The use of GMOs as a prevention strategy for mycotoxin formation

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Abstract

A variety of environmental stress factors increase susceptibility of corn plants to infection with various fungi that produce mycotoxins. These stress factors include insect damage, heat and drought stress, nitrogen deficiency and genetic susceptibility. For example, insect feeding injures corn kernels, creating ports of entry for fungi that produce ear rot and mycotoxins. Biotechnology is helping to provide season-long protection of corn plants (Bt corn) against corn borer damage through the introduction of insect control proteins derived from *Bacillus thuringiensis.* Reduction of insect feeding damage in Bt corn has led to lower contamination with fumonisin mycotoxins in most locations it has been tested around the world. Biotechnology is also being used to develop healthier corn plants by making them less susceptible to drought stress, increasing nitrogen utilisation, and protecting plants against a wider variety of insect pests that feed on the ears, stalks and roots. In the future, combining these traits will further improve yield and should reduce corn plant susceptibility to environmental stress factors that contribute to mycotoxin contamination in the field.

Keywords: biotechnology, *Bacillus thuringiensis*, fumonisins, corn borers, insect protection

1. Introduction

Corn (*Zea mays* L.) can be infected with fungi that produce toxic secondary metabolites called mycotoxins. *Fusarium verticillioides* (Sacc.) Nirenberg (synonym = *F. moniliforme* J. Sheld.) and *F. proliferatum* (T. Matsushima) Nirenberg, which produce fumonisin mycotoxins, are the most common fungi that infect corn wherever it is grown. Various environmental factors such as insect damage, heat and drought stress, nitrogen deficiency and genetic susceptibility predispose corn plants to infection with fungi (Miller, 2001; Munkvold, 2003). Dietary exposure to fumonisins can cause a variety of adverse health effects in farm and laboratory animals (CAST, 2003). Epidemiological studies suggest a link between high dietary intake of fumonisins and elevated rates of liver and/or oesophageal cancer in certain regions of Africa, China, Italy and Brazil (CAST, 2003; van der Westhuizen *et al.*, 2003). As a consequence, regulatory agencies have recommended limits for fumonisin contamination of corn intended for animal feed and human food use (CAST, 2003).

Control of insect pest damage to corn can reduce fungal infection since insect feeding provides ports of entry for fungi. Some insect pests also serve as vectors for fungal infection (Sobek and Munkvold, 1999). An effective insect pest control strategy has been developed with the introduction of coding sequences for the Cry1Ab protein derived from *B. thuringiensis* into corn plants (event MON 810, YieldGard Cornborer®, trademark of Monsanto Technology LLC) (Betz *et al.*, 2000). The Cry1Ab protein controls lepidopteran insect pests such as the European corn borer (ECB), *Ostrinia nubilalis* Hübner, the most important stalk-boring and ear damaging insect pest of corn in the U.S. Corn Belt (Pilcher *et al.*, 1997). The CaMV 35S gene promoter enables constitutive expression of the Cry1Ab protein throughout the growing season, thus providing season-long protection against corn borers. The *B. thuringiensis*-based (Bt) microbial pesticides that contain Cry proteins such as Cry1Ab have been used commercially in agriculture for over 40 years to control larval insect pests (Betz *et al.* 2000; IPCS, 1999). They have an exemplary safety record because their insecticidal mode of action is highly specific against target lepidopteran insect pests. The Cry 1Ab protein has no activity against non-target organisms such as mammals and birds (Betz *et al.*, 2000; IPCS, 1999).

Munkvold and co-workers were the first to report that corn hybrids protected with the Cry1Ab protein had significantly lower fumonisin mycotoxin levels in corn (Munkvold *et al.*, 1999). This was most evident in corn plants that expressed Cry1Ab protein constitutively during the growing season. Additional field trials have been conducted in countries that permitted field testing of YieldGard Cornborer hybrids to assess their impact on fumonisin levels under local conditions. Results are presented from field trials in Italy, France, Turkey and Argentina that compared the levels of fumonisins in YieldGard Cornborer varieties with their near-isogenic controls.

2. Materials and methods

2.1. Turkey

Field trials with YieldGard Cornborer varieties were carried out in Adana province, in the East Mediterranean Cukurova region where corn is planted as a second crop after wheat. Damage from corn boring insects pests is most severe in the second corn crop. The hybrids used for the study were DK626 Bt, its near-isogenic control (DK626) and a conventional hybrid traditionally grown in the region. The trials were carried out under conditions of natural insect infestation. Trials in 2000 and 2001 were set up using a randomised split plot design with four replicates. Each of the four blocks was divided into two sub-blocks. Four sub-blocks received three applications of the insecticide lamba-cyhalotrine at two to three week intervals, starting the third week of July. The other sub-blocks were not treated with insecticide. Test plots consisted of eight corn rows 20 m in length. At harvest, mycotoxin concentrations were compared across treatments. ELISA test kits (quantitative kit for fumonisin, Veratox, Neogen Corp., Lansing, MI, USA) were used to determine fumonisin levels in accordance with the kit instructions.

2.2. Italy

In 1999, field trials were carried out in northern Italy at 30 different locations with four YieldGard Cornborer varieties of varying genotype and their respective near-isogenic controls. The trials were carried out under conditions of natural insect infestation, as there is significant ECB infestation in these regions. Approximately 93 samples of corn were randomly collected from one to 10 weeks post-harvest from the various locations and sent to the School of Agriculture, U.C.S.C. Piacenza, Italy, for analysis using published procedures (Shephard *et al.*, 1990).

2.3. France

During 1997 to 1999, the levels of fumonisin mycotoxins were measured in YieldGard Cornborer varieties and their near-isogenic controls at 25 field trial locations in France. The majority of sites were in the Southwest of France where ECB is normally present. The trials were carried out under conditions of natural insect infestation. In non-replicated field trials, the plot size was a minimum of 20 meters x 8 rows. In replicated field trials, there were four randomised blocks in two plots (Bt and non-Bt), in plots 12 meters by six rows. For non-replicated trials, corn was collected from 20 consecutive plants in two adjacent rows at four locations in the plot. For replicated trials, corn was collected from 20 consecutive plants in two adjacent rows for each replicate. Corn harvested in 1999 trials was analysed for fumonisins at INRA, Nantes, France according to published methods (Bakan *et al.*, 2002). Corn samples harvested in 1998 were analysed for fumonisins at IEEB (European Institute of Environment at Bordeaux) using established methods (Shephard *et al.*, 1990). For corn harvested in 1997, samples were analysed at AGPM (French Corn Growers Association) using ELISA methods (Diffchamb France, Transia® plate Fumonisins).

2.4. Argentina

A YieldGard Cornborer hybrid (DK696) and its near-isogenic control (DK696) were grown in 57 different locations in Buenos Aires province in Argentina during 2000. This province is an important corn growing region for Argentina and corn pests such as *Diatraea saccharalis* (corn borer) and *Helicoverpa zea* (ear worm) are frequently found in this region. The field trials were carried out under conditions of natural insect infestation. Corn samples were collected from each of the sites at harvest and submitted for fumonisin analysis at the Laboratory Technology of Uruguay (LATU) in Montevideo using published methods (Visconti *et al.*, 2001).

3. Results

As shown in Table 1 and Figures 1-3, many of the field locations had lower fumonisin levels in YieldGard Cornborer hybrids compared to their near isogenic controls. These results confirm that insect feeding is an important contributor to fumonisin contamination of corn under conditions where there is significant corn borer presence. Protecting corn against insect feeding damage can reduce the opportunity for fungi to infect kernels. YieldGard Cornborer hybrids have

Table 1. Lower fumonisin levels in YieldGard Cornborer hybrid DK 626 grown in Turkey, 2001-2002.

Varieties	Fumonisin level (mg/kg)				Average
	1st Rep.[c]	2nd Rep.	3rd Rep.	4th Rep.	
Field trial 2001					
DK626 Bt (-)[a]	3.5	2.0	2.5	2.0	2.5
DK626 Bt (+)[b]	3.7	2.3	1.8	2.8	2.6
DK626 (-)	16.8	15.1	18.3	19.9	17.5
DK626 (+)	14.1	15.6	15.4	17.4	15.6
Conventional (-)	18.8	16.7	18.0	18.8	18.1
Conventional (+)	17.2	16.4	15.0	17.9	16.6
Field trial 2002					
DK626 Bt (-)	0.8	1.0	0.4	0.8	0.78
DK626 Bt (+)	0.5	0.4	0.8	0.7	0.63
DK626 (-)	17.3	18.7	18.2	12.8	16.75
DK626 (+)	5.5	10.6	18.0	16.7	12.7
Conventional (-)	18.7	17.5	16.6	18.0	17.7
Conventional (+)	12.8	12.2	15.6	17.8	14.6

[a]Non-insecticide treated plots (-).
[b]Insecticide treated plots (+).
[c]Cobs were wounded artificially.

been reported to have lower fungal contamination based on measurements of ergosterol levels in corn (Bakan *et al.*, 2002; Pietri and Piva, 2000).

When the levels of fumonisins were averaged across all sites for trials in each country (Table 2), the YieldGard Cornborer hybrids had reductions ranging from 61 to 97% compared to their near isogenic controls. In field trials carried out across the U.S. in 2000 and 2001, fumonisins levels were approximately 50% lower when averaged across all locations (180 total sites) for each year (Hammond *et al.*, 2004). Additional studies have been completed and recently published demonstrating a similar reduction of fumonisin levels in the corn

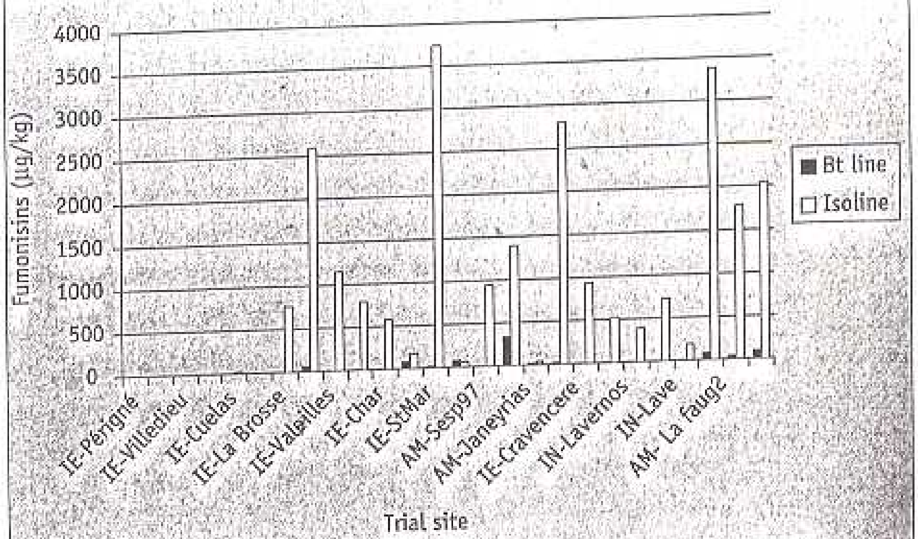

Figure 1. Field trials with YieldGard Cornborer in France, 1997-1999.

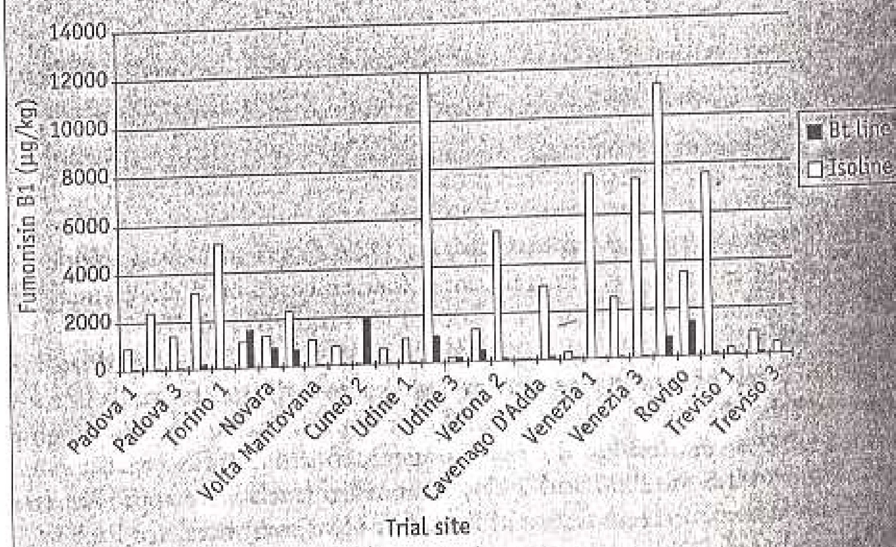

Figure 2. Field trials with YieldGard Cornborer in Italy, 1999.

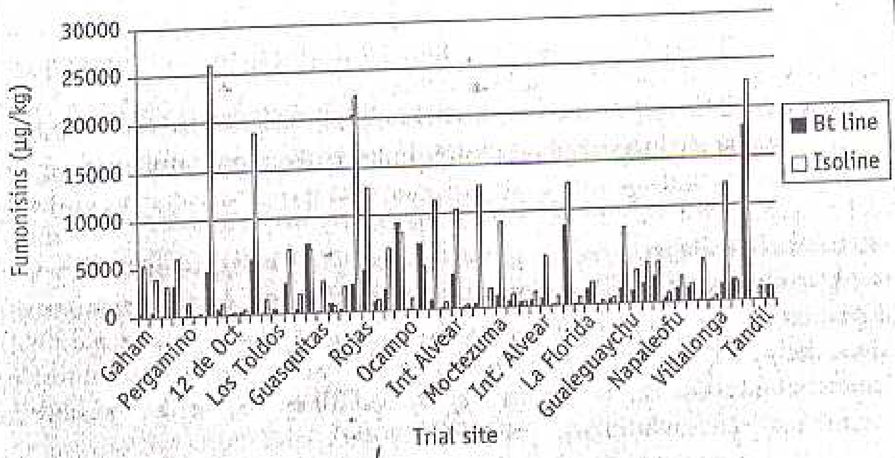

Figure 3. Field trials with YieldGard Cornborer in Argentina, 2000.

Table 2. Percentage reduction in fumonisin levels observed in field trial studies with YieldGard Cornborer by country.

Country	Year	Sites	Mean fumonisins (mg/kg)		% Reduction in mean fumonisins
			Non-Bt hybrids	Bt hybrids	
Turkey	2001-2002	2	17.5	2.6	85
France	1997-1999	26	1.0	0.03	97
Italy	1999	30	2.9	0.35	88
Argentina	2000	57	5.03	1.95	61

of YieldGard Cornborer varieties. These included field trials in Argentina (de la Campa *et al.*, 2005) and in Germany (Papst *et al.*, 2005). At some locations where YieldGard Cornborer was tested, the reduction in fumonisin levels was large enough to make the difference between an unacceptable crop and one that was safe for consumption.

4. Discussion

Significant fumonisin contamination of corn intended for human consumption continues to occur as evidenced by recent reports of its presence in corn meal (mean level 2,200 μg/kg) and corn-based infant food (de Castro *et al.*, 2004). Other scientists are investigating possible links between fumonisin exposure in early pregnancy and increased incidence of neural tube defects in new-borns (Marasas *et al.*, 2004). Fumonisin and aflatoxin co-contamination of corn has also been reported in some locations (Bankole and Mabekoje, 2004), which may be of concern since fumonisins promote the liver carcinogenic activity of aflatoxin in animal models (Carlson *et al.*, 2001; Gelderblom *et al.*, 2002). Given the widespread dietary exposure to fumonisins alone or in combination with other mycotoxins, there is an urgent need for more research to assess the potential impacts on human health (IPCS, 2000).

The economic impact of mycotoxin contamination of food crops has been estimated to range from 0.5 to 1.5×10^9 US dollars per year in the U.S. alone (CAST, 2003). In other world areas, the economic impact of mycotoxin contamination has not been well studied, but is expected to be significant. The economic impact resulting from human health consequences has not been included in the aforementioned estimates. As a consequence of the significant economic impact of mycotoxins, scientists around the world are actively engaged in finding ways to reduce mycotoxin contamination of food crops. Biotechnology is providing tools for researchers to understand the complex dynamics involved in fungal infection and production of mycotoxins. The fungal synthetic pathways for mycotoxins such as aflatoxin, fumonisin and deoxynivalenol are complex, involving multiple enzymatic steps. Biotechnology has helped to identify the genes involved in mycotoxin synthesis. Gene-knockout fungal strains are being used to determine key enzymatic steps in mycotoxin production (Proctor *et al.*, 2003; Alexander, 2004). The regulation of genes involved in mycotoxin production has been investigated using RNA silencing targeted against mycotoxin biosynthetic pathway-activating transcription factors (McDonald *et al.*, 2005). All of these research efforts may help to identify critical control points in mycotoxin production that could provide research leads for future mycotoxin reduction. Some have proposed introducing genes into plants that produce enzymes to degrade mycotoxins (Duvick, 2001). Others are using biotechnology to help identify natural resistance factors to fungal infection that are present in plants including corn (Payne *et al.*, 2004). Once identified, the coding sequences for these resistance factors may be introduced into susceptible varieties of corn to see if they improve protection against fungal infection.

Biotechnology is also being used to help develop healthier corn plants, which can withstand environmental stress factors that increase susceptibility to fungal infection in the field. Results were presented for fumonisin reduction in corn plants protected against corn borers. Building on this initial success, the next generation of Bt corn will produce Cry proteins that will provide better protection against a wider variety of insect pests such as fall armyworm, corn earworm in addition to corn borers (EPA, 2006). The overall strategy is to produce corn varieties that are much less susceptible to environmental stress factors in the field and therefore improve yield for farmers. A secondary benefit of these improvements should be reduced susceptibility to fungal infection and mycotoxin contamination.

An example of reduced environmental stress followed the recent introduction of another Bt corn variety protected against corn rootworm feeding (YieldGard Rootworm®). This variety demonstrated superior performance in 2005 across parts of the midwest U.S. afflicted with prolonged drought (Rice and Oleson, 2005). The maintenance of a healthy root system afforded better absorption of water and nutrients thus protecting the plant against drought stress compared to conventional corn varieties that were less protected against rootworm damage (Wenzel, 2006). The introduction of herbicide resistant corn varieties can also improve nutrient availability by reducing weed competition, which competes for the same nutrients such as nitrogen. Increasing the availability of nitrogen has been reported to reduce aflatoxin contamination in corn (Munkvold, 2003). The combination of root, stalk and ear protection against insect feeding as well as herbicide tolerance in the same corn plant can reduce plant stress significantly. Ongoing research is identifying genes that reduce drought stress and others that improve the efficiency of nitrogen uptake from the roots (Schulze, 2005). Combining these important agronomic traits with those already available will further enhance the ability of improved corn varieties to withstand a broad variety of plant stress factors. It is anticipate that these improvements will also lead to further reduced fungal infection and mycotoxin contamination. Thus, biotechnology has opened up many different research opportunities, which may lead to significant reduction of mycotoxin contamination of food and feed crops.

5. Conclusion

Fumonisin contamination occurs wherever corn is grown. Field trial experiments in locations where corn borers are prevalent have consistently found lower fumonisin contamination in the grain of YieldGard Cornborer hybrids. At some locations, the reduction in fumonisin levels was large enough to make the difference between an unacceptable crop and one that was safe for consumption. Biotechnology will provide many tools to help understand the dynamics of environmental stress factors and their impact on fungal interactions with the corn plant resulting in mycotoxin production. This may lead to new intervention strategies to reduce mycotoxin contamination. Developing healthier corn plants to better withstand environmental stress factors will also have secondary benefits of reducing mycotoxin contamination. The application of the many biotechnology tools that are now available should significantly improve crop yield and improve feed and food security, which is of critical economic importance to the world community.

References

Alexander, N.J., McCormick, S.P., Proctor, R.H. and Harris, L.J., 2004. Functional analysis of trichothecene biosynthetic genes via heterologous expression in a trichothecene-nonproducing *Fusarium* species. In: Proceedings of the 2nd International Symposium on *Fusarium* Head Blight, edited by S.M. Canty, T. Boring, J. Wardwell and R.W. Ward, 11-15 December 2004, Orlando, FL, USA, pp. 552.

Bakan, B., Melcion, D., Richard-Molard, D. and Cahagnier, B., 2002. Fungal growth and *Fusarium* mycotoxin content in isogenic traditional maize and genetically modified maize grown in France and Spain. Journal of Agricultural and Food Chemistry 50:728-731.

Bankole, S.A. and Mabekoje, O.O., 2004. Occurrence of aflatoxins and fumonisins in preharvest maize from south-western Nigeria. Food Additives and Contaminants 21:251-255.

Betz, F.S., Hammond, B.G. and Fuchs, R.L., 2000. Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regulatory Toxicology and Pharmacology 32:156-173.

Carlson, D.B., Williams, D.E., Spitsbergen, J.M., Ross, P.F., Bacon, C.W., Meredith, F.I. and Riley, R.T., 2001. Fumonisin B_1 promotes aflatoxin B_1 and N-methyl-N'-nitro-nitrosoguanidine-initiated liver tumors in rainbow trout. Toxicology and Applied Pharmacology 172:29-36.

CAST (Council for Agricultural Science and Technology), 2003. Mycotoxins: risks in plant, animal, and human systems. Task Force Report No. 139, Ames, IA, USA.

de la Campa, R., Hooker, D.C., Miller, J.D., Schaafsma, A.W. and Hammond, B.G., 2005. Modeling effects of environment, insect damage, and Bt genotypes on fumonisin accumulation in maize in Argentina and the Philippines. Mycopathologia 159:539-552.

de Castro, M.F., Shephard, G.S., Sewram, V., Vicente, E., Mendonca, T.A. and Jordan, A.C., 2004. Fumonisins in Brazilian corn-based foods for infant consumption. Food Additives and Contaminants 21:693-699.

Duvick, J. 2001. Prospects of reducing fumonisin contamination of maize through genetic modification. Environmental Health Perspectives109:337-342.

EPA (U.S. Environmental Protection Agency), 2006. Experimental use permit; receipt of application. Federal Register: 26 May 2006 (Vol. 71, No. 102), pp. 30403-30404.

Gelderblom, W.C.A, Marasas, W.F.O., Lebepe-Mazur, S., Swanevelder, S., Vessey, C.J. and de la Hall, P., 2002. Interaction of fumonisin B_1 and aflatoxin B_1 in a short-term carcinogenesis model in rat liver. Toxicology 171:161-173.

Hammond, B.G., Campbell, K.W., Pilcher, C.D., Degooyer, T.A., Robinson, A.E., McMillen, B.L., Spangler, S.M., Riordan, S.G., Rice, L.G. and Richard, J.L., 2004. Lower fumonisin mycotoxin levels in the grain of Bt corn grown in the United States in 2000-2002. Journal of Agricultural and Food Chemistry 52:1390-1397.

IPCS (International Programme on Chemical Safety), 1999. Environmental Health Criteria 217: *Bacillus thuringiensis*. World Health Organization, Geneva, Switzerland, pp. 36-66.

IPCS (International Programme on Chemical Safety), 2000. Environmental Health Criteria 219: fumonisin B_1. World Health Organization, Geneva, Switzerland, p. 88.

Marasas W.F., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C., Allegood, J., Martinez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A.V., Voss, K.A., Wang, E. and Merrill, Jr., A.H., 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. Journal of Nutrition 134:711-716.

McDonald, T., Brown, D., Keller, N.P. and Hammond, T.M., 2005. RNA silencing of mycotoxin production in *Aspergillus* and *Fusarium* species. Molecular Plant-Microbe Interactions 18:539-545.

Miller, J.D., 2001. Factors that affect the occurrence of fumonisin. Environmental Health Perspectives 109 (Suppl.2):321-324.

Munkvold, G.P., Hellmich, R.L. and Rice, L.G., 1999. Comparison of fumonisin concentration in kernels of transgenic Bt maize hybrids and nontransgenic hybrids. Plant Disease 83:130-138.

Munkvold, G.P., 2003. Mycotoxins in corn- occurrence, impact, and management. In: Corn Chemistry and Technology, edited by P.J. White and L.A. Johnson, American Association of Cereal Chemists, St. Paul, MN, USA, pp. 811-881.

Papst, C., Utz, H.F., Melchinger, A.E., Elder, J., Magg, T., Klein, D. and Bohn, M., 2005. Mycotoxins produced by *Fusarium* spp. in isogenic Bt *vs.* non-Bt maize hybrids under European corn borer pressure. Agronomy Journal 97:219-224.

Payne, G.A., Holmes, R.A. and Boston, R.S., 2004. Inhibition of aflatoxin production by corn seed proteins. In: Proceedings of the 4th Annual Fungal Genomics, 5th Annual Multi-Crop Fumonisin, 17th Annual Multi-Crop Aflatoxin Elimination Workshops, 25-28 October 2004, Sacramento, CA, USA, USDA, pp. 18.

Pietri, A. and Piva, G., 2000. Occurrence and control of mycotoxins in maize grown in Italy. In: Proceedings of the 6th International Feed Production Conference, Food safety, current situation and perspectives in the European Community, edited by G. Piva and F. Masoero, 27- 28 November 2000, Piacenza, Italy, pp. 226-236.

Pilcher, C.D., Rice, M.E., Obrycki, J.J. and Lewis, L.C., 1997. Field and laboratory evaluations of transgenic *Bacillus thuringiensis* corn on secondary Lepidopteran pests (Lepidoptera: Noctuidae). Journal of Economic Entomology 90:669-678.

Proctor, R.H., Brown, D.W., Plattner, R.D. and Desjardins, A.E., 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genetics Biology 38:237-249.

Rice, M.E and Oleson, J.D., 2005. Three-year summary of corn rootworm control products. Integrated Crop Management, 12 December 2005. http://www.ipm.iastate.edu/ipm/icm/2005/12-12/rootworm.html. (accessed 11 July 2006)

Schulze, L.D., 2005. Monsanto developing drought tolerance. The Label, November 2005, Vol. 17, No. 11. http://pested.unl.edu/thelabel/tlnov05.htm. (accessed 11 July 2006).

Shephard, G.S., Sydenham, E.W., Thiel, P.G. and Gelderblom, W.C.A., 1990. Quantitative determination of fumonisin B_1 and B_2 by high performance liquid chromatography with fluorescence detection. Journal of Liquid Chromatography 13:2077-2087.

Sobek, E.A. and Munkvold, G.P., 1999. European corn borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. Journal of Economic Entomology 2:503-509.

Van der Westhuizen, L., Shephard, G.S., Scussel, V.M., Costa, L.L., Vismer, H.F., Rheeder, J.P. and Marasas, W.F., 2003. Fumonisin contamination and *Fusarium* incidence in corn from Santa Carina, Brazil. Journal of Agricultural and Food Chemistry 51:5574-5578.

Visconti, A., Solfrizzo, M. and De Girolamo, A., 2001. Determination of fumonisins B_1 and B_2 in corn and corn flakes by liquid chromatography with immunoaffinity column clean-up: collaborative study. Journal of AOAC International 84:1828-1837.

Wenzel, W., 2006. Root Rescuers. Agweb, 11 July 2006. http://www.agweb.com/get_article.asp?pageid=127305&src=fscrn. (accessed 11 July 2006).