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FUMONISINS CONCENTRATIONS IN MAIZE AS AFFECTED BY PHYSICO-CHEMICAL, ENVIRONMENTAL AND AGRONOMICAL CONDITIONS

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ABSTRACT - The objective of the present work was to investigate factors influencing fumonisins concentrations in maize collected from 58 fields located in seven producing areas of the River Ebro Valley (north-eastern Spain). Factors studied were: moisture content and water activity of the grains, climatic conditions during growth, the use of transgenic (insect-resistant) maize seeds, and commercial drying. Fumonisins were detected in maize at harvest in 48.3% samples at a mean concentration of 632 µg/kg. Warm temperature during flowering coupled with wet weather in the last months preceding harvest were key factors to explain differences in fumonisins concentrations between the growing areas. Also, grain moisture content at harvest above a critical value of 20% (0.93 a_W) was a significant condition for fumonisins production in mature grains. Results indicated that concentrations of fumonisins may not be reduced in transgenic maize hybrids under low pressure infestation by corn borers. Damp maize after harvest was delivered to seven grain dryers located in the same production areas, and it was subjected to commercial drying until 14% moisture content. The incidence of fumonisins in dried maize was 85.7%, with a mean concentration of 352 µg/kg, indicating that this mycotoxin remained mostly stable from harvesting to drying.

KEY WORDS: Fumonisins; Mycotoxins; Maize; Agronomy.

INTRODUCTION

In the temperate climatic conditions prevailing in Europe, *Fusarium* fungi are important in the cereal food chain and can reduce crop yields and contaminate grain with mycotoxins. *Fusarium verticillioides* is generally regarded as the most important colonizer of maize grains and has the ability to produce the fumonisins mycotoxins (Logrieco *et al.*, 2002; Frisvad *et al.*, 2007). In the River Ebro Valley (northeast Spain), maize diseases caused by *Fusarium* have been reported since the early 1980s (Palazón and Palazón, 1982). This is a high-risk area for fumonisins contamination of maize, which levels during the period 1999-2003 were more than 250% above the European mean of 1.59 mg/kg (GLM, 2006). Monitoring of the recent harvests revealed that maize can be very highly contaminated by fumonisins, although variations in fumonisins concentrations have been noted between years, indicative of the substantial impact of climatic and agronomical conditions (GENVCE, 2007). In the River Ebro Valley a significant surface of maize crops is planted with insect-resistant genetically modified varieties. For the purpose of comparison, total surface of maize crops in Spain was 355,000 ha during 2007 season, including 75,148 ha of transgenic MON-810 maize, 78.3% of which (58,873 ha) was planted in northeast Spain (MARM, 2008).

Fumonisins have been involved in several animal diseases and implicated in esophageal cancer in humans (Marasas *et al.*, 2000). Fumonisin B_1 (FB_1) is possibly carcinogenic to humans (group 2B of the International Agency for Research on Cancer classification), and the Scientific Committee on Foods has established a tolerable daily intake of 2 µg/kg of body weight for the total of FB_1, FB_2, and FB_3 (EC, 2003). In October 2007, compulsory limits of fumonisins were established by Commission Regulation (EC) No. 1126/2007, with maximum limits set at 4,000 µg/ kg for unprocessed maize and 1,000 µg/ kg for maize intended for direct human consumption.

Good agricultural practices (GAP) represent the primary line of defence in controlling the contamination of cereals by *Fusarium* toxins, followed by the implementation of Good Manufacturing practices (GMP) during the handling, storage, process-

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ing, and distribution of cereals for human food and animal feed, as indicated in Commission Recommendation 2006/583/EC on the prevention and reduction of *Fusarium* toxins in cereals and cereal products. Several agronomical practices are known to affect infection and mycotoxin production by toxigenic fungi in maize (BARRIER-GUILLOT *et al.*, 2007; MAIORANO *et al.*, 2009). In a previous field trial study in the River Ebro Valley, the present authors investigated the effect of some farm management practices on the contamination of maize by fumonisins (ARIÑO *et al.*, 2009). Wet planting and the removal of debris from the previous crop significantly lowered the risk of fumonisins in maize, while higher levels of nitrogen fertilizer had a tendency to increase fumonisins levels. However, the effect of some physico-chemical, environmental and agronomical conditions needs further study.

The objective of the present work was to investigate factors influencing fumonisins concentrations in maize collected from 58 fields located in seven producing areas of the River Ebro Valley (northeastern Spain). The factors studied were moisture content and water activity of the grains, climatic conditions during growth, and the use of transgenic (insect-resistant) maize seeds. Damp maize was delivered to the corresponding seven grain dryers and it was subjected to commercial drying until 14% moisture to compare fumonisins levels from harvesting to drying.

FIGURE 1 - Map showing sampling locations of seven maize-growing areas (58 fields sampled) in the River Ebro Valley (northeast Spain).

MATERIALS AND METHODS

Maize fields

Freshly harvested maize samples were collected from 58 maize fields located in seven producing areas of the River Ebro Valley (north-eastern Spain) during the 2007 harvest year (see Fig. 1 for sampling locations). Eighty-four point five percent (84.5%) of fields were planted with conventional maize seeds, and the remaining fields (15.5%) were sown with insect protected transgenic Monsanto MON-810 seeds (identity confirmed by Trait Bt1 Test Kit, Strategic Diagnostics, Newark, DE).

In this area of the River Ebro Valley, maize is usually planted in late April to early May, flowering takes place around the beginning of July, and the crop is harvested from late October to November. The meteorological indicators of harvest season 2007 in the maize-growing areas under study are available in Table 1.

The sampling protocol was based on the principles laid down in Commission Regulation (EC) No. 401/2006 for static bulk grain lots of less than 50 tons. Sampling was done at the trailers loaded with the freshly harvested maize. To obtain representative samples, ten 250-g incremental samples were collected at 10 different points of the trailer load and combined to form the aggregate sample (2,5 kg). Each aggregate sample was thoroughly mixed to obtain a single 500-g laboratory sample, so this data set contained a total of 58 laboratory samples collected at harvest. Samples were ground in an EG-43 mill (Mahlkönig, Hamburg, Germany), thoroughly homogenized, and kept at -21°C until mycotoxin analysis.

Grain dryers

Grain must be dried to suitable moisture, usually 14% in maize, in order to prevent excessive spoilage during prolonged storage. Damp maize from the 58 fields investigated was delivered to the corresponding seven grain dryers located in the same producing areas. Prior to drying maize grains were subjected to cleaning by cleaner-separator devices to eliminate impurities, plant and insect wastes, and fractured kernels. Grain dryers have a capacity ranging from 20 to 55 tons/hour, and they used hot air (95-120°C) forced through the grain mass until final 14% moisture content was reached. Then, air-dried maize was transferred to vertical storage silos.

Sampling at the seven grain dryers was carried out 24 hours after drying, when maize kernels had reached ambient temperature. The sampling protocol consisted of collecting ten incremental samples, which were combined, thoroughly mixed, and divided into three 500-g laboratory samples per drying unit. Then, this data set contained a total of 21 laboratory samples (collected in triplicate from each of the 7 drying units). Samples were ground in an EG-43 mill (Mahlkönig, Hamburg, Germany), thoroughly homogenized, and kept at -21°C until mycotoxin analysis.

Analysis of moisture and water activity

Moisture content of samples was measured using the conventional air oven AACC Method 44-15.02 (AACC, 2000). Water activity (a_W) measurement was carried out at 25°C with an Aqualab Model CX-2 (Decagon Devices, Inc., Pullman, WA) instrument that allows temperature-controlled measurements of a_W.

Mycotoxin analysis

The analysis of fumonisins was carried out with the ROSA (Rapid One Step Assay) quantitative fumonisins test based on lateral flow immunoassay (Charm Sciences, Lawrence, MA) accord-

TABLE 1 - *Mean temperature (°C), total rainfalls (mm) and relative humidity (%) during flowering and ripening of maize fields under study in harvest season 2007.*

Mean temperature	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**
April	13.6	13.7	13.7	13.7	13.0	13.6	14.1
May	16.9	17.5	17.5	17.2	15.9	16.3	17.9
June	20.7	21.4	21.4	21.6	19.9	20.7	22.2
July	22.6	23.7	23.7	23.9	21.8	22.3	24.6
August	21.5	22.6	22.6	22.7	20.9	21.4	23.3
September	18.6	19.2	19.2	19.2	18.0	18.5	19.8
October	14.2	14.3	14.3	14.1	13.6	14.2	15.2
November	6.2	6.0	6.0	4.6	7.5	8.3	8.4
Total rainfalls	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**
April	92.8	94.2	94.2	55.0	126.8	127.4	92.9
May	39.6	28.0	28.0	33.8	36.2	46.2	3.9
June	33.6	17.8	17.8	14.4	21.6	18.7	8.0
July	0.6	10.4	10.4	2.8	4.4	0.6	1.1
August	21.0	5.8	5.8	11.2	22.6	42.6	1.2
September	22.8	3.6	3.6	11.8	0.6	8.8	10.6
October	13.0	12.2	12.2	10.4	23.2	102.2	6.5
November	7.6	7.2	7.2	7.8	3.6	1.8	2.9
Relative humidity	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**
April	72.6	71.9	71.9	78.0	74.6	72.6	71.2
May	59.6	57.6	57.6	64.2	64.8	63.1	55.7
June	57.8	55.0	55.0	58.1	58.5	55.4	49.7
July	53.5	48.1	48.1	52.9	52.1	50.7	43.6
August	58.0	52.2	52.2	57.1	57.0	54.8	47.9
September	63.5	59.2	59.2	65.2	62.0	60.4	56.8
October	67.6	65.9	65.9	71.6	69.4	67.5	63.2
November	67.0	65.2	65.2	75.4	62.2	59.0	58.4

Data from http://oficinaregante.aragon.es
Location of maize fields: (1) Lalueza, (2) Sariñena, (3) Villanueva de Sijena, (4) Binefar-Monzón, (5) Ejea de los Caballeros, (6) Alagón, and (7) Fuentes de Ebro

ing to the instructions of the manufacturer. A representative sample of 50 g of ground maize was extracted with 100 ml of 70% methanol using a Vibromatic rocking mixer (JP Selecta, Barcelona, Spain) for 2 min. The extract was filtered through a Whatman No. 2V filter (Whatman, Clifton, NJ), and 100 µl of filtrate was diluted with 1 ml of ROSA FUMQ dilution buffer to prepare the diluted extract; a second diluted extract was prepared by pipetting 300 µl of diluted extract to a predispensed 1 ml of ROSA FUMQ dilution buffer. A test strip was placed on the ROSA-M incubator, and 300 µl of second diluted extract was placed in a sample compartment. The resealed test strip was incubated at 45°C for 10 min, and results were read on the ROSA-M reader using the FUM channel, 3 line mode. A negative and positive control were run daily to verify performance of equipment and test strips based on internal quality assurance standards.

The ROSA strip test measures the major types of fumonisins (FB1, FB2 and FB3) and it is approved for corn by USDA-GIPSA (Certificate No. FGIS 2009-102). The analytical method was validated in-house for precision and recovery using a matrix reference material (Romer Labs, Union, MO) consisting of maize flour containing 2,406 µg/kg fumonisin B_1 and 630 µg/kg fumonisin B_2. Recovery of total fumonisins was 100%, the precision relative standard deviation (RSD) was 11%, and the limit of detection (LOD) was 100 µg/kg. The performance characteristics for the fumonisins were within the acceptable margins outlined in Commission Regulation (EC) No. 401/2006. A comparison of the ROSA test and HPLC method EN-13585 (CEN, 2001) showed a good correlation (r = +0.94), in a study with 25 maize samples presented at a national conference (Herrera *et al.*, 2009).

Statistical analysis

As stated above, this paper considers two sets of data: a total of 58 maize samples collected at harvest and a total of 21 maize samples acquired at drying units (collected in triplicate from each of the 7 grain dryers). The results from the fumonisins analyses were subjected to descriptive and comparative statistical analyses according to SACHS (1978). The incidence of fumonisins in the samples was expressed as the percentage of samples containing fumonisins at levels above the LOD (100 µg/kg). Results were recorded as the mean ± standard error, which was calculated using a value of zero for results lower than the LOD. Differences between samples were compared by ANOVA (Fisher PLSD) using StatView SE_Graphics (1988, Abacus Concepts, Inc., Berkeley, CA) for Macintosh personal computers.

RESULTS AND DISCUSSION

The frequency of fumonisins detection in field samples of maize was 48.3%, and concentrations ranged from LOD to 5,823 µg/kg. Only one sample exceeded the maximum limit for fumonisins (the sum of FB_1 and FB_2) of 4,000 µg/kg for unprocessed maize, set by the Commission Regulation (EC) No 1126/2007. Fumonisins were detected in maize at harvest in 5 out of 7 growing areas at a mean concentration of 632 ± 152 µg/kg (Table 2), ranging from not detected (Sariñena and Villanueva de Sijena areas) to 1,529 ± 431 µg/kg in Binéfar-Monzón area (ANOVA $P < 0.05$).

The significant differences observed in fumonisins incidence and levels between the seven maize-producing areas were influenced by weather conditions during the growing season. Thus, higher fumonisins levels in maize grown in Binéfar-Monzón area (1,529 µg/kg) was related to a warm temperature of 23.9°C during maize flowering in July (Table 1), although the relative humidity was moderate (52.9%), typical of the semi-arid climate during summer in this area of the River Ebro Valley. However, the mentioned Binéfar-Monzón area showed the highest humidity values during the critical months of October (71.6%) and November (75.4%), while in the other areas the relative humidity never exceeded 70%. For instance, the growing area of Fuentes de Ebro showed slightly higher temperature during flowering in July (24.6°C), but much lower humidity in July (43.6%), October (63.2%), and November (58.4%), and consequently fumonisins levels were lower (619 µg/kg). In summary, warm temperature during flowering coupled with wet weather in the last months preceding harvest were key factors to explain differences in fumonisins concentrations between the growing areas. Also, as showed in Table 2, grain moisture content at harvest was the highest in Binéfar-Monzón area (21.1%), which could have favored *F. verticillioides* growth and fumonisins production in mature grains.

Fumonisins content in conventional maize (673 ± 179 µg/kg, n = 49) was numerically higher than that in transgenic maize (405 ± 120 µg/kg, n = 9), indicating that plants protected against borer insects were somewhat less susceptible to *Fusarium* infection and concomitant mycotoxin contamination, but the difference was not significant (ANOVA $P > 0.05$). Extensive work in the United States had revealed that the implementation of genetically engineered maize hybrids containing the insecticidal genes from *Bacillus thuringiensis* can reduce *Fusarium* spp. infection and subsequent fumonisins contamination of maize indirectly by reducing insect damage (MUNKVOLD, 2003; KENDRA and DYER, 2007). In Argentina, BARROS *et al.* (2009) reported that fumonisins levels were significantly lower in transgenic than in conventional maize under conditions of natural insect infestation. Fungi may gain entrance to maize ears through wounds caused by insects, and European corn borer (ECB), *Ostrinia nubilalis*, is the dominant insect that can wound maize ears in the north-eastern production areas in Spain. However, the lack of significant differences in fu-

TABLE 2 - *Mean ± standard error of grain moisture (%), water activity (a_W) and fumonisins levels (µg/kg) in the maize fields under study in harvest season 2007.*

Location of fields	Fields sampled	Grain moisture[1]	Water activity of grain[1]	Fumonisins levels[1]
Lalueza	7	18.0 ± 0.7 a	0.88 ± 0.02 a	323 ± 201 a
Sariñena	8	17.8 ± 1.0 a	0.88 ± 0.03 a	<LOD[2] a
Villanueva de Sijena	4	19.0 ± 1.3 ab	0.91 ± 0.03 a	<LOD a
Binéfar-Monzón	17	21.1 ± 0.2 c	0.95 ± 0.01 b	1,529 ± 431 b
Ejea de los Caballeros	10	19.4 ± 0.5 ab	0.92 ± 0.01 ab	210 ± 153 a
Alagón	8	20.9 ± 0.4 bc	0.95 ± 0.01 b	395 ± 121 a
Fuentes de Ebro	4	17.5 ± 1.4 ab	0.87 ± 0.05 a	619 ± 216 ab
Mean total	**58**	**19.5 ± 0.3**	**0.91 ± 0.01**	**632 ± 152**

[1] Means followed by the same letter in a column are not significantly different (P=0.05) according to Fisher's PLSD test.
[2] LOD (limit of detection) = 100 µg/kg.

monisins levels between maize kernels grown from conventional and transgenic seed may be explained by the low degree of ECB damage during 2007 harvest year. The historic data of ECB in infected areas of the River Ebro Valley, expressed as number of borers/100 stalks, are: 345 in 2003, 125 in 2004, 155 in 2005, 140 in 2006, and 123 during 2007 (C. MARTIN, personal communication).

The moisture content in the analyzed maize samples ranged from 17.5 to 21.1%, correlative to water activity values of 0.87 and 0.95, respectively (Table 2). These results should be interpreted considering the warm and dry conditions of the summer during harvest season 2007 in most growing areas. Under those conditions, maize grains matured faster, and kernels rapidly reached the 20% critical moisture limit for *F. verticillioides* to synthesize fumonisins in kernels. Only in two maize-growing areas the moisture content at harvest was above 20% (Binefar-Monzón and Alagón), and one of them showed the highest fumonisins concentrations as mentioned above. It is well known that *Fusarium* species invade seeds and other plant tissues in the field. *F. verticillioides* is the most common fungi associated with maize in Spain (SANCHIS *et al.*, 1995; ARIÑO *et al.*, 2007). According to MARIN *et al.* (2004), the threshold water activity (a_W) for fumonisins production by *F. verticillioides* is 0.93. In our maize samples, a water activity of 0.93 corresponded to a moisture content of around 20%. Therefore, in the River Ebro Valley area, when grains have moisture content of 20% or higher, fungal development and fumonisins production can occur in the moist crop. This is in agreement with GOLOB (2007) in a recent FAO training manual indicating that if the moisture content for maize is 20%, the grain will not be safe to store and moulds will grow. For comparison, in a study in the Po-Valley of northern Italy, MAIORANO *et al.* (2009) indicated that the critical moisture limit for *F. verticillioides* to synthesize fumonisins in maize kernels was 22%.

In the last part of the study, samples of air-dried maize from the corresponding 7 grain dryers located in the same production areas were analyzed for fumonisins concentrations to compare levels from harvesting to drying. The incidence of fumonisins in dried maize was 87.5%, at a mean concentration of 352 ± 90 µg/kg, indicating that this mycotoxin remained mostly stable from harvesting to drying and storage (Table 3).

The apparent decrease in fumonisins concentrations from freshly harvested maize (632 µg/kg) to dried maize (352 µg/kg) could be attributable to the cleaning, sorting, mixing and homogenization during the drying processing, but the differences were not significant (ANOVA $P > 0.05$). The moisture content in all analyzed samples of dried maize was 14%, corresponding to a water activity of 0.71, making dried maize a shelf stable commodity thanks to a low a_W that is very restrictive for germination and growth of mycotoxigenic fungi.

CONCLUSIONS

In conclusion, the field situation with regard to fumonisins contamination in maize is complex, depending on multiple interacting factors. Results indicated that concentrations of fumonisins may not be reduced in transgenic maize hybrids under conditions of low pressure infestation by corn borers. Moreover, unforeseeable environmental factors, such as temperature, rainfall, and atmospheric humidity, were key factors to explain differences in fumonisins concentrations among the growing areas. Moisture control at harvest is extremely important to maintain the quality of the grain, as being particu-

TABLE 3 - *Comparison of fumonisins (µg/kg, mean ± std. error) in freshly harvested maize and air-dried maize at corresponding grain dryers.*

Maize-producing area	Maize at Harvest	Maize at grain-dryers	ANOVA P-values
Lalueza	323 ± 201	183 ± 116	0.5760
Sariñena	<LOD[1]	200 ± 163	0.3693
Villanueva de Sijena	<LOD	<LOD	0.4980
Binéfar-Monzón	1,529 ± 431	703 ± 160	0.2424
Ejea de los Caballeros	210 ± 153	533 ± 239	0.2500
Alagón	395 ± 121	386 ± 149	0.9617
Fuentes de Ebro	619 ± 216	457 ± 111	0.4733
Mean total	**632 ± 152**	**352 ± 90**	**0.1647**

[1] LOD (limit of detection) = 100 µg/kg.

larly relevant to damp maize with moisture content above 20% (a_W >0.93), which seems to be critical for fumonisins production. The incidence of fumonisins in freshly harvested maize was similar to that found in dried maize at corresponding grain dryers, indicating that this mycotoxin remained mostly stable from harvesting to drying.

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