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Homozygosis of Bt locus increases Bt protein expression and the control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize hybrids

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ABSTRACT

Spodoptera frugiperda (J.E. Smith) is the main pest of maize in Brazil and its control has been targeted by Bt maize hybrids. Generally, Bt maize hybrids possess the transgenic locus in a hemizygous condition, that is, containing only one copy of the Bt allele. However, studies have shown that maize hybrids with an additional transgenic allele, namely, in a homozygous state, increases transgenic protein expression. Our aim in this study was to evaluate whether the transgenic event TC1507 x MON89034 x NK603 in homozygosis increases Bt protein expression levels and, consequently, reduces *S. frugiperda* leaf-feeding injury and larval survival which affects maize grain yield. Leaf-feeding injury of *S. frugiperda* was 29% lower on homozygous hybrids relative to their isogenic hemizygous versions. Isogenic homozygous and hemizygous hybrids did not differ in grain yield in this study. *S. frugiperda* survivorship on homozygous hybrids was significantly lower than on their hemizygous isogenic versions (16.9% and 38.5%, respectively). Homozygous hybrids presented higher Cry1F, Cry1A.105, and Cry2Ab2 protein expression levels relative to their isogenic hemizygous versions (approximately 1.5-, 2.0-, and 2.5-fold, respectively). The Bt maize event TC1507 x MON89034 x NK603 in a homozygous state increases Bt protein expression levels and the control of *S. frugiperda*. Therefore, the deployment of homozygous transgenic maize hybrids to farmers is more desirable than the hemizygous versions.

1. Introduction

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) is the primary pest affecting maize in Brazil, causing leaf injury and yield losses up to 57.6% (Cruz and Turpin, 1983; Cruz et al., 1999; Waquil et al., 2002; Niu et al., 2014). Genetically-modified (GM) maize hybrids with *Bacillus thuringiensis* (Bt) genes can decrease *S. frugiperda* infestation levels on the field, increase yield (Siebert et al., 2012; Storer et al., 2012; Frizzas et al., 2014; Moraes et al., 2015; Santos-Amaya et al., 2015), and diminish insecticide use worldwide (Meissle et al., 2010; Benbrook, 2012).

In the last decade, *S. frugiperda* resistance to Bt protein has increased due to the intense use of Bt maize hybrids (Storer et al., 2010; Tabashnik et al., 2013; Jakka et al., 2014; Huang et al., 2014), and studies have reported *S. frugiperda* field-evolved resistance to Cry1F (Farias et al., 2014) and Cry1Ab (Omoto et al., 2016) in Brazil. In 2010, the Brazilian Technical Bio-Safety Commission (CTNBio) approved pyramided events in Brazil expressing multiple toxins to increase efficacy and delay resistance evolution (Zhao et al., 2003; Hardke et al., 2011; Huang et al., 2014). One of these pyramided events, TC1507 x MON89034 x NK603, contains three Bt proteins: Cry1F, Cry1A.105 and Cry2Ab2 (ISAAA, 2019). Due to protein similarity, reports have shown cross-resistance between Cry1F and other Cry1 proteins, such as Cry1A.105 and Cry1Ab; however, Cry1F did not present cross-resistance with Cry2Ab2 (Bernardi et al., 2015; Horikoshi et al., 2016). Therefore, high-dose pyramided Bt events and the use of refuge are key strategies for the resistance management of *S. frugiperda* (Shelton et al., 2000; Andow, 2008; Farias et al., 2015).

The commercialization of Bt maize hybrids in Brazil started in 2008 (James, 2009). Brazil produces more than 90 million tons of maize on approximately 17 million hectares (CONAB, 2019), and the overall use of genetically modified (GM) maize hybrids is responsible for approximately 90% of the production (ISAAA, 2017). Breeding companies develop transgenic maize hybrids that are hemizygous for the transgene

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(Guadagnuolo et al., 2006; Burkness et al., 2010, 2011; Caprio et al., 2015), because the introgression of transgenic traits is a costly and time-consuming process when all elite inbred lines of a breeding program are taken into consideration (Morris et al., 2003). In hemizygous F1 maize hybrids, the transgenic locus presents only one copy of the transgenic allele, while the other allele is considered null (Guadagnuolo et al., 2006). This null allelic version in the transgenic locus occurs because a Bt trait in maize result from the insertion of an exogenous Bt allele in the maize genome; therefore, the genome does not possess an alternative allelic variation for the transgenic locus.

An elite inbred line is generated by transgene introgression through the backcrossing method. It involves the crossing of a transgenic donor genotype and a recurrent parent (the elite inbred line) for several cycles of backcrossing. In each generation of backcrossing occurs the selection of inbred lines with the presence of the desired transgenic trait and greater proportion of the recurrent parent through marker-assisted selection (Venkatesh et al., 2015). After the recovery of the recurrent parent with the desired transgenic trait, the selected transgenic elite inbred line is self-pollinated so that the transgenic locus is homozygous. A hemizygous transgenic hybrid is obtained by crossing two inbred lines: a homozygous inbred line for the transgenic trait with a non-GM inbred line. This approach does not provide the highest levels of transgenic protein expression (Caligari et al., 1993; James et al., 2002; Clough et al., 2006; Hood et al., 2012; Howard and Hood, 2014). However, a homozygous transgenic version of maize hybrids is obtained by crossing two transgenic inbred lines. This results in maize hybrids with the transgenic locus in a homozygous state, that is, with two transgenic alleles. Homozygous transgenic hybrids may present their maximum transgenic protein accumulation due the presence of an additional transgenic allele (Caligari et al., 1993; Streatfield et al., 2002; Clough et al., 2006). Previous studies have shown that the transgene zygosity of a plant in a homozygous condition increases the transgenic protein expression up to four fold when compared to a hemizygous version (James et al., 2002; Law et al., 2006; Hood et al., 2012).

Zygosity studies in maize hybrids have been made considering cross-pollination and its impact on maize kernels (Chilcutt and Tabashnik, 2004; Burkness et al., 2011), but it is still unclear whether the zygosity of Bt transgenes might influence Bt protein expression in maize plants (Caprio et al., 2015). To our knowledge, Bt homozygous maize hybrids are not commercialized yet. Thus, this study aimed to assess whether the number of Bt alleles in maize hybrids increases the expression levels of Bt proteins and *S. frugiperda* control, and affects grain yield.

2. Materials and methods

2.1. Genetic material

The genetic material comprised five maize hybrids (A, B, C, D, and E) belonging to Corteva Agriscience. Each hybrid was generated in two isogenic versions: homozygous (HO) and hemizygous (HE) for the event TC1507 x MON89034 x NK603. In the pyramid, TC1507 is responsible for the production of Cry1F protein (*cry1Fa2* gene) and tolerance to glufosinate herbicide (*pat* gene); MON89034 confers resistance to lepidopteran insects by the product of *cry1A.105* and *cry2Ab2* genes; and NK603 is responsible for glyphosate tolerance (*cp4 epsps* gene). In addition, one non-GM hybrid was defined as control (ACO), which is a conventional isogenic version of the hybrid A. Therefore, the 11 hybrids were designated as ACO, AHO, AHE, BHO, BHE, CHO, CHE, DHO, DHE, EHO, and EHE.

To present two, one or no copies of the TC1507 x MON89034 x NK603 event, i.e., homozygous, hemizygous, and non-GM, respectively, each hybrid was produced in a specific manner. Transgenic homozygous hybrids were obtained by crossing two TC1507 x MON89034 x NK603 homozygous inbred lines, while the hemizygous hybrids were obtained by crossing a transgenic homozygous inbred line with a non-GM inbred line. To obtain the non-GM version of hybrid A, two non-GM inbred lines were crossed. For instance, the isogenic versions of hybrid A were generated by the same parental inbred lines. To generate AHO, both parental inbred lines were transgenic; for AHE, only one parental inbred line was transgenic; for ACO, both parental inbred lines were non-GM. Thus, the difference between the versions of hybrid A is the presence of the transgenic trait in their parental inbred lines.

2.2. Field experiments

Two field experiments were carried out: one in the first growing season (sowing date: Sep 30th, 2015) and the other in the second growing season (sowing date: Feb 2nd, 2016), at the Experimental Farm of São Paulo State University, Campus of Jaboticabal (UNESP/FCAV), Brazil (21°14′S and 48°17′W). Jaboticabal has a rainy summer and dry winter type of climate, classified as Aw (Rubbel and Kottek, 2010). Weather data were provided by the São Paulo State University Agro-meteorological Station, which is about 1 km away from the site where the experiments were conducted (Supplementary Table 1).

The experimental design was a complete randomized block with three replications. Each plot consisted of four 5-m-long rows, spaced 0.5 m apart, and with 60 plants per plot, representing a population density of 60,000 plants ha^{-1}. The management of the experiments followed the maize crop recommendations for the region (Fornasieri Filho, 2007). Leaf-feeding injury of *S. frugiperda* in the field, under natural infestation, was assessed using a 0–9 scale, which the lowest score represents the absence of *S. frugiperda* infestation, while the highest score refers to the total destruction of the plant (Davis et al., 1992). The scores of leaf-feeding injuries were given according to the average of 10 random plants of the two central lines of each plot, in the V6/V7 growth stage, approximately 35 days after sowing each experiment. There was no insecticide application. After harvesting, at the R6 stage, the first and second growing season (March 2nd and July 4th, respectively), grain yield of each plot was weighed, standardized by correcting grain moisture to 13% and converted to kg ha^{-1}.

2.3. Leaf tissue bioassays

Bioassays were installed and conducted approximately 30 days after sowing each experiment at the Applied Ecology Laboratory of UNESP/FCAV. *Spodoptera frugiperda* egg masses were collected in maize fields at the Experimental Farm of UNESP/FCAV, five days prior to sowing the field experiments. The egg masses were taken to the laboratory and maintained at 25 ± 1 °C, under a daily photoperiodic cycle of 12:12 h (light: dark) during all phases of the bioassays. Larvae were reared using bean based diet similarly to Oliveira et al. (2006) until pupation. Pupae were separated by sex and five couples were placed per mating cage (10 cm diameter x 22 cm height). Adults were fed with a 9:1 solution of water and honey until oviposition. This field-collected population was used in all bioassays.

Once the second generation of *S. frugiperda* was obtained, the bioassays were conducted. The newest leaf with collar of the plants of each hybrid (V6/V7 growth stage) was used for each bioassay replication. Leaves were identified and kept in paper bags, taken to the laboratory and immersed in water solution with 0.5% of NaClO, rinsed in running water and dried with paper towels to avoid larval contamination by field pathogens. Afterwards, leaves were cut into pieces (approximately 5.0 cm × 3.5 cm) and used to feed neonate larvae (<24 h) in the bioassays.

For each bioassay, a deep well of a plastic tray (5.0 cm × 3.5 cm x 4.0 cm) was used with a *S. frugiperda* neonate and a piece of leaf with the same dimension of the well's bottom surface. The bioassay of the first season had three replicates and 10 individualized larvae per tray (total of 330 larvae), while the bioassay of the second season was performed with five replicates and 16 individualized larvae per tray (total of 880 larvae). The bioassay design was a completely randomized block, because we could not accommodate all trays in a single shelf. After seven

days, we assessed survivorship of *S. frugiperda*, and growth inhibition by the number of larvae with length below 1.5 cm. Larvae that did not move at the touch of a fine camel-hair brush were considered as dead.

2.4. Analysis of Bt protein abundance in maize leaves

Absolute Cry1F protein concentration levels were quantified using 10 plants from a separate plot of each homozygous and hemizygous maize hybrid at the V6/V7 growth stage. The samples were assayed with an ELISA test using the QualiPlate kit for Cry1F detection in maize and cotton (cat# AP-016, Envirologix, Portland, ME, USA). Quantification of Cry1F was performed by setting up a 7-point standard curve, in duplicate, with two blanks, along with three positive and three negative controls to determine the concentration of Cry1F in each sample. The Cry1F protein standard was provided by Corteva Agriscience, coded internally as TSN 302442.

The relative expression of the gene products Cry1A.105 and Cry2Ab2 was determined by peptide spectrometry analysis (HPLC-MS/MS). Only one replicate was used as the aim of the analysis was solely to confirm the high expression of the target proteins in the homozygous versions of the hybrids. Each replicate was comprised by twelve young plants (V2/V3) of each hybrid. A pool of the leaves of each hybrid was collected and pulverized in liquid nitrogen. Proteins were then extracted with a sample buffer (125 mM Tris pH 6.8; 20% Glycerol; 1% SDS and 1% DTT) and precipitated in cold acetone for 16 h at −30 °C. Protein quantification was determined based on Bradford (1976) protocol using bovine serum albumin (BSA) as standard. Prior to mass spectrometry analysis, the protein extract was digested with trypsin for 4 h at 37 °C. The extracted peptides were cleaned using C-18 spin columns (Thermo Scientific) according to the manufacturer's instructions and then were dried down under vacuum centrifugation. Tryptic peptides were separated in a chromatographic gradient of 60 min under constant flow rate of 400 nL/min (EASY nLC 1000, Thermo Scientific) using a C18 nano-column (15 cm 2 μm, 100 Å). Mass spectrometer (Q-Exactive, Thermo Scientific) was operated in the data-dependent acquisition mode for the ten most abundant peptide ions. Protein identification was carried out by spectral correlations approach against *Zea mays* genome and Cry1A.105 and Cry2Ab2 protein sequences. We used Sequest-HT as a search tool and the data were normalized according to NSAF (normalized spectral abundance factors) described in Paoletti et al. (2006). Protein relative expression was determined by spectral counting. The fold-change was calculated by dividing the relative expression of homozygous versions by relative expression of hemizygous versions.

2.5. Data analysis

Two orthogonal contrasts were applied to leaf-feeding injury of *S. frugiperda* in the field, grain yield, and survival of first-instar *S. frugiperda*. The first contrast compared the group of transgenic hybrids (homozygous and hemizygous) with the non-GM hybrid, following the model: $Y_1 = m_1 + m_2 - 2m_3$, while the second contrast, $Y_2 = m_1 - m_2$, compared homozygous and hemizygous transgenic hybrids, where Y_1 and Y_2 = contrasts between hybrids means; m_1 = homozygous hybrids mean; m_2 = hemizygous hybrids mean; and m_3 = non-GM hybrid mean, by t-test. Each season was considered separately. Concentrations of Cry1F, Cry1A.105, and Cry2Ab2 of all homozygous and hemizygous versions were submitted to t-test. Expressions of Cry1F protein between homozygous and hemizygous versions of each hybrid were submitted to t-test. However, Cry1A.105 and Cry2Ab2 expressions were not submitted to t-test because there was only one replicate per hybrid, since we used the HPLC-MS/MS analysis only to confirm that the homozygous versions accumulated more of the proteins Cry1A.105 and Cry2Ab2 than the hemizygous versions. In addition, a multi-year analysis was performed combining both seasons for leaf-feeding injury of *S. frugiperda* in the field, grain yield, and survival of *S. frugiperda* neonates. The degrees of freedom of the hybrids and the interaction between hybrids and seasons were divided into three other sources of variation: homozygous, hemizygous, and the combination of the three groups (homozygous, hemizygous, and non-GM), designated as groups. All analyses were performed in R 3.3.1 (R Core Team, 2016).

3. Results

3.1. Cry1F, Cry1A.105, and Cry2Ab2 protein expression in the leaf

All homozygous hybrids presented higher Cry protein concentrations when compared to their hemizygous isogenic versions. The mean leaf concentration of Bt proteins in homozygous hybrids were 54% for Cry1F ($t_{1,\ 98} = 4.06$, $p < 0.0001$), 151% for Cry1A.105 ($t_{1,\ 8} = 7.12$, $p < 0.01$), and 146% for Cry2Ab2 ($t_{1,\ 8} = 3.35$, $p < 0.0001$) higher than the hemizygous versions (Fig. 1). Fold-change between homozygous and hemizygous hybrids for Cry1F, Cry1A.105, and Cry2Ab2 ranged from 1.49 to 1.94, 1.81 to 7.83, and 2.31 to 2.62, respectively (Supplementary Table 2).

3.2. Leaf injury in the field trials

Leaf-feeding injury on homozygous transgenic hybrids was 29% lower than on their hemizygous versions ($t_{2,\ 30} = -3.97$, $p < 0.0005$) during the first growing season. Conversely, in the second season, such a difference was not observed. Transgenic hybrids were less injured than the non-GM hybrid regardless of the season (first: $t_{2,\ 30} = 9.63$, $p < 0.0001$; second: $t_{2,\ 30} = 4.12$, $p < 0.0003$) (Fig. 2A) (Supplementary Tables 3 and 4).

3.3. Survivorship of S. frugiperda in the leaf tissue bioassays

Survivorship of first-instar *S. frugiperda* was significantly reduced on homozygous hybrids when compared to their hemizygous versions in both seasons (first: $t_{2,\ 30} = -2.41$, $p = 0.0224$; second: $t_{2,\ 52} = -7.19$, $p < 0.0001$). Moreover, mortality of first-instar *S. frugiperda* was higher on the transgenic hybrids than on the non-GM hybrid for both seasons (first: $t_{2,\ 30} = 8.33$, $p < 0.0001$; second: $t_{2,\ 52} = 10.63$, $p < 0.0001$) (Fig. 2B). Average survivorship for homozygous, hemizygous, and the non-GM hybrid was $16.9 \pm 1.6\%$, $38.5 \pm 2.08\%$, and $94.5 \pm 2.18\%$, respectively, combining the results of both seasons (Supplementary Tables 3 and 4).

3.4. Grain yield

Grain yield of homozygous and hemizygous did not differ in both seasons (first: $t_{2,\ 30} = 0.512$, $p = 0.6122$; second: $t_{2,\ 30} = -0.247$, $p = 0.807$). The yield of transgenic hybrids was higher than that of the non-GM hybrid in the first season ($t_{2,\ 30} = -2.26$, $p = 0.0311$). The yield loss of the non-GM hybrid in relation to the homozygous and hemizygous hybrids was approximately 25% and 23%, respectively, during the first season. The hybrids did not differ in grain yield in the second season ($t_{2,\ 30} = -0.937$, $p = 0.3560$) (Fig. 2C) (Supplementary Tables 3 and 4).

4. Discussion

Spodoptera frugiperda control and leaf concentration of Cry1F, Cry1A.105, and Cry2Ab2 were higher on the homozygous hybrids than on their hemizygous versions, showing that the additional transgenic allele TC1507 x MON89034 x NK603 directly influenced the expression of Bt proteins. Indeed, an increase in the transgene zygosity of the maize hybrids caused an additive effect on the levels of Cry proteins in the leaf. The additive effect has also been reported for other transgenes (Caligari et al., 1993; James et al., 2002; Hood et al., 2012).

Although the concentration of Bt proteins in the leaf increased, there was no decrease in grain yield in the homozygous hybrids, that is to say, the additional TC1507 x MON89034 x NK603 allele in the transgenic

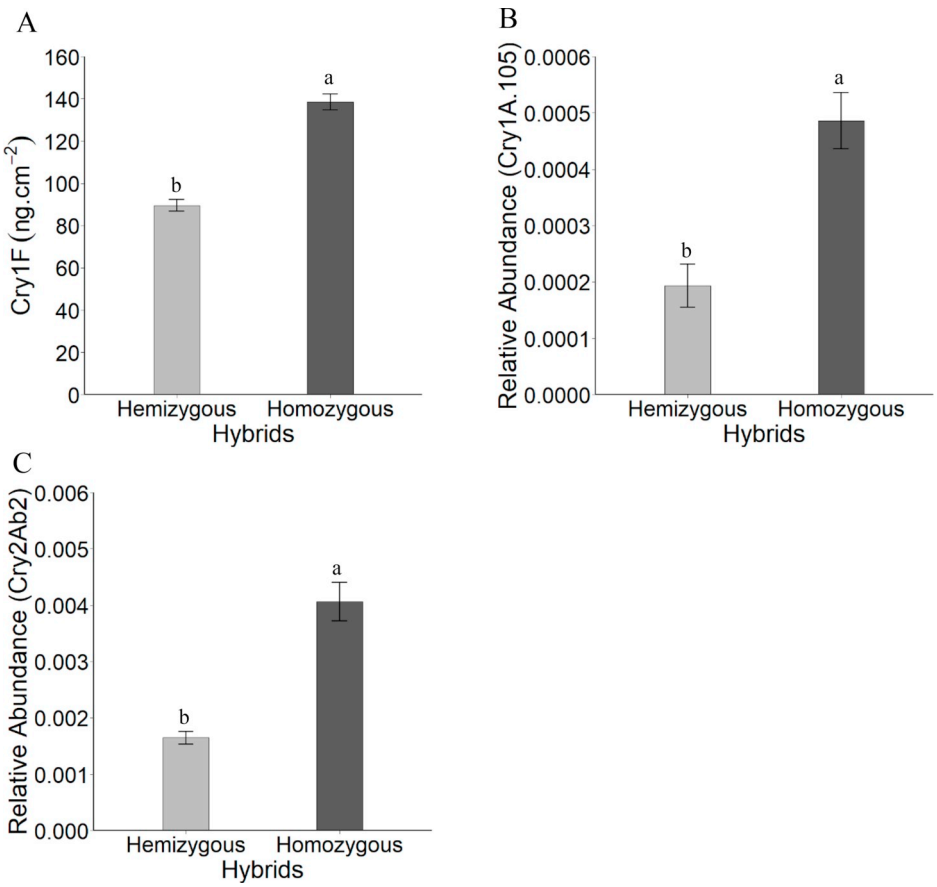

Fig. 1. Overall means (±SEM) of Cry1F (ng cm^{-2}) (A), Cry1A.105 (relative abundance) (B), and Cry2Ab2 (relative abundance) (C) expression in leaves of homozygous and hemizygous TC1507x89034xNK603 maize hybrids. Each zygosity with different letters on top of the bars differ by t-test ($p < 0.0001$ for Cry1F, $p < 0.01$ for Cry1A.105, and $p < 0.0001$ for Cry2Ab2).

locus did not cause a detectable energy cost for the plant that reduced grain production. In fact, grain yield of the homozygous hybrids was higher than the non-GM hybrid and similar to the hemizygous hybrids, revealing that Bt alleles protected maize grain yield against injuries caused by *S. frugiperda*. Indeed, in the absence of a selection pressure from *S. frugiperda* leaf injury, the transgene becomes a neutral factor to the fitness of the plant (Guadagnuolo et al., 2006), and as a result neither transgenic zygosity impairs maize grain yield based on our data.

Despite high infestation levels of *S. frugiperda* during the first season, there was no difference in the grain yield of hemizygous and homozygous hybrids. Nevertheless, the use of homozygous hybrids could be incorporated in the resistance management of *S. frugiperda*, since their levels of Cry proteins expressions are higher than hemizygous hybrids, causing higher mortality rates of *S. frugiperda*. Although, in this study, neither zygosity of TC1507 x MON89034 x NK603 met the criteria of a high-dose event (Andow, 2008), homozygous versions could still be useful for delaying *S. frugiperda* field-evolved resistance to Cry proteins due to its increase in larval control.

However, to better understand the effect of Bt zygosity in maize plants, new studies considering the effect of Bt transgenes on leaves and kernels, as well as on plant morphology and pollen viability should be considered, since a transgene could affect the phenotype of the plant (Saxena and Stotzky, 2001). To our knowledge, this is the only study that examines the effect of Bt zygosity on Bt expression levels in leaves of maize hybrids, though we did not examine other Bt proteins such as Cry1Ab and Vip3Aa20, and the effect of Bt pollen on non-Bt maize kernels in refuge areas. The effect of Bt zygosity in maize kernels has been studied, but Bt protein concentrations were not assessed (Burkness et al., 2011; Yang et al., 2014; Caprio et al., 2015). As kernel pericarps are an exclusively maternal tissue, and embryo and endosperm are tissues of maternal and paternal origin, more studies are required to assert precisely which tissue is expressing Bt proteins, and if distinct tissues of the kernel are expressing a sufficient amount of Bt protein that could fit the high-dose strategy. To clarify that, Burkness et al. (2011) did reciprocal crosses between hemizygous Bt11 maize hybrids with non-Bt hybrids as male and female parents. They found higher mortality of *Ostrinia nubilalis* (Hübner) on kernels of hemizygous Bt11 plants that had non-Bt plants as pollen donors, when compared to mortality on kernels of crosses of non-Bt plants with hemizygous Bt plants as pollen donors. Based on these authors' results, we believe that the hemizygosity of the pericarp caused higher mortality on ears of the hemizygous Bt plants, since kernels of non-Bt hybrids present non-Bt pericarps. Correspondingly, in our study we found lower larval survival on leaves of homozygous hybrids in comparison to hemizygous versions that shows the effect of the additional transgenic allele, causing a double allelic TC1507 x MON89034 x NK603 dose, without the confounding factors of maternal and paternal tissues found in the kernels. Furthermore, although there are no studies considering the increase of Bt protein expression due to additional Bt alleles in maize hybrids, some studies acknowledge a four-fold increase in the amount of transgenic human antibodies (Law et al., 2006) and industrial enzymes in homozygous versions of maize hybrids (Hood et al., 2012).

A more severe infestation of *S. frugiperda*, resulting in higher scores of leaf-feeding injury, decreased grain production on the non-GM hybrid in the first season, though grain production of homozygous and hemizygous hybrids remained the same. Due to lack of rain, which is an important natural mortality factor, higher infestations of *S. frugiperda* typically take place during the second season in Brazil (Varella et al.,

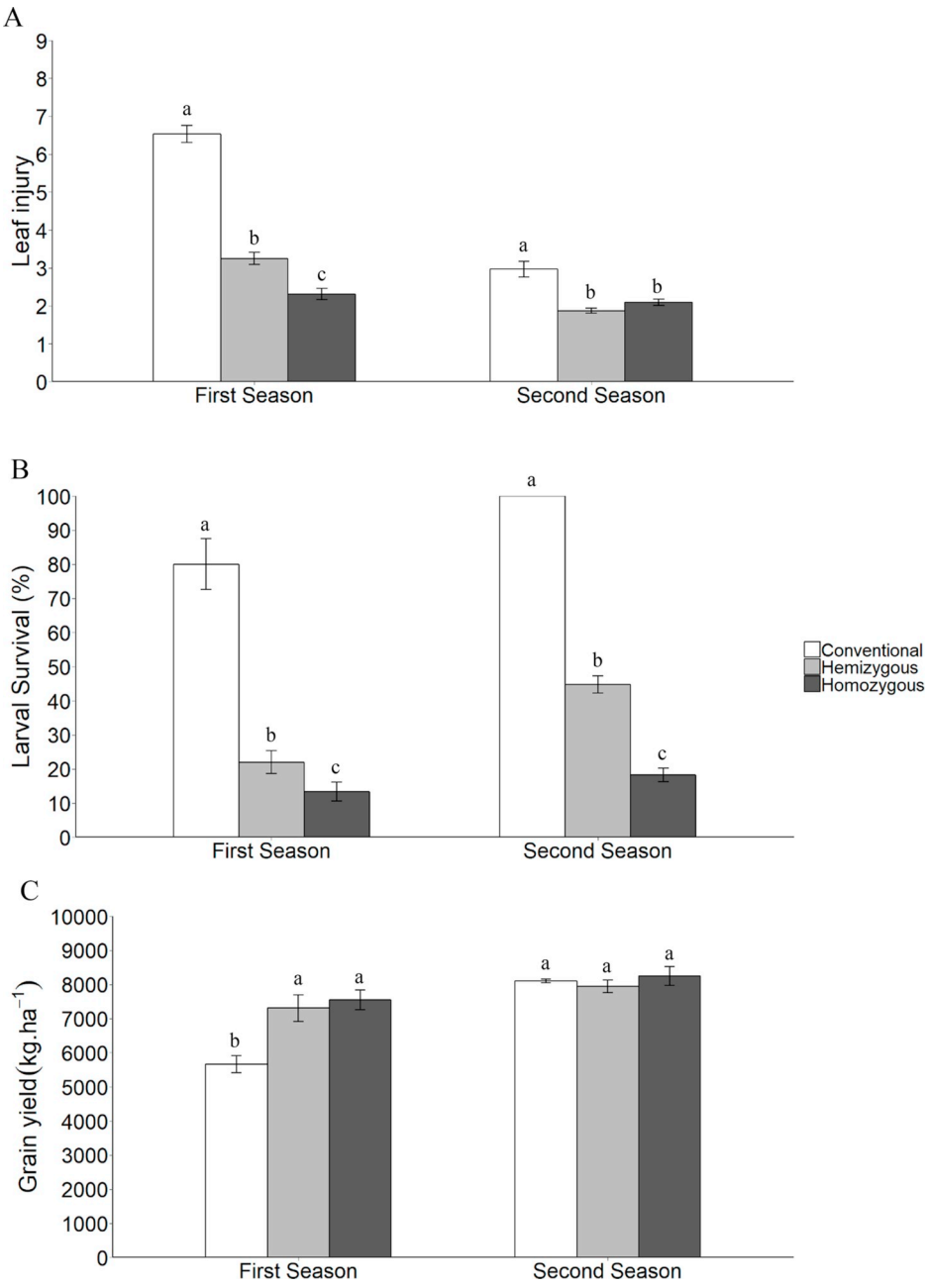

Fig. 2. Means (±SEM) of leaf-feeding injury of *Spodoptera frugiperda* (score 0–9) (A), survival of first instar *S. frugiperda* (%) (B), and grain yield (kg ha^{-1}) of homozygous and hemizygous TC1507x89034xNK603 maize hybrids (C) and non-Bt hybrid (conventional), during the first and second seasons of 2015/2016, Jaboticabal, Brazil. Each season was submitted to t-test separately. Different letters on top of the bars differ each treatment by t-test ($p < 0.05$) according to orthogonal contrast between transgenic hybrids, homozygous and hemizygous, and the conventional hybrid; and orthogonal contrast between homozygous and hemizygous hybrids.

2015). Notably, the main mortality factors in neonatal *S. frugiperda* larvae are predation, drowning, and dislodgement by rainfall (Varella et al., 2015). Contrary to the usual weather, the 2015/2016 growing season presented an initial drier period and a late wetter period. To illustrate, in the first season, we evaluated leaf-feeding injury of *S. frugiperda* in the beginning of November, when there was less rainfall in the previous days (i.e., in October there were only eight days with rain, totaling 149.7 mm of precipitation), which may have contributed to an increase in *S. frugiperda* infestation, and, therefore higher leaf injury. However, in the second season, infestation was lower than in the first season, and for instance, the assessment of leaf-feeding injury of *S. frugiperda* was performed in the beginning of March, when the number of days with rain and precipitation in February were 14 and 201 mm, respectively (Supplementary Table 1). Thus, abiotic factors, mainly rainfall, likely contributed to higher mortality of *S. frugiperda* eggs and neonatal larvae, leading to a lower rate of leaf-feeding injury of *S. frugiperda* in the second season in comparison to the first season.

Resistance to Cry1F maize has occurred widely in Brazil due to the intensive use of TC1507 maize and low refuge compliance (Farias et al., 2014), and because TC1507 is not a high-dose event (Farias et al., 2015). Furthermore, control failures with *S. frugiperda* on Cry1F maize have been reported since 2013 (Farias et al., 2014; Monnerat et al., 2015), and cross-resistance has been identified with other Cry proteins, such as Cry1Ab and Cry1A.105 (Bernardi et al., 2015). Since 2013, Brazilian farmers would rather buy Bt pyramided maize hybrids and seed companies in Brazil reduced considerably their production of single maize Bt events, such as the case of TC1507 expressing Cry1F (Cruz et al., 2013; Pereira Filho and Borghi, 2016). In this view, Cry2Ab2 likely had more of an impact on *S. frugiperda* than Cry1F and Cry1A.105 in the TC1507 x MON89034 x NK603 pyramid, since Cry2Ab2 does not present cross-resistance with Cry1 proteins (Bernardi et al., 2015; Horikoshi et al., 2016). Additionally, further studies with other Bt transgenes, single or pyramided, will determine a more precise relation between the additive effect of the additional transgenic allele and *S. frugiperda*

control. Although the mortality of *S. frugiperda* did not reach the levels of the high-dose strategy (Huang et al., 2011), we observed that all larvae fed on homozygous and hemizygous hybrids presented growth inhibition, with length less than 1.5 cm, whereas more than 85% of the larvae fed on the non-GM were longer than 1.5 cm.

Our results revealed that homozygous transgenic maize hybrids increased the control of *S. frugiperda* because of the presence of an additional TC1507 x MON89034 x NK603 allele, which was the main reason for the higher concentration levels of Cry1F, Cry1A.105, and Cry2Ab2 proteins in the leaves. To the best of our knowledge, this is the first study investigating the influence of the number of Bt alleles in maize hybrids on Bt expression levels and *S. frugiperda* control. Therefore, the deployment of homozygous maize hybrids may be more desirable than the use of hemizygous versions, since control of *S. frugiperda* is enhanced while maintaining grain yield. In fact, the homozygous approach could be considered as a novel tool for insect resistance management strategies, since the extra Bt allele increases the concentration of Bt proteins in the leaf of homozygous maize hybrids, and as a consequence leaf-feeding injury and survivorship of *S. frugiperda* were reduced without diminishing the grain yield of the plants. To obtain homozygous hybrids for the Bt transgenes, both parental inbred lines of each hybrid must be converted to isogenic transgenic versions, presenting the transgenic locus in a homozygous state. This is a time- and money-consuming process that might restrain breeding companies from generating homozygous maize hybrids, especially when pyramided events are involved. Nonetheless, of the use of homozygous hybrid seeds presents several benefits, such as the reduction of *S. frugiperda* infestation and insecticide application, because both parental inbred lines will be carrying Bt transgenes.

Declarations of interest

None.

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Appendix A. Supplementary data

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