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Bacillus Thuringiensis Derived Vegetative Insecticidal Protein Vip3Aa20 as a Potential Aflatoxin Mitigation Tool in Maize (*Zea mays*)

Mays DT[1], Medrano EG[2], Setamou M[3] and Schuster GL[3*]

[1]Department of Entomology, Texas A&M AgriLife Extension Service, Hillsboro, TX, USA

[2]Department of Agriculture, Insect Control and Cotton Disease Research Unit, College Station, TX, USA

[3]Department of Agriculture, Agribusiness, and Environmental Sciences, Texas A&M University-Kingsville, Kingsville, TX, USA

[*]**Corresponding author:** Greta L Schuster, Department of Agriculture, Agribusiness, and Environmental Sciences, Texas A&M University-Kingsville, Kingsville, TX, USA, E-mail: tyler.mays@ag.tamu.edu

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Abstract

Aflatoxin contamination is an annual profitability challenge for maize (*Zea mays*) producers in Texas and other portions of the Southern United States of America. Insects are known to play a key role in the maize infection process by *Aspergillus flavus* that produce aflatoxin. Transgenic maize hybrids produce *Bacillus thuringiensis* (Bt) toxins to reduce the level of aflatoxin contamination. This project evaluated the potential effectiveness of incorporating the Vegetative Insecticidal Protein Vip3Aa20 in an integrated aflatoxin mitigation program. Maize variety trait packages evaluated consisted of a non-Bt, Genuity VT Double pro, Herculex, Genuity SmartStax, and Leptra which contained the Vip3Aa20 Bt protein. During the 2014 growing season aflatoxin concentrations ranged from 1.408 µg/kg in the Leptra trait package to a high of 45.97 µg/kg in the non-Bt trait package. Maize that harbored the Leptra trait package significantly reduced the level of aflatoxin compared to all other treatments. In 2015, aflatoxin concentrations ranged from non-detected (<0.0 µg/kg) in the Herculex trait package to a high of 5.64 µg/kg in the Genuity VT double pro trait package; statistical differences between these treatments were not found. Aflatoxin concentrations in 2016 ranged from <0.0 µg/kg in the Herculex treatment to a high of 45.58 µg/kg in the non-Bt trait package; all Bt trait packages had statistically less aflatoxin contamination than the non-Bt trait suite. Aflatoxin concentrations in the Leptra trait package was lower than the non-Bt by an average of 91.8, 51.64 and 95% during the 2014, 2015 and 2016 growing seasons, respectively. Results from this research illustrated that utilization of the Vip3Aa20 Bt-protein reduced aflatoxin contamination in maize and thus, has a high economic potential.

Keywords: Aflatoxin; Bacillus thuringiensis; Corn earworm; Mycotoxin; Vip3A

Introduction

Aflatoxin is a secondary metabolite produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, both of which are known to cause *Aspergillus* ear rot of maize (*Zea mays*). Aflatoxin contamination of maize is a yearly problem for producers in the southern United States of America (USA). The contamination of maize with aflatoxin is a major concern due to the negative health effects it can have on animals and humans [1]. Due to these adverse health effects the amount of aflatoxin in maize grain is regulated by governmental agencies around the world. In the USA, aflatoxin contamination is regulated by the Food and Drug Administration (FDA) that permits only 20 µg/kg for human consumption and varying levels for livestock depending on the species. Mitchell et al. estimated the potential economic loss from aflatoxin contamination to be between USD $52.1 million and USD $1.68 billion.

Since the discovery that aflatoxin contamination can occur prior to harvest in the mid-1970s research has been conducted on factors affecting aflatoxin accumulation and potential mitigation tactics [2]. Based on recent work [3], there is a consensus that aflatoxin contamination is a result of plant stress caused by heat, drought, nutrient deficiencies, growing hybrids not fit for an environment, and insect damage to the stalk and/or ear. Temperatures above 30°C are unfavorable for maize growth and development, and as temperatures continue to increase above 30°C stresses the plant becomes more susceptible to diseases and reduced yields [4]. Temperatures between 36°C and 38°C are optimal for *A. flavus* infection and development. In Texas and most of the southern USA, temperatures above 30°C are often accompanied by dry weather, and often occur during the reproductive growth stages. Expectantly, drought conditions cause a loss in turgor pressure producing an increased maize susceptibility to *A. flavus* infections. Plant fertility also affects the ability of the plant to defend itself against diseases. Research conducted in the 1970s found the risk of fungal infection and mycotoxin contamination is reduced when plants have adequate levels of nitrogen [5]. Damage caused by insect pests have also been associated with aflatoxin contamination by facilitating infection of the ear with *A. flavus* [6,7]. Lillehoj et al. [8] found that ears damaged by *Ostrinia nubilalis*, *Helicoverpa zea*, and/or *Spodoptera frugiperda* had significantly more aflatoxin compared to undamaged ears. Based on the aforementioned reports, reducing the amount of insect damage to the plant in turn decreases aflatoxin contamination levels.

Maize producers address aflatoxin issues through planting data, utilization of maize hybrids genetically modified to express toxins from the soil bacterium *Bacillus thuringiensis* (Bt), and the application of atoxigenic *A. flavus* strains. Weaver et al. [9] found that the application of atoxigenic fungal strains to mitigate aflatoxin contamination is both effective and economical. Maize transformed to express Bt toxins were first released in the late 1990s, and up until 2011 the crystalline proteins used consisted of Cry1Ab, Cry1F, Cry1A.105, and Cry2Ab2 [10]. In 2011, the Vegetative Insecticidal Protein (VIP) product was commercially released. The original release of Bt maize targeted boring insect pests such as the European corn borer (*O. nubilalis*) and southwestern corn borer (*Diatraea grandiosella*), but it was subsequently found to suppress corn earworm (*H. zea*) and fall armyworm (*S. frugiperda*) [11]. There have been multiple studies focused on aflatoxin control using Bt maize hybrids. Pruter et al. [12] and Abbas et al. [13] observed lower insect damage and aflatoxin in samples from Bt maize hybrids than the non-Bt hybrids. However, only Pruter et al. include the Vip3Aa20 protein, and results indicated further research is needed to determine if the Vip3Aa20 Bt protein can limit aflatoxin concentrations. Additionally, over the last decade reports of field evolved resistance of H. zea to these crystalline Bt proteins have increased. To date there are reports of field evolved practical resistance in *H. zea* populations to Cry1Ab, Cry1Ac, Cry1A.105, and Cry2Ab2 [14], all of which are used in Bt maize trait packages. These reports of resistance leave only the Vip3Aa20 Bt protein as a reliable option for reduced insect damage and aflatoxin contamination. The primary objectives of this project were to examine the ability of the Vip3Aa20 Bt protein to yield lower aflatoxin contamination and to measure its effectiveness as a tool for an integrated aflatoxin mitigation strategy.

Materials and Methods

Field trials were conducted during the 2014, 2015 and 2016 growing seasons at the Texas A&M University-Kingsville school farm in South Texas. Planting, fertilization and pest management other than for *Lepidopteran* insect pests were conducted following the recommendations of Texas A&M agrilife extension service. Varieties included a non-Bt hybrid, Genuity VT Double Pro, Herculex I and Leptra. The Genuity SmartStax variety was included in the trial during the 2015 and 2016 growing seasons (Table 1). During all three years treatments were replicated four times implementing a randomized complete block design. For statistical analysis the data was log transformed using the formula Log (x+1), where x was the aflatoxin concentration or area of insect damage. An ANOVA with a confidence limit of 95% (α=0.95). Mean separations were conducted using Tukey's HSD with and a 95% confidence limit (p=0.05).

Trait package	Cry1Ab	Cry1A.105	Cry1F	Cry2Ab2	Vip3Aa20
Non-Bt					
Genuity VT Double Pro		□		□	
Genuity smart stax		□	□	□	
Herculex			□		
Leptra	□		□		□

Table 1: List of *Bacillus thuringiensis* (Bt) toxin packages and Bt proteins expressed by maize hybrids included trials at Kingsville, TX, USA in 2014, 2015, and 2016.

The area of insect damage was collected during the 2014 and 2016 growing seasons by hand harvesting 10 ears per plot and measuring the amount of insect damage to the ear in square centimeters. Damage was divided into the top and lower portions of the ear to putatively distinguish between *H. zea* and *S. frugiperda*. Due to limited damage to the lower ear, both the upper ear and lower ear injury was combined for statistical analysis. Aflatoxin concentrations were quantified by shelling and grinding the 10 ears used to measure insect attack. Maize was ground using a Thomas Wiley Mill model 4 (Thomas Scientific, Swedesboro, NJ, USA) to pass through a 20-mesh screen. A standard subsample of 200 g was collected and sent to a plant pathology laboratory at Texas A&M University at college station, TX, USA for aflatoxin quantification. The concentration of aflatoxin was determined by using the AflaTest immunoaffinity column from VICAM. Manufacturer standard operating procedures were followed and allowed for detection of aflatoxin between 0 µg/kg and 500 µg/kg.

Results

In 2014, aflatoxin concentrations ranged from 1.40 µg/kg to a high of 45.97 µg/kg for Leptra and the non-Bt trait package, respectively. Aflatoxin concentrations were statistically significantly lowered by the Leptra Bt trait package compared to all other treatments (df=3,9; F=4.397; p=0.0364; Table 2). The Genuity VT Double Pro and Herculex Bt trait packages did not differ from the non-Bt. The addition of the Vip3Aa20 protein reduced aflatoxin by 97% compared to the non-Bt and between 95 and 96 percent compared to the Herculex and Genuity VT Double Pro, respectively (Figure 1). The Vip3Aa20 protein contained in the Leptra Bt trait package significantly reduced the amount of insect damaged compared to all other treatments. The Genuity VT Double Pro treatments significantly reduced the amount of insect damage to the ear compared to non-Bt and Herculex Bt trait package yet, it did not translate into a reduction in aflatoxin contamination. The Leptra Bt trait package reduced the amount of insect damage by between 92.5% and 96.4% compared to the Genuity VT Double Pro and non-Bt hybrid, respectively. In 2014, there were 23 days where the average temperature exceeded 30°C, and 82 days where the high temperature surpassed the upper temperature threshold (Table

3). There were 15 days during the growing season where the daily high temperature was within the *A. flavus* optimum temperature range. This region of Texas on average receives 39.42 cm of precipitation during the growing season. In 2014, the amount of precipitation that occurred during the growing season was 1.6-fold lower than the average and totaled only 19.71 cm.

Treatment	2014	2015	2016
Non-Bt	45.97 a[1]	3.04	32.27 a
Genuity VT double pro	37.54 ab	5.67	1.60 b
Herculex	28.38 ab	0	0.00 b
Leptra	1.40 b	1.47	0.73 b
Genuity smart stax	Nd[2]	0.34	0.82 b
P>F	0.0364	0.4459	0.0043

Table 2: Mean maize aflatoxin concentrations in µg/kg from Kingsville, TX, USA field trials during 2014, 2015 and 2016. [1]: Means in the same column followed by the same letter are not statistically different based on F-protected LSD (p=0.05). [2]: Genuity Smart Stax was not included in the 2014 study.

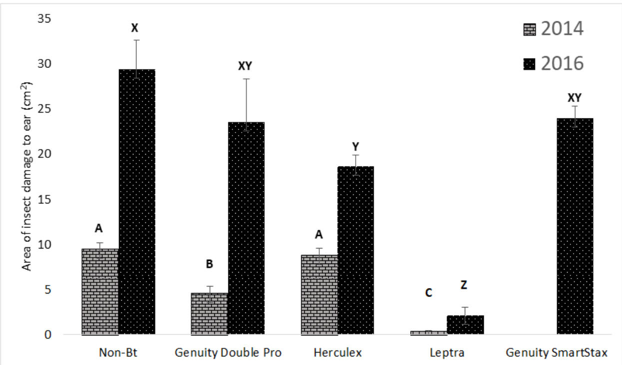

Figure 1: Area of ear damage cause by *Helicoverpa zea* during the 2014 and 2016 growing seasons in Kingsville, TX, USA. Treatments with the same letters are not statistically different based on an F-protected LSD (p=0.05).

Weather Parameter	2014	2015	2016
Average low temperature	19.6°C	21°C	21.6°C
Average high temperature	29.7°C	28.6°C	30.07°C
Average temperature	24.6°C	24.8°C	25.8°C
Precipitation	19.71 cm	73.6 cm	45.46 cm
Days averaging ≥ 30°C	23	12	21
Days with highs ≥ 30°C	82	73	85
Days with highs ≥ 36°C ≤ 38°C	15	7	5

Table 3: Weather data for Kingsville, TX, USA from 2014-2016 growing seasons. Weather data was compiled from Texas Mesonet.

Aflatoxin contamination was lower in 2015 compared to 2014 and averaged 0.40 µg/kg across all treatments. Levels of contamination ranged from 0.0 µg/kg for the Herculex Bt trait package to a high of 5.67 µg/kg for the Genuity VT Double Pro treatment (Table 2). Concentrations of aflatoxin during the 2015 growing season was not significantly impacted by the presence of any Bt proteins, and the Genuity VT Double Pro trait package had over a 86% increase in aflatoxin compared to the non-Bt. The Herculex, Leptra, and Genuity Smart Stax Bt trait packages all had aflatoxin levels below that of both the non-Bt and Genuity VT Double Pro. The Vip3Aa20 protein containing Leptra Bt trait package reduced aflatoxin by 51.6% compared to the non-Bt and 74% compared to the Genuity VT Double Pro Bt trait package. Insect damage to the ear caused by *H. zea* and/or *S. frugiperda* was not measured during the 2015 growing season. In the 2015 growing season, there was a total of 12 days where the average daily temperature was equal to or higher than 30°C, and 73 days where the daily high exceeded 30°C (Table 3). During the latter part of the growing season there was a total of 43 consecutive days with a high above 30°C, and of those 31 had high temperatures above 32°C. Additionally, during the growing season there was a total of 7 days where the high temperatures were between 36°C and 38°C, which favored the growth of *A. flavus* and not maize. Rainfall for the 2015 growing season totaled 73.6 cm which was 2-fold higher than the average rainfall for the region [15]. However, during the reproductive stages which has the highest evapotranspiration demand of the growing season there was a period of 29 consecutive days without rainfall.

The average aflatoxin concentration across all treatments in 2016 was 0.61 µg/kg and was the lowest of the three years. Aflatoxin concentrations were significantly reduced by the Bt trait packages which ranged from 0.0 µg/kg for the Herculex Bt trait package to a high of 32.27 µg/kg for the non-Bt (Table 2). Statistical differences were observed between all Bt trait packages and the non-Bt, but there were no significant differences observed between the various Bt trait packages. The area of the ear damaged by *H. zea* ranged from 1.65 cm2 in the Leptra Bt trait package to a high of 28.87 cm^2 for the non-Bt (Figure 1). The Leptra trait package had significantly less insect damage than the non-Bt, Genuity VT Double Pro, Herculex and Genuity Smart Stax Bt trait suites. The average temperature during the 2016 growing season was 25.8°C and was measurably warmer than the 2014 (24.6°C) and 2015 (24.8°C) growing season (Table 3). There was a total of 21 days during the growing season where the average daily temperature exceeded the 30°C threshold for favorable corn growth, and 85 days where the daily high temperature was equal to or exceeded 30°C. Precipitation received during the growing season totaled 45.46 cm which was above the average rainfall for the growing season in this region of Texas [15].

Discussion

Aflatoxin concentrations in the Vip3Aa20 containing Leptra Bt trait package ranged from 37.3% (2015) to 95.6% (2016) lower than the non-Bt (Table 4, dashes represent no reduction in aflatoxin compared to the untreated control). Reduction in aflatoxin during 2014 was likely due to a decrease in the amount of maize ear damaged caused by *H. zea* and *S. frugiperda*, that ranged from 0.34 cm^2 for the Leptra Bt trait package to a high of 9.35 cm^2 in the non-Bt (Figure 1). The significant reduction in aflatoxin observed during the 2014 and 2016 growing seasons appeared to be related to a decrease in insect damage to the ear. The results from 2014 and 2016 in this study align with observed reductions in mycotoxin contamination in hybrids genetically modified to express the Cry-proteins as reported previously [3,16-18]. However, in 2015 a statistically significant reduction in aflatoxin caused by the various Bt trait packages evaluated was not detected. The lower aflatoxin concentrations in 2015 are likely due to more rainfall events and the fact that the study location was flooded and most of the fourth replication died prematurely due to waterlogged soils. Temperatures above 30°C are not conducive for growth and development of maize [4], and *Aspergillus flavus* development is optimal at temperatures between 36°C and 38°C [2]. Notably, there was an apparent benefit to utilization of maize containing Vip3Aa20 Bt trait packages in aflatoxin mitigation based on either significantly or numerically (2015) lower toxin detection than the non-Bt by 91.8, 37.3, and 95.6 percent in 2014, 2015, and 2016 respectively (Table 4).

Treatment	2014	2015	2016
Non-Bt	--	--	--
Genuity VT Double Pro	[1]	--	75.30%
Herculex	--	100%	100%
Leptra	91.87%	37.30%	95.61%
Genuity Smart Stax	Nd[2]	70.37%	96.97%

Table 4: Percent reduction in maize aflatoxin contamination relative to the Non-Bt. [1]: No reduction in aflatoxin relative to the non-Bt treatment. [2]: Genuity Smart Stax was not included in the 2014 study.

These results also indicated that there may be other factors affecting aflatoxin accumulation in maize grain other than the amount of insect damage to the ear. A total of 5 days during the growing season had high temperatures that were optimum of *A. flavus* development with temperatures between 36°C and 38°C [2]. The development of *A. flavus* is favored by temperatures between 36°C and 38°C. Since the discovery of preharvest aflatoxin contamination several biotic and abiotic factors have been suspected as potential factors affecting aflatoxin contamination. When temperatures exceed 30°C the weather is no longer favorable for corn growth [2], and as temperatures continue to increase past 30°C there can be a negative impact on the health and yield of the plant. Stress factors include drought and high temperatures, fertility, growing hybrids not suited for the region, planting and harvest dates, plant populations, and insects [19-22]. Research conducted by Ni et al. found relationships between aflatoxin and other corn insect pest not currently controlled by Bt proteins including the maize weevil (*Sitophilus zeamais*) and the brown stink bug (*Euschistus servus*); these parameters were not measured in this work. Another factor that was not assessed but could have impacted the results is the development of practical resistance in the natural *H. zea* populations to multiple Bt proteins evaluated in this study. To date, Vip3Aa resistance alleles have been detected in several insects including *H. zea* (10,23-28]. In Texas, Yang et al. observed that commercially used maize hybrids with Bt trait packages containing Cry1 and Cry2 Bt proteins had little efficacy against *H. zea* and suggested that the insect population have developed practical resistance to the toxins. Additionally, Yang et al. observed a low level of resistance alleles to the Vip3Aa20 protein in *H. zea* collected from infested ears of Leptra corn outside of college station, Texas, USA.

Results from this study indicated that Bt trait packages that contain the Vip3Aa20 protein have the potential to significantly reduce aflatoxin contamination. Thus, these Bt trait packages should be regarded as a critical component to minimizing insect damage and mitigating aflatoxin issues. The exploitation of Bt trait packages, particularly those containing the Vip3Aa20 protein can increase the selection pressure for resistant alleles to the Bt proteins and thus, favors development of toxin immunity. A successful aflatoxin mitigation program should be multifaceted and include: (1) Planting to avoid temperature and/or moisture stress during the reproductive stages of crop development; (2) Provide adequate soil nutrition to propagate healthy plants; (3) Planting at populations that minimize intra-plant competition; and (4) The application of atoxigenic *A. flavus* strains. The incorporation of atoxigenic *A. flavus* strains to maize fields in the *Mississippi Delta* was found to be cost effective at reducing aflatoxin contamination [9]. Our results along with those of Pruter et al. [12] and Ni et al. [22] indicated that further research is needed across various agroecosystems and their varying environmental conditions to develop a systematic determination of the role of Lepidopteran and other insect pests of maize play in the *A. flavus* infection process and the subsequent accumulation of aflatoxin.

Conclusion

The presented results suggested that the significant reduction in insect damage to the maize ear may be related to addition of the Vip3Aa20 protein in the Leptra Bt trait package and thus, associated with reduced aflatoxin contamination. The data also showed that even though insect damage effects are present, environmental factors like moisture and temperature stress may exacerbate high aflatoxin accumulations. Together, the data determined that the inclusion of Vip3Aa20 protein into Bt trait packages may provide protection against elevated aflatoxin levels under certain conditions. Based on the data collected and weather conditions in 2016, the utilization of Bt technology to reduce the amount of insect damage to the ear, may also significantly reduce aflatoxin accumulation in the corn grains. Collectively, results from this investigation demonstrated that

Vip3Aa20 Bt maize hybrid technology is an effective component to an integrated aflatoxin mitigation program.

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