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Field evaluation of soybean transgenic event DAS-81419-2 expressing Cry1F and Cry1Ac proteins for the control of secondary lepidopteran pests in Brazil

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A B S T R A C T

Genetically modified soybean with transgenic event DAS-81419-2 (Conkesta™ technology), expresses two *Bacillus thuringiensis* (*Bt*) derived proteins, Cry1Ac and Cry1F. Event DAS-81419-2 is a proposed new Integrated Pest Management (IPM) tool with demonstrated high efficacy for controlling the primary lepidopteran pests affecting soybean production in South America. Studies were conducted in Brazil from 2011 to 2016 to assess the efficacy of DAS-81419-2 against secondary lepidopteran pests including *Elasmopalpus lignosellus*, *Agrotis ipsilon* and *Helicoverpa armigera*, using artificial infestations to ensure uniform pest pressure. Results from research trials across nine localities showed that compared to a non-*Bt* isoline, DAS-81419-2 significantly reduced the seedling stage plant mortality caused by *E. lignosellus* and the feeding damage caused by *H. armigera* during both vegetative and reproductive crop growth stages. Event DAS-81419-2 showed moderate activity on *A. ipsilon*, and additional control tactics such as a chemical insecticide applied as a seed treatment or spray during early crop stages may be needed against this pest to provide consistently higher levels of protection in fields that are at risk of infestation. Collectively, the efficacy of soybean transgenic event DAS-81419-2 for the control of secondary lepidopteran pests, added to its high efficacy on primary lepidopteran pests suggest this new, dual protein technology will be an important tool where primary and secondary pests affect soybean production.

1. Introduction

Soybean, *Glycine max* (L.) Merrill (Fabaceae: Phaseoleae) is one of the most important legume crops worldwide. It has experienced the highest percentage increase in planted area since the 1970s compared to any other major crop and is now grown in approximately 6% of the world's arable land (Hartman et al., 2011). The planted area in North and South America represents about 80% of total global soybean production (Chang et al., 2015). Brazil is the second largest producer, following the United States (US), with 96.5 million tons and an area of approximately 33 million hectares during the 2015–2016 growing season (USDA, 2016). The potential productivity of the soybean crop is influenced and often limited by various abiotic and biotic factors, including edapho-climatic conditions and the occurrence of diseases and pests throughout the crop cycle.

Hemiptera and Lepidoptera are the main orders of insect pests that attack the soybean crop and cause significant yield losses by reducing stand density (from plant death) and causing defoliation and feeding damage to pods (Sosa-Gómez et al., 2014). The most important and widely distributed defoliating lepidopteran pests of the soybean crop in Brazil include the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae) (Bernardi et al., 2012) and the soybean looper, *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae) (Bueno et al., 2011). However, an increasing number of reported outbreaks of secondary

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lepidopteran pests including *Elasmopalpus lignosellus* (Zeller, 1848) (Viana, 2007; Hoffmann-Campo et al., 2012; Afonso-Rosa, 2013), *Agrotis ipsilon* (Hufnagel) (Hoffmann-Campo et al., 2012; Ferreira et al., 2015) and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Specht et al., 2013; Pomari-Fernandes et al., 2015) have been recently observed in Brazil. While not occurring regularly or extensively at economically-damaging levels, these secondary pests have significant potential to cause yield losses in localized areas. In the case of *H. armigera*, a recently introduced pest (Czepak et al., 2013), it is considered an emerging key pest in some Brazilian crop producing areas and requires close attention in terms of monitoring and control tactics.

The lesser cornstalk borer, *E. lignosellus*, is a polyphagous and cosmopolitan soil insect pest. It is more abundant in sandy soils and more damaging during hot and dry climatic conditions (Smith and Barfield, 1982; Funderburk and Mack, 1994). The larval stage is semi-subterranean and causes damage on seedlings and mature soybean plants (Leuck, 1966). The attack occurs in localized spots within a field, and can compromise up to 50% of the crop's production (Hoffmann-Campo et al., 2012). Another soil pest, *A. ipsilon*, is a polyphagous insect with nocturnal habits. The larvae cause damage to soybean plants, cutting off seedlings and causing plant death, which leads to economic losses (Hoffmann-Campo et al., 2012; Sharaby and El-Nojiban, 2015; Xu et al., 2016). *Helicoverpa armigera* feeds on the aerial parts of the plant. It was recently reported in the main soybean producing regions of Brazil, causing losses by severely damaging leaves and pods (Czepak et al., 2013; Specht et al., 2013).

Until recently, lepidopteran pest management in soybeans relies primarily on the use of synthetic insecticides. However, the effectiveness of these products can be limited by an increase in insecticide resistance and the difficulty in reaching target insects at their resting and feeding sites, as they remain protected in the soil or within the plant canopy (Bernardi et al., 2012). An additional tool within an Integrated Pest Management (IPM) program is the use of genetically modified plants that express crystal (Cry) proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*). The mode of action of Cry proteins has been documented in several studies (Bravo et al., 2007; Soberón et al., 2009). These proteins bind to specific receptors on the midgut epithelial cells of sensitive lepidopteran larvae, leading to pore formation, osmotic imbalance, and septicemia. The affected cells rupture and cause death of the insect (Federici and Siegel, 2008; Soberón et al., 2010; Bravo et al., 2013).

The use of *Bt* in genetically modified plants has been widely adopted in many crops such as corn and cotton across multiple countries, including Brazil (Romeis et al., 2008; Naranjo, 2014). In Brazil, genetically modified maize plants that express the *Bt* protein (*Bt* corn) have been commercialized since 2008 (Mendes et al., 2009; Omoto et al., 2016). *Bt* soybean plants expressing the Cry1Ac protein were recently approved for cultivation in Brazil and launched during the crop season of 2013–2014 (Bernardi et al., 2014; Yano et al., 2015). Since then, *Bt* soybeans have been considered as an important tool in lepidopteran insect-management programs (Bernardi et al., 2012, 2014; Yu et al., 2013; Bernardi et al., 2014; Bortolotto et al., 2014; Azambuja et al., 2015; Yano et al., 2015). Soybean transgenic event DAS-81419-2, known as Conkesta™ technology (Dow AgroSciences, LLC, Indianapolis, IN), was developed using an *Agrobacterium*-mediated transformation process to express Cry1Ac, Cry1F, and phosphinothricin acetyltransferase (PAT) proteins (Fast et al., 2015). Event DAS-81419-2 combines two *Bt* proteins in soybeans, Cry1Ac and Cry1F, derived from *Bacillus thuringiensis* subspecies *kurstaki* and *Bacillus thuringiensis* subspecies *aizawai*, respectively. They are highly similar to those expressed in cotton transformation events DAS-21023-5 and DAS-24236-5 respectively, combined through breeding in WideStrike™ cotton (Dow AgroSciences, LLC, Indianapolis, IN), previously approved for cultivation in the United States (US EPA, 2005) and Brazil (CTNBio, 2009). Marques et al. (2016) reported higher efficacy of DAS-81419-2 in soybeans against *A. gemmatalis*, *C. includens*, *Heliothis virescens* (Fabricius) and *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidae) than commercial foliar insecticides sprayed in soybean fields. There are limited reports of field studies evaluating the efficacy of *Bt* soybean to control other lepidopteran pests that are considered of secondary importance. The objective of the study was to assess the efficacy of the soybean transgenic event DAS-81419-2 expressing Cry1F and Cry1Ac to control *E. lignosellus*, *A. ipsilon*, and *H. armigera*.

2. Materials and methods

Field and laboratory trials were designed to evaluate insect damage by *E. lignosellus*, *A. ipsilon*, and *H. armigera* to DAS-81419-2 (Conkesta™ technology) soybeans (Dow AgroSciences LLC, Indianapolis, IN, USA) and an isoline non-*Bt* soybean. Trials were conducted between 2011 and 2016 in southern and central Brazil (Table 1). Feeding damage to the soybean line expressing Cry1F and Cry1Ac was compared to that of a non-*Bt* isogenic soybean line. Each field experiment consisted on a randomized complete block design with four replications, except in 2011 trials which had three replications. Plots sizes across locations ranged from four to 20 rows wide (45.0–50.0-cm row centers) by 5.0–20.0 m in length. The germplasm used for these experiments was Maverick (Sleper et al., 1998) or Maverick crossed with DM16 (Don Mario Seeds, Buenos Aires, Argentina). All trials followed strict adherence to Brazilian regulatory requirements for confined field trials and were conducted at accredited certified field research sites. Field plots received no soil or foliar insecticide applications. Commercial herbicides and fungicides were applied in accordance with local weed and disease control management practices.

2.1. Insects

Natural insect infestations of secondary pest in Brazil vary across localities and growing seasons. Infestation pressure at specific research field sites is unpredictable and may be non-uniform. Therefore, artificial infestations of *A. ipsilon*, *E. lignosellus*, and *H. armigera* were performed on field trials to ensure pest pressure across plots in all locations. All of the insects used in these studies were obtained from a laboratory-reared colony (SGS, Jaboticabal – São Paulo State, Brazil). The insect colonies were reared on artificial diet and maintained at room temperature of 25 ± 3 °C, 60 ± 10% RH, and a photoperiod of 14:10 h (L:D). Colonies were invigorated by introducing new field collected larvae every year from non-*Bt* fields in Brazil.

2.2. Field trials

2.2.1. Elasmopalpus lignosellus

A total of 10–20 plants per plot, randomly selected from one of the center rows of each plot, was infested with two third-instar larvae per plant at the VC (cotyledon) soybean growth stage (Ritchie et al., 1982). Larvae were confined to an individual plant using a cut polyvinyl chloride (PVC) pipe (15 cm in diameter and 12 cm in height) placed at soil level. Prior to infestation, larvae were transferred from diet cups to Eppendorf tubes (one larva per tube). Two open Eppendorf tubes were positioned near the base of the plant inside the PVC pipe arena, allowing the larvae to exit the tubes and infest the plant. Plant injury was assessed 28 days after infestation (DAI) by counting dead plants that resulted from insect feeding and tunneling plants at the soil level. Percent plant

Table 1
List of pests, trial locations and soybean growth stage that at which pests were artificially infested, from 2011 to 2016 in several Brazilian regions.

Pest	Location (city, state)	Year (# of trials)[a]	Infestation method/soybean growth stage
Elasmopalpus lignosellus	Cascavel, PR	2012, 2014	Artificial infestations in VC stage soybean
	Castro, PR	2013, 2014, 2015	
	Cravinhos, SP	2011, 2012, 2013	
	Jaboticabal, SP	2016 (laboratory trial)	
	Indianópolis, MG	2011, 2012 (3), 2013 (2), 2015 (2)	
	Mogi Mirim, SP	2015 (2)	
	Montividiu, GO	2012 (2), 2013 (2), 2014 (2)	
	Palotina, PR	2014, 2015	
	Uberlândia, MG	2012 (3)	
Agrotis ipsilon	Cascavel, PR	2012, 2014	Artificial infestations in VC stage soybean
	Castro, PR	2013, 2015	
	Cravinhos, SP	2012 (2), 2013	
	Indianópolis, MG	2012, 2013 (2), 2015	
	Mogi Mirim, SP	2015 (2)	
	Montividiu, GO	2012, 2013 (2), 2014 (3)	
	Palotina, PR	2014, 2015	
	Uberlândia, MG	2012	
Helicoverpa armigera	Castro, PR	2015 (1, V4/1, R4)[b]	Artificial infestation in V4 and/or R4 stage soybean
	Indianópolis, MG	2015 (2, R4)	
	Mogi Mirim, SP	2015 (2, V4/1, R4)	
	Montividiu, GO	2014 (1, V4)	
	Palotina, PR	2014 (1, V4), 2015 (1, R4)	

[a] If not specified then only one trial was conducted in the year listed.
[b] Number of trials per location and soybean growth stages.

mortality for each plot was calculated based on the number of dead plants versus the total number of infested plants.

2.2.2. *Agrotis ipsilon*

Between 10 and 20 plants were artificially infested at the VC soybean growth stage by placing one or two third-instar larvae at the base of each plant using a pair of soft forceps. Larvae were confined to the area of the seedling plants by placing barriers made of polyethylene plastic (20 cm in height, 1 m length and 25 cm width) around each plot. Plant injury was assessed 15 DAI by counting the plants that presented insect damage, which is usually at soil level. Percent plant mortality in each plot was calculated based on the number of dead (cut) plants versus the total number of infested plants.

2.2.3. *Helicoverpa armigera*

Larvae were artificially infested at V4 (four node) and R4 (full pod) stages of soybean growth (Ritchie et al., 1982). A total of 8–15 plants per plot was randomly selected from one of the central four rows of each plot during each growth stage. The number of plants selected depended on the number of larvae available for infestation. Plants were marked and infested with 10 first-instar larvae or 5 third-instar larvae per plant. Infestations were performed manually using a camel's hair brush and then each individual plant was covered with a fine-mesh cage to prevent larvae from escaping. Field evaluations were conducted 10 DAI at the two selected plant growth stages. The percentage of live larvae per plot and a visual estimation of defoliation percentage were assessed.

2.3. Laboratory trial

A replicated laboratory trial was conducted to assess the efficacy of the DAS-81419-2 soybean on *E. lignosellus*. Laboratory conditions were 27 ± 1 °C, relative humidity 60± 10% and a 14:10 h light:dark photoperiod. A completely randomized design was used, with six replicates per treatment. Each replicate consisted of six pots, with four seeds of either DAS-81419-2 or non-*Bt* isoline sown per pot. The number of emerged plants varied between 20 and 24 per replicate. One larva at the third instar was infested per plant three days after seedling emergence. Each pot was placed on top of a tray filled with water to provide needed moisture without disrupting the larva. In addition, pots were individually covered with a fine mesh bag to prevent larval escape. Plant mortality was assessed 10 and 35 DAI by counting the percentage of dead plants that resulted from insect feeding and tunneling plants at the soil level.

2.4. Statistical analyses

The percentage plant defoliation caused by *H. armigera* was analyzed with a linear mixed model: $\eta_{ijk} = \eta + Treatment_i + Trial_j + Treatment \times Trial_{ij} + Block_{k(j)} + nlarvaeX_{ijk}$ with observations normally distributed, $y_{ijk} \sim N(\mu_{ijk}, \sigma^2)$, and identity link function $\eta_{ijk} = \mu_{ijk}$; $nlarvaeX_{ijk}$ is a covariate to account for the number of larvae used in the artificial infestation on each plant.

The percentage plant mortality caused by *E. lignosellus* and *A.ipsilon*, and the percentage of live *H. armigera* larvae were analyzed with the generalized linear mixed model:

$$\eta_{ijk} = \eta + Treatment_i + Trial_j + Treatment \times Trial_{ij} + Block_{k(j)} + nlarvaeX_{ijk}$$

with observations binomially distributed, $y_{ijk} \sim Binomial(N_{ijk}, \pi_{ijk})$. The link function for the binomial distribution is the logit function $\eta_{ijk} = log\left[\pi_{ijk}/1 - \pi_{ijk}\right]$.

In both mixed models, treatment is considered a fixed effect while trial, block (trial) and the interaction treatment × trial are considered random effects. Linear mixed model was estimated by restricted maximum likelihood (REML) and Kenward Rodgers method for degrees of freedom calculation. For the generalized linear mixed model the estimation method was maximum likelihood with Laplace approximation. The significance of treatment effect was evaluated with F-approximate test ($\alpha = 0.05$) and least square means from different treatments were compared with Tukey's test. The proportions of variance explained by random effects were calculated and their significance levels were determined with a likelihood ratio test at $\alpha = 0.05$ (Stroup, 2012). Linear mixed models and generalized linear mixed models were estimated with

SAS PROC MIXED and SAS PROC GLIMMIX respectively (SAS Institute, 2011).

3. Results

DAS-81419-2 soybean significantly ($F_{1,21} = 73.68$, $P < 0.0001$) reduced plant mortality caused by *A. ipsilon* (Fig. 1A). DAS-81419-2 plant mortality was 7.22% compared to 63.16% non-*Bt* soybean mortality (Table 2).

Plant mortality during the seedling stage of soybean caused by *E. lignosellus* was significantly lower on *Bt* soybeans containing the DAS-81419-2 event, compared with the non-*Bt* isogenic line 28 DAI ($F_{1,28} = 127.29$, $P < 0.0001$). DAS-81419-2 soybean was highly effective at protecting plants and averaged only 1.16% plant mortality, even under a high infestation level and larval confinement on the plant (Fig. 1B and Table 2). In the laboratory trial, DAS-81419-2 soybean also provided consistent protection at 10 and 35 DAI and significantly reduced the percentage plant mortality, exhibiting 2.04 and 7.14% plant mortality compared to 55.43 and 57.61% observed for the non-*Bt* treatment at 10 DAI and 35 DAI, respectively (10 DAI: $F_{1,68} = 30.16$, $P < 0.0001$; 35 DAI: $F_{1,68} = 41.57$, $P < 0.0001$) (Fig. 2).

The effect of *H. armigera* larval instar (L1 and L3) on treatment comparisons was not statistically significant both at vegetative and reproductive stage for any of the evaluations (% Defoliation V4, $F_{1,3} = 1.97$, $P = 0.25$; % Defoliation R4, $F_{1,3} = 0.21$, $P = 0.67$; % Live larvae V4, $F_{1,18} = 0.99$, $P = 0.33$; % Live larvae R4, $F_{1,18} = 1.45$, $P = 0.24$), therefore the data were combined for subsequent analysis and graphical representation. Levels of defoliation caused by *H. armigera* and observed in DAS-81419-2 soybeans were significantly lower compared to those in the non-*Bt* isogenic treatment both at vegetative and reproductive stages (V4, $F_{1,6.8} = 45.07$, $P = 0.0003$; R4, $F_{1,4} = 51.43$, $P = 0.002$) (Fig. 1C). Defoliation in the non-*Bt* treatment reached 62.60% during the vegetative stage and 36.56% in the reproductive stages, both of which are above the economic threshold levels (Table 3). *H. armigera* was highly susceptible to DAS-81419-2 soybeans at the vegetative and reproductive soybean crop stages evaluated (Fig. 1D). *H. armigera* survival on DAS-81419-2 ranged from 0.64% to 0.05% compared to 37.7%–20.06% on the non-*Bt* treatment in the vegetative and reproductive stages, respectively, with significant differences between both treatments (V4, $F_{1,8} = 32.13$, $P = 0.0005$; R4, $F_{1,19} = 37.31$, $P < 0.0001$) (Table 3).

Random variation was mainly explained by trial and trial× treatment effects. For those cases where variance components were significant (likelihood ratio tests χ^2_{1df}, P < 0.05), percentages of variance explained by trial ranged from 39 to 61% and percentage of variance explained by trial× treatment interaction ranged from 26 to 70%. The relevance of both variance components was mainly due to the variation in the response of the non-*Bt* isoline treatment across trials, as can be observed in Fig. 1. In general, the performance of event DAS-81419-2 was consistent across trials for plant mortality, defoliation and surviving larvae. Damage caused by *A. ipsilon* and *E. lignosellus* was observed in certain trials to cause up to about 40% and 20% plant mortality, respectively, to DAS-81419-2 plots but was consistently lower than in the non-*Bt* treatment.

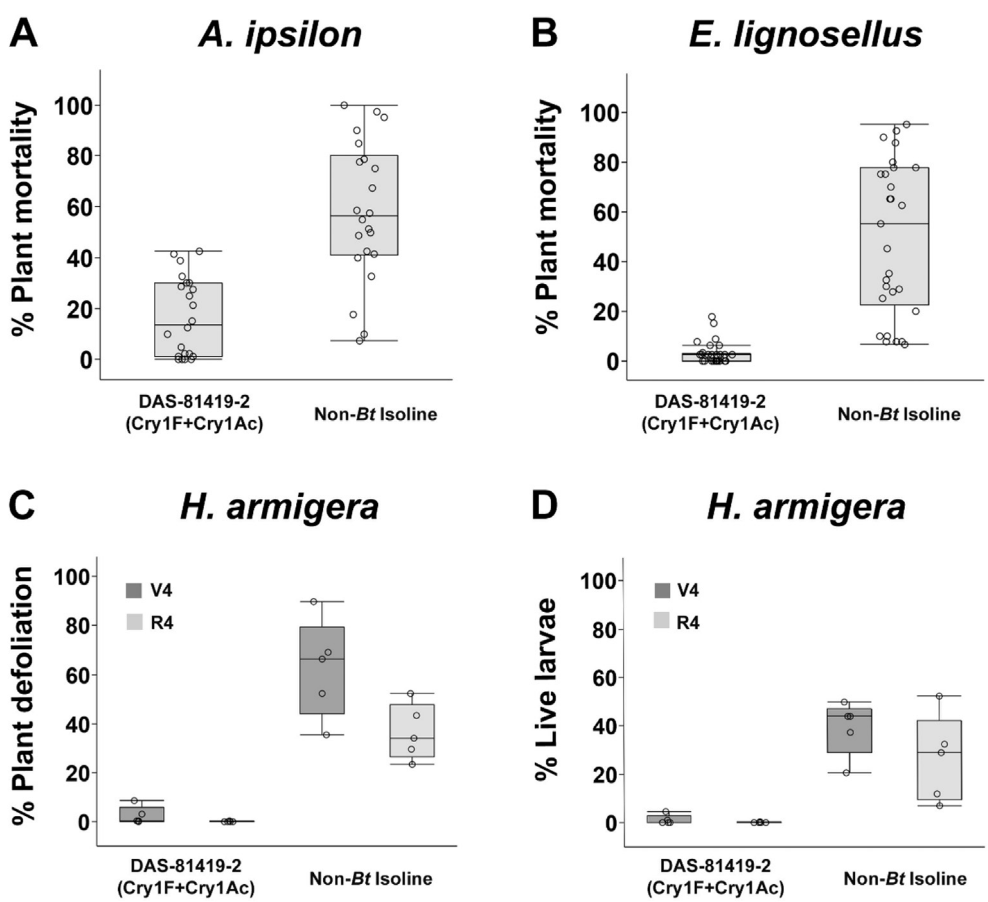

Fig. 1. Percentage plant mortality caused by *A. ipsilon* (A) and *E. lignosellus* (B) in field trials. Plant defoliation (%) by *H. armigera* (C) and percentage of live larvae of *H. armigera* (D) in field trials at V4 and R4 soybean growth stages (Dot markers represent individual trial mean values).

Table 2
Percentage plant mortality in *Bt* (event DAS-81419-2) and non-*Bt* soybean caused by *E. lignosellus* and *A. ipsilon* in field trials.

Pest species	Treatment	No. of trials	% Plant mortality	
			Lsq mean	SE
E. lignosellus	DAS-81419-2 (Cry1F + Cry1Ac)	29	1.16	0.43 b
	Non-*Bt* isoline		49.09	7.35 a
A. ipsilon	DAS-81419-2 (Cry1F + Cry1Ac)	22	7.22	2.75 b
	Non-*Bt* isoline		63.16	9.06 a

Values with the same letter in each column, within species, are not significantly different (Tukey's test, P > 0.05).

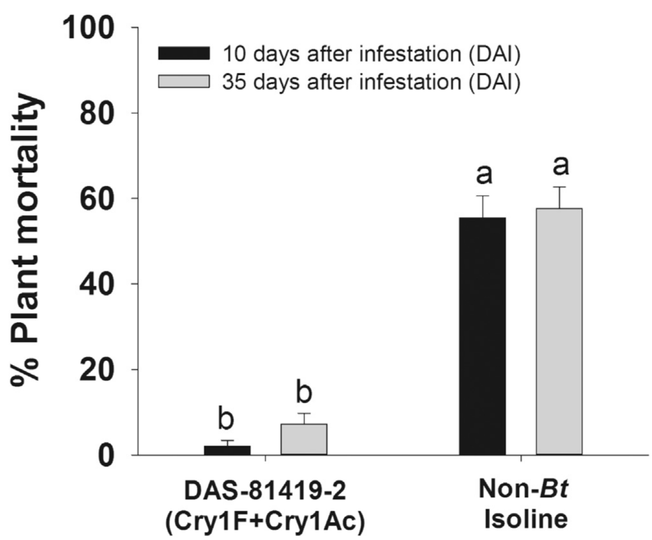

Fig. 2. Percentage plant mortality in *Bt* and non-*Bt* soybean caused by *E. lignosellus* under laboratory conditions (lsq mean ± SE; statistical comparison performed by evaluation time, Tukey's test, P < 0.05).

4. Discussion

Our studies conducted from 2011 to 2016 in Brazil present the first field data of a biotech soybean crop expressing two *Bt* proteins against pests that currently are considered secondary in importance. Recent studies from South America indicate that *Bt* transgenic technology in soybeans are highly effective against the main lepidopteran pests (Bernardi et al., 2012, 2014; Marques et al., 2016). The efficacy of the combined Cry1Ac and Cry1F insecticidal proteins expressed by soybean transgenic event DAS-81419-2 was characterized under a range of field environment conditions in several Brazilian regions. DAS-81419-2 reduced significantly the percent plant mortality caused by *E. lignosellus* and *A. ipsilon* as well as feeding damage caused by *H. armigera* during both vegetative and reproductive crop growth stages. The consistent results observed with DAS-81419-2 across the most important soybean producing geographies in Brazil are of extreme importance. Albeit these pests are not widely distributed at economically important densities, they have demonstrated a high potential to cause locally severe yield losses (Viana, 2007; Hoffmann-Campo et al., 2012; Ávila et al., 2013).

Our results using a soybean transgenic event with dual *Bt* protein expression, Cry1Ac and Cry1F, agree with those of previous authors using single protein. Walker et al. (2000) reported a non-*Bt* soybean variety exhibited four-times higher infestation of *E. lignosellus* under natural conditions compared to a *Bt* soybean isogenic variety expressing the single Cry1Ac protein. In addition, Siebert et al. (2012) and Okumura et al. (2013) reported field maize expressing single protein Cry1F to provide high levels of control against *E. lignosellus* both in the U.S. and Brazil, respectively.

Soybean transgenic event DAS-81419-2 demonstrated moderate reductions in plant mortality caused by the black cutworm, *A. ipsilon*, compared to a non-*Bt* treatment. This result agrees with previous work showing that *A. ipsilon* is relatively insensitive or less susceptible to many current *Bt* Cry toxins such as Cry1Ac, Cry1Fa, and Cry1Fb (de Maagd et al., 2003). Cry1F in maize inflicted only 40% mortality but caused significant weight reduction to *A. ipsilon* larvae compared to a non-*Bt* maize (Binning et al., 2014). Seedling feeding laboratory bioassays using Cry1F maize revealed partial efficacy against black cutworm. However, better results with Cry1F maize were observed in field-strip trials. The authors indicated the addition of a seed treatment to the *Bt* maize contributed to achieve a higher larval mortality and reduced larval mortality of survivors and concluded that Cry1F maize alone or in combination with a seed treatment may be suitable for suppressing *A. ipsilon* in no-tillage corn fields (Kullik et al., 2011). In addition, previous work by Yu et al., (2013) evaluating the susceptibility of two *Bt* soybean lines expressing a single Cry1Ac protein provided almost no resistance to *A. ipsilon*. Rule et al. (2014) reported that Cry1F in maize protected plants and maintained mean plant stand densities of >85%, even despite high infestation levels of *A. ipsilon* under confinement conditions with the plants. These results may suggest better activity on *A. ipsilon* observed with Cry1F than with Cry1Ac proteins. Willrich et al. (2005) reported a good efficacy against low-level infestations of black cutworm using WideStrike™ cotton that expresses Cry1Ac and Cry1F protein and similar to our study on soybeans suggest compatibility of both proteins to manage *A. ipsilon* at moderate levels. However, Binning et al. (2014) demonstrated an initial, post-ingestive aversive response of *A. ipsilon* to Cry1F maize. This behavior may offer a possible

Table 3
Percentage live larvae and defoliation by *H. armigera* at V4 and R4 soybean growth stages.

Pest species	Treatment	Crop Stage/No. of trials	% Live larvae		% Defoliation	
			Lsq mean	SE	Lsq mean	SE
H. armigera	DAS-81419-2 (Cry1F + Cry1Ac)	V4/5	0.64	0.45 b	2.42	6.38 b
	non-*Bt* isoline		37.71	9.98 a	62.60	6.38 a
	DAS-81419-2 (Cry1F + Cry1Ac)	R4/5	0.05	0.06 b	0.08	3.80 b
	non-*Bt* isoline		20.06	8.16 a	36.56	3.80 a

Values with the same letter in each column, within species, are not significantly different (Tukey's test, P > 0.05).

explanation of the moderate levels of protection observed with the DAS-81419-2 soybean line. The two proteins expressed in the DAS-81419-2 event may elicit a feeding deterrent behavior with black cutworm larvae. Similar results were reported by Harris et al. (1997) in light brown apple moth, *Epiphyas postvittana* (Walker), where neonate larvae abandoned diet containing *Bt* endotoxins Cry1Ac and Cry1Ba in choice feeding bioassays after initial ingestion. Despite the moderate levels of control of *A. ipsilon* observed, our studies and existing literature indicate additional control methods such as seed treatments or foliar insecticides, following close monitoring, may be needed when DAS-81419-2 soybeans are planted in areas prone to black cutworm infestations.

Results presented herein document the first detailed report for the susceptibility of *H. armigera* larvae to *Bt* soybean expressing two proteins with the DAS-81419-2 transgenic event. Our studies showed that DAS-81419-2 provides high efficacy against *H. armigera*, consistent low levels of defoliation damage, and high levels of mortality of *H. armigera* larva. Our results suggest that the probability of survival of this pest to adult stages in the field will be extremely low. The high efficacy exhibited by soybean transgenic event DAS-81419-2 event against *H. armigera* is in accordance with previous studies testing *Bt* soybean expressing a single Cry1Ac protein (Yu et al., 2013; Azambuja et al., 2015; Dourado et al., 2016). Laboratory studies testing purified protein showed high toxicity of Cry1Ac (Babu et al., 2002) and significant larval growth inhibition with Cry1Fa1 on *H. armigera* (Avilla et al., 2005). Additional studies by Ibargutxi et al. (2008) on *Bt* cotton concluded that Cry1Ac was significantly more toxic than Cry1Fa against *H. armigera*. However, when both proteins were expressed in the same plant, they showed an additive effect against this pest.

The concurrent expression of several toxins in the same plant has been recommended as one of the most effective strategies to delay insect resistance development to *Bt* plants (Ibargutxi et al., 2008). However, binding studies conducted by Hernández and Ferré (2005) revealed the occurrence of a common receptor for Cry1Ac and Cry1Fa in *H. armigera* larvae. Therefore, as with any *Bt* transgenic technology, a sound Insect Resistance Management (IRM) strategy, adapted to local conditions, must be implemented and adopted to promote a long duration of *Bt* technologies involving these two proteins. IRM strategies based on planting areas of non-*Bt* refuge crops alongside the *Bt* crop are designed specifically to delay the development of resistance in the primary target pests. Refuge strategies are targeted at delaying resistance in primary pests, and will need to be augmented with best management practices that reduce the level of infestation and include scouting for unexpected damage by primary or secondary pests followed by supplemental insecticide treatments if necessary.

Secondary pest outbreaks are a well-known phenomenon, resulting in unexpected yield losses to agricultural production in several localized areas. However, because they are not widely distributed, and often are referred to as occasional pests, they are generally not the focus of transgenic crop research. Abundant research documents *Bt* crops as highly effective at controlling target key pests even though they may not be as effective at controlling other pests that historically pose less or even no threat across large areas, but are important locally. Over several years, suppression of primary pests using *Bt* technology and associated changes in crop production practices may allow secondary pests to increase in importance (Ho et al., 2009). For example, the problem of secondary pests may be exacerbated because they no longer will be controlled in the absence of broad-spectrum pesticide applications that previously were aimed at controlling key pests (Sharma and Ortiz, 2000).

Our results on the efficacy of soybean transgenic event, DAS-81419-2, to manage secondary lepidopteran pests, combined with the high efficacy on key lepidopteran pests (Marques et al., 2016) suggest this new technology will be an important tool that has a fit across soybean areas where either or both key and secondary pests affect a soybean crop. To extend the benefit offered by DAS-81419-2 event as with any other *Bt* technology, the product should be used as the basis of IPM programs, and strong IRM plans should be implemented that are designed to maintain the benefits and extend the lifetime of *Bt* transgenic crops against key and secondary pests.

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