Occurrence of Corn Mycotoxins in Galicia (Northwest Spain)

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A systematic study of the presence and concentration of pathogenic fungi and mycotoxins in corn in Galicia was carried out during a 3-year period with special attention to the influence of the storage method. Toxicity assays (*Artemia salina* and rabbit skin test) followed by chromatographic analysis showed the presence of zearalenone (0.8–9.6 ppm) and deoxynivalenol (43–315 ppb) in a significant number of samples while aflatoxins, T-2 toxin, and diacetoxyscirpenol were not detected. Ten different fungus species were isolated from the toxic samples. Eight of these species were *Fusarium*, of which *Fusarium monoliforme* and *Fusarium poae* were the most common. Although the 1984 collection was found to be more toxic than those of 1985 and 1986, no relationship could be established between the toxicity of samples, the general climatic data, and the hybrid of autochthonous character of corn. However three geographical areas were found to have a higher incidence of toxic samples. In addition, the storage procedure was found to greatly influence the development of mycotoxins.

In Galicia, a region in northwest Spain, the production of corn is of significant economical importance. In 1988 a total of 541 000 tons of cereals were grown, of which corn accounted for 413 000 tons. The majority of this corn (ca. 70%) was produced in the western provinces of A Coruña and Pontevedra. The harvest and storage of corn are still carried out in a traditional fashion on family smallholdings. The freshly collected ears are stored for several months without further processing in small buildings called horreos until they are used as animal feed. In addition, a relatively small quantity is used to prepare traditional breads for human consumption. Horreos are ventilated store houses built from granite and raised from the ground to prevent attack by rodents.

Due to the high air humidity and the general climatic conditions in Galicia, there is a certain risk of attack of the corn by several fungi. The presence of these fungi may result in the generation of mycotoxins that are toxic to both livestock and man (Ueno, 1983).

In this paper we report the result of a 3-year study of the incidence, concentration, and identity of the mycotoxins present in corn stored in horreos. In addition, a taxonomic investigation of the sources of fungal metabolites was carried out.

EXPERIMENTAL SECTION

Collection of Samples. A total of 55 samples of corn were taken directly from the field from the provinces of A Coruña and Pontevedra in October and November 1983. Further samples were collected annually between 1984 and 1986 during April and May (110 samples in 1984, 20 samples in 1985, 25 samples in 1986) from horreos in the above provinces.

For this study four or five ears per sample (about 1 kg) were chosen at random. The ears were husked and shelled, and the grains were dried for several days on a tray at 40 °C. The samples were then stored in paper bags at 0–4 °C until required for analysis.

The moisture of the corn as harvested was 25–35%, and the moisture of the corn after storage was 15–20%.

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Standards and Solvents. Standards of aflatoxins, zearalenone (**1**), T-2 toxin (**4**), and diacetoxyscirpenol (**3**) were obtained from Spectrix. Deoxynivalenol (**2**) was a gift from Dr. Peter M. Scott. Dieldrin (Polyscience Corp., analytical standards) was used as internal standard for GLC.

Cyclohexane for GLC was purchased from Carlo Erba. Acetonitrile, methanol, and the other solvents were HPLC quality.

Toxicity Assays. All the samples were tested by the *Artemia salina* toxicity assay as described: The ground sample (25 g) was poured into a 250-mL Erlenmeyer flask, and then chloroform (75 mL) was added. This mixture was stirred for 30 min and filtered through a rapid-flow filter paper. The filtrate was dried and concentrated under reduced pressure, and the residue was dissolved in acetone (1 mL). Aliquots (100, 25, 5 μL) of this solution were transferred to three cavities in a porcelain plate for spot test analysis. Standards of the different toxins were placed in other cavities of the plate.

The toxicity was assayed according to the method of Harwig and Scott (1971). Those samples showing a death rate greater than 80% in the 5-μL assay were considered toxic.

The toxic samples were submitted to the rabbit skin test according to the method of Chung et al. (1974). The extract (2 μL) was dissolved in ethyl acetate (1 mL) and was applied to the closely clipped skin of a rabbit. The skin reactions were read at 1, 2, and 3 days. In order to obtain homogeneous results, only rabbits of the California variety were employed.

Isolation of Fungi. Single-spored isolates from infected ears were cultured on autoclaved and toxin-free corn that had previously been washed with sodium hypochlorite solution (1%) and then with sterile physiological serum. Five grains were placed on a Petri dish containing Sabouraud and potato dextrose agar media. These cultures were maintained at 25 °C and were examined after 5 and 7 days. Grown colonies were then introduced in glass tubes containing Sabouraud honey and potato dextrose agar media.

The taxonomic determination of the fungi was carried out after macro- and microscopic examination according to Booth's key (1971).

TLC Screening of Mycotoxins. Aflatoxins B_1, B_2, G_1, and G_2, zearalenone, deoxynivalenol, T-2 toxin, and diacetoxyscirpenol were qualitatively analyzed by TLC according to the method of Gimeno (1979).

Two aliquots of each sample extract (10 μL) were chromatographed on two different silica gel 60 TLC plates (Merck 5721).

Aflatoxins were determined on the first plate by comparison with aflatoxin standards B_1, B_2, G_1, and G_2 by visualization

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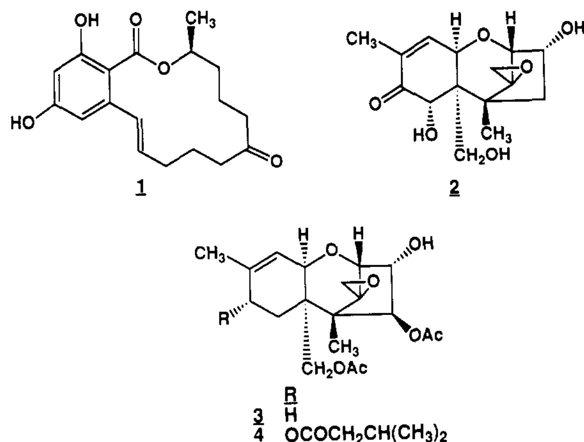

with ultraviolet light after developing with ethyl ether and then with chloroform–acetone (22:3) or with chloroform–ethyl ether–acetic acid (17:3:1).

Zearalenone and tricothecenes were determined on the second plate as follows. Initially, the plate was developed with hexane–ethyl ether–acetic acid (70:30:2), and zearalenone was visualized under ultraviolet light at 366 and 254 nm. Subsequently the plate was developed with hexane–ethyl acetate (1:3), then sprayed with ethanolic solution of aluminum chloride (20%), and heated to 110 °C for 10 min. At this stage deoxynivalenol appeared as a blue fluorescent spot under 366-nm ultraviolet light. Finally the plate was sprayed with sulfuric acid–methanol (1:4) and heated to 130 °C for 10 min. T-2 toxin and diacetoxyscirpenol gave greenish blue and dark gray fluorescence spots, respectively.

Quantitation of Deoxynivalenol by GLC. The samples were extracted and purified according to the method of Gimeno (1979). These samples were transferred to a silica gel column with toluene–acetone (95:5) and then eluted with ethyl acetate–toluene–methanol (20:5:1). The solvent was evaporated under reduced pressure, and the solid residue was dissolved in toluene–acetonitrile (49:1, 1 mL). An aliquot (100 μL) of this solution was transferred to a 4-mL tube, and then toluene–acetonitrile (49:1, 1 mL) and heptafluorobutyric anhydride (50 μL) were added. The tube was sealed and heated to 60 °C for 30 min. At the conclusion of this period, the tube was cooled to room temperature, then an aqueous solution of sodium bicarbonate (3%, 1 mL) was added, and the mixture was stirred for 2 min and centrifuged. A portion of the organic layer (0.5 mL) was concentrated under reduced pressure and dissolved in 1 mL of a solution containing 1 ppm of dieldrin in cyclohexane as the internal standard.

The accuracy of the method was determined by the assay of known concentrations of pure deoxynivalenol.

GLC analyses were carried out on a Konik 3000 HRGC gas chromatograph equipped with an electron capture detector and a fused silica capillary column (30 m × 0.25 mm) packed with DB-5.

Chromatographic parameters: carrier gas, H_2 at 1.2 mL/min; oven temperature, 180 °C for 1 min and then programmed to 228 °C at 4 °C/min followed by heating to 254 °C at 1°C/min. The final temperature was maintained for a further 3 min. Under these conditions the retention times were 8.8 min for deoxynivalenol and 11.0 min for dieldrin.

Quantitation of Zearalenone by HPLC. The extracts containing zearalenone were dissolved in 1 mL of benzene–acetonitrile (49:1), and this solution was applied to a silica gel 60 preparative TLC plate. Elution with chloroform–methanol (97:3), extraction of the band coincident with authentic zearalenone with chloroform–methanol (9:1), and evaporation of the solvent afforded the crude zearalenone. The resulting residue was dissolved in acetonitrile–methanol–water (1:1:1) (5 mL) and submitted to HPLC analyses on a Perkin-Elmer Series 10 chromatograph equipped with a C_{18} Spherisorb column (15 cm × 4.6 mm) and a Perkin-Elmer LS-3 spectrofluorimeter as detector. The mobile phase was acetonitrile–methanol–water (1:1:1) with a flow rate of 1 mL/min. A calibration curve was previously established by injecting 2.5, 5, 10, 15, and 20 μg/mL standard solutions of pure zearalenone. Under these conditions zearalenone had a retention time of 10 min.

Table I. Distribution of Toxins and Toxicity of Corn

year	procedence	no. samples	AS[a]	2[b]	1[c]
1983	field	55	3		
1984	horreos	110	30	8	15
1985	horreos	20	6		1
1986	horreos	24	5	1	2

[a] Positive samples in the *Artemia salina* test. [b] Number of samples containing deoxynivalenol. [c] Number of samples containing zearalenone.

Table II. Species of Fungi Isolated from Toxic Corn Samples

species	no. isolates
F. moniliforme	4
F. moniliforme var. subglutinans	4
F. poae	6
F. tricintum	1
F. sambucinum	3
F. solani	2
F. graminearum	3
F. culmorum	1
Trichoderma	3
P. karoo	1

RESULTS AND DISCUSSION

Samples (55 in total; 25–35% moisture) of corn freshly harvested during the autumn of 1983 were taken from several locations in the Galicia provinces of A Coruña and Pontevedra. In addition, samples of corn stored for several months in horreos (15–20% moisture) during 1984 (110 samples, from the 1983 crop), 1985 (20 samples, from the 1984 crop), and 1986 (24 samples, from the 1985 crop) were also collected around the country.

As a general methodology, the toxicity of all samples was assayed by the *A. salina* test as described for mycotoxins, and in certain cases the rabbit skin test was also used. All the samples that proved to be toxic were submitted to a chromatographic screening of mycotoxins, and this showed the presence of zearalenone and deoxynivalenol and the absence of aflatoxins, diacetoxyscirpenol, and T-2 toxin. Furthermore, the toxic samples were analyzed quantitatively by HPLC for zearalenone and by GLC for deoxynivalenol.

Table I shows the result of the general screening and toxicity of the samples. It is observed that only a few samples of freshly harvested corn are toxic, while between 20% and 30% of the stored corn samples gave positive results in the *A. salina* test. The mycotoxins detected by TLC, HPLC, and GLC in the toxic samples were zearalenone and deoxynivalenol. It was found also that there is a poor correlation between the positive *A. salina* results and the chemical detection of the toxins. However, this is not an unexpected observation since *A. salina* larvae are sensitive to a wide range of different compounds.

Toxic corn samples were also studied from a mycological point of view, and the fungi-producing toxins were determined. Table II shows the results of these mycological studies. All but 4 of the 28 toxic samples examined along those years were found to be infected by *Fusarium* species. The most frequently found fungi were *Fusarium moniliforme* and *Fusarium moniliforme* var. subglutinans; however *Fusarium graminearum* was present in the most toxic samples.

The low toxicity of zearalenone toward *A. salina* and the abundance of *Fusarium* species prompted us to sub-

Table III. Quantitation of 1 and 2 in Toxic Samples of Corn with Indication of the Fungi Isolated

sample (year)	1, ppm	2, ppb	fungus
186 (1984)	0.8		
205 (1984)	6.3	180	*F. sambucinum*
208 (1984)	9.9	315	*F. graminearum*
210 (1984)	5.8		
213 (1984)	4.9		*F. miniliforme*
214 (1984)	9.6	81	*F. moniliforme*
216 (1984)	0.7		
217 (1984)	1.2		*F. moniliforme*
251 (1984)	1.2	43	*Trichoderma*
253 (1984)	1.0	158	*F. moniliforme* var. subglutinans
260 (1984)	7.0	67	*F. solani*
272 (1984)	1.4	74	*F. graminearum*
276 (1984)	2.0	59	*Trichoderma*
282 (1984)	0.9		
283 (1984)	1.2		*F. moniliforme* var. subglutinans
25 (1985)	0.7		*F. sambucinum*
20 (1986)	1.1	60	*F. graminearum*
13 (1986)	1.0		

mit the toxic *A. salina* samples to the rabbit skin test. This skin test is a specific assay for tricothecenes and in our hands indicated that about one-third of the samples toxic to the *A. salina* larvae gave a positive reaction, suggesting the presence of trichothecenes. Interestingly, only deoxynivalenol was detected by chemical procedures in those samples (Table I).

Table III shows the results of the quantitative analysis of zearalenone and deoxynivalenol in 18 samples, with indication of the fungi producing the toxins. All samples where deoxynivalenol was present were also shown to contain zearalenone. The concentration of these toxins agrees well with the data reported in the literature (Eppley et al., 1974; Mirocha et al., 1979). In our case the amount of zearalenone varies between 9.9 and 0.7 ppm, but the 1984 collection is consistently more toxic than the others. Thus, two 1984 samples present a zearalenone concentration greater than 9 ppm and six more than 5 ppm while the 1985 and 1986 collections contain 0.7–1.1 ppm.

Interestingly, none of the samples infected by *Fusarium poae*, *Fusarium tricintum*, *Fusarium culmorum*, and *Phytomices karoo* were shown to contain toxins.

A laboratory culture of *F. graminearum* was recently reported to produce some deoxynivalenol and zearalenone derivatives (Muñoz et al., 1989). This fungus had been selected because of its occurrence in the most toxic sample (Table III, sample 208).

A comparative study of the toxicity and the autochthonous or hybrid character of the corn examined indicated that no apparent relationships exist between the presence of toxins and the fungal infection on the different varieties of cultured corn.

Analogously, an analysis of the general climatic data, temperature, rainfall, and humidity, at seven selected locations did not indicate a relationship with toxicity.

On the contrary, some geographical relationships can be found, and in fact three main areas of fungal infection and higher toxicity can be identified: They are As Mariñas, O Condado, and the area between the rivers Tambre and Ulla. The low altitude and high air humidity due to the close proximity of several rivers may apparently favor the development of fungi.

Finally, the much higher incidence of toxicity in samples taken from horreos is also noteworthy, and this together with the significantly lower humidity of the stored corn seems to indicate that infections or at least fungal growth occurs during storage.

CONCLUSIONS

In summary, our results show the incidence of *Fusarium* infection in corn harvested in Galicia. The toxins analysis showed that zearalenone was the most frequently found mycotoxin, with deoxynivalenol also being present. However, no aflatoxins, T-2 toxin, and diacetoxyscirpenol were detected. No relationship was found between the toxicity and the climatic data or the variety of cultured corn. A connection between the traditional storage method, the geographical location of the samples, and the presence of fungi infection and toxin development was demonstrated.

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LITERATURE CITED

Booth, C. The Species of *Fusarium*. In *The genus Fusarium*; Commonwealth Mycology Institute: Kew, Sunny, England, 1971.

Chung, C. W.; Truchksess, M. W.; Giles, A. L., Jr.; Friedmann, L.; Rabbit skin test for stimation of T-2 toxin and other skin-irritating toxins in contaminated corn. *J. Assoc. Off. Anal. Chem.* **1974**, *57*, 1121–27.

Eppley, R. M.; Stoloff, L.; Trucksess, M. W.; Chung, C. W. Survey of corn for *Fusarium* toxins. *J. Assoc. Off. Anal. Chem.* **1974**, *57*, 632–5.

Gimeno, A. Thin layer chromatographic determination of aflatoxins, ochratoxins, sterigmatocystin, zearalenone, citrinin, T-2 toxin, diacetoxyscirpenol, penicillic acid, patulin and penitrem A. *J. Assoc. Off. Anal. Chem.* **1979**, *62*, 579–85.

Harwig, J.; Scott, P. M. Brine Schrimp (*Artemia salina*) larvae as a screening system for fungal toxins. *Appl. Microbiol.* **1971**, *21*, 1011–16.

Mirocha, C. J.; Schauerhamer, B.; Christensen, C. M.; Kommedahl, T. Zearalenone, deoxynivalenol and T-2 toxin associated with stalk rot in corn. *Appl. Environ. Microbiol.* **1979**, *38*, 557–58.

Muñoz, L.; Castro, J. L.; Cardelle, M.; Castedo, L.; Riguera, R.; Acetylated mycotoxins from *Fusarium graminearum*. *Phytochemistry* **1989**, 83–85.

Ueno, Y. *Trichothecenes—chemical, biological and toxicological aspects*; Elsevier Science: Amsterdam, The Netherlands, 1983.

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