UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CÂMPUS DE JABOTICABAL

BT ZYGOSITY IN MAIZE HYBRIDS

Kian Eghrari Moraes
Agronomist

2020

UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

BT ZYGOSITY IN MAIZE HYBRIDS

Kian Eghrari Moraes

Advisor: Prof. Dr. Gustavo Vitti Môro

Co-advisor: Prof. Dr. Odair Aparecido Fernandes

Co-advisor: Prof. Dr. Bruno Henrique Sardinha de Souza

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Agronomia (Genética e Melhoramento de Plantas)

2020

M827b Moraes, Kian Eghrari
Bt zygosity in maize hybrids / Kian Eghrari Moraes. -- Jaboticabal, 2020
96 p. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientador: Gustavo Vitti Môro
Coorientador: Odair Aparecido Fernandes

1. Breeding. 2. Spodoptera. 3. Transgenic organisms. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: BT ZYGOSITY IN MAIZE HYBRIDS

AUTOR: KIAN EGHRARI MORAES
ORIENTADOR: GUSTAVO VITTI MÔRO
COORIENTADOR: ODAIR APARECIDO FERNANDES
COORIENTADOR: BRUNO HENRIQUE SARDINHA DE SOUZA

Aprovado como parte das exigências para obtenção do Título de Doutor em AGRONOMIA (GENÉTICA E MELHORAMENTO DE PLANTAS), pela Comissão Examinadora:

Prof. Dr. GUSTAVO VITTI MÔRO
Departamento de Produção Vegetal / Faculdade de Ciências Agrárias e Veterinárias - UNESP Jaboticabal

p/
Dr. RENATO ASSIS DE CARVALHO (VIDEOCONFERÊNCIA)
Monsanto do Brasil - Matriz / São Paulo/SP

p/
Prof.ª Dr.ª JANETE APPARECIDA DESIDERIO (VIDEOCONFERÊNCIA)
Biologia Aplicada à Agropecuária / Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal - UNESP

p/
Prof. Dr. GUSTAVO HUGO FERREIRA DE OLIVEIRA (VIDEOCONFERÊNCIA)
Universidade Federal de Sergipe / Nossa Senhora da Glória/SE

p/
Pesquisador Dr. GUSTAVO BARNABÉ BIUDES (VIDEOCONFERÊNCIA)
Syngenta Proteção de Cultivos (Latin America Trait Product Manager) / Piracicaba/SP

Jaboticabal, 24 de setembro de 2020

AUTHOR'S INFORMATION

Kian Eghrari Moraes, born on July 2nd, 1990, in Araguari, MG, Brazil, has a bachelor's degree in Agronomy from the Federal University of Uberlandia (2014). In his master's dissertation he was a pioneer in the study of Bt zygosity in maize hybrids and got his degree in Agronomy (genetics and plant breeding) at the Sao Paulo State University (2017). Kian was a visiting and research scholar for one year at the University of Illinois at Urbana-Champaign (2019) conducting research on genomics, maize breeding, statistics, and genome editing via CRISPR/Cas9. Currently (2020), Kian is a Technical Research Assistant at Corteva Agriscience focused on new traits, native or transgenic, to protect cotton, maize, and soybean from pests and diseases.

EPIGRAPH

Science may be likened to a mirror wherein the images of the mysteries of outer phenomena are reflected. It brings forth and exhibits to us in the arena of knowledge all the product of the past. It links together past and present. The philosophical conclusions of bygone centuries, the teachings of the Prophets and wisdom of former sages are crystallized and reproduced in the scientific advancement of today. Science is the discoverer of the past. From its premises of past and present we deduce conclusions as to the future. Science is the governor of nature and its mysteries, the one agency by which man explores the institutions of material creation. All created things are captives of nature and subject to its laws. They cannot transgress the control of these laws in one detail or particular. The infinite starry worlds and heavenly bodies are nature's obedient subjects. The earth and its myriad organisms, all minerals, plants and animals are thralls of its dominion. But man through the exercise of his scientific, intellectual power can rise out of this condition, can modify, change and control nature according to his own wishes and uses. Science, so to speak, is the breaker of the laws of nature.

'Abdu'l-Bahá, The Promulgation of Universal Peace, US Bahá'í Publishing Trust, 1982 second edition

ACKNOWLEDGEMENTS

I thank God with the words of Bahá'u'lláh, "Praise be unto Thee, O our God, that Thou hast sent down unto us that which draweth us nigh unto Thee, and supplieth us with every good thing sent down by thee in Thy Books and Thy Scriptures."

I thank my parents that encouraged my decisions to get a masters and a PhD degree and supported me material and spiritually during the last five years. I extend my thanks to my brothers, Naim and Nabil, and my little sister, Elisa. For my extended family, I appreciate all their support.

Everything that was accomplished regarding the high level of the experiments and discussions, I owe to my advisor, co-advisors, co-authors, and several researchers that contributed for them.

To my advisor, Gustavo Vitti Môro, I am grateful for his knowledge, guidance, and giving me independence over this research. Also, for his patience in our discussions about genetics and statistics that helped me consolidate some knowledge and write this thesis.

To my co-advisor, Odair Aparecido Fernandes, for his openness and patience when I showed up out of the blue knowing nothing about collecting, rearing, and conducting experiments with *S. frugiperda*. His standard of excellence affected me profoundly, when he showed that in the writing process it is important to check everything from the title to each one of the references. Also, for his patience while reviewing and editing the manuscripts, and solidarity when showing up at the "churrascos".

To my co-advisor, Bruno Henrique Sardinha de Souza, for encouraging me to conduct all experiments, accompanying me in the reviewing process of the articles and inspiring me to always have a strong rectitude of conduct. Moreover, for teac
me by example while conducting part of my thesis' experiments in Lavras.

I would like to thank Professor Julio Viglioni Penna for being my mentor in plant breeding, and André Brito who was my mentor in the transgene zygosity in maize hybrids during my internship at Dow AgroSciences in 2014. Julio Fatoretto for making our partnership between UNESP and Syngeta feasible. Bruna Queiroz, Serena Capriogli, Fabricio Francischini, and Betiana Parody for helping in the conduction,

discussion, and execution of the experiments. Gustavo Biudes for generating all seeds used in the experiments. Amanda Nascimento and Larah Martins for conducting the experiments in Lavras and collaborating with the investigations.

My friends from NEGEMM who always helped me with my experiments, even during the weekends. They are people that I can always count on and that helped me think deeply about my research and how to always improve it. Thank you, Flávia Alves, Naiara Zancanari, Luiz Bertasello, Elcio Samecima, Lucas Revolti, Sophia Mangussi, Camila Baptista, Gustavo Hugo, Carlos Giorgenon, Rodolfo Buzinaro, Kevyn Belossi, Marcela Marconato, and Carlos Caprio. Thank you, Elba!

I would also like to thank Dr. Steve Moose for accepting me as a research scholar in his lab at the University of Illinois at Urbana-Champaign for one year and allowing me to learn more about genomics and statistics. And to my friends Brian Rhodes, Eddie Ross, and Praveena Kanchupati who selflessly taught me how to work in a molecular biology lab.

I also thank the APECOLAB staff that supported me in my bioassays and in teaching me how to rear *Spodoptera frugiperda*. To FEPE staff and Marcelo Scatolin for helping me out with the field experiments.

I thank the defense committee composed by Renato Carvalho, Gustavo Biudes, Janete Desidério, Gustavo Hugo and Gustavo Môro who added valuable considerations for this thesis. I also thank the anonymous reviewers from the Journal of Pest Science and their relevant contributions.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. This study was also financed in part by the National Council for Scientific and Technological Development - Brasil (CNPq).

Special thanks to my co-op friends both in Brazil and in the USA, Ábner, Carlão, Murilo, Felipe, and Steffan, a.k.a., batman.

And, finally, I thank my wife material, Nadine, for making the last year of my PhD sweeter.

CONTENTS

ABSTRACT iii
RESUMO iv
CHAPTER 1 – General considerations 1
1. INTRODUCTION 1
2. LITERATURE REVIEW 2
2.1. Generating hybrids of different Bt zygosity 2
2.2. Bt zygosity effects on maize leaves 4
2.3. Xenia and Bt zygosity effects on kernels 5
2.4. Bt zygosity effects on refuge strategies 9
2.5. *Spodoptera frugiperda* as a model to study Bt zygosity in maize 10
3. REFERENCES 11
CHAPTER 2 - The implications of homozygous vip3Aa20- and cry1Ab-maize on *Spodoptera frugiperda* control 22
ABSTRACT 23
KEY MESSAGE 24
1. INTRODUCTION 25
2. MATERIALS AND METHODS 27
2.1. Genetic material 27
2.2. Field experiment design 27
2.3. Quantification of Cry1Ab and Vip3Aa20 proteins in the leaf 28
2.4. Field evaluation of *S. frugiperda* leaf injury 29
2.5. Leaf tissue bioassays 30
2.6. R3-grain bioassays 31
2.7. Statistical analysis 32
3. RESULTS 32
3.1. Quantification of Cry1Ab and Vip3Aa20 in the leaf 32
3.1.1. Cry1Ab quantification 32
3.1.2. Vip3Aa20 quantification 34
3.2. Evaluation of *S. frugiperda* leaf injury 35
3.3. Leaf tissue bioassays 36
3.4. R3 grain bioassays 36
4. DISCUSSION 37

ACKNOWLEDGEMENTS....43
REFERENCES....44
FIGURE LEGENDS....53
Supplementary table 1 56
Supplementary table 2 57
CHAPTER 3 - Xenia effects of Bt pollen and mortality of *Spodoptera frugiperda* on maize kernels 64
ABSTRACT 64
1. INTRODUCTION 65
2. MATERIALS AND METHODS 66
2.1. Maize genetic material 66
2.2. Detection of Vip3Aa20 in R3 maize kernels 67
2.3. *S. frugiperda* origin and rearing....67
2.4. Laboratory *S. frugiperda* population feeding on maize kernels 67
2.5. Simulating movement of field *S. frugiperda* larvae fed with non-Bt or Bt11 kernels onto MIR162 kernels 68
2.6. Simulating movement of field *S. frugiperda* from non-Bt or Bt11 leaves to MIR162 maize leaves....68
2.7. Data analysis....69
3. RESULTS 69
3.1. Detection of Vip3Aa20 in maize kernels....69
3.2. Survivorship of laboratory *S. frugiperda* larvae on Vip3Aa20 R3 maize kernels 69
3.3. Survivorship of field *S. frugiperda* larvae transferred from non-Bt and Bt11 kernels to MIR162 kernels 70
3.4. Survivorship of field *S. frugiperda* larvae transferred from non-Bt and Bt11 leaves to MIR162 leaves....71
4. DISCUSSION 72
5. REFERENCES 75
APPENDIX – Supplementary tables....83

BT ZYGOSITY IN MAIZE HYBRIDS

ABSTRACT - The effect of transgene zygosity has been addressed in various plant species. Nevertheless, only in 2019 the effect of the number of Bt alleles in maize was investigated. In this thesis, we used near-isogenic maize hybrids that were hemizygous and homozygous for the Bt transgenes *cry1Ab* and *vip3Aa20* to investigate their impacts on 1) Bt protein concentration in leaves and detection of Bt proteins in F2 kernels; 2) leaf-feeding injury of *Spodoptera frugiperda* in the field; 3) efficiency in controlling field-collected *S. frugiperda* larvae in different instars feeding on leaves and kernels; and 4) insect resistance management (IRM) strategies. We assessed 1,920 plants to quantify Cry1Ab and Vip3Aa20 concentrations in the leaves; performed 2,465 evaluations of leaf-feeding injury of *S. frugiperda* in four field experiments; and assessed the survivorship of 3,526 larvae of different instars in leaf and kernel bioassays. Our results showed that 1) Bt concentrations of homozygous hybrids are around 1.5- to 3-fold higher than that of hemizygous versions in leaves, and that kernels express Bt proteins when they originate from non-Bt silks fertilized by Bt pollen and self-fertilized hemizygous plants; 2) Vip3Aa20 hybrids leaves were barely scratched, while in Cry1Ab hybrids *S. frugiperda* leaf-injuries accounted for ~81% of those found on the non-Bt version, likely due to the resistance of the field populations of *S. frugiperda* to Cry1Ab; 3) on either *vip3Aa20* zygosity, *S. frugiperda* larvae in different instars were killed when fed with Vip3Aa20 leaves and kernels, while for Cry1Ab survival rates of *S. frugiperda* were ~90% and 98% of the larval survival found in leaves and kernels of the non-Bt version, respectively.

Keywords: *Bacillus thuringiensis*, fall armyworm, insect resistance management, xenia, *Zea mays* L.

ZIGOSIDADE BT EM HÍBRIDOS DE MILHO

RESUMO - O efeito da zigosidade de transgenes tem sido estudada em várias espécies de plantas. Contudo, apenas em 2019 investigou-se o efeito do número de alelos Bt no milho. Nesta tese, usamos híbridos de milho isogênicos hemizigotos e homozigotos para os transgenes Bt *cry1Ab* e *vip3Aa20* a fim de investigar os seus impactos em 1) concentração de proteínas Bt nas folhas e a detecção das proteínas em grãos F2; 2) injúria de *Spodoptera frugiperda* em folhas a nível de campo; 3) eficiência no controle de populações de campo de *S. frugiperda* em diferentes ínstares que se alimentaram de folhas e grãos; e 4) estratégias para o manejo de resistência de insetos. Foram avaliadas 1.920 plantas para quantificar as concentrações de Cry1Ab e Vip3Aa20 nas folhas; realizadas 2.465 avaliações de injúria de *S. frugiperda* em quatro experimentos de campo; e contabilizada a sobrevivência de 3.526 lagartas em diferentes ínstares em bioensaios de folhas e grãos. Os resultados mostraram que 1) concentrações de proteínas Bt em híbridos homozygous varia de 1,5 a 3 vezes a mais quando comparadas com versões hemizigotas em folhas, e que grãos expressam proteínas Bt quando originados de estilos-estigmas não Bt que foram fertilizados por pólen Bt, ou de autofecundação de plantas Bt hemizigotas; 2) folhas de híbridos Vip3Aa20 quase não foram danificadas, enquanto híbridos Cry1Ab tiveram injúrias de *S. frugiperda* que representaram ~81% daquelas encontradas na versão não Bt, provavelmente pela resistência das populações de *S. frugiperda* a Cry1Ab; 3) considerando *vip3Aa20* em qualquer zigosidade, lagartas de *S. frugiperda* em diferentes ínstares morreram quando alimentadas de folhas e grãos Vip3Aa20, enquanto para Cry1Ab, as taxas de sobrevivência de *S. frugiperda* foram ~90% e 98% da sobrevivência em folhas e grãos da versão não Bt, respectivamente.

Palavras-chave: *Bacillus thuringiensis*, lagarta-do-cartucho-do-milho, manejo de resistência de inseto, xenia, *Zea mays* L.

CHAPTER 1 – General considerations

1. INTRODUCTION

On average 200 million hectares of transgenic crops are cultivated annually (ISAAA, 2017). By the year 2000, transgenic plants were being commercialized in less than 15 countries, and ten years later, more than 30 countries were adopting the cultivation of genetically modified plants (ISAAA, 2017). Currently, 33 countries approved the cultivation of *Bacillus thuringiensis* (Bt) maize with ~100 transgenic events (ISAAA, 2017).

As concerns with pests increased with monoculture, genetically modified plants with the insertion of Bt genes were launched to alleviate the damage caused by lepidopteran and coleopteran pests (Tabashnik et al., 2013), and to reduce the use of chemical insecticides (Naranjo, 2009; Lu et al., 2012). Due to high control efficiency over a range of primary pests, Bt maize conveyed the impression of solving natural pest infestation in agriculture (Jaffe, 2003), and began to be the main approach adopted by farmers to control leaf-, ear-, and root-feeding pests (Hutchison et al., 2010; Fernandez-Cornejo and Wechsler, 2012; Cullen et al., 2013). New Bt transgenes, with new modes of action, were being launched and stacked with those that were already in the market (Fatoretto et al., 2017). Nevertheless, with the increase of Bt maize use, and lack of compliance with insect resistance management (IRM) strategies, reports of field-evolved resistance of insect species to Bt proteins began to appear around the world (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014; Omoto et al., 2016).

As discussions on the subject gained momentum, the recommended approaches to delay evolution of resistance were deeply investigated. Among the main IRM strategies, those involving high-dose events and refuge approaches stood out (Chilcutt and Tabashnik, 2004; Andow, 2008). However, most investigations did not mention the number of Bt alleles in transgenic Bt loci, which influences Bt protein concentration in the plant and gene flow from Bt to non-Bt maize plants (Yang et al., 2014; Caprio et al., 2016; Eghrari et al., 2019).

Thus, this thesis uses near-isogenic versions of maize hybrids that are hemizygous and homozygous for Bt traits to investigate the zygosity effects on 1) Bt protein concentration in leaves; 2) detection of Bt proteins in kernels and xenia effects; 3) leaf-feeding injury of *S. frugiperda* in the field; 4) efficiency in controlling field-collected *S. frugiperda* larvae in different instars feeding on leaves and kernels; and 5) the impact on IRM strategies.

2. LITERATURE REVIEW

2.1. Generating hybrids of different Bt zygosity

Transgenic maize hybrids could be either hemizygous or homozygous for the transgene (Guadagnuolo et al., 2006; Burkness et al., 2011; Caprio et al., 2016; Eghrari et al., 2019). Currently, only hemizygous Bt hybrids are produced (Eghrari et al., 2019; Carrière et al., 2020). The term hemizygous characterizes the presence of one transgenic allele in the transgenic locus of transformed plants (James et al., 2002; Guadagnuolo et al., 2006; Sridevi et al., 2006; Hood et al., 2012). In other words, hemizygous transgenic loci do not carry an alternative allelic version for the exogenous gene, i.e. transgene, in the genome of the transformed species (Guadagnuolo et al., 2006). In fact, hemizygous locus has a distinct meaning from heterozygous, which the latter is a locus that possesses allelic variants (Roessler et al., 2019).

With the advent of transgenic events in agriculture, the effects of transgene zygosity have been studied regarding biomass conversion for biofuel production (Hood et al., 2012), relative fitness of wild maize relatives (Guadagnuolo et al., 2006) and even for the production of therapeutic proteins (Law et al., 2006). Although zygosity in Bt maize has been addressed before (Chilcutt and Tabashnik, 2004; Chilcutt et al., 2007; Burkness et al., 2011; Yang et al., 2014; Caprio et al., 2016), its effects started to be investigated in leaves of maize plants only in 2019 (Eghrari et al., 2019; Carrière et al., 2020).

To produce near-isogenic maize hybrids with different Bt zygosity, Bt and non-Bt near-isogenic versions of the parental inbred lines are necessary (Sridevi et al., 2006; Hood et al., 2012; Hood and Requesens, 2016). Figure 1 shows that hemizygous Bt hybrids are generated by crossing a homozygous Bt inbred line with a non-Bt inbred

line, while the near-isogenic homozygous version is produced by crossing two homozygous transgenic inbred lines. To obtain near-isogenic inbred lines that are transgenic, Bt traits are introgressed to the elite inbred lines by five-seven cycles of backcrossing through classical breeding (Hood and Requesens, 2016) or two-three cycles when molecular marker assisted selection is utilized (Ribaut and Ragot, 2007). This is a laborious, time- and money-consuming process that encumber breeding programs (Morris et al., 2003; Chen and Ow, 2017). However, new approaches, such as CRISPR-Cas9, could facilitate trait stacking by precisely inserting new traits in near positions in the genome (Gao et al., 2020). Besides the cumbersomeness of incorporating transgenes into elite inbred lines, transgene silencing when the locus is in homozygosis (James et al., 2002) and seed piracy, involving transgene stacking of trait combinations that are not commercially approved, could be some of the reasons why breeding companies do not produce homozygous hybrids for the transgenes.

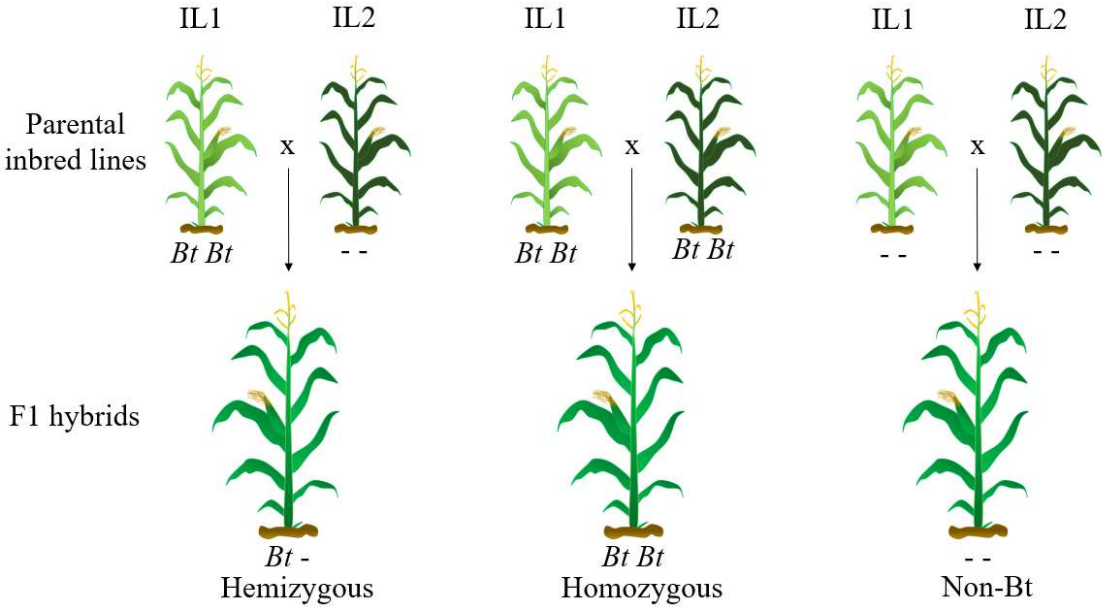


Figure 1. Crossing design to generate near-isogenic hemizygous, homozygous, and non-Bt maize hybrids. IL1, inbred line 1. IL2, inbred line 2. *Bt*, presence of the Bt allele. -, absence of the Bt allele. *Bt* -, Bt-hemizygous. *Bt Bt*, Bt-homozygous. - -, non-Bt.

In the production fields of Bt-hemizygous maize hybrid seed, one of the parental inbred lines is non-Bt and the other is Bt-homozygous (Eghrari et al., 2019). Because

hybrid seed production fields are composed of lines of transgenic and non-transgenic plants, management practices of the crop are adopted according to the traits that the genotypes possess. For instance, non-Bt plants are not protected against lepidopteran pests like Bt lines are (Hutchison et al., 2010; Kiesselbach et al., 2019). Thereby, the benefits that transgenic events dispose for plants could be useless regarding the non-application of insecticides on a field of hybrid seed production, because the non-Bt line might needs be protected against key pests that are controlled by Bt traits.

2.2. Bt zygosity effects on maize leaves

It has been found that increasing the allelic dosage of transgenes in their respective loci, from hemizygous to homozygous, increases the transgene's product concentration (Caligari et al., 1993; James et al., 2002; Eghrari et al., 2019). That increase could be up to four times when the transgene is in a homozygous state (Hood et al., 2012; Hood and Requesens, 2016). In addition, choosing the right hybrid background could also increase transgenic proteins concentrations (Hood et al., 2012; Trtikova et al., 2015), however previous results did not show differences between Cry1Ab concentrations in different genetic backgrounds (Lohn et al., 2020). Moreover, an increase in allelic dose in a determined locus is different than increasing the number of transgene copies, i.e., insertions, in the genome (Hobbs et al., 1990; Gao and Nielsen, 2013). Complex segregation patterns (Travella et al., 2005), unstable transgene expression (Dai et al., 2001; Travella et al., 2005), transgene silencing (James et al., 2002; Travella et al., 2005), and cumbersomeness in obtaining the final product (Lowe et al., 2009) are some disadvantages of having multiple copies of a transgene compared to a single copy in the genome of targeted species.

In maize leaves, quantification of transgenes' products, such as Bt proteins, have been extensively performed (Badea et al., 2010; Bernardi et al., 2015; Trtikova et al., 2015), albeit few of those investigations considered the effects of Bt zygosity on the resulting proteins concentrations in maize leaves. The pioneer study of Eghrari et al. (2019) assessed the effects of hemizygous and homozygous Bt traits expressing the proteins Cry1F, Cry1A.105, and Cry2Ab2 in the pyramided event TC1507xMON89034xNK603. Shortly after that, the response of zygosity was evaluated on pyramided and singular Bt11 and MIR162 events, expressing the

proteins Cry1Ab and Vip3Aa20, respectively (data not published). The fold-change of homozygous hybrids over their hemizygous near-isogenic versions shows the increase of Bt proteins concentrations (Figure 2). Cry1Ab and Cry1F had a fold-change below 2, while Cry1A.105, Cry2Ab2, and Vip3Aa20 doubled their expression when their genes are in a homozygous state (Figure 2).

2.3. Xenia and Bt zygosity effects on kernels

Xenia in a broad sense is the immediate effect of non-maternal pollen on the formation and development of kernels (Kiesselbach, 1960; Poelman, 1986). The effect of foreign pollen on the formation of maize kernels has been studied to discover bottlenecks related to the increase of grain production and kernel quality (Feil et al., 2003; Munsch et al., 2010; Kahriman et al., 2017). Techniques employing cytoplasmatic male sterile (CMS) hybrids fertilized by a distinct pool of hybrids, known as plus-hybrid system, showed to be promising by increasing productivity up to 20% (Weingartner et al., 2002; Munsch et al., 2010).

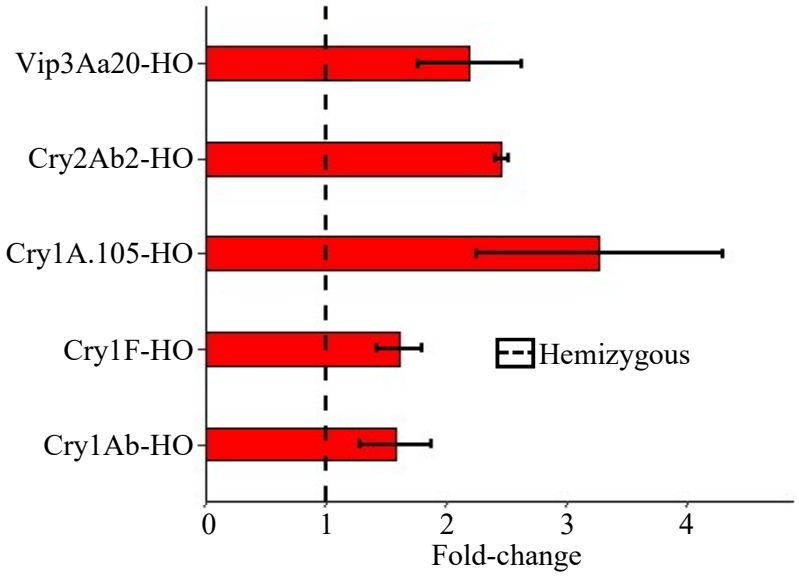


Figure 2. Fold-change (mean ± SE) of Bt proteins expressed in homozygous maize leaves relative to their near-isogenic hemizygous versions. HO, homozygous. Cry1F, Cry1A.105, and Cry2Ab2 concentrations were extracted from Eghrari et al. 2019, and Cry1Ab and Vip3Aa20 concentrations are available in first article of this thesis.

However, with the advent of transgenic plants, xenia effects began to be better explored regarding the effects of transgenic pollen on adjacent non-transgenic crops and wild relatives (Gathmann et al., 2006; Guadagnuolo et al., 2006). Some studies, notwithstanding, explored xenia using synonyms like gene flow (Chilcutt and Tabashnik, 2004), cross-pollination (Kang et al., 2012), transgenic pollen contamination (Yang et al., 2017), and outcrossing (Dively et al., 2020) .

Regarding Bt events, studies mostly focused on the entomological aspect, with their goals more directed to the impact of Bt pollen on insect resistance management strategies (Yang et al., 2014, 2017; Caprio et al., 2016). Likewise, entomological studies also assessed the effects of Bt pollen on target (Burkness et al., 2011, 2015) and non-target insects (Li et al., 2008; Perry et al., 2010), barely focusing on Bt pollen effects in each tissue of the kernel during its formation (Caprio et al., 2016). Consequently, zygosity of Bt traits had been considered indirectly in maize kernel studies (Chilcutt and Tabashnik, 2004; Burkness et al., 2011; Yang et al., 2014, 2017). To our knowledge, only in 2020 a study mentioning zygosity on Bt kernels was published (Carrière et al., 2020). As to Bt pollen effects on maize kernels, they have been described as gene flow (Chilcutt and Tabashnik, 2004) and cross-pollination (Yang et al., 2020).

Exploring the cytology, genetics, physiology, and anatomy of maize kernels helps clarify the relationship of zygosity and xenia effects caused by Bt pollen. Clarifying such concepts is important in order that the understanding of segregation of Bt traits in kernels be not limited to what occur in the embryo (Chilcutt and Tabashnik, 2004; Chilcutt et al., 2007; Burkness et al., 2011; Yang et al., 2014; Carrière et al., 2020).

Thereupon, maize kernels are composed of three main tissues: embryo, endosperm, and pericarp. Embryo and endosperm are filial tissues, resulted from paternal and maternal components, whereas pericarp is exclusively of maternal origin. Kernel development begins when a pollen grain germinates on an ear silk, thereby developing the pollen tube. Each pollen tube bears two sperms, one fertilizing the egg, syngamy (2n), and the other fertilizing the polar nuclei of the embryo sac, triple fusion (3n), constituting the embryo and endosperm, respectively (Sprague, 1931; Kiesselbach, 1960). Despite that, in exceptional cases, embryo and endosperm could

be genetically distinct by two reasons: egg and polar nuclei of the same embryo sac present different genetic constitution and are fused by genetic identical sperms, or genetically equal egg and polar nuclei fuse with sperms with distinct genotypes (Sprague, 1931).

Bt zygosity effects on Bt protein concentrations in maize kernels have not been evaluated yet. Nevertheless, knowledge of the maize genotypes' Bt zygosity and the use of crossing designs could indicate the relative levels of Bt expression (Carrière et al., 2020). For instance, as Bt concentrations increased in maize leaves when doubling Bt zygosity in the plant (Eghrari et al., 2019), Bt zygosity on the tissues of the kernel could also contribute to differential Bt expressions in the kernel, since the proportion of embryo, endosperm, and pericarp in the kernel is 0.7, 86.4, and 12.9%, respectively, 14 days after fertilization (Kiesselbach, 1960), and 11, 82, and 6%, respectively, at physiological maturity (Tollenaar and Dwyer, 1999; García-Lara et al., 2019). In figure 3, we present examples of distinctive zygosity in the embryo, endosperm, and pericarp of maize kernels of F1 hybrids according to different crossing designs.

For instance, a selfed hemizygous for Bt hybrid (Bt-HE) has a segregation of 1:2:1 of homozygous, hemizygous, and non-Bt for the embryo of the kernel, respectively. Regarding the endosperm, a 3n tissue, the segregation would also be 1:2:1 for three Bt alleles, one or two Bt alleles, and no Bt allele, respectively. The genotype of the endosperm with one or two Bt alleles depends on the genotype of the polar nuclei and the sperm. If the polar nuclei (2n, *BtBt*) are fertilized by a non-Bt pollen (n, -), then the genotype of the endosperm is *BtBt*-, i.e., with two Bt alleles. Conversely, if the non-Bt polar nuclei (2n, --) are fertilized by a Bt pollen (n, *Bt*), the genotype of the endosperm presents only one Bt allele, that is, *Bt*--. As for the pericarp, its genotype has the same genetic configuration of its maternal plant, that is, the F1 hybrid. For instance, in refuge plants the genotype of the F2 kernels pericarp is non-Bt, regardless of the genotype of the pollen that fertilized the silks. Whereas in Bt maize hybrids, the genotype of the pericarp is hemizygous for the Bt trait, because it has the same genetic configuration of the F1 hybrid, which is, in general, hemizygous for the Bt trait, i.e., *Bt*- (Chilcutt and Tabashnik, 2004; Caprio et al., 2016; Eghrari et al., 2019; Carrière et al., 2020) (Figure 3A).

Differential Bt allelic dosage is also present in reciprocal crosses between Bt-HE and non-Bt hybrids. The presence or absence of one Bt allele in the endosperm or pericarp's genotype could also contribute to different levels of Bt protein expression (Carrière et al., 2020). For instance, a non-Bt maize fertilized by Bt-HE hybrids have a 1:1 segregation of Bt and non-Bt kernels. As for the 50% Bt ones, they carry one Bt allele in the embryo and one in the endosperm, and no Bt allele in the pericarp. In the reciprocal cross, the segregation ratio is going to be the same, 1:1, only when embryo and endosperm are considered, because the pericarp has the same genotype as the plant that generated it. On these terms, maternal effects increase Bt dosage in the kernel likely increasing Bt expression, since kernels with Bt endosperm present two alleles and the pericarp also contains the transgene (Figure 3B and 3C). Similar results with reciprocal crosses of Bt and non-Bt maize hybrids showed higher mortality of *Ostrinia nubilalis* and *Helicoverpa* zea on kernels of Bt-hemizygous maize pollinated by non-Bt maize (Burkness et al., 2011), indicating the influence of the maternal effect on the endosperm and pericarp.

If Bt homozygous hybrids would be deployed, although F2 kernels would segregate for other traits, they would not segregate for the Bt trait. In other words, kernels would be homozygous for the embryo, endosperm, and pericarp (Figure 3D). In a cross of non-Bt and Bt-HO hybrids, with the latter as pollen donor, F2 embryos would be hemizygous for the Bt trait, endosperm would have only one Bt allele, and pericarp would be non-Bt (Figure 3E). In the reciprocal cross, the embryo will present the same Bt allelic dosage, whereas endosperm and pericarp will have, each one, two Bt alleles (Figure 3F).

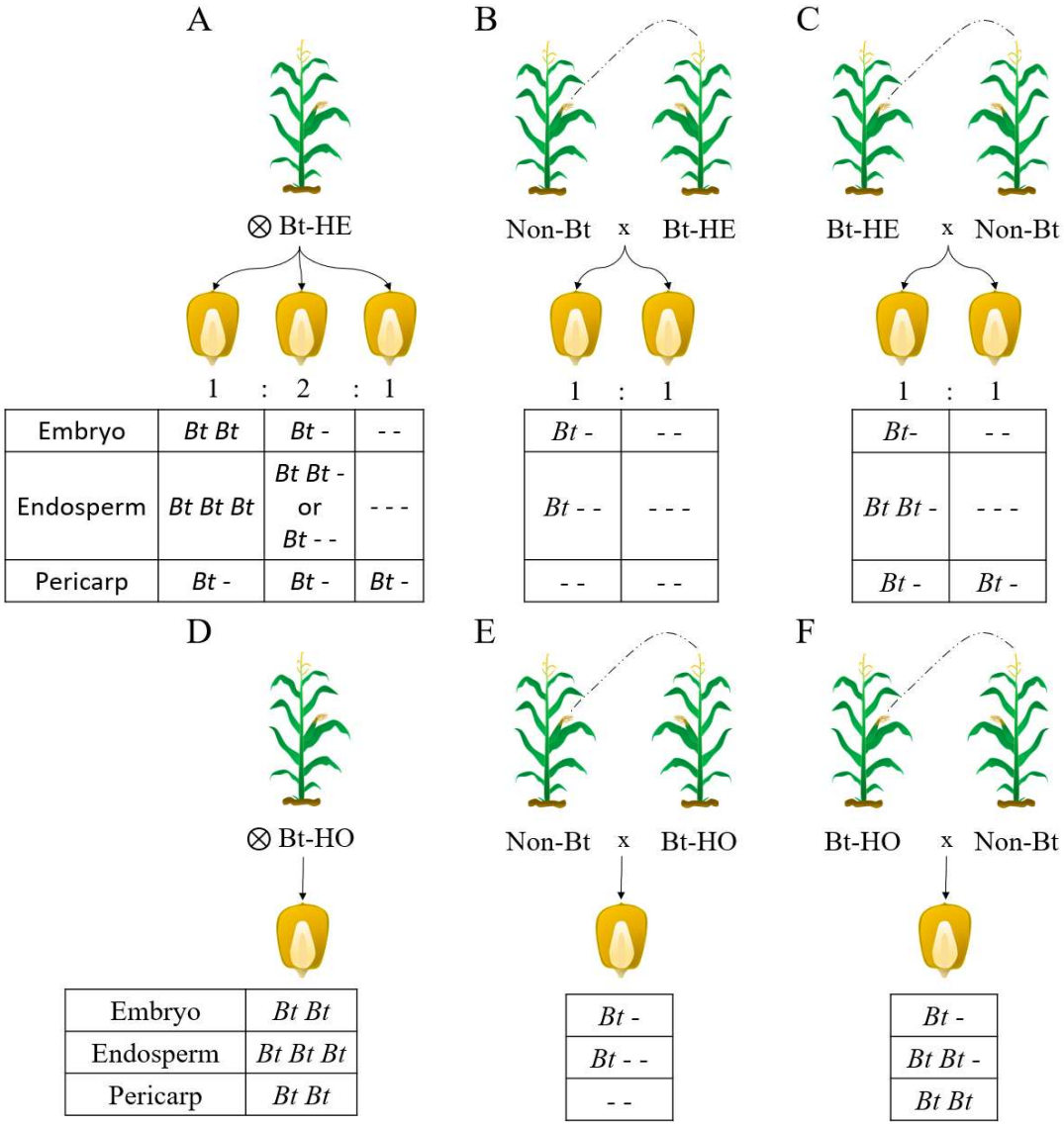


Figure 3. Bt allele segregation on kernels of F1 maize hybrids in six designs: self-fertilized hemizygous for Bt (A); reciprocal crosses of non-Bt with Bt-HE (B and C); self-fertilized Bt-homozygous hybrid (D); and reciprocal crosses of non-Bt with Bt-HO (E and F). Bt allele is represented by the term "*Bt*". ⊗, self-fertilized. HE, hemizygous. HO, homozygous.

2.4. Bt zygosity effects on refuge strategies

Compliance with refuge strategies is crucial to extend the effectiveness of Bt maize on targeted Lepidopteran pests (Andow, 2008; Huang et al., 2011). Two main refuge strategies are used to delay the evolution of resistance to Bt proteins: structured refuge and seed mixture (Carroll et al., 2012; Dively et al., 2020). The choice of the

best refuge strategy should consider its impacts on leaf- and ear-feeding pests (Onstad et al., 2011; Caprio et al., 2016; Yang et al., 2017).

On reproductive tissues, Bt transgenes segregate and non-Bt kernels could express Bt traits if fertilized by Bt pollen, i.e., the genotype depends on the genotype of the female and male gametes (Huang et al., 2014; Caprio et al., 2016; Oyediran et al., 2016; Carrière et al., 2020). Therefore, for ear-feeding pests, contamination of refuge ears could contribute to increase dominance of resistance to Bt traits (Yang et al., 2017). If the farmers would prefer to use seed mixture instead of structured refuge, they might have to increase the percentage of non-Bt plants in their field (Yang et al., 2020).

Refuge plants in a seed mixture refuge would be surrounded by Bt plants and would had their non-Bt silks fertilized by Bt pollen (Oyediran et al., 2016; Dively et al., 2020), which will eventually express Bt proteins (Burkness et al., 2011). In a seed mixture scenario, non-Bt plants could not be considered a real refuge for ear-feeding pests, and therefore adopting structured refuge is more efficient to delay insect resistance evolution to Bt proteins (Huang et al., 2014; Yang et al., 2017, 2020).

2.5. *Spodoptera frugiperda* as a model to study Bt zygosity in maize

Fall armyworm, *Spodoptera frugiperda*, is one of most dreaded pests in the maize crop and its damage could impact maize production if integrated pest management strategies are not employed (Baudron et al., 2019; Santos et al., 2020). *S. frugiperda* is mainly a pest that feeds on the maize whorl (Sisay et al., 2019; Santos et al., 2020), but it can also feed on silks, ears, tassels, and seedlings (Pannuti et al., 2016; Reay-Jones et al., 2016). *S. frugiperda* recent spread around the world has worried farmers and the scientific community (Li et al., 2020; Nboyine et al., 2020). Although transgenics were apparently the solution to control insects and protect maize production, *S. frugiperda* populations in the American continents evolved resistance to the some Bt proteins (Farias et al., 2014; Huang et al., 2014; Omoto et al., 2016)

Due to *S. frugiperda* abundance in a multitude of environments and rearing facility, studies have been using *S. frugiperda* as a model to better understand the behavior of lepidopteran pests in maize Bt crops (Yang et al., 2017; Zhu et al., 2020). These investigations encompass the insect's movement in the field (Pannuti et al.,

2016; Malaquias et al., 2020), preference of oviposition (Gonçalves et al., 2020), effectiveness of refuge strategies (Yang et al., 2017), resistance characterization to the Bt proteins available in the market (Amaral et al., 2020), and the effect of Bt zygosity on *S. frugiperda* mortality in leaves (Eghrari et al., 2019) and kernels (Carrière et al., 2020).

3. REFERENCES

Amaral FSA, Guidolin AS, Salmeron E, et al (2020) Geographical distribution of Vip3Aa20 resistance allele frequencies in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. **Pest Management Science** 76:169–178. https://doi.org/10.1002/ps.5490

Andow DA (2008) The risk of resistance evolution in insects to transgenic insecticidal crops. **Collection of Biosafety Reviews** 4:142–199

Badea EM, Chelu F, Lăcătuşu A (2010) Results regarding the levels of Cry1Ab protein in transgenic corn tissue (MON810) and the fate of Bt protein in three soil types. **Romanian Biotechnological Letters** 15:55–62

Baudron F, Zaman-Allah MA, Chaipa I, et al (2019) Understanding the factors influencing fall armyworm (*Spodoptera frugiperda* J.E. Smith) damage in African smallholder maize fields and quantifying its impact on yield. A case study in Eastern Zimbabwe. **Crop Protection** 120:141–150. https://doi.org/10.1016/j.cropro.2019.01.028

Bernardi O, Bernardi D, Amado D, et al (2015) Resistance risk assessment of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and *Diatraea saccharalis* (Lepidoptera: Crambidae) to Vip3Aa20 insecticidal protein expressed in corn. **Journal of Economic Entomology** 108:2711–2719. https://doi.org/10.1093/jee/tov219

Burkness EC, Cira TM, Moser SE, Hutchison WD (2015) Bt maize seed mixtures for

Helicoverpa zea (Lepidoptera: Noctuidae): larval movement, development, and survival on non-transgenic maize. **Journal of Economic Entomology** 108:2762–2769. https://doi.org/10.1093/jee/tov253

Burkness EC, O'Rourke PK, Hutchison WD (2011) Cross-pollination of nontransgenic corn ears with transgenic bt corn: efficacy against lepidopteran pests and implications for resistance management. **Journal of Economic Entomology** 104:1476–1479. https://doi.org/10.1603/ec11081

Caligari PDS, Yapabandara YMHB, Paul EM, et al (1993) Field performance of derived generations of transgenic tobacco. **Theoretical and Applied Genetics** 86:875–879. https://doi.org/10.1007/BF00212615

Caprio MA, Martinez JC, Porter PA, Bynum E (2016) The impact of inter-kernel movement in the evolution of resistance to dual-toxin Bt-corn varieties in *Helicoverpa zea* (Lepidoptera: Noctuidae). **Journal of Economic Entomology** 109:307–319. https://doi.org/10.1093/jee/tov295

Carrière Y, Degain BA, Harpold VS, Unnithan GC, Tabashnik BE (2020) Gene Flow Between Bt and Non-Bt Plants in a Seed Mixture Increases Dominance of Resistance to Pyramided Bt Corn in Helicoverpa zea (Lepidoptera: Noctuidae). **Journal of Economic Entomology** 20:1-11. https://doi.org/10.1093/jee/toaa138

Carroll MW, Head G, Caprio M (2012) When and where a seed mix refuge makes sense for managing insect resistance to Bt plants. **Crop Protection** 38:74–79. https://doi.org/10.1016/j.cropro.2012.02.015

Chen W and Ow DW (2017) Precise, flexible and affordable gene stacking for crop improvement. **Bioengineered** 8:451-456. https://doi.org/10.1080/21655979.2016.1276679

Chilcutt CF, Odvody GN, Correa JC, Remmers J (2007) Effects of *Bacillus*

thuringiensis transgenic corn on corn earworm and fall armyworm (Lepidoptera: Noctuidae) densities. **Journal of Economic Entomology** 100:327–334. https://doi.org/10.1603/0022-0493(2007)100[327:eobttc]2.0.co;2

Chilcutt CF, Tabashnik BE (2004) Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. **Proceedings of the National Academy of Sciences of the United States of America** 101:7526–7529. https://doi.org/10.1073/pnas.0400546101

Cullen EM, Gray ME, Gassmann AJ, Hibbard BE (2013) Resistance to Bt corn by western corn rootworm (Coleoptera: Chrysomelidae) in the U.S. corn belt. **Journal of Integrated Pest Management** 4:1–6. https://doi.org/10.1603/ipm13012

Dai S, Zheng P, Marmey P, et al (2001) Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. **Molecular Breeding** 7:25–33. https://doi.org/10.1023/A:1009687511633

Dively GP, Huang F, Oyediran I, et al (2020) Evaluation of gene flow in structured and seed blend refuge systems of non-Bt and Bt corn. **Journal of Pest Science** 93:439–447. https://doi.org/10.1007/s10340-019-01126-4

Eghrari K, Humberto de Brito A, Baldassi A, et al (2019) Homozygosis of Bt locus increases Bt protein expression and the control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize hybrids. **Crop Protection** 124:. https://doi.org/10.1016/j.cropro.2019.104871

Farias JR, Andow DA, Horikoshi RJ, et al (2014) Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. **Crop Protection** 64:150–158. https://doi.org/10.1016/j.cropro.2014.06.019

Fatoretto JC, Michel AP, Filho MCS, Silva N (2017) Adaptive potential of fall armyworm (Lepidoptera: Noctuidae) limits Bt trait durability in Brazil. **Journal of Integrated Pest**

Management 8:17. https://doi.org/10.1093/jipm/pmx011

Feil B, Weingartner U, Stamp P (2003) Controlling the release of pollen from genetically modified maize and increasing its grain yield by growing mixtures of male-sterile and male-fertile plants. **Euphytica** 130:163–165. https://doi.org/10.1023/A:1022843504598

Fernandez-Cornejo J, Wechsler S (2012) Revisiting the impact of Bt corn adoption by U.S. farmers. **Agricultural and Resource Economics Review** 41:377–390. https://doi.org/10.1017/S1068280500001337

García-Lara S, Chuck-Hernandez C, Serna-Saldivar SO (2019) Development and structure of the corn kernel. In.: Saldívar SROS (Eds.) **Corn Chemistry and Technology.** Duxford: Woodhead Publishing, p. 147-163.

Gao H, Mutti J, Young JK, Yang M, Schroder M, Lenderts B, Wang L, Peterson D, St Clair G, Jones S, Feigenbutz L (2020) Complex Trait Loci in Maize Enabled by CRISPR-Cas9 Mediated Gene Insertion. **Frontiers in Plant Science** 11:535. https://doi.org/10.3389/fpls.2020.00535

Gao C and Nielsen KK (2013) Comparison between *Agrobacterium*-mediated and direct gene transfer using the gene gun. In: Sudowe S, Reske-Kunz A. (Eds) **Biolistic DNA Delivery. Methods in Molecular Biology (Methods and Protocols)**, vol 940. Humana Press: Totowa p. 3-16

Gathmann A, Wirooks L, Hothorn LA, et al (2006) Impact of Bt maize pollen (MON810) on lepidopteran larvae living on accompanying weeds. **Molecular Ecology** 15:2677–2685. https://doi.org/10.1111/j.1365-294X.2006.02962.x

Gonçalves J, Rodrigues JVC, Santos-Amaya OF, et al (2020) The oviposition behavior of fall armyworm moths is unlikely to compromise the refuge strategy in genetically modified Bt crops. **Journal of Pest Science** 93:965–977.

https://doi.org/10.1007/s10340-020-01219-5

Guadagnuolo R, Clegg J, Ellstrand NC (2006) Relative fitness of transgenic vs. non-transgenic maize x teosinte hybrids: A field evaluation. **Ecological Applications** 16:1967–1974. https://doi.org/10.1890/1051-0761(2006)016[1967:RFOTVN]2.0.CO;2

Hobbs SLA, Kpodar P, DeLong CMO (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. **Plant Molecular Biology** 15:851–864. https://doi.org/10.1007/BF00039425

Hood EE and Requesens DV (2016) Commercial plant-produced recombinant cellulases for biomass conversion. In.: Howard JA, Hood EE (Eds) **Commercial Plant-Produced Recombinant Protein Products.** Springer: Berlin, Heidelberg, p. 231-246.

Hood EE, Devaiah SP, Fake G, et al (2012) Manipulating corn germplasm to increase recombinant protein accumulation. **Plant Biotechnology Journal** 10:20–30. https://doi.org/10.1111/j.1467-7652.2011.00627.x

Huang F, Andow DA, Buschman LL (2011) Success of the high-dose ／ refuge resistance management strategy after 15 years of Bt crop use in North America. 1–16. https://doi.org/10.1111/j.1570-7458.2011.01138.x

Huang F, Qureshi JA, Meagher RL, et al (2014) Cry1F resistance in fall armyworm *Spodoptera frugiperda*: Single gene versus pyramided Bt maize. **PLoS ONE** 9:. https://doi.org/10.1371/journal.pone.0112958

Hutchison WD, Burkness EC, Mitchell PD, et al (2010) Areawide suppression of European corn. **Science** 330:222–225. https://doi.org/10.1126/science.1190242

ISAAA (2017) **Global Status of Commercialized Biotech/GM Crops in 2017: Biotech Crop Adoption Surges as Economic Benefits Accumulate in 22 Years. ISAAA Brief No. 53.** ISAAA: Ithaca, NY, p. 143.

Jaffe G (2003) Planting trouble: Are farmers squandering Bt corn technology. Center for Science in the Public Interest, Washington, DC, available at: http://cspinet. org/new/pdf/bt_corn_report. pdf.

James VA, Avart C, Worland B, et al (2002) The relationship between homozygous and hemizygous transgene expression levels over generations in populations of transgenic rice plants. **Theoretical and Applied Genetics** 104:553–561. https://doi.org/10.1007/s001220100745

Kahriman F, Şerment M, Haşlak M, Kang M (2017) Pollen effect (xenia) for evaluating breeding materials in maize. **Genetika** 49:217–234. https://doi.org/10.2298/GENSR1701217K

Kang J, Onstad DW, Hellmich RL, et al (2012) Modeling the impact of cross-pollination and low toxin expression in corn kernels on adaptation of european corn borer (Lepidoptera: Crambidae) to transgenic insecticidal corn. **Environmental Entomology** 41:200–211. https://doi.org/10.1603/en11133

Kiesselbach TA (1960) The significance of xenia effects on the kernel weight of corn. (Research Bulletin: Bulletin of the Agricultural Experiment Station of Nebraska No. 191)

Law RD, Russell DA, Thompson LC, et al (2006) Biochemical limitations to high-level expression of humanized monoclonal antibodies in transgenic maize seed endosperm. **Biochimica et Biophysica Acta - General Subjects** 1760:1434–1444. https://doi.org/10.1016/j.bbagen.2006.05.006

Li XJ, Wu MF, Ma J, et al (2020) Prediction of migratory routes of the invasive fall armyworm in eastern China using a trajectory analytical approach. **Pest Management Science** 76:454–463. https://doi.org/10.1002/ps.5530

Li Y, Meissle M, Romeis J (2008) Consumption of Bt maize pollen expressing Cry1Ab

or Cry3Bb1 does not harm adult green lacewings, *Chrysoperla carnea* (Neuroptera: Chrysopidae). **PLoS ONE** 3:. https://doi.org/10.1371/journal.pone.0002909

Lohn AF, Trtikova M, Chapela I, Van den Berg J, Plessis H, Hilbeck A (2020) Transgene behavior in Zea mays L. crosses across different genetic backgrounds: Segregation patterns, cry1Ab transgene expression, insecticidal protein concentration and bioactivity against insect pests. PloS One. https://doi.org/10.1371/journal.pone.0238523

Lowe BA, Prakash NS, Way M, et al (2009) Enhanced single copy integration events in corn via particle bombardment using low quantities of DNA. **Transgenic Research** 18:831–840. https://doi.org/10.1007/s11248-009-9265-0

Lu Y, Wu K, Jiang Y, et al (2012) Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. **Nature** 487:362–365. https://doi.org/10.1038/nature11153

Malaquias JB, Caprio MA, Godoy WAC, et al (2020) Experimental and theoretical landscape influences on *Spodoptera frugiperda* movement and resistance evolution in contaminated refuge areas of Bt cotton. **Journal of Pest Science** 93:329–340. https://doi.org/10.1007/s10340-019-01145-1

Morris M, Dreher K, Ribaut JM, Khairallah M. (2003) Money matters (II): costs of maize inbred line conversion schemes at CIMMYT using conventional and marker-assisted selection. Molecular Breeding 11:235-247. https://doi.org/10.1023/A:1022872604743

Munsch MA, Stamp P, Christov NK, et al (2010) Grain yield increase and pollen containment by plus-hybrids could improve acceptance of transgenic maize. **Crop Science** 50:909–919. https://doi.org/10.2135/cropsci2009.03.0117

Naranjo SE (2009) Impacts of Bt crops on non-target invertebrates and insecticide use

patterns. **CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources** 4:. https://doi.org/10.1079/PAVSNNR20094011

Nboyine JA, Kusi F, Abudulai M, et al (2020) A new pest, *Spodoptera frugiperda* (J.E. Smith), in tropical Africa: Its seasonal dynamics and damage in maize fields in northern Ghana. **Crop Protection** 127:104960. https://doi.org/10.1016/j.cropro.2019.104960

Omoto C, Bernardi O, Salmeron E, et al (2016) Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil. **Pest management science** 72:1727–1736. https://doi.org/10.1002/ps.4201

Onstad DW, Mitchell PD, Hurley TM, et al (2011) Seeds of change: corn seed mixtures for resistance management and integrated pest management. **Journal of Economic Entomology** 104:343–352. https://doi.org/10.1603/ec10388

Oyediran I, Dively G, Huang F, Burd T (2016) Evaluation of European corn borer *Ostrinia nubilalis* (Lepidoptera: Crambidae) larval movement and survival in structured and seed blend refuge plantings. **Crop Protection** 81:145–153. https://doi.org/10.1016/j.cropro.2015.12.008

Pannuti LER, Paula-Moraes SV, Hunt TE, et al (2016) Plant-to-plant movement of *Striacosta albicosta* (Lepidoptera: Noctuidae) and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Maize (Zea mays). **Journal of Economic Entomology** 109:1125–1131. https://doi.org/10.1093/jee/tow042

Perry JN, Devos Y, Arpaia S, et al (2010) A mathematical model of exposure of nontarget Lepidoptera to Bt-maize pollen expressing Cry1Ab within Europe. **Proceedings of the Royal Society B: Biological Sciences** 277:1417–1425. https://doi.org/10.1098/rspb.2009.2091

Poehlman, JM (1986) Breeding field crops. 3nd. Van Nostrand Reinhold: New York, p. 724

Reay-Jones FPF, Bessin RT, Brewer MJ, Buntin DG, Catchot AL, Cook DR, et al (2016) Impact of lepidoptera (Crambidae, Noctuidae, and Pyralidae) pests on corn containing pyramided Bt traits and a blended refuge in the Southern United States. Journal of Economic Entomology 109:1859-1871. https://doi.org/10.1093/jee/tow109

Ribaut JM, Ragot M (2007) Marker-assisted selection to improve drought adaptation in maize: The backcross approach, perspectives, limitations, and alternatives. **Journal of Experimental Botany** 58:351–360. https://doi.org/10.1093/jxb/erl214

Roessler K, Muyle A, Diez CM, et al (2019) The genome-wide dynamics of purging during selfing in maize. **Nature Plants** 5:980–990. https://doi.org/10.1038/s41477-019-0508-7

Santos LF, Ruiz-sánchez E, Andueza-noh RH, et al (2020) Leaf damage by *Spodoptera frugiperda* J . E . Smith (Lepidoptera : Noctuidae) and its relation to leaf morphological traits in maize landraces and commercial cultivars. **Journal of Plant Diseases and Protection** 127:103–109. https://doi.org/10.1007/s41348-019-00276-y

Sisay B, Simiyu J, Mendesil E, Likhayo P, Ayalew G, Mohamed S, et al (2019). Fall armyworm, Spodoptera frugiperda infestations in East Africa: Assessment of damage and parasitism. Insects 10:1-11. https://doi.org/10.3390/insects10070195

Sprague GF (1932) The nature and extent of hetero-fertilization in maize. **Genetics** 17: 358

Sridevi G, Parameswari C, Rajamuni P, Veluthambi K (2006) Identification of hemizygous and homozygous transgenic rice plants in T1 generation by DNA blot analysis. **Plant Biotechnology** 23:531–534. https://doi.org/10.5511/plantbiotechnology.23.531

Storer NP, Babcock JM, Schlenz M, et al (2010) Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico.

Journal of Economic Entomology 103:1031–1038. https://doi.org/10.1603/ec10040

Tabashnik BE, Brévault T, Carrière Y (2013) Insect resistance to Bt crops: Lessons from the first billion acres. **Nature Biotechnology** 31:510–521. https://doi.org/10.1038/nbt.2597

Tollenaar M and Dwyer LM (1999) Physiology of maize. In.: Smith DL, Hamel C (Eds.) **Crop Yield.** Berlin: Springer, p. 169-204.

Travella S, Ross SM, Harden J, et al (2005) A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. **Plant Cell Reports** 23:780–789. https://doi.org/10.1007/s00299-004-0892-x

Trtikova M, Wikmark OG, Zemp N, et al (2015) Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. **PLoS ONE** 10:1–9. https://doi.org/10.1371/journal.pone.0123011

Weingartner U, Kaeser O, Long M, Stamp P (2002) Combining cytoplasmic male sterility and xenia increases grain yield of maize hybrids. **Crop Science** 42:1848–1856. https://doi.org/10.2135/cropsci2002.1848

Yang F, Kerns DL, Brown S, et al (2017) Pollen contamination in seed mixture increases the dominance of resistance to Bt maize in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). **Pest Management Science** 73:2379–2385. https://doi.org/10.1002/ps.4631

Yang F, Kerns DL, Head GP, et al (2014) A challenge for the seed mixture refuge strategy in Bt maize: Impact of cross-pollination on an ear-feeding pest, corn earworm. **PLoS ONE** 9:1–9. https://doi.org/10.1371/journal.pone.0112962

Yang F, Kerns DL, Head GP, et al (2020) Extended evaluation of Bt protein cross-pollination in seed blend plantings on survival, growth, and development of *Helicoverpa*

zea feeding on refuge ears. **Pest Management Science** 76:1011–1019. https://doi.org/10.1002/ps.5611

Zhu G, Chereddy SCRR, Howell JL, Palli SR (2020) Genome editing in the fall armyworm, *Spodoptera frugiperda*: Multiple sgRNA/Cas9 method for identification of knockouts in one generation. **Insect Biochemistry and Molecular Biology** 103373. https://doi.org/10.1016/j.ibmb.2020.103373

CHAPTER 2 - The implications of homozygous vip3Aa20- and cry1Ab-maize on *Spodoptera frugiperda* control[1]

[1] This chapter corresponds to the scientific article submitted to the Journal of Pest Science and is currently under review for publication.

Kian Eghrari[1*]; Serena Capriogli Oliveira[2]; Amanda Maria Nascimento[3]; Bruna Queiroz[4]; Julio Fatoretto[2]; Bruno Henrique Sardinha de Souza[3]; Odair Aparecido Fernandes[1]; Gustavo Vitti Môro[1]

[1]São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, Jaboticabal, SP, Brazil.

[2]Syngenta Crop Protection, Av. Nações Unidas 18001, São Paulo 04795-900, Brazil.

[3]Federal University of Lavras, Lavras, MG, Brazil.

[4]Departamento de Entomologia e Acarologia, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ/USP), Av. Pádua Dias 11, Piracicaba, São Paulo 13418-900, Brazil.

[*]Corresponding author: K Eghrari or GV Môro, Department of Agricultural Production Sciences, FCAV/UNESP – Rod. Prof. Paulo Donato Castelane, km 5, Jaboticabal, SP, Brazil, 14884-900. E-mail: kianem@gmail.com (K. Eghrari); gv.moro@unesp.br (GV Môro).

Orcid of the authors: KE: 0000-0003-0132-1175; SCO: 0000-0002-3801-514X; AN: 0000-0002-0020-1350; BQ: 0000-0002-0636-752X; JF: 0000-0001-6840-3584; BHSS: 0000-0002-2802-2688; OAF: 0000-0003-3489-4754; GVM: 0000-0001-7709-7814.

ABSTRACT

Historically, Bt maize hybrids have been produced with the Bt alleles in a hemizygous state. Homozygous Bt transgenes increase the expression of Bt proteins in plants; however, recent studies have only briefly explored the zygosity of Bt transgenes in maize hybrids and its effects on the control of lepidopteran pests. In this study we investigated whether an additional allelic dose of the single Bt events Bt11 and MIR162, and a pyramided version (Bt11 + MIR162 + GA21) would impact Bt protein expression in leaves and increase the control of fall armyworm, *Spodoptera frugiperda*, in leaves and grains of near-isogenic maize hybrids. Our results revealed that homozygous Bt hybrids had higher Cry1Ab and Vip3Aa20 protein concentrations than their hemizygous versions in leaves. However higher concentrations of Bt proteins in homozygous versions did not increase the control of *S. frugiperda*. Hybrids expressing Cry1Ab, hemizygous and homozygous for the transgene, had similar mortality on *S. frugiperda* that fed on leaves and grains as the non-Bt near-isogenic version, likely due to resistance of the pest to Cry1Ab. High susceptibility to Vip3Aa20 caused all larvae to die on hemizygous and homozygous MIR162 leaves. Also, larvae that survived on Vip3Aa20 grains did not gain weight after four days of feeding, regardless of the Bt zygosity of Vip3Aa20-expressing grains. Additional implications of Bt zygosity in maize hybrids on insect resistance management strategies are discussed.

Keywords: Bt protein quantification; Bt pyramiding; fall armyworm; high-dose event; kernel; *Zea mays*.

KEY MESSAGE

- Homozygous Bt11 and MIR162 events, either singular or pyramided, increased the expression of Cry1Ab and Vip3Aa20 proteins in maize leaves.
- The Bt11 event in hemizzygous or homozygous state was not efficient in controlling field populations of *S. frugiperda*, likely due to their resistance to Cry1Ab.
- Regardless of MIR162's zygosity and pyramiding with Bt11, the presence of Vip3Aa20 controlled all *S. frugiperda* larvae on leaves and kernels, likely because of their high susceptibility to Vip3Aa20.

Declarations

Funding: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, and the National Council for Scientific and Technological Development - Brasil (CNPq).This study was also financed in part by Syngenta Crop Protection.

Conflicts of interest/Competing interests: Not applicable

Availability of data and material: Data will be available if needed.

Code availability: R scripts will be provided if needed.

Authors' contributions: KE, JF, BHSS, OAF and GVM conceived and designed research. KE, SCO, BQ, AMN and BHSS conducted experiments. KE, AMN, JF, BHSS, OAF and GVM analyzed data. KE, BHSS, OAF and GVM wrote the manuscript. All authors read and approved the manuscript.

1. INTRODUCTION

The main pest of maize in the Americas, fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), has been detected in new territories, such as African and Asian countries (Botha et al. 2019; Li et al. 2020), and, therefore, increased the concern of farmers and scientists worldwide (Silver 2019; Amaral et al. 2020). Maize is the most important cereal crop in the world (FAO 2018), and the use of Bt proteins is key to safeguarding its production (Hutchison et al. 2010; Storer et al. 2012; Amaral et al. 2020). Before the transgenics era, *S. frugiperda* was mainly controlled using chemical insecticides (Young 1979; Yu 1991), which can cause mortality of beneficial insects (Tavares et al. 2010) and contaminate the soil (Silva et al. 2019).

Although Bt maize plays a leading role in *S. frugiperda* control in the Americas, this technology is not available in every country (ISAAA 2019). Furthermore, field-evolved resistance of *S. frugiperda* to Cry1 proteins has been reported in countries such as Puerto Rico (Storer et al. 2010), United States (Huang et al. 2014), and Brazil (Farias et al. 2014; Omoto et al. 2016); however, Vip3Aa20 still controls *S. frugiperda* efficiently (Amaral et al. 2020). Therefore, deployment of insect resistance management (IRM) strategies is necessary to ensure that Bt proteins continue to be effective in controlling *S. frugiperda* (Tabashnik et al. 2013). Additionally, the evolution of *S. frugiperda* resistance to Bt proteins accelerates with non-compliance of the refuge strategy (Carrière et al. 2016), inaccurate use of Bt proteins that do not meet the high-dose event criterion (Andow 2008), occurrence of initial high resistant alleles frequency in the field (Farias et al. 2016), and the continued use of single Bt events (Ives et al. 2011; Santos-Amaya et al. 2015).

Zygosity of Bt transgenes in maize hybrids has been recently and only briefly explored (Caprio et al. 2015; Eghrari et al. 2019; Carrière et al. 2020). Predominantly, Bt maize hybrids are hemizygous for their Bt genes (Law et al. 2006; Burkness et al. 2010; Caprio et al. 2015; Eghrari et al. 2019; Carrière et al. 2020) in countries where transgenic maize crops are grown. However, it is possible to produce homozygous transgenic hybrids (Law et al. 2006; Hood et al. 2012; Eghrari et al. 2019). The production of homozygous Bt hybrids requires the introgression of Bt genes not only to one parental inbred line, but both (Howard and Hood 2014).

Transgenes in homozygosis usually increase the expression of their protein products in the plant (Caligari et al. 1993; James et al. 2002; Clough et al. 2006; Hood et al. 2012; Howard and Hood 2014). In Bt hybrids, expression of the proteins Cry1F, Cry1A.105, and Cry2Ab2 increased 56%, 114%, and 146%, respectively, with correspondingly increase in *S. frugiperda* control both in the field and laboratory conditions (Eghrari et al. 2019). To our knowledge, Bt zygosity has been scarcely mentioned regarding refuge strategies, Bt gene flow, proportion of Bt and non-Bt kernels in maize ears, and insect mortality in maize kernels (Carrière et al. 2020). All these issues are very important to consider and have implications on IRM. Thus, this study aimed to assess the implications of Bt zygosity on Vip3Aa20 and Cry1Ab expressions in maize and its effect on *S. frugiperda* control. Additionally, we discuss some of the implications of Bt zygosity on insect resistance management strategies.

2. MATERIALS AND METHODS

2.1. Genetic material

The genetic material comprised seven isogenic versions of the Status (Syngenta Seeds, São Paulo, Brazil) hybrid: conventional (non-Bt); hemizygous (HE) and homozygous (HO) versions of the Bt11 event, expressing Cry1Ab (Bt11-HE and Bt11-HO, respectively); HE and HO versions of the event MIR162, which express Vip3Aa20 (Vip-HE and Vip-HO, respectively); and HE and HO versions of the pyramided event Bt11 x MIR162 x GA21, expressing the Bt proteins Cry1Ab and Vip3Aa20 (Vip3-HE and Vip3-HO, respectively).

2.2. Field experiment design

Four experiments were carried out in four growing seasons, as follows: two in Jaboticabal, SP, Brazil (first and second seasons of 2017/2018; sowing dates: DEC/04/2017 and JAN/23/2018; coordinates 21°14'53"S 48°17'08"W) and two in Lavras, MG, Brazil (first season of 2017/2018 and first season of 2018/2019; sowing dates: DEC/16/2017 and NOV/14/2018, respectively; coordinates 21°13'40"S 44°58'38"W). Plots of each experiment consisted of two 5-m rows in length spaced 0.5 m apart, with 15 plants per row, corresponding to a population of 60,000 plants ha^{-1}. The design was a randomized complete block with four replicates. The field plots were utilized for leaf sampling aiming at absolute Bt protein quantification (section 2.3), field evaluation of leaf injury caused by *S. frugiperda* larvae (section 2.4), and sampling for leaf tissue bioassays (2.5).

2.3. Quantification of Cry1Ab and Vip3Aa20 proteins in the leaf

Leaf collection was performed at both V4/V5 and V8/V9 growth stages (Magalhães and Durães 2006) in the first and second growing seasons of 2017/2018 in Jaboticabal. In Lavras, leaves were collected at the V5/V6 and V9/V10 growth stages, in the first growing seasons of 2017/2018 and 2018/2019. Each replicate was comprised of 10 plants of each hybrid version. Four leaf discs (0.8 cm diameter) were sampled from each maize whorl and pooled for quantification. Each experiment had four replicates, totaling 40 quantifications per hybrid in each growth stage. Leaf samples were kept in -80ºC and lyophilized before quantification. A total of 1920 samples were analyzed: six hybrids, 10 plants per replicate, four replicates per season, two growth stages per season, and four growing seasons. The presence of Cry1Ab and Vip3Aa20 proteins were assessed in all transgenic hybrids except in the non-Bt version.

Samples were prepared by adding steel beads to the 96-well blocks containing lyophilized tissue. The samples were ground using a Kleco grinder (Visalia, CA, USA) followed by adding 0.5 ml phosphate buffer saline containing 0.05% Tween-20 (PBST) and mixing well. The blocks were centrifuged at 3500 x g for 5 min. Total soluble protein (TSP) of each sample extract supernatant was measured using the Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL). The extract supernatants were diluted in ELISA diluent (PBS containing 1% BSA and 0.05% Tween-20) for subsequent testing in the ELISA.

To determine the expression levels of Cry1Ab and Vip3A, quantitative ELISA was performed using a duplex method to measure the amounts of Cry1Ab and Vip3A proteins in the same sample at the same time. High-binding 96-well plates (Nunc

Maxisorp) were coated with 2 μg/ml rabbit anti-Cry1Ab plus 2 μg/ml goat anti-VIP3A in 25 mM borate, 75 mM NaCl, pH 8.5 and incubated at 4°C overnight (100 µl/well). Plates were washed three times with phosphate buffer saline pH 7.3 (PBS) containing 0.05% Tween-20 (PBST). Samples or standards in ELISA diluent (PBST containing 1% bovine serum albumin) were added to the plate (100 μl/well), incubated for 1 h at room temperature (RT) with shaking, and washed five times. One hundred μl/well of 0.5 µg/mL HRP-rabbit anti-VIP3A, 5 µg/mL biotin-sheep anti-Cry1Ab, and 0.2 µg/mL streptavidin-AP (Jackson ImmunoResearch Labs, West Grove, PA) in ELISA diluent was added to the plate, incubated for 1 h at RT/shaking, and washed as before. Substrate p-nitrophenyl phosphate (SurModics, Eden Prairie, MN) was added (100 μl/well) and allowed to develop for 30 min at room temperature. Absorbance was measured at 405 nm using a microplate reader (BioTek Powerwave XS2, Winooski, VT) for quantifying Cry1Ab. The plate was washed as before. Substrate tetramethylbenzidine (SurModics, Eden Prairie, MN) was added (100 μl/well) and allowed to develop for 30 min at room temperature with shaking. The reaction was stopped using 1 N HCl (100 µl/well). Absorbance was measured at 450 nm for detecting Vip3A. The standard curve used a four-parameter curve fit to plot the concentrations versus the absorbance. To normalize for extraction efficiency, the concentration of the analyte (Cry1Ab or Vip3A) was divided by the concentration of the total soluble protein (TSP).

2.4. Field evaluation of *S. frugiperda* leaf injury

Leaf injury by *S. frugiperda* larvae in the field was assessed following a modified 0-9 injury score scale (Davis et al. 1992), in which the lowest score represents no injury

and the highest score refers to destruction of the maize whorl. In Jaboticabal, each plot consisted of 16 plants assessed at the V4/V5 and V8/V9 growth stages, while in Lavras each plot consisted of 12 plants assessed at the V5/V6 and V9/V10 growth stages. The experiments were designed in randomized complete block with four replications. Evaluations of *S. frugiperda* injury, under natural infestation, took place in three growing seasons, being two in Jaboticabal and one in Lavras (first season 2017/2018).

2.5. Leaf tissue bioassays

Bioassays were conducted during the first and second growing seasons of 2017/2018 in Jaboticabal using V4/V5 and V8/V9 maize leaf tissues. In Lavras, the bioassays were installed at the V5/V6 and V9/V10 growth stages in the first season of 2017/2018. Leaves were collected from the hybrids in the field, stored in styrofoam boxes with ice at the bottom and taken to the laboratory. Leaf tissues were washed in 0.5% sodium hypochlorite solution, rinsed in tap water, and dried with paper towels.

Natural populations of *S. frugiperda* from each site were used for the bioassays. Nearly 15 egg masses in each site were collected from the border maize plants of the field experiments. The egg masses were allowed to hatch in laboratory condition and neonates were fed on artificial diet (Kasten et al. 1978) until pupation. After emergence, adults were kept in Polyvinyl chloride (PVC) cages (10 cm diameter x 25 cm high) covered with voile fabric for mating. The inner wall of the cages was covered with white paper sheet as substrate for oviposition. F1 generation of neonates was used in the leaf tissue bioassay.

Replicates of the bioassays consisted of 12 individualized *S. frugiperda* neonates (< 24-h old) in Petri dishes with a piece (~5 x 3 cm) of maize leaf placed over a sheet of

paper towel moistened with distilled water. The design was a randomized complete block with five replications, and all Petri dishes were kept in ambient controlled room (25±1ºC, 60±10% RH, 12L:12D). Larval survival and weight were determined after seven days of feeding. Larvae were considered dead when they did not move after a delicate poke with a fine paintbrush. Weight of surviving larvae was assessed with the aid of an analytical scale (E02140, Ohaus Corporation, Parsippany, USA).

2.6. R3-grain bioassays

Separate plots of maize hybrids in the field were used for R3-grain bioassays. Each plot consisted of a 5-m row with 15 plants, spaced at 0.5 m. During the first growing season of 2018/2019, each maize hybrid was selfed to avoid pollen contamination of the ears and to simulate a F1 hybrid maize field. The main ear of each plant was protected with a plastic bag (10 x 20 cm x 0.6 mm) before silking, and tassels were covered with paper bags (17.5 x 38 cm) on the first day of anthesis. On the second day of anthesis, pollination took place by removing the plastic bag protecting each ear and using the paper bag covering the tassel of the same plant to self-fertilize it. Approximately 18-day-old ears in the R3 growth stage were harvested.

Each replicate of the bioassays consisted of 12 third/fourth-instar *S. frugiperda* larvae, collected in Jaboticabal maize fields during the same year and previously reared on artificial diet (Kasten Jr. et al. 1978) for one generation, with five R3-stage kernels placed on a dry sheet of paper towel in a Petri dish. Each kernel was carefully detached from the maize ear to avoid rupture of the pericarp. Each larva was weighed right before being released in the dish.

Two bioassays were conducted in Jaboticabal in randomized complete block with five replicates (n = 60). In the first bioassay, three corn ears were used: one for

the first replicate, the second for replicates two and three, and the third for replicates four and five. In the second bioassay, five ears were available, and kernels of each ear represented a replicate. Survival and weight of surviving larvae were recorded after four days of feeding (25ºC ± 1, 60 ± 10% RH, 12L:12D).

2.7. Statistical analysis

ANOVA was performed and followed by LSD tests to compare mean concentrations of Cry1Ab and Vip3Aa20 proteins in leaves, and means of leaf injury of *S. frugiperda* in the field among the near-isogenic hybrids within each season and growth stage. Data of larval survival for the leaf-tissue and R3-grain bioassays were submitted to a binomial generalized linear model followed by analysis of deviance to identify the simplest and more complete binomial model. Confidence intervals (95%) were estimated based on the fitted binomial generalized linear model and used to compare the means of larval survival on each hybrid version within each bioassay. Mean weight of surviving larvae on each near-isogenic version was compared using LSD tests to show weight gain at the end of each bioassay. Statistical analysis was performed in R software (R Core Team 2019).

3. RESULTS

3.1. Quantification of Cry1Ab and Vip3Aa20 in the leaf

3.1.1. Cry1Ab quantification

Cry1Ab concentrations between HE and HO for Bt11 did not differ in the first season (2017/2018) at the Jaboticabal site at the V4 growth stage. Cry1Ab concentrations were also not different between the HE and HO versions of the

pyramided Vip3 near-isogenic hybrid. Among pyramided and single event near-isogenic hybrids, Cry1Ab expression was higher in Vip3-HO than in Bt11-HE plants at the V4 growth stage ($t_{3, 12}$ = 2.179, P < 0.05). At V8 growth stage, Vip3-HO had higher Cry1Ab concentrations than the other near-isogenic versions ($t_{3, 12}$ = 2.179, P < 0.05) (Fig. 1a). Expression of Cry1Ab at V8 was 3.87-fold higher than at V4 for Bt11-HE ($t_{1, 6}$ = 5.959, P < 0.001), 2.47 for Vip3-HE ($t_{1, 6}$ = 5.959, P < 0.001), and 3.00 for Vip3-HO ($t_{1, 6}$ = 5.959, P < 0.001), but did not differ for Bt11-HO.

Similarly to the first season in Jaboticabal, Cry1Ab of Bt11-HE and Bt11-HO did not differ in the second season (2018) at V4. The Vip3-HO version had higher Cry1Ab concentrations, 1.53-fold, than its HE version ($t_{3, 12}$ = 3.054, P < 0.01). At V8, concentrations of Cry1Ab were higher in the HO versions of Bt11 and Vip3 when compared to their HE versions ($t_{3, 12}$ = 3.054, P < 0.01). Vip3-HO expressed more Cry1Ab than the other near-isogenic versions ($t_{3, 12}$ = 3.054, P < 0.01) (Fig. 1b). The HE and HO versions of Bt11 hybrids expressed greater Cry1Ab protein concentrations at V8 relative to V4 stage, 1.66- and 1.88-fold, respectively ($t_{1, 6}$ = 5.959, P < 0.001). The same trend was observed for Vip3 hybrids, with the HE version expressing 1.64-fold ($t_{1, 6}$ = 3.707, P < 0.01) and the HO version 2.22-fold ($t_{1, 6}$ = 5.959, P < 0.001) at V8 relative to V4 stage.

In the 2017/2018 season in Lavras, Bt11-HO had higher concentrations of Cry1Ab than Bt11-HE at V5 ($t_{3, 12}$ = 3.054, P < 0.01). At V9, Vip3-HO expressed higher Cry1Ab concentrations than Vip3-HE, Bt11-HO, and Bt11-HE at V5 and V9 ($t_{3, 12}$ = 3.054, P < 0.01) (Fig. 1c). Protein expressions were higher at V5 than at V9 ($t_{1, 6}$ = 2.447, P < 0.05).

In the following season in Lavras (2018/2019), Cry1Ab concentrations of Bt11-HO were higher than Bt11-HE at V5 and V9 ($t_{3, 12}$ = 2.179, P < 0.05). For Vip3 versions, Cry1Ab concentrations did not differ from HE and HO versions at V5 and V9. However, Vip3-HO Cry1Ab concentrations were higher than the HE and HO Bt11 versions at V5 ($t_{3, 12}$ = 2.179, P < 0.05), and higher than Bt11-HE at V9 ($t_{3, 12}$ = 3.054, P < 0.01) (Fig. 1d). Only Bt11-HO expressed more Cry1Ab at V9 than at V5 ($t_{1, 6}$ = 3.054, P < 0.01). The concentrations of Cry1Ab did not differ between V5 and V9 for the other near-isogenic hybrids.

3.1.2. Vip3Aa20 quantification

In the first season in Jaboticabal at V4, Vip-HO had higher Vip3Aa20 concentrations in the leaves than Vip-HE ($t_{3, 12}$ = 4.318, P < 0.001), while the pyramided Vip3-HE and Vip3-HO versions did not differ. At V8, Vip3-HO expressed more Vip3Aa20, 4.14-fold, than its HE version ($t_{3, 12}$ = 4.318, P < 0.001). Additionally, at V4 Vip-HO had higher Vip3Aa20 concentrations than any other near-isogenic version ($t_{3, 12}$ = 4.318, P < 0.001), while at V8 Vip3-HO was the version that had the highest Vip3Aa20 concentration ($t_{3, 12}$ = 4.318, P < 0.001) (Fig. 2a). Comparing Vip3Aa20 concentrations of each version at V4 and V8, all near-isogenic hybrids expressed more protein at V8 ($t_{1, 6}$ = 5.959, P < 0.001), ranging from 3.94- to 15.63-fold.

In the second season in Jaboticabal, Vip-HO did not differ from Vip-HE, but Vip3-HO expressed higher Vip3Aa20 concentrations than Vip3-HE and the other Vip near-isogenic hybrids at V4 ($t_{3, 12}$ = 3.054, P < 0.01). At V8 stage, the concentration of Vip3Aa20 in Vip-HO and Vip3-HO was 3.58- and 5.22-fold higher than their respective HE versions ($t_{3, 12}$ = 3.054, P < 0.01) (Fig. 2b). Except for Vip3-HE, V8-stage plants expressed more Vip3Aa20 than V4-stage plants ($t_{1, 6}$ = 3.054, P < 0.01).

In both seasons at Lavras, Vip-HO and Vip3-HO had higher concentrations than their HE versions at V5 and V9 ($t_{3, 12}$ = 3.054, P < 0.01). On average, Vip-HO and Vip3-HO expressions were 2.21- and 1.98-fold higher than Vip-HE and Vip3-HO, respectively (Fig. 2c). In 2017/2018, Vip3Aa20 concentrations were higher at V5 in Vip-HE and Vip3-HO than at V9 ($t_{1, 6}$ = 2.447, P < 0.05), while they did not differ for Vip-HO and Vip3-HE. In 2018/2019, all hybrid versions expressed more Vip3Aa20 at V9 (2.78-fold higher on average) than at V5 ($t_{1, 6}$ = 3.054, P < 0.01).

3.2. Evaluation of *S. frugiperda* leaf injury

In Jaboticabal during the first season, there was no significant difference in injury scores between the HO and HE versions of the hybrids Bt11, Vip, and Vip3 at V4. The non-Bt hybrid showed higher injury scores due to *S. frugiperda* herbivory than the transgenic hybrids ($t_{6, 21}$ = 2.831, P < 0.01), with 5.50 ± 0.33 mean injury score, and Bt11 versions had higher injury scores than hybrids expressing Vip3Aa20 ($t_{6, 21}$ = 2.831, P < 0.01) (Fig. 3a). At the V8 stage, HO and HE hybrids of single and pyramided events did not differ for *S. frugiperda* injury. The non-Bt hybrid did not differ from the HO and HE versions of Bt11, although non-Bt and Bt11 versions had higher injury than Vip3Aa20-expressing hybrids ($t_{6, 21}$ = 2.831, P < 0.01) (Fig. 3b).

During the second season in Jaboticabal, leaf injury caused by *S. frugiperda* did not differ between HO and HE versions of Bt11, Vip, and Vip3 hybrids at V4 and V8. The non-Bt hybrid presented higher injury than the other hybrids but Bt11-HO at V4 and Bt11-HE at V8 ($t_{6, 21}$ = 2.831, P < 0.01) (Fig. 3c and 3d). Hybrids expressing Vip3Aa20 did not present mean injury scores higher than 2 at V4 and 1 at V8.

In Lavras at V5 and V9, mean injury scores did not differ between HO and HE versions of the transgenic hybrids. Injury score on the non-Bt and Bt11-HE was higher at V5 ($t_{6, 21}$ = 2.080, $P \leq 0.05$) (Fig. 3e), and higher on Bt11-HE at V9 ($t_{6, 21}$ = 3.819, $P < 0.001$), when compared to plants expressing Vip3Aa20 (Fig. 3f).

3.3. Leaf tissue bioassays

In both V4 and V8 bioassays in both seasons at Jaboticabal, survival of *S. frugiperda* larvae did not differ between HO and HE versions of any transgenic versions. Larval survival on the non-Bt hybrid, Bt11-HE, and Bt11-HO did not differ in the bioassays conducted in Jaboticabal during both seasons as well at V4 and V8, and was above 60% (Fig 4a-4d). In Lavras, HE and HO versions did not differ for larval survival at V5 and V9 (Fig. 4e and 4f). At V9, *S. frugiperda* larval survival in non-Bt leaves was higher than in the Bt11 hybrids (95% CI) (Fig. 4f). Vip3Aa20-expressing hybrids caused complete larval mortality of *S. frugiperda* in both sites, seasons and growth-stages (Fig. 4).

The weight of surviving *S. frugiperda* larvae did not differ between Bt11-HE and Bt11-HO at any bioassay (Fig. 5), except at V5 in Lavras when larvae fed with Bt11-HE gained more weight than Bt11-HO ($t_{2, 11}$ = 4.437, $P < 0.001$) (Fig. 5e). Also, weight of larvae fed with the non-Bt version was higher than those fed with Bt11 hybrids in V5 Jaboticabal and V9 Lavras ($t_{2, 11}$ = 3.106, $P < 0.01$) (Fig. 5c and 5f).

3.4. R3 grain bioassays

Survival of *S. frugiperda* third/fourth instars did not differ between HO and HE versions of the Bt11 and Vip hybrids in the two R3-grain bioassays conducted in

2018/2019 at Jaboticabal (Fig. 6a and 6b). For Vip3 hybrids, larval survival did not differ in the first bioassay (Fig. 6a), however it was lower on HO selfed kernels (100% mortality) than on HE kernels in the second bioassay (95% CI) (Fig. 6b). Survivorship did not differ between the non-Bt selfed kernels and Bt11 kernels. Selfed kernels of Vip3Aa20-expressing hybrids had lower larval survival than Bt11 and non-Bt selfed kernels in both bioassays (95% CI).

The mean larval weight of surviving *S. frugiperda* did not differ between HO and HE versions of the transgenic hybrids, except for the Vip3-HO treatment that had 100% larval mortality in the second bioassay ($t_{6,\ 28}$ = 2.048, P < 0.05) (Fig. 6c and 6d). Surviving larvae on kernels originated from Vip3Aa20-expressing hybrids did not gain weight after four days and had less than 10 times the weight of larvae fed with kernels from Bt11 versions and the non-Bt ($t_{6,\ 28}$ = 2.048, P < 0.05). The mean weight of larvae fed with Bt11 versions and the non-Bt did not differ, except for the Bt11-HO treatment that had lower larval weight than the non-Bt in the first bioassay ($t_{6,\ 28}$ = 2.048, P < 0.05).

4. DISCUSSION

The additional allele of the Bt11 and MIR162 events increased the concentrations of Cry1Ab and Vip3Aa20 in the leaves, respectively. Despite that, more expression of Cry1Ab and Vip3Aa20 proteins in HO plants did not result in differences in leaf injury or mortality of *S. frugiperda* in field and laboratory conditions. Thus, contrary to our previous results with Bt pyramided (Cry1F + Cry1A.105 + Cry2Ab2) maize hybrids (Eghrari et al. 2019), this study suggests that an increase in Bt zygosity

may not contribute to higher control of *S. frugiperda*, likely due to *S. frugiperda* resistance to Cry1Ab and high-susceptibility to Vip3Aa20.

Although the expression of Cry1Ab increased ~1.5-fold in the HO version with only the Bt11 event, it was neither sufficient to reduce injury of *S. frugiperda* in the field experiments nor to increase mortality in the bioassays with leaves and grains. Compared to the non-Bt version, HO and HE Bt11 hybrids showed similar leaf injury in the field and survival rates in both the leaves and R3-grains under laboratory conditions. Cry1Ab protein was the first Bt protein commercially released for maize crop in Brazil targeting lepidopteran pests in the MON810 (CTNBio 2007) and Bt11 (CTNBio 2008) maize events. However, the effectiveness against *S. frugiperda* has decreased over the past years (Bernardi et al. 2015a), and therefore, the use of maize hybrids expressing Cry1Ab is not an effective strategy for the control of this insect pest (Omoto et al. 2016). Our results strongly suggest that the field populations of *S. frugiperda* used in this study were resistant to Cry1Ab. New studies with susceptible (SS) and heterozygous (RS) *S. frugiperda* fed with HO and HE Cry1Ab maize are needed to indicate if zygosity would contribute to differences in larval mortality, like it was reported for HO hybrids expressing Cry1F + Cry1A.105 + Cry2Ab2 (Eghrari et al. 2019).

MIR162 event expressing the Vip3Aa20 protein, regardless of the transgene's zygosity, allowed to maintain low levels of *S. frugiperda* field infestation resulting in injury scores below 2 on average, with only superficial scrape injury of less than 1 cm in length on the leaves. In addition, leaves of all evaluated MIR162 hybrids caused total mortality of *S. frugiperda* neonates, which also stresses the efficacy of HE MIR162 hybrids in controlling *S. frugiperda*, even though they present lower Vip3Aa20 protein

concentrations in the leaves when compared to HO versions. In contrast, results of the R3-grains bioassays showed a rather high survival of third/fourth instars (30.00 ± 2.80% for the first and 5.83 ± 2.85% for the second bioassay). However, larvae that were fed MIR162 R3-grains did not gain weight, and, therefore, the Vip3Aa20 protein might have deterred the third/fourth-instar larvae from feeding on the grains because of toxic effects and high susceptibility of the populations to Vip3Aa20. In contrast, the surviving larvae on Bt11 R3-grains gained at least 30 mg within four days of feeding, which represent three times their initial weight and ~64% of the weight observed on larvae fed on non-Bt grain.

Protein concentrations of transgenes in maize vary according to the season and environment (Trtikova et al. 2015; Gutha et al. 2018), growth-stage (Nguyen and Jehle 2007; Székács et al. 2010), tissues (CTNBio 2008; 2009), and zygosity (Eghrari et al. 2019; Carrière et al. 2020). We noticed that Cry1Ab expression always increased from V4 to V8 in Jaboticabal, whereas in Lavras usually the concentration of Cry1Ab at V9 was lower than V5. Although we did not weighed our leaf samples, leaf thickness plays an important role in increasing Bt protein content (Nguyen and Jehle 2007; Székács et al. 2010), our results are consistent with studies that show that Cry1Ab concentrations peak after five weeks of cultivation, at V5/V6, and rapidly decline after V8 (Székács et al. 2010; Bernardi et al. 2015b). Regarding Vip3Aa20, we observerd an increase in concentrations from V4 to V8 in Jaboticabal, and from V5 to V9 in Lavras. Our results are consistent with higher concentrations of Vip3Aa20 at V6 than at V10 as reported by Bernardi et al. (2015b).

The concept of high-dose event requires that a Bt plant produces sufficient Bt protein concentration to kill more than 95% of the progeny originated between a

resistant (RR) and a susceptible (SS) insect strain (Andow 2008; Tabashnik et al. 2009; Huang et al. 2011). When high-dose Bt protein is associated with the refuge strategy, resistance allele frequency and the dominance of the resistant trait is assumed to remain low enough so that heterozygous individuals (RS) of a target pest will not be able to pass their resistant alleles to the next generation (Huang et al. 2011). The current investigation revealed that increasing Cry1Ab expression was inefficient to control field populations of *S. frugiperda*. Furthermore, in leaf bioassays, the Bt11 events in either zygosity had similar performance as the non-Bt hybrid, indicating that *S. frugiperda* populations from Jaboticabal and Lavras, which are located in different states of Brazil, are resistant to Cry1Ab, as it was already reported in other regions of the country (Omoto et al. 2016). To illustrate that, even though HO Bt11 hybrids increased Cry1Ab expression, there was no increased mortality in the bioassays with leaves and grains, nor reduced leaf injury in the field experiments. Nevertheless, it was reported that Cry1F, Cry1A.105, and Cry2Ab2 concentrations increased ~1.5-, 2.0-, and 2.5-fold, respectively, in HO hybrids relative to their near-isogenic versions, and, as a result, *S. frugiperda* control increased 22% (Eghrari et al. 2019). Despite the reported increase in Bt proteins expressions and *S. frugiperda* control using HO Bt hybrids (Eghrari et al. 2019), it was not enough to comply with the high-dose strategy requirements (Andow 2008). On the other hand, our results showed that Vip3Aa20 killed all neonate larvae after seven days. Although resistant (RR) and heterozygous (RS) larvae of *S. frugiperda* were not considered in our bioassays, the results show the effectiveness of Vip3Aa20 in killing all evaluated larvae (n = 1440) from two field populations, likely susceptible to Vip3Aa20, that survived approximately 76, 73, and 82% on leaves of near-isogenic Bt11-HE, Bt11-HO, and non-Bt hybrids, respectively.

New studies are needed to observe the impacts on dominance of RS larvae fed with HO Bt hybrids.

The methodology used in the R3-grain bioassay comprised self-pollination of each F1 hybrid to simulate the F2 segregation in the kernels of an open-pollinated maize hybrid field. When the methodology was designed, we were not concerned about the different origin of the kernel tissues, but whether Bt R3-grains would express sufficient concentrations of Bt proteins to kill *S. frugiperda* larvae. Contrary to what was observed with larvae fed with Cry1Ab kernels, the concentration of Vip3Aa20 in the grains was sufficient to kill all field third/fourth-instar *S. frugiperda*. Nevertheless, we cannot infer that Vip3Aa20 expression in grains meets the requirements of a high-dose protein because heterozygous resistant larvae (RS) were not considered here (Andow 2008), but resistance might be recessive because Vip3Aa20 kernels killed more than 99.9% of a likely susceptible populations (US EPA 1998; Tabashnik and Carriere 2017). Contrary to the 5- to 10-fold lower levels of Cry1Ab concentrations in grains relative to leaves of Bt11 (CTNBio 2008), Vip3Aa20 grains present half of the highest concentration found in the plant (CTNBio 2009). Additionally, since third/fourth-instar *S. frugiperda* did not gain weight after four days, or died when feeding on Vip3Aa20 R3-grains, our results showed that Vip3Aa20 expression levels in the kernels efficiently control *S. frugiperda* larvae at least up to forth instar.

Third/fourth-instar *S. frugiperda* larvae did not gain weight after being fed with R3 kernels produced from self-pollinated HE and HO Vip3Aa20 hybrids. Since Bt traits follow a mendelian segregation, it is expected a 75% Bt expression in the filial tissues, embryo and endosperm, of the kernels of self-pollinated Bt HE plants (CTNBio 2008; Long et al. 2012; Lohn et al. 2020). However, Bt expression is present in all F2 kernels

of a HE Bt hybrid, as the pericarp is a maternally produced tissue (Caprio et al. 2015; Carrière et al. 2020). Consequently, since commercial hybrids are HE for the Bt transgenes (Law et al. 2006; Burkness et al. 2010; Caprio et al. 2015; Eghrari et al. 2019), all their kernels will express Bt proteins because the pericarp has the same genotype as the maternal plant, i.e., HE for the Bt trait (Carrière et al. 2020). The pericarp of kernels originated from HO Bt hybrids will also be HO for the Bt transgene, and might have higher Bt concentrations than HE pericarp, as there is a positive correlation between Bt zygosity and Bt protein concentrations in maize (Eghrari et al. 2019), however there are no studies considering Bt protein expression in the pericarp. Embryo and endosperm, which are both maternal and paternal tissues, are likely to increase their Bt concentrations with the increase of Bt zygosity (Carrière et al. 2020). Regardless of the zygosity of the embryo, endosperm, and pericarp, in this study there was no difference in *S. frugiperda* mortality between HE and HO self-pollinated ears for both Bt events.

As non-Bt plants pollinated by Bt pollen will generate Bt kernels (Chilcutt and Tabashnik 2004; Yang et al. 2014; Carrière et al. 2016; Carrière et al. 2020) and half of the pollen produced by HE Bt plants are Bt (Carrière et al. 2020), in a seed mixture refuge scenario with HO Bt hybrids the percentage of non-Bt plants with Bt kernels might be higher. Since all pollen generated by HO plants are Bt, Bt pollen contamination of refuge plants could contribute to increase the dominance of resistance of ear-feeding pests to Bt maize (Yang et al. 2017). We believe that further studies of Bt zygosity with kernels will elucidate whether HO Bt versions of maize hybrids constitute an important tool for *S. frugiperda* resistance management.

The methodology applied in this research focused more on the effect of Bt zygosity on Bt proteins concentrations in maize leaves; however, Bt zygosity in maize hybrids could have implications before, during and after the crop season. Some of these implications that can be further explored involve: 1) introgression of Bt traits in both parental inbred lines to produce HO hybrids that could increase costs and workload of a breeding program (Morris et al. 2003); 2) protection against lepidopteran pests is likely to increase and insecticide use may decline in hybrid seed production fields, once both parental inbred lines are introgressed with Bt traits; 3) novel designs of refuge strategies could be defined for ear-feeding pests considering cross-pollination between Bt and non-Bt plants (Yang et al. 2020), mosaic of Bt and non-Bt kernels in maize ears (Chilcutt and Tabashnik 2004; Yang et al. 2014; Carrière et al. 2016), number of Bt alleles in kernel tissues (Carrière et al. 2020), and segregation of single and pyramided Bt traits on F2 kernels (Yang et al. 2014); 4) farmers saving grains of the F2 generation of HO hybrids since all seeds inherit the Bt traits; and 5) risk of increasing seed piracy, e.g., creation of new Bt combinations that might not be approved for cultivation, feed, and food.

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, and the National Council for Scientific and Technological Development - Brasil (CNPq). We thank Syngenta Seeds Brazil and Gustavo Biudes for generating and providing the seeds, and Syngenta Corporation US for Bt quantification; Betyana Parody and Fabrício Francischini regarding the conduction of our partnership; Larah Martins for helping

with conducting the experiments in Lavras; Naiara Zancanari, Flávia Alves, Luiz Bertasello for helping in the experiments in Jaboticabal; and, finally, Betiana Parody, Renato Carvalho, Gustavo Biudes, Brian Rhodes, Eddie Ross, Nadine Guyot, Douglas Sumerford, Larry Zeph, and the anonymous reviewers of this journal for the valuable contributions to this article.

REFERENCES

Amaral FSA, Guidolin AS, Salmeron E, Kanno RH, Padovez FE, Fatoretto JC, Omoto C (2020) Geographical distribution of Vip3Aa20 resistance allele frequencies in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. Pest Manag Sci. https://doi.org/10.1002/ps.5490

Andow DA (2008) The risk of resistance evolution in insects to transgenic insecticidal crops. Colln Biosaf Rev 4:142-199. https://www.icgeb.org/wp-content/uploads/2019/01/4-The-Risk-of-Resistance-Evolution-in-Insects-to-Transgenic-Insecticidal-Crops-David-A.-Andow.pdf. Accessed 31 October 2020

Bernardi D, Salmeron E, Horikoshi RJ, Bernardi O, Dourado PM, Carvalho RA, Martinelli S, Head GP, Omoto C (2015a) Cross-resistance between Cry1 proteins in fall armyworm (*Spodoptera frugiperda*) may affect the durability of current pyramided Bt maize hybrids in Brazil. PLoS One 10:1-15. https://doi.org/10.1371/journal.pone.0140130

Bernardi O, Bernardi D, Amado D, Sousa RS, Fatoretto J, Medeiros FC, Conville J, Burd T, Omoto C (2015b) Resistance risk assessment of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and *Diatraea saccharalis* (Lepidoptera: Crambidae) to Vip3Aa20 insecticidal protein expressed in corn. J Econ Entomol 108:2711-

2719. https://doi.org/10.1093/jee/tov219

Botha AS, Erasmus A, du Plessis H, Van den Berg J (2019) Efficacy of Bt maize for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in South Africa. J Econ Entomol 112:1260–1266. https://doi.org/10.1093/jee/toz048

Burkness EC, Dively G, Patton T, Morey AC, Hutchison WD (2010) Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions: implications for resistance management. GM Crops 1:337-343. https://doi.org/10.4161/gmcr.1.5.14765.

Burkness EC, O'Rourke PK, Hutchison WD (2011) Cross-pollination of nontransgenic corn ears with transgenic Bt corn: efficacy against lepidopteran pests and implications for resistance management. J Econ Entomol 104:1476-1479. https://doi.org/10.1603/EC11081

Caligari PDS, Yapabandara YMHB, Paul EM, Perret J, Roger P, Dunwell JM (1993) Field performance of derived generations of transgenic tobacco. Theor Appl Genet 86:875-879. https://doi.org/10.1007/BF00212615.

Caprio MA, Martinez JC, Porter PA, Bynum E (2015) The impact of inter-kernel movement in the evolution of resistance to dual-toxin Bt-corn varieties in *Helicoverpa zea* (Lepidoptera: Noctuidae). J Econ Entomol 109:307-319. https://doi.org/10.1093/jee/tov295

Carrière Y, Degain BA, Harpold VS, Unnithan GC, Tabashnik BE (2020) Gene Flow Between Bt and Non-Bt Plants in a Seed Mixture Increases Dominance of Resistance to Pyramided Bt Corn in *Helicoverpa zea* (Lepidoptera: Noctuidae). J Econ Entomol 20:1-11. https://doi.org/10.1093/jee/toaa138

Carrière Y, Fabrick JA, Tabashnik BE (2016) Can pyramids and seed mixtures delay resistance to Bt crops? Trends Biotechnol 34:291-302. https://doi.org/10.1016/j.tibtech.2015.12.011

Chilcutt CF, Tabashnik BE (2004) Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. PNAS 101:7526-7529. https://doi.org/10.1073/pnas.0400546101

Clough RC, Pappu K, Thompson K, Beifuss K, Lane J, Delaney DE, Harkey R, Drees C, Howard JA, Hood EE (2006) Manganese peroxidase from the white-rot fungus *Phanerochaete chrysosporium* is enzymatically active and accumulates to high levels in transgenic maize seed. Plant Biotechnol J 4:53-62. https://doi.org/10. 1111/j.1467-7652.2005.00157.x.

Comissão Ténica Nacional de Biossegurança, CTNBio (2007) Parecer Técnico Nº 1100/2007. http://ctnbio.mcti.gov.br/liberacao-comercial. Accessed 17 July 2019

Comissão Ténica Nacional de Biossegurança, CTNBio (2008) Parecer Técnico Nº 1255/2008. http://ctnbio.mcti.gov.br/liberacao-comercial. Accessed 16 July 2019

Comissão Ténica Nacional de Biossegurança, CTNBio (2009) Extrato de Parecer Técnico N° 2042/2009. http://ctnbio.mcti.gov.br/liberacao-comercial. Accessed 16 July 2019

Davis FM, Ng SS, Williams WP (1992) Visual rating scales for screening whorl-stage corn resistance to fall armyworm. Mississippi State University, Mississipi State, USA.

Eghrari K, Brito AH, Baldassi A, Balbuena TS, Fernandes OA, Môro GV (2019) Homozygosis of Bt locus increases Bt protein expression and the control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize hybrids. J Crop Prot.

https://doi.org/10.1016/j.cropro.2019.104871

Farias JR, Andow DA, Horikoshi RJ, Sorgatto RJ, Fresia P, dos Santos AC, Omoto C (2014) Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. J Crop Prot 64:150-158. https://doi.org/10.1016/j.cropro.2014.06.019

Farias JR, Andow DA, Horikoshi RJ, Bernardi D, Ribeiro RDS, Nascimento ARD, Santos AC, Omoto C (2016) Frequency of Cry1F resistance alleles in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. Pest Manag Sci 72:2295-2302. https://doi.org/10.1002/ps.4274

Food and Agriculture Organization of the United Nations, FAO (2018) FAOSTAT Database. http://www.fao.org/faostat/en/#data/QC. Accessed 20 June 2020.

Gutha LR, Purushottam D, Veeramachaneni A, Tigulla S, Kodappully V, Enjala C, Rajput H, Anderson J, Hong B, Schmidt J, Bagga S (2018) Expression of endogenous proteins in maize hybrids in a multi-location field trial in India. Transgenic Res 27:331-42. https://doi.org/10.1007/s11248-018-0077-y

Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh KT, Requesens DV, Hayden C, Hood KR, Pappu KM, Carrol J, Howard JA (2012) Manipulating corn germplasm to increase recombinant protein accumulation. Plant Biotechnol J 10:20-30. https://doi.org/10.1111/j.1467-7652.2011.00627.x

Howard JA, Hood EE (2014) Commercial plant-produced recombinant protein products: Case studies. Springer-Verlag, Berlin.

Huang F, Andow DA, Buschman LL (2011) Success of the high-dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entomol Exp Appl 140:1-16. https://doi.org/10.1111/j.1570-7458.2011.01138.x

Huang F, Qureshi JA, Meagher RL, et al (2014) Cry1F resistance in fall armyworm *Spodoptera frugiperda*: Single gene versus pyramided Bt maize. PLoS One 9:. https://doi.org/10.1371/journal.pone.0112958

Hutchison WD, Burkness EC, Mitchell PD, Moon RD, Leslie TW, Fleischer SJ, Abrahamson M, Hamilton KL, Steffey KL, Gray ME, Hellmich RL (2010) Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330:222-225. https://doi.org/10.1126/science.1190242

International Service for the Acquisition of Agri-Biotech Applications, ISAAA (2019) GM Approval Database. http://www.isaaa.org/gmapprovaldatabase. Accessed 22 August 2019.

Ives AR, Glaum PR, Ziebarth NL, Andow DA (2011) The evolution of resistance to two-toxin pyramid transgenic crops. Ecol Appl 21:503-515. https://doi.org/10.1890/09-1869.1

James VA, Avart C, Worland B, Snape JW, Vain P (2002) The relationship between homozygous and hemizygous transgene expression levels over generations in populations of transgenic rice plants. Theor Appl Genet 104:553-561. https:// doi.org/10.1007/s001220100745.

Kasten Jr P, Precetti AACM, Parra JRP (1978) Dados biológicos comparativos de *Spodoptera frugiperda* (J.E. Smith, 1797) em duas dietas artificiais e substrato natural. Rev Agri 53:69-78.

Law RD, Russel DA, Thompson LC, Schroeder SC, Middle CM, Tremaine MT, Jury TP, Delannay X, Slater S (2006) Biochemical limitations to high-level expression of humanized monoclonal antibodies in transgenic maize seed endosperm.

Biochim Biophys Acta 1760:1434-1444. https://doi.org/10.1016/j.bbagen.2006.05.006.

Li XJ, Wu MF, Ma J, et al (2020) Prediction of migratory routes of the invasive fall armyworm in eastern China using a trajectory analytical approach. Pest Manag Sci 76:454–463. https://doi.org/10.1002/ps.5530

Lohn AF, Trtikova M, Chapela I, Van den Berg J, Plessis H, Hilbeck A (2020) Transgene behavior in Zea mays L. crosses across different genetic backgrounds: Segregation patterns, cry1Ab transgene expression, insecticidal protein concentration and bioactivity against insect pests. PloS One. https://doi.org/10.1371/journal.pone.0238523

Long N, Bottoms J, Meghji M, Hart H, Que Q, Pulliam D (2012) Corn event MIR162. U.S. Patent 8,232,456, issued July 31, 2012. https://patentimages.storage.googleapis.com/93/97/af/668ff4ca3fdfab/US8232456.pdf Accessed 17 July 2019

Magalhães PC, Durães FO (2006) Fisiologia da produção de milho. Embrapa Milho e Sorgo-Circular Técnica (INFOTECA-E). https://www.embrapa.br/busca-de-publicacoes/-/publicacao/490408/fisiologia-da-producao-de-milho. Accessed 25 April 2020

Morris M, Dreher K, Ribaut JM, Khairallah M. (2003) Money matters (II): costs of maize inbred line conversion schemes at CIMMYT using conventional and marker-assisted selection. Mol Breed 11: 235-247. https://doi.org/10.1023/A:1022872604743

Nguyen HT, Jehle JA (2007) Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize Mon810. J Plant Dis Protect 114:82-

87. https://doi.org/10.1007/BF03356208

Omoto C, Bernardi O, Salmeron E, Sorgatto RJ, Dourado PM, Crivellari A, Carvalho RA, Willse A, Martinelli S, Head GP (2016) Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil. Pest Manag Sci 72:1727-1736. https://doi.org/10.1002/ps.4201

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org

Santos-Amaya OF, Rodrigues JV, Souza TC, Tavares CS, Campos SO, Guedes RN, Pereira EJ (2015) Resistance to dual-gene Bt maize in *Spodoptera frugiperda*: selection, inheritance, and cross-resistance to other transgenic events. Sci Rep. https://doi.org/10.1038/srep18243

Székács A, Lauber É, Juracsek J, Darvas B (2010) Cry1Ab toxin production of MON 810 transgenic maize. Environ Toxicol Chem 29:182-190. https://doi.org/10.1002/etc.5

Silva V, Mol HG, Zomer P, Tienstr, M, Ritsema CJ, Geissen V (2019) Pesticide residues in European agricultural soils–A hidden reality unfolded. Sci Total Environ 653:1532-1545. https://doi.org/10.1016/j.scitotenv.2018.10.441

Silver A (2019) Caterpillar's devastating march across China spurs hunt for native predator. Nature 570:286. https://doi.org/10.1038/d41586-019-01867-3

Storer NP, Babcock JM, Schlenz M, Meade T, Thompson GD, Bing JW, Huckaba RM (2010) Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J Econ Entomol 103:1031-1038. https://doi.org/10.1603/EC10040

Storer, NP, Kubiszak ME, King JE, Thompson GD, Santos AC (2012) Status of

resistance to Bt maize in *Spodoptera frugiperda*: lessons from Puerto Rico. J Invertebr Pathol 110:294-300. https://doi.org/10.1016/j.jip.2012.04.007

Tabashnik BE, Brévault T, Carrière Y (2013) Insect resistance to Bt crops: lessons from the first billion acres. Nat Biotechnoly 31:510. https://doi.org/10.1038/nbt.2597

Tabashnik BE and Carrière Y (2017) Surge in insect resistance to transgenic crops and prospects for sustainability. Nat Biotechnoly 35:926-935. https://doi.org/10.1038/nbt.3974

Tabashnik BE, Van Rensburg JBJ, Carrière Y (2009) Field-evolved insect resistance to Bt crops: definition, theory, and data. J Econ Entomol 102:2011-2025. https://doi.org/10.1603/029.102.0601

Tavares WS, Costa MA, Cruz I, Silveira RD, Serrao JE, Zanuncio JC (2010) Selective effects of natural and synthetic insecticides on mortality of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and its predator *Eriopis connexa* (Coleoptera: Coccinellidae). J Environ Sci Heal B 45:557-561. https://doi.org/10.1080/03601234.2010.493493

Trtikova M, Wikmark OG, Zemp N, et al (2015) Transgene expression and bt protein content in transgenic bt maize (MON810) under optimal and stressful environmental conditions. PLoS ONE 10:1–9. https://doi.org/10.1371/journal.pone.0123011

US Environmental Protection Agency, US EPA (1998) Final Report of the subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management. https://archive.epa.gov/scipoly/sap/meetings/web/pdf/finalfeb.pdf.

Yang F, Kerns DL, Brown S, Head GP, Huang F (2017) Pollen contamination in seed

mixture increases the dominance of resistance to Bt maize in Spodoptera frugiperda (Lepidoptera: Noctuidae). Pest Manag Sci 73:2379-2385. https://doi.org/10.1002/ps.4631

Yang F, Kerns DL, Head GP, Leonard BR, Levy R, Niu Y, Huang F (2014) A challenge for the seed mixture refuge strategy in Bt maize: impact of cross-pollination on an ear-feeding pest, corn earworm. PLoS One. https://doi.org/10.1371/journal.pone.0112962

Yang F, Kerns DL, Head GP, Price PA, Levy R, Niu Y, Huang F (2020) Extended evaluation of Bt protein cross-pollination in seed blend plantings on survival, growth, and development of Helicoverpa zea feeding on refuge ears. Pest Manag Sci 76:1011-1019. https://doi.org/10.1002/ps.5611

Young JR (1979) Fall armyworm: control with insecticides. Fla Entomol 130-133.

Yu SJ (1991) Insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (JE Smith). Pestic Biochem Phys 39:84-91.

FIGURE LEGENDS

Fig. 1 Concentration (means ± SE) of Cry1Ab in leaves of maize hybrids at V4 and V8 growth stages in the first (A) and second (B) seasons of 2017/2018 in Jaboticabal, Brazil, and at V5 and V9 growth stages in the first season of 2017/2018 (C) and first season of 2018/2019 (D) in Lavras, Brazil. Bars topped with the same letters are not significantly different by t-test (Figure A and D: $p < 0.05$; Figures B and C: $p < 0.01$). Means of each growth stage (V4, V5, V8, or V9) were compared separately by t-tests. Seven isogenic versions of the Status maize hybrid: Non-Bt (Conventional version); Bt11 (expressing Cry1Ab) hemizygous (HE); Bt11 homozygous (HO); Vip-HE (expressing Vip3Aa20); Vip-HO; Vip3-HE (expressing Cry1Ab and Vip3Aa20); and Vip3-HO. Asterisk (*) indicates that protein Cry1Ab was not detected in Bt11-HE.

Fig. 2 Concentration (means ± SE) of Vip3Aa20 in leaves of maize hybrids at V4 and V8 growth stages in the first (A) and second (B) seasons of 2017/2018 in Jaboticabal, Brazil, and at V5 and V9 growth stages during the first season of 2017/2018 (C) and first season of 2018/2019 (D) in Lavras, Brazil. Bars topped with the same letters are not significantly different by t-test (Figure A: $p < 0.001$; Figures B, C, and D: $p < 0.01$). Means of each growth stage (V4, V5, V8, or V9) were compared separately by t-tests. Seven isogenic versions of the Status maize hybrid: Non-Bt (Conventional version); Bt11 (expressing Cry1Ab) hemizygous (HE); Bt11 homozygous (HO); Vip-HE (expressing Vip3Aa20); Vip-HO; Vip3-HE (expressing Cry1Ab and Vip3Aa20); and Vip3-HO.

Fig. 3 Leaf injury (0-9) of *Spodoptera frugiperda* (FAW) in the first growing season of 2017/2018 in Jaboticabal at V4/V5 (A) and V8/V9 (B) growth stages; in the second growing season at V4/V5 (C) and V8/V9 (D) growth stages; and in the first growing season of 2017/2018 in Lavras at V5/V6 (E) and V9/V10 (F) growth stages of seven isogenic versions of the Status maize hybrid: Non-Bt (Conventional version); Bt11 (expressing Cry1Ab) hemizygous (HE); Bt11 homozygous (HO); Vip-HE (expressing Vip3Aa20); Vip-HO; Vip3-HE (expressing Cry1Ab and Vip3Aa20); and Vip3-HO. Black dots represent the mean values with SE. Each jitter point represents an injured plant (Figures A, B, C, and D: n = 64; Figures E and F: n = 48). Colors red, green, and blue represent non-Bt, hemizygous, and homozygous versions, respectively. Means followed by the same letter are not significantly different by t-test (Figures A, B, C, and D: $p < 0.01$; Figures E and F: $p < 0.05$ and $p < 0.001$, respectively).

Fig. 4 Survivorship (means ± SE expressed in %) of *Spodoptera frugiperda* in Jaboticabal in the first season at V5 (A) and V8 (B), in the second season at V5 (C) and V8 (D) of 2017/2018, and in Lavras in the first season of 2017/2018 at V5 (E) and V9 (F) (n = 48) on leaves of seven isogenic versions of the Status maize hybrid: non-Bt (Conventional); Bt11 (expressing Cry1Ab) hemizygous (HE); Bt11 homozygous (HO); Vip-HE (expressing Vip3Aa20); Vip-HO; Vip3-HE (expressing Cry1Ab and Vip3Aa20); and Vip3-HO. Bars with the same letter are not significantly different according to confidence intervals (95%) overlaps generated by a generalized linear model. Asterisk (*) indicates null larval survival and no variability.

Fig. 5 Larval weight (means ± SE expressed in mg) of surviving *Spodoptera frugiperda* in Jaboticabal in the first season at V5 (A) and V8 (B), in the second season at V5 (C) and V8 (D) of 2017/2018, and in Lavras in the first season of 2017/2018 at V5 (E) and V9 (F) (n = 48) on leaves of seven isogenic versions of the Status maize hybrid: non-Bt (Conventional); Bt11 (expressing Cry1Ab) hemizygous (HE); Bt11 homozygous (HO); Vip-HE (expressing Vip3Aa20); Vip-HO; Vip3-HE (expressing Cry1Ab and Vip3Aa20); and Vip3-HO. Bars with the same letter are not significantly different by t-test (Figures A, B, C, and D: $p < 0.05$; Figures E and F: $p < 0.001$). Asterisk (*) indicates null larval survival and no variability.

Fig. 6 Survivorship (± SE) of third/fourth instar *Spodoptera frugiperda* (A and B) and larval weight (± SE) (C and D) after four days of feeding on R3 grains of seven isogenic versions of the Status maize hybrid: Non-Bt (Conventional); Bt11 (expressing Cry1Ab) hemizygous (HE); Bt11 homozygous (HO); Vip-HE (expressing Vip3Aa20); Vip-HO; Vip3-HE (expressing Cry1Ab and Vip3Aa20); and Vip3-HO in two bioassays. Bars of survivorship (%) with the same letter are not significantly different when confidence intervals (95%) overlap. Bars of final weight with the same letter are not significantly different by t-test ($p < 0.05$).

Supplementary table 1 Concentration (means ± SE expressed in ng cm^{-2}) of Cry1Ab and Vip3Aa20 proteins in leaves of six transgenic versions of the Status maize hybrid: Bt11 (expressing Cry1Ab) hemizygous (HE); Bt11 homozygous (HO); Vip-HE (expressing Vip3Aa20); Vip-HO; Vip3-HE (expressing Cry1Ab and Vip3Aa20); and Vip3-HO in the Jaboticabal site. Leaves were collected in the first and second seasons of 2017/2018 in Jaboticabal, Brazil, at the V4 and V8 growth stages.

First season 2017/2018 Jaboticabal						
Hybrid versions	Cry1Ab (ng cm^{-2})			Vip3Aa20 (ng cm^{-2})		
	V4	V8	Fold-change (V8/V4)[a]	V4	V8	Fold-change (V8/V4)[a]
Bt11-HE	27.03 ± 2.01	104.55 ± 6.68	3.87^{***}	-	-	-
Bt11-HO	46.44 ± 12.34	78.95 ± 8.25	1.70^{ns}	-	-	-
Fold-change (HO/HE)[a]	1.72^{ns}	0.76^{ns}	-	-	-	-
Vip-HE	-	-	-	13.01 ± 1.41	105.28 ± 6.43	8.09^{***}
Vip-HO	-	-	-	38.91 ± 2.80	153.29 ± 13.92	3.94^{***}
Fold-change (HO/HE)[a]	-	-	-	2.99^{***}	1.46^{*}	-
Vip3-HE	41.85 ± 2.40	103.21 ± 9.31	2.47^{***}	19.21 ± 0.82	93.45 ± 9.84	4.86^{***}
Vip3-HO	49.24 ± 5.45	147.84 ± 2.37	3.00^{***}	24.86 ± 1.35	388.76 ± 26.96	15.63^{***}
Fold-change (HO/HE)[a]	1.18^{ns}	1.43^{**}	-	1.29^{*}	4.16^{***}	-
Second season 2017/2018 Jaboticabal						
Hybrid versions	Cry1Ab (ng cm^{-2})			Vip3Aa20 (ng cm^{-2})		
	V4	V8	Fold-change (V8/V4)[a]	V4	V8	Fold-change (V8/V4)[a]
Bt11-HE	65.52 ± 3.68	108.92 ± 4.43	1.66^{***}	-	-	-
Bt11-HO	93.77 ± 18.15	176.48 ± 11.11	1.88^{***}	-	-	-
Fold-change (HO/HE)[a]	1.43^{**}	1.62^{**}	-	-	-	-
Vip-HE	-	-	-	65.22 ± 5.91	89.22 ± 2.40	1.37^{**}
Vip-HO	-	-	-	107.87 ± 12.81	319.87 ± 20.40	2.97^{***}
Fold-change (HO/HE)[a]	-	-	-	1.65^{*}	3.59^{***}	-
Vip3-HE	73.45 ± 9.60	120.47 ± 5.97	1.64^{**}	75.11 ± 12.81	100.09 ± 8.20	1.33^{ns}
Vip3-HO	112.55 ± 6.95	249.61 ± 14.64	2.22^{***}	226.39 ± 10.37	522.88 ± 72.96	2.31^{**}
Fold-change (HO/HE)[a]	1.53^{*}	2.07^{***}	-	3.01^{***}	5.22^{**}	-

[a] Fold-change followed by asterisk differed by t-test (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$).

Supplementary table 2 Concentration (means ± SE expressed in ng cm^{-2}) of Cry1Ab and Vip3Aa20 proteins in leaves of six transgenic versions of the Status maize hybrid: Bt11 (expressing Cry1Ab) hemizygous (HE); Bt11 homozygous (HO); Vip-HE (expressing Vip3Aa20); Vip-HO; Vip3-HE (expressing Cry1Ab and Vip3Aa20); and Vip3-HO in the Lavras site. Leaves were collected in the first season of 2017/2018 and the first season of 2018/2019 in Lavras, Brazil, at the V5 and V9 growth stages.

First season 2017/2018 Lavras						
Hybrid versions	Cry1Ab (ng cm^{-2})			Vip3Aa20 (ng cm^{-2})		
	V5	V9	Fold-change (V9/V5)[a]	V5	V9	Fold-change (V9/V5)[a]
Bt11-HE	90.57 ± 6.30	0.00	0.00	-	-	-
Bt11-HO	169.51 ± 13.49	104.52 ± 7.09	0.62^{**}	-	-	-
Fold-change (HO/HE)[a]	2.02^{***}	0.00	-	-	-	-
Vip-HE	-	-	-	234.40 ± 10.88	154.07 ± 12.35	0.66^{**}
Vip-HO	-	-	-	407.66 ± 13.99	375.54 ± 25.72	0.92^{ns}
Fold-change (HO/HE)[a]	-	-	-	1.74^{***}	2.44^{***}	-
Vip3-HE	151.90 ± 5.45	108.54 ± 7.01	0.71^{**}	191.19 ± 7.78	148.22 ± 19.93	0.78^{ns}
Vip3-HO	214.73 ± 13.30	180.82 ± 2.97	0.84^{*}	317.46 ± 14.97	253.38 ± 13.97	0.80^{*}
Fold-change (HO/HE)[a]	1.41^{**}	1.67^{***}	-	1.66^{***}	1.71^{**}	-
First season 2018/2019 Lavras						
Hybrid versions	Cry1Ab (ng cm^{-2})			Vip3Aa20 (ng cm^{-2})		
	V5	V9	Fold-change (V9/V5)[a]	V5	V9	Fold-change (V9/V5)[a]
Bt11-HE	55.76 ± 10.03	81.34 ± 28.37	1.46^{ns}	-	-	-
Bt11-HO	179.87 ± 8.05	267.30 ± 14.50	1.49^{**}	-	-	-
Fold-change (HO/HE)[a]	3.23^{***}	3.29^{**}	-	-	-	-
Vip-HE	-	-	-	190.23 ± 6.93	612.50 ± 106.06	3.22^{**}
Vip-HO	-	-	-	532.02 ± 27.69	1312.86 ± 106.20	2.47^{***}
Fold-change (HO/HE)[a]	-	-	-	2.87^{***}	2.14^{**}	-
Vip3-HE	249.47 ± 49.53	219.65 ± 32.13	0.88^{ns}	289.15 ± 40.30	708.20 ± 90.40	2.45^{**}
Vip3-HO	296.02 ± 27.32	246.13 ± 18.33	0.83^{ns}	500.01 ± 34.04	1573.71 ± 106.38	3.15^{***}
Fold-change (HO/HE)[a]	1.19^{ns}	1.09^{ns}	-	1.73^{**}	2.22^{***}	-

[a] Fold-change followed by asterisk differed by t-test (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$).

Fig. 1

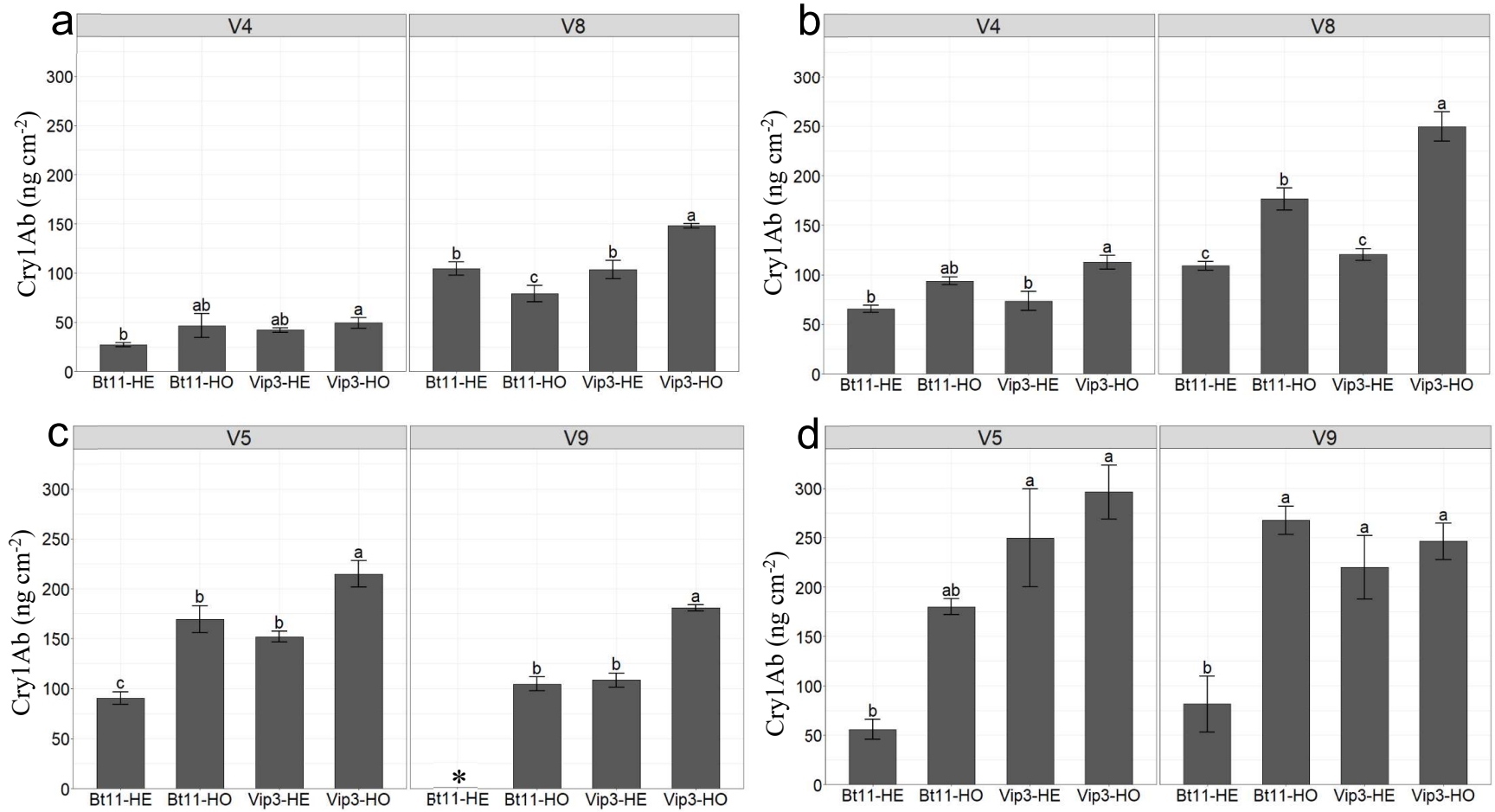

a
V4
V8
Cry1Ab (ng cm-2)
0
50
100
150
200
250
300
b
ab
ab
a
b
c
b
a
Bt11-HE
Bt11-HO
Vip3-HE
Vip3-HO
b
V4
V8
Cry1Ab (ng cm-2)
b
ab
b
a
c
b
c
a
Bt11-HE
Bt11-HO
Vip3-HE
Vip3-HO
c
V5
V9
Cry1Ab (ng cm-2)
c
b
b
a
*
b
b
a
Bt11-HE
Bt11-HO
Vip3-HE
Vip3-HO
d
V5
V9
Cry1Ab (ng cm-2)
b
ab
a
a
b
a
a
a
Bt11-HE
Bt11-HO
Vip3-HE
Vip3-HO

Fig. 2

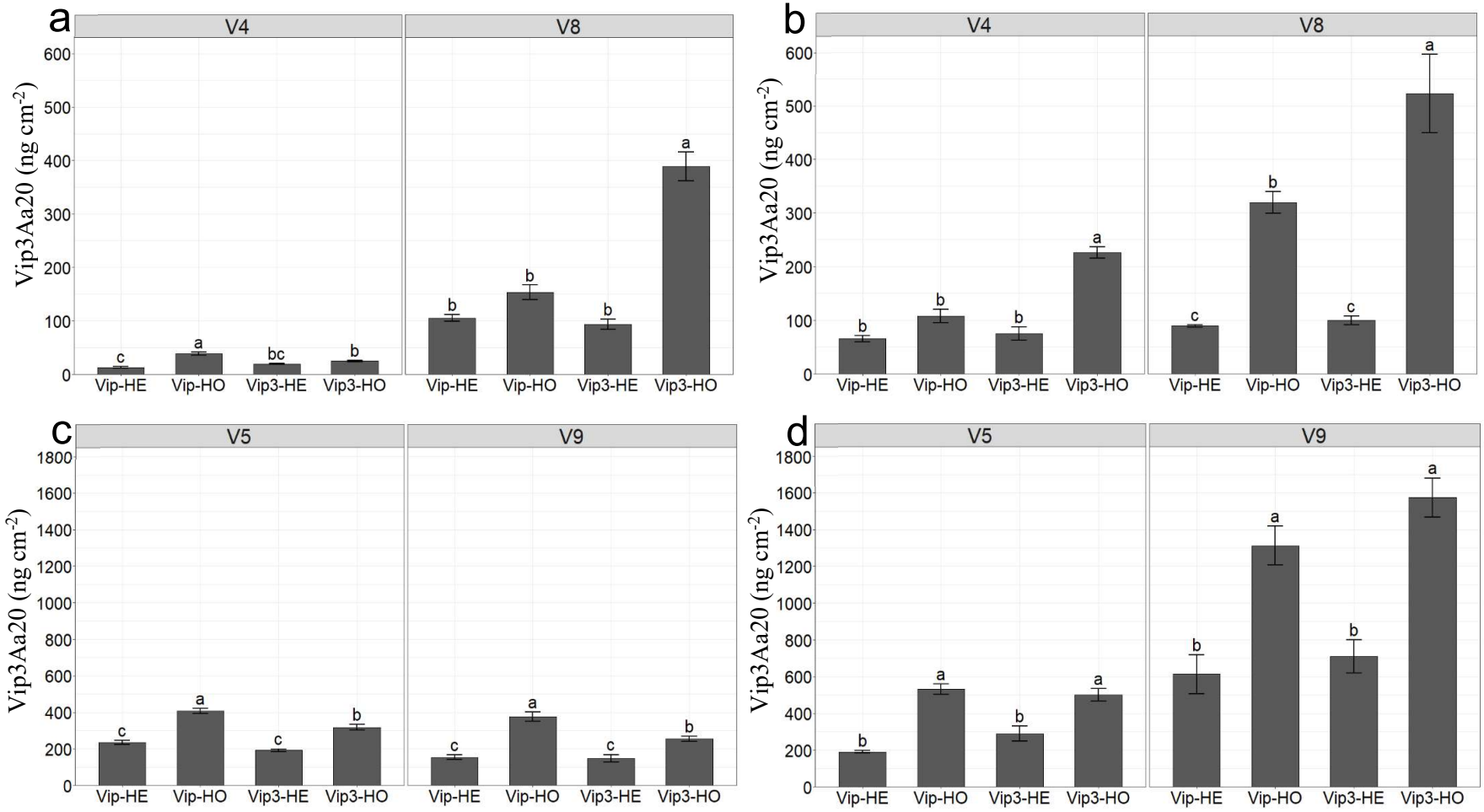

a
V4
V8
b
V4
V8
c
V5
V9
d
V5
V9
Vip3Aa20 (ng cm-2)
Vip-HE
Vip-HO
Vip3-HE
Vip3-HO

Fig. 3

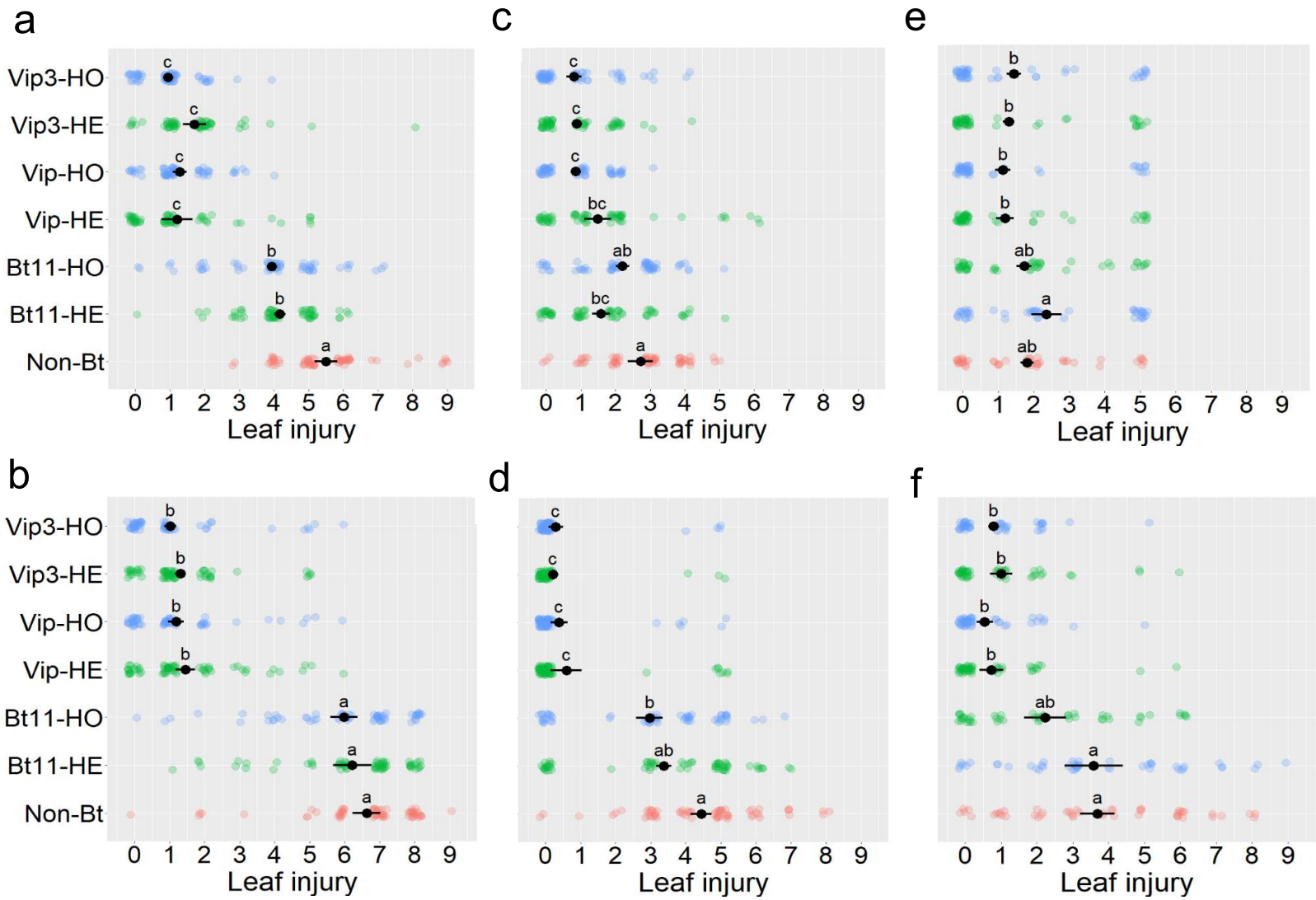

a
c
e
b
d
f
Vip3-HO
Vip3-HE
Vip-HO
Vip-HE
Bt11-HO
Bt11-HE
Non-Bt
0 1 2 3 4 5 6 7 8 9
Leaf injury

Fig. 4

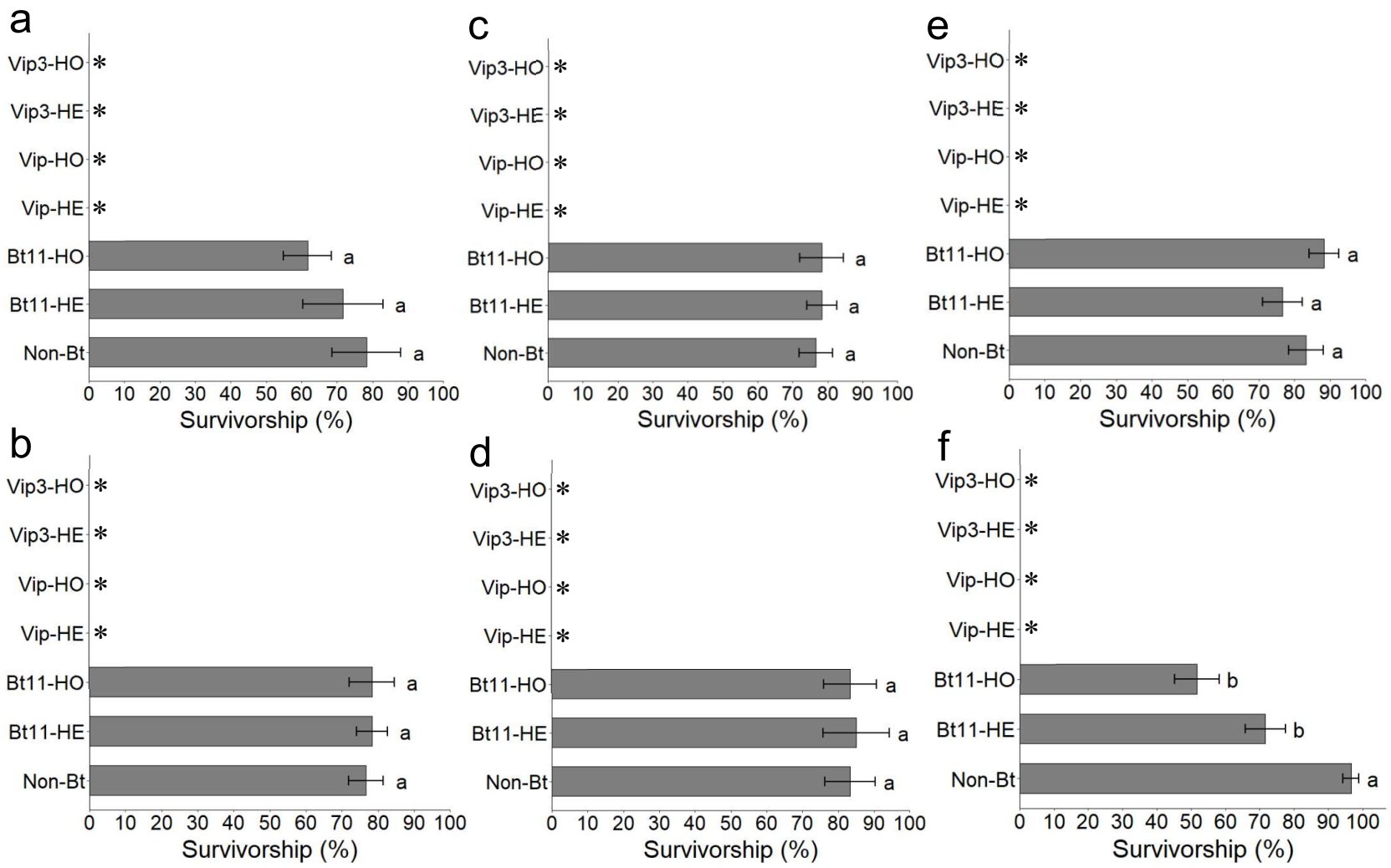

a
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO a
Bt11-HE a
Non-Bt a
0 10 20 30 40 50 60 70 80 90 100
Survivorship (%)
c
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO a
Bt11-HE a
Non-Bt a
0 10 20 30 40 50 60 70 80 90 100
Survivorship (%)
e
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO a
Bt11-HE a
Non-Bt a
0 10 20 30 40 50 60 70 80 90 100
Survivorship (%)
b
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO a
Bt11-HE a
Non-Bt a
0 10 20 30 40 50 60 70 80 90 100
Survivorship (%)
d
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO a
Bt11-HE a
Non-Bt a
0 10 20 30 40 50 60 70 80 90 100
Survivorship (%)
f
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO b
Bt11-HE b
Non-Bt a
0 10 20 30 40 50 60 70 80 90 100
Survivorship (%)

Fig. 5

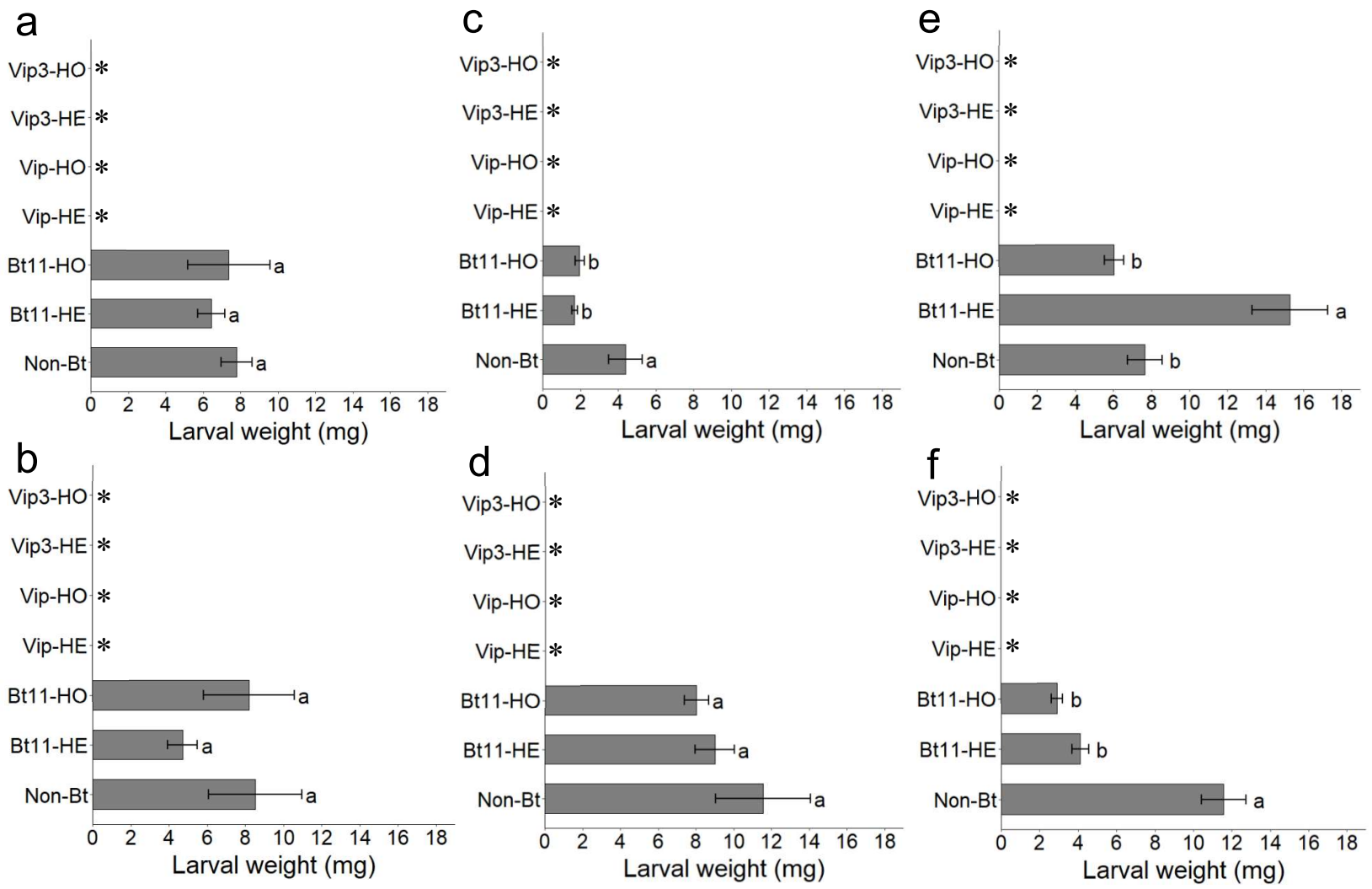
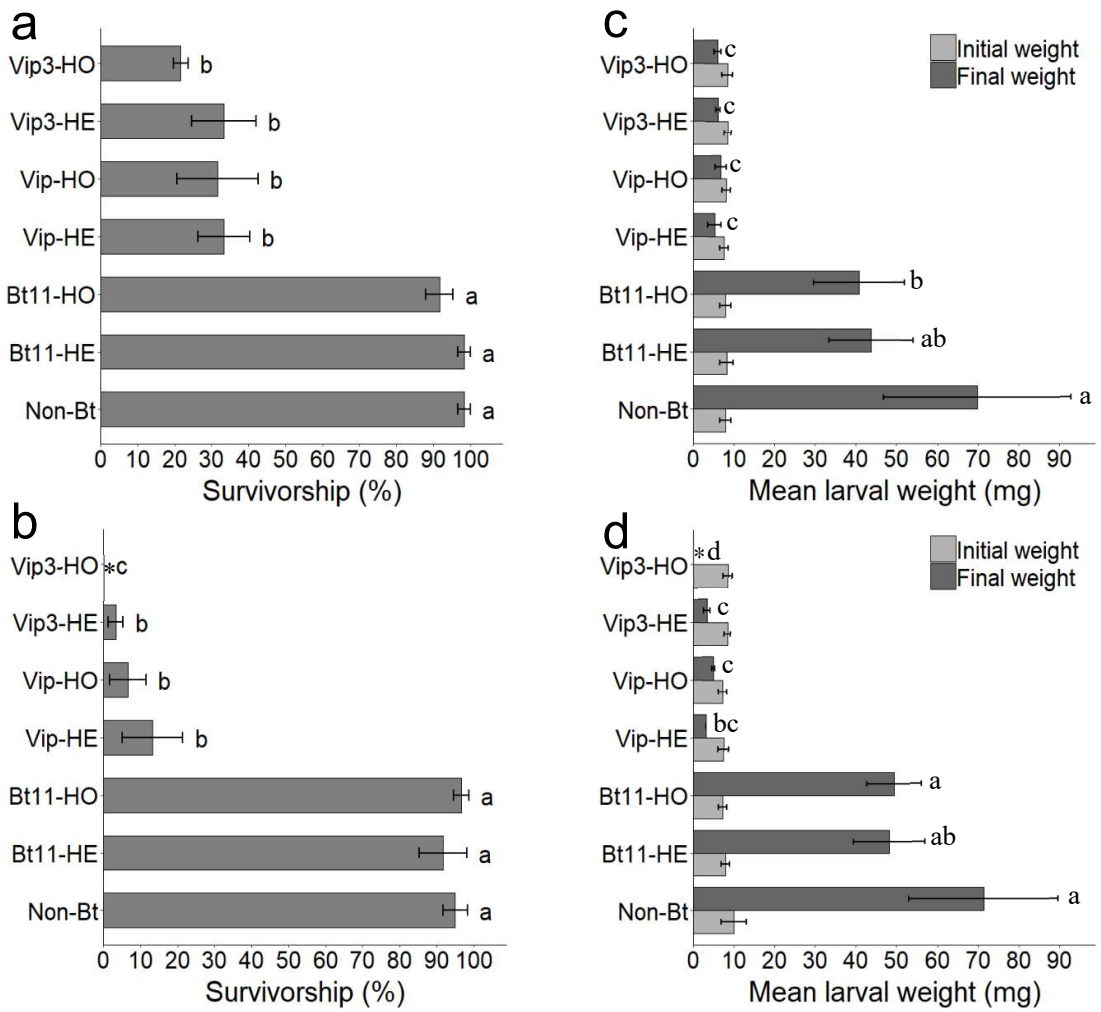

a
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO a
Bt11-HE a
Non-Bt a
0 2 4 6 8 10 12 14 16 18
Larval weight (mg)
c
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO b
Bt11-HE b
Non-Bt a
0 2 4 6 8 10 12 14 16 18
Larval weight (mg)
e
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO b
Bt11-HE a
Non-Bt b
0 2 4 6 8 10 12 14 16 18
Larval weight (mg)
b
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO a
Bt11-HE a
Non-Bt a
0 2 4 6 8 10 12 14 16 18
Larval weight (mg)
d
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO a
Bt11-HE a
Non-Bt a
0 2 4 6 8 10 12 14 16 18
Larval weight (mg)
f
Vip3-HO *
Vip3-HE *
Vip-HO *
Vip-HE *
Bt11-HO b
Bt11-HE b
Non-Bt a
0 2 4 6 8 10 12 14 16 18
Larval weight (mg)

Fig. 6


a
Vip3-HO
Vip3-HE
Vip-HO
Vip-HE
Bt11-HO
Bt11-HE
Non-Bt
b
b
b
b
a
a
a
0 10 20 30 40 50 60 70 80 90 100
Survivorship (%)
b
Vip3-HO *c
Vip3-HE b
Vip-HO b
Vip-HE b
Bt11-HO a
Bt11-HE a
Non-Bt a
0 10 20 30 40 50 60 70 80 90 100
Survivorship (%)
c
Initial weight
Final weight
Vip3-HO c
Vip3-HE c
Vip-HO c
Vip-HE c
Bt11-HO b
Bt11-HE ab
Non-Bt a
0 10 20 30 40 50 60 70 80 90
Mean larval weight (mg)
d
Initial weight
Final weight
Vip3-HO *d
Vip3-HE c
Vip-HO c
Vip-HE bc
Bt11-HO a
Bt11-HE ab
Non-Bt a
0 10 20 30 40 50 60 70 80 90
Mean larval weight (mg)

CHAPTER 3 - Xenia effects of Bt pollen and mortality of *Spodoptera frugiperda* on maize kernels

ABSTRACT - *Spodoptera frugiperda* is the main pest of maize and has been used as an insect model to investigate insect resistance management practices. Studying Bt pollen effect on non-Bt plants and the simulated movement of *S. frugiperda* from non-Bt to Bt diets, consisting of leaves and kernels, helps clarify means to extend the efficiency of Bt maize against target pests. In this study we investigated the presence of Bt proteins in kernels of non-Bt maize plants fertilized by Bt pollen and mortality of *S. frugiperda* being moved from non-Bt and Cry1Ab diet to MIR162 tissues expressing Vip3Aa20 protein. Vip3Aa20 was detected in all kernels at the R3 growth stage that originated from silks of non-Bt maize fertilized by MIR162 pollen and selfed ears of hemizygous for MIR162. Field larvae of *S. frugiperda*, likely resistant to Cry1Ab, with three to eight days old, died when were moved from non-Bt, or Cry1Ab hybrids, to feed on Vip3Aa20-maize leaves and kernels. Effects of non-maternal pollen on kernel formation, xenia, suggests that structured refuge is more adequate than seed mixture to delay resistance of ear-feeding pests to Bt proteins, as refuge plants in the seed mixture approach are surrounded by Bt plants and more exposed to Bt pollen, which can prevent the survival of susceptible insects.

Keywords: cross-pollination; fall armyworm; gene flow; simulated movement; outcrossing; refuge strategies.

1. INTRODUCTION

Bacillus thuringiensis (Bt) maize was devised to protect grain production by reducing damage caused by lepidopteran and coleopteran pests (Devos et al., 2013; Frizzas et al., 2014), without deploying chemical insecticides for the control (Clark et al., 2000; Naranjo, 2009). However, less than 15 years after the commercial release of Bt maize in the market, control failures of target pests started to appear in some regions of the globe (Huang et al., 2007; Storer et al., 2010; Huang et al., 2014; Souza et al., 2019).

One of the target pests of Bt-maize is the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), a primary pest of the crop that has been spreading worldwide (Martinelli et al., 2017; Botha et al., 2019; Jing et al., 2019). The maize whorl is the main plant target of *S. frugiperda* feeding (Sisay et al., 2019; Santos et al., 2020). The consequences of injuries caused by the pest, if not counteracted, could cause the destruction of the plant and consequently production loss (Baudron et al., 2019; Sisay et al., 2019). Secondary feeding targets of *S. frugiperda* include ear shoot and kernels, silks, tassel, and emerging seedlings (Pannuti et al., 2016; Reay-Jones et al., 2016).

Currently, *S. frugiperda* resistance alleles to Cry1Ab, Cry1F, Cry1A.105, Cry2Ab2, and Vip3Aa20 have been reported (Huang et al., 2014; Omoto et al., 2016; Yang et al., 2018; Eghrari et al., 2019). Resistance evolution is mainly caused by the lack of compliance with resistance management strategies such as the utilization of high-dose Bt events (Andow, 2008; Burkness et al., 2010), combining proteins of different modes of action to target the same pest (Bernardi et al., 2015; Niu et al., 2016), and the adoption of non-Bt plants as refuge for the survival and reproduction of susceptible insects (Chilcutt and Tabashnik, 2004; Jin et al., 2015). There are two main refuge approaches recommended in insect resistance management. The seed mixture, or refuge-in-the-bag approach, is the random distribution of Bt and non-Bt seeds in the field at different rates, e.g., 5:95% and 20:80% (non-Bt:Bt plants) (Carrière et al., 2016; Yang et al., 2017). In the structured refuge approach, a ratio of non-Bt and Bt seeds is also used, but they are disposed in separate blocks in the field (Onstad et al., 2011; Yang et al., 2020). Regarding the control of leaf- and root-feeding pests, the seed mixture strategy could delay insect resistance evolution to Bt proteins as well as be more adopted by farmers compared to the structured refuge strategy (Head et al., 2014). However, the use of structured refuge is preferred to delay the

resistance evolution of ear-feeding pests to Bt proteins, since there is a decrease of non-Bt plants being fertilized by Bt pollen (Brévault et al., 2015; Dively et al., 2020; Carrière et al., 2020).

The effects of non-maternal pollen on the formation of filial tissues, embryo and endosperm, of F_1 kernels are known as xenia (Kiesselbach, 1960; Weingartner et al., 2002). Xenia effects caused by Bt pollen fertilizing refuge plants could have two main consequences: 1) kernels of non-Bt plants will express Bt proteins and will not be a real refuge for ear-feeding pests (Yang et al., 2017; Carrière et al., 2020), and 2) a mosaic of Bt and non-Bt kernels will occur and this could facilitate the evolution of resistance to Bt proteins (Dively et al., 2020). For instance, survivorship of lepidopteran pests feeding on refuge kernels contaminated by Bt pollen decrease significantly when compared to pure non-Bt kernels (Yang et al., 2014; 2017; 2020). Although *S. frugiperda* is not a primary ear-feeding pest, it has been used as a model for studying the implications of Bt pollen on maize kernels (Yang et al., 2017).

In this context, this study investigated the effects of Bt pollen on the expression of Bt proteins in maize kernels and whether *S. frugiperda* could survive after being moved from non-Bt and Cry1Ab diet to Vip3Aa20 diet.

2. MATERIALS AND METHODS

2.1. Maize genetic material

Plant genetic material comprised seven near-isogenic versions of the one-way Status hybrid (Syngenta Proteção de Cultivos, Brazil). Six near-isogenic versions contained single or pyramided Bt traits in hemizygosis (HE) and homozygosis (HO). The Bt traits utilized were Bt11 and MIR162, which express the proteins Cry1Ab and Vip3Aa20, respectively. The seven near-isogenic versions were non-Bt, HE for Bt11 (Bt11-HE), HO for Bt11 (Bt11-HO), HE for MIR162 (MIR162-HE), HO for MIR162 (MIR162-HO), HE for Bt11 and MIR162 (MIR162 + Bt11-HE), and HO for Bt11 and MIR162 (MIR162 + Bt11-HO). Kernels at the R3 growth-stage and leaves at the VT growth-stage (Magalhães and Durães, 2006) were collected from these plants to compose bioassays with *S. frugiperda*.

R3-kernels were obtained following specific crossing-designs described in the following sections. Ears were covered with a plastic bag (10 x 20 cm x 0.6 mm) before silking. When pollen was shedding and silks were available, tassels were collected on

paper bags (17.5 x 38 cm) and deposited on the silking ears, covering them with the pollen bag until reaching the R3 growth stage.

2.2. Detection of Vip3Aa20 in R3 maize kernels

Kernels were generated by three crosses of near-isogenic hybrids: selfed non-Bt hybrid, selfed MIR162-HE hybrid, and non-Bt hybrid fertilized by MIR162-HO hybrid. Plants were crossed in different moments, according to the availability of pollen and silks. Ears were harvested when reached the R3 reproductive stage.

ELISA strip tests for the detection of Vip3Aa20 in the event MIR162 (Cat# AS 085, Envirologix, Portland, ME, USA) were utilized to determine the presence of the protein in R3 maize kernels. Each strip was used for one kernel only, after imbibing it in the buffer provided in the ELISA kit and grinding it with mortar and pestle. One hundred, 41, and six kernels of the selfed MIR162-HE hybrid, non-Bt hybrid pollinated by MIR162-HO, and selfed non-Bt hybrid were tested, respectively.

2.3. *S. frugiperda* origin and rearing

Two *S. frugiperda* populations were used in the bioassays. One was a laboratory population never exposed to Bt events which have been reared with non-Bt artificial diet similar to Oliveira et al. (2006) over the last 10 years. The other was a field population with egg masses and larvae collected in non-Bt and Bt Cry-expressing maize fields in the municipality of Jaboticabal, Brazil (coordinates 21°14’53”S 48°17’08”W) in 2018. Neonate larvae of both lab and field populations were reared on artificial diet (Oliveira et al., 2006) until third instar to compose kernel and leaf-tissue bioassays.

2.4. Laboratory *S. frugiperda* population feeding on maize kernels

S. frugiperda lab population was reared with non-Bt artificial diet (Oliveira et al., 2006) until third instar. These larvae were then fed with F_1 R3-kernels from four crossing designs with near-isogenic versions of the Status hybrid: selfed non-Bt, selfed MIR162-HE, selfed MIR162-HO, and non-Bt fertilized by MIR162-HO pollen.

Each replicate consisted of 12 *S. frugiperda* larvae in a Petri dish containing five R3-kernels over a piece of dry paper towel. The larvae were weighed prior to being released in the dishes. Each kernel was carefully detached from the maize ear to avoid rupture of the pericarp and spilling of the internal material. The design was a

randomized complete block with four replicates (n = 48). Survivorship and individual weight evaluations took place after four days of feeding (12-hour photoperiod at 25 ± 1 ºC). Surviving larvae of treatments with MIR162 were fed with non-Bt maize leaves to evaluate late Vip3Aa20 effects.

2.5. Simulating movement of field *S. frugiperda* larvae fed with non-Bt or Bt11 kernels onto MIR162 kernels

S. frugiperda field population was fed artificial diet (Oliveira et al., 2006) until the third instar. Third-instar *S. frugiperda* larvae were randomly transferred to Petri dishes to feed on selfed R3-kernels of seven near-isogenic versions (section 2.1.) for four days.

Seven-day-old *S. frugiperda* larvae that survived were offered selfed R3-kernels of only two out of the seven near-isogenic hybrids: non-Bt and MIR162-HE. Each replicate comprised 13 *S. frugiperda* in Petri dishes with five R3-kernels over a dry sheet of paper towel. The design was a randomized complete block with five replicates (n = 65). Survivorship and larval individual weight were assessed after four days (12-hour photoperiod at 25 ± 1 ºC). Surviving larvae on MIR162 kernels were fed with leaves of the non-Bt maize to evaluate late Vip3Aa20 effects.

2.6. Simulating movement of field *S. frugiperda* from non-Bt or Bt11 leaves to MIR162 maize leaves

Field *S. frugiperda* population was fed non-Bt artificial diet (Oliveira et al., 2006) until the third-instar. Larvae were then fed with leaves of seven near-isogenic versions of the Status hybrid (section 2.1.). Each replicate of the leaf bioassay consisted of 12 weighed larvae in a Petri dish over a sheet of paper towel moistened with distilled water and a piece of leaf (5.0 x 3.5 cm) at the VT growth stage to reduce leaf desiccation. Leaves were replaced every two days. The bioassay design was a randomized complete block with five replications (n = 60). Larval mortality and individual weight of surviving larvae were assessed after five days (12-hour photoperiod at 25ºC ± 1).

Eight-day-old larvae that survived were fed with leaves of non-Bt and MIR162-HE hybrids. Each larva was again weighed before being transferred to Petri dish. Each replicate consisted of 13 *S. frugiperda* with leaves of each treatment in the VT growth stage over a sheet of paper towel moistened with distilled water. The design was a

randomized complete block with five replicates (n = 65). Larval mortality and weight of surviving larvae were assessed after five days (12-hour photoperiod at 25 ± 1 ºC). Surviving larvae were fed with non-Bt leaves to observe the occurrence of pupation and late effects of Vip3Aa20.

2.7. Data analysis

Means and standard errors (SE) of surviving larvae were calculated in R (R Core Team, 2019). The package ggplot2 (Wickham, 2016) was used to plot the data. Survivorship and weight of surviving larvae data were submitted to ANOVA followed by pairwise LSD test to compare means using the agricolae package (Mendiburu, 2020) in R.

3. RESULTS

3.1. Detection of Vip3Aa20 in maize kernels

All kernels of the selfed MIR162-HE hybrid expressed the Vip3Aa20 protein. Also, Vip3Aa20 was found on kernels of non-Bt silks fertilized by MIR162 pollen. Vip3Aa20 was not detected in R3-kernels of the selfed near-isogenic non-Bt version (Table 1).

Table 1. Elisa strip test to detect the protein Vip3Aa20 expressed by the MIR162 maize event.

Kernel	Number of kernels tested	Positive (%)
Non-Bt⊗	6	0
MIR162-HE⊗	100	100
♀Non-Bt x ♂ MIR162-HO	41	100

MIR162 expresses the protein Vip3Aa20. HE - hemizygous; HO - homozygous; ⊗ - self-fertilization; ♀ - female plant; ♂ - male plant.

3.2. Survivorship of laboratory *S. frugiperda* larvae on Vip3Aa20 R3 maize kernels

Less than 10% of third-instar *S. frugiperda* larvae from the laboratory colony (never exposed to Bt proteins) survived after being fed for four days with MIR162 kernels (Figure 1A), but died two days later after being fed with non-Bt maize leaves (Supplementary Table 1). All larvae fed with non-Bt R3-kernels survived and

presented a mean weight 10 times higher than that of surviving larvae on MIR162-HE kernels after four days of feeding (Figure 1B).

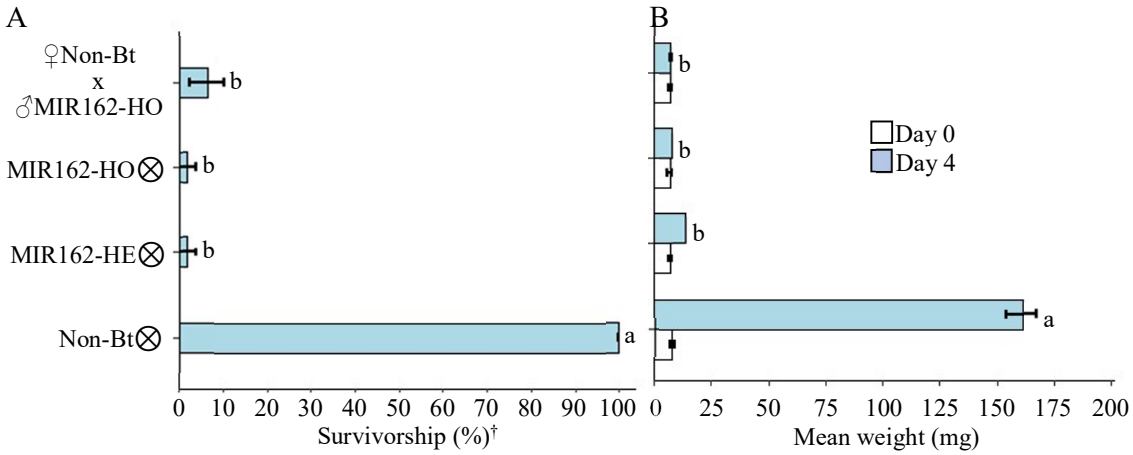


Figure 1. Survivorship (mean ± SE, %) of third instar *S. frugiperda* fed with R3 maize kernels for four days (A) and mean weight (mg) ± SE of surviving larvae before and after being fed with R3-kernels (B). † *S. frugiperda* subjected to MIR162 died after being fed with non-Bt maize leaves for two days. MIR162 expresses the protein Vip3Aa20. HE, hemizygous. HO, homozygous. ⊗, self-fertilization. Bars with the same letter are not significantly different by pairwise LSD test ($p < 0.001$).

3.3. Survivorship of field *S. frugiperda* larvae transferred from non-Bt and Bt11 kernels to MIR162 kernels

All third-instar larvae fed with MIR162 R3-kernels died, while third-instar larvae fed with non-Bt, Bt11-HE, and Bt11-HO R3-kernels survived after four days. This surviving larvae, seven-day-old *S. frugiperda*, were then transferred to MIR162-HE and non-Bt R3-kernels. The survivorship of the seven-day-old *S. frugiperda* was ~20% on selfed R3-kernels of MIR162-HE hybrid. The remaining ~20% surviving larvae on MIR162-HE died after two days when transferred to non-Bt leaves, which were barely scratched (Supplementary Table 2). On the other hand, larval survival was greater than 90% on selfed R3-kernels of the non-Bt version (Figure 2A). For larval weight, surviving larvae on MIR162-HE were ~2.5 times lighter than larvae fed with the non-Bt version (Figure 2B).

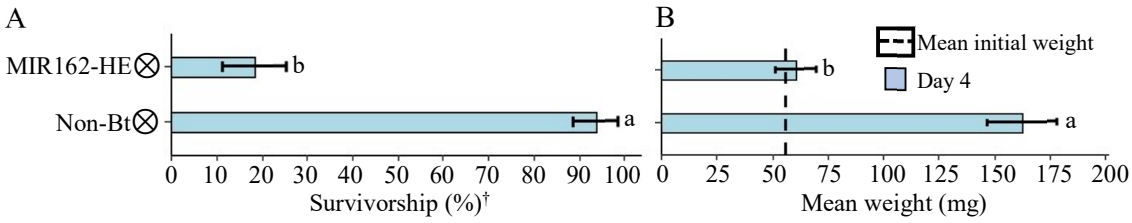


Figure 2. Survivorship (mean ± SE, %) of seven-day old *S. frugiperda* larvae fed with R3 maize kernels of two near-isogenic versions of the Status maize hybrid for four days (A) and mean weight (mg) ± SE of *S. frugiperda* larvae at day four (B). † *S. frugiperda* subjected to MIR162 died after being fed with non-Bt maize leaves for two days. MIR162 expresses the protein Vip3Aa20. HE, hemizygous. ⊗, self-fertilization. Bars with the same letter are not significantly different by pairwise LSD test ($p < 0.001$).

3.4. Survivorship of field *S. frugiperda* larvae transferred from non-Bt and Bt11 leaves to MIR162 leaves

All third-instar *S. frugiperda* fed with MIR162 leaves died, and 85.00 ± 7.64%, 73.33 ± 10.34%, and 77.67 ± 10.00% survived on non-Bt, Bt11-HE, and Bt11-HO VT leaves, respectively (Figure 3A, Supplementary table 3). Larvae that survived gained on average 37.35, 43.4, 49.25 mg after being fed with non-Bt, Bt11-HE, and Bt11-HO leaves in five days, respectively (Figure 3B, Supplementary Table 3).

Eight-day-old surviving larvae fed with non-Bt, Bt11-HE, and Bt11-HO versions died on MIR162-HE VT-leaves at the VT growth-stage and survived on the non-Bt version (Figure 3C). The mean weight of the surviving larvae tripled in five days (Figure 3D, Supplementary Table 4).

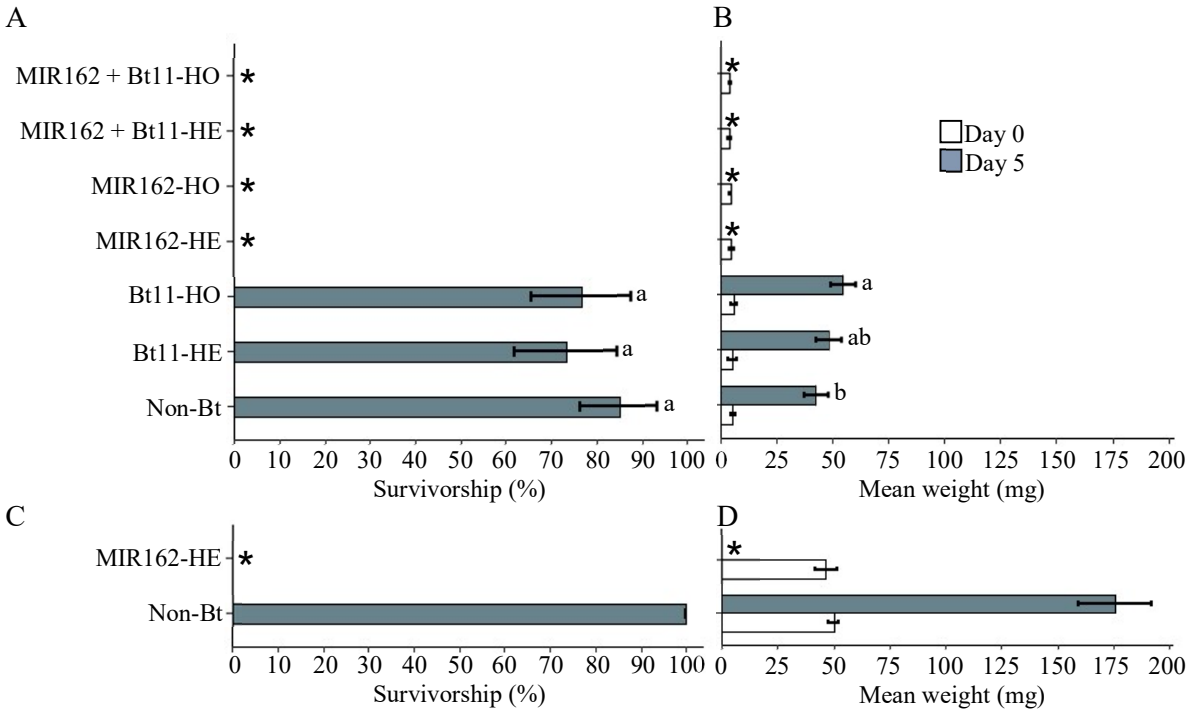


Figure 3. Survivorship (mean ± SE, %) of third instar *S. frugiperda* fed with VT leaves of seven near-isogenic versions of the Status maize hybrid for five days (A); Mean weight (mg) ± SE of third instar *S. frugiperda* at day 0 and after five days (B); Survivorship (%) ± SE of eight-day old surviving fed with VT leaves of non-Bt and MIR162-HE near-isogenic versions of the Status hybrid (C); and mean weight (mg) ± SE of eight-day old *S. frugiperda* day 0 and after five days (D). Values equal to zero are represented by an asterisk (*). MIR162 and Bt11 express the proteins Vip3Aa20 and Cry1Ab, respectively. HE, hemizygous. HO, homozygous. Bars with the same letter are not significantly different by pairwise LSD test ($p < 0.01$).

4. DISCUSSION

Xenia was evidenced by the detection of Vip3Aa20 protein in kernels of non-Bt maize fertilized by Bt pollen, and by the mortality of *S. frugiperda* fed on those kernels. Our results also show that Bt zygosity has an important role in the number of Bt alleles and the concentration levels of Bt proteins in the main tissues of the kernel (embryo, endosperm, and pericarp) (Eghrari et al., 2019; Carrière et al., 2020). Additionally, a high susceptibility to Vip3Aa20 was observed on both lab and field *S. frugiperda* populations, and an apparent resistance to Cry1Ab was detected in the field population.

As for the Bt zygosity in kernel tissues, kernels originated from female non-Bt x male MIR162-HO likely expressed Vip3Aa20 in the embryo and endosperm of each kernel, but not in the pericarp (Wright et al., 1989; Carrière et al., 2020). On the other hand, selfed HE Bt hybrids always express Bt proteins in the pericarp, due to its maternal origin, whereas embryo and endosperm follow a mendelian segregation (Carrière et al., 2020). The proportion of each tissue of the kernel changes as they reach physiological maturity. Embryo, endosperm, and pericarp correspond to 0.7, 86.4, and 12.9% of the entire kernel weight 14 days after fertilization (Kiesselbach, 1960), i.e., approximately at the R3 growth stage used in our bioassays (Magalhães and Durães, 2006). Therefore, concentrations of Bt proteins on pericarp and endosperm could be higher at the R3 growth stage than at physiological maturity, when embryo, endosperm, and pericarp account for approximately 11, 82, and 6% of the dry weight of the kernel, respectively (Tollenaar and Dwyer, 1999; García-Lara et al., 2019). Thus, regardless of the kernel phenological stage, MIR162 allele could be widely expressed in kernels of non-Bt maize plants pollinated by MIR162-HO hybrids because the endosperm accounts for more than 80% of the mass of the kernel

(Kiesselbach, 1960; Tollenaar and Dwyer, 1999; García-Lara et al., 2019). As for kernels originated from hemizygous plants, in a situation where embryo and endosperm do not present any Bt allele, the pericarp is likely to have a considerable impact on kernel-feeding pests, since the percentage of the pericarp is higher at the initial stages of kernel development (Kiesselbach, 1960).

Xenia effects caused by Bt pollen, also referred to as impact of cross-pollination (Yang et al., 2014; 2020) and gene flow (Chilcutt and Tabashnik, 2004; Dively et al., 2020), have been found and discussed elsewhere in the context of the seed mixture and structured refuge strategies. In a 5:95% (non-Bt:Bt-HE) seed mixture strategy, the Bt plants produce the majority of the pollen, which, on average, 50% is Bt. As maize is an allogamous species, more than 95% of the kernels of maize plants are fertilized by adjacent plants (Poehlman, 1987), thereby approximately half of the kernels in non-Bt refuge could be Bt (Yang et al., 2014). Dively et al. (2020) reported the occurrence of 18.6% of Bt kernels in non-Bt plants in a 5:95% (non-Bt:Bt) seed mixture scheme, and suggested that the lack of synchrony between pollen shedding and silking could have affected the low percentage of Bt kernels produced.

In this context, resistance management of ear-feeding pests could be jeopardized by the use of the seed mixture approach because kernels of non-Bt hybrids might not be serving as a real refuge for pests, since they would be expressing Bt proteins, and therefore could kill susceptible larvae (Yang et al., 2014), increasing consequently the selection pressure. Additionally, if homozygous Bt hybrids would be deployed (Miraldo et al., 2016) in a seed mixture condition of 5:95% (non-Bt:Bt), ~95% of the kernels from the refuge plants could express Bt proteins, and thus the likelihood of having refuge kernels for grain-feeding pests would decrease even more relative to a condition with the trait in a hemizygous state. When pyramided traits are included into the seed mixture scenario, 94.4% of refuge kernels express at least one Bt protein from the surrounding Bt pyramided plants in a 5:95% (non-Bt:Bt) strategy (Yang et al, 2014). At high compliance rates, structured refuge has been shown to be more effective in delaying resistance evolution in insect pests to Bt proteins than the seed mixture strategy (Carroll et al., 2012; Yang et al., 2017). In light of our results, structured refuge would also serve better as a refuge for grain-feeding pests, because with separate blocks of Bt and non-Bt plants in the field less non-Bt silks could be fertilized by Bt pollen (Yang et al., 2020).

Regarding the reciprocal cross in a 5:95% (non-Bt:Bt) seed mixture approach, that is, few silks of Bt-hemizygous plants fertilized by non-Bt maize pollen (Yang et al., 2014), the genotype of the resulting kernels might resemble that of selfed Bt hemizygous hybrids (Burkness et al., 2011; Yang et al., 2014; Dively et al., 2020). As our results revealed, kernels of selfed MIR162-HE hybrids controlled 100% of three- to eight-day-old *S. frugiperda* larvae. Theoretically, selfed kernels of hemizygous for Bt maize plants would have a 1:2:1 mendelian segregation for the Bt trait in a homozygous, hemizygous, and non-Bt state, respectively, that is, on average 75% of the kernels would have the Bt allele (Wang et al., 1999). Chilcutt and Tabashnik (2004) presented that expected mendelian segregation, with ~75% of maize kernels of selfed Bt11-HE maize plants expressing Cry1Ab. We hypothesize that one of the reasons why Cry1Ab concentrations in the pericarp might not have been sufficient to be detected by the ELISA strip test might be due to the promoter of the Bt11 construct, 35S CaMV (CTNBio, 2008), which is weaker than the promoter of the MIR162 transgene, polyubiquitin ZmUbiINT (Christensen et al., 1992; CTNBio, 2009; Bernardi et al., 2015. Indeed, Chilcutt and Tabashnik's results (2004) might have detected the presence of the Cry1Ab protein only in the embryo and endosperm of each kernel, which are filial tissues that follow the mendelian pattern of segregation (Wang et al., 1999). Furthermore, actual segregation of Bt traits could be seen by genotyping the embryo or tissues of the plants that germinate from the kernels.

In that view, our study showed that all kernels of selfed MIR162-HE plants expressed the Vip3Aa20 protein. Similar results with 100% Bt detection in kernels of pure Bt fields suggested that the hybrids were homozygous for the trait (Yang et al., 2014). Contrary to that assumption, since we knew the zygosity of our hybrids, we believe that detection of Vip3Aa20 in all kernels might have been due to presence of the Vip3Aa20 in the pericarp (Carrière et al., 2020).

Three- to eight-day-old *S. frugiperda* were killed when transferred from a non-Bt diet (artificial diet, leaves, or kernels) to MIR162 leaves or kernels. Our results show the efficiency of the MIR162 event, which is a protein that complies with the high-dose strategy for *S. frugiperda* for both leaves and kernels (Andow, 2008; Burkness et al., 2010). In more than 80% of the cases, it took less than five days for larvae to be killed when feeding on MIR162 diet. Those larvae that survived on MIR162 were transferred to non-Bt leaves and eventually died after two days of feeding due to the late effect of the Vip3Aa20 protein (Horikoshi et al., 2016; Yang et al., 2016).

Decreased susceptibility to Bt proteins as lepidopteran larvae advance development has been reported (Sorgatto et al., 2015; Miraldo et al., 2016; Silva et al., 2018). In the current study, however, we revealed that *S. frugiperda* larvae of three, seven, or eight days old previously fed with non-Bt diet died once they were transferred to MIR162 leaves or kernels. The high mortality of *S. frugiperda* on Vip3Aa20 tissues could be an indication of both low frequency of resistant alleles to Vip3Aa20 and the power-killing of the protein on the population collected in Jaboticabal, Brazil, since Cry1F-susceptible *S. frugiperda* presented higher mortality in mosaic maize ears than Cry1F-heterozygous and resistant larvae (Yang et al., 2017). Conversely, similar larval survival on Bt11 and non-Bt tissues suggests that the Jaboticabal *S. frugiperda* population is resistant to Cry1Ab, resistance that has been found also in other regions of Brazil (Omoto et al., 2016). In fact, the *S. frugiperda* field population of this study was collected in the same region where survivorship of *S. frugiperda* on Cry1F, Cry1A.105, and Cry2Ab2 proteins has been reported (Eghrari et al., 2019).

5. REFERENCES

Andow DA (2008) The risk of resistance evolution in insects to transgenic insecticidal crops. **Collection of Biosafety Reviews** 4:142-199.

Baudron F, Zaman-Allah MA, Chaipa I, Chari N, Chinwada P (2019) Understanding the factors influencing fall armyworm (*Spodoptera frugiperda* J.E. Smith) damage in African smallholder maize fields and quantifying its impact on yield. A case study in Eastern Zimbabwe. **Crop Protection** 120:141-150.

Bernardi O, Bernardi D, Amado D, Sousa RS, Fatoretto J, Medeiros FCL, et al (2015) Resistance risk assessment of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and *Diatraea saccharalis* (Lepidoptera: Crambidae) to Vip3Aa20 insecticidal protein expressed in corn. **Journal of Economic Entomology** 108:2711-2719.

Bernardi D, Salmeron E, Horikoshi RJ, Bernardi O, Dourado PM, Carvalho RA, et al (2015) Cross-resistance between Cry1 proteins in fall armyworm (*Spodoptera frugiperda*) may affect the durability of current pyramided bt maize hybrids in Brazil. **PLoS One** 10:1-15.

Botha AS, Erasmus A, du Plessis H, Van den Berg J (2019) Efficacy of Bt maize for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in South Africa. **Journal of Economic Entomology** 112:1260-1266.

Brévault T, Tabashnik BE, Carrière Y (2015) A seed mixture increases dominance of resistance to Bt cotton in *Helicoverpa zea*. **Scientific Reports** 5:1-7.

Burkness EC, Dively G, Patton T, Morey AC, Hutchison WD (2010) Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions: Implications for resistance management. **GM Crops** 1:337-343.

Burkness EC, O'Rourke PK, Hutchison WD (2011) Cross-pollination of nontransgenic corn ears with transgenic Bt corn: Efficacy against lepidopteran pests and implications for resistance management. **Journal of Economic Entomology** 104:1476-1479.

Carrière Y, Degain BA, Harpold VS, Unnithan GC, Tabashnik BE (2020) Gene Flow Between Bt and Non-Bt Plants in a Seed Mixture Increases Dominance of Resistance to Pyramided Bt Corn in *Helicoverpa zea* (Lepidoptera: Noctuidae). **Journal of Economic Entomology** 20:1-11. https://doi.org/10.1093/jee/toaa138

Carrière Y, Fabrick JA, Tabashnik BE (2016) Can pyramids and seed mixtures delay resistance to Bt crops? **Trends in Biotechnology** 34:291-302.

Carroll MW, Head G, Caprio M (2012) When and where a seed mix refuge makes sense for managing insect resistance to Bt plants. **Crop Protection** 38:74-79.

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. **Plant Molecular Biology** 18:675-689.

Clark TL, Foster JE, Kamble ST, Heinrichs EA (2000) Comparison of Bt (*Bacillus thuringiensis* Berliner) maize and conventional measures for control of the European Corn Borer (Lepidoptera: Crambidae). **Journal of Entomological Science** 35:118-128.

Chilcutt CF and Tabashnik BE (2004) Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. **Proceedings of the National Academy of Sciences of the United States of America** 101:7526-7529.

CTNBio (2009) **Extrato de parecer técnico 2042/2009.** Brasília, 4 p.

CTNBio (2008) **Parecer técnico 1255/2008.** Brasília, 8 p.

Devos Y, Meihls LN, Kiss J, Hibbard BE (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.

Dively GP, Huang F, Oyediran I, Burd T, Morsello S (2020) Evaluation of gene flow in structured and seed blend refuge systems of non-Bt and Bt corn. **Journal of Pest Science** 93:439-447.

Eghrari K, Brito AH, Baldassi A, Balbuena TS, Fernandes OA, Môro GV (2019) Homozygosis of Bt locus increases Bt protein expression and the control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize hybrids. **Crop Protection** 124.

Frizzas MR, Silveira Neto S, Oliveira CM, Omoto C (2014) Genetically modified corn on fall armyworm and earwig populations under field conditions. **Ciência Rural** 44:203-209.

García-Lara S, Chuck-Hernandez C, Serna-Saldivar SO (2019) Development and structure of the corn kernel. In.: Saldívar SROS (Eds.) **Corn Chemistry and Technology.** Duxford: Woodhead Publishing, p. 147-163.

Head G, Campbell LA, Carroll M, Clark T, Galvan T, Hendrix WM, et al (2014) Movement and survival of corn rootworm in seed mixtures of SmartStax® insect-protected corn. **Crop Protection** 58:14-24.

Horikoshi RJ, Bernardi D, Bernardi O, Malaquias JB, Okuma DM, Miraldo LL, et al (2016) Effective dominance of resistance of Spodoptera frugiperda to Bt maize and cotton varieties: implications for resistance management. **Scientific Reports** 6: 1-8.

Huang F, Qureshi JA, Meagher RL, Reisig DD, Head GP, Andow DA, et al (2014), Cry1F resistance in fall armyworm *Spodoptera frugiperda*: Single gene versus pyramided Bt maize. ***PLoS One*** 9.

Huang F, Leonard BR, Wu X (2007) Resistance of sugarcane borer to *Bacillus thuringiensis* Cry1Ab toxin. **Entomologia Experimentalis et Applicata** 124:117-123.

Jin L, Zhang H, Lu Y, Yang Y, Wu K, Tabashnik BE, et al (2015) Large-scale test of the natural refuge strategy for delaying insect resistance to transgenic Bt crops. **Nature Biotechnology** 33:169-174.

Jing DP, Guo JF, Jiang YY, Zhao JZ, Sethi A, He KL, et al (2019) Initial detections and spread of invasive *Spodoptera frugiperda* in China and comparisons with other noctuid larvae in cornfields using molecular techniques. **Insect Science**:1-11.

Kiesselbach TA, The significance of xenia effects on the kernel weight of corn (1960) **Historical Research Bulletins of the Nebraska Agricultural Experiment Station** 143.

Magalhães PC and Durães FO (2006) **Fisiologia da produção de milho.** Sete Lagoas: Embrapa: Embrapa Milho e Sorgo, 10 p. (Circular Técnica, 76).

Martinelli S, Carvalho RA, Dourado PM, Head GP (2017) Resistance of *Spodoptera frugiperda* to *Bacillus thuringiensis* proteins in the Western Hemisphere. In.: Fiuza LM, Polanczyk RA, Crickmore N (Eds.) ***Bacillus thuringiensis* and *Lysinibacillus***

***sphaericus*.** Cham: Springer, pp. 273-288.

Mendiburu F (2020) agricolae: Statistical Procedures for Agricultural Research.

Miraldo LL, Bernardi O, Horikoshi RJ, Amaral FSA, Bernardi D, Omoto C (2016) Functional dominance of different aged larvae of Bt-resistant *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on transgenic maize expressing Vip3Aa20 protein. **Crop Protection** 88:65-71.

Naranjo SE (2009) Impacts of Bt crops on non-target invertebrates and insecticide use patterns. **CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources** 4.

Niu Y, Head GP, Price PA, Huang F (2016) Performance of Cry1A.105-selected fall armyworm (Lepidoptera: Noctuidae) on transgenic maize plants containing single or pyramided Bt genes. **Crop Protection** 88:79-87.

Oliveira R, Carneiro TR, Fernandes OA (2006) Criação de *Telenomus remus* Nixon (Hymenoptera: Scelionidae) para o controle da lagarta-do-cartucho, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae). In.: Bortoli S, Boiça Jr AL, Oliveira J (Eds.) **Agentes de controle biológico - Metodologias de criação, multiplicação e uso.** Jaboticabal: Funep, Jaboticabal, p. 151–166.

Omoto C, Bernardi O, Salmeron E, Sorgatto RJ, Dourado PM, Crivellari A, et al (2016) Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil. **Pest Management Science** 72:1727-1736.

Onstad DW, Mitchell PD, Hurley TM, Lundgren JG, Porter RP, Krupke CH, et al (2011) Seeds of change: Corn seed mixtures for resistance management and integrated pest management. **Journal of Economic Entomology** 104:343-352.

Pannuti LER, Paula-Moraes SV, Hunt TE, Baldin ELL, Dana L, Malaquias JV (2016) Plant-to-plant movement of *Striacosta albicosta* (Lepidoptera: Noctuidae) and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize (*Zea mays*). **Journal of**

Economic Entomology 109:1125-1131.

Poehlman JM (1987) Breeding corn (maize). In.: Poehlman JM (Eds.) **Breeding field crops.** Dordrecht: Springer, p. 451-507.

R Core Team (2019) R: A Language and Environment for Statistical Computing. Vienna, Austria.

Reay-Jones FPF, Bessin RT, Brewer MJ, Buntin DG, Catchot AL, Cook DR, et al (2016) Impact of lepidoptera (Crambidae, Noctuidae, and Pyralidae) pests on corn containing pyramided Bt traits and a blended refuge in the Southern United States. **Journal of Economic Entomology** 109:1859-1871.

Santos LF, Ruiz-sánchez E, Andueza-noh RH, Garruña-hernández R, Latournerie-moreno L, Mijangos-cortés JO (2020) Leaf damage by *Spodoptera frugiperda* J. E. Smith (Lepidoptera : Noctuidae) and its relation to leaf morphological traits in maize landraces and commercial cultivars. **Journal of Plant Diseases and Protection** 127:103-109.

Silva IHS, Goméz I, Sánchez J, Castro MDL, Valicente FH, Soberón M, et al (2018) Identification of midgut membrane proteins from different instars of *Helicoverpa armigera* (Lepidoptera: Noctuidae) that bind to Cry1Ac toxin. **PLoS One** 13:1-16.

Sisay B, Simiyu J, Mendesil E, Likhayo P, Ayalew G, Mohamed S, et al (2019). Fall armyworm, *Spodoptera frugiperda* infestations in East Africa: Assessment of damage and parasitism. **Insects** 10:1-11.

Sorgatto RJ, Bernardi O, Omoto C (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** 44:186-192.

Souza CSF, Silveira LCP, Pitta RM, Waquil JM, Pereira EJG, Mendes SM (2019) Response of field populations and Cry-resistant strains of fall armyworm to Bt maize

hybrids and Bt-based bioinsecticides. **Crop Protection** 120:1-6.

Storer NP, Babcock JM, Schlenz M, Meade T, Thompson GD, Bing JW, et al (2010) Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. **Journal of Economic Entomology** 103:1031-1038.

Tollenaar M and Dwyer LM (1999) Physiology of maize. In.: Smith DL, Hamel C (Eds.) **Crop Yield.** Berlin: Springer, p. 169-204.

Wang SC, Wang GY, Ding QX, Zhang H, Xie YJ, Dai JR (1999) Studies of transgene segregation and integration in maize. **Yi Chuan Xue Bao** 26:254-261.

Weingartner U, Kaeser O, Long M, Stamp P (2002) Combining cytoplasmic male sterility and xenia increases grain yield of maize hybrids. **Crop Science** 42:1848-1856.

Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. New York: Springer-Verlag.

Wright AD and Neuffer MG (1989) Orange pericarp in maize: Filial expression in a maternal tissue. **Journal of Heredity** 80:229-233.

Yang F, Kerns DL, Brown S, Head GP, Huang F (2017) Pollen contamination in seed mixture increases the dominance of resistance to Bt maize in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). **Pest Management Science** 73:2379-2385.

Yang F, Morsello S, Head GP, Sansone C, Huang F, Gilreath RT, et al (2018) F2 screen, inheritance and cross-resistance of field-derived Vip3A resistance in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) collected from Louisiana, USA. **Pest Management Science** 74:1769-1778.

Yang F, Kerns DL, Head GP, Price PA, Levy R, Niu Y, et al (2020) Extended evaluation of Bt protein cross-pollination in seed blend plantings on survival, growth, and development of *Helicoverpa zea* feeding on refuge ears. **Pest Management Science**

76:1011-1019.

Yang F, Kerns DL, Head GP, Leonard BR, Levy R, Niu Y, et al (2014) A challenge for the seed mixture refuge strategy in Bt maize: Impact of cross-pollination on an ear-feeding pest, corn earworm. **PLoS One** 9:1-9.

Yang F, Kerns DL, Brown S, Kurtz R, Dennehy T, Braxton B, et al (2016) Performance and cross-crop resistance of Cry1F-maize selected *Spodoptera frugiperda* on transgenic Bt cotton: implications for resistance management. **Scientific Reports** 6: 1-7.

APPENDIX – Supplementary tables

Supplementary Table 1. Survivorship and weight (mean ± SE) of lab third-instar *S. frugiperda* larvae fed with R3-kernels.

Hybrid	Number of larvae Day 0	Number of survivors Day 4	Survivorship (%) Day 4	Larval weight (mg) Day 0	Larval weight (mg) Day 4
Non-Bt	48	48	100.00 ± 0.00	8.08 ± 0.78	161.04 ± 7.02
MIR162-HE⊗	48	1	2.08 ± 2.08†	7.14 ± 0.73	13.70 ± 0.00
MIR162-HO⊗	48	1	2.08 ± 2.08†	6.91 ± 0.84	7.50 ± 0.00
♀Non-Bt x ♂MIR162-HO	48	3	6.25 ± 3.99	6.96 ± 0.73	7.40 ± 0.15

† *S. frugiperda* subjected to MIR162 died after being fed with non-Bt maize leaves for two days.

Supplementary Table 2. Survivorship and weight (mean ± SE) of field *S. frugiperda* larvae fed with R3-kernels.

Hybrid	Number of larvae Day 0	Number of survivors Day 4	Survivorship (%) Day 4	Larval weight (mg) Day 0‡	Larval weight (mg) Day 4
Non-Bt⊗	65	65	93.85 ± 5.01	56.39 ± 6.44	162.58 ± 15.57
MIR162-HE⊗	65	12	18.46 ± 6.99†	56.39 ± 6.44	60.65 ± 9.22

† *S. frugiperda* subjected to MIR162 died after being fed with non-Bt maize leaves for two days. ‡ Third-instar larvae were previously fed with R3-kernels of the non-Bt, hemizygous for Bt11, and homozygous for Bt11 of near-isogenic versions of the Status maize hybrid for four days (Eghrari et al. 2020 Syngenta).

Supplementary Table 3. Survivorship and weight (mean ± SE) of field third-instar diet-reared *S. frugiperda* fed with VT leaves of seven isogenic versions of the maize hybrid Status for five days.

Hybrid	Number of larvae Day 0	Number of survivors Day 5	Mean survivorship (%)	Mean weight (mg) Day 0	Mean weight (mg) Day 5
Non-Bt	60	51	85.00 ± 7.64	5.49 ± 1.22	42.84 ± 4.87
Bt11-HE	60	44	73.33 ± 10.34	5.21 ± 1.74	48.61 ± 5.43
Bt11-HO	60	46	76.67 ± 10.00	5.85 ± 1.53	55.10 ± 5.14
MIR162-HE	60	0	0.00 ± 0.00	4.76 ± 0.96	0.00 ± 0.00
MIR162-HO	60	0	0.00 ± 0.00	4.46 ± 0.29	0.00 ± 0.00
MIR162 + Bt11-HE	60	0	0.00 ± 0.00	3.85 ± 0.35	0.00 ± 0.00
MIR162 + Bt11-HO	60	0	0.00 ± 0.00	4.04 ± 0.37	0.00 ± 0.00

Supplementary Table 4. Survivorship and weight (mean ± SE) of third-instar diet-reared *S. frugiperda* fed with maize leaves at the VT growth-stage of two near-isogenic versions of the Status hybrid for 5 days.

Hybrid	Number of larvae Day 0	Number of survivors Day 5	Mean survivorship (%)	Mean weight (mg) Day 0	Mean weight (mg) Day 5
Non-Bt	65	65	100.00 ± 0.00	50.02 ± 2.73	179.19 ± 16.63
MIR162-HE	65	0	0.00 ± 0.00	45.52 ± 4.55	0.00 ± 0.00