

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

**Dynamics of lepidopteran pests and a phylogeographic approach of
three *Spodoptera* species (Lepidoptera: Noctuidae) on soybean
fields in Brazil**

Renato Jun Horikoshi

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

**Piracicaba
2022**

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Spodoptera species (Lepidoptera: Noctuidae) on soybean fields in Brazil**

versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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Science. Area: Entomology

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2022

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Horikoshi, Renato Jun

Dynamics of lepidopteran pests and a phylogeographic approach of three *Spodoptera* species (Lepidoptera: Noctuidae) on soybean fields in Brazil / Renato Jun Horikoshi - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2022.

88 p.

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

1. Soja Cry1Ac 2. Planta *Bt* 3. MIP 4. Genética de população I. Título

ACKNOWLEDGMENTS

To Escola Superior de Agricultura “Luiz de Queiroz” from the University of São Paulo for the opportunity to study and learn to be a better professional.

To the Graduate Program in Entomology and Professors for lessons, trainings and opportunities during my time as student.

To Professor Dr. Alberto Soares Corrêa for guidance and friendship during my time in the University.

To Patrick M. Dourado for supporting me during my journey at ESALQ and being fundamental to start my course at ESALQ.

To Professor Dr. Celso Omoto for contributing to the development of thesis research.

To Frederico Nanini for helping with the research conducted in laboratory.

To the Entomology Department of ESALQ for the support and possibility to study.

To Bayer Crop Science, with special thanks to Geraldo U. Berger, Luciano B. Fonseca and Ramiro Ovejero for the possibility to start my course at ESALQ.

To the Laboratório de Ecologia Molecular de Artrópodes da ESALQ - USP (ENTOMOL) team for friendship: Alan Saldanha, Ana Wengrat, Cristina Marquesini, Daniela Maggio, Davi Fernandes, Denise Alves, Eduardo Shimbori, Frederico Hickmann, Maria Gabriela, Mateus Aurélio, Sarah Garcia, Tais Cabrera, Tamara Moraes, Victória Rossetti.

To all my colleagues from the Entomology Department for the friendship.

To all my colleagues from Bayer for the friendship, celebrating every accomplishment.

To my girlfriend Ana Letícia Zero for friendship and support during my journey.

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RESUMO

Dinâmica de lepidópteros-praga e abordagem filogeográfica de três espécies de *Spodoptera* (Lepidoptera: Noctuidae) na cultura da soja no Brasil

A soja é uma das fontes mais importantes de óleo e proteína em todo o mundo. A área plantada com soja aumentou significativamente nos últimos 50 anos e, atualmente, o Brasil é o maior produtor mundial. Várias inovações agrícolas foram necessárias para aumentar a produção, tais como o sistema de plantio direto, correção da acidez do solo e adubação, novas variedades adaptadas a várias latitudes, inoculação de bactérias fixadoras de nitrogênio, sistema de multiculturas com pelo menos duas safras por ano e o uso de plantas geneticamente modificadas. A tecnologia de soja MON 87701 × MON 89788, que expressa a proteína Cry1Ac e confere a tolerância ao herbicida glifosato, vem sendo amplamente adotada no Brasil desde 2013. No entanto, mudanças na composição de pragas ou a evolução de resistência podem reduzir os benefícios desta tecnologia. Apesar da importância no Manejo Integrado de pragas, o conhecimento sobre a dinâmica populacional, mudanças na composição e a estrutura genética das populações de pragas agrícolas no Brasil ainda são incipientes. Neste trabalho, são apresentadas evidências da redução regional no uso de inseticidas em áreas no Brasil onde a soja Cry1Ac é cultivada, com redução de até 50% no número de aplicações de inseticidas para o manejo de lepidópteros-praga em soja não-Bt, observado em locais específicos. Além disso, um número reduzido de adultos de *Chrysodeixis includens* foi capturado ao longo dos anos em um estudo de monitoramento de pragas em quatro mesorregiões. O número de adultos de *Helicoverpa* spp. capturados também foi reduzido em três mesorregiões. Em uma amostragem em larga escala de lagartas em lavouras comerciais de soja durante as safras de 2019 e 2020, *C. includens* foi o principal lepidóptero-praga presente em áreas não-Bt. Mais de 98% das lagartas encontradas na soja Cry1Ac foram representadas por *Spodoptera* spp., embora os números de *Spodoptera* spp. foram semelhantes entre os campos de soja Cry1Ac e não-Bt. A soja Cry1Ac resultou em um alto nível de proteção contra *Anticarsia gemmatalis*, *C. includens*, *Chloridea virescens* e *Helicoverpa* spp. Para investigar a diversidade genética, estrutura populacional e demografia de *Spodoptera eridania*, *Spodoptera cosmioides* e *Spodoptera frugiperda*, foram realizadas análises baseadas na sequência do COI mitocondrial. Alta diversidade genética foi observada para *S. eridania* amostrada em soja no Brasil. O índice de diversidade genética de *S. eridania* foi superior em relação aos valores observados para *S. cosmioides* e *S. frugiperda*. Baixa estrutura genética foram observadas para as três espécies de *Spodoptera*. O maior Φ_{ST} observado foi para *S. cosmioides* (0,058) seguido por *S. eridania* (0,058) e *S. frugiperda* (0,017). Há evidências de que as três espécies de *Spodoptera* avaliadas neste estudo estão em expansão demográfica e espacial. Os resultados do estudo também sugerem que a linhagem milho é a principal linhagem de *S. frugiperda* que ocorre na cultura da soja no Brasil. Esta pesquisa clarificou o conhecimento sobre as populações de insetos-praga que atacam a cultura da soja no Brasil. Foi observado uma supressão regional de lepidópteros-praga e redução do uso de inseticidas relacionado com a alta adoção da soja Cry1Ac, trazendo benefícios econômicos e ambientais. *Chrysodeixis includens* e *A. gemmatalis* continuam sendo os principais lepidópteros-praga da soja no Brasil e a soja Cry1Ac continua a oferecer proteção contra as pragas-alvo. O aumento da abundância relativa de *Spodoptera* spp. em soja não-Bt e Cry1Ac confirmou o aumento da

importância deste gênero na soja. Por fim, estas espécies de *Spodoptera* apresentam baixa estrutura genética e estão em expansão demográfica e espacial, provavelmente impulsionadas pela expansão da agricultura, principalmente com lavouras de soja.

Palavras-chave: Soja Cry1Ac, Planta *Bt*, MIP, Genética de população

ABSTRACT

Dynamics of lepidopteran pests and a phylogeographic approach of three *Spodoptera* species (Lepidoptera: Noctuidae) on soybean fields in Brazil

Soybean is one of the most important sources of oil and protein worldwide. The area planted with soybean significantly increased in the last 50 years, and currently, Brazil is the largest producer. Several agricultural innovations were needed to increase production such as no-tillage system cultivation, soil acid correction and fertilization, new crop varieties adapted to a range of latitudes, inoculation of nitrogen-fixing bacteria, multi-crop system with at least two cropping seasons per year and the use of genetically modified plants. The soybean technology MON 87701 × MON 89788, expressing Cry1Ac and conferring tolerance to glyphosate has been widely adopted in Brazil since 2013. However, pest shifts or resistance evolution could reduce the benefits of this technology. Despite the importance to Integrated Pest Management, knowledge on the population dynamics, shifts and genetic structure of agriculture pest populations in Brazil are still incipient. Here, is presented evidence of the regional reduction in insecticide use across areas in Brazil where Cry1Ac soybean is grown, with up to 50% reduction in the number of insecticide applications for managing lepidopteran pests on non-*Bt* soybean observed at specific locations. Furthermore, a reduced number of *Chrysodeixis includens* moths were captured across years in a pest-monitoring study in four mesoregions. The number of *Helicoverpa* spp. moths captured also were reduced at three mesoregions. In a large-scale sampling of larvae on commercial soybean fields during the 2019 and 2020 crop seasons, *C. includens* was the main lepidopteran pest in non-*Bt* fields. More than 98% of larvae found in Cry1Ac soybean were *Spodoptera* spp., although the numbers of *Spodoptera* spp. were similar between Cry1Ac soybean and non-*Bt* fields. Cry1Ac soybean provided a high level of protection against *Anticarsia gemmatalis*, *C. includens*, *Chloridea virescens* and *Helicoverpa* spp. To investigate the population genetic diversity, population structure, and demographic pattern of *Spodoptera eridania*, *Spodoptera cosmioides* and *Spodoptera frugiperda*, a mitochondrial COI sequence-based analyzes were performed. High genetic diversity was observed for *S. eridania* sampled on soybean in Brazil. The genetic diversity index of *S. eridania* was higher than that observed for *S. cosmioides* and *S. frugiperda*. Low genetic structure was observed for the three *Spodoptera* species. The highest Φ_{ST} observed was for *S. cosmioides* (0.058) followed by for *S. eridania* (0.058) and *S. frugiperda* (0.017). There is evidence that the three *Spodoptera* species evaluated in this study are in demographic and spatial expansion. The results of the study also suggest that corn strain is the major lineage of *S. frugiperda* occurring in soybean in Brazil. This research clarified and updated the knowledge of insect pest populations attacking soybean in Brazil. A regional suppression of lepidopteran pests and reduced insecticide use with the widespread adoption of Cry1Ac soybean has been observed, bringing economic and environmental benefits. *Chrysodeixis includens* and *A. gemmatalis* continue to be primary lepidopteran pests of soybean in Brazil and Cry1Ac soybean continues to effectively manage the target lepidopteran pests. Increase in the relative abundance of non-target *Spodoptera* spp. larvae in both non-*Bt* and Cry1Ac soybeans confirmed the emerging importance of this genera in soybean. Finally, these *Spodoptera* species show low genetic structure and are at demographic and spatial expansion, probably driven by agriculture expansion, especially soybean fields.

Keywords: Cry1Ac soybean, *Bt* plant, IPM, Population genetics

1. INTRODUCTION

Soybean is the main crop planted in Brazil and one of the most important sources of oil and protein worldwide (USDA 2022). Brazil is responsible for approximately 23% of global production, followed by the United States, China, and Argentina, with 20, 10 and 8%, respectively (USDA 2022). A turning point of the Brazilian agriculture was the expansion to the Cerrado, allowing the growth of soybean cultivated area from approximately 1.3 million hectares in 1970 to more than 38 million hectares in the 2020/21 season, with an estimated production of 135.9 million tons of soybean grain (CONAB 2022). The advance of agriculture in Cerrado was possible with innovations and, in a period of 50 years, several changes were observed: no-tillage system cultivation, soil correction and fertilization, new crop varieties adapted to a range of latitudes with increased yield, inoculation of nitrogen-fixing bacteria, the multi-crop system with at least two cropping season per year and the use of genetically modified plants are some examples (Fatoretto et al. 2017; Cattelan and Dall'Agnol 2018; Umburanas et al. 2022). As soybean production in Brazil transformed into a highly structured and organized large-scale business operation primarily targeting export markets, the need to reach high yield implies adopting good agricultural practices and consequently pest management.

Intensive and large use of agricultural land creates an environment conducive to the buildup of relatively large insect pest populations (Fatoretto et al. 2017; Silva et al. 2020). "Tropical agriculture" such as that practiced in Brazil is typically based on two or more crop seasons per year, allowing pest populations to go through multiple generations per year potentially under selection to control tactics pressure such as insecticides and *Bt* crops (Fatoretto et al. 2017). Insect populations in tropical agriculture might also present rapid plastic responses to environmental changes, as observed in *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) (Silva-Brandão et al. 2017). Rapid responses, adaptations and high pest pressure are some of the reasons for the difficulty in managing pests in Brazil.

In this context, the soybean technology MON 87701 × MON 89788 (Intacta RR2 PRO®), expressing the Cry1Ac insecticidal protein (event MON 87701) and conferring tolerance to glyphosate (event MON 89788), was commercially launched and became available to farmers in Brazil in 2013. The adoption of Cry1Ac soybean by Brazilian farmers increased from 1.2 million hectares in the 2013/14 cropping season to 30 million hectares in the 2020/21 cropping season (CIB and AGROCONSULT 2018; SPARK 2021). The rapid adoption of MON 87701 × MON 89788 soybean has been driven by the significant yield advantage of varieties containing this technology and the high levels of protection against the primary soybean lepidopteran pests in Brazil. MON 87701 × MON 89788 soybean provides adequate protection against larval feeding by *Anticarsia gemmatilis* (Hübner, 1818) (Lepidoptera: Erebidae), *Chrysodeixis includens* (Walker, [1858]), *Chloridea virescens* (Fabricius, 1781) and *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae) (Bernardi et al. 2012, 2014; Dourado et al. 2016; Horikoshi et al. 2021).

Beyond the direct benefit of controlling target pests, *Bt* crops such as Cry1Ac soybean have the potential to provide additional benefits to insect management in agricultural systems, including a reduction in insecticide use (Brookes 2018), compatibility with biocontrol measures (Edgerton et al.

2012; Lu et al. 2012), and regional suppression of insect pest populations (Carrière et al. 2003; Hutchison et al. 2010; Wu et al. 2008; Dively et al. 2018). Regional pest suppression might occur when *Bt* plants account for a significant amount of available host plants for a pest species in the agricultural landscape, reducing the pest population (Tabashnik 2010). Suppression of target pests after a long period of use of highly efficacious *Bt* technologies has been documented in *Pectinophora gossypiella* (Saund., 1844) (Lepidoptera: Gelechiidae), *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Pyralidae) and *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) in the USA (Carrière et al. 2003; Hutchison et al. 2010; Dively et al. 2018) and *H. armigera* in China (Wu et al. 2008). Similarly, the high efficacy of Cry1Ac soybean against lepidopteran pests such as *C. includens* and *A. gemmatilis*, the leading soybean pests in Brazil (Bernardi et al. 2012), resulted in fewer insecticide sprays to manage lepidopteran larvae after five years of commercial use in Brazil (Brookes 2018).

In addition, resistance evolution by target pest populations can reduce the benefits of *Bt* crops (Gould 1998). Understanding the performance of a *Bt* crop against target pests at the field level is an important component to manage resistance and drive pest management strategies, as pest abundance might be directly affected. In some situations, where a *Bt* technology is ineffective against non-target secondary pest species and or broad-spectrum insecticide use has decreased due to highly effective control of the target species, secondary pests may increase in abundance over time (Lu et al. 2010; Zhao et al. 2011). Determining whether non-target pests are increasing in abundance can inform the need of adoption of appropriate Integrated Pest Management (IPM) practices in Brazil.

Among the non-target pest species of Cry1Ac soybean, the genus *Spodoptera* is highlighted. *Spodoptera* genus is along with *Helicoverpa* genus, a major problematic and widespread Lepidopteran pest genus worldwide (Kergoat et al. 2021). In Brazil, the most known species of this genus is the fall armyworm, *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), the main corn pest (Blanco et al. 2016; Faretto et al. 2017) and the representative that is in the spotlight recently with the invasion of eastern hemisphere (Georgen et al. 2016, Kalleshwaraswamy et al. 2018; Jing et al. 2019). However, other pest species of this genera: *Spodoptera eridania* (Stoll, 1782) (Lepidoptera: Noctuidae) and *Spodoptera cosmioides* (Walker, 1898) (Lepidoptera: Noctuidae) are also economically important pests in Brazil (Montezano et al. 2014; Specht, Roque-Specht 2016; Horikoshi et al., 2021). Along with *S. frugiperda*, these three species are commonly referred to as *Spodoptera* complex and are the main *Spodoptera* species that attacks soybean in Brazil (Horikoshi et al., 2021). That complex was not considered a significant threat to the soybean crop until recently, and we can consider them as emerging pests of soybean crop (Panizzi, Corrêa-Ferreira 1997; Sosa-Gómez et al. 2014, Horikoshi et al. 2021). In addition, Cry1Ac soybean does not confer protection to these three *Spodoptera* species, which is why it needs to be monitored (Bernardi et al. 2014).

Phylogeography is the field of study to understand the principles and processes governing the geographic distribution of genealogical lineages (Freeland, 2011). Comprehend how historical events are influencing the shape of geographical dispersion of genes, population and species is the objective of phylogeography (Freeland, 2011). Changes in agricultural landscapes may affect the population dynamics. As agricultural practices modify the landscape and usually simplify the environment structure over large areas, it affects the gene flow among populations (Altieri 1999;

Gauffre et al. 2015; Alvarado-Serrano et al. 2019). Gene flow might result from dispersal, that is the movement of individuals and holds a central role in population dynamics and structure (Ronce 2007). Understanding population genetics is an essential piece of integrated pest management (IPM) as lineages or strains might be present in a country of continental proportions. *S. frugiperda* corn and rice strains are an example of the presence of host adapted lineages in the American continent (Pashley, Martin 1987; Nagoshi, Meagher 2008; Siva-Brandão et al. 2018). Recently, lineages were also found in *Euschistus heros* (Hemiptera: Pentatomidae) in Brazil, with an older and more diverse strain arising in the northern regions and a younger and less diverse strain occurring in the southern region (Soares et al. 2018). The presence of strains in the landscape might directly affect the response to control tactics, as different susceptibility is observed for insecticides and *Bt* proteins (Ríos-Díez, Saldamando-Benjumea 2011; Ingber et al. 2018).

The study of the dynamic of soybean lepidopteran pest species is essential to the implementation of effective pest management in Brazil. The expansion of agriculture in Cerrado along with changes in agricultural practices in the last 50 years, and more recently the introduction of *Bt* soybean in Brazil revolutionized pest management practices. Understanding the Cry1Ac soybean performance and its impacts on pest management is important to assess the benefits of technology in the production system. Also, clarifying the current scenario of pest abundance and distribution will aid in the refinement of pest management at a regional level, as large countries might have particularities regarding pest populations. Studies are necessary to evaluate the soybean pest dynamics to verify whether changes occurred after changes in agricultural practices in the last years. Moreover, the information on population diversity and structure is lacking for the emerging pests of soybean *S. eridania*, *S. cosmioides* and *S. frugiperda*. Therefore, the major goal in the current research were:

- a) Assess whether the widespread adoption of Cry1Ac soybean in Brazil has promoted the regional suppression of natural populations of its target pests;
- b) Evaluate Cry1Ac soybean performance and impacts on soybean pest management, assessing the relative abundance of lepidopteran pest species attacking soybean fields and comparing these results to data collected before the commercial introduction of Cry1Ac soybean;
- c) Understand the genetic diversity and population dynamics of emerging pests of soybean *S. eridania*, *S. cosmioides* and *S. frugiperda* in Brazil.

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2. REGIONAL PEST SUPPRESSION ASSOCIATED WITH ADOPTION OF Cry1Ac SOYBEAN BENEFITS PEST MANAGEMENT IN TROPICAL AGRICULTURE

ABSTRACT

Bt crops have been adopted worldwide, providing high-level protection from insect pests. Furthermore, *Bt* crops preserve natural enemies, promote higher yield, and economically benefit farmers. Although regional pest suppression by widespread *Bt* crop adoption has been observed in temperate regions, this possibility remains uncertain in tropical areas due to the high diversity of alternative hosts and mild winters. Here, we present evidence of regional reduction in insecticide use across areas in Brazil where Cry1Ac soybean is grown since 2013, with up to 50% reduction in the number of insecticide sprays for managing lepidopteran pests on non-*Bt* soybean observed at specific locations from 2012 to 2019. We also present pest-monitoring data in four mesoregions across five years of commercial plantings of Cry1Ac soybean from December 2014 to July 2019. Reduced numbers of *Chrysodeixis includens* moths were captured in pheromone traps across years at all locations. The number of *Helicoverpa* spp. moths captured also were reduced at three locations. Thus, we provide evidence for regional suppression of lepidopteran pests and reduced insecticide use with the widespread adoption of Cry1Ac soybean, bringing economic, social and environmental benefits.

Keywords: Intacta soybean, *Chrysodeixis includens*, *Helicoverpa*, IPM

2.1. Introduction

Soybean is one of the most important crops worldwide, accounting for more than half of the global demand for oil and vegetable protein^{1,2}. Of 127.8 million of hectares globally cultivated with this crop, Brazil is the biggest producer with soybean occupying more than 38 million hectares in the 2020/21 season^{3,4}. Despite producing 38.5 million tons in 2020/21 season⁴, soybean yield in Brazil has constantly been reduced by a diverse group of pest species⁵. Among those pests, Lepidoptera and Hemiptera have been the primary taxa requiring frequent insecticide sprays to protect yield⁶. Therefore, finding ways to reduce the load of insecticides used in soybeans in Brazil is of great theoretical and practical interests.

Genetically modified plants expressing *Bacillus thuringiensis* (*Bt*) genes have been used to manage crop pests worldwide. The adoption of *Bt* technologies has been extensive, mainly due to their efficacy against the major lepidopteran pests on maize, cotton, and soybean crops^{7,8}. Therefore, the *Bt* soybean MON 87701 × MON 89788 (Intacta RR2 PRO[®]), which expresses the Cry1Ac insecticidal protein derived from *B. thuringiensis*, which is effective at controlling major lepidopteran pests including *Anticarsia gemmatalis* (Lepidoptera: Erebiidae), *Chrysodeixis includens* (Lepidoptera: Noctuidae), *Chloridea virescens* (Lepidoptera: Noctuidae), and *Helicoverpa armigera* (Lepidoptera: Noctuidae)^{9,10,11,12} was commercially launched in Brazil in 2013/14. Initially deployed on 1.2 million hectares, Cry1Ac soybean has shown rapid increase in adoption by farmers, achieving 30 million hectares cultivated with this technology in the 2020/21 crop season in Brazil¹³.

Several benefits are reported to be associated with the adoption of *Bt* plants. The direct benefit is the effective management of key pests, but *Bt* crops promote other positive impacts such as preserving natural enemies due to a likely reduction in insecticide sprays and increases in yield and income to farmers^{14,15,16}. Regional suppression of target pests promoted by widespread adoption of *Bt* crops, resulting in less damage in other crop fields, is another benefit¹⁷. The widespread adoption of *Bt* maize in the USA, where it was introduced in 1996, suppressed natural populations of *Ostrinia nubilalis* (Lepidoptera: Crambidae), and *Helicoverpa zea* (Lepidoptera: Noctuidae) on a regional scale^{17,18}. Regional suppression was also observed following the widespread adoption of *Bt* cotton in the USA and China, reducing populations of *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) in both countries and *H. armigera* in China (Lepidoptera: Gelechiidae)^{14,19,20}.

Two of the major lepidopteran pests of soybean in Brazil are *C. includens* and *H. armigera*, both of which are polyphagous insects with wide distribution across the country^{21,22,23,24}. *Chrysodeixis includens* is a native species of the Americas and the most important defoliator pest of soybean in Brazil^{23,25,26,27}. *Helicoverpa armigera* is an invasive pest that causes severe damage to soybean and other cultivated crops and has become the most abundant species of genus *Helicoverpa* in Brazil^{21,24,28}. For both species, soybean is one of the most suitable host plants^{24,29}. However, Cry1Ac soybean has provided effective control of *C. includens* and *Helicoverpa* spp. in soybean fields throughout Brazil, which could promote the regional suppression of these pests²⁷.

Although regional pest suppression by *Bt* crops has been observed in subtropical/temperate areas such as the USA and China, until now there have been no reports of such an outcome in a tropical environment such as Brazilian agriculture. Most of the agricultural territory of Brazil are within a tropical zone, allowing for the cultivation of up to three crop seasons per year. Furthermore, the dynamics of polyphagous pests in tropical environments are shaped by the broad availability and diversity of cultivated and non-cultivated hosts: when combined with the typical mild winters in such regions, these dynamics favor high reproductive and dispersal rates year-round^{24,30}. Here we hypothesized that the widespread adoption of Cry1Ac soybean in Brazil has promoted the regional suppression of natural populations of its target pests. We compiled the numbers of insecticide sprays for lepidopteran pest control on soybean crops from 2012 to 2019 and associated them with the regional adoption of Cry1Ac soybean. At the field level, we collected adult individuals of *C. includens* and *Helicoverpa* spp. at four locations by using pheromone traps weekly from 2014 (early years of commercial planting of Cry1Ac soybean) to 2019.

2.2. Results

2.2.1. Cry1Ac soybean adoption and insecticide spraying

The analysis of 43 to 46 Brazilian mesoregions demonstrated that increases in Cry1Ac adoption significantly reduced the number of insecticide sprays against lepidopteran pests across both total soybean area ($F = 322.0$, $df = 1, 311$, $R^2 = 0.51$, $P < 0.0001$) (Fig 1A) and non-*Bt* soybean area ($F = 88.3$, $df = 1, 311$, $R^2 = 0.22$, $P < 0.0001$) (Fig 1B). The slopes of regression indicated that the number

of insecticide sprays was reduced by 0.2252 and 0.1418 (from a mean of 4 sprays in the case of 0% adoption) for every 10% increase in Cry1Ac adoption for total soybean area and non-*Bt* soybean area across mesoregions, respectively. In other words, for every 10% increase in Cry1Ac adoption, insecticide reduction of 5.6% and 3.5% was observed for total soybean area and non-*Bt* soybean area across mesoregions, respectively.

When four of the mesoregions were analyzed separately, the number of sprays for controlling lepidopteran pests across the total soybean area was significantly reduced with Cry1Ac soybean adoption in the following regions: West Bahia, from 6.8 (2013) to 2.0 (2019) ($F = 93.79$, $df = 1, 5$, $R^2 = 0.94$, $P = 0.0002$) (Fig. 2A); North Mato Grosso, from 4.5 (2013) to 3.0 (2019) ($F = 38.54$, $df = 1, 5$, $R^2 = 0.88$, $P = 0.0016$) (Fig. 2B); and North Central Paraná, from 2.7 (2013) to 1.7 (2019) ($F = 22.42$, $df = 1, 5$, $R^2 = 0.81$, $P = 0.0052$) (Fig. 2C). In contrast, there was no significant reduction in the application of insecticides (~ 2.7 sprays/season) in the Northwest Rio-Grande ($F = 6.23$, $df = 1, 5$, $R^2 = 0.55$, $P = 0.0547$) (Fig. 2D). When the number of sprays on non-*Bt* soybean was analyzed, a significant reduction in insecticide sprays against lepidopteran pests was also detected in West Bahia, from 6.8 (2013) to 3.4 (2019) ($F = 31.73$, $df = 1, 5$, $R^2 = 0.86$, $P = 0.0024$) (Fig. 2E); North Mato Grosso, from 4.5 (2013) to 3.6 (2019) ($F = 15.85$, $df = 1, 5$, $R^2 = 0.41$, $P = 0.0105$) (Fig. 2F); and North Central Paraná, from 2.7 (2013) to 1.8 (2019) ($F = 22.66$, $df = 1, 5$, $R^2 = 0.82$, $P = 0.0051$) (Fig. 2G). As observed in the analysis of total soybean area, no significant reduction in insecticide sprays was observed in Northwest Rio-Grande ($F = 1.22$, $df = 1, 5$, $R^2 = 0.19$, $P = 0.3184$) (Fig. 2H).

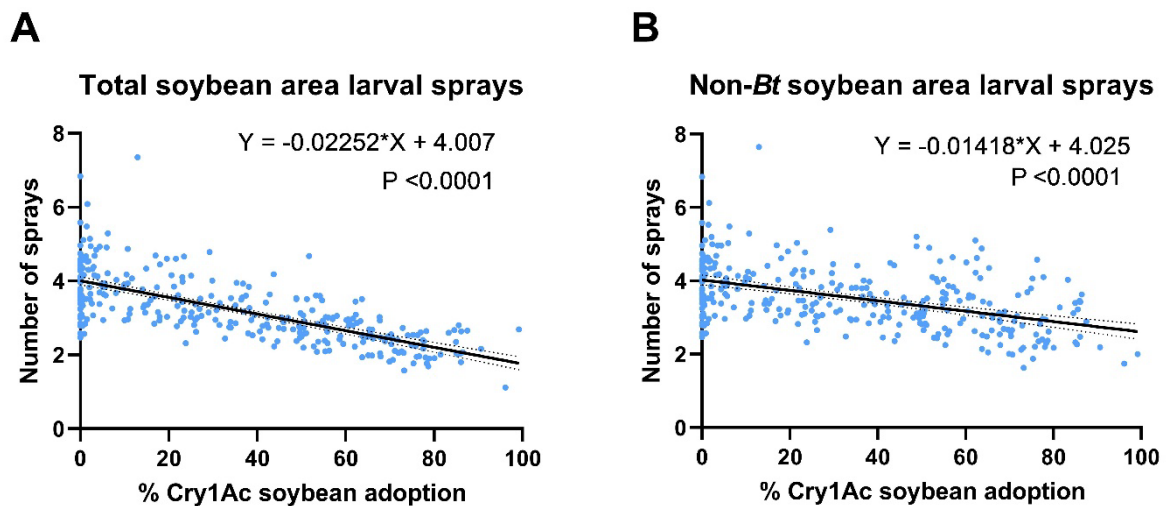


Fig. 1 Number of insecticide sprays for controlling lepidopteran pests in soybean in mesoregions of Brazil. A) Total soybean area (*Bt* + non-*Bt*) versus percentage adoption of Cry1Ac soybean. B) Non-*Bt* soybean area versus percentage adoption of Cry1Ac soybean.

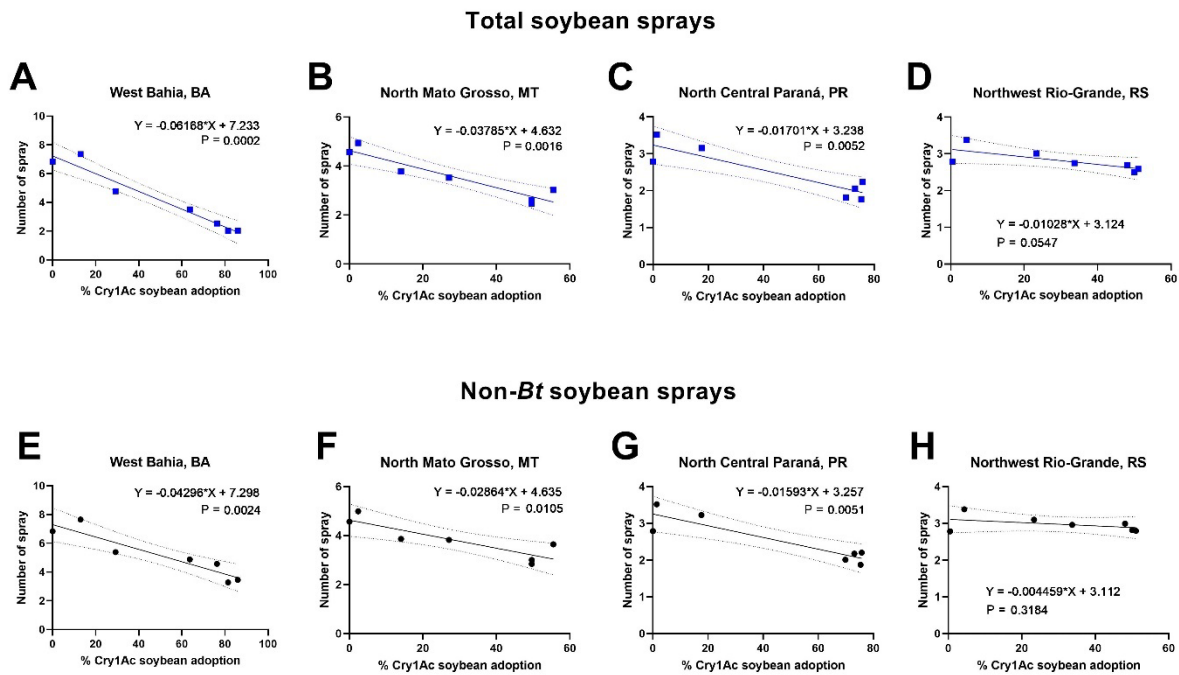


Fig. 2 Number of insecticide sprays for controlling lepidopteran pests. Total soybean area (*Bt* + non-*Bt*) versus percentage adoption of Cry1Ac soybean (A–D) and non-*Bt* soybean area (E–H) versus percentage adoption of Cry1Ac soybean in four mesoregions of Brazil.

2.2.2. Adult sampling with pheromone traps

Our results showed wave-like fluctuations of *C. includens* and *Helicoverpa* spp. populations throughout the year. With few exceptions, the peaks in numbers of *C. includens* and *Helicoverpa* spp. overlapped with the presence of a soybean crop in the field in all regions (SI Figs. 1 and 2).

The number of *C. includens* adult moths captured in pheromone traps declined over the years in all mesoregions evaluated (Fig. 3). West Bahia, average moths captured per year decreased from 71.9 in 2015 to 32 in 2019 ($df = 391$, residual deviance = 23926, slope = -0.021 , $P < 0.0001$) (Fig. 5A); North Mato Grosso, 97.9 in 2015 to 27.2 in 2019 ($df = 391$, residual deviance = 23958, slope = -0.020 , $P < 0.0001$) (Fig. 3B); North Central Paraná, 143.1 in 2015 to 114.3 in 2019 ($df = 379$, residual deviance = 39691, slope = -0.012 , $P < 0.0001$) (Fig. 3C) and Northwest Rio-Grande, 32.2 in 2015 to 11.1 in 2019 ($df = 379$, residual deviance = 39708, slope = -0.012 , $P < 0.0001$) (Fig. 3D)

A decrease in number of *Helicoverpa* spp. adults trapped over the years was also observed in West Bahia, with average moths captured per year decreasing from 22.4 in 2015 to 17.9 in 2019 ($df = 391$, residual deviance = 7333, slope = -0.008 , $P < 0.0001$) (Fig. 4A); North Mato Grosso, 22.9 in 2015 to 9.9 in 2019 ($df = 410$, residual deviance = 9223, slope = -0.021 , $P < 0.0001$) (Fig. 4B) and Northwest Rio-Grande, 11.9 in 2015 to 0.5 in 2019 ($df = 410$, residual deviance = 2810, slope = -0.134 , $P < 0.0001$) (Fig. 4D). In contrast, an increase in moth capture per year was observed in North Central Paraná, with 8.9 moths captured in 2015 and 15.2 in 2019 ($df = 371$, residual deviance = 4304, slope = 0.002 , $P = 0.0006$) (Fig. 4C).

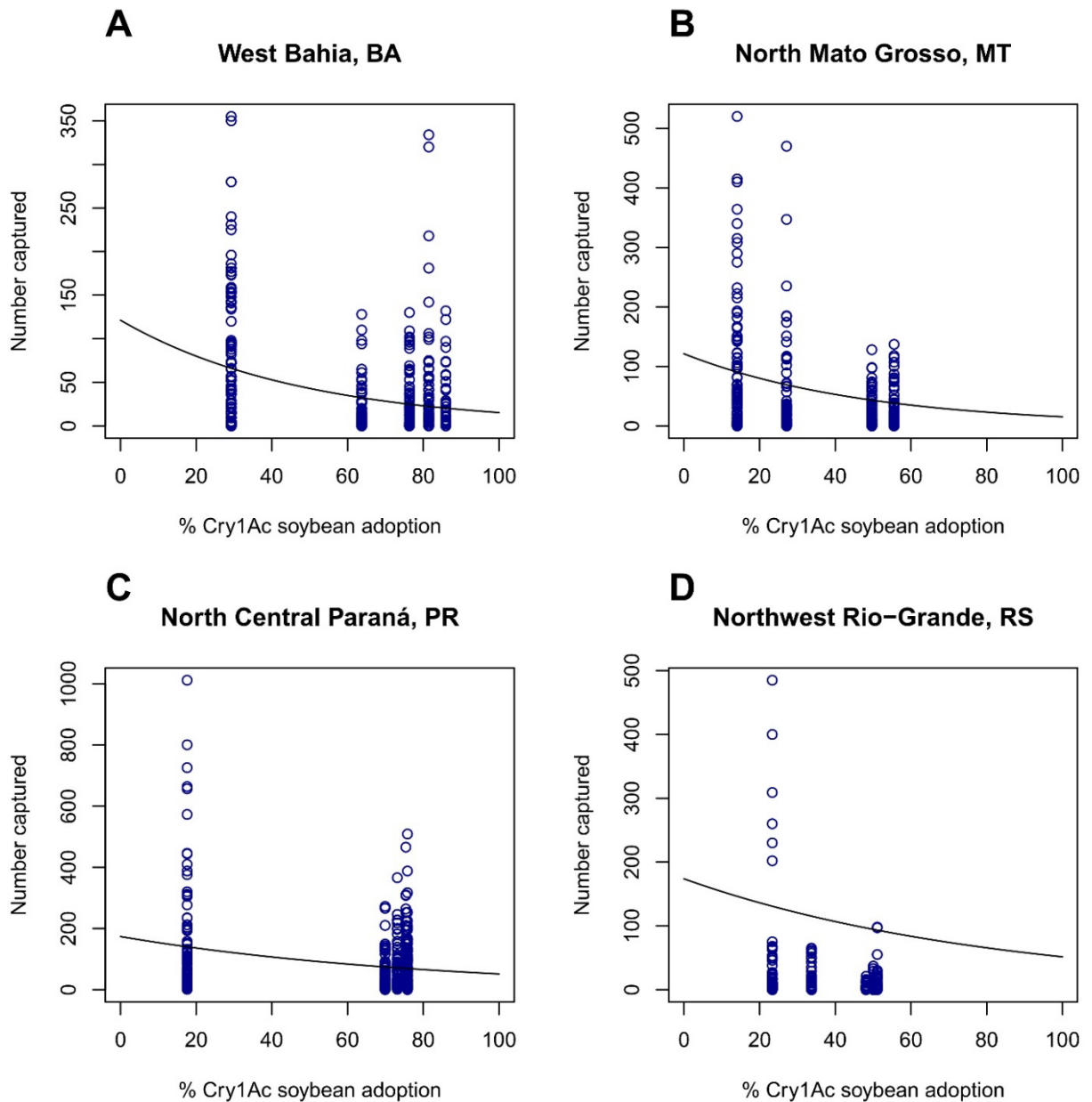


Fig. 3. *C. includens* adult sampling with pheromone traps. Regression analysis of the number of *C. includens* captured as a function of Cry1Ac soybean adoption in four regions of Brazil: West Bahia (A), North Mato Grosso (B), North Central Paraná (C) and Northwest Rio-Grande (D).

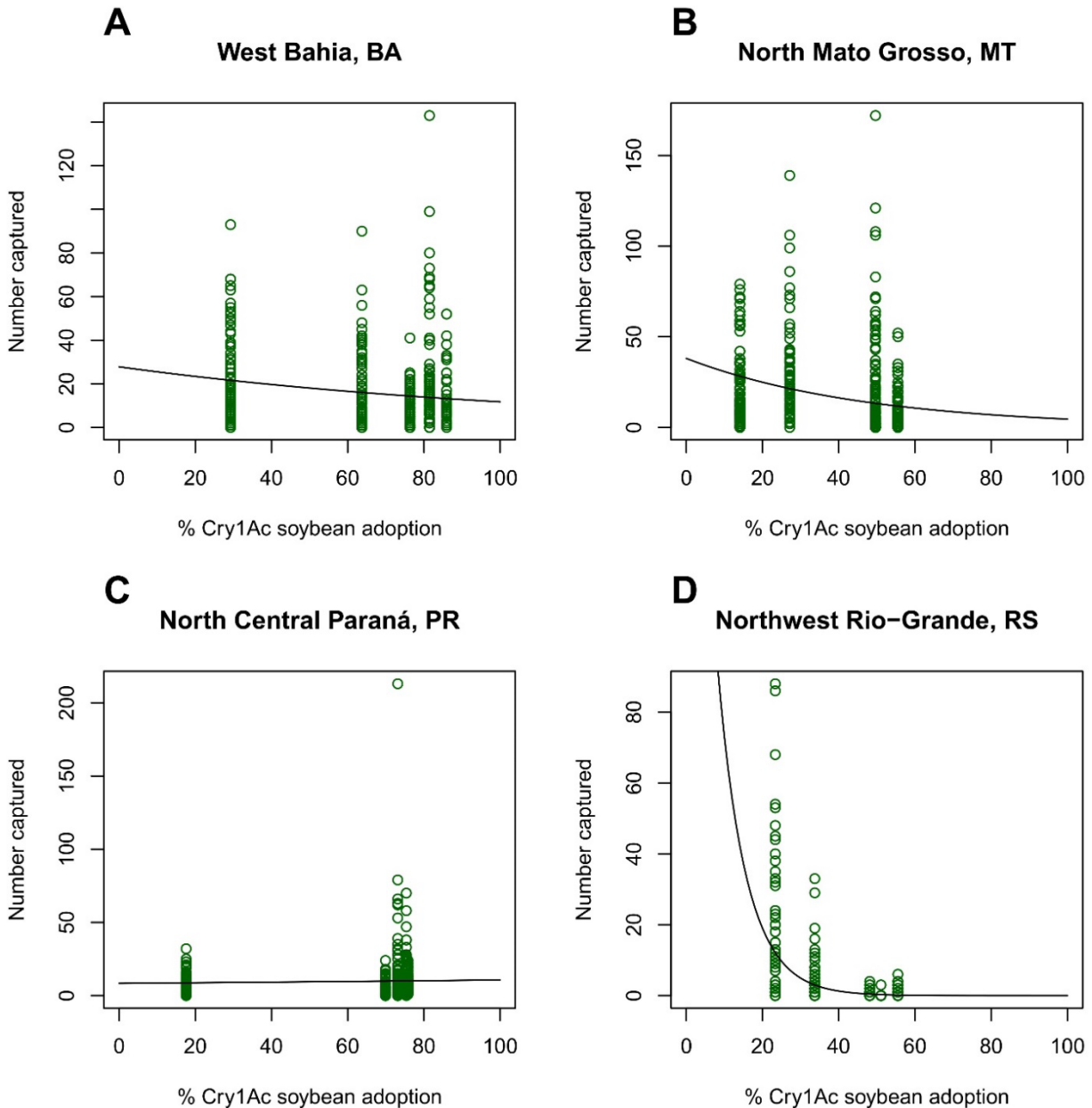


Fig. 4. *Helicoverpa* spp. adult sampling with pheromone traps. Regression analysis of the number of *Helicoverpa* spp. captured as a function of Cry1Ac soybean adoption in four regions of Brazil: West Bahia (A), North Mato Grosso (B), North Central Paraná (C) and Northwest Rio-Grande (D).

2.2.3. Climatic variables

The climatic variables from 2010 to 2018 showed no significant differences in the linear regression analysis of annual precipitation, maximum temperature, and minimum temperature in West Bahia, North Mato Grosso, North Central Paraná, and Northwest Rio-Grande ($P > 0.05$), suggesting that these factors had no relevant effects on the population densities of the lepidopteran pests studied.

2.3. Discussion

The widespread adoption of Cry1Ac soybean in Brazil was associated with reductions in the number of insecticide sprays for lepidopteran control on total soybean area and non-*Bt* soybean area over Brazilian mesoregions. Up to 46 mesoregions of Brazil were analyzed from 2012 to 2019, and reductions in insecticide sprays were observed in both total soybean and non-*Bt* soybean areas. The reduction in the number of insecticide sprays against lepidopteran pests in Cry1Ac soybean fields was not a surprise due to the efficacy of technology to control major soybean pests²⁷. However, the reduction in insecticide sprays also expanded to non-*Bt* soybean and represents an enormous beneficial impact on soybean crops leading to lower production cost, lower exposure of non-target organisms to pesticides, and lower contamination of soil, water, and atmosphere^{16,31,32}. When specific mesoregions were assessed to verify the effects in a single mesoregion, the extensive adoption of Cry1Ac soybean in Brazil was also associated with reduction in the number of insecticide sprays to lepidopteran control on total soybean and non-*Bt* soybean in three of the four Brazilian mesoregions studied. In addition, non-significant reduction of the number of insecticide sprays in Northwest Rio-Grande was observed. This mesoregion is in the southern part of Brazil, which has colder winters, and pest abundance is usually reduced compared to northern mesoregions of Brazil. Consequently, the number of insecticide sprays is historically lower in this mesoregion than in northern regions and a reduction is not evident because insecticide sprays are still required for non-target pests of this technology.

We confirmed the population decline of two key pests, *C. includens* and *Helicoverpa* spp., associated with the widespread adoption of the Cry1Ac soybean in tropical agricultural areas. Theoretically, natural pest populations can be greatly reduced when *Bt* plants account for most of the available host plants³³. Our data on population fluctuation suggest that soybean crops are the primary host for both *C. includens* and *Helicoverpa* spp., with a positive effect on insect abundance in the field (see Figs. 3 and 4)^{24,34}. Thus, the widespread adoption of Cry1Ac soybean from 2014, in addition to its high control efficacy²⁷, triggered *C. includens* and *Helicoverpa* spp. population suppression. Our climate analysis did not reveal any climate variations that would have significantly reduced the populations of these two lepidopteran species. Instead, from 2014 to 2019, other lepidopteran pest species that are not targeted by Cry1Ac soybean (e.g., *Spodoptera* species) increased in abundance in soybean crops in Brazil²⁷.

When we compared the population fluctuation of both species, the most intensive suppression occurred in *C. includens*, probably associated with the higher dependency of *C. includens* on soybean compared to *H. armigera*^{23,24,34}. The lack of reduction in *Helicoverpa* spp. moth populations observed in North Central Paraná could be explained by the higher polyphagia of these species, indicated by the large number of insects sampled in the soybean off-season. The presence of *H. zea* moths in the traps could also mask a potential reduction in the presence of *H. armigera*. Although *H. armigera* is the most abundant *Helicoverpa* species in Brazil, some regions have large areas of maize, which is the main host of *H. zea* but not a usual host of *H. armigera*^{21,35}. This landscape can favor the *H. zea* population, increasing the collection of this species in the pheromone traps and thus affecting the number of *Helicoverpa* spp. collected, as observed by Dourado et al.²⁴.

Host preference suggests that *H. armigera* would be more susceptible to reduction in population size by Cry1Ac soybean than would *H. zea*. Indeed, low abundance of *H. armigera* in soybean has previously been observed, evidencing its population decline related to the broad adoption of Cry1Ac soybean²⁷.

Both *C. includens* and *Helicoverpa* spp. are polyphagous pests, causing damage in other cultivated crops such as common bean, tomato, sorghum, and cotton grown in areas adjacent to soybean during the soybean season and in the soybean off-season. In these cases, farmers would benefit from the regional suppression of both pests promoted by widespread adoption of Cry1Ac soybean. In the USA and China, the widespread adoption of *Bt* crops reduced the occurrence of target pests on non-*Bt* crop areas, including row crops, vegetable crops, and organic farms^{17,19,20}.

Our results confirm the association that widespread adoption of Cry1Ac soybean in different mesoregions of Brazil significantly reduced insecticide sprays on soybean fields. Direct and indirect benefits in pest management are achieved through the pest suppression caused by widespread adoption of *Bt* crops^{14,17,19,20}. However, it is important to note that the maintenance of these benefits depends on the insect control efficacy of the technology. Therefore, following insect resistance management recommendations is essential to maintain the efficacy of Cry1Ac soybean, which is still providing benefits eight years after the first commercial launch in Brazil²⁷. Stewardship practices to maintain the efficacy of Cry1Ac soybean against target pests and preserve its other benefits also build the foundation for deploying future generations of transgenic plants that would contribute to sustainability of agriculture in tropical regions.

2.4. Material and Methods

2.4.1. Cry1Ac soybean adoption and insecticide spraying

Data reporting the adoption of the Cry1Ac soybean technology and use of insecticide sprays for managing lepidopteran pests from 2012 (before commercial planting of Cry1Ac soybean) to 2019 were obtained from market research companies Kynetec (2012–2018) and Spark - BIP soybean (2019). For each year, 43 to 46 mesoregions in soybean-planting areas of Brazil were used for analysis (SI Fig. 3). Mesoregions are geographical divisions within each Brazilian State³⁶.

To evaluate the relationship between insecticide sprays and *Bt* adoption, a linear regression analysis was performed with the number of insecticide sprays as a function of Cry1Ac soybean adoption. Cry1Ac soybean adoption is based on the area planted (hectares) with Cry1Ac soybean divided by the total area planted with soybean in a mesoregion. Two distinct analyses were performed, the first one with the total number of insecticide sprays without differentiating Cry1Ac soybean from non-*Bt* soybean (total soybean area) and the second considering only the number of insecticides sprays over non-*Bt* soybean. Separate analyses using this same methodology were also made for four individual mesoregions: West Bahia, North Mato Grosso, North Central Paraná, and Northwest Rio-Grande. Linear regressions were performed in GraphPad Prism 8 – version 8.1.2³⁷.

2.4.2. Adult sampling with pheromone traps

Adult moths were sampled with traps (McPhail type) using synthetic sex pheromone of *C. includens* (Bio Pseudoplusia, Fuji Flavor Co, Ltd.) and *Helicoverpa* spp. (Bio Helicoverpa, ChemTica Internacional, S.A.). The sampling was performed from December 2014 (early years of commercial planting of Cry1Ac soybean) to July 2019 (>64% adoption of Cry1Ac soybean in Brazil). During this period, five soybean seasons were cultivated in every region sampled. Pheromone traps were placed in four mesoregions representing distinct environments and crop systems in Brazil: West Bahia (Luís Eduardo Magalhães/BA) (−11.959167, −46.119167), North Mato Grosso (Sorriso/MT) (−12.462417, −55.68425), North Central Paraná (Rolândia/PR) (−23.313056, −51.491389), and Northwest Rio-Grande (Carazinho/RS) (−28.32273, −52.7125) (SI Fig. 4). In each location, two independent pheromone traps were placed for each species. The total numbers of adults of *C. includens* and *Helicoverpa* spp. trapped were recorded weekly, traps emptied, and pheromone baits replaced.

To evaluate change in adult capture with the increase of Cry1Ac soybean adoption, a generalized linear model (GLM) with Poisson link was fit to the weekly moth sampling data as function of Cry1Ac soybean adoption for each mesoregion (West Bahia, North Mato Grosso, North Central Paraná and Northwest Rio-Grande). Then, a regression curve was estimated for each mesoregion where insects were sampled. Data from each pest species and mesoregion (West Bahia, North Mato Grosso, North Central Paraná and Northwest Rio-Grande) were analyzed separately. The analyses were performed in R statistical software - R version 4.0.2³⁸.

2.4.3. Climatic variables

Three climatic variables (precipitation, maximum temperature, and minimum temperature) from each site containing traps were taken from the WorldClim website: (www.worldclim.org/). To determine the effect of climatic conditions on insect collections, a linear regression analysis was performed with the annual precipitation, maximum temperature, and minimum temperature. Annual precipitation was obtained from the sum of monthly precipitation within a year, while maximum and minimum temperature were obtained from the average maximum or minimum monthly temperature within a year. Analyses were performed with climatic variable as a function of year, using GraphPad Prism 8 version 8.1.2³⁷.

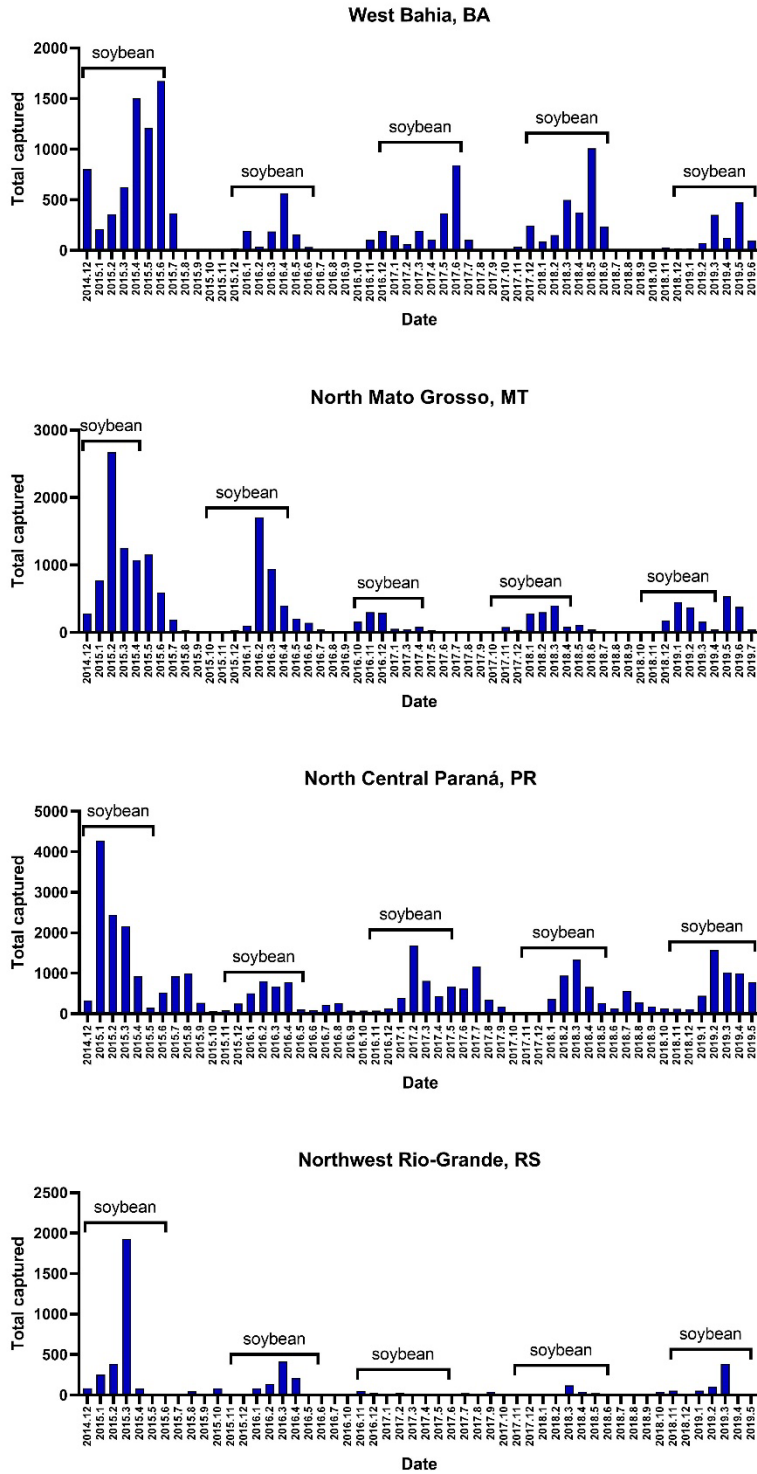
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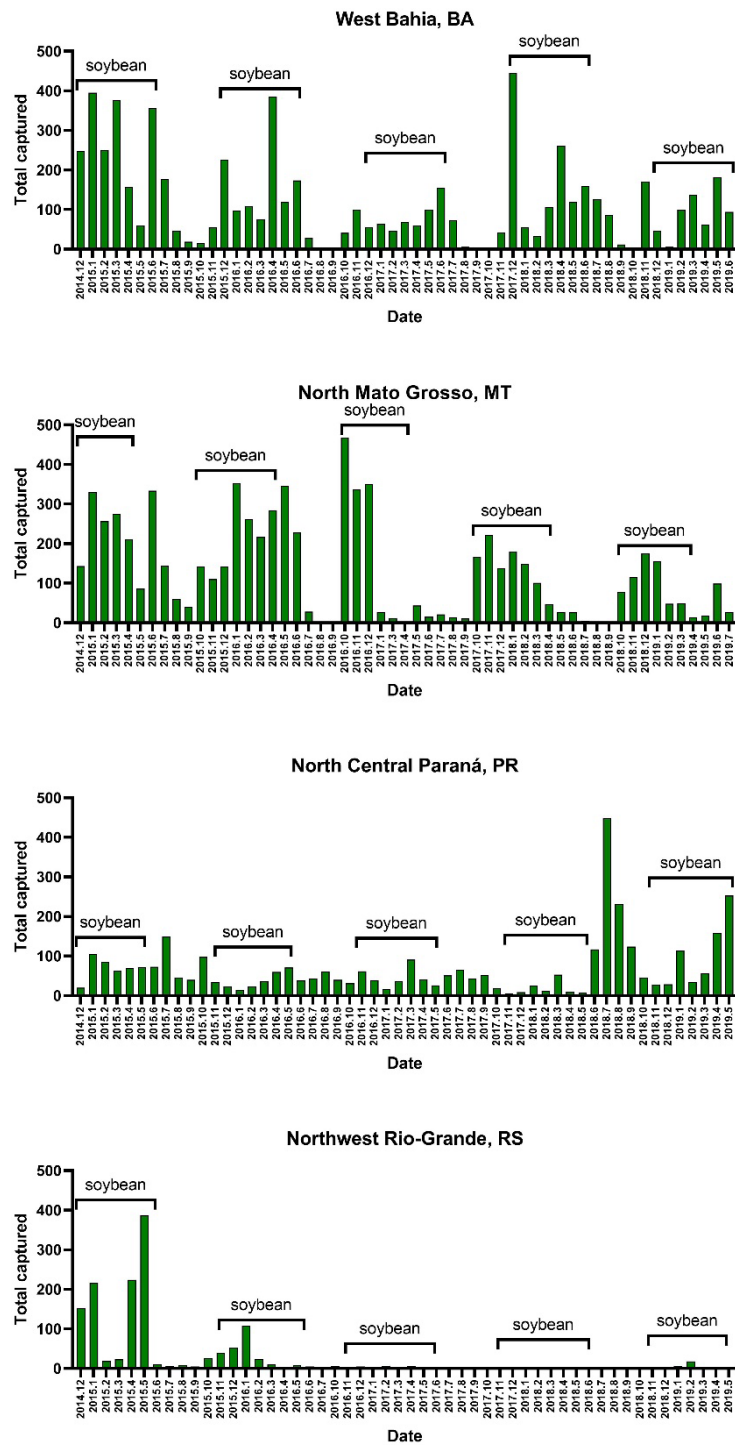
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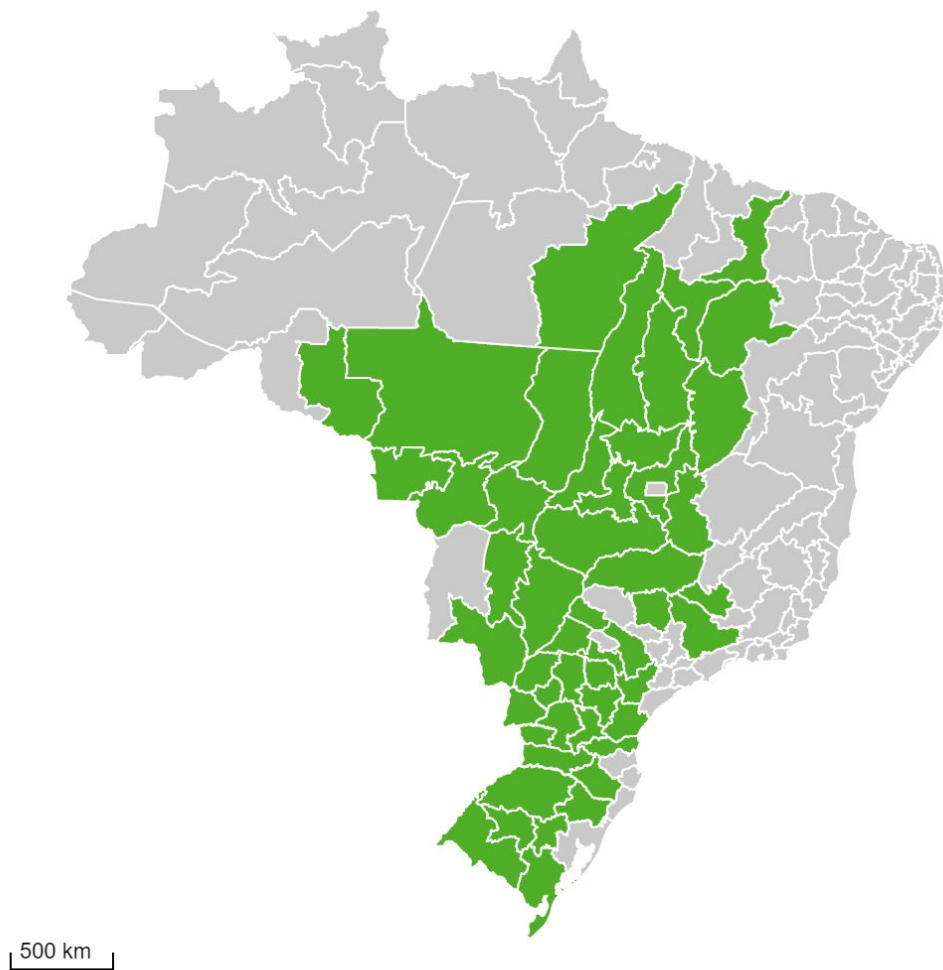
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Supporting Information (SI). Fig. 1 Monthly total numbers of *C. includens* adults captured from 2014 to 2019 in four mesoregions. Lines marked “soybean” indicate the soybean crop windows for each mesoregion.



Supporting Information (SI). Fig. 2 Monthly total numbers of *Helicoverpa* spp. adults captured from 2014 to 2019 in four mesoregions. Lines marked “soybean” indicate the soybean crop window for each mesoregion.



Supporting Information (SI). Fig. 3. Brazilian mesoregions used for Cry1Ac soybean adoption and insecticide spray analysis. Map was generated using TIBCO Spotfire® Analyst 10.10.2 LTS (<https://www.tibco.com/products/tibco-spotfire>).



Supporting Information (SI). Fig. 4. Blue areas represent mesoregions in Brazil where pheromone traps were used to collect *C. includens* and *Helicoverpa* spp. (West Bahia, Northwest Rio-Grande, North Central Paraná, and North Mato Grosso). Map was generated using TIBCO Spotfire® Analyst 10.10.2 LTS (<https://www.tibco.com/products/tibco-spotfire>).

3. LARGE-SCALE ASSESSMENT OF LEPIDOPTERAN SOYBEAN PESTS AND EFFICACY OF Cry1Ac SOYBEAN IN BRAZIL

ABSTRACT

The soybean technology MON 87701 × MON 89788, expressing Cry1Ac and conferring tolerance to glyphosate, has been widely adopted in Brazil since 2013. However, pest shifts, or resistance evolution could reduce the benefits of this technology. To assess Cry1Ac soybean performance and understand the composition of lepidopteran pest species attacking soybeans, we implemented large-scale sampling of larvae on commercial soybean fields during the 2019 and 2020 crop seasons to compare with data collected prior to the introduction of Cry1Ac soybeans. *Chrysodeixis includens* was the main lepidopteran pest in non-Bt fields. More than 98% of larvae found in Cry1Ac soybean were *Spodoptera* spp., although the numbers of *Spodoptera* were similar between Cry1Ac soybean and non-Bt fields. Cry1Ac soybean provided a high level of protection against *Anticarsia gemmatilis*, *C. includens*, *Chloridea virescens* and *Helicoverpa* spp. Significant reduction in insecticide sprays for lepidopteran control in soybean were observed from 2012 to 2019. Our study showed that *C. includens* and *A. gemmatilis* continue to be primary lepidopteran pests of soybean in Brazil and that Cry1Ac soybean continues to effectively manage the target lepidopteran pests. However, there was an increase in the relative abundance of non-target *Spodoptera* spp. larvae in both non-Bt and Cry1Ac soybeans.

Keywords: MON 87701 × MON 89788, Plusiinae, *Spodoptera*, Integrated Pest Management (IPM), Bt plant, pest abundance

3.1. Introduction

Brazil is a top producer of soybean (*Glycine max* (L.) Merrill)¹, and a turning point in the commercial cultivation of soybean in Brazil was the expansion of soybean production from the South (subtropical climate) into areas in the savannahs (i.e., Cerrado) in the Central-West region of the country (tropical climate)². This expansion was largely enabled by technological advances ranging from better soil management and fertilization practices to the development of soybean varieties adapted to equatorial latitudes³. Moreover, the expansion of no-till systems, the use of better planting and harvesting equipment, and the adoption of transgenic soybeans to assist in weed control made important contributions to increases in the national average soybean yield in Brazil^{1,3,4,5}. As soybean production in Brazil transformed into a highly structured and organized large-scale business operation primarily targeting export markets, the need to adopt simple, low-cost agronomic practices for controlling insects caused an increase in the number of insecticide sprays required^{6,7,8}.

In this context, the soybean technology MON 87701 × MON 89788 (Intacta RR2 PRO[®]), expressing the Cry1Ac insecticidal protein (event MON 87701) and conferring tolerance to glyphosate (event MON 89788), was commercially launched and became available to farmers in Brazil in 2013. The adoption and use of Cry1Ac soybean by Brazilian farmers increased from 1.2 million hectares in the 2013/14 cropping season to 21.9 million hectares in the 2017/18 cropping season⁹. The

acceptance of this soybean technology by Brazilian farmers can be attributed to the cost-effective and simpler weed control enabled by the tolerance to glyphosate, coupled with higher yields from a combination of better pest and weed management¹⁰. Cry1Ac soybean provides high-level protection against the major soybean lepidopteran pests, including *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Erebiidae), *Chrysodeixis includens* (Walker [1858]) (Lepidoptera: Noctuidae), *Chloridea virescens* (Fabricius, 1777) (Lepidoptera: Noctuidae) and *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae)^{11,12,13,14}. Despite its benefits to soybean pest management, Cry1Ac soybean does not confer protection against the main species of *Spodoptera* found in Brazil: *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), *Spodoptera eridania* (Stoll, 1782) (Lepidoptera: Noctuidae) and *Spodoptera cosmioides* (Walker, 1898) (Lepidoptera: Noctuidae)¹⁵.

Beyond the direct benefit of controlling target pests, *Bt* crops such as Cry1Ac soybean have the potential to provide additional benefits to insect management in agricultural systems, including reduction in insecticide use¹⁰, compatibility with biocontrol measures^{16,17}, and regional suppression of insect pest populations^{18,19,20,21}. In particular, suppression of target pests after a long period of use of highly efficacious *Bt* technologies has been documented in *Pectinophora gossypiella* (Saund., 1844) (Lepidoptera: Gelechiidae), *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Pyralidae) and *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) in the USA^{18,19,21} and *H. armigera* in China²⁰. Similarly, high efficacy of Cry1Ac soybean against lepidopteran pests such as *C. includens* and *A. gemmatalis*, the main soybean pests in Brazil¹², resulted in fewer insecticide sprays to manage lepidopteran larvae after five years of commercial use in Brazil¹⁰. However, where a *Bt* technology is ineffective against non-target secondary pest species and or broad-spectrum insecticide use has decreased due to highly effective control of the target species, secondary pests may increase in abundance over time^{22,23}.

In addition, resistance evolution by target pest populations can reduce the benefits of *Bt* crops²⁴. The high-dose expression and refuge strategy was proposed to manage resistance of target pest populations to Cry1Ac soybean in Brazil¹². Nevertheless, poor compliance with refuge recommendations has been a common factor in most cases of documented field-evolved resistance to *Bt* crops^{25,26,27,28,29}. Moreover, the intensive use of agricultural land creates an environment conducive to the buildup of relatively large insect pest populations and multiple generations of pests potentially under selection of *Bt* crops or insecticides^{30,31}. "Tropical agriculture" such as that practiced in Brazil is typically based on two or more crop seasons per year, allowing pest populations to go through multiple generations per year on *Bt* crops and consequently increasing selection pressure³⁰. Therefore, understanding the performance of a *Bt* crop against target pests at the field level and determining whether non-target pests are increasing in abundance can inform the need for the adoption of appropriate Integrated Pest Management (IPM) practices in Brazil.

In this study, we carried out a two-year large-scale assessment on commercial soybean fields in Brazil after eight years of Cry1Ac soybean use with the goals of (a) evaluating Cry1Ac soybean performance and impacts on soybean pest management and (b) assessing the relative abundance of lepidopteran pest species attacking soybean fields and comparing these results to data collected prior to the commercial introduction of Cry1Ac soybeans.

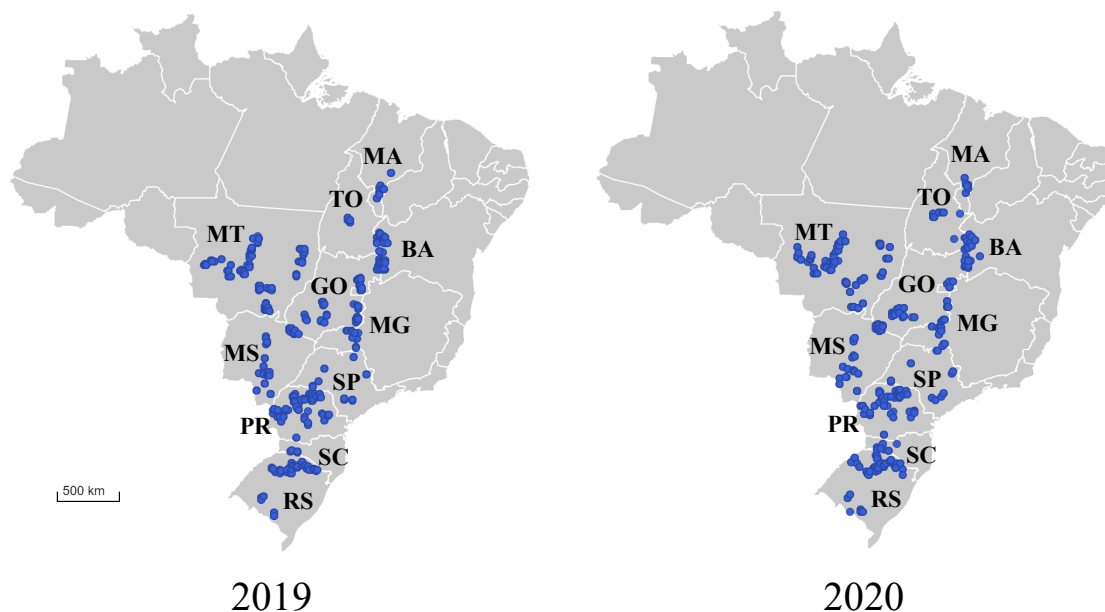
3.2. Methods

3.2.1. Insect sampling and data collection.

All insect collections were in accordance with the approval granted by the System of Authorization and Information on Biodiversity (SISBIO) of the Ministry of Environment to a contracted company responsible for the field sampling (PROMIP, Permit for scientific purpose activity: 61826, 61824).

Sampling prior to commercialization of Cry1Ac soybean. From 2011 (2010/11) to 2014 (2013/14), field sampling of lepidopteran larvae was carried out in plots of non-*Bt* (Roundup Ready - RR) soybean. Samples consisted of 10 beat sheets (length = 1 meter) per location followed by identification of larvae. A total of 829 samples were taken across the states of Bahia (BA), Distrito Federal (DF), Goiás (GO), Mato Grosso (MT), Mato Grosso do Sul (MS), Minas Gerais (MG), Paraná (PR), Rio Grande do Sul (RS), Santa Catarina (SC), São Paulo (SP) and Tocantins (TO). These data from 2011–2014 provided a baseline for descriptive comparisons with the subsequent sampling described below but were not analyzed statistically.

Sampling after commercialization of Cry1Ac soybean. Lepidopteran larvae were sampled from commercial 399 soybean fields during the 2019 (2018/19) and 387 fields in 2020 (2019/20) cropping seasons (Fig. 1). Each location had a non-*Bt* (Roundup Ready - RR) soybean field and a Cry1Ac soybean (MON 87701 × MON 89788, Intacta RR2 PRO®) field. Samplings were conducted at early reproductive stages (R1–R4) and late reproductive stages (R5–R7). Larvae were sampled with a beat sheet (length = 1 meter) and the sampling unit consisted of 10 beats in a zig-zag pattern per soybean reproductive stages. Additionally, for each beat sheet sampling, the level of defoliation in soybean was evaluated. To avoid border effects, sampling was initiated at a minimum of 20 meters from the edge of the soybean fields in the Southern region of Brazil, where farms are smaller (average size of farms less than 100 ha), and 100 meters in the Central-West and Northeast regions, where larger farms are common (average size of farms greater than 150 ha). For each location, sampling was done first in the non-*Bt* field: if at least 1 larva per meter was obtained, then samples were also taken from a nearby Cry1Ac soybean field at a similar plant growth stage to have a pair of neighboring fields with comparable incidence of lepidopteran pests. All fields were checked for Cry1Ac expression using QuickStix kits (Envirologix) to confirm the presence of *Bt* protein in Cry1Ac soybean plants and absence of this protein in non-*Bt* soybean plants. Larvae were transferred to 50-mL labeled conical centrifuge tubes containing propylene glycol. All tubes were then sent to the laboratory and kept in a freezer (–20 °C) until identification. All lepidopteran larvae were identified based on Herzog³², Sosa-Gómez et al.³³ and Gilligan and Passoa³⁴.



Soybean reproductive stage	2019		2020	
	Cry1Ac soybean	Non- <i>Bt</i> soybean	Cry1Ac soybean	Non- <i>Bt</i> soybean
Early (R1–R4)	195	194	195	195
Late (R5–R7)	204	205	192	192
Total	399	399	387	387

Figure 1. Locations and number of lepidopteran larvae samplings in commercial soybean fields in 2019 and 2020. Maps were generated using TIBCO Spotfire® Analyst 10.10.2 LTS <https://www.tibco.com/products/tibco-spotfire>.

3.2.2. Lepidopteran species composition sampled from non-*Bt* fields prior and after commercialization of Cry1Ac soybean.

A descriptive analysis was made with the total insects sampled prior (2011-2014) and after (2019-2020) commercialization of Cry1Ac soybean. The relative number of each species for 2011-14 and 2019-20 was represented as a percentage of total.

3.2.3. Comparison of pest abundance on Cry1Ac soybean and non-*Bt* soybean.

Fields with Cry1Ac soybean were paired with neighboring non-*Bt* fields (see “Insect sampling and data collection”). To compare larval counts in Cry1Ac soybean fields with counts in non-*Bt*

soybean fields, a generalized linear mixed model with Poisson link was fit to the data separately for each species and plant growth stage (combined across seasons 2019 and 2020). If Y_{ij} is the count for the i th field type in the j th pair, then $E(\log(Y_{ij})) = u_j$ for non-*Bt* fields, and $E(\log(Y_{ij})) = \tau + u_j$ for Cry1Ac soybean fields, where $u_j \sim N(0, \sigma^2)$ is the effect of the j th pair and $\exp(\tau)$ is the relative larvae count in Cry1Ac soybean fields. Analyses were performed with R statistical software - R version 4.0.2³⁵.

3.2.4. Pest species contributions to defoliation.

To determine the relative contribution of individual species to defoliation, multiple regression was performed of defoliation against counts of *A. gemmatalis*, *C. includens*, *C. virescens*, *Helicoverpa* spp., *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae), *S. cosmioides*, *S. eridania* and *S. frugiperda*. Regression was conducted separately for Cry1Ac soybean and non-*Bt* soybean, and for early and late growth reproductive stages, combined across the 2019 and 2020 seasons. The regression coefficient for a given species can be interpreted as the percentage increase in defoliation for each individual larva present. Thus, species with large coefficients contributed more to defoliation than did species with small coefficients. Analyses were performed with R statistical software - R version 4.0.2³⁵.

3.2.5. Geographic variation in soybean pest abundance.

Sampling locations were grouped according to Embrapa's soybean variety regionalization³⁶. These groupings are called "edaphoclimatic regions" and "soybean macroregions" and are based on agroecological zones, Köppen climate classification for Brazil, technical recommendations for soybean production, soybean research meeting documents, and contributions of research institutes³⁶. To characterize geographic variation in pest abundance, random effects for edaphoclimatic regions were estimated using a linear mixed-effects model for larval count data with Poisson link, with edaphoclimatic region nested within soybean macroregion. The abundance was estimated based on non-*Bt* soybean larval sampling. The edaphoclimatic region estimates were color-coded in choropleth maps. Analyses were performed with R statistical software - R version 4.0.2³⁵.

3.2.6. Insecticide use on soybean fields in Brazil.

Data on use of insecticide sprays to manage lepidopteran larvae across mesoregions for the 2013 to 2019 cropping seasons were obtained from the AMIS Kleffmann Group database (2013–2018) and BIP Spark (2019). A linear regression analysis was performed with number of insecticide sprays as a function of cropping season. The number of insecticide sprays for lepidopteran control in every mesoregion of soybean planting area was log-transformed. Analyses were performed in GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3.3. Results

3.3.1. Lepidopteran species composition sampled from non-*Bt* fields

The percentage of each pest species relative to total larvae sampled in commercial (non-*Bt*) soybean fields prior to the commercialization of Cry1Ac soybean (2011 to 2014 cropping seasons; hereafter “pre-commercial period”) and total larvae sampled in commercial non-*Bt* soybean fields during the post-commercial period of Cry1Ac soybean (2019 and 2020 cropping seasons; hereafter “post-commercial period”) is shown in Fig. 2. Of the 16,277 lepidopteran larvae sampled in non-*Bt* fields during the pre-commercial period, more than 90% were *C. includens* and *A. gemmatalis* (Fig. 2A). The importance of these two species in non-*Bt* soybean fields was maintained during the post-commercial period, in which *C. includens* and *A. gemmatalis* represented more than 70% of 12,676 insects sampled (Fig. 2B). Small numbers of other species such *Helicoverpa* spp., *R. nu* and *C. virescens* were present, together representing less than 6% and less than 5% of the total samples during the pre- and post-commercial periods, respectively. *Spodoptera* species, represented mostly by *S. frugiperda*, *S. eridania* and *S. cosmioides*, also were found in low numbers during the pre-commercial period. Representing only 3% of 12,676 insects sampled during the pre-commercial period, *S. eridania* was the predominant species sampled. However, *Spodoptera* species represented more than 23% of the total insects sampled in non-*Bt* soybean fields during the post-commercial period (Fig. 2B).

The absolute numbers of *S. cosmioides*, *S. eridania* and *S. frugiperda* found in Cry1Ac and non-*Bt* soybean were similar (Fig. 3A). Of the 1,376 and 1,122 total insects sampled in Cry1Ac soybean at the early and late reproductive stages, respectively, more than 98% were *S. cosmioides*, *S. eridania* and *S. frugiperda* (Fig. 3B). More *S. frugiperda* were observed at the early reproductive stage and more *S. eridania* at the late reproductive stage in both soybean types (Fig. 3B). For non-*Bt* soybean, 12,676 larvae were identified, of which 51.32% and 68.26% were *C. includens* at the early and late reproductive stages, respectively (Fig. 3C). *Spodoptera frugiperda* and *A. gemmatalis* represented similar percentages of the total insects from non-*Bt* soybean at the early reproductive stage (18.21% and 18.24%, respectively) and late reproductive stage (7.95% and 5.93%, respectively) (Fig. 3C).

The median percentage defoliation in Cry1Ac soybean fields was lower than in non-*Bt* soybean fields in all but one comparison. For 2019, the Cry1Ac soybean median defoliation was 2.5% and 4.7% at the early and late reproductive stages, respectively, whereas median defoliation in non-*Bt* soybean fields was 8.6% and 13.7% at the early and late reproductive stages, respectively (Fig. 4). The 75th percentiles for defoliation in Cry1Ac soybean were 5% and 6.5% and for non-*Bt* soybean were 13.3% and 21.5% at the early and late reproductive stages, respectively (Fig. 4). In 2020, median defoliation in both Cry1Ac soybean and non-*Bt* soybean was 5% at the early reproductive stage, and 5% and 10%, respectively, at the late reproductive stage (Fig. 4). The 75th percentiles for defoliation at the late reproductive stage were 9.8% and 15% for Cry1Ac soybean and non-*Bt* soybean, respectively (Fig. 4).

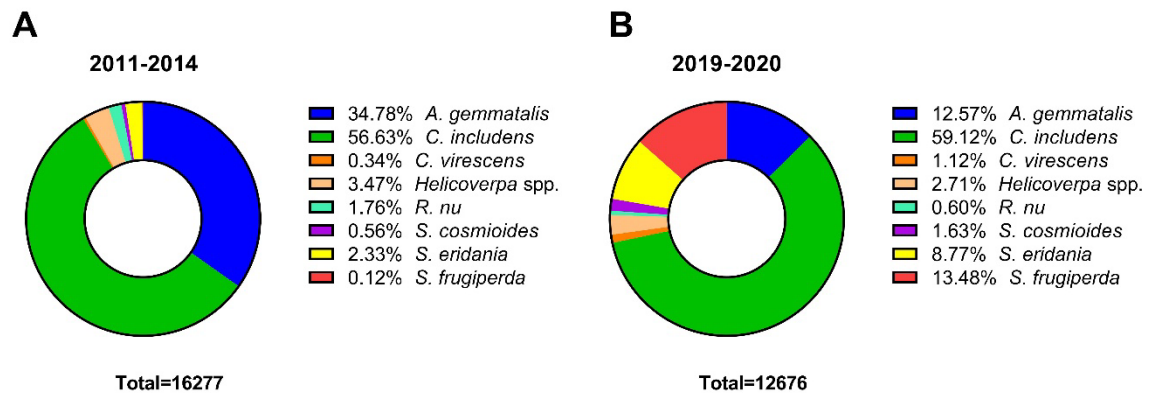
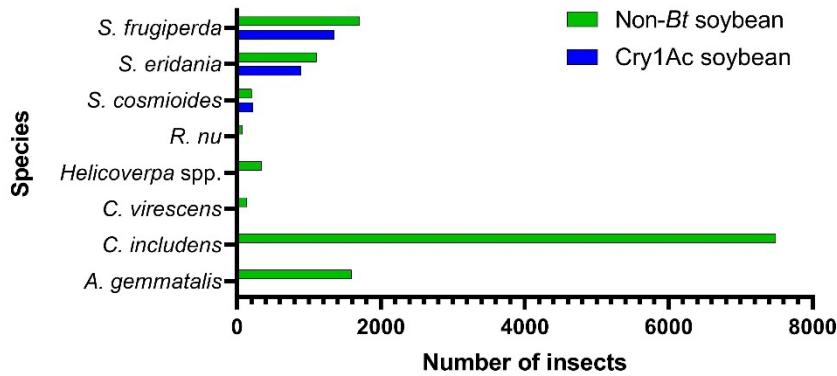


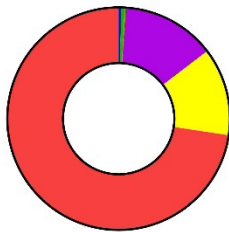
Figure 2. Lepidopteran species composition of sampled larvae from non-*Bt* fields during (A) pre-commercial (2011–2014) and (B) post-commercial Cry1Ac soybean (2019 and 2020) cropping seasons.

A

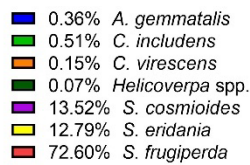


B

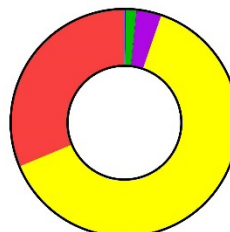
Cry1Ac soybean Early



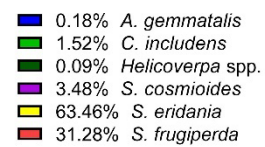
Total=1376



Cry1Ac soybean Late

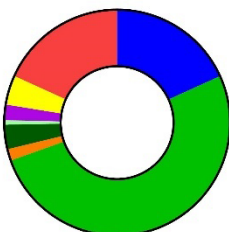


Total=1122

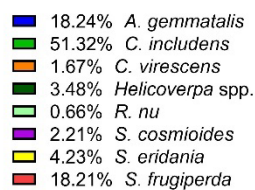


C

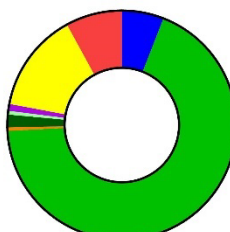
Non-Bt soybean Early



Total=6838



Non-Bt soybean Late



Total=5838

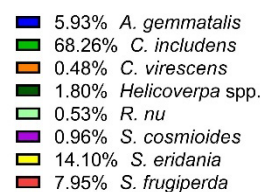


Figure 3. Lepidopteran species composition of sampled larvae from Cry1Ac soybean and non-Bt fields in the 2019 and 2020 cropping seasons. (A) Total number of lepidopteran larvae sampled in Cry1Ac soybean and non-Bt soybean fields. (B) Larvae species composition from Cry1Ac soybean fields in early and late stage. (C) Larvae species composition from non-Bt soybean fields in early and late stage.

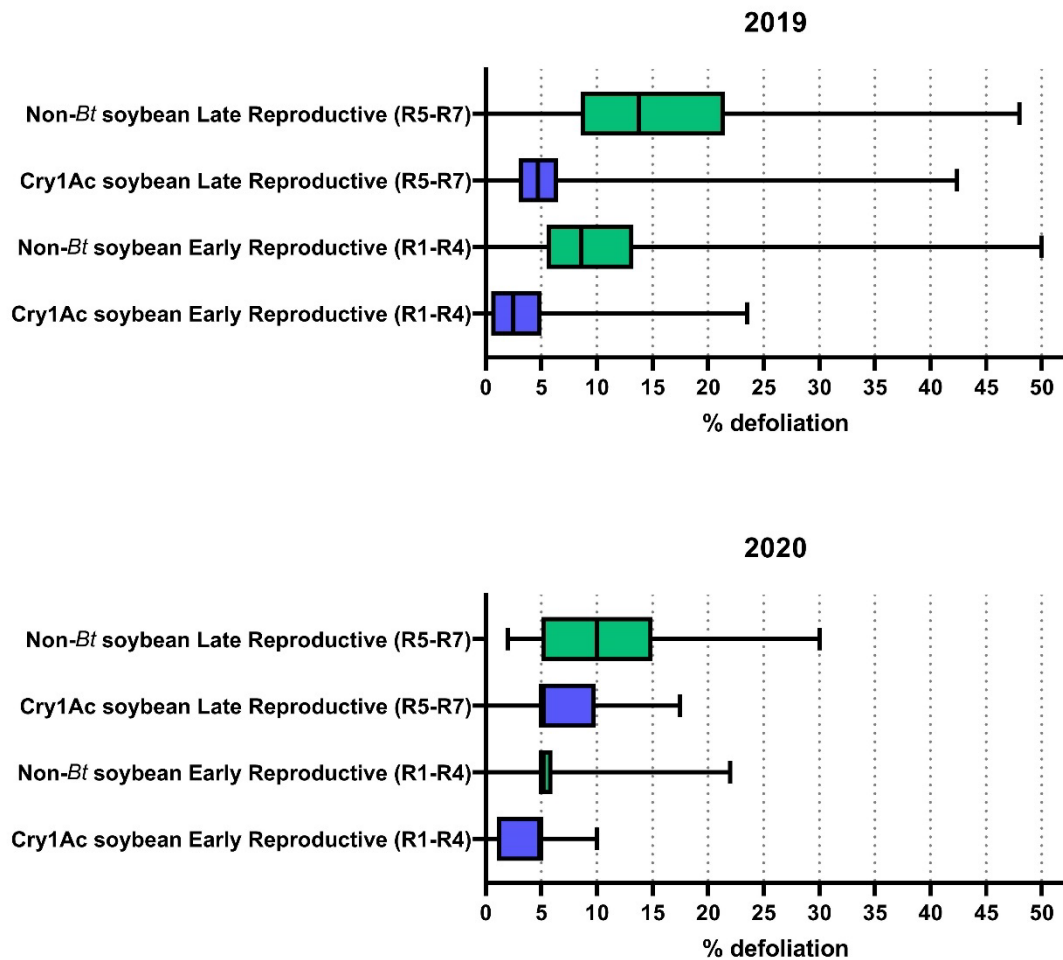


Figure 4. Defoliation in Cry1Ac soybean and non-*Bt* soybean fields in the 2019 and 2020 seasons. The middle vertical line within each box represents the median; the left and right edges of the boxes represent the 25th and 75th percentiles, respectively.

3.3.2. Comparison of pest abundance on Cry1Ac soybean and non-*Bt* soybean

Larval abundance in Cry1Ac soybean fields relative to non-*Bt* soybean fields in the 2019 and 2020 seasons, and 95% confidence intervals, are presented in Table 1. Based on these analyses, Cry1Ac soybean provided high control (relative abundance <0.02) of *A. gemmatalis*, *C. includens*, *C. virescens* and *Helicoverpa* spp.; minimal control of *S. eridania* and *S. frugiperda*; and no control of *S. cosmioides* (Table 1). *R. nu* larvae were rare in samples from both non-*Bt* and Cry1Ac soybean fields. The relative Cry1Ac soybean abundance values were similar among early and late reproductive stages within each species, with the exception that *S. cosmioides* was more prevalent at the early reproductive stage (Table 1).

Table 1. Relative abundance of lepidopteran larvae in Cry1Ac soybean fields combined across the 2019 and 2020 seasons.

Species	Relative abundance ¹	LCL ²	UCL ²
Early reproductive stage (R1–R4)			
<i>A. gemmatalis</i>	0.004	0.002	0.01
<i>C. includens</i>	0.002	0.001	0.004
<i>C. virescens</i>	0.018	0.004	0.071
<i>Helicoverpa</i> spp.	0.004	0.001	0.03
<i>R. nu</i>	0	0	Inf
<i>S. cosmioides</i>	1.231	0.993	1.526
<i>S. eridania</i>	0.609	0.505	0.734
<i>S. frugiperda</i>	0.802	0.738	0.872
Late reproductive stage (R5–R7)			
<i>A. gemmatalis</i>	0.006	0.001	0.023
<i>C. includens</i>	0.004	0.003	0.007
<i>C. virescens</i>	0	0	Inf
<i>Helicoverpa</i> spp.	0.01	0.001	0.068
<i>R. nu</i>	0	0	Inf
<i>S. cosmioides</i>	0.697	0.463	1.048
<i>S. eridania</i>	0.865	0.783	0.957
<i>S. frugiperda</i>	0.758	0.66	0.871

¹ Relative abundance in Cry1Ac soybean vs non-*Bt* soybean

² LCL, lower confidence limit; UCL, upper confidence limit (95% confidence interval)

3.3.3. Pest species contributions to defoliation

Table 2 gives regression coefficient estimates for early and late reproductive stage non-*Bt* and Cry1Ac soybean. As described earlier, these coefficients represent the percentage increase in defoliation caused by each larva of a species. In non-*Bt* soybean fields, *A. gemmatalis*, *C. includens*, *Helicoverpa* spp., *S. cosmioides*, *S. eridania* and *S. frugiperda* contributed to defoliation at both early and late reproductive stages ($P < 0.05$) (Table 2). The highest coefficients observed for non-*Bt* soybean were for *S. cosmioides*: 1.385 and 2.136 for early and late reproductive stages, respectively (Table 2). Comparing *S. cosmioides* with *S. frugiperda* on early-reproductive-stage soybean, for example, the estimated coefficients were 1.385 and 0.245, respectively, indicating that an individual *S. cosmioides* larva caused $1.385/0.245 = 5.65$ times the damage caused by an individual *S. frugiperda* larva. By that same logic, *S. cosmioides* caused 1.7 to 6.7 times the damage per larva caused by *A. gemmatalis*, *C. includens*, *Helicoverpa* spp. and *S. eridania* at the early reproductive stage. At the late reproductive stage, *S. cosmioides* caused 1.9 to 4.3 times the damage per larva caused by *A. gemmatalis*, *C. includens*, *Helicoverpa* spp., *S. eridania* and *S. frugiperda*.

For Cry1Ac soybean fields, only *S. cosmioides*, *S. eridania* and *S. frugiperda* contributed to defoliation ($P < 0.05$) (Table 2). The other species were controlled by Cry1Ac soybean, as described in the previous section. *S. cosmioides* had the highest coefficients in Cry1Ac soybean: 0.623 and 2.121 for early and late reproductive stages, respectively. Each *S. cosmioides* larva caused 2.0 and 4.5 times the damage at the early reproductive stage and 4.9 and 2.7 times the damage at the late reproductive stage caused by individual *S. eridania* and *S. frugiperda* larvae, respectively.

Table 2. Relative contribution of lepidopteran species to early- and late-reproductive-stage defoliation in soybean fields, combined across the 2019 and 2020 seasons.

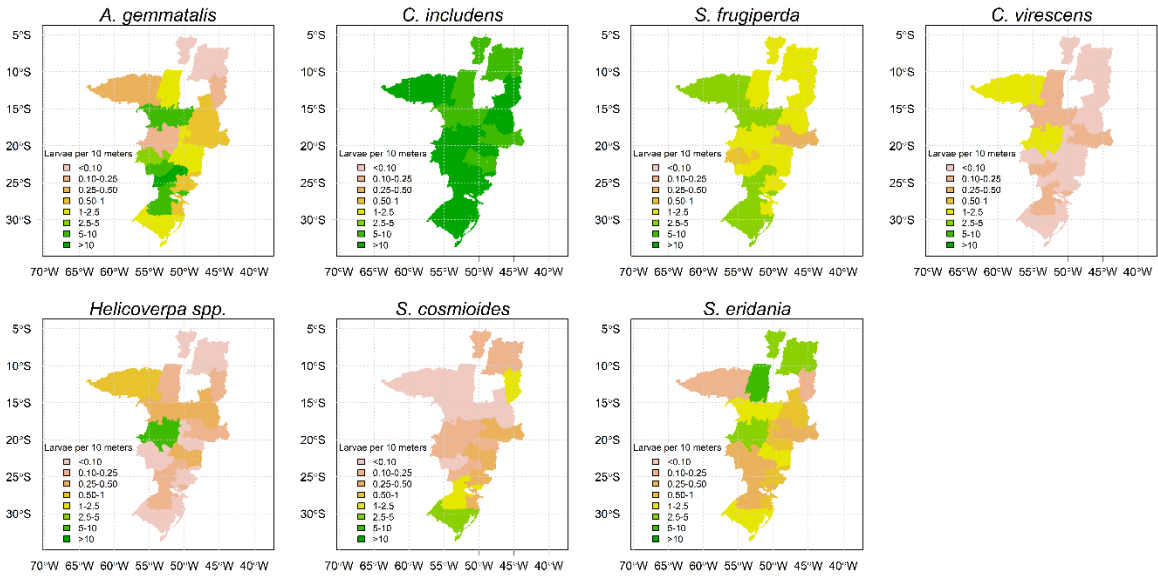
Technology	Species	Estimate ¹	Std. Error	t value	P(> t)
Non-Bt soybean	Early Reproductive Stage (R1–R4)				
	<i>A. gemmatalis</i>	0.204	0.026	7.748	<0.0001
	<i>C. includens</i>	0.376	0.023	16.085	<0.0001
	<i>C. virescens</i>	0.139	0.228	0.611	0.541
	<i>Helicoverpa</i> spp.	0.304	0.118	2.565	0.011
	<i>R. nu</i>	0.119	0.345	0.347	0.729
	<i>S. cosmioides</i>	1.385	0.246	5.632	<0.0001
	<i>S. eridania</i>	0.81	0.139	5.824	<0.0001
	<i>S. frugiperda</i>	0.245	0.055	4.452	<0.0001
	Late Reproductive Stage (R5–R7)				
	<i>A. gemmatalis</i>	0.699	0.142	4.93	<0.0001
	<i>C. includens</i>	0.649	0.044	14.717	<0.0001
	<i>C. virescens</i>	0.718	0.656	1.095	0.274
	<i>Helicoverpa</i> spp.	1.118	0.498	2.247	0.025
	<i>R. nu</i>	0.419	1.129	0.371	0.711
<i>S. cosmioides</i>	2.136	0.642	3.326	0.001	
<i>S. eridania</i>	0.501	0.101	4.95	<0.0001	
<i>S. frugiperda</i>	0.619	0.19	3.259	0.001	
Cry1Ac soybean	Early Reproductive Stage (R1–R4)				
	<i>A. gemmatalis</i>	0.643	1.715	0.375	0.708
	<i>C. includens</i>	0.125	1.53	0.081	0.935
	<i>C. virescens</i>	2.431	1.954	1.245	0.214
	<i>Helicoverpa</i> spp.	-1.147	3.967	-0.289	0.773
	<i>S. cosmioides</i>	0.623	0.092	6.797	<0.0001
	<i>S. eridania</i>	0.309	0.077	3.99	<0.0001
	<i>S. frugiperda</i>	0.137	0.027	5.162	<0.0001
	Late Reproductive Stage (R5–R7)				
	<i>A. gemmatalis</i>	4.899	4.71	1.04	0.299
	<i>C. includens</i>	1.157	0.918	1.261	0.208
	<i>Helicoverpa</i> spp.	7	6.66	1.051	0.294
	<i>S. cosmioides</i>	2.121	0.712	2.978	0.003
	<i>S. eridania</i>	0.425	0.054	7.812	<0.0001
	<i>S. frugiperda</i>	0.778	0.134	5.789	<0.0001

¹Estimate of the regression coefficient for a given species, which can be interpreted as the percentage increase in defoliation for each individual larva present.

3.3.4. Geographic variation in soybean pest abundance

Visualization of pest abundance in non-*Bt* soybean by species in Figs 5–6 shows that *C. includens* was present at high levels in all of the soybean-producing regions sampled in Brazil. *A. gemmatalis* and *S. eridania* abundance varied among seasons and growth stages but both species were often present at high levels. *S. frugiperda* abundance was lower in 2019 than in 2020, when there was high infestation in northern regions (Figs 5–6). Abundances of *C. virescens*, *Helicoverpa* spp. and *S. cosmioides* were lower across the regions evaluated when compared to *C. includens*, *A. gemmatalis*, *S. eridania* and *S. frugiperda* (Figs 5–6).

2019 Early reproductive stage



2019 Late reproductive stage

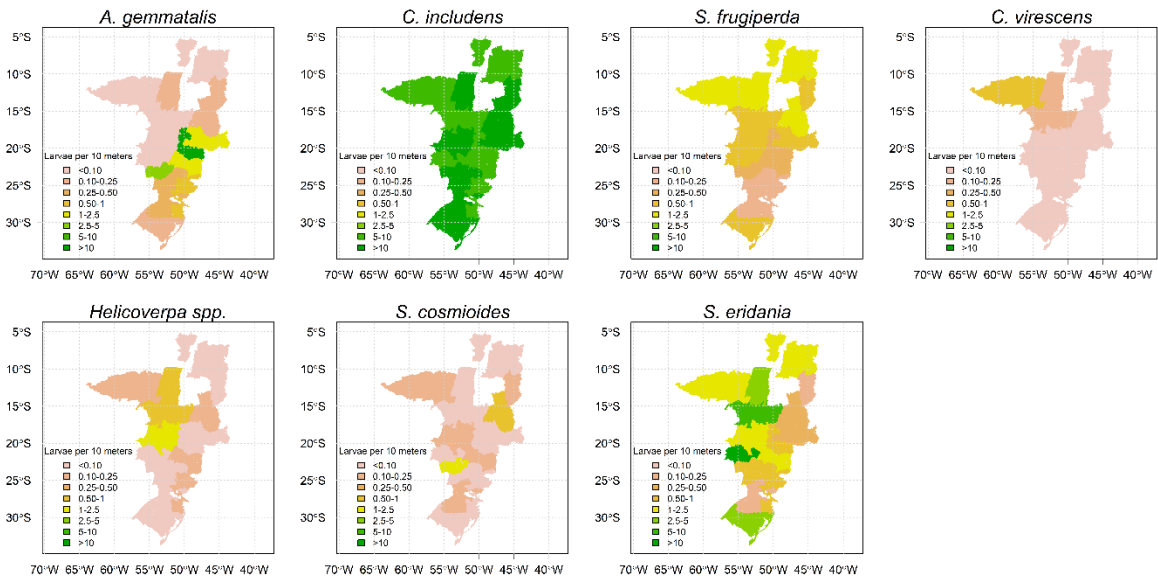
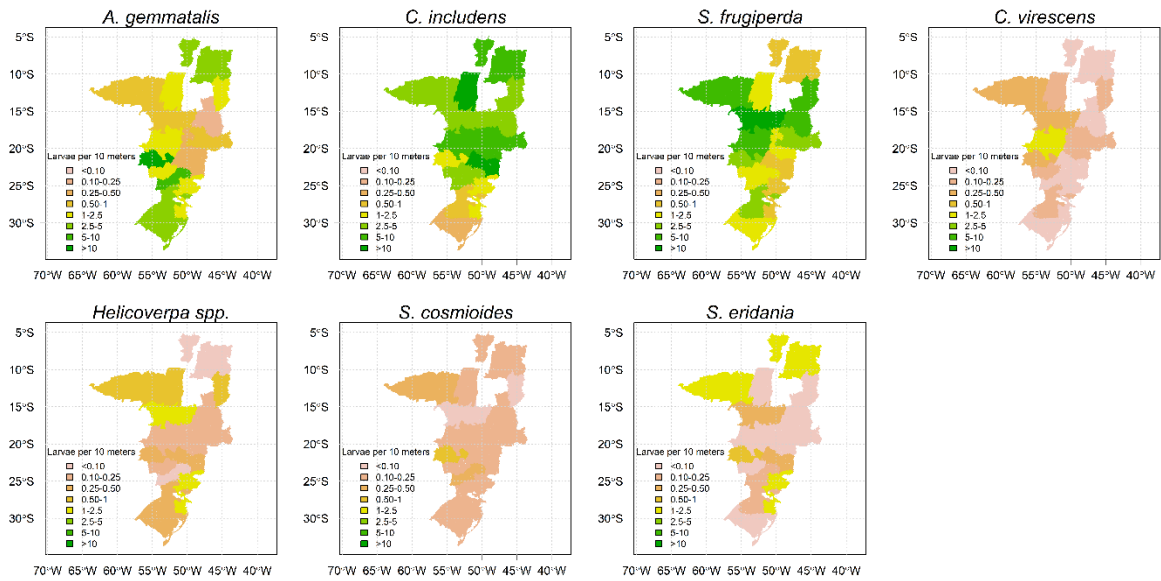


Figure 5. Pest abundance (larvae per 10 meters of beat sheet) in non-Bt soybean by geographic region in 2019 season. Maps were generated using R statistical software - R version 4.0.2 <https://www.R-project.org/>.

2020 Early reproductive stage



2020 Late reproductive stage

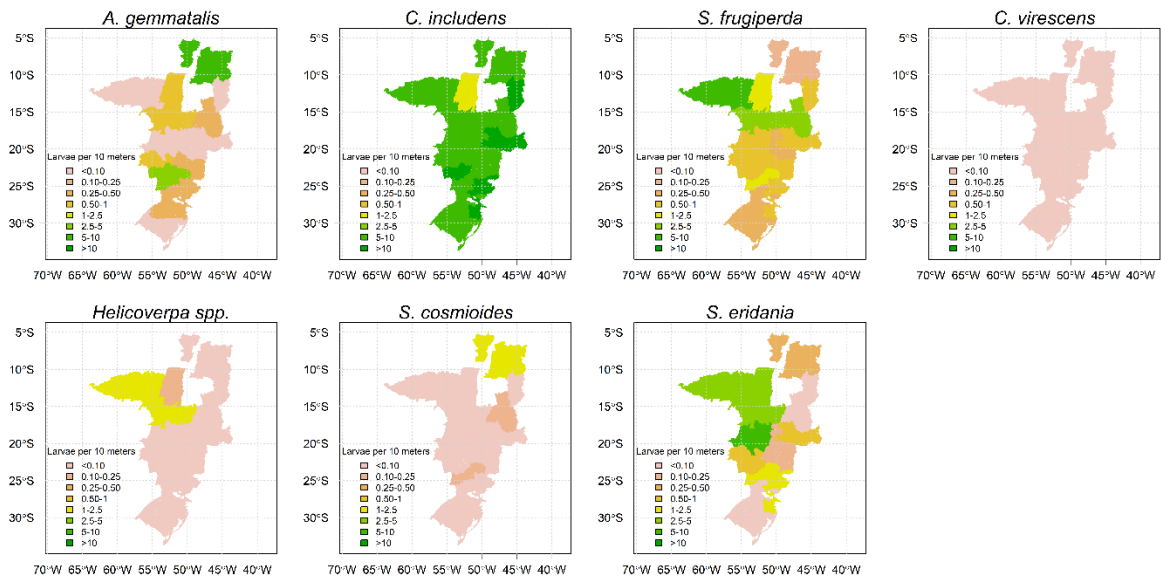


Figure 6. Pest abundance (larvae per 10 meters of beat sheet) in non-*Bt* soybean by geographic region in 2020 season. Maps were generated using R statistical software - R version 4.0.2 <https://www.R-project.org/>.

3.3.5. Insecticide spray usage on soybeans fields in Brazil

The number of sprays for management of lepidopteran larvae over mesoregions decreased from an average of 3.5 in 2012 to 2.45 in 2019 cropping season ($F = 182.5$, $df = 1,354$, $R^2 = 0.34$, $P < 0.0001$) (Fig 7).

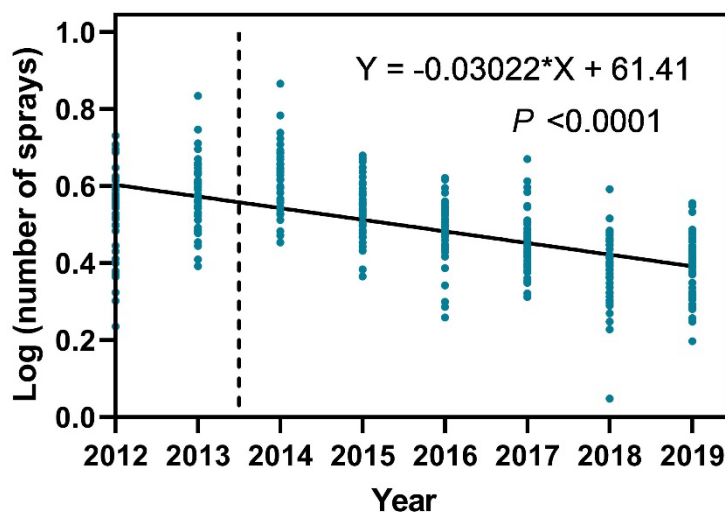


Figure 7. Number of insecticide sprays used to manage lepidopteran larvae across mesoregions and crop seasons in Brazil. Dashed line represents the start of commercial planting of Cry1Ac soybean in Brazil. Data on use of insecticide sprays to manage lepidopteran larvae across mesoregions for the 2013 to 2019 cropping seasons were obtained from the AMIS Kleffmann Group database (2013–2018) and BIP Spark (2019).

3.4. Discussion

This work presents the most extensive geographic assessment of lepidopteran pests on Brazil's soybean fields that covers more than 35 million hectares. *C. includens* was the main lepidopteran species occurring in non-*Bt* soybean (RR) fields in our study, being present in all the regions evaluated. This species was considered a secondary pest of soybean until the early 2000s. Its relevance in soybean likely increased because of changes in cultivation systems (i.e., no-till and cultivation of multiple crop and non-crop hosts of this species) and a decline in the adoption of IPM practices^{7,37}. Both cotton and soybean have been documented as suitable hosts of *C. includens*^{38,39,40,41}. The large increase in soybean cultivation area in Brazil seems to be a particularly important component in *C. includens* adaptation³¹. Soybean farms now are predominant in the agricultural landscape, narrowing the host plant availability to *C. includens* in some regions³¹. Increases in cultivation of other crop and non-crop hosts of *C. includens* also may have created a “green bridge” favoring the growth and spread of populations^{7,31}. This likely increased the selective pressure of insecticides and *Bt* soybean plants, leading to higher resistance risk for these control tactics. *C. includens* prefers to feed on the lower and mid canopies of soybean plants, making it difficult to manage with insecticide sprays in the first place⁴². The resistance of *C. includens* populations to pyrethroids and chitin synthesis inhibitors has further contributed to the increased prevalence of this pest^{43,44}. However, even under this high-resistance-risk scenario, our data showed that Cry1Ac soybean continues to be effective at controlling this pest. The near-high-dose level of the

Cry1Ac *Bt* soybean MON 87701 × MON 89788 against *C. includens*¹² and the low initial resistance allele frequency⁴⁵ in *C. includens* have been key to managing the risk of *Bt* resistance in this pest.

A. gemmatalis was recognized as a major defoliating insect associated with soybean fields in Brazil, requiring an average of 2 insecticide applications every season⁴⁶. Our results showed that the abundance of *A. gemmatalis* was lower than that of *C. includens* before and after the commercial launch of Cry1Ac soybean (2011–2014 and 2019–2020 cropping seasons). In the 2011 to 2014 sampling, *A. gemmatalis* was the second most abundant pest after *C. includens*, confirming that these two were the major pests of soybean in the early part of the decade. *A. gemmatalis* feeds primarily on leguminous plants (at least 34 species within Fabaceae family) and on only three other families (Begoniaceae, Poaceae and Malvaceae), with five species in these families serving as larval hosts^{47,48}. This relatively narrow host range, in combination with the high efficacy of Cry1Ac soybean against *A. gemmatalis*¹², may have contributed to reduced abundance of this species in Brazil. Our analysis showed that *A. gemmatalis* made a significant contribution to defoliation and is widely distributed in non-*Bt* soybean fields, so it is important to monitor for this species. In contrast, the low relative abundance of *A. gemmatalis* in Cry1Ac soybean showed that the pest is being effectively controlled by this technology in Brazil eight years after commercial launch.

Helicoverpa spp. was found at relatively low abundance. Within the *Helicoverpa* species found in Brazil, *H. armigera* are prevalent on dicotyledonous hosts such as soybean and cotton and *H. zea* on maize^{49,50,51,52}. Therefore, most of the *Helicoverpa* spp. larvae in our collections are likely to be *H. armigera*. Although this species was first reported causing damage in soybean in Brazil⁵³, the suitability of cotton as a host plant seems to be higher than soybean, as evidenced by higher larval viability and net reproductive rate^{52,54}. The broad cultivation of row crops (e.g. soybean, cotton and maize) and availability of non-crop hosts across Brazil throughout the year, in combination with the high polyphagia of *H. armigera*, may be shaping the dynamics of this pest^{5,52}. The relatively low abundance of this pest in soybean in Brazil also may be related to the broad adoption of Cry1Ac soybean, which is highly efficacious against this pest^{14,52}.

The prevalence of *S. frugiperda* in non-*Bt* soybean was higher in 2019–2020 than in 2011–2014, increasing from 0.12% to more than 13%. *S. frugiperda* is a major pest of maize and cotton^{30,55,56,57}, but recently has been also reported as a pest of soybean in Brazil^{6,58,59}. The occurrence of *S. frugiperda* on soybean is favored by its ability to develop on several host plants⁶⁰, high dispersal and migratory capacity^{61,62}, high reproductive potential⁶³, adaptation to Brazilian crop systems with availability of suitable hosts throughout the year³⁰, and resistance to several classes of insecticides^{64,65,66,67,68,69}. Although soybean plants produce proteinase inhibitors, *S. frugiperda* can adapt by altering the composition of proteolytic enzymes in the midgut⁷⁰. *S. frugiperda* also expresses detoxification gene families that enable rapid response to plant secondary metabolites⁷¹. The abundance of green plant material provided by winter cover crops such as millet, which are highly suitable for *S. frugiperda*⁵⁷, can also contribute to keeping populations of this pest at reasonably high levels throughout the year, creating a “green bridge” enabling dispersal and/or migration among hosts. Another factor that may be influencing *S. frugiperda* population growth and increasing its occurrence in soybean is the recent increase in winter maize area, where maize is rotated with soybean, and

decrease in summer maize acreage in Brazil⁵. Currently, winter maize is planted on more than 13 million hectares, representing most of the maize planted in Brazil; in contrast, summer maize represented 4.3 million hectares in 2020⁵. The removal of a significant number of maize plants from the landscape during the summer season may have triggered *S. frugiperda* to more frequently explore and colonize other suboptimal but readily available hosts such as soybean. Our analyses indicate that *S. frugiperda* could contribute to defoliation in soybean fields, though its capacity to defoliate soybean is lower than some other *Spodoptera* species.

S. eridania was more abundant than *S. frugiperda* during the late reproductive stages of soybean. *S. eridania* is also a polyphagous pest, reported to be capable of feeding on 202 host plant species⁷². Compared to cotton, soybean is a less suitable host for *S. eridania*, leading to lower pupal survivorship when consumed exclusively⁷³. However, in the soybean–cotton farming system in the Cerrado region of Brazil (in the Central-West of the country), this pest may be of greater importance because it can find a continuous source of food in these two crops⁷³. Sampling of lepidopteran larvae from soybeans at four locations in Mato Grosso do Sul State in 2011/12 showed that *Spodoptera* species represented about 10% of lepidopteran larvae in the samples⁷⁴. Another study at one location in 2015/16 showed that *Spodoptera* accounted for 5% of the total lepidopteran larval sample: among these larvae, more than 50% were *S. eridania*⁷⁵. In addition to feeding on leaves, *Spodoptera* species can feed on soybean pods⁶, which may have contributed to the higher density of *S. eridania* observed at the late reproductive stage in our collections.

S. cosmioides was at lower abundance than the other two *Spodoptera* species mentioned above, but its capacity to defoliate the soybean crop was greater than that of any other species in our collections. Its high capacity to cause damage has been demonstrated under laboratory conditions. For example, *S. cosmioides* was able to defoliate nearly twice the area defoliated by *A. gemmatilis*, *S. eridania* or *S. frugiperda*⁶. *S. cosmioides* is also a polyphagous pest capable of feeding on 126 plant species⁷⁶. Soybean and cotton are conducive to development of this species, but maize does not allow its larval development^{77,78}. Soybean and cotton also are preferred hosts for oviposition of the species when compared to oats, wheat and maize⁷⁸. Therefore, the monitoring of this pest in soybean and cotton fields is important to prevent yield loss due to significant defoliation or pod damage.

The *Spodoptera* species are not controlled by Cry1Ac soybean, so their presence is expected in both Cry1Ac soybean and non-*Bt* soybean fields¹⁵. Larvae of *Spodoptera* species predominated in Cry1Ac soybean fields in our study, and the numbers of these three species were similar between Cry1Ac soybean and non-*Bt* soybean fields. Therefore, any differences in lepidopteran control tactics adopted by growers in Cry1Ac soybean and non-*Bt* soybean fields have not resulted in an increase in density of these *Spodoptera* species on Cry1Ac soybean relative to non-*Bt* soybean fields.

Abundance of both *C. virescens* and *R. nu* was low in our samples from the 2019 and 2020 seasons. Both species are considered pests of soybeans in Brazil³³. Combined, these species represented less than 2.3% and 1.0% of the samples at the early and late reproductive stages in non-*Bt* soybean, respectively. Low abundance of these species was also observed in the 2011–2014 samples. *C. virescens* is a major pest in cotton and used to be observed attacking soybean in the

central region of Brazil⁷⁹. Cry1Ac soybean meets the high-dose concept for *C. virescens*¹³ and continues to provide effective control according to our results. In the USA, this pest is also considered an important pest of cotton, and the high adoption of *Bt* cotton in the USA may have reduced its abundance over large areas⁸⁰. In Brazil, high adoption of both *Bt* cotton and Cry1Ac soybean could also be influencing the abundance of *C. virescens*. *R. nu* occurs predominantly in southern South America, and this pest is an important defoliator of soybean in Argentina⁸¹. *R. nu* is more adapted to subtropical and temperate regions than to tropical regions and is favored by higher latitudes and altitudes⁴¹. However, this pest has been reported from southern (Rio Grande do Sul and Paraná) to central regions of Brazil (Distrito Federal)^{75,82,83,84}.

Despite fluctuations in lepidopteran pest abundance across regions and cropping seasons, *C. includens* and *A. gemmatalis* continue to be the main lepidopteran pests on non-*Bt* soybean in Brazil. The absence or very low density of these two species and reduced levels of defoliation on *Bt* soybean observed across regions and seasons indicate that Cry1Ac soybean still provides effective protection against these species. A significant reduction in the number of insecticide sprays to manage lepidopteran larvae has occurred over mesoregions and crop seasons, indicating that increasing adoption of Cry1Ac soybean has effectively managed and apparently suppressed *C. includens* and *A. gemmatalis* populations across soybean-growing regions. Assessing the environmental impact of this reduction in lepidopteran sprays would be worthwhile. Suppression of target pests after a long period of use of *Bt* technologies has been documented in *P. gossypiella*, *O. nubilalis* and *H. zea* in the USA^{18,19,21} and *H. armigera* in China²⁰. However, *Spodoptera* species are not controlled by Cry1Ac soybean¹⁵, and consequently they can be found on both Cry1Ac and non-*Bt* soybean. The occurrence of *Spodoptera* species, which were historically considered as secondary pests of soybeans in Brazil, on Cry1Ac and non-*Bt* soybeans may be associated with the high efficacy of the Cry1Ac soybean against target species (i.e., *C. includens* and *A. gemmatalis*) and the resulting reduction in insecticide use in soybeans fields in Brazil (Fig. 7). The challenge posed by secondary pests such as *Spodoptera* species highlights the need to develop *Bt* soybean technologies with novel modes of action^{59,85,86}. When available, *Bt* soybean technologies with diverse modes of action will enhance pest management systems for soybean in Brazil.

It is also important to emphasize that the planting of refuge is crucial to the management of *Bt* crop pests. In Brazil, soybean, maize and cotton are planted simultaneously or in succession within the Cerrado landscape³⁰. The proteins used in *Bt* soybean, maize and cotton overlap to a large degree and several of the most important target pests feed on two or all three of these crops e.g., *S. frugiperda*, *H. armigera* and *C. includens*, as discussed herein. Cross-crop resistance is a threat to their management. Indeed, resistance of *S. frugiperda* to *Bt* maize is already affecting the efficacy of *Bt* soybean and cotton due to cross resistance resulting from shared or similar *Bt* proteins among technologies^{59,87}. Therefore, adherence to refuge recommendations for *Bt* maize, cotton and soybean is necessary to enhance durability of current and future *Bt* technologies in this multi-crop agroecosystem.

Overall, our study provides a large-scale assessment of Cry1Ac soybean field efficacy and demonstrates that its pest control benefits are being sustained. Cry1Ac soybean has provided

Brazilian farmers with eight years of consistent protection against damage from the primary lepidopteran soybean pests (*C. includens* and *A. gemmatalis*). However, Cry1Ac soybean needs to be viewed as one tool within the pest management toolbox and should be integrated with other effective control tactics.

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4. GENETIC STRUCTURE AND DEMOGRAPHIC HISTORY OF EMERGING SOYBEAN PESTS *Spodoptera eridania*, *Spodoptera cosmioides* AND *Spodoptera frugiperda*

ABSTRACT

Spodoptera genus is a major problematic and widespread Lepidopteran pest genus worldwide. Understanding the population genetics is an important piece of integrated pest management as lineages or strains might be present in a country of continental proportions. The current study objective was to (i) confirm the larvae identification, using COI marker, and define the genealogic relationship among *Spodoptera* species collected in soybean fields in Brazil; (ii) estimate the genetic diversity and population structure of *Spodoptera eridania*, *Spodoptera cosmioides* and *Spodoptera frugiperda* in different soybean macroregions; and (iii) investigate the population demographic of *S. eridania*, *S. cosmioides* and *S. frugiperda* in different soybean macroregions. A total of 89 *S. eridania*, 32 *S. cosmioides* and 29 *S. frugiperda* were identified and 33, 10 and 14 haplotypes were found for each species, respectively. High genetic diversity was observed for *S. eridania* sampled on soybean in Brazil. The genetic diversity indexes of *S. eridania* was higher than that observed for *S. cosmioides* and *S. frugiperda*. Low genetic structure was observed for the three *Spodoptera* species. The highest Φ_{ST} observed was for *S. cosmioides* (0.058) followed by for *S. eridania* (0.058) and *S. frugiperda* (0.017). Our results evidence that the populations of the three *Spodoptera* species evaluated in this study are in demographic and spatial expansion. The results also suggests that corn strain is the major lineage of *S. frugiperda* occurring in soybean in Brazil. This work increased our understanding of population dynamics of *S. eridania*, *S. cosmioides* and *S. frugiperda*.

Keywords: Phylogeography, *Spodoptera* complex, pest management, ecology

4.1. Introduction

Spodoptera genus is along with *Helicoverpa* genus, a major problematic and widespread Lepidopteran pest genus worldwide (Kergoat et al., 2021). In Brazil, the most known species of this genus is the fall armyworm, *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae), the main corn pest (Blanco et al., 2016; Fatoreto et al., 2017) and the representative that is in spotlight recently with invasion of eastern hemisphere (Georgen et al., 2016, Kalleshwaraswamy et al., 2018; Jing et al., 2019). However, other pest species of this genera: *Spodoptera eridania* (Stoll, 1782) (Lepidoptera: Noctuidae) and *Spodoptera cosmioides* (Walker, 1858) (Lepidoptera: Noctuidae) are also economically important pest in Brazil (Montezano et al., 2014; Specht; Roque-Specht, 2016; Horikoshi et al., 2021). Along with *S. frugiperda*, these three species are commonly referred as *Spodoptera* complex and are the main *Spodoptera* species that attacks soybean in Brazil (Horikoshi et al., 2021). That complex was not considered a major threat to the soybean crop until recently, and we can consider them as emerging pests of soybean crop (Panizzi, Corrêa-Ferreira, 1997; Sosa-Gómez et al., 2014, Horikoshi et al., 2021).

Soybean is the main crop planted in Brazil and one of the most important sources of oil and protein worldwide (USDA, 2022). The area planted with soybean in Brazil was 38.5 million hectares in 2020/21 season, with an estimate production of 135.9 million ton of soybean grain (CONAB, 2022). Brazil is the top producer of soybean and responsible for approximately 23% of global production, followed by United States, China, and Argentina, with 20, 10 and 8%, respectively (USDA, 2022). However, this was quite different decades ago, when soybean was a minor crop in Brazil with approximately 1.3 million hectares planted in 1970 (Cattelan, Dall'Agnol, 2018). In a period of 50 years, several changes were observed in the Brazilian agriculture: no-tillage system cultivation, advance of plantings in Cerrado region, new crop varieties, multi-crop system with at least two cropping season per year and use of genetically modified plants are some examples (Fatoretto et al., 2017; Cattelan, Dall'Agnol, 2018).

Changes in agricultural landscapes may affect the population dynamics. As agricultural practices modify the landscape and usually simplifies the environment structure over large areas, it affects the current distribution, demography, and genetic structure of the populations (Altieri, 1999; Gauffre et al., 2015; Alvarado-Serrano et al., 2019). Understanding the population genetics is an important piece of integrated pest management (IPM) as lineages or strains might be present in a country of continental proportions. *Spodoptera frugiperda* corn and rice strain are an example of the presence of host adapted lineages in American continent (Pashley, Martin, 1987; Nagoshi, Meagher, 2008; Siva-Brandão et al., 2018). Recently, lineages were also found in *Euschistus heros* (Hemiptera: Pentatomidae) in Brazil, with an older and more diverse lineage occurring in the northern regions and a younger and less diverse lineage occurring in the southern region (Soares et al., 2018). The presence of lineages in the landscape might directly affect the response to control tactics, as different susceptibility is observed for insecticides and *Bt* proteins (Ríos-Díez, Saldamando-Benjumea, 2011; Ingber et al., 2018).

Despite the importance to IPM, knowledge on the genetic diversity and structure of agriculture pest populations in Brazil are still incipient. Besides *S. frugiperda*, no information is available for *S. eridania* and *S. cosmioides*. Here we used a mitochondrial COI sequence-based analyzes to investigate the population genetic diversity, population structure, and demographic history of *S. eridania*, *S. cosmioides* and *S. frugiperda* collected on soybean fields in Brazil. Specifically, our objectives are: (i) confirm the larvae identification, using COI marker, and define the genealogic relationship among *Spodoptera* species collected in soybean fields in Brazil; (ii) estimate the genetic diversity and population structure of *S. eridania*, *S. cosmioides* and *S. frugiperda* in different soybean macroregions; and (iii) investigate the population demographic of *S. eridania*, *S. cosmioides* and *S. frugiperda* in different soybean macroregions. The objectives are to have the first insights to start understanding the genetic diversity and population dynamics of these emerging pests of soybean in Brazil.

4.2. Material and Methods

4.2.1. Sample collection and DNA extraction

Spodoptera eridania, *S. cosmioides* and *S. frugiperda* larvae were sampled during 2020 and 2021 from soybean fields in Brazil (Supplementary Figure 1). Larvae were initially sampled in propylene glycol and in laboratory transferred to a 99.9% ethanol and stored in a freezer at -20°C. The larvae were morphologically identified according to Herzog (1980), Sosa-Gómez et al. (2014) and Gilligan and Passoa (2014). After, DNA were extracted using the modified CTAB protocol (Doyle and Doyle, 1987). Each sample was isolated in a 1.5 mL tube and macerated with liquid nitrogen. Then 500 µl of CTAB buffer (100 mM of Tris HCl; 1.4 M of NaCl; 0.02 M of EDTA pH 8.0; 2% CTAB), 2 µl of β-mercaptoethanol and 10 µL of proteinase K (20.0 mg/mL) were added. The samples were homogenized, and the tubes were incubated in a water bath (65 °C) for 3 hours. Subsequently, 2 µl of RNase (20 mg/mL – pure) was added and the tubes were kept in a water bath for another 2 hours. After this period, the tubes were centrifuged for 10 min at 14,000 rpm to form the supernatant, which was transferred to a new tube (1.5 mL). 500 µl of CIA (Chloroform – isoamyl alcohol 24:1) were added to new tube, vortexed, centrifuged at 14,000 rpm for 20 min and the supernatant transferred to a new tube (1.5 ml). The processes of adding CIA, centrifugation, removing the supernatant and passing to a new tube were repeated and then 400 µl ice cold (-20°C) isopropanol was added to each tube. The tubes were gently shaken and kept overnight in the freezer (-20°C). After overnight, tubes were centrifuged at 14,000 rpm for 30 min and then supernatant removed, keeping the pellet (DNA) which was first washed with 500 µL of Ethanol 70% and then with Ethanol 95%. The pellet was placed to dry at room temperature in a hood. Subsequently, the pellet was resuspended in 40 µl of ultrapure water and the DNA was stored in a freezer at -20 °C.

4.2.2. PCR amplification and sequencing

The mitochondrial *cytochrome c oxidase subunit I* (COI) gene fragment was amplified by polymerase chain reaction (PCR) using the specific primers SpoF1 (Forward) (5'-TG TAGAAAATGGAGCAGGAAC-3') and SpoR1 (Reverse) (5'-CTGAATATCGACGAGGTATACC-3') which were designed from COI gene sequence of *S. frugiperda*, *S. eridania* and *S. cosmioides* found on Popset 310617289 (Kergoat et al., 2012) available at NCBI. The PCR reaction were performed in 25 µL total volume: 2 µL of DNA (50 ng/µL); 19.25 µL Mili-Q water; 0.25 µl 10X PCR Buffer Mg²⁺ free (Thermo Fisher Scientific™); 1.25 µl MgCl₂ (50 mM) (Thermo Fisher Scientific™); 0.125 µl dNTP (10 mM) (Sinapse Inc[®]); 1 µl of each primer (5 µM) and 0.125 µl Platinum® Taq DNA Polymerase (5 U µl⁻¹) (Thermo Fisher Scientific™). The program of thermocycler to PCR amplification was 94 °C for 3 min for primary denaturation, then 35 cycles of 94 °C for 30 s, 53°C for 45 s, 72 °C for 2 min, with a final extension at 72 °C for 10 min. To confirm the PCR reaction, amplified fragments were separated in agarose electrophoresis gel stained with SYBR Safe (Life Technologies) and visualized in UV light. Then, the PCR products (amplicon) were purified using 2.0 µl EXO-SAP (Cellco Biotec[®]) to each 5 µl

of amplicon. The purification was made in the thermocycler at 37 °C for 30 min, followed by 80 °C for 15 min. The bidirecional Sanger sequencing was carried out at Laboratório de Biotecnologia Agrícola (CEBTEC) from Universidade de São Paulo (USP), Piracicaba, Brazil.

4.2.3. Data assembly and insect molecular identification

All sequences were manually edited using the software Sequencher v.4.0.1. After editing and aligning the mitochondrial COI sequences, the length of sequence used for the analyses of *S. eridania*, *S. cosmioides* and *S. frugiperda* were 867, 896 and 890 bp, respectively. Each mitochondrial COI sequence were blasted in BOLD Systems (BOLD Systems, <https://www.boldsystems.org>) to molecular identification.

Additionally, to confirm the species identification and the mitochondrial lineages among *Spodoptera* species, a Bayesian phylogenetic tree was estimated using the MrBayes v3.2 software program (Ronquist et al., 2012). Bayesian analysis were performed with 25 million generations. *Helicoverpa armigera* (GenBank Accession number: AB620129), *Elaphria agrtoina* (our data), *Spodoptera exigua* (JB064572) and *Spodoptera litura* (JQ064568) sequences were included as outgroups for the Bayesian analysis. Some sequences of *S. frugiperda* corn and rice strains withdrawn from Nagoshi et al. (2011) were added to compare with our sequences and determine which lineage(s) were found in current study (GenBank Accession numbers: HM136587, HM136588, HM136593, HM136596, HM136599 and HM136601).

4.2.4. Population diversity, demography, and structure

For population diversity analysis, the individuals of *S. eridania*, *S. cosmioides* and *S. frugiperda* were grouped according to the geographical location of sampling in soybean macroregions (MRS1, MRS2, MRS3, MRS4, MRS5) (Kaster, Farias, 2012). Additionally, *S. frugiperda* was also grouped by the host plant that larvae were sampled in the field (Corn and Soybean). The number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π) and average number of nucleotide differences (k) were estimated using DNASp v.6 (Rozas et al., 2017). The genealogical relationship of COI mitochondrial gene fragment was reconstructed by a network of median-joining haplotypes. The haplotype network was generated using PopArt v1.7 software (Leigh, Bryant, 2015).

Analysis of molecular variance (AMOVA) was performed with two and three hierarchical levels. For three hierarchical level analysis, the individuals were grouped as described earlier (soybean macroregion and host). Analysis was performed in Arlequin with parametric bootstrap (2000 replicates) with 5% significance (Excoffier et al., 2005).

The Tajima's D and Fu's Fs neutrality tests were performed in Arlequin v3.1 software (Excoffier et al., 2005). For both tests, significance was determined with 1000 permutations in coalescent simulations. Tests were made for the same individual groups mentioned above. Fu's Fs statistics were significant at 5% when p-value was <0.02. Significant negative values of Tajima's D or

Fu's F_s indicates abundance of low frequency haplotypes and supports the hypothesis of population expansion or purifying selection while significant positive values support the hypothesis of population bottleneck.

To test the hypothesis of population expansion, a mismatch distribution analysis using a spatial expansion model was performed. The sum of square deviation (SSD), raggedness index (r) and associated p-value were calculated in Arlequin (Excoffier et al., 2005). If SSD p-value > 0.05 , the population expansion hypothesis cannot be rejected. Raggedness index p-value > 0.05 indicates a good fit of data to the model.

A Bayesian Skyline Plot was used to reconstruct the demographic history of the species, based on mitochondrial COI sequences in Beast v.1.8.4 software. We used a strict molecular clock model to estimate the substitution rate and coalescent tree priors set to the constant size model. The insect molecular clock corresponds to 3.54% divergence per million years (Papadopoulou et al., 2010). Three independent runs of 100 million generations, sampling every 5000 steps and 20% were discarded as burn-in. Convergence, effective sample size (ESS) and mean with 95% highest posterior density interval (HPD) for divergence times were calculated in TRACER v.1.7.1.

4.3. Results

4.3.1. Molecular identification of *Spodoptera* spp. samples

A total of 153 individuals were identified. Among these, 89 *S. eridania*, 32 *S. cosmioides* and 29 *S. frugiperda* were identified and 33, 10 and 14 haplotypes were found for each species, respectively (Supplementary Tables 1, 2, 3). Individuals of *S. eridania*, *S. cosmioides* and *S. frugiperda* were observed in all regions of sampling, evidencing that they are widely distributed (Supplementary Figs. 1A, 1B and 1C). A single individual of *E. agrotina*, *S. dolichos* and *S. ornithogalli* were found among the samples (Supplementary Table 4). The *S. dolichos* and *S. ornithogalli* were previously misidentified as *S. eridania*.

The tree generated by Bayesian analysis divided the *Spodoptera* species of the current study into clades, confirming the species that was used for analysis (Fig. 1). All *S. frugiperda* haplotypes identified on soybean fields were grouped with the corn strain (Fig. 1).

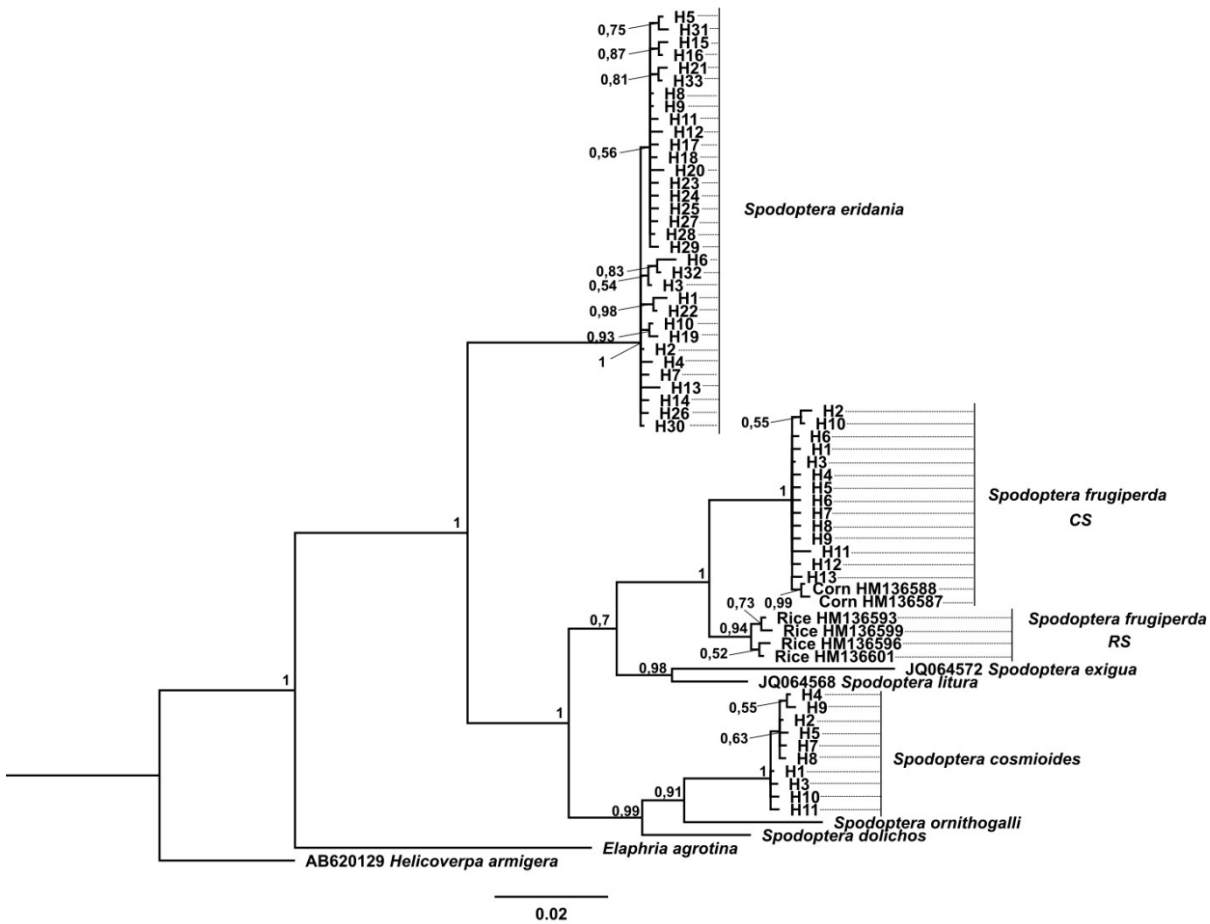


Figure 1. Bayesian phylogenetic tree of *Spodoptera* species sampled in Brazil. The tree is based on mitochondrial COI sequences and includes *Spodoptera litura* and *Spodoptera exigua* sequences. *Helicoverpa armigera*, *Elaphria agrtoina*, *Spodoptera exigua* and *Spodoptera litura* sequences were included as outgroups for the Bayesian analysis. *S. frugiperda* CS and RS means corn strain and rice strain, respectively.

4.3.2. Diversity, structure and demographic analysis

4.3.2.1. *Spodoptera eridania*

An 867bp region of COI from 89 individuals were used for analysis. A total of 33 haplotypes were found and 24 of them (72%) were found in only one individual. The overall haplotype diversity, nucleotide diversity and mean number of nucleotide differences were $H_d = 0.922$, $\pi = 0.00346$ and $k = 3.00255$, respectively. Haplotype diversity, nucleotide diversity and average number of nucleotide differences were similar among macroregions MRS2, MRS3 and MRS4 (H_d ranging from 0.913 to 0.938, π ranging from 0.00345 to 0.00384 and k ranging from 2.9904 to 3.3260). Lower values were observed for MRS1 ($H_d = 0.873$, $\pi = 0.00243$ and $k = 2.1090$) and MRS5 ($H_d = 0.722$, $\pi = 0.00199$ and $k = 1.7222$) (Table 1).

Haplotype network analysis did not show predominant haplogroups for *S. eridania* (Fig. 2). The most frequent haplotype among the samples were H2 and H8, with 17 (19.1%) and 12 (13.5%) haplotypes, respectively. The major haplotypes were widely distributed across macroregions (Fig. 2).

4.3.2.2. *Spodoptera cosmioides*

An 896bp region of COI from 32 individuals were used for analysis. A total of 10 haplotypes were found and 6 of them (60%) were found in only one individual. The overall haplotype diversity, nucleotide diversity and mean number of nucleotide differences were $H_d = 0.798$, $\pi = 0.00153$ and $k = 1.3689$, respectively. Haplotype diversity, nucleotide diversity and average number of nucleotide differences were similar among macroregions MRS2, MRS3 and MRS4 (H_d ranging from 0.810 to 0.952, π ranging from 0.00138 to 0.00223 and k ranging from 1.2381 to 2.0000) (Table 1). Lower values were observed in MRS1 ($H_d = 0.536$, $\pi = 0.00060$ and $k = 0.5357$) and no individuals of *S. cosmioides* from MRS5 were used in current analysis (Table 1).

The network analysis showed two haplotypes that were more frequent: H04 (12) and H02 (8), representing 37.5 and 25% from the total, respectively (Figure 3). The major haplotypes were widely distributed across macroregions (Fig. 3).

Table 1. Sample size, haplotype number and genetic diversity of *Spodoptera* spp. samples according to the groups established.

Geographic region group	Sample size (N)	Haplotype number (H)	Haplotype diversity (Hd)	Nucleotide diversity (π)	Average number of nucleotide differences (k)
<i>S. eridania</i>					
Pooled	89	33	0.922	0.00346	3.0025
MRS1	11	7	0.873	0.00243	2.1090
MRS2	21	14	0.938	0.00345	2.9904
MRS3	24	14	0.928	0.00384	3.3260
MRS4	24	13	0.913	0.00354	3.0724
MRS5	9	5	0.722	0.00199	1.7222
<i>S. cosmioides</i>					
Pooled	32	10	0.798	0.00153	1.3689
MRS1	8	2	0.536	0.00060	0.5357
MRS2	10	6	0.889	0.00166	1.4888
MRS3	7	4	0.810	0.00138	1.2381
MRS4	7	6	0.952	0.00223	2.0000
MRS5	-	-	-	-	-
<i>S. frugiperda</i>					
Pooled	29	14	0.813	0.00157	1.3940
Corn	13	7	0.833	0.00164	1.4615
Soybean	16	9	0.817	0.00153	1.3583
MRS1	2	2	1.000	0.00112	1.0000
MRS2	11	5	0.764	0.00151	1.3454
MRS3	7	4	0.810	0.00118	1.0476
MRS4	7	5	0.857	0.00193	1.7142
MRS5	2	2	1.000	0.00225	2.0000

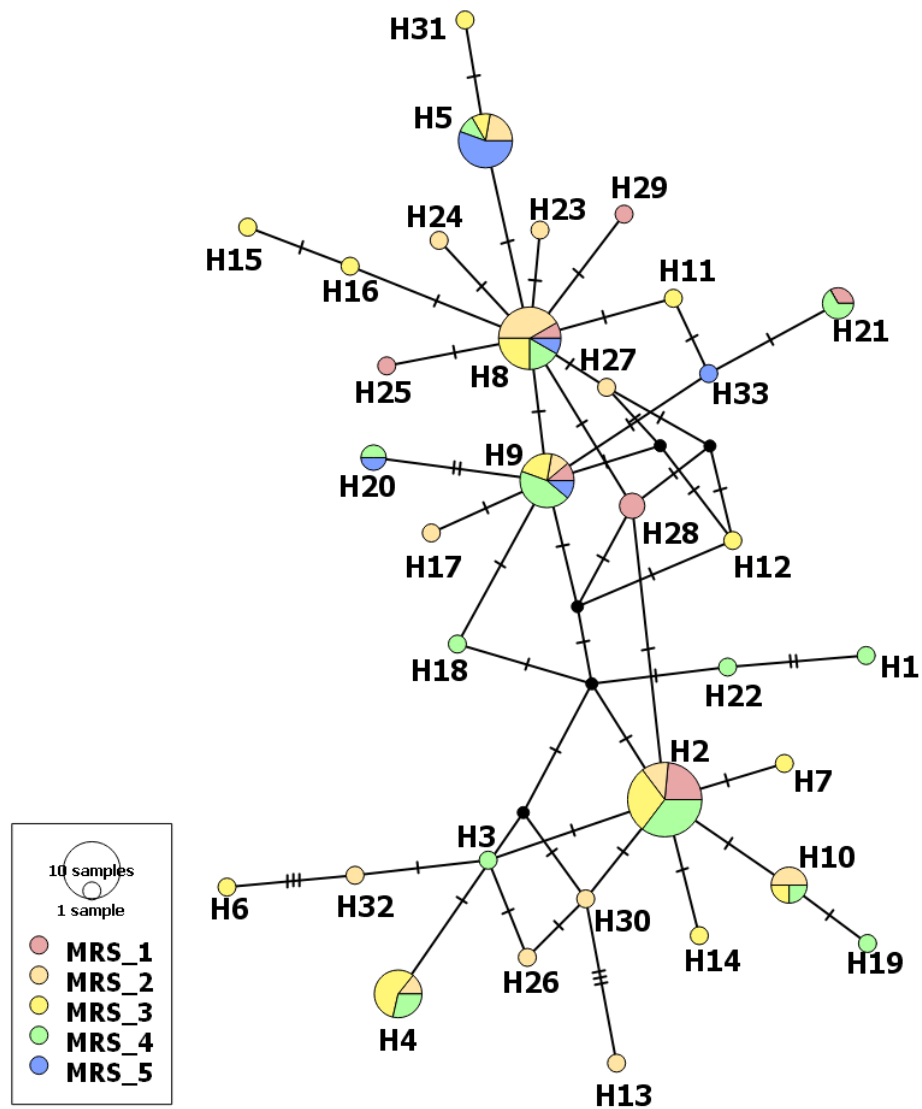


Figure 2. Haplotype network of 89 mitochondrial COI sequences of *S. eridania* sampled in Brazil. Haplotype circle size represents sample size and dark circles represents missing haplotype. Colors indicates the macroregion where individuals were sampled. Tick marks reflects the number of mutation steps.

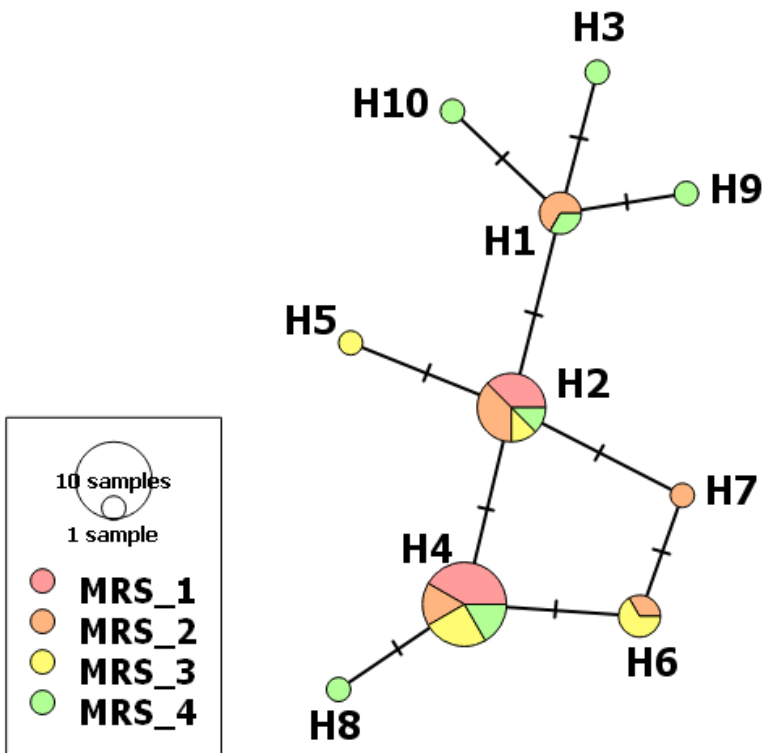


Figure 3. Haplotype network of 32 mitochondrial COI sequences of *S. cosmioides* sampled in Brazil. Haplotype circle size represents sample size. Colors indicates the macroregion where individuals were sampled. Tick marks reflects the number of mutation steps.

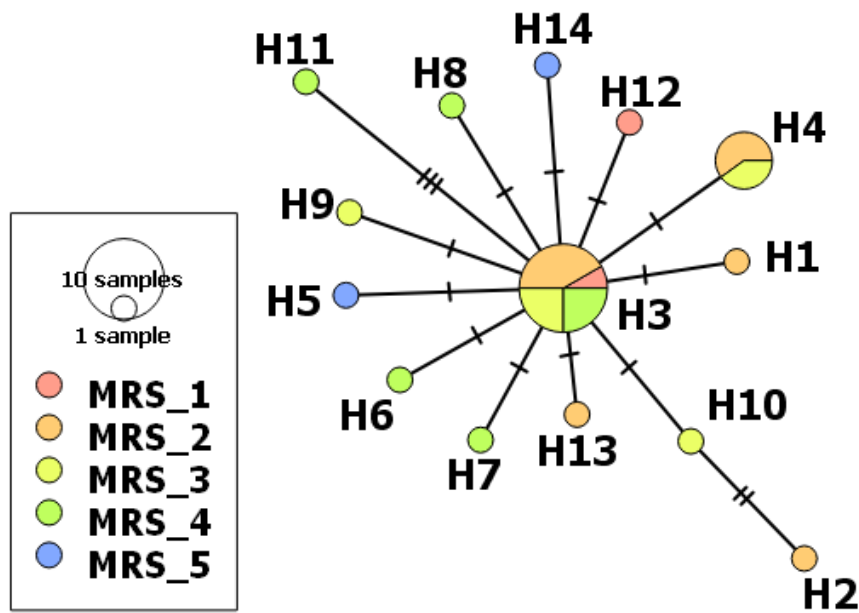


Figure 4. Haplotype network of 29 mitochondrial COI sequences of *S. frugiperda* sampled in Brazil. Haplotype circle size represents sample size. Colors indicates the macroregion where individuals were sampled. Tick marks reflects the number of mutation steps.

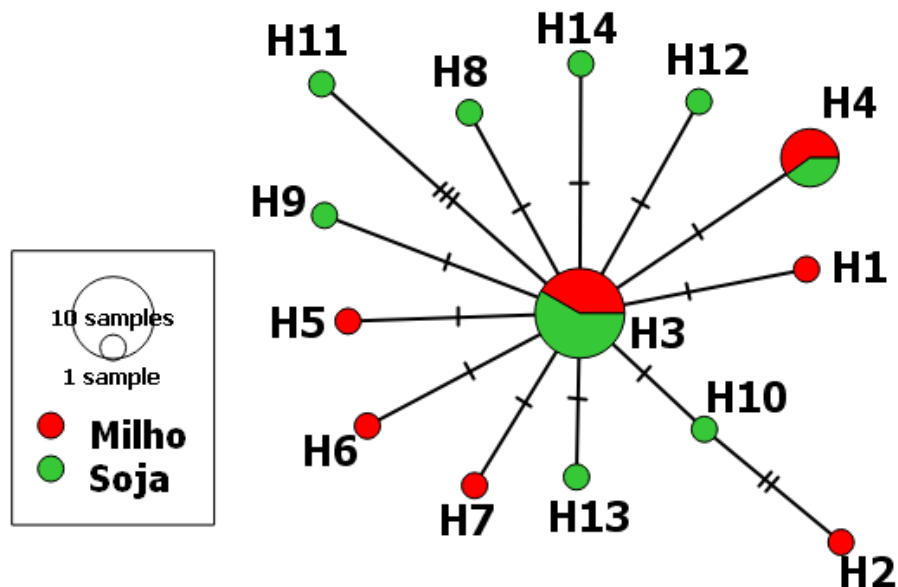


Figure 5. Haplotype network of 29 mitochondrial COI sequences of *S. frugiperda* sampled in Brazil. Haplotype circle size represents sample size. Colors indicates the host plant that individuals were sampled. Tick marks reflects the number of mutation steps.

4.3.2.3. *Spodoptera frugiperda*

An 890bp region of COI from 29 individuals were used for analysis. A total of 14 haplotypes were found and 12 of them (85%) were found in only one individual. The overall haplotype diversity, nucleotide diversity and mean number of nucleotide differences were $H_d = 0.813$, $\pi = 0.00157$ and $k = 1.3940$, respectively. Similar haplotype diversity, nucleotide diversity and average number of nucleotide differences to the overall analysis were observed when individuals were separated by host (sampled crop) (Table 1). Haplotype diversity, nucleotide diversity and average number of nucleotide differences showed higher variation among macroregions (H_d ranging from 0.764 to 1.000, π ranging from 0.00112 to 0.00225 and k ranging from 1.0000 to 2.0000) (Table 1).

The haplotype network analysis resulted in a central single predominant haplotype surrounded by low frequency haplotypes (Figs. 4 and 5). The H3 haplotype were observed in 12 out of 29 samples (41.3%). The major central haplotype was widely distributed across macroregions and host plant (Figs. 4 and 5).

4.3.3. Population structure

4.3.3.1. *Spodoptera eridania*

The analysis of molecular variance (AMOVA) with two hierarchical level showed that majority of variation was accounted for differences within populations (94.8% percentage variation), $\Phi_{ST} = 0.051$ ($p = 0.0075$) (Table 2). The AMOVA with three hierarchical levels to test the hypothesis that population was structured by macroregions was not significant to any of tested group $\Phi_{CT} = 0.047$ ($p = 0.0996$) (Table 2).

Table 2. Analysis of molecular variance (AMOVA) based on mitochondrial COI sequences for *S. eridania*, *S. cosmioides* and *S. frugiperda* sampled in Brazil.

Source of variation	d.f.	Variance components	Percentage variance	Fixation indices (p -value)
<i>S. eridania</i>				
Among populations	4	0.0789 Va	5.2	$\Phi_{ST} = 0.051$ (0.0075)
Within populations	84	1.4395 Vb	94.8	
Total	88	1.5184		
Among groups	4	0.0724 Va	4.77	$\Phi_{CT} = 0.047$ (0.0996)
Among populations within groups	10	0.0152 Vb	1.01	$\Phi_{SC} = 0.010$ (0.3176)
Within populations	74	1.4306 Vc	94.22	$\Phi_{ST} = 0.057$ (0.0583)
Total	88			
<i>S. cosmioides</i>				
Among populations	3	0.0407 Va	5.87	$\Phi_{ST} = 0.058$ (0.1319)
Within populations	28	0.6531 Vb	94.13	
Total	31	0.6939		
Among groups	3	-0.0461 Va	-6.65	$\Phi_{CT} = -0.066$ (0.6263)
Among populations within groups	6	0.1716 Vb	24.73	$\Phi_{SC} = 0.231$ (0.0593)
Within populations	22	0.5687 Vc	81.93	$\Phi_{ST} = 0.180$ (0.0327)
Total	31	0.6941		
<i>S. frugiperda</i> (MRS)				
Among populations	4	0.0119 Va	1.70	$\Phi_{ST} = 0.017$ (0.3363)
Within populations	24	0.06880 Vb	98.30	
Total	28	0.6999		
Among groups	4	0.0172 Va	2.46	$\Phi_{CT} = 0.024$ (0.2424)
Among populations within groups	5	-0.0119 Vb	-1.70	$\Phi_{SC} = -0.017$ (0.3805)
Within populations	19	0.6947 Vc	99.25	$\Phi_{ST} = 0.007$ (0.444)
Total	28	0.7000		
<i>S. frugiperda</i> (Host)				
Among populations	1	-0.0098 Va	-1.43	$\Phi_{ST} = -0.014$ (0.7019)
Within populations	27	0.7021 Vb	101.43	
Total	28	0.6922		
Among groups	1	-0.0145 Va	-2.10	$\Phi_{CT} = -0.020$ (0.6867)
Among populations within groups	14	0.0478 Vb	6.91	$\Phi_{SC} = 0.067$ (0.3202)
Within populations	13	0.6589 Vc	95.18	$\Phi_{ST} = 0.048$ (0.3244)
Total	28	0.6923		

4.3.3.2. *Spodoptera cosmioides*

The AMOVA with two hierarchical level showed that 94.1% of variation was observed within population ($\Phi_{ST} = 0.058$, $p = 0.1319$) (Table 2). The three hierarchical level divided the population by macroregions was not significant ($\Phi_{CT} = -0.066$, $p = 0.6263$) (Table 2).

4.3.3.3. *Spodoptera frugiperda*

The AMOVA for both macroregion and host groups for *S. frugiperda* do not indicate genetic structure, value higher than 98% of variance were observed within populations. When, we tested the genetic structure by soybean macroregions ($\Phi_{CT} = 0.024$ $p = 0.2424$) and host, corn and soybean ($\Phi_{ST} = 0.014$, $p = 0.7019$), non-significant results were found (Table 2).

4.3.4. Demographic statistics

4.3.4.1. *Spodoptera eridania*

Negative values were observed in both Tajimas' D and Fu's F_s neutrality tests, although significances observed only in Fu's F_s test (Table 3), indicating population expansion or purifying selection. Only the MRS2 and MRS5 were not significative for Fu's F_s test ($p > 0.02$) (Table 3). The non-significant values of SSD and r (raggedness) ($p > 0.49$) supports the hypothesis of spatial expansion of *S. eridania* populations in all groups analyzed (Table 3). The Extended Bayesian Skyline Plot (BSP) analysis showed demographic equilibrium with an expansion approximately 400 years ago (Fig. 6A).

4.3.4.2. *Spodoptera cosmioides*

Pooled analysis indicates that *S. cosmioides* population are in expansion of purifying selection considering the Fu's F_s test ($p = 0.002$) (Table 3). MRS2 and MRS4 were also significant for macroregion analysis on Fu's F_s test ($p = 0.007$) (Table 3). Tajima's D test did not showed significance for any of groups analyzed (Table 3).

Non-significant values of SSD and r (raggedness) ($p > 0.49$) supports the hypothesis of spatial expansion of *S. cosmioides* (Table 3). The Extended Bayesian Skyline Plot (BSP) analysis showed demographic equilibrium with an expansion approximately 100 years ago (Fig. 6B).

4.3.4.3. *Spodoptera frugiperda*

Significant negative values were observed in pooled analysis with both Tajima's D and Fu's Fs neutrality tests (Table 3). When individuals were split in host plant, significant negative values were also observed in both Tajima's D and Fu's Fs test (Table 3), indicating population expansion of purifying selection. For macroregion analyses, no significantly negative values were observed for Fu's Fs test ($p > 0.02$) (Table 3).

The values of SSD and r (raggedness) were non-significant ($p > 0.17$), supporting the hypothesis of spatial expansion of *S. frugiperda* (Table 3). The Extended Bayesian Skyline Plot (BSP) analysis showed demographic equilibrium with an expansion approximately 400 years ago (Fig. 6C).

Table 3. Neutrality test statistics and mismatch distribution analysis based on mitochondrial COI sequences for *S. eridania*, *S. cosmioides* and *S. frugiperda* sampled in Brazil

Geographic region group	Sample size (N)	Tajima's D test (p value)	Fu's Fs test (p value)	SSD (p -value)	r (p -value)
<i>S. eridania</i>					
Pooled	89	-1.333 (0.065)	-24.943 (<0.001)	0.0006 (0.879)	0.0201 (0.791)
MRS1	11	-0.476 (0.343)	-2.706 (0.024)	0.0036 (0.852)	0.0446 (0.829)
MRS2	21	-1.031 (0.165)	-8.038 (<0.001)	0.0025 (0.730)	0.0239 (0.817)
MRS3	24	-0.796 (0.231)	-6.156 (0.001)	0.0068 (0.494)	0.0349 (0.567)
MRS4	24	-0.830 (0.220)	-5.297 (0.005)	0.0039 (0.687)	0.0329 (0.657)
MRS5	9	-0.270 (0.410)	-1.185 (0.115)	0.0025 (0.947)	0.0293 (0.999)
<i>S. cosmioides</i>					
Pooled	32	-0.930 (0.181)	-4.869 (0.002)	0.0050 (0.284)	0.0764 (0.310)
MRS1	8	1.166 (0.924)	0.866 (0.579)	-	-
MRS2	10	0.203 (0.649)	-2.647 (0.013)	-	-
MRS3	7	0.050 (0.573)	-1.058 (0.091)	-	-
MRS4	7	-0.099 (0.471)	-3.273 (0.007)	-	-
MRS5	-	-	-	-	-
<i>S. frugiperda</i>					
Pooled	29	-2.249 (0.002)	-11.825 (<0.001)	0.0083 (0.200)	0.1024 (0.174)
Corn	13	-1.677 (0.036)	-3.334 (0.006)	-	-
Soybean	16	-2.048 (0.006)	-6.050 (<0.001)	-	-
MRS1	2	0.000 (1.000)	0.000 (0.240)	-	-
MRS2	11	-1.358 (0.095)	-1.329 (0.100)	-	-
MRS3	7	-0.654 (0.322)	-1.389 (0.046)	-	-
MRS4	7	-1.524 (0.035)	-1.889 (0.039)	-	-
MRS5	2	0.000 (1.000)	0.693 (0.358)	-	-

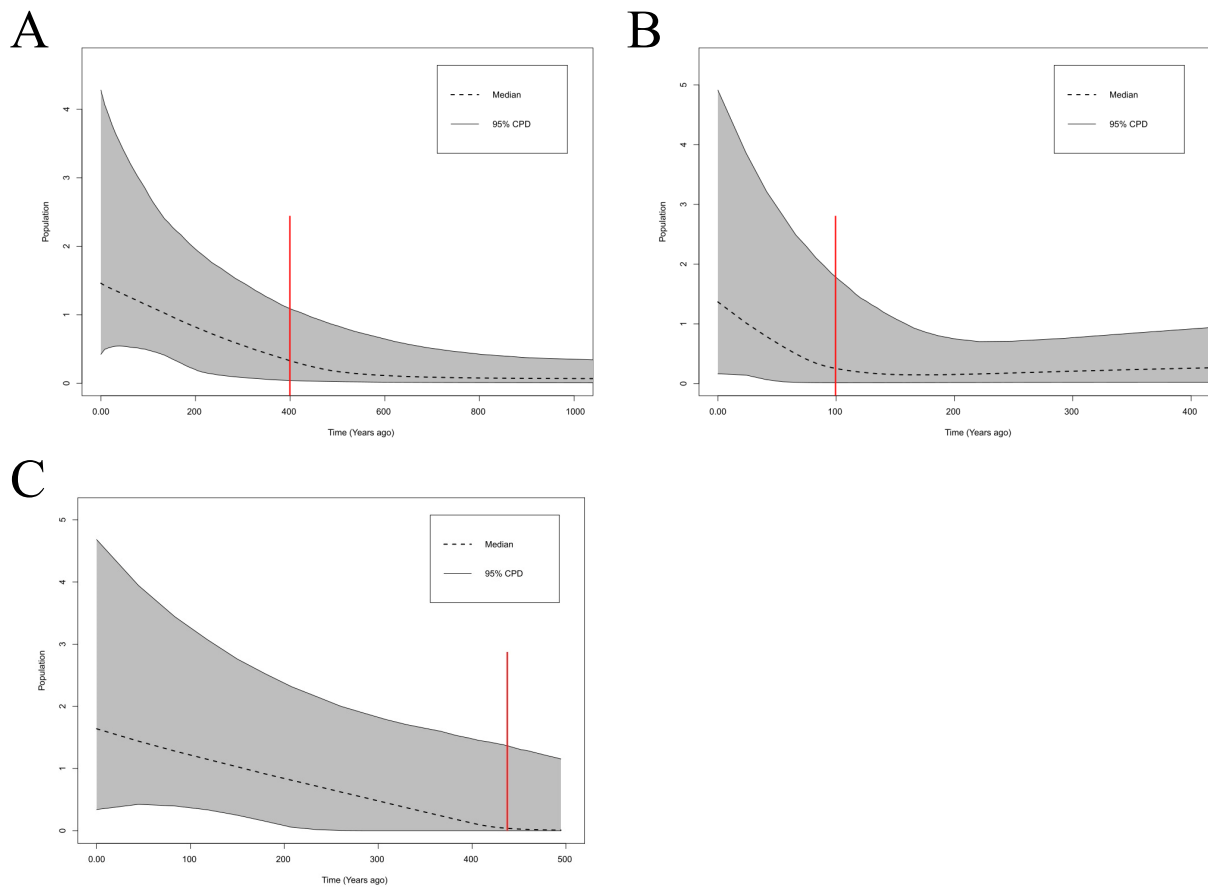


Figure 6. Bayesian skyline plot (BSP) for the populations of (A) *S. eridania*; (B) *S. cosmioides*, (C) *S. frugiperda*. The figures represent the effective population size as a function of time. Dashed lines represent median BSP estimate and gray are the 95% posterior density limits.

4.4. Discussion

The molecular tools successfully confirmed the identification of *Spodoptera* larvae to the species level. The Bayesian phylogenetic tree confirmed that the different haplotypes we used in analysis belongs to the correct *Spodoptera* species. Interesting to note that a single *S. ornithogalli* and *S. dolichos* were identified when the COI sequence were blasted in BOLD. These 2 species were previously misidentified as *S. eridania*. For DNA extraction, we try to select smaller larvae whenever possible due to lower amount of fat to obtain better DNA quality. As the first identification tier was based on morphology, small larvae could be subject to misidentification. These two species are native to the Western hemisphere and only *S. ornithogalli* is classified as a pest species (Kergoat et al., 2021). Although, there are some reports of *S. dolichos* occurrence on cultivated crops (Montezano et al., 2016). To date, both *S. ornithogalli* and *S. dolichos* are not listed as pest species of economic importance in soybean (Hoffmann-Campo et al., 2000; Moscardi et al., 2012; Sosa-Gómez et al., 2014).

High genetic diversity was observed for *S. eridania* sampled on soybean in Brazil. The genetic diversity indexes of *S. eridania* was higher than that observed for *S. cosmioides* and *S.*

frugiperda. Similar genetic diversity was found in *S. frugiperda* by Arias et al. (2019) in individuals collected on corn crops in Paraguay and Brazil. When we compare diversity to other Noctuidae, *Chrysodeixis includens*, a main pest of soybean, *S. eridania*, *S. cosmioides* and *S. frugiperda* showed higher diversity (Silva et al., 2020), however when we compared with *H. zea*, the *Spodoptera* species showed a lower diversity (Leite et al., 2014).

The three species of *Spodoptera* of current study are native to the western hemisphere (Kergoat et al., 2021). The *Spodoptera* genus origin dates from 17-18 million years ago, while the origin of *S. eridania*, *S. cosmioides* and *S. frugiperda* are estimated to be 11.04, 0.56 and 10.61 million years ago (Kergoat et al., 2021). Although *S. eridania* seems to be as old as *S. frugiperda*, *S. eridania* did not present a clear differentiation of lineages or strains as observed for *S. frugiperda* (corn and rice strains) (Pashley; Martin, 1987; Dumas et al., 2015; Silva-Brandão et al., 2018). Although, we would need additional samples of *S. eridania* from host plants other than soybean to confirm whether host speciation could be present. The diversity indexes of *S. cosmioides* were close to the observed for *S. frugiperda*. *S. cosmioides* are one of the youngest species within *Spodoptera* genus, being dated around 0.56 million years ago (Kergoat et al., 2021). The divergence among the two host strains of *S. frugiperda* is dated around 2.5 million years ago (Kergoat et al., 2021). As we analyzed only corn strains in our study, it seems to be reasonable that diversity indexes are similar among these two *Spodoptera* species, considering the proximity among divergence time.

Low genetic structure was observed for the three *Spodoptera* species. The highest Φ_{ST} observed was for *S. cosmioides* (0.058) followed by for *S. eridania* (0.058) and *S. frugiperda* (0.017). Generally, values of Φ_{ST} at 0-0.05 are indicative of little genetic differentiation, while 0.05-0.25 moderate and >0.25 are considered high (Freeland, 2011). According to that, based on our samples, the genetic structure observed for the three species are low. The AMOVA result showed that the large source of variation is found within the population for *S. eridania*, *S. cosmioides* and *S. frugiperda*. This is evident when we observe the haplotypes being widely distributed across all macroregions in haplotype network. The lack of genetic structure could lead to a panmixia hypothesis but given the large distance of collections across the country this is still initial insights as mitochondrial markers might fail in detect genetic structure and a fine scale gene flow among populations (Wirth, Bernatchez, 2001). Additional studies are needed to confirm this hypothesis, but within Noctuidae family, *Helicoverpa zea* is an example of crop pest that apparently are in panmixia due to low genetic differentiation among populations (Leite et al., 2017; Cordeiro et al., 2020).

Our results evidence that the populations of the three *Spodoptera* species evaluated in this study are in demographic and spatial expansion. In a recent history of Brazil, soybean was a minor crop in 1960's and became the main crop planted in Brazil nowadays (Cattelan, Dall'Agnol, 2018). This large expansion of planting, from 1.3 Mha in 1970 to > 38 Mha in 2021 was greatly associated with the expansion of soybean cultivation towards the Cerrado, in the central region of Brazil (Cattelan, Dall'Agnol, 2018, CONAB, 2021). In this path, changes in insect dynamics were expected as new environments had started to be occupied. Along with agricultural area expansion, changes in the production system were also observed, with new technologies such as: no-tillage systems, use of selective insecticides and GM crops (Panizzi, Corrêa-Ferreira, 1997). These changes eventually might

lead to different levels of insect adaptation, main pests might become less abundant while other secondary pests increase in importance (Panizzi, Corrêa-Ferreira, 1997; Horikoshi et al., 2021). As effective population size is increasing to the three species of *Spodoptera*, we can expect rapid adaptation of these pests to changes in environment. We hypothesize that the three *Spodoptera* species in the current study are in demographic and spatial expansion driven by the agriculture expansion. Similar outcomes were observed for *Chrysodeixis includens*, with soybean and cotton within the agriculture landscape are influencing the genetic diversity in this species (Silva et al., 2020).

The results of our study also suggests that corn strain is the major lineage of *S. frugiperda* occurring in soybean in Brazil. All samples that we sequenced in current study was associated with corn strain. Although rice strain can be found in corn plants, its frequency is low when compared to corn strain in Brazil (Arias et al., 2019). This implies that if we increase our sample size, we could eventually find some rice strain, but essentially the haplotypes that we found in our study are shared among host plants. This is an indicative that *S. frugiperda* populations that feed on corn are the same that feed on soybean in Brazil. The occurrence of *S. frugiperda* in soybean was rare or nor even reported in a recent past, but it increased in number in 2018-2019 (Panizzi, Corrêa-Ferreira, 1997, Horikoshi et al., 2021). As hypothesized in Horikoshi et al., 2021, the increase soybean area in summer with subsequent reduction of summer corn and increase of winter corn might be contributing to this host adaptation of *S. frugiperda*.

This study increased our understanding of population dynamics of *S. eridania*, *S. cosmioides* and *S. frugiperda*. These three species are increasing in importance in soybean crop in Brazil (Horikoshi et al., 2021). Further studies using more advanced molecular techniques such as genotyping by sequencing (GBS) or whole genome sequence (WGS) are needed to deeply explore the relationship of populations in Brazil. Generating knowledge of how these populations were distributed across the country is fundamental to have refined recommendations of pest management. Fundamentally, our data suggested a population expansion is occurring along the agriculture expansion and this probably will continue to evolve and shaping the population dynamics from now onward.

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Supplementary Table 1. *Spodoptera eridania* sample location and haplotypes.

City	State	Host	MRS	Code	mtDNA haplotype	Latitude	Longitude
Jaborandi	BA	Soybean	4	21BA13B1	H03	-14,08928735	-45,81742566
Barreiras	BA	Soybean	4	19BA311	H04	-11,43703098	-46,15823518
Barreiras	BA	Soybean	4	21BA01B1	H01	-12,14228238	-45,45319919
Correntina	BA	Soybean	4	21BA10B1	H02	-13,50195891	-45,68113008
Correntina	BA	Soybean	4	21BA10B2	H02	-13,47748593	-46,68931545
Chapadão do Céu	GO	Soybean	3	20GO01B1	H05	-18,42793200	-52,72902100
Montividiu	GO	Soybean	4	20GO12B1	H02	-17,23466200	-51,19536000
Montividiu	GO	Soybean	3	20GO13B1	H10	-17,19326300	-50,82623400
Formosa	GO	Soybean	3	21GO18B1	H02	-15,23099120	-47,38756165
Chapadão do Céu	GO	Soybean	3	20GO02B2	H04	-18,44684400	-52,90600000
Cristalina	GO	Soybean	3	21GO04B1	H04	-16,83263272	-47,54034147
Jataí	GO	Soybean	3	20GO05B1	H07	-17,56878900	-51,71945600
Jataí	GO	Soybean	3	20GO05B2	H06	-17,55620700	-51,71982500
Jataí	GO	Soybean	3	20GO06B1	H08	-17,56196000	-51,52339500
Santa Helena de Goiás	GO	Soybean	3	20GO08B1	H02	-17,94752500	-50,42491000
Rio Verde	GO	Soybean	3	20GO10B2	H02	-17,80648100	-50,76199300
Montividiu	GO	Soybean	4	20GO11B2	H09	-17,50422000	-51,13209700
Montividiu	GO	Soybean	3	20GO13B2	H08	-17,20123000	-50,83214300
Balsas	MA	Soybean	5	21MA01B1	H09	-7,23301046	-45,97516243
Tasso Fragoso	MA	Soybean	5	21MA03B1	H08	-8,04406771	-45,98628802
Uberaba	MG	Soybean	3	21MG01B1	H11	-19,74747377	-47,76005923
Araguari	MG	Soybean	3	20MG02B1	H12	-18,83209600	-47,99156300
Uberlândia	MG	Soybean	3	20MG06B1	H09	-19,07684000	-48,19228500
Araguari	MG	Soybean	3	20MG07A2	H08	-18,61856900	-48,24327400
Tupaciguara	MG	Soybean	3	21MG09A1	H02	-18,58865433	-48,67376327
Rio Brilhante	MS	Soybean	2	20MS07B2	H09	-21,60112200	-55,00265800
São Gabriel do Oeste	MS	Soybean	3	20MS12B2	H14	-19,42448500	-54,59617400
Chapadão do Sul	MS	Soybean	3	20MS14B1	H16	-18,83180300	-52,67229600
Rio Brilhante	MS	Soybean	2	20MS01B2	H13	-21,90830800	-54,22257800
Campo Grande	MS	Soybean	2	21MS05B1	H08	-20,46219300	-54,84470000
Rio Brilhante	MS	Soybean	2	21MS11B1	H08	-21,86619200	-54,30676900
Chapadão do Sul	MS	Soybean	3	20MS13B2	H15	-18,78416200	-52,51943300
Maracaju	MS	Soybean	2	21MS15A1	H17	-21,74472700	-55,18037300
Chapadão do Sul	MS	Soybean	3	20MS16B1	H04	-18,71096900	-52,90383400

Supplementary Table 1 (continuing). *Spodoptera eridania* sample location and haplotypes.

City	State	Host	MRS	Code	mtDNA haplotype	Latitude	Longitude
Querência	MT	Soybean	4	20MT1131	H02	-12,54647450	-52,53109210
Querência	MT	Soybean	4	20MT1151	H09	-12,44572650	-52,61332590
Sorriso	MT	Soybean	4	20MT801	H08	-13,00042790	-55,87823740
Sorriso	MT	Soybean	4	20MT821	H18	-12,61058650	-55,64540230
Campo Novo do Parecis	MT	Soybean	4	20MT911	H08	-13,66460920	-57,87561610
Campo Novo do Parecis	MT	Soybean	4	20MT92A1	H19	-13,54847600	-57,87146640
Sapezal	MT	Soybean	4	21MT10A1	H21	-13,40343878	-56,63544874
Canarana	MT	Soybean	4	20MT1122	H20	-13,55185860	-51,93501840
Rondonópolis	MT	Soybean	4	21MT19B1	H02	-16,74716700	-54,60414500
Itiquira	MT	Soybean	4	21MT20B1	H10	-17,07760800	-54,23017700
Diamantino	MT	Soybean	4	21MT22B1	H05	-14,00412300	-56,58404300
Canarana	MT	Soybean	4	21MT31B1	H22	-13,47894900	-52,23597600
Canarana	MT	Soybean	4	21MT32B1	H09	-13,52824000	-52,24696700
Tangará da Serra	MT	Soybean	4	21MT41A1	H21	-14,54329000	-57,49523700
Campo Novo do Parecis	MT	Soybean	4	20MT58A1	H02	-13,66483070	-57,87559130
Campo Novo do Parecis	MT	Soybean	4	20MT912	H09	-13,66470950	-57,87704440
Nova Mutum	MT	Soybean	4	20MT971	H04	-13,83695710	-58,02805360
Tibaji	PR	Soybean	1	21PR06A2	H08	-24,75864000	-50,47768700
Cafelândia	PR	Soybean	2	20PR08B1	H26	-24,65824280	-53,35864140
Terra Roxa	PR	Soybean	2	20PR13B1	H02	-24,21105149	-53,89513277
Piraí do Sul	PR	Soybean	1	21PR03A2	H25	-24,47219200	-49,85135900
Palotina	PR	Soybean	2	21PR12B2	H27	-24,27068500	-53,86008300
Palotina	PR	Soybean	2	21PR12B1	H02	-24,26732500	-53,86340100
Peabiru	PR	Soybean	2	21PR18B2	H23	-23,86478800	-52,31511000
Goioerê	PR	Soybean	2	21PR20A2	H24	-24,22461900	-53,05005000
Paraíso do Norte	PR	Soybean	2	20PR21B2	H08	-23,26586300	-52,67952300
Jussara	PR	Soybean	2	21PR21B1	H10	-23,61361900	-52,43433564
Londrina	PR	Soybean	2	20PR31B2	H10	-23,60086065	-51,15001655
Passo Fundo	RS	Soybean	1	20RS02A1	H28	-28,29128841	-52,33364786
Sananduva	RS	Soybean	1	20RS04A1	H28	-27,93135038	-51,79828011
Sananduva	RS	Soybean	1	20RS05A1	H02	-27,99343928	-51,81539755
Sananduva	RS	Soybean	1	20RS06A1	H02	-27,87411824	-51,75798504
Cruz Alta	RS	Soybean	1	21RS23B1	H09	-28,61138400	-53,66733600
Cruz Alta	RS	Soybean	1	21RS24B1	H29	-28,66987600	-53,61644300

Supplementary Table 1 (continuing). *Spodoptera eridania* sample location and haplotypes.

City	State	Host	MRS	Code	mtDNA haplotype	Latitude	Longitude
Cruz Alta	RS	Soybean	1	21RS24B2	H21	-28,67061800	-53,61285000
Rosário do Sul	RS	Soybean	1	21RS26B2	H02	-30,32632700	-55,00477100
Xanxerê	SC	Soybean	1	21SC01A1	H02	-26,84736400	-52,39794700
Itaí	SP	Soybean	2	21SP01A1	H32	-23,56499700	-48,91737000
Casa Branca	SP	Soybean	2	20SP04A1	H30	-21,77088372	-47,10466898
Mogi Guaçu	SP	Soybean	2	21SP04B1	H04	-22,28665550	-47,11541997
Angatuba	SP	Soybean	2	21SP08A1	H08	-23,55310420	-48,31773230
Angatuba	SP	Soybean	2	21SP08A2	H05	-23,54538930	-48,27237410
Guaíra	SP	Soybean	3	21SP11A1	H04	-20,23591300	-48,35583800
Guaíra	SP	Soybean	3	21SP11B1	H02	-20,23591300	-48,35523800
Guaíra	SP	Soybean	3	20SP12A2	H09	-20,23838700	-48,40777500
Birigui	SP	Soybean	2	21SP15A2	H05	-21,40880200	-50,49471300
Birigui	SP	Soybean	2	21SP16B1	H08	-21,35781100	-50,47138400
Guaíra	SP	Soybean	3	20SP06A2	H31	-20,26388069	-48,25491680
Porto Nacional	TO	Soybean	5	20TO01A1	H20	-10,19305085	-48,61977073
Porto Nacional	TO	Soybean	5	20TO02B2	H05	-10,45005932	-48,55815394
Palmas	TO	Soybean	5	20TO03A1	H05	-10,14765203	-47,84500494
Palmas	TO	Soybean	5	20TO03B1	H05	-10,13515604	-47,84992117
Palmas	TO	Soybean	5	20TO03A2	H05	-10,12210626	-47,86239797
Palmas	TO	Soybean	5	20TO04B2	H05	-10,11163961	-48,18874394
Porto Nacional	TO	Soybean	5	21TO04B2	H33	-10,47522331	-48,34421776

Supplementary Table 2. *Spodoptera cosmioides* sample location and haplotypes

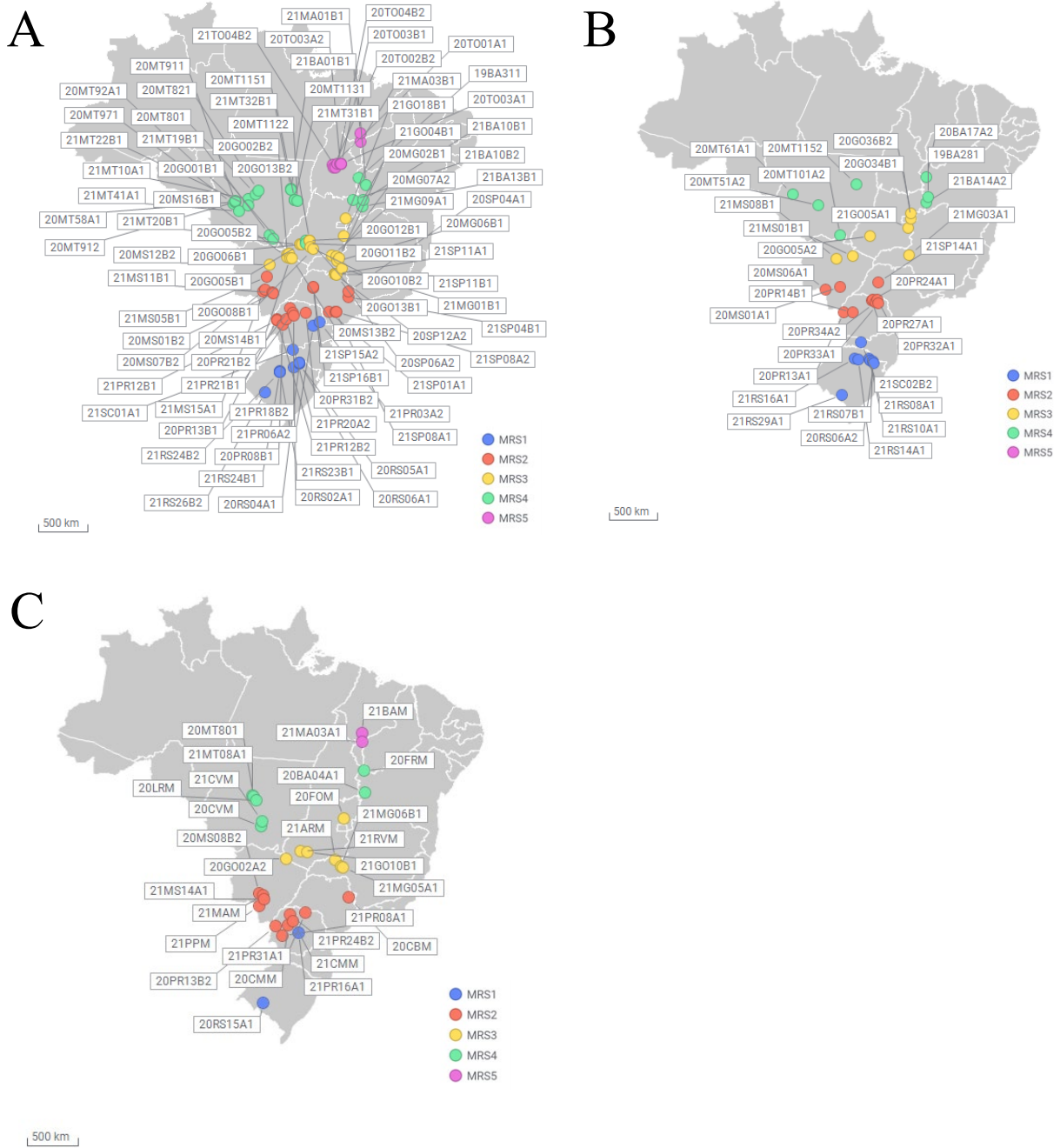
City	State	Host	MRS	Code	mtDNA		
					haplotype	Latitude	Longitude
Jaborandi	BA	Soybean	4	21BA14A2	H01	-14,20666907	-45,90022353
Barreiras	BA	Soybean	4	20BA17A2	H02	-11,79130523	-45,84450650
Rosário	BA	Soybean	4	19BA281	H03	-13,67004613	-45,69181412
Jataí	GO	Soybean	3	20GO05A2	H04	-17,29171700	-51,35360100
Cristalina	GO	Soybean	3	21GO05A1	H05	-16,58055876	-47,60561247
Formosa	GO	Soybean	3	20GO34B1	H04	-15,67832696	-47,38983787
Formosa	GO	Soybean	3	20GO36B2	H02	-15,22925057	-47,38632859
Nova Ponte	MG	Soybean	3	21MG03A1	H06	-19,05831627	-47,58955590
Rio Brillhante	MS	Soybean	2	20MS01A1	H07	-21,90014330	-54,22941660
Paraíso das Águas	MS	Soybean	3	21MS01B1	H04	-19,11129990	-53,04176000
Ponta Porã	MS	Soybean	2	20MS06A1	H06	-22,20753370	-55,63974900
São Gabriel do Oeste	MS	Soybean	3	21MS08B1	H06	-19,34829300	-54,60036600
Querência	MT	Soybean	4	20MT1152	H09	-12,44214310	-52,62187880
Sapezal	MT	Soybean	4	20MT61A1	H04	-13,41569550	-58,78259440
Itiquira	MT	Soybean	4	20MT101A2	H08	-17,19225484	-54,21637532
Diamantino	MT	Soybean	4	20MT51A2	H04	-14,39478010	-56,34217610
Terra Roxa	PR	Soybean	2	20PR13A1	H01	-24,21105149	-53,89513277
Goioerê	PR	Soybean	2	20PR14B1	H10	-24,20783526	-53,04491707
Sertanópolis	PR	Soybean	2	20PR24A1	H04	-23,05434800	-51,05661700
Cornélio Procopio	PR	Soybean	2	20PR32A1	H01	-23,09308818	-50,59810559
Nova Fátima	PR	Soybean	2	20PR33A1	H04	-23,38125063	-50,53025880
Assaí	PR	Soybean	2	20PR34A2	H02	-23,35152655	-50,80618789
Cambé	PR	Soybean	2	20PR27A1	H02	-23,12131600	-51,20197500
Vacaria	RS	Soybean	1	20RS06A2	H04	-28,56628000	-51,02339700
Vacaria	RS	Soybean	1	21RS07B1	H02	-28,41423500	-51,04306000
Lagoa Vermelha	RS	Soybean	1	21RS08A1	H04	-28,35833800	-51,30193000
Lagoa Vermelha	RS	Soybean	1	21RS10A1	H02	-28,23031500	-51,43202800
Passo Fundo	RS	Soybean	1	21RS14A1	H04	-28,33053100	-52,46627000
Carazinho	RS	Soybean	1	21RS16A1	H04	-28,23527500	-52,81133700
Bagé	RS	Soybean	1	21RS29A1	H04	-31,29755700	-54,02329900
Faxinal dos Guedes	SC	Soybean	1	21SC02B2	H02	-26,85257200	-52,23586200
Piacatu	SP	Soybean	2	21SP14A1	H02	-21,54761500	-50,57145100

Supplementary Table 3. *Spodoptera frugiperda* sample location and haplotypes

City	State	Host	MRS	Code	mtDNA haplotype	Latitude	Longitude
Roda velha	BA	Soybean	4	20BA04A1	H08	-12,64214757	-45,71580453
Serra Coceral	BA	Corn	4	20FRM	H06	-10,63153910	-45,76659900
Chapadão do Céu	GO	Soybean	3	20GO02A2	H09	-18,44687400	-52,90608900
Rio Verde	GO	Soybean	3	21GO10B1	H03	-17,87744300	-51,01864100
Formosa	GO	Corn	3	20FOM	H03	-14,92951520	-47,59859490
Rio verde	GO	Corn	3	21RVM	H04	-17,82322970	-51,64444020
Tasso Fragoso	MA	Soybean	5	21MA03A1	H14	-8,04958522	-45,97761078
Balsas	MA	Corn	5	21BAM	H05	-7,25424216	-45,97921500
Nova Ponte	MG	Soybean	3	21MG05A1	H10	-19,18331924	-47,73357658
Nova Ponte	MG	Soybean	3	21MG06B1	H03	-19,12235951	-47,93000427
Araguari	MG	Corn	3	21ARM	H04	-18,52160170	-48,42718780
Maracaju	MS	Soybean	2	20MS08B2	H04	-21,43437400	-55,38046000
Dourados	MS	Soybean	2	21MS14A1	H03	-21,94077900	-54,97943600
Maracaju	MS	Corn	2	21MAM	H03	-21,62321200	-55,04352800
Ponta Porã	MS	Corn	2	21PPM	H04	-22,48470600	-55,37989600
Lucas do Rio Verde	MT	Soybean	4	21MT08A1	H03	-12,90790054	-56,00398956
Sorriso	MT	Soybean	4	20MT801	H11	-13,00042790	-55,87823740
Campo Verde	MT	Corn	4	21CVM	H03	-15,21449470	-55,09546100
Campo Verde	MT	Corn	4	20CVM	H03	-15,64048207	-55,24582691
Lucas do Rio Verde	MT	Corn	4	20LRM	H07	-13,29014302	-55,64747701
Cascavel	PR	Soybean	2	21PR08A1	H03	-24,97763800	-53,31570300
Terra Roxa	PR	Soybean	2	20PR13B2	H13	-24,21185150	-53,89542785
Pitanga	PR	Soybean	1	21PR16A1	H12	-24,72875300	-51,77335600
Cambé	PR	Soybean	2	21PR31A1	H03	-23,10548350	-51,20071970
Campo Mourão	PR	Corn	2	21CMM	H03	-23,81512300	-52,27715900
Campo Mourão	PR	Corn	2	20CMM	H02	-24,11066481	-52,73942087
Paraíso do Norte	PR	Soybean	2	21PR24B2	H04	-23,25672900	-52,57540100
Rosário do Sul	RS	Soybean	1	20RS15A1	H03	-30,43150856	-55,03817604
Casa Branca	SP	Corn	2	20CBM	H01	-21,80502577	-47,18251620

Supplementary Table 4. Sample location for *Elaphria agrotina*, *Spodoptera dolichos* and *Spodoptera ornithogalli*.

Species	City	State	Host	MRS	Code	Latitude	Longitude
<i>E. agrotina</i>	Barreiras	BA	Soybean	4	20BA06B2	-11,98053772	-46,20230706
<i>S. dolichos</i>	Honório Serpa	PR	Soybean	1	20PR07A1	-26,14669608	-52,28395915
<i>S. ornithogalli</i>	Bagé	RS	Soybean	1	20RS18A1	-31,29414315	-54,01941421



Supplementary Figure 1. Sample location for (A) *Spodoptera eridania*, (B) *Spodoptera cosmioides* and (C) *Spodoptera frugiperda*.

5. FINAL CONSIDERATIONS

The agriculture in Brazil expanded significantly in the last 50 years, bringing new technologies along this time. Insect populations are also adapting to the new agriculture landscapes and this thesis are a step forward to the understanding of population dynamics in Brazil. Here, regional pest suppression of two soybean lepidopteran pests were observed with the increase adoption of *Bt* soybean in Brazil. The lower abundance of insects in the field resulted in reduced insecticide sprays at a farm level. This is a first report of such benefit in a tropical environment and brings a lot of value for farmers to manage pests. The confirmation of pest suppression is an important finding as we can see the how the recently introduced *Bt* crop can bring direct and indirect benefits in the system.

In a large-scale sampling of larvae on commercial soybean fields during the 2019 and 2020 crop seasons, *C. includens* was the main lepidopteran pest in non-*Bt* fields. These results indicated that even with *C. includens* suppression by *Bt* soybean, this species is still an important pest in soybean fields. Probably, if *Bt* soybean were not present in the system, the abundance of this species could be even higher. Cry1Ac soybean have provided a high level of protection against *A. gemmatalis*, *C. includens*, *C. virescens* and *H. armigera* evidencing that this technology continues to be effective in manage pests in the field. In a near future, if the adoption of Cry1Ac soybean continue high and resistance alleles frequency remained low for these species, the pest suppression will probably continue to be present and will regionally benefit farmers in Brazil.

We also observed that 98% of larvae found in Cry1Ac soybean were *Spodoptera* spp., although the numbers of *Spodoptera* spp. were similar between Cry1Ac soybean and non-*Bt* fields. It is expected to observe larvae of this genera in Cry1Ac soybean as this technology does not confer protection to these species. However, our data showed an increase in the abundance of this species group when compared to the recent past. One hypothesis is that changes in production system might be related to that shift, e.g., winter corn area growth and species adaptation to soybean plants.

These emerging pests of soybean, *S. eridania*, *S. cosmioides* and *S. frugiperda*, have a potential to become a major pest in the soybean, but little information of population genetics is available to these species. We investigated the population genetic diversity, population structure, and demographic pattern, using the mitochondrial COI sequences. A high genetic diversity was observed for *S. eridania* sampled on soybean in Brazil. The genetic diversity index of *S. eridania* was higher than that observed for *S. cosmioides* and *S. frugiperda*. Weak genetic structure was observed for the three *Spodoptera* species. There is evidence that the three *Spodoptera* species evaluated in this study are in demographic and spatial expansion. The results of study also suggest that *S. frugiperda* corn strain is predominant on soybean in Brazil. These are initial steps to future investigations of the *Spodoptera* population genetics on soybean crops, where more abundant markers will be essential to understand this dynamic and guide new management strategies.

Overall, this thesis brought new insights to the population dynamics of the main lepidopteran pests present in soybean crop. After 50 years of agriculture expansion, Brazil became a top producer of soybean. In the current agriculture model, every detail is important to obtain the best result, following best agronomic practices along with sustainability. Further research is still needed to

understand better how the insects will respond to the agriculture landscape changes and the intensification of production systems, but the comprehension of insect dynamics will be a fundamental cornerstone.