NILSON RODRIGUES DA SILVA

BT TOXIN IN THE SOYBEAN LOOPER: SUSCEPTIBILITY, FITNESS & SELECTION

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de Doctor Scientiae.

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Altair Arlindo Semeão

Haddi Khalid

Raul Narciso Carvalho Guedes

Eugênio Eduardo de Oliveira
(Coorientador)

Eliseu José Guedes Pereira
(Orientador)

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SILVA, Nilson Rodrigues da, D.Sc., Universidade Federal de Viçosa, Julho de 2015. **Bt toxin in the soybean looper: susceptibility, fitness & selection**. Orientador: Eliseu José Guedes Pereira. Coorientador: Eugênio Eduardo de Oliveira.

A lagarta-falsa-medideira, Chrysodeixis includens e a lagarta-das-maçãs, Heliothis virescens, (Lepidoptera: Noctuidae), causam perdas significativas em soja e algodão no Brasil e são alvo de cultivares transgênicas produzindo as toxinas Cry1A e Cry2A de Bacillus thuringiensis (Bt). Neste estudo, três experimentos foram realizados para determinar: i) a susceptibilidade de populações brasileiras de C. includens a Cry1Ac e Cry2Aa, bem como a toxicidade destas toxinas para H. virescens, ii) a mortalidade e o efeito no desempenho demográfico de C. includens causadas por algodão ou soja Bt que produzem uma ou duas toxinas Cry, e iii) a resposta à seleção para resistência a Cry1Ac, a herdabilidade e a resistência cruzada à outras toxinas Cry em C. includens. Os cultivares de soja e algodão usados foram duas isolinhas não-Bt, dois eventos de algodão e soja Bt com um único gene Bt (algodão e soja Cry1Ac), e dois algodões Bt piramidados (Cry1Ac + Cry2Ab2 e Cry1Ac + Cry1Fa). Bioensaios usando toxina Bt aplicada na superfície da dieta mostraram que a suscetibilidade de C. includens a Cry1Ac e Cry2Aa foi variável, apresentado valores de CL_{50} de 16 a 241 ng/cm² para Cry1Ac e de 3 a 24 ng/cm² para Cry2Aa. Bioensaios com folhas de soja Cry1Ac e algodão Bt piramidados causaram 100% mortalidade das larvas de C. includens com dois dias de exposição. O algodão Cry1Ac causou baixa mortalidade, mas afetou negativamente diversas variáveis da história de vida de C. includens, severamente reduzindo a taxa reprodutiva líquida (R_0) e a taxa intrínseca de crescimento (r_m) em comparação aos indivíduos criados em algodão não-Bt. Seleção em laboratório para resistência a Cry1Ac em C. includens com contínua exposição ao algodão Cry1Ac durante o desenvolvimento larval ou à proteína purificada Cry1Ac por sete dias resultou em um aumento na resistência à toxina de 31 e 126 vezes com herdabilidade de 0,78 e 0,41, respectivamente, em 12 gerações de seleção. Esse nível de resistência das populações selecionadas foi insuficiente para permitir sobrevivência das larvas na folhagem da soja Bt que produz Cry1Ac. Além disso, as larvas das duas populações selecionadas em laboratório apresentaram também resistência a Cry1Ab, mas não a Cry2Aa nem a Cry1Fa em relação à população suscetível controle, não selecionada. Conclui-se que as populações de C. includens apresentam variação natural na susceptibilidade a Cry1Ac e Cry2Aa, mas a soja Bt e os algodões Bt piramidados

causaram alta mortalidade larval, indicando que variedades dessas plantas Bt devem ser eficazes para o manejo de populações desta espécie em condições de campo. Este estudo demostra que C. includens tem potencial para evoluir níveis consideráveis de resistência a Cry1Ac, mas o aumento de cerca de 130 vezes na resistência ainda não permite sobrevivência das lagartas na soja Bt Cry1Ac. Além disso, seleção para resistência a Cry1Ac não levou a resistência cruzada às toxinas Cry2A e Cry1F, sugerindo que essas toxinas são compatíveis para o manejo de resistência de C. includens.

ABSTRACT

SILVA, Nilson Rodrigues da, D.Sc., Universidade Federal de Viçosa, July, 2015. **Bt toxin in the soybean looper: susceptibility, fitness & selection**. Adviser: Eliseu José Guedes Pereira. Co-adviser: Eugênio Eduardo de Oliveira.

The soybean looper, Chrysodeixis includens, and the cotton bollworm, Heliothis virescens, (Lepidoptera: Noctuidae), cause significant losses in soybean and cotton in Brazil and are targeted by transgenic Bacillus thuringiensis (Bt) cultivars producing Cry1A and Cry2A toxins. In this study, three experiments were conducted to determine: i) the susceptibility of Brazilian populations of C. includens to Cry1Ac and Cry2Aa as well as the toxicity of these toxins to H. virescens, ii) the lethality and fitness effects on C. includens caused by Bt soybean or cotton producing one or the two toxins, and iii) the response to selection for Cry1Ac resistance to assess the risk of evolution of resistance and cross-resistance to Cry toxins in C. includens. Cotton and soybean cultivars used included two non-Bt isolines, two single-gene Bt cultivars (Cry1Ac cotton and soybean), and two pyramided Bt cottons: (Cry1Ac + Cry2Ab2 and Cry1Ac + Cry1Fa). Diet-surface bioassays showed that susceptibility of C. includens to Cry1Ac and Cry2Aa was variable among the populations tested. LC_{50} values ranged from 16 to 241 ng/cm² for Cry1Ac and from 3 to 24 ng/cm² for Cry2Aa, respectively. Leaf-tissue bioassays with Cry1Ac soybean and pyramided Bt cotton caused 100% mortality of C. includens larvae after two days of exposure. Cry1Ac cotton caused low mortality but negatively affected life-history traits and strongly reduced the net reproductive rate (R_0) as well as the intrinsic rate of increase (r_m) compared with controls. Selection for Cry1Ac resistance in C. includens using continuous exposure to Cry1Ac cotton during larval development or purified Cry1Ac protein for seven days resulted in 31- and 126-fold resistance with realized heritability of 0.78 and 0.41, respectively, after 12 generations of selection. Importantly, this level of resistance in the Cry1Ac-selected populations was insufficient to allow for survival of larvae in foliage of the Bt soybean producing Cry1Ac. Additionally, soybean loopers of the two laboratory-selected populations showed resistance to Cry1Ab too, but not the Cry2Aa or Cry1Fa in relation to the control, non-selected susceptible population. Taken together, the results of this investigation show that there is natural variation in susceptibility to Cry1Ac and Cry2Aa in the C. includens populations, but the single-toxin Bt soybean and the two-toxin Bt cottons efficiently killed their larvae, indicating that cultivars of these Bt crops should be effective for managing populations of this pest species in field settings. In

addition, this study shows that C. includens have potential to evolve relevant resistance levels to Cry1Ac, yet the increase of about 130 fold in the resistance does not allow for survival of neonate soybean loopers in the Bt Cry1Ac soybean. Furthermore, selection for resistance to Cry1Ac did not lead to cross-resistance to Cry2A and Cry1F, suggesting that these two Bt toxins are compatible for resistance management of C. includens.

GENERAL INTRODUCTION

The ability transfer foreign genes to plant genomes represents a major technological advance in modern agriculture (James 2009). Bt crops are plants genetically modified that produce Bacillus thuringiensis (Bt) proteins. Transgenic Bt tobacco was the first plant modified to express the ∂-endotoxins from B. thuringiensis with Cry1Ab gene in 1987 (Tabashnik et al. 2000). Transgenic Bt plants began to be used commercially in 1996, and since then these plants became one of the main tactics for pest management of Lepidoptera in field crops. Worldwide, the area cultivated with genetically modified plants exceeds 181,5 million hectares, and Brazil is ranked second with 42.2 million hectares, emerging as a global leader in biotech crops (James 2014a). This widespread adoption of Bt crops have increased the need for effective and locally adapted insect resistance management (IRM) plans to ensure the sustainable use of Bt crops (Gould 1998, Tabashnik et al. 2008).

Cry1Ac Bt cotton was adopted for the first time in the United States in 1996 and was widely adopted by growers even with warnings that several lepidopteran pests were not efficiently controlled by this technology alone (Bacheler and Mott 1997, Smith 1998, Adamczyk et al. 2001). In Brazil, cotton was the first Bt crop to be commercially and released in 2005, followed by maize in 2008, and soybean in 2010 (CTNBio 2015). Bt soybean event MON 87701 x MON 89788 (Bt/RR2) producing the Cry1Ac insecticidal Bt protein has Anticarsia gemmatalis and Chrysodeixis includens (Lepidoptera: Noctuidae) as primary target pests. This event also produces a especial type of 5-enolpyruvylshikimate-3-phosphate synthase, which confers herbicide tolerance to glyphosate. Although this event was commercially approved in 2010, it was adopted for the first time only in 2013/14 harvest (James 2014a). Cry1Ac cotton is considered very effective to control pests highly susceptible to Cry1Ac, such as the tobacco budworm, Heliothis virescens and the pink bollworm, Pectinophora gossypiella, but supplemental foliar insecticide applications are be necessary to control population of less sensitive species such as the soybean looper C. includens.

Chrysodeixis includens is one of the most economically important defoliator pest in many crops, such as soybean and cotton. Its occurrence is restricted to the Western Hemisphere, from the US to southern South America, and Australia (CAB 2014, Palma et al. 2015). Chrysodeixis includens belongs to the subfamily Plusiinae, being that this is one of the most abundant caterpillars of species which attack soybeans. This pest has a wide host range of more than 73 plants from 29 different families. Among the plants

are crops of great economic interest, such as soybeans, cotton, beans, tobacco, sunflower, tomato and other vegetables (Eichlin and Cunningham 1978, Herzog 1980a).

Feeding by soybean lopper reduce foliar area leading to substantial economic losses. Infestations occur in different phonological stages and parts of the plant, especially leaves of the lower canopy (Degrande and Vivian 2007). Female C. includens has a high reproductive capacity (Jost and Pitre 2002), and development times from neonate to adult is about 26 days, with 5 to 6 larval stages (Mitchel 1967), potentially producing three generations per growing season of soybean. Outbreaks of C. includens have often been observed in Brazil, which is sometimes associated with outbreaks of the velvetbean caterpillar, Anticarsia gemmatalis (Papa and Celoto 2007, Guedes et al. 2010).

Because of recent advances in agricultural biotechnology with commercial release of new transgenic events in Brazil, pest management for C. includens is currently in a transition period between the use of synthetic insecticides and Bt plants. Cry1Ac cotton has been used since 2005 against C. includens as secondary target insect, but does not provides good control efficacy. This Bt cotton help to reduce defoliation by causing some level of larval mortality, reducing insect body size, and increasing developmental time (Weiland et al. 1997, Ashfaqb et al. 2001, Funichello et al. 2013b). Host-plant effects in these life-history traits may increase exposure to natural mortality factors, including natural enemies (Weseloh 1984, Sedaratian et al. 2013). In contrast, they may increase fitness of insects heterozygous for Cry1Ac resistance or and consequently increase rates of resistance evolution in the field. Although C. includens populations differ significantly in susceptibility to B. thuringiensis (Mascarenhas and Boethel 1997), in Brazil it has not been mandatory determination frequency of resistance alleles prior to release commercial transgenic events. In this scenario, data on geographic susceptibility, sublethal effects caused by Bt plants, and response to selection for resistance in the laboratory may provide important information to devise and improve IRM strategies for Bt cotton and soybean cultivars.

Prior to the commercialization of any Bt crop it is important to know the susceptibility of target insect pests to Bt proteins produced in transgenic cultivars. In Brazil, there is not a specific resistance management program to characterize and monitor susceptibility of C. includens to Cry1Ac cotton. In the US, in seven years of resistance monitoring in Helicoverpa zea an increase in resistance to Cry1Ac toxin of more than 100-fold was found (Luttrell and Ali 2009), indicating a significant increase in the frequency resistance allele. To study of geographical susceptibility in the

laboratory, the first step is choose appropriate bioassay techniques and establish of baseline susceptibility data among populations across the geographic range of the target species. Throughout statistical analysis of this data is possible the determine the diagnostic concentrations, which constitute an effective way of detecting resistant phenotypes (Marçon et al. 1999b). The susceptibility of insects also may be monitored throughout bioassays with flesh Bt plant leaves. Recently, some studies have determine baseline susceptibility of Brazilian lepidopteran populations, such as H. virescens for Cry1Ac (Albernaz et al. 2013), Diatraea saccharalis for Cry1Ab (Girón-Pérez et al. 2014) and Spodoptera frugiperda and D. saccharalis for Vip3Aa20 (Bernardi et al. 2014). However, there are no published data on the variation of C. includens response to Cry toxins. In addition, data on interpopulation varition allows assessment of the risk of resistance evolution in field populations of C. includens.

Lethality is only a partial measure of the potential deleterious effects of Bt toxins on insects. One must also consider toxin sublethal effects on arthropod physiology and behavior for a more complete analysis (Desneux et al. 2007). Several studies only report effects on survivorship and development of C. includens larvae feeding on Cry1Ac cotton, but these studies not characterized sublethal effects in other life-history traits. These effects may interfere in the reproductive biology, host-finding, feeding behavior, enzyme modulation, among other (Moriarty 1969, Haynes 1988). According to Stark and Bank, (Stark and Banks 2003), the estimating population growth rates allows more accurate assessments because measures of population growth rates combine lethal and sublethal effects. Thus, the combination of life table statistics and characterization of life-history traits for C. includens in single and dual gene Bt cotton allow better understanding of impact of Bt cultivars on the soybean looper. Understanding sublethal effects of Cry1Ac on this species contributes to the development of more appropriate resistance management strategies locally adapted to the Brazilian agricultural scenario, which is likely to work more efficiently to delay resistance evolution in the field.

That insects can evolve resistance to Bt toxins is evidenced in six different insects species (Van Rensburg 2007, Tabashnik et al. 2008, Storer et al. 2010, Dhurua and Gujar 2011a, Gassmann et al. 2011, Zhang et al. 2011a), and the phenomenon is practical implications because it can lead to control failures in the field. For the soybean looper, however, there is no report of resistance to Bt or synthetic insecticides in Brazil to date although high levels of pyrethroid resistance has been reported in US populations (Thomas et al. 1996, Portillo and Pitre 1997, Palma et al. 2015). In this context, laboratory selection of Bt resistant populations is an important step to develop

sound IRM for transgenic plants (Van Rie et al. 1989, Carrière et al. 2015). The availability of laboratory-selected populations allows for characterization of resistance mechanisms that can evolve in field settings, making possible, for example, to determine the mode of inheritance and other factors to assess the risk of resistance evolution (Gould et al. 1997b, Pittendrigh et al. 2000) as well as potential development of molecular or phenotypic bio-indicators as tools to monitor resistance evolution in the field (Jurat-Fuentes et al. 2011). All this information will contribute to improve IRM strategies that allows for more effective delay on mitigation of resistance in the field.

Some studies with C. includens have been conducted lately as it is one of the main targets of Bt soybean commercially released in Brazil (Bernardi et al. 2012). For example, molecular variability and genetic structure (Palma et al. 2015), incidence of outbreaks in different bean species (Baldin et al. 2014), survival and development in Bt cotton cultivars (Funichello et al. 2013b, Sorgatto et al. 2015), control efficacy of Bt soybean against C. includens in the laboratory and field (Macrae et al. 2005, Bernardi et al. 2012), and incidence as well as crop injury in Bt soybean (McPherson and MacRae 2009) have been determined. Nevertheless, currently no published studies assessed geographical susceptibility to Cry1Ac and its sublethal effects, response to selection in the laboratory, cross-resistance to Bt toxins in C. includens, which were the focus of the present research.

The present thesis was written in three chapters, which report results of three different studies. First, I determined the geographical variation in susceptibility in C. includens populations to Cry1Ac and Cry2Aa. Second, I studied lethal and sublethal effect on the soybean looper caused by either single- or two-toxin Bt plants. Third, selection for Cry1Ac resistance and characterization of cross-resistance to other Cry toxins were assessed. Finally the main conclusions and implications of this is presented in the Summary and Conclusions section.

REFERENCES

Adamczyk, J., L. Adams, and D. Hardee. 2001. Field efficacy and seasonal expression profiles for terminal leaves of single and double Bacillus thuringiensis toxin cotton genotypes. Journal of Economic Entomology 94: 1589-1593.

Albernaz, K., B. Merlin, S. Martinelli, G. Head, and C. Omoto. 2013. Baseline susceptibility to Cry1Ac insecticidal protein in Heliothis virescens (Lepidoptera: Noctuidae) populations in Brazil. Journal of Economic Entomology 106: 1819-1824.

Ashfaqb, M., S. Y. Young, and R. W. McNew. 2001. Larval Mortality and Development of Pseudoplusia includens (Lepidoptera: Noctuidae) Reared on a Transgenic Bacillus thuringiensis-Cotton Cultivar Expressing CryIAc Insecticidal Protein. Journal of Economic Entomology 94: 1053 - 1058.

Bacheler, J. S., and D. W. Mott. 1997. Efficacy of growermanaged Bt cotton in North Carolina., pp. 858-861. In P. Dugger and D. Richter (eds.), In Proceedings, Beltwide Cotton Conference National Cotton Council, Memphis, TN.

.

Baldin, E. L. L., A. L. Lourenção, and E. C. Schlick-Souza. 2014. Outbreaks of Chrysodeixis includens (Walker) (Lepidoptera: Noctuidae) in common bean and castor bean in São Paulo state, Brazil. Bragantia 73: 458-461.

Bernardi, O., D. Amado, R. S. Sousa, F. Segatti, J. Fatoretto, A. D. Burd, and C. Omoto. 2014. Baseline Susceptibility and Monitoring of Brazilian Populations of Spodoptera frugiperda (Lepidoptera: Noctuidae) and Diatraea saccharalis (Lepidoptera: Crambidae) to Vip3Aa20 Insecticidal Protein. Journal of Economic Entomology 107: 781-790.

Bernardi, O., G. S. Malvestiti, P. M. Dourado, W. S. Oliveira, S. Martinelli, G. U. Berger, G. P. Head, and C. Omoto. 2012. Assessment of the high-dose concept and level of control provided by MON 87701× MON 89788 soybean against Anticarsia gemmatalis and Pseudoplusia includens (Lepidoptera: Noctuidae) in Brazil. Pest management science 68: 1083-1091.

CAB. 2014. International. Crop Protection Compendium, http://www.cabicompendium.org/cpc/home.asp.

Carrière, Y., N. Crickmore, and B. E. Tabashnik. 2015. Optimizing pyramided transgenic Bt crops for sustainable pest management. Nature biotechnology 33: 161-168.

CTNBio. 2015. - Comissão Técnica Nacional de Biossegurança. Resumo Geral de Plantas Geneticamente modificadas aprovadas para Comercialização.

Degrande, P. E., and L. M. Vivian. 2007. Pragas da Soja., Boletim de Pesquisa da Soja. Fundação MT.

Desneux, N., A. Decourtye, and J.-M. Delpuech. 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52: 81-106.

Dhurua, S., and G. T. Gujar. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, Pectinophora gossypiella (Saunders)(Lepidoptera: Gelechiidae), from India. Pest management science 67: 898-903.

Eichlin, T. D., and H. B. Cunningham. 1978. The Plusiinae (Lepidoptera: Noctuidae) of America north of Mexico, emphasizing genitalic and larval morphology, Department of Agriculture, Agricultural Research Service.

Funichello, M., J. F. J. Grigolli, B. H. S. de Souza, A. L. B. Junior, and A. C. Busoli. 2013. Effect of transgenic and non-transgenic cotton cultivars on the development and survival of Pseudoplusia includens (Walker)(Lepidoptera: Noctuidae). African Journal of Agricultural Research 8: 5424-5428.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLoS ONE 6: e22629.

Girón-Pérez, K., A. Oliveira, A. Teixeira, R. Guedes, and E. Pereira. 2014. Susceptibility of Brazilian populations of Diatraea saccharalis to Cry1Ab and response to selection for resistance. Crop Protection 62: 124-128.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annual review of entomology 43: 701-726.

Gould, F., A. Anderson, A. Jones, D. Sumerford, D. G. Heckel, J. Lopez, S. Micinski, R. Leonard, and M. Laster. 1997. Initial frequency of alleles for resistance to Bacillus thuringiensis toxins in field populations of *Heliothis virescens.* Proceedings of the National Academy of Sciences of the United States of America 94: 3519 – 3523.

Guedes, J. V. C., C. S. Stecca, R. B. Rodrigues, and M. Bigolin. 2010. Cultiv Gd Cult. , Nova dinâmica.

Haynes, K. F. 1988. Sublethal effects of neurotoxic insecticides on insect behavior. Annual Review of Entomology 33: 149-168.

Herzog, D. C. 1980. Sampling soybean looper on soybean, pp. 140-168. . In M. Kogan and D. C. Herzog (eds.), Sampling methods in soybean entomology. Springer-Verlag, New York.

James, C. 2009. Brief 41: Global status of commercialized biotech/GM crops: 2009. ISAAA Brief. Ithaca, NY: International Service for the Acquisition of Agri-biotech Applications: 290.

James, C. 2014. Global Status of Commercialized Biotech/GM Crops: 2014., ISAAA Brief Ithaca, NY.

Jost, D. J., and H. N. Pitre. 2002. Soybean looper (Lepidoptera: Noctuidae) oviposition on cotton and soybean of diferente growth stages: Influence of olfactory stimuli. Journal of Economic Entomology 95: 286-293.

Jurat-Fuentes, J. L., L. Karumbaiah, S. R. K. Jakka, C. Ning, C. Liu, K. Wu, J. Jackson, F. Gould, C. Blanco, and M. Portilla. 2011. Reduced levels of membrane-bound alkaline phosphatase are common to lepidopteran strains resistant to Cry toxins from Bacillus thuringiensis. PLoS One 6: e17606.

Luttrell, R., and M. Ali. 2009. Variability in the response of Helicoverpa zea and Heliothis virescens (Lepidoptera: Noctuidae) to Cry1Ac and Cry2Ab2 in diet incorporation assays. Resistant Pest Management Newsletter 19: 31-34.

Macrae, T. C., M. E. Baur, D. J. Boethel, B. J. Fitzpatrick, A.-G. Gao, J. C. Gamundi, L. A. Harrison, V. T. Kabuye, R. M. Mcpherson, and J. A. Miklos. 2005. Laboratory and field evaluations of transgenic soybean exhibiting high-dose expression of a synthetic Bacillus thuringiensis cry1A gene for control of Lepidoptera. Journal of Economic Entomology 98: 577-587.

Marçon, P. C. R. G. Y., L.J., K. L. Steffey, and B. D. Siegfried. 1999. Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to Bacillus thuringiensis toxins. Journal of Economic Entomology 92: 279 - 285.

Mascarenhas, R., and D. Boethel. 1997. Responses of field-collected strains of soybean looper (Lepidoptera: Noctuidae) to selected insecticides using an artificial diet overlay bioassay. Journal of Economic Entomology 90: 1117-1124.

McPherson, R. M., and T. C. MacRae. 2009. Assessing lepidopteran abundance and crop injury in soybean lines exhibiting a synthetic Bacillus thuringiensis cry1A gene. Journal of entomological science 44: 120-131.

Mitchel, E. R. 1967. Life history of Chrysodeixis includens (Walker) (Lepidoptera: Noctuidae). . Journal of the Georgia Entomological Society 2: 53-57.

Moriarty , F. 1969. The sublethal effects of synthetic insecticides on insects. Bioi. Rev. 44: 321-357.

Palma, J., K. Maebe, J. V. C. Guedes, and G. Smagghe. 2015. Molecular Variability and Genetic Structure of Chrysodeixis includens (Lepidoptera: Noctuidae), an Important Soybean Defoliator in Brazil. PLoS ONE 10.

Papa, G., and F. J. Celoto. 2007. Lagartas na soja., Available: http://www.ilhasolteira.com.br/colunas/index.php?acao = verartigo&idartigo=1189090532.

.

Pittendrigh, B. R., P. Gaffney, and L. L. Murdock. 2000. Deterministic modeling of negative cross-resistance strategies for use in transgenic host-plant resistance. Journal of theoretical biology 204: 135-150.

Portillo, H. E., and H. N. Pitre. 1997. Pyrethroid Resistance Levels in Two Generations of Soybean Looper (Lepidoptera: Noctuidae) on Soybean in Mississippi.

Sedaratian, A., Y. Fathipour, R. Talaei-Hassanloui, and J. Jurat-Fuentes. 2013. Fitness costs of sublethal exposure to Bacillus thuringiensis in Helicoverpa armigera: a carryover study on offspring. Journal of Applied Entomology 137: 540-549.

Smith, R. H. 1998. Year two of Bollgard behind boll weevil eradication: Alabama observations, pp. 965-966. In P. Dugger and D. Richter (eds.), Proceedings, 2000 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Sorgatto, R. J., O. Bernardi, and C. Omoto. 2015. Survival and Development of Spodoptera frugiperda and Chrysodeixis includens (Lepidoptera: Noctuidae) on Bt Cotton and Implications for Resistance Management Strategies in Brazil. Environmental Entomology: nvu018.

Stark, J. D., and J. E. Banks. 2003. Population-level effects of pesticides and other toxicants on arthropods. Annual review of entomology 48: 505-519.

Storer, N. P., J. M. Babcock, M. Schlenz, T. Meade, G. D. Thompson, J. W. Bing, and R. M. Huckaba. 2010. Discovery and characterization of field resistance to Bt maize: Spodoptera frugiperda (Lepidoptera: Noctuidae) in Puerto Rico. Journal of Economic Entomology 103: 1031-1038.

Tabashnik, B. E., Y.-B. Liu, R. A. de Maagd, and T. J. Dennehy. 2000. Cross-resistance of pink bollworm (Pectinophora gossypiella) to Bacillus thuringiensis toxins. Applied and environmental microbiology 66: 4582-4584.

Tabashnik, B. E., A. J. Gassmann, D. W. Crowder, and Y. Carrière. 2008. Insect resistance to Bt crops: evidence versus theory. Nature Biotechnology 26: 199-202.

Thomas, J. D., J. A. Ottea, D. J. Boethel, and S. Ibrahim. 1996. Factors influencing pyrethroid resistance in a permethrin-selected strain of the Soybean Looper, Pseudoplusia includens (Walker). Pesticide biochemistry and physiology 55: 1-9.

Van Rensburg, J. 2007. First report of field resistance by the stem borer, Busseola fusca (Fuller) to Bt-transgenic maize. South African Journal of Plant and Soil 24: 147-151.

Van Rie, J., S. Jansens, H. Höfte, D. Degheele, and H. Van Mellaert. 1989. Specificity of Bacillus thuringiensis delta-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target
insects. Eur. J. Biochem. 186: 239–247.

Weiland, R. T., P. T. McDonald, and M. K. Kish. Year. Published. Efficacy of Dimilin (Diflubenzuron) and transgenic Bt cotton on several lepidopteran species., pp. 1095-1099. In P. Dugger and D. Richter (eds.), Proceedings, Beltwide Cotton Production Research Conferences, 1997, New Orleans, LA. National Cotton Council of America, Memphis, TN.

Weseloh, R. M. 1984. Effects of the feeding inhibitor Plictran and low Bacillus thuringiensis Berliner doses on Lymantria dispar (L.)(Lepidoptera: Lymantriidae): implications for Cotesia melanoscelus (Ratzeburg)(Hymenoptera: Braconidae). Environmental entomology 13: 1371-1376.

Zhang, H., W. Yin, J. Zhao, L. Jin, Y. Yang, S. Wu, B. E. Tabashnik, and Y. Wu. 2011. Early warning of cotton bollworm resistance associated with intensive planting of Bt cotton in China. PLoS ONE 6: e22874-e22874.

CHAPTER 1 – Cry1Ac Susceptibility in Brazilian Populations of the Soybean Looper, Chrysodeixis includens

ABSTRACT

The variation in susceptibility of the soybean looper, Chysodeixis includens (Lepidoptera: Noctuidae), to Cry1Ac and Cry2Aa toxins of Bacillus thuringiensis (Bt) was studied to set a baseline to detect future changes in the frequency of resistant individuals that might occur upon increased use of genetically modified Bt crops in Brazil. Populations of C. includens were collected from three Brazilian states, and neonates of the F_3 were exposed to graded Bt protein concentrations applied on the surface of the artificial diet. A population of cotton bollworm Heliothis virescens, known to be susceptible to Cry1Ac, was also biossayed as check for the Bt proteins toxicity. Soybean looper neonates were quite sensitive to the Bt proteins studied; LC_{50} values ranged from 16 to 241 ng/cm² for Cry1Ac and from 2.9 to 24 ng/cm² for Cry2Aa, which corresponded to 15 and 9-fold variation in susceptibility among the populations, respectively. For H. virescens, the Cry2Aa protein showed a LC_{50} value of 12 ng/cm² and was 10 times more toxic than Cry1Fa, for which the LC_{50} value was 128 ng/cm². These estimates indicate that there is a 10-fold variation in toxicity ratios for both species and indicate that Cry1A is more potent than Cry2A toxins against C. includens and H. virescens. These results suggest that differences in susceptibility and in toxicity observed reflect natural variation of these species to different toxins from B. thuringiensis. Therefore, C. includens and H. virescens apparently are susceptible to B. thuringiensis toxins, and the LC_{50} values should be considered in resistance management programs in order to detect susceptibility changes in both populations.

Keywords: Soybean looper, tobacco budworm, Bacillus thuringiensis, resistance monitoring, Cry1Ac and Cry2Aa toxins.

INTRODUCTION

Genetically modified plants producing insecticidal proteins from the bacteria Bacillus thuringiensis Berliner (Bt), have been used as integral part of pest management for nearly two decades. Currently, the cultivation of transgenic plants occupies a global area bigger than the 181 million hectares, and Brazil is ranked second with 42.2 million hectares and is emerging as a strong global leader in biotech crops (James 2014a). Bt Cry1Ac cotton (Bollgard I) was released in Brazil for the first time in 2005 (CTNBio 2005a) followed by the Bt soybean (event MON 87701 x MON 89788) in 2010 (CTNBio 2010), which also produces the Bt Cry1Ac insecticidal protein.

Among soybean and cotton pest species, the soybean looper, Chrysodeixis includens (Lepidoptera: Noctuidae), and the tobacco budworm, Heliothis virescens (Lepidoptera: Noctuidae), stand out as the main lepidopteran species pests to date. Besides the tobacco budworm is considered a key pest in cotton, it has recently become an important pest of soybean in Brazil (Tomquelski and Maruyama 2009, Domingues et al. 2012). However, C. includens is one of the most important defoliator, given its rapid rise in population density and high frequency of outbreaks in recent years (Bueno et al. 2012b). In addition, Cry1Ac cotton (Bollgard I event 531) has high control efficiency for H. virescens, but not against species less sensitive such as C. includens. The risk of development of resistance to Cry1Ac toxin should be considered, especially in situations where H. virescens and C. includens populations are exposed to both Cry1Ac cotton and Bt soybean.

The selection of resistant insect populations is the main threat to the sustainable use of Bt plants (Onstad 2008b, Tabashnik 2008, Tabashnik et al. 2013a), which can lead to loss of control efficacy by these plants. Laboratory selection experiments demonstrated that some lepidopteran species such as H. virescens, Pectinophora gossypiella and Helicoverpa armigera have high potential to evolve resistance to Cry1Ac (Gould et al. 1995, Gould et al. 1997b, Tabashnik et al. 2002, Xu et al. 2005). However, at least three cases of field resistance have been reported for the following species H. zea in the United States (Tabashnik et al. 2008), H. armigera in China (Zhang et al. 2011b) and P. gossypiella in India (Dhurua and Gujar 2011b). However in Brazil, to date, there is no report of resistance to this toxin. One of the most common methods to identify and monitor resistance in insect populations is the use of the dose-response bioassays, perhaps because it is relatively inexpensive and simple (Everich et al. 1992, Bolin et al. 1998, Mascarenhas et al. 1998b, Marçon et al. 1999b, Hardee et al. 2001, Huang 2006).

Dose-response bioassays are important tools to establish baseline susceptibility, to determine the diagnostic dose-concentration, to confirm the presence of resistance in insect populations and to assess the relative toxicity of different Bt toxins. In addiction the effective monitoring, surveillance and early detection of resistance, as well as studies of characterization of baseline of susceptibility to insect pest populations target of Bt proteins constitute one of the essential steps for the insect resistance management (IRM) programs to Bt crops (Tabashnik et al. 2008). Recently, some studies of baseline susceptibility have been reported in Brazil with different lepidopteran species, such as H. virescens for Cry1Ac (Albernaz et al. 2013), Diatraea saccharalis for Cry1Ab (Girón-Pérez et al. 2014), and Spodoptera frugiperda and D. saccharalis for Vip3Aa20 (Bernardi et al. 2014). However, there are no published data available on the extent of the susceptibility variation on C. includens in response to Cry1A and Cry2A toxins or even on the relative toxicity of different Cry toxin against H. virescens.

In this context, here We report data from two experiments conducted to: (i) determine background susceptibility of Brazilian populations of C. includens to Cry1Ac and Cry2Aa toxins, and (ii) determine the relative toxicity of toxins representative of those produced by Bt cotton events in Brazil, used against H. virescens. The results obtained will be useful for monitoring pest susceptibility and therefore represent a basis to monitor the efficacy of resistance management strategies employed in the country.

MATERIAL AND METHODS

Insects

Five populations of C. includens and one of H. virescens were collected in distinct geographic regions in Brazil, from January to May 2013 (Table 1). All C. includens populations were originated from collections in conventional soybean fields except one that was collected in a dry bean field in Coimbra county, Minas Gerais state (population Cb). Approximately 450-500 late-instars per location were obtained. For H. virescens, approximately 50-150 late-instars were collected per location in fields of non-Bt cotton in the main cotton-producing regions of the state of Mato Grosso, Brazil, in 2013. All C. includens populations were collected before commercial release of Bt soybean in Brazil.

After the field-sampling, the larvae were brought to laboratory and reared individually in 16-well plastic trays (Advento do Brasil, Diadema, São Paulo). Larvae were fed daily with soybean leaves, and kept in a rearing room at 27 ± 1°C, 80% relative humidity, and photoperiod of 14 h light until pupation. Pupae (80♂ + 80♀) were placed in cages made of polyvinylchloride (PVC), 20 cm diameter and 30 cm

height, lined internally with sulfite paper as substrate for oviposition. Adults were fed daily with 10% honey solution in water, and eggs were collected daily and stored in an incubator until hatching. Neonates were transferred to trays with artificial diet as described in (Greene et al. 1976b) with slight modifications and kept in the rearing room. The F1 generation neonate larvae were used for bioassays with Cry1 and Cry2 toxins. A few F1 neonates were used to raise F2 if the F1 neonate number was insufficient for bioassays.

Diet overlay bioassays

Cry1Ac, Cry1Fa and Cry2Aa toxins were obtained from Dr. Marianne P. Carey (Case Western Reserve University, OH). Proteins were activated with trypsin, purified on HPLC, shipped as lyophilized powder and stored at -80 ^{o}C.

Susceptibility of C. includens and H. virescens larvae were determined using diet-surface bioassays (Marçon et al. 1999b). Toxin dilutions were prepared in 0.1% Triton-X-100, and bioassays were performed in duplicate on two different dates and included at least seven graded concentrations of toxin, plus a control (0.1% Triton-X-100). Thirty milliliters of toxin solution were applied on the surface of the diet in each well of a 128-well tray (CD International, Pitman, NJ), and were allow to air dry. A single neonate (< 24 h hatching) was placed in each well, and trays were held in a growth chamber at 27 ºC, 24 h scotophase, and 80% R.H. for seven days until larval mortality was assessed (Marçon et al. 1999b, Pereira et al. 2008b). Larvae that did not molt to second instar or weighted less than 0.1 mg were considered dead. Larval weight of survivors was also recorded to calculate percentage of inhibition of growth relative to control, unexposed larvae (Marçon et al. 1999b).

Statistical analysis

Bioassay data were subjected to probit analysis using Polo-Plus software (Russell et al. 1977, Robertson et al. 2007b) to estimate the LC_{50} and EC_{50} values, line slopes and their standard errors, as well as lethal concentration ratios and confidence intervals. Mortality was adjusted relative to controls when necessary. Lethal concentration ratios were considered significantly different ($P < 0.05$) when their confidence limits did not include the value one.

RESULTS

Susceptibility of C. includens populations to Cry1Ac

Mortality and growth inhibition data were suitably modeled with probit analysis as indicated by non-significant lack-of-fit chi-square values ($\chi^2 < 9.5$, $P > 0.05$; Tables 2, 3), thus allowing valid estimates for lethal or effective concentrations and resistance

ratios with 95% confidence intervals (Tables 2 and 3). The Cry1Ac protein showed relatively high toxicity against all populations of C. includens. LC_{50} values ranged from 16 ng/cm² (CB) to 241 ng/cm² (SP), which corresponds to a variation of approximately 15 fold in the LC_{50} resistance ratio (Table 2). Similar results were obtained using larval growth inhibition as response variable, with some variation regarding intra-population heterogeneity; resistance rations reached values of 11 fold for the MT population (Table 3). The MS population presented resistance ratio values of 2 and 5 fold for mortality and larval growth inhibition, respectively (Tables 2 and 3).

Susceptibility of populations of C. includens to Cry2Aa

The Cry2Aa protein also exhibited high insecticidal activity against the populations of C. includens (Table 2 and 3). LC_{50} values ranged from 2.9 ng/cm^2 (CB) to 24.3 ng/cm² (VÇ), which corresponds to a variation of approximately 9 fold in the proportions of resistance ratio (Table 2); the values for growth inhibition ranged from 0.5 to 8.8 ng/cm², corresponding to a variation of 18.7 fold for the population of MT (Table 3). There was a high variation of intra-population and heterogeneity among populations, with the concentration-mortality curves varying 1.32-4.03 (Table 2); similar results were obtained on larval growth, which ranged from 1.03-3.15 (Table 3). The mixed population presented values of 6.90 and 12.0 fold resistance ratio for mortality and larval growth inhibition, respectively (Tables 2 and 3).

Susceptibility of H. virescens to different toxins Cry

Variation in susceptibility of H. virescens when exposed to different Bt toxins was observed, as indicated by their concentration-response curves (Table 4). The bioassay data were adequately described by the probit model as indicated by non-significant ($P > 0.05$) chi-square values for lack-of-fit of the model (Table 4). Cry2Aa was the most toxic Bt protein with LC_{50} values of 12.4 ng/cm² (Table 4). The toxicity of Cry1Fa and Cry1Ac was 10 and 5 fold lower than that of Cry2A, respectively, as indicated by their toxicity ratios (Table 4). For growth inhibition, a similar pattern of toxicity was observed (Table 4) although the EC_{50} value of Cry1Ac was the highest among the toxins and the magnitude of the variation was relatively small (i.e., 2-6 fold).

DISCUSSION

The results of this study indicate moderate inter-population variation in susceptibility of C. includens to CrylAc and to Cry2Aa. The magnitude of these differences for both toxins was statistically significant, ranging from 11 to 18 fold, for both variables used. In the bioassays of relative toxicity to H. virescens, Cry2Aa was the most toxic Bt protein. The slope of the concentration-mortality curves of C. includens

for Cry1Ac was significantly different from those for Cry2Aa, suggesting higher genetic variation in the field collected populations. In contrast, the slopes of concentration-mortality curves for H. virescens were not significantly different, suggesting lower genetic variation in response to the toxins in the population. It should be noted, however, that the slope of concentration-response line is associated with phenotypic deviation in susceptibility in the population, and genetic and environmental factors underlying such variation (Hoskins 1960, Abbas et al. 2014b). Additionally, they are not good indicators of genetic variation in susceptibility as environmental variation and errors of estimation are also included (Chilcuit and Tabashnik 1995).

In this current study, the Cry2Aa protein demonstrated high toxicity than Cry1Ac protein against all C. includens populations, with a mean LC_{50} values below 17 ng/cm². We also observed moderate inter-population variation to both toxins. Beside the populations tested represented a small sample, taken at one point in time, and of a multivoltine species (Sims et al. 1996, Marçon et al. 1999b), our results suggested moderate genetic natural variation in Brazilian populations of the soybean looper to Cry2Aa and Cry1Ac protein. In study with eight soybean looper populations from eight states of Brazil indicated high levels of gene flow among populations, as well as low genetic diversity and low genetic structure (Palma et al. 2015). However, the inter-population variation in the susceptibility to chemical or microbial insecticides is a common phenomenon that may occur when bioassays are repeated (Robertson et al. 1995).

Studies carried out in several countries around the world indicated a high natural variability in susceptibility to Cry1Ac and Cry2Ab proteins among populations of H. virescens in the US (16 fold) (Stone and Sims 1993), as well as H. armigera in China (100 fold) (Wu et al. 1999), West African countries (ranging from 23 to 44 fold for Cry1Ac and from 10 to 40 fold for Cry2Ab) (Brévault et al. 2009), and India (5 fold for Cry2Ab) (Jalali et al. 2014). However, in a study with Brazilian populations of H. virescens was found only a 4-fold variation in the susceptibility to Cry1Ac (Albernaz et al. 2013). For Brazilian populations of D. saccharalis there was a variation of 33 fold in the response to Cry1Ab (Girón-Pérez et al. 2014). Here, the Cry1Ac susceptibility of C. includens populations varied, respectively, 15 and 11 fold for LC_{50} and EC_{50} values. Likewise, for Cry2Aa, the variation was 9 and 19 fold on LC_{50} and EC_{50} values, respectively. Overall, there was no indication of increased tolerance to Cry1Ac toxin as result of the prior exposure to Bt Cry1Ac cotton in Brazil as the lethal and effective concentrations were low although one needs to have data on background susceptible to

comfortably argue on this issue. Even so, the results reported in this study support the hypothesis that the soybean looper is very susceptible to Cry1Ac and Cry2Aa from B. thuringiensis.

Other Bt toxins such as Cry1Fa and Cry2Ab are expressed in other events of Bt cotton in Brazil, but published studies on their relative toxicity to H. virescens are unavailable. In the diet-surface bioassays carried out, here Cry1 and Cry2 toxins showed different degree of toxicity to H. virescens, for both LC_{50} (ng/cm²) [(Cry2Aa (12.3) > Cry1Ac (27.2) > Cry1Fa (127.6)] and EC_{50} values (ng/cm²) [(Cry2Aa (2.7) > Cry1Fa (5.8) > Cry1Ac (15.3)] (see Table 4). The variation in LC_{50} values among Brazilian populations of H. virescens, seen in the current study, is high (about 3-64-fold) when compared to the variability reported from the US a standard susceptible laboratory colony: Cry1Ac (0.5) > Cry1Fa (1.9) > Cry2Aa (4.3) (Jurat-Fuentes and Adang 2001, Jurat-Fuentes et al. 2003). According to our results, Cry1Ac and Cry2Aa may be equally effective against H. virescens, and Cry1F is much less toxic than these Cry toxins tested. Comparatively seems probable that the differences in response Cry proteins being more likely to represent the natural variation of the susceptibility of H. virescens populations, and may be not caused by previous exposure to selection pressure to Bt cotton. However, for understanding this reason should be a research topic for future experiments.

Lethal and effective concentration values reported in this paper suggest that Cry2Aa and Cry1Ac toxins are highly toxic to C. includens and H. virescens populations, indicating that both toxins are promising for use in pyramided Bt events. Modeling studies suggest that plants expressing two different Bt toxin genes (pyramided plants) have the potential to slow resistance evolution more effectively than plants expressing a single toxin gene (Zhao et al. 2003). In addition, studies have shown that by inserting a gene coding a second Bt Cry2Ab protein in the Cry1Ac cotton contributed in reducing survival of Lepidoptera less sensitive to Cry1Ac (Greenplate et al. 2000, Adamczyk et al. 2001, Stewart et al. 2001). One challenge for using Bt cotton events is the variation in Cry toxin expression levels throughout the season and canopy (Olsen et al. 2005, Dong and Li 2007). Thus, the introduction of Cry1Ac + Cry2Ab cotton should increase plant protection against C. includens and H. virescens, as well as Helicoverpa armigera, based on the experience with this event in other agroecosystems (Kranthi et al. 2009). In addition, given the higher levels of cry2Ab gene expression in cotton, pyramided Cry1Ac + Cry2Ab cotton and soybean crops should improve control efficacy against Lepidoptera species that are intrinsically more tolerant to Cry1Ac,

although care should be taken to not lose Cry1Ac toxicity due to resistance evolution, as it can compromise the efficacy of pyramided events containing it.

From an IRM perspective, the variation in susceptibility of C. includens populations is an indication of the genetic and physiological capacity of insects for adapting to Bt in the face of more intensive exposures. Especially when the gene flow between populations allows repeated colonization of Bt fields provides opportunities for rapid local adaptation (Carrière et al. 2010). In this context, the two strategies may adopted for delaying resistance the refuge and pyramid strategies. Although both can reduce heritability of resistance, but pyramids can also delay resistance by reducing genetic variation for resistance (Carrière et al. 2010). In this current study, the H. virescens population was more susceptible to Cry2Aa and Cry1Ac than Cry1F protein. However, the value of a toxin in transgenic plants hinges not on its potency relative to other toxins, but its ability to kill the target pests when produced by the plants (Tabashnik et al. 2013b). For a more effective monitoring of resistance in the C. includens and H. virescens populations are necessary are necessary more dose-response bioassays to establishing of diagnostic dose to confirming the presence of resistance in insect populations in the field (Huang 2006). On the other hand, the bioassay technique used in this study was adequate with the IRM programs to evaluate the susceptibility of C. includens and H. virescens populations to the Cry protein as reported for other Bt proteins and insect pests (Marçon et al. 1999b, Liao et al. 2002, Siegfried et al. 2006, Ali and Luttrell 2011).

In summary, we observed a 15-fold variation in susceptibility of populations of C. includens to Cry1Ac and Cry2Aa using larval mortality and growth inhibition as response variable in diet-overlay bioassays. To our knowledge, this is the first study to determine the susceptibility of C. includens to Cry1A and Cry2A toxins using diet-surface bioassays in Brazil before large-scale commercial use of Bt soybean. This study shows that larval growth inhibition is useful response variable in bioassays and may enhance the detection of early changes in susceptibility or evolution of resistance (Sims et al. 1996). In diet-surface bioassays, H. virescens were highly susceptible to Cry2Aa and Cry1Ac toxin. Because of the recent release of Bt Cry1Ac soybean, more work is needed to understand if the variation in Cry1Ac susceptibility here observed in the soybean looper has a genetic basis, which can be tested in a selection experiment. Finally, it is important to continue the efforts to preserve susceptibility of target species to Cry1A and Cry2A toxins, especially in regions with high adoption rates of Bt

soybean and cotton, and to invest on research to develop more appropriate tactics to maximize the sustainable use of these transgenic crops.

REFERENCES

Abbas, N., H. A. A. Khan, and S. A. Shad. 2014. Cross-resistance, genetics, and realized heritability of resistance to fipronil in the house fly, Musca domestica (Diptera: Muscidae): a potential vector for disease transmission. Parasitology research 113: 1343-1352.

Adamczyk, J., L. Adams, and D. Hardee. 2001. Field efficacy and seasonal expression profiles for terminal leaves of single and double Bacillus thuringiensis toxin cotton genotypes. Journal of Economic Entomology 94: 1589-1593.

Albernaz, K., B. Merlin, S. Martinelli, G. Head, and C. Omoto. 2013. Baseline susceptibility to Cry1Ac insecticidal protein in Heliothis virescens (Lepidoptera: Noctuidae) populations in Brazil. Journal of Economic Entomology 106: 1819-1824.

Ali, M., and R. Luttrell. 2011. Susceptibility of Helicoverpa zea and Heliothis virescens (Lepidoptera: Noctuidae) to Vip3A insecticidal protein expressed in VipCot™ cotton. Journal of invertebrate pathology 108: 76-84.

Bernardi, O., D. Amado, R. S. Sousa, F. Segatti, J. Fatoretto, A. D. Burd, and C. Omoto. 2014. Baseline Susceptibility and Monitoring of Brazilian Populations of Spodoptera frugiperda (Lepidoptera: Noctuidae) and Diatraea saccharalis (Lepidoptera: Crambidae) to Vip3Aa20 Insecticidal Protein. Journal of Economic Entomology 107: 781-790.

Bolin, P., W. Hutchison, D. Andow, and K. Ostlie. 1998. Monitoring for European corn borer (Lepidoptera: Crambidae) resistance to Bacillus thuringiensis: logistical considerations when sampling larvae. Journal of agricultural entomology (USA).

Brévault, T., P. Prudent, M. Vaissayre, and Y. Carrière. 2009. Susceptibility of Helicoverpa armigera (Lepidoptera: Noctuidae) to Cry1Ac and Cry2Ab2 insecticidal proteins in four countries of the West African cotton belt. Journal of Economic Entomology 102: 2301-2309.

Bueno, R. C. O. D., J. R. P. B. Parra, and A. D. Bueno. 2012. Trichogramma pretiosum parasitism of Pseudoplusia includens and Anticarsia gemmatalis eggs at different temperatures. Biological Control 60: 154-162.

Carrière, Y., D. W. Crowder, and B. E. Tabashnik. 2010. Evolutionary ecology of insect adaptation to Bt crops. Evolutionary Applications 3: 561-573.

Chilcuit, C. F., and B. E. Tabashnik. 1995. Evolution of Pesticide Resistance and Slope of the oncentration-Mortality Line: Are They Related?, vol. 88.

CTNBio, -. C. T. N. d. B. 2005. Parecer Técnico Nº 513/2005. http://www.ctnbio.gov.br/index.php/content/view/12526.html. http://www.ctnbio.gov.br/index.php/content/view/12526.html

CTNBio, C. T. N. d. B. 2010. Liberação comercial de soja geneticamente modificado resistente a insetos e tolerante a herbicida MON 8771 x MON 89788. In Parecer Técnico Prévio Conclusivo Nº 2542/2010. (http://www.ctnbio.gov.br/index.php/content/view/12884.html).

Dhurua, S., and G. T. Gujar. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, Pectinophora gossypiella (Saunders) (Lepidoptera: Gelechiidae) from India. Journal of Pest Science 67: 898 - 903.

Domingues, F. A., K. L. Silva-Brandão, A. G. Abreu, O. P. Perera, C. A. Blanco, F. L. Cônsoli, and C. Omoto. 2012. Genetic structure and gene flow among Brazilian populations of Heliothis virescens (Lepidoptera: Noctuidae). Journal of economic entomology 105: 2136-2146.

Dong, H., and W. Li. 2007. Variability of endotoxin expression in Bt transgenic cotton. Journal of Agronomy and Crop Science 193: 21-29.

Everich, R., G. Dively, and J. Linduska. 1992. Baseline monitoring of Colorado potato beetle sensitivity to Bacillus thuringiensis and associations with pyrethroid resistance. Resistant pest management (USA).

Girón-Pérez, K., A. Oliveira, A. Teixeira, R. Guedes, and E. Pereira. 2014. Susceptibility of Brazilian populations of Diatraea saccharalis to Cry1Ab and response to selection for resistance. Crop Protection 62: 124-128.

Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar. 1995. Selection and genetic analysis of a Heliothis virescens (Lepidoptera: Noctuidae) strain with high levels of resistance to Bacillus thuringiensis toxins. Journal of Economic Entomology 88: 1545 - 1559.

Gould, F., A. Anderson, A. Jones, D. Sumerford, D. G. Heckel, J. Lopez, S. Micinski, R. Leonard, and M. Laster. 1997. Initial frequency of alleles for resistance to Bacillus thuringiensis toxins in field populations of *Heliothis virescens.* Proceedings of the National Academy of Sciences of the United States of America 94: 3519 – 3523.

Greene, G. L., N. C. Lepla, and W. A. Dickerson. 1976. Velvetbean caterpillar: a rearing procedure and artificial medium. Journal of Economic Entomology 69: 488 - 497.

Greenplate, J., S. Penn, Z. Shappley, M. Oppenhuizen, J. Mann, B. Reich, J. Osborn, P. Dugger, and D. Richter. Year. Published. Bollgard II efficacy: quantification of total lepidopteran activity in a 2-gene product, pp. 1041-1043. In, 2000 Proceedings Beltwide Cotton Conferences, San Antonio, USA, 4-8 January, 2000: Volume 2., 2000. National Cotton Council.

Hardee, D. D., L. C. Adams, W. L. Solomon, and D. V. Sumerford. 2001. Tolerance to Cry1Ac in populations of Helicoverpa zea and Heliothis virescens (Lepidoptera: Noctuidae): three-year summary. Journal of Agricultural and Urban Entomology 18: 187-197.

Hoskins, W. 1960. Use of the dosage-mortality curve in quantitative estimation of insecticide resistance. Misc. Publ. Entomol. Soc. Am 2: 85-91.

Huang, F. 2006. Detection and monitoring of insect resistance to transgenic Bt crops. Insect Science 13: 73-84.

Jalali, S. K., L. Yadavalli, R. Ojha, P. Kumar, S. B. Sulaikhabeevi, R. Sharma, R. Nair, R. C. Kadanur, S. P. Kamath, and M. S. Komarlingam. 2014. Baseline sensitivity of maize borers in India to the Bacillus thuringiensis insecticidal proteins Cry1A. 105 and Cry2Ab2. Pest management science.

James, C. 2014. Global Status of Commercialized Biotech/GM Crops: 2014., ISAAA Brief Ithaca, NY.

Jurat-Fuentes, J. L., and M. J. Adang. 2001. Importance of Cry1 δ-endotoxin domain II loops for binding specificity in Heliothis virescens (L.). Applied and environmental microbiology 67: 323-329.

Jurat-Fuentes, J. L., F. L. Gould, and M. J. Adang. 2003. Dual resistance to Bacillus thuringiensis Cry1Ac and Cry2Aa toxins in Heliothis virescens suggests multiple mechanisms of resistance. Applied and environmental microbiology 69: 5898-5906.

Kranthi, S., C. Dhawad, S. Naidu, A. Bharose, A. Chaudhary, V. Sangode, S. Nehare, S. Bajaj, and K. Kranthi. 2009. Susceptibility of the cotton bollworm, Helicoverpa armigera (Hubner)(Lepidoptera: Noctuidae) to the Bacillus thuringiensis toxin Cry2Ab before and after the introduction of Bollgard-II. Crop Protection 28: 371-375.

Liao, C., D. G. Heckel, and R. Akhurst. 2002. Toxicity of Bacillus thuringiensis insecticidal proteins for Helicoverpa armigera and Helicoverpa punctigera (Lepidoptera: Noctuidae), major pests of cotton. Journal of invertebrate pathology 80: 55-63.

Marçon, P. C. R. G. Y., L.J., K. L. Steffey, and B. D. Siegfried. 1999. Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to Bacillus thuringiensis toxins. Journal of Economic Entomology 92: 279 - 285.

Mascarenhas, V., J. Graves, B. Leonard, and E. Burris. 1998. Susceptibility of field populations of beet armyworm (Lepidoptera: Noctuidae) to commercial and experimental insecticides. Journal of Economic Entomology 91: 827-833.

Olsen, K., J. Daly, H. Holt, and E. Finnegan. 2005. Season-long variation in expression of Cry1Ac gene and efficacy of Bacillus thuringiensis toxin in transgenic cotton against Helicoverpa armigera (Lepidoptera: Noctuidae). Journal of Economic Entomology 98: 1007-1017.

Onstad, D. W. 2008. Insect Resistance Management: Biology, Economics, and Prediction, Elsevier, San Diego.

Palma, J., K. Maebe, J. V. C. Guedes, and G. Smagghe. 2015. Molecular Variability and Genetic Structure of Chrysodeixis includens (Lepidoptera: Noctuidae), an Important Soybean Defoliator in Brazil. PLoS ONE 10.

Pereira, E. J. G., B. A. Lang, N. P. Storer, and B. D. Siegfried. 2008. Selection for Cry1F resistance in the European corn borer and cross-resistance to other Cry toxins. Entomologia Experimentalis et Applicata 126: 115 - 121.

Robertson, J., H. Preisler, S. Ng, L. A. Hickle, and W. Gelernter. 1995. Natural variation: a complicating factor in bioassays with chemical and microbial pesticides. Journal of Economic Entomology 88: 1-10.

Robertson, J. L., N. E. Savin, H. K. Preisler, and R. M. Russell. 2007. Bioassays with Arthropods, CRC press.

Russell, R. M., J. L. Robertson, and N. E. Savin. 1977. POLO: a new computer program for probit analysis. Bulletin of the Entomological Society of America 23: 209 - 213.

Siegfried, B. D., T. Spencer, A. Crespo, E. Pereira, and P. Marçon. Year. Published. Ten years of Bt resistance monitoring in the European corn borer: what we know, what we don't know, and what we can do better, pp. 168-171. In, Proceedings of The 9th International Symposium on the Biosafety of Genetically Modified Organisms, Jeju Island, Korea, 2006.

Sims, S. B., J. T. Greenplate, T. B. Stone, M. A. Caprio, and F. L. Gould. 1996. Monitoring strategies for early detection of Lepidoptera resistance to Bacillus thuringiensis insecticidal proteins.

Stewart, S., J. Adamczyk, K. Knighten, and F. Davis. 2001. Impact of Bt cottons expressing one or two insecticidal proteins of Bacillus thuringiensis Berliner on growth and survival of noctuid (Lepidoptera) larvae. Journal of Economic Entomology 94: 752-760.

Stone, T. B., and S. R. Sims. 1993. Geographic susceptibility of Heliothis virescens and Helicoverpa zea (Lepidoptera: Noctuidae) to Bacillus thuringiensis. Journal of Economic Entomology 86: 989-994.

Tabashnik, B. E. 2008. Delaying insect resistance to transgenic crops. Proceedings of the National Academy of Sciences 105: 19029 - 19030.

Tabashnik, B. E., T. Brévault, and Y. Carrière. 2013a. Insect resistance to Bt crops: lessons from the first billion acres. Nature Biotechnology 31: 510 - 521.

Tabashnik, B. E., A. J. Gassmann, D. W. Crowder, and Y. Carrière. 2008. Insect resistance to Bt crops: evidence versus theory. Nature Biotechnology 26: 199-202.

Tabashnik, B. E., Y.-B. Liu, T. J. Dennehy, M. A. Sims, M. S. Sisterson, R. W. Biggs, and Y. Carrière. 2002. Inheritance of Resistance to Bt Toxin Cry1Ac in a Field-Derived Strain of Pink Bollworm (Lepidoptera: Gelechiidae), vol. 95.

Tabashnik, B. E., J. A. Fabrick, G. C. Unnithan, A. J. Yelich, L. Masson, J. Zhang, A. Bravo, and M. Soberón. 2013b. Efficacy of genetically modified Bt toxins alone and in combinations against pink bollworm resistant to Cry1Ac and Cry2Ab.

Tomquelski, G. V., and L. C. T. Maruyama. 2009. Lagartada-da-maçã em soja. Revista Cultivar. 117: 20-22.

Wu, K., Y. Guo, and N. Lv. 1999. Geographic variation in susceptibility of Helicoverpa armigera (Lepidoptera: Noctuidae) to Bacillus thuringiensis insecticidal protein in China. Journal of Economic Entomology 92: 273-278.

Xu, X., L. Yu, and Y. Wu. 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of Bacillus thuringiensis in Helicoverpa armigera. Applied and environmental microbiology 71: 948-954.

Zhang, H., W. Yin, J. Zhao, L. Jin, Y. Yang, S. Wu, B. E. Tabashnik, and Y. Wu. 2011. Early warning of cotton bollworm resistance associated with intensive planting of Bt cotton in China. PLoS ONE 6: 1 - 8.

Zhao, J. Z., J. Cao, Y. X. Li, H. L. Collins, R. T. Roush, E. Earle, D., and A. M. Shelton. 2003. Transgenic plants expressing two Bacillus thuringiensis toxins delay insect resistance evolution. Nature Biotechnology 21: 1493 - 1497.

TABLES AND FIGURES

Table 1. Details of the field-collected populations of C. includens sampled from distinct geographic regions in Brazil, in 2013.

County	Population code	Longitude	Latitude	Sampling date
Viçosa/ MG	Vç	20° 45′ 14″ S	42° 52′ 55″ W	Feb.-Mar. 2013
Coimbra/ MG	Cb	20º 51' 24" S	42º 48' 10" W	Feb.-Apr. 2013
Primavera do Leste /MT	MT	15º 27' 57" S	54º 17' 06" W	Apr.-Mar. 2013
Sorriso/ MT	Ms	12º 32' 43" S	55º 42' 41" W	Feb.-Mar. 2013
Conchal/ SP	SP	22º 19' 49" S	47º 10' 21" W	Mar.-Apr. 2013

Table 2. Susceptibility of C. includens populations to Cry toxins from B. thuringienis. Mortality was assessed after seven days of exposure.

Bt toxin	Population code	Slope ± SE	LC_{50} (95% CL)[1] --- ng/cm^2 ---	LC_{90} (95% CL) --- ng/cm^2 ---	χ^2	P	RR (95% CL)[2]
Cry1Ac	VÇ	2.15 ± 0.19	54.5(46.3 - 63.3)	215.7 (170.8 - 292.7)	4.73	0.450	3.4 (2.6 - 4.4)
	MS	2.34 ± 0.28	28.6 (22.5 - 35.8)	101.0 (74.9 - 155.4)	1.33	0.931	1.8 (1.3 - 2.4)
	SP	1.78 ± 0.24	240.9 (138.5 - 433.7)	1267.3 (625.3 - 8316.3)	7.47	0.113	14.9 (10.7 - 20.7)
	MT	2.76 ± 0.22	46.5 (40.3 - 53.5)	135.5 (111.5 - 173.6)	3.74	0.587	2.9 (2.3 - 3.7)
	CB	1.85 ± 0.14	16.1 (13.2 - 19.7)	79.8 (60.7 - 112.5)	1.94	0.857	1
Cry2Aa	VÇ	4.03 ± 0.68	24.3 (19.5 - 30.6)	50.7 (38.7 - 80.0)	0.23	0.998	8.7 (6.2 - 12.2)
	MS	3.80 ± 0.61	19.3 (15.4 - 24.0)	41.9 (32.2 - 65.0)	0.37	0.996	6.9 (4.9 - 9.7)
	MT	3.33 ± 0.40	21.4 (16.4 - 27.8)	51.9 (38.0 - 88.1)	5.65	0.341	7.6 (5.5 - 10.5)
	CB	1.32 ± 0.11	2.9 (1.6 - 4.4)	26.3 (14.9 - 64.7)	9.35	0.096	1

[1]LC_{50} and LC_{90}, Lethal concentration causing 50% and 90% of mortality, respectively. The values are presented with the 95% confidence limits estimated by probit regression using Polo-Plus (Robertson et al.. 2007). [2]Resistance ratio = highest LC_{50}/lowest LC_{50}. Indicates how many times the population is less susceptible than the one with the lowest LC_{50} value. In parentheses are the 95% confidence limits for the resistance ratio (Robertson et al.. 2007).

Table 3. Susceptibility of C. includens populations to Cry toxins from Bacillus thuringienis. Growth inhibition was assessed after seven days of exposure.

Bt toxin	Population code	Slope ± SE	EC_{50} (95% CL)[1] --- ng/cm^2 ---	EC_{90} (95% CL) --- ng/cm^2 ---	χ^2	P	RR_{50} (95% CL)[2]
Cry1Ac	VÇ	2.09 ± 0.22	15.9 (10.5 - 20.9)	65.5 (50.6 - 96.2)	5.85	0.320	7.50 (5.0 - 11.2)
	MS	2.04 ± 0.19	10.3 (8.6 - 12.0)	44.1 (36.4 - 56.5)	1.99	0.849	4.86 (3.3 - 7.1)
	SP	1.03 ± 0.07	19.6 (11.5 - 33.0)	341.3 (167.4 - 987.9)	7.03	0.134	9.20 (6.0 - 14.1)
	MT	2.18 ± 0.15	23.3 (19.0 - 28.1)	90.7 (70.4 - 127.5)	5.77	0.328	11.0 (7.7 - 15.7)
	CB	1.21 ± 0.11	2.1 (1.4 - 2.8)	24.3 (17.7 - 36.5)	3.42	0.634	1
Cry2Aa	VÇ	3.12 ± 0.32	6.8 (5.9 - 7.8)	17.7 (14.7 - 22.9)	2.04	0.842	14.6 (8.5 - 25.1)
	MS	3.15 ± 0.35	5.6 (4.9 - 6.5)	14.4 (11.9 - 18.8)	0.76	0.942	12.0 (7.0 - 20.7)
	MT	2.94 ± 0.28	8.8 (7.5 - 9.9)	24.0 (20.5 - 29.5)	0.60	0.963	18.7 (10.8 - 32.1)
	CB	1.03 ± 0.11	0.5 (0.1 - 0.8)	8.3 (5.0 - 17.3)	6.52	0.258	1

[1]EC_{50} and EC_{90}, Effective concentration causing 50% and 90% of larval inhibition of the population, respectively. Values are presented with the 95% confidence limits estimated by probit regression using Polo-Plus (Robertson et al.. 2007). [2]Resistance ratio = highest EC_{50}/lowest EC_{50} and indicates how many times the population is less susceptible than the one with the lowest EC_{50} value. In parentheses are the 95% confidence limits for the resistance ratio (Robertson et al.. 2007).

Table 4. Toxicity of Bt toxins to H. virescens from the state of Mato Grosso, Brazil, at seven days of exposure.

Response variable	Toxins	Slope ± SE	LC_{50} or EC_{50} (95% CL)[1] --- ng/cm^2 ---	Toxicity ratio (95% CL)[2]	χ^2	P
Mortality	Cry2Aa	2.90 ± 0.34	12.3 (10.1 - 15.0)	10.3 (7.6 - 14.1)	0.73	0.981
	Cry1Ac	1.63 ± 0.33	27.2 (20.8 - 35.6)	4.7 (3.3 - 6.7)	1.94	0.856
	Cry1Fa	2.25 ± 0.35	127.6 (98.7 - 174.5)	1	3.07	0.688
Growth inhibition	Cry2Aa	2.59 ± 0.33	2.7 (2.0 - 3.2)	5.7 (4.4 - 7.5)	0.42	0.994
	Cry1Fa	1.29 ± 0.11	5.8 (4.3 - 7.5)	2.6 (1.9 - 3.5)	3.42	0.635
	Cry1Ac	3.50 ± 0.33	15.3 (13.7 - 17.4)	1	0.71	0.690

[1]LC or EC, Lethal or effective concentration causing 50 or 90% of mortality or growth inhibition, respectively. Values are presented with 95% confidence limits estimated by probit regression using Polo-Plus (Robertson et al. 2007). [2]Toxicity ratio = 1/(lowest LC_{50}/ highest LC_{50}); indicates how many times the toxin is more potent than the one with the highest LC_{50} value. In parentheses are 95%confidence limits for the toxicity ratio (Robertson et al.. 2007).

CHAPTER 2 - Mortality and fitness of the soybean looper (Chrysodeixis includens) exposed to single- and dual-gene transgenic Bt crops

ABSTRACT

The effects of transgenic events producing either one or two Bt proteins against target species are scarcely known, especially for the soybean looper, Chrysodeixis includens. Here, mortality and sublethal effects of single-gene Bt cotton (Event MON 531), Bt soybean (MON 87701 x MON 89788), as well as dual-gene (i.e., pyramided) Bt cotton producing either Cry1Ac + Cry2Ab (MON 15985) or Cry1Ac + Cry1Fa (DAS 281-24-236/3006-210-23) were investigated on life-history traits of C. includens under laboratory conditions. The results revealed that single-gene soybean and pyramided cotton led to 100% mortality of C. includens larvae after two days of exposure in leaf-tissue bioassays. Single-gene Bt cotton caused low larval mortality, but negatively affected life-history traits such as extended development time, body size (i.e., weight of larvae and pupae), viability of pupae, adult longevity, and skewed sex ratio. These sublethal effects on larval fitness components reflected in significant differences with the lower values of net reproductive rate (R_0) and intrinsic rate of population increase (r_m) in relation to larvae reared on non-Bt cotton. In addition, higher mean generation time (T) and doubling time (Dt) were observed in insects exposed to the Bt cotton compared with control. This research demonstrate significant fitness effects on C. includens resulting from larval development on single-gene Bt cotton; Bt soybean and pyramided cotton caused a high mortality of the soybean looper. These effects should be considered when devising resistance management programs.

Keywords: Sublethal effects, Bacillus thuringiensis, intrinsic rate of population increase, Cry1Ac cotton, insect resistance management, transgenic soybean and cotton

INTRODUCTION

The soybean looper, Chrysodeixis includens (Walker) (Lepidoptera: Noctuidae), is one of the most important defoliator pests in many crops from the northen US to southern South America (Eichlin and Cunningham 1978, Alford and Hammond 1982). Direct damages are caused by this species in crops of great economic interest, such as soybeans, cotton, beans, tobacco, sunflower, tomato and other vegetables (Eichlin and Cunningham 1978, Herzog 1980b) . This specie has a high potential for population increase and high frequencies of outbreaks have been observed in recent years (Bueno et al. 2012a). Furthermore, C. includens has important life-history characteristics such as polyphagy, high mobility and fecundity, as well as relatively low susceptibility to

Cry1 toxins, some synthetic insecticides, and Bt formulations (Morales et al. 1995, Mascarenhas et al. 1998a, Bernardi et al. 2012). This has made management of its populations a challenging task with substantial increases in control expenditures. Until recently, synthetic insecticides were the main control method used against C. includens, but concern of their deleterious effects on natural enemies and evolution pest resistance have attracted attention for implementation of more environmentally-safe methods to manage the soybean looper.

In the last two decades, advances in agricultural biotechnology have allowed for development of new tools for pest control. In this scenario, transgenic plants expressing one or more genes of Bacillus thuringiensis (Bt) have been developed and successfully implemented for control of destructive lepidopteran pests worldwide, becoming an essential component of pest management in many agroecosystems. Among the transgenic events marketed in Brazil, Bt cotton producing either Cry1Ac + Cry2Ab (MON 15985) or Cry1Ac + Cry1Fa (DAS 281-24-236/3006-210-23), and Bt soybean (MON 87701 x MON 89788) showed effective control of C. includens (Tindall et al. 2009, Bernardi et al. 2012, Sorgatto et al. 2015). Cry1Ac cotton (MON 531) does not provide efficient control of this pest species according to a number of field reports, but their sulethal effect on fitness components of the soybean looper is still unknown.

There are different views about the negative effects of trade maintenance of Cry1Ac cotton, but in Brazil this technology is still concurrently planted with others Bt crops, and sublethal effects caused by this transgenic event is often overlooked. The deployment of Bt plants that produces low toxin levels or that reduces synthesis of Bt toxins with senescence can allow for survival of the target insects and may negatively affect their physiological homeostasis with wide consequences for population growth (Eizaguirre et al. 2005), especially less-susceptible pests such as C. includens. Transgenic cotton events are known to have large variation in toxin gene expression, which is associated with environment and phenological factors (Kranthi et al. 2005, Olsen et al. 2005, Rochester 2006, Pawade et al. 2015). This variation is well known for the Cry1Ac toxin (Kranthi et al. 2009). Sublethal exposure to these events may increase the fitness of resistant heterozygotes and increases rates of resistance evolution (Siegfried et al. 2001). In general, the cases of field resistance are associated with failure to reach high-dose expression of toxin in Bt cultivars (Huang et al. 2011), or lack of sufficient refuge that may compromise the integrity of high-dose/refuge strategy.

A way to better understand the effects of sublethal Bt intoxication on fitness of a target insect is to construct life tables, which is powerful approach for analyzing and

understanding the impact of treatments on insect development and survival, reproduction, and rate of population increase (Sandhu et al. 2010, Barrionuevo et al. 2012). Some studies about the sublethal effects of Bt toxins have been reported for Ostrinia nubilalis in maize , for Helicoverpa zea (Boddie) in cotton (Jackson et al. 2004), and for H. zea in maize (Reisig and Reay-Jones 2015). In these studies, the main changes caused by sublethal effects of Bt toxins can be associated with reduced developmental rates, fertility, fecundity, changes in sex ratio, and behaviors, such as feeding and oviposition. However, there is no detailed experimentation regarding the sublethal effects of Bt cotton events on the demographic performance of C. includens, which was this aim of this work.

Here, We tested two hypotheses: first, that Bt soybean and dual-gene Bt cotton are more lethal than single-gene Bt cotton to larvae of C. includens, a specie relatively tolerant to Bt (Bernardi et al. 2012), and given that, the second hypothesis was that Bt cotton producing a single toxin is not so lethal against soybean loopers although adverse effects of sublethal exposure reduce the fitness of C. includens. Life tables were developed to detect and quantify the consequences of sublethal exposure to Bt toxin on population increase of the soybean looper.

MATERIAL AND METHODS

Insect rearing

A strain of soybean looper was established in the laboratory from field-collected insects (1000 late-instar larvae) obtained from common beans, Phaseolus vulgaris, in Coimbra County, Minas Gerais, Brazil, in April 2014. The larvae were brought to the laboratory and reared individually in 16-well plastic trays (Advento do Brasil, Diadema, São Paulo). Larvae were fed daily with soybean leaves, and kept in a rearing room at 27 ± 1°C, 80% relative humidity, and photoperiod of 14 h light until pupation. Pupae (80♂ + 80♀) were placed in cages made of polyvinylchloride (PVC), 20 cm diameter and 30 cm height, lined internally with sulfite paper as substrate for oviposition. Adults were fed with 10% honey solution in water, and eggs were daily collected and stored in an incubator until hatching. Neonates were transferred to 16-well trays with artificial diet adapted from Greene et al. (1976a) and kept in the rearing room. After establishing the colony, individuals from the 2nd to the 3th generation were used in the experiments. This prevented to avoid colony adaptation to laboratory rearing conditions.

Neonates (< 24 h hatching) were individually reared in 16-well plastic trays with each well containing a leaf piece and lined with moistened filter paper to prevent desiccation. The treatments consisted of the following transgenic events: single-gene

Cry1Ac cotton (MON 531, Monsanto do Brasil, São Paulo, SP), Cry1Ac soybean (MON 87701 x MON 89788, Monsanto do Brasil, São Paulo, SP), as well as dual-gene (i.e., pyramided) Bt cotton producing either Cry1Ac + Cry2Ab (MON 15985, Monsanto do Brasil, São Paulo, SP) or Cry1Ac + Cry1Fa (DAS 281-24-236/3006-210-23, Dow AgroSciences, São Paulo, SP). Control treatments were non-Bt isoline cotton (Delta OPAL, Monsanto do Brasil, São Paulo, SP) and the soybean isoline (MSOY8866, Monsanto do Brasil, São Paulo, SP). Plant leaves were replaced daily until the larvae reached the pupal stage. The pupae were maintained individually in 50-ml plastic containers lined with moistened filter paper until adult emergence. Two groups of approximately 320 larvae were used to measure life cycle duration and sex proportion. Furthermore, these individuals were followed during one generation to collect data for survival analysis. From the adults obtained, at least ten couples of each treatment were randomly paired to determine longevity, reproduction and population growth parameters.

Plants

Cotton and soybean were grown in the greenhouse. Seeds were sown every two months in 15-L pots containing substrate composed of 3 parts of soil, 2 parts cattle manure, and 2 parts of sand to produce plants with normal levels of Bt protein synthesis (Olsen et al. 2005), which was qualitatively checked with immunoassay test strips (AgraStrip, Union, MO, EUA). Plants were irrigated two to three times a day depending on soil moisture conditions, and leaves were collected from cotton plants approximately 45-50 days after germination. Soybean leaves were used when plants reached R2-R4 phenological stages. Soil fertilization was carried out according to recommendations for the cotton (Richetti et al. 2003) and soybean crops (EMBRAPA 2011). Plants were daily inspected to prevent arthropod infestation, and whenever needed, mechanical pest control was used with no application of pesticides.

Life-history traits

All treatments daily were evaluated to determine the developmental time and survivorship in larva, pupa, and adult stages. Also, the sex ratio on each treatment was determined by sexing the pupae (Angulo and Weigert 1975). Oviposition period, fecundity (number of eggs deposited by a female during her entire life period), and fertility (percentage of eggs hatching) were estimated by placing 15 couples in PVC cages (10 cm height and 10 cm diam.) lined internally with sulfite paper as substrate for oviposition. Adults were fed daily with 10% honey solution in water, and eggs were collected daily and stored in an incubator until hatching to calculate the total fertility.

Each female represented a replicate. Each couple was formed by one virgin female and one virgin male (< 24 h old). Moths were maintained in their cages until the female died. These parameters were daily recorded in all treatments for each female until death.

Growth rate

For C. includens, 100 1st instar larvae were weighed collectively and 320 C. includens larvae were individually reared in 16-well plastic trays with each well containing a leaf piece and lined with moistened filter paper to prevent desiccation, as describe previously. The treatments consisted of the following event: Cry1Ac cotton (MON 531, Monsanto do Brasil, São Paulo, SP), non-Bt isoline cotton (Delta OPAL, Monsanto do Brasil, São Paulo, SP) and the soybean isoline (MSOY8866, Monsanto do Brasil, São Paulo, SP). Plant leaves were replaced daily until the larvae reached the pupal stage. Pupae were removed, weighed (after 1 day) and placed in separate Eppendorf tubes (1.5 ml). The mean relative growth rate (MRGR) was calculated using the formula (Radford 1967):

$$MRGR = [In\ W_2(mg) - In\ W_1(mg)] / T$$

where W_1 = initial larval weight; W_2 = pupal weight; T = time to pupal stage.

Life table construction

The above data obtained during development and reproduction were used to construct life tables and estimate population growth parameters. The methodology described by Carey (1993) was use to estimate the following parameters: survivorship (lx), life table entropy (H), net reproductive rate (R0), intrinsic rate of increase (rm), finite rate of increase (k), doubling time (DT) and mean generation time (T).

Statistical analyses

The results of the time-mortality bioassays were subjected to survival analysis using the non-parametric procedure LIFETEST and stratifying the survival differences for transgenic events (SAS 2001). This procedure allows for estimation of survival curves obtained through Kaplan–Meier estimator median.

The life-history traits measured were analyzed through the one-way ANOVA to detect the sublethal effect of Bt cotton on duration of life stages, pupal weight, total fecundity and egg viability (PROC GLM, (SAS 2003)). Means were separated using the Tukey test ($P < 0.05$). In addition, we used linear regression analysis to determine the relationship between the sublethal effects caused Bt plant exposure and larval weight (PROC REG; SAS Institute, 2001). A chi-square test was computed to determine whether there was any deviation from the expected sex ratio of 1 : 1(SAS 2001) .

Life-table parameters were estimated using the jackknife procedure, which allows the calculation of confidence intervals for all estimated parameters, as well as t-tests to perform pairwise or multiple comparison between groups (Maia et al. 2000).

RESULTS

3.1. Time-mortality bioassays

Survival analysis of C. includens larvae exposed to different transgenic and non-transgenic plants indicated significant differences between among treatments (Log-rank test, $\chi^2 = 951.4$, df = 5, $P < 0.0001$). Survival curves for larvae on Cry1Ac cotton differ significantly from that for larvae feeding on non-Bt soybean ($\chi^2 = 31.9$, df= 1, P = 0.0001), but no significant differences between Cry1Ac cotton and non-Bt cotton ($\chi^2 = 3.04$, df = 1, P = 0.156) was found. Transgenic cotton events producing Cry1Ac + Cry2Ab, Cry1Ac + Cry1Fa, and Cry1Ac soybean caused 100% mortality of C. includens larvae after 2 days of exposure, while the same effect was not observed for larvae feeding on Cry1Ac cotton leaves (Figure 1). Such differences reflected in the mean survival time observed for each transgenic event, in which Cry1Ac cotton took more than 10 days to cause 50% mortality (Figure 2).

3.2. Life-history traits

The oviposition periods were 7.4, 6.4, and 3.5 days for insects fed on non-Bt soybean, non-Bt cotton, and Cry1Ac cotton, respectively; total fecundity during this period were 590, 662, and 325 eggs per female, ranging from 132-1536, 112-1016, and 35-530 eggs per female (N=15), respectively. Larval developmental time of C. includens varied significantly among treatments ($F = 129.84$; df = 2, 39; $P < 0.0001$), as did female pupal weight ($F = 5.62$; df = 2, 118; $P = 0.0052$). Larvae feeding on soybean leaves completed development 3 days before those feeding on cotton leaves; Cry1Ac cotton delayed larval development 12 or 8 days relative to non-Bt soybean or cotton, respectively. In addition, the sublethal effects of Cry1Ac cotton on C. includens larvae reduced 48% fecundity of the moths and 99% of their fertility, that is, there was a strong reduction on egg production and viability. For the other life-history traits, there was no difference ($P > 0.1729$) among treatments (Table 1).

Life tables

Table 3 shows that all four population parameters associated with the fertility life table (Ro, T, r_m and Dt) were significantly different ($P < 0.05$) for individuals reared on the plant types compared. In the conditions of the experiment, the number of times that the C. includens population would multiply per generation was estimated to be 45, 41 and 0.3, on non-Bt soybean, non-Bt cotton, and Cry1Ac cotton, respectively (Table

3). While soybean was the best host-plant leading to better fitness of C. includens, Cry1Ac cotton was the worst host plant in all life-table parameters. The intrinsic rate of increase (r_m) and the net reproductive rate (R_0) for the Cry1Ac cotton treatment was significantly lower compared to non-Bt cotton. Sublethal effect caused by Cry1Ac cotton significantly increased the mean generation time (T) and doubling time (Dt) of C. includens.

Pupal weight

The weights of the pupae were significantly different between gender ($F = 11.11$; $df = 1, 363$; $P = 0.0009$), and between those developed from the larvae that fed on non-Bt soybean and different cotton leaves ($F = 8.11$; $df = 2, 338$; $P = 0.0004$), but significant interaction between gender and cotton leaf treatment ($F = 3.35$; $df = 2, 363$; $P = 0.0365$) (Table 2) was found. The pupae from the larvae that fed on Cry1Ac cotton leaves were significantly heavier than those from the larvae that fed on non-Bt soybean and non-Bt cotton leaves (males: $F = 3.06$; $df = 2, 179$; $P = 0.0490$; females: $F = 8.98$; $df = 2, 183$; $P < 0.0002$). Of the pupae from the larvae that fed on Cry1Ac Cotton leaves, the male pupae were significantly heavier than the female pupae ($F = 5.19$; $df = 1, 83$; $P = 0.0334$). In contrast, male and female pupae from the larvae that fed on non-Bt cotton leaves were not significantly different in weight ($F = 0.42$; $df = 1, 119$; $P = 0.5201$). However, significantly different in weight in male and female pupae from the larvae that fed on non-Bt soybean leaves were found ($F = 5.84$; $df = 1, 134$; $P = 0.0185$).

DISCUSSION

In this study, we confirm the hypotheses that Bt soybean and dual-gene Bt cotton are more lethal than single-gene Bt cotton to larvae of C. includens, and that Cry1Ac cotton producing a single toxin is not so lethal against soybean loopers although adverse effects of sublethal exposure reduce the fitness of C. includens. Our results indicated more than 60% mortality of soybean looper larvae and that only moderate levels of Cry1Ac is produced by the Cry1Ac cotton, which should not be enough to provide good protection from feeding by C. includens larvae, but their sublethal effects do affect negatively the reproductive output of adults by reducing egg production and viability.

Our bioassays result confirmed that Cry1Ac-expressing soybean plants provide excellent protection against C. includens (Bernardi et al. 2012), which is also consistent with results for Cry1Ac-expressing in pyramided cotton (Cry1Ac + Cry2Ab cotton and Cry1Ac + Cry1Fa cotton (Tindall et al. 2009, Sorgatto et al. 2015). These results indicate that Bt cotton producing two Bt toxins should provide better control of C.

includens compared to Cry1Ac cotton, which expresses a single Bt gene in low levels relative to Cry1Ac soybean that produces high levels of the Bt toxin (Miklos et al. 2007). First- and second-instar lepidopteran larvae are normally more susceptible to Bt toxins than advanced instar larvae (Sneh et al. 1981, Bai et al. 1993), possibly because the gut juice of advanced larval instars (3^{rd}-5^{th}) exhibit very high proteolytic activities which lead to a complete degradation of Cry proteins (Keller et al. 1996). As Bt Cry1Ac toxin produced in Bollgard I cotton does not cause much lethality of neonate soybean loopers, this Bt technology by itself may not provide good protection against defoliation by C. includens larvae in the field, although sublethal effects on pest population growth should also be considered.

Exposure to Cry1Ac cotton delayed larval development time and increased larval and pupal weight of the soybean looper. This observation is consistent with earlier reports on other Lepidoptera such as Helicoverpa zea, Spodoptera frugiperda, Spodoptera exigua and C. includens in the field and laboratory (Stewart et al. 2001, Adamczyk Jr and Gore 2004, Funichello et al. 2013a). Average development time for C. includens larvae reared on non-Bt soybean, non-Bt cotton (isoline) and Cry1Ac cotton were respectively 18, 20 and 29 days; hence, Cry1Ac-producing Bt cotton delayed the development time 12 and 9 days compared to non-Bt events of soybean and cotton. The developmental delay has been attributed to a decrease in food intake or acquisition of low-quality, and the increase in the number of larval instars, a cause that could partially explain the development delay in larvae fed with sublethal Bt concentrations (Danks 1987, Eizaguirre et al. 2005). Although speculative, this commonly reported delayed larval development after Bt intoxication may reflect physiological alterations resulting from increase in the immune response and production of pro-coagulants that recognize and form specific aggregates around the Bt toxin (Rahman et al. 2004, Ma et al. 2005, Rahman et al. 2007, Bravo and Soberón 2008). Even as, the increase levels of digestive proteins that allow the insects to avoid exposure to high levels of Bt toxin (Oppert et al. 1997), which also diverts energy and resources from growth and development.

Curiously the pupal weight of larvae that fed on non-Bt and on Cry1Ac Cotton were not significantly different, but larvae that fed on non-Bt Soybean shown pupal weight were significantly lower compare to non-Bt and Cry1Ac Cotton. This observation is consistent with the adverse effect of B. thuringiensis var. kurstaki on pupal weight earlier reports on other insect pests (Erb et al. 2001, Khalique and Ahmed 2002, Aly et al. 2011, Sedaratian et al. 2013). The pupae from the larvae that fed on Cry1Ac cotton and non-Bt Cotton leaves are ~10 % heavier than larvae that fed on non-

Bt Soybean leaves. The male pupae were 12 and 7% heavier when the larvae fed on non-Bt Cotton and non-Bt Soybean leaves, respectively. The female pupae were 6 and 15% heavier when the larvae fed on non-Bt Cotton leaves than those when the larvae fed Cry1Ac Cotton and non-Bt Soybean leaves, respectively. Pupal weight has been positively correlated with fecundity in Trichoplusia ni (Milks et al. 1998), but this association was not found here.

Exposure to Cry1Ac cotton significantly reduced the reproductive potential of C. includens. The sublethal effect of the larval feeding on Cry1Ac cotton did not change the sex ratio but was associated with reduction in egg production and viability. Similar results were reported with other Lepidoptera (Salama and Zaki 1986, Bauce et al. 2006, Sedaratian et al. 2013). According to our results, only 1% eggs hatching when the C. includens larvae fed on Cry1Ac cotton, however when the larvae were reared with non-Bt cotton and non-Bt Soybean leaves approximately 28 and 35 % eggs hatching, respectively. Reduced egg hatchability also reported when larvae of H. virescens and P. xylostella were fed the Vip3A toxin (Gulzar and Wright 2015). Sedaratian et al. (2013) also reported that the oviposition period and eggs hatching were significantly reduced when the H. armigera larvae treated with sublethal concentrations of the B. thurigiensis var krustak. Although it was not possible to assess changes in physiology or reproductive behavior of C. includens in this study, clear evidence was shown that exposure sublethal exposure to Cry1Ac can affect adult reproductive success of this species.

Life table experiments showed a high capacity of C. includens to increase the population when larvae are not exposed to Bt cotton or soybean. The Cry1Ac cotton negatively affected all life table parameters of C. includens, especially the net reproductive rate (R_0), and intrinsic rate of increase (r_m) as expected from the strong effect observed on larval development time, adult fecundity, and egg viability. Ecotoxicological analysis based on population growth rate results in more accurate assessments of the impacts of toxicants because measures of population growth rate combine lethal and sublethal effects (Stark and Banks 2003). In life table results, the sublethal effect from the exposure to Cry1Ac Cotton showed an 81 and 86 % decrease in C. includens intrinsic rate of increase (r_m) respectively compared with insects reared with fresh non-Bt cotton and non-Bt soybean leaves. This is in agreement with several previous studies which examined the sublethal effects on other lepidopteran species such as Helicoverpa armigera with BTK (Sedaratian et al. 2013), Heliothis virescens and Plutella xylostella with Vip3A toxin (Gulzar and Wright 2015). The results

obtained in this study provide clear evidence of the sublethal effect of Cry1Ac cotton on the C. includens population growth. The alteration of population growth parameters can directly affect management tactics adopted for this species in the field.

These results suggest that Cry1Ac cotton technology can be used in insect resistance management programs (IRM) as a supplementary tactic to control C. includens as a moderate resistant plant. However, this technology will be dependent on locally adapted IRM programs, such adoption of refuge areas to help to maintain susceptible individuals in the population. As adoption of refuge by the growers is usually low in Brazil (Bernardi et al. 2014), and the tropical climate provide conditions for growing crops as well as for survival of high population densities of pest species almost year around, such a scenario of the intense selection pressure may rapidly lead to evolution of resistance of C. includens. The developmental asynchrony between adults carrying Cry1Ac resistance alleles (i.e., survivors of sublethal exposure) and the homozygous susceptible adults emerging from non-Bt plants tend to favor non-random mating and this could reduce the expected benefits of the refuge. Besides, the use of Cry1Ac-expressing soybean plants Cry1Ac soybean tend to increase selection pressure for Bt resistance in C. includens and other Lepidoptera that colonizes both crops.

In summary, the first hypothesis of higher lethality of Bt soybean and pyramided Bt cotton on C. includens is supported by the results of this study. The findings also provided support to the second hypothesis on the importance of sublethal effects for population growth of the C. includens and contribute to a better understanding of sublethal effects of Bt toxins on insects. Evidence for the above hypotheses come from reduced mortality rates, net reproductive rate (R_0), and intrinsic rate of increase (r_m), as well as longer generation time (T) and doubling time (Dt) when C. includens larvae were reared on leaves Cry1Ac cotton. Also, delayed larval development time and reduction on female pupal weight were alterations observed in C. includens exposed to Cry1Ac cotton. These results are clear evidence for adverse, sublethal effects of Cry1Ac cotton on C. includens and may also reflect potential reduction in fitness of individuals surviving sublethal exposure to Bt crops. Future efforts should be concentrated on dissecting these effects on male and female reproductive biology and to investigate the physiological impairment associated with the sublethal exposure to Bt toxins in the soybean looper.

REFERENCES

Adamczyk Jr, J., and J. Gore. 2004. Laboratory and field performance of cotton containing Cry1Ac, Cry1F, and both Cry1Ac and Cry1F (widestrike®) against beet armyworm and fall armyworm larvae (Lepidoptera: Noctuidae). Florida Entomologist 87: 427-432.

Alford, R. A., and A. Hammond. 1982. Plusiinae (Lepidoptera: Noctuidae) populations in Louisiana soybean ecosystems as determined with looplure-baited traps. Journal of Economic Entomology 75: 647-650.

Aly, M. Z. Y., M. M. M. Soliman, E. E. E. Mohamed, H. F. Dahi, and S. A. R. Salem. 2011. Assessments the toxic effects of entomopathogenic bacterium, Bacillus thuringeinsis subsp. kurstaki, and methomyl insecticide on larval instars of the greater sugarcane borer; Sesamia cretica (Lederer). Egypt Acad. J. Biolog. Sci. 3: 1–9.

Angulo, A. O., and G. T. H. Weigert. 1975. Estados inmaduros de Lepidópteros Noctuidos de importancia económica en Chile y claves para su identificación (Lepidoptera: Noctuidae). Soc. Biol. Concepción: 153.

Bai, C., D. Degheele, S. Jansens, and B. Lambert. 1993. Activity of insecticidal proteins and strains of Bacillus thuringiensis insecticidal proteins and strains of Bacillus thuringiensis against Spodoptera exempta (Walker). J. Invertebr. Pathol 62: 211-215.

Barrionuevo, M. J., M. G. Murúa, L. Goane, R. Meagher, and F. Navarro. 2012. Life table studies of Rachiplusia nu (guenée) and Chrysodeixis (= Pseudoplusia) includens (Walker)(lepidoptera: noctuidae) on artificial diet. Florida Entomologist 95: 944-951.

Bauce, E., N. Carisey, and A. Dupont. 2006. Carry over effects of the entomopathogen Bacillus thuringiensis ssp. Kurstaki on Choristoneura fumiferana (Lepidoptera: Tortricidae) progeny under various stressful environmental conditions. Agricultural and Forest Entomology 8: 63-76.

Bernardi, O., D. Amado, R. S. Sousa, F. Segatti, J. Fatoretto, A. D. Burd, and C. Omoto. 2014. Baseline Susceptibility and Monitoring of Brazilian Populations of Spodoptera frugiperda (Lepidoptera: Noctuidae) and Diatraea saccharalis (Lepidoptera: Crambidae) to Vip3Aa20 Insecticidal Protein. Journal of Economic Entomology 107: 781-790.

Bernardi, O., G. S. Malvestiti, P. M. Dourado, W. S. Oliveira, S. Martinelli, G. U. Berger, G. P. Head, and C. Omoto. 2012. Assessment of the high-dose concept and level of control provided by MON 87701× MON 89788 soybean against Anticarsia gemmatalis and Pseudoplusia includens (Lepidoptera: Noctuidae) in Brazil. Pest management science 68: 1083-1091.

Bravo, A., and M. Soberón. 2008. How to cope with insect resistance to Bt toxins? Trends in biotechnology 26: 573-579.

Bueno, F., R. C. Oliveira, W. S. Parra, and A. J. R. P. Freitas Bueno. 2012. Trichogramma pretiosum parasitism of Pseudoplusia includens and Anticarsia gemmatalis eggs at different temperatures. Biological Control 60: 154-162.

Danks, H. V. 1987. Insect dormancy: an ecological perspective, Biological Survey of Canada (Terrestrial Arthropods).

Eichlin, T. D., and H. B. Cunningham. 1978. The Plusiinae (Lepidoptera: Noctuidae) of America north of Mexico, emphasizing genitalic and larval morphology, Department of Agriculture, Agricultural Research Service.

Eizaguirre, M., S. Tort, C. Lopez, and R. Albajes. 2005. Effects of sublethal concentrations of Bacillus thuringiensis on larval development of Sesamia nonagrioides. Journal of Economic Entomology 98: 464-470.

EMBRAPA, E. B. D. P. A. 2011. Tecnologias de produção de soja–Região Central do Brasil 2012 e 2013. Londrina, Embrapa Soja/Embrapa Cerrados/Embrapa Agropecuária Oeste.

Erb, S. L., R. S. Bourchier, K. Van Frankenhuyzen, and S. M. Smith. 2001. Sublethal effects of Bacillus thuringiensis Berliner subsp. kurstaki on Lymantria dispar (Lepidoptera: Lymantriidae) and the Tachinid parasitoid Compsilura concinnata (Diptera: Tachinidae). . Environ. Entomol. 30: 1174–1181.

Funichello, M., J. F. Grigolli, B. H. S. de Souza, A. L. B. Junior, and A. C. Busoli. 2013. Effect of transgenic and non-transgenic cotton cultivars on the development and survival of Pseudoplusia includens (Walker)(Lepidoptera: Noctuidae). African Journal of Agricultural Research 8: 5424-5428.

Greene, G., N. Leppla, and W. Dickerson. 1976. Velvetbean caterpillar: a rearing procedure and artificial medium. Journal of Economic Entomology 69: 487-488.

Gulzar, A., and D. J. Wright. 2015. Sub-lethal effects of Vip3A toxin on survival, development and fecundity of Heliothis virescens and Plutella xylostella. Ecotoxicology: 1-8.

Herzog, D. C. 1980. Sampling soybean looper on soybean, pp. 141-168, Sampling Methods in Soybean Entomology. Springer.

Huang, F., D. A. Andow, and L. L. Buschman. 2011. Success of the high-dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entomologia Experimentalis et Applicata 140: 1-16.

Jackson, R. E., J. R. Bradley, J. W. Van Duyn, and F. Gould. 2004. Comparative Production of Helicoverpa zea (Lepidoptera: Noctuidae) from Transgenic Cotton Expressing Either One or Two Bacillus thuringiensis Proteins with and without Insecticide Oversprays, vol. 97.

Keller, M., B. Sneh, N. Strizhov, E. Prudovsky, A. Regev, C. Koncz, J. Schell, and A. Zilberstein. 1996. Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of Spodoptera littoralis to CryIC. Insect biochemistry and molecular biology 26: 365-373.

Khalique, F., and K. Ahmed. 2002. Retarding effect of spore-dendotoxin complex of Bacillus thuringiensis (Berliner) strains on the development of Helicoverpa armigera (Hubner). . Pak. J. Biol. Sci. 5: 853–857.

Kranthi, K. R., S. Naidu, C. Dhawad, A. Tatwawadi, K. Mate, E. Patil, A. Bharose, G. Behere, R. Wadaskar, and S. Kranthi. 2005. Temporal and intra-plant variability of Cry1Ac expression in Bt-cotton and its influence on the survival of the cotton bollworm, Helicoverpa armigera (Hubner)(Noctuidae: Lepidoptera). Current Science-Bangalore 89: 291.

Kranthi, S., C. Dhawad, S. Naidu, A. Bharose, A. Chaudhary, V. Sangode, S. Nehare, S. Bajaj, and K. Kranthi. 2009. Susceptibility of the cotton bollworm, Helicoverpa armigera (Hubner)(Lepidoptera: Noctuidae) to the Bacillus thuringiensis toxin Cry2Ab before and after the introduction of Bollgard-II. Crop Protection 28: 371-375.

Ma, G., H. Roberts, M. Sarjan, N. Featherstone, J. Lahnstein, R. Akhurst, and O. Schmidt. 2005. Is the mature endotoxin Cry1Ac from Bacillus thuringiensis inactivated by a coagulation reaction in the gut lumen of resistant Helicoverpa armigera larvae? Insect biochemistry and molecular biology 35: 729-739.

Maia, A. d. H. N., A. J. B. Luiz, and C. Campanhola. 2000. Statistical Inference on Associated Fertility Life Table Parameters Using Jackknife Technique: Computational Aspects, vol. 93.

Mascarenhas, R., D. Boethel, B. Leonard, M. Boyd, and C. Clemens. 1998. Resistance monitoring to Bacillus thuringiensis insecticides for soybean loopers (Lepidoptera: Noctuidae) collected from soybean and transgenic Bt-cotton. Journal of economic entomology 91: 1044-1050.

Miklos, J. A., M. F. Alibhai, S. A. Bledig, D. C. Connor-Ward, A.-G. Gao, B. A. Holmes, K. H. Kolacz, V. T. Kabuye, T. C. MacRae, M. S. Paradise, A. S. Toedebusch, and L. A. Harrison. 2007. Characterization of Soybean Exhibiting High Expression of a Synthetic Transgene That Confers a High Degree of Resistance to Lepidopteran Pests. Crop Science 47: 148-157.

Milks, M. L., I. Burnstyn, and J. H. Myers. 1998. Influence of Larval Age on the Lethal and Sublethal Effects of the Nucleopolyhedrovirus of Trichoplusia niin the Cabbage Looper. Biological Control 12: 119-126.

Morales, L., F. Moscardi, J. Kastelic, D. Sosa-Gomez, F. Paro, and I. Soldorio. 1995. Suscetibilidade de Anticarsia gemmatalis Hübner e Chrysodeixis includens (Walker)(Lepidoptera: Noctuidae), a Bacillus thuringiensis (Berliner). Annais da Sociedade Entomológica do Brasil.

Olsen, K., J. Daly, H. Holt, and E. Finnegan. 2005. Season-long variation in expression of Cry1Ac gene and efficacy of Bacillus thuringiensis toxin in transgenic cotton against Helicoverpa armigera (Lepidoptera: Noctuidae). Journal of Economic Entomology 98: 1007-1017.

Oppert, B., K. J. Kramer, R. W. Beeman, D. Johnson, and W. H. McGaughey. 1997. Proteinase-mediated insect resistance to Bacillus thuringiensis toxins. Journal of Biological Chemistry 272: 23473-23476.

Pawade, V., S. Thakare, A. Thakare, and B. Ghodaki. 2015. In-season variation in cry1ac expression in various plant parts of different BT cotton hybrids in India. International Journal of Environmental Sciences 5: 675.

Radford, P. J. 1967 Growth analysis formulae-their use and abuse. Crop Sci. 7: 171–175.

Rahman, M. M., H. L. Roberts, and O. Schmidt. 2007. Tolerance to Bacillus thuringiensis endotoxin in immune-suppressed larvae of the flour moth Ephestia kuehniella. Journal of invertebrate pathology 96: 125-132.

Rahman, M. M., H. L. Roberts, M. Sarjan, S. Asgari, and O. Schmidt. 2004. Induction and transmission of Bacillus thuringiensis tolerance in the flour moth Ephestia kuehniella. Proceedings of the National academy of Sciences of the United States of America 101: 2696-2699.

Reisig, D. D., and F. P. F. Reay-Jones. 2015. Inhibition of Helicoverpa zea (Lepidoptera: Noctuidae) Growth by Transgenic Corn Expressing Bt Toxins and Development of Resistance to Cry1Ab.

Richetti, A., A. E. Araújo, C. L. Morello, C. A. D. Silva, C. Lazarotto, D. M. P. Azevedo, E. C. Freire, E. M. Arantes, F. M. Lamas, F. S. Ramalho, F. P. Andrade, G. A. Melo Filho, G. B. Ferreira, J. C. F. Santana, J. A. B. Amaral, J. C. Medeiros, J. R. C. Bezerra, P. J.R., K. L. Silva, L. A. Staut, L. C. Silva, L. G. Chitarra, M. A. L. Barros, M. C. S. Carvalho, M. J. Silva e Luz, N. E. M. Beltrão, N. D. Suassuna, O. R. R. F. Silva, P. F. Ferreira, R. F. Santos, and R. G. Fonsêca. 2003. Cultura do Algodão no Cerrado.

Rochester, I. J. 2006. Effect of genotype, edaphic, environmental conditions, and agronomic practices on Cry1Ac protein expression in transgenic cotton. Journal of cotton Science.

Salama, H., and F. Zaki. 1986. Effect of Bacillus thuringiensis Berliner on prepupal and pupal stages of Spodoptera littoralis (Boisd.)(Lepidoptera: Noctuidae). Insect science and its application 7: 747-749.

Sandhu, H. S., G. S. Nuessly, S. E. Webb, R. H. Cherry, and R. A. Gilbert. 2010. Life table studies of Elasmopalpus lignosellus (Lep. Pyralidae) on sugarcane. Environmental Entomology 39: 2025-2032.

SAS. 2001. SAS/STAT User's Guide, v.8. SAS Institute. Cary, NC, USA.

SAS. 2003. A guide to statistical and data analysis, version 9.1. SAS Institute, Cary.

Sedaratian, A., Y. Fathipour, R. Talaei-Hassanloui, and J. Jurat-Fuentes. 2013. Fitness costs of sublethal exposure to Bacillus thuringiensis in Helicoverpa armigera: a carryover study on offspring. Journal of Applied Entomology 137: 540-549.

Siegfried, B. D., A. C. Zoerb, and T. Spencer. 2001. Development of European corn borer larvae on Event 176 Bt corn: influence on survival and fitness. Entomologia Experimentalis et Applicata 100: 15-20.

Sneh, B., S. Schuster, and M. Broza. 1981. Insecticidal activity of Bacillus thuringiensis strains against the egyptian cotton leaf worm Spodoptera littoralis [Lep.: Nocutidae]. Entomophaga 26: 179-190.

Sorgatto, R. J., O. Bernardi, and C. Omoto. 2015. Survival and Development of Spodoptera frugiperda and Chrysodeixis includens (Lepidoptera: Noctuidae) on Bt Cotton and Implications for Resistance Management Strategies in Brazil. Environmental Entomology: nvu018.

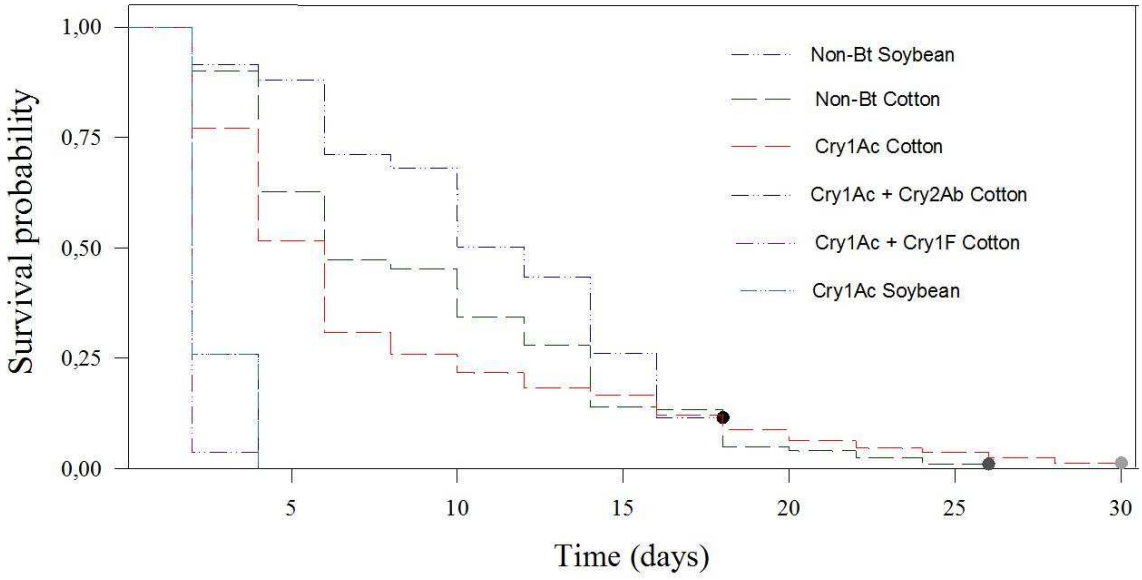
Stark, J. D., and J. E. Banks. 2003. Population-level effects of pesticides and other toxicants on arthropods. Annual review of entomology 48: 505-519.

Stewart, S., J. Adamczyk, K. Knighten, and F. Davis. 2001. Impact of Bt cottons expressing one or two insecticidal proteins of Bacillus thuringiensis Berliner on growth and survival of noctuid (Lepidoptera) larvae. Journal of Economic Entomology 94: 752-760.

Tindall, K., M. W. Siebert, B. Leonard, J. All, and F. Haile. 2009. Efficacy of Cry1Ac: Cry1F proteins in cotton leaf tissue against fall armyworm, beet armyworm, and soybean looper (Lepidoptera: Noctuidae). Journal of economic entomology 102: 1497-1505.

FIGURES AND TABLES

a)



b)

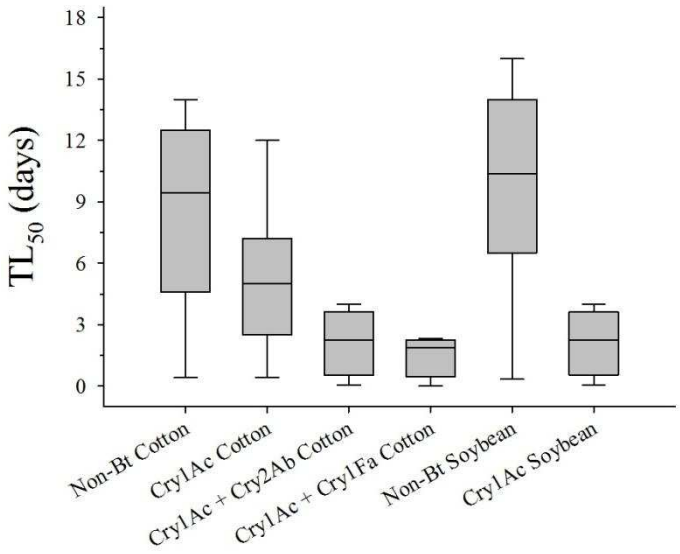


Figure 1. Lethality of single-gene Bt soybean and single- and dual-gene Bt cotton on Chrysodeixis includens larvae. (**a**) Estimated survival curves (product-limit survivor function estimates) and (**b**) LT_{50} ± standard error.

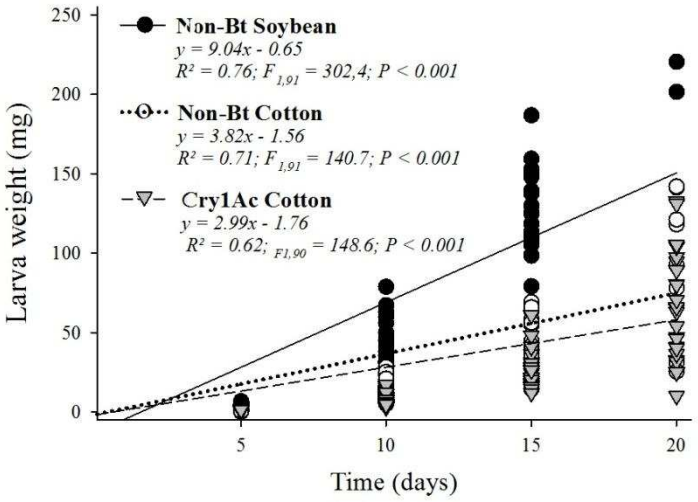


Figure 2. Sublethal effects of Bt Cry1Ac cotton leaves on development of the soybean looper, Chrysodeixis includens. Larval weight gain.

Table 1. Duration of life stages, pupal weight and total fecundity (mean ± SE) of Chrysodeixis includens larvae reared on leaves of different soybean and cotton cultivars.

Treatment	Larva development (± SE) (day)	Fecundity (± SE) (egg/female)	Fertility % (± SE) (neonate/female)
On non-Bt soybean	17.5 ± 0.21 ^{A}	589 ± 174 ^{A}	35 ± 15 ^{A}
On non-Bt cotton	20.5 ± 0.23 ^{B}	662 ± 176 ^{A}	28 ± 10 ^{A}
On Cry1Ac cotton	29.1 ± 0.50 ^{C}	325 ± 115 ^{B}	1 ± 0 ^{B}

Means in the same row followed by same uppercase letters letter in the row do not significantly different at P < 0.05 (Tukey's HSD test, SAS Institute, 2005).

Table 3. The weight of Chrysodeixis includens pupae developed from the larvae that fed on non-Bt soybean, non-Bt cotton and Cry1Ac Cotton leaves.

Treatment	Weight per pupa (mg ± SE[a])			MRGR[b]
	Average	Male	Female	
On non-Bt soybean	146.87 ± 3.1^{Ab}	156.54 ± 5.4 ^{Ab}	135.99 ± 6.3 ^{Bb}	0.42
On non-Bt cotton	162.69 ± 2.8^{Aa}	165.34 ± 5.7 ^{Aab}	160.22 ± 5.5 ^{Aa}	0.36
On Cry1Ac cotton	162.67 ± 4.4^{Aa}	177.71 ± 11.2 ^{Aa}	151.11 ± 11.7 ^{Bab}	0.25

[a]Means in the same row followed by same uppercase letters or the means in the same column followed by the same lowercase letter are not significantly different at P < 0.05 (Tukey's HSD test, SAS Institute, 2005). [b]Mean relative growth rate.

Table 2. Sex ratio of Chrysodeixis includens as affected by developing on Cry1Ac cotton. Chi-square goodness-of-fit to a 1 : 1 (female/male) ratio.

	Observed frequency		Expected frequency				
Treatment	Female	Male	Female	Male	X^2(df = 1)	P	Female/Male
Non-Bt Soybean	44	49	46.5	46.5	0.27	0.604	0.89
Non-Bt Cotton	47	44	45.5	45.5	0.10	0.753	1.07
Cry1Ac cotton	17	13	15	15	0.53	0.465	1.30

Table 3. Estimated life-table parameters (mean ± 95% confidence interval) for the soybean looper, Chrysodeixis includens reared on different events, transgenic and conventional cotton and soybean.

Life-table parameter	Treatment		
	Non-Bt soybean	Non-Bt cotton	Cry1Ac cotton
R_0 (female offspring)	53.99 ± (13.4-94.6) ^{A}	39.50 ± (10.8-68.2) ^{A}	0.31 ± (0.14-0.48) ^{B}
r_m (per day)	0.197 ± (0.16-0.24) ^{A}	0.144 ± (0.11-0.17) ^{B}	-0.027 ± (-0.04-0.01) ^{C}
T (day)	20.2 ± (19.9-20.6) ^{C}	25.5 ± (24.0-27.1) ^{B}	42.5 ± (41.6-43.3) ^{A}

Values followed by same letter within a column are not significantly different according to Student's t-test (P > 0.05). R_o: Net reproductive rate, T: Time interval between generations (days) and r_m: Intrinsic rate of population increase (females/female/day). For each parameter, values followed by the same letter do not differ significantly (P > 0.05) by t-tests using jackknife method to estimate variances using SAS software (SAS Institute, 2011; Maia et al., 2001).

CHAPTER 3 - Resistance to Cry1Ac in the soybean looper, Chrysodeixis includens: laboratory selection, cross-resistance, and heritability

ABSTRACT

High selection pressure with transgenic cotton and soybean producing the Cry1Ac protein from Bacillus thuringiensis in Brazil represents a scenario of high risk of resistance evolution in target insect-pest populations, especially in the soybean looper, Chrysodeixis includens (Lepidoptera: Noctuidae), which is relatively less susceptible to Cry1Ac than other soybean and cotton pests. Hence, to anticipate and characterize resistance that could evolve in field settings, two soybean looper strains obtained from field collections in 2013 were selected for resistance to Cry1Ac in the laboratory. Using chronic exposure to Bt Cry1Ac cotton leaves throughout larval development or exposure to increasing concentrations of purified Cry1Ac on the artificial diet for seven days, resistance levels of 31 or 126-fold were obtained in 12 generations of selection, respectively. Both selected strains also showed resistance to Cry1Ab but not to Cry2Aa and Cry1Fa compared to a control, unselected strain. Realized heritability values were 0.78 and 0.41 for soybean looper strains selected with Bt cotton leaves and purified Cry1Ac toxin, respectively. Interestingly, the Cry1Ac-selected strains became more susceptible to Cry2Aa and Cry1Fa, indicating clear lack cross-resistance between them. The results of this investigation have important implications for resistance management of C. includens, indicating that Cry1 and Cry2 as well as Cry1Ac and Cry1Fa are compatible for use in pyramided Bt events against the soybean looper.

Keywords: soybean, cotton, Bacillus thuringiensis, pyramided Bt events, resistance management.

INTRODUCTION

Transgenic plants producing Bacillus thuringiensis (Bt) proteins have been commercially used since 1996 and in 2014 were planted in an global area of more than 181.5 million hectares (James 2014b), making them an integral component of pest management in many agroecosystems. In Brazil, cotton was the first Bt crop to be commercially released in 2005, followed by maize in 2008, and soybean in 2010 (CTNBio 2015). The Bt soybean event MON 87701 x MON 89788 (Monsanto do Brasil, Ltda) produces the Cry1Ac toxin, which is also expressed in the Bollgard I cotton, event MON 531. The Cry1Ac expressed in Bollgard I cotton is highly effective against a number relatively susceptible lepidopteran pest species (CTNBio 2005b), but additional applications of synthetic insecticides is required to control the soybean looper, Chrysodeixis includens (Lepidoptera: Noctuidae), which is relatively less susceptible to Cry1Ac.

Successive and overlapping cropping systems are common in Brazil, where the warm climate allows year around cultivation and provides conditions and resources for continuous insect development, leading to multiple and overlapping generations a year, a scenario that intensifies pest problems. Large scale and successive cultivation of Bt plants impose a strong selective pressure for Bt resistance in insects-pests (Porta et al. 2011). It has been hypothesized that soybean and cotton events producing the same Bt toxin tend to increase selection pressure for Bt resistance in insect species that colonizes both crops. Among soybean and cotton pest species, C. includens is one of the most important defoliator, given its rapid rise in population density and high frequency of outbreaks in recent years (Bueno et al. 2012a). In addition to its lower susceptibility to Cry1 toxins and Bt formulations (Morales et al. 1995, Mascarenhas et al. 1998a, Bernardi et al. 2012) the Cry1Ac expression level exhibited by Bollgard I cotton is relatively low, allowing survival of some resistant heterozygotes, which can increase the rate of resistance evolution and compromise the durability of Cry1Ac soybean that was recently released.

Resistance evolution is considered the main threat to the sustainable use of Bt plants given their high selection pressure imposed to target pest populations (Onstad 2008a, Tabashnik et al. 2008, Tabashnik et al. 2013a), a phenomenon that can lead to loss of efficacy of these plants for pest control (Tabashnik et al. 2013a). The high potential for insect-pests to develop resistance to Bt toxins is evident in the laboratory (Tabashnik 1994b, Ferré and Van Rie 2002, Pereira et al. 2008a) and field settings (Tabashnik et al. 2013a). Curiously, of the seven cases of field-evolved resistance, three

refer to Cry1Ac cotton (Tabashnik et al. 2008, Dhurua and Gujar 2011a, Zhang et al. 2011b).

To delay resistance evolution, it is recommended to adopt resistance management strategies, such as the high dose/refuge, that is more effective if events producing two or more toxins with different modes of action against target pests are available (Zhao et al. 2003). For proper use of these strategies research is needed to validate some assumptions associated with them, including low frequency of resistance alleles, recessive inheritance of resistance and lack of cross-resistance between Bt proteins used in combination in pyramided events (Tabashnik 1992). In this context, laboratory selection of resistant strains allows us to characterize resistance mechanisms ahead of time, characterize Cry receptors (Jurat-Fuentes et al. 2011), determine the biochemical and physiological basis of resistance (Siqueira et al. 2006, Pereira et al. 2008a) and estimate frequency of resistance alleles in the field (Gould et al. 1997a). Furthermore strains that exhibit high levels of resistance to a toxin can be used especially in validating and improving resistance management strategies (Carrière et al. 2015).

Laboratory selection of Bt resistant insect strains has been achieved using different approaches, including leaves of Bt protein-producing plants, and Bt contaminated diet, as well as other alternatives (Gould et al. 1995, Ferré and Van Rie 2002, Pereira et al. 2008a). However, there is evidence for an effect of different plant tissues with different amounts of secondary compounds as gossypol in cotton on the potency of Cry1Ac (Carrière et al. 2004, Dong and Li 2007, Gahan et al. 2010). Here we report the potential of C. includens to evolve resistance to Cry1Ac toxin under laboratory conditions. Three strains were subjected to different regimes of selection to Cry1Ac toxin: chronic exposure to Cry1Ac-expressing on transgenic cotton Bollgard (MON 531), exposure to increasing concentrations of purified Cry1Ac on the artificial diet for seven days and an unexposed control (Control strain). After selection, we performed bioassays to characterized evidence of cross-resistance and realized heritability. The survival of both C. includens selected strains in Bt-soybean transgenic, event MON 87701 x MON 89788 (Cry1Ac-expressing), were also assessed.

MATERIAL AND METHODS

Insects and plants

Chrysodeixis includens strains were originated from collections of approximately 450-500 larvae in conventional soybean fields located in the Experimental Station of the main campus of the Federal University of Viçosa, Viçosa,

Minas Gerais, Brazil, in January and March of 2013. Another susceptible strain that was used in this study was originated from a collection of approximately 1000 late-instar larvae in bean crops in Coimbra county, Minas Gerais, Brazil, in April 2014. After collection, the larvae were brought to laboratory and reared individually in 16-well plastic trays (Advento do Brasil, Diadema, São Paulo). Larvae were fed daily with soybean leaves, and kept in a rearing room at 27 ± 1°C, 80% relative humidity, and photoperiod of 14 hours light until pupation. Pupae (80♂ + 80♀) were placed in cages made of polyvinylchloride (PVC), 20 cm diameter × 30 cm height, lined internally with sulfite paper as substrate for oviposition. Adults were fed daily with 10% honey solution in water, and eggs were collected daily and stored in an incubator until hatching. Neonates were transferred to trays with artificial diet as in Greene et al. (1976a) with slight modifications and kept in the rearing room. In the F_1 generation, a portion of the larvae was used for selection with purified Cry1Ac, a portion was exposed to MON 531 cotton leaf tissue, and third portion was used for mass rearing of the colony using methods described above without insecticide exposure and maintaining population size around 200 adults per generation.

Transgenic Bollgard I cotton (event MON 531, Monsanto do Brasil, São Paulo, SP), the transgenic soybean (event MON 87701 x MON 89788, Monsanto do Brasil, São Paulo, SP) and its non-transgenic cotton isoline (Delta OPAL, Monsanto do Brasil, São Paulo, SP) and the soybean isoline (MSOY8866, Monsanto do Brasil, São Paulo, SP) were grown in the greenhouse. Seeds were sown every three months in 15-L pots with substrate composed of 3 parts of soil, 2 parts cattle manure, and 2 parts of sand to produce plants with normal levels of Bt protein expression (Olsen et al. 2005), which was qualitatively checked with immunoassay test strips (AgraStrip, Union, MO, EUA). Plants were irrigated two to three times a day depending on soil moisture conditions, and leaves were collected from cotton plants approximately 45-50 days after germination. Soybean leaves were used when plants reached R2-R4 phenological stages. Soil fertilization was carried out according to recommendations for the cotton (Richetti et al. 2003) and soybean crops (EMBRAPA 2011). Plants were daily inspected to prevent arthropod infestation, and whenever needed, mechanical pest control was used with no application of pesticides.

Selection using cotton leaf tissue

Chronic selection experiments using cotton leaves started in the first generation after establishment of the field-collected colony in the laboratory, in 2013. In each generation of selection, approximately 1600 neonates were separated in batches of 10 and

transferred to each well of a 16 well-plastic tray (Advento do Brasil, Diadema, São Paulo), containing a section of cotton leaf. After three days, surviving larvae were transferred to new 16-well trays leaving 2-3 larvae per well, depending on size. The larvae were fed cotton leaves every three days until pupation and then collected, sexed, and transferred to mating PVC cages. Adults were held as described previously. The selection experiment took place during twelve generations. A control strain was reared in parallel in conventional, non-Bt cotton leaves to estimate natural mortality in the absence of selection. In each round of selection, the selection gain was estimated using bioassays with purified Cry1Ac protein as described in the following text.

Selection using purified Cry1Ac

This experiment was initiated in parallel with the selection using plant leaf-tissue. We used methods adapted from Gould et al. (1995) and Pereira et al. (2008a). We carried out initial bioassays to determine the strain susceptibility to Cry1Ac and used the same protein source in the selection. Bioassays were done using graded Cry1Ac concentrations applied on the surface of artificial diet (Marçon et al. 1999a). Larval mortality was recorded after seven days of exposure and analyzed by probit regression to determine lethal concentration to kill 90% of the larvae (LC_{90}), which was used as the starting concentration applied for selection. Toxin concentration was increased each generation of selection to obtain the LC_{90} value to be used in the next round of selection (Table 1). However, as neonates were exposed for only seven days, only larvae with sizes similar to those of the control were used to start the next generation. This procedure was repeated in each generation of selection. At least 2500 neonates were transferred to the untreated artificial diet until pupation, and adults were held in mating cages as previously described. The gain by selection was estimated by assaying a portion of the larvae from the parents that were selected in the previous generation.

Bioassays

Bioassays were conducted in duplicate on two dates and included at least seven different graded concentrations of purified Cry1Ac plus a control (0.1% Triton-X 100 only, applied to the diet surface) (Marçon et al. 1999a). The Cry1Ac protein used was obtained from Dr. Marianne P. Carey (Case Western Reserve University, OH). The protein was activated with trypsin, purified on HPLC, shipped as lyophilized powder and stored at -80 ^{o}C. The toxicity of the Cry1Ac stock used in our bioassays is similar to that reported elsewhere (Litchefield and Wilcoxon 1949). A single neonate (< 24 hours after hatching) was placed in each well of a 128 well-tray (CD International, Pitman,

NJ), and held for seven days at 2727 ± 1 ºC, 24 h photophase, and 80% R.H. until larval mortality and growth inhibition was evaluated (Marçon et al. 1999a, Pereira et al. 2008a). Larval weight of survivors was also determined to obtain the percentage of inhibition of growth inhibition relative to control larvae (Marçon et al. 1999a).

Leaf tissue assays

Cry1Ac-expressing (MON 87701 x MON 89788) soybean plants and near isoline plants leaf tissue squares (2 cm^2) were cut from individual leaves and placed in individual cells of a 16 well-plastic tray (Advento do Brasil, Diadema, São Paulo). Approximately 400 neonates were separated in batches of 10 and transferred to each well containing a section of soybean leaf and keeping under laboratory conditions, as describe previously. After three days, the assays were evaluated for surviving larvae. The assays were replicated with 20 leaf squares from different plants and both the selected strains on Cry1Ac cotton and Cry1Ac on diet and an unselected strain control were evaluated on Cry1Ac-expressing and non-Bt plant tissue.

Cross-resistance bioassays

Cross-resistance bioassays were conducted with Cry1Ab, Cry1Fa, and Cry2Aa using the methods described above. All insecticidal proteins used in this bioassay were also obtained from Dr. Marianne P. Carey (Case Western Reserve University, OH). These proteins were activated with trypsin, purified on HPLC, shipped as lyophilized powder, and stored at -80 ^oC. Due of low population levels of the strain selected with Cry1Ac toxin, it was not possible to perform Cry1Fa bioassays with larvae of this strain when these experiments were conducted.

Realized of heritability

Calculation of realized heritability (h^2) was done according to Falconer (1989) and Tabashnik (1992) as: h^2 = Selection response – Selection differential. In this equation, selection response (R) was calculated as: R = (Log final LC_{50} – Log initial LC_{50})/N. Here, the final LC_{50} = LC_{50} of population after 8 and 7 generations of selection with event MON 531 cotton and Cry1Ac toxin, respectively. Initial LC_{50} = LC_{50} of parental population before selection. In our case one single base population of C. includens was submitted to two different regimes of selection, and for this reason the same value of Initial LC_{50} was used for both strains selected. N = number of generations selected with Bollgard I cotton or Cry1Ac toxin.

Selection differential was calculated as follows: Selection differential = i x σp, where: I = intensity of selection calculated according to Falconer (1989); σp =

phenotypic standard deviation calculated as follows: σp = [(initial slope + final slope) x 0,5]$^{-1}$. Based on the response of both C. includens strains selected in the laboratory, the number of generations required for a tenfold increase in LC_{50} (G) was calculated as: G = 1/R.

Statistical analysis

The bioassay data were submitted to probit analysis using Polo-Plus software (Robertson et al., 2007) to estimate the LC_{50} and EC_{50} values, line slopes and their standard errors, as well as confidence intervals and lethal concentration ratios. Mortality was adjusted relative to controls when necessary. Lethal concentration ratios were considered significantly different ($P < 0.05$) when they do not include the value one.

Regression analysis was used to study the relationship between the gain in selection in the parental strain and offspring-parent. Statistical significance was declared when $P < 0.05$. The heritability data were subjected to regression analysis PROC REG, SAS Institute (2008).

However, data from leaf tissue assays were analyzed with a two-way analysis of variance

(ANOVA) using the SAS Institute (2002). The two main factors were C. includens strains and plant material (Bt soybean or non-Bt soybean). Treatment means were separated using Tukey tests with $P<0.05$. Before proceding with the statistical analysis, the survival data were transformed to sqrt(x+1). After transformation, the data were considered to be normally distributed and transformed data were used in the subsequent statistical analysis. Statistical calculations were performed using SAS Institute (2001).

RESULTS

Selection of resistant strains

Estimates of survival rates over the generations of selection (Figure 1) indicate that C. includens larvae showed a significant increase in tolerance to Cry1Ac when exposed to MON 531 cotton leaf tissues throughout larval development. However, from the sixth generation afterwards, no significant gain in tolerance to Cry1Ac was obtained, as indicated by similar survival rates for selected and control insects (Figure 1A). Selection with Cry1Ac overlaid on the surface of the diet resulted in higher gains of resistance in each round of selection (Figure 2). This regime allowed selection with higher Cry1Ac concentrations imposing a greater intensity of selection (Figure 1B). With only two generations of selection using purified Cry1Ac (Figure 2), we achieved an increase in the LC_{50} values similar to that achieved in six generations of selection using MON 531 cotton leaves (Figure 1). Offspring-parent regression for Cry1Ac

susceptibility (i.e., relationship between LC_{50} values for parents and offspring) indicated that the strain of C. includens selected with purified toxin showed greater selection gain for resistance relative to the strain exposed in Cry1Ac cotton (Figure 2).

Level of resistance

Bioassays using purified Cry1Ac toxin revealed that both strains selected under different regimes developed significant levels of resistance after 12 generations of selection (Table 2). A lower resistance level was obtained for the strain selected with Cry1Ac cotton leaves relative to the strain selected on Cry1Ac toxin (Table 2). The resistance ratio obtained for the strain selected with Cry1Ac cotton leaves were respectively 31 and 20 times greater than LC_{50} and EC_{50} values for the control (Table 2). The strain selected on Cry1Ac toxin showed resistance levels of 127 and 22 fold based on LC_{50} and EC_{50} values, respectively (Table 2).

Leaf tissue assays

Neonate larvae from the Cry1Ac cotton and Cry1Ac on diet selected strains exhibited significantly lower rates of survival on Cry1Ac-expressing in soybean leaves tissue (Figure 2) relative to the non-Bt soybean (control). Significant survival effects on soybean tissue ($F_{1,110} = 13545.8$, $P<0.001$) was detected, but not significant effects on survival between selected and unselected strains ($F_{2,109} = 0.01$, $P = 0.9884$) was found. There was not significant interaction between selected strains and survival on soybean tissue ($F_{2,110} = 1.373$, $P = 0.315$) was found.

The level of surviving by the Cry1Ac cotton and Cry1Ac on diet selected strains on the Cry1Ac-expressing leaves tissues was not significantly different from unselected strain survival. The Cry1Ac-expressing leaf tissue exhibited a higher efficiency control when infested with the both selected strains and unselected strain, and all insects from the selected and unselected strains were dead after 3 days. Surving to isoline plant tissue was similar for both selected strains, as well as unselected control.

Level of cross-resistance

Both selection regimes yielded strains showing increases of 7-9 fold in the Cry1Ab LC_{50} values, indicating that Cry1Ab has cross-resistance to Cry1Ac in C. includens (Table 3). In contrast, reduction in Cry2Aa and Cry1Fa LC_{50} values of 4 and 5 fold were respectively observed for larvae selected with Bt cotton leaves; likewise, a 6-fold decrease in Cry2Aa LC_{50} value was observed in the strain selected with Cry1Ac toxin. These results indicate negative cross-resistance between Cry1Ac and Cry2Aa or Cry1Fa in C. includens (Table 3).

Realized heritability

After 8 generations of selection with Bt cotton event MON 531, Cry1Ac LC_{50} values increased from 16 to 503 ng/cm^2, and the slope of the probit line increased from 1.84 to 2.34, while after 7 generations of selection with Cry1Ac, toxin LC_{50} values increased from 16 to 2048 ng/cm^2 and the slope increased from 1.84 to 2.11 (Table 4). Realized heritability (h^2) estimates were 0.41 and 0.78 for the selected strains (Table 4). The number of generations needed for a 10-fold increase in LC_{50} values of strain selected with Cry1Ac cotton and Cry1Ac toxin were estimated to be approximately 3 and 6 generations (Table 4).

DISCUSSION

Results with the Cry1Ac cotton-selected and Cry1Ac on diet-selected strains suggested studies laboratory selection using purified Cry toxin (Gould et al. 1995, Pereira et al. 2008a, Gong et al. 2010) and Cry toxin expressing plants leaves tissue (Meihls et al. 2008, Girón-Pérez et al. 2014) shown similar efficiency to achievement resistant strains. Although we only have one strain per treatment, our data suggest that chronic exposure to Cry1Ac cotton leaves produced 31-fold resistance after 12 generations of selection while continuous selection pressure with purified Cry1Ac led to 126-fold resistance with only seven generations of selection. These results suggest that evolution of resistance to Cry1Ac in C. includens is associated with the intensity of the selection (Falconer, 1989) exerted by the toxin. However, the selection with purified toxin provided a response to selection not different ($P > 0.05$) than using Bt Cry1Ac cotton leaves (Figure 2), probably because of the experimental error associated with bioassays (Robertson et al. 1995) although selection with increasing doses of toxin seemed advantageous because of the lack of secondary metabolites or other compounds that would confound the response to selection when using Bt plants. Variation in the levels of Cry1Ac protein synthesis in cotton plants was previously reported (Olsen et al. 2005, Dong and Li 2007, Sivasupramaniam et al. 2008), but here care was taken to grow the plants and select young terminal leaves to minimize such variation. Most important, this is the first report of selection of Bt resistant strains of C. includens. Despite the Cry1Ac on diet-selected strains showed levels superior to 126-fold resistance, this selected strain not exhibited the ability to feed and survive on Cry1Ac-expressing soybean plant tissue (Figure 3).

Selection for Cry1Ac resistance resulted in different levels of cross-resistance to other Cry toxins in both selected strains. The highest level of cross-resistance was obtained for Cry1Ab, which may be associated with altered receptor binding sites in midgut wells (Gahan et al. 2001) and similarity of amino acid sequences of domains II

and III of toxins (Carrière et al. 2015). Competition binding assays have shown that Cry1Ac, Cry1Ab and Cry1Fa share binding sites in H. virescens (Jurat-Fuentes and Adang 2001), as well as Ostrinia nubilalis and S. frugiperda (Hernández-Rodríguez et al. 2013). Furthermore, comparisons between Cry1Ac and Cry1Ab revealed that both proteins share 99 and 51% similarity of amino acid sequences of domains II and III, respectively (Carrière et al. 2015). In a study with O. furnacalis, a Cry1Ab-selected strain also exhibited high levels of cross-resistance to Cry1Ac (Zhang et al. 2014). All these published studies corroborate with this present study, indicating that Cry1Ab and Cry1Ac probably share binding sites in receptor proteins of C. includens.

The lack of cross-resistance between Cry1Ac and Cry1Fa or Cry2Aa was evident in both selected strains, indicating that this phenomenon occur independently of the resistance level. These cross-resistance patterns suggest that the increase of resistance to Cry1Ac may negatively correlated with increase of cross-resistance to Cry1Fa or Cry2Aa toxin in C. includens (Table 3). Selection experiments for Cry1Ac resistance have been conducted in H. virescens (Gould et al. 1995, Jurat-Fuentes et al. 2002) and P. gossypiella (Tabashnik et al. 2000); in common, they reported high cross resistance to Cry1Ab, Cry1Aa and Cry1Fa, as well as moderate cross-resistant to Cry2Aa. These earlier findings contrast with those obtained with the strains here selected in which they became more susceptible to Cry1Fa and Cry2Aa. The lack of cross-resistance to Cry1Fa and Cry2Aa suggests that these toxins do not share binding sites in receptor proteins (Lee et al. 1995) and is especially relevant because Cry2A and Cry1F toxins are pyramided with Cry1Ac in second generation Bt cotton. Most importantly, these results indicate that Cry2A and Cry1F could be pyramided with Cry1Ac in second generation Bt soybean for resistance management of C. includens.

Realized heritability (h^2) is considered a tool for resistance risk assessment, an attempt to predict the rate at which a pest will evolve resistance as a function of toxin use (Chambers et al. 1991, Tabashnik 1992, 1994a). The h^2 is the amount of phenotypic variation and additive genetic variation (Falconer 1989, Abbas et al. 2014a). Phenotypic variation in laboratory may come from gene mutation and selection pressure, but in the field this variation may come also from another factors, such as migration, varied insecticide use, and environmental factors (Abbas et al. 2014a). The h^2 values for Cry1Ac resistance estimated here were quite high (0.41 and 0.78) and indicates that C. includens have high potential to develop resistance to Cry1Ac. Such high values of heritability means that most of phenotypic variation for Cry1Ac resistance in C. includens is due to genetic variation (Tabashnik 1992). Our data clearly shows that high

selection intensity with high concentration of Cry1Ac used C. includens strain selected increased significantly the LC_{50} value of generation 7 compared with LC_{50} at generation 1 (Table 4). Additionally, the short time for 10-fold increase in resistance, higher value of h^2, even as the high LC_{50} value indicate that gain selection with Cry1Ac toxin selection is superior to cotton leaf selection (Table 4).

In this study, we explored the potential of C. includens to evolve resistance to plant-produced Bt-toxins from the first generation of Cry1Ac Cotton (MON 153) and Cry1Ac toxin. Although the intensity and pattern of selection may be similar in the field and the laboratory (Groeters and Tabashnik 2000), it is still not clear that laboratory selection can predict exactly what mechanisms of Bt resistance will appear in the field (Ffrench-Constant 2013). However, laboratory selection programs have often produced levels and mechanisms (e.g. increased metabolism) of resistance qualitatively similar to those of field strains (Roush and McKenzie 1987). Our results indicate that pre-existing Cry1Ac toxin resistance alleles could be common in C. includens, but the risk of this specie evolve in the field should be more studied. Evidence (including laboratory, greenhouse selection data) indicates that the selection study was efficient in predict the risk of resistance evolution in field in species as Spodoptera frugiperda in Brazil (Farias et al. 2014, Leite et al. 2015), Diabrotica virgifera virgifera in US (Meihls et al. 2008, Gassmann et al. 2011, Meihls et al. 2011, Oswald et al. 2011, Gassmann 2012). Laboratory-selected resistant strains are not confined to studies of resistance but also relate to elucidating different steps in the mode of action of Bt, estimating levels of field resistance and likely the fitness of resistance genes, critical to the successful establishment of a resistance management strategy (Devos et al. 2013).

Taken together, our results suggested that both selected C. includens strains showed increase tolerance and cross-resistance to Cry1Ab. Our biggest surprise was the negative association observed between increased tolerances to Cry1Ac and decreases in the susceptibility to Cry2Aa and Cry1Fa. These data suggest that the increase of tolerance to Cry1Ac may induce negative cross-resistance to Cry2Aa and Cry1Fa. Furthermore, this phenomenon has implications for resistance management in C. includens, especially with regard to the use of pyramided events. However, estimated h^2 values give indication that C. includens have a potential to develop higher levels of resistance than reported in this study. The selection of strains for high degree of resistance with purified toxin has provided material for the study of resistance mechanisms in several species of Lepidoptera (Siqueira et al. 2006, Pereira et al. 2008a), the selection of strains of C. includens is an opportunity to broaden the

knowledge of these mechanisms. Although, the laboratory conditions are different from those found in the field, the laboratory results allow the study of resistance mechanisms of biochemical and molecular nature, the extrapolation of forecast mathematical models, estimating resistance allele frequencies in field and the fitness costs of resistance genes, that is a critical component to the successful establishment of a resistance management strategy.

REFERENCES

Abbas, N., H. A. A. Khan, and S. A. Shad. 2014. Resistance of the house fly Musca domestica (Diptera: Muscidae) to lambda-cyhalothrin: mode of inheritance, realized heritability, and cross-resistance to other insecticides. Ecotoxicology 23: 791-801.

Bernardi, O., G. S. Malvestiti, P. M. Dourado, W. S. Oliveira, S. Martinelli, G. U. Berger, G. P. Head, and C. Omoto. 2012. Assessment of the high-dose concept and level of control provided by MON 87701× MON 89788 soybean against Anticarsia gemmatalis and Pseudoplusia includens (Lepidoptera: Noctuidae) in Brazil. Pest management science 68: 1083-1091.

Bueno, F., R. C. Oliveira, W. S. Parra, and A. J. R. P. Freitas Bueno. 2012. Trichogramma pretiosum parasitism of Pseudoplusia includens and Anticarsia gemmatalis eggs at different temperatures. Biological Control 60: 154-162.

Carrière, Y., N. Crickmore, and B. E. Tabashnik. 2015. Optimizing pyramided transgenic Bt crops for sustainable pest management. Nature biotechnology 33: 161-168.

Carrière, Y., C. Ellers-Kirk, R. Biggs, D. M. Higginson, T. J. Dennehy, and B. E. Tabashnik. 2004. Effects of gossypol on fitness costs associated with resistance to Bt cotton in pink bollworm. Journal of Economic Entomology 97: 1710-1718.

Chambers, J. A., A. Jelen, M. P. Gilbert, C. S. Jany, T. B. Johnson, and C. Gawron-Burke. 1991. Isolation and characterization of a novel insecticidal crystal protein gene from Bacillus thuringiensis subsp. aizawai. Journal of bacteriology 173: 3966-3976.

CTNBio. 2005. - Comissão Técnica Nacional de Biossegurança. Liberação comercial de Parecer Técnico Prévio Conclusivo Nº 513/2005. (http://www.ctnbio.gov.br/index.php/content/view/12526.html).

CTNBio. 2015. - Comissão Técnica Nacional de Biossegurança. Resumo Geral de Plantas Geneticamente modificadas aprovadas para Comercialização.

Devos, Y., L. N. Meihls, J. Kiss, and B. E. Hibbard. 2013. Resistance evolution to the first generation of genetically modified Diabrotica-active Bt-maize events by western corn rootworm: management and monitoring considerations. Transgenic research 22: 269-299.

Dhurua, S., and G. T. Gujar. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, Pectinophora gossypiella (Saunders)(Lepidoptera: Gelechiidae), from India. Pest management science 67: 898-903.

Dong, H., and W. Li. 2007. Variability of endotoxin expression in Bt transgenic cotton. Journal of Agronomy and Crop Science 193: 21-29.

EMBRAPA, E. B. D. P. A. 2011. Tecnologias de produção de soja–Região Central do Brasil 2012 e 2013. Londrina, Embrapa Soja/Embrapa Cerrados/Embrapa Agropecuária Oeste.

Falconer, D. S. 1989. Introduction to quantitative genetics, Longman, London.

Farias, J. R., D. A. Andow, R. J. Horikoshi, R. J. Sorgatto, P. Fresia, A. C. dos Santos, and C. Omoto. 2014. Field-evolved resistance to Cry1F maize by Spodoptera frugiperda (Lepidoptera: Noctuidae) in Brazil. Crop protection 64: 150-158.

Ferré, J., and J. Van Rie. 2002. Biochemistry and Genetics of Insect Resistance to Bacillus thuringiensis. Annual review of entomology 47: 501-533.

Ffrench-Constant, R. H. 2013. The molecular genetics of insecticide resistance. Genetics 194: 807.

Gahan, L. J., F. Gould, and D. G. Heckel. 2001. Identification of a gene associated with Bt resistance in Heliothis virescens. Science 293: 857-860.

Gahan, L. J., Y. Pauchet, H. Vogel, and D. G. Heckel. 2010. An ABC transporter mutation is correlated with insect resistance to Bacillus thuringiensis Cry1Ac toxin.

Gassmann, A. J. 2012. Field-evolved resistance to Bt maize by western corn rootworm: predictions from the laboratory and effects in the field. Journal of invertebrate pathology 110: 287-293.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLoS ONE 6: e22629.

Girón-Pérez, K., A. Oliveira, A. Teixeira, R. Guedes, and E. Pereira. 2014. Susceptibility of Brazilian populations of Diatraea saccharalis to Cry1Ab and response to selection for resistance. Crop Protection 62: 124-128.

Gong, Y., C. Wang, Y. Yang, S. Wu, and Y. Wu. 2010. Characterization of resistance to Bacillus thuringiensis toxin Cry1Ac in Plutella xylostella from China. Journal of invertebrate pathology 104: 90-96.

Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar. 1995. Selection and genetic analysis of a Heliothis virescens (Lepidoptera: Noctuidae) strain with high levels of resistance to Bacillus thuringiensis toxins. Journal of Economic Entomology 88: 1545 - 1559.

Gould, F., A. Anderson, A. Jones, D. Sumerford, D. Heckel, J. Lopez, S. Micinski, R. Leonard, and M. Laster. 1997. Initial frequency of alleles for resistance to Bacillus thuringiensis toxins in field populations of Heliothis virescens. Proceedings of the National Academy of Sciences 94: 3519-3523.

Greene, G., N. Leppla, and W. Dickerson. 1976. Velvetbean caterpillar: a rearing procedure and artificial medium. Journal of Economic Entomology 69: 487-488.

Groeters, F. R., and B. E. Tabashnik. 2000. Roles of selection intensity, major genes, and minor genes in evolution of insecticide resistance. Journal of economic entomology 93: 1580-1587.

Hernández-Rodríguez, C. S., P. Hernández-Martínez, J. Van Rie, B. Escriche, and J. Ferré. 2013. Shared midgut binding sites for Cry1A. 105, Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa proteins from Bacillus thuringiensis in two important corn pests, Ostrinia nubilalis and Spodoptera frugiperda. PloS one 8: e68164.

Institute, S. 2008. SAS/STAT User's Guide. SAS Institute, Cary, NC, USA. computer program, version By Institute, S.

James, C. 2014. Global Status of Commercialized Biotech/GM Crops: 2014., ISAAA Brief Ithaca, NY.

Jurat-Fuentes, J. L., and M. J. Adang. 2001. Importance of Cry1 δ-endotoxin domain II loops for binding specificity in Heliothis virescens (L.). Applied and environmental microbiology 67: 323-329.

Jurat-Fuentes, J. L., F. L. Gould, and M. J. Adang. 2002. Altered glycosylation of 63-and 68-kilodalton microvillar proteins in Heliothis virescens correlates with reduced Cry1 toxin binding, decreased pore formation, and increased resistance to Bacillus thuringiensis Cry1 toxins. Applied and Environmental Microbiology 68: 5711-5717.

Jurat-Fuentes, J. L., L. Karumbaiah, S. R. K. Jakka, C. Ning, C. Liu, K. Wu, J. Jackson, F. Gould, C. Blanco, and M. Portilla. 2011. Reduced levels of membrane-bound alkaline phosphatase are common to lepidopteran strains resistant to Cry toxins from Bacillus thuringiensis. PLoS One 6: e17606.

Lee, M. K., F. Rajamohan, F. Gould, and D. Dean. 1995. Resistance to Bacillus thuringiensis CryIA delta-endotoxins in a laboratory-selected Heliothis virescens strain is related to receptor alteration. Applied and environmental microbiology 61: 3836-3842.

Leite, N. A., S. M. Mendes, O. F. Santos-Amaya, C. A. Santos, T. P. M. Teixeira, R. N. C. Guedes, and E. J. G. Pereira. 2015. Rapid selection for Cry1F resistance in a Brazilian strain of fall armyworm and its characterization. Entomologia Experimentalis et Applicata.

Litchefield, J. a., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. Journal of pharmacology and experimental therapeutics 96: 99-113.

Marçon, P. C., L. J. Young, K. L. Steffey, and B. D. Siegfried. 1999. Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to Bacillus thuringiensis toxins. Journal of Economic Entomology 92: 279-285.

Mascarenhas, R., D. Boethel, B. Leonard, M. Boyd, and C. Clemens. 1998. Resistance monitoring to Bacillus thuringiensis insecticides for soybean loopers (Lepidoptera: Noctuidae) collected from soybean and transgenic Bt-cotton. Journal of economic entomology 91: 1044-1050.

Meihls, L. N., M. L. Higdon, M. Ellersieck, and B. E. Hibbard. 2011. Selection for resistance to mCry3A-expressing transgenic corn in western corn rootworm. Journal of economic entomology 104: 1045-1054.

Meihls, L. N., M. L. Higdon, B. D. Siegfried, N. J. Miller, T. W. Sappington, M. R. Ellersieck, T. A. Spencer, and B. E. Hibbard. 2008. Increased survival of western corn rootworm on transgenic corn within three generations of on-plant greenhouse selection. Proceedings of the National Academy of Sciences 105: 19177-19182.

Morales, L., F. Moscardi, J. Kastelic, D. Sosa-Gomez, F. Paro, and I. Soldorio. 1995. Suscetibilidade de Anticarsia gemmatalis Hübner e Chrysodeixis includens (Walker)(Lepidoptera: Noctuidae), a Bacillus thuringiensis (Berliner). Annais da Sociedade Entomológica do Brasil.

Olsen, K., J. Daly, H. Holt, and E. Finnegan. 2005. Season-long variation in expression of Cry1Ac gene and efficacy of Bacillus thuringiensis toxin in transgenic cotton against Helicoverpa armigera (Lepidoptera: Noctuidae). Journal of Economic Entomology 98: 1007-1017.

Onstad, D. 2008. Insect Resistance Management: Biology, Economics, and Prediction, Academic Press, London.

Oswald, K. J., B. W. French, C. Nielson, and M. Bagley. 2011. Selection for Cry3Bb1 resistance in a genetically diverse population of nondiapausing western corn rootworm (Coleoptera: Chrysomelidae). Journal of Economic Entomology 104: 1038-1044.

Pereira, E. J., B. A. Lang, N. P. Storer, and B. D. Siegfried. 2008. Selection for Cry1F resistance in the European corn borer and cross-resistance to other Cry toxins. Entomologia experimentalis et applicata 126: 115-121.

Porta, H., G. Jiménez, E. Cordoba, P. León, M. Soberón, and A. Bravo. 2011. Tobacco plants expressing the Cry1AbMod toxin suppress tolerance to Cry1Ab toxin of Manduca sexta cadherin-silenced larvae. Insect Biochemistry and Molecular Biology 41: 513-519.

Richetti, A., A. E. Araújo, C. L. Morello, C. A. D. Silva, C. Lazarotto, D. M. P. Azevedo, E. C. Freire, E. M. Arantes, F. M. Lamas, F. S. Ramalho, F. P. Andrade, G. A. Melo Filho, G. B. Ferreira, J. C. F. Santana, J. A. B. Amaral, J. C. Medeiros, J. R. C. Bezerra, P. J.R., K. L. Silva, L. A. Staut, L. C. Silva, L. G. Chitarra, M. A. L. Barros, M. C. S. Carvalho, M. J. Silva e Luz, N. E. M. Beltrão, N. D. Suassuna, O. R. R. F. Silva, P. F. Ferreira, R. F. Santos, and R. G. Fonsêca. 2003. Cultura do Algodão no Cerrado.

Robertson, J., H. Preisler, S. Ng, L. A. Hickle, and W. Gelernter. 1995. Natural variation: a complicating factor in bioassays with chemical and microbial pesticides. Journal of Economic Entomology 88: 1-10.

Robertson, J. L., N. Savin, H. K. Preisler, and R. M. Russell. 2007. Bioassays with arthropods, CRC press.

Roush, R. T., and J. A. McKenzie. 1987. Ecological genetics of insecticide and acaricide resistance. Annual review of entomology 32: 361-380.

Siqueira, H. A., J. González-Cabrera, J. Ferré, R. Flannagan, and B. D. Siegfried. 2006. Analyses of Cry1Ab binding in resistant and susceptible strains of the European corn borer, Ostrinia nubilalis (Hübner)(Lepidoptera: Crambidae). Appl Environ Microbiol 72: 5318-5324.

Sivasupramaniam, S., W. Moar, L. Ruschke, J. Osborn, C. Jiang, J. Sebaugh, G. Brown, Z. Shappley, M. E. Oppenhuizen, and J. Mullins. 2008. Toxicity and characterization of cotton expressing Bacillus thuringiensis Cry1Ac and Cry2Ab2 proteins for control of lepidopteran pests. Journal of Economic Entomology 101: 546-554.

Tabashnik, B. E. 1992. Resistance risk assessment: realized heritability of resistance to Bacillus thuringiensis in diamondback moth (Lepidoptera: Plutellidae), tobacco budworm (Lepidoptera: Noctuidae), and Colorado potato beetle (Coleoptera: Chrysomelidae). Journal of Economic Entomology 85: 1551-1559.

Tabashnik, B. E. 1994a. Evolution of resistance to Bacillus thuringiensis. Annual Review of Entomology 39: 47 - 79.

Tabashnik, B. E. 1994b. Evolution of resistance to Bacillus thuringiensis. Annual review of entomology 39: 47-79.

Tabashnik, B. E., T. Brévault, and Y. Carrière. 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nature Biotechnology 31: 510 - 521.

Tabashnik, B. E., Y.-B. Liu, R. A. de Maagd, and T. J. Dennehy. 2000. Cross-resistance of pink bollworm (Pectinophora gossypiella) to Bacillus thuringiensis toxins. Applied and environmental microbiology 66: 4582-4584.

Tabashnik, B. E., A. J. Gassmann, D. W. Crowder, and Y. Carrière. 2008. Insect resistance to Bt crops: evidence versus theory. Nature Biotechnology 26: 199-202.

Zhang, H., W. Yin, J. Zhao, L. Jin, Y. Yang, S. Wu, B. E. Tabashnik, and Y. Wu. 2011. Early warning of cotton bollworm resistance associated with intensive planting of Bt cotton in China. PLoS ONE 6: 1 - 8.

Zhang, T., M. He, A. M. Gatehouse, Z. Wang, M. G. Edwards, Q. Li, and K. He. 2014. Inheritance patterns, dominance and cross-resistance of Cry1Ab-and Cry1Ac-selected Ostrinia furnacalis (Guenée). Toxins 6: 2694-2707.

Zhao, J. Z., J. Cao, Y. X. Li, H. L. Collins, R. T. Roush, E. Earle, D., and A. M. Shelton. 2003. Transgenic plants expressing two Bacillus thuringiensis toxins delay insect resistance evolution. Nature Biotechnology 21: 1493 - 1497.

FIGURES AND TABLES

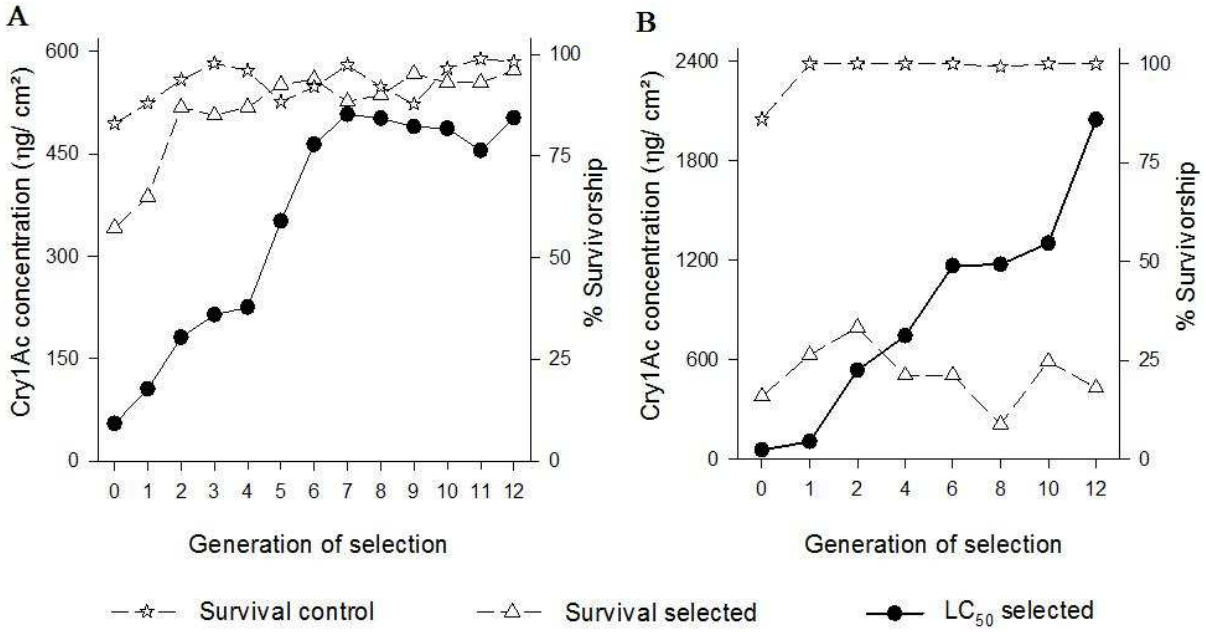


Figure 1. Response to selection for Cry1Ac resistance in Chrysodeixis includens subjected to two regimes of artificial selection. (A) Chronic exposure to Cry1Ac cotton leaves showing the increase in the median lethal concentration (LC_{50}) for selected larvae and survival rates assessed at the end of larval stage for the selected and control strains. (B) Exposure to Cry1Ac overlaid on the surface of the diet for seven days showing the increase in the median lethal concentration (LC_{50}) for selected larvae and survival rates for the selected and control strains.

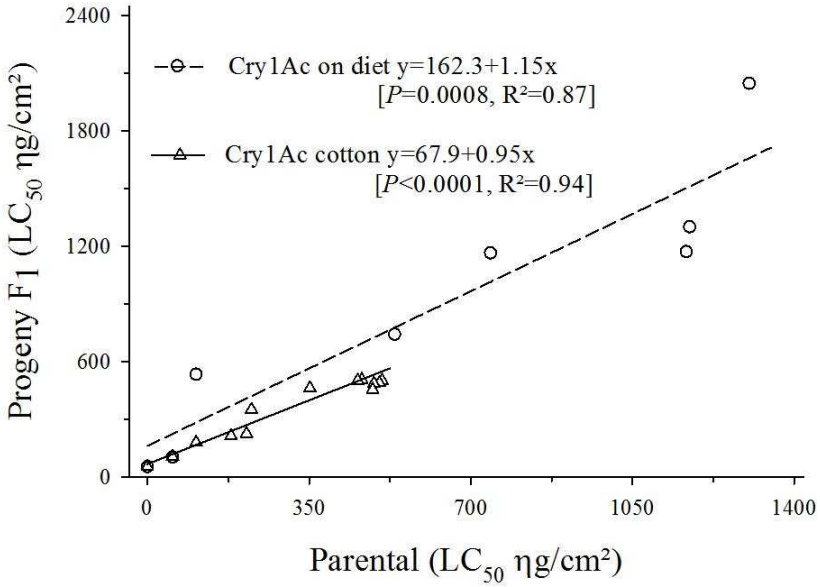


Figure 2. Offspring-parent regression for median lethal concentration data (LC_{50}) showing the significant gain by selection after each generation of selection in two strains of the soybean looper exposed to Bt Cry1Ac cotton leaves or artificial diet overlaid with purified Cry1Ac protein.

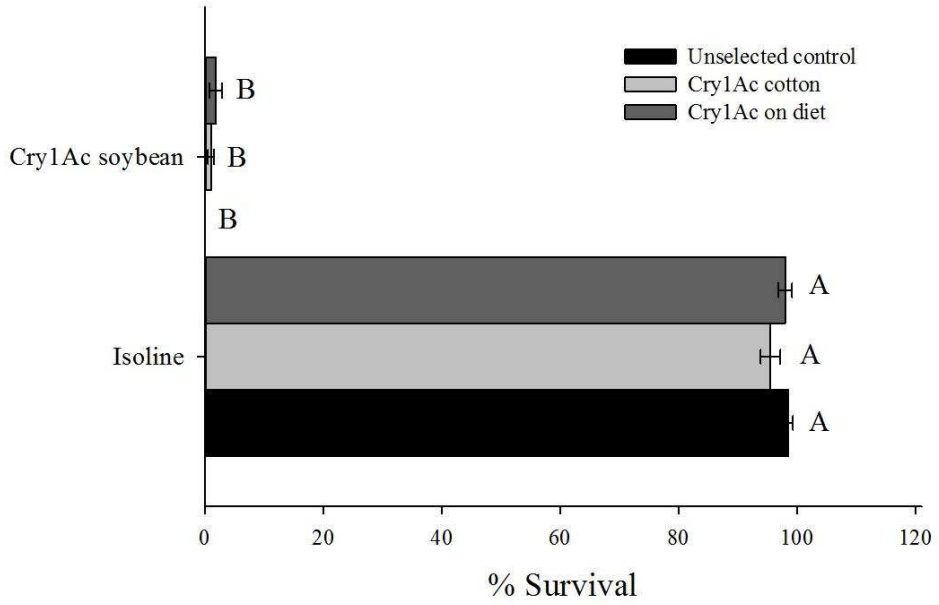


Figure 3. Mean (± SE) survival of Chrysodeixis includens selected on Cry1Ac cotton and Cry1Ac on diet, and control (n = 200) neonate larvae on tissue derived from Cry1Ac-expressing and near-isoline plants. Treatment means were separated using Tukey tests at the $\alpha = 0.05$ level.

Table 1. Results of diet-surface bioassays with Cry1Ac for a strain of Chrysodeixis includens selected for resistance to Cry1Ac using the Bt toxin on the artificial diet.

Generation of selection	Slope ± SE	LC_{90} (95% CL) [1]	[2] χ^2	P
1	2.15 ± 0.19	215.7 (176.7 - 276.8)	4.73	0.450
2	1.82 ± 0.21	535.0 (288.6 - 1768.1)	7.70	0.173
3	2.74 ± 0.32	1568.4 (1180.3 - 2378.5)	1.33	0.931
4	3.20 ± 0.41	1868.2 (1410.6 - 2711.7)	1.21	0.943
5	3.12 ± 0.42	3003.6 (2276.3 - 4469.0)	1.85	0.763
6	2.38 ± 0.27	4057.9 (2457.5 - 10716.0)	8.52	0.130
7	Nd	> 2000	Nd	Nd

[1] LC_{90} (Lethal concentration that causes 90% mortality of the population) was estimated through the probit analysis using Polo-Plus (Robertson et al. 2007a) . The concentrations were expressed as ng/ cm^2. [2] χ^2 is the statistic χ^2 with its P value. Nd = not determined due to insufficient concentration-response.

Table 2. Comparative toxicity of Cry1Ac in two Chrysodeixis includens strains, one selected with Cry1Ac cotton leaves (BGI-Selected) and the other selected with Cry1Ac toxin on the artificial diet (Cry1Ac-Selected). The bioassays were done at the end of the section experiment.

Response variable	Insect strain	Slope ± SE	LC_{50} or EC_{50} (95% CL)[1]	Resistance ratio (95% CL)[2]	χ^{2} [3]	P	N[3]
Mortality	Control	1.85 ± 0.14	16.1 (13.2 - 19.7)	1	2.66	0.752	545
	BGI-Selected	2.34 ± 0.27	502.8 (398.5 - 636.5)	31.1 (22.8 - 42.8)	1.27	0.937	236
	Cry1Ac-Selected	2.11 ± 0.23	2048.4 (1567.7 - 2893.4)	126.6 (94.1 - 170.5)	5.43	0.365	437
Growth inhibition	Control	1.21 ± 0.11	2.1 (1.4 - 2.8)	1	3.42	0.634	545
	BGI-Selected	1.33 ± 0.13	42.1 (27.9 - 56.8)	19.7 (12.2 - 31.9)	2.03	0.994	236
	Cry1Ac-Selected	0.86 ± 0.10	46.5 (5.9 - 113.0)	21.8 (10.3 - 46.1)	6.68	0.153	437

Concentrations values are in ng/cm². [1]LC_{50} (Lethal concentration causing 50% mortality) and EC_{50} (effective concentration causing 50% growth inhibition) were estimated by probit analysis using POLO-PLUS (Robertson et al. 2007a). [2]Resistance ratio = LC_{50} /LC_{50} for the control strain. It indicates the level of resistance, that is, how many times the selected strain is less susceptible than the control, non-selected strain; values in parentheses represent the 95% confidence limits for the resistance ratio (Robertson et al. 2007a). [3]Chi-square statistic with its P value for df = 5. [3]Number of insects tested in the bioassays.

Table 3. Cross-resistance to Bt toxins in two Chrysodeixis includens strains, one selected with Cry1Ac cotton leaves (BGI-Selected) and the other selected with purified Cry1Ac protein on the artificial diet (Cry1Ac-Selected).

Toxin	Insect strain	Slope ± SE	LC_{50} (95% CL)[1]	Cross-resistance ratio (95% CL)[2]	χ^{2} [3]	P	N[4]
Cry2Aa	Control	4.03 ± 0.68	24.3 (19.5 - 30.6)	1	0.23	0.998	248
	BGI-Selected	3.92 ± 0.54	6.1 (5.1 - 7.3)	0.3 (0.2 - 0.3)	3.28	0.657	252
	Cry1Ac-Selected	2.52 ± 0.31	3.9 (2.5 - 5.8)	0.2 (0.1 - 0.2)	10.36	0.065	443
Cry1Ab	Control	3.39 ± 0.64	86.4 (64.2 - 107.5)	1	2.21	0.697	230
	BGI-Selected	1.25 ± 0.16	576.9 (321.3 - 1763.0)	6.7 (4.1 - 10.8)	9.02	0.108	359
	Cry1Ac-Selected	0.76 ± 0.10	748.0 (304.9 - 3863.4)	8.7 (3.9 - 18.9)	6.67	0.246	301
Cry1Fa	Control	5.43 ± 0.81	117.1 (101.2 - 135.8)	1	2.24	0.691	256
	BGI-Selected	2.77 ± 0.30	22.1 (18.3 - 25.7)	0.2 (0.1 - 0.2)	0.97	0.965	539

Concentration values are in ng/cm². [1]LC_{50}, (Lethal concentration causing 50% mortality) was estimated by probit analysis using POLO-PLUS (Robertson et al. 2007a). [2]Cross-resistance ratio = LC_{50} /LC_{50} for control strain. It indicates the level of cross-resistance, that is, how many times the selected strain is less susceptible than the control, non-selected strain to a particular toxin; values in parentheses represent the 95% confidence limits for the resistance ratio (Robertson et al. 2007a). [3]Chi-square statistic with its P value for df = 5. [3]Number of insects tested in the bioassays.

Table 4. Estimates of realized heritability (h^2) and number of generations (G) to a10-fold increase in resistance based on initial LC_{50} for two strains of soybean looper selected for resistance to Cry1Ac in the laboratory.

Estimate of mean response per generation					Estimate of mean selection differential per generation							
Strain	Gs[a]	Initial Log LC_{50}[b]	Final Log LC_{50}[b]	R[c]	P[d]	I[e]	Initial slope	Final slope	σp[f]	S[g]	h^2[h]	G[i]
BG1-Selected	8	1.28	2.70	0.17	90	0.4	1.04	2.49	0.56	0.22	0.78	5.64
Cry1Ac-Selected	7	1.28	3.31	0.33	24	1.3	1.04	2.11	0.63	0.82	0.41	2.96

[a] Gs Number generations selected
[b] Initial (G1) and final (G8 and G7) LC_{50} values were calculated in ng/cm².
[c] R selection response
[d] p average percentage survival of C. includens strains after selection
[e] i intensity of selection according to Falconer (1989)
[f] σp phenotypic deviation
[g] S selection differential
[h] h^2 realized heritability
[i] G Numbers of generations necessary for 10-fold increase in $LC_{50.}$

SUMMARY, CONCLUSIONS, AND IMPLICATIONS OF THE RESEARCH

The adoption of transgenic crops expressing insecticidal protein genes from Baciilus thuringiensis (Bt) in Brazil is becoming a major tactic of insect pest management, a technology that is advantageous because of its specificity, direct killing only target pests while preserving non-target insect species. Field-evolved resistance development in target pest populations has increased the concern of technology providers and regulation agencies about the future utility of Bt crops. In Brazil, field-evolved resistance to Bt maize was already reported, but currently no published study has tackled Bt resistance in C. includens, one of most destructive pests in soybean and cotton production. The present study represents a first step to characterize Cry1Ac resistance in C. includens, a Bt toxin which is currently produced in Bt cotton and soybean cultivars. The research reports results on variation in geographical susceptibility, lethal and sublethal effects of main Bt transgenic events used against C. includens, as well as selection of Cry1Ac-resistant strains under laboratory conditions.

The characterization of the geographic susceptibility of target insect pest populations to the Bt proteins is an important step for development of rational insect resistance management plan for Bt crops. This is the first work to report natural variation in response to B. thuringiensis among C. includens populations before widespread commercial use of soybean in Brazil. Diet-surface bioassays indicated that larvae from C. includens were highly sensitive to Cry1Ac and Cry2Aa toxins. The variation found in larval mortality and growth inhibition was similar, suggesting that both variables can be used in bioassays to detect evolution of resistance in the field. In dose response bioassays, Cry2Aa or Cry1Ac toxins were highly toxic to H. virescens, which indicates that the deployment Cry1Ac cotton cultivars in Mato Grosso, Brazil does not seemed to have affected the susceptibility of H. virescens, but more work with more populations is needed to confirm this hypothesis because this specie has already evolved resistance to Cry1Ac and other Bt-derived proteins in the laboratory. Although Cry1Ac is one of the most used Bt proteins against these species in the American continent, there is no published evidence that the use of Cry1Ac cotton cultivars is contributing to increase its tolerance to the toxin.

Single-gene Bt soybean and dual-gene Bt cotton were very effective in controlling the C. includens in leaf bioassays. The results suggest that these transgenic plants may be used as an effective tool for managing C. includens populations in the field. There are a number field reports about the low control efficiency of Cry1Ac

cotton against C. includens. In fact, this event reduced significantly the value of the net reproductive rate (R_0) and the intrinsic rate of increase (r_m), allowing a portion of larvae to complete development on the Bt plants. Also, Cry1Ac cotton extended the developmental time of C. includens larvae, and these fitness effects can result in asynchronous moth emergence. Future efforts should be focus on studying the influence of Bt sublethal exposure on the reproductive biology of the soybean looper to know better if mating behavior of adults is altered under exposure to Bt, which can have consequences for resistance evolution in the field.

This study evidenced the high potential of C. includens populations to evolve resistance to Cry1Ac. The results suggest that there is a positive correlation between selection pressure and increase in resistance to Cry1Ac in C. includens strains. Further research is needed to determine the mode of inheritance of Cry1Ac resistance, the underlying mechanism(s) of this resistance, and its effects on the fitness of resistant individuals both in transgenic and non-transgenic Bt plants. Regarding the cross-resistance bioassays, our data indicates that development of resistance to Cry1Ac in C. includens did not reduce its susceptibility to Cry1F or to Cry2A toxins, and hence these toxins can be used in multiple toxin approach for resistance management of the soybean looper in Bt crops. However, he high realized heritability values suggests that the looper has potential to rapidly evolve resistance to Cry1Ac under field conditions if no resistance management is adequately adopted.

Due the recent release of Bt Cry1Ac soybeans in Brazil, future efforts should concentrate on developing and establishing diagnostic bioassays to effectively monitor the resistance status of C. includens field populations in both Bt cotton and Bt soybean crops. One concern is that exposure to Cry1Ac cotton may result in increased fitness of resistant heterozygotes and increased rates of resistance evolution to pyramided Bt events that also produces the Cry1Ac toxin. Additionally, as in Brazil refuge adoption has been low and the recent C. includens outbreaks increase concerns about resistance evolution in populations of this specie. The lack of cross-resistance patterns in C. includens need to be considered when devising effective insect resistance management strategies. In this context, the adoption of pyramided crops expressing multiple Bt toxin genes should be combined with other insect pest control tactics to avoid fast development of resistance in soybean looper populations in Bt soybean and cotton crops.