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CARACTERIZAÇÃO DE MOVIMENTO LARVAL DE IMPORTANTES NOCTUÍDEOS-PRAGA EM SOJA *Bt* E NÃO-*Bt*

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LARVAL MOVEMENT CHARACTERIZATION OF IMPORTANT NOCTUID PESTS IN *Bt* and NON-*Bt* SOYBEAN

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TÍTULO DA TESE: CARACTERIZAÇÃO DE MOVIMENTO LARVAL DE IMPORTANTES NOCTUÍDEOS-PRAGA EM SOJA Bt E NÃO Bt

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I Dedicate

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RESUMO

Embora o Brasil seja o segundo maior produtor de soja [*Glycine max* (L.) Merrill] do mundo, a produtividade desse grão é afetada diretamente pela incidência de insetos-praga. Dentre as pragas, os lepidópteros *Chrysodeixis includens* (Walker) e *Spodoptera eridania* (Stoll) (Noctuidae) têm causado danos expressivos nos últimos anos. As práticas de controle mais empregadas no manejo dessas pragas envolvem a aplicação de inseticidas sintéticos e o uso da tecnologia *Bt*. No entanto, a movimentação larval dessas espécies pode comprometer essas estratégias, com impactos relevantes sobre o Manejo Integrado de Pragas (MIP) e o Manejo de Resistência de Insetos (MRI). Para a espécie *S. eridania*, existe um agravante, visto que a mesma apresenta baixa suscetibilidade à toxina Cry1Ac. Nesse sentido, esse estudo teve como objetivo caracterizar a movimentação de diferentes ínstares larvais dessas duas espécies e o possível impacto que esse comportamento tem sobre o MIP e MRI. Assim, foram realizados estudos de movimentação larval na planta e entre plantas em cultivares de soja *Bt* (TMG 7062 IPRO) e não *Bt* (TMG 7262 e 92Y83 “Pioneer”) em período reprodutivo sob condições de laboratório, casa de vegetação e campo. Nos estudos de movimentação na planta. Foram avaliadas a distribuição larval em três posições (superior, mediano e basal) das plantas e em diferentes períodos, além da permanência dos insetos sobre as plantas. Os estudos de movimentação larval entre plantas tiveram como objetivo caracterizar a distância percorrida pelas lagartas entre as ruas de plantio de soja, bem como o padrão de movimentação e distribuição. Exceto pelo experimento de movimentação larval na planta conduzido no Brasil na safra 2016/2017 com *C. includens*, os demais foram instalados sob infestação artificial. Quanto à movimentação na planta, as lagartas em ínstares iniciais de ambas espécies permaneceram no local da infestação. Com lagartas mais desenvolvidas em plantas não-*Bt*, os insetos foram encontrados preferencialmente na região mediana das plantas. Na cultivar *Bt*, não houve diferença quanto à porcentagem de lagartas desenvolvidas recuperadas entre os setores superior e mediano para *S. eridania*. A permanência das lagartas dessa espécie foi superior em 35,0 % nessa cultivar, quando comparada com a tecnologia não-*Bt* (2.1%). A permanência das lagartas desenvolvidas em soja não-*Bt* após a infestação com *C. includens* foi de 38,3 % na última avaliação, sendo o aumento na desfolha um possível fator responsável por esse comportamento. Quanto à movimentação entre plantas, as lagartas de ambas espécies demonstraram habilidade em se movimentar entre as ruas e plantas de soja, sendo esse comportamento constatado também na cultivar *Bt* com *S. eridania*. As duas espécies aparentemente exibem um padrão de movimento não-direcional. Os resultados sugerem que a movimentação larval desses dois insetos-praga nas plantas é influenciada pelos ínstares em que se encontram e, no caso de *S. eridania*, a tecnologia *Bt* evidencia melhor essa característica devido a um possível efeito adverso da proteina Cry1Ac sobre a biologia do inseto. Os resultados obtidos poderão auxiliar na escolha do momento ideal para pulverizações com inseticidas e também no uso de outras estratégias de manejo, contribuindo com o manejo integrado de pragas (MIP). A capacidade de movimentação larval dessas espécies entre as plantas de soja sugere que o refúgio “no saco” pode não ser uma estratégia adequada, visando ao manejo de resistência a inseticidas (MRI). Neste caso, o refúgio estruturado poderia ser um modelo mais eficiente. Os ensaios realizados poderão servir como modelo para futuros estudos envolvendo estratégias de MIP e MRI para insetos-praga na cultura da soja.

Palavra-chave: Comportamento; *Glycine max*; Movimentação larval; MIP; MRI

ABSTRACT

Brazil is the second largest soybean [*Glycine max* (L.) Merrill]) producer in the world, and the productivity of this grain is directly affected by the incidence of insects. Among the pests, two species of Lepidoptera have caused significant damage in recent years, *Chrysodeixis includens* (Walker) and *Spodoptera eridania* (Stoll). The most widely used control practices for these pests are the application of synthetic insecticides and the use of *Bt* technology. However, larval movement can directly affect these strategies, influencing Integrated Pest Management (IPM) and Insect Resistance Management (IRM), particularly for *S. eridania*, because it has low susceptibility to the currently deployed *Bt* toxin, Cry1Ac. Thus, the objective of this study was to determine the instar-specific movement of these two species and their possible impact on IPM and IRM. On-plant and plant-to-plant larval movement studies were carried out on *Bt* (TMG 7062 IPRO) and non-*Bt* (TMG 7262 and 92Y83 “Pioneer”) reproductive stage soybean cultivars under laboratory, greenhouse and field conditions. In the on-plant movement studies, the objective was to determine the larval distribution in three plant sectors (upper, middle and lower), at different periods and their permanence on the infested plant. Plant-to-plant larval movement studies aimed to characterize larval their movement pattern and distribution in the soybean from an infestation point, including the distance traveled by larvae across the soybean rows. Biological parameters, such as head capsule size and larval length, and defoliation percentage were evaluated in order to contribute to the understanding of the movement of these insects. Except for the on-plant larval movement experiment conducted in Brazil in the 2016/2017 season with *C. includens*, the others studies were conducted with artificial infestation. For on-plant larval movement, the early instars remained at the infestation site for both species. For the most developed larvae on non-*Bt* plants, the insects were found preferentially in the middle sector of the plants. When considering *Bt* cultivar with older instars, there was no difference regarding the percentage of larvae recovered between the upper and middle sectors for *S. eridania*. The larval permanence of this species was higher than 35.0% in this cultivar, when compared with non-*Bt* cultivar (2.1%). As for plant-to-plant larval movement in the non-*Bt* cultivar, both species have the ability to move across the rows, and this behavior was also observed in the *Bt* cultivar with *S. eridania*. The movement patterns of both species in this study appear to be nondirectionally oriented. The results suggest the larval movement of these two insects is influenced by the instars they meet, and in the case of *S. eridania*, *Bt* technology influences this behavior by a possible adverse effect of Cry1Ac on insect biology. This knowledge may contribute to IPM by being used to select the appropriate time to apply insecticides to control these pests, and also in the use of other management strategies. The larval movement ability of these species across soybean plants and rows suggest that use of a seed mixture is not an appropriate IRM strategy, and that the use of structured refuge may be a better strategy. These experiments can contribute as a model for future IPM and IRM studies for these two species and also for other soybean insect pests, contributing to the sustainable development of our agriculture.

Keywords: Behavior; *Glycine max*; Larval movement; IPM; IRM

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SUMÁRIO

GENERAL INTRODUCTION

Based on data from the 2018/2019 season, Brazil is the second largest soybean producer in the world, with 35.87 million hectares sown and 115.07 million tons of grain produced, being the main exporting country of this oilseed (CONAB, 2019). However, its production is hampered by abiotic and biotic factors, such as the incidence of numerous pest insects.

The extensive agricultural system, involving crop succession (maily soybean, corn and cotton), the excessive pesticide application, presence of invasive plants in adjacent cultivation areas, allied with the use of inadequate management methods have led to the occurrence of outbreaks of insect pests previously considered secondary (SANTOS et al., 2009; SOUZA et al., 2014). Within this context, the larvae complex of *Spodoptera* spp., such as *Spodoptera eridania* (Cramer, 1784), *Spodoptera cosmioides* (Walker, 1858) and *Spodoptera frugiperda* (JE Smith, 1797) have received special attention as pest species limiting to these crops in the main agricultural regions of the Brazilian Cerrado (JESUS et al., 2013; SILVA et al., 2017).

The *Spodoptera* spp. larvae acquire economic importance, especially from the beginning of the reproductive stage of soybean (GAZZONI; YORINORI, 1995), when they feed on the leaves and pods. The attack of these lepidopterans can also occur on newly germinated plants, when more advanced instars cut the plants close to the ground, similar to the damage caused by *Agrotis ipsilon* (Hufnagel, 1767) (Lepidoptera: Noctuidae), causing stand reduction. This type of injury is more common in areas with a prevalence of *S. frugiperda* (SOSA-GÓMEZ et al., 1993). Compared to the other two species mentioned above, *S. eridania* has been reported more frequently and with higher population densities in soybean and cotton (SANTOS et al., 2009; SANTOS et al., 2010; FAVETTI et al., 2015).

The adults of *S. eridania* are moths that have an approximately 40 mm wingspan, with forewings grayish or brown with a blackish point in the center, and white hindwings. The females in their lifetime lay about 800 greenish eggs (egg mass). The larvae are brown or green and have a whitish longitudinal lateral band on the back. On either side of the body they have a white or yellow stripe that is discontinued by a dark spot on the first uromer. The larvae usually go through six instars, depending on the host, and the biological cycle is completed between 30 and 40 days (CAPINERA, 1999; SANTOS et al., 2005).

Spodoptera spp. management in soybean crops has been systematically carried out by spraying with synthetic insecticides (organophosphates, carbamates and pyrethroids, among other molecules as spinosyn and diamide insecticides). However, the efficiency of this practice has decreased due to the selection of resistant populations to these compounds (CARVALHO et al., 2013; OKUMA et al., 2018; BOLZAN et al., 2019). The inappropriate use of these insecticides in field has also contributed negatively to the increase in the production cost and elimination of natural enemies, besides having a potential impact on environment. Among the alternative methods to this control, the use of genetically modified plants (GMOs) has been highlighted (SANTOS et al., 2009). In 2013/14 season, genetically modified soybean (MON 87701 x MON89788), which expresses the protein Cry1Ac, was launched in Brazil. This biotechnology is an important tool for integrated lepidopterous pest management and was quickly accepted by farmers (YANO et al., 2016).

However, similar to that was found with the inappropriate use of insecticides, the rapid adoption of *Bt* crops without proper management around the world has also caused strong selection pressure of Cry proteins on pests, which has resulted in the selection of resistant populations, with consequent control failures (HUANG et al., 2011; FARIAS et al., 2014). In the case of soybeans, the low susceptibility of *S. eridania*, *S. cosmioides* and *S. frugiperda* larvae to the Cry1Ac protein (BERNARDI et al., 2014) has been reported, generating important questions regarding the future management of these lepidopterans on *Bt* technology.

Competition in a given host may play an important role in the population dynamics of herbivorous insects, as documented in the literature (KAPLAN; DENNO, 2007). However, the importance of status change involving primary and secondary pests from technological advances has been the subject of a limited number of studies (DENNO et al., 1995; HARDIN et al., 1995). The survival of secondary species (not susceptible to *Bt* technology) may increase as a result of the decrease of insecticide use in the field. In addition, this problem can be progressively aggravated by elimination of interspecific competition that existed in this habitat prior to the advent of transgenic species, favoring secondary species not susceptible to the technology (ZHAO et al., 2011).

In the US, the reduction of populations of lepidopteran *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) after introduction of transgenic maize has been documented, and this scenario of reduced competition has opened a feeding niche for

Striacosta albicosta (Lepidoptera: Noctuidae) (DORHOUT; RICE, 2010; HUTCHISON et al., 2010). This could also explain the increasing incidence of *Spodoptera* spp. in Brazilian soybean fields, where these species were considered secondary pests (BERNARDI et al., 2014).

Secondary pests becoming more primary pests does not only apply to the *Spodoptera* spp. complex. Currently, *C. includens* has been considered a primary pest in Brazil (HOFFMANN-CAMPO et al., 2012). *C. includens* moths have a wingspan of approximaterly 35 mm, brown forewings with a small silver design in the center, and brown hindwings (SOSA-GÓMEZ et al., 2010 b). Oviposition is isolated on the leaves and eggs range from light cream to bright yellow, darkening and becoming light brown near hatching. (MITCHELL, 1967; YOUNG; YEARIAN, 1982; BEACH; TOOD, 1985).

The occurrence of *C. includes* in all Brazilian states, high polyphagia (175 species of host plants), inappropriate use of insecticides and fungicides, and resistance to control measures in soybean are factors which have contributed to the increase of this insects' populations, making it one of the most important soybean pests in Brazil (SOSA-GÓMEZ et al., 2010 a; BALDIN et al., 2014; SPECHT et al., 2015).

To date, no population of *C. includens* resistant to Cry1Ac has been found in the country. This pest shows high susceptibility and low allele resistance frequency to Cry1Ac across the main Brazilian soybean-producing regions. It is important to maintain a low allele resistance frequency to Cry1Ac in *C. includens* populations (YANO et al., 2016).

In theory, three key factors favor the success of refuge strategies: recessive resistance inheritance, low frequency of resistance alleles, abundant refuges from non-Bt host plants near Bt crops (GOULD, 1998; TABASHNIK et al., 2009). Fitness cost and incomplete resistance are two additional factors that contribute to retarding this resistance (GASSMANN et al., 2009; CARRIÈRE et al., 2010).

Knowledge about larval movement of pest insects in agricultural production areas is of critical importance in managing resistance to Cry or other transgenic toxins (e.g. VIP toxins). Regarding the practices adopted in this management, the refuge strategy has been the main approach used to retard or decrease the resistance to *Bt* crops (TABASHNIK, 2008).

The knowledge about the movement of insect is essential for pest management, especially due to the lack of information (ALLEN; SINGH, 2016). Larval dispersal has been studied considering the resistance risk that seed mixing formulation (refuge-in-a-bag “RIB”) presents. Larvae may move a longer distance than expected towards non-*Bt* cotton plants when stimulated by *Bt* plants, and resistance could evolve within a few years (RAMALHO et al., 2014). For pests with low larval movement, RIB can be a good alternative strategy in delaying the evolution of resistance (WANGILA et al., 2012).

Larval dispersal and survival may also directly influence sampling methods used in IPM (ROSS; OSTLIE, 1990; PAULA-MORAES et al., 2013; PANNUTI et al., 2016). Thus, more accurate knowledge about on-plant and plant-to-plant larval movement may improve levels of control and decrease economic damage (both of which are still quite variable for these noctuid pests). Bueno et al. (2012) stressed that there are important emerging pests in soybean, such as pod-eating larvae, for which better control is necessary.

Knowledge about the position of caterpillars on different plant structures throughout the day may also aid IPM, making insecticide application more effective by better directing of droplets to the insect body. Difonzo et al. (2015) found that fifth-instar *S. albicosta* (Smith) (Lepidoptera: Noctuidae), a major US dry and green bean pest, remain lower on the plant, or even on the ground, during the hottest periods of the day (morning-afternoon), while in the cooler periods (evening-night) they disperse to leaves and pods.

Considering the importance of soybean and the expansion of these pests in conventional and *Bt* cultivation areas, it is becoming increasingly important to deepen the knowledge regarding the behavior of these important lepidopterous pests in soybean. This information can be useful, aiding decision making and strategies to be adopted for IPM, as well as for the refuge strategy to be adopted for IRM. Thus, this project proposes to analyze the on-plant and plant-to-plant larval movement of *S. eridania* and *C. includens* in *Bt* and non-*Bt* soybeans.

Chapter 1

Understanding the larval movement of *Chrysodeixis includens* in soybean: its importance in IPM and IRM

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Abstract

Chrysodeixis includens (Walker) is one of the main soybean pests in Brazil. The larval behavior in conventional and *Bt* soybeans has not been fully elucidated, and although *Bt* soybean is currently an effective control, it is necessary to adopt strategies that will maintain the larval susceptibility to current *Bt* proteins (Cry1Ac). In view of this reality, this study aimed to understand the baseline larval movement of this species in non-*Bt* soybean in order to improve management, as well as to develop resistance management strategies designed to minimize the resistance evolution to Cry1Ac and future transgenic proteins. In the on-plant larval movement assay, the positioning of larvae on soybean plant was directly related to the stage of larval development. All larvae in initial instars that were infested in the apical region of the plant were later found in upper sector of plants and on infested leaflet. Older larvae preferred the middle sector of plant. For plant-to-plant larval movement, the larvae abandoned the infested plants and moved to other plants. It appears that movement of this species is random and nondirectional. This study provides information regarding *C. includens* larval behavior in soybean which will contribute to the effective management of this pest by more accurate insecticide applications. In addition, the extensive plant-to-plant movement of this species is problematic for a mixed refuge (i.e., refuge-in-a-bag) resistance management strategy.

Key words: insect behavior, soybean looper, larval dispersal, refuge, time to spray

INTRODUCTION

Transgenic crops expressing the *cry* or *vip* genes of *Bacillus thuringiensis* Berliner have been adopted worldwide for the control of important pests. Although there are still doubts about the possible environmental impact that these materials can cause, it is necessary to emphasize that *Bt* crops have provided enormous benefits to the environment (Sanahuja et al. 2011), with positive socio-economic and environmental impacts around the world (Romies et al. 2006, Yu et al. 2011, APA 2018). Negative impacts on non-target insects are generally lower in *Bt* crops when compared to those caused by commonly used synthetic insecticides (Naranjo 2009, Tian et al. 2015). GMO crops have allowed farmers to use insecticides and herbicides more strategically, reducing their environmental impact by 18.4% in agricultural areas since 1996 (ISAAA 2018).

The global area with transgenic technology in 2017 reached 189.9 million hectares, compared to 185.1 million in 2016; *Bt* soybean cultivars representing 50% of this area. Brazil, with planted areas in the 2017/2018 season of 35,139.6 million hectares, is among the countries with more than 80% adoption of this biotechnology in soybean (CONAB 2018, ISAAA 2018). Although genetically modified crops are currently the main tools for the management of lepidopteran pests in major crops, the over-use of this technology may favor the evolution of insect resistance by decreasing their susceptibility to these toxins (Tabashnik et al. 2009). In corn, the reduction in control efficiency has already been documented due to the development of resistance in populations of *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) to Cry1F toxin (Huang et al. 2014, Santos-Amaya et al. 2016). Reports of allelic resistance to Cry1Ac have also been documented in cotton with *Helicoverpa armigera*

(Hübner) (Lepidoptera: Noctuidae) (Liu et al. 2010, Zhang et al. 2011, Kukanur et al. 2018).

Although not yet released in the United States, genetically modified soybean (MON 87701 x MON89788), which expresses the Cry1Ac protein, was made available in Brazil for the 2013/2014 season, and is being readily accepted by producers (Yano et al. 2016). Although there are still no reports of resistance of important noctuids, such as *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae) and *H. armigera* (Dourado et al. 2016, Yano et al. 2016) to this technology, it is necessary to adopt management strategies that contribute to the maintenance of Cry1Ac susceptibility.

One of the main strategies is the adoption of refuge areas for high-dose events. This strategy is based on the planting of non-resistant cultivars inside or around *Bt* crop fields, allowing for the survival of susceptible individuals (Tabashnik et al. 2013). Some studies have suggested that the model of structured refuge (e.g. block refuges) may be most efficient in delaying the evolution of resistance; others suggest that a seed mix (e.g. refuge-in-a-bag) can be as efficient as the structured refuge (Tyutyunov et al. 2008, Pan et al. 2011, Zancanaro et al. 2012). However, the decision of which model to adopt is related to the behavior and biology of the target pest species. For insects that have high larval dispersion capacity and can move readily from plant to plant, the seed mix model may not be adequate because the possible movement of larvae from susceptible plants to the transgenic plants and vice versa, making the resistance functionally less recessive (Mallet et al. 1992, Davis et al. 2000).

C. includens is one of the main pests of soybean, being found in several soybean producing regions in Brazil and the USA. Factors such as overuse of non-selective insecticides, increased use of fungicides after the introduction of soybean rust (consequent reduction in the occurrence of entomopathogenic fungi), resistance to the active ingredients, and insecticide application at the beginning of soybean reproductive stages (less efficiency of the insecticide application) contributed to the change of this insect’s status from secondary to a primary pest in Brazil (Bernardi et al. 2012, Moscardi et al. 2013, Sosa-Gómez et al. 2013). To date, *Bt* soybean has been highly efficient in controlling this pest. The high susceptibility to Cry1Ac and the low frequency of resistant alleles in Brazilian populations of *C. includens* are important requirements for the successful management of resistance of this pest to this biotechnology (Bernardi et al. 2012, Yano et al. 2016). A better understanding of *C. includens* larval dispersal and behavior is necessary to help design resistance management strategies to maintain *C. includens* susceptibility to *Bt* toxins.

Pannuti et al. (2016) point out that studies of larval movement in conventional plants are essential for the improvement of pest management and pest resistance to toxins in *Bt* crops. A more precise knowledge of position of these larvae in the plant canopy may improve management by informing insecticide application methodology, such as more precise targeting of the pesticide application. A more efficient application, with better droplet targeting could reduce the number of sprays and contribute to the adoption of better management techniques that improve natural mortality factors, as recommended by IPM (Naranjo et al. 2005, Difonzo et al. 2015).

Therefore, the objective of this research was to characterize *C. includens* larval on-plant and plant-to-plant movement in non-*Bt* soybean. The results of this study could improve the management of this pest in soybean, as well as contribute to the permanence of *Bt* technology in the field, with positive impacts to IPM and IRM.

MATERIALS AND METHODS

On-plant and plant-to-plant larval movement studies in non-*Bt* soybean were conducted during the 2016/2017 and 2017/2018 cropping seasons in Brazil (São Paulo State University/Botucatu, SP), and in the 2017 season in the US (University of Nebraska Haskell Agricultural Laboratory/Concord, NE). The cultivars TMG 7262 and 92Y83 (Pioneer) were used in Brazil and in the US, respectively. The daily wind direction data in the US were collected on the Weather Underground site at two different weather stations near the experimental area: Wayne Municipal Station and Loberg Farms Station. All soybean fields were grown under conventional agronomic practices appropriate for each region.

C. includens larvae and eggs used in the experiments in Brazil and in the US were commercially acquired from Pragas.com insumos biológicos, Piracicaba, SP and Benzon Research, Carlisle, PA, respectively. The *C. includens* eggs were placed in cylindrical acrylic cages (30 cm in diameter x 15 cm in height) until they reached the larval development stage desired for the experiments. The cages had a hole covered with Organdy cloth to maintain adequate aeration. Soybean leaves from the same cultivar used in the experiments were removed from the field and installed inside the cages. A piece of cotton moistened in water was wrapped around the base of the petioles to maintain turgidity. The tissue containing insect eggs was placed on the leaves, which served as food for the newly hatched larvae. The cages were kept in an environmental chamber (temperature = 25°C; photoperiod of 14:10 [D: E]). The moisture cotton was checked daily, and water added when needed. The leaves were changed periodically to provide adequate food for the larvae. Thirty larvae per cage were maintained through the third instar.

On-plant larval movement

In Brazil, two experiments (22º82'48'' S, 48º26'05 '' W) were conducted to characterize *C. includens* larval distribution on soybean at the R2/R3 phenological stage (Fehr and Caviness 1977). The first experiment was carried out in the 2016/2017 season under natural pest infestation. The row spacing was 0.45 m with 15 plants per linear meter. The experimental units consisted of three sectors of the plant (upper, middle and bottom), totaling three treatments. Fifty plants were randomly selected in an experimental area that was nine rows x 5 m. Each plant represented a repetition. The plants were selected in the five central rows, leaving the two external rows on each side of the area as borders.

The second experiment was carried out in the 2017/2018 season under greenhouse conditions. For this, two soybean seeds per cell were planted in polystyrene seed trays with 82 cells containing soil and commercial substrate "Tropstrato HT" (1:3). When they reached the phenological stage V1/V2, the seedlings were transferred to pots (2.5 L). After thinning the less developed, one plant per pot was maintained. The plants received standard cultural treatments until the initiation of the experiment. At R2/R3, 14 pots were transported to the field and they were arranged in two rows with seven plants spaced 0.45 m from one another. Two types of artificial infestation were performed with neonates (0 - 24 h old). Seven plants were infested in the apical region, and another seven in the middle region. Each plant represented a repetition. The experimental units consisted of three sectors of the plant (upper, middle and bottom). In order to keep the plants free of infestations of other insects, metallic frames (2 m length x 2 m width x 2 m height) coated with screen (16 mesh) were installed.

In the US, the experiment (42°22'44.5" N, 96°57'16.7" W) was designed to characterize the larval distribution throughout the day in the three different sectors of the plant. Twenty plants in R2/R3 arranged in a row were selected randomly in relation to the other rows (spaced 0.76 m). To avoid contact between plants and possible movement of larvae between them, thinning was done to isolate each plant, which were spaced 0.50 m apart. Each plant was supported with a bamboo stake and artificially infested (using forceps) with 12 fifth instars in the middle sector of the plant at dusk the day before the evaluations. The larval instar was determined by measuring its cephalic capsules size with a stereomicroscope (Nikon - Stereo Zoom Microscope SMZ 645, Tokyo, Japan) and a high precision manual meter from BioQuip Products, California, USA (Strand 1990).

All three studies were conducted in a completely randomized design. In Brazil, under natural infestation, larval size (larvae ≤ 1.0 cm, larvae ≥ 1.0 cm) and percentage in the different sectors of the plant were evaluated. Barrionuevo and Blas (2016) reported that 3rd instar larvae of *C. includens* are 7.49 ± 0.99 mm long and 4th instar larvae are 11.70 ± 1.55 mm long. In the case of artificial infestation, the percentage of larvae recovered in different sectors and the percentage of larvae remaining on the plants 48 h after infestation were also evaluated.

In the US, the position of larvae on plants was observed for three consecutive days after infestation. For the first day, the larval count was done every 2.5 hours for the different sectors of the plants (except the last evaluation to avoid night darkness), totaling six evaluations (8:00 am, 10:30 am, 1:00 pm, 3:30 p.m., 6:00 pm and 7:15 p.m.). On the second day, the assessments were made at 8:00 am and 3:30 pm; on the last day the evaluation was made at 8:00 am to confirm the pattern of the larval movement. The data were first submitted to analysis of repeated evaluations on the same plant through time (longitudinal data/temporal pseudoreplication). Then, a punctual analysis of the percentage of larvae and defoliation percentage in the different sections of the plant in each evaluation period was carried out, totaling nine isolated evaluations with three treatments and 20 replicates. The data referring to percentage of larvae were obtained by the quotient of the total number of larvae recovered per plant and the amount counted in each sector. The percentage of larval population remaining on the infested plants in each period was also evaluated from the quotient between the total number of larvae recovered by the amount infested per plant (12 larvae).

Plant-to-plant larval movement

This study was performed in the central US (42°23'01.1" N, 96°57'17.4" W, Concord, NE) to avoid significant natural *C. includens* infestation. To approximate the spacing commonly used in Brazil (0.45 m x 0.45 m), the plants were spaced 0.38 m x 0.38 m (one of several row spacings common in the US). The experiment consisted of six plots arranged in a randomized block design. The plots were 3.0 m long x 2.3 m wide, totaling 315 plants per plot (15 plants/m). The plants were previously inspected to confirm the absence of natural *C. includens* infestation. An artificial infestation was performed, releasing 75 neonates (0 - 24 h old) in each of four locations on the central plant of each plot, totaling 300 neonates per plant. The infestation was done using soybean leaflets infested of the rearing cages. Leaflets were selected with at least 75 neonates, and the excess were removed with a delicate paintbrush. In the field, the infested leaflets were fixed on the plant leaflet surface with a small piece of adhesive tape, completely covering them, and taking care so that the neonates did not stick to the tape. This technique allowed easier contact between the neonates with the infested plant. This methodology reduced stress to the neonates, increasing the efficiency of the infestation process.

At 14 days after infestation, destructive sampling was carried out on all plants. Characterization of the larval movement was based on the presence of the insect on each plant in each plot, and percent survival was calculated by comparing the number of larvae recovered by the number of released neonates (Pannuti et al. 2016). The proportion of larvae present on infested plants and rows was also evaluated, and their distribution at different on-plant sectors.

The plants followed the north (N) and south (S) orientation in the same row related the infested plant, and east (E) and west (W) across rows. The soybean rows were in a N–S orientation. The position of the larvae was designated zero for the infested plant, and the position where the larvae were found during destructive sampling was demarcated. The distance from the release point and the number/frequency of larvae were established by the position in which the insects were found in different quadrants: northeast (NE), southeast (SE), northwest (NW), and southwest (SW). Plants from the center (infested) row and across rows directly adjacent from the infested plant were north (N) or south (S), and east (E) or west (W), respectively. The mean and maximum distance covered by the larvae was calculated by the Euclidean distance. The most distant larvae in each quadrant and orientation was considered the maximum distance (Pannuti et al. 2016).

If a larva was recovered in the last row that bounded the plot, the adjacent rows were also evaluated, maximizing the evaluation of larval movement among the soybean plants. The position where the plants with larvae presence, as well as the number of insects in each plant sector of the plant, were demarcated in the field by "small identification flags" on wooden sticks. This demarcation allowed determination of the distance covered by the insect(s) in the different geographic orientations.

Statistical Analyses

There are a limited number of studies of lepidopteran larval movement in large part because of experimental methodological difficulties (Zalucki et al. 2002). Therefore, this research adopted novel statistical analyses not often used for this type of study in an attempt to provide a more appropriate analysis of the data; for example, use of GLMMs for the on-plant study with longitudinal data to verify some statistical differences between the treatments in an overall design, and then to investigate this difference in punctual analyses.

In the last decade, data from ecological studies have begun to give more importance to random variation in time and space and non-normal data, such as counts or proportions that often challenge classical statistical procedures. GLMMs provide a more flexible approach to analyzing non-normal data when there are random effects. These models combine the properties of two statistical models widely used in ecology and evolution studies: linear mixed models (incorporating random effects) and generalized linear models (which manipulate non-normal data using link functions for exponential families, such as distributions of Poisson or binomial) (Bolker et al. 2009).

For all assays the normality was verified by the Shapiro-Wilk test and homogeneity by the Levene test (Winer et al. 1991). In the analysis of temporal pseudoreplication, it was adopted the generalized linear mixed models (GLMMs) with the glmmTMB function of the "glmmTMB" package, due our data has many zeros, increasing the probability of overdispersion (Brooks et al. 2017). The models were fit by AIC, considering models with zero-inflated Poisson distribution, zero-inflated negative binomial distribution and excluding zero-inflation with Poisson distribution. The better model was the one excluding zero-inflation.

The three treatments (upper, middle and bottom sector of the plant) were adopted as fixed effect in relation to the variable response (percentage of larvae in these sectors) and continuous (9 periods assessed) and categorical (the plant related to their respective sector = subject) were adopted as random effects, totaling 20 observations for each sector of the plant with 9 replicates, resulting in 540 observations (180 for each treatment). The random-effects formula indicated that the periods of measurement represented pseudoreplication within each individual plant. Significant values from the fixed effects were calculated from type II Wald chi-square tests using the function ‘Anova’ (deviance analysis) in the package “car” (Shiojiri et al. 2017). Then, the results obtained by the GLMMs were submitted to the LSmean post hoc test ($p \leq 0.05$) by the "lsmeans" package (Lenth 2016).

For the on-plant larval movement experiments with natural infestation, artificial infestation by neonates and large larvae in the median site of plants, the normal distribution assumption was violated. The one-time analysis data were submitted to Kruskal-Wallis analysis, followed by Dunn's multiple comparison test ($p \leq 0.05$). The data relating to the size of the larvae were compared by the Wilcoxon Matched-Pairs Signed-Ranks Test.

In relation to the on-plant larval movement for artificial infestation with neonates, the data regarding the larval permanence and the mean percentage of larvae recovered in the different sectors of the plant, when infestation was made at the apical region, obeyed the normality supposition and the larval presence was analyzed by the unpaired t test and the mean percentage of larvae by the F test (ANOVA) with the means compared by the Tukey test ($p \leq 0.05$). When the 9 periods were considered as treatments, with the aim of comparing the mean percentage of the larval population in each period, the data also presented a normal distribution and were submitted to ANOVA by the F test and the means compared by the Tukey test ($p \leq 0.05$).

For the plant-to-plant larval movement experiment, data were analized using a generalized linear model (GLM). A gaussian error distribution was used for the distance data and binomial or quasibinomial (overdispersion) for proportions. Count data were analyzed using a poisson error distribution. Significant values from count and binomial data were calculated from type II Wald chi-square tests using the function ‘Anova’ in the package “car”, with pairwise comparations from LSmeans with Tukey adjustment ($P \geq 0.05$), using “lsmeans” package. In case of overdispersion, an F test was performed. For other analysis, ANOVA by the F test was done to detect difference between the means, and when necessary separated by the Tukey test ($p \leq 0.05$), using the "multicomp" and "sandwich" packages. All analyses were performed using software R 3.4.1 (Achim 2004, 2006, Hothorn et al. 2008, Herberich et al. 2010, Lenth 2016, R Core Team 2017).

RESULTS

The number of *C. includens* larvae found on soybean plants in the different experiments allowed an adequate statistical analysis. In Brazil, 175 larvae were found on 50 plants with a mean of 3.5 insects per plant. In the US, 225 larvae were recovered with a mean of 37.5 larvae per plot and larval survival percentage >12%. Most of the captured larvae found were 4th instar (Table 1).

On-plant larval movement in Brazil

For the on-plant larval movement, experiment carried out in 2016, there was a difference in the size of the larvae found on soybean plants. The mean percentage of large and small larvae was 93.8% (median equal to 100) and 6.2% (median equal to 0.0), respectively (Fig. 1A). The mean percentage of larvae was higher in the middle sector of the plants (mean 65.9% - median 66.7%), followed by the upper (mean 22.3% - median 6.3%) and bottom (mean 11.8% - median 0.0%) (Fig. 1B).

In 2017, the mean percentage of remaining neonates infested in the apical region of the plants (72.0%) differed significantly from the remaining neonates infested in the median region (58.9%) (Fig. 2A). There was a significant difference in the mean percentage of larvae present in the different plant sectors in both infestations. The infestation in the middle sector (mean 74.2% - median 69.2%) did not differ from the basal sector (mean 23.8% - median 30.8%) when infestation occurred in the middle of the plants (Fig. 2B). However, when they were infested in the apical region of the plants, the highest percentage of larvae was found in the upper sector (mean 91.8% - median 94.1%), differing from the middle (mean 6.6% - median equal to 5.3%) and bottom sectors (mean 1.6% - median equal to 0.0) (Fig. 2C).

On-plant larval moviment (temporal pseudoreplication)

When analyzing the random effects, the variation in intercepts between the subjects (0.8574) explained about 162 times the variation in the number of larvae present in different sectors than that occurred in time (0.0053). There was a significant difference between fixed effects. The highest average number of larvae was found in the middle sector (mean 6.6), followed by upper (mean 1.5) and bottom (mean 0.2) (Table 2).

On-plant larval movement (distribution of larvae during and across days)

Concerning the periods as treatments, there was difference in the mean larval percentage on the 20 soybean plants between the penultimate period evaluated in the second day (67.9%) and the first period evaluated in the first day (83.2%), and between the evaluation of the third day with the other days. The mean larval population remaining on the plants after 48 h of the beginning of the evaluations was 38.3%, and in that period the variability was highest (minimum value 8.3% - maximum value 75.0%, median 41.7%), with 75.0% of this population (Q3) reaching a mean larval percentage of approximately 45.0% (Fig. 3).

Concerning *C. includens* larval movement on the soybean plant throughout the day, the middle sector always presented the highest mean percentage of larvae, followed by the upper and bottom sectors. This pattern was maintained throughout the experiment. The mean percentage of larvae declined from the first to last evaluation in the middle sector (84.4% to 73.4%), while in the upper sector there was an increase from 11.6% to 24.9%. The mean percentage of defoliation also increased during the evaluations, reaching a common action threshold during the last evaluation (15.5%) (Fig. 4).

Plant-to-Plant larval movement

Concerning *C. includens* larval movement among soybean plants, differences were observed between directions, with more insects moving to SW. A difference in mean distance was observed in the infested row; distance moved to the S orientation (71.6 cm) being higher than to the N orientation (43.4 cm). However, except for the cross row parameter (E and W axes) and orientations (N and S) for the mean maximum distances moved, there were significant differences among the diffrent quadrants (SW, NW, S and NE) and within infested row (N and S axes). The highest mean maximum distance moved was in the SW quadrant (125.3 cm), differing to NE quadrant (80.5 cm). Within infested row, the highest value was observed to the S orientation (108.2 cm) (Table 3).

There were statistical differences for the mean number of *C. includens* larvae recovered among the different quadrants and orientations. The larval frequency varied from 41.8% (SW quadrant) to 15.7% (NE quadrant). The highest frequencies were found in the S orientation (65.8 %), within infested row (S = 72.5 %), and W orientation (64.1%) (Table 3). Except for one plot (plot F; Fig. 5), most of the larvae were found in the S orientation.

Larval dispersal varied according to the location of the infestation. Most of the larvae did not remain on the infested plant, dispersing to the adjacent plants (99.1%). Only one larva remained in the infested plant of plots C and E (Fig. 5 and 6). As for the movement of larvae across soybean rows from the release point, there was difference in most treatments except in Row 4. Approximately 75% of the larvae were found in the rows where the infestation occurred (Row 5 - 29.8%) and in the rows parallel to it (Row 4 - 31.1% and Row 6 - 13.7%). The highest variability of the data collected in all plots was observed in Row 5 (minimum value 8.0% - maximum value 54.2%, median 29.4%) (Fig. 7).

There was difference in the positioning of larvae in the different sectors of the plant (upper, middle, bottom). The middle sector presented the highest percentage of larvae (59.9%), differing from upper (29.1%) and bottom (11.0%) sectors (Fig. 8).

DISCUSSION

Many studies related to insects movement consider only adult stage (Fitt et al. 1995, Caprio 1998, Caprio et al. 2001). Research designed to understand lepidopteran pest larval movement is relatively limited. It has been suggested that this is because of the difficulty of establishing an adequate design and the lack of appropriate methodology to analyze the data (Zalucki et al. 2002, Pannuti et al. 2016). Following the advent of *Bt* technology, the need for information on larval movement and dispersion has gained greater relevance, both for integrated pest management (IPM) and insect resistance management (IRM) (Paula-Moraes et al. 2012, Difonzo et al. 2015). Our study characterizes *C. includens* baseline movement in non-*Bt* soybean, and allows comparison of larval movement of this species in different agronomic realities.

The on-plant larval movement experiments performed in Brazil and the USA showed that more advanced *C. includens* instars (4th and 5th) are found in the middle sector of the plant. The temporal analysis with pseudoreplications and punctual analysis demonstrated that the larvae in these stages present this behavior independently of the random factor "time". However, when the neonate dispersion was analyzed, this behavior was different. Newly hatched larvae infested the upper sector of the plants, remaining on the infested leaflet, and when they moved around in the upper region of the plant. However, when infestation occurred in the middle sector, more larvae left the infestation site, without significant differences between the middle and bottom sectors (Fig. 2). Similar results were observed in pea plants with *H. armigera* when comparing the larval movement of this species after oviposition in three different plant sectors (upper, middle and bottom) (Perkins et al. 2010). The authors suggested that movement of 1st instars from the infested site was greater when oviposition occurred in the bottom sector due to the lower concentration of nitrogen in the leaves of this region. This behavior reinforces that the nitrogen and water content from food are important determinants of young larvae performance (Zalucki et al. 2002).

Whereas Zulin et al. (2018) observed that eggs were deposited by the *C. includens* moths in greater quantity in the lower soybean plant sector, other authors observed the oviposition of this pest occurs in the upper and middle sectors of the soybean plant, and larval feeding is more evident in the lower one-half to two-thirds of the apical region the plant (Mascarenhas and Pitre 1997, Herzog 1980, Jost and Pitre 2002, Valverde 2007). According to Kogan and Cope (1974), the small larvae of this species feed preferentially on new leaves, which have low fiber content and high digestibility, while the more developed larvae feed on older leaves with a lower concentration of nutrients and nitrogen located in the central region of the plant. It is common to see older larvae move from the apical to the middle region in search of older leaves. These reports corroborate observations of the present research, and reinforce that *C. includens* larval movement is directly related to the larval development phase.

Chrysodeixis includens is considered difficult to control in soybean with insecticides. This difficulty is not only related to a greater resistance to some chemical groups of insecticides, but also to the low insecticide exposure at the time of application because the larvae have a habit of remaining in the middle part of the canopy (Bernardi et al. 2012). The ideal time for insecticide application would be when the larvae were more exposed and vulnerable to the insecticides. This time would coincide with the earlier instars that are concentrated in the upper region of the canopy. Additionally, neonates are more susceptible to the action of foliar insecticides, or toxins expressed in the plant (secondary metabolites or *Bt* proteins), due to their lower detoxification capacity at this stage. According to Zalucki et al. (2002), first instars are more susceptible to the action of these metabolites compared to that of more advanced instars larvae. For example, the lectin protein has been shown to be highly toxic to neonates of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Czapla and Lang 1990). Several studies have also reported a decrease of suscetibility to *Bt* protein when *Heliothis virescens* (Fabricius, 1781) (Lepidoptera: Noctuidae), *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) and *C. includens* larvae were in more advanced instars (Halcomb et al. 1996, Li et al. 2006, Sorgato et al. 2015). Thus, it is essential to sample at the correct time, that is, when *C. includens* moths begin to mate and oviposit. Regardless of the genotype tested, adults prefer to oviposit when soybeans are in the reproductive stage, which may be related to a higher leaf density present in the old plants compared to the younger plants (Felland et al. 1992, Mascarenhas and Pitre 1997).

Ineffective management of *C. includens* (e.g. missing the neonate period) can result in significant defoliation, reducing the productive capacity of the plant. For example, in Brazil the recommended economic threshold for soybean defoliation is 15% in the reproductive stages (Bueno et al. 2011). The foliar consumption from the fourth to sixth instar represents approximately 96% of the total consumed by all instars (Trichilo and Mack 1989). The high leaf consumption observed in this research was in the larval movement experiment with 5th larvae, where a mean defoliation percentage was higher than 15% (the recommended ET) after 48 h, supporting the need to manage *C. includes* early while larvae are still in the upper part of the canopy.

The movement of early instar Lepidoptera on host plants largely determines where feeding sites become established (Zalucki et al. 2002, Perkins et al. 2008). In the case of *C. includes,* application of insecticides during this period would result in more insecticide reaching the target, and result in greater susceptibility of the insect to said control practices. In addition, other biotic and abiotic mortality factors, such as natural enemies and rainfall, are typically more effective in the control of young larvae (Zalucki et al. 2002, Naranjo and Ellsworth 2005). Pereira et al. (2018) reported that the natural mortality by biotic and abiotic factors in the immature stages of *C. includens* was high, highlighting the egg and first instar stages. The main factors involved in mortality were parasitism, predation and rainfall.

In the experiment of larval plant-to-plant movement, low *C. includens* larval survival was observed (12.5%) (Table 1). Similar results were also observed with *Striacosta albicosta* (Lepidoptera: Noctuidae) and *S. frugiperda* on maize plants (Pannuti et al. 2016). Larval mortality observed in initial instars of Lepidoptera can often reach 95%. It is difficult to determine the exact causes of mortality, but among the associated factors are predation, climatic conditions, dispersion, and failure to establish in the host (Zalucki et al. 2002). Cornel and Hawkins (1995) suggest action by natural enemies is the main cause of this high mortality. During the present study, few natural enemy species were observed in the experimental area, and these were represented by low populations of arachnids. However, the mean number of remaining larvae per plot was relatively high (37.5), and therefore adequate for statistical analysis (Table 1).

The greatest mean distance from the infestation site was observed in the S and W orientations and their respective quadrants. The same happened with the parameters larval mean number and mean maximum larval distance. Although a significant difference was observed in these two parameters with predominant larval frequencies in the S and W orientations and in the SW quadrant, it can not be said that this species presents a type of directional movement. The larvae likely exhibit nondirectional movement. The reason for this conclusion is explained by the wind direction during the experiment. In the first two days after infestation, the predominance of the wind was from the north-northeast, influencing the movement of larvae preferentially to the S and W orientations, and SW quadrant. The influence of wind was also reported by Pannuti et al. (2016) as possibly affecting *S. frugiperda* larval dispersion in maize.

It is believed that *C. includens* neonates exhibit the ability to randomly disperse among soybean plants by behavior called ballooning. In ballooning, larvae are displaced from the initial site by wind currents and repositioned in another location by silk threads produced by the labial silk glands. This behavior has been observed in numerous lepidopteran families, such as Cossidae, Geometridae, Lymantriidae, Noctuidae, Psychidae, and Pyralidae (Zalucki et al. 2002). The insect orientation ability may also be of the directional type that is governed by different biotic and abiotic factors: chemical gradients of pheromones or odor from feeding sources that can direct phytophagous insects to a host plant; sound waves; and visual location (Bell 1990, Rojas et al. 2018). However, as previously described, *C. includens* appears to be oriented non-directionally, and the abiotic factor "wind" was probably the major factor responsible for the displacement of larvae during the initial instars. The experimental plots were homogeneous during the experiment (free of disease and other pests, adequatelyly fertilized, plants at same phenological stage, etc.), so it is unlikely that biotic factors were responsible for the displacement of the larvae.

Although the total number of larvae was higher in the S orientation, it can be considered that *C. includens* dispersion was random, that is, without the existence of sensory information that favored its movement to a specific location. Without the effect of wind, there would likely be no statistical difference between the mean number of larvae in the different geographic positions, as well as in the maximum distance covered by the insect. However, in experiments conducted under field conditions it is difficult to isolate the effect of factors such as wind and rain, which directly affect larval survival and behavior. The Poisson distribution is the probabilistic model that best describes the random or random arrangement of insects (Elliott 1977, Rabinovich 1980). Shepard and Carner (1976) reported after a study of more than three years that the pattern of spatial distribution for numerous species in soybean fields, including *C. includens*, is a Poisson probabilistic model. The larval dispersion graphs (Fig. 5), together with the persistence analysis of remaining larvae on infested plants (Fig. 6), reinforce this type of random special dispersion for this species.

Other authors report different results with other insect and crop species. Panutti et al. (2016) reported that *S. frugiperda* and *S. albicosta* exhibited an aggregate and symmetrical distribution on corn plants, with most of the larvae found on the initial infested plants. Trindade et al. (2017) reported that the *S. frugiperda* larval dispersal in maize is related to larvae size. Small larvae present an aggregate behavior, while medium and large larvae are in a random pattern. This last work is similar to the results of *C. includens* dispersion in the present research, where it was observed that the larvae in initial instars (48 h of age) did not move from the infested leaflet, whereas in the plant-to-plant experiment it was observed that larvae moved randomly in all plots evaluated 14 days after infestation.

Understanding larval movement can be a valuable tool for IPM and IRM. The use of *Bt* soybean is an important technology in pest control, including for *C. includens*; however, a significant risk of failure to this technology is in the resistance evolution if the IRM strategies are not correctly adopted (Mallet and Porter 1992, Davis and Onstad 2000, Godoy et al. 2015). This research determined that *C. includens* presents a high dispersion capacity from the infestation site, moving among plants. About 30% of the larvae were recovered in the infested row, and 70% in the other rows, especially in the rows parallel to the infested one (Fig. 7). The larvae covered at least 11 plants in each orientation (N, S, E, and W), considering 15 plants/linear meter. A single larva on the originally infested plant was found in only two plots (Fig. 5). In view of this scenario, the mixed refuge strategy (i.e. RIB) for *C. includens* could be inadequate because practically all larvae moved away from the originally infested plant. According to Davis and Ostad (2000), insects that show high capacity of larval movement between plants are bad candidates for the adoption of a mixed refuge. The seed mix may result in the larvae to be stimulated on *Bt* plants to move to non-*Bt* plants, and also the more developed larvae that have fed on the non-*Bt* material moving to other plants, including *Bt* plants. These older larvae may be less susceptible to these toxins (greater detoxification capacity), and resistance may occur in a few years (Felland et al. 1992, Ramalho et al. 2014, Sorgato et al. 2015,). It is critical to understand larval movement when designing a refuge configuration to be implemented.

The knowledge of insect pest population dynamics in crop production systems can contribute significantly to the development of pest management strategies by optimizing sampling methods and insecticide application technology, and contributing to the effective design of IRM for transgenic pest management technologies (e.g. *Bt* maize and soybean, dimensioning of refuge areas) (Ross and Ostlie 1990, Onstad 2006, Paula-Moraes et al. 2012, Tabashnik et al. 2013). Our on-plant larval movement experiments suggest that the optimal time to control *C. includens* in soybean is when the larvae are found in the early instars due to their increased exposure and vulnerability. More developed larvae preferentially inhabit the middle sector of the plants, making their control by foliar spraying difficult. Considering the high capacity of

C. includens larval movement, a structured refuge would likely be the best option to maintain low frequencies of *Bt* resistance alleles *C. includens* populations in Brazil and other areas where *Bt* soybean is grown.

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Table 1. Number of *C. includens* larvae observed/recovered, larval survival (%), mean number of larvae per plant/plot and mean size (±SE) of head capsule of larvae recovered from soybean plants.

Experiments	**Number of larvae recovered**	**% larval survival**	**Mean number of larvae per plant[†] or plot[‡]**	**Mean head capsule size in mm (±SE)[§]**
On-Plant (BRA)	175[†]	--------	3.5[†]	--------
Plant-to-Plant (US)	225[‡]	12.5	37.5[‡]	0.8 (±0.01)

[†]Mean number of larvae per plant.
[‡] Mean number of larvae per plot.
[§]Head capsule width (mm) after destrutive evalaution.

Table 2. Analysis of deviance (Type II Wald chi-square tests) of the fixed effects (3 different plant sectors) on the number of larvae (±SE) recovered on soybean plants at different periods (random effects).

Analysis of deviance			
Fixed effects	**df**	**χ^2**	**P-value**
Sectors	2	224.67	< 2.2e-16
Random effects		**Variance**	
Subjects (Intercept)		0.857408	
Periods		0.005329	
Post hoc test			
Upper	1.5 ± 0.1 b		
Middle	6.7 ± 0.2 a		
Bottom	0.2 ± 0.1 c		

Number of obs: 540, groups: 20 “each plant sector was considered as a subject”

Table 3. Mean number (±SE) and frequency of recovered *C. includens* larvae, and mean and maximum distance (±SE) covered by larvae at different positions related to the infested soybean plant, Concord, NE - 2017.

POSITION	**Mean number recovered**[† or ‡]	**Frequency (%)**[§]	**Mean distance (cm)** [‡]	**Maximum distance mean (cm)**[‡]
NW	5.7 ± 1.8 ab	22.2	75.7 ± 6.5 a	108.2 ± 15.5 ab
SW	10.7 ± 1.3 a	41.8	80.5 ± 10.4 a	125.3 ± 12.5 a
NE	4.0 ± 0.8 b	15.7	64.5 ± 5.7 a	80.5 ± 10.5 b
SE	5.2 ± 1.5 ab	20.3	73.7 ± 6.8 a	104.6 ± 9.1 ab
df	3		3	3
Chisq[†] **/ F**[‡]	3.6214[‡]		1.457	4.169
p-value	0.038		0.2661	0.0157
ORIENTATIONS				
General				
N	12.7 ± 1.4 b	34.2	69.4 ± 3.8 a	114.5 ± 11.7 a
S	24.3 ± 4.1 a	65.8	73.0 ± 6.9 a	109.2 ± 20.0 a
df	1		1	1
Chisq[†] **/ F**[‡]	8.6075[‡]		0.345	0.081
p-value	0.03249		0.582	0.787
Infested row				
N	3.2 ± 0.9 b	27.5	43.4 ± 4.5 b	58.8 ± 6.6 b
S	8.3 ± 3.0 a	72.5	71.6 ± 9.2 a	108.2 ± 11.8 a
df	1		1	1
Chisq[†] **/ F**[‡]	8.1320[†]		11.154	11.051
p-value	0.004349		0.0206	0.0209
Across rows				
W	16.3 ± 1.9 a	64.1	74.9 ± 6.3 a	127.3 ± 13.0 a
E	9.2 ± 2.0 b	35.9	69.1 ± 6.1 a	105.4 ± 9.4 a
df	1		1	1
Chisq[†] **/ F**[‡]	11.7545[†]		1.023	3.708
p-value	0.000607	22.5	0.3582	0.112

Count data (Analysis of deviance - [†] Chisquare test and [‡] F test). The means with the same letter are not significantly different (Pairwise comparations from LSmeans with Tukey adjustment - *P* ≥ 0.05) by GLM.

[§] Frequency (%) of *C. includens* larvae in each position.

[‡] Mean and maximum distance covered by *C. includens* larvae. Distance was calculated by Euclidean distance. The means with the same letter are not significantly different (*P* ≥ 0.05 - Tukey Contrasts) by GLM (ANOVA by test F).

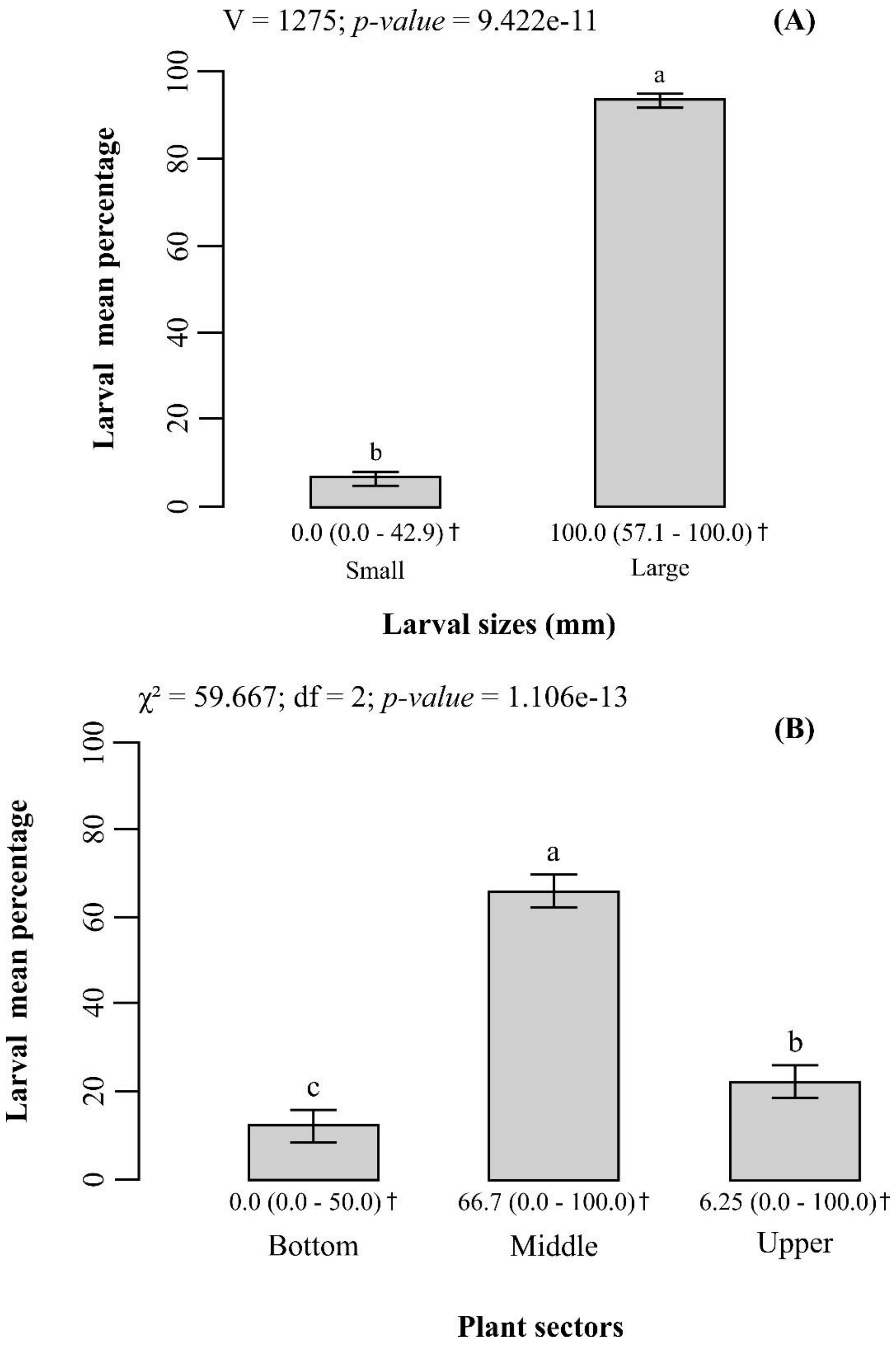


Fig. 1. Barplots resuts regarding the parameters evaluated for the *C. includens* larval on-plant movement study on 50 soybean plants. (A) Mean percentage (±SE) of small and large larvae recovered on the plants, (B) Mean percentage larval recovered in three different plant sectors. Means ± standard errors followed by the same letter are not significantly different {Wilcoxon test (A) and Kruskall-Walis (B) test, α = 0.05}. † Median (minimum and maximum values) from each treatment.

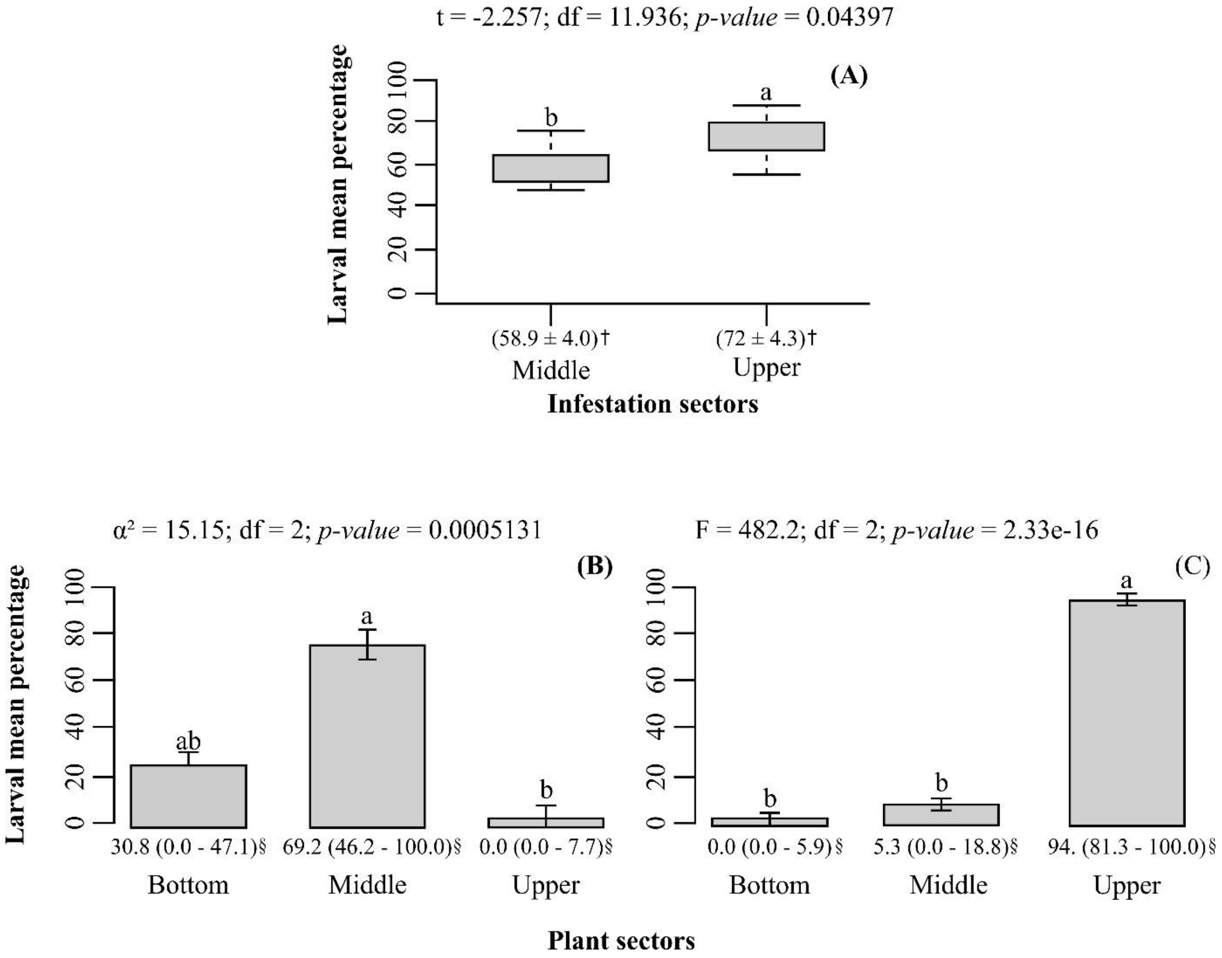


Fig. 2. Results regarding the parameters evaluated for the *C. includens* larval on-plant movement study on 7 x 7 soybean plants. (A) Boxplot results regardin the percentage of early instar larvae that remain in plants after infestation, (B) Barplot results regardin the mean percentage larval in three different plant sectors when the neonates were infested in middle of the plants. (C) Bar Plots results regardin the mean percentage larval in three different plant sectors when the neonates were infested in top of the plants. Means ± standard errors followed by the same letter are not significantly different {t test (A), Kruskall-Walis (B) and F test (C), α = 0.05}. † Means ± standard errors and § Median (minimum and maximum values) from each treatment.

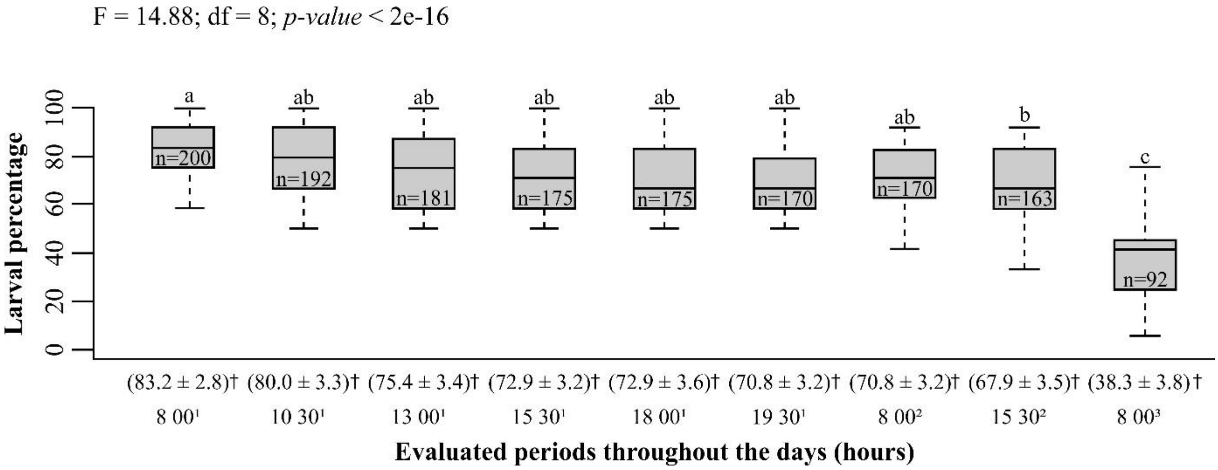
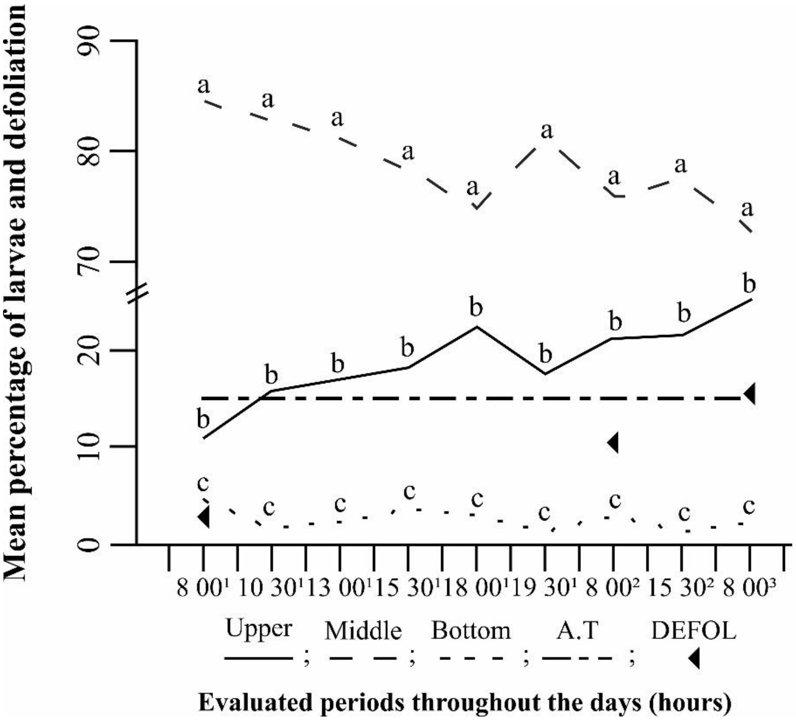


Fig. 3. Boxplot results regarding the *C. includens* larval on-plant movement study on 20 soybean plants. Larval percentage remaining after infestation considering the periods as treatments and the plants as repetitions. The total number of larvae recovered in 20 plants in that specific period is represented in each box. The total number of larvae infested per period was 240. The same letters are not significantly different (P ≥ 0.05 - Tukey Contrasts) by ANOVA test F. † (Mean ±SE) of each treatment.



Kruskal-Walis statistical analysis

Periods	**$\varkappa^2$**	**df**	**p-value**
8 00^{1}	44.2	2	2.572e-10
10 00^{1}	47.6	2	4.656e-11
13 00^{1}	45.7	2	1.202e-10
15 30^{1}	45.0	2	1.66e-10
18 00^{1}	47.6	2	4.563e-11
19 30^{1}	49.5	2	1.785e-11
8 00^{2}	46.3	2	8.862e-11
15 30^{2}	50.0	2	1.418e-11
8 00^{2}	49.4	2	1.927e-11

Fig. 4. Results (analyses at each period) regarding the *C. includens* larval on-plant movement study on 20 soybean plants. Mean larval percentage at three different plant sectors (Upper, Middle, Bottom) and defoliation (DEFOL). A.T. = example action threshold for defoliation to soybean during reproductive stages. Each plant sector was considered a treatment. Means followed by the same letter are not significantly different (Kruskall-Walis test, α = 0.05).

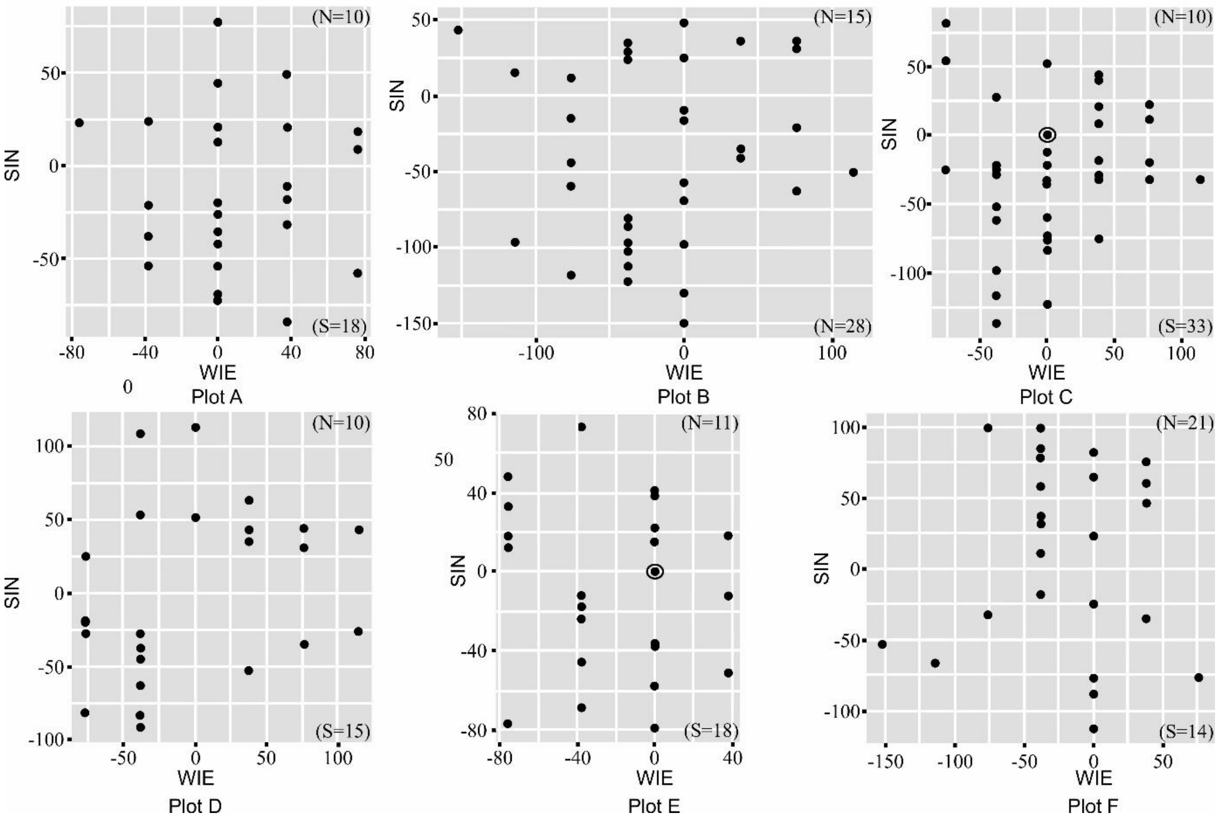


Fig. 5. Dispersion graphs (cm) regarding to the the *C. includens* larval plant-to-plant movement study on soybean plants (6 plots). The black spots represent the plants where the larvae were found. S|N and W|E are the orientations. Each plot has the total number of larvae found in the N and S orientations. These graphs were constructed by softwere R 3.4.1 using the ggplot2 package.

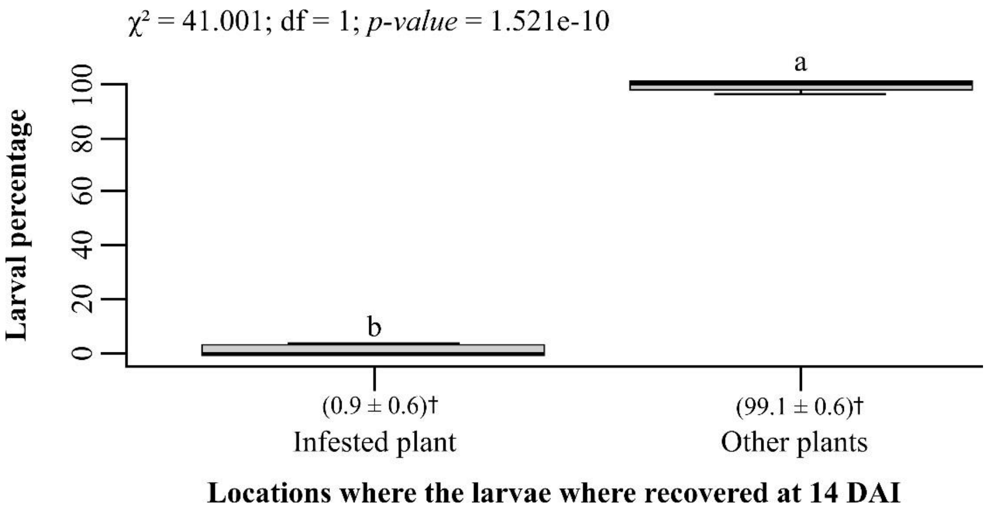


Fig. 6. Boxplot results regarding the *C. includens* plant-to-plant larval movement study on soybean plants (6 plots). Larval percentage on infested plant and on other plants. The same letter are not significantly different ($P \geq 0.05$ - Tukey Contrasts) by GLM. † (Mean ±SE) of each treatment.

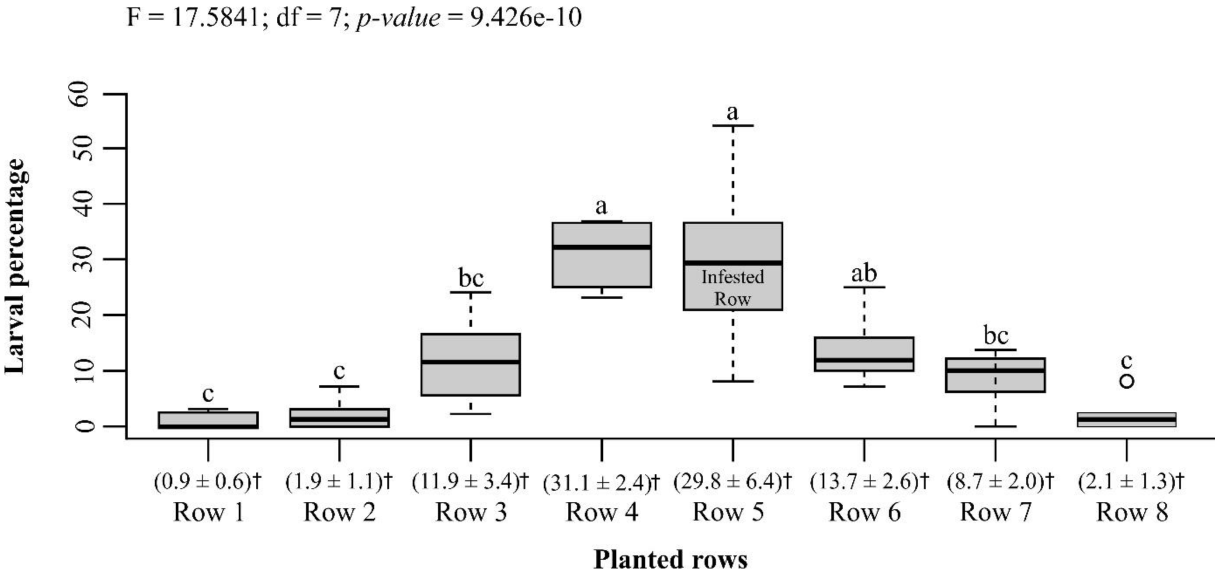


Fig. 7. Boxplot results regarding the *C. includens* plant-to-plant larval movement study on soybean plants (6 plots). Larval percentage in each different soybean row. The same letter are not significantly different (P ≥ 0.05 - Tukey Contrasts) by GLM. † (Mean±SE) of each treatment.

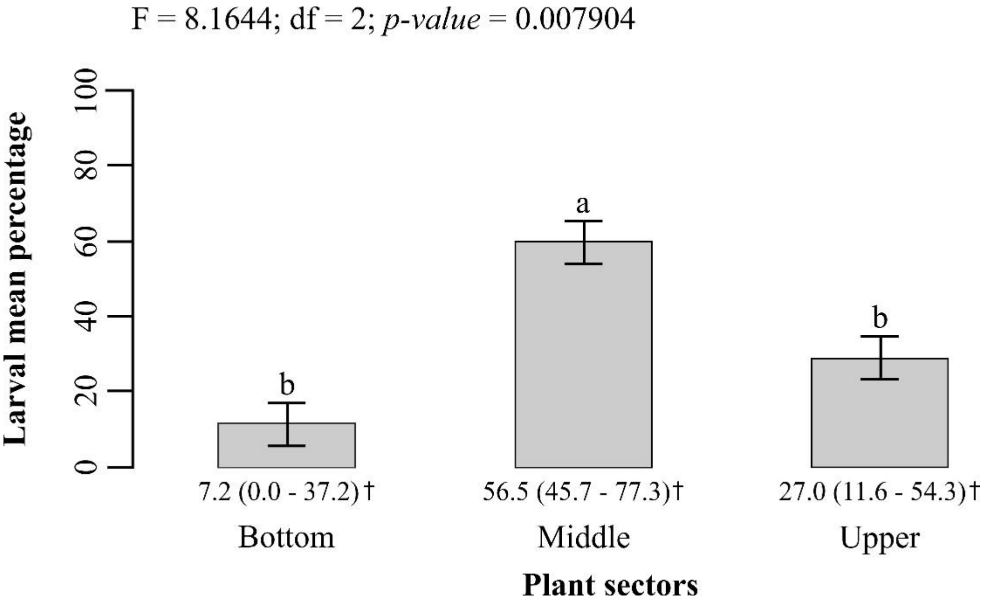


Fig. 8. Barplots resuts regarding the *C. includens* plant-to-plant larval movement study on soybean plants (6 plots). Larval mean percentage in three different plant sectors (Upper, Middle, Bottom). The same letter are not significantly different ($P \geq 0.05$ - Tukey Contrasts) by GLM. † Median (minimum and maximum values) from each treatment.

Chapter 2

Baseline plant-to-plant larval movement of *Spodoptera eridania* in *Bt* and non-*Bt* soybean and its possible impacts on IRM

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Abstract

The widespread use of transgenic technologies has led to the emergence of insect populations resistant to *Bt* toxins. Some lepidopteran pest species also appear to naturally have some level of tolerance to certain proteins, such as some *spp*. of *Spodoptera* to Cry1Ac. One of the main strategies to manage resistance is the use of refuge areas, the success of which is in part dependent on larval movement of the target pest. Thus, in order to assess the viability of a refuge strategy addressing *Spodoptera eridania* in *Bt* soybean, it was evaluated the larval movement across plants in *Bt* and non-*Bt* soybean, as well as the larval development and mortality on *Bt* and non-*Bt* soybean cultivars. We concluded that apparent *S. eridania* incomplete resistance resulting from high larval mortality and low adaptability on *Bt* plants, high larval dispersal, non-directional larval movement, and random larval spatial dispersion suggest that structured refuge is more suitable than mixed refuge for managing resistance in *S. eridania* populations.

Keywords Southern armyworm, *Glycine max*, Behavior, Incomplete resistance, Refuge strategy

INTRODUCTION

Brazil is approximately 860 Mha, of which 38% is used for agricultural systems (Cattelan and Dall'Agnol 2018). Soybean is the most valuable Brazilian agricultural product, and is planted to 35.5 Mha, primarily in center-west Brazil (16Mha) (CONAB 2019). In 2018, soybean gross production value (GPV) reached R$ 142.36 billion, corresponding to 25.0% of the country's agricultural production (Cattelan and Dall'Agnol 2018, MAPA 2018). However, soybean cultivation is made difficult by the incidence of numerous lepidopteran pests, mainly in the superfamily Noctuoidae, that cause significant economic damage (Formentini et al. 2015).

One of the techniques that has been widely used in the management of Lepidoptera in Brazil is the use of transgenic varieties that express the Cry1Ac toxin. In 2017, 33.7 Mha of transgenic soybeans were planted, followed by maize and cotton (ISAAA 2017). The use of this technology is primarily aimed at controlling *Anticarsia gemmatalis* (Hübner, 1818), *Chrysodeixis includens* (Walker, [1858]), and species belonging to the Heliothinae subfamily (Luz et al. 2018). However, this toxin presents low efficacy for some species of the *Spodoptera* genus, such as *Spodoptera eridania* (Stoll), *Spodoptera cosmioides* (Walker), and *Spodoptera frugiperda* (JE Smith) (Bernardi et al. 2014). These species have been gaining importance due the defoliation and damage caused on soybean flowers and pods (Panizzi et al. 2013,).

Low susceptibility to Cry1Ac toxin in some *Spodoptera* species may favor the evolution of resistance on MON 87701 x MON 89788 (Bernardi et al. 2014); therefore, resistance management measures designed for this technology must be address for these species. An important strategy for insect resistance management (IRM) to *Bt* toxins is the use of refuge areas with high-dose events (high dose/refuge strategy), which aims to promote the survival of susceptible insects to mate with any possible resistant insects, thus reducing the proportion of resistant phenotypes in the population. However, other important factors favor the success of the refuge strategy, such as recessive inheritance of resistance, low resistance allele frequency and fitness costs (Carriére et al. 2010, Tabashnik et al. 2013).

Initial high dose/refuge strategies designed to delay resistance to *Bt* crops utilized structured refuge as a block planting or in strips through a field, but the use of seed mixtures (refuge in the bag “RIB”) has been suggested to manage resistance to *Bt* toxins (Carroll et al. 2013). However, while RIB may present some advantages, such as greater ease of implementation, the use of this strategy may favor the field-evolved resistance due to the biological and behavioral characteristics that a pest species may present in a particular crop. For example, seed mixtures would not be recommended for species that present high larval movement capacity (Davis and Onstad 2000, Carroll et al. 2013). Larval movement can expose the larvae to sublethal doses of *Bt* toxins, accelerating the evolution of resistance. Some early instars are able to feed in *Bt* plants and move to non-*Bt* plants, or they can move from non-*Bt* to *Bt* plants as older instars (Head et al. 2014, Ramalho et al. 2014). In addition, larvae with some tolerance to *Bt* proteins, and stimulated by *Bt* plants, may move longer distances toward non-*Bt* plants (Malaquias et al. 2017).

In comparison with *S. cosmioides* and *S. frugiperda*, the frequency of *S. eridania* infestations in soybean is increasing season by season (SANTOS et al. 2010); thus, the importance of understanding its larval dispersion capacity in soybean as it relates to possible resistance evolution to *Bt* toxins has increased. The low susceptibility of this pest to Cry1Ac toxin, in comparation to other species, may impact the selection of resistance by allowing selection of incomplete resistance (Bernardi et al. 2014). Incomplete resistance is an important factor to delaying resistance. It occurs when resistant insects can complete development on *Bt* plants, but are at a disadvantage (lower fitness) compared to those developing on corresponding non-*Bt* plants, reducing selection for resistance on *Bt* plants (Gassmann et al. 2011, Carrière et al. 2016).

Information regarding *S. eridania* larval behavior on *Bt* and non-*Bt* soybean plants is scarce to non-existent, but important for IRM programs for transgenic soybean that expresses Cry1Ac protein. This research aimed to understand *S. eridania* larval movement in plant-to-plant movement studies in *Bt* and non-*Bt* soybean commercial cultivars. The results obtained in this research will serve as a baseline for future *S. eridania* studies, in IRM design concerning the choice of the best refuge strategy, and in probabilistic models which have been developed to address the complexity of examining refuge configurations (Ives et al. 2011, Garcia et al. 2016). Model assumptions, such as larval movement capacity and the biology of the target pest, need to be better understood to improve the predictive ability of these models (Onstad 2006).

MATERIALS AND METHODS

Plant-to-plant larval movement studies in *Bt* and non-*Bt* soybean cultivars were conducted during the 2016/2017 and 2017/2018 cropping seasons in Brazil (São Paulo State University/Botucatu, SP) (22º82'48'' S, 48º26'05'' W). The cultivars TMG 7262 (non-*Bt*) and TMG 7062 IPRO expressing *Bacillus thuringiensis* (*Bt*) protein Cry1Ac, recommended for the regions of the São Paulo State with altitudes above 600 m, were used in the experiments. The row spacing was 0.45 m with 15 plants per linear meter, and the soybean stage evaluated was R2/R3 (Fehr and Caviness 1977). All soybean fields were grown under conventional agronomic practices appropriate for each region.

Insects

S. eridania eggs were commercially acquired from Pragas.com insumos biológicos, Piracicaba, SP. They were purchased close to hatching in order to standardize infestations of the plants in the field with neonates (0-24 h old). Egg incubation and hatching was conducted under controlled conditions (25 ± 2 °C, 70 ± 10% RH and 14 h photophase). Soybean leaves were removed from the field (same cultivars planted to this experiment) and installed inside the plastic cages (21.5 x 14.5 x 3.5 cm). A piece of cotton moistened in water was wrapped around the base of the petioles to maintain leaf turgidity. Individual egg masses were cut from organza cloth where the adults oviposited, and then placed on leaves of each soybean cultivar until they hatched. After hatching, 45 neonates per leaflet were kept and isolated in a Gerbox (11 x 11 x 3.5 cm) until infestation. The excess larvae were removed with a delicate paintbrush. The field infestation occurred at dusk in order to reduce stress to the larvae and maximize the effectiveness of the infestation.

Larval movement

The experiments consisted of four plots arranged in a randomized block design, except for 2016/2017 where there were three plots of the non-*Bt* cultivar. The plots were five 3.0 m long rows, totaling 225 plants per plot (15 plants/m). To avoid natural enemies, all plots were covered by metallic cages (16 mesh) (3.0 m long x 3.0 m wide x 2.5 m height). The cages were installed 20 days before infestation, along with the application of deltamethrin (Decis, 200 ml/ha, Bayer) to kill any other larvae species which could interfere with *S. eridania*.

In 2016/2017, artificial infestation was performed by releasing 45 neonates at four locations in the middle portion of the central plant of each plot, totaling 180 neonates per plant. The infestation location and larval total of 180 per plant was selected because *S. eridania* moths prefer to oviposit in the middle third of the soybean plant (200 to 280 eggs / mass), with variable egg viability (50% to 80%) (Santos et al. 2005, Souza et al. 2014, Silva et al. 2017), so we infested based on 65% egg viability. In 2017/2018, the same infestation level was used for non-Bt plots; however, 280 neonates were infested per plant in Bt plots due to high mortality in 2016/2017. The infestation was performed by fixing infested leaflets from the rearing cages to the upper surface of a central plant leaflet with a small piece of adhesive tape, taking care so that the neonates did not stick to the tape. This technique reduced stress to the neonates, resulting in efficient transfer of the neonates to the infested plant.

Destructive sampling of all plants was carried out in each plot at 14 days after infestation. The number/frequency of larvae were established by the position in which the insects were found in relation to the infested central plant of each plot. Larval position was demarcated in the field by "small identification flags" on wooden sticks placed at the plant base. Larval movement was categorized based on the presence of each insect on each plant in each plot, and percent survival was calculated by dividing the number of larvae released by the number recovered (Pannuti et al. 2016). The proportion of larvae present on infested plants and in rows was also evaluated.

The rows ran north to south, and larval orientation was categorized as north (N) and south (S) in the same row with respect to the infested plant, and east (E) and west (W) when directly across rows from the infested plant. The position of the larvae was designated as zero when on the infested plant. For larvae not directly N, S, E, or W of the infested plant, the position from the release point was categorized by quadrant: northeast (NE), southeast (SE), northwest (NW), and southwest (SW). Larval mean and maximum distance from the infested plant was determined by Euclidean distance.

The most distant larvae in each quadrant and orientation was considered the maximum distance (Pannuti et al. 2016).

In order to determine a possible interaction among larval size and soybean rows where the larvae were recovered, two larval sizes (small < 1.0 cm and large ≥ 1.0 cm) and three or five rows (with one more “external row” in 2016/2017 non-*Bt* plots for larvae which were found off the plant on the ground at the plot edge) were considered. Larval size was measured by a digital paquimeter (Mitutoyo 150 mm Digital Caliper 0.01 mm, Metric). In 2016/2017 the larval size was measured only by the separation of large and small larvae. Because *S. eridania* lives gregariously between the first and third instars (Silva et al. 2017), a small trial (data not published) was done with non-*Bt* soybean in an acclimatized greenhouse (25 ± 2 °C, 70 ± 10% RH and 14 h photophase) to verify the mean size of third instar *S. eridania*. One non-*Bt* plant was infested with 25 neonates and after 7 days, the period at which the larvae would be late third instar (Santos et al. 2005, Montezano et al. 2014), larval length was measured

Protein effect on larval survival and biological parameters

As this species has some level of tolerance to MON 87701 x MON 89788 soybean containing the protein Cry1Ac (Bernardi et al. 2014), results regarding the percentage of live larvae observed between the cultivars was used to calculate the larval survival component of incomplete resistance using the formula [larval survival on *Bt* plants / larval survival on non-*Bt* plants] (Tabashnik et al. 2005).

Cephalic capsule size (mm), instar, and percentage of small larval were recorded to evaluate the *S. eridania* larval fitness for *Bt* and non-*Bt* soybean in the 2017/2018. Instar was determined by measuring cephalic capsules (Capinera 1999) with a stereomicroscope (Nikon - Stereo Zoom Microscope SMZ 645, Tokyo, Japan) and a high precision manual meter from BioQuip Products, California, USA.

Statistical analysis

Larval distance parameters and proportion data were analyzed using a generalized linear model (GLM) with gaussian and binomial or quasibinomial (overdispersion) error distributions, respectively. Count data were analyzed using generalized linear mixed models (GLMM) with Poisson or negative binomial (overdispersion) distributions, considering the plots as random effects and the treatments (number of larvae in each quadrant and orientations, number of larvae in each 20 cm distance interval), and the interaction among soybean rows and larval size in *Bt* and non-*Bt* soybean as fixed effects using the function 'glmer' with package "lme4". Too many zeros can increase the probability of overdispersion in count data (Brooks et al. 2017), so when many zeros occurred in the response variables, the analyses were modeled with zero-inflated GLMM with 'glmmTMB' function with "glmmTMB, bbmle and ggplot2" packages. The models were fit by AIC, considering models with zero-inflated Poisson distribution and excluding zero-inflation with Poisson distribution.

Statistically significant values from count and binomial data were calculated from type II Wald chi-square tests using the function 'Anova' in the package "car" with pairwise comparison from LSmeans with Tukey adjustment ($p \geq 0.05$), using "lsmeans" package. In case of overdispersion, the F test was performed. For gaussian distribution data, ANOVA by the F test was done to detect difference between the means, and when necessary separated by the Tukey test ($p \leq 0.05$), using the "multicomp" package.

Prior to conducting the correlation analyzes by linear regression (size = dependent variable and cephalic capsule = independent variable), the normality was verified by the Shapiro-Wilk test and homogeneity by the Breusch-Pagan test using the function ‘bptest’ with package “lmtest”. Logarithmic transformation was done in the response variable if the subsequent assumptions were not reached. Correlation analysis between the residuals was then performed by Durbin-Watson test using the function ‘dwtest’ with package “lmtest”. The models in which autocorrelation was detected in the residuals were not considered in the analyses. Linear regression was performed with replicated values of the explanatory variable (cephalic capsules), and the models were tested for lack of fit. If a lack of fit was found ($p \leq 0.05$), a new model would be needed. After checking all these steps, the linear models were submitted to Pearson correlation analysis and descriptive statistical analysis. All analyses were performed using software R 3.5.2 (R Development Core Team 2018).

RESULTS

Larval movement

There were no statistical differences for mean and maximum distances covered by larvae in all seasons and cultivars in the quadrants and orientations, although the highest values were observed in non-*Bt* plants. For *Bt* plants, in 2016/2017, it was only possible to evaluate the within infested row orientation (N and S axes) because larval recovery was low. In 2017/2018, larval recovery was sufficient to do all evaluations (Table 1).

There were significant differences for mean number of larvae recovered at each distance interval for all evaluations (Table 2). For non-*Bt* plants, the larvae moved more in 2016/2017 than in 2017/2018 (furthest larvae recovered at 200 to 220 cm, versus 120 to 140 cm in 2017/2018), with the highest number of larvae (16.3) recovered at 80 to 100 cm. In 2017/2018, the highest number of larvae (25.0) was observed in the first interval. For *Bt* plants, the overall and mean number of larvae recovered in each interval was lower than non-*Bt* plants, and the highest number of larvae was observed in the first interval for both seasons (3.0 and 9.8, respectively).

For non-*Bt* plants in 2016/2017, except for Row3 small larvae, statistical differences were observed in the other interactions between sizes and rows. The mean number of large larvae in all rows was higher than for small larvae. The highest number of small larvae were recovered in Row 3 (4.3), and large larvae in Row 2 (12.7) and Row 4 (11.3) (Table 3). For 2017/2018, there was a significant interaction between sizes and rows. The highest number of larvae was observed in Row 3 for small larvae (31.0) (Table 4).

On *Bt* plants, in 2016/2017, the larvae were recovered only on the infested row (Row 3) (Fig. 1). In 2017/2018, there was no significant difference of the interaction between sizes and rows (Table 5); however, there was a difference between these factors separately. The mean number of small larvae (6.5) was higher than large larvae (0.9), and most larvae were in Row 3 (8.3). Different from non-Bt plants, the larvae on Bt plants moved only to the first adjacent rows (Row 2 and Row 4) from the infested row (Table 5 and Fig. 1).

The *S. eridania* larvae moved similarly for all quadrants and orientations in both seasons and for both cultivars, with no significant differences observed (Table 6 and Fig. 1). For non-*Bt* plants in 2016/2017, there were no significant differences among the percentage of larvae recovered in the different rows and out of them (external), although numerically the highest frequency was observed in Row 3 (25.4%) (Fig. 2).

For *Bt* plants in 2016/2017, larvae were only recovered in the infested row (Row 3) (Fig. 1). In 2017/2018 the frequencies in Row 3 were similarly higher in both non-*Bt* and *Bt* plants (62.4% and 78.9%, respectively), and significantly different than the other rows. Except for 2016/2017 on *Bt* plants, more larvae left than remained on the infested plant (Fig. 3).

Protein effect on larval survival and biological parameters

The adjusted coefficient of determination (R2) for *Bt* plants was lower than for non-*Bt* plants (Table 7). For non-*Bt* plants, only plots B and C satisfied assumptions to run a linear regression. The linear regression model was significant for *Bt* plant plots A and D, and non-*Bt* plant plots B and C.

The mean percentage of small larvae in both seasons was higher on *Bt* plants, with more than 82.0% of the larvae recovered on *Bt* plants being small. The head capsule size and larval sizes were lower on *Bt* plants than on non-*Bt* plants. The larvae on *Bt* plants were in initial 4th instar, while in non-*Bt* plants they were in transition from 4th to 5th instar (Capinera 1999). Larval survival on non-*Bt* plants was higher than on *Bt* plants for both seasons, with larval survival component values of 0.07 and 0.19, respectively (Table 8).

DISCUSSION

Considering that a high-dose event should kill 99.99% of a target insect population (Tabashnik et al. 2013), the survival of *S. eridania* larvae on *Bt* soybean plants (~3.0% in 2016/2017 and ~8.0% in 2017/2018) in the present research indicates the high-dose requirement is not met. Bernardi et al. (2014) observed that *S. eridania* has low susceptibility to Cry1Ac. The survival of these insects in the field can be a potential risk to this crop. Santos et al. (2009) confirmed in Brazil the low efficacy of Cry1Ac on *S. eridania* in cotton, necessitating the use additional control tactics. Therefore, maintaining the susceptibility of *S. eridania* to this technology will be important to ensure the sustainability of this control strategy (Bernardi et al. 2014). However, Cry1Ac affected *S. eridania* behavior and development when compared to larvae on non-*Bt* soybean in practically all evaluated parameters (e.g. shorter distances moved, reduced larval survival), which may result in increased exposure of larvae to biotic and abiotic factors (facilitating the use of other control methods) (Romeis et al. 2006, Bernardi et al. 2014), and have implications for resistance management strategies (Tabashnik et al. 2005).

Although *S. eridania* larvae mean/maximum distance covered and mean number in different positions on *Bt* soybean were lower than on non-*Bt* soybean, the larvae were observed to move similarly in all directions independent of the season or cultivar. Thus, movement of this species appears to be non-directional and independent of biotic or abiotic factors (Bell 1990, Pannuti et al. 2016).

The spatial distribution of *S. eridania* larvae in *Bt* and non-*Bt* soybean appears to be random. Except for the 2016/2017 on *Bt* plants, the mean percentage of larvae found on the infested plants was significantly lower than on the other plants. This distribution is different among species and host plants. Pannuti et al. (2016) reported that *S. frugiperda* and *Striacosta albicosta* (Smith) exhibited an aggregate and symmetrical distribution on maize, with most of the larvae recovered on the initial infested plants. Instar and larval behavior contribute to species and host plant specific spatial distribution. Small *S. frugiperda* larvae present an aggregate behavior, while medium and large *S. frugiperda* larvae exhibit a random pattern in maize (Trindade et al. 2017). As a result of competition for space or food resources, *Helicoverpa armigera*

(Hübner) larvae dispersed on *Chrysanthemum* when they reached high larval density, exhibiting a uniform spatial distribution (Kakimoto et al. 2003).

Spodoptera eridania larvae dispersed more from the infested plant in non-*Bt* soybean than in *Bt* soybean, where they were located only in the infested and adjacent rows. In all evaluations, the highest percentage of larvae was in the infested row. The movement across plants can be different for different crops. Larval movement is higher within rows where more leaves are in contact, such as with common bean (*Phaseolus vulgaris* L.) (Blickenstaff 1983).

Higher dispersion in non-*Bt* soybean is also reflected by the higher mean number of recovered larvae in each distance interval. Larval dispersion was higher in 2016/2017 with more developed larvae (larvae ≥ 1.0 cm), where the proportion of larger larvae was significantly higher than the smaller larvae, with no differences among the rows for larger larvae. On non-*Bt* soybean, less developed larvae moved less and were found in greater number in the infested row at 0 to 20 cm. Although larval dispersion was lower in 2017/2018 on non-*Bt* plants, it was higher than that observed on *Bt* plants. In the case of *Bt* plants, in both seasons the proportion of smaller larvae was higher, and as on non-*Bt* soybeans during 2017/2018, they were recovered primarily in the infested row at 0 to 20 cm. Therefore, it appears larval development directly affects larval dispersal in soybean, and the Cry1Ac toxin contributes to lower larval movement by adversely affecting larval development.

Spodoptera eridania larvae do not exhibit cannibalism (Hass et al. 2012), and the higher dispersion of more developed larvae may be related to the search for food; the larger they become, more defoliation occurs. The highest leaf consumption during the last instars is related to a more rapid growth of the larvae to attain adequate nutritional reserves for later a transformation processes during the pupal phase (Pratissoli et al. 2002).

Even though *S. eridania* presents reduced susceptibility to Cry1Ac (Bernardi et al. 2014), their reduced movement on *Bt* plants may be due adverse effects caused by the toxin. Lynch et al. (1999) found that *Helicoverpa zea* (Boddie) larvae surviving on *Bt* sweet corn were generally lethargic, with reduced feeding and therefore weight, when compared to larvae that had fed on non-*Bt* sweet corn. The negative effect of *Bt* toxin to *S. eridania* is demonstrated by the low linear correlation between size and larval cephalic capsule, as well as lower larval survival, smaller body and cephalic capsule size, and increased proportion of early instars in the assessments of fitness and incomplete resistance.

Although *S. eridania* has reduced susceptibility to Cry1Ac, the protein effect on larval behavior and biological parameters observed in the present research suggests incomplete resistance to this toxin. When incomplete resistance occurs, the insects surviving on *Bt* plants often develop more slowly and have higher mortality than insects on non-*Bt* plants (Huang et al. 2011). The higher percentage of live larvae on plants that do not express *Bt* toxins, increasing the number of *Bt* susceptible individuals, is desired in IRM and falls within the concept of high-dose/refuge, which requires the survival of susceptible individuals to mate with any possible resistant individuals (Tabashnik et al. 2013).

However, incomplete resistance is not an independent factor contributing to the dilution of resistance alleles from a resistant population. It is dependent on the interaction among different key parameters: type and size of refuge, fitness cost, and rate of larval dispersion (Tabashnik et al. 2005, Garcia et al. 2016). Modeling research with pink bollworm (*Pectinophora gossypiella*) shows that the resistance allele frequency probably decreases with increases in fitness costs and refuge percentage (Tabashnik et al. 2005).

During 2016/2017 in non-*Bt* soybean, where a larger proportion of more developed larvae were found, *S. eridania* larvae could move to 13 plants (N and S orientations) and across at least two rows from the infested row (W and E orientations) in a stand of 15 plants/meter. Even in *Bt* soybean, larvae moved to 6 plants (N and S orientations) and to the rows adjacent to the infested row at high infestation levels (2017/2018).

Although RIB can be an option in IRM, insects that exhibit high larval movement across plants generally favor the evolution of resistance in a seed mixture refuge, since such movement exposes insects to sublethal doses of the toxins, especially for single-gene *Bt* events (Head et al. 2014, Garcia et al. 2016). Seed mixtures can increase the dominance of resistance by increasing survival of heterozygous larvae relative to homozygous susceptible larvae when individual larvae feed on both *Bt* and non-*Bt* plants (Mallet and Porter 1992). Therefore, this strategy may not be ideal for resistance management of *S. eridania* to the Cry1Ac toxin; a more appropriate strategy being the use of strip or block refuge.

There are studies with other pest species which present low susceptibility to a certain *Bt* toxins that demonstrate RIB is not appropriate when the event tested expresses a single toxin. *Helicoverpa zea* presents mobile larvae and low susceptibility to Cry1Ac, and on *Bt* and non-*Bt* cotton provides evidences that the dominance of pest resistance to a *Bt* crop was higher in a seed mixture than in a block refuge scenario (Brévault et al. 2015). In a field study conducted with seed mixes of non-*Bt* and *Bt* pyramid maize, *Diatraea saccharalis* (Fabr.) larvae were able to move from infested plants, as well as to adjacent rows, so larvae could feed on non-*Bt* maize until they reached a size that allowed their survival on *Bt* maize (Wangila et al. 2013).

The possible existence of incomplete resistance in *S. eridania* indicates that RIB for *Bt* soybean with a single toxin should not be adopted. Garcia et al. (2016), in theoretical ecological models, observed that larval movement directly affects the evolution of resistance of *S. frugiperda* in *Bt* maize, and adequate management depends of the resistance type which the pest presents, as well as the configuration and size of the refuge areas. In this study the authors concluded that the border between *Bt* and non-*Bt* crops plays an important role in resistance evolution. They observed that RIB strategy was not adequate, considering complete and incomplete resistance, because the larval movement favored the occupation of non-Bt by the resistant larvae. Considering all results, the authors found that the best configuration for all these conditions was the strip refuge. The block strategy was not appropriate due to spatial isolation, even for high or intermediate larval movement rates. This last observation (larval movement rate) applies in the current study, because *S. eridania* larvae presented different dispersion rates in the non-*Bt* and *Bt* soybean, which could also lead to spatial isolation with block refuge.

The choice of refuge configuration in IRM for a particular species depends on several factors that involve both the biology and behavior of the insect and the target crop, as well as the phonological plant stage that directly affects insect behavior and development. The ideal refuge is the result of a combination of studies between pest and transgenic crop (Carrière et al. 2010, Tabashnik and Carrière 2017). Research under natural conditions that aim at understanding behavior and larval biology under specific conditions (e.g. in a specific host or cropping system) are necessary to develop IRM, as are ecological models that aim to elucidate the relationship between larval movement and the evolution of resistance of the target insect (Onstad 2006, Pannuti et al. 2016).

On December 19, 2018, by the normative instruction nº 59, the Brazilian Ministry of Agriculture, Livestock and Food Supply instituted, the practice of structured refuge as a phytosanitary measure for the management of insect resistance in genetically modified cropping systems (DOF 2018). Because RIB does not appear to be appropriate for *S. eridania* in current *Bt* transgenic soybean, studies should be conducted to identify which IRM strategy would be most suitable (e.g. what structured refuge configuration would be best). In the case of Brazil, the choice of which structured refuge strategy should be adopted for IRM is essential. Additional *S. eridania* biological and ecological studies should be conducted to contribute to future modeling studies as new transgenic events and/or pyramids become available.

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Table 1. Mean and maximum distance (± SE) covered by *S. eridania* larvae in different positions and directional orientations relative to the infested plant in non-*Bt* and *Bt* soybean, Botucatu, SP- 2016/2017 and 2017/2018 seasons.

	Non-*Bt* plants				***Bt* plants**			
	1º season		**2º season**		**1º season**		**2º season**	
Quadrants	**Mean distance (cm)**	**Maximum distance (cm)**	**Mean distance (cm)**	**Maximum distance (cm)**	**Mean distance (cm)**	**Maximum distance (cm)**	**Mean distance (cm)**	**Maximum distance (cm)**
NW	97.8 ± 6.1 a	168.5 ± 29.6 a	83.4 ± 5.6 a	112.1 ± 11.6 a	---	---	56.0 ± 1.6 a	58.8 ± 4.4 a
SW	89.9 ± 5.0 a	158.7 ± 11.4 a	70.3 ± 3.1 a	100.3 ± 9.8 a	---	---	53.7 ± 6.9 a	54.0 ± 6.5 a
NE	108.5 ± 13.7 a	163.3 ± 31.9 a	73.6 ± 5.1 a	110.3 ± 11.3 a	---	---	52.2 ± 0.4 a	52.8 ± 0.3 a
SE	85.6 ± 6.4 a	127.8 ± 33.9 a	76.2 ± 8.4 a	110.5 ± 10.2 a	---	---	46.8 ± 0.6 a	48.0 ± 0.6 a
P value	0.319	0.619	0.431	0.6029	---	---	0.286	0.383
F	1.449	0.635	1.012	0.649	---	---	1.806	1.327
Df	3	3	3	3	---	---	3	3
ORIENTATION								
N	87.6 ± 6.8 a	194.0 ± 08.7 a	49.8 ± 5.7 a	120.4 ± 10.9 a	---	---	40.2 ± 8.1 a	50.4 ± 11.6 a
S	78.6 ± 8.2 a	167.7 ± 16.9 a	52.3 ± 5.9 a	113.4 ± 9.7 a	---	---	35.7 ± 3.2 a	46.4 ± 6.4 a
P value	0.0746	0.0879	0.820	0.532	---	---	0.570	0.643
F	11.93	9.902	0.062	0.497	---	---	0.405	0.264
DF	1	1	1	1	---	---	1	1
Within Infested Row								
N	50.2 ± 7.3 a	104.2 ± 18.3 a	25.2 ± 2.7 a	60.1 ± 20.1 b	40.0 ± 10.5 a	---	34.5 ± 6.1 a	40.4 ± 9.4 a
S	49.1 ± 7.4 a	76.8 ± 6.7 a	38.1 ± 9.4 a	86.6 ± 20.7 a	58.8 ± 30.3 a	---	27.4 ± 5.1 a	31.8 ± 4.2 a
P value	0.44194	0.182	0.190	0.0532	0.450	---	0.465	0.381
F	0.905	4.038	2.851	9.625	0.75	---	0.699	1.048
Df	1	1	1	1	1	---	1	1
Across rows								
W	89.4 ± 5.5 a	177.5 ± 20.6 a	73.1 ± 1.3 a	112.1 ± 11.6 a	---	---	54.8 ± 1.6 a	60.5 ± 4.0 a
E	100.9 ± 13.1 a	164.5 ± 30.7 a	73.1 ± 4.6 a	118.3 ± 10.2 a	---	---	48.7 ± 0.6 b	51.2 ± 1.6 a
P value	0.561	0.771	1.000	0.654	---	---	0.030	0.084
F	0.479	0.110	0.000	0.245	---	---	31.803	10.419
Df	1	1	1	1	---	---	1	1

Distance was calculated by Euclidean distance. The means with the same letter are not significantly different (*P* ≥ 0.05 - Tukey Contrasts) by GLM (ANOVA by test F).

Table 2. Mean number (±SE) of larvae in each distance interval (cm) by *S. eridania* in non-*Bt* and *Bt* soybean, Botucatu, SP- 2016/2017 and 2017/2018 seasons.

	Non-*Bt* plants				***Bt* plants**			
Intervals (cm)	**1º season**[a]	**Rep.**	**2º season**[a]	**Rep.**	**1º season**[b]	**Rep.**	**2º season**[c]	**Rep.**
$0 \le x \le 20$	04.7 ± 1.2 bc	3	25.0 ± 9.1 a	4	3.0 ± 0.8	4	9.8 ± 4.1 a	4
$20 < x \le 40$	02.3 ± 0.9 c	3	14.0 ± 4.7 ab	4	1.0 ± 0.7	4	4.8 ± 3.2 ab	4
$40 < x \le 60$	17.3 ± 7.5 a	3	16.5 ± 7.4 ab	4	0.8 ± 0.8	4	6.8 ± 3.2 a	4
$60 < x \le 80$	06.0 ± 3.6 bc	3	06.5 ± 3.0 bc	4	---	---	0.8 ± 0.3 b	4
$80 < x \le 100$	16.3 ± 5.9 a	3	07.0 ± 4.1 bc	4	0.3 ± 0.3	4	---	---
$100 < x \le 120$	09.7 ± 6.7 ab	3	06.3 ± 4.4 c	4	---	---	---	---
$120 < x \le 140$	05.7 ± 3.2 bc	3	02.0 ± 1.4 c	4	---	---	---	---
$140 < x \le 160$	03.3 ± 2.3 c	3	---	---	---	---	0.3 ± 0.3 b	4
$160 < x \le 180$	02.0 ± 0.6 c	3	---	---	---	---	---	---
$180< x \le 200$	02.0 ± 1.5 c	3	---	---	---	---	---	---
$200 < x \le 220$	01.7 ± 1.0 c	3	---	---	---	---	---	---
Chisq	119.95	---	52.33	---	---	---	32.91	---
P-value	< 2.2e-16	---	1.6e-09	---	---	---	1.246e-06	---
Df	10	---	6	---	---	---	4	---

[a] Data were overdispersed and they were modeled using negative binomial distribution GLMM. [b] Data does not contain information to estimate the parameters. [c] Data assumed Poisson distribution and were modeled with zero-inflated GLMM, without excluding zero-inflation (zero-inflated Poisson model with a single zero-inflation parameter applying to all observations). Pairwise t-test comparations it was done by Least Squares Means adjusted by Tukey ($p \le 0.05$).

Table 3. Analysis of deviance among 5 different soybean rows plus one external row, and two sizes of larvae regarding the mean number of larvae (±SE) recovered on non-*Bt* soybean, Botucatu, SP- 2016/2017 season.

Treatments	**Analysis of deviance**		
	Df	**Chisq†**	**P-value**
Row	5	4.7039	0.453068
Size	1	53.8978	2.112e-13
Row x Size	5	18.0773	0.002851
	Decomposition of the Interaction		
	Small	**Large**	**Rep.**
Row 1	0.3 ± 0.3 aB	9.7 ± 4.8 aA	3
Row 2	1.6 ± 1.2 aB	12.7 ± 5.8 aA	3
Row 3	4.3 ± 2.9 aA	9.6 ± 0.7 aA	3
Row 4	0.7 ± 0.3 aB	11.3 ± 6.5 aA	3
Row 5	0.3 ± 0.5 aB	9.6 ± 6.5 aA	3
External	0.3 ± 0.3 aB	9.7 ± 6.5 aA	3

	Fixing the factor Small and their rows						
		Large					
	Small	**Row 1**	**Row 2**	**Row 3**	**Row 4**	**Row 5**	**External**
Row 1	A	B	B	B	B	B	B
Row 2	A	B	B	B	B	B	B
Row 3	A	A	B	A	A	A	A
Row 4	A	B	B	B	B	B	B
Row 5	A	B	B	B	B	B	B
External	A	B	B	B	B	B	B

Data assumed Poisson distribution and they were modeled with GLMM. The pairwise t-test comparations it was done by Least Squares Means adjusted by Tukey ($p \leq 0.05$). Means within a column followed by the same lowercase letter and in each row followed by the same uppercase letter are not significantly different.

Table 4. Analysis of deviance among 5 different soybean rows and two sizes of larvae regarding the mean number of larvae (±SE) recovered on non-*Bt* soybean, Botucatu, SP- 2017/2018 season.

Treatments	**Analysis of deviance**		
	Df	**Chisq†**	**P-value**
Row	4	175.42	< 2.2e-16
Size	1	1.5602	0.2116
Row x Size	4	29.5284	6.105e-06
	Decomposition of the Interaction		
	Small	**Large**	**Rep.**
Row 1	1.0 ± 0.4 bA	4.8 ± 3.2 bcA	4
Row 2	4.5 ± 1.0 bcA	8.0 ± 06.7 abA	4
Row 3	31.0 ± 10.6 aA	16.8 ± 13.9 aB	4
Row 4	5.8 ± 1.3 bA	4.0 ± 2.6 bcA	4
Row 5	0.3 ± 0.3 bA	1.3 ± 0.8 cA	4

	Fixing the factor Small and their rows					
		Large				
	Small	**Row 1**	**Row 2**	**Row 3**	**Row 4**	**Row 5**
Row 1	A	A	B	B	A	A
Row 2	A	A	A	B	A	A
Row 3	A	B	B	B	B	B
Row 4	A	A	A	B	A	A
Row 5	A	A	B	B	A	A

Data assumed Poisson distribution and were modeled with zero-inflated GLMM, without excluding zero-inflation (zero-inflated Poisson model with a single zero-inflation parameter applying to all observations). The pairwise t-test comparations it was done by Least Squares Means adjusted by Tukey ($p \leq 0.05$). Means within a column followed by the same lowercase letter and in each row followed by the same uppercase letter are not significantly different.

Table 5. Analysis of deviance among 3 different soybean rows and two sizes of larvae regarding the mean number of larvae (±SE) recovered on *Bt* soybean, Botucatu, SP- 2017/2018 season.

Analysis of Deviance			
Treatments	**Df**	**Chisq**	**P-value**
Row	2	47.1216	5.857e-11
Size	1	29.8461	4.677e-08
Row x Size	2	4.7569	0.09269

Each Factor					
Factor Rows		**Rep.**	**Factor Sizes**		**Rep.**
Row 2	1.3 ± 0.6 b	8	**Small**	6.5 ± 02.6 a	12
Row 3	8.3 ± 3.8 a	8	**Large**	0.9 ± 00.3 b	12
Row 4	1.7 ± 0.6 b	8		---	12

Data assumed Poisson distribution and they were modeled by GLMM. The pairwise t-test comparations it was done by Least Squares Means adjusted by Tukey (P ≤ 0.05). Means within a column followed by the same lowercase letter are not significantly different. To *Bt* plants in the first season all larvae were found on the infested row. So, no analysis with these parameters to this season.

Table 6. Mean number (± SE) and frequency of *S. eridania* larvae recovered in different positions and directional orientations relative to the infested plant in non-*Bt* and *Bt* soybean, Botucatu, SP- 2016/2017 and 2017/2018 seasons.

	Non-Bt plants				**Bt plants**			
	1º season		**2º season**		**1º season**		**2º season**	
Quadrants	**Mean[a]**	**Frequency (%)**	**Mean[a]**	**Frequency (%)**	**Mean[b]**	**Frequency (%)**	**Mean[a]**	**Frequency (%)**
NW	13.7 ± 7.7 a	26.3	9.0 ± 2.7 a	31.3	---	---	1.5 ± 0.7 a	26.1
SW	12.7 ± 5.8 a	24.4	7.8 ± 2.8 a	27.0	---	---	0.8 ± 0.5 a	13.1
NE	14.0 ± 9.0 a	26.9	5.5 ± 1.8 a	19.1	---	---	1.8 ± 1.0 a	30.4
SE	11.7 ± 7.8 a	22.4	6.5 ± 2.7 a	22.6	---	---	1.8 ± 0.9 a	30.4
P value	0.8556	---	0.2771	---	---	---	0.3333	---
Chisq	0.7744	---	3.8589	---	---	---	3.4049 [c]	---
Df	3	---	3	---	---	---	3	---
ORIENTATION								
N	34.3 ± 18.4 a	53.9	31.5 ± 14.1 a	50.0	---	---	7.8 ± 3.0 a	47.7
S	29.3 ± 14.4 a	46.1	31.5 ± 19.9 a	50.0	---	---	8.5 ± 3.8 a	52.3
P value	0.2766	---	1.000	---	---	---	0.7066	---
Chisq	1.184	---	0.000	---	---	---	0.1417	---
DF	1	---	1	---	---	---	1	---
Within Infested Row								
N	07.0 ± 2.0 a	58.3	17.5 ± 6.7 a	49.3	1.5 ± 0.9 a	75.0	4.5 ± 1.7 a	57.1
S	05.0 ± 1.2 a	41.7	18.0 ± 7.5 a	50.7	0.5 ± 0.3 a	25.0	6.0 ± 2.8 a	42.9
P value	0.3184	---	0.8659	---	0.1785	---	0.8647	---
Chisq	0.9956	---	0.0285	---	1.8104	---	0.029 [c]	---
Df	1	---	1	---	1	---	1	---
Across rows								
W	28.7 ± 13.7 a	51.5	12.0 ± 4.3 a	58.3	---	---	3.7 ± 1.0 a	39.1
E	27.0 ± 17.2 a	48.5	16.8 ± 9.1 a	41.7	---	---	2.3 ± 1.1 a	60.9
P value	0.6977	---	0.0756	---	---	---	0.3011	---
Chisq	0.1509	---	3.1563	---	---	---	1.0695	---
Df	1	---	1	---	---	---	1	---

[a] Data assumed Poisson distribution and were modeled with GLMM. [b] Data assumed Poisson distribution and they were modeled with zero-inflated GLMM, excluding zero-inflation.[c] Data were overdispersed and they were modeled using negative binomial distribution GLMM.

Table 7. Linear regression analysis to examine the correlation between the larval cephalic capsule (mm) and larval length size (cm) of *S. eridania* in *Bt* and Non-*Bt* soybean in different plots, Botucatu, SP-2017/2018 season.

Plots	***R***	**Equation**	R^2	**F**	**P-value**[a]	**P-value**[b]	**P-value**[c]	**N**
				***Bt* plants**				
A	0.63	$\hat{Y} = 2.257 + 4.687x$	0.2975	3.965	0.09356	0.984	0.5606	8
B	0.55	$\hat{Y} = 2.930 + 4.648x$	0.0746	1.323	0.3335	0.746	0.0950	5
C	0.65	$\hat{Y} = 2.347 + 5.019x$	0.4052	20.07	0.000123	0.728	0.3294	29
D	0.69	$\hat{Y} = 3.289^{0.6887x}$	0.4621	37.08	3.233e-07	0.904	0.6417	42
				Non-*Bt* plants				
A	---	---	---	---	---	0.000	---	94
B	0.9660	$\hat{Y} = 2.199^{1.2055x}$	0.9237	97.82	2.3e-05	0.492	0.4239	9
C	0.7860	$\hat{Y} = 2.224 + 4.629x$	0.6092	71.14	9.682e-11	0.236	0.8677	46
D	---	---	---	---	---	0.000	---	148

[a] P-value of the linear regression model. [b] P-value for autocorrelation analysis. [c] Test for lack of fit. P-values ($p \leq 0.05$) indicates that there is a lack of fit.

Table 8. Empirical estimates of protein effect on survival and larval biological parameters of *S. eridania* in *Bt* and Non-*Bt* soybean, Botucatu, SP- 2016/2017 and 2017/2018 seasons.

Treatments	**1º Season**	**2º Season**		
	Fitness			
	% small larvae	**% small larvae**	**C.C. (mm)**	**Instar**
***Bt* plots**	82.3 ± 06.3	89.6 ± 03.0	0.94 ± 0.001	4^{th}
Non-*Bt* plots	9.8 ± 01.2	71.8 ± 13.8	1.12 ± 0.105	$4/5^{th}$
	Incomplete Resistance			
Larval survival on *Bt* plots	2.8 ± 00.6	8.0 ± 02.8		
Larval survival on non-*Bt* plots	38.9 ± 15.8	43.1 ± 14.6		
Larval survival component	0.07	0.19		

The means (± SE) are the average of the means from all plots of each season.

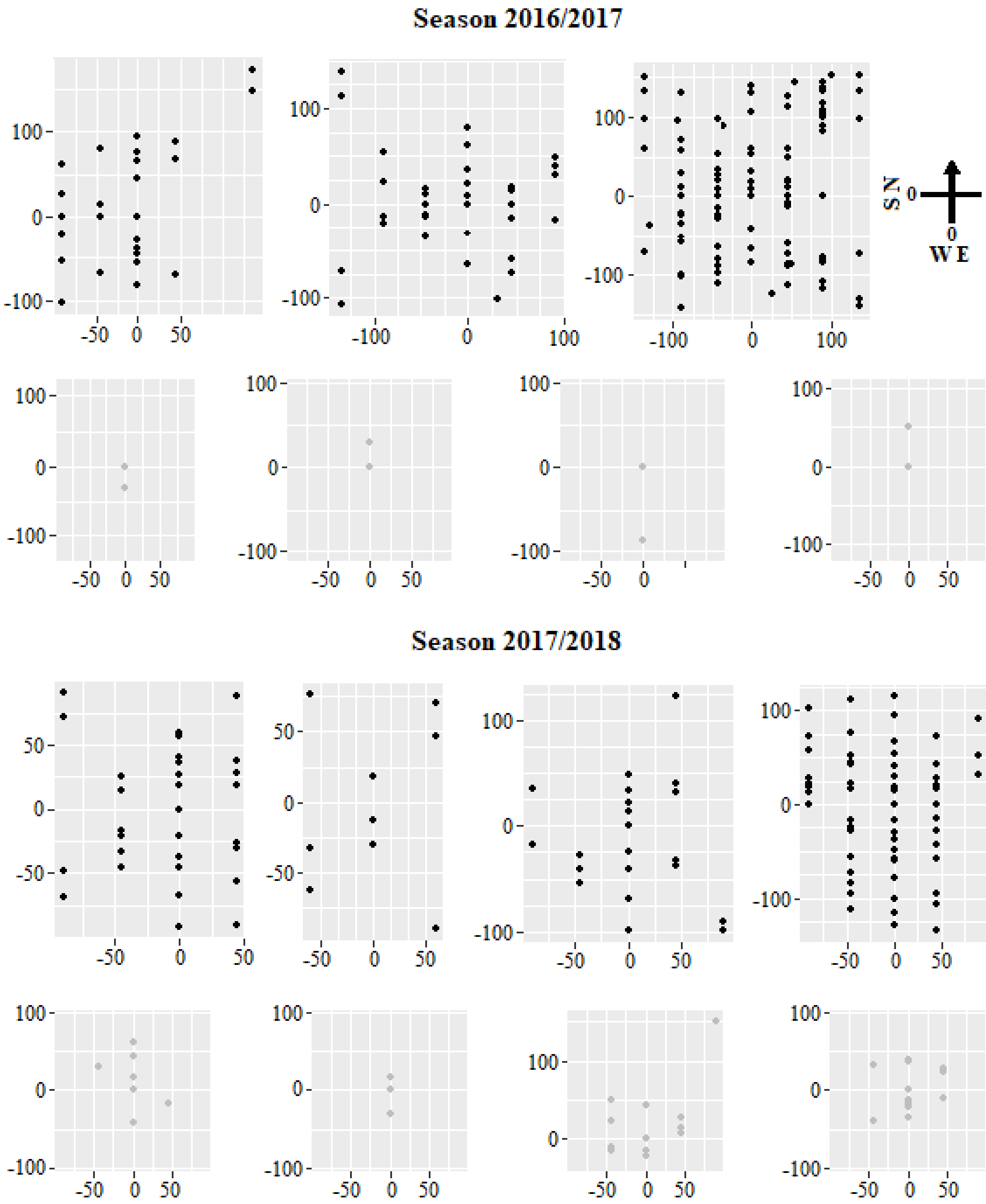

Fig. 1. Dispersion graphs (cm) regarding *S. eridania* larval plant-to-plant movement in non-*Bt* (black spots) and *Bt* (grey spots) soybean. The spots represent the plants where the larvae were found. S|N and W|E are the orientations. These graphs were constructed by software R 3.4.1 using the ggplot2 package.

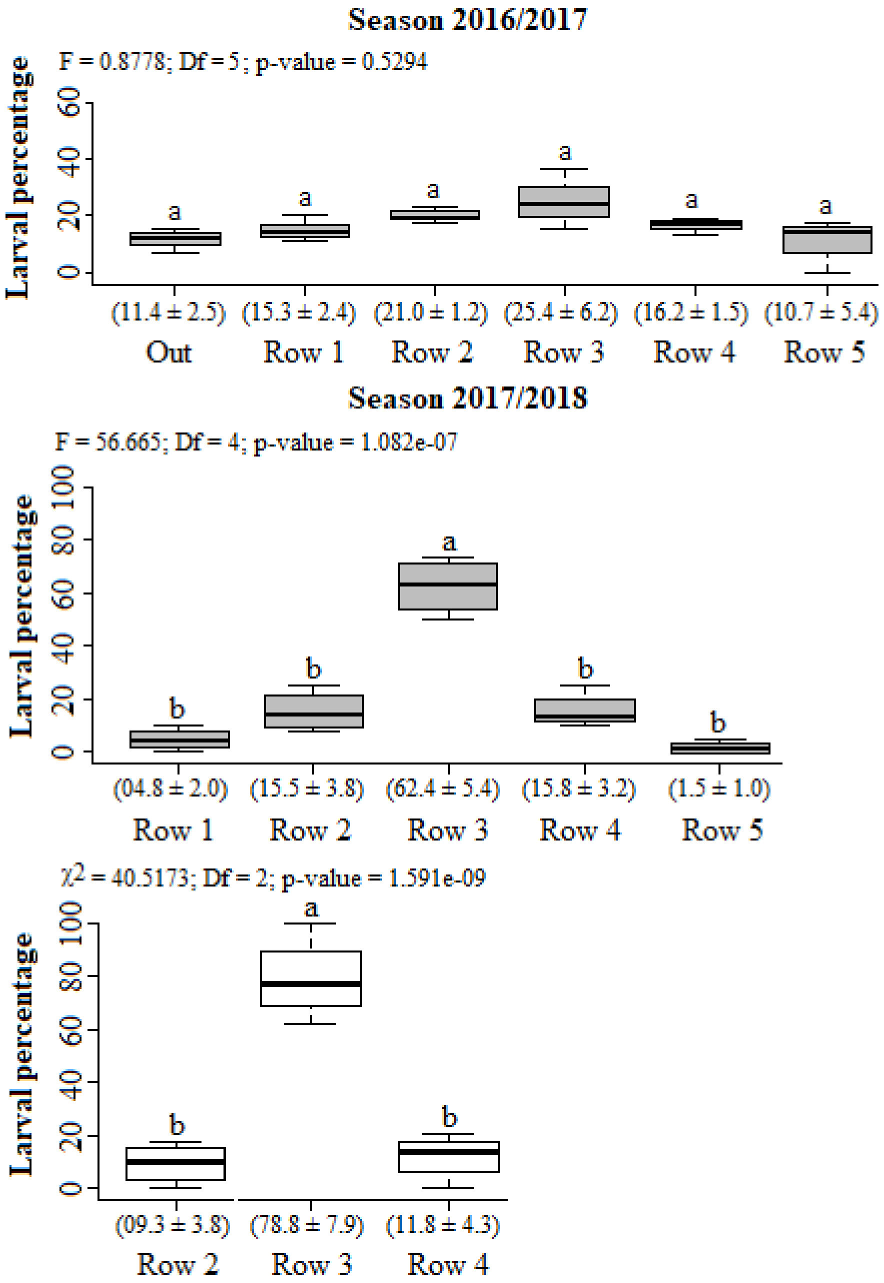

Fig. 2. Boxplot results regarding the larval percentage in each different soybean row (grey box for non-*Bt* and white box for *Bt* plants). Data assumed binomial distribution or quasibinomial (overdispersion) and they were modeled by GLMM. The pairwise t-test comparations it was done by Least Squares Means adjusted by Tukey ($p \leq 0.05$). ^{a} (Mean percentage ± SE) of each treatment.

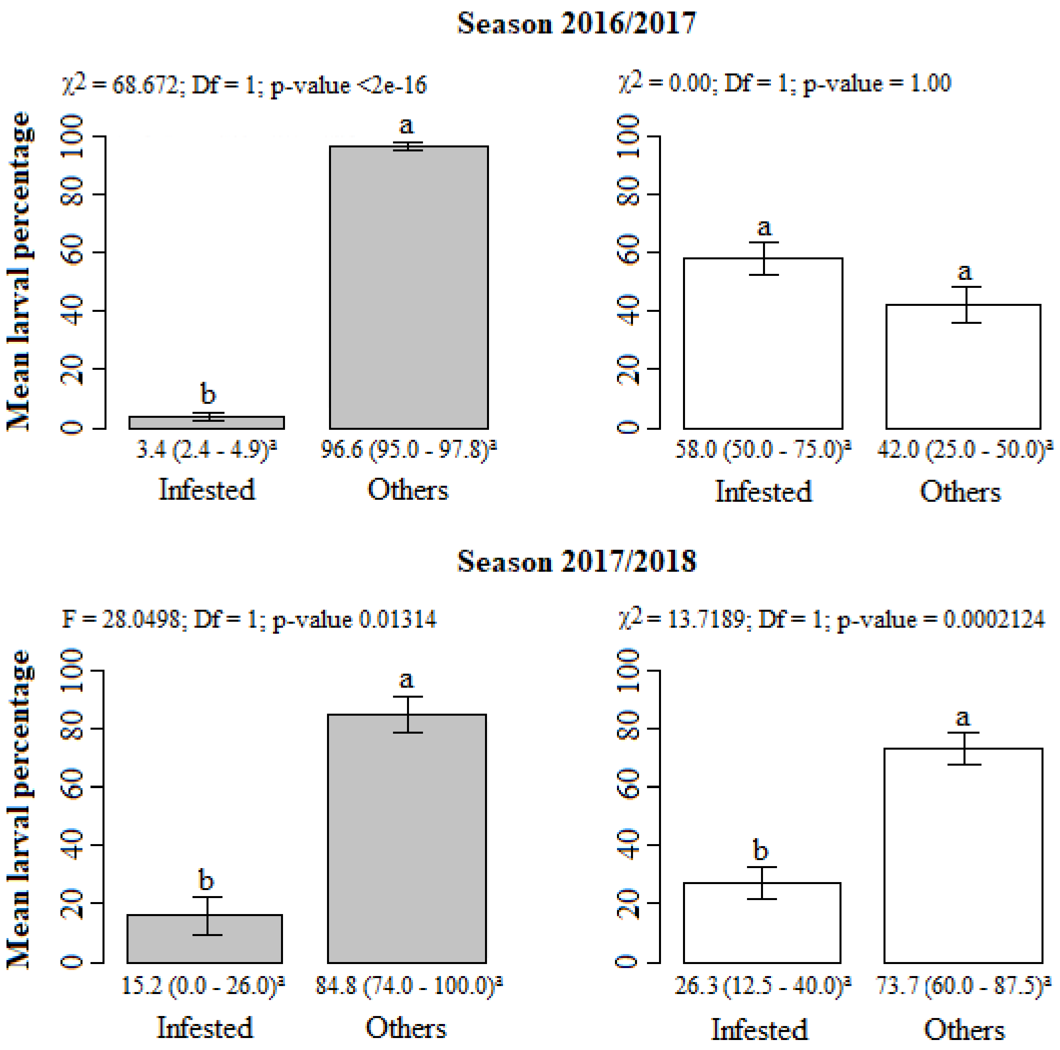

Fig. 3. Mean percentage (±SE) of *S. eridania* larvae recovered in different seasons on infested plants or other plants. Non-*Bt* plots (grey bars) and *Bt* plots (white bars). Data assumed binomial distribution or quasibinomial (overdispersion) and they were modeled by GLMM. The pairwise t-test comparisons was done by Least Squares Means adjusted by Tukey (p ≤ 0.05). ª Median (minimum and maximum values).

Chapter 3

On-Plant larval movement of *Spodoptera eridania* (Lepidoptera: Noctuidae) in reproductive stage non-*Bt* and *Bt* soybean plants

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Abstract

Larval dispersal of lepidopteran species is often influenced by host plant. Research has shown that soybean plants expressing Cry proteins impact insect behavior when compared to non-*Bt* plants. Here, we evaluated on-plant larval movement and select biological parameters (head capsule and body length) of southern armyworm, *Spodoptera eridania* (Stoll) in *Bt* and non-*Bt* soybean cultivars. Experiments were conducted under laboratory, greenhouse and field conditions between 2016 and 2018 with neonates and 5th instars. The larval movement was influenced by the type of cultivar (*Bt* and non-*Bt*) and instar. Although the larvae showed a gregarious habit in early instars for both cultivar types, larval dispersal in non-*Bt* plants was higher over time; larvae moved from the infestation site to other plant regions. The remaining larvae on these plants were more developed compared to those recovered in the *Bt* plants. Later instar permanence was higher on *Bt* plants, with larvae found in the upper and middle section of the plant, whereas on non-*Bt* plants they were recovered primarily in the middle section of the canopy. Although *S. eridania* larvae has low susceptibility to Cry1Ac, this protein adversely affected its ability to move, which can directly impact IPM and IRM strategies.

Key words: transgenic crop, southern armyworn, insect behavior, larval dispersion, management strategies

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is an important oilseed crop cultivated worldwide (Silva et al. 2014). The estimated world production is 360.99 million metric tons (mmt). The United States and Brazil are the most important soybean producers with estimated production at 123.66 and 117 mmt for 2018/19, respectively, Mato Grosso being the most important State producer in any South American country (USDA 2019). However, due to the favorable climatic conditions and the large production area, the Brazil has been faced with a significant huge challenge in the control of insect pests (Catellan and Dall’ Agnol 2018).

Intensive crop-production and inadequate pest management in Brazil has favored the occurrence of outbreaks insect pests previously considered secondary pests (Santos et al. 2009, Souza et al. 2014). *Spodoptera* spp., once considered relatively minor pests of soybean, have become a limiting factor in soybean cultivation in the main agricultural regions of the Brazilian cerrado (Jesus et al. 2013).

The southern armyworm [*Spodoptera eridania* (Stoll) (Lepidoptera: Noctuidae)] has emerged as a significant pest each season in relation to the other species (Santos et al. 2009, Santos et al. 2010). Due its high capacity for defoliation and pod consumption, as well as high population densities, this species became an important pest of soybean causing significant damage and economic losses (Bueno et al. 2011). It has assumed an important role at the beginning of the soybean reproductive phase (Favetti et al. 2015).

The use of genetically modified soybean (event MON 87701 x MON89788), which expresses the Cry1Ac protein, is an important lepidopteran pest management tool that was quickly accepted by producers (Yano et al. 2016). However, *S. eridania* presents low susceptibility to this toxin, which may favor the evolution of resistance and consequent population increase in the field (Sivasupramaniam et al. 2008, Bernardi et al. 2014). In addition, with the decrease in the use of synthetic insecticides due to the use of *Bt* plants, this ecological change involving primary and secondary pests may be aggravated by the elimination of interspecific competition that existed prior to the advent of transgenic technology, favoring secondary species, such as *S. eridania,* not as susceptible to the biotechnology (Zhao et al. 2011, Formentini et al. 2015).

Given the importance that *S. eridania* has gained in soybean production areas (Silva et al. 2017), in addition to the advent of *Bt* soybean, information regarding larval movement and the insect’s biology become important for its management. Understanding larval movement is essential for the effective implementation of pest management strategies (IPM) (Ross and Ostlie 1990; Paula-Moraes et al. 2012), contributing to synthetic insecticides control (Difonzo et al. 2015).

The location of a suitable feeding site is essential for larval development (Foster and Howard 1999), and dispersal of early instar Lepidoptera on host plants largely determines where feeding sites become established. Control of lepidopteran pests by either biotic or abiotic means is facilitated when they are in early instars (Zalucky et al. 2002).

Larval movement is also important for the management of pest resistance to *Bt* toxins (IRM). On-plant movement can influence the evolution of resistance because toxin concentrations differ among plant tissues, so an insect can ingest a greater or lesser amount of protein depending on its feeding site (Paula-Moraes et al. 2012, Pannuti et al. 2016). In general, *Bt* protein expression levels are highest in young plants and newly formed tissues, and tend to decrease with age (Wang et al. 2014), as described for Cry1Ac content in cotton leaves (Adamczyk and Meredith 2004).

Integrated refuges, as part of an IRM strategy, are not recommended for species that have high dispersal behavior within rows (Davis and Onstad 2000). Feeding on *Bt* plant tissue can stimulate larval dispersal to other plants, including to non-*Bt* plants, which can compromise refuge strategy (Ramalho et al. 2014, Malaquias et al., 2017). Therefore, understanding the factors associated with larval dispersal, such as when it occurs and how far larvae can disperse, should be better understood.

Because *S. eridania* has been increasing in importance in soybean producing areas and larval behavior is necessary for effective IPM and IRM, this study characterized on-plant larval movement of this pest in early and later instars on *Bt* and non-*Bt* reproductive stage soybean under laboratory, field and greenhouse conditions, as well as described select biological parameters that may be associated with movement.

MATERIALS AND METHODS

On-plant larval movement studies were performed in the laboratory at the University of Nebraska-Lincoln during 2017, and in the greenhouse in 2017/2018 and under field conditions during the 2016/2017 and 2017/2018 (22º50'41'' S, 48º26'05 '' W) seasons in Brazil (São Paulo State University/Botucatu, SP). The soybean cultivar 92Y83 (Pioneer) was used in the laboratory study, and the cultivars TMG 7262 (non-*Bt*) and TMG 7062 IPRO (MON 87701 x MON89788) (*Bt*) expressing Cry1Ac *Bacillus thuringiensis* protein was utilized in the greenhouse and field studies (cultivars recommended for regions of São Paulo State with altitudes above 600 m). The soybean stage evaluated was R2/R3, according classificarion of Fehr and Caviness 1977, and the field was grown under standard management practices.

Insects

Eggs or larvae of *S. eridania* used in the experiments were commercially acquired from Pragas.com Biological Products Inc., Piracicaba, SP, Brazil and Benzon Research Inc., Carlisle, PA, USA. Eggs acquired were close to hatching and maintained in the laboratory (25 ± 2 °C, 70 ± 10% RH and 14 h photophase). In the field experiment using older larvae, the larvae were reared on artificial diet until the appropriate instar for the experiments was reached.

Exploratory trial with early instars

Prior to conducted the main on-plant early instar larval movement study, a preliminary test was performed in the laboratory (environmental conditions as noted above) in order to better understand the proportion of neonates that dispersed from the infested leaf to other plant tissues at 12, 36 and 60 hours after hatching. The study was conducted in a completely randomized design with repeated analyzes on the same plant (random effect) at different periods (treatments).

Seeds of conventional Pioneer 92Y83 soybean were planted in 2-liter plastic pots containing fertilized soil and maintained in the greenhouse until initiation of the study when they were transferred to the laboratory. Because field and greenhouse studies using *S. eridania* was prohibited in the state of Nebraska, this experiment was conducted under strict containment conditions (Permit Number: P526P-17-02337) in the laboratory.

Infestation was done by attaching a piece of oviposition substrate with an egg mass of approximately 200 near to hatch eggs to a leaflet in the middle region of the plant with a small piece of adhesive. The percentage of larvae remaining on the infested leaf was calculated by considering the total number of live larvae recovered on the plants for each evaluation period. Each plant containing one egg mass represented one replicate (six in a total).

Greenhouse study - early instar movement

Based on the pattern observed in the laboratory study, the following greenhouse experiment was designed to characterize neonate dispersion on *Bt* and non-*Bt* plants during a 9 day period. The study was conducted as a completely randomized design with six replications (plants) and an artificial infestation was performed, releasing 30 neonates (0 - 24 h old) per plant.

Twenty five evaluations were made through time (2h, 5h, 20h, 24h, 29h, 44 h, 48 h, 53 h, 68 h, 72 h, 77 h, 92 h, 96 h, 101 h, 116 h, 120 h e 125 h, 140 h, 144 h, 149 h, 164 h, 168 h, 173 h, 188 h e 197 h after infestation). We recorded the percentage of larvae recovered on plants on each day (always considering the last evaluated period); percentage of larvae recovered in the infested leaflet at 5 h and 197 h after infestation; and punctual analyzes regarding the larval percentage in each plant sector (upper, middle and lower).

The percentage of larvae present on the plants for each evaluation day, always considering the last evaluated period, was obtained by the quotient between the number of larvae present on the plant and the total released. In the other evaluations, this proportion was calculated by the quotient between the number of recovered larvae and the total of them on the plant. The artificial infestation with neonates on the leaflets in the middle section of plants was done using delicate paintbrush, infesting six plants per cultivar.

Biological parameters, larval and head capsule size, were also recorded at 197 h after infestation in order to analyze larval development between *Bt* (Cry1Ac toxin) and non-*Bt* plants. The larval instar (Capinera 1999) was determined by measuring its cephalic capsules with a stereomicroscope (Nikon - Stereo Zoom Microscope SMZ 645, Tokyo, Japan) and a high precision manual meter from BioQuip Products, California, USA. The larval size was measured by a digital caliper (Mitutoyo 0.01 mm-150mm, Metric). In these evaluations, we used the larval and head capsule mean for larva found per plant, considering each plant a repetition. Larval leaf consumption on different cultivars was quantified using the software ImageJ.

The study was conducted in a factorial design with 2 soybean cultivars (*Bt* and non-*Bt*) by 2, 3 or 9 periods, regarding the percentage of larvae recovered on the infested plants and infested leaflet. The plants were considered as a random effect and the factors as fixed effects. In the case of punctual assessments, the treatment factorial design was a 2 (soybean cultivars) by 3 different sectors of the plants. In experiments referring to insect biology, each plant of each cultivar was considered one replicate (6 in a total).

Field study - later instar movement

Two types of on-plant larval movement experiments were conducted under field conditions. The first one was conducted as a completely randomized design in 2017/2018. For this, two experimental areas of the same proportions were established for both commercial soybean cultivars. Prior to initiating the experiment, five rows (spaced 0.45 m) at 15 plants/m were grown under conventional agronomic practices. To avoid contact between plants and possible movement of larvae between the rows, thinning was done to isolate each plant. Final spacing was two rows (spaced 0.90 m) with 8 plants (replicates) each spaced 0.50 m apart. Each plant was supported with a bamboo stake. Plants were artificially infested (using forceps) with 12 fifth instars in the middle sector of the plant at dusk (6 pm) the day before the evaluations.

The *Bt* and non-*Bt* plots were covered by metal cages (16 mesh), (4.5 m long x 2.0 m wide x 2.0 m high) to exclude natural enemies. They were installed 20 days before infestation, along with the application of deltamethrin (Decis®, 200 ml/ha, Bayer) to eliminate other larvae species. The evaluations were done at 14 h, 16 h, 18 h, 20 h, 22 h and 24 h after infestations, regarding the percentage of larvae remained in the infested plants in each evaluation period.

The second experiment was conducted during the 2016/2017 and 2017/2018 seasons. Experiments consisted of four plots arranged in a randomized block design, except for the first season where there were three plots of the non-*Bt* cultivar. The plots were five 3.0 m long rows with 15 plants/m. All plots were covered by metallic cages (16 mesh), (3.0 m long x 3.0 m wide x 2.5 m height).

In 2016/2017, artificial infestation was performed by releasing 180 neonates in the middle sector of the central plant of each plot. In 2017/2018 the same infestation level was used for non-*Bt* plots; however, 280 neonates were released per plant in *Bt* plots due to high mortality observed in first season. The infestation was performed by fixing infested leaflets with neonates with a small piece of adhesive tape (Fanela et al. 2019, submitted). Destructive sampling was carried out for all plants in each plot. The number of larvae per plant sector (upper, middle, bottom) was recovered 14 days after infestation.

Statistical Analysis

For analysis with repeated measures in exploratory and greenhouse study, the generalized linear mixed models (GLMMs) with the glmmTMB function of the "glmmTMB" package, excluding zero-inflamation by default ziformula=~0 (Magnusson et al. 2019) with binomial distribution was used. For the greenhouse neonate dispersal in punctual analysis and the plant-to-plant larval movement experiment, data were analyzed using a generalized linear model (GLM). In all experiments a binomial or quasibinomial (overdispersion) distribution was assumed. Significant values data were calculated from type II Wald chi-square tests using the function ‘Anova’ in the package “car”, with pairwise comparisons from LSmeans with Tukey adjustment ($P \geq 0.05$), using “lsmeans” package. ANOVA by the F test was done in case of overdispersion.

Prior to conducting the correlation analyzes by linear regression, the normality was verified by the Shapiro-Wilk test and homogeneity by the Breusch-Pagan test using the function ‘bptest’ with package “lmtest” and the correlation analysis between the residuals was performed by Durbin-Watson test using the function ‘dwtest’ with package “lmtest”. The linear models were submitted to Pearson correlation analysis and descriptive statistical analysis.

Regarding the comparison of the head capsules and sizes of the larvae, and leaf consumption, the normality was verified by the Shapiro-Wilk test and homogeneity by Levene test. When these assumptions were violated, the data were compared by the Wilcoxon Matched-Pairs Signed-Ranks Test, otherwise they were compared by T-test. Analyses were performed using software R 3.5.2 (R Development Core Team 2018).

RESULTS

Exploratory trial with early instars

In the laboratory study egg masses did not hatch or most neonates died right after hatch on three plants, so 6 plants (replications) were considered for analysis. There was no significant period effect for mean percentage of *S. eridania* recovered on infested leaf after egg mass hatched in non-*Bt* soybean plants, with more than 98.0 % of larvae remaining on the leaf 60 hours after infestation (HAI) (Table 1).

Greenhouse study - early instar movement

There was no significant interaction between cultivars and periods. In general, more than 72.0 % were recovered on infested *Bt* and non-*Bt* plants (Table 2). There was a significant interaction between cultivars and periods for the mean percentage of larvae recovered on the infested leaflet. Regardless of the cultivar analyzed, the percentage of larvae remaining on the infested leaflet was lower at the final evaluation (197 HAI). However, at this period, the highest value was observed on the *Bt* plants (52.7 %) (Table 3).

Regardless of the period and cultivar, the larvae were more concentrated in the middle section of the plants. Except at 197 HAI in non-*Bt* plants, in which the larvae showed greater dispersion to the other sections of the plant, the percentage of larvae recovered in the middle section was greater than 90.0%. Considering *Bt* cultivar, more than 95.0% of the larvae were recovered in the middle section for all periods (Figure 1).

There was a significant interaction between cultivars and sites only at 197 HAI. The percentage of larvae recovered in the middle section on *Bt* plants (96.4%) was higher than non-*Bt* (84.3%), and when analyzed separately for each cultivar, the middle section differed from the others. Although there was no statistical difference in relation to the upper section of the plant among the cultivars, due to the low larval proportion found in this site, no larvae (0.0%) moved to the upper section in the *Bt* plants (Table 4).

The adjusted coefficient of determination (R^2) for *Bt* plants (0.6157) was lower than for non-*Bt* plants (0.9804). For non-*Bt* plants the value of Pearsons's correlation coefficient (0.9902) was significant (Table 5).

Mean head capsule size and mean larval body length were higher on the non-*Bt* plants (0.73 mm and 7.25 mm, respectively) than *Bt* plants (0.55 mm and 4.69 mm, respectively) (Table 6). Although the highest value of mean leaf consumption was observed on non-*Bt* plants (31 cm^2), there were no significant difference between the cultivars (Table 7).

Field study - later instar movement

There was significant interaction between cultivars and periods in the percentage of larvae recovered on the plants. A significantly higher percentage of larvae on *Bt* plants was observed at 14 h (62.0 %) and 24 h (35.4 %) after release, when compared with non-*Bt* plants (30.2 % and 2.1 %, respectively) (Table 8). The percentage of larvae recovered on *Bt* plants was more than double than on non-*Bt* plants in all evaluations (Figure 2).

Except in the 2016/2017 season for non-*Bt* plants, there was significant plant site effect for mean percentage of *S. eridania* larvae recovered for both soybean cultivars. Due to the low amount of larvae recovered on *Bt* plants in the first season, this evaluation was disregarded for this cultivar. In all evaluations for non-*Bt* plants, the percentage of larvae in the middle section was higher than the other sites, with more than 60.0 % in the second season. For *Bt* plants, the highest value was observed in the upper section (54.7 %), and no larvae were observed in the bottom section (Table 9).

DISCUSSION

Spodoptera eridania early instars have a habit of remaining at the initial infestation site for at least four days. This species has a high acceptance rate for this host, regardless of the technology adopted. This behavior was also confirmed by the percentage of larvae remaining on the plants at 197 h after infestation, which did not differ from the first evaluation at 5 h AI. However, in most lepidopteran species a prefeeding movement phase is common, and this is also observed on *Bt*-expressing host plants (Zalucki et al. 2002). This effect of larval dispersal stimulated by *Bt* toxins is also influenced by the exposure time of neonates to these toxins (Razze et al. 2011).

Malaquias et al. (2017) verified that the *S. frugiperda* neonate dispersal of the susceptible and Cry1F-resistant strains on *Bt* and non-*Bt* cotton plants varied according to the exposure time. According to authors, the host acceptance rate for the susceptible strain was higher from 0–6 h after infestation and lower from 12–18 h. The Cry1F-resistant strain showed a similar pattern of host acceptance among exposure times, which differs from the results obtained in this study, where S. *eridania* neonates or early instars present sedentary behavior. According to Silva et al. (2017), this *S. eridania* feed gregariously between the first and third instars.

The high proportion of live larvae recovered in *Bt* plants demonstrates that this species has a natural tolerance to Cry1Ac protein. Bernardi et al. (2014) also verified the low susceptibility of *S. eridania* on Cry1Ac soybean. However, although more than 70.0 % of neonates were recovered on *Bt* plants, the effect of this toxin on insect development can be verified by the delay in the phenological stage with larvae still in 2nd instar, larvae 1.5 times smaller than larvae on non-*Bt* plants, and reduced leaf consumption. Lynch et al. (1999) found that *Bt* sweet corn negatively affected the development of *Helicoverpa zea* (Boddie) by compromising its movement (lethargic effect).

This adverse effect of Cry1Ac on *S. eridania* larval biology may be the reason why early and late instar larvae show less dispersal in *Bt* soybean plants. In both cultivars most remaining larvae were observed at the infestation site (middle), except for older larvae, which were primarily located in the upper section of the plant (54.7 %).

Larval dispersion is a major factor in resistance management (IPM) studies. Knowledge about the movement of Lepidoptera larvae between *Bt* and non-*Bt* plants is essential to design refuge strategies (Pannuti et al. 2016; Vélez et al. 2016). Considering refuge options, the seed mixture, or “refuge-in-the-bag” (RIB strategy), may be adequate for species with low larval mobility (Wangila et al. 2012). Structured refuges are indicated for insects that have high mobility and tend to disperse within rows (Petzold-Maxwell et al. 2013, Pannuti et al. 2016). Our data show that early instar *S. eridania* have low mobility, remaining on the infested plant and infestation site for non-*Bt* and *Bt* cultivars. Therefore, the RIB strategy could be appropriate for this pest. However, this is valid only if dispersion is low for all instars.

Larval movement during the last instars from non-*Bt* plants to *Bt* plants can favor the evolution of resistance because of the brief exposure to *Bt* toxin in which the insect does not ingest a sufficient amount of toxin to cause mortality (Ramalho et al. 2014, Miraldo et al. 2016). When analyzing *S. eridania* larval dispersion for later instars, the percentage of larvae remaining in non-*Bt* plants at 24 h after infestation was only 2.0%, about 17 times lower than that observed in *Bt* plants (35.4%).

Although the percentage of larvae recovered from *Bt* plants was higher, approximately 75.0% also abandoned these plants. This characteristic should also be considered for designing resistance management, as this species has low susceptibility to Cry1Ac (Bernardi et al. 2014). Larvae can grow on *Bt* plants, and move to non-*Bt* plants at more advanced instars. Larval movement among *Bt* and non-*Bt* plants could result in sublethal exposure, favoring resistance evolution by increasing the survival of the resistant heterozygotes or individuals carrying minor resistance alleles (Wanglia et al. 2012).

Considering that first instar *S. eridania* are more susceptible to insecticide action (Zalucki et al. 2002) and its behavior to feed in the middle section of the plant from oviposition (Silva et al. 2017) until the 3rd instar, management can be difficult by foliar insecticide application. This middle canopy feeding behavior of another important pest in soybean plants, *C. includens*, also results in management difficulties with foliar insecticide application (Bernardi et al. 2012).

In an IPM context, different insect control strategies beneficial to the environment and human health need to be applied in synergy, and the interaction between conventional or transgenic needs to be compatible with all control methods (Sousa et al. 2019). One of the strategies that can be implemented is the use of biological control by egg parasitoids. However, it is also essential to understand the impact of *Bt* plants on the natural enemies, although the *Bt* risks seem to be lower than with chemical technologies and benefits seem to be greater (Wolfenbarger et al. 2008, Naranjo 2009). No adverse effects of *Bt* soybean (MON 87701 ×MON 89788) were observed for the egg parasitoid *Telenomus remus* Nixon, which can help to prevent *S. eridania* outbreaks (Bortolotto et al. 2014).

When compared to non-*Bt* soybean the effect of Cry1Ac toxin on *S. eridania* biology may favor the control of more developed larvae, because the largest larval proportion of later instars was found in the upper third of plants. According to Bernardi et al. 2014, the effect of Cry1Ac protein after 14 days on *Spodoptera* species can increase the larvae exposure to biotic and abiotic factors, including insecticide application.

Based on our results, we conclude that regardless of cultivar analyzed, *S. eridania* feeding site choice is established during the first instar, and larvae remain at the infestation site (eggs or neonates) for at least four days. Older larvae appear to initiate movement to other plants, and this behavior is more pronounced on non-*Bt* plants. Variable larval movement between instars may directly impact IPM and IRM, so additional studies involving plant-to-plant larval movement are still needed in different agronomic scenarios (e.g. narrow row soybean, drilled soybean) to fully understand instar-specific *S. eridania* larval movement in non-*Bt* and *Bt* soybean.

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Table 1. Analysis of deviance of repeated measures for the fixed effect periods after *S. eridania* infestation (12 h, 36 h and 60 h) for larval mean percentage (±SE) recovered on the infested leaf of soybean plants (plants = random effects).

Analysis of deviance			
Fixed effects	**Df**	**Chisq**	**Pr(>Chisq)**
Periods	2	0.0244	0.9879

Mean percentage (±SE) of larvae recovered	
12 h after infestation	100.0 ± 0.0 a
36 h after infestation	99.5 ± 0.5 a
60 h after infestation	98.7 ± 0.7 a

Number of observations: 18, groups: Plants, 6 (non-*Bt*). Data were modeled with GLMM. The pairwise t-test comparisons it was done by LS Means adjusted by Tukey (*p* ≤ 0.05).

Table 2. Analysis of deviance of repeated measures for the fixed effects cultivars (non-*Bt* and *Bt*) and different periods (9 days) after *S. eridania* infestation regarding the mean percentage of larvae (±SE) recovered on the infested soybean plants (plants = random effects).

Analysis of deviance

Fixed	**Df**	**Chisq**	**Pr(>Chisq)**
Cultivars	1	0.1312	0.7172
Periods	8	8.9885	0.3433
Cultivars x Periods	8	0.9778	0.9984

Mean percentage of larvae on two different cultivars

Periods	**Non-*Bt***	***Bt***
5 h After Infestation	100.0 ± 0.0 a	97.2 ± 1.3 a
29 h After Infestation	93.9 ± 2.5 a	91.7 ± 1.1 a
53 h After Infestation	86.1 ± 2.5 a	91.7 ± 1.1 a
77 h After Infestation	82.8 ± 5.1 a	88.9 ± 0.7 a
101 h After Infestation	81.1 ± 4.8 a	87.2 ± 1.0 a
125 h After Infestation	80.5 ± 4.8 a	86.1 ± 1.3 a
149 h After Infestation	80.0 ± 4.4 a	81.7 ± 0.7 a
173 h After Infestation	79.4 ± 4.0 a	80.0 ± 0.9 a
197 h After Infestation	78.3 ± 3.9 a	72.2 ± 2.2 a

Number of observations: 108, groups: Plants, 12 (6 *Bt* and 6 Non-*Bt* plants). Data were modeled with GLMM. The pairwise t-test comparisons it was done by LS Means adjusted by Tukey ($p \leq 0.05$).

Table 3. Analysis of deviance of repeated measures for the fixed effects soybean cultivars (non-*Bt* and *Bt*) and three different periods (5, 101 and 197 hours after *S. eridania* infestation - HAI) regarding the mean percentage of larvae (±SE) recovered on the infested leaflet of each plant (plants = random effects).

Analysis of deviance

Fixed	**Df**	**Chisq**	**Pr(>Chisq)**
Cultivars	1	1.4768	0.22428
Periods	2	41.2817	1.086e-09
Cultivars x Periods	2	7.0646	0.02924

Decomposition of the interaction (cultivars x periods)

Cultivars	**5 x 101 HAI**		**5 x 197 HAI**		**101 x 197 HAI**	
Non-*Bt*	99.4 ± 0.6 aA	79.2 ± 5.5 aA	99.4 ± 0.6 aA	20.7 ± 8.5 aB	79.2 ± 5.5 aA	20.7 ± 8.5 aB
Bt	100.0 ± 0.0 aA	76.9 ± 8.1 aA	100.0 ± 0.0 aA	52.7 ± 7.5 aB	76.9 ± 8.1 aA	52.7 ± 7.5 aA

Number of observations: 36, groups: Plants, 12 (6 *Bt* and 6 Non-*Bt* plants). Data were modeled with GLMM. The pairwise t-test comparisons it was done by LS Means adjusted by Tukey ($p \le 0.05$).). Means within a column followed by the same lowercase letter and in each row followed by the same uppercase letter are not significantly different.

Table 4. Two-way analysis of variance among plant sections (superior, middle and bottom) and soybean plants (non-*Bt* and *Bt*) in 25 different periods after *S. eridania* infestation (HAI) regarding the mean percentage of larvae (±SE) recovered on the plants.

Periods	**Cultivars**			**Sectors**			**Cultivars x Sectors**			**Test**
	Df	**Chisq / F**	**P-value**	**Df**	**Chisq / F**	**P-value**	**Df**	**Chisq / F**	**P-value**	**Chisq[a] / F[b]**
2 h AI	1	0.0000	1.0000	2	614.49	<2e-16	2	0.0000	1.0000	a
5 h AI	1	0.0000	1.0000	2	614.49	<2e-16	2	0.0000	1.0000	a
20 h AI	1	0.0000	1.0000	2	597.23	<2e-16	2	0.0000	1.0000	a
24 h AI	1	0.0000	1.0000	2	588.6	<2e-16	2	0.0000	1.0000	a
29 h AI	1	0.0000	0.9691	2	564.28	<2e-16	2	1.41	0.4948	a
44 h AI	1	0.0100	0.9375	2	560.50	<2e-16	2	1.41	0.4947	a
48 h AI	1	0.0000	0.9688	2	531.02	<2e-16	2	0.36	0.8372	a
53 h AI	1	0.0000	0.9665	2	522.48	<2e-16	2	0.40	0.8180	a
68 h AI	1	0.0000	0.9665	2	513.94	<2e-16	2	0.40	0.8204	a
72 h AI	1	0.0000	0.9969	2	504.61	<2e-16	2	0.00	0.9987	a
77 h AI	1	0.0000	0.9956	2	496.11	<2e-16	2	0.01	0.9975	a
92 h AI	1	0.0009	0.9749	2	485.63	<2e-16	2	0.13	0.9352	a
96 h AI	1	0.0000	0.9744	2	483.94	<2e-16	2	0.14	0.9328	a
101 h AI	1	0.0000	0.9748	2	478.86	<2e-16	2	0.13	0.9355	a
116 h AI	1	0.0000	0.9748	2	478.86	<2e-16	2	0.13	0.9355	a

120 h AI	1	0.0000	0.9748	2	475.47	<2e-16	2	0.13	0.9357	a
125 h AI	1	0.0009	0.9748	2	475.47	<2e-16	2	0.13	0.9357	a
140 h AI	1	0.0000	0.9737	2	472.08	<2e-16	2	0.14	0.9308	a
144 h AI	1	0.0000	0.9987	2	453.66	<2e-16	2	0.00	0.9999	a
149 h AI	1	0.0000	0.9981	2	451.97	<2e-16	2	0.00	0.9997	a
164 h AI	1	0.0000	0.9709	2	444.05	<2e-16	2	0.13	0.9384	a
168 h AI	1	0.0040	0.8478	2	405.64	<2e-16	2	4.63	0.0988	a
173 h AI	1	0.0200	0.8783	2	412.07	<2e-16	2	3.23	0.1990	a
188 h AI	1	0.0200	0.8994	2	380.97	<2e-16	2	2.98	0.2249	a
197 h AI	1	0.3093	0.58222	2	166.4271	<2e-16	2	5.9995	0.00643	b

Decomposition of the interaction (cultivars x Sectors) to recovered larvae (%) at 197 HAI

Cultivars	**Upper x Bottom**		**Upper x Middle**		**Middle x Bottom**	
Non-*Bt*	06.9 ± 3.1 aA	08.8 ± 3.3 aA	06.9 ± 3.1 aB	84.3 ± 3.6 bA	84.3 ± 3.6 bA	08.8 ± 3.3 aB
Bt	0.0 ± 0.0 aA	3.6 ± 1.8 aA	0.0 ± 0.0 aB	96.4 ± 1.8 aA	96.4 ± 1.8 aA	03.6 ± 1.8 aB

Data in each period were modeled with GLM. The pairwise t-test comparisons it was done by LS Means adjusted by Tukey ($p \leq 0.05$). [a] Chisq test was used without overdispersion. [b] F test was used in overdispersion case. Means within a column followed by the same lowercase letter and in each row followed by the same uppercase letter are not significantly different.

Table 5. Linear regression analysis used to examine the interaction between *S. eridania* larval head capsule size (mm) of and body length (cm) on non-*Bt* and *Bt* soybean plants.

Cultivars	***R***	**Equation**	R^2	**F**	**P-value**[a]	**P-value**[b]	**N**[c]
Non-*Bt*	0.9902	$\hat{Y} = 0.3533 + 0.0520x$	0.9804	200.1	0.0002	0.762	6
Bt	0.7846	$\hat{Y} = 0.0051 + 0.1166x$	0.6157	6.408	0.0645	0.858	6

[a] *P*-value of the linear regression model. [b] P-value for autocorrelation analysis. [c] Number of plants where was recovered the larvae to measure the mean number of head capsule and body size regarding the total of larvae recovered in each plant.

Table 6. Comparison between soybean cultivars (non-*Bt* and *Bt*) regarding the mean sizes (±SE) of larval head capsules and body length recovered on soybean plants in the last evaluation.

	Head capsule (mm)					
Cultivars	**Mean (±SE)**	**Df**	**t-value**	**P-value**	**N[a]**	**Instar**
Non-*Bt*	0.73 ± 0.02 a	9.0375	4.3999	0.001703	6	3th
Bt	0.55 ± 0.03 b					2nd/3th

	Body length (mm)					
Cultivars	**Mean (±SE)**	**Df**	**W-value**	**P-value**	**N[a]**	**---**
Non-*Bt*	7.25 ± 0.45 a	-----	0	0.002165	6	---
Bt	4.69 ± 0.22 b					

Means with the same letter are not different ($p \leq 0.05$). [a] Number of plants where was recovered the larvae to measure the mean number of head capsule and body size regarding the total of larvae recovered in each plant.

Table 7. Mean defoliation (cm^2±SE) by *S. eridania* on soybean cultivars (non-*Bt* and *Bt*) at 197 hours after infestation.

	Leaf consumption (cm^2)		
Cultivars	**Mean (±SE)**	**W-value**	**P-value**
Non-*Bt*	31.0 ± 5.8 a	12	0.3939
Bt	20.1 ± 3.4 a		

Means with the same letter are not different ($p \leq 0.05$).

Table 8. Analysis of deviance of repeated measures for the fixed effects cultivars (non-*Bt* and *Bt*) and two different periods (14 and 24 hours after *S. eridania* infestation - HAI) regarding the percentage of larvae recovered on the plants.

Fixed	**Df**	**Chisq**	**Pr(>Chisq)**
Cultivars	1	29.131	6.765e-08
Periods	1	20.728	5.295e-06
Cultivars x Periods	1	14.368	0.0001503

Decomposition of the interaction (cultivars x periods)

Cultivars	**14 HAI**	**24 HAI**
Non-*Bt*	30.2 ± 4.3 bA	02.1 ± 0.9 bB
Bt	62.0 ± 3.6 aA	35.4 ± 3.2 aB

Number of observations: 64, groups: Plants, 32 (16 *Bt* and 16 Non-*Bt* plants). Data were modeled with GLMM. The pairwise t-test comparisons it was done by LS Means adjusted by Tukey ($p \leq 0.05$).). Means within a column followed by the same lowercase letter and in each row followed by the same uppercase letter are not significantly different.

Table 9. Mean percentage (± SE) of *S. eridania* larvae recovered in different plant sections and seasons on non-*Bt* and *Bt* soybean plants in different seasons.

Secttors	**Season 2016/2017**	**Season 2017/2018**	
	Non-*Bt*	**Non-*Bt***	***Bt***
Upper	31.9 ± 1.9 a	12.1 ± 2.1 c	54.7 ± 5.6 a
Middle	43.0 ± 3.2 a	60.7 ± 3.2 a	45.3 ± 5.8 a
Bottom	25.1 ± 1.3 a	27.2 ± 3.1 b	0.0 ± 0.0 b
N^a	60.0 ± 23.4	77.3 ± 26.2	22.3 ± 8.0
Df	2	2	2
Chisq^b/F^c	4.2707^b	23.0089^c	62.184^b
Pr(>Chisq)	0.1182	0.001535	3.139e-14

Data in each season were modeled with GLM. [a] Mean number of larvae recovered on the plants in the plots. It was used Glm. [b] Chisq test was used without overdispersion. [c] F test was used in overdispersion case.

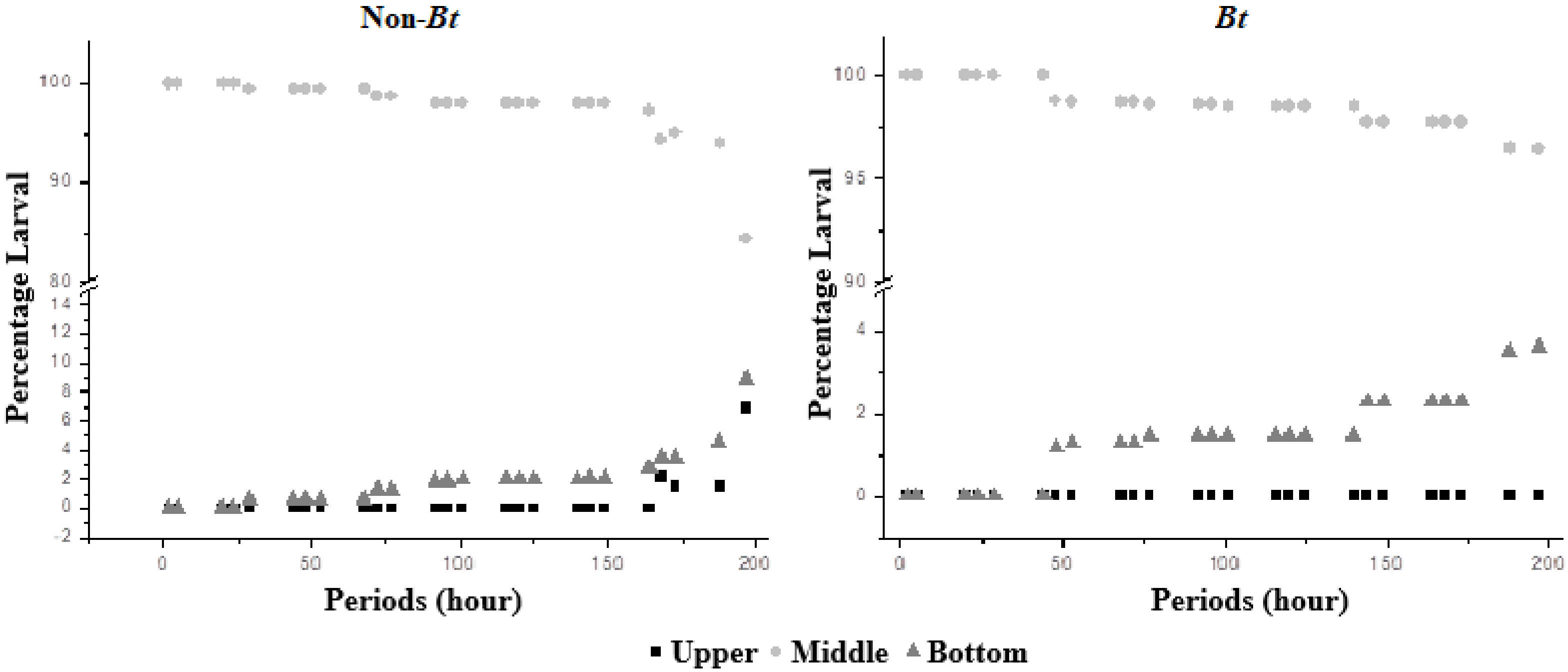

Fig. 1. Mean percentage of *S. eridania* larvae recovered in different periods and plant sections after infestation on non-*Bt* and *Bt* soybean plants.

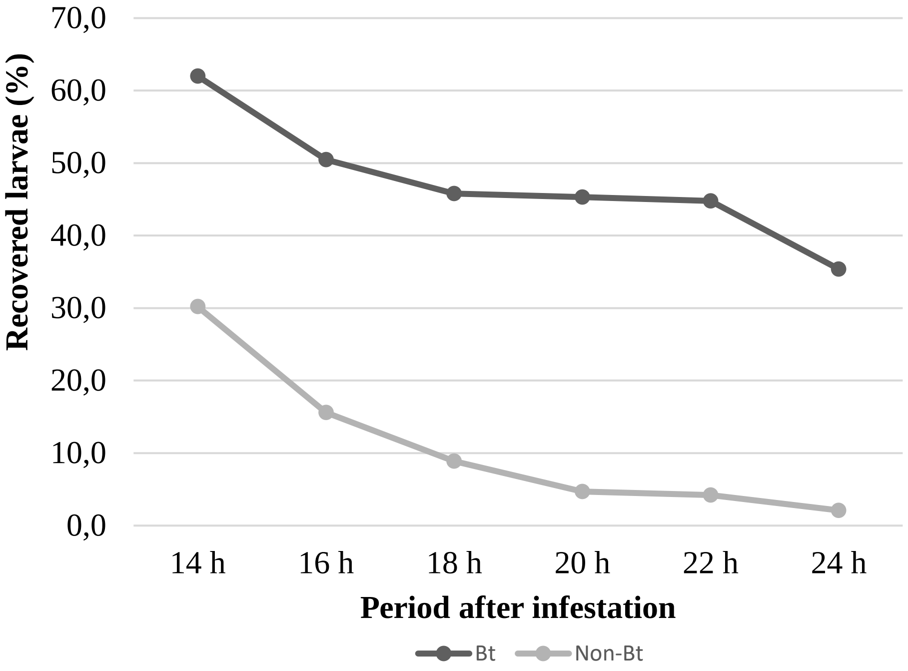

Fig. 2. Mean percentage of *S. eridania* larvae recovered in different periods after infestation on non-*Bt* and *Bt* soybean plants.

FINAL CONSIDERATIONS

The importance of *Spodoptera eridania* and *Chrysodeixis includens* have increasing importance in recent years in the Brazilian soybean pest complex. An uninterrupted crop succession (soybean, corn and cotton), the advent of *Bt* technology that expresses Cry1Ac protein, the presence of weeds, larval behavior and the low efficiency of some insecticides are some of the factors that have favored the increase of these Lepidoptera pests, which were previously considered secondary pests.

Given this scenario, it becomes important to better understand the behavioral aspects of Lepidoptera pests, as well as their movement patterns, since they share the same feeding guild in soybean. This knowledge can assist in soybean IPM and IRM strategies. The objective of this work was to characterize the *S. eridania* and *C. includens* "on-plant" and "plant-to-plant" larval movement in soybean and their possible impact on IPM and IRM, as well as to characterize some biological parameters to help us understand said movement.

Regarding the data obtained in the preliminary test of Chapter 1 with the on-plant larval movement assay, the positioning of the *C. includes* larvae on the plants was directly related to the stage of larval development. The larvae in early instars were recovered mainly in the infested sectors, and they preferred to remain in the upper plant sector where oviposition occurs and the leaves have better physical and nutritional characteristics for the development of neonates. This period is the best moment to implement chemical contro under an IPM strategy. The older larvae were recovered in the middle sector of the plant. For plant-to-plant larval movement, the larvae present a high dispersion capacity and moved to other plants, suggesting that the seed mixture strategy (RIB) is not suitable for IRM designed for this insect.

In the second study, results demonstrad that *S. eridania*, as with *C. includens*, has high dispersion capacity and moved to other plants, regardless of the technology adopted, although the distance traveled by larvae between *Bt* plants was shorter than in non-*Bt* plants. It appears that *S. eridania* also exhibited nondirectional movement. The results also demonstrate the possible existence of incomplete resistance in this species, because some insects can develop on *Bt* plants. However, larvae developing on *Bt* soybean incurred a fitness cost when compared to those developing on corresponding non-*Bt* soybean. These results may help in the design and implementation of refuge areas for IRM. Our data also suggest the RIB strategy for *Bt* soybean with a single toxin Cry1Ac is inappropriate for *S. eridania*.

Based on the results presented in the on-plant experiment with *S. eridania*, although the larval movement was higher in non-*Bt* plants, the early instars had low mobility, remaining on the infested plants, as well as in the infested sector. The percentage of older instars remaining on plants was low, especialy on non-*Bt* plants. Therefore, the older instars can move to other plants, and as this species has low susceptibility to Cry1Ac, this behavior should be considered when designing resistance management strategies. Larval movement among *Bt* and non-*Bt* plants can favor the evolution of resistance by exposing the larvae to lower levels or sub-lethal doses of the toxin. We also observed that the Cry1Ac protein adversely affected insect biology and its ability to move.

CONCLUSIONS

- On-plant larval movement

- For both Lepidoptera species on non-*Bt* cultivars, larvae (early instars) are found in the infested plant sectors;

- The increase in defoliation observed on the last day of evaluation reflects the larval dispersion observed in older *C. includes* larvae;

- Regarding *S. eridania* on *Bt* soybean, this technology affects the larval biology, directly reflected in insect behavior.

- There is no defoliation difference between the technologies. However, the percentage of early *S. eridania* larvae in the middle plant sector is higher than in non-*Bt* plants at 197 h after infestation;

- The percentage of older *S. eridania* larvae remaining on *Bt* plants is higher than on non-*Bt* plants at 24 h after infestation;

- There is no difference for the larval percentage recovered between the upper and middle sectors of *Bt* soybean.

- Plant-to-plant larval movement

- In both species, the larvae show high dispersion capacity and move across plants, regardless of the technology adopted;

- *Spodoptera eridania* and *C. includens* exhib nondirectional movement;

- Larval development (size) directly affects larval dispersal in soybean;

- The reduced larval movement of *S. eridania* on *Bt* soybean may be due to some adverse effect caused by the toxin;

- A possible existence of incomplete resistance was observed in *S. eridania*.

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