IDENTIFICATION OF RESISTANCE ALLELES TO BT PROTEINS IN *HELICOVERPA ZEA*

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Abstract

Bt crops have improved agricultural pest control, increasing yields with reduced insecticide applications. Nevertheless, reports of control failures raise the concerns of pests evolving resistance to the Bt toxins. More information regarding the status of Bt resistance is imperative for the development of strategies to delay its evolution. F_2 screens were conducted on populations of *Helicoverpa zea* to detect alleles conferring resistance to Cry1Ac, Cry2Ab2 and Vip3Aa51. Twelve F_2 families were established from populations collected in College Station, Muleshoe and Amarillo TX. Ten of those families (83.33%) survived on Cry1Ac (10µg/cm^2) after 7 days of exposure to the toxin and surpassed the homozygous resistance frequency of 6.25%. Five families (42.00%) survived on Cry2Ab2 (10µg/cm^2) after 7 days exposed to the toxin and surpassed the homozygous resistant frequency. None of the F_2 families met the criteria of a potential resistant strain after exposure to Vip3Aa51 (1µg/cm^2). However, one F_2 family had an unexpectedly high percentage of larval survivorship reaching 2nd instar. Full range bioassay confirmed significant resistance ratios for 2 F_2 strains. The Cry1Ac-F_2 strain A19, resulted 311.60-fold more resistant to Cry1Ac than the Benzon susceptible strain (BZ-SS). The Cry2Ab2-F_2 strain A41, resulted 56.50-fold more resistant to Cry1Ac and 17.70 times more resistant to Cry2Ab2 than BZ-SS. Despite the small sample size, we identified resistance to Cry1Ac and Cry2Ab2 toxins in *H. zea* in Texas. This finding may suggest that occurrence of alleles conferring resistance to these toxins in the species could be high in the collection region.

Introduction

H. zea (Lepidoptera: Noctuidae) is a destructive pest of crops of economic importance (Cunningham and Zalucki 2014, CABI 2019). The main damage provoked by *H. zea* consists on holes and lesions inflicted by the larval stage when feeding on flowering and fruiting structures of the plants (Bergvinson 2005). Its feeding habit appear to discriminate for the high protein content in meristematic tissues and in consecuence has a detrimental effect on the yield (Hardwick 1965, Fitt 1989). In cotton, the larvae provokes damage on bolls, squares and blossoms boring these tissues (Mally 1892). Severely affected pinhead squares and small cotton bolls may fall from the plant (Pitre et al. 1979). Larger larvae penetrate formed bolls and feed on carpels. The excrement left by the larva stains any remaining fiber on bolls. This creates a conducive environment for fungal infection leading to boll losses (Quaintance and Brues 1905). In corn, the damage provoked by *H. zea* significantly reduce grain quality and marketability (Archer and Bynum 1994, Archer and Bynum 1998). Furthermore, it facilitates infection of ears by pathogenic fungi capable of producing toxic metabolites on grains, which represents significant risks to human and animal health (Mc Millian et al. 1985, Abbas et al. 2009)

The use of insecticides is one of the primary methods for controlling *H. zea*. However, the effectiveness of the control may be highly variable because of the pest's cryptic feeding behavior. Growers depend on scouting the fields frequently to detect pest activity early in the season, and target eggs and first instars which are the most vulnerable pest stages. Once *H. zea* gains access to fruiting structures, it is largely protected from sprays (Sparks and Mitchell 1979, Green and Lyon 1989, Matthews 1989). The introduction of transgenic crops expressing insecticidal toxins from the soil-dwelling bacteria *Bacillus thuringiensis* has overcome many of the limitations for controlling *H. zea.* Insect pathogenic bacteria *Bacillus thuringiensis* produces Vip and Cry proteins during its vegetative and sporulation life stages respectively. These proteins are solubilized in the digestive tract of susceptible insects, releasing a toxin active form. After protease processing, the toxin passes to the peritrophic membrane and binds to specific receptors located on the brush border membrane of the insect midgut cells. These leads to pore formations on the epithelial wall and cell lysis, which favors infection by opportunistic organisms and the eventual insect death (Ferré and Van Rie 2002, Chakroun et al. 2016, Banerjee et al. 2017). Through DNA recombinant technology, the genes encoding the bacterial insecticidal toxins has been introduced directly into many crops such as cotton and corn, providing protection against important insect pests (Rani and Usha 2013).
Among the advantages on the use of transgenic crops expressing Bt toxins includes an improved efficacy controlling important plant pests, a high selectivity against a reduced number of target pests (Jurat-Fuentes and Crickmore 2017)

and no persistency in the environment (Koch et al. 2015). In addition, it has represented higher profits for growers due to an increase in crop yields with reduced pesticide applications (Fernandez-Cornejo et al. 2014). It is estimated that the use of Bt crops in the US have reduced insecticide applications by 123 million pounds, representing additional benefits for human health and the environment in general. (US-EPA 2016). These facts have been translated into a significant increase in the use of the technology worldwide. The area planted with Bt crops went from 1.1 million hectares in 1996 to 98.5 million in 2016, accounting for a percentage increase of 88.54% (Tabashnik and Carrière 2017). In the US, the areas planted with Bt cotton increased from 15% in 1997 to 85% in 2018. Similar trends are noted for corn, where Bt corn increased from 8% in 1997 to 82% in 2018 (USDA-ERS 2018).

Unfortunately, the large acceptance of the technology has driven selection pressure towards resistant individuals over time, which has been evidenced by reports of unusual damage provoked by insects in Bt crops (Tabashnik and Carrière 2017). Furthermore, it's considered that there is evidence of field evolved resistance of *H. zea* to the most common Cry toxins expressed in both transgenic corn and cotton in the southeastern US (Dively et al. 2016, Reisig et al. 2018). More information regarding the status of Bt resistance in *H. zea* is of crucial need to determine which are the possible crop production risks and delineate strategies to delay its evolution.

Resistance monitoring conform a key element of the overall strategies for delaying Bt resistance (Venette et al. 2000). The use of systematic monitoring plans has the objective of collecting valuable information to make important crop and insect management decisions before resistance become prevalent (Andow and Ives 2002). It could assess the success of the insect resistance management strategies already in place, by detecting changes in the susceptibility of an insect population to a Bt toxin and its resistant allelic frequency in the selection environment. This could benefit in understanding possible shifts and create adaptive plans to counteract the phenomena (Venette et al. 2000, Hutchison 2015). Proactive monitoring activities should be conducted with a sensitive and informative detection method to better plan a response in case of resistance evolution. The F_2 screen (Andow and Alstad 1998) is considered as a core method for monitoring insect resistance to Bt crops, due to its accuracy and sensitiveness detecting rare resistant alleles (Venette et al. 2000, Andow and Ives 2002, Huang 2006). It consists of collecting mated adult feral females, establishing iso-lines from the females by rearing its progeny (F_1) separately, sib-mating the F_1 generation and screen the F_1 progeny or F_2 generation neonates on diet containing the Bt toxin or Bt plant tissues. If the sampled females carry an allele conferring resistance, 6.25% of the F_2 progeny is expected to be homozygous for the resistant allele on the screen when resistance is recessive. Resistance is confirmed by conducting an appropriate statistical analysis (Andow and Alstad 1998, Huang 2006).

The objective of our study is to conduct a modified version of the F_2 Screen on *Helicoverpa zea* populations to detect alleles conferring resistance to Cry1Ac, Cry2Ab2 and Vip3Aa51 toxins commonly expressed in transgenic cotton and corn. Information resulting from this research could contribute to monitoring and resistance management efforts, thus supporting sustainability of Bt technologies.

Materials and Methods

Insect Source

H. zea feral larvae were collected from June to August 2018, from host plants at College Station, Muleshoe and Amarillo, TX. Larvae were placed on 29 ml plastic condiment cups (Solo®) containing *Heliothis* diet (WARD'S Stonefly Heliothis diet, Rochester, NY) and closed with plastic lids. The cups with the living larvae were identified per collection site, placed in plastic trays (Bio-Serv, Frenchtown, NJ) and transported to the Texas A & M Entomology Research Laboratory, College Station, TX. Larvae were reared into an environmentally controlled chamber (28 ºC, 50% RH and 14:10h (L:D) photoperiod) (Percival Scientific, Perry, LA) until reaching pupal stage. The pupae were taken out of the cups and classified by sex. Female pupae were selected and placed into 3.8L paper containers with approximately 60g of vermiculite (SunGro®, Pine Bluff, AR). An 87 mL plastic cup was embedded at the vermiculite and half filled with a solution of 10% honey water. Paper towel was rolled up and paced into the honey water, absorbing part of the solution. The towel covers the liquid surface and exceed the top of the cup. This prevents the moths from drowning and provides them with surface to settle and feed. The top opening of the paper container is covered with a cotton gauze (Casselman Global Enterprises, Toronto, Ontario) to prevent moth from escaping. After adult emergence, feral female moths are placed into the refrigerator at -20ºC for 2 minutes to diminish activity. After this period, the moths are taken out and marked carefully with a color marker for further identification. Then, marked female moths were transferred to 20L wire baskets (Seville Classics, Inc., Torrance, CA) containing 200 g of vermiculite at the bottom and 10% honey water. Adult *H. zea* males from a standard susceptible laboratory colony

(BZ-SS) (Benzon Research Inc., Carlisle, P.A.), were placed with the feral females (>30 :30) and allowed to mate for 10 days. After that period, feral females were taken out and transferred individually into 0.36L paper containers with vermiculite and 10 % honey water solution. The top opening of the paper container was covered with a cotton gauze, which prevents the moth from escaping and provides a surface for egg laying. Fertile eggs were collected and separately reared as F_1 iso-families. Each F_1 iso-family was sib-mated following the procedure described previously and its eggs were collected as the F_2 generation for the screen.

F_2 Screens

The susceptibility of each *H. zea* F_2 family was tested on diagnostic concentrations of the Bt toxins Cry1Ac, Cry2Ab2, and Vip3Aa51. Discriminatory concentrations were based on previous studies conducted by (Yang et al. 2017). Cry1Ac and Cry2Ab2 proteins were provided by Bayer Crop Science as a lyophilized MVPII powder (20.0% AI) and a lyophilized freeze-dried Bt-corn leaf powder (~ 4 mg of Cry2Ab2 protein/g) respectively. BASF Co. provided the Vip3Aa51 protein (2.9mg Vip3Aa51/mL). Four plastic trays (128-well -trays C-D International, Pitman, N.J.), one for each toxin and a control treatment were used per F_2 family. 800 µL of meridic liquid diet (Corn earworm diet; Southland Products Inc.) were pipetted per well. Once solidified, a solution of each Bt protein and Triton (0.1%) was overlayed on the diet surface in a proportion of 10µg Cry1Ac/cm^2, 10µg Cry2Ab2/cm^2 and 1µg Vip3Aa51/cm^2. The volume of the solution used per well for the Cry1Ac and Vip3Aa51 proteins was 40µL, and 200µL for the Cry2Ab2. 40µL of Triton (0.1%) was overlayed per well for the control treatments.

After the protein solution air dried over the diet, one *H. zea* F_2 neonate (< 24 h) was placed per well using a damped small painting brush. The wells were covered with vented plastic lids (C-D International, Pitman, NJ) and the trays were placed into an environmentally controlled chamber at 28 ºC, 50% RH, and a 14:10 (L:D) h photoperiod. The experimental setting had 4 replicates of 32 neonates each, for a total of 128 observations per F_2 family/toxin. Larval mortality, percent of larval survivorship and larval instar were recorded after 7 days exposed to the Bt toxins. Potential positives were defined as strains resulting with 5 or more living larvae, at 3rd instar or above after the treatment. Survivors were reared and sib-mated as described on the first section. Progeny from this sib-mating event was tested in a full concentration range bioassay for resistance confirmation.

Resistance Confirmation

Full range bioassays for resistance confirmation was performed on F_2 strains that met and exceeded the criteria for potential positives in the F_2 screens. Descriptions of the Bt proteins used for the bioassays, trays and diet preparation follow those of the previous section. Each bioassay included 6 or 7 concentrations per toxin plus one untreated control. The concentrations of Cry1Ac protein used for the diet-overlay were 0, 0.0316, 0.1, 0.316, 1.0, 3.16, 10.0 and 31.6 µg/cm^2. For Cry2Ab2, the concentrations were 0, 0.0316, 0.1, 0.316, 1.0, 3.16 and 10.0 µg/cm^2. At last, the concentrations used for the Vip3aA1 protein were 0, 0.01, 0.0316, 0.1, 0.316, 1.0 and 3.16 µg/cm^2. A solution of each Bt protein and protein concentration plus Triton (0.1%) was overlayed on the diet surface. As before, the volume of the solution used per well for the Cry1Ac and Vip3Aa51 proteins was 40µL, and 200µL for Cry2Ab2. 40µL of Triton (0.1%) was overlayed per well for the 0 µg/cm^2 treatments. After protein solution air dried over the diet, one *H. zea* neonate (< 24 h) was placed per well and covered with vented plastic lids. Trays were placed into an environmentally controlled chamber at 28 ºC, 50% RH, and a 14:10 (L:D) h photoperiod for 7 days.

We used an experimental setting for the full range bioassays having 4 replicates of 16 neonates per concentration/Bt protein. That is 64 observations/concentration=384 observations on full range bioassays with 6 concentrations and 448 for those with 7 concentrations. Larval mortality and larval instar were recorded after this period.

Data Analysis

The percent of larval survivorship of the F_2 strains tested with diagnostic concentrations of the Bt toxins was calculated as the number of living larvae at 3rd instar or above/total larvae assayed. The resulting value was corrected using the survivorship of the control treatment (Abbott 1925) and multiplied by 100. Resulting percentage value was compared with the theoretical homozygous resistant frequency of 6.25%. Strains resulting with this value or above were advanced for Bt resistance confirmation.

Larval mortality was calculated based on the number of dead larvae plus survivors that were still in the first instar (mortality = dead+L1) divided by the total number of insects assayed and was used to determine LC_{50} values and the

corresponding 95% confidence limits (CL). Larval mortality at each concentration was first corrected based on the control mortality (Abbott 1925), followed by Probit analysis (SAS/STAT-_software_version_9.4® 2017) to determine the LC_{50} that caused 50% mortality and the corresponding 95% CL. Resistance ratio was calculated using the LC_{50} of the tested strain divided by the LC_{50} of the BZ-SS susceptible strain where the LC_{50} 95% CL for tested strains did not overlap with the LC_{50} 95% CL of the BZ-SS. If the LC_{50} value of an insect population was smaller than that of the BZ-SS, a negative sign was assigned to the resistance ratio.

Results

We established 12 *H. zea* F_2 families for the diagnostic screens. Larval survivorship percentages resulted from the Cry1Ac-F_2 screen, ranged from 0 to 43.02% (Table 1.). From the established strains, 10 (83.33 %) met the criteria for potential positives (e 5 living larvae, e 3rd instar) after 7 days exposed to Cry1Ac (10µg/cm^2) and surpassed the theoretical homozygous resistant frequency of 6.25%. For the Cry2Ab2-F_2 screen, the larval survivorship percentages ranged from 0 to 21.10%. 5 of the F_2 strains (42.00%) met the criteria for potential positives after 7 days exposed to Cry2Ab2 (10µg/cm^2) and surpassed the theoretical homozygous resistant frequency. Larval survivorship percentages values for the Vip3Aa51-F_2 screen, ranged from 0 to 0.81%. None of the F_2 strains met the criteria of potentially resistant strains after the exposure to Vip3Aa51 (1µg/cm^2). However, one F_2 family resulted with an unexpectedly high survivorship of larvae reaching 2nd instar after the screen. Considering larvae reaching 2nd instar and above, the larval survivorship percent of this strain (D24) resulted in 17% after 7 days exposed to Vip3Aa51 (1µg/cm^2) (Table 2).

Table 1. Percentage (%) of *H. zea* F_2 larval survivorship after 7 days of exposure to diagnostic concentrations of selected Bt proteins (3rd instar and above)

F2 strains	Strain name	Cry1Ac (10 µg/cm^2)	Cry2Ab2 (10 µg/cm^2)	Vip3Aa51 (1 µg/cm^2)	Untreated check
1	A41	29.22	8.12	0.00	
2	A19	43.02	11.36	0.00	
3	A10	8.93	4.87	0.00	
4	D24	0.00	0.81	0.81	
5	E54	31.66	19.48	0.00	
6	G13	25.97	4.87	0.00	96.25
7	G40	10.55	8.93	0.00	
8	G30	0.00	0.00	0.00	
9	G17	26.79	2.44	0.00	
10	A16	7.31	0.00	0.00	
11	B10	15.42	0.81	0.00	
12	H1	27.60	21.10	0.00	

Table 2. Percentage (%) of *H. zea* F_2 larval survivorship after 7 days of exposure to diagnostic concentrations of selected Bt proteins (2nd instar and above)

F2 strains	Strain name	Cry1Ac (10 µg/cm^2)	Cry2Ab2 (10 µg/cm^2)	Vip3Aa51 (1 µg/cm^2)	Untreated check
1	A41	43.83	11.36	5.68	
2	A19	56.82	34.09	0.00	
3	A10	31.66	21.10	0.81	
4	D24	0.00	9.74	17.05	
5	E54	44.64	34.90	0.00	
6	G13	46.27	34.09	0.00	96.25
7	G40	43.02	37.34	0.81	
8	G30	0.00	0.00	1.62	
9	G17	52.76	19.48	0.00	
10	A16	9.74	0.00	0.00	
11	B10	31.66	4.87	0.00	
12	H1	37.34	47.08	4.06	

Two F_2 strains, one from the Cry1Ac- F_2 screen (strain A19) and other from the Cry2Ab2- F_2 screen (strain A41) were selected for resistance confirmation. Based on the criteria of non-overlapping confidence limits of the LC_{50} values,

strain A19, resulted significantly more resistant to the Cry1Ac and Cry2Ab2 toxins relative to BZ-SS. It also resulted to be significantly more susceptible to the Vip3Aa51 toxin compared to the BZ-SS (Table 3). Similarly, strain A41 resulted significantly more resistant to the Cry1Ac and Cry2Ab2 toxins than BZ-SS, but significantly more susceptible to Vip3Aa51 than the susceptible reference strain. Resistance ratios for the A19 strain relative to BZ-SS resulted in 311.60 for Cry1Ac, 2.75 to Cry2Ab2 and -20.00 for Vip3Aa51. Strain A41 resulted in resistance ratios of 56.50 for Cry1Ac, 17.70 to Cry2Ab2 and -1.81 for Vip3Aa51 relative to the susceptible reference strain (Table 3).

Table 3. Median lethal concentration (LC_{50}) of two *H. zea* strains derived from an F_2 screen to Bt proteins overlayed in diet bioassays

Bt protein	Strain name	N[a]	LC_{50} (95% CL) (µg/cm^2)[b]	Slope ± SE	□^{2}	DF	Resistance ratio[c]
Cry1Ac	BZ-SS	958	0.10 (0.08, 0.11)	1.55 ± 0.09	28.40	26	1.00
	A19	448	31.16 (16.66, 82.20	0.73 ± 0.10	32.23	22	311.60*
	A41	448	5.65 (2.78, 15.33)	0.69 ± 0.11	63.14	26	56.50*
Cry2Ab2	BZ-SS	958	0.20 (0.17, 0.24)	1.61 ± 0.09	30.70	26	1.00
	A19	384	0.55 (0.41, 0.74)	1.78 ± 0.18	25.36	22	2.75
	A41	384	3.54 (1.61, 13.35)	0.63 ± 0.12	44.27	22	17.70*
Vip3Aa51	BZ-SS	958	0.20 (0.16, 0.26)	1.48 ± 0.12	21.80	22	1.00
	A19	448	0.01 (0.01, 0.02)	1.63 ± 0.21	9.46	22	-20.00
	A41	448	0.11 (0.09, 0.14)	2.20 ± 0.20	23.35	22	-1.81

[a]Total number of neonates assayed.
[b]Larval mortality were calculated based on the number of dead larvae plus survivors that were still in the first instar (mortality = dead+L1) divided by the total number of insects assayed.
[c]Resistance ratio for Bt protein were calculated by dividing the LC_{50} value of an insect population by that of the susceptible strain (BZ-SS). If the LC_{50} an insect population was smaller than that of the BZ-SS, a negative sign was assigned to the resistance ratio.
*Indicates significant resistance ratios >10-fold.

Conclusion

Based on (Tabashnik 1994), resistance ratios above 10-fold are more likely to reflect Bt resistance. Statistically significant resistance ratios obtained from strain A19 (311.60) and A41 (56.50) in the Cry1Ac bioassay, and from strain A41 (17.70) in the Cry2Ab2 bioassay, provides a strong evidence of a genetically based reduction in the susceptibility of the strains to the Bt toxins under study. Based on our results, Vip3Aa51 proteins appears highly toxic to the cotton bollworm. However, a high survivorship of 2nd instar larvae after 7 days exposed to Vip3Aa51 (1 µg/cm^2) toxin is unexpected and deserves more attention in monitoring efforts. In conclusion, despite the small sample size managed in the experiment, we were able to detect Bt resistance to Cry1Ac and Cry2Ab2 toxins in *Helicoverpa zea* in Texas. The probability of finding a Bt resistant individual increases when resistance is common and also by analyzing a very large sample size (Venette et al. 2000). Thus, our findings may suggest that the occurrence of alleles conferring resistance to these toxins in *H. zea* could be high in the collection region. Pest control failures could be observed when there is a high frequency of Bt resistant alleles within an insect pest population (Andow and Ives 2002). If that is the case, growing transgenic crops expressing these and/or similar Bt proteins could be at risk of failure due to a significant reduction on the susceptibility of *H. zea* to the toxins.

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