Fusarium and Fumonisin Occurrence in Argentinian Corn at Different Ear Maturity Stages

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The distribution of *Fusarium* species (*Liseola* section) and fumonisins B_1, B_2, and B_3 (FB_1, FB_2, and FB_3) in corn at the field stage to harvest time has been investigated. Corn samples were obtained from the 45th day after flowering to harvest stage, with 15 day intervals. A good correlation between fungal species and fumonisin contamination was observed. In particular, fumonisin contamination was higher in samples with fungal infection represented mainly by *Fusarium moniliforme* and *Fusarium proliferatum*, two well-known producers of fumonisins. Low levels of fumonisins were detected at the first and second samplings, when mainly *Fusarium subglutinans*, a fumonisin nonproducer species, was recorded. The three samplings after physiological maturity showed predominant *F. moniliforme* and *F. proliferatum* infection and a considerable contamination with fumonisins. In these samples the levels of FB_1 averaged above 1 μg/g and FB_2 and FB_3 levels showed similar values but lower than FB_1 values. This is the first report of fumonisins in Argentinian corn at field stages.

Keywords: *Fumonisins; Fusarium; corn; Argentina*

INTRODUCTION

Corn is one of the main crops grown in Argentina; the province of Cordoba supports about 25% of the national production.

Fusarium species are common pathogens of the maize plant (*Zea mays* L.); among them, *Fusarium moniliforme* Sheldon and *Fusarium proliferatum* (Matsushima) Nirenberg are two species of the section *Liseola* that occur worldwide associated with corn diseases. Both species are known to produce a new group of mycotoxins, the fumonisins. Fumonisins B_1, B_2, and B_3 (FB_1, FB_2, and FB_3, respectively) account for most of the fumonisins that are found in naturally contaminated corn samples (Nelson et al., 1993).

The occurrence of fumonisins in corn-based feeds has been associated with field outbreaks of equine leukoencephalomalacia (ELEM) and porcine pulmonary edema (Ross et al., 1990; Thiel et al., 1992; Caramelli et al., 1994). Also, FB_1 has been shown to possess cancer-promoting activity in rats (Gelderblom et al., 1991). Fumonisin contamination in Argentina was reported in corn harvested in Buenos Aires province at levels ranging between 1585 and 9990 ng/g (Sydenham et al., 1993). Relatively low fumonisin levels were found in Argentinian corn samples (1991 crop) exported to South Africa (Rheeder et al., 1994). *F. proliferatum* high-level fumonisin producers were isolated from corn harvested in Cordoba province (Chulze et al., 1995).

This investigation was undertaken to evaluate the contamination with *Fusarium* section *Liseola* species and fumonisins in corn harvested in Cordoba province at different maturity stages.

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EXPERIMENTAL PROCEDURES

Sampling. For sampling, a stratified random sampling design (SRSD) was followed (Delp et al., 1986). The population in the study (5000 plants) was divided into 10 sectors of uniform size (50 m—10 plants × furrow).

Sample sites were randomly located in each sector, and sampling was done periodically at 15 day intervals from the 45th day after flowering until harvest time (105th day). Physiological maturity was reached after 60 days. Each sample consisted of 10 consecutive plants in the same furrow (10 ears). The ears were hand-harvested, the kernels were shelled and pooled, and a subsample of 100 g was taken to determine fungal and fumonisin contamination.

Fungal Isolation and Identification. From each sample of 10 corn ears, a subsample of 100 corn kernels was surface-disinfected with NaClO (1%) for 1 min and rinsed with distilled water (three times). One hundred kernels were plated (10 kernels per Petri dish) onto a modified pentachloronitrobenzene medium (PNBC). The PNBC plates were incubated at 24 °C for 7 days under 12/12 h photoperiod cold white and black fluorescent lamps. *Fusarium* species that developed from the kernels were then identified according to the guidelines of Nelson et al. (1983).

Representative cultures of the species isolated were grown from single conidia for 10—14 days on Petri dishes of carnation leaf agar (CLA), KCl medium, potato dextrose agar (PDA) slants, and soil agar for 30 days at 24 °C with a 12/12 h photoperiod under cold white and black fluorescent lamps.

Fumonisin Production. Erlenmeyer flasks containing 100 g of corn kernels and 35 mL of distilled water were autoclaved twice for 30 min at 121 °C. After cooling, corn was inoculated with an aqueous suspension of conidia (1 mL) of 10^6 spores obtained from CLA culture and incubated in the dark at 25 °C for 28 days. To avoid clump formation, the cultures were hand-shaken during the first days of incubation and thereafter as necessary. Corn cultures were then dried at 50 °C, and finely ground with a laboratory mill, and stored at 4 °C until fumonisin analysis.

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Table 1. Distribution[a] of *Fusarium* Species in Corn Ears at Different Maturity Stages

sampling (days)[b]	sample	total *Fusarium* spp. in sample	*Fusarium* species (section *Liseola*) isolated			
			F. moniliforme	*F. proliferatum*	*F. subglutinans*	others[c]
first (45)	I2B	100		74	10.5	15.5
	I3B	100	40	23	31	6
	I4B	71	24	14	62	
	I5B	100	12.5		75	12.5
	I6B	100			89.5	10.5
	I9B	100	21		79	
	I10B	100			82	18
total			**14**	**16**	**61**	**9**
second (60)[d]	I1C	54		20	80	
	I3C	100		10	30	60
	I4C	93		21	71	8
	I5C	50	16	9	75	
	I6C	60	10	20	60	10
	I8C	62.5	73	9	18	
	I10C	62.5			100	
total			**14**	**13**	**62**	**11**
third (75)	I1D	100		100		
	I2D	100	25	65	10	
	I5D	100	13	57	30	
	I6D	50		49	14	37
	I7D	100	7	28.5	28.5	36
	I8D	100	34	61		5
	I9D	75		41		59
	I10D	100	29	59	6	6
total			**13**	**58**	**11**	**18**
fourth (90)	I1E	75	50	25	12.5	2.5
	I2E	65		60	30	10
	I5E	92		80	10	10
	I6E	65	53	12	23	12
	I7E	85	25	15	60	
	I8E	73	40	40	20	
	I9E	71	21	46	33	
total			**27**	**40**	**27**	**6**
fifth (105)[e]	I1F	100	90.5	5	4.5	
	I2F	100	18	36	41	5
	I3F	100	90		7	3
	I4F	100	85	8	7	
	I6F	78	90		10	
	I7F	80	30	30	40	
	I8F	92	95		5	
	I9F	75	58	17	25	
total			**70**	**12**	**17**	**1**

[a] Data are expressed as percentage. [b] Days after flowering. [c] *Fusarium* of the other sections. [d] Physiological maturity. [e] Harvest.

Fumonisin Analysis. The following procedure, mainly based on the method originally reported by Shephard et al. (1990), has been used for the analysis of fumonisins in corn samples and cultures.

Subsamples of about 100 g were finely ground in a Buehler laboratory mill and thoroughly mixed. Aliquots of the ground subsamples (25 or 10 g for corn or culture, respectively) were shaken with 50 mL of methanol/water (3:1) for 30 min and filtered through Whatman No. 4 filter paper. While the flow rate was maintained below 2 mL/ min, 10 mL of the filtered extract was applied to a Bond-Elut strong anion-exchange (SAX) cartridge (Varian, Harbor City, CA) fitted to a Supelco solid-phase extraction (SPE) manifold (Supelco, Bellefonte, PA), previously conditioned by the successive passage of methanol (5 mL) and methanol/water (3:1, 5 mL). The cartridge was then washed with methanol/water (3:1, 8 mL) followed by methanol (3 mL), and fumonisins were eluted with 0.5% acetic acid in methanol (14 mL). The eluate was evaporated to dryness at 40 °C, under a moderate stream of nitrogen, and stored dry at 4 °C until HPLC analysis.

The residue after cleanup was redissolved in 200 μL of acetonitrile/water (1:1). An aliquot (50 μL) of this solution was derivatized with 200 μL of *o*-phthaldialdehyde (OPA) solution obtained by adding 5 mL of 0.1 M sodium tetraborate and 50 μL of 2-mercaptoethanol to 1 mL of methanol containing 40 mg of OPA. The fumonisin OPA derivatives (20 μL solution) were analyzed using a reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of a 2150 LB pump (Bromma, Sweden) connected to a Perkin-Elmer MPF-44 B spectrofluorometric detector and a Perkin-Elmer LCI/ 100 integrator (Perkin-Elmer, Norwalk, CT). Chromatographic separations were performed on a stainless steel Supelcosil LC-ABZ, C_{18} reversed-phase column (150 × 4.6 mm i.d., 5 μm particle size; Supelco) connected to a Supelguard LC-ABZ precolumn (20 × 4.6 mm i.d., 5 μm particle size; Supelco). Methanol/0.1 M sodium dihydrogen phosphate (75: 25) solution adjusted to pH 3.35 with orthophosphoric acid was used as mobile phase, at a flow rate of 1.5 mL/min. Fluorescence of the fumonisin OPA derivatives was recorded at excitation and emission wavelengths of 335 and 440 nm,

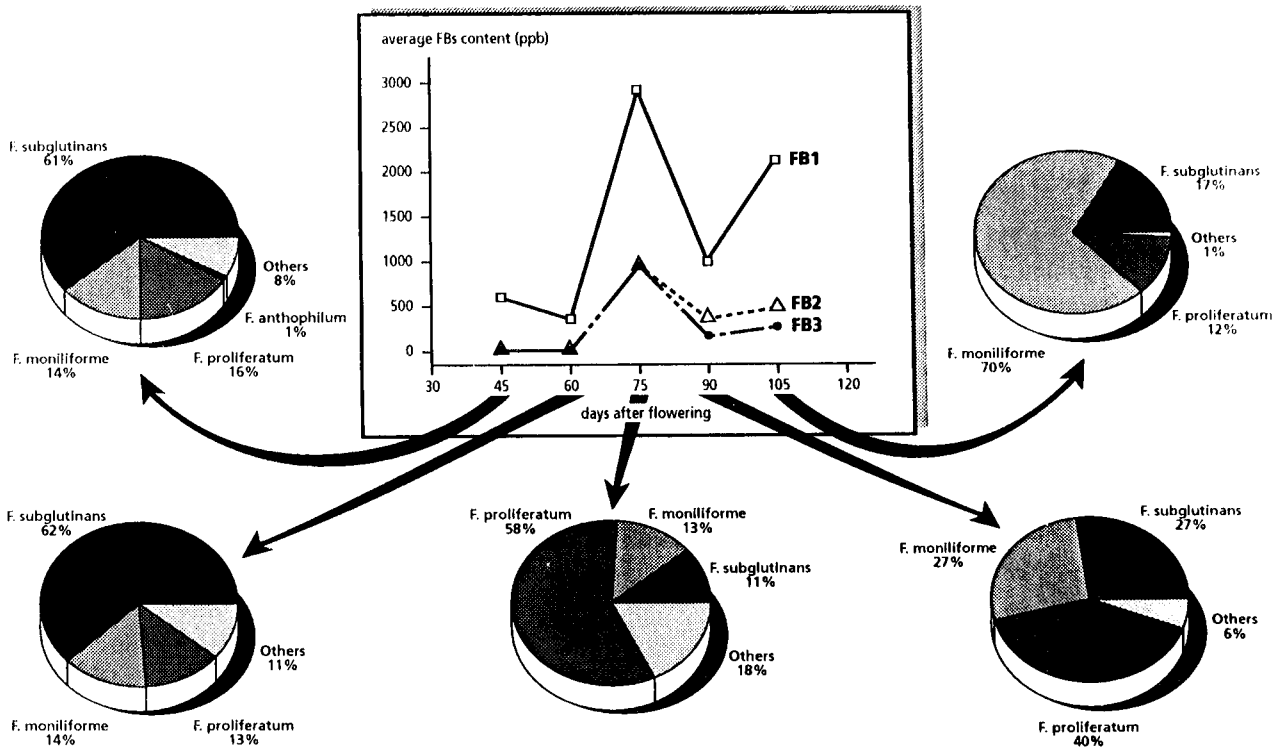

Figure 1. Fumonisin occurrence and fungal contamination at different stages of corn ear development (sample I1D has not been considered in the calculation of the average FB content at 75 days because of the exceptionally high FB_2 content).

respectively. Fumonisin quantification was performed by peak height measurements and comparison with reference standard solutions. The standard solution was obtained by dissolving pure FB_1 and FB_2 (CSIR, Division of Food Science and Technology, Pretoria, South Africa) in acetonitrile/water (1:1), at concentrations of 100 and 50 μg/mL, respectively. FB_3 concentrations were estimated on the basis of the FB_2/FB_3 signal ratio derived from a reference solution provided as part of a AOAC/IUPAC interlaboratory study (PROMEC, Tygerberg, South Africa). Appropriate dilutions of standards and/or sample extracts were made with acetonitrile/water (1:1). The limit of detection of the analytical method was 20 ng/g for the toxins.

Statistics. The GraphPAD InStat (Sigma, St. Louis, MO) was used for regression analysis and to determine differences between means (unpaired *t* test) and medians (Kruskal-Wallys nonparametric ANOVA test) of groups of samples with different levels of infection by *F. moniliforme* and *F. proliferatum*.

RESULTS AND DISCUSSION

The evolution of fungal contamination at different stages of corn maturity showed that *F. subglutinans* (Wollenw & Reinking) Nelson, Toussoun & Marasas, was the prevalent species during the first stage (60 days after flowering), while *F. moniliforme* Sheldon became the major species in the later maturity stages (from 90 days after flowering to harvest). *F. proliferatum* (Matsushima) Nirenberg was found with higher frequency in the intermediate stages, i.e., 75−90 days after flowering (Table 1). Fumonisin occurrence at different stages of corn development is reported in Table 2.

A good agreement was found between fungal contamination and fumonisin occurrence (Figure 1). In particular, the high levels of fumonisins at the medium and late stages of maturity are related with the occurrence of *F. proliferatum* and *F. moniliforme*, both good producers of fumonisins (Ross et al., 1990; Nelson et al., 1993; Visconti and Doko, 1994). On the other hand, in the first period after flowering the lower occurrence of fumonisins is determined by the predominance of *F. subglutinans*, a nonproducer of fumonisins (Nelson et al., 1993; Visconti and Doko, 1994).

Statistical analysis of the overall data showed a good correlation between fumonisin-producing species and fumonisins content. In particular, the regression analysis of the percent of fumonisin-producing strains vs logarithm of total fumonisins ($n = 35$, after two outliers, I8C and I9F, were excluded) showed an extremely significant correlation ($P < 0.001$; $r = 0.61$). Moreover, the mean and the median of the fumonisin content in samples with *F. moniliforme* and *F. proliferatum* infection levels higher than 67% were significantly higher ($P < 0.05$) than in groups of samples with 0−33% and 33−67% infection by these species.

Although *F. moniliforme* and *F. proliferatum* are responsible for the formation of fumonisins at harvest time, the occurrence of *F. subglutinans* at an earlier stage should not be neglected, due to the ability of this species to produce other toxins such as moniliformin and beauvericin (Logrieco et al., 1993).

The average levels of fumonisins found at the third sampling were unexpectedly higher than in other samplings. Also, a wide dispersion was observed among the results of this particular sampling time. Besides, an exceptionally high concentration of FB_2, higher than that of FB_1, was detected in sample I1D, showing an enormous increase of the FB_2 average (Table 2). This unusual pattern of toxin distribution could be tentatively explained by the occurrence of peculiar fungal strains of *F. proliferatum* producing very high amounts of FB_2 and minor amounts of other fumonisins. This behavior has previously been shown for fumonisin producing strains isolated in Argentina (Chulze et al., 1995) as well as in other countries (Plattner, 1995). An investigation on fungal isolates from samples colleted at this particular maturity stage (third sampling) showed that all tested strains (four *F. moniliforme* and

Table 2. Fumonisin Occurrence at Different Stages of Corn Ear Development

		concn of fumonisins (ng/g)			
sampling	sample	FB_1	FB_2	FB_3	total
first	I2B	500	nd[a]	nd	500
	I3B	1100	nd	nd	1100
	I4B	378	nd	nd	378
	I5B	788	184	618	1590
	I6B	591	nd	nd	591
	I9B	427	nd	nd	427
	I10B	378	nd	nd	378
second	I1C	685	nd	nd	685
	I3C	126	nd	nd	126
	I4C	631	nd	nd	631
	I5C	408	nd	nd	408
	I6C	583	nd	nd	583
	I8C	162	nd	nd	162
	I10C	643	nd	nd	643
third	I1D	4973	11300	487	16760
	I2D	6535	3212	3537	13284
	I5D	4565	1250	722	6537
	I6D	371	42	97	510
	I7D	2119	500	292	2911
	I8D	3531	775	575	4881
	I9D	538	88	313	939
	I10D	2498	606	819	3923
fourth	I1E	2331	500	444	3275
	I2E	1206	306	167	1679
	I5E	1152	972	167	2291
	I6E	290	59	54	403
	I7E	675	95	45	815
	I8E	1020	278	180	1478
	I9E	281	72	36	389
fifth	I1F	306	163	50	519
	I2F	275	78	nd	353
	I3F	8791	2267	980	12038
	I4F	513	98	91	702
	I6F	5541	1530	746	7817
	I7F	272	156	50	478
	I8F	1265	375	169	1809
	I9F	85	nd	nd	85

[a] nd, nondetected, <20 ng/g.

Table 3. Fumonisin Production by *Fusarium* Strains Isolated from Corn Collected at the Third Sampling (75 Days after Flowering)

	concn (μg/g)			
strain	FB_1	FB_2	FB_3	total
F. moniliforme				
I10D001	2197	889	900	3986
I10D007	2216	589	417	3222
I10D008	1032	204	231	1467
I10D009	1903	524	358	2785
F. proliferatum				
I1D003	293	1216	11	1520
I1D012	250	1749	13	2012
I5D009	1107	747	112	1966
I5DO11	1180	746	117	2043
I10D011	298	2062	15	2375
I10D012	1103	342	45	1490
I10D014	1037	487	139	1663

[a] Detection limit FB: 20 ng/g.

seven *F. proliferatum*) produced high levels of fumonisins (1.5−4.0 mg/g) and that three of them (all belonging to *F. proliferatum* species) produced more fumonisin B_2 than fumonisin B_1 (Table 3). Two of these three high fumonisin B_2 producers (I1D003 and I1D012) were indeed the only representatives isolated of sample I1D, and this strongly supports the unusually high content of fumonisin B_2 (also in comparison to fumonisin B_1) in this sample (Table 2).

This is the first report on the occurrence of fumonisins in corn at the field stages in Argentina. Fumonisin occurrence in corn grown in Cordoba province was detected at early stages of maturity until harvest time. The predominant fumonisin was FB_1, although FB_2 and FB_3 were also found. It is noticeable that, with the exception of one sample, FB_2 and FB_3 could be detected after the second sampling. This indicates that FB_2 and FB_3 are formed in the field at a later stage compared to FB_1.

The Mycotoxin Committee of the American Association of Veterinary Laboratory Diagnosticians recommended that concentrations greater than 5, 10, 50, and 50 ppm should not be feed to horses, pigs, beef cattle, and poultry, respectively (Riley et al., 1993). Following these advisory levels, at least two samples of corn at harvest time should be considered hazardous to feed to horses and pigs.

The toxin levels detected at harvest time were comparable to those of Buenos Aires province (Sydenham et al., 1993). Although corn is one of the major crops grown in Argentina, human dietary consumption within the country is relatively low compared to that of wheat-based products. Nevertheless, the levels of fumonisins found in corn are a matter of concern because the health implication of low levels of fumonisins in human foods is unknown.

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