

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Management of target pests of Bt soybean in refuge areas, ecological risk to
ground-dwelling predators and their implications for insect resistance
management**

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Thesis presented to obtain the degree of Doctor in
Science. Area: Entomology

**Piracicaba
2021**

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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Dados Internacionais de Catalogação na Publicação

DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Barros, Lucas Silva

Management of target pests of Bt soybean in refuge areas, ecological risk to ground-dwelling predators and their implications for insect resistance management / Lucas Silva Barros - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2021.

118 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Manejo integrado de pragas 2. Refúgio estruturado 3. Soja transgênica 4. Capacidade de voo 5. Efeito subletais de inseticidas 6. Monitoramento de risco ambiental I. Título

DEDICATION

To God for the wonderful life and to be on my side in the most difficult moments during this journey.

To my parents, Benilton José de Barros and Mariza Antônia da Silva Barros, and to my sister Aline Silva Barros. Thank you for your endless love, sacrifices, dedication, prayers, support and advice.

To my fiancée Carolina Pucci Moraes for all the love and support in times of trouble and distress. Without your support, the path would certainly be more difficult. I am truly blessed to have you as my lifemate.

My dear aunts Maria José (Zezé), Emília Vitória (Piu) and Maria Vitória (Totoia) who always cheered, supported and encouraged my studies since child.

To my dear grandmother Vitória Lucinda da Silva who passed way recently (*in memorian*). I will never forget your joy and affection for me. I love you grandma.

ACKNOWLEDGEMENTS

To the graduate program in Entomology from College of Agriculture "Luiz de Queiroz" (ESALQ/USP) for allowing the completion of the doctoral study.

To the National Council for Scientific and Technological Development (CNPq) for financial support.

To the Department of Entomology and Acarology of ESALQ/USP for the infrastructure and technical support, especially to the employee Vitor Celso da Silva during the weekly trips to Campina do Monte Alegre, SP, and the employee Neide Graciano Zério for their friendship and companionship, always making insect pests and artificial diets for laboratory bioassays.

My dear advisor Pedro Takao Yamamoto, who entrusted me with this project, especially during the exchange period with the United States Department of Agriculture (USDA). I am extremely grateful to Professor Pedro for his guidance, patience, teachings and time dedicated to my professional and personal development. I thank him for the opportunity to work in his laboratory and learn from him.

To all professors in the Department of Entomology and Acarology, especially senior professors Sinval Silveira Neto and Octavio Nakano. It was great to learn from you during informal conversations.

To friend and professor Rodrigo Neves Marques, who on behalf of the Federal University of São Carlos, Lagoa do Sino Campus, allowed all the field experimentation of my thesis to be possible.

To the several interns' students who helped me to conduct the field and laboratory assays, especially to Rodrigo Faria (Pepa Pig), Matheus Calheiros (CPI), Henrique Sanches (Boi), Christian Price, Felipe Carneiro, Leandro Santos, Gustavo Barbosa, Anna Lydhia, ...

To my dear friend member from IPM Laboratory, specially to Gabriel Rugno, Aline Franco, Cynthia Jacob, Thais Fagundes Matioli, Camila Gachter Skanata, Inana Schutze, Mariana Rosa, Júlia Aleixo, Ana Clara, Fernando Iost... As it was cool to take a coffee and spend time talking about many funny issues...

To the friends of the Resistance Laboratory Rogério (Goiás), Dyrson, Anderson, Boquinha, Detona, Pãozinho, Ewerton, for the moments of relaxation, soccer, coffee, barbecue, ...

To the friends Natasha, Diandra, Cleane, Mayara and Laura for their friendship, for their help and opportunity to work together at some point during their doctoral period.

To the PROMIP company for having provided the egg parasitoid *Trichogramma pretiosum* for field mass release and Bayer for supporting and collaborating with this project goals.

To researcher Steve Naranjo and his entire team at the Arid Land Agricultural Research Center at the United States Department of Agriculture (USDA).

To the friends from Goiânia Leonardo Buys, Jardel Barbosa, Humberto Oliveira, Tiago Carvalhais and Bruno Alves Rosa Soares (*in memoriam*) who made positive influence during my undergraduate and internship period in IPM Laboratory from UFG – Goiânia, to follow the Entomology area, in addition to my dear supervisor and mom Cecília Czepak, who I had the privilege of working with and have as an example of dedication and passion for the profession.

To the friends I met in Botucatu during the master's degree, especially Bruna Favetti, Ana Laura Favoreto (Porteira), Nádia Bueno, João Paulo, Joáz Dorneles, as well as the old friends from the fraternity Domina&Cama for their friendship and companionship...good times that never come back!!!

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RESUMO

Manejo de pragas-alvo da soja Bt em áreas de refúgio, risco ecológico para predadores de solo e suas implicações para o manejo da resistência a insetos

A estratégia de alta dose/refúgio é o plano de Manejo de Resistência a Insetos (MRI) mais significativa para retardar a evolução da resistência das pragas-alvo às culturas transgênicas inseticidas. Além disso, as Avaliações de Risco Ambiental (ARA) representa uma importante etapa para monitorar o impacto ecológico de plantas Bt para Organismos Não Alvos (ONA's). Para subsidiar programas de MRI à soja transgênica MON87701 × MON89788 que expressa em alta dose a proteína inseticida Cry1Ac de *Bacillus thuringiensis* (Bt), foram realizados estudos para entender quais são as melhores estratégias de Manejo Integrado de pragas (MIP) a serem aplicadas em áreas de refúgio de soja Bt visando a máxima produção de indivíduos suscetíveis em paralelo viabilidade econômica, assim como estudos para verificar os efeitos subletais dos inseticidas flubendiamide e chlorantraniliprole no desenvolvimento e desempenho de vôo de pragas-alvo da soja Bt e suas implicações para o manejo da área de refúgio da soja Bt, bem como o risco ecológico da soja Bt nos artrópodes predadores habitantes do solo (APHS's) e o potencial impacto na evolução da resistência. Nossos resultados demonstraram que de acordo com a abundância de larvas (indicador de produção de mariposas suscetíveis), apenas o manejo de percevejos-praga (tratamento controle) nas áreas de refúgio da soja Bt proporcionou de forma satisfatória, tanto o fornecimento dos potenciais indivíduos suscetíveis, bem como os melhores indicadores de viabilidade econômico-financeira e produtividade. Os efeitos subletais dos inseticidas flubendiamida e clorantraniliprole na dinâmica populacional de *Chloridea virescens* podem levar à assincronia de geração e implicar no fornecimento insuficiente de mariposas suscetíveis quando aplicados em áreas de refúgio. No entanto, a distância e a duração do voo podem ainda ser suficientes para garantir o acasalamento de populações potencialmente resistentes e suscetíveis em campo. Em relação à ARA, não encontramos impacto ecológico da soja Bt na comunidade de artrópodes predadores do solo, ou diferenças significativas em sua diversidade. A maioria das espécies encontradas na soja Bt e não Bt foram constituídas por membros das famílias Carabidae, Formicidae, Anisolabididae e o grupo Araneidae. Não foi possível concluir o potencial dos APHS's dominantes em retardar ou acelerar a evolução da resistência. Portanto, esse fatores ainda devem ser investigados profundamente.

Palavras-chave: Soja Bt, Campos de refúgio, Dinâmica populacional, Tabela de vida, Controle de pragas, Diamidas, Capacidade de voo

ABSTRACT

Management of target pests of Bt soybean in refuge areas, assessment of ecological risk to ground-dwelling predators and their implications for insect resistance the management

The high-dose/refuge strategy is the most Insect Resistance Management (IRM) plan to delay evolution of resistance in target pest to transgenic insecticidal crop. Also, the Environmental Risk Assessments (ERA) represent the important step to monitoring the ecological impact of Bt plants to Non-Target Organism (NTO's). Thus, to support IRM programs to transgenic soybean MON87701 × MON89788 expressing high concentration of Cry1Ac protein from *Bacillus thuringiensis* (Bt), was carried out a studies to understand the best Integrated Pest Management (IPM) strategies to apply on refuge areas of Bt soybean aiming for the maximum generation of a susceptible individual in parallel with economically viability, as well as the sublethal effects of insecticides on development and flight performance of target-pest of Bt soybean and their implications for Bt soybean refuge area management, as well as the ecological risk of Bt soybean on Ground-Dwelling Predatory (GDP's) arthropods, and the potential impact on resistance evolution. According with larvae abundance as source of generation susceptible moth, our results showed that only the management of pest stink bugs in refuge areas of Bt soybean (control treatment) provided satisfactorily the potential susceptible individuals, as well as the best indicators of economic-financial viability and productivity. The sublethal effects of flubendiamide and chlorantraniliprole insecticides on *Chloridea virescens*' population dynamics could lead to generation asynchrony and provide insufficient susceptible moths when sprayed on refuge crops. However, the distance and duration of flight may still be enough to ensure mixing of potentially resistant and susceptible populations from refuge plots. In relation to ERA, we did not find ecological impact of Bt soybean on the ground-dwelling predatory arthropod community, or significant differences in their diversity. The most species in Bt and non-Bt soybean areas consisting of members of the families Carabidae, Formicidae, Anisolabididae and the Araneidae group. The potential of dominant GDP's to delay or accelerate the evolution of resistance could be not concluded and must be investigate more.

Keywords: Bt soybean, Refuge fields, Population dynamics, Life history, Pest control, Diamide insecticide, Flight performance

1. INTRODUCTION

The soybean, *Glycine max* (L.) Merrill (Fabaceae: Phaseoleae), is the main agricultural commodity of Brazil. In the 2018/2019 growing season, Brazil produced around 117 million tons of grains and achieved the status of largest exporter (USDA, 2019). In recent years, the sown of the genetically modified (GM) soybean event MON 87701×MON 89788 (INTACTA RR2 PRO[®]) have endorsed these improvements. This Bt soybean has genes encode the expression of Cry1Ac protein from *Bacillus thuringiensis* (Bt) (Berliner) (Bacillales: Bacillaceae) and confer resistance against the main lepidopteran pests of soybean, including *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Erebiidae), *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), *Chloridea virescens* (Fabricius) (Lepidoptera: Noctuidae) and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Bernardi *et al.* 2012; Bernardi *et al.* 2014a,b; Yano *et al.* 2015, Dourado *et al.* 2016). In addition, the transgenic Bt soybean expresses the protein 5-enolpyruvylshikimate 3-phosphate synthase (EPSP's) from *Agrobacterium* sp. which provide tolerance to the herbicide glyphosate (CTNBio, 2010). Thus, the Bt soybean represents an important tool to Integrated Pest Management (IPM) programs (Martins-Salles *et al.* 2017). However, there is a strong economic threat from the propensity of insects involve resistance and Bt soybean control efficiency failures (Luca *et al.* 2020). In worldwide, several cases about field-evolved insect resistance to Bt crops have been reported (Tabashnik *et al.* 2009; Tabashnik and Carrière, 2017), for instance in Brazil on Bt maize expressing the single Bt proteins Cry1Ab and Cry1F to *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) (Farias *et al.* 2014; Omoto *et al.* 2016). In this way, insect resistance management (IRM) programs for Bt traits must be designed and implemented to delay resistance evolution to avoid this scenario, proactive (MAPA, 2021).

In literature, the main approach used to slow the evolution of resistance is the “high-dose/refuge” (Campagne *et al.* 2016). The “high-dose/refuge” strategy is based on the assumptions that Bt proteins have high toxicity against the target pest by express high concentration of Cry toxin around 25 to 50 times the lethal dose to kill 99.99% of susceptible insects, the inheritance the resistance be functionally recessive, the resistant alleles be rare and the refuge areas (isoline/conventional sister line of non-Bt plants) may be cultivated at least 0.5 miles (800 meters) from Bt crops (Gould, 1998; Tabashnik *et al.* 2009; Tabashnik and Carrière, 2017). In Brazil, the refuge area must comprise 20% of the total soybean crop cultivated under different spatially arranged layouts obeying this distance limit (MAPA,

2019). Overall, the purposes of the refuge are the production of susceptible individuals (alleles) from the target pests of Bt crops (mentioned above) (Tabashnik *et al.* 2008, 2013). The individuals generated in the refuge areas should disperse and mate with rare resistant from Bt crop, which the resulting heterozygous offspring will be kill on Bt crop (Gould, 1998). In Brazil, studies were conducted to assess the risk of resistance evolution in insects to GM soybean MON 87701 × MON 89788 in order to subsidize IRM programs (Bernardi *et al.*, 2012; França *et al.*, 2017). Despite this, assessment approaching the appropriate pest management strategies to apply on refuge area of Bt soybean (Cry1Ac) have not yet been conducted. In Australia, for instance, Baker *et al.* (2008) assessed the production of larvae, pupae, and adults in conventional sister line and Bt cotton expressing one (Cry1Ac) or two Bt proteins (Cry1Ac and Cry2Ab) under insecticide treated and non-treated fields. The authors found that Bt cotton generated similar number of bollworms *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae compared with non-Bt cotton, but the Bt variety produced significantly fewer bollworm adults than conventional cotton.

Brazil is a tropical country characterized by intensive agricultural production system (overlapping or succession of annual crops) and constant polyphagous pest pressure (Garcia *et al.* 2018). Thus, the Biotechnology subunit group from Insecticide Resistance Action Committee (IRAC) in Brazil has proposed IPM tactics for pest management on refuge area of Bt soybean through the insecticide sprays according to economic thresholds (IRAC, 2017). Although Bt soybean is highly effective against the major soybean lepidopteran pests, the supplementary control in areas of refuge through the spraying foliar insecticide can be necessary to manage the targets-pest of Bt soybean, as well as the non-target pest of Bt soybean such as *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae), *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidae) and *S. frugiperda* (Bernardi *et al.* 2014b; Azambuja *et al.* 2015; Silva *et al.* 2016).

Since refuge effectiveness will depend on the degree of movement and mating propensity of moths from areas of refuge, as well as the production of enough susceptible moths, an important point raise up is related to the potential lethal (mortality) and sublethal effects (i.e.: life table parameters and flight performance) of any insecticide to Bt soybean target pest and their impact on IRM and the “high-dose/refuge” strategy (Zhang *et al.* 2013; França *et al.* 2015; Mahmoudvand and Moharramipour, 2015; Malaquias *et al.* 2017). By contrast, the applied biological control through the mass release of egg parasitoid *Trichogramma* spp., the use of entomopathogenic microbiological insecticides based on virus

composition, the natural biological control, or even integrating IPM methods and evaluating combined control effects can be useful and complementary alternatives strategies to adopted on refuge area (Bueno *et al.* 2011; Parra *et al.* 2015; Sanches, 2019; Karlsson Green *et al.* 2020).

In addition to field-evolved resistance to Bt soybean, another major concern related to Bt crops are their potential impact on non-target organisms (NTO's) (EFSA, 2010; Hilbeck *et al.* 2011). Under field conditions, the NTO's (i.e.: pollinators and natural enemies) can be exposed to many pathways and routes, for example directly by ingestion or feeding Bt nectar flowers, by feeding contaminated prey, by contact with Bt toxins in the soil plant exudates and/or as well as indirectly through reductions in prey/host populations in function of Bt crop insecticide activity (Peterson *et al.* 2009; 2011). For this, regulatory environmental risk assessment in worldwide should be performed to monitoring these possible effects on NTO's (EFSA, 2010; Lu *et al.* 2012; Romeis *et al.* 2013; Bauer-Panskus and Then, 2014). Although in most of literature cases have been widely reported no detrimental effects of Bt crops to NTO's, currently there is no post commercial release information of Bt soybean expressing Cry1Ac toxin to the arthropod soil predators (Yaqoob *et al.* 2016; Krogh *et al.* 2020). In parallel, among the issues that also arises from the perspective of the IRM is the ecological impact of Bt crops over natural enemies in Bt and/or non-Bt plants (refuge area) into delay or accelerate the evolution of resistance process. These perspective are linked with the potential of natural enemies to eliminate the Bt soybean target pest on Bt soybean field (presence of individuals carrying the resistance alleles), as well on refuge area (presence of carrying susceptible alleles in the refuge area) (Lawrence *et al.* 2007; Sanvido *et al.* 2012; Liu *et al.* 2014; Devos *et al.* 2015; Romeis *et al.* 2019). Given this contextualization, this thesis had the following goals:

(i) to analyze the IPM strategies to apply on refuge area of Bt soybean (Cry1Ac) aiming maximum generation of potential susceptible individual associated with satisfactory soybean yield and economically viable to subsidize IRM programs in Brazil (**second chapter**).

(ii) to evaluate the sublethal effects of flubendiamide and chlorantraniliprole, a diamide insecticides group, on life history traits of target-pest of Bt soybean on life table parameters, and flight performance in order to better understand the implications for management of refuge area (**third chapter**).

(iii) to assess the ecological risk of Bt soybean (Cry 1Ac) on ground-dwelling predatory arthropods, approaching their composition of community in Bt and non-Bt soybean

fields, the faunistic and ecological diversity indexes, the predominant or dominant species and the tritrophic interactions and potential species for biological control into impact the evolution of resistance (**fourth chapter**).

Each chapter of this thesis was written according to the wish manuscript submission guidelines: Crop Protection (**Chapter 2**), Insects (**Chapter 3**) and Bulletin of Entomological Research (**Chapter 4**).

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2. EVALUATION OF ECONOMICALLY VIABLE IPM STRATEGIES TO REFUGE AREAS OF BT SOYBEAN (CRY 1AC) AIMING TO GENERATE THE MAXIMUM TARGET SUSCEPTIBLE INDIVIDUALS

Abstract

The Bt (*Bacillus thuringiensis*) soybean expressing a high-dose of Cry1Ac protein confers resistance against the main lepidopteran-pest and represents an important tool for Integrated Pest Management (IPM). In Brazil, the Bt soybean is largely cultivated and there is a concern to field-evolved resistance. The most effective Insect Resistance Management (IRM) strategy to delay resistance evolution is the high-dose/refuge. The ‘refuge’ represents the cultivated area with non-Bt plants to provide a source of susceptible moths to ensure matings with resistant survivors from Bt crops. Still, there is no evidence about pest control strategies to apply in the refuge area of Bt soybean in Brazil. Thus, the performance of IPM strategies for refuge areas, aiming the maximum generation of susceptible lepidopteran target pests of Bt soybean associate with economic viability, was assessed. Five treatments (IPM strategies) and four replicates (plots) under complete randomized block design were used. Each plot had 350 m² (35 × 100 m). The treatments were: T₁ – Specific and selectivity insecticide (Insecticide - I) to control lepidopteran pests + mass release of *T. pretiosum* (Biological Control - BC); T₂ – Mass release of *Trichogramma pretiosum* (BC); T₃ – Specific and selectivity insecticide to control lepidopteran pests (I); T₄ – Unsprayed targeting lepidopteran pest (Check - C) and T₅ – Soybean growers or farmers lepidopteran pest management (Farmers - F). The control of stink bugs pest and soybean diseases were standardized for all treatments and controlled by soybean growers. The larvae abundance was used as an indicator of potential moth production. In general, during the 2016/2017 and 2017/2018 growing seasons, although larvae production in all IPM refuge strategies of Bt soybean were not significant, the economic viability was favorable to Check treatment. Thus, this treatment seems to be the best refuge option to apply in a refuge area of Bt soybean.

Keywords: IRM; *Bacillus thuringiensis*; High-dose/Refuge; Bt plants; Population dynamics.

2.1. Introduction

The soybean crop [*Glycine max* (L.)] is cultivated in a range of countries worldwide. In Brazil, this oilseed plant represents the largest agricultural commodity of economic importance. In the 2018/2019 growing season, Brazil was the largest exporter reaching out around 117 million tons of grains production (USDA, 2019). Among the factors that support it, the widespread planting of genetically modified soybean (GM) MON 87701 × MON 89788 (Intacta RR2/PRO[®]) stands out (Céleres, 2017; Brookes, 2018; Cattelan and Dall'Agnol, 2018). This GM soybean expresses a high-dose of insecticidal Cry1Ac protein from *Bacillus thuringiensis* (Bt) providing protection against main lepidopteran pests of soybean, such as *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), *Anticarsia gemmatalis* (Hübner)

(Lepidoptera: Erebidae), *Chloridea virescens* (Fabricius) (Lepidoptera: Noctuidae) and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Bernardi et al., 2012; Bernardi et al., 2014a, b; Yano et al., 2015, Dourado et al., 2016). Therefore, this Bt soybean represents an important tool for integrated pest management (IPM) (Martins-Salles et al., 2017).

Several cases about field involving resistance have been reported worldwide. In Brazil, for example, the remarkable report to *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) on Bt maize (Cry1F and Cry1Ab) emerged after two years of commercially available (Farias et al., 2014; Omoto et al., 2016; Tabashnik and Carrière, 2017). The poor refuge areas (non-Bt plants) compliance and the Bt crops that was not “high-dose” are the main factors in the selection of the resistant populations in most of the cases worldwide (Tabashnik et al., 2009; Tabashnik and Carrière, 2017). Although Bt soybean attends the high-dose concept to the main lepidopteran pests, there is a strong economic concern about the propensity of insects evolving resistance and the loss of pest control efficacy of Bt soybean technology (Tabashnik et al., 2019). Thus, to delay the evolution of resistance, the most Insect Resistance Management (IRM) strategy known has been the high-dose concept of Bt toxin (kill at least 99.99% of susceptible insects) and planting refuge areas (non-Bt plants) (Gould, 1998; Tabashnik et al., 2009; Tabashnik and Carrière, 2017). However, to be effective, the refuge area should produce large numbers of Bt-susceptible moths and reduce the likelihood of evolving field resistance to Bt crops (Tabashnik and Carrière, 2017). Therefore, refuge areas act as a source of susceptible alleles from Bt target pests that support slow the evolution of resistance by providing susceptible mates for resistant insects (Tabashnik et al., 2008, 2013).

According to the guidance of the U.S. Environmental Protection Agency (EPA), to be effective, around 500 susceptible (SS) individuals are required for each insect carrying at least one Bt resistance allele (SAP, 1998). Nevertheless, there are few studies under field conditions that assess the abundance of susceptible target pest of Bt crops and understand the potential of production in refuge areas (Jackson et al. 2004; Baker et al. 2008; Peres et al., 2012; Justiniano et al., 2014; Jin et al., 2016). Since currently there are no studies approaching the IPM strategies vs. potential generation of susceptible individuals in Brazil, and the sowing refuge area is not mandatory yet, the absence of pest control strategies to apply on soybean refuge areas accelerate the risk of field-evolved resistance and loss Bt soybean technology. In this sense, this study assessed IPM strategies to apply on refuge areas of Bt soybean (Cry1Ac)

aiming for the maximum generation of a potentially susceptible individual in parallel with satisfactory soybean yield and economically viable to subsidize IRM programs.

2.2. Material e methods

2.2.1 Study site

The experiments were carried out under field conditions on two consecutive soybean growing seasons (2016/2017 and 2017/2018). The studies were carried out at UFSCar, Lagoa do Sino Campus, municipality of Buri, São Paulo state, under the global position of longitude (W) 48°32'59.54" and latitude (S) 23°36'28.07" (Figure 1). The randomized block experimental design was used with 5 treatments (IPM strategies) and four replications. Each replication was 35 meters wide (70 rows under 0.5 meter of plant row spacing) per 100 meters long, totaling 14,000 m² of the treatment plot area.

The non-Bt soybean variety sown in the refuge area in both growing seasons was 'NA5909RG' with maturity group 5.9 and indeterminate growth habit. The Bt soybean variety was 'DM6563RSF IPRO' with maturity group 6.3 and indeterminate growth habit. The Bt soybean was planted around the field refuges to support the influence of bioecological factors, as well as the realistic condition of structural refuge.

2.2.2 Soybean arthropods sampling

During 2016/2017 and 2017/2018 growing seasons, the sampling of arthropods-pests and natural enemies started around 21 days after emergence (DAE). The beat cloth method (1.0 × 1.5 m) was used weekly to sample the target and non-target immature pests of Bt soybean, as well as the natural enemies at 10 random points in each replication. There were performed total of 15 assessments in 2016/2017 growing season and 13 in the 2017/2018 growing season. The insects were identified at the species level and grouped into target and non-target pests of Bt soybean. The lepidopteran larvae were classified in small size (<1.5 cm) and large size (> 1.5 cm), and the bugs were categorized in nymphs (3rd to 5th instar) and adults. However, to performer statistical data analyses all pests' categories size or development stage were grouped. In addition, the natural enemies were grouped in parasitoids, predators and entomopathogens diseases (fungi, bacteria and viruses) (Bueno et al., 2013; Punithavalli et al., 2014). The adults of moth population were monitored using 3 delta traps with *C. includens*, *S. frugiperda* and *Helicoverpa* spp. synthetic sex pheromones. The traps were separated by 100 meters of distance each one to avoid olfactory chemical

response. The traps were evaluated weekly and the moths were counted by species. The pheromone lures were changed every 2 weeks.

2.2.3 Soybean refuge area management strategies

The following treatments were assessed on soybean refuge area pest management: T₁ – Insecticide [Spray harmless insecticide to control lepidopteran-pest according to Economic Threshold – (ET)] and Biological Control (Mass release of *Trichogramma pretiosum* according to lepidopteran-pest eggs densities) (I + BC); T₂ – Biological Control (Mass release of *Trichogramma pretiosum* according to lepidopteran-pest eggs densities) (BC); T₃ – Insecticide (Spray harmless insecticide to control lepidopteran-pest according to ET) (I); T₄ – Check (Control soybean stink bugs complex, but not lepidopteran-pest) (C); T₅ – Farmer (Soybean grower or farmers phytosanitary practices management) (F).

In treatments that were sprayed with insecticides (T₁ and T₃), the spraying decision was made following the ET recommendation. Also, the insecticide was chosen according to its target pest specificity and natural selectivity profile (Bueno et al., 2017). To confirm field identification of the Heliiothinae complex (*H. armigera* and *C. virescens*), 50 larvae were randomly collected in the vegetative stage and 50 in the reproductive stage of soybean growth in all treatments and subjected to molecular analysis. The identification of the species was confirmed through the amplification of the mitochondrial COI gene using PCR techniques through the specific primers and the restriction enzyme BstZ17I which electrophoretic profiles of *H. armigera* were obtained with the primers COI-F and COI-R (Behere et al., 2008).

Regarding the treatments involving Biological Control, the density threshold of 0.1 egg per plant was used to initiate mass field release of the egg parasitoid *T. pretiosum* (Favetti, 2017). For this, 15 plants were randomly collected in each replication of treatments. The number of lepidopteran pest eggs was counted in the laboratory in all plant structures. For field release, the rates used were 120,000 parasitoids per hectare (Favetti, 2017). The *T. pretiosum* were manually released through the cardboards (fictitious parasitized eggs of *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae) whose adults of *T. pretiosum* were close to the emergence or with few emerged). The cardboards were homogeneously spread in the field according to the capacity of dispersion of *T. pretiosum* in soybean crop (Zachrisson and Parra, 1998; Bueno et al., 2011; Parra et al., 2015).

All releases were made between 07:00 am and 07:30 am, except for the 5th release, which was carried out between 5:30 pm and 6:30 pm when the temperature and humidity were favorable to the establishment and action of parasitoids in the area. The cardboards were placed in the bottom of soybean plant to avoid biotic and abiotic stresses. In addition, to double check *T. pretiosum* emergency, samples of cardboards were maintained under laboratory (25 ± 2 °C and $50 \pm 10\%$ RH) and field environmental conditions. To assess the efficiency of *T. pretiosum*, parasitized lepidopteran eggs (black color) were searched in 60 plants (15 plants collected by each treatment replication) as previously described.

Regarding to the Farmers treatment (F), the management adopted followed the soybean grower decision. This pest control management is traditionally characterized by no previous scouting for IPM decisions, using an insecticide spray schedule, usually with the use of those with a broad spectrum and harmful to natural enemies. For Check treatment (C), was not applied any IPM strategy to control lepidopteran pests in soybean refuge areas.

In all treatments, the management of stink bugs complex was standardized by neonicotinoids (4A) and pyrethroids (3A) chemical groups of insecticides (Tables 1 and 2) because they are more specific and lightly harmless to natural enemies than organophosphates (acephate). The practices of fertilization and plant protection management, as soybean plant diseases and weeds, were also standardized for all treatments.

2.2.4 Seed soybean yield

For each treatment, 4 m² were manually harvested from each replication (subdivided in 4 random repetitions of 1 m² – totaling 16 m²/treatment) upon the full maturity soybean grains (R8 plant growth stage). Then, the samples were weighed, and the moisture corrected to 13%, and the productivity data (kg ha⁻¹) and mass of 1000 grains (g) estimated.

2.2.5 Profitability and economic viability

The profitability and economic viability adopted followed the one proposed by Carvalho et al. (2016). The methodology proposed by Mello et al. (1988) was used to elaborate the production system of IPM strategies applied to the refuge area, the technical data from each treatment were collected in field conditions (performance of spray insecticide machine per hectare or hour, person for field manual release of *T. pretiosum*, etc). Resellers were contacted in the study's local region, as well as the manufacturing companies to obtain prices of inputs and other consumables supplies through budgets. With this information, the

methodology of Martin et al. (1998) was applied to calculate the Production Costs (e.g.: Effective Operating Cost - Hand labor, machinery, equipment and supplies, etc), and Total Operating Cost (machinery and equipment depreciation, social charges, etc). The profitability indicators (e.g.: Gross Revenue, Gross Profits Margin, Break Even Points and Operating Profit) were estimated according to Lazzarini Neto (1995) and Martin et al. (1998). The Benefit-Cost ratio (%) was estimated in accordance with Vitale and Miranda (2010). To calculate the Gross Revenue and Net Profit, the Yield of each treatment in the two-growing seasons was determined according to mentioned in item 2.4. The sale price of soybean production was obtained at the Agricultural Economics Institute's website, using as reference the month of March (harvest period). The exchange rate of Brazilian Real currency (R\$) and US dollars (\$) was converted using the average historical price during the period from 09/01/2016 to 04/30/2017 (first growing season) and 09/01/2017 to 04/30/2018 (second growing season).

2.2.6 Statistical data analyses

Quasi-Poisson generalized linear models (Demétrio et al., 2014) were fitted to insect count data, split per time point and species, including the additive effects of block and treatment in the linear predictor. The significance of the effects was assessed through F-test, since the dispersion parameter was estimated. Multiple comparisons were performed by comparing the 95% confidence intervals for the true model coefficients. Linear models were fitted according Gaussian distribution to the yield (kg ha⁻¹) data, including the effects treatment in the linear predictor. The significance of the effects was assessed through F-tests. Multiple comparisons were performed using Tukey's test at 5% significance level. Linear mixed-effects model was fitted to the 1000-weight (g) data including the fixed effects of treatment and random plot effects in the linear predictor, since observations taken on the same plot were correlated. The significance of the effects was assessed through likelihood-ratio (LR) tests between tested models. The goodness-of-fit of all fitted models was assessed using half-normal plots with a simulated envelope (Moral et al., 2017). All statistical analyses were carried out using the R statistical software environment (R Core Team, 2020). In addition, two-way ANOVA (analysis of variance) was used to test moths and eggs abundance using JMP v5 software (SAS Institute Inc., Cary, NC). Multiple comparisons of the means were done by the Tukey-Kramer HSD test ($\alpha=0.05$).

2.3 Results

2.3.1 First growing season – 2016/2017

2.3.1.1 Abundance of lepidopteran target pests of Bt soybean

At the beginning of monitoring (V5, V6, and V7 soybean growth stages) the presence of velvetbean caterpillar, *A. gemmatalis*, was not observed. However, in the R1 soybean growth stage (flowering period) an increase in the pest population was observed. The peak of *A. gemmatalis* population was observed in the R6 soybean growth stage, corresponding to population densities ranging from 0.27 ± 0.13 to 1.72 ± 0.24 larvae per meter. In general, there were no significant differences in the *A. gemmatalis* larvae population in most of soybean growth stage, except at the plant growth stages R4/R5 ($F_{4, 12} = 5.22$; $P = 0.011$) and R6/R5 ($F_{4, 12} = 4.11$; $P = 0.025$) when the Farmer (F), Biological Control (BC), and Check (C) treatments showed the higher populations densities (Figure 2A). Moreover, this species did not achieve the ET.

Regarding soybean looper, *C. includens*, its presence has been observed since the vegetative soybean growth stage. However, only at R2/R3 plant growth stage were found significant differences among treatments ($F_{4, 12} = 3.70$; $P = 0.034$) (Fig. 2B). During this period, the Biological Control (BC) treatment showed the highest population density while the Insecticide treatment (I) the lowest. The population peak of *C. includens* was observed between R1/R2 to R5.1/R5.3, ranging from 0.97 ± 0.16 to 2.42 ± 0.22 larvae per meter (Fig. 2B), but did not achieve the ET.

The old world bollworm, *H. armigera*, was the only target pest of Bt soybean that achieved the ET level at R2/R3 plant growth stage (Fig. 1C). Therefore, the pest control measures were adopted in the I + BC (Insecticide + Biological Control) and I (Insecticide) treatments by spraying Belt[®] insecticide (Contain 480 g/L of flubendiamide active ingredient, IRAC Group 28) at the rate of 70 mL ha^{-1} (Table 1 and Fig. 2C). The population peak of *H. armigera* was observed in this same period, and the pest population ranged from 1.12 ± 0.15 to 1.72 ± 0.45 larvae per meter. In general, there were no significant differences among treatments in any of the periods evaluated (Fig. 2C).

Concerning the tobacco budworm, *C. virescens* (Heliiothinae), showed the lowest population abundance and low frequency during sampling. Thus, *C. virescens* also did not achieve the ET. However, the population peak of *C. virescens* was observed at R5.1/R5.3 ranging from 0.25 ± 0.09 to 0.80 ± 0.18 larvae per meter. No significant differences were observed among treatments in any of the plant growth stage (Fig. 2D).

Although only *H. armigera* achieved the ET at R2/R3 plant growth stage, the F (Farmer) treatment was sprayed 4 times with the following insecticides targeting lepidopteran pests: Curyom 550 EC[®] (Contain 550 g/L of profenofos + 50 g/L of lufenurom, IRAC Group 1B + 15) at the rate of 0.25 L ha⁻¹ in the R5.4/R5.5 plant growth stage and Orthene[®] (Contain 750 g/Kg of acephate, IRAC Group 1B), 3 times, at the rate of 0.5 kg ha⁻¹ in the R1, R5.3/R5.4 and R5.4/R5.5 plant growth stages (Table 1).

2.3.1.2 Abundance of non-target lepidopteran pest of Bt soybean

The fall armyworm, *S. frugiperda*, the black armyworm, *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidae), and the southern armyworm, *Spodoptera eridania* (Stoll) (Lepidoptera: Noctuidae), were the main non-target lepidopteran pests of Bt-soybean across sampling. In general, there were no significant differences among treatments for these species (Figs. 3A, 3B and 3C), except for *S. frugiperda* during the soybean growth stage R4/R5 ($F_{4, 12} = 3.90$; $P = 0.0295$) (Fig. 3A). In this period, the Farmer (F) treatment showed the highest population density (0.70 ± 0.16) and the Insecticide (I) + Biological Control (BC) the lowest (0.35 ± 0.09). Among the non-target lepidopteran pests, *S. eridania* showed the highest density of larvae per meter over the sampling period. The population peaked at the R7.3 plant growth stage ranging from 1.40 ± 0.47 to 3.97 ± 1.20 larvae per meter (Fig. 3B). None of these species reached the ET level to imply control measures in none of the treatments (Fig. 3).

2.3.1.3 Abundance of moths and eggs

The *H. armigera* showed two population peaks, at R1/R2 ($n = 15$) and R4/R5 ($n = 16$) plant growth stages, in which larger numbers of adults were captured. The adults of velvetbean caterpillar, *A. gemmatalis*, showed low abundance and frequency, with population peak at R5.3/R5.4 ($n = 4$) (Fig. 4) while *C. includens* had the highest captures in R4/R5 ($n = 10$) and R5.5/R5.6 ($n = 10$). The non-target pest of Bt soybean, *S. frugiperda*, also had two adult population peaks at R1/R2 ($n = 26$) and R5.3/R5.4 ($n = 17$) growth stage. The capture of *S. eridania* in the traps of *S. frugiperda* was occasional ($n = 1$) in R5.5/R5.6 (Fig. 4).

Parallel to the capture of adults in the area, the peak population peaks of lepidopterans pests' eggs was also observed (Fig. 5). For I + BC, the greatest abundance of eggs was observed at R2/R3 (0.18 ± 0.08 eggs/plant), R5.1/R5.3 (0.15 ± 0.05 eggs / plant) and R6 (0.21 ± 0.16 eggs/plant). The BC (0.23 ± 0.11 eggs/plant) and C (0.23 ± 0.08 eggs/plant) treatments

had egg population lower than that found in the I + BC treatment, except in the plant growth stage R7.1 (Fig. 5).

Thus, the egg parasitoid *T. pretiosum* was released 5 times, subdivided into 2 different moments according to the threshold adopted (average of 0.1 egg/plant). The first release occurred in the reproductive soybean plant growth stage R4/R5, and the average egg's density per plant was 0.12. The parasitoids were released at 3 sequential times in treatments with 7 days of intervals. The second release occurred in reproductive plant growth stage R7.1 and 0.14 eggs per plant density. Two consecutive parasitoid releases were performed in the same intervals as the previous one (Fig. 5).

2.3.1.4 Stink bugs pests

The brown stink bug, *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae), and the green-belly stink bug, *Diceraeus* (= *Dichelops*) (Dallas) (Hemiptera: Pentatomidae), were observed in the experimental area. *E. heros* was predominant comparing to *D. melacanthus* (Figs. 6A and 6B), during the sampling period the population of *E. heros* increased from the V7 plant growth stage and the population peaked from R6 to R8. During this period, the number of stink bugs per meter ranged from 2.02 ± 0.25 to 10.05 ± 0.81 (Fig. 6A). Significant differences were observed among treatments at the plant growth stage R2/R3 ($F_{4, 12} = 3.86$; $P = 0.030$), when the Biological Control (BC) showed the highest population density per meter (0.52 ± 0.14) and the Insecticide (I) treatment the lowest (0.12 ± 0.05) (Fig. 6A). No significant differences were found in the other soybean plant growth stages for *E. heros* (Fig. 6A) and *D. melachantus* (Fig. 6B) among treatments.

2.3.1.5 Coleopteran pests

During the sampling were observed the presence of the cucurbit beetle, *Diabrotica speciosa* Germar (Coleoptera: Chrysomelidae), and the leaf beetle *Maecolaspis calcarifera* Bechyné (Coleoptera: Chrysomelidae). Both species showed similar population dynamics according to the soybean growth stage. No significant differences were observed between the treatments in none of the periods evaluated to *D. speciosa* (Fig. 6C) and *M. calcarifera* (Fig. 6D).

2.3.1.6 Natural enemies

Several natural enemies were observed during sampling in all treatments, highlighting predators and entomopathogens through the beat cloth. The main species of predatory insects were: lady bugs *Eriopsis connexa* (Germar) (Coleoptera: Coccinellidae) and *Hippodamia convergens* (Guérin-Meneville) (Coleoptera: Coccinellidae), green lacewing *Chrysoperla externa* (Hagen) (Neuroptera: Chrysopidae), assassin bug *Nabis* spp., soldier beetles *Callida* spp. and *Calosoma* spp., soldier bug *Podisus nigrispinus* (Dallas) (Hemiptera: Pentatomidae), earwig *Doru luteipes* (Scudder) (Dermaptera: Forficulidae), minute pirate bug *Orius* spp., big-eyed bug *Geocoris* spp., ants *Solenopsis* sp., rove beetles member of Staphylinidae family (Fig. 7A), and predatory spider (Fig. 7C), this last natural enemy had predominance and the highest contribution to natural enemies community composition (Fig. 7D). For the entomopathogens, larvae infected by *Metarhizium rileyi* Farlow (Hypocreales: Clavicipitaceae) (formerly *Nomuraea rileyi*) were more observed than by *Baculovirus* spp. (Fig. 7B). In general, there were no significant differences among treatments for predatory insects, spiders and *M. rileyi* (Figs. 7A, 7B and 7C). However, at the R8 plant growth stage, a significant difference was observed concerning the community of natural enemies (Fig 7D), when the Biological Control (BC) treatment showed the highest population density (0.45 ± 0.18 individuals per meter) ($F_{4, 12} = 3.52$; $P \leq 0.05$) (Fig. 7D).

2.3.1.7 Soybean yield and mass grain

The weight of 1000 grains ranged between 148.64 to 156.01 g (Table 3), and yield between 4,753 kg ha⁻¹ to 4,929 kg ha⁻¹ (Table 4). However no significant differences were observed in relation to the weight of 1000 grains ($\chi^2_{4, 15} = 4.46$; $P = 0.33$) (Table 3) or yield (kg ha⁻¹) ($F_{4, 15} = 0.0818$; $P = 1.27$) (Table 4).

2.3.1.8 Production costs and economic viability

The treatment I + BC showed the highest gross revenue in comparison to the other pest management programs to the refuge of Bt soybean (yield ranging from 4921.67±241.12 kg ha⁻¹) (Table 5). However, the Insecticide + Biological Control (I + BC) showed the highest values of effective operating cost (664.35 US\$ ha⁻¹) and total operating cost (647.49 US\$ ha⁻¹). On the contrary, the Insecticide (I) treatment showed the lowest gross revenue (1,746.09 US\$ ha⁻¹) and Check (C) the lowest value of effective operating cost (369.02 US\$ ha⁻¹) and total operating cost (578.3 US\$ ha⁻¹), as well as the highest gross profit margin of effective operating cost (EOC) (374.31 US\$ ha⁻¹) and Total Operating Cost (TOC) (202.66 US\$ ha⁻¹).

The Check (C) treatment (66.96% and 3.03%), followed by Farmer (63.63% and 2.75%), and Insecticide (60.64% and 2.54%) showed the best indicator of financial viability (Margin of profitability and Benefit–cost ratio) (Table 5).

2.3.2 Second growing season – 2017/2018

2.3.2.1 Abundance of lepidopteran target pests of Bt soybean

The presence of the main lepidopteran target pests of Bt soybean was observed in all sampling periods, except *C. virescens*, which was very rare during sampling (Fig. 8). Significant differences were observed to the velvetbean caterpillar among treatments only at the plant growth stages R5.1/R5.2 ($F_{4, 12} = 4.49$; $P = 0.018$), R5.3/R5.4 ($F_{4, 12} = 10.11$; $P = 0.0001$), and R5.4/R5.5 ($F_{4, 12} = 6.46$; $P = 0.0516$) (Fig. 8A). At the R5.1/R5.2 soybean growth stage, the treatments Check (0.95 ± 0.21), Insecticide (0.87 ± 0.18), and Biological Control (0.70 ± 0.31) showed the highest abundance of larvae per meter, while the producer management showed the lowest density population (0.12 ± 0.15). At the soybean growth stage R5.3/R5.4 and R5.4/R5.5, the treatments Insecticide + Biological Control, Biological Control, Insecticide, and Check presented the highest population densities, with values ranging from 0.42 ± 0.08 to 2.67 ± 0.32 and 0.32 ± 0.08 to 1.55 ± 0.31 larvae per meter, respectively in each sampling periods. The population peaks of velvetbean caterpillar occurred at the soybean growth stage V7/R1 and R5.3/R5.4, respectively, with 1.35 ± 0.19 to 1.57 ± 0.22 and 0.42 ± 0.08 to 2.67 ± 0.32 larvae per meter (Fig. 8A).

The soybean looper, *C. includens*, occurred frequently in all sampled periods, but significant differences were observed among treatments only at the plant growth stages R1/R2 ($F_{4, 12} = 5.07$; $P = 0.012$) and R5.1/R5.2 ($F_{4, 12} = 6.38$; $P = 0.005$) (Fig. 8B). The population peak of *C. includens* was noted at the R5.1/R5.2 soybean growth stage, in which the treatments I + BC treatment showed the highest abundance of larvae per meter (4.20 ± 0.37) and Farmer (0.975 ± 0.14) the lowest (Fig. 8B).

In opposite to the 2016/2017 growing season, *H. armigera* did not reach the ET. Thus, it was not necessary to apply pest management programs to the refuge of Bt soybean proposed by treatments. In general, there were no significant differences among treatments in none of the periods evaluated for *H. armigera* (Fig. 8C). However, the population peaks were observed at the stages R5.3/R5.4 and R5.4/R5.5, in which the number of larvae per meter ranged between 0.4 ± 0.1 to 0.75 ± 0.12 and 0.35 ± 0.13 to 0.67 ± 0.10 , respectively.

Although in the 2017/2018 growing season was not necessary to apply any IPM tactics involving insecticides to control lepidopteran pest, the following insecticides were sprayed in the Farmers treatment (F): Pirate[®] (Chlorfenapyr, IRAC Group 13) at the rate of 0.4 L ha⁻¹, and Bazuka[®] (Methomyl, IRAC Group 1A) at the rate 0.8 L ha⁻¹ at V4/V5 plant growth stage, and Orthene 750 BR[®] (Acephate, IRAC Group 1B) at the rate 1 L ha⁻¹ at R2/R3 plant growth stage (Table 2).

2.3.2.2 Abundance of non-target lepidopteran pests of Bt soybean

As the first growing season (2016/2017), the *Spodoptera* complex was the most predominant lepidopteran pest during sampling period (Fig. 9). The southern armyworm, *S. eridania*, showed higher population density compared to *S. frugiperda* and *S. cosmioides* (Fig. 9B). The highest abundances of *S. eridania* were observed between R6 and R7.2 growth plant stages. Farmer and Biological Control + Insecticide treatments showed the highest population density at the R7.2 soybean growth stage (2.10 ± 0.40 and 1.77 ± 0.3 larvae per meter, respectively). Despite this, no significant differences were found among treatments in none assessed periods, except at R6 ($F_{4, 12} = 4.74$; $P = 0.001$) where the Biological Control + Insecticide treatment showed the highest abundance of larvae per meter (1.45 ± 0.86) and the Insecticide the lowest value (0.05 ± 0.03) (Fig. 9B).

The fall armyworm, *S. frugiperda*, was the most frequent species during the sampling period, but its population density was lower than *S. eridania* (Fig. 9B). Significant differences were observed among treatments only at the soybean growth stage R5.3/R5.4 ($F_{4, 12} = 4.18$; $P = 0.023$) when the Biological Control + Insecticide treatment showed the highest density pest population (0.45 ± 0.17) (Fig. 9A). Still, the Check treatment showed the lowest abundance (0.05 ± 0.05) in the same period. On the other hand, *S. cosmioides* was the non-target lepidopteran pest that showed the lowest population densities during sampling periods, compared to other species in the complex. No significant differences were found among treatments at any of the plant growth stages (Fig. 9C).

2.3.2.3 Abundance of moths and eggs

As in the 2016/2017 growing season, the highest population density of adults captured were *S. frugiperda*, and population peaks were observed at R2/R3 ($n = 23$), R5.3/R5.4 ($n = 34$), and R6 ($n = 34$). About *H. armigera*, the density of adults caught in the 2017/2018 growing season was lower than the previous season. Population peaks were noted in the plant

growth stages R5.3/R5.4 (n = 9) and R7.2 (n = 14). In the 2017/2018 growing season, the presence of low frequency of non-target lepidopteran pests, as *Elaphria agrotina* (Guenée) (Lepidoptera: Noctuidae) and *Argyrotaenia* sp. Meyrick (Lepidoptera: Tortricidae), was also observed in the Bt soybean, with population peaks at R2/R3 (n = 8) and R7.1 (n = 14), respectively (Fig. 10).

In parallel, variations were observed concerning the abundance of eggs between treatments, but without significant differences (Fig. 11). For the I + BC treatment, population peak was observed at V5 (0.20 ± 0.16 eggs/plant), as well as for the BC treatment at R1/R2 (0.13 ± 0.06 eggs/plant). For the Check (C) treatment, 3 peaks were observed, at V7/R1 (0.16 ± 0.16 eggs/plant), R2/R3 (0.11 ± 0.07 eggs/plant), and R5.4/R5.5 (0.10 ± 0.03 eggs/plant). For BC and C treatments, the greatest abundance of eggs was observed at R7.1 (Fig. 11).

2.3.2.4 Stink bugs pests

Significant differences were observed among treatments in relation to the brown stink bug *E. heros* at the R2/R3 ($F_{4, 12} = 3.63$; $P = 0.0366$), R5.4/R5.5 ($F_{4, 12} = 4.48$; $P = 0.019$), and R5.5/R5.6 ($F_{4, 12} = 3.74$; $P = 0.003354$) plant growth stages (Fig 12A). The Farmer and Biological Control treatments showed the highest abundances (1.45 ± 0.2) at the R2/R3 plant growth stage, and Biological Control + Insecticide showed the lowest population densities (0.85 ± 0.19). In contrast, at the plant growth stages R5.4/R5.5 and R5.5/R5.6, the highest abundances of *E. heros* were found in the Insecticide (5.75 ± 0.62) and Biological Control + Insecticide (19.17 ± 1.06) treatments. In these same periods, the lower population density was found in the Farmer treatment, in which the peak population of *E. heros* occurred at the R6 plant growth stage with the values ranging from 19.55 ± 1.57 to 29.60 ± 1.56 stink bugs per meter (Fig. 12A).

About the green-belly stink bug, *D. melacanthus*, no significant differences were found among treatments (Fig. 12B). *D. melacanthus* showed low frequency over the sampling periods, but at the R6 and R7.1 plant growth stage was found in all treatments with densities ranging from 0.025 ± 0.025 to 0.05 ± 0.03 , and 0.025 ± 0.025 to 0.10 ± 0.04 stink bugs per meter, respectively (Fig. 12B).

2.3.2.5 Coleopteran pests

Differently from the first season (2016/2017), the predominance of the leaf beetle *M. calcarifera* over *D. speciosa* was found. The coleopteran pest *M. calcarifera* showed the

greatest abundance, and *D. speciosa* showed low population densities (Figs. 12C and 12D). There were no significant differences between treatments in the periods evaluated for *D. speciosa* (Fig. 12C), but for *M. calcarifera* was found significant difference at R5.3/R5.4 plant growth stage ($F_{4, 12} = 12.77$; $P = 0.0003$), with Biological Control and Insecticide treatments presenting the highest population densities (Fig. 12D).

2.3.2.6 Natural enemies

The composition of the natural enemies' community was similar to the first growing season (2016/2017), highlighting the species of predators *E. connexa*, *C. externa*, *Calosoma* spp., *P. nigrispinus*, *D. luteipes*, and *Solenopsis* sp. (Fig. 13A), as well as the presence of predatory spiders, which showed the most prevalent and abundant among natural enemies (Fig. 13C), and infected larvae by the entomopathogen *Metarhizium rileyi* (Fig. 13B). Nevertheless, the incidence was lower when compared to the first growing season (2016/2017). There were no significant differences among treatments in any of the evaluated periods to predators and entomopathogen *M. rileyi* (Figs. 13A and 13B). However, for spider there was a significant difference among treatments at the plant growth R2/R3 ($F_{4, 12} = 5.15$; $P = 0.012$) (Fig. 13C). During this period, the Farmer treatment showed the lowest abundance of arthropods per meter (0.05 ± 0.03), while the Biological Control and Insecticide treatments had the highest population densities. The community of natural enemies in that same plant growth stage also showed significant differences ($F_{4, 12} = 5.76$; $P = 0.007$) (Fig. 13D), in which the Biological Control and Insecticide treatments had the highest population densities. The population peak of natural enemies was observed in the plant growth stages R5.3/R5.4 and R6, ranging from 0.35 ± 0.10 to 1.05 ± 0.23 , and 0.50 ± 0.11 to 0.92 ± 0.17 arthropods per meter (Fig. 13D).

2.3.2.7 Soybean yield and mass grain

No significant differences were observed among treatments in relation to the yield ($F_{4, 15} = 0.986$; $P = 0.321$) and weight of 1000 grains ($\chi^2_{4, 15} = 5.02$; $P = 0.284$) (Tables 3 and 4). The yield ranged between $4,081.00 \text{ kg ha}^{-1}$ and $4,478.09 \text{ kg ha}^{-1}$, and the weight of 1000 grains ranged from 156.01 g to 162.73 g (Tables 3 and 4).

2.3.2.8 Production costs and economic viability

The highest value of gross revenue was found in the Farmer treatment (1,880.77 US\$ ha⁻¹), while the lowest value in the I + BC (1,714.19 US\$ ha⁻¹). The highest effective operating cost was observed in the I + BC treatment (591.27 US\$ ha⁻¹), and the highest total operating cost in the Farmer management (384.98 US\$ ha⁻¹). In contrast, the highest values of the gross profit margin of EOC (322.1 US\$ ha⁻¹) and TOC (528.47 US\$ ha⁻¹) were observed in the Check treatment, as well as the lowest values concerning the break even point of EOC (766.85 US\$ ha⁻¹) and TOC (1,258.19 US\$ ha⁻¹). The Check treatment also had the lowest value in cost per unit of EOC (0.08 US\$ ha⁻¹) and TOC (0.13 US\$ Kg ha⁻¹), besides the largest operating profit (1,246.46 US\$), the best margin of profitability (70.23 US\$), and the best benefit–cost ratio (3.36%) (Table 5).

2.3. Discussion

The Bt (Cry1Ac) variety is an important tool in IPM-soybean. However, there is a great concern regarding the field evolving resistance and the loss of this technology. In this sense, the main strategy proposed by IRM to Bt crops has been the high-dose expression of the insecticidal protein Bt and the planting of refuge areas (non-Bt plants), since the insufficient compliance with refuge requirements are the main causes of the field-evolved resistance (Sumerford et al., 2013). Thus, the purpose of refuge areas is the production of many susceptible individuals (SS) of target pests to mate with rare resistant (RR) from Bt crop areas, resulting in heterozygous offspring (RS), which will be killed by Bt crops high-dose expression (Gould, 1998).

During monitoring pests and natural enemies' population in refuge areas (non-Bt soybeans), in general, there were no significant differences among treatments regarding the potential supply of susceptible individuals to the target pests of Bt soybean (insects per meter) in both growing seasons. However, differences in pest and natural enemies' population dynamics were observed between the two growing seasons, probably associated with abiotic (eg.: precipitation and temperature) and biotic (natural enemies) factors (Huang and Hao, 2020). Also, considerable variability in the abundance and frequency of lepidopteran larvae of Bt target pest sampled in space and time was noted, which may be related to their behavior or food preference.

Remarkably, the highest abundance of lepidopteran target pest of Bt soybean was found in the reproductive stages. Justiniano et al. (2014), assessing the abundance and

diversity of population dynamics on the refuge area of Bt soybean, also found the highest lepidopteran pest abundance at the reproductive stage. Prolonged flowering times were the most attractive to egg-laying moths and could, therefore, produce more Bt-susceptible moths than the other potential soybean growth stage. The host selection and the oviposition preference of *H. armigera*, for example, are strongly influenced by the flowering of hosts (Cunningham et al. 1998, 1999; Wu et al. 2004). Thus, this information must be considered when assessing potential refuge areas from the perspective of IPM and IRM (e.g., insecticide application, parasitoid release vs. its possible consequences by limiting the supply of susceptible individuals, and crop productivity).

In Brazil, soybean crop can be attacked by several economically important insect pests. Thus, to reduce pests damage and yield losses, soybean growers traditionally spray pesticides (Stacke et al., 2020). However, the extensive use of insecticides can limit refuge purposes (Bueno et al., 2020). According to Onstad et al. (2013), the efficacy of Bt broccoli refuge areas can be compromised by spraying insecticide, resulting in negative effects of long-term control of *P. xylostella* populations. Baker et al. (2008) observed that unsprayed refuges of Bt cotton produced more pupae of *H. armigera* and *Helicoverpa punctigera* Wallengren (Lepidoptera: Noctuidae) than sprayed non-Bt cotton and Bt cotton. In the current study, the old world bollworm, *H. armigera*, was the only target pest of Bt soybean that reached ET. According to Pitta and Netto (2016), the ET to control *Helicoverpa* spp. and *C. virescens* in the reproductive soybean growth stage is 2 larvae per meter. This species is considered an important lepidopteran pest in soybean crop and has been causing significant damage in both the vegetative and the reproductive plant growth stage (Stacke et al., 2018).

On the opposite, in Mato Grosso do Sul state, Brazil, *A. gemmatalis* and *C. includens* were the most abundant lepidopteran target pest of Bt soybean sampled on non-Bt soybean (Justiniano et al., 2014). In this case, the lepidopteran pests achieved the ET leading to spray methomyl (IRAC Group 1A) and flubendiamide insecticides (IRAC Group 28). In situations where the population reaches the ET in refuge areas, has been recommended the preferential use of specific insecticides to the target insect and selective to natural enemies to reduce the risk of bioecological imbalance and the impacts on the generation of potentially susceptible individuals in the refuge area (Bernardi et al., 2016). In this context, the careful choice of insecticides is highlighted, given their lethal and sublethal effects on the target pests, since the refuge function is mainly determined by the abundance of Bt-susceptible moths produced and

by the mating synchrony between moths from Bt-cotton and refuge crops (Tabashnik et al. 2009; Huang et al. 2011; Barros et al., 2020).

In this study, besides releasing *T. pretiosum*, the insecticide flubendiamide was used, considered selective (harmless) to natural enemies and specific to lepidopteran pests (Bueno et al., 2011; Khan et al., 2015; Paiva et al., 2018; Adams et al., 2016; Bolzan et al., 2019). However, it was observed that both strategies were inefficient, since the population of *H. armigera* in the treatments that were not sprayed also reduced, as well as the number of eggs of lepidopteran pests in the control treatment, where *T. pretiosum* was not released. We hypothesized that the low performance of the release of *T. pretiosum* might be related to bad weather conditions at the time of release (rain), the low density of eggs, and the predatory effect of ants (Favetti, 2017). Moreover, the natural or conservative biological control of pests by natural enemies also contributed to the reduction of lepidopteran populations in the area, both immature (eggs and larvae) and adult (moths) phases. In this study, several natural enemies with the potential to reduce the pest population of lepidopteran pests were observed, such as *E. connexa*, *C. externa*, *Calosoma* spp., *P. nigrispinus*, *D. luteipes*, *Solenopsis* sp., and predatory spiders.

The direct benefits of natural biological pest control in Bt and non-Bt cultivation areas have been widely demonstrated (Naranjo et al., 2011; Romeis et al., 2019). Therefore, it is believed that the low abundance of lepidopteran pests and their eggs in the treatments might be associated with the “halo effect”, a phenomenon in which Bt plants act as a dead-end trap crop, conferring some protection for non-transgenic plants (Wan et al., 2012; Dively et al. 2018). In the Midwest of the US, was observed a regional suppression of *O. nubilalis* after a long period of Bt maize cultivated, which has benefited Bt maize growers as well those who did not grow Bt maize (Hutchison et al., 2010). Naranjo et al. (2011) highlight that Bt cotton has led to large reductions in the abundance of targeted pests and benefited non-Bt cotton adopters and even growers of other crops affected by polyphagous target pests. Wan et al. (2012) revealed that Bt cotton decreased significantly population density of pink bollworm in China, 91% of eggs and 95% of larvae on non-Bt cotton after 11 years of Bt cotton widely adoption. Thus, the dead-end trap Bt soybean serves as a sink for pests, preventing their movement from the Bt soybean to the refuge area.

In this sense, secondary pests species have become more prevalent in Bt crops worldwide (Naranjo, 2011), such as whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), and soybean stem fly *Melanagromyza sojae* Zehntner (Diptera: Agromyzidae)

in Brazil (Bernardi et al., 2014; Silva et al., 2014; Bortolotto et al., 2015; Czepak et al., 2018; Machado et al., 2020; Pozebon et al., 2020). In North of China, field trials showed that mirid bugs have progressively increased population sizes and acquired pest status in cotton (Lu et al. 2012). Similarly, in South Dakota, USA, the Bt maize continuous cultivation has favored the increase of *Striacosta albicosta* (Smith) (Lepidoptera: Noctuidae) (Catangui and Berg, 2016). Here we found the predominance in the refuge area of *Spodoptera* spp. and stink bugs complex as the most important non-target pests of current commercial Bt soybean. Although, only *E. heros* achieved the ET in both growing seasons.

In the management of soybean refuge areas, it is important to avoid pods/grains damage and yield losses by *E. heros*. The reproductive stage of soybean when the stink bug complex can affect the yield is also when the higher concentration of the main lepidopteran target pests of Bt soybean are found in the refuge area. Thus, considering the restricted active ingredients available in the Brazilian market to control the stink bug complex, where most are wide control spectrum and non-selective to natural enemies, there is a possible reduction of susceptible individuals' generation after spraying the refuge areas. In this case, biological control is strongly suggested, such as the egg parasitoids *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae) and *Trissolcus basalus* Wollaston (Hymenoptera: Platygasteridae) (Silva et al., 2014; Zantedeschi et al., 2018). Another alternative is the use of the Bt protein that confers resistance to hemipteran pests. For example, the transgenic event expressing Cry51Aa2.834_16, that targets hemipteran and thysanopteran pests such as *Lygus* and thrips on cotton (Akbar et al., 2019).

The *Spodoptera* spp. complex consists of important species that attack soybean in the vegetative and reproductive growth stages (Bernardi et al., 2014b; Machado et al., 2020). The most important species found in current study were *S. frugiperda*, *S. eridania*, and *S. cosmioides*, all of them are non-target of Bt soybean. In the study, *S. eridania* was predominant in the samples. This pest can cause damage to pod and affects soybean yield. The *Spodoptera* complex, as well as stink bugs, can reduce the effectiveness of the refuge area if they achieve an ET and chemical control measures, such as insecticide application, are taken. Fortunately, for lepidopteran pests, several specific and selective insecticides are recommended for use as they do not disturb the beneficial organisms. The IPM propose to soybean that can support *Spodoptera* spp. complex management is the pyramided Bt soybean expressing Cry1A.105, Cry2Ab2, and Cry1Ac from Bt toxin that is approved for commercial use in Brazil (Carrière et al., 2016; Bacalhau et al., 2020). Therefore, aiming at future studies

and prospection in GMO systems with the interaction of non-target pest species and Bt soybean technology, it is suggested the pyramidation of insecticidal proteins with different modes of action for the control of *Spodoptera* spp. complex and pest bugs, as well as the new plant breeding techniques RNAi and CRISPR-Cas9 (Naranjo et al., 2020).

In addition to the bioecological purpose of the refuge area, it is also essential to consider the cost of production and the economic viability of the pest management adopted. For soybean growers, the refuge area implies an intertemporal economic trade-off, which they must forego the theoretical short-run benefits of Bt soybean to preserve the efficacy of technology over a longer period (Frisvold and Reeves, 2008; Alphey and Bonsall, 2018). Carvalho et al. (2016) analyzed the production cost and economic viability of Bt (Cry 1Ac) and RR soybean cultivation (glyphosate-tolerant soybean and non-Bt) and found that Bt soybean showed better economic performance compared to RR soybean due to lower outgoing with labor, charges, pesticides, and others.

Despite this, these authors did not analyze the economic aspects of IPM proposals for refuge areas (non-Bt soybean). In our study, it was observed that although the gross revenue (US \$ ha⁻¹) was lower than most of the treatments in 2016/2017 growing season, the Check treatment showed the best indicators of profitability and economic feasibility in both growing seasons, which were much more attractive and promising such as the Gross Profit Margin of EOC and TOC (US \$ ha⁻¹), the Margin of Profitability (%) and the Benefit-cost ratio (%). Also, the Check treatment proved to be more interesting compared to the others because it provided social and environmental benefits (less use and/or reduced application of insecticides, labor and machinery for spraying insecticides, less exposure of man to agrochemicals and less contamination of the groundwater). These factors, combined with the satisfactory production of susceptible individuals of target pest of Bt soybean, suggest that Check treatment is the best pest management strategy to be adopted in the refuge areas of Bt soybean. However, the economic performance of refuge area also depends on the company's marketing incentive and the culture of the countries in which Bt crops are deployed, such as ongoing education and extension support for soybean growers (MAPA, 2017).

2.4. Conclusion

If we accept the larvae abundance as source of generation susceptible moth, our findings suggest that the Check treatment through without the adoption of IPM strategies and methods in the refuge areas for the Bt soybean pest lepidopterans, but only the the

management of pest stink bugs in refuge areas of Bt soybean associated with natural biological control provided satisfactorily the potential susceptible individuals (larvae by meter of target pests of soybean), as well as the best indicators of economic-financial viability and productivity.

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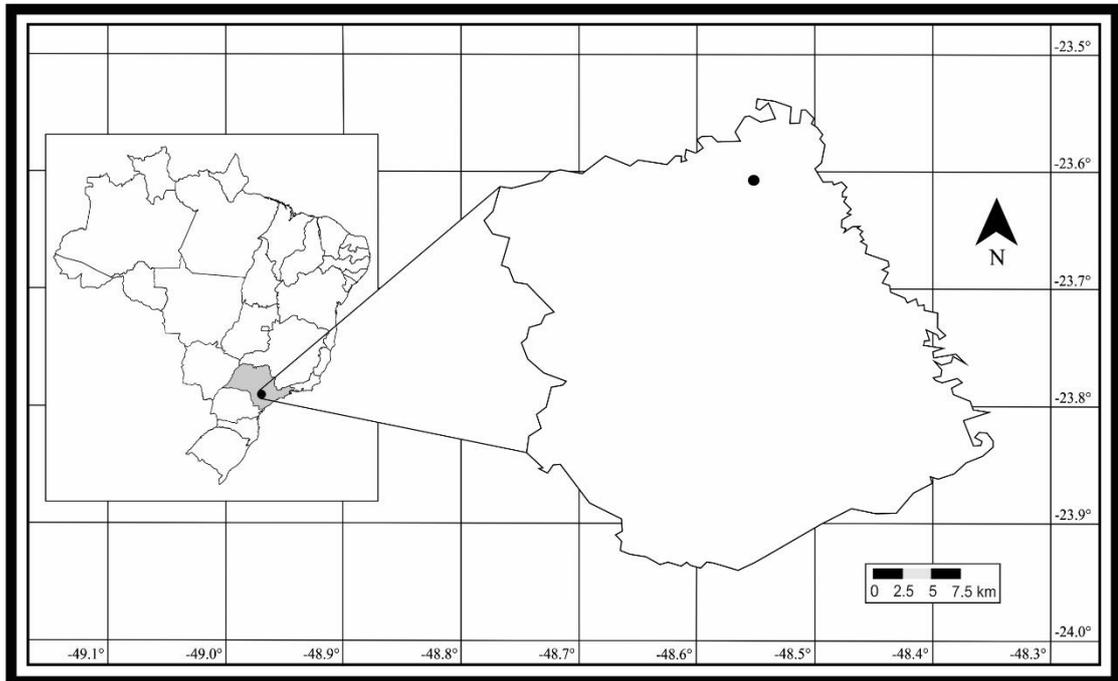
Figure and Table Legends

Figure 1. Location of the study site in the municipality of Buri, southeast of São Paulo state, Brazil.

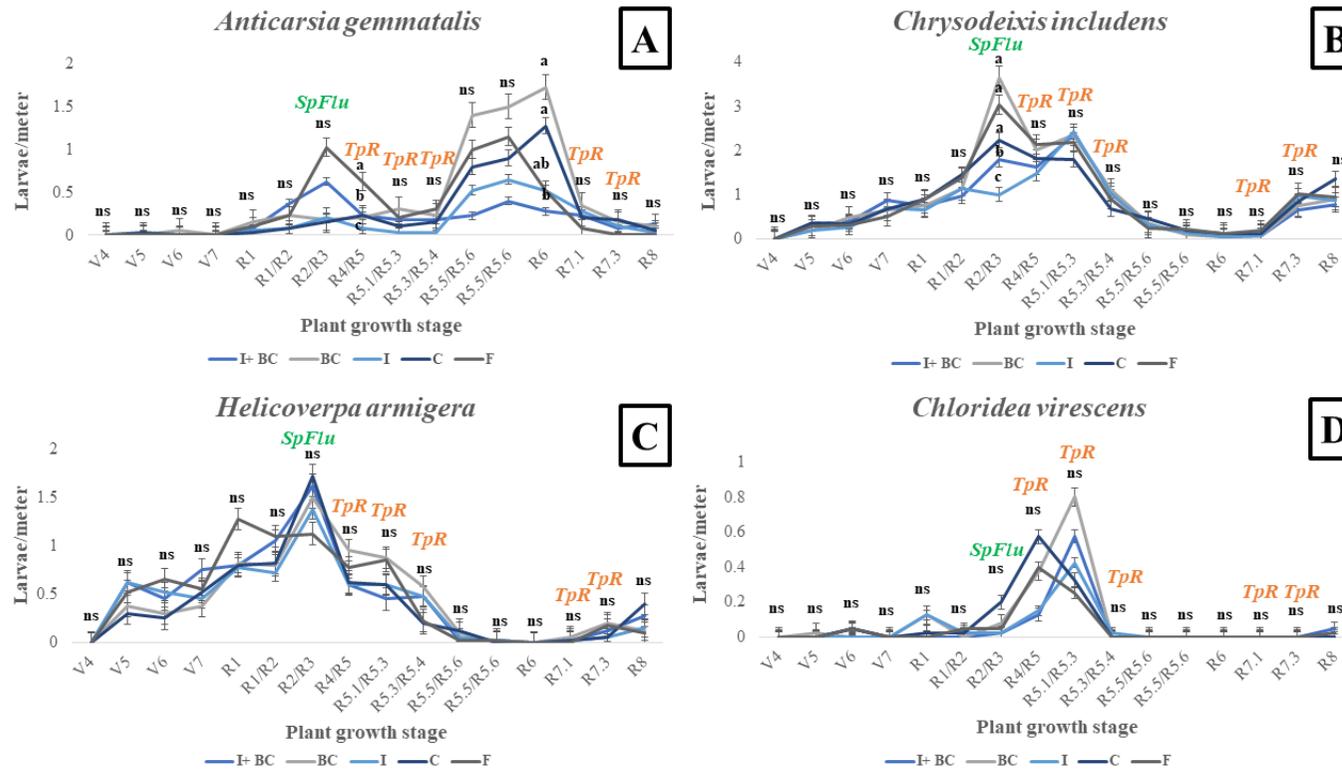


Figure 2. Mean population dynamics (\pm SE) of the lepidopteran target pests of Bt soybean across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2016/2017 growing season in Buri, São Paulo state, Brazil.

*I + BC: Insecticide (Spray to control lepidopteran-pest according ET and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according ET), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); SpFlu: Spray flubendiamide insecticide (70 mL of Belt® per hectare) aiming control species of Heliothinae complex (*H. armigera* and *C. virescens*); TpR: Mass field release of *Trichogramma pretiosum* egg parasitoid at rate of 120.000 individuals per hectare to control lepidopterans-pest; Means (\pm SE) followed by the same letter in the graph plant growth stage period don't differ statistically from each treatment according 95% confidence intervals (C.I) ($\alpha=0.05$); ns: Not significant ($\alpha=0.05$).

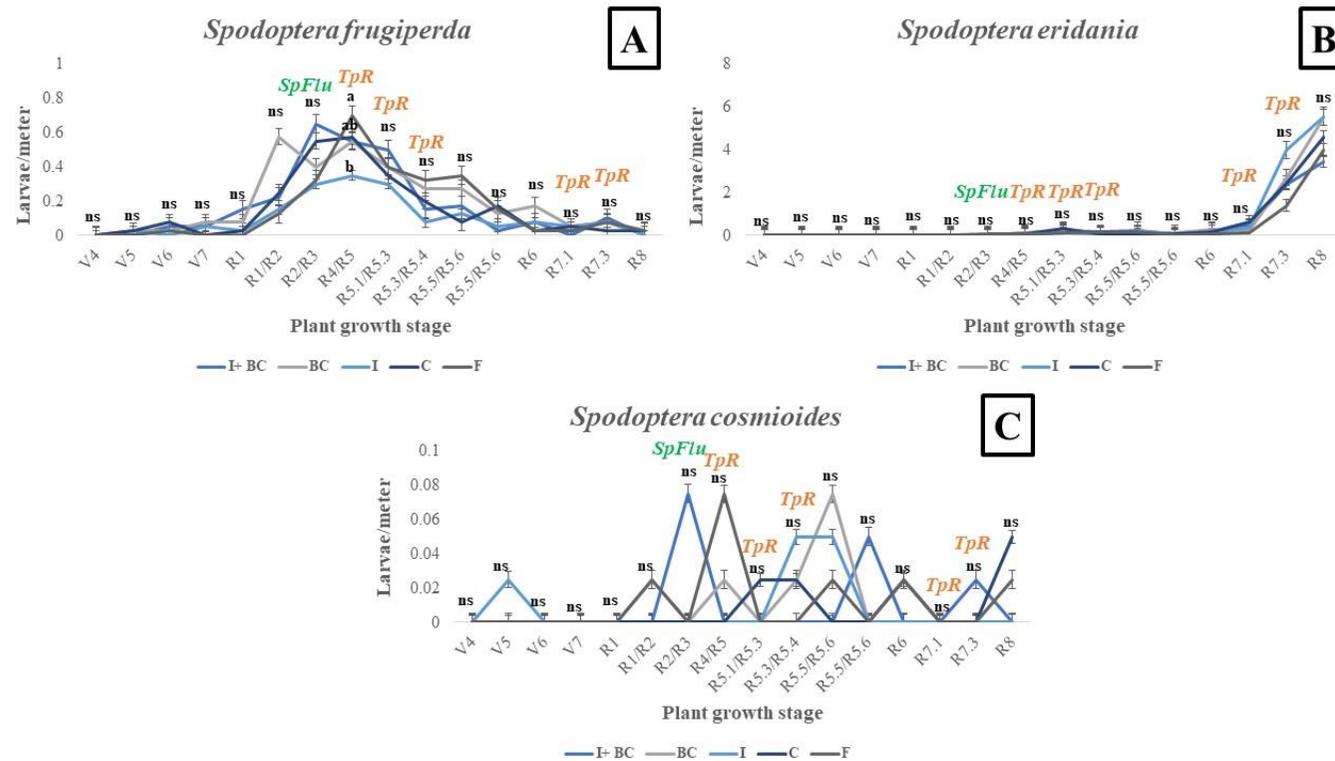


Figure 3. Mean population dynamics (\pm SE) of the lepidopteran non-target pests of Bt soybean across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2016/2017 growing season in Buri, São Paulo state, Brazil.

*I + BC: Insecticide (Spray to control lepidopteran-pest according economic threshold) and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according economic threshold), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); SpFlu: Spray flubendiamide insecticide (70 mL of Belt® per hectare) aiming control species of Heliothinae complex (*H. armigera* and *C. virescens*); TpR: Mass field release of *Trichogramma pretiosum* egg parasitoid at rate of 120.000 individuals per hectare to control lepidopterans-pest; Means (\pm SE) followed by the same letter in the graph plant growth stage period don't differ statistically from each treatment according 95% confidence intervals (C.I) ($\alpha=0.05$); ns: Not significant ($\alpha=0.05$).

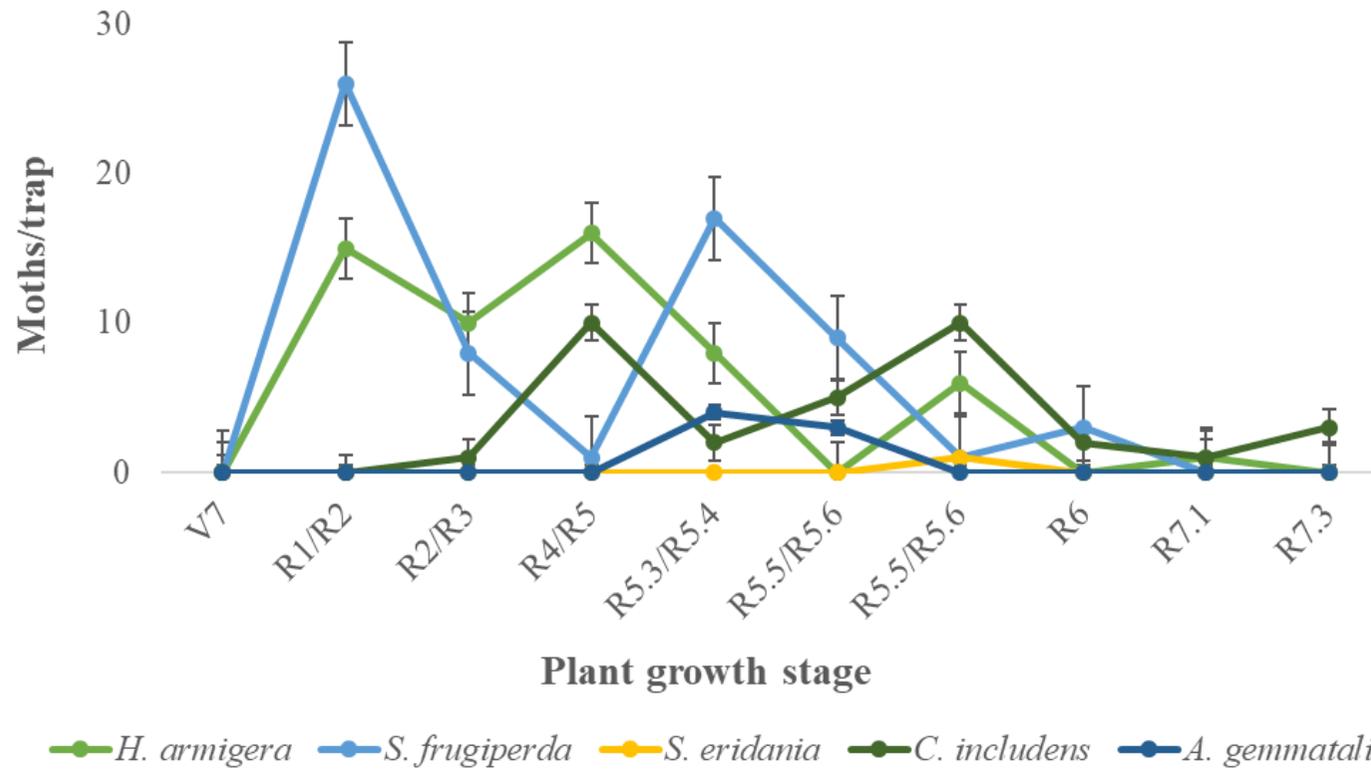


Figure 4. Mean population dynamics (\pm SE) of the main lepidopteran pest adults of soybean crop caught on the surface of plastic delta trap with pheromone baits to *S. frugiperda*, *C. includens*, and *H. armigera* on non-Bt soybean across the plant growth stage on 2016/2017 growing season in Buri, São Paulo state, Brazil.

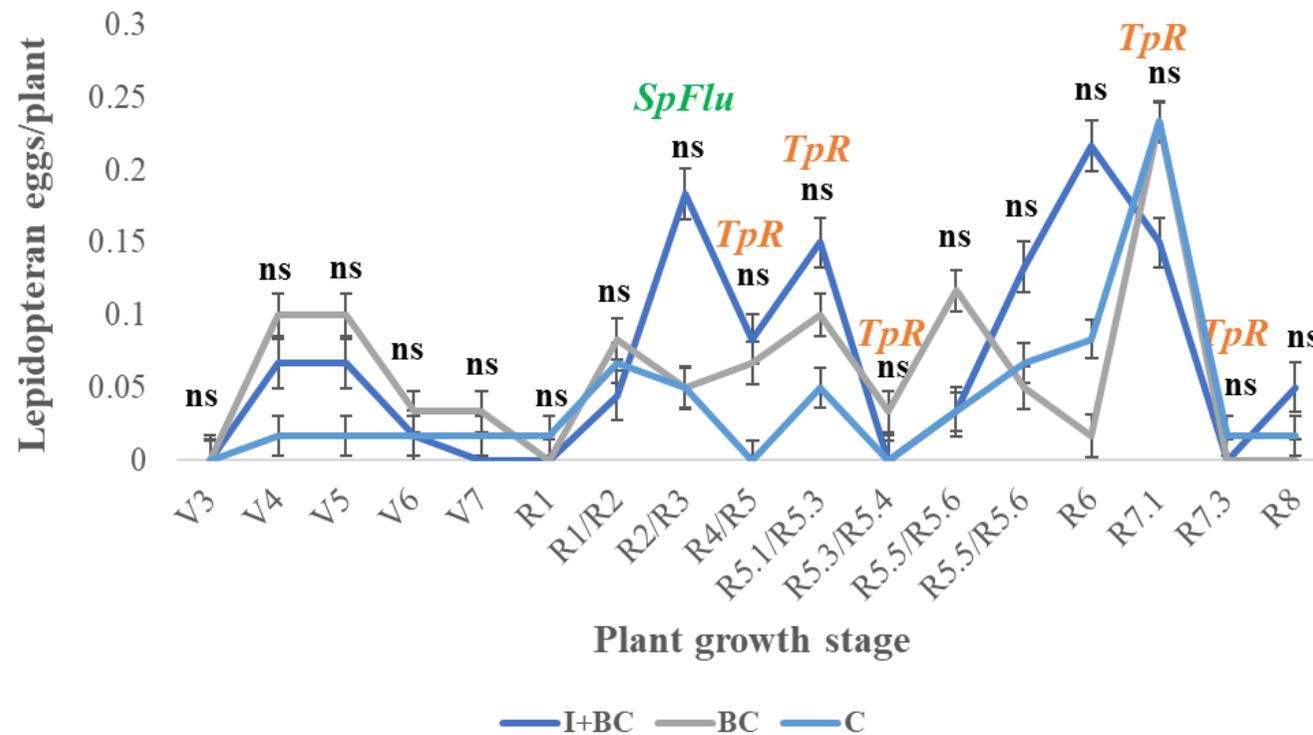


Figure 5. Mean population dynamics (\pm SE) of lepidopteran pest eggs on non-Bt soybean across the plant growth stage according to pest management strategies for refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) during 2016/2017 growing season in Buri, São Paulo state, Brazil.

*I + BC: Insecticide (Spray to control lepidopteran-pest according ET) and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according ET), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); SpFlu: Spray flubendiamide insecticide (70 mL of Belt® per hectare) aiming control species of Heliothinae complex (*H. armigera* and *C. virescens*); TpR: Mass field release of *Trichogramma pretiosum* egg parasitoid at rate of 120.000 individuals per hectare to control lepidopterans-pest; Means (\pm SE) followed by the same letter in the graph plant growth stage period don't differ statistically from each treatment according Tukey's test ($P \leq 0.05$).; ns: Not significant according Tukey's test ($P > 0.05$).

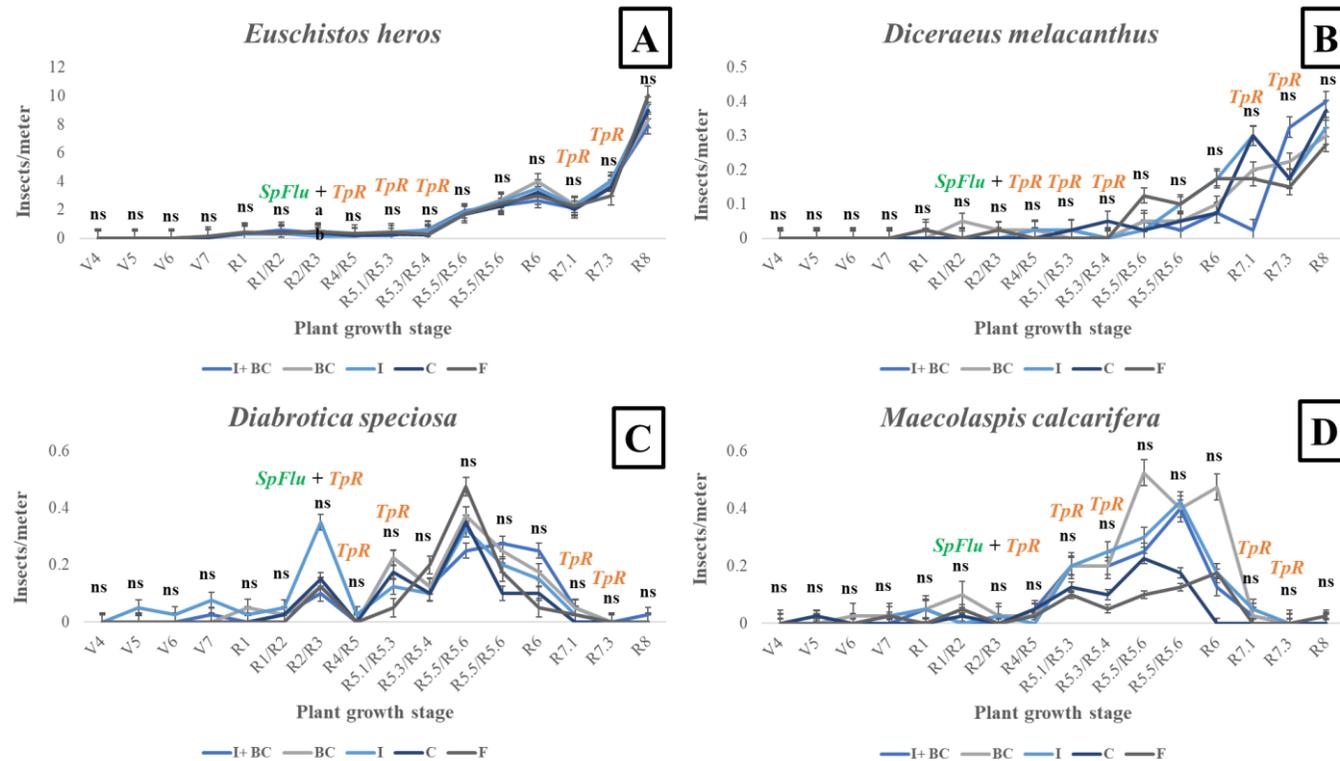


Figure 6. Mean population dynamics (\pm SE) of the non-target pest of Bt soybean (Cry 1Ac) hemipterans stink bugs (*E. heros* and *D. melacanthus*) and coleopterans leaf beetle (*D. speciosa* and *M. calcarifera*) across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2016/2017 growing season in Buri, São Paulo state, Brazil.

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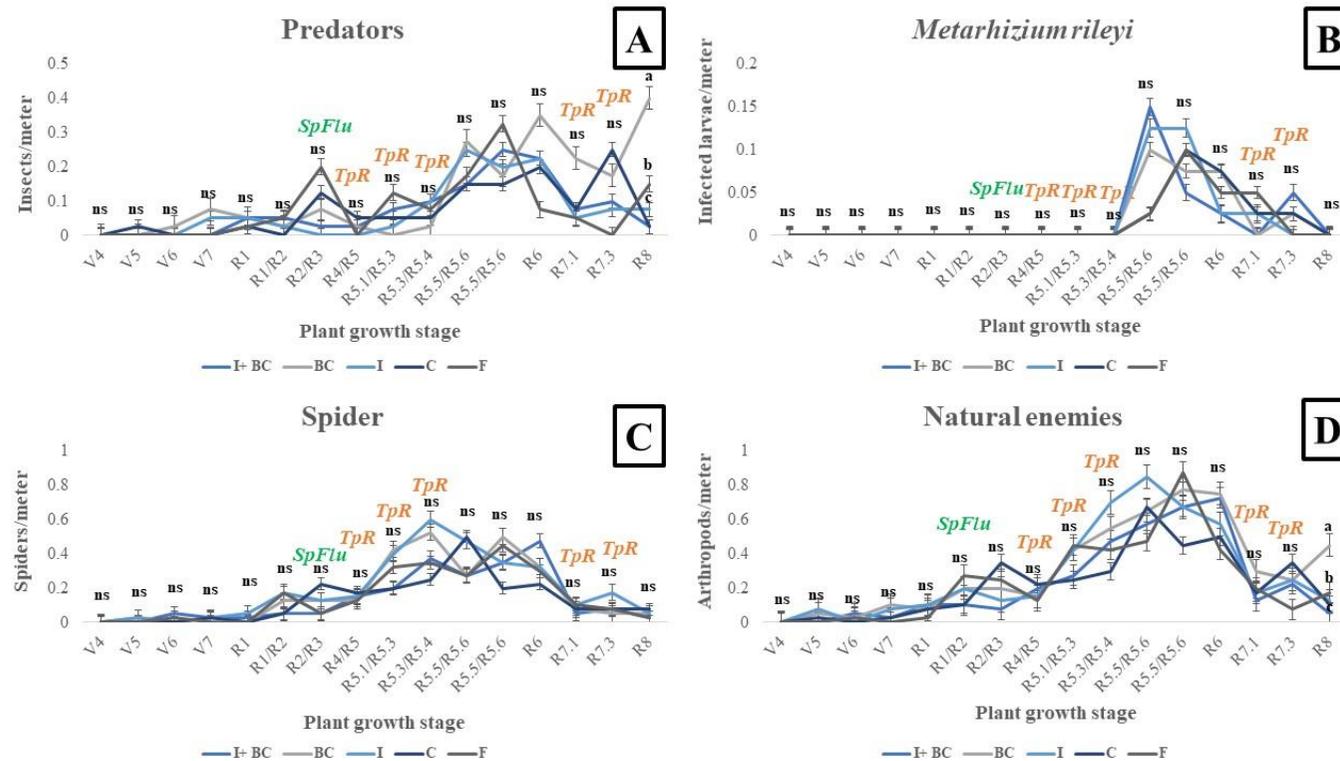


Figure 7. Mean population dynamics (\pm SE) of the beneficial arthropods composed of predators (ladybugs, green lacewings, assassin bugs, soldier beetle, earwigs, big-eyed bugs, rove beetles, ants), entomopathogen fungi, spider, and total natural enemy community (predators, *M. rileyi* and spiders) on non-Bt soybean across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2016/2017 growing season in Buri, São Paulo state, Brazil.

*I + BC: Insecticide (Spray to control lepidopteran-pest according ET) and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according ET), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); SpFlu: Spray flubendiamide insecticide (70 mL of Belt® per hectare) aiming control species of Heliothinae complex (*H. armigera* and *C. virescens*); TpR: Mass field release of *Trichogramma pretiosum* egg parasitoid at rate of 120.000 individuals per hectare to control lepidopterans-pest; Means (\pm SE) followed by the same letter in the graph plant growth stage period don't differ statistically from each treatment according 95% confidence intervals (C.I) ($\alpha=0.05$); ns: Not significant ($\alpha=0.05$).

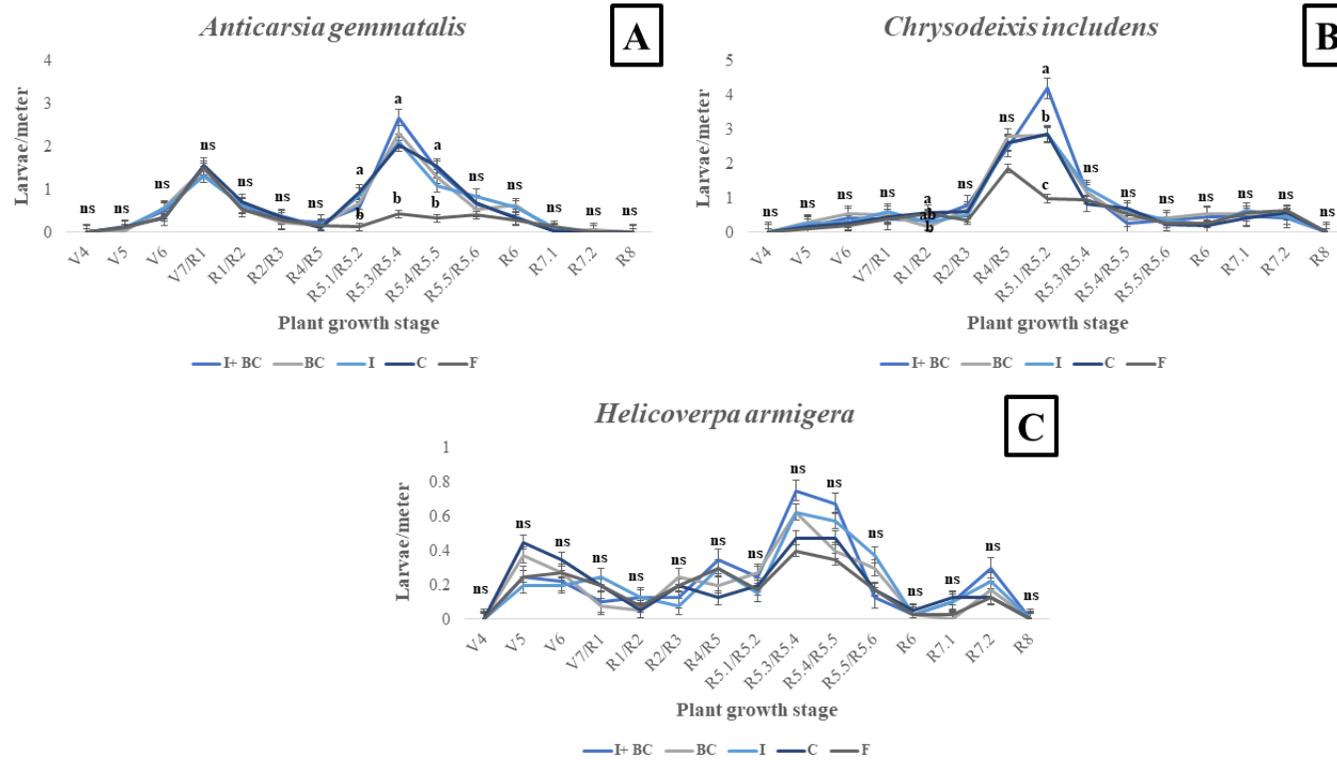


Figure 8. Mean population dynamics (\pm SE) of the lepidopteran target pests of Bt soybean across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2017/2018 growing season in Buri, São Paulo state, Brazil.

*I + BC: Insecticide (Spray to control lepidopteran-pest according ET) and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according ET), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); Means (\pm SE) followed by the same letter in the graph plant growth stage period don't differ statistically from each treatment according 95% confidence intervals (C.I) ($\alpha=0.05$); ns: Not significant ($\alpha=0.05$).

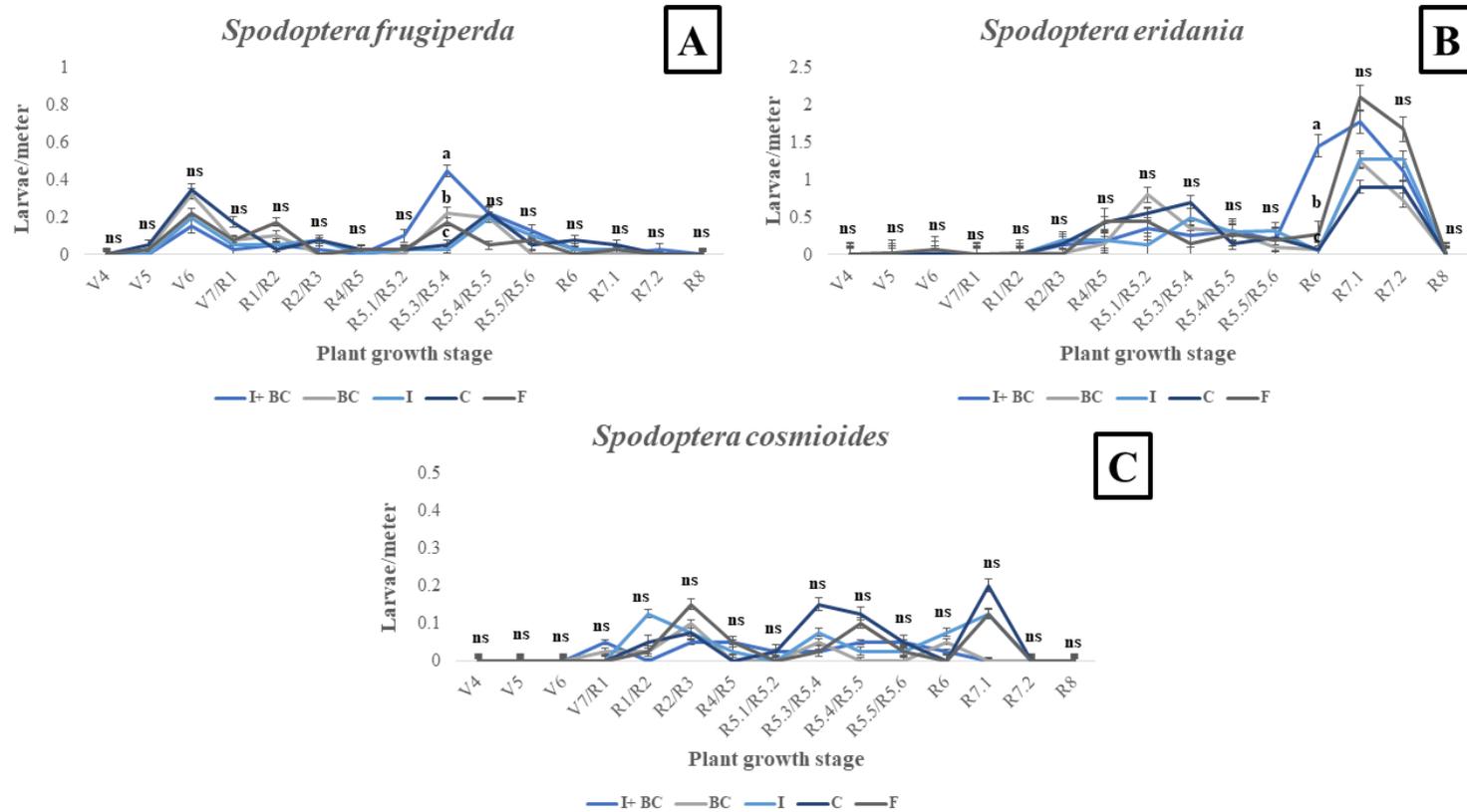


Figure 9. Mean population dynamics (\pm SE) of the lepidopteran non-target pests of Bt soybean across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2017/2018 growing season in Buri, São Paulo state, Brazil.

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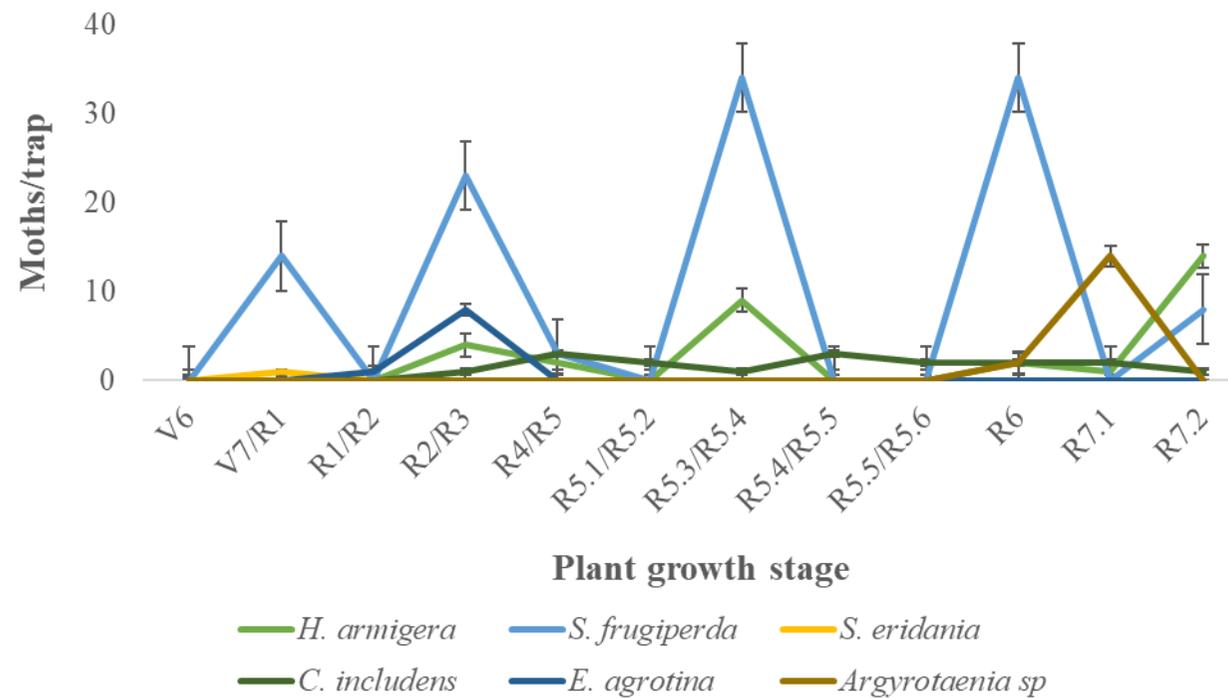


Figure 10. Mean population dynamics (\pm SE) of the main lepidopteran pest adults of soybean crop caught on the surface of plastic delta trap with pheromone baits to *S. frugiperda*, *C. includens*, and *H. armigera* on non-Bt soybean across the plant growth stage on 2017/2018 growing season in Buri, São Paulo state, Brazil.

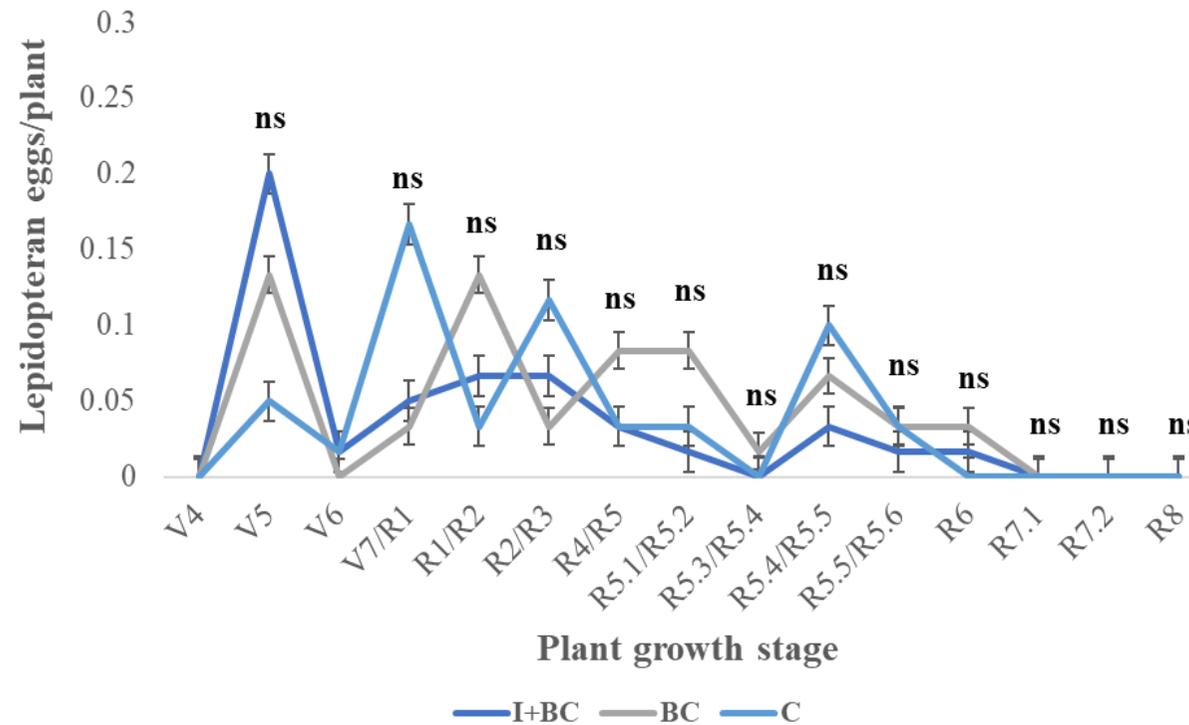


Figure 11. Mean population dynamics (\pm SE) of lepidopteran pest eggs on non-Bt soybean across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2017/2018 growing season in Buri, São Paulo state, Brazil.

*I + BC: Insecticide (Spray to control lepidopteran-pest according ET) and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according ET), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); Means (\pm SE) followed by the same letter in the graph plant growth stage period don't differ statistically from each treatment according Tukey's test ($P \leq 0.05$); ns: Not significant according Tukey's test ($P > 0.05$).

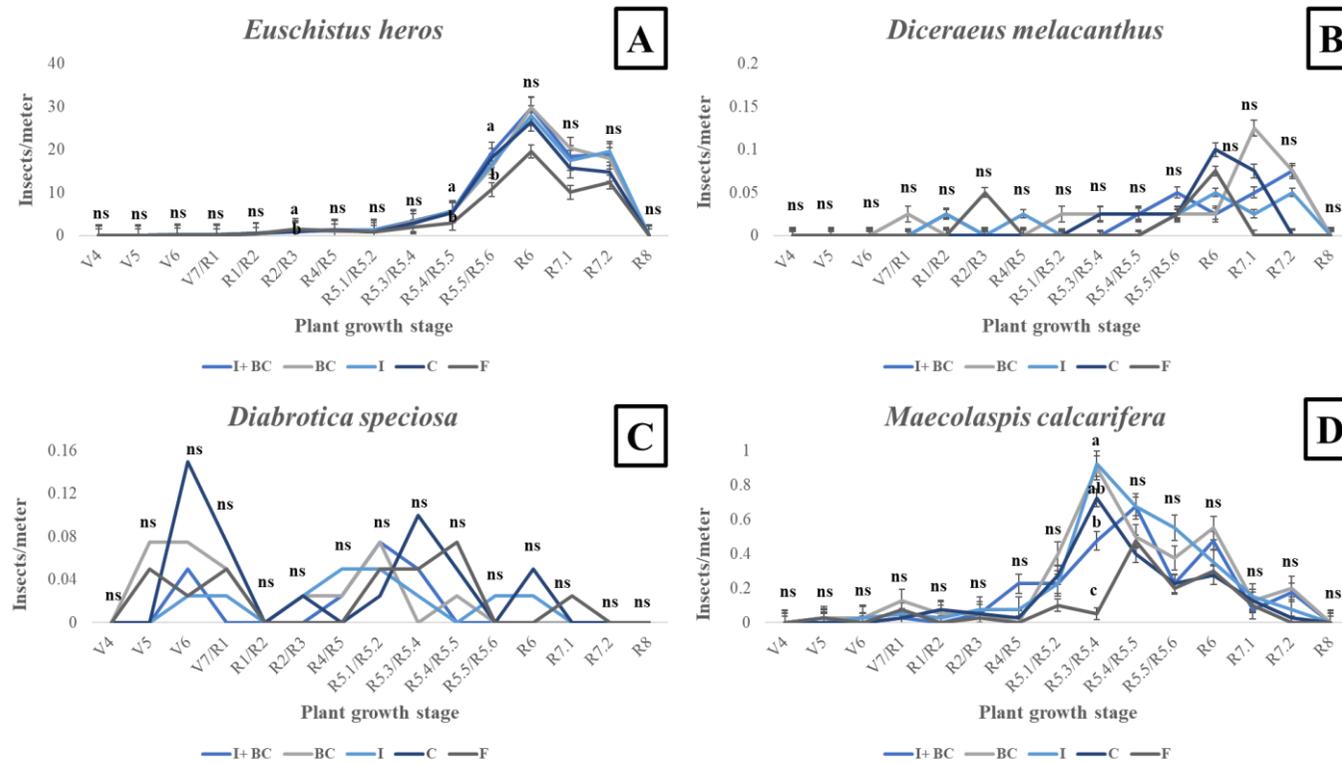


Figure 12. Mean population dynamics (\pm SE) of the non-target pest of Bt soybean (Cry 1Ac) hemipterans stink bugs (*E. heros* and *D. melacanthus*) and coleopterans leaf beetle (*D. speciosa* and *M. calcarifera*) across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2017/2018 growing season in Buri, São Paulo state, Brazil.

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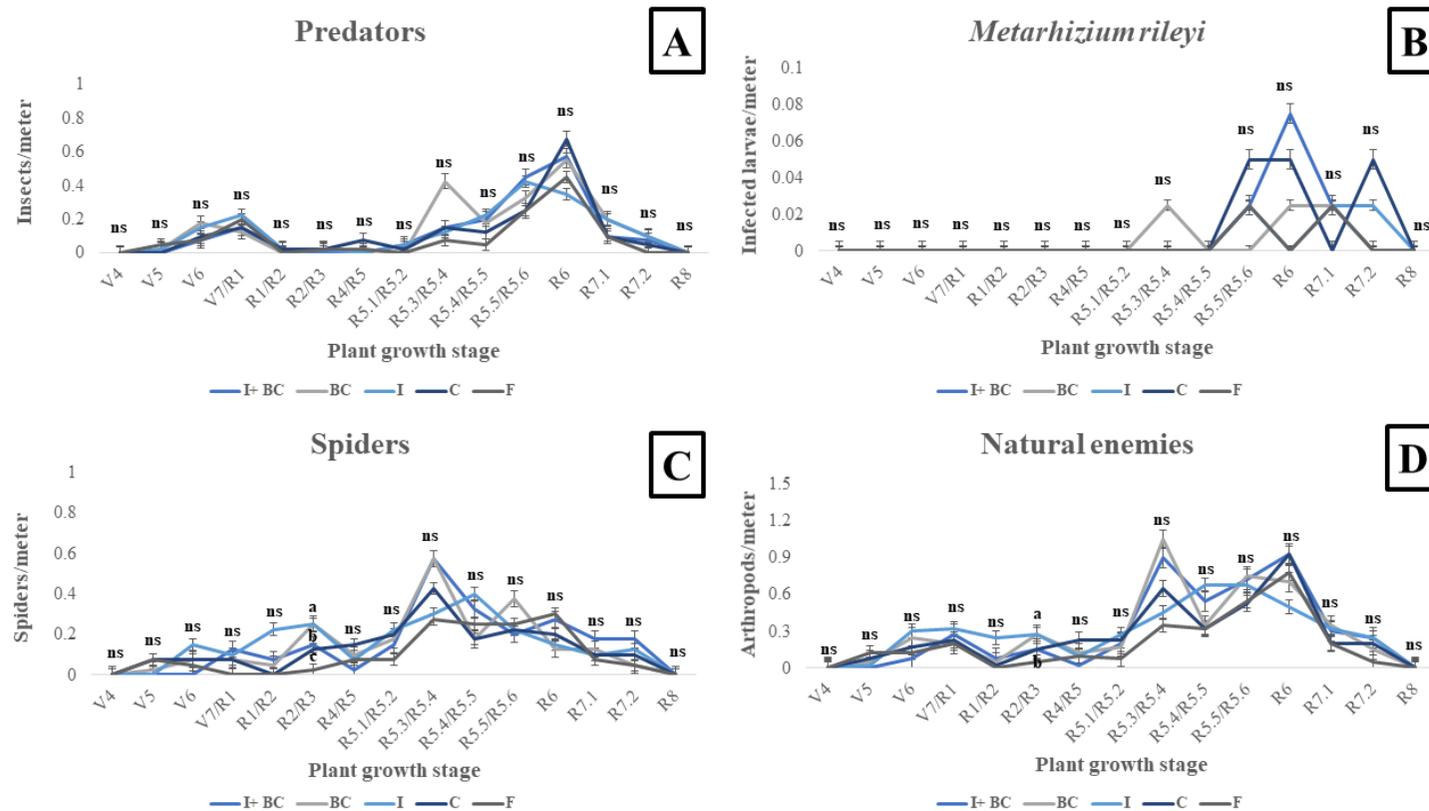


Figure 13. Mean population dynamics (\pm SE) of the beneficial arthropods composed of predators (ladybugs, green lacewings, assassin bugs, soldier beetle, earwigs, big-eyed bugs, rove beetles, ants), entomopathogen fungi, spider, and total natural enemy community (predators, *M. rileyi* and spiders) on non-Bt soybean across the plant growth stage according to pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2017/2018 growing season in Buri, São Paulo state, Brazil.

*I + BC: Insecticide (Spray to control lepidopteran-pest according ET) and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according ET), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); Means (\pm SE) followed by the same letter in the graph plant growth stage period don't differ statistically from each treatment according 95% confidence intervals (C.I) ($\alpha=0.05$); ns: Not significant ($\alpha=0.05$).

Table 1. Phytosanitary products sprayed during the 2016/2107 growing season on refuge area treatments of Bt soybean (Cry 1Ac).

Commercial product	Active ingredient	Field rate (L ha ⁻¹)	Plant growth stages	Targets
Unizeb Gold	Mancozeb (75%)	1.5	R1	Soybean foliar disease ¹
Orthene 750 BR	Acephate (75%)	0.5	R1	Stink bugs and lepidopterans-pest ^{3,4}
Fox	Trifloxistrobin (15%) + Prothioconazol (17.5%)	0.4	R1	Soybean foliar disease
Aureo	Soybean oil methyl ester (72%)	0.25	R1	Adjuvant ²
Belt	Flubeandiamide (48%)	0.07	R2/R3	Lepidopterans-pest ³
Elatus	Azoxistrobin (30%) + Benzovindiflupir (15%)	0.2	R5.1/R5.2	Soybean foliar disease
Aureo	Soybean oil methyl ester (72%)	0.25	R5.1/R5.2	Adjuvant
Orthene	Acephate (75%)	0.5	R5.1/R5.2	Stink bugs and lepidopterans-pest
Elatus	Azoxistrobin (30%) + Benzovindiflupir (15%)	0.2	R5.3	Soybean foliar disease
Eforia	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	R5.3	Stink bugs
Aureo	Soybean oil methyl ester (72%)	0.2	R5.3/R5.4	Adjuvant
Orthene 750 BR	Acephate (75%)	0.5	R5.3/R5.4	Stink bugs and lepidopterans-pest
Orthene 750 BR	Acephate (75%)	1.0	R5.4/R5.5	Stink bugs and lepidopterans-pest
Curyom 550 EC	Profenofos (50%) + Lufenuron (5%)	0.25	R5.4/R5.5	Lepidopterans-pest
Score flexi	Propiconazol (25%) + Difenconazol (25%)	0.2	R5.4/R5.5	Soybean foliar disease
Aureo	Soybean oil methyl ester (72%)	0.5	R5.4/R5.5	Adjuvant
Score flexi	Propiconazol (25%) + Difenconazol (25%)	0.2	R6	Soybean foliar disease
Eforia	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	R6	Stink bugs
Aureo	Soybean oil methyl ester (72%)	0.5	R6	Adjuvant

¹Soybean rust (*Phakopsora pachyrhizi*) and late-season soybean diseases (*Microsphaera diffusa*, *Corynespora cassicola*);²Spreader-sticker surfactant to improve fungicide performance;³ Neotropical brown stink bug (*E. heros*);⁴Heliothinae complex (*Helicoverpa armigera* and *Chloridea virescens*).

Table 2. Phytosanitary products sprayed during the 2017/2018 growing season on refuge area treatments of Bt soybean (Cry 1Ac).

Commercial product	Active ingredient	Field rate (L ha ⁻¹)	Plant growth stages	Targets
Pirate	Clorfenapyr (24%)	0.4	V4/V5	Lepidopterans-pest ⁴
Bazuka	Methomyl (21.6%)	0.8	V4/V5	Lepidopterans-pest
Fox	Trifloxistrobin (15%) + Prothioconazol (17.5%)	0.4	V6/V7	Soybean foliar disease
Vegetal Oil EC	Esters of vegetable fatty acids (93%)	0.4	V6/V7	Adjuvant ²
Eforia	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	V6/V7	Stink bugs ³
Orthene 750 BR	Acephate (75%)	1.0	R2/R3	Stink bugs and lepidopterans-pest
AG - Bem	Adhesive resin (37.5%) + Anionic surfactant (12.5%)	0.025	R2/R3	Adjuvant
Fox	Trifloxistrobin (15%) + Prothioconazol (17.5%)	0.4	R4/R5	Soybean foliar disease ¹
Vegetal Oil EC	Esters of vegetable fatty acids (93%)	0.5	R4/R5	Adjuvant
AG - Bem	Adhesive resin (37.5%) + Anionic surfactant (12.5%)	0.025	R4/R5	Adjuvant
Galil SC	Bifentrin (5%) + Imidacloprid (25%)	0.25	R5.4/R5.5	Stink bugs
Actara 250 WG	Tiametoxan (25%)	1.4	R5.4/R5.5	Stink bugs

¹Soybean rust (*Phakopsora pachyrhizi*) and late-season soybean diseases (*Microsphaera diffusa*, *Corynespora cassicola*);²Spreader-sticker surfactant to improve fungicide performance;³ Neotropical brown stink bug (*E. heros*);⁴Heliothinae complex (*Helicoverpa armigera* and *Chloridea virescens*).

Table 3. The yield (kg ha⁻¹) of soybean crop under different pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2016/2017 and 2017/2018 growing seasons in Buri, São Paulo state, Brazil.

	2016/2017	2017/2018
Treatments	Yield (kg ha ⁻¹)	
I + BC	4921.67±241.12 ns	4081±109.04 ns
BC	4803.21±158.94	4109.16±190.39
I	4753.17±158.28	4163.23±102.64
C	4763.81±258.04	4225.69±71.27
F	4789.45±321.66	4478.09±187.02
F	0.0818	1.2792
P	0.9868	0.3218
df treat	4	4
df resid	15	15

*I + BC: Insecticide (Spray to control lepidopteran-pest according ET) and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according ET), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); Means (±SE) followed by the same letter in the column don't differ statistically from each treatment according Tukey's test ($P \leq 0.05$).; ns: Not significant according Tukey's test ($P > 0.05$).

Table 4. The 1000-grains weight (g) of soybean crop under different pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) on 2016/2017 and 2017/2018 growing seasons in Buri, São Paulo state, Brazil.

	2016/2017	2017/2018
Treatments	1000-grain weight (g)	
I + BC	156.43±1.60 ns	156.68±0.89 ns
BC	148.64±1.09	161.02±1.12
I	152.26±1.24	157.54±0.99
C	156.01±1.22	158.83±0.69
F	152.93±1.55	162.73±1.36
Chi-square	4.5616	5.0256
P	0.3353	0.2847
df treat	4	4
df resid	15	15

*I + BC: Insecticide (Spray to control lepidopteran-pest according ET) and Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), BC: Biological Control (*T. pretiosum* egg parasitoid mass release according eggs densities), I: Insecticide (Spray to control lepidopteran-pest according ET), C: Check (Control soybean stink bugs complex) and F: Farmer (Soybean grower phytosanitary practices management); Means (±SE) followed by the same letter in the column don't differ statistically from each treatment according Tukey's test ($P \leq 0.05$).; ns: Not significant according Tukey's test ($P > 0.05$).

Table 5. Indicators of profitability and economic feasibility of pest management strategies on refuge area (non-Bt soybean) of Bt soybean (Cry 1Ac) during 2016/2017 and 2017/2018 growing seasons in Buri, São Paulo state, Brazil.

	2016/2017					2017/2018				
	I + BC	BC	I	C	F	I + BC	BC	I	C	F
Gross revenue (US\$ ha ⁻¹) ¹	1,808.02	1,764.38	1,746.09	1,750.28	1,758.87	1,714.19	1,726.04	1,748.72	1,774.93	1,880.77
Effective operating cost (US\$ ha ⁻¹)	664.35	647.49	446.01	369.02	427.77	591.27	585.12	495.15	322.1	384.98
Total operating cost (US\$ ha ⁻¹)	925.1	897.17	687.26	578.3	639.63	826.49	821.54	732.4	528.47	663.01
Gross profit margin of EOC (US\$ ha ⁻¹) ²	172.15	172.5	291.49	374.31	311.18	189.92	194.99	253.17	451.06	388.54
Gross profit margin of TOC (US\$ ha ⁻¹) ³	95.44	96.66	154.07	202.66	174.98	107.41	110.1	138.76	235.86	183.67
Break even point of EOC (kg ha ⁻¹)	1,808.73	1,762.81	1,214.28	1,004.66	1,164.61	1,407.72	1,393.08	1,178.86	766.85	916.57
Break even point of TOC (kg ha ⁻¹)	2,518.61	2,442.58	1,871.08	1,574.43	1,741.42	1,967.72	1,955.94	1,743.73	1,258.19	1,578.52
Equilibrium price of TOC (US\$ kg ⁻¹) ⁴	0.19	0.19	0.14	0.12	0.13	0.2	0.2	0.18	0.13	0.15
Cost per unit of EOC (US\$ kg ha ⁻¹)	0.13	0.13	0.09	0.08	0.09	0.14	0.14	0.12	0.08	0.09
Cost per unit of TOC (US\$ kg ha ⁻¹)	0.19	0.19	0.14	0.12	0.13	0.2	0.2	0.18	0.13	0.15
Operating profit of TOC (US\$ ha ⁻¹) ⁵	882.92	867.21	1,058.84	1,171.98	1,119.24	887.71	904.5	1,016.31	1,246.46	1,217.76
Margin of profitability (%) ⁶	48.83	49.15	60.64	66.96	63.63	51.79	52.4	58.12	70.23	64.75
Benefit–cost ratio (%) ⁷	1.95	1.97	2.54	3.03	2.75	2.07	2.1	2.39	3.36	2.84

¹Gross revenue=Yield x Price;^{2,3}Gross profit margin of EOC or TOC= (GR – EOC or TOC/ EOC or TOC);⁴Equilibrium price of EOC or TOC= EOC or TOC/Yield;⁵Operating profit= Gross revenue – TOC;⁶Margin of profitability= Operating profit of TOC/Gross revenue;⁷Benefit-cost ratio= Gross revenue/TOC.

*The economic parameters were calculate using the average of historical exchange rate during the period from 09/01/2016 to 04/30/2017 for 2016/2017 (US\$ 3.25) and 09/01/2017 to 04/30/2018 for 2017/2018 growing seasons (US\$ 3.21).

3. SUBLETHAL EFFECTS OF DIAMIDES INSECTICIDES ON DEVELOPMENT AND FLIGHT PERFORMANCE OF *Chloridea virescens* (LEPIDOPTERA: NOCTUIDAE): IMPLICATIONS FOR BT SOYBEAN REFUGE AREA MANAGEMENT

Abstract

High-dose and refuge are the most important strategies for delaying resistance evolution in Bt crops. Insecticide sprays in refuge areas could be necessary and may limit refuge effectiveness. Here, we evaluated the sublethal effects of two diamide insecticides (chlorantraniliprole and flubendiamide) on *Chloridea virescens* life history traits and flight performance. Sublethal concentrations of chlorantraniliprole and flubendiamide increased larval and pre-pupal development times and decreased larval weight; flubendiamide increased pupal development times. Chlorantraniliprole increased adult male longevity and reduced female fertility, while flubendiamide reduced fecundity. Overall life table parameters were negatively impacted by both treatments. Males exposed to either insecticide showed significant reductions in flight duration and distance for unsustained flights (<30 min). The duration and distance of the first flights were reduced when exposed to chlorantraniliprole. Sustained flights (>30 min) were generally unaffected by insecticide exposure and both sexes flew >6400 m in a single flight. The sublethal effects of flubendiamide and chlorantraniliprole on *C. virescens*' population dynamics could lead to generation asynchrony and provide insufficient susceptible moths when sprayed on refuge crops. However, the distance and duration of flight may still be sufficient to ensure mixing of potentially resistant and susceptible populations from refuge plots.

Keywords: tobacco budworm; flubendiamide; chlorantraniliprole; life history; flight mill; Bt soybean

*This chapter has already been published at *Insects*

Barros, L. S., Yamamoto, P. T., Merten, P., Naranjo, S. E. (2020). Sublethal effects of diamide insecticides on development and flight performance of *Chloridea virescens* (Lepidoptera: Noctuidae): implications for Bt soybean refuge area management. *Insects*, 11(5).

3.1. Introduction

The tobacco budworm, *Chloridea* (= *Heliothis*) *virescens* Fabricius [Lepidoptera: Noctuidae] is an important economic pest of several crops in southern Canada, the United States, and throughout South America, except for Chile and southern Argentina [1–3]. In Brazil, the pest attacks cotton and the vegetative (leaves and stems) and reproductive structures (flower buds and pods) of soybeans, causing yield loss [4]. In the 2013–2014 growing season, genetically modified soybeans MON 87701 × MON 89788 (Intacta RR2 PRO[®]) were commercially introduced in Brazil. This soybean event expresses genes encoding

the insecticidal protein Cry1Ac of *Bacillus thuringiensis* Berliner (Bt) and the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) protein of *Agrobacterium* sp. that confers tolerance to the herbicide glyphosate (RR) [5].

Previous work revealed that soybean MON 87701 × MON 89788 provides a “high dose” against major lepidopteran pests such as *C. virescens* [6,7]. The high-dose strategy requires that Bt plants express high enough concentrations of *B. thuringiensis* insecticidal proteins to ensure mortality of more than 95% of heterozygous insects [8,9]. Because of this high efficacy and associated yield protection, soybean MON 87701 × MON 89788 has been widely adopted by growers in most regions of Brazil. As a consequence, there is a high risk for the evolution of resistance in this pest [10,11].

To manage evolution of resistance, the main insecticide resistant management (IRM) strategies are “high dose” and planting of “structured refuge” (non-Bt soybean). The structured refuge areas provide sources of *Bt*-susceptible pests, which can mate with rare survivors from the Bt crop field, decreasing the abundance of the resistant insects [8,9]. Recent global monitoring revealed a sustained susceptibility for populations of nine species of lepidopteran pests from six countries after at least 10 years of exposure to Bt crops [12]. However, in tropical countries such as Brazil, structured refuge adoption and pest management are challenging because of the high pest pressure [4,13,14]. Therefore, although Bt soybean has been highly effective against *C. virescens*, supplemental foliar insecticide applications to control and reduce pest populations could be necessary in non-Bt soybean refuge areas [14,15]. Thus, the Insecticide Resistance Action Committee (IRAC) biotechnology subunit group in Brazil has proposed integrated pest management (IPM) tactics for management of refuge fields, including windows of insecticide sprays when population densities exceed economic thresholds [15].

Diamides (IRAC Group 28) are the most recent chemical group introduced to the insecticide market for use in soybean refuge areas to manage *C. virescens* [16–18]. Two representatives from this class of insecticides are flubendiamide, a phthalic acid diamide, and chlorantraniliprole, an anthranilic diamide [19]. Diamide insecticides have a unique mode of action as modulators of ryanodine receptors (RyRs), which are located in the membrane of the sarcoplasmic reticulum of muscle tissues. These channels work to rapidly release Ca²⁺ from intracellular stocks, a process necessary for muscle contraction. In intoxicated insects, symptoms begin with cessation of feeding and uncoordinated muscle contraction, eventually causing mortality [20–24].

In the field, it is likely that in addition to direct mortality (lethal effect), some target pests may be exposed to sublethal concentrations, where they survive but suffer negative biological effects [25,26]. Management of target pests in refuge crops is expected and the size of the refuge crop relative to the Bt crop is adjusted so that sufficient susceptible insects are generated [9,15]. Sublethal effects on target pest biology are not necessarily accounted for in the refuge strategy [27]. Thus, it is important to evaluate and understand how the sublethal effect of any insecticide might affect the overall resistance management strategy [28–31]. Diamide insecticides can affect muscle contraction and release of neurotransmitters. Thus, it is possible that dispersal and other biological attributes could be negatively affected [29–37]. This, in turn, might interfere with the ability of susceptible moths to disperse and mate with resistant moths arising from Bt crop fields and disrupt the high-dose and refuge IRM strategy. We, therefore, used *C. virescens* in Bt soybeans as a model system to study potential effects of sublethal insecticide exposure on aspects of a resistance management system. Our objectives were to evaluate the sublethal effects of flubendiamide and chlorantraniliprole on life history traits of *C. virescens*, life table parameters, and flight performance in order to better understand the implications for soybean refuge area management, and by inference, refuge management strategies in other Bt crops.

3.2. Material e methods

3.2.1. Insect rearing

Susceptible strains of *C. virescens* were obtained from Benzon Research (Carlisle, Pennsylvania). The larvae were reared on tobacco budworm artificial diet purchased from Southland Products (Lake Village, Arkansas). This diet was a dry premix and was prepared following the manufacturer's suggested protocol. The *C. virescens* adults were fed a 10% honey solution and water via cotton wicks in Petri dishes placed on the bottom of adult cages. All insect stages were maintained in chambers at constant environmental conditions (25 ± 2 °C, $60 \pm 10\%$ RH [relative humidity], and 14h Light: 10h Dark).

3.2.2. Insecticides

The commercial formulations of diamide insecticides used in the bioassays were Prevathon[®] 5 SC (chlorantraniliprole, 5% active ingredient [a.i.]) supplied by DuPont and Belt[®]SC (flubendiamide, 39% a.i.) by Bayer CropScience.

3.2.3. Larval toxicity bioassay

For determination of sublethal concentrations, eight concentrations of chlorantraniliprole (0.56 to 32.0 ng mL⁻¹) and nine concentrations of flubendiamide (1.8 to 180 ng mL⁻¹) on a logarithmic scale were tested on newly molted third instar larvae of *C. virescens* using a diet-incorporated bioassay [16]. Insecticide concentrations were dissolved in distilled water to create a stock solution and then serial dilutions of desired concentrations were performed. Forty mL of insecticide solutions at desired concentrations were added to diet to yield 400 mL of diet when it dropped to 55 °C. Control diets were produced with the same procedure using 40 mL of distilled water. Three mL of diet was placed into 30-mL clear plastic cups. Individual third instar *C. virescens* larvae were placed in each cup after the diet cooled. Ninety third instar larvae were tested per treatment. Insect mortality was evaluated daily for seven days following exposure to treated diets. Larvae were considered dead if they did not show head movement or peristaltic contractions when touched with a paintbrush. Moribund larvae were scored as alive [38]. The mortality of the treated insects was corrected using the control treatment mortality according to Abbott's formula [39]. The corrected data were submitted to Probit analysis, using the Polo-Plus program (LeOra Software®, Berkeley, CA) to analyze the concentration-mortality relationship [40]. The lethal concentration [LC]₅₀, LC₄₀, LC₃₀ and the corresponding confidence intervals (95% CI) for chlorantraniliprole and flubendiamide were estimated and the values were considered different when there was no overlap of the 95% CI (confidence interval).

3.2.4. Sublethal Effects on *C. virescens* on Life History Traits and Life Table Parameters

Based on previous research, the LC₃₀ was selected as a representative sublethal concentration to assess effects on life history traits and life table parameters of *C. virescens* [30,31]. Newly molted third instar larvae were exposed for 7 days on treated diet. Studies were scheduled in subsets over time, to ensure sufficient numbers of adult survivors (male and female) on different days for tethered flight bioassays (see below). Thus, 1200, 600, and 950 larvae were reared for chlorantraniliprole LC₃₀, flubendiamide LC₃₀ and for the control treatment, respectively. Surviving larvae were then placed on untreated diet until pupation. New diet was provided as needed. A randomly selected, representative number of surviving larvae in each treatment were weighed after 7 days on the treated diets using an analytical balance. Larvae placed on untreated diet were weighed after another 4 and 11 days.

Larval mortality and development were monitored daily. After pupation, each insect was sexed and weighed. Pupae were individually placed in plastic cups (30 mL) and examined daily for adult emergence. A representative number of newly emerged adults (<24 h) were weighed before they fed. The numbers of normal and deformed adults were recorded. Adults were considered deformed if they were unable to shed the pupal exuvium or had wing deformities. A pair of nondeformed, newly emerged moths (<24 h) was introduced into a rearing cage. The rearing cage was 12-cm-high with top and bottom diameters of 16 and 14 cm, respectively, and contained an inner sheet of paper and a transparent fabric top, both of which served as oviposition substrates. A Petri dish (6 cm diameter) was placed on the cage bottom with a wick of cotton soaked in a 10% honey solution for adult food. Oviposition substrates and food supplies were replaced as needed. In all treatments, at least 24 pairs of moths were used for studies. Eggs laid by each pair were counted daily until female death.

Male and female adult longevity was recorded. Males were not replaced if they died before the female. To evaluate fertility (percentage of eggs that hatched), a minimum of 800 eggs from five random pairs per treatment were collected on the third day of oviposition. Immature development and survival, female oviposition period, fecundity, fertility, and female longevity were used to construct life tables for each treatment. Parameters measured included the intrinsic rate of increase (r), the finite rate of increase (λ), net reproductive rate (R_0), and mean generation time (T).

3.2.5. Sublethal effects on *C. virescens* flight performance

The sublethal effects of chlorantraniliprole and flubendiamide on *C. virescens*' flight performance were estimated using tethered flight on automated flight mill apparatus [41–43]. The flight mill consisted of a wooden base with a lightweight, aerodynamic, stainless arm (30 cm length, 0.95 m circumference) with a Teflon rod pivot and magnetic levitation that essentially eliminated friction. Flight rotations were counted with a magnetic sensor (Optec, Inc., Lowell, MI, USA) that was monitored continuously by a computer via a digital input/output board (National Instruments, Austin, TX). The device consisted of 24 flight mills that were run simultaneously. For flight assays, newly emerged (<24 h old) unmated adults of *C. virescens* that were previously exposed to insecticide treatments as detailed above were randomly selected from treatment cages. These moths were separate from those used in life history and life table studies. Only adults with nondeformed wings were assayed. Males and

females of *C. virescens* were flown on different days to avoid any disturbance from sex pheromone.

Adults were fed with a 10% honey solution for 4 h before tethering while being maintained in environmental chambers (25 ± 2 °C and $60 \pm 10\%$ RH). Moths were anesthetized in a freezer (-15 °C) for 5–7 min. Stainless-steel entomological pins (number 00) with the nylon head removed were used to tether the moths. The cut end was inserted into one end of a computer connector pin that could then be connected to the flight mill arm. A small cork (ca. 2 mm square) attached to the pointed end of the pin served as the tethering point. Gel super glue with bond activator (Loctite®; Henkel Corporation, Düsseldorf, Germany) was used to attach the tether to the moth's prothorax after clearing the scales with a small paintbrush. To minimize wing movement and stress before the flight tests, the moth was placed in a Styrofoam box and positioned, so its legs were in contact with the substrate. Moths were refrigerated (4 °C) for 10 min before the start of the flight assay. Due to variable effects of the insecticides on insect development and the large number of insects that needed to be reared, treatments could not be blocked over time in the flight chamber. To the degree possible, the insects flown on any given day were all either male or female from one insecticide treatment and the control.

The flight mill system was located in an environmentally controlled room (25 ± 2 °C and $60 \pm 10\%$ RH) and assays were conducted from 7 p.m. to 7 a.m. during the dark phase of the daily cycle. Each moth was flown a single night. A custom LabView (National Instruments, Austin, TX) computer program automatically recorded data for each of 24 stations including the clock time of the beginning and end of each flight, and the number of revolutions of each flight. These data were then used to calculate flight duration (s), flight distance (m, one rotation = 0.95 m), and flight speed (m/s). The time between flight bouts also was calculated (arrest time in s). Additional calculations were made to estimate total flight time, total distance, and total arrest time over the 12-h assay.

For analyses, *C. virescens*' individual flight durations were categorized as sustained (>30 min) or unsustained (≤ 30 min). This delineation was consistent with a gap in the distribution of flight durations and has been used by many other researchers to delineate sustained flights in various insect species [43–45]. Analyses also examined the timing and duration of the first flight of each moth, again delineated as sustained or unsustained.

3.2.6 Data analysis

The data were subjected to exploratory analyses to assess the assumptions of normality of residuals [46], homogeneity of variance of treatments and additivity of the model [47]. The flight data were log (duration, distance, speed, and arrest) or square-root transformed (sum of duration, distance, and arrest) as required prior to application of ANOVA. These transformations adequately normalized the residuals. The design was a completely randomized two-factor model with insecticides and sex as fixed effects for flight data and most of the life history data. Data were analyzed using Proc GLIMMIX (generalized linear mixed models) [48], and the SLICEDIFF (simple effects test) options within LSMEANS (least square means) were used to examine simple effects. Mean separation was done using the Tukey option, which controls for experiment-wise error rates. One-way models were used for some life history data where sex was not a factor (e.g., fecundity). Proportional data were analyzed with one-way ANOVA in JMP (SAS Institute, Cary, NC, USA) and the effects were analyzed with a Chi-square test (χ^2 ; $\alpha = 0.05$) [48].

Life table statistics for *C. virescens* were estimated using a matrix model approach in Pop Tools [49]. Matrices for each treatment were parameterized as detailed in Naranjo [50] to estimate λ , the finite growth rate (insects/female/day); r , intrinsic rate of increase; R_0 , net reproductive rate; and T , generation time (days) for each treatment. Confidence intervals were estimated by bootstrap resampling with 5000 iterations. Permutation testing was used to compare life table parameters between treatments with a Bonferroni correction for multiple comparisons. The test statistic was simply the difference between treatment parameters and the p -value was estimated as the number of times the resampled test statistic exceeded the original test statistic out of 5000 iterations.

3.3. Results

3.3.1. Larval toxicity bioassay

Chloridea virescens' third instar larvae were more susceptible to the lethal concentrations of chlorantraniliprole than flubendiamide. The LC_{50} of chlorantraniliprole was 4.819 ng mL^{-1} and corresponded to about a 7-fold lower concentration than the flubendiamide LC_{50} ($27.972 \text{ ng mL}^{-1}$). Likewise, chlorantraniliprole LC_{40} and LC_{30} concentrations (4.007 and 3.289 ng mL^{-1}) were approximately 4.5- and 4-fold lower than flubendiamide LC_{40} and LC_{30} concentrations (18.583 and $11.997 \text{ ng mL}^{-1}$) (Table 1).

3.3.2 Sublethal Effects on *C. virescens*' Life History and Life Table Parameters

Significant differences were found among treatments for larval survival when fed on treated diets for the first 7 days (F-value = 34.83; df (degrees of freedom) = 2, 2547; $p < 0.0001$; Figure 1). *C. virescens*' survival was lowest in the chlorantraniliprole treatment during this period, in comparison with flubendiamide and the control. After placing exposed larvae on untreated diets, significant differences also were observed among treatments, but here insecticide exposure reduced survival compared with the control. (F = 72.17; df = 2, 2034; $p < 0.0001$) (Figure 1).

Both male and female larval development times were longer with exposure to sublethal insecticide doses and the effect was more pronounced with chlorantraniliprole (Table 2). Likewise, pre-pupae development times for both sexes were longer following insecticide exposure. Female pupal stage duration did not differ among treatments, but male pupal duration was longest with exposure to flubendiamide (Table 2). Larval survival was highest in the controls compared with the insecticide treatments (Table 3). However, neither pupal survival nor the sex ratio of resulting adults differed among treatments (Table 3).

Following 7 days of exposure to insecticides, larval weight was much lower compared with the control (Table 4). For exposed larvae allowed to feed for 4 additional days on untreated diet, those initially on chlorantraniliprole diets weighed less compared with those exposed to flubendiamide (Table 4). Comparisons to the control were not possible because after 11 days the control larvae had already reached the pre-pupal or pupal stage. Pupal weight did not differ between chlorantraniliprole and the control; insects exposed to flubendiamide weighed the least for both sexes (Table 4). A similar pattern was observed for adult weight.

Adult male longevity was highest with exposure to chlorantraniliprole compared with flubendiamide and the control, but female longevity was unaffected (Table 5). Flubendiamide reduced fecundity (total number of eggs/female) compared to chlorantraniliprole or the control. Chlorantraniliprole reduced egg fertility compared to flubendiamide and the control (Table 5). Overall, sublethal doses of chlorantraniliprole and flubendiamide, compared with the control, reduced the finite rate of increase (λ), the innate capacity of population increase (r), net reproductive rate (R_0), and the resultant generation time (T) (Table 6). Net reproductive rates also differed between the two insecticide exposures.

3.3.3 Sublethal Effects on *C. virescens*' Flight Performance

For the duration of continuous individual sustained flights greater than 30 min, differences among treatments were observed for males ($F = 3.00$; $df = 2, 202$; $p = 0.05$), but not females ($p = 0.62$) (Figure 2A). Males in the flubendiamide treatments exhibited the longest mean flight duration while the shortest was observed with chlorantraniliprole.

The mean flight distance for females ranged from 6451–8151 and males 6664–9311 m. There were no treatments effects on flight distance, flight speed, the rest period between flights (Figure 2B–D), or the number of flights and total flight distance over the 12-h assay period for either sex (Figure 3B,D). In contrast, the mean total flight duration over the 12-h assay period was higher for males in the flubendiamide treatment compared with chlorantraniliprole or the control ($F = 3.18$; $df = 2, 202$; $p = 0.0436$; Figure 3A).

For continuous flights less than 30 min, treatments once again only affected male moths. Males in the control treatment had the longest flight durations and those in the chlorantraniliprole treatment had the shortest durations ($F = 3.41$; $df = 2, 481$; $p = 0.0337$) (Figure 4A). Control males also flew the longest distance while males exposed to chlorantraniliprole flew the shortest distance ($F = 3.83$; $df = 2, 481$; $p = 0.0224$; Figure 4B). The flight speed of males was fastest in the control and slowest in the chlorantraniliprole treatment ($F = 5.71$; $df = 2, 481$; $p = 0.0036$; Figure 4C). Rest periods between flights did not differ by treatment (Figure 3D). No differences among treatments were observed in the mean cumulative duration, distance, rest periods, and number of flight attempts over the entire 12-h assay (Figure 5A–D).

For first flights greater than 30 min in duration, no differences were observed in either sex for duration, distance, speed, or rest periods (Figure 6A–D). However, for first flight durations less than 30 min, differences were observed for flight duration ($F = 6.31$; $df = 2, 406$; $p = 0.002$; Figure 7A), distance ($F = 7.61$; $df = 2, 406$; $p = 0.0006$; Figure 7B), and flight speed for males ($F = 8.24$; $df = 2, 406$; $p = 0.0003$; Figure 7C). Males flew about twice as long and twice the distance in the control compared with the insecticide treatments (Figure 7A,B). Males flew the slowest in the flubendiamide treatment (Figure 7C). No differences were observed in male or female rest periods (Figure 7D).

A high percentage of tethered moths flew in the assay system, ranging from 69–95% depending on treatment. The proportion of tethered moths engaged in unsustained flights differed among treatments for females ($\chi^2 = 12.15$; $df = 2, 280$; $p = 0.0023$) and males ($\chi^2 = 10.56$; $df = 2, 319$; $p = <0.0001$) (Figure 8A). Averaged over both sexes, moths exposed to

chlorantraniliprole had the highest propensity for flight (90%) followed by the control (84%) and flubendiamide (66%). There was no difference in the propensity of moths engaged in sustained flight ($p > 0.10$, Figure 8B). Over all treatments, about 35% of moths engaged in sustained flights greater than 30 min in duration.

3.4. Discussion

In Brazil, Bt soybean has been highly effective in controlling *C. virescens* [6,7]. Nevertheless, foliar sprays could be necessary for lepidopteran pest management in refuge areas [14,15,27]. Such sprays would reduce the abundance of susceptible moths through direct mortality, but there may be additional sublethal effects on surviving insects as the concentrations of insecticides degrade after application [25,26]. Models of the high-dose, refuge strategy assume at least 500 susceptible adults will emerge for every resistant moth, then randomly mate with rare and resistant insects (RR) from the Bt fields, resulting in susceptible heterozygous progeny (RS) [9,51,52]. While these models account for direct mortality of the target pest through the size and placement of the refuge (e.g., 20% of Bt crop), it is less clear if they also account for the more subtle biological and behavioral effect that could impact survivors exposed to sublethal insecticide doses [53,54]. Here, we demonstrated that flubendiamide and chlorantraniliprole, two diamide insecticides that are popular insect control choices for growers in Brazil [55,56], are toxic to *C. virescens* larvae. In addition, sublethal concentrations of these insecticides at a dose represented by the LC₃₀, were associated with changes in life history traits that ultimately affected population growth characteristics and phenology. Additional effects were observed in the flight behavior of male moths.

These life history trait effects included reductions in larval, pupal, and adult weight; prolongation of larval and pupal development times; reductions in adult male longevity; and reductions in female fertility and fecundity. There also were additional reductions in larval survival, which is usual in studies of this nature, but suggests that our sublethal doses may have been slightly too high. Overall, our results are consistent with other studies on diamide sublethal effects [29–31,57,58]. Nonetheless, the insect suffered multiple biological changes as a result of exposure to what would represent degraded insecticide concentrations in the field [59,60]. The biological traits affected are interrelated and so each effect does not represent an independent outcome of sublethal stress. Life table statistics allowed us to integrate these observed effects into more meaningful metrics. We observed reductions in net reproductive rates and intrinsic rates of increase, and prolonged generation times due to

sublethal exposure. These changes could potentially lead to asynchronous target pest population growth and phenology between Bt and non-Bt soybeans, and, ultimately, reduce the probability of random matings between susceptible and putatively resistant insects.

Several additional factors need to be considered in determining whether and to what degree these sublethal effects will disrupt resistance management. For example, diamides insecticides have been shown to have low toxicity to natural enemies [18,61]. Thus, target pests in refuge crops could be subject to higher levels of predation and parasitism from conserved natural enemies. This additional mortality may further reduce the number of susceptible moths generated in refuge fields and the effect could be further enhanced by the increased susceptibility of sublethally affected prey to natural enemy attack [62]. In contrast, there may be fitness costs associated with resistance to Bt proteins in the target pest that could affect life history traits in much the same way as the sublethal effects observed here. This might negate the impacts associated with asynchronicity in population growth and phenology previously discussed [63]. However, the transgenic soybean events used in Brazil express a high dose of the Cry 1Ac, and studies suggest that *C. virescens*' larvae from first through fifth instar are highly susceptible to Cry 1Ac, leading to 100% mortality [6,7]. Thus, it is unclear if fitness costs would be relevant given essentially no survivors in Bt soybean at the present time. Finally, natural enemy activity in the Bt crop might also contribute to delays in resistance evolution [64,65]. Overall, there are many interacting factors influencing the outcome of the high-dose, refuge strategy that may need to be more carefully studied and modelled.

Given the need for both sufficient numbers of susceptible moths and populations synchronized to produce adults during the same time period in Bt and non-Bt fields, the dispersal ability of susceptible moths should be sufficient to ensure cross mating of susceptible and resistant moths [9,51,52,66]. IRAC technical recommendations in Brazil recommend at least a 20% structured refuge area within a maximum distance of 800 m (0.5 mile) from Bt soybeans [15,67]. We found that sublethal flubendiamide exposure appeared to reduce the flight propensity of moths while chlorantraniliprole increased flight propensity relative to the control. These differences were apparent only for unsustained flights <30 min in duration; the propensity for sustained flight was unchanged by treatment. For insects that flew, we observed effects of sublethal exposure on several aspects of flight behavior, but only for male moths. Why female moth flight was unaffected is not clear. One hypothesis is that there was a trade-off between flight capacity and fecundity, which was reduced with sublethal

exposure [68]. Insect flight and reproduction are critical ecological processes that are both energetically costly [69,70]. Flight fuel production can compete with energy used for ovarian development and oogenesis [69–71]. *C. virescens* females may have invested in flight energy at the expense of reproduction, but additional work will be needed to test this hypothesis.

Sublethal concentrations of flubendiamide reduced pupal and adult weight, in addition to fecundity. Studies have shown that the binding of flubendiamide on *C. virescens*' thoracic muscles was four times higher than cyantraniliprole, another anthranilic similar to chlorantraniliprole [72]. Therefore, we hypothesized that the absence of negative impact of chlorantraniliprole LC₃₀ on *C. virescens* fecundity might be associated with differential ryanodine binding on the RyR complex [72,73]. This differential effect of the two insecticides may also help explain the lower flight propensity observed for moths exposed to flubendiamide. It should be noted that we tested only newly emerged and unmated moths. These individuals may use their lipid energy sources for flights rather than for egg maturation [68,74], corroborating the lack of sublethal effects' exposure on flight capacity. It is also possible that females have more efficient detoxification systems for metabolizing insecticides [75]. Thus, the muscle-related effects of diamides may have only affected males [24]. Future research is needed to test these hypotheses on differential female and male effects.

Flight mills have been widely used to measure flight behavior in a wide diversity of insects and to examine the effects of a wide array of biological and ecological factors [32–36,43–45]. Still, the approach represents a highly artificial system that makes it difficult to tie behavior in the laboratory to that in the field [43,76–78]. The best approach is to use flight mill results in a comparative fashion [43] and/or to think of flight mill results as a measure of the biological potential of a species [79–81]. Here, despite the negative impact of sublethal exposure on some flight performance aspects of *C. virescens* males, both sexes flew approximately 1.7 to 2.7 times the maximum distance (800 m) required between Bt soybean fields and refuge areas when engaged in unsustained flight in a single 12-h assay period. This figure jumped to 10- to 14-fold for moths engaged in sustained flight, although only about a third of moths do so. Thus, both sexes have the potential to more than adequately move the required 800 m. Still, fewer moths exposed to flubendiamide would be expected to engage in unsustained flight, while those exposed to chlorantraniliprole seemed to be more active than the control. The overall impact of these differences is difficult to judge in the field, but flubendiamide use could reduce the number of moths traveling the required 800 m. Previous work has demonstrated that *C. virescens* adults can fly more than 7.5 km in their lifetimes

[1,3,60,82], although the moth engages in facultative migration behavior that is modified by the environment and other biological factors [76–78]. Only the mean distance of individual male flights less than 30 min were reduced by sublethal exposure. Mean distance of flight over 30 min and cumulative flight distance over the 12-h assay did not differ between insecticide treatments or sex. Thus, if we assume that unexposed moths are capable of reaching the refuge fields (based on the refuge distance requirement), then there is no indication that sublethally affected moths could not do the same, albeit the total number doing this could be reduced with use of flubendiamide.

3.5. Conclusion

Overall, we found that sublethal exposure of *C. virescens* to several common diamide insecticides can reduce population growth and alter phenological timing. This could potentially disrupt random mating between susceptible moths from the refuge and resistant moths from Bt fields and interfere with resistance mitigation. The extent of the impact would likely depend on the actual sublethal dose the insects would encounter and this would certainly change over time [25,26,59,60,83,84]. As noted, these effects also could be blunted by fitness-related costs in resistant moths. Our flight mill results suggest that this sublethal exposure would not be expected to reduce the moths' ability to disperse an adequate distance from non-Bt refuges, but additional research will be needed to define the extent of sublethal exposure in the field. Our results may be applicable to other Bt crops in which *C. virescens* is an economically important pest, such as Bt cotton in Mexico, Puerto Rico, Colombia, and Brazil [85], and to situations where diamides insecticides are used in refuge areas of other crops for tobacco budworm population control. It is likely that the life history effects demonstrated here are not unique to diamide insecticides or even the specific target species and crop examined. Instead, this may be a concern for any effective insecticide used to manage target pests in refuge crops and further work may be needed to examine the underlying assumptions of the high-dose refuge strategy, especially with reference to the number of susceptible moths required to be generated for effective resistance management.

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Figures and Tables Legends

Table 1. Toxicity of flubendiamide and chlorantraniliprole to third instar larvae of *Chloridea virescens* in diet bioassays.

Insecticides	n	Slope (SE) ^a	LC ₅₀ (ng mL ⁻¹) ^b	LC ₄₀ (ng mL ⁻¹)	LC ₃₀ (ng mL ⁻¹)	χ^2 (df) ^c	P
Flubendiamide	900	1.43 (0.10)	27.972 (18.08–39.64)	18.583 (11.71–25.09)	11.997 (8.562–16.014)	11.064 (6)	0.09
Chlorantraniliprole	810	3.16 (0.20)	4.819 (4.02–5.79)	4.007 (3.302–4.786)	3.289 (2.645–3.943)	12.194 (6)	0.06

^a Standard error, ^b 95% confidence limits, ^c Chi-square value (χ^2) and degrees of freedom (df) as calculated by probit analysis.

Table 2. Sublethal effects of flubendiamide (Flu) and chlorantraniliprole (Chlo) on larval, pre-pupal, and pupal development time of *Chloridea virescens* under controlled laboratory environmental conditions (25 ± 2 °C, 60 ± 10% RH, and 14L: 10D).

Treatments	Larval Stage (Days)		Pre-Pupal Stage (Days)		Pupal Stage (Days)	
	Female	Male	Female	Male	Female	Male
Control	8.72 ± 0.09 c	8.85 ± 0.08 c	2.02 ± 0.07 b	1.95 ± 0.07 b	11.44 ± 0.03 a	12.81 ± 0.03 b
Flu LC ₃₀	12.56 ± 0.16 b	12.53 ± 0.13 b	2.54 ± 0.13 a	2.67 ± 0.11 a	11.58 ± 0.07 a	13.02 ± 0.06 a
Chlo LC ₃₀	14.60 ± 0.083 a	14.71 ± 0.08 a	2.75 ± 0.07 a	2.63 ± 0.07a	11.55 ± 0.03 a	12.81 ± 0.03 b
F	1090.65	1208.32	23.98	26.880	2.820	5.440
df	2, 1835	2, 1835	2, 1835	2, 1835	2, 1734	2, 1734
p	<0.0001	<0.0001	<0.0001	<0.0001	0.06	0.004

All values are means ± standard error (SE). Means in same column followed by different letters are significantly different (Tukey test, $p < 0.05$).

Table 3. Sublethal effects of flubendiamide (Flu) and chlorantraniliprole (Chlo) on larvae and pupal survival and the sex ratio of resulting adult *Chloridea virescens* under controlled laboratory environmental conditions (25 ± 2 °C, 60 ± 10% RH, and 14L: 10D).

Treatments	(%) Larval Survival ¹	(%) Pupal Survival ²		(%) Sex Ratio
		Female	Male	
Control	81.47 ± 1.42 a	93.58 ± 1.12 a	93.50 ± 1.11 a	0.48 a
Flu LC ₃₀	71.02 ± 2.22 b	93.96 ± 2.02 a	92.50 ± 1.76 a	0.42 a
Chlo LC ₃₀	67.43 ± 1.26 b	96.67 ± 1.10 a	96.70 ± 1.08 a	0.48 a
F	27.8578	2.0812	3.0694	1.7601
df	2, 2547	2, 878	2, 982	2, 1863
p	<0.0001	0.1254	0.0469	0.1723

¹ Larvae that became pupae, ² pupae that became adults. All values are means ± standard error (SE). Means in same column followed by different letters are significantly different (Tukey test, $p < 0.05$).

Table 4. Sublethal effects of flubendiamide (Flu) and chlorantraniliprole (Chlo) on larval weight after 7 days of exposure to treated diets, after 4 additional days on untreated diet (11 days total), and on pupal and adult weight of *Chloridea virescens* under controlled laboratory environmental conditions (25 ± 2 °C, $60 \pm 10\%$ RH, and 14L: 10D).

Treatments	Larval Weight (g)		Pupal Weight (g)		Adult Weight (g)	
	7 Days	11 Days ^a	Female	Male	Female	Male
Control	0.342 ± 0.01 a	*	0.268 ± 0.01 a	0.279 ± 0.01 a	0.152 ± 0.01 a	0.142 ± 0.01 a
Flu LC ₃₀	0.086 ± 0.01b	0.243 ± 0.01 a	0.246 ± 0.01 b	0.247 ± 0.01 b	0.141 ± 0.03 b	0.124 ± 0.01 b
Chlo LC ₃₀	0.026 ± 0.001 c	0.171 ± 0.01 b	0.276 ± 0.01 a	0.282 ± 0.01 a	0.156 ± 0.01 a	0.145 ± 0.01 a
<i>F</i>	1073.38	23.4	20.27	40.63	8.77	20.340
<i>df</i>	2, 578	1, 329	2, 727	2, 727	2, 396	2, 396
<i>p</i>	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001

^aLarval survival after 7 days on treated diet and 4 additional days on untreated diet. * Comparable weight was not possible because larvae on untreated diets completed development before 11 days. All values are means ± standard error (SE). Means in same column followed by different letters are significantly different (Tukey test, $p < 0.05$ for 3 mean comparisons).

Table 5. Sublethal effects of flubendiamide (Flu) and chlorantraniliprole (Chlo) on adult longevity, fecundity, and fertility of *Chloridea virescens* under controlled laboratory environmental conditions (25 ± 2 °C, $60 \pm 10\%$ RH, and 14L: 10D).

Treatments	Longevity (Days)		Fecundity ¹	Fertility ² (%)
	Female	Male		
Control	10.00 ± 0.93 a	10.83 ± 0.93 b	1289.45 ± 80.22 a	87.37 ± 1.49 a
Flu LC ₃₀	9.73 ± 0.89 a	10.11 ± 0.89 b	873.54 ± 80.22 b	83.78 ± 1.20 ab
Chlo LC ₃₀	11.00 ± 0.85 a	13.38 ± 0.85 a	1193.75 ± 72.98 a	81.63 ± 1.27 b
<i>F</i>	0.59	3.88	7.46	4.2987
<i>df</i>	2, 152	2, 152	2, 74	2, 2357
<i>p</i>	0.5553	0.023	0.0011	0.0137

¹Total number of eggs laid per female, ²percent of eggs hatching. All values are means ± standard error (SE). Means in same column followed by different letters are significantly different (Tukey test, $p < 0.05$).

Table 6. Life table parameters for *C. virescens* (mean, 95% CI) exposed to sublethal concentrations of flubendiamide (Flu) and chlorantraniliprole (Chlo) under controlled laboratory environmental conditions (25 ± 2 °C, $60 \pm 10\%$ RH, and 14L: 10D).

Life Table Parameters	Treatments		
	Control	Flu LC ₃₀	Chlo LC ₃₀
λ ^a	1.312 (1.301–1.323) a	1.237 (1.219–1.256) b	1.257 (1.247–1.267) b
<i>R</i> ^b	0.272 (0.263–0.280) a	0.213 (0.198–0.228) b	0.229 (0.221–0.236) b
<i>R_o</i> ^c	422.7 (377.8–464.1) a	213.5 (167.9–263.6) b	313.2 (285.4–340.8) c
<i>T</i> ^d	37.5 (36.7–38.4) a	40.9 (39.1–42.8) b	43.2 (41.7–44.7) b

^a λ , finite growth rate (insects/female/day); ^b*r*, intrinsic rate of increase; ^c*R_o*, net reproductive rate; ^d*T*, generation time (days). Confidence intervals estimated by bootstrap analysis and mean comparisons done with permutation testing (5000 repetitions) corrected for multiple comparisons. Means followed by different letters within a row are significantly different ($p < 0.0167$).

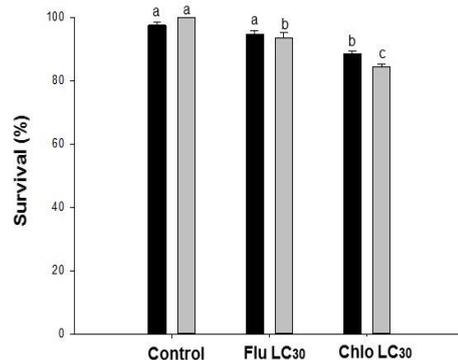


Figure 1. Survival (%) of *Chloridea virescens* larvae (mean \pm SE) when exposed for 7 days on treated diet (black bars) with sublethal concentrations of flubendiamide (Flu) and chlorantraniliprole (Chlo), and after feeding on untreated diet four additional days (grey bars). The corresponding sample sizes were: Control (950, 775), flubendiamide (390, 296), and chlorantraniliprole (1210, 775). Mean survival with different letters within the same color of bars are significantly different (Tukey test, $p < 0.05$).

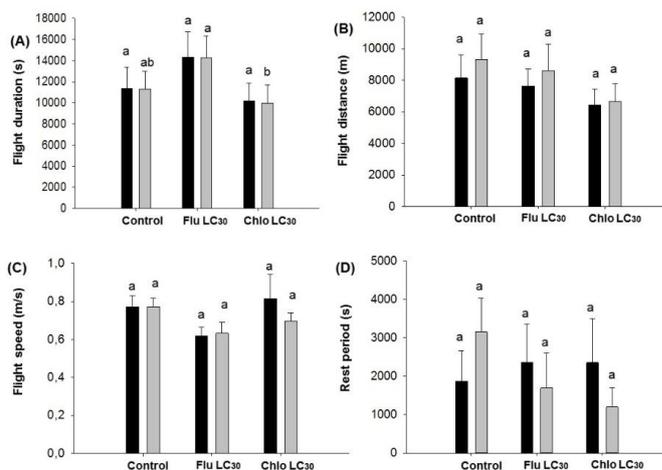


Figure 2. *Chloridea virescens* sustained flights greater than 30 min: (A) Mean flight duration, (B) mean flight distance, (C) mean flight speed, (D) mean rest period. The black bars represent female and grey bars represent male moths. Different letters above the standard error bars indicate significant differences based on Tukey–Kramer test ($p < 0.05$).

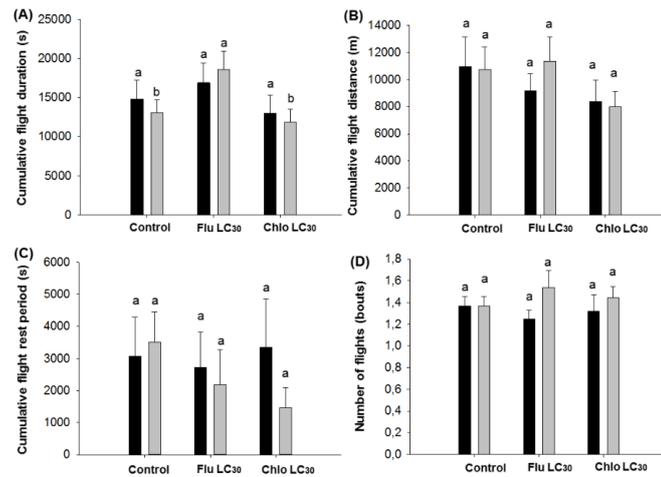


Figure 3. *Chloridea virescens* sustained flights greater than 30 min: (A) Cumulative flight duration, (B) cumulative flight distance, (C) cumulative flight rest period, and (D) number of flights (bouts). Black bars represent female and grey bars represent male moths. Different letters above the standard error bars indicate significant differences based on Tukey–Kramer test ($p < 0.05$).

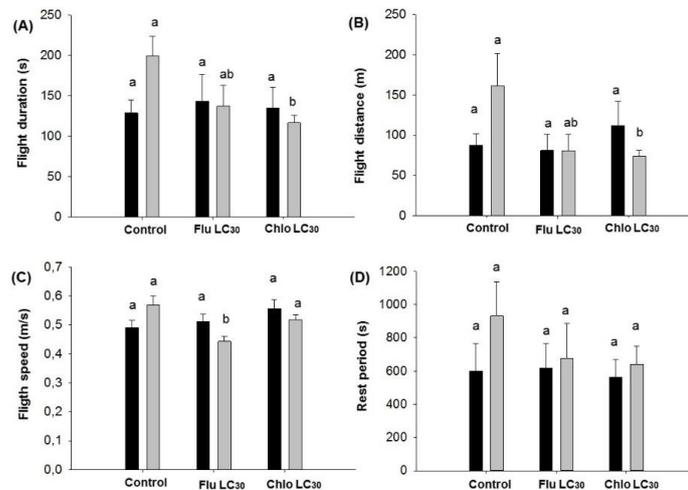


Figure 4. *Chloridea virescens* unsustained flights less than 30 min: (A) Mean flight duration, (B) mean flight distance, (C) mean flight speed, and (D) mean rest period. Black bars represent female and grey bars represent male moths. Different letters above the standard error bars indicate significant differences based on Tukey–Kramer test ($p < 0.05$).

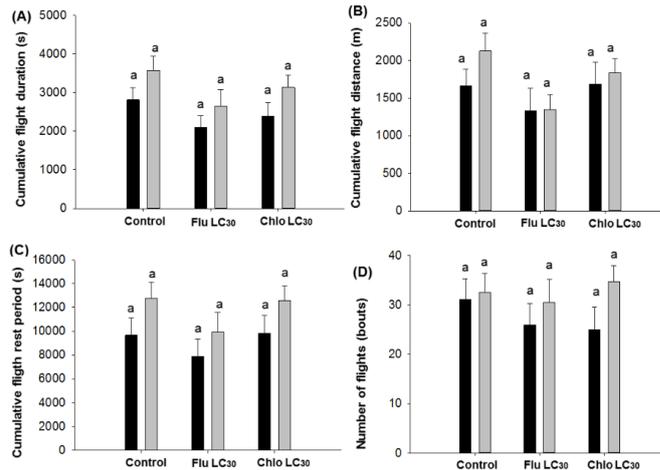


Figure 5. *Chloridea virescens* unsustained flights less than 30 min: (A) Cumulative flight duration, (B) cumulative flight distance, (C) cumulative flight rest period, and (D) number of flights (bouts). Black bars represent female and grey bars represent male moths. Different letters above the standard error bars indicate significant differences based on Tukey–Kramer test ($p < 0.05$).

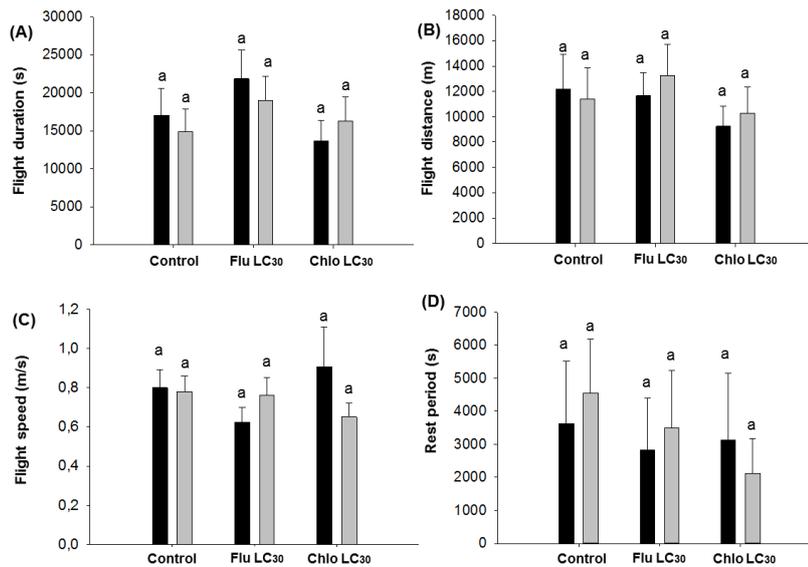


Figure 6. First flight taken by *Chloridea virescens* greater than 30 min: (A) Mean flight duration, (B) mean flight distance, (C) mean flight speed, and (D) mean rest time. Black bars represent female and grey bars represent male moths. Different letters above the standard error bars indicate significant differences based on Tukey–Kramer test ($p < 0.05$).

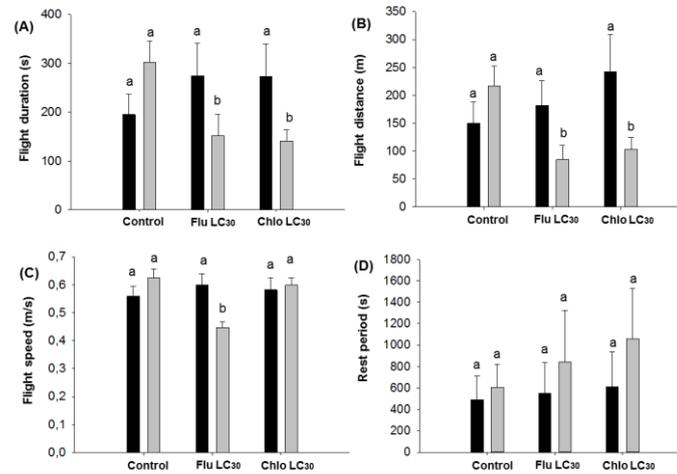


Figure 7. First flights of *Chloridea virescens* less than 30 min: (A) Flight duration, (B) flight distance, (C) flight speed, and (D) rest period. Black bars represent female and grey bars represent male moths. Different letters above the standard error bars indicate significant differences based on Tukey–Kramer test ($p < 0.05$).

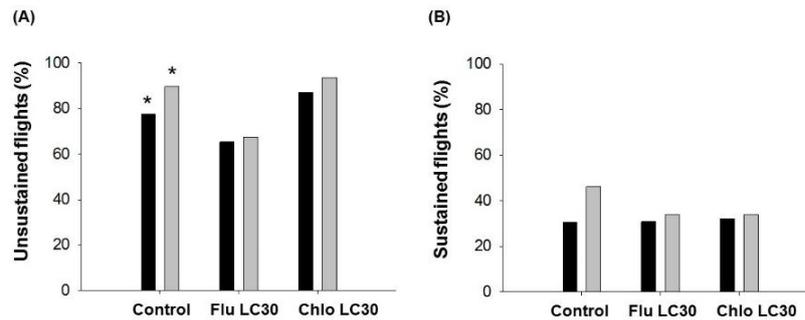


Figure 8. Proportion of *Chloridea virescens* that (A) engaged in unsustained (<30 min) or (B) sustained flights (>30 min). Black bars represent female and grey bars represent male moths. The asterisks indicate significant differences among treatments for a given comparison (χ^2 , $\alpha = 0.05$).

4. ECOLOGICAL RISK OF BT SOYBEAN ON GROUND-DWELLING PREDATORY ARTHROPODS AND THE POTENTIAL IMPACT ON RESISTANCE EVOLUTION

Abstract

To evaluate the ecological risk of soybean expressing the Cry1Ac endotoxin from *Bacillus thuringiensis* (Berliner) (Bt soybean) on ground-dwelling predatory arthropods (GDP's), a field experiment was carried out over two crop seasons (2016–2017 and 2017–2018). The experimental design used paired blocks (Bt and non-Bt soybean) with 15 repetitions (pitfall traps). To determine the GDP communities and the environmental risk assessment of Bt soybean to GDP, a faunistic analysis was conducted and ecological diversity indexes (EDI's) were calculated. The abundances of predominant and dominant species were compared and explored using univariate and multivariate approaches. During the cropping season, the numbers of GDP's sampled ranged from 216 to 637 in Bt, and 211 to 674 GDP in non-Bt soybean areas, most of them were members of Carabidae, Formicidae, Anisolabididae and Araneidae. In terms of biodiversity, the results indicated no ecological risk of Bt soybean on the GDP community, and no significant difference in the EDI's of abundance (N), richness (S), Simpson's (D), Shannon-Wiener (H') and Pielou's evenness (J'). In general, the univariate and multivariate analyses showed that the interactions of certain predators are specific for each soybean cultivar and this current relationship with predator species could be dependent on the prey and season. *Megacephala brasiliensis*, *Odontocheila nodicornis*, *Calosoma granulatum*, *Pheidole* sp. and spiders showed relatively high predominance and dominance, indicating a higher potential to regulate pest population in Bt soybean or in refuge areas. *Euborellia* sp. is more abundant in non-Bt soybean fields. The potential of dominant GDP's to delay or accelerate the evolution of resistance must be investigate more due the dependence and interaction with cultivar and season, and the complex genetic and ecological factors involved.

Keywords: Environmental risk assessment, Transgenic Bt soybean, Non-target organisms, Biodiversity, Biocontrol, Insect Resistance Management.

4.1. Introduction

The genetically modified (GM) soybean cultivar MON 87701 × MON 89788 [*Glycine max* (L.)] expresses the Cry1Ac endotoxin from *Bacillus thuringiensis* Berliner (Bt), which controls main soybean lepidopteran pests (Martins-Salles et al., 2017). Since its commercial release, Bt soybean has been widely planted, resulting increases in yields and reduction of insecticide applications (Céleres, 2017; Brookes, 2018; Brookes & Barfoot, 2018; Cattelan & Dall'Agnol, 2018). However, issues involving the evolution of resistance by target pests (Gould, 1998; Tabashnik & Carrière, 2017; Malaquias et al., 2017a; 2020) and the ecological risk to non-target organisms (NTO's), including natural enemies of pest, pollinators, microorganism and mammals, have been discussed in the international scientific community

(Capalbo et al., 2003; Andow & Hilbeck, 2004; Devos et al., 2015; Davison & Ammann, 2017). For this, the use of high-dose concept of GM plants expressing a high concentration of the insecticidal Bt toxins combined with refuge plantings (non-Bt plants) (Gould, 1998) and monitoring the ecological impact of Bt plants on NTO's are the main strategies for insect resistance managing (IRM) and for assess environmental risks (Andow & Hilbeck, 2004; 2006; Romeis et al., 2008; Bauer-Pankus & Then, 2014).

Natural enemies, such as parasitoids and predators, are responsible for the biological control and natural mortality of arthropod pests in agroecosystems (Cardinale et al., 2003; Romeis et al., 2006; Straub & Snyder, 2008; Naranjo et al., 2015). In Bt crop systems, natural enemies can contribute positively to delay the process of resistance evolution, by preying on individuals carrying Bt resistance genes (Liu et al., 2014; Romeis et al., 2019); or can contribute negatively by reducing the number of susceptible individuals in refuge areas (Mallampalli et al. 2005; Heimpel et al., 2005; Lawrence et al., 2007; Baker et al., 2008; Baker & Tann, 2014, 2017). To increase the likelihood of predation on resistant insect pests, the Bt crop should not negatively impact communities of natural enemies (Cardinale et al., 2003; Straub & Snyder, 2008; Griffin et al., 2013; Magurran, 2013; Jonsson et al., 2017; Romeis et al., 2019). However, natural enemies can be negatively impacted directly and/or indirectly by Bt plants in the fields (Hilbeck et al., 1998; Poppy & Sutherland, 2004; Saxena et al., 2004; O'Callaghan et al., 2005; Lundgren et al., 2005; Andow et al., 2006; Romeis et al., 2006; Torres & Ruberson, 2006, 2008; Madliger et al., 2011; Yu et al., 2014; Peterson et al., 2016).

Of the several groups of natural enemies that attack soybean pests, generalist ground-dwelling predatory arthropods are extremely important. Among these, members of the orders Coleoptera (carabids and staphylinids), Dermaptera (earwigs), Hymenoptera (ants) and Araneae (spiders) are ecologically important because of their abundance and high predation capacities (Symondson et al., 2002; Cividanes, 2002; Cividanes & Cividanes, 2008; Cividanes et al., 2009; Panizzi et al., 2012; Cividanes et al., 2014). The carabids *Calosoma granulatum* Perty and *Galerita brasiliensis* Dejean control several key soybean lepidopteran pests (Cividanes et al., 2018; Martins et al., 2018). In relation to the ants, besides being generalist predators and contributing to natural pest mortality, they are also considered bioindicator species (Risch, 1981; Eubanks, 2001; Gerlach et al., 2013; Fernandes et al., 2019).

A large body of literature has demonstrated the environmental safety of Bt crops to

natural enemies and other NTO's compared with insecticides (Lu et al., 2012; Guo et al., 2014; Klümper & Qaim, 2014; Comas et al., 2014; Assis et al., 2018). However, the insecticidal Bt proteins are constantly exposed in the leaf tissues of Bt plants, have some soil persistence (Madliger et al., 2011), and negative effects to natural enemies have been reported (Hilbeck et al., 1998; Zwahlen et al., 2003; CERA, 2011). Furthermore, the safety of these proteins remains controversial and studies to assess the potential risk to NTOs are recommended and needed, even though Bt crops have been released in the market (Andow & Hilbeck, 2004; Hilbeck et al., 2011; Abbas, 2018).

Given the contextualization, here in our article, biodiversity and biological control potential of GDP's were discussed in the context of management resistance evolution strategy "high-dose/refuge". This study evaluated the ecological risk of Bt soybean expressing the insecticidal protein Cry1Ac on ground-dwelling predatory arthropods. We posed the following questions: (i) What is the composition of ground-dwelling predatory arthropod communities in Bt and non-Bt soybean areas? (ii) Are there differences in the faunistic and ecological diversity indexes? (iii) Which species are predominant or dominant? (iv) Are there differences in abundance between predominant and dominant species? (v) Are there tritrophic interactions and potential species for biological control that could affect the evolution of resistance?

4.2. Material e methods

4.2.1. Experimental design

Two field studies were carried out during the 2016–2017 and 2017–2018 crop seasons in a commercial agricultural area in Buri municipality, São Paulo (23°36'28.07"S and 48°32'59.54"W). The experimental design was a paired block with two treatments (Bt and non-Bt soybean) and 15 replications. The replications dimensions were 18 × 18 m (324 m²) and 13.5 × 15 m (202.5 m²) in the 2016–2017 and 2017–2018 crop seasons, respectively. Therefore, the correspondent total block area was 4,860 m² and 3,037.5 m². There was a distance of 18 m and 15 m between Bt and non -Bt blocks in the 2016–2017 and 2017–2018 crop seasons, respectively, to avoid any possible flow of arthropods between treatments (adapted from Lima Junior et al., 2013). The Bt soybean cultivar sown was "DM 6563 RSF IPRO" with maturity group 6.3 and indeterminate growth habit, and the non-Bt soybean cultivar was "NA 5909 RG" with maturity group 6.4 and indeterminate growth habit. The both cultivars were sown in both crop seasons and expressing tolerance to the herbicide

glyphosate. The soybean plantings were managed within the traditional Brazilian crop system cycle (soybean, corn and wheat crops), and with conservation soil-management practices (no tillage). Fertilization, weeding and diseases control were performed following the technical recommendations for soybean, except for pests that were managed in Bt and non-Bt soybean according to the different pest pressures and commercial grower practices (Tables 1 and 2) to simulate a real field condition (Andow & Hilbeck, 2004; IRAC, 2017).

4.2.2. Arthropod sampling

In the 2016–2017 crop season, sampling for ground-dwelling predatory arthropods in Bt soybean began at phenological growth stages V6–V7 and for non-Bt soybean at R1/R2 (Fehr & Caviness, 1977). Sampling continued on 14-day intervals. In the 2017–2018 crop season, sampling began when the soybean crop was in the R2/R3 phenological growth stages, with 7-day intervals between sampling periods. During both seasons, sampling ended when the crop was in senescence, close to harvest. Six samples in the 2016–2017 and nine samples in the 2017–2018 crop season were conducted. Ground-dwelling arthropods were sampled with ground-level pitfall traps installed in the center of each experimental plot, totaling 15 traps per treatment. The traps consisted of 500 mL plastic cups (12 cm diameter × 15 cm high), 2/3 filled with a 0.1% aqueous sodium hypochlorite solution to conserve the insects captured. In addition, 1% neutral detergent was added to break the water surface tension and prevent insects from escaping. To avoid contamination of the solution by plant residues and flooding by rainwater, a removable plastic cover was placed above each pitfall trap. The cover consisted of a plastic plate supported by wooden sticks and placed at a sufficient height to allow insects to enter the trap (Lima Junior et al., 2013). At each collection, the aqueous sodium hypochlorite solution was discarded and replaced with fresh solution. The insects were removed and transferred to new containers filled with 70% ethanol for preservation. The samples were preliminarily identified in the field and later classified in the laboratory by Prof. Dr. Sinval Silveira Neto, using the entomological reference collections at the Museum of Entomology of the Department of Entomology and Acarology of Esalq/USP.

Complementary assessment was performed on the 2017–2018 crop season in order to investigate the possible correlation between pest and natural enemies on treatments. The foliar-dwelling pest and natural enemies population abundance was measured on Bt and non-Bt soybean treatments using the beat cloth method (1.0 m long x 1.5 m large) (Stürmer et al., 2012). The cloth was positioned horizontally on the ground, parallel to soybean rows. The

samples were near to correspondent pitfall traps points and the number of samples and frequency were similar as previously described for ground-dwelling predatory arthropods in the 2017–2018 crop season.

4.2.3. Specimen identification

To evaluate the composition of the ground-dwelling predatory arthropod community, a faunistic analysis was performed according to Silveira Neto et al. (1976), using the ANAFAU software (Moraes et al. 2003). Values of dominance, abundance, frequency and constancy of arthropods collected in the Bt and non-Bt soybean areas were obtained. The dominant species were the most abundant and had the highest indexes of frequency, constancy and dominance (Silveira Neto et al., 1995). Ecological diversity abundance (N), richness (S), Shannon-Wiener (H'), Simpson's (D) and Pielou's evenness (J') indexes were calculated using Paleontological Statistics Software (PAST) version 3.26 (Hammer et al., 2001).

To compare the abundance of the most important species based on the results of the faunistic analysis, individuals that showed dominance and predominance (species that obtained the highest faunal indexes) in the Bt and non-Bt soybean areas according previous results of faunistic analysis were selected and analyzed using Generalized Linear Models (GLM) (Nelder & Wedderburn, 1972). Analyses were performed according to data distribution. The goodness of fit of the statistical models was assessed with the “hnp” package, which generated half-normal plots (Moral et al., 2017). Account data were analyzed with the Poisson, Quasi-Poisson and Negative Binomial distribution models, with the p-value obtained by Chi-square test in the case of Poisson, and F test for Quasi-Poisson and Negative Binomial models. When data were overdispersed, the negative binomial model or the quasi-Poisson model were used (Hinde & Demétrio, 1998), with the p-value obtained by F test.

In order to explore the interactions among the most important ground-dwelling predatory arthropods in the Bt and non-Bt soybean areas in the 2016–2017 and 2017–2018 crop seasons, we used a multivariate principal component analysis (PCA) of predominant species and dominance on the covariance matrix to compare them regarding sampling date, soybean growth stages and crop season. The criteria adopted to select species were based on the results provided by the faunistic analysis and according to the eigenvalues from the correlation matrix (Kaiser, 1974; Malaquias et al., 2017b). Data were standardized by dividing the difference between each observation and the arithmetic mean of each variable of interest by the standard deviation of the same variable, using R software (Studio version

3.6.1). Path analysis modelling was used to explain the interactions involving abundance of pests and natural enemies in Bt and non-Bt soybean.

4.3. Results

4.3.1. Faunistic analysis

In the 2016–2017 crop season, 15 ground-dwelling predatory arthropod species were collected in Bt and non-Bt soybean, totaling 216 and 211 individuals, respectively (Table 3). *Kleter* sp., *Camponotus* sp. and *Corydalus* sp. occurred only in the Bt soybean area, and *Tachys* sp., *Cyphomyrmex* sp. and *Timula* sp. only in non-Bt soybean (Table 3). With the exception of *Kleter* sp., which was dominant, and *Corydalus* sp., which was very frequent, the other species were non-dominant, rare, or scattered, showing that their occurrences were random, with low population densities and not influenced by Bt soybean. For species that occurred in both treatments, except *Scarites* sp., *Euborellia* sp. and *Odontomachus* sp., the faunistic indexes were similar. Despite differences in some faunistic parameters for *Scarites* sp., *Euborellia* sp. and *Odontomachus* sp., the numbers and frequency of the sampled individuals were insufficiently different to indicate any influence of Bt soybean on populations of these arthropods. *Euborellia* sp. predominated in both the Bt and non-Bt soybean areas, while *Pheidole* sp. predominated only in Bt soybean (Table 3).

In the 2017–2018 crop season, 637 and 674 individuals were captured in the Bt and non-Bt soybean areas, representing 20 and 25 species, respectively (Table 4). *Brachinus fuscicornis* (Dejean) (Coleoptera: Carabidae), *Notiobia* sp., *Scarites* sp., *Labidura xanthopus* (Stal) (Dermaptera: Labiduridae), and *Cyphomyrmex* sp. occurred only in the Bt soybean area, and *Athrostictus speciosus* Dejean, 1829 (Coleoptera: Carabidae), *Galerita collaris* (Dejean), *Polpochila impressifrons* (Dejean) (Coleoptera: Carabidae), *Aleochara* sp., *Eulissus chalybaeus* (Mannerheim) (Coleoptera: Staphylinidae), *Cabro* morph., *Eciton* sp. and *Hoplocrates* sp. in non-Bt soybean (Table 4). Only *Cyphomyrmex* sp. was dominant and frequent. The other species were non-dominant, rare, or scattered, and accidental, indicating that their occurrences were random, with low population densities and not influenced by Bt soybean.

The faunistic indexes were similar for the species that occurred in both treatments, except for *C. granulatum*, *M. brasiliensis*, *Odontocheila nodicornis* (Dejean) (Coleoptera: Carabidae), *Scarites* sp., *Selenophorus* sp., *Euborellia* sp. and *Odontomachus* sp. Although there were differences in some faunistic parameters for these species, the participation and

frequency of the individuals collected were not sufficiently different to indicate an influence of Bt soybean on their populations. Spiders and *Pheidole* sp. were predominant in both the Bt and non-Bt soybean areas, and *O. nodicornis* only in the non-Bt soybean area (Table 4).

4.3.2. Ecological diversity index

Sampling in the 2016–2017 crop season revealed higher richness (S) and abundance (N) of the species sampled in the Bt soybean compared to the non-Bt area, except for the richness (S) in the second sampling and abundance (N) in the first two samplings. No significant differences were found between treatments for Simpson's diversity (D), Shannon-Wiener diversity (H') and Pielou's evenness (J') indexes on the sampling dates. Insufficient individuals were obtained in non-Bt soybean in the last collection to run analyses (Table 5).

In the 2017–2018 crop season, richness (S) of species was 55.55% higher and abundance of individuals (N) was 44.44% higher in the non-Bt than in the Bt. Simpson's diversity (D) and Pielou's evenness (J') indexes did not differ significantly among the treatments. The Shannon-Wiener diversity index (H') differed significantly in the sixth collection (Table 6).

4.3.3. Principal components analysis

Based on the Kaiser criterion (eigenvalues higher than 1), three main components were selected from the correlation matrix of data collected for arthropod predators in the 2016–2017 and 2017–2018 cropping seasons.

PC1 and PC2 explain 24.10% and 14.00% of the total variation in the populations, respectively (Figure 1). PC3 explains very small percentages (less than 12%) of the total variation, therefore the relationship involving PC1 and PC2 with PC3 was not significantly informative or revealed apparent patterns, thus the plots about PC1 versus PC3 and PC2 versus PC3 are available in the supplementary material.

The first principal component (PC1) is represented by the weighted average of the Araneae (eigenvector value= 0.4501), *Euborellia* sp. (eigenvector value= 0.3515), *Odontomachus* sp. (eigenvector value= 0.3105), *Camponotus* sp. (eigenvector value= 0.3104) and *M. brasiliensis* (eigenvector value= 0.3054) (Fig. 1). PC2 includes the contrast between the occurrence of *M. brasiliensis* (eigenvector value= 0.4634), *Odontomachus* sp. (eigenvector value= 0.4103) and the weighted averages of *Kleter* sp. (eigenvector value= -0.3660) and *Scarites* sp. (eigenvector value= -0.3719) (Fig. 1).

There was overlapping among confidence ellipses (95%); however, it was possible to visualize a significant characterization of the natural enemies (vectors) between seasons. Great part of the occurrence of *Euborellia* sp., *Camponotus* sp. and *Kleter* sp., was evidenced in non-Bt soybean. On the other hand, *M. brasiliensis* and *Odontomachus* sp. had more abundance in Bt soybean in the second season. Non-Bt soybean in the first season was characterized by high values of *P. impressifrons*, *Athrostictus* sp. and *C. granulatum*, and Bt soybean was characterized by the same species and with addition *Scarites* sp. (Fig. 1).

The third principal component (PC3) is represented by the contrast between the weighted average of the number of *Selenophorus* sp. (eigenvector value= 0.5517) and *Athrostictus* sp. (eigenvector value= 0.4630) and *Pheidole* sp. (eigenvector value= - 0.4160) (Fig. 1). In general, the biplot graphs showed that the interactions of certain predators are specific for each soybean cultivar. Univariate tests (see abundance comparison section) were applied to evidence the significance effect of the factors: cultivar and season, and the interactions between them.

4.3.4. Path analysis with pests and natural enemy abundance

For the path analysis in non-Bt soybean, the pest species *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae), *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), *Spodoptera* spp., *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), *Piezodorus guildinii* (Westwood), *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae) and *Diabrotica speciosa* (Germar) (Coleoptera: Chrysomelidae) were included as explanatory variables. In Bt soybean, we excluded the variables *A. gemmatalis* and *P. guildinii*, because the non-occurrence of these species. Natural enemy (Araneae, Cicindelinae and *Euborellia* sp.) were included in the path models as both independent variables, because we tried to explain expected effect of the pests in Bt and non-Bt areas to natural enemy abundance. The number that are associated with paths between pests and natural enemies are path coefficients presented as standardized values, these values are scaled by the standard deviations of the variables.

The path models explained 67.30% (Bt soybean) and 58.60% (non-Bt soybean) of the variation in the total number of Araneae, 58.10% (Bt soybean) and 57.60% (non-Bt soybean) of Cicindelinae, and 14.20% (Bt soybean) and 65.70% (non-Bt soybean) of *Euborellia* sp. (Figures 2 and 3). Based on structural equation models, the significant relations involving Araneae are with *D. speciosa* ($P= 0.0001$, $\delta= 0.783$) and *Spodoptera* spp. ($P= 0.002$, $\delta= -$

0.573) in Bt soybean; and with only *Spodoptera* spp. ($P= 0.0001$, $\delta= -0.940$) in non-Bt soybean (Figs. 2 and 3).

Expressive interactions were observed between Cicindelinae with *E. heros* ($P= 0.0001$, $\delta= 0.707$) and *D. speciosa* ($P= 0.0001$, $\delta= 0.690$), in Bt and non-Bt soybean, respectively (Fig. 2 and 3). In relation to *Euborellia* sp., it had only significant interaction in non-Bt soybean involving the following pest species *C. includens* ($P= 0.024$, $\delta= 0.421$), *E. heros* ($P= 0.0001$, $\delta= 0.750$) and *H. armigera* ($P= 0.005$, $\delta= -0.517$) (Figs. 2 and 3).

4.3.5. Abundance comparison

The lowest abundance of *O. nodicornis* and *C. granulatum* was recorded in Bt soybean in the first (2016–2017) and second season (2017–2018), respectively, while *Pheidole* sp. was less abundant during the first season in non-Bt soybean (2016–2017). For these three species there was interaction between season *versus* soybean cultivar ($df= 1$; $P < 0.05$) (Fig. 4).

No significant interaction ($df= 1$; $P < 0.05$) or statistical differences between year ($df= 1$; $P < 0.05$) or cultivar ($df= 1$; $P < 0.05$) were evidenced in *Kleber* sp., *Scarites* sp. and *Selenophorus* sp. (Fig. 5). *M. brasiliensis*, *Camponotus* sp. and *P. impressifrons* occurrence differed significantly between seasons ($df= 1$; $P < 0.05$); however, no interactions ($df= 1$; $P > 0.05$) and difference between cultivars ($df= 1$; $P > 0.05$) were evidenced. *M. brasiliensis* and *Camponotus* sp. had higher abundance in the second season (2017-2018), and the opposite was observed in *P. impressifrons* (Fig. 5).

The abundance of *Euborellia* sp. differed significantly between cultivars, and between seasons; however, no interactions were observed between these factors. *Euborellia* sp. was more abundant in non-Bt soybean than Bt soybean, with more expressive abundance in the first season (2016-2017) (Fig. 5).

4.4. Discussion

This study showed that there is no ecological impact of Bt soybean (Cry1Ac) on the ground-dwelling predatory arthropod community. Marques et al. (2018) evaluated the ecological impact of Bt soybean (Cry1Ac and Cry1F) on the diversity and abundance of foliar-dwelling and ground-dwelling natural enemies and found no adverse effects. Justiniano et al. (2014) found no negative ecological impact of Bt soybean (Cry1Ac) on the abundance and diversity of the natural enemy community for foliar dwelling insect any soybean growth

stage. Besides, absence of negative ecological impact on predator species were found on Bt cotton producing Cry1Ac and Cry2Ab toxins (Thomazoni et al., 2013; Lima Junior et al., 2013; Zhao et al., 2016).

Bt crops have been favoring the action of natural enemies of agricultural pests by reducing the the broad-spectrum insecticide inputs that have historically caused high degree of mortality on natural enemies (Romeis et al., 2019; Snyder, 2019). Here, we found important species for biological pest control in both the Bt and non-Bt soybean plots, such as *Kleter* sp., *Scarites* sp. and *Selenophorus* sp. *O. nodicornis*, *C. granulatum*, *Pheidole* sp, had variation between cultivars according to season, and *M. brasiliensis*, *Camponotus* sp. and *P. impressifrons* occurrence differed significantly between seasons. These natural enemies have an important ecological role in agroecosystems and are responsible for natural pest mortality (Cardinale et al., 2003; Straub & Snyder, 2008; Naranjo et al., 2015). The importance of the carabids *C. granulatum*, *O. nodicornis*, *Galerita* sp., *Scarites* sp. and the staphylinid *Eulissus chalybaeus* as control agents of the soybean pest *A. gemmatalis* was reported by Cividanes et al. (2014). The frequency of the ants *Pheidole* sp. and *Odontomachus* sp. has been highlighted by their importance for pest control and as bioindicator species for assessing environmental risks (Risch, 1981; Gerlach et al., 2013; Fernandes et al., 2019).

Our path analysis revealed that the unique significant interaction that was simultaneous for Bt and non-Bt soybean were those involving Araneae and *Spodoptera* spp. Some natural enemies had significant interactions with pest only in one of the cultivars (Bt or non-Bt), as observed in *Euborellia* sp. in non-Bt soybean. Therefore, the models from path analysis revealed that for some species the soybean cultivar could be a primary factor to identify the impact of availability of prey to influence the strength of these hypothesized relationships. In fact, *Euborellia* sp. had more abundance in non-Bt soybean and being more expressive in the first season (2016-2017). *Euborellia* sp. is considered an efficient predator of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) eggs and larvae, an important non-target pest that damages the leaves and pods of Bt soybean (Silva et al., 2009a, b; Martins-Salles et al., 2017). Spiders were abundant and frequent in the Bt and non-Bt soybean areas in both crop seasons in our study; and it has contributed to natural control of several groups of agricultural pests (Peterson et al., 2011; Silva et al., 2014; Michalko et al., 2019).

Interestingly, although our findings suggested a high similarity to ground-dwelling predatory arthropods between treatments, the faunistic analyses showed that some species occurred only in Bt soybean areas or in non-Bt areas during the cropping seasons. The

importance and use of faunistic and ecological diversity indexes in environmental risk assessment must be emphasized (Silveira Neto et al., 1995; Candolfi et al., 2004; Silveira Neto et al., 2014; Marques et al., 2018; Fernandes et al., 2019). This analysis makes it possible to explore the impact of Bt soybean on ground-dwelling predatory arthropods and to assess ecological risks. Richness (number of species), abundance (evenness) and homogeneity of individuals are main components of the biodiversity of a community (Olfert et al., 2002; Magurran, 2013). Communities with a higher diversity of natural enemies and higher ecological diversity indexes have greater potential to support biological control (Jonsson et al., 2017; Greenop et al., 2018; Snyder, 2019).

In this study, although individual species differed in abundance (N) and richness (S) during the sampling (Tables 5 and 6), these differences do not indicate a negative ecological impact of Bt soybean. The response of this community was probably more closely related to the effects of insecticides and to environmental or weather conditions rather than to an effect of Bt soybean, as well as to poor host quality (Naranjo, 2009; Resende et al., 2016; Marques et al., 2018). Some authors have suggested that differences in species abundance greater than 50% are a reasonable threshold to compare ecological impact (Perry et al., 2003; Torres & Ruberson, 2005). In addition, the abundance of ground-dwelling beetles can be influenced by the variation of lepidoptera larvae population between treatments. Furthermore, although significantly different abundances were found for *Pheidole* sp. and *C. granulatum*, these did not indicate adverse ecological effects. This hypothesis is supported by the ecological diversity indexes during the sampling period (Tables 5 and 6). Besides that, these values refer to only two species and do not correspond to the entire community of ground-dwelling predatory arthropods, agreeing with the findings of Frizzas et al. (2017). Thus, our values do not indicate a negative impact of Bt soybean on the ecological diversity indexes, supporting the hypothesis that Bt crops and biological control have synergism and can be compatible in integrated pest management programs.

With respect to insect resistance management, the absence of a negative ecological impact of Bt soybean on ground-dwelling predatory arthropods can help to delay the evolution of resistance to Bt soybean target pests, as a function of their potential capacity to predate and eventually eliminate resistant individuals (Guo et al., 2014; Liu et al., 2014; Romeis et al., 2019). In Bt broccoli cultivar, *Coleomegilla maculata* (De Geer) (Coleoptera: Coccinellidae) combined with non-Bt and an unsprayed refuge area delayed the development of resistance in *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) compared to

treatments without the predator (Liu et al., 2014). The opposite effect can be also occurring in non-Bt soybean areas, but few studies have addressed the potential negative effects of natural enemies on the effectiveness of refuges. Assessing the best refuge option and refuge crop performance, the authors mentioned the potential negative impact of the parasitoid ichneumonid wasp *Heteropelma scaposum* (Morley) (Hymenoptera: Ichneumonidae) on reducing the production of pupae of susceptible individuals of the lepidopteran moth *Helicoverpa* spp. (Baker et al., 2008; Baker & Tann, 2014, 2017).

In agroecosystems with a high biodiversity of natural enemies, the potential mortality rates of eggs, larvae and adult moths are higher and can have potentially detrimental effects on the production of susceptible moths in refuges (Whitehouse et al., 2005; Lawrence et al., 2007). The main role of refuge areas is the production of susceptible individuals to mate with the rare resistant individuals from Bt areas and reduce the likelihood of Bt resistance. Refuges, to be most effective, must produce sufficient numbers of susceptible insects. For each resistant individual, at least 500 susceptible individuals must be generated in refuge areas (Gould, 1998; SAP, 1998). Thus, when predators kill the susceptible Bt target pest, the likelihood of providing enough individuals from refuge areas to mate with resistant individuals from Bt soybean areas can be reduced.

Here, *O. nodicornis*, *Galerita* sp., *Scarites* sp., *C. granulatum*, *Kleter* sp., *Euborellia* sp., *L. riparia*, spiders and other ground-dwelling predatory arthropods that occur exclusively in non-Bt soybean areas (Tables 3 and 4) have the potential to negatively affect the effectiveness of refuge areas, as demonstrated by their high predation capacity (Martins et al., 2012; Cividanes et al., 2014; Michalko et al., 2019). In practical terms, considering the mortality rates of the target and non-target Bt-soybean pests predated by other groups of natural enemies, together with the population suppression of pests in non-Bt areas (Wan et al., 2012; Dively et al., 2018), the provision of susceptible individuals in refuge field may be reduced (Baker et al., 2008; Baker & Tann, 2014, 2017).

Since the potential benefit of predatory natural enemies poses a dilemma, complementary studies of this kind are needed. Furthermore, we suggest the application of ecological models using data on the predatory capacity of predominant, dominant and exclusively occurring species; the relationship between predator and prey (independent, synergistic or antagonistic) and among predators (cooperative, competitive, intraguild predation and anti-predator responses) (Rosenheim et al., 1993; Sih et al., 1998; Vance-Chalcraft & Soluk, 2005; Rudolf, 2007; McCoy et al., 2012); the size and spatial

configuration of the refuge area; the application rate and chemical group of insecticides and their impact on survival of target pests; and the lethal and sublethal effect on the prey and predator, as important aspects to validate this potential scenario.

It is important to highlight some limitations in this study, such as the absence of a treatment without insecticide application and the simplification of spider classification into a single taxonomic group. On the other hand, this study was conducted under the actual conditions of phytosanitation practices and commercial soybean cultivation in Brazil. The lack of negative impact by Bt crops on spiders has been reported in other studies (Peterson et al., 2011; Silva et al., 2014); however, knowledges of the richness of spider species and their bioecological characteristics is important for biological control and environmental risk assessments, which must be applied in future studies.

To conclude, we found no negative ecological impact of Bt soybean (Cry1Ac) on the ground-dwelling predatory arthropod community, or significant differences in their diversity. The most species in Bt and non-Bt soybean areas consisting of members of the families Carabidae, Formicidae, Anisolabididae and the Araneidae group, from orders Coleoptera, Hymenoptera, Dermaptera and spiders. Some ground-dwelling predatory arthropods were non-dominant, scattered or rare, less frequent, accidental, and/or occurred only in Bt or non-Bt areas. Our analyses showed that the interactions of certain predators are specific for each soybean cultivar and this current relationship with predator species could be dependent on the prey and season. *Euborellia* sp. is more abundant in non-Bt soybean, and it had only significant prey-interaction in non-Bt soybean, involving the following lepidopteran species *C. includens* and *H. armigera*. Others showed predominance and dominance, with a significant participation in biological pest control and a potential to delay or accelerate the evolution of resistance by preying on target pests that are resistant and/or susceptible to Bt soybean. However, studies using ecological modeling are recommended to simulate scenarios and provide an additional information.

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Figures and Tables Legends

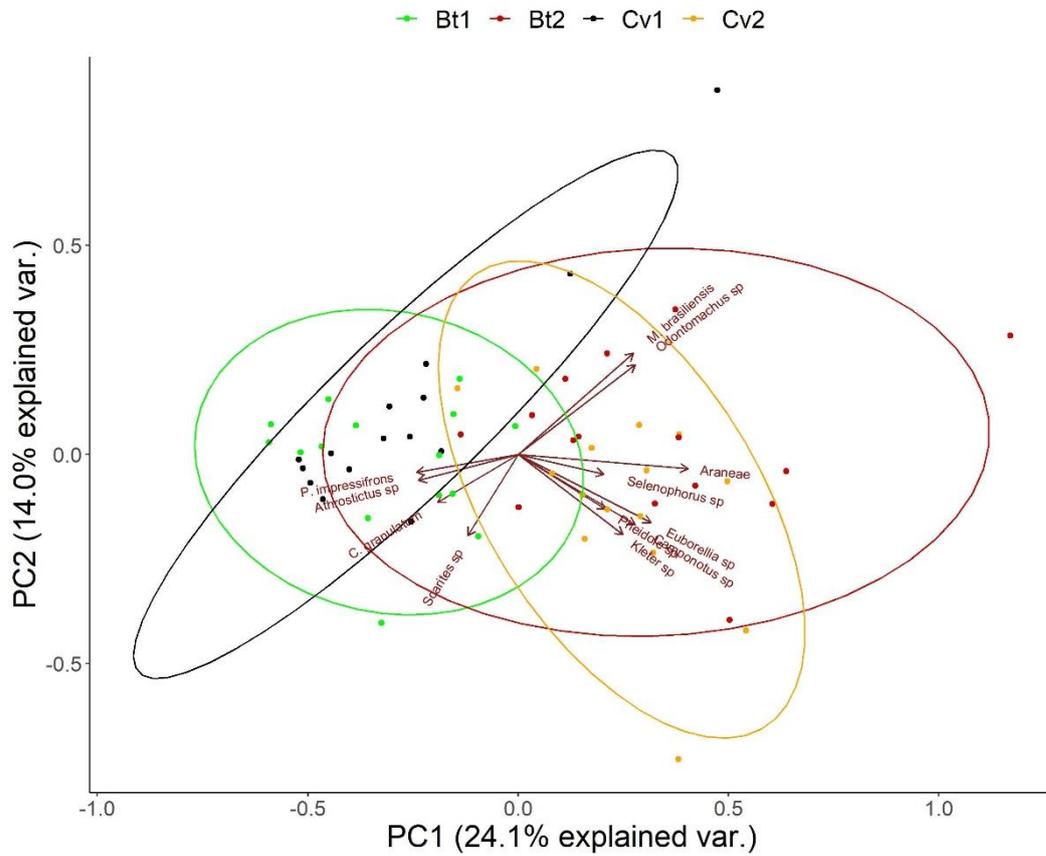


Figure 1. Distribution of dominant ground-dwelling predatory arthropods (plotted as vectors) sampled in the Bt and non-Bt (Cv) soybean areas during the 2016–2017 (1) and 2017–2018 (2) crops season in the principal components analysis biplot (PCA).

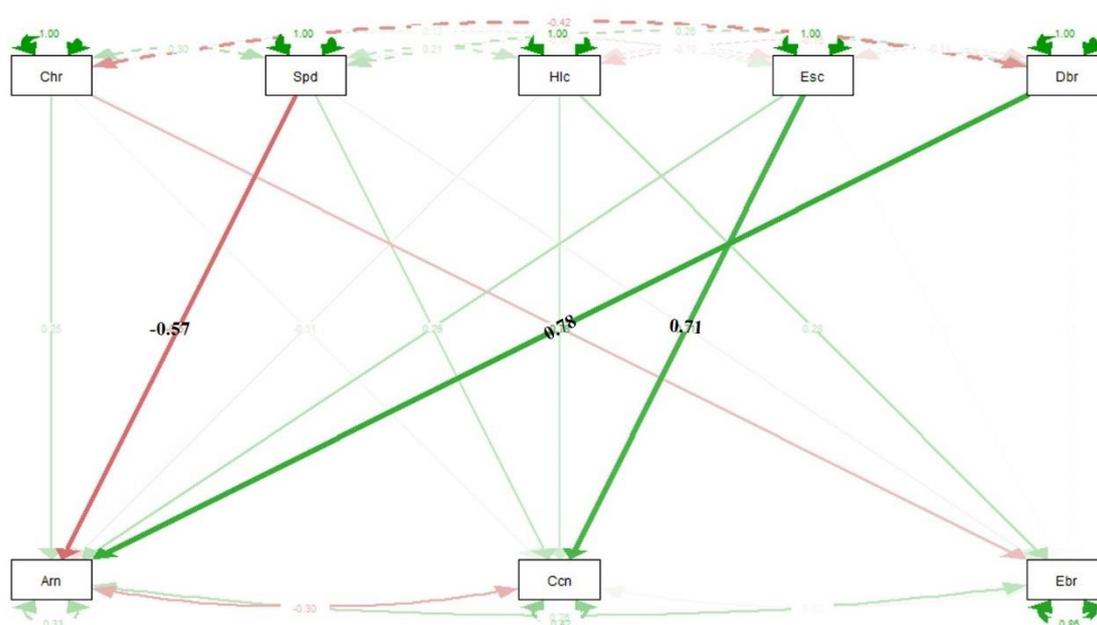


Figure 2. Path analysis model to describe natural enemy abundance [Araneae (Arn), Cicindelidae (Ccn) and *Euborellia* sp. (Ebr)] according to occurrence of the pest species *Chrysodeixis includens* (Chr), *Spodoptera* spp. (Spd), *Helicoverpa armigera* (Hlc), *Euschistus heros* (Esc) and *Diabrotica speciosa* (Dbr) (independent variables) in Bt soybean. The thickness of lines and arrows is proportional to effect size to response variable; numbers are path coefficients (range between -1 to 1).

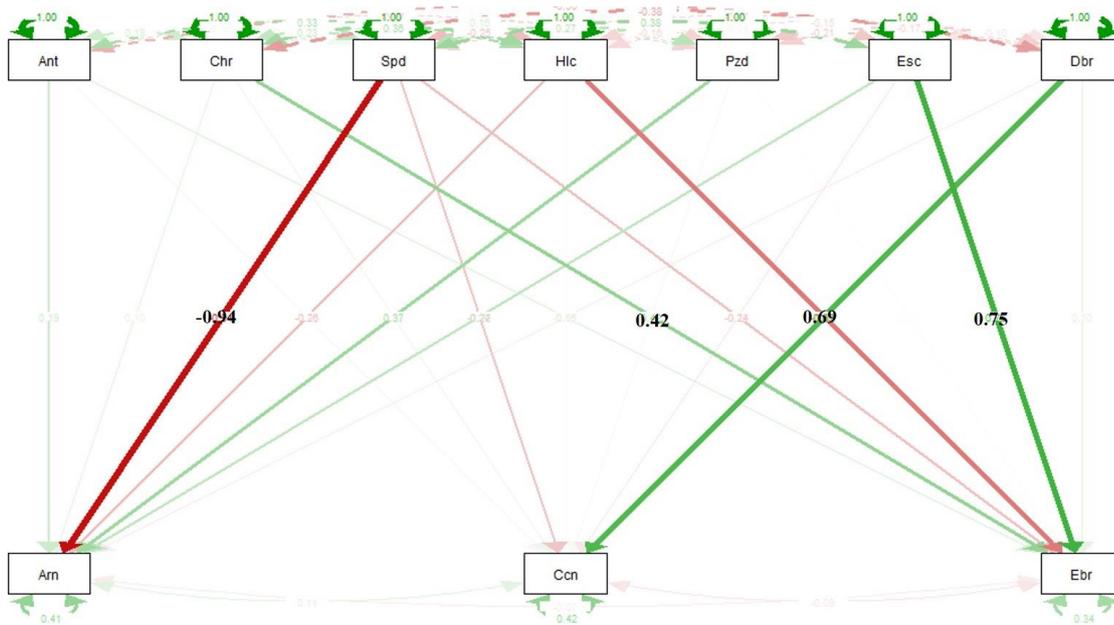


Figure 3. Path analysis model to describe natural enemy abundance [Araneae (Arn), Cicindelinae (Ccn) and *Euborellia* sp. (Ebr)] according to occurrence of the pest species *Anticarsia gemmatilis* (Ant), *Chrysodeixis includens* (Chr), *Spodoptera* spp. (Spd), *Helicoverpa armigera* (Hlc), *Piezodorus guildinii* (Pzd), *Euschistus heros* (Esc) and *Diabrotica speciosa* (Dbr) (independent variables) in non-Bt soybean. The thickness of lines and arrows is proportional to effect size to response variable; numbers are path coefficients (range between -1 to 1).

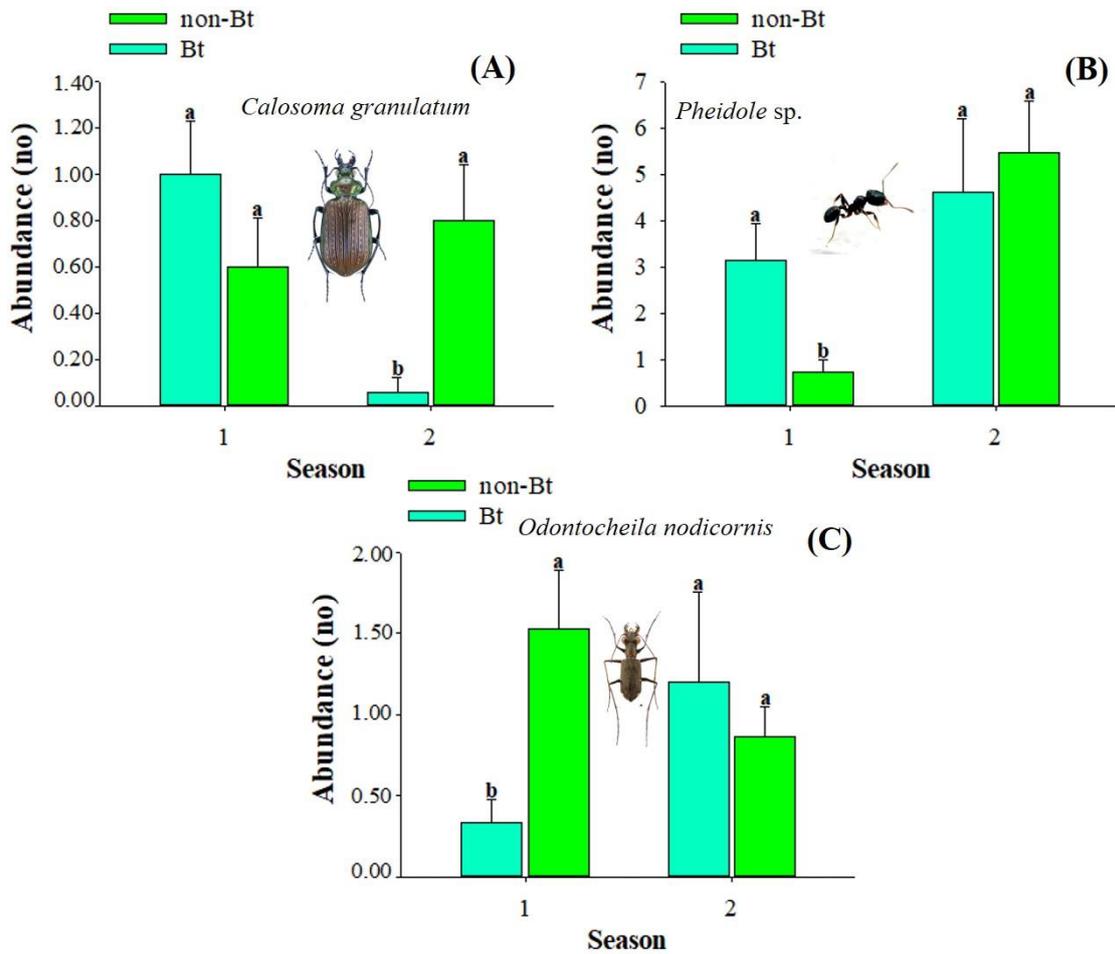


Figure 4. Comparison between abundances (mean ± standard error) of *Calosoma granulatum*, *Pheidole sp.* and *Odontocheila nodicornis* in Bt and non-Bt soybean during two seasons.

***Statistically significant *P*-value ($\alpha = 0.05$).

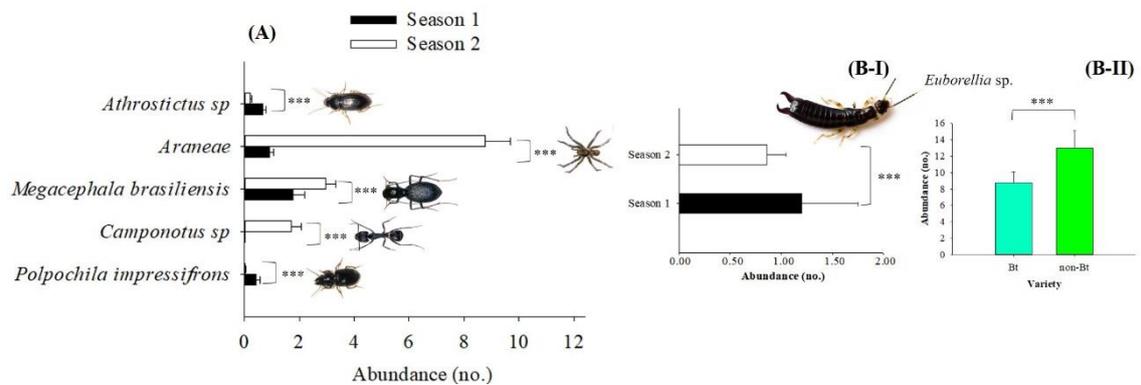


Figure 5. Comparison of abundances (mean ± standard error) of *Athrostictus sp.*, *Araneae*, *Megacephala brasiliensis*, *Camponotus sp.* and *Polpochila impressifrons* between seasons (A) and *Euborellia sp.* between season (B-I) and cultivar (B-II).

***Statistically significant *P*-value ($\alpha = 0.05$).

Table 1. Phytosanitary products sprayed during the crop season 2016/2107 on Bt and non Bt-soybean according phenological growth stages.

Treatments	Comercial product	Active ingrediente	Field rate (L ha ⁻¹)	Phenological growth stages	Targets
Bt soybean	Fox	Trifloxistrobin (15%) + Protiocozol (17.5%)	0.4	V7/R1	Soybean foliar disease ¹
	Aureo	Soybean oil methyl ester (72%)	0.25	V7/R1	Adjuvant ²
	Engeo pleno	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	V7/R1	Stink bugs ³
	Elatus	Azoxistrobin (30%) + Benzovindiflupir (15%)	0.2	R5.3/R5.4	Soybean foliar disease
	Aureo	Soybean oil methyl ester (72%)	0.5	R5.3/R5.6	Adjuvant
	Engeo pleno	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	R5.3/R5.5	Stink bugs
Non-Bt soybean	Unizeb Gold	Mancozeb (75%)	1.5	R1	Soybean foliar disease
	Orthene 750 BR	Acephate (75%)	0.5	R1	Stink bugs and lepidopterans-pest ⁴
	Fox	Trifloxistrobin (15%) + Protiocozol (17.5%)	0.4	R1	Soybean foliar disease
	Aureo	Soybean oil methyl ester (72%)	0.25	R1	Adjuvant
	Elatus	Azoxistrobin (30%) + Benzovindiflupir (15%)	0.2	R5.1/R5.2	Soybean foliar disease
	Aureo	Soybean oil methyl ester (72%)	0.25	R5.1/R5.2	Adjuvant
	Orthene	Acephate (75%)	0.5	R5.1/R5.2	Stink bugs and lepidopterans-pest
	Elatus	Azoxistrobin (30%) + Benzovindiflupir (15%)	0.2	R5.3	Soybean foliar disease
	Eforia	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	R5.3	Stink bugs
	Aureo	Soybean oil methyl ester (72%)	0.2	R5.3/R5.4	Adjuvant
	Orthene 750 BR	Acephate (75%)	0.5	R5.3/R5.4	Stink bugs and lepidopterans-pest
	Orthene 750 BR	Acephate (75%)	1.0	R5.4/R5.5	Stink bugs and lepidopterans-pest
	Curyom 550 EC	Profenofos (50%) + Lufenuron (5%)	0.25	R5.4/R5.5	Lepidopterans-pest
	Score flexi	Propiconazol (25%) + Difenocozol (25%)	0.2	R5.4/R5.5	Soybean foliar disease
	Aureo	Soybean oil methyl ester (72%)	0.5	R5.4/R5.5	Adjuvant
	Score flexi	Propiconazol (25%) + Difenocozol (25%)	0.2	R6	Soybean foliar disease
Eforia	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	R6	Stink bugs	
Aureo	Soybean oil methyl ester (72%)	0.5	R6	Adjuvant	

¹Soybean rust (*Phakopsora pachyrhizi*) and late-season soybean diseases (*Microsphaera diffusa*, *Corynespora cassicola*);²Spreader-sticker surfactant to improve fungicide performance;³ Neotropical brown stink bug (*Euschistus heros*);⁴Heliothinae complex (*Chloridea virescens*, *Helicoverpa armigera*).

Table 2. Phytosanitary products sprayed during the crop season 2017/2018 on Bt and non Bt-soybean according phenological growth stages.

Treatment	Comercial product	Active ingredient	Field rate (L ha ⁻¹)	Phenological growth stages	Targets
Bt soybean	Fox	Trifloxistrobin (15%) + Protiocanazol (17.5%)	0.4	V6/V7	Soybean foliar disease ¹
	Vegetal Oil EC	Esters of vegetable fatty acids (93%)	0.4	V6/V7	Adjuvant ²
	Eforia	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	V6/V7	Stink bugs ³
	Fox	Trifloxistrobin (15%) + Protiocanazol (17.5%)	0.4	R4/R5	Soybean foliar disease
	Vegetal Oil EC	Esters of vegetable fatty acids (93%)	0.5	R4/R5	Adjuvant
	AG - Bem	Adhesive resin (37.5%) + Anionic surfactant (12.5%)	0.025	R4/R5	Adjuvant
	Galil SC	Bifentrin (5%) + Imidacloprid (25%)	0.25	R5.4/R5.5	Stink bugs
	Actara 250 WG	Tiametoxan (25%)	1.4	R5.4/R5.5	Stink bugs
	Galil SC	Bifentrin (5%) + Imidacloprid (25%)	0.25	R5.5/R5.6	Stink bugs
	Actara 250 WG	Tiametoxan (25%)	1.4	R5.5/R5.6	Stink bugs
	Elatus	Azoxistrobin (30%) + Benzovindiflupir (15%)	0.2	R5.5/R5.6	Soybean foliar disease
	AG - Bem	Adhesive resin (37.5%) + Anionic surfactant (12.5%)	0.025	R5.5/R5.6	Adjuvant
	Vegetal Oil EC	Esters of vegetable fatty acids (93%)	0.25	R5.5/R5.6	Adjuvant
Non Bt soybean	Pirate	Clorfenapyr (24%)	0.4	V4/V5	Lepidopterans-pest ⁴
	Bazuka	Methomyl (21.6%)	0.8	V4/V5	Lepidopterans-pest
	Fox	Trifloxistrobin (15%) + Protiocanazol (17.5%)	0.4	V6/V7	Soybean foliar disease
	Vegetal Oil EC	Esters of vegetable fatty acids (93%)	0.4	V6/V7	Adjuvant
	Eforia	Lambda-cyhalothrin (10.6%) + Thiametoxan (14.1%)	0.2	V6/V7	Stink bugs
	Orthene 750 BR	Acephate (75%)	1.0	R2/R3	Stink bugs and lepidopterans-pest
	AG - Bem	Adhesive resin (37.5%) + Anionic surfactant (12.5%)	0.025	R2/R3	Adjuvant
	Fox	Trifloxistrobin (15%) + Protiocanazol (17.5%)	0.4	R4/R5	Soybean foliar disease
	Vegetal Oil EC	Esters of vegetable fatty acids (93%)	0.5	R4/R5	Adjuvant
	AG - Bem	Adhesive resin (37.5%) + Anionic surfactant (12.5%)	0.025	R4/R5	Adjuvant
	Galil SC	Bifentrin (5%) + Imidacloprid (25%)	0.25	R5.4/R5.5	Stink bugs
	Actara 250 WG	Tiametoxan (25%)	1.4	R5.4/R5.5	Stink bugs

¹Soybean rust (*Phakopsora pachyrhizi*) and late-season soybean diseases (*Microsphaera diffusa*, *Corynespora cassicola*);²Spreader-sticker surfactant to improve fungicide performance;³ Neotropical brown stink bug (*Euschistus heros*);⁴Heliothinae complex (*Chloridea virescens*, *Helicoverpa armigera*).

Table 3. Faunistic analysis of ground-dwelling predatory arthropods captured by pitfall traps in Bt and non-Bt soybean during the 2016–2017 crop season.

Species	Order	Family	Bt soybean (n = 216)						non-Bt soybean (n = 211)					
			%PI ³	%FI ⁴	D	A	F	C	%PI	%FI	D	A	F	C
Spiders	Araneida	–	6.94 (15)	100 (6)	D	c	F	W	5.69 (12)	83.33 (5)	D	c	F	W
<i>Athrostictus</i> sp.	Coleoptera	Carabidae	5.55 (12)	66.67 (4)	D	c	F	W	3.80 (8)	50.0 (3)	D	c	F	W
<i>Calosoma granulatum</i>	Coleoptera	Carabidae	6.94 (15)	66.67 (4)	D	c	F	W	4.26 (9)	83.33 (5)	D	c	F	W
<i>Megacephala brasiliensis</i> ²	Coleoptera	Carabidae	10.18 (22)	100 (6)	D	c	F	W	14.70 (31)	66.67 (4)	D	va	MF	W
<i>Polpochila impressifrons</i>	Coleoptera	Carabidae	3.24 (7)	66.67 (4)	D	c	F	W	2.84 (6)	50.0 (3)	D	c	F	W
<i>Scarites</i> sp.	Coleoptera	Carabidae	1.85 (4)	33.33 (2)	ND	s	LF	Y	3.31 (7)	50.0 (3)	D	c	F	W
<i>Selenophorus</i> sp.	Coleoptera	Carabidae	1.85 (4)	16.67 (1)	ND	s	LF	Z	0.94 (2)	16.67 (1)	ND	r	LF	Z
<i>Tachys</i> sp.	Coleoptera	Carabidae	–	–	–	–	–	–	0.94 (2)	16.67 (1)	ND	r	LF	Z
<i>Euborellia</i> sp. ^{1,2}	Dermaptera	Anisolabididae	33.33 (72)	83.33 (5)	D	va	LF	W	46.92 (99)	100 (6)	SD	sa	SF	W
<i>Kleter</i> sp.	Dermaptera	Forficulidae	2.78 (6)	50 (3)	D	c	F	W	–	–	–	–	–	–
<i>Labidura xanthopus</i>	Dermaptera	Labiduridae	0.46 (1)	16.67 (1)	ND	r	LF	Z	0.47 (1)	16.67 (1)	ND	r	LF	Z
<i>Cyphomyrmex</i> sp.	Hymenoptera	Formicidae	–	–	–	–	–	–	0.47 (1)	16.67 (1)	ND	r	LF	Z
<i>Camponotus</i> sp.	Hymenoptera	Formicidae	0.46 (1)	16.67 (1)	ND	r	LF	Z	–	–	–	–	–	–
<i>Dinoponera</i> sp.	Hymenoptera	Formicidae	1.38 (3)	50.0 (3)	ND	s	LF	W	1.42 (3)	16.67 (1)	ND	s	LF	Z
<i>Odontomachus</i> sp.	Hymenoptera	Formicidae	2.31 (5)	83.33 (5)	ND	c	F	W	8.53 (18)	33.33 (2)	D	va	MF	Y
<i>Pheidole</i> sp. ¹	Hymenoptera	Formicidae	21.76 (47)	66.67 (4)	D	va	VF	W	5.21 (11)	50.0 (3)	D	C	F	W
<i>Timulla</i> sp.	Hymenoptera	Mutillidae	–	–	–	–	–	–	0.47 (11)	16.67 (1)	ND	R	LF	Z
<i>Corydalus</i> sp.	Megaloptera	Corydalide	0.92 (2)	33.33 (2)	ND	s	VF	Y	–	–	–	–	–	–

¹Predominant species in Bt soybean; ²Predominant species in non-Bt soybean; ³%PI: Participation of individuals sampled (%) followed by the number of insects sampled in parentheses; ⁴%FI: Frequency of individuals sampled (%) followed by the number of times collected in parentheses; D: Dominance – superdominant (SD), dominant (D), non-dominant (ND); A: Abundance – superabundant (sa), very abundant (va), abundant (a), rare (r), common (c) and scattered (s); F: Frequency – superfrequent (SF), very frequent (VF), frequent (F) and less frequent (LF); C: Constancy – constant (W), accessory (Y) and accidental (Z).

Table 4. Faunistic analysis of ground-dwelling predatory arthropods captured by pitfall traps in Bt and non-Bt soybean during the 2017–2018 crop season (Conitnue).

Species	Order	Family	Bt soybean (n = 637)						non-Bt soybean (n = 674)					
			%PI	%FI	D	A	F	C	%PI	%FI	D	A	F	C
Spiders ^{1,2}	Araneida	–	22.13 (141)	100.0 (9)	D	va	VF	W	18.10 (122)	100 (9)	D	va	VF	W
<i>Aspidoglossa crenata</i>	Coleoptera	Carabidae	0.15 (1)	11.11 (1)	ND	r	LF	Z	0.29 (2)	22.22 (2)	ND	s	LF	Z
<i>Athrostictus</i> sp.	Coleoptera	Carabidae	0.62 (4)	22.22 (2)	ND	s	LF	Z	0.29 (2)	11.11 (1)	ND	s	LF	Z
<i>Athrostictus speciosus</i>	Coleoptera	Carabidae	–	–	–	–	–	–	0.14 (1)	11.11 (1)	ND	s	LF	Z
<i>Brachinus fuscicornis</i>	Coleoptera	Carabidae	0.31 (2)	22.22 (2)	ND	r	LF	Z	–	–	–	–	–	–
<i>Brzoskaicheila hispidula</i>	Coleoptera	Carabidae	0.47 (3)	11.11 (1)	ND	s	LF	Z	0.29 (2)	11.11 (1)	ND	s	LF	Z
<i>Calosoma granulatum</i>	Coleoptera	Carabidae	0.15 (1)	11.11 (1)	ND	r	LF	Z	1.78 (12)	66.67 (6)	D	c	F	W
<i>Camptodontus</i>	Coleoptera	Carabidae	0.15 (1)	11.11 (1)	ND	r	LF	Z	0.14 (1)	11.11 (1)	ND	s	LF	Z
<i>Galerita collaris</i>	Coleoptera	Carabidae	–	–	–	–	–	–	0.14 (1)	11.11 (1)	ND	s	LF	Z
<i>Megacephala brasiliensis</i>	Coleoptera	Carabidae	7.70 (49)	77.78 (7)	D	c	F	W	5.93 (40)	55.56 (5)	D	va	VF	W
<i>Notiobia</i> sp.	Coleoptera	Carabidae	0.15 (1)	11.11 (1)	ND	r	LF	Z	–	–	–	–	–	–
<i>Odontocheila nodicornis</i> ²	Coleoptera	Carabidae	8.95 (57)	88.89 (8)	D	a	VF	W	1.92 (46)	66.67 (6)	D	va	VF	W
<i>Polpochila impressifrons</i>	Coleoptera	Carabidae	–	–	–	–	–	–	0.14 (1)	11.11 (1)	ND	s	LF	Z
<i>Scarites</i> sp.	Coleoptera	Carabidae	0.15 (1)	11.11 (1)	ND	r	LF	Z	0.44 (3)	33.33 (3)	ND	s	LF	Y
<i>Selenophorus</i> sp.	Coleoptera	Carabidae	1.41 (9)	33.33 (2)	D	s	LF	Y	0.44 (3)	22.22 (2)	ND	s	LF	Z
<i>Aleochara</i> sp.	Coleoptera	Staphylinidae	–	–	–	–	–	–	0.14 (1)	11.11 (1)	ND	s	LF	Z
<i>Eulissus chalybaeus</i>	Coleoptera	Staphylinidae	–	–	–	–	–	–	0.14 (1)	11.11 (1)	ND	s	LF	Z
<i>Euborellia</i> sp. ^{1,2}	Dermaptera	Anisolabididae	29.98 (191)	100.0 (9)	D	va	VF	W	43.17 (291)	100 (9)	SD	sa	SF	W
<i>Kleter</i> sp.	Dermaptera	Forficulidae	2.04 (13)	55.56 (5)	D	c	F	W	2.07 (14)	44.44 (4)	D	c	F	Y
<i>Labidura xanthopus</i>	Dermaptera	Labiduridae	0.15 (1)	11.11 (1)	ND	r	LF	Z	–	–	–	–	–	–
<i>Cabro morph.</i>	Hymenoptera	Cabronidae	–	–	–	–	–	–	0.14 (1)	11.11 (1)	ND	s	LF	Z
<i>Camponotus</i> sp.	Hymenoptera	Formicidae	3.92 (25)	55.56 (5)	D	c	F	W	3.85 (26)	55.56 (5)	D	c	F	W
<i>Camponotus rufipes</i>	Hymenoptera	Formicidae	–	–	–	–	–	–	0.59 (4)	55.55 (5)	ND	s	LF	W
<i>Cyphomyrmex</i> sp.	Hymenoptera	Formicidae	6.43 (41)	22.22 (2)	D	c	F	Z	–	–	–	–	–	–
<i>Odontomachus</i> sp.	Hymenoptera	Formicidae	3.61 (23)	77.78 (7)	D	c	F	W	6.82 (46)	88.89 (8)	D	c	F	W
<i>Eciton</i> sp.	Hymenoptera	Formicidae	–	–	–	–	–	–	0.29 (2)	11.11 (1)	ND	s	LF	Z
<i>Pheidole</i> sp. ^{1,2}	Hymenoptera	Formicidae	10.83 (69)	77.78 (7)	D	va	VF	W	12.16 (82)	77.78 (7)	D	va	VF	W

Table 4. Faunistic analysis of ground-dwelling predatory arthropods captured by pitfall traps in Bt and non-Bt soybean during the 2017–2018 crop season (Concluded).

Species	Order	Family	Bt soybean (n = 637)					non-Bt soybean (n = 674)						
			%PI	%FI	D	A	F	C	%PI	%FI	D	A	F	C
<i>Hoplocrates</i> sp.	Hymenoptera	Mutillidae	–	–	–	–	–	–	0.14 (1)	11.11 (1)	ND	s	LF	Z
<i>Pomp morph.</i>	Hymenoptera	Pompilidae	0.62 (4)	22.22 (2)	ND	s	LF	Z	0.29 (2)	11.11 (1)	ND	s	LF	Z

¹Predominant species in Bt soybean; ²Predominant species in non-Bt soybean; ³%PI: Participation of individuals sampled (%) followed by the number of insects sampled in parentheses; ⁴%FI: Frequency of individuals collected (%) followed by the number of times collected in parentheses; D: Dominance – superdominant (SD), dominant (D), non-dominant (ND); A: Abundance – superabundant (sa), very abundant (va), abundant (a), rare (r), common (c) and scattered (s); F: Frequency – superfrequent (SF), very frequent (VF), frequent (F) and less frequent (LF); C: Constancy – constant (W), accessory (Y) and accidental (Z).

Table 5. Ecological diversity indices of richness (S), abundance (N), Simpson's diversity index (D), Shannon-Wiener diversity index (H') and Pielou's evenness index (J) of non-target ground-dwelling predatory arthropods sampled by pitfall traps in non-Bt and Bt soybean during the 2016–2017 crop season.

Samplings ¹	S		N		D		H'		J'	
	Bt	non-Bt	Bt	non-Bt	Bt	non-Bt	Bt	non-Bt	Bt	non-Bt
1	11	8	52	88	0.676 (0.574 – 0.792)	0.541 (0.439 – 0.645)	1.635 (1.379 – 1.936)	1.181 (0.982 – 1.410)	0.681 (0.592 – 0.808)	0.568 (0.472 – 0.678)
2	8	10	46	66	0.755 (0.679 – 0.815)	0.745 (0.665 – 0.810)	1.641 (1.466 – 1.843)	1.717 (1.509 – 1.922)	0.789 (0.704 – 0.886)	0.745 (0.655 – 0.834)
3	6	6	40	26	0.708 (0.590 – 0.777)	0.713 (0.615 – 0.789)	1.460 (1.201 – 1.628)	1.452 (1.223 – 1.657)	0.814 (0.670 – 0.908)	0.810 (0.686 – 0.924)
4	10	8	50	15	0.740 (0.658 – 0.832)	0.817 (0.666 – 0.853)	1.750 (1.560 – 2.000)	1.876 (1.297 – 1.991)	0.760 (0.677 – 0.872)	0.902 (0.789 – 0.964)
5	9	7	25	15	0.771 (0.636 – 0.844)	0.853 (0.755 – 0.844)	1.789 (1.428 – 2.009)	1.934 (1.587 – 1.899)	0.814 (0.699 – 0.926)	0.993 (0.862 – 0.975)
6	3	1	3	1	0.666	–	1.099	–	1	–

¹The sampling periods correspond to soybean growth stages: Bt Area – (1) V6–V7, (2) R2, (3) R5.3, (4) R5.3/R5.5, (5) R6 and (6) R8; non-Bt area – (1) R2, (2) R5.1/R5.2, (3) R5.5/R5.6, (4) R6, (5) R7.2 and (6) R8; Results using Bootstrap technique, resampling 9,999 times with 95% confidence intervals.

Table 6. Ecological diversity indices of richness (S), abundance (N), Simpson's diversity index (D), Shannon-Wiener diversity index (H') and Pielou's evenness index (J) of ground-dwelling predatory arthropods sampled by pitfall traps in non-Bt and Bt soybean during the 2017–2018 crop season.

Samplings ¹	S		N		D		H'		J'	
	Bt	non-Bt	Bt	non-Bt	Bt	non-Bt	Bt	non-Bt	Bt	non-Bt
1	12	17	76	137	0.759 (0.691 – 0.821)	0.667 (0.599 – 0.750)	1.800 (1.620 – 2.019)	1.702 (1.541 – 1.945)	0.724 (0.654 – 0.813)	0.601 (0.548 – 0.688)
2	6	4	52	63	0.736 (0.654 – 0.784)	0.688 (0.609 – 0.724)	1.498 (1.317 – 1.630)	1.254 (1.098 – 1.332)	0.836 (0.734 – 0.911)	0.904 (0.795 – 0.960)
3	7	11	62	90	0.764 (0.703 – 0.805)	0.696 (0.626 – 0.772)	1.618 (1.451 – 1.752)	1.591 (1.430 – 1.821)	0.831 (0.746 – 0.900)	0.663 (0.596 – 0.761)
4	8	12	87	105	0.738 (0.665 – 0.793)	0.649 (0.579 – 0.737)	1.629 (1.448 – 1.779)	1.495 (1.366 – 1.746)	0.783 (0.692 – 0.856)	0.601 (0.551 – 0.706)
5	11	10	147	114	0.695 (0.628 – 0.751)	0.651 (0.580 – 0.717)	1.589 (1.417 – 1.753)	1.405 (1.219 – 1.595)	0.662 (0.590 – 0.732)	0.610 (0.537 – 0.692)
6	13	9	128	101	0.807 (0.773 – 0.840)	0.764 (0.719 – 0.798)	1.924 (1.808 – 2.080)	1.653 (1.507 – 1.790)	0.750 (0.703 – 0.810)	0.752 (0.689 – 0.815)
7	7	7	35	27	0.800 (0.705 – 0.829)	0.782 (0.675 – 0.820)	1.748 (1.452 – 1.849)	1.674 (1.319 – 1.809)	0.898 (0.767 – 0.949)	0.860 (0.756 – 0.934)
8	5	4	31	19	0.734 (0.584 – 0.780)	0.670 (0.482 – 0.731)	1.467 (1.165 – 1.561)	1.234 (0.897 – 1.352)	0.911 (0.724 – 0.970)	0.889 (0.680 – 0.975)
9	5	5	19	18	0.664 (0.432 – 0.764)	0.580 (0.296 – 0.728)	1.340 (0.826 – 1.517)	1.165 (0.556 – 1.440)	0.832 (0.575 – 0.949)	0.723 (0.506 – 0.898)

¹The sampling periods correspond to soybean growth stages: Bt and non-Bt area – (1) R2/R3, (2) R4/R5, (3) R5.1/R5.2, (4) R5.3/R5.4, (5) R5.4/R5.5, (6) R5.5/R5.6, (7) R6, (8) R7.1 and (9) R7.2; Results using Bootstrap technique, resampling 9,999 times with 95% confidence intervals.

5. GENERAL CONCLUSIONS

In general, although our result has shown that only the management of pest bugs in the soybean refuge areas has shown the best performance, both from an ecological point of view and from the insect resistance management (greater supply of susceptible individuals from Bt soybean target pests), as well as the economic viability (lower cost of production, yield and greater indicators of the benefit-cost ratio), the monitoring of pests and natural enemies should not be denied in the Bt soybean refuge areas, since there is an influence and interaction of several factors (biotic, abiotic, regional and at the level of agricultural landscape) that can promote and direct to another scenario.

In addition, under conditions that require chemical interventions through the application of insecticides for the control of lepidopteran pests in areas of refuge (according to the economic threshold), although the sublethal concentrations of chlorantranilipole and flubendiamide insecticides have negative implications on life history traits and life table parameters of the tested model species *C. virescens* and could lead to generation asynchrony and provide insufficient susceptible moths when sprayed on refuge crops, such insecticides do not impact the flight capacity of adults from this species, which achieved the maximum distance recommendations of 800 meters (0.5 miles) between refuge areas (non-Bt soybean) and Bt soybeans.

In relation to the ecological risk assessment of Bt soybean to soil predatory arthropods, was not observed environmental impact on the community through fauna analysis and ecological indices. However, predominant and dominant species have been noted that may have the potential to accelerate or delay the evolution of pest resistance in the Bt soybean crop due to its predatory capacity and the higher potential to regulate pest population in Bt soybean or in refuge areas. For this, more detailed studies involving simulations and/or mathematical modeling should be explored to find more conclusive results.