Bt maize: a tool for improving food safety of grains at harvest

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Abstract

A new EU (European Union) regulation came into force in 2007 with Regulation (EC) No. 1126/2007 which established maximum levels for fumonisins B_1 and B_2 (4000 ppb), deoxynivalenol (1750 ppb) and zearalenone (350 ppb) in maize and maize products. In order to evaluate French maize food safety, studies were carried out by the national Biological Risk Monitoring (BRM) Network. In this study, field trials involving 84 plots were conducted with Bt maize (MON 810) and its isogenic non-Bt counterpart in 2005 and 2006 in South-western France. Mycotoxin levels were determined in grain at harvest. Fumonisins B_1 and B_2, deoxynivalenol, and zearalenone were analyzed by LC-MS-MS and the results treated statistically using non parametric tests for mycotoxins and analysis of variance test 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 showed that all mycotoxin families were not impacted in the same way. The efficacy of *Bt* maize reduced mycotoxins more than 90% for fumonisins and more than 50% for zearalenone although deoxynivalenol was lightly increased. Therefore a competition between the different *Fusarium* spp. which produced fumonisins or trichothecenes is hypothesized. According to Regulation (EC) No. 1126/2007, 93% of the maize of Bt maize plots were able to be commercialized compared to only 45% for non-Bt maize plots. The results of this work showed that Bt maize improved food safety and constituted an useful tool to reduce significantly mycotoxin levels in harvested and stored grains.

Keywords: *Bt* (MON810) maize, Fumonisins B_1 and B_2, Deoxynivalenol (DON), Zearalenone, EC regulation 1126/2007 thresholds

1. Introduction

Maize (*Zea mays* L.) faces up to 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 kept up in grain-derived products, i.e. in human food and animal feeds, These 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 to face these contaminations (Miller, 2008). The main mycotoxins contaminating maize in France are: (i) fumonisins B_1 and B_2 [FB1-B2] 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 [DON] and (iii) zearalenone [ZEA] produced by *F. graminearum* Schwabe, and *F. culmorum* (Wm. G. Sm.) Sacc. (syn. *F. roseum* Link. Because DON, fumonisins and ZEA do not impact human and animal health in the same way and with the same intensity, it is important to determine when a fungal contamination occurs in a crop, which kind of mycotoxins still remain at harvest time and then in stored grains. The European Union (EU) decided to establish maximum levels of mycotoxins in cereals for human consumption and animal feed. The new EC regulation No. 1126/2007 (modifying EC regulation

No. 1881/2006) establishes the regulatory thresholds for fumonisins B_1 and B_2, deoxynivalenol (DON) and zearalenone. Since October 2007, the thresholds (maximum trace tolerable limit, MTL) were set at 4000 ppb for FB1-B2, 1750 ppb for DON and 350 ppb for ZEA.

Within this framework, and in order to maintain maize production in France below the EC mycotoxin threshold values, studies were carried out by the national Biological Risks 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 $g.ha^{-1}$), which controlled the two main maize borers - *Ostrinia nubilalis* Huebner [Lepidoptera: Crambidae], and *Sesamia nonagrioides* Lefebvre [Lepidoptera: Noctuidae] was more efficient than the fungicide tebuconazole (250 $g.ha^{-1}$) to reduce mycotoxins levels in maize grains (Folcher et al., 2009a). According to Sobek and Munkvold (1999), insect borer damages help ear rot disease development by spore transport and kernel epidermis alteration. Beside temperature and relative humidity, genetic parameters impact the susceptibility of maize varieties for borer infestation and *Fusarium* spp. *Bt* maize, which was genetically modified (GM) for controlling Lepidoptera, reduced significantly *Fusarium* ear rot infection in kernels (Munkvold et al., 1997).

To complete 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 several *Bt* maize events (Bt 176 and MON 810) were evaluated. These two *Bt* events encode for the same protein Cry1Ab but their promoter genes differ, and the control levels of the insects were different. The results showed that MON 810 controlled efficiently in the field the two corn borer pests both in ears and in stems (Folcher et al., 2006). Then, a reduction of mycoflora *Fusarium* spp. was observed with *Bt* (MON 810) maize (Folcher et al, 2009b).

In this study, the experimental work focused on a comparative experimentation involving *Bt* maize (MON 810) *vs*. its isogenic counterpart for evaluating the mycotoxin levels at the harvest. The following points were taken into account: (i) it was carried out under non differential conditions of cultivations for *Bt* and *non-Bt* maize to avoid any interference other than the one produced by the transgenic event; (ii) chemical analysis was conducted to quantify separately DON, ZEA and FB1-B2 levels. We aimed to determine if all mycotoxins were reduced within Bt maize vs its non Bt counterpart and, if not, which mycotoxin families were more reduced. One of the expected results was also to compare them to the new UE thresholds.

2. Materials and methods

Our experiments involved two varieties of maize (*Zea mays* L.): a *non-Bt* PR33P67 and its GM counterpart PR33P66 transformed by MON 810 event. The choice of these cultivars was guided by the duration of crop development (forwardness factor) in the Southwestern France (the so-called Région Midi-Pyrénées). Experiments were located in 21 sites in 5 departments: Haute-Garonne (9 sites), Tarn (4), Tarn et Garonne (5), Gers (2), and Ariège (1).

The bioassays were conducted under natural conditions twice in summers (2005 and 2006). The 21 fields (>1 ha), i.e. 84 twin plots (42 *non-Bt vs*. 42 GM *Bt* maize plots) for the two years, were seeded over a period of 20 days beginning April 15, 2005 and 2006. No insecticide neither fungicide treatments were done during cropping. The meteorological data (temperature, relative humidity (r.h.) and rainfalls) were recorded to verify if conditions were ensured for *Fusarium* spp. growing.

These trials were located in an area where by maize borers *O. nubilalis* and *S. nonagrioides* regularly infested maize. In this area, both pests are multivoltine. 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. Two 1kg samples of kernels were taken from 20 mixed ears collected randomly in each plot (i.e. 4 kg per field) and analyzed for mycotoxins. One hundred and sixty-eight samples were gathered. Mycotoxins were analysed by LC-MS-MS. DON, Fumonisin B1, Fumonisin B2 and ZEA were checked.

The following variables were subjected to statistical analyses (84 data per variable): FB1-B2, DON, ZEA and Mycotox (total mycotoxins). The weather variables involved temperature, rainfall and relative humidity (r.h.). The Shapiro normality tests (Shapiro and Wilk, 1965) indicated that the weather variables (rainfall, temperature and r.h.) 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-Whiney (Noted *U* test) tests (Siegel and Castellan**,** 1998) to analyse these variables. ANOVA test 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:

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

in which 'control' and 'treatment' were the mycotoxin levels in respectively *non-Bt* and *Bt* plots. Efficacy may be negative if value in average for Treatment is upper than in Control.

3. Results

The comparison of *Bt vs*. *non-Bt* samples revealed distinct patterns for each mycotoxin family. The amounts of fumonisins B1 and B2 was significantly lower in *Bt* than in *non-Bt* maize for both year 2005 ($Tw = 231$; N = 21; $P < 10^{-4}$) and year 2006 ($Tw = 3$; N = 21; $P < 10^{-4}$) (Table 1). The efficacy E was respectively 95.66% for year 2005 and 92.44% for year 2006. DON levels were significantly lower for *non-Bt* maize than *Bt* cultivars for both year 2005 ($Tw = 65$; N = 21; $P = 0.04$) and year 2006 ($Tw = 51$; N = 21; $P = 0.01$). Parameter E was negative in this case with -31.04% for year 2005 and -75.52% for year 2006. A reduction of ZEA level was observed with *Bt* maize, but this reduction was smaller than the one observed for FB1-B2. It was not statistically significant for either of the two years (year 2005: $Tw = 64$; N = 21; $P = 0.24$, and year 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 GM *Bt* maize: respectively ($Tw = 231$; N = 21; $P < 10^{-4}$) and ($Tw = 212$; N = 21; $P = 3.98.10^{-4}$). Efficacy E was 92.6% for year 2005 and 76.3% for year 2006.

Table 1 Comparison of mycotoxin levels (mean ± SE in ppb) of GM *Bt* maize *vs*. its isogenic *non-Bt* counterpart.

Mycotoxin	Cultivars	2005	2006	*U* test[2]
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[1]	*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* = 3.980x10^{-4}	

Legend:[1] *Tw* test*:* Results of Wilcoxon signed rank test ($P < 0.05$); [2] *U* test*:* Results of Mann-Whitney test ($P < 0.05$).

The inter-annual variability of mycotoxin levels in 2005 and 2006 is detailed in Table 1. The Mann-Whitney test showed no significant difference between the levels of fumonisins B1 and B2 for non-Bt maize ($U = 259$; N = 21; $P = 0.17$) neither for Bt-maize ($U = 265$; N = 21; $P = 0.12$). Consequently, FB1-B2 levels of the two cultivars could be considered as homogenous for the two years. Conversely the DON 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 underlined that DON biosynthesis was more important in 2006 than in 2005. Regarding ZEA, the level was significantly higher for *non-Bt* cultivar in 2005 compared to 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 ZEA levels. The level of the mycotoxins FuB1B2, DON and ZEA taken all together was not statistically different for the two years 2005 and 2006 for *non-Bt* maize (U = 259; N = 21; $P = 0.17$) neither for *Bt* maize (U = 193; N = 21; $P = 0.24$).

These mycotoxins are biosynthesized by *Fusarium* spp. So it was important to consider if meteorological data set up environmental conditions to develop *Fusarium* spp. Several rainfalls higher than 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 (Marin et al., 1995; Brennan et al., 2005). Consequently, the number of days with an average moisture higher than 90% was considered over the 4 months covering the trials (beginning of June until the end of October) as well as the number of days with rainfalls higher than 5 mm (spore release). The percentage of days suitable for spore release and germination was calculated for each trial location. Table 2 shows that mean temperatures varied from 18.9°C and 20.6°C. Recorded rainfalls during the bioassays varied from 259 mm to 345.5 mm with a number of rainy days (with rainfall > 5 mm) between 12 to 21. In all locations, the percentage of favorable days for spore release varied from 6.52 to 11.41% during the summers although the percentage of favorable days for spore germination fluctuated between 1.09 to 3.80%. In all cases, conditions required for fungi development were met (Table 2).

Table 2 Meteorological data within Region Midi-Pyrénées area during the summers (May 1st to October 31th 2005 and 2006).

Sites location (Department)	Year	Temp. mean (°C)	Rain fall (mm)	Raining day number (>5mm)	Favourable days for spore release (%)	Hygrometry days number (>90%)	Favourable days for spore germination (%)
Haute-Garonne	2005	19.75	285.5	18	9.78	7	3.80
Haute-Garonne	2006	20.58	259	12	6.52	4	2.17
Tarn	2005	19.22	345.5	16	8.70	2	1.09
Tarn	2006	20.00	309.5	15	8.15	2	1.09
Tarn et Garonne	2005	19.21	318.4	17	9.24	7	3.80
Tarn et Garonne	2006	19.96	335.2	21	11.41	2	1.09
Gers	2005	18.86	341	19	10.33	5	2.72
Gers	2006	19.69	266	16	8.70	3	1.63

The climatic conditions in the bioassays, temperature, r.h. and rainfall, were favorable to the development of *F. verticillioides* and *F. proliferatum* as well as *F. graminearum* and *F. culmorum.* The climate changes with rainfall and drought events interfered with DON and fumonisin production (Abbas et al., 2007). Regarding the levels of mycotoxins and because field trials were conducted in strictly identical conditions (except the transgenic event introduced into the maize variety), we verified that climate was homogenous inside the experimental area (Region Midi-Pyrénées). Table 3 gave the conclusion that no statistical difference should be noted. Moreover, in each field, *Bt* maize and *non-Bt* counterparts growing in twin plots were rigorously submitted to similar weather conditions. Consequently, the MON 810 event introduced into the maize was the key parameter which differed between the two twin plot series. As the climate in the experimental area was similar for the two years of the study, the difference of mycotoxins levels observed within the *non-Bt* maize and its *Bt* counterpart can be considered as a consequence of the transgenic event.

Table 3 Climatic conditions in Region Midi-Pyrénées during the summers (May 1st to October 31th) 2005 and 2006 according to sites and years.

Variables	Site			Year			Site / Year		
	F^1	df	P^2	F^1	df	P^2	F^1	df	P^2
Temperature	0.190	3,40	0.9025	0.887	1,40	0.3519	0.001	3,40	1.0000
Rainfall	0.237	3,40	0.8703	0.318	1,40	0.5758	0.124	3,40	0.9453
Relative Humidity	0.522	3,40	0.6696	1.506	1,40	0.2269	0.261	3,40	0.8530

Legend: [1] *F* value; [2] Results of Two-Way ANOVA ($P < 0.05$)

4. Discussion

The level of total mycotoxins was significantly reduced with MON 810, but difference was observed according to mycotoxin families: FuB1B2 were strongly reduced, ZEA also but not significantly, DON was even increased compared to non-Bt maize.

Some field trials, conducted in USA in 2000-2002, demonstrated that fumonisins 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 DON concentrations were reduced in Bt maize but mainly dependent of *O. nubilalis* density in the field (Schaafsma et al., 2002). A mycotoxin characterization campaign on maize conducted in the whole 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. The Northern was characterized by predominance of DON or ZEA. Southern France with a more limited contamination by trichothecenes suffered high fumonisin levels (Delos et al., 2007). The higher levels of FB1-B2 in our bioassays corroborated this conclusion.

The complexity of the relations between insects, fungi and mycotoxins is well known. It was established that the level of infestation of maize ears by both *F. verticillioides* and *Aspergillus flavus* Fresen was affected by a competition correlated with insect activity that damaged the plant (Cardwell, 2000). Reid et al. (1999) observed from an evaluation of ergosterol (a metabolite biosynthesized by fungi and considered to be a biomarker of fungal activity), that *F. graminearum* developed a higher activity than *F. verticillioides*. Contamination of the grains by different species of fungi gave an idea of the competition between species colonizing the ears of maize (Velluti et al., 2000).

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

Regarding the thresholds requirements of EC regulation No. 1126/2007 for food safety, it was observed that for FB1-B2, 9 plots were below the 4000 ppb threshold and 12 above for year 2005 and also 10 plots below the threshold and 11 above for year 2006. But for GM *Bt* maize, all the plots were below the threshold for year 2005 and one plot was above the threshold for year 2006. All the plots with non-Bt maize were below the 1750 ppb threshold for both 2005 and 2006 for DON, although all the GM maize plots were below the threshold for 2005, but 3 of 21 plots were above the threshold for year 2006. Considering ZEA, all the twin plots had levels below the EC threshold (Figure 1).

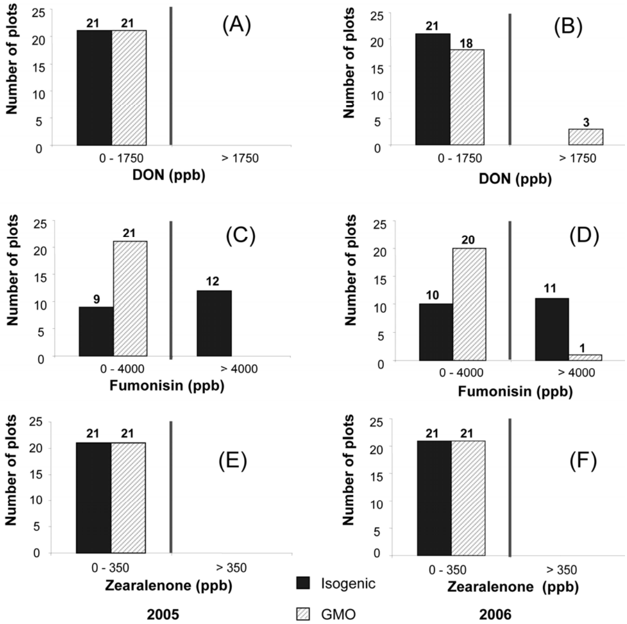

Figure 1 Grain deoxynivalenol (A & B), fumonisins B1 and B2 (C & D) and zearalenone (E & F) levels at harvest in Bt and non-Bt (isogenic) maize plots for 2005 and 2006 years. Red bars represent the present EC thresholds according the EC Regulation 1126/2007 of 2007-09-28.

Consequently, if this experimental crop would be carried on the market, the balance for the two years would be 7% of uncommercialized maize for *Bt* maïze compared to 55% i.e. more than half of the production that could not be commercialized for conventional *non-Bt* maize without any treatment. The loss for *Bt* maize was undoubtedly lower than for *non-Bt* maize with all economic consequences that could be induced by this situation.

From these results, it can be concluded that Bt maize improves the food safety of maize harvested grains and limits the risk of mycotoxins. It is also an useful tool to reduce the economic impact of such contamination for harvested and stored grains.

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