1: The distribution of mid-point lethality volumes (LV_{50}) to BHK-21 cells exposed to *F. moniliforme* culture extracts. Values represent the mean of six replicates.

Table 2: LV_{50} and NR_{50} data for *F. moniliforme* culture extracts and fumonisins (from regression line).

Extract	LV_{50} ± sd (µl/ml)	Fumonisin standard	NR_{50} ± sd (µg/ml)
1	60.0 ±4.0	FB_1	60.0 ± 2.2
2	60.0 ± 3.0	FB_2	74.0 ± 3.3
23	56.0 ± 3.4		
33	71 ± 3.2		

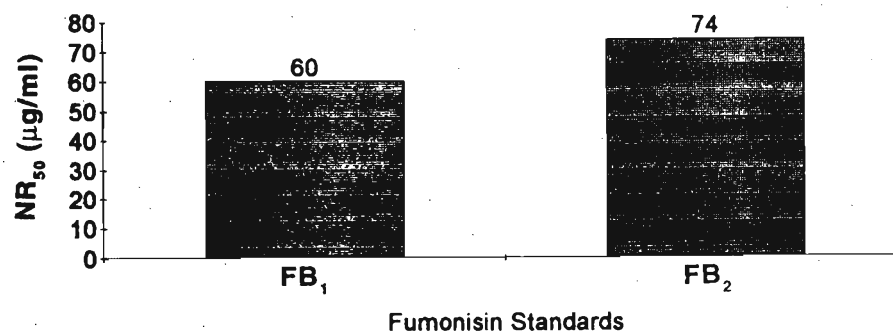

Figure 2: The distribution of mid-point neutral red values (NR_{50}) to BHK-21 cells exposed to fumonisin standards. Values represent the mean of six replicates.

FB_1 and FB_2 standards were both toxic to BHK-21 cells. FB_1 level needed to cause 50% cell death (NR_{50}) was 60 ± 2.2 µg/ml. A slightly higher level of FB_2 (74 ± 3.3 µg/ml) was needed to cause NR_{50} to BHK-21 cells compared to the amount of FB_1 needed indicating FB_1 is more potent.

All *F. moniliforme* culture extracts have been analyzed for the presence of fumonisins using HPLC-fluorescence detection.[7] FB_1 levels associated with the culture extracts were 216,177,160 and 50 µg/ml in 1,2,23 and 33 respectively.[7] In the same extracts FB_2 levels were 35, 50, 28 and 14 µg/ml. The cytotoxic effect on BHK-21 cells could most probably be due to the combined effect of FB_1 and FB_2. Even though fairly high levels of extracts (containing fumonisin) were needed to demonstrate LV_{50}, considerably low levels of standard FB_1 was needed to demonstrate 50% cell death to the same cells. This could be attributed to the presence of many interfering compounds such as fusarins, zearalenone and other metabolites produced by the fungus.

The present bioassay further indicated that FB_2 is slightly less toxic to BHK-21 cells (by requiring higher amount to cause 50% cell death). However a similar survey indicated that FB_2 is three to four times more toxic to turkey lymphocytes.[8] The two contrasting findings indicate that toxicity demonstrated by fumonisins B_1 and B_2 may depend on the cell line on which their effect is tested. The similarity in cytotoxicity of *F. moniliforme* from LEM-associated and non-LEM corn points out the variability in fumonisin production by various strains of the same fungus, therefore doing microbial analysis such as identification of strains and testing for known metabolites only in corn samples suspected of being toxic, would not be reliable. Of greater value is the use of mammalian cell line bioassays to detect any toxic metabolites in food or feed samples before chemical analysis is done to test for known mycotoxins. Neutral red is a rapid bioassay which gives quantitative and precise results that could be adapted to screen fungal extracts for the presence of toxic metabolites.

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