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Fungal Growth and *Fusarium* Mycotoxin Content in Isogenic Traditional Maize and Genetically Modified Maize Grown in France and Spain

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Fungi of the genus *Fusarium* are common fungal contaminants of maize and are also known to produce mycotoxins. Maize that has been genetically modified to express a Bt endotoxin has been used to study the effect of insect resistance on fungal infection of maize grains by *Fusarium* species and their related mycotoxins. Maize grain from Bt hybrids and near-isogenic traditional hybrids was collected in France and Spain from the 1999 crop, which was grown under natural conditions. According to the ergosterol level, the fungal biomass formed on Bt maize grain was 4−18 times lower than that on isogenic maize. Fumonisin B_1 grain concentrations ranged from 0.05 to 0.3 ppm for Bt maize and from 0.4 to 9 ppm for isogenic maize. Moderate to low concentrations of trichothecenes and zearalenone were measured on transgenic as well as on non-transgenic maize. Nevertheless, significant differences were obtained in certain regions. The protection of maize plants against insect damage (European corn borer and pink stem borer) through the use of Bt technology seems to be a way to reduce the contamination of maize by *Fusarium* species and the resultant fumonisins in maize grain grown in France and Spain.

KEYWORDS: ***Fusarium*; mycotoxins; ergosterol; transgenic maize; *Ostrinia*; *Sesamia***

INTRODUCTION

Fusarium species are common contaminants of maize (*Zea mays* L.). Infection may be associated with yield reduction, but *Fusarium* species may also infect the plant without any symptoms (*1*, *2*). Several *Fusarium* species are also able to produce mycotoxins such as fumonisins, zearalenone, and trichothecenes, which may be dangerous for both human and animal health (*3*). The contamination of cereals by these fungi and their related mycotoxins is a worldwide problem (*4*, *5*).

Numerous factors may influence the infection of maize kernels by *Fusarium*. It has been demonstrated that damage resulting from insect feeding provides preferential sites for the penetration of the fungi, and some insects can even operate as vectors of mycotoxigenic fungi (*2*, *6*, *7*). Recent studies carried out in Italy indicated that genetic engineering of maize to protect against insect pests may affect fumonisin concentration and fungal growth (*8*). However, other experiments have indicated that significant reduction of fumonisin grain content was obtained only under certain conditions. Hybrid brands and insect infestation levels also have an effect on fumonisin concentration in maize grain (*9*, *10*).

It has also been shown that the environmental conditions found in the specific area of cultivation play an important role in the accumulation of fumonisins in maize (*11*).

The aim of this work was to study whether insect resistance can affect *Fusarium* growth and mycotoxin concentration in maize grains under natural conditions of maize culture conducted in France and Spain.

In the present study the *cry*1A(b) gene from *Bacillus thuringiensis* was introduced into maize to produce the MON 810 maize hybrid. The Cry-1A(b) protein is expressed throughout the MON 810 plant, protecting it against insect pests such as the European corn borer (*Ostrinia nubilalis*) and the pink stem borer (*Sesamia nonagrïoides*) (*12*).The larvae of these insects can be found throughout the maize-growing regions of France and Spain, where they feed the aerial parts of the plants.

Bt MON 810 hybrids and near-isogenic nonmodified hybrids, which have a genetic background similar to that of MON 810, were grown in three locations of southwestern France and in two locations of northern Spain in the same agricultural conditions. Fungal infection, assessed by the isolation of *Fusarium* strains and quantified through the ergosterol content in grain, and concentration of mycotoxins (fumonisins, trichothecenes, and zearalenone) grain content were compared.

MATERIALS AND METHODS

Maize Hybrids. The genetic modification of the MON 810 hybrids allows a constitutive expression of the Cry1-A(b) protein at all growth stages of the plant and in all plant tissues. In field experiments carried out in Europe in 1995, the average content of Cry1-A(b) protein in the kernels was 0.46 μg/g of fresh tissue versus 9.26 μg/g in the leaves (Monsanto, unpublished data). Transgenic (Bt) maize hybrids and their near-isogenic traditional hybrids (N) used in the trials were supplied,

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Table 1. Insect Feeding and Contamination of Maize Kernels by *Fusarium* Species

expt	origin	maize hybrid	mean no. of insects per plant[a]	kernels infected by *Fusarium* sp. (%)	% kernels infected by			
					F. verticillioides	*F. proliferatum*	*F. graminearum*	*F. poae*
O25	France	N	3.65	88	31	57	10	5
		Bt	0.1	47	24	22	8	1
O30	France	N	3.4	100	28	72	7	0
		Bt	0.05	67	24	40	2	0
O32	France	N	5.31	92	44	56	1	0
		Bt	0.15	43	16	27	0	0
SP1	Spain	N	2.2	100	41	61	0	0
		Bt	0.35	22	14	12	0	0
SP2	Spain	N	5.6	85	24	67	0	0
		Bt	0.4	33	11	19	0	0

[a] Number of live and dead insect larvae (*Ostrinia nubilalis* and *Seamia nonagrioides*) per plant determined when the whole plant was dissected at harvest maturity.

respectively, by RAGT for varieties DK512(N) and DK513 (Bt) and by Monsanto Spain for varieties Actor (N) and Actor Bt (Bt).

Field Experiments. Three field experiments were conducted in 1999 in southwestern France (experiments 025, 030, and 032); two were conducted in Spain, in the Catalunya province (experiment SP1) and the Aragon province (experiment SP2). The near-isogenic traditional hybrids and the Bt hybrids were sown side-by-side in 40 m long plots of 12 rows. Infestation by insects or *Fusarium sp*. was exclusively natural; no manual treatment took place.

Just before harvest, 20 randomly selected plants were dissected to measure infestation by insects, assessed through the number of dead and alive larvae of the European corn borer and pink stem borer per plant (**Table 1**). A 5 kg ear sample was collected from each plot.

Fifty kernels were used for mycological analysis. The remaining ears were dried at 60 °C with forced ventilation to 15% moisture (on a wet basis). After shelling, the kernels were ground for ergosterol and mycotoxin analyses.

The ergosterol and mycotoxin results correspond to the average of 10 samples taken after grinding. Mean values from Bt and near-isogenic hybrids were compared according to Student's *t* test.

Isolation of *Fusarium*. Maize kernels (50) were surface disinfected with 1% NaOCl for 3 min, rinsed three times with sterile water, and plated out on a *Fusarium*-selective medium, dichloran chloramphenicol peptone agar (DCPA) (*13*). After 7 days of incubation in constant daylight at 20 °C, *Fusarium* species were identified on the basis of conidial morphology according to the method of Nelson et al. (*14*). The isolated strains of *Fusarium* were thereafter purified and kept on potato dextrose agar (PDA) at 4 °C.

Determination of Kernel Moisture Content. Maize kernels (10 g) were ground and then dried in an oven at 130 °C for 4 h as previously described (*15*).

Ergosterol Analysis. The extraction, purification, and assay of ergosterol were carried out as described in Cahagnier et al. (*16*). Briefly, grain (50 g) was ground in 150 mL of methanol. Ergosterol was extracted after saponification of the sample in a mixture of methanol (150 mL), ethanol (50 mL), and potassium hydroxide (20 g). After filtration, a 20 mL aliquot of filtrate was purified on a solid-phase extraction column (Extrelut 20 R, Merck, Darmstadt, Germany). The organic phase (*n*-hexane) collected from the column was evaporated to dryness, and the residue was resuspended in the mobile phase. Ergosterol was assayed by HPLC on a silica column (SI60, 5 μm) and detected at 282 nm. Quantification was performed using external standard (Merck). The results were expressed in micrograms per gram of dry matter or parts per million.

Fumonisin Analyses. Fumonisins were measured by using the method of Sydenham et al. (*17*). This method quantifies fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3). Fumonisin was coupled to *o*-phthaldialdehyde (OPA) and assayed using an HPLC column equipped with a fluorescence detector (Shimadzu, Kyoto, Japan). Quantification using external standard was expressed in micrograms per gram of dry matter (parts per million). Fumonisin B_1 standard was purchased from Sigma Chemical Co. (St. Louis, MO).

Zearalenone Analysis. Zearalenone analysis was performed by HPLC as described in Langseth et al. (*18*), using a C8 (Equisorb, 150 × 4.6 mm, 5 μm phase thickness) column equipped with an LDC fluorescence detector (Milton Roy, Pont-Saint-Pierre, France). Quantification was performed using external standard (Sigma). The results were expressed in nanograms per gram of dry matter (parts per billion).

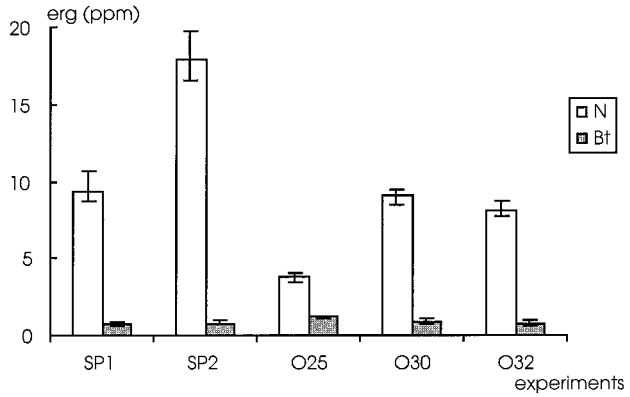

Figure 1. Ergosterol grain content from field experiments conducted in Spain (SP1 and SP2) and in France (025, 030, and 032). Errors bars represent minimum and maximum values. N, normal maize hybrids; Bt, genetically modified maize hybrids.

Trichothecene Analysis. The extraction of the trichothecenes was carried out with a mixture of acetonitrile and water (84 + 16, v/v). The purification columns were composed of a mixture of activated charcoal (60/80, Interchim, Montlucon, France) and aluminum oxide (0.63−0.20 mesh Merck) as previously described (*19*). The trichothecenes (type B) were silylated (TMSI) and then separated by GC-ECD on an HP5 column. Deoxynivalenol (DON) and nivalenol (NIV) standards were purchased from Sigma Chemical Co. The results were expressed in nanograms per gram of dry matter (parts per billion).

RESULTS

In all field experiments, mycological analysis revealed that the percentage of kernels infected by *Fusarium* was higher in traditional maize than in transgenic Bt maize (**Table 1**). The predominant *Fusarium* species isolated from Bt hybrids as well as from non-Bt hybrids were *F. verticillioides* (formally named *F. moniliforme*) and *F. proliferatum*. *F. graminearum* and *F. poae* were present only in maize grains originating from France.

The ergosterol grain contents are reported in **Figure 1**. All samples of maize grain from Bt hybrids had a content of ergosterol 4−18 times lower than grains from near-isogenic hybrids, which indicates a very low fungal contamination in Bt maize grains and corroborates the results of the mycological analysis.

Fumonisins were the major mycotoxins present in the samples. This result is also in accordance with the results of the mycological analysis, because *F. proliferatum* and *F.*

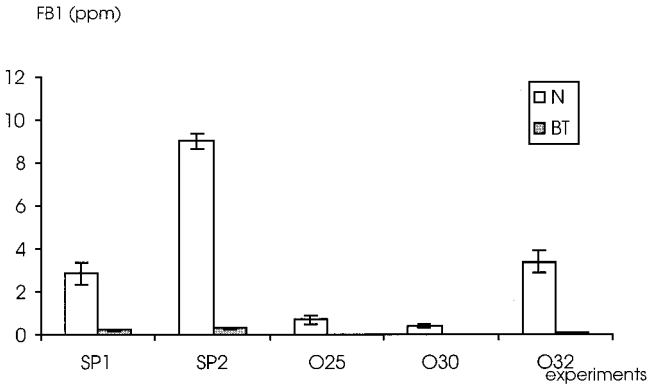

Figure 2. Fumonisin B_1 grain content from field experiments conducted in Spain (SP1 and SP2) and in France (025, 030, and 032). Errors bars represent minimum and maximum values. N, normal maize hybrids; Bt, genetically modified maize hybrids.

Table 2. Trichothecene and Zearalenone Grain Contents

		mycotoxin content[a] (ng/g)		
expt	hybrid	DON	NIV	ZEN
O25	N	472*	100	3
	Bt	729	80	tr
O30	N	751*	64	33*
	Bt	332	90	4
O32	N	179	21	3
	Bt	181	45	4
SP1	N	82*	20*	7
	Bt	17	tr	3
SP2	N	271*	87*	4
	Bt	20	tr	3

[a] Asterisks indicate a significant difference ($p < 0.01$) between isogenic and transgenic hybrids. tr < 3 ppb.

verticillioides, which were the predominant fungal species detected, are well-known fumonisin-producing species (*20*, *21*). As previously reported (*9*), we observed that FB_1 represented ~70% of the total fumonisins. FB_1 concentrations are presented in **Figure 2**. FB_1 grain concentrations ranged from 0.05 to 0.3 ppm for Bt maize and from 0.4 to 9 ppm for near-isogenic maize. In all cases, the concentrations of FB_1 were significantly ($p < 0.01$) lower in Bt maize kernels than in the corresponding isogenic hybrid.

The trichothecene and zearalenone contents of maize grains are reported in **Table 2**. DON, NIV, and ZEN concentrations measured in this study are in accordance with previously reported quantities found in maize grain from France (*22*). Significantly ($p < 0.01$) lower amounts of DON were observed in Bt hybrids for the 032, SP1, and SP2 experiments. Conversely, Bt hybrids from experiment 025 contained significantly ($p < 0.01$) higher amounts of DON than the near-isogenic maize grains. NIV concentrations were not significantly different in the 025, 030, and 032 experiments. The quantities of zearalenone measured in the different samples were very low. However, significantly ($p < 0.01$) higher concentrations of ZEN were observed in the traditional, non-Bt hybrids of the 030 experiment.

DISCUSSION

The mycological analysis of the kernels showed that *Fusarium* species from the Liseola section, including *F. proliferatum* and *F. verticillioides*, predominated in naturally contaminated maize. This result is in accordance with the literature (*23*, *24*). These *Fusarium* species produce large quantities of microconidia, which most probably allow them to disseminate and easily contaminate maize ears in the field.

In these experiments, the concentrations of fumonisins in nonmodified maize were important. Similar FB_1 contents in commercial maize collected in Spain have recently been reported (*25*). Conversely, these FB_1 concentrations are higher than those recently reported in commercial maize produced in France (*26*). Nevertheless, in all cases, the Bt transgenic maize kernel contents of fumonisins are significantly ($p < 0.01$) lower than those of the nonmodified maize kernels and are below the 1 ppm limit, which was proposed by Switzerland (*27*). On the total number of samples, the contents of zearalenone are low and below the 200 ppb limit recommended in France. The concentration of trichothecenes in all of the analyzed samples is also below 1 ppm, which corresponds to the guideline limit found in many countries (*28*).

Ergosterol is a specific component of the fungal membrane. Its analysis is commonly used to estimate the fungal biomass formed on natural solid substrates (*16*). In the different experiments, the contents of ergosterol in Bt maize kernels are 4−10 times lower than those observed on kernels from the near-isogenic control hybrids. The concentration of ergosterol in all Bt maize grain was <3 ppm, which is considered as an acceptable limit for market quality of maize grain (*29*, *30*).

This result shows that kernels which are protected from insect pests are less likely to be invaded by fungi. The differences in fungal biomass between Bt maize and near-isogenic maize hybrids most probably account for the observed differences in the fumonisin content of grain. The biosynthesis of fumonisin is influenced by the composition of the substrate (*31*). One could hypothesize that a difference of biochemical composition of the kernel between the MON 810 Bt and near-isogenic hybrids could account for the differences in the contents of fumonisins. However, on these varieties of maize, Masoero et al. (*8*) measured the contents of protein, soluble nitrogen, starch, and sugars and did not find any significant difference in composition between the MON 810 hybrids and their near-isogenic controls. Similar results have also been reported by Betz et al. (*32*).

In addition, it has been shown that without European corn borer infection, the concentrations of FB_1 were not significantly different for the Bt MON 810 and nonmodified maize (*9*). Similar results were obtained by Windham et al. (*33*) in their study of the content of aflatoxins on Bt and non-Bt maize kernels. These data indicate that Bt toxin has no toxic effect on these fungi.

Genetic engineering of maize to protect it against insect damage seems to be a way to considerably reduce the contamination of maize by *Fusarium* and fumonisin contamination of grain in France and Spain.

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