The impact of *Bacillus thuringiensis* technology on the occurrence of fumonisins and other mycotoxins in maize

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Abstract

In many developing countries, maize is both a staple food crop and a widely-used animal feed. However, adventitious colonization or damage caused by insect pests allows fungi to penetrate the vegetative parts of the plant and the kernels, the latter resulting in mycotoxin contamination. Maize seeds contaminated with fumonisins and other mycotoxins pose a serious threat to both humans and livestock. However, numerous studies have reported a significant reduction in pest damage, disease symptoms and fumonisin levels in maize hybrids expressing the *Bacillus thuringiensis* (Bt) gene *cry1Ab*, particularly in areas where the European corn borer is prevalent. When other pests are also present, the *cry1Ab* gene alone offers insufficient protection, and combinations of insecticidal genes are required to reduce damage to plants caused by insects. The combination of Cry1Ab protein with other Cry proteins (such as Cry1F) or Vip proteins has reduced the incidence of pests and, indirectly, mycotoxin levels. Maize hybrids expressing multiple Bt genes, such as SmartStax®, are less susceptible to damage by insects, but mycotoxin levels are not routinely and consistently compared in these crops. Bt maize has a greater economic impact on *Fusarium* toxins than aflatoxins. The main factors that determine the effectiveness of Bt hybrids are the type of pest and the environmental conditions, but the different fungal infection pathways must to be also considered. An alternative strategy to reduce mycotoxin levels in crops is the development of transgenic plants expressing genes that protect against fungal infection or reduce mycotoxin levels by *in situ* detoxification. In this review article, we summarize what is known about the relationship between the cultivation of Bt maize hybrids and contamination levels with different types of mycotoxin.

Keywords

Fumonisins, aflatoxins, deoxynivalenol, *Bacillus thuringiensis*, European corn borer

Abbreviations: AFs, aflatoxins; FBs, fumonisins; FB_1, fumonisin B_1; FB_2, fumonisin B_2; DON, deoxynivalenol; ZEA, zearalenone; MON, moniliformin; Bt, *Bacillus thuringiensis*; ECB, European corn borer; CEW, Corn earworm; WBC, Western bean cutworm; BCW, Black cutworm; FAW, Fall armyworm; SWCB, Southwestern corn borer.

Introduction

Maize (*Zea mays* L.) is the most common source of fumonisins (FBs) in human and animal diets. Maize seeds are often contaminated with FBs produced primarily by *Fusarium verticillioides* and *F. proliferatum.* These fungi can infect the seeds, or the silks can be contaminated by airborne or waterborne conidia, systemic infections can be caused by contamination of the roots, or pest insects can injure the plants allowing fungal penetration (Munkvold and Desjardins, 1997). Other mycotoxins may be present alone or together with FBs, including aflatoxins (AFs), deoxynivalenol (DON), zearalenone (ZEA), and some recently-discovered *Fusarium* metabolites collectively known as emergent mycotoxins, such as moniliformin (MON), beauvericin, enniatins and fusaproliferin (Desjardins, 2006; Marín *et al.,* 2013). AFs are mainly produced by *Aspergillus flavus,* which synthesizes type B AFs as well as cyclopiazonic acid (CPA) depending on the strain, and by *A. parasiticus*, which synthesizes both type B and type G AFs, but not CPA. DON and ZEA are mainly produced by *F. graminearum* and *F. culmorum* and they are often found as co-contaminants. Among the emergent mycotoxins, MON is the most prevalent and can be produced by several *Fusarium* species, including *F. avenaceum*, *F. tricinctum*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (Marín *et al*., 2013).

The consumption of mycotoxin-contaminated kernels is associated with a range of diseases and disorders in humans and domestic animals, including cancer, immune system dysfunction and metabolic disorders (Marasas, 2001; Marín *et al*., 2013; Sobrova *et al*., 2010; Williams *et al*., 2004). FBs and AFs are carcinogens (AFB_1 is the most carcinogenic natural compound known), and this creates a strong impetus to restrict the exposure of human and animal populations as far as possible (IARC, 1993, 2002, 2012). In 2001, several countries submitted information on the concentration of FBs in maize and maize-derived foods, and FBs were detected in more than 60% of all food products (JECFA, 2001). These data are supported by a European Union report on the exposure of the EU population to *Fusarium* toxins. Among samples of raw maize material, 67% were positive for FB_1 (total = 801) and a 51% were positive for FB_2 (total = 544) (SCOOP, 2003).

The European Commission (EC) and European Parliament (EP) have set maximum levels for mycotoxins in maize and maize products. When such products are intended for human consumption, these values are currently 200–1000 µg/kg for FBs, 750 µg/kg for DON, 100 µg/kg for ZEA, 3 µg/kg for ochratoxin A (OTA), 5 µg/kg for AFB_1, and 10 µg/kg for total AFs. Recently, indicative levels of 100 µg/kg in maize were also established for the total content of the trichothecene mycotoxins T-2 and HT-2 (EC, 2006b, 2007, 2010, 2012, 2013a). Only the level of AFB_1 in animal feed is currently regulated in the EU (maximum 0.005–0.02 mg/kg, depending on the type of feed). Other important mycotoxins in feed are delimited by guidance values that vary with the species of livestock: 0.9–12 mg/kg for DON, 0.1–3.0 mg/kg for ZEA, 0.05–0.25 mg/kg for OTA and 5–60 mg/kg for FBs (FB_1+FB_2). Recently, indicative levels of 250–2000 µg/kg in cereal products for feed and compound feed have been specified for the total content of T-2 and HT-2, with the exception of feed for cats, for which the guidance value is 50 µg/kg (EC, 2003, 2006a, 2013a, 2013b; EP, 2002).

In contrast, the United States Food and Drug Administration (FDA) has proposed a guideline for total FB levels in food of 2–4 mg/kg (depending on the product), and total AF levels of 20 µg/kg in maize and maize products for human consumption. For animal feed, the levels vary from 5 to 100 mg/kg for FBs and from 20 to 300 µg/kg for AFs, depending on the animal species (FDA, 2000, 2001).

Transgenic maize and mycotoxins

Factors that affect mycotoxin occurrence

The presence of mycotoxins in maize results from the interaction of several factors, including temperature and humidity, nutrient availability, the presence of other fungi, stress, and physical damage caused by pest insects. Before harvest, important factors include the weather (temperature, humidity and rainfall), exposure to insect pests, fungi and other pathogens, planting dates, the maize genotype and cropping system. FB contamination in maize is directly associated with *Fusarium* pink ear rot (mainly produced by *F. verticilliodes*) and its incidence depends on both environmental conditions and pest damage. Kernel damage caused by insects exposes the kernels to fungal spores, although there are several additional infection pathways. Fungal growth and mycotoxin accumulation can also be stimulated post-harvest by poor storage conditions such as high humidity and the presence of other pests (Marín *et al.*, 2004; Miller, 2001).

Fungal growth and mycotoxin production are affected by multiple ecophysiological factors. The main factors that control FB production in grain are temperature and water activity (a_w). *F. verticillioides* and *F. proliferatum* germinate at 5–37ºC when a_w exceeds 0.88, although the growth range fluctuates in the range 7–37ºC when a_w exceeds 0.90. The optimum conditions for FB production by *F. verticillioides* are 30ºC at 0.97 a_w and for and *F. proliferatum* the corresponding values are 15ºC at 0.97 a_w. Physicochemical and nutritional factors such as pH and carbon/nitrogen ratio can also affect FB production. The presence of other fungi, such as *A. flavus* and *A. niger*, can affect the growth of *Fusarium* species, which is most competitive at 15ºC and 0.98 a_w. At high a_w values, FB production can be stimulated by *A. niger* and other species (Marín *et al.*, 1999; Picot *et al.*, 2010; Sanchis *et al.*, 2006).

Suppression of insect pests using Bt technology

Injuries caused by insects are common sites of fungal infection on maize ears and stalks. The fungi can be airborne or may be suspended in water droplets that splash the wound, but insects can also act as vectors. One of the most prevalent examples is the European corn borer (ECB) (*Ostrinia nubilalis* Hübner), a maize pest that not only injures plants and exposes them to infection, but also vectors fungal spores, particularly *F. verticillioides* and *F. proliferatum*. ECB therefore promotes *Fusarium* infection of maize kernels and stalks, and may reduce yields by increasing the incidence of stalk rot (Munkvold and Desjardins, 1997; Munkvold *et al.*, 1997; Sobek and Munkvold, 1999). *F. verticillioides* is the most prevalent fungal pathogen of maize but fungicides are only partially effective, with efficacy depending on the pathogen strain and the fungicide mechanism of action (Falcão *et al.*, 2011). Therefore, pest insects are more appropriate targets than fungi for the development of strategies to reduce mycotoxin levels in maize.

In many parts of the world, the management of ECB now relies on transgenic hybrid maize lines expressing the *cry1Ab* gene from the Gram-positive soil bacterium *Bacillus thuringiensis* (Bt). This gene encodes a potent pro-toxin that is activated in the alkaline environment of the insect gut and is highly specific towards particular insect species. These insecticidal crystal proteins are also named δ-endotoxins or Cry proteins. *B. thuringiensis* has been used since 1938 to produce an insecticidal spray, but Bt transgenic plants resistant to ECB larvae were first made available in the USA in 1996 and in the EU in 1998. Different strains of the bacterium express different *cry* genes producing different pro-toxins that can protect plants against many different pests, including the corn rootworm complex (*Diabotrica virgifera*) (EPA, 2011; Höfte and Whiteley, 1989; Koziel *et al*., 1993; Schnepf *et al*., 1998). Therefore, alternative Bt genes (such as *cry1F*) have also been expressed in maize to protect against further lepidopteran pests (Abbas *et al*., 2013; Bowers *et al*., 2013; Koziel *et al*., 1993). Economically-important maize pests that can be partially controlled using Bt hybrids include the corn earworm (CEW; *Helicoverpa zea*), common stalk borer (*Papiapema nebris*), southwestern corn borer (SWCB; *Diatraea grandiosella*) and western bean cutworm (WBC; *Striacosta albicosta*), whereas this strategy has proven less efficient against the fall armyworm (FAW; *Spodoptera frugiperda*) and black cutworm (*Agrotis ipsilon*) (Bowers *et al*., 2013, 2014; Dowd, 2000; Munkvold and Hellmich, 1999; Williams *et al*., 2002, 2005, 2006). Table 1 lists the Bt events targeting lepidopteran pests and the corn rootworm complex that are currently commercially available in the USA.

Since its adoption in the USA, Bt maize has become the second most widely cultivated GM crop worldwide, after herbicide-tolerant soybean. About 30% of global maize production in 2014 (184 million ha) was represented by GM varieties (55.2 million ha) (James, 2014). However, the EU has a strict, complex and contradictory legislative framework for GM crops, with only the Mon810 maize event currently authorized for cultivation (EC, 1998; EP, 2003, 2008).

FB contamination in Bt and non-Bt maize

The ability of Bt genes to protect maize against ECB and other lepidopteran pests means that Bt maize tends to suffer a lower frequency of fungal infections and the infections that occur are often less severe or even symptomless. In the case of FBs, there is a large body of evidence to support the benefits of Bt maize, as shown by the comparison of FB concentrations in Bt and non-Bt maize hybrids in different field locations (Table 2).

Munkvold *et al*. (1999) published a fundamental study concerning the effect of Bt maize on disease management and concluded that transgenic hybrids expressing *cry1Ab* were less susceptible to ECB, suffered less from *Fusarium* ear rot and had lower FB levels than their non-transgenic counterparts. However, if other insect pests were present alone or concurrent with ECB then the levels of FBs remained high (Dowd, 2000; Clements *et al*., 2003; Hammond *et al*., 2004; Papst *et al*., 2005).

Many studies of natural infestations confirm the significant reduction in FB levels associated with Bt hybrids (Abbas *et al*., 2013; Ostry *et al*., 2010; Pazzi *et al*., 2006). These studies were carried out at different times in different countries, including Italy (Masoero *et al*., 1999; Pietri and Piva, 2000), France (Bakan *et al*., 2002; Folcher *et al*., 2010; Pinson *et al*., 2002), Spain (Bakan *et al*., 2002), Argentina (Barros *et al*., 2009; De la Campa *et al*., 2005) and the USA (Abbas *et al*., 2006, 2007, 2013; Bruns and Abbas, 2006; Dowd, 2001).

Importantly, Bowers *et al*. (2013) confirmed that lower levels of FBs were present in Bt hybrids exposed to ECB, but found that a *cry1Ab* x *vip3Aa* hybrid was more resistant to ECB, CEW and WBC than the *cry1Ab* hybrid and the non-Bt hybrid in all of the years covered by the study. The vegetative insecticidal protein Vip3Aa can therefore be combined with other toxins such as Cry1Ab to target additional lepidopteran pests. Unlike the *cry* genes, which are expressed during sporulation, *vip* genes are expressed during the *B. thuringiensis* vegetative growth phase and they do not share sequence homology with *cry* genes (Lee *et al*., 2003; Schnepf *et al*., 1998). The *cry1Ab* x *vip3Aa* hybrid also showed a lower level of pest damage, a lower incidence of *Fusarium* ear rot and lower levels of FBs when infested with ECB, whereas *cry1F* hybrids were better protected against WCB because *cry1F* specifically targets this pest (Bowers *et al*., 2014). A comparison of eight commercially available Bt hybrids expressing multiple genes found no significant differences in the content of FBs among the hybrids (Abbas *et al*., 2013). Recent studies of SmartStax® maize, which produces Cry1F, Cry1A.105+Cry2Ab2, Cry34Ab1/Cry35Ab1 and Cry3Bb1 to protect against common lepidopteran pests and the corn rootworm complex, reported less pest damage compared with single Bt hybrids and non-Bt hybrids, but did not consider mycotoxin levels (Rule *et al*., 2014; Head *et al*., 2014).

AF contamination in Bt and non-Bt maize

Whereas the link between Bt maize and lower FB levels is clearly established, the data for AF contamination are more contentious (Ostry *et al*., 2014). Windham *et al*. (1999) showed a significant correlation between fungal and insect exposure, inoculation or infestation dates and AF contamination. They found that Bt hybrids suffered less damage from insects and had lower AF levels than the other hybrids studied (*A. flavus* resistant and *A. flavus* susceptible hybrids and a non-Bt isogenic hybrid) when manually infested with SWCB. More recent studies have focused on the inoculation technique, showing that inoculation with *A. flavus* by kernel wounding, which facilitates fungal penetration, results in high-level AF contamination regardless of the hybrids used. There were no differences among the hybrids when infected with fungi alone because lower AF levels in the Bt hybrids reflected the reduction in insect damage, which indirectly reduced fungal contamination. In contrast, a non-wounding inoculation technique combined with SWCB infestation resulted in significantly lower levels of AF in the Bt hybrids (Williams *et al*., 2002, 2005; 2006). A testcross involving AF-resistant and AF-susceptible lines crossed with Bt and non-Bt maize revealed lower AF levels in the Bt testcrosses, but the difference for individual lines was significant in only two of 10 lines investigated. The low insect pressure during the experiment could explain these results, because the higher the insect pressure, the greater the differences between hybrids (Williams *et al*., 2010).

In a 3-year study, Wiatrak *et al*. (2005) observed significantly lower AF levels in Bt compared to non-Bt hybrids during the first year of the experiment, but there was no difference with a tropical non-Bt hybrid. In the second year, significantly lower levels of AFs were detected in the Bt hybrids than the tropical non-Bt hybrid, but there were no differences compared to the non-Bt hybrids. In the final year, there were no differences in AF contamination among the hybrids. Another 3-year study reported lower levels of AFs in Bt than non-Bt hybrids, but only in one of the years (Abbas *et al*., 2006; 2007; Bruns and Abbas, 2006). Nevertheless, a subsequent study showed that AF contamination was significantly reduced in the Bt hybrid compared to its non-Bt isoline (Abbas *et al*., 2008). The authors continued these field trials until 2009, reporting lower mycotoxin levels in Bt maize, but the difference was not significant, perhaps due to the continuous cultivation (Abbas *et al*., 2013).

In the USA, Odvody *et al*. (2000) observed less insect damage in Bt hybrids but AF levels were not consistent. In a subsequent study of different Bt hybrids, the lowest level of insect damage was observed in the Mon840 event (*cry2Ab*) correlating with significantly lower AF levels compared to non-Bt and *cry1Ab* hybrids in 2000, but only compared to the non-Bt hybrid in 2001 (Odvody and Chilcutt, 2002). Different *cryAb* events were also evaluated, revealing less insect damage in the Bt hybrids Mon810 and Bt11 compared to non-Bt hybrids, but AF levels were also inconsistent in this experiment (Odvody and Chilcutt, 2003). Similarly, Maupin *et al*. (2001) did not find significant differences in the levels of ear rot and AF accumulation when comparing Bt and non-Bt hybrids inoculated with *A. flavus*. Buntin *et al*. (2001) found no significant differences in AF levels between Bt and non-Bt maize, but the Bt hybrids suffered less severe FAW infestations. These data indicate that insect damage is strongly correlated with FB levels but not AF levels, suggesting that other factors such as drought stress and individual hybrid vulnerability may play a more dominant role than insect damage in the determination of AF levels. The field experiments concerning AF levels in Bt and non-Bt hybrids are summarized in Table 3.

Contamination with other mycotoxins in Bt and non-Bt maize

The results obtained with other mycotoxins are also controversial. Significantly lower levels of DON were observed in Bt compared to non-Bt hybrids in some studies (Magg *et al*., 2002; Schaafsma *et al*., 2002; Selwet, 2011; Valenta *et al*., 2001), whereas in other cases the mycotoxin levels appeared to be location-dependent (Bakan *et al*., 2002; Papst *et al*., 2005; Pinson *et al*., 2002) or there was no difference between Bt and non-Bt hybrids (Barros *et al*., 2009). A few studies have even found evidence for slightly higher DON levels in Bt hybrids, although the location was an important confounding effect (Folcher *et al*., 2010; Bakan *et al*. 2002). Schaffsma *et al*. (2002) analyzed 102 commercial maize fields in Canada, reporting a reduction in DON levels in Bt hybrids depending on the severity of ECB infestation in each field. This was supported by a study carried out in Germany showing a reduction in DON levels in Bt compared to non-Bt hybrids (Valenta *et al*., 2001). In another study, also in Germany, Magg *et al*. (2002) found significantly lower concentrations of DON in Bt maize in one of the two years of the experiment. Pinson *et al.* (2002) described differences in DON and ZEA levels between plots in two different fields in south and central France. Lower levels were observed in two Bt hybrids, but two others contained significantly higher levels of both DON and ZEA compared to the corresponding non-Bt cultivars. Bakan *et al.* (2002) reported low ZEA levels in their study, but significantly higher concentrations were observed in a traditional cultivar in France. In all the studies, the Bt hybrids expressed *cry1Ab* (Table 4).

Only one study has investigated the impact of Bt on MON levels, and the Bt hybrids showed significantly lower levels of MON than non-Bt hybrids (isogenic and commercial hybrids) when infested with ECB. MON levels were significantly higher following the manual infestation of unprotected plants (296 µg/kg) compared to those treated with insecticide (66.2 µg/kg). In the infested plots, the MON concentrations were 153.5, 336.7 and 266.1 µg/kg for the transgenic, isogenic and commercial hybrids, respectively. In contrast, in the protected plots, the MON concentrations were 49.1, 99.3 and 42.9 µg/kg for the transgenic, isogenic and commercial hybrids, respectively (Magg *et al.*, 2003).

Economic impact of mycotoxin reduction in Bt maize

The main goal of Bt technology is to reduce pest damage and promote higher yields. However, indirect benefits such as the reduction of FB levels also increase the percentage of maize grain that meets US and/or EU regulatory limits, which can have a significant economic impact and may also reduce the prevalence and severity of human and animal diseases (Bowers *et al.*, 2013; Folcher *et al.*, 2010; Hammond *et al.*, 2004; Magg *et al.*, 2002, 2003; Munkvold *et al.*, 1997, 1999; Pinson *et al.*, 2002).

Estimations for the cost of crop losses due to mycotoxin contamination in the USA range from $US 500,000 to $US 1.5 billion, reflecting variations in contamination levels, regulatory limits, price variations and production outputs (Vardon *et al.*, 2003). However, in the USA most losses are regulatory in nature (i.e. based on the rejection of grain based on quality) rather than actual harvest losses, and maize grains rejected for food and feed use may still be suitable for industrial processes such as biofuel production. On the other hand, the economic benefit of Bt maize in the USA has been specifically valued at $US 8.8 million in terms of preventing losses caused by FBs, and similarly $US 8.1 million for DON and $US 14 million for AFs, even though the AF levels depend on the predominant pest species (Wu, 2006, 2007; 2014; Wu *et al.*, 2004). These data based on studies carried out over a decade ago do not take into account the increased adoption of Bt maize, which has risen from 30% in 2005 to more than 80% in 2015 (USDA, 2015). A recent study of the Thailand maize market estimated the economic losses due to AFs. A loss of US$ ~6.9 million per annum was estimated assuming low levels of AF contamination (data from harvest and dried maize supplied by a pet company) but this increased to US$ ~100 million per annum assuming higher AF levels (data from retail markets). The rejection of AF-contaminated maize by the livestock sector is the most influential factor contributing to economic losses. Thus, the selection of high-quality maize (by the pet company) reflects lower levels of AF contamination, and lower economic losses (Lubulwa *et al.*, 2015). Bt technology can improve the quality of maize by reducing mycotoxin levels as an indirect consequence of preventing infestations with insect pests. This technology also reduces the need for chemical insecticides, resulting in lower levels of pesticide residues in food and water and less environmental impact (Qaim, 2009; Brookes and Barfoot, 2013).

Alternative strategies to reduce mycotoxin levels in maize

Although targeting pest insects can help to reduce opportunistic fungal infections of maize, other transgenic approaches have emerged more recently in which the fungus itself is the target. For example, Kant *et al*. (2012) reported a field study of transgenic maize expressing a modified rice *Rp13* gene encoding ribosomal protein L3, a primary target of DON. They developed two transgenic maize lines expressing the *Rp13* gene, one using the constitutive CaMV 35S promoter and the other using the silk-specific ZmGRP5 promoter. Both plants were less susceptible to *F. graminearum* than wild-type plants, and those containing the silk-specific promoter were the most tolerant, with the mildest symptoms under field conditions (DON levels were not evaluated). The difference in efficacy may reflect the broader activity of the silk promoter in the seed pericarp tissue. Maize silks are the primary route used by *F. graminearum* to infect the kernels. Expression of the modified *Rpl3* gene in silk tissue may therefore help to reduce *Gibberella* ear rot and hence DON levels.

Maize plants expressing the α-amylase inhibitor protein from *Lablab purpurea* (AILP) can also be used to reduce fungal infection. Fungal amylases liberate fermentable sugars from kernel starch which are essential for mycotoxin production. Kernel screening assays in AILP-transgenic maize plants revealed AF levels 56% lower than controls. AILP expression therefore appears to reduce both fungal growth on the kernels and AF accumulation (Chen *et al*., 2015).

Another promising approach to reduce mycotoxin contamination is enzymatic mycotoxin detoxification *in situ*, which converts the mycotoxins into less toxic compounds. The ZEA lactone ring is sensitive to hydrolysis by the fungus *Clonostachys rosea*, which synthesizes an alkaline lactonohydrolase responsible for detoxification (Kimura *et al*., 2006; Takahashi-Ando *et al*., 2002). The corresponding gene (*zhd101*) was able to reduce ZEA levels *in vitro* and in field-grown plants compared to non-transgenic controls, even when infected with *F. graminearum*. The ability of transgenic seeds to degrade ZEA was evaluated by immersing the seeds in 50 µg/ml ZEA for 48 h. The kernel tissues were then analyzed by HPLC, showing that wild-type seeds contained 24.6 ± 1.7 µg ZEA/g whereas the transgenic seeds contained only 1.6 ± 0.4 µg ZEA/g. The detoxification of ZEA in *Fusarium*-infected transgenic kernels was evaluated after inoculation with *F. graminearum*. The wild-type seeds contained 15.4 ± 3.7 ng ZEA/g whereas the level of ZEA in non-inoculated seeds and inoculated transgenic seeds was below the detection threshold (Igawa *et al*., 2007). Similarly, the yeasts *Exophiala spinifera* and *Rhinocladiella atrovirens*, and the Gram-negative bacterium ATCC 55552, can produce enzymes that metabolize FBs. Duvick *et al*. (2003) patented a fumonisin esterase produced by these yeasts which can hydrolyze the tricarballylate esters of FB_1, and this is active in transgenic maize. The esterase gene reduced FB levels in *Fusarium*-infected grain from 1.522 mg/kg without the enzyme to 0.379 mg/kg in esterase positive plants. AF detoxification in transgenic plants has yet to be reported (Duvick, 2001; Hartinger and Moll, 2011; Jard *et al*., 2011).

Concluding remarks

Several transgenic strategies can be used to reduce mycotoxin contamination in food and feed but Bt maize hybrids are widely grown and several studies have confirmed the reduction in pest damage, disease symptoms and FB levels, particularly when ECB is the predominant pest. This is because FB levels are reduced in Bt hybrids if the *Fusarium* population is dominated by species whose colonization of the plant is promoted by ECB damage (Miller, 2001).

Among the commercial Bt hybrids, Mon810 and Bt11 (which express *cry1Ab*) were associated with a reduction in the occurrence of *Fusarium* ear rot and FBs due to the lower level of kernel damage caused by susceptible lepidopteran pests (Dowd, 2000, 2001; Magg *et al*., 2001; Munkvold and Hellmich, 1999; Papst *et al*., 2005). Conversely, no difference has been reported between Bt 176 hybrids (Bt event 176 was withdrawn in 2001) and non-Bt maize (Magg *et al.*, 2002; Schaafsma *et al*., 2002), and it has even been proposed that resistance against ECB and *Fusarium* ear rot may be inherited independently (Magg *et al*., 2002; Miller, 2001).

The pest species and its abundance are key determinants of mycotoxin levels, particularly if the main pest is CEW which is unaffected by Cry1Ab (Clements *et al*., 2003; Dowd, 2000; Hammond *et al*., 2004). Lower levels of AFs occur in Bt maize if the main pest is SWCB or CEW, but there is no difference between Bt and non-Bt maize if the pest is FAW (Buntin *et al*., 2001; Williams *et al*., 2002, 2005, 2006, 2010). When other pests are present, hybrids expressing Cry1Ab are inefficient and must be combined with further Cry proteins (such as Cry1F) or Vip proteins to increase the level of protection (Bowers *et al*., 2013; 2014). Thus, if Bt events are not selected by taking into account prevalent insect pests and environmental conditions in each field, Bt technology will not be effective. Hence the importance of Bt hybrids expressing multiple genes, whose performance in the presence of different pests and climate conditions has yet to be studied in detail.

The impact of Bt maize on the accumulation of AFs, DON and ZEA is inconclusive because the extent of contamination depends on many interrelated factors. The different fungal infection pathways must be considered to understand the effect of Bt hybrids. The most common route used by *F. verticillioides* to infect the kernel is through the silks or through wounds caused by insect pests, whereas *F. graminearum* primarily reaches the kernels via the silks (Munkvold, 2003; Munkvold and Desjardins, 1997). *A. flavus* can infect maize kernels through the silks too. This may explain why Bt maize hybrids that are resistant to insect pests have less *Fusarium* ear rot and lower FB levels, whereas the relationship with DON, ZEA and AF levels is not so clear cut. Further studies are necessary to determine the interaction between Bt maize and the different fungal species that produce these toxins.

Mycotoxin contamination is frequently linked with drought, heat stress and insects. Drought favors the accumulation of FBs more than heat stress (Miller, 2001). A 2-year study was carried out by Traore *et al*. (2000) to characterize the effect of drought stress on Bt maize. Water deficit during the vegetative period delayed leaf emergence, reduced the leaf area and caused stunted growth in both Bt and non-Bt hybrids. It also reduced grain and biomass yields and kernel number per ear during both years. However, the Bt hybrids had greater biomass in 1997 and greater grain yields in 1998 because they were not so severely infected by second-generation ECB. Therefore, Bt maize continues to play an important role in insect resistance under drought stress.

The first commercially available drought-tolerant GM maize variety (MON87460) expresses a bacterial cold shock protein B (CspB), a molecular chaperone derived from *Bacillus subtilis*, which may provide a yield advantage under limited water availability. A recent field study confirmed the higher grain yield of MON87460 under drought conditions compared to a conventional hybrid (Nemali *et al*., 2015). The combination of Bt and drought-tolerant maize should therefore achieve even higher yields and lower mycotoxin levels because it will be protected against two major environmental stress factors.

Studies that considered AFs and FBs simultaneously reported variable results, suggesting that diverse environmental conditions may prevent the control of both mycotoxins in the same crops (Abbas *et al*., 2002, 2006, 2007, 2008; Bruns and Abbas, 2006). More studies are needed to determine whether AF resistance traits can be crossed into Bt hybrids. AF-resistant germplasm tend**s** to possess undesirable agronomic traits such as tight husk coverage and late maturity. Breeding programs aiming to achieve the introgression of AF resistance into Bt hybrids could remove these undesirable characteristics while reducing AF contamination (Williams *et al*., 2008; Williams *et al*., 2010). Bt maize has a greater economic impact on *Fusarium* mycotoxins than AFs (Wu, 2006, 2007; Wu *et al*., 2004). Further studies are needed to evaluate the effect of both Bt hybrids expressing multiple genes and Bt hybrids combined with maize lines that are resistant to the accumulation of other mycotoxins, especially AFs.

A potential risk that must be borne in mind comes in the form of mycotoxin derivatives (modified mycotoxins) that escape routine analytical techniques but may be digested by animals triggering toxic effects comparable to free mycotoxins. These derivatives should be included in the total mycotoxin allowances, and future legislation must consider their presence even though this would increase the stringency of testing and rejection, resulting in further economic loss. This highlights the benefits of Bt maize, which would reduce the levels of mycotoxins and potentially their derivatives, although the impact of Bt hybrids on the accumulation of modified mycotoxins needs to be addressed in more detail (De Boevre *et al*., 2012, 2014; De Saeger and van Egmond, 2012; Wu, 2006). Finally, the development of transgenic plants expressing genes that protect against fungal infection (e.g. the modified *Rp13* gene and the AILP transgene) or reduce mycotoxin levels by *in situ* detoxification (e.g. *zhd101* and fumonisin esterase) could provide an additional strategy to control mycotoxins (Chen *et al*., 2015; Duvick, 2001; Duvick *et al*., 2003; Igawa *et al*., 2007; Kant *et al*., 2012) and could be combined with Bt hybrids to provide additive or even synergistic protection against mycotoxin-producing fungal pathogens.

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Table 1. Current Bt maize varieties against Lepidopteran pests and Corn rootworm complex (EPA, 2011).

Pest	**Bt event**	**Protein(s) expressed**	**Target pests**[a]	**Registrant**
Lepidopteran pests	Bt11	Cry1Ab	ECB	Syngenta
	Mon810	Cry1Ab	ECB	Monsanto
	TC1507	Cry1F	ECB, BCW, FAW, SWCB	Dow/Mycogen Pionner/Dupont
	Mon89034	Cry1A.105 + CryAb2	ECB, SWCB, CEW, FAW	Monsanto
	MIR162	Vip3Aa20	CEW, FAW, BCW, WBC	Syngenta
Coleopteran pests	DAS-59122-7	Cry34Ab1 + Cry35Ab1	WCRW, NCRW, MCRW	Pionner/Dupont
	Mon88017	Cry3Bb1	WCRW, NCRW, MCRW	Monsanto
	MIR604	Cry3A	CRW	Syngenta

[a] ECB: European corn borer; CEW: Corn earworm, WBC: Western bean cutworm; BCW: Black cutworm; FAW: Fall armyworm; SWBC: Southwestern corn borer; CRW: Corn rootworm; WCRW: Western corn rootworm; NCRW: Northern corn rootworm; MCRW: Mexican corn rootworm.

Table 2. Summary of studies comparing FB levels in Bt and non-Bt hybrids.

Bt events tested	**Cry proteins expressed**	**Year (no. locations)**	**Location**	**Mold inoculation**	**Insect infestation**	**Area**	**FB levels (mg/kg) in Bt hybrids**[b]		**FB levels (mg/kg) in non-Bt hybrids**[b]		**Reference**
Mon810 Bt11 Bt176 DBT418 CBH351	Cry1Ab Cry1Ab Cry1Ab Cry1Ac Cry9C	1995 (1)	USA (Iowa)	Natural	Natural	-	FB_1	≈ 2-2.5	FB_1	≈ 1	Munkvold *et al.* (1999)[d]
					Manual: ECB[a]	-	FB_1	≈ 3-6.5	FB_1	≈ 9	
		1996 (1)	USA (Iowa)	Natural	Natural	-	≈ 0.3-2.7		≈ 0.7-2.7		
					Manual: ECB[a]	-	≈ 1.7-5.5		≈ 3-12		
		1997 (1)	USA (Iowa)	Natural Manual: *F. verticillioides*	Natural	-	≈ 2-13		≈ 4-19		
					Manual: ECB[a]	-	≈ 2-12		≈ 10.5-24		
-	Cry1Ab	1997(3)	Northern Italy	Natural	Natural	-	1.97		20.05		Masoero *et al.* (1999)
Mon810	Cry1Ab	1997 (3)	Northern Italy	Natural	Natural	-	2.02		19.76		Pietri and Piva (2000)
		1998 (4)	Northern Italy	Natural	Natural	-	5.45		31.63		
		1999 (30)	Northern Italy	Natural	Natural	-	1.39		39.02		
Mon810 Bt11 Bt176	Cry1Ab Cry1Ab Cry1Ab	1996 (1)	USA (Illinois)	Natural Manual: *A. flavus*, *F. graminearum*	Natural	Peoria	< 0.35		< 0.35		Dowd (2000)
					Manual: ECB[a]	Peoria	2		8.8 ± 6		
		1997 (1)	USA (Illinois)	Natural Manual	Natural Manual: ECB[a]	Peoria	2.8 ± 1.5		4.8 ± 1.9		
		1998 (1)	USA (Illinois)	Natural Manual	Natural Manual: ECB[a]	Peoria	Not evaluated		Not evaluated		
Mon810 Bt11 Bt176	Cry1Ab Cry1Ab Cry1Ab	1998 (1)	USA (Illinois)	Natural	Natural	Manito	II	0.022	I	0.4	Dowd (2001)[c]
						Easton	II IV V	0.74 0.37 0.6	III	1.08	
		1999 (1)	USA (Illinois)	Natural	Natural	Kilbourne	VII	0.16	VI	0.5	
						Bath	II IV	1.08 0.42	-	-	
						Easton	II	0.89	-	-	

							VIII	0.14			
Mon810	Cry1Ab	2000-2001 (2)	USA (Illinois)	Natural Manual: *F. verticillioides*, *F. graminearum*	Natural Manual: ECB[a], CEW[a]	Urbana and Monmouth	8 ± 13		10 ± 20		Clements *et al.* (2003)
Mon810	Cry1Ab	2000-2002 (several locations)	USA	Natural	Natural Manual: ECB[a]	Field trials	≈0.2-2		≈2-15.8		Hammond *et al.* (2004)[d]
Mon810 Bt176	Cry1Ab Cry1Ab	2001 (3)	East and South Germany	Natural	Natural	-	Not detected		I II	0.016 0.032	Papst *et al.* (2005)
					Manual: ECB[a]	-	0.569		I II	4.85 2.71	
Mon810	Cry1Ab	2000 (4)	Argentina	Natural	Natural	-	2.46		6.29		De la Campa *et al.* (2005)
		2001 (4)	Argentina	Natural	Natural	-	0.56		3.06		
			Phillippines	Natural	Natural	-	0.81		0.97		
		2002 (2)	Phillippines	Natural	Natural	-	0.25		0.45		
-	Cry1Ab	2002 (7)	Argentina	Natural	Natural	-	0.043		0.173		Barros *et al.* (2009)
		2003 (5)	Argentina	Natural	Natural	-	0.2		0.633		
Mon810	Cry1Ab	2005 (21)	Southwestern France	Natural	Natural	-	0.26		6.11		Folcher *et al.* (2010)
		2006 (21)	Southwestern France	Natural	Natural	-	0.43		5.62		
	Cry1Ab Cry1Ab xVip3Aa	2008 (1) 2009 (1) 2011 (1)	USA (Iowa)	Natural	Natural	-	Cry1Ab Cry1Ab x Vip3Aa	2.2 0.61	4.23		Bowers *et al.* (2013)
					Manual: ECB[a]	-	Cry1Ab Cry1Ab x Vip3Aa	1.52 0.57	6.75		
					Manual: CEW[a]	-	Cry1Ab Cry1Ab x Vip3Aa	2.55 0.61	7.51		
					Manual: WBC[a]	-	Cry1Ab Cry1Ab x Vip3Aa	2.08 0.39	2.37		
Mon810 TC1507	Cry1Ab Cry1F	2008 (1)	USA (Iowa)	Natural	Natural	-	Cry1Ab Cry1F	1.45 1.26	0.87		Bowers *et al.* (2014)
					Manual: ECB[a]	-	Cry1Ab Cry1F	1.4 1.99	12.34		
		2009 (1)			Natural	-	Cry1Ab Cry1F	0.15 0.04	0.12		
					Manual: ECB[a]	-	Cry1Ab	0.3	1.25		

							Cry1F	0.2		
					Manual: WBC[a]	-	Cry1Ab Cry1F	0.78 0.23	0.64	
		2010 (1)			Natural	-	Cry1Ab Cry1F	0.29 0.61	0.42	
					Manual: ECB[a]	-	Cry1Ab Cry1F	0.69 0.56	1	
					Manual: WBC[a]	-	Cry1Ab Cry1F	0.55 0.12	0.86	

[a] ECB: European corn borer; CEW: Corn earworm; WBC: Western bean cutworm.

[b] Roman numerals indicate maize varieties, but they are different among the studies.

[c] Study in commercial fields, the remainder are field experiments.

[d] The data that correspond with this study have been interpreted from a graphic.

Table 3. Summary of studies comparing AF levels in Bt and non-Bt hybrids.

Bt events tested	**Cry proteins expressed**	**Year (no. locations)**	**Location**	**Mold inoculation**	**Insect infestation**	**Area**	**Treatment**		**AF levels (µg/kg) in Bt hybrids[b]**	**AF levels (µg/kg) in non-Bt hybrids[b]**	**Reference**
Bt11	Cry1Ab	1995-1997 (1)	USA (Mississippi) (only Bt hybrid in 1997)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Day 7	*A. flavus*	166	45	Windham *et al.* (1999)
								SWCB	4	19	
								A. flavus + SWCB	83	136	
						-	Day 21	*A. flavus*	145	45	
								SWCB	5	41	
								A. flavus + SWCB	290	650	
						-	Control		17	6	
Mon810	Cry1Ab	1999 (2)	USA (South Texas)	Natural Manual: *A. flavus*	Natural	CC	Dryland		1136	601	Odvody *et al.* (2000)
						CC	Irrigated		423	243	
		2000 (3)	USA (South Texas)	Natural Manual: *A. flavus*	Natural	CC	Dryland		1399	1166	
						CC	Irrigated		1078	979	
						BEE	Dryland		187	347	
-	Cry1Ab	2000 (2)	USA (Indiana and Ilinois)	Natural Manual: *A. flavus*	Natural	-	-		Data not available	Data not available	Maupin *et al.* (2001)
Mon810 Bt11	Cry1Ab Cry1Ab	1998 (3)	USA (Georgia)	Natural	Natural	A	-		≈300	≈425	Buntin *et al.* (2001)[c]
						P	1st planting		≈875	≈700	
							2nd planting		≈75	≈100	
							3rd planting		≈25	≈25	
Mon810 Bt11	Cry1Ab Cry1Ab	2000	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Needle *A. flavus*		942	936	Williams *et al.* (2002)
							Spray *A. flavus*		311	646	
							Spray + SWCB		360	945	
							Control		115	328	
		2001 (1)	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Needle *A. flavus*		530	808	
							Spray *A. flavus*		61	237	
							Spray + SWCB		138	408	
							Control		9	23	

Mon810 Mon851 Mon840 Mon84006	Cry1Ab Cry1Ab Cry2Ab Cry2Ab	2000-2001 (2)	USA (South Texas)	Natural Manual: *A. flavus*	Natural	-	-	Data not available	Data not available	Odvody *et al.* (2002)
Mon810 Bt11 Bt176	Cry1Ab Cry1Ab Cry1Ab	2001-2002 (3)	USA (South Texas)	Natural Manual: *A. flavus*	Natural	-	-	Data not available	Data not available	Odvody *et al.* (2003)
-	Cry1Ab	2001 (1)	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Needle *A. flavus*	637	784	Williams *et al.* (2005)
							Spray *A. flavus*	140	192	
							Spray + SWCB	110	417	
							Control	15	24	
		2002 (1)	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Needle	261	391	
							Spray	5	5	
							Spray + SWCB	5	22	
							Control	2	4	
-	Cry1Ab	1998 (1)	USA (North Florida)	Natural	Natural	-	-	314	Normal 634 Tropical 470	Wiatrak *et al.* (2005)
		1999 (1)	USA (North Florida)	Natural	Natural	-	-	70	Normal 86 Tropical 259	
		2000 (1)	USA (North Florida)	Natural	Natural	-	-	55	Normal 36 Tropical 48	
-	-	1998 (1)	USA (Arkansas)	Natural	Natural	CB	-	196	227	Abbas *et al.* (2006)
		1999 (1)	USA (Arkansas)	Natural	Natural	CB	-	3.6	26.6	
		2001 (2)	USA (Arkansas)	Natural	Natural	CB	-	< 5	10	
						PT		< 5	6.5	
-	-	2002-2004 (1)	USA (Mississippi)	Natural	Natural	-	-	12.4 (data 2003)	45.3 (data 2003)	Bruns and Abbas (2006)
-	Cry1Ab	2003-2005 (1)	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	-	I 10 II 4	I 57 II 33	Williams *et al.* (2006)
-	-	2002 (1)	USA (Arkansas)	Natural	Natural	CB	Planting in April Planting in May	0.18 11.5	1.3 0.28	Abbas *et al.* (2007)
		2004 (1)	USA (Arkansas)	Natural	Natural	CB	Planting in April Planting in May	1.1 75.7	17 20	
		2005 (1)	USA (Arkansas)	Natural	Natural	CB	Planting in April Planting in May	2.1 12.9	5.2 8.5	

Mon810	Cry1Ab	2006 (1)	USA (Mississippi)	Natural	Natural	-	-	109	211	Abbas *et al.* (2008)
-	Cry1Ab	2009 (3)	USA (North Carolina)	Manual: *A. flavus*	Natural	-	-	249	382	Williams *et al.* (2010)
			USA (Georgia)	Manual: *A. flavus*	Natural	-	-	287	398	
			USA (Mississippi)	Manual: *A. flavus*	Natural	-	-	259	332	
-	Cry1Ab	2008 (1)	USA (Mississippi)	Natural	Natural	-	No-till	775	2381	Abbas *et al.* (2013)
							Tillage	272	266	
		2009 (1)	USA (Mississippi)	Natural	Natural	-	No-till	631	1457	
							Tillage	755	2381	
Mon88017 + Mon89034 GA21 TC1507	Cry1A.105 Cry2Ab CP4 EPSPS Cry3Bb CP4 EPSPS CP4 EPSPS PAT Cry1F	2010-2012 (1)	USA (Mississippi)	Manual: *A. flavus*	Natural	-	-	Data not available	Data not available	Bruns and Abbas (Un-published data)

[a] SWCB: Southwestern corn borer.

[b] Roman numerals indicate maize varieties, but they are different among the studies.

[c] The data that correspond with this study have been interpreted from a graphic.

Table 4. Summary of studies comparing DON levels in Bt and non-Bt hybrids.

Bt events tested	**Cry proteins expressed**	**Year (no. of locations)**	**Location**	**Mold inoculation**	**Insect infestation**	**Area**	**DON levels (μg/kg) in Bt hybrids**[b]		**DON levels (μg/kg) in non-Bt hybrids**[b]		**Reference**
Mon810	Cry1Ab	1999 (12)	South Germany	Natural	Manual: ECB[a]	-	152		873		Valenta *et al.* (2001)
Bt176	Cry1Ab			Natural	Natural		51		77		
Mon810	Cry1Ab	1999 (5)	South-western France	Natural	Natural	O25	729		472		Bakan *et al.* (2002)
						O30	332		751		
						O32	181		179		
			Northern Spain	Natural	Natural	SP1	17		82		
						SP2	20		271		
Mon810 Bt176	Cry1Ab Cry1Ab	1999 (4)	Germany (Upper Rhine Valley)	Natural	Natural Manual: ECB[a]	-	69		I II	138 136	Magg *et al.* (2002)
		2005 (5)	Germany (Upper Rhine Valley and Bavaria)	Natural	Natural Manual: ECB[a]	-	717		I II	867 765	
Bt11 Bt176	Cry1Ab	1996-1999 (102)	Canada (Ontario)	Natural	Natural	-	0.57 μg/g		0.96 μg/g		Schaafsma *et al.* (2002)[c]
Mon810	Cry1Ab	France 2000 (4)	Southern France	Natural	Natural	32	I II	2 4	I II	5 3	Pinson *et al.* (2002)
				Natural	Natural	82	I II	10 123	I II	38 43	
			Central France	Natural	Natural	41	III IV	5 5	III IV	10 12	
				Natural	Natural	45	III IV	37 14	III IV	110 23	
Mon810	Cry1Ab	2008 (1)	Poland (Wroclaw)	Natural	Natural	-	I	41	II III IV V	213 254 198 201	Selwet (2011)
		2009 (1)	Poland (Wroclaw)	Natural	Natural	-	I	32	II III IV V	201 243 186 199	

[a] ECB: European corn borer.

[b] Roman numerals indicate maize varieties, but they are different among the studies. The results are expressed in μg/kg, except in one study, Schaafsma *et al*. (2002), where are expressed in μg/g.

[c] Study in commercial fields, the remainder are field experiments.