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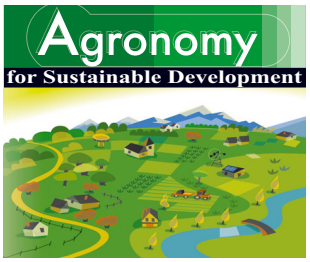

Research article

Lower mycotoxin levels in Bt maize grain

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Abstract – Mycotoxins produced by *Fusarium* spp. during plant cultivation induce severe diseases in animal and humans. In 2007 a European Union regulation set maximum concentrations of mycotoxins in maize and derivatives of 4000 ppb for fumonisins B_1 and B_2, 1750 ppb for deoxynivalenol, and 350 ppb for zearalenone. To assess the safety of French maize food, investigations are currently being carried out by the national Biological Risk Monitoring network. Here, 84 plots were cropped with the Bt maize MON 810 and its isogenic non-Bt counterpart in 2005 and 2006 in Southwestern France. Mycotoxin levels were measured in grain at harvest. Fumonisins B_1 and B_2, deoxynivalenol, and zearalenone were analysed by liquid chromatography-mass spectrometry. The data were analysed statistically using non-parametric tests for mycotoxins and analysis of variance for weather variables. As the climate was homogenous inside the experimental area, the transgenic event introduced into the maize was the only key parameter which differed between Bt and non-Bt maize plots. Our results show that Bt maize decreased concentrations of fumonisins by 90% and zearalenone by 50%, whereas the concentration of deoxynivalenol was slightly increased. Those findings suggest a competition among *Fusarium* species that produce fumonisins or trichothecenes. According to the European regulation, 93% of the Bt maize crops can be sold, compared with only 45% for non-Bt maize plots. Our results thus show that Bt maize improved food safety by greatly reducing mycotoxin levels in field crops in Southwestern France.

Bt MON810 maize / fumonisins B_1 and B_2 / deoxynivalenol / zearalenone / Regulation 1126/2007

1. INTRODUCTION

Mycotoxins cause several severe diseases in animals and humans. A recent review described the main diseases which were observed in the past provoked by the chronic contamination of small grains and maize, and updated the challenges of facing this contamination (Miller, 2008). In France, maize (*Zea mays* L.) was cultivated for food and seed production on more than 1.5 million hectares in 2007, out of a total of 9 million hectares for all cereals. Most of the maize fields for this are concentrated in Southwestern France. Like other cereals, this crop faces infestations by toxigenic fungi including *Fusarium* spp. These fungi cause diseases - e.g., ear rot - that affect plant growth, yield and crop quality. Toxins produced by *Fusarium* spp. were identified within the grain and remained in grain-derived products, i.e. in human food and animal feeds (Weidenbörner, 2007). The main mycotoxins contaminating maize in France are: (i) fumonisins B_1 and B_2, produced by *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* J. Sheld.) and *F. proliferatum* (T. Matsushima) Nirenberg, (ii) several trichothecenes, for the most part deoxynivalenol, and (iii) zearalenone, produced by *F. graminearum* Schwabe, and *F. culmorum* (Wm. G. Sm.) Sacc. (syn. *F. roseum* Lk. Emend Snyd. & Hans).

It is well known that deoxynivalenol, fumonisins and zearalenone do not develop similar pathologies and symptoms. Wild and Hall (1996) reviewed that fumonisins caused leukoencephalomalacia in horses, pulmonary oedema in swine and hepatotoxicity and nephrotoxicity in several animals,

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and have been shown to be carcinogenic for rats and mice. High contamination levels of fumonisins in food may cause oesophageal cancer in humans (Avantaggiato et al., 2002). Associated with acute mycotoxicosis in Asian populations during the 1980s, DON was identified as an immunosuppressor. Cellular studies when deoxynivalenol was inhaled or ingested suggested that its toxic effect might target human alveolar macrophages and epithelial cells in the lungs and colon, but did not increase the allergic response to allergens (Instanes and Hetland, 2004). The reproductive disorders caused by zearalenone are known in domestic animal species, particularly swine and cattle, with oestrogenic syndrome and male infertility. Zearalenone administered at a low dose decreased performance and impacted haematological parameters of mares (Raymond et al., 2005). Consequently, because deoxynivalenol, fumonisins and zearalenone do not impact human and animal health in the same way and with the same intensity, it is important to determine if a fungal contamination occurs in a crop and which kinds of mycotoxins remain at harvest time.

Efforts to control *Fusarium* spp. fungal infections and to prevent or to eliminate the presence of their mycotoxins in food have not yet been very successful, but the risks have been taken into account by the authorities for two reasons. First, in the recent past, exposure of young children has been close to the Provisional Maximum Tolerable Daily Intake (PMTDI) in the Netherlands (Pieters et al., 2004) and in Denmark (Rasmussen et al., 2007). Secondly, the Joint Expert Committee on Food Additives and Contaminants of the World Health Organization/Food and Agricultural Organization recommendations for deoxynivalenol and fumonisin PMTDI are now considered to be major achievements (Raymond et al., 2005). So, the European Union decided to establish maximum levels of mycotoxins in cereals for human consumption and animal feed. The recent Regulation (EC) No. 1126/2007 amended the Regulation (EC) No. 1881/2006 and established the regulatory thresholds for fumonisins B_1 and B_2, deoxynivalenol, and zearalenone. Since October 2007, the thresholds have been set at 4000 ppb for fumonisins, 1750 ppb for deoxynivalenol and 350 ppb for zearalenone.

Within this framework, and in order to maintain maize production in France below the EU mycotoxin threshold values, studies were carried out by the national Biological Risk Monitoring (BRM) Network supervised by the French Ministry of Agriculture. Field trials were conducted in France to compare the incidence of several plant protection approaches including prophylactic plant protection methods, agrochemicals and Bt technology.

In a previous study, we observed that field trials conducted with agrochemicals showed that the insecticide deltamethrine (20 $g{\cdot}ha^{-1}$), which controlled the two main maize borers *Ostrinia nubilalis* Hübner [Lepidoptera: Crambidae] and *Sesamia nonagrioides* Lefebvre [Lepidoptera: Noctuidae], was more efficient than the fungicide tebuconazole (250 $g{\cdot}ha^{-1}$) in reducing mycotoxin levels in maize fields (Folcher et al., 2009a). According to Sobek and Munkvold (1999), insect borer damage promoted ear rot disease development by spore transport and by alteration of the kernel epidermis. However, other experiments indicated that a significant reduction of mycotoxin grain content was obtained under genetic conditions (Dowd, 2000). De la Campa et al. (2005) showed that the weather parameters, temperature and rainfall affected fumonisin accumulation in maize. Bakan et al. (2002) compared mycotoxin contents in maize grains collected from Bt hybrids and near-isogenic traditional hybrids growing under natural conditions in France and Spain. They found that the fungal biomass formed on Bt maize grain was 4–18 times lower than that on near-isogenic maize. They determined that fumonisin B_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. Differences were seen between France and Spain because the countries do not have the same climate. Besides temperature and relative humidity, genetic parameters impact the susceptibility of maize varieties for borer infestation and *Fusarium* spp. Crickmore et al. (2010) reviewed that *Bacillus thuringiensis* produced more than 250 genes involved in the biosynthesis of Cry proteins. These crystal proteins are insecticidal for lepidopteran insects. They have many applications in agriculture through biopesticide Bt soap formulations (Fargues and Bourguet, 2005; Sanchis and Bourguet, 2008) but also with genetically modified plants expressing Bt genes introduced by transgenesis. Bt maize, which was genetically modified for controlling Lepidoptera, significantly reduced *Fusarium* ear rot infection in kernels and stalks (Munkvold et al., 1997).

In order to expand upon these observations, field trials with Bt and non-Bt corn were conducted in Southwestern France by the national BRM Network. The biocontrol of *O. nubilalis* and *S. nonagrioides* by two Bt maize events (Bt 176 and MON 810) were evaluated.These two Bt events encode for the same protein, Cry1Ab, but their gene promoters differ. The control levels of the insects also differed. The results showed that MON 810 efficiently controlled the two corn borer pests in the ears and in the stems (Folcher et al., 2006). A reduction of mycoflora *Fusarium* spp. was also observed with Bt (MON 810) maize (Folcher et al., 2009b).

In the present study, experiments involving a comparison between Bt maize (MON 810) and its isogenic counterpart evaluated the mycotoxin levels at harvest. The following points were taken into account: (i) the experiment was carried out under non-differential conditions of cultivation for Bt and non-Bt maize to avoid any interference other than that produced by the transgenic event; (ii) chemical analysis was conducted to quantify separately deoxynivalenol, zearalenone, and fumonisin B_1 and B_2 levels. We aimed to determine if all mycotoxins were reduced within Bt maize and its non-Bt counterpart, and if not which mycotoxin families were more reduced. The results are discussed with regard to the efficacy of the Bt trait in controlling the maize borers *O. nubilalis* and *S. nonagrioides* and to a hypothesised fungal competition between *Fusarium* spp. One of the expected results was also to determine the efficacy of the Bt variety and its non-Bt isogenic counterpart for reducing the level of total mycotoxins and to compare them with the new EU thresholds. Considering each family of *Fusarium* spp. mycotoxins, this study aims to evaluate if the benefit/risk ratio is similar in Bt maize and in its

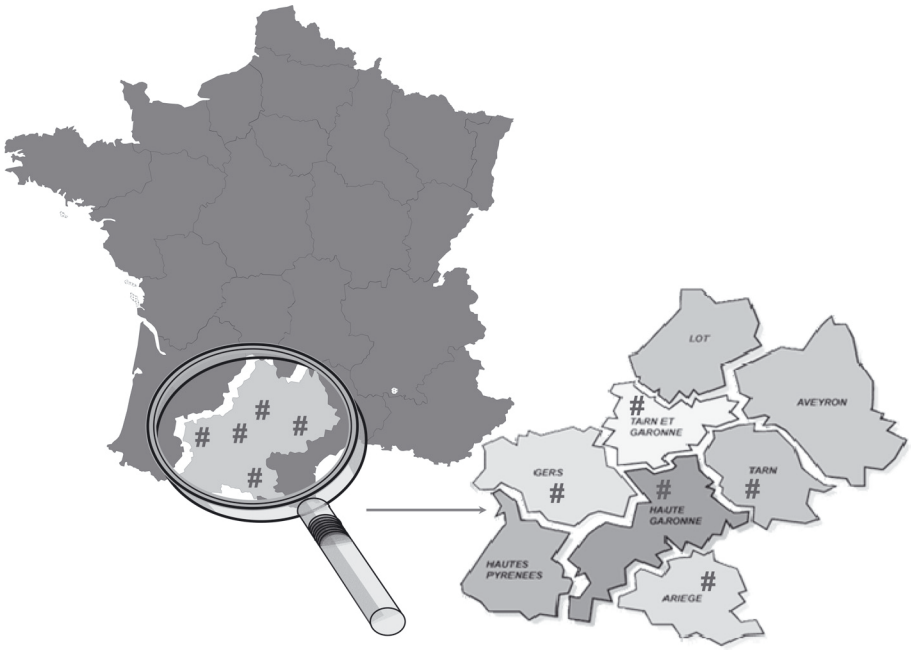

Figure 1. Geographical location of field trials in Southwestern France ("Midi-Pyrénées" Region). Legend: # Field trial locations. Experiments were located in 21 sites, i.e. 5 administrative subdivisions of the Region, the departments Haute-Garonne (9 sites), Tarn (4 sites), Tarn et Garonne (5 sites), Gers (2 sites) and Ariège (1 site).

isogenic non-Bt counterpart in Southwestern France, where maize borer pressure is very high.

2. MATERIAL AND METHODS

2.1. Field trials

Our experiments involved two varieties of maize (*Zea mays* L.), the non-Bt PR33P67 and its genetically modified counterpart PR33P66 transformed by the MON 810 event. The choice of these cultivars was guided by the duration of crop development (earliness factor) in Southwestern France (the "Midi-Pyrénées" Region). Experiments were located in 21 sites, i.e. 5 administrative subdivisions of the Region (departments), Haute-Garonne (9 sites), Tarn (4), Tarn et Garonne (5), Gers (2) and Ariège (1). These departments are located close to each other (Fig. 1).

The bioassays were conducted under natural conditions in the summers of 2005 and 2006. The 21 fields (>1 ha) were divided into plots, resulting in 42 twin plots being examined per year, i.e. 84 twin plots (42 non-Bt and 42 Bt maize plots) for the two years. Fields were seeded over a period of 20 days beginning on April 15, 2005 and 2006. No insecticide or fungicide treatments were given during cropping. The meteorological data (temperature, relative humidity [RH] and rainfall) were recorded to verify if conditions were ensured for *Fusarium* spp. growth and were homogenous over the field trials.

These trials were located in an area regularly infested by maize borers. In this area, both pests are multivoltine. *O. nubilalis* larvae resume development after the winter diapause ending in April or at the beginning of May. The first flight occurs in early June, and the second one during the first half of August. The *S. nonagrioides'* cycle occurs approximately 3 weeks earlier. There are 5 (*O. nubilalis*) and 7 (*S. nonagrioides*) larval stages. Rearing insects caught from the field allowed a precise monitoring of their reproductive cycle during the cropping.

During cultivation and at the harvest, the infestation by caterpillars of the two borers was checked by dissection of 20 stalks and ears from each plot. For mycotoxin analysis two 1 kg samples of kernels were taken from 20 mixed ears collected randomly in each plot (i.e. 4 kg per field). Consequently, 168 samples were gathered and kept at 4 °C to avoid post-harvest development of *Fusarium* spp. between the field and the laboratory.

2.2. Mycotoxin analysis

Mycotoxins were analysed by LC-MS-MS. The following mycotoxins were identified: deoxynivalenol, fumonisin B_1, fumonisin B_2 and zearalenone. All reagents, acetonitrile, methanol (SDS and Carlo Erba,Val de Rueil 27, France), pure water, acetic acid LC-MS grade (Fluka, Buchs, Switzerland) were analytical HPLC (High Performance Liquid Chromatography) grade. Standard products and calibrant solutions were obtained from Biopure and Sigma. Stock solutions were prepared in acetonitrile and were solubilised with 0.01% of acetic acid for LC-MS/MS calibration. One kilogram of grains per sample was ground and sifted through a 0.5-mm particle size filter following the protocols of the

European Regulation No. 2006/401/EC. Then, 5 g were mixed with 100 µL of internal standard solutions and extracted with 20 mL of acetonitrile/water with 2 h of reversed agitation, after which a 3-mL aliquot of the aqueous phase was centrifuged and dried in a rotoevaporator. The final product was dissolved in a solution of 0.01% acetic acid and methanol (2/1 v/v). The solution (50 µL) was pipetted with a syringe equipped with a filter, and directly injected into a LC-MS/MS. HPLC (Hewlett Packard, Eybens 38, France, 1100 type) analysis was carried out using a C_{18} column (VWR, Pessac 33, France, 250 * 4.6 mm). The mobile phase was ammonium acetate 1 nM, 0.0001% acetic acid/methanol and 1% acetonitrile, with a linear gradient over 40 min at a flow rate of 1 mL·min^{-1}. The detection was performed with a quadrupole tandem mass spectrometer, API 4000 (Applied Biosystems, Foster City, CA, USA), with Analyst-Applied Biosystems software for control and data processing. The detection threshold was 10 µg·kg^{-1}. TURBO Ion Spray® from Applied Biosystems with positive and negative ionisation mode was used. The detection conditions were Interchangeable Turbolon Spray (ITS) in positive and negative mode interface with 500 °C of source temperature and – 4500 V and 4500 V for ion spray voltage. Multiple Reaction Monitoring (MRM) was used, and identification and quantification were carried out on two or three transitions for each mycotoxin. The protocol was in accordance with AFNOR V03-110. AFNOR, the "Association Française de Normalisation", is the French official association for product standardisation.

The full scan (Total Ionic Current, TIC) and specific chromatograms (noted XIC) were recorded. The full scan chromatogram provided the sum of ionic responses produced by both ionised molecules extracted from the organic maize sample. The specific MRM chromatogram from mass/mass spectrometry (i.e. MRM transition) of each mycotoxin allowed identification of each molecule by its spectral response such as chromatographic peak and retention time. The quantification was carried out with the peak area which was proportional to the solute concentration in the linearity range. This specific MRM transition on a molecular mass and chemical structure basis of each mycotoxin was extracted from the full scan to give a precise and sensitive identification of fumonisin B_1 and fumonisin B_2, deoxynivalenol, and zearalenone (Fig. 2).

2.3. Data and statistical analysis

The statistical unit was a couple of plots that involved analysis of paired comparison. The following variables were subjected to statistical analyses (84 data per variable): (i) mycotoxins: fumonisins B_1 and B_2, deoxynivalenol, zearalenone, and total mycotoxins; (ii) the weather variables involved: temperature, rainfall and relative humidity (RH).

The Shapiro normality tests (Shapiro and Wilk, 1965) indicated that the weather variables (Rainfall, Temperature and RH) followed a normal distribution but the mycotoxin variables did not ($P < 0.05$). Consequently, we used non-parametric bilateral Wilcoxon signed-rank tests (noted *Tw* test) and Mann-Whitney (Noted *U* test) tests (Siegel and Castellan, 1998) to analyse these variables. ANOVA (Analysis of Variance) was used for normally distributed variables. Differences are considered significant if $P < 0.05$. Analysis was conducted using the software StatBox (Version 6.1).

The efficacy (E) parameter was calculated on the average of all plots as follows:

$$\text{“E(\%)} = [(\text{Control} - -\text{Treatment})/\text{Control}] \times 100\text{”}$$

in which Control and Treatment were the mycotoxin levels in, respectively, non-Bt and Bt plots. Efficacy may be negative if the value on average for Treatment is higher than in Control.

3. RESULTS AND DISCUSSION

The results are indicated then discussed in the following. After giving the levels found for each family of mycotoxins within Bt and non-Bt maize, we examine the variability of these mycotoxins for each year and under the weather conditions. Then, we consider the mycotoxin levels with regard to the EU thresholds.

The bioassays were conducted with twin plots in field trials combining a non-Bt (isogenic) with its Bt counterpart maize. The comparison of Bt vs. non-Bt samples revealed distinct patterns for each mycotoxin family. The amounts of fumonisins B_1 and B_2 were significantly lower in Bt than in non-Bt maize for both the year 2005 ($Tw = 231$; N = 21; $P < 10^{-4}$) and the year 2006 ($Tw = 3$; N = 21; $P < 10^{-4}$) (Tab. I). The efficacy E was, respectively, 95.66% for the year 2005 and 92.44% for the year 2006. Deoxynivalenol levels were significantly lower for non-Bt maize than Bt cultivars for both the year 2005 ($Tw = 65$; N = 21; $P = 0.04$) and the year 2006 ($Tw = 51$; N = 21; $P = 0.01$). Parameter E was negative in this case, with –31.04% for the year 2005 and –75.52% for the year 2006. A reduction of zearalenone level was observed with Bt maize, but this reduction was smaller than that observed for fumonisins B_1 and B_2. It was not statistically significant for either of the two years (2005: $Tw = 64$; N = 21; $P = 0.24$ and 2006: $Tw = 99$; N = 21; $P = 0.27$). Efficacy E was, respectively, 50% and 54%. Considering total mycotoxin levels, for both 2005 and 2006, the reduction was highly significant for genetically modified Bt maize: respectively ($Tw = 231$; N = 21; $P < 10^{-4}$) and ($Tw = 212$; N = 21; $P = 3.980.10^{-4}$). Efficacy E was 92.6% for the year 2005 and 76.3% for the year 2006.

The inter-annual variability of mycotoxin levels in 2005 and 2006 is detailed in Table I. The Mann-Whitney test showed no significant difference between the levels of fumonisins B_1 and B_2 for non-Bt maize ($U = 259$; N = 21; $P = 0.17$) and for Bt maize ($U = 265$; N = 21; $P = 0.12$). Consequently, the levels of fumonisins B_1 and B_2 of the two cultivars could be considered as homogenous for the two years. Conversely, the deoxynivalenol levels were significantly higher in 2006 than in 2005 for both non-Bt ($U = 94$; N = 21; $P < 10^{-4}$) and Bt maize ($U = 146$; N = 21; $P = 0.03$). It was also to be noted that deoxynivalenol biosynthesis was more significant in 2006 than in 2005. Regarding zearalenone, the level was significantly higher for the non-Bt cultivar in 2005 compared with

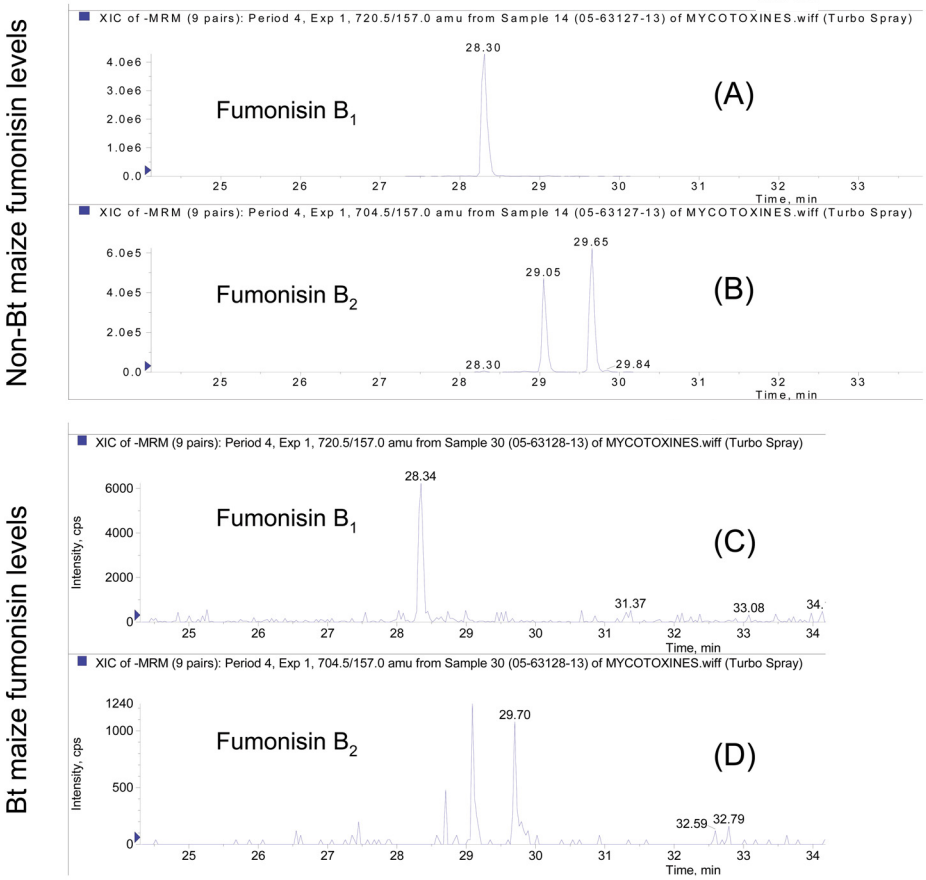

Figure 2. Detection and determination of mycotoxins by LC-MS-MS, e.g. comparative levels of fumonisins B_1 and B_2 within isogenic non-Bt (A, B) and Bt maize (C, D) harvested samples. For fumonisin B_1, we observed 4×10^6 cps value in isogenic non-Bt maize (Fig. 2, A) versus 6000 cps in Bt maize (Fig. 2, C). For fumonisin B_2, 6×10^5 cps were noted in isogenic non-Bt maize (Fig. 2, B) versus 1240 cps recorded in Bt maize (Fig. 2, D).

the year 2006 (U = 121; N = 21; $P = 0.005$), but not for Bt maize (U = 163; N = 21; $P = 0.07$). In both cases, Bt maize gave a reduction of the zearalenone levels. The level of the mycotoxins fumonisins B_1 and B_2, deoxynivalenol, and zearalenone taken all together was not statistically different for the two years 2005 and 2006 for non-Bt maize (U = 259; N = 21; $P = 0.17$) or for Bt maize (U = 193; N = 21; $P = 0.24$).

The level of total mycotoxins was significantly reduced with MON 810, but the difference was observed according to mycotoxin families: fumonisins B_1 and B_2 were strongly reduced, zearalenone also but not significantly, though deoxynivalenol was even increased compared with non-Bt maize.

Our results corroborated other field experiments conducted in other countries. Some field trials, conducted in the USA in 2000–2002, demonstrated that fumonisin levels were frequently lower in grains of Bt hybrids than in non-Bt varieties (Hammond et al., 2004). Other experiments carried out in central Europe concluded that Bt maize hybrids slightly reduced the fusariotoxin level of maize (Magg et al., 2002). Field studies conducted in Ontario (Canada) showed that deoxynivalenol concentrations were reduced in Bt maize but were mainly dependent on *O. nubilalis* density in the field (Schaafsma et al., 2002). The MON 810 event encodes for a Cry1Ab protein which is an δ-endotoxin highly toxic to lepidopteran larvae. However, Clements et al. (2003) observed that the Cry1Ab protein was associated with reduced fumonisin concentration in grain when *O. nubilalis* was the predominant insect pest but not when *Helicoverpa zea* (Boddie) was the prevalent one. The susceptibility of the insect species to the proteins had to be taken into consideration because it impacted the mycotoxin levels.

It was also important to consider if meteorological conditions were conducive to providing the environmental conditions necessary for the growth of *Fusarium* spp. Several rain showers of >5 mm are required to release the spores of fungi from the ascospores and a daily RH up to 90% is required to induce spore germination. Thermal conditions favourable to toxin genesis need to be between 15 °C and 20 °C (Marin et al., 1995; Brennan et al., 2005). Consequently, the number of days with an average moisture of >90% was considered

Table I. Comparison of mycotoxin levels (mean ± SE in ppb) of GM Bt maize vs. its isogenic non-Bt counterpart (N = 21 GM[2] Bt[1] replicates vs. 21 non-GM Bt replicates/year).

Mycotoxin	Cultivars	2005	2006	Utest[a]
Fumonisins B_1/B_2	GM	265.621 ± 114.062	425.076 ± 249.144	$U = 265$, $P = 0.120$
	Isogenic	6114.931 ± 1292.660	5620.036 ± 1453.458	$U = 259$, $P = 0170$
	Tw test[b]	$Tw = 231$, $P < 10^{-4}$	$Tw = 3$, $P < 10^{-4}$	
Deoxynivalenol	GM	185.691 ± 46.763	975.605 ± 471.796	$U = 146$, $P = 0.030$
	Isogenic	113.576 ± 57.199	238.805 ± 56.096	$U = 93.5$, $P < 10^{-4}$
	Tw test	$Tw = 65$, $P = 0.040$	$Tw = 51$, $P = 0.010$	
Zearalenone	GM	9.373 ± 3.030	1.567 ± 1.422	$U = 163$, $P = 0.070$
	Isogenic	18.954 ± 8.857	3.471 ± 2.313	$U = 121$, $P = 0.005$
	Tw test	$Tw = 64$, $P = 0.240$	$Tw = 99$, $P = 0.270$	
Total mycotoxins	GM	460.685 ± 116.457	1402.248 ± 573.679	$U = 193$, $P = 0.240$
	Isogenic	6247.461 ± 1282.183	5862.312 ± 1466.050	$U = 259$, $P = 0.170$
	Tw test	$Tw = 231$, $P < 10^{-4}$	$Tw = 212$, $P = 3.980 \times 10^{-4}$	

[a] U test: results of Mann-Whitney test ($P < 0.05$).
[b] Tw test: results of Wilcoxon signed-rank test ($P < 0.05$).
[1] Bt, *Bacillus thuringiensis*.
[2] GM, genetically modified.

over the 6 months covering the trials (beginning of May until the end of October) as well as the number of days with rain showers of >5 mm (spore release).

According to these observations we calculated the percentage of days suitable for spore release and germination for each trial location. We observed that mean temperatures varied from 18.86 °C to 20.58 °C. Recorded rainfalls during the bioassays varied from 259 mm to 345.5 mm with a number of rainy days (>5 mm) between 12 and 21. In all locations, the percentage of favourable days for spore release varied from 6.52 to 11.41% during the summers, although the percentage of favourable days for spore germination fluctuated between 1.09 and 3.80%. In all cases, conditions required for fungi development were met. The climatic conditions in the bioassays, temperature, RH and rainfall, were favourable to the development of *F. verticillioides* and *F. proliferatum* as well as *F. graminearum* and *F. culmorum.*

A mycotoxin characterisation campaign on maize conducted in all of France in 2004 by the national BRM network underlined a geographical distribution of *Fusarium* spp. The two main species producing fumonisins, *F. verticillioides* and *F. proliferatum*, need warmer temperatures to develop than *F. graminearum* and *F. culmorum*. They predominate in Southwestern France, while *F. graminearum* and *F. culmorum* are prevalent in Northern and Eastern France. Northern France was characterised by the predominance of deoxynivalenol and zearalenone. Southern France, with a more limited contamination by trichothecenes, suffered high fumonisin levels (Delos et al., 2007). Our work confirmed this conclusion, with high levels of fumonisins in non-Bt maize (Tab. I). Besides this observation, we also identified in a preliminary work a wide range of *Fusarium* spp in Southwestern France including both the main *Fusarium* spp. producing fumonisins and those which biosynthesised trichothecenes (Folcher et al., 2009b).

The complexity of the relationship between fungi is well known and it has a consequence for mycotoxins. Insects play a role in it. Reid et al. (1999) observed from an evaluation of ergosterol (a metabolite biosynthesised by fungi and considered to be a biomarker of fungal activity) that *F. graminearum* had a greater amount of activity than *F. verticillioides*. Contamination of the grains by different species of fungi gave an idea of the competition between species colonising the ears of maize (Velluti et al., 2000). It has also been established that the level of infestation of maize ears by both *F. verticillioides* and *Aspergillus flavus* Fresen was affected by competition correlated with insect activity that damaged the plant (Cardwell, 2000).

In this present study, the competition occurring between *F. verticillioides* or *F. proliferatum* and *F. graminearum* within the maize grains is hypothesised. The control of lepidopteran larvae by the MON 810 event decreased the fumonisin levels, but increased the deoxynivalenol level in Bt maize, higher than the non-Bt variety. Following this observation, we hypothesised that the control of insects limited the invasion of *F. verticillioides* or *F. proliferatum*, an opportunist fungus, and, as a consequence, favoured the development and the activity of *F. graminearum*, which infested the plant. This phenomenon was observed on wheat spikes and has been named "flora inversion" (Ioos et al., 2004). However, because the level of deoxynivalenol compared with fumonisin was not so high, the development or the activity of *F. graminearum* would be lower than *F. verticillioides* or *F. proliferatum*. To verify/falsify this hypothesis, further work taking into account a qualitative and quantitative evaluation of occurring *Fusarium* spp. ought to be carried out. The ecological dimension of *Fusarium* spp., and their geographical distribution as well as gene control, should also be taken into consideration (Yates and Sparks, 2008).

Another point to consider is the climate. In fact, climate changes with rainfall and drought events interfered with deoxynivalenol and fumonisin production (Abbas et al., 2007). Although Bt maize and non-Bt counterparts growing in twin plots were rigorously submitted to similar weather conditions

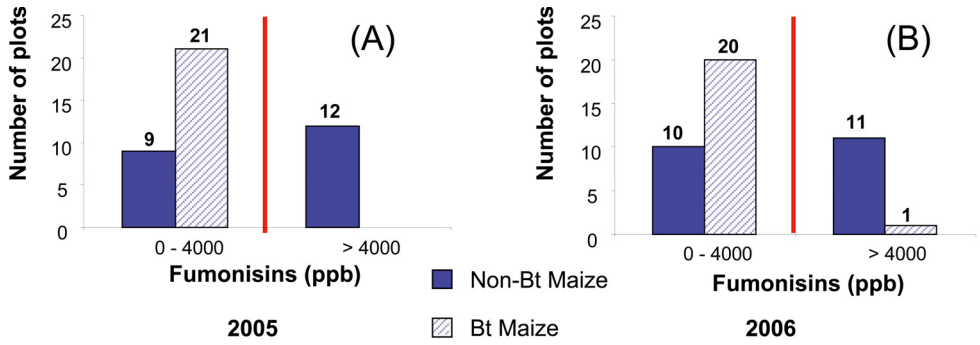

Figure 3. Grain levels of fumonisins B_1 and B_2 at harvest in Bt and non-Bt (isogenic) maize plots for 2005 (A) and 2006 (B). Red bars represent the 4000 ppb threshold according to the Regulation (EC) No. 1126/2007.

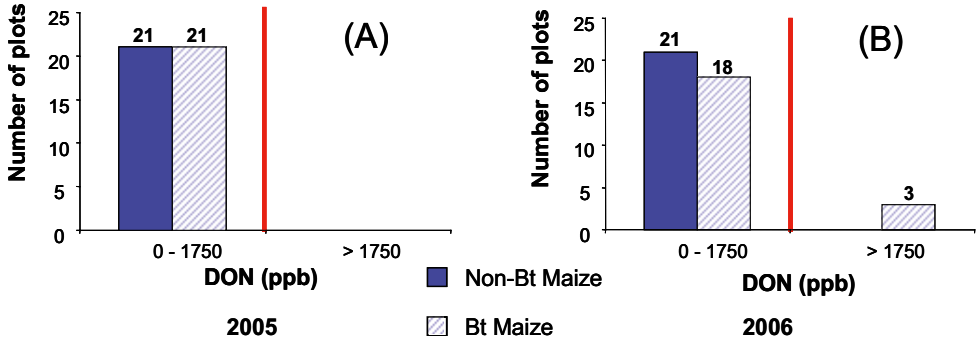

Figure 4. Grain deoxynivalenol (DON) levels at harvest in Bt and non-Bt (isogenic) maize plots for 2005 (A) and 2006 (B). Red bars represent the 1750 ppb threshold according to the Regulation (EC) No. 1126/2007.

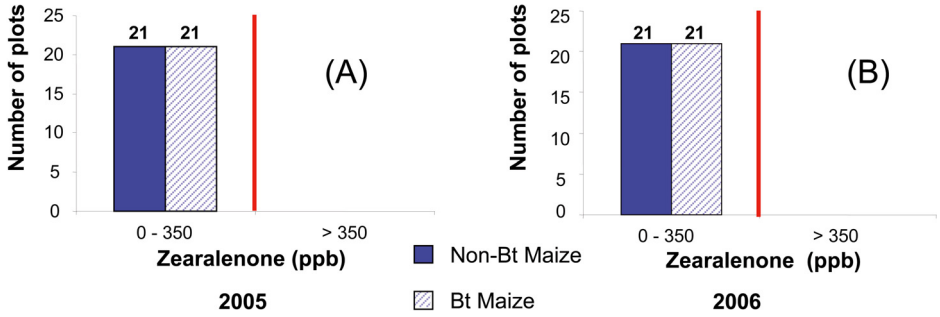

Figure 5. Grain zearalenone levels at harvest in Bt and non-Bt (isogenic) maize plots for 2005 (A) and 2006 (B). Red bars represent the 350 ppb threshold according to the Regulation (EC) No. 1126/2007.

in each field, we needed to verify if the climate was homogenous inside the whole experimental area during cropping. A two-way analysis of variance gave the conclusion that no statistical difference between sites was noted for: (i) temperature ($F = 0.19$; $Df = 3, 40$; $P = 0.90$), (ii) rainfall ($F = 0.24$; $Df = 3, 40$; $P = 0.87$) and (iii) relative humidity ($F = 0.52$; $Df = 3, 40$; $P = 0.67$). Consequently, the Bt event introduced into the maize was the only key parameter which differed between the two twin plot series for all trials during the summer.

We also considered if there was any change between the two years 2005 and 2006. The climate in the experimental area was similar for the two years, showing no significant difference for temperature ($F = 0.89$; $Df = 1, 40$; $P = 0.35$), rainfall ($F = 0.32$; $Df = 1, 40$; $P = 0.58$) and relative humidity ($F = 1.51$; $Df = 1, 40$; $P = 0.23$). Consequently, mycotoxin levels observed within the non-Bt maize and its Bt counterpart can be considered in this study as a consequence of the transgenic event.

Finally, we examined the results according to the EU thresholds. Did the mycotoxin levels meet the threshold requirements of Regulation (EC) No. 1126/2007 (4000 ppb for fumonisins B_1 and B_2, 1750 ppb for deoxynivalenol, and 350 ppb for zearalenone)? What would be the consequences of such thresholds with regard to the economic prospects of the crops?

Considering plots and EU thresholds, mycotoxins were distributed as following:

- Fumonisins B_1 and B_2.

In non-Bt maize trials, 9 plots were below the 4000 ppb threshold and 12 above for the year 2005 (Fig. 3, A); and 10 plots below the threshold and 11 above for the year 2006 (Fig. 3, B).

In Bt maize trials, all the plots were below the threshold for the year 2005 (Fig. 3, A) and one plot was above the threshold for the year 2006 (Fig. 3, B).

- Deoxynivalenol.

In non-Bt maize trials, all plots were below the 1750 ppb threshold for both years 2005 and 2006 (Fig. 4, A&B).

In Bt maize trials, all plots were below the threshold for 2005 (Fig. 4, A), but 3 of 21 plots were above the threshold for the year 2006 (Fig. 4, B).

Regarding zearalenone, all plots, Bt and non-Bt maize as well, were below the threshold for both years (Fig. 5, A&B).

From these results, it is shown that 97.5% of Bt maize plots were below the EU threshold for fumonisins B1 and B2 but only 45% of the non-Bt maize plots (Fig. 3). Regarding deoxynivalenol, 7% of Bt maize plots, all grown in 2006, were above the EU deoxynivalenol threshold for the two years, while all non-Bt maize plots were below the threshold for both years (Fig. 4). For zearalenone, all plots, Bt and non-Bt maize as well, were below the threshold for both years (Fig. 5).

Consequently, if this experimental crop were carried on the market, 57% of non-Bt maize would not be commercialised because of high fumonisin levels for the year 2005, while all Bt maize would be commercialised. For the year 2006, 52% of non-Bt maize would not be commercialised because of fumonisin levels, and 15% of Bt maize would not be commercialised because of the deoxynivalenol level being above the threshold.

For a farmer who seeded Bt maize, the balance for the two years would be 7% of uncommercialised maize compared with 55%, i.e. more than half of the production that could not be commercialised if he seeded conventional maize without any treatment. The loss for Bt maize was undoubtedly lower than for non-Bt maize with all the economic consequences that could be induced by this situation.

However, the incidence is not only economic but also ecological. In Southwest France, where pest pressure is particularly high compared to Northern and Eastern France and where borers develop several generations during the summer, seeding Bt maize controlling borers would limit the use of insecticides. In fact, the control of these borers by conventional insecticides requires treating the fields several times during the cropping. Bt MON 810 maize, which biosynthesises the Cry1Ab Protein throughout its lifetime, is better protected against the damage of the borers and avoids conventional insecticide treatment. However, our trials nevertheless showed that this control could be over in a few cases. The increase in deoxynivalenol we observed did not seem to be a constant phenomenon, as it was shown in Ontario (Canada), that the levels of trichothecenes decreased in Bt maize fields (Schaafsma et al., 2002). Several points ought to be considered: the density as well as the biological reproductive cycle of the insects in the field, but also the susceptibility of the cultivar to pathogenic fungi, especially *Fusarium graminearum*. This point needs further experiments to be answered.

4. CONCLUSION

This study showed a significant reduction (more than 75%) of the mycotoxin amounts taken overall with Bt maize. However, contrasting results were observed regarding each mycotoxin family. The efficacy of Bt maize in reducing mycotoxins was more than 90% for fumonisins and more than 50% for zearalenone, although deoxynivalenol was slightly increased. According to the European Union regulation (EC) No. 1126/2007, 93% of the Bt maize plots were able to be commercialised compared with only 45% for non-Bt maize plots. Considering the consumption quality with respect to EU mycotoxin level thresholds, Bt maize MON810 would constitute a useful tool to significantly reduce mycotoxin levels in field crops in Southwestern France and to improve the food safety of this major crop growing in Southern Europe. The adoption of more severe limits for mycotoxin toxicity in the EU promotes the reconsideration of maize agricultural practices including alternative methods and biotechnologies such as Bt maize. This would reduce the pesticide use in agriculture, especially in areas where the lepidopteran pressure is particularly high because of multivoltinism. In these areas, the insect pest control necessitates several treatments during the summer. Bt maize (MON 810), that biosynthesised in a continuous way an insecticidal protein, decreased the necessity of chemical treatments. This approach would fit in with the new Directive No. 2009/128/EC, establishing a framework for Community action to achieve the sustainable use of pesticides.

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