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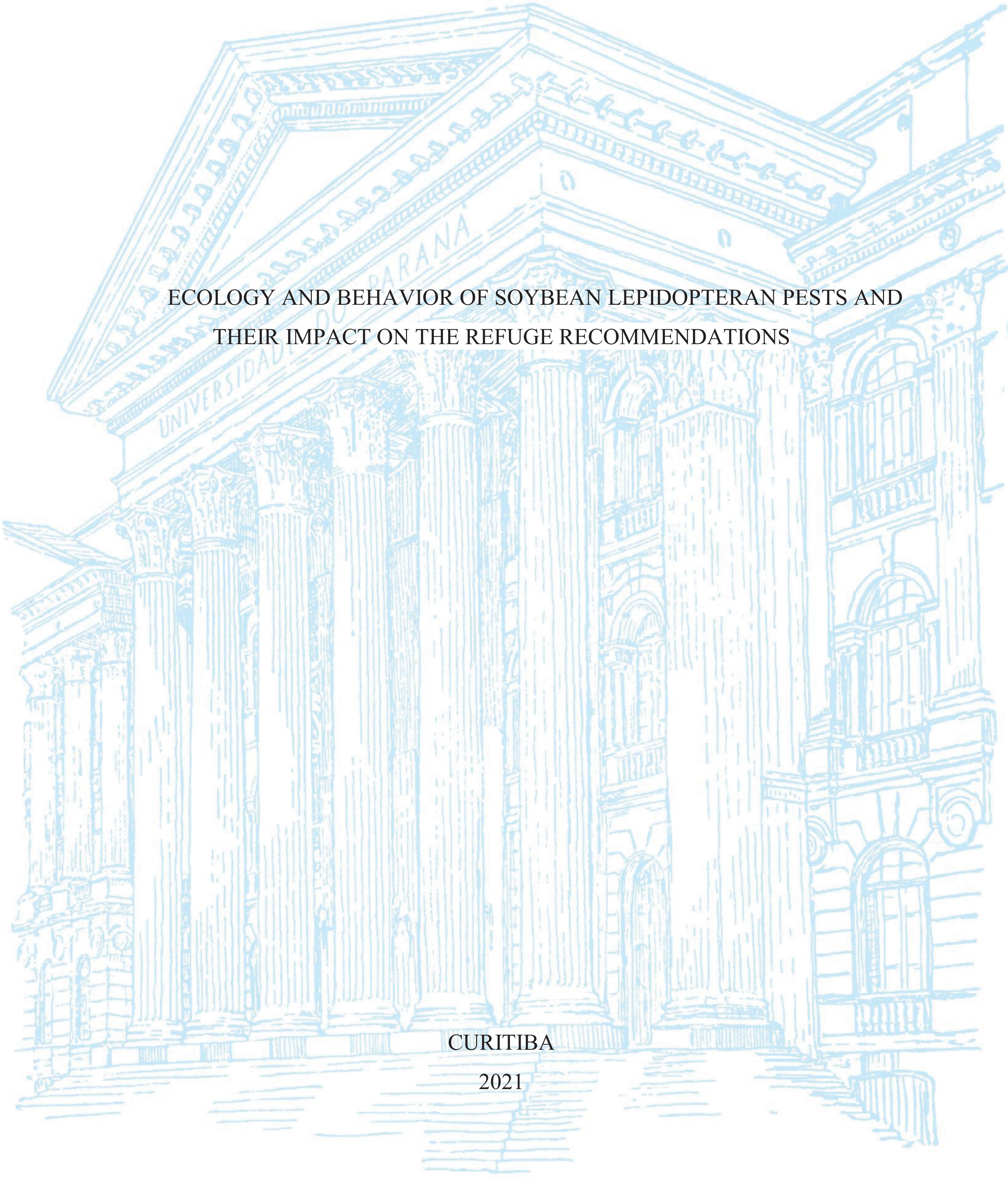

ECOLOGY AND BEHAVIOR OF SOYBEAN LEPIDOPTERAN PESTS AND THEIR IMPACT ON THE REFUGE RECOMMENDATIONS

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JACIARA GONÇALVES

ECOLOGY AND BEHAVIOR OF SOYBEAN LEPIDOPTERAN PESTS AND THEIR IMPACT ON THE REFUGE RECOMMENDATIONS

Tese apresentada à Coordenação do curso de Pós-graduação em Ciências Biológicas, Área de concentração em Entomologia, do Setor de Ciências Biológicas da Universidade Federal do Paraná, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas.

Orientador: Prof. Dr. Adeney de Freitas Bueno

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIAS BIOLÓGICAS (ENTOMOLOGIA) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **JACIARA GONÇALVES** intitulada: **Ecology and behavior of soybean lepidopteran pests and their impact on the refuge recommendations**, sob orientação do Prof. Dr. ADENEY DE FREITAS BUENO, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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“Commit your way to the LORD; trust in Him and He will act”.

(Psalm 37:5)

RESUMO

A estratégia de refúgio é uma das ferramentas do Manejo de Resistência de Insetos (MRI) implementada para retardar a evolução da resistência de lepidópteros desfolhadores, como *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae) e *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae) à soja Cry1Ac. No entanto, informação sobre biologia e comportamento ainda são cruciais para desenvolver as recomendações de MRI, incluindo injúria nas plantas não-Bt do refúgio cultivadas adjacentes às áreas Bt. Assim como, diferenças na data de plantio da soja Bt e o correspondente refúgio, causando assincronia no estádio fenológico das duas culturas, podem influenciar a oviposição das mariposas, e a distância máxima recomendada para plantar o refúgio é baseada em estudos que documentam a capacidade de voo de *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) e *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae). Neste trabalho, conduziu-se experimentos com escolha de oviposição para investigar se há preferência por plantas Bt ou não-Bt, com ou sem injúria larval e diferentes estádios fenológicos. Também se conduziu experimentos para determinar a distância de voo das duas espécies em área de produção de soja, através da técnica de marcação, liberação e recaptura. Os resultados indicam que a capacidade de voo de *A. gemmatalis* é maior que os 800 metros recomendados, mas a maioria das mariposas de *C. includens* foram capturadas a menos de 800 metros. Fêmeas das duas espécies estavam acasaladas mesmo quando recapturadas próximo ao ponto de liberação. Preferência de oviposição por plantas Bt foi observada quando o refúgio foi semeado 5 dias depois da área Bt, e por plantas Bt quando o refúgio teve maior porcentagem de desfolha que as plantas Bt. Os resultados deste estudo reforçam a necessidade de MRI em soja Bt, considerando a escolha da cultivar para o refúgio com fenologia semelhante, plantio no mesmo dia que a soja Bt e adoção dos níveis de ação no refúgio para reduzir o comportamento de evitar plantas com injúria pelas mariposas.

Palavras-chave: Cry1Ac 1. Resistência 2. Injúria 3. Oviposição 4. IRM 5. MIP 6.

ABSTRACT

Refuge strategy is one of the Insect Resistance Management (IRM) tools to delay the evolution of resistance of the lepidopteran defoliators, such as, *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Erebidae) and *Chrysodeixis includens* (Walker, 1858) (Lepidoptera: Noctuidae) to Cry1Ac soybean. However, biology and behavior information is still critical in the development of IRM recommendations, including feeding in refuge non-Bt plants cultivated with Bt fields. In addition, differences in planting date of Bt field and corresponded refuge, and desynchronization of crop phenology of Bt and non-Bt cultivars may influence the moth oviposition, and the recommended maximum distance to plant refuge is based on studies documenting the moth capacity of *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) and *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae). Here, choice experiments were performed to investigate if there is oviposition preference for Bt or non-Bt plants, with or without larval injury, and at different phenological growth stages. Also, to determine the flight distance of both species in a soybean production area, by the mark-release-recapture technique. The results indicated that flight capacity of *A. gemmatalis* is more than the 800 meters that is recommended, but prevalent recapture of *Chrysodeixis includens* were less than 800 meters. Females of both species were mated even very close to the release point. Oviposition preference was observed for Bt plants when refuge was sown 5 days after the Bt area for Bt plants when the refuge had a higher defoliation percentage. Furthermore, the results of this study reinforce the need of the IRM in Bt soybean, considering the selection of cultivar for refuge with similar growth phenology, planting at the same date the Bt crop and refuge area, and adoption of economic threshold in refuge area to reduce moth oviposition avoidance in injured plants.

Keywords: Cry1Ac 1. Resistance 2. Injury 3. Oviposition 4. IRM 5. IPM 6.

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CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1. GENERAL INTRODUCTION

Soybean has historically been impacted by pests, such as the *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae), common name velvetbean caterpillar, which larvae can cause up to 100% defoliation (Moscardi et al., 2012). Another important defoliator that has arisen concern is *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), commonly known as the soybean looper (Bernardi et al., 2012). One of the approaches to control these soybean defoliators is the use of Bt soybean, which is a soybean cultivar that had the insertion of a gene isolated from the soil bacteria *Bacillus thuringiensis,* and express the Cry1Ac protein (name maintained in the new nomenclature) (Jurat-Fuentes et al., 2021), that is toxic for some lepidopteran species. Cry1Ac was the only toxin expressed on soybean cultivars (Intacta®), since 2013, and it has provided efficient high level of field efficacy against *A. gemmatalis* and *C. includens* (Horikoshi et al., 2021a).

Nevertheless, the adoption of Bt soybean has been increasing, and Brazil is the country with the largest Bt soybean area, as 20.2 million hectares were planted in 2018 (ISAAA, 2018), this area increased even more in the 2020-2021 crop season, achieving more than 30 million hectares (Adeney de Freitas Bueno, personal information). This high adoption raises a concern about the possible selection of Bt resistant population in field, which will be no longer controlled by the technology (Andow, 2008). In this context, *Rachiplusia nu* Guenée (Lepidoptera: Noctuidae) and *Crosidosema aporema* (Walsingham) (Lepidoptera: Tortricidae), species with reports of susceptibility to Cry1Ac (Macrae et al., 2005; Yano et al., 2012), were already documented surviving in Cry1Ac soybean fields during 2020-2021 crop season in some Brazilian regions (Bueno and Sosa-Gómez, 2021; Nardon et al., 2021; Horikoshi et al., 2021b).

Therefore, the demand of an Insect Resistance Management (IRM) program in soybean is critical. IRM principles are based on the following assumptions: (a) resistance in insects are usually recessive or incompletely dominant (Tabashnik, 1994); (b) the Bt high dose expressed in the plants is 25-fold the toxin concentration to kill all susceptible individuals and more than 95% of the heterozygotes (U.S. Environmental Protection Agency, 1998); (c) refuges are plants that do not express the toxin, serving as a source of susceptible individuals, which randomly mate with rare homozygotes resistant ones that are not killed by the high dose (Gould, 1994). Structure refuges for Bt soybean consist of planting 20% of the field with non-Bt cultivar in a way that all Bt plants of the field are not further than 800 meters from the closest non-Bt plant of the refuge.

Although the recommendation is established, the 800-m distance is supposed to reflect or be inferior the dispersal capacity of moths of all lepidopteran species targeted by Bt soybean. Instead of this, it has been based on *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae) flying capacity in studies carried out in maize fields (Vilarinho et al., 2011). A validation of this distance was attempted for *A. gemmatalis*, which detected more than 10% of recaptured moths were able to fly 800 meters or more (Caixeta, 2014). However, the landscape was composed by sugarcane cultivated together with soybean, which might have negatively influenced the moths dispersal. Thus, it is necessary to validate this recommendation for Bt soybean considering the target lepidopteran species for this technology. Another aspect related to the behavior of the moths, is a possible difference in oviposition preference between refuge and Bt plants. It has been reported for others species, such as moths of *Chloridea virescens* Fabricius (Lepidoptera: Noctuidae) (De Moraes et al., 2001) and *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), which were demonstrated to avoid tobacco and soybean plants, respectively, that have larval injury and defoliation. In the Bt and refuge fields, a higher defoliation will probably occur in the non-Bt plants from the refuge, thus the moths could oviposit preferentially in the Bt plants, which could increase the larvae exposure to Bt Cry toxins, as previously investigated for the main corn pest (Gonçalves et al., 2020; Téllez-Rodríguez et al., 2014).

Important to mention that refuge areas for Bt soybean are planted with non-Bt cultivars that are not isogenic. Although the recommendation is to select a cultivar that is similar regarding the architecture and maturity group, which reflects the growth phenology, differences may occur in the growth of the Bt and non-Bt cultivars. In addition, Brazilian production areas of soybean are extensive fields, an average of 1,000 hectares but many operate more than 100,000 hectares, especially in the savannah region (Steinweg et al., 2017), which may take several days to conclude planting. Since 80% of the soybean area is cultivated with Bt soybean, farmers usually gives priority of sowing the Bt cultivar. Growers that adopt refuge sow this area later in the plant season, once has concluded the planting of the Bt fields. Differences in growth phenology of the Bt and non-Bt cultivars may be discriminated by the moths of target species, and could cause oviposition preference towards less infested plants in Bt crop. Therefore, it is of theoretical and practical interest to understand how the target lepidopteran pests interact with the Bt and refuge areas, and then to provide more information to improve refuge implementation in an IRM program to Bt soybean. This is important mainly taking into consideration that adoption of refuge is one of the foundation for maintaining the effectiveness of the Bt technology.

1.1. OBJECTIVES

1.1.1. General objective

Deepen the knowledge about the main biologic and behavioral aspects of the primary lepidopteran defoliators and targeted pest of Bt-soybean, *A. gemmatalis* and *C. includens* that influence refuge effectiveness.

1.1.2. Specific objectives

- Investigate the impact of differences in soybean growth phenology in Bt-soybean and refuge non-Bt soybean in the oviposition behavior of *A. gemmatalis* and *C. includens*;
- Investigate whether there is oviposition preference for different soybean cultivars by *A. gemmatalis* and *C. includens*;
- Investigate whether there is oviposition preference by *A. gemmatalis* and *C. includens* between undamaged Bt and damaged non-Bt soybean;
- Document the flight capacity range and mating behavior of *A. gemmatalis* and *C. includens* moths and their compatibility with the current 800 meters recommendation distance between any Bt-soybean from the closest non-Bt plant.

2. LITERATURE REVIEW

2.1. Current status of Bt technology worldwide adoption

Transgenic crops have been a fasted adopted technology, as the global planted area increased 113 times in the last 23 years, achieving 191.7 million hectares in 2018. The USA is the country with the largest planted area (75 million hectares) followed by Brazil (51.3 million hectares), with a 93% adoption rate considering the total agricultural area of the country. Considering all the transgenic crops adopted in Brazil, in 2018 insect-resistant crops comprised most of the biotech crops as cotton (83.2%) maize (95.8%), and soybean (58%) (ISAAA, 2018), which was first adopted in Brazil in 2013 (James, 2013) and later in other South America and Asia countries (ISAAA, 2020).

Transgenic crops are crops that express toxins of the *Bacillus thuringiensis*, a gram-positive, spore-forming bacterium that exists in the soil and forms a parasporal crystal during sporulation. The crystals are formed by one or more δ-endotoxins or crystal (Cry) proteins (De Maagd et al., 1999). The gene coding for the insecticidal toxin (Bt toxin) was then inserted into a crop plant, making it resistant to feeding damage by target pests (Prado et al., 2014). The DNA insertion into a plant genome from a single transformation process is called a genetically modified event (Pilacinski et al., 2011). When only one event is present in a plant, it is called a single event, which is the case of the first transgenic plants. When the transgenic events are combined in a single variety, aiming at controlling the same pest, the variety is called pyramided, whereas, when two or more transgenes are not related and do not aim to control the same pest species, the variety is called stacked (Andow, 2008).

The Bt technology in the soybean crop in Brazil was first approved by the National Biosafety Technical Committee (CTNBio) in 2010. The event MON 87701 x MON 89788 expressing a single Bt toxin, Cry1Ac toxin and tolerance to glyphosate and became available to growers in 2013, and for 8 years it was the only commercially soybean trait available. In 2016, the pyramided event DAS-81419-2 expressing Bt toxins Cry1Ac and Cry1F, and in 2018, the pyramided MON 87751 x MON 87708 x MON 87701 x MON 89788 event expressing the Bt toxins Cry1A.105, Cry2Ab2, and Cry1Ac, were approved by the CTNBio (CTNBio, 2020). MON 87751 x MON 87708 x

MON 87701 x MON 89788 is already available for growers to sow this 2021-2022 crop seasons. The pyramided event expressing Bt toxins Cry1Ac and Cry1F showed significantly less defoliation levels when compared to the non-Bt soybean by the defoliators species *A. gemmatalis*, *C. includens*, *C. virescens*, and *Spodoptera cosmioides* Walker (Lepidoptera: Noctuidae) in field experiments with artificially infested plants (Marques et al., 2016). The pyramided event expressing the Bt toxins Cry1A.105, Cry2Ab2, and Cry1Ac was highly effective at protecting soybean against *A. gemmatalis*, *C. includens*, and *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) in leaf disc bioassays and field conditions (Bacalhau et al., 2020).

Bt soybean is nowadays an important technology in the Integrated Pest Management (IPM) of the most important lepidopteran pest species of the soybean (Bueno et al., 2021). The soybean cultivars that express Cry1Ac toxin have shown efficacy in the management of *A. gemmatalis*, *C. includens* (Bernardi et al., 2012; Yano et al., 2016), *C. virescens*, and *C. aporema* (Macrae et al., 2005). In addition, high susceptibility to Cry1Ac of the old-world bollworm *H. armigera* (Dourado et al., 2016; Yu et al., 2013) has been reported, which contributed to the decline of the populations of these invasive species in Brazil (Paula-Moraes et al., 2017).

Among the benefits provided by Bt crops, the reduction of yield loss caused by insect economic damage has been significant (Sanglestsawai et al., 2014), as well as the reduction of insecticide spraying and therefore its side effects on beneficial organisms. In Brazil, a total of 41.5 million kg of active ingredients were not applied because of Bt crops, comprised of 26.6, 13.2, and 1.7 million kg of active ingredients not used in Bt maize, Bt soybeans and Bt cotton, respectively (Brookes and Barfoot, 2020). Moreover, Bt crops are target-specific, managing only the target pests (Romeis et al., 2019).

Although growers can benefit from this technology, its high adoption associated with low refuge compliance might imply on an adverse effect: the selection of resistant insect populations, no longer controlled by the Bt crop. Field-evolved resistance consists of a genetic reduction in susceptibility to a toxin in a population caused by continuous exposure to the toxin over time (Tabashnik et al., 2014). Some field-resistant cases of target species to corn and cotton Bt plants have been reported in five countries, United States, Brazil, Argentina, South Africa, and India (Tabashnik and Carrière, 2017). One important case is the worldwide maize pest, the fall armyworm *S. frugiperda* which, has been selected for resistance in the field to Bt toxins in two countries, Brazil and the USA (Farias et al., 2014; Huang et al., 2014; Omoto et al., 2016; Storer et al., 2010). More recently, two soybean species have been reported to survive in Cry1Ac soybean, *R. nu* and *C. aporema*, species initially controlled by Cry1Ac (Macrae et al., 2005; Yano et al., 2012) during 2020-2021 crop season in some Brazilian regions (Bueno and Sosa-Gómez, 2021; Nardon et al., 2021).

2.2. Insect Resistance Management (IRM)

The risk of resistance evolution of target pests by the Bt technology demands the adoption of strategies in an IRM program, which aims to prevent or at least delay the occurrence of control failures. The IRM is based on some assumptions: (a) resistance in insects are usually recessive or incompletely dominant (Tabashnik, 1994); (b) the Bt high dose expressed in the plants is 25-fold the toxin concentration to kill all susceptible individuals and more than 95% of the heterozygotes (U.S. Environmental Protection Agency, 1998); (c) refuges are plants that do not express the toxin, serving as a source of susceptible individuals, which randomly mate with rare homozygotes resistant ones that are not killed by the high dose (Gould, 1994).

Based on IRM assumptions, according to Andow (2008), there are some approaches to practically delay the evolution of resistance: (a) preserve phenotypes to the Bt toxins by maintaining refuge areas, and then reduce the selection pressure on the target pest; (b) reduce the fitness of the resistant phenotypes from Bt areas, by suppressing them with other controlling tactics, such as biological control or insecticides; (c) reduce the heterozygote fitness by using a high-dose event, making it a susceptible phenotype; (d) manage the movement of specific sex and then the mating frequency to delay the evolution of resistance (Andow and Ives, 2002).

2.2.1. High-dose refuge strategy

A combination of the first and third approaches mentioned above is the high-dose refuge strategy. As already previously mentioned, the strategy consists of the planting of a non-Bt field near the Bt area, known as refuge, which will serve as a source of susceptible individuals, that will randomly mate with possible resistant individuals from the Bt area. This mating will result in heterozygotes, which are expected to be susceptible, assuming that the resistance is recessive, and then will be killed by the Bt plants (Gould, 1994). The initial frequency of resistance alleles should be less than 10^{-3}, which means nearly all alleles will be heterozygotes genotypes and can be killed by the high dose Bt crop. Also, mating of individuals from Bt crop and refuge must be sufficient to ensure that females from Bt fields are likely to mate with males from the refuge (Andow, 2008).

In the USA, the main consumer of Bt maize and cotton, the refuge approach varies depending on the crop and pest, for example, in southeastern region, structured refuge is not mandatory for cotton, as alternative host plants play an important role as natural refuges, thus contributing to maintaining susceptible moths (Gould et al., 2002; U.S. Environmental Protection Agency, 2007). On the other hand, in the Corn Belt, the implementation of structured refuge in corn is mandatory, and the developer of the technology is the responsible to inspect the execution of refuge planting. The compliance of refuge adoption is regulated by the EPA (Environmental Protection Agency) and no adoption of refuge results in farmers being prevented from buying seeds for the next crop seasons (Carrière et al., 2019). Industry has been adopting an educational program, which has a "phased compliance approach", that is a warning letter from the registrant, together with additional IRM education and assistance when the growers do not comply with the refuge requirements. But, when the grower has not complied for two consecutive years, then the grower can lose access to the Bt seeds in the next crop season (U.S. EPA, 2021)

Currently in Brazil, the Normative Instruction nº 59, published on December 19 of 2018 by the Ministry of Agriculture, Livestock, and Supply (MAPA) established the structured refuge as a phytosanitary measure to manage the insect resistance to Bt. The document confers to the technology developer the responsibility to provide scientifically based information of the refuge area size and distance to each crop and toxin (Ministério da Agricultura Pecuária e Abastecimento - MAPA, 2018).

Although Brazil does not have regulatory tools that mandates farmers to adopt IRM recommendations such as refuge, the Brazilian Insecticide Resistance Action Committee (IRAC Brazil), composed of members of the industry, academy, and the Ministry of Agriculture, Livestock and Supply (MAPA), have produced publications that aim to guide consultants and growers on how to adopt the IRM strategies in an IPM framework. The refuge recommendation includes (IRAC Brazil, 2018): 1) The refuge area needs to be at least 10% for maize and 20% for soybean and cotton; 2) Use cultivars or hybrids of the same or similar vegetative cycle planted at the same time of the Bt crop; 3) The maximum distance between any plant from the Bt area and the refuge area must not be higher than 800 meters; 4) In-field strip refuges or refuges planted within the Bt field are recommendable to increase the refuge efficacy to delay resistance; 5) Refuge must be grown in the same property of the Bt crop and be managed by the same grower; 6) Follow the seed company's orientations on the leaf spraying on the refuge: not more than 2 insecticide sprays up to V6 in maize, and follow action thresholds recommendations for cotton and soybean.

Despite the detailed recommendation from IRAC, some of those recommendations has not been validated for the target soybean pests, such as the distance that moths disperse in the field, and the recommendations have been based on studies with other species (Hunt et al., 2001; Vilarinho et al., 2011). Also, the influence of the refuge area, expected under high infestation and consequently high defoliation in the attractiveness (Gonçalves et al., 2020; Téllez-Rodríguez et al., 2014) for moth oviposition still needs validation in the target pests of Bt-soybean *A. gemmatalis* and *C. includens.*

2.2.2. Fitness reduction of the resistant phenotypes

The second approach on the IRM relies on reducing the fitness of the resistant phenotypes from Bt areas, by suppressing them with other controlling tactics, such as biological control or insecticides (Andow, 2008). Which means, a control tactic, other than the Bt plant, is applied only on the Bt area, in order to decrease the potential of resistant phenotypes to multiplicate.

In fact, mathematical models have shown that natural enemies that decrease differential fitness between susceptible and resistant can delay the resistance evolution, whereas natural enemies that increase this differential fitness could accelerate the resistance (Gould et al., 1991). Empirical data have confirmed this models, such as the case of *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae), a ladybird predator that combined with refuge plants delayed the resistance of *Plutella xyllostella* (L.) (Lepidoptera: Plutellidae) to Cry1Ac broccoli (Liu et al., 2014). In addition, the presence of the entomopathogenic nematode *Steinernema riobrave* (Rhabditida: Steinernematidae) caused a higher mortality of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) Cry1Ac resistant than susceptible strain, explained by a potentially lower ability to defend against the nematode infection, as trade-off to the Cry1Ac resistance (Gassmann et al., 2006).

Johnson et al. (1997) investigated the effect of the parasitoid wasp *Campoletis sonorensis* (Cameron) and the entomopathogenic fungus *Metarhizium rileyi* on susceptible and resistant *C. virescens* to tobacco plants expressing CryIA(b) toxin. Their conclusion was that the parasitoid would likely delay the development of resistance to Bt tobacco plants, while the fungus would likely promote the development of resistance, due to a higher and lower susceptibility of Bt CryIA(b) resistant larvae to the natural enemies, respectively, comparing the susceptible *C. virescens* strains.

2.2.3. Manage the movement of specific sex

This approach is related to the adult behavior of pests, by managing the movement of specific sex, the mating frequency is supposed to decrease and delay the evolution of resistance (Andow and Ives, 2002). The authors mention some tactics based on simulation models, such as attracting males into Bt fields with female pheromones and mass releasing of susceptible males.

2.3. Role of volatile organic compounds in moth attraction and its influence in the refuge strategy

Volatile organic compounds (VOC's) from plants play an important role in the host search by the moths (Renwick and Chew, 1994). Particularly, when the plants are under a pest attack, this injury triggers herbivore-induced plant volatile (HIPVs) production. On one hand, the HIPVs might serve as a cue to the moth, that it should not oviposit in that plant to avoid future competition for its progeny, diminish the probability to find natural enemies, and avoid plants with induced resistance and low nutritional value (De Moraes et al., 2001). On the other hand, some studies demonstrated that the HIPVs had the opposite effect, attracting females and males to plants damaged by conspecific larvae (El-Sayed et al., 2016), which means, these compounds may play different roles in each specific insect-plant interaction.

Considering the fact that Bt plants carry genes that express toxic proteins, they might cause some differences on the VOC's profile. However, similar VOC's profiles of Bt and non-Bt plants were detected in rice (Sun et al., 2013) and cotton (Yan et al., 2004). This similarity might explain the non-discrimination by moths between Bt and non-Bt plants in maize (Obonyo et al., 2008; Van Den Berg and Van Wyk, 2007), cotton (Hardke et al., 2012; Torres and Ruberson, 2006), cabbage (Kumar, 2004), broccoli (Yi et al., 2015), and rice (Sun et al., 2013). In contrast, maize plants under herbivory tend to modify their HIPVs profile, which can be detected by fall armyworm moths (Pinto-Zevallos et al., 2016), and could explain their avoidance behavior observed by Signoretti et al. (2012) in olfactometer bioassays. In addition, when comparing Bt and non-Bt maize hybrids under herbivory, the non-Bt isogenic hybrid emitted higher amounts of the same HIPVs (Turlings et al., 2005), which means, even the VOC's profile of the different hybrids is similar, under herbivory, there might be differences in the amounts of HIPVs produced by each hybrid.

Téllez-Rodríguez et al. (2014) investigated the fall armyworm behavior in Bt maize and its refuge in Cuba, and found a strong oviposition preference for Bt maize in the field, and associated this egg-laying bias to the higher injury caused by conspecific larvae in the refuge plants. This oviposition avoidance behavior to injured plants was observed in other species of the same family (Noctuidae), in greenhouse tests for *Tricoplusia ni* Hübner, (Lepidoptera: Noctuidae) in soybean plants (Coapio et al., 2016) and *C. virescens* in tobacco plants (De Moraes et al., 2001). However, various results have been observed in Brazilian populations of fall armyworm, that do not discriminate between Bt and non-Bt maize in field and greenhouse experiments, even when the plants were under injury by conspecifics (Gonçalves et al., 2020).

Despite the reported information, knowledge about the Bt soybean and its primary defoliators is lacking. A deeper understanding on how female moths of *A. gemmatalis* and *C. includens* behave in the context of Bt soybean and structured refuge could support the insect resistance management of both species, and contribute to the longevity of this technology, which has been efficient at suppressing pest population in Brazil (Horikoshi et al., 2021a).

2.4. Impact of plant stage on moth oviposition choice

Despite the significant number of studies addressing plant injury on moth host location, the information on differences of plant phenological stage are limited. This information is fundamental to support the recommendation of refuge planting at the same time as the Bt crop.

A recent study was performed to investigate whether the rice leaf folder (RLF), *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae) would prefer rice plants at the seedling, tillering, and booting stages. Their findings demonstrated that the moths preferred ovipositing at the more mature plants, at tillering and booting stages (Liu et al., 2021). Another study investigated the preference of the cabbage looper *T. ni* of young and mature leaves of different hosts, has also concluded that this moth species is more likely to oviposit on older leaves (Coapio et al., 2018).

Concerning the Bt crop and refuge planting, it is expected that if any unpredictable event occurs, the grower will first plant the Bt crop, and later the refuge. This situation would lead to the Bt and refuge fields being at different stages, leading to a possible oviposition preference choice by the moths to either younger or older fields. That was the case of *O. nubilalis*, which laid between 50 and 100% of the eggs in the early corn planting during the first generation (Pilcher and Rice, 2001). For soybean areas that are typically large, such as observed in the Brazilian savannah, the effect of different Bt soybean stage and refuge soybean stage is crucial, as the largest area planted is Bt, there are high chances that both areas will not be sowed at the same time, as the sowing operation can last days to be complete.

2.5. Implications of the moth movement on refuge effectiveness

The success of the refuge strategy is based on the moths dispersion in the field and random mating behavior (Gould, 1994). The Environmental Protection Agency (EPA) of the USA recommends that the maximum distance to plant the maize and cotton refuge is approximately 804 meters (half-mile) from any plant from the Bt field in order to promote random mating and dilution of homozygous resistant insects in the population (U.S. EPA, 2021).

Scientific data on moth dispersal have demonstrated differences for the same pest. Adults of *O. nubilalis*, were recovered 23-49 km from the release point in Iowa, USA (Showers et al., 2001). Nevertheless, in Nebraska, released adults tended to remain near the irrigated maize (Hunt et al., 2001). In Kansas 99% of the European corn borer moths were recaptured at 350 meters from the release point, which is less than the recommended distance, but the authors agree that it might be not the real situation for wild moths, as they have captured them in transgenic fields, i.e., they must have flown from refuge fields that were at longer distances (Qureshi et al., 2005). Similar distance and conclusions were observed by the same authors to another maize borer, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae) (Qureshi et al., 2006).

In Brazil, the same maximum distance was recommended to plant the refuge fields, although with no previous data to confirm the moth dispersal in maize, cotton, and soybean. Because of that, some studies were carried out, and for the main maize pest, fall armyworm (*S. frugiperda*) the 800 meters seemed adequate, as the authors recaptured moths at 806 and 608 meters, males and females, respectively (Vilarinho et al., 2011). The same conclusion was found for the sugarcane borer, *Diatraea saccaralis* Fabricius (Lepidoptera: Crambidae), in sugarcane fields (Caixeta, 2010).

In soybean, up to now, a single study was carried out to address the moth dispersal of *A. gemmatalis*. The results reported that more than 10% of recaptured moths were found at 800 meters or further from the release point of the marked moths (Caixeta, 2014). However, the author highlighted that the sugarcane cultivated together with soybean in the experiments might have negatively influenced the moth dispersal. Therefore, information on the main moth species targeted by Bt soybean in Brazil is still lacking.

2.5.1. Marking-release-recapture technique

Studies focused on insect dispersal need to apply a methodology that provides reliable information on the distances that individuals have moved. The mark-release-recapture technique is reliable method for insect dispersal studies. Insects are marked, released in the field, and then recapture at known distances. The marker on the insects will differentiate them from the wild ones (Hagler and Jackson, 2001).

Insects can be marked with various methodologies, such as dust or powders (Culbert et al., 2020), oil-soluble dyes (Vilarinho et al., 2011), pollen (Hartstack et al., 1982), protein markers such as chicken egg albumin (Tavares et al., 2019). To make the best marker choice, some aspects of the marker need to be taken into account, such as, to be identifiable and retained on the insect for all the time that it is expected to analyze its dispersal. In addition, the marker must not adversely affect the insect (behavior, growth, reproduction, and life span) and the environment (Hagler and Jackson, 2001). Dusts are commonly used for external marking, and adequate to mark large insects with hairy bodies, which is the case of moths, such as *A. gemmatalis* and *C. includens*, both species studied in this work.

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CHAPTER 2: OVIPOSITION BEHAVIOR OF TWO PRIMARY SOYBEAN DEFOLIATORS IN BT SOYBEAN AND NON-BT

ABSTRACT

BACKGROUND: The adoption of refuge areas in combination of the high dose Bt traits are critical components for an Insect resistance management (IRM) program for *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae) and *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae). However, there is no information on how refuge implementation might affect the oviposition choice of the moths. This study aimed at understanding the oviposition behavior of *A. gemmatalis* and *C. includens* in Bt and refuge plants, under larval injury, different growth phenology, and cultivars in semi-field and field experiments.

RESULTS: Females of *A. gemmatalis* did not discriminate between soybean cultivars for oviposition, however they showed preference to oviposit on Bt plants when refuge sowing was delayed 5 and 10 days after the Bt field in semi-field experiments. A three-year field experiment exploring the oviposition of natural populations of *A. gemmatalis* in Bt and refuge, planted side by side, showed that a higher number of eggs were laid in Bt plants which were less defoliated. On the other hand, females of *C. includens* did not show oviposition preference towards Bt or refuge at any scenario explored in the study, in the semi-field and field experiments.

CONCLUSION: Female oviposition behavior is crucial when designing refuge recommendations for target pests of Bt soybean, such as *A. gemmatalis*. The results of the present study validated the current recommendation to structured refuge for Bt soybean. In addition, the recommendation of refuge should be in an Integrated Pest Management (IPM) framework, and includes choosing similar maturity groups of Bt and non-Bt soybean cultivars, synchronization of planting dates of Bt crop and corresponding refuge area, and adoption of defoliation thresholds to manage target pests in the refuge area in a way to keep its effectiveness as a source of susceptible population.

Keywords: Cry1Ac, oviposition behavior, defoliation in refuge, IRM, IPM

Running title: Oviposition behavior of target pests in Bt soybean and structured refuge

1. INTRODUCTION

Genetically modified soybean has been widely adopted, totaling 95.9 million hectares around the world, comprised of 69.3 million hectares of RR soybean (glyphosate-tolerant) and 26.6 million hectares stacked soybean with RR and insect resistance, expressing Cry1Ac toxin. Brazil had the largest area planted with IR/HT soybean, 20.2 million hectares in 2018[1]. Since 2013, the Bt soybean technology MON 87701 x MON 89788, expressing the Cry1Ac toxin and tolerance to glyphosate, has been adopted by growers. More recently, the pyramided event MON 87751 x MON 87708 x MON 87701 x MON 89788, expressing the Bt toxins Cry1A.105, Cry2Ab2 and Cry1Ac that was approved in 2018, became available for growers to plant in the 2021-2022 crop season [2]. Cry1Ac expresses high levels of control of the two main lepidopteran defoliators, the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, (Lepidoptera: Erebidae) and the soybean looper, *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae). This toxin is a high dose event prior technology release, for *A. gemmatalis* [LC50 (FL 95%)=0.23 (0.15–0.34) µg Cry1Ac mL^{-1}], and even if it is not a high dose for *C. includens*, it shows high levels of control [LC50 (FL 95%)=3.72 (2.65–4.86) µg Cry1Ac mL^{-1}] [3]. This high efficacy has been maintained even after 8 years of pest exposure in the field. Recent report have indicated suppression of *A. gemmatalis* and *C. includens* since the adoption of MON 87701 x MON 89788 soybean in Brazil [4].

However, the risk of selection of resistant individuals to Bt technology should not be ignore, even for high dose events such the MON 87701 x MON 89788 soybean, due to the high exposure of the target defoliator pests to Cry1Ac [5]. The recommendation of refuge in Bt soybean is 20% of the field with non-Bt soybean field, which should have the maximum distance between any plant from the Bt area and the refuge area no higher than 800 meters. This non-Bt soybean field will serve as a source of susceptible individuals, that is expected to randomly mate with possible resistant individuals that survived from the Bt area. This mating will result in susceptible heterozygotes, assuming that the resistance is recessive, and then killed by the Bt plants in next generations [6].

Genetic factors of target populations by Bt technology are important components in the risk of resistance evolution. In addition, there are other aspects that might influence the selection of resistance to Bt technology, such as moth dispersal capacity and mating populations from refuge and Bt fields [7]. Ovipositon behavior of moths is also an key component that needs to be considered when designing refuge reccomendatios. Plants emit volatile organic compounds (VOCs) that play an important role in the search for the host by moths [8]. Thus the presence of Bt and non-Bt plants at the same time and field could impact moths of target pests of the Bt technology if they have differences in the VOCs production. In rice and cotton, no differences among Bt and non-Bt VOCs have been documented [9,10]. This similarity in plant volatiles might explain the absence of oviposition preference between Bt and non-Bt plants of cotton [11,12], cabbage [13], maize [14,15], rice [9], canola [16], and broccoli [17].

Nevertheless, plants under herbivory tend to modify their VOC profile, as the larvae feeding prompts the plant to produce herbivore-induced plant volatile (HIPVs) [18]. This situation could lead to an oviposition avoidance behavior of plants with higher defoliation (what can be expected in the refuge area) as observed in some noctuid species [19–21], This moth behavior prevents future competition for their progeny, diminishes the probability to find natural enemies, and avoid plants with induced resistance and low nutritional value [20].

Feeding of *A. gemmatalis* or *C. includens* in soybean plants increases the emissions of VOC's [22,23]. Structured refuge with non-Bt soybean have higher level of defoliation when compared with Bt soybean field and a high level of egg deposition by female moths of both of these target pests may occur. This hypothesis is due to the fact that females moths may detect differences in the VOC's profile, avoiding the refuge. However, no data have been reported to test this hypothesis of higher oviposition on non-Bt soybean, which is critical for the refuge recommendation in soybean.

In addition, soybeans are planted in large areas, such as in Brazil, where approximately 40K hectares were cultivated with soybean in the 2020-2021 crop season [24]. In extensively large areas, the planting operation can be challenging and growers usually give priority to plant the Bt crop, the expected profitable crop, resulting in delays for the planting of refuge area. This lack of synchronization between Bt and refuge planting dates may lead to a possible differential moth oviposition of target pests to either younger or older fields. Limited studies on oviposition preference on plants of different phenological stages have indicated that *Trichoplusia ni* (Hubner) (Lepidoptera: Noctuidae), *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Pyralidae), and *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) prefer to oviposit on more mature plants [25–27]. The effect of delay of refuge planting and consequent desynchronization of the crop phenology of the Bt crop is a critical aspect when designing refuge recommendations, due to the possible impact in oviposition choice.

In addition, the cultivars available for planting refuge are not isogenic of the Bt cultivars. Then, recommendation of Insecticide Resistance Action Committee in Brazil (IRAC-Brazil) is to select cultivars of the same or similar vegetative phenological growth stage [28]. However, plant morphological differences might occur and there is no information on moth oviposition behavior in a scenario of different cultivars being planted in the Bt and refuge fields.

A possible scenario of oviposition preference of the target pest by Bt soybean towards the Bt fields, either due to avoidance of damaged plants from the refuge, late mature development of Bt fields or cultivar preference could jeopardize the refuge effectiveness. It would increase larvae exposure to Bt crops and accelerate the rate of resistance evolution to Bt soybean. Therefore, this study aimed at understanding the oviposition behavior of *A. gemmatalis* and *C. includens* in Cry1Ac Bt and refuge plants, under larvae injury, different growth stages and cultivars in semi-field and field experiments.

2. MATERIAL AND METHODS

2.1. Semi-field experiments

2.1.1. Plants

Plants of Bt (DM61i59 IPRO and BRS 1010 IPRO) and non-Bt (BRS 413 RR) soybean cultivars were sown in 5 L pots containing substrate and soil (1:1) inside the greenhouse with irrigation as necessary, for all the multi-choice experiments. DM61i59 IPRO and BRS 1010 IPRO were from maturity group 6.1 and 6.2, respectively and indeterminate growth habit. BRS 413 RR from maturity group 6.2 and indeterminate growth habit. The plants were transferred to 5 x 4 x 2.5 m cages placed in the field and right after the moths were released into the cages, by opening the lids of the acrylic cages, allowing the moths naturally fly out. The number of plants per treatment varied according to each experiment. The number of moths released into each cage was 50 pairs of *A. gemmatalis* and 30 pairs of *C. includens*.

2.1.2. Insects

Dual and multi-choice oviposition preference experiments were performed with populations of *A. gemmatalis* and *C. includens*. Two *A. gemmatalis* populations were used in the oviposition preference test in order to have representative data from both scenarios and allowing understanding whether the origin of *A. gemmatalis* population could affect the oviposition behavior. One colony of *A. gemmatalis* had been kept in the laboratory for over 10 years and the other population was collected in a non-Bt soybean field (±1000 larvae), in Campo Verde, Mato Grosso, Brazil, in December of 2018. Larvae was transferred to the Insect Rearing laboratory at Embrapa Soja, Londrina, Paraná, Brazil. One population of *C. includens* was established for this study, collected in Campo Verde, Mato Grosso, in 2018 and transferred to the laboratory.

The colony of both species and populations were kept in laboratory and larvae were fed with an artificial diet previously described [29] inside 200 ml plastic cups until 3rd instar, when every 3 larvae were transferred to 50 ml plastic cups until pupate. Pupae were placed inside transparent acrylic cages (Criartshop, Londrina, Brazil) (32 x 45 x 30 cm) for adult emergence, mating, and egg collection from the sulfite paper (Chamex®, Mogi-Guaçu, São Paulo, Brazil) placed in the inner walls. Eggs were then placed inside plastic cups (200 ml) for larva hatching until the 3rd instar, when every larva was transfer to the plastic cup. For the experiments, at the pupa stage of each species, the insects were separated by sex and placed inside transparent acrylic cages (30 x 32 x 35 cm) for adult emergence. After emergence, the same number of females and males was transferred to third cage. Fifty pairs of *A. gemmatalis* and 30 pairs of *C. includens* were placed in each cage for mating, containing 2 petri dishes with cotton embedded with water and sugar based liquid diet as food source. When the first eggs were observed on the cage wall (around 3 days after adult emergence), the couples were transferred to cages placed in the field with soybean plants, where they remained for 24 hours, and then plants were cut and taken to the laboratory for egg counting [30].

2.1.3. Dual oviposition choice preference between Bt and refuge at different crop growth stages

Dual-choice oviposition preference tests were independently performed for *A. gemmatalis* and *C. includens* in cages placed in the experimental fields. The aim of the experiments was to investigate the effect of the planting delay of the refuge area and consequently oviposition choice in soybean plants in different phenological stages. The experiment was performed in a randomized completely block design, with three treatments. Each treatment corresponded to a dual-choice between Bt and non-Bt plants, where only the non-Bt plants varied at the phenological stage among treatments, as they were sowed 0, 5, and 10 days after Bt plant sowing, respectively for treatment 1, 2 and 3. The experiment were repeated 4 times (replications) and fifteen plants of Bt soybean cultivar BRS 1010 IPRO (Bt) and 15 plants of BRS 413 RR (non-Bt) were placed inside each cage, totaling 30 plants per cage.

In the oviposition preference choice experiment with *A. gemmatalis*, each cage contained Bt plants at the reproductive stage R1. The non-Bt plants were in the phenological growth stages of R1, V8, and V6, in the treatments 1, 2, and 3, respectively. In the oviposition preference choice experiment with *C. includens*, each cage contained Bt plants at the reproductive stage R2 and non-Bt plants in R2, R1, and V7, in the treatments 1, 2, and 3, respectively.

2.1.4. Multi-choice oviposition preference between Bt and non-Bt soybean cultivars

Multi-choice oviposition preference tests were independently performed for *A. gemmatalis* and *C. includens* in cages placed in the field in a randomized completely block design. The cultivars DM61i59 (Bt), BRS 1010 IPRO (Bt), and BRS 413 RR (non-Bt) were used to test a possible oviposition preference towards one or more soybean cultivars. The experiment was conducted when the experimental field with the three cultivars were at V6 stage. Inside each cage, fifteen plants of each cultivar were placed, totaling 45 plants per cage. Each cage was considered one block composed by three treatments (cultivars), and the experiment were repeated four times for each species.

2.1.5. Multi-choice oviposition preference between injured and non-injured soybean plants

Multi-choice oviposition preference tests were independently performed for *A. gemmatalis* and *C. includens* in cages placed in the field in a randomized completely block design. Three experiments were performed with *A. gemmatalis*, one with the laboratory colony and two with the field-derived colony. Two experiments were carried out with *C. includens*. Plants of the soybean cultivars, BRS 1010 IPRO (Bt) and BRS 413 RR (non-Bt) were used only in the experiment with the laboratory colony and cultivars DM61i59 IPRO(Bt) and BRS 413 RR (non-Bt) were used in the other experiments. Part of the non-Bt plants were infested with 4th larva instar of *A. gemmatalis*, 24 hours before moth releasing. Four trifoliates per plant were infested with 3 larvae per trifoliate, contained by organza voile bags (25 x 30 cm) to avoid larva escape. Treatments were: (1) Bt plants without injury; (2) non-Bt plants without injury; and (3) injured non-Bt plants. Fifteen plants of soybean per treatment were placed inside each cage, which was considered one block, that was repeated three or five times depending on the experiment (Table 1).

2.2. Field experiments

Oviposition preference experiments in field were performed for three consecutive crop seasons, 2018-2019, 2019-2020, and 2020-2021. The field experiments were conducted side by side at the experimental field of Embrapa Soja

(23º11’47” S 51º10’53”W), Londrina, Paraná. For detailed information on cultivars, dates and growth stages evaluated, see table 2. Bt and refuge plots of soybean were sown side-by-side in blocks. Each plot was 25 meters long and 9 meters width, 225 m^2 area, 18 planting rows 0.5 meters between rows and 0.14 between plants. Each block was repeated 4 times for each experiment (Fig. S1). The oviposition of natural populations of *A. gemmatalis* and *C. includens* in Bt soybean and its correspondent structured refuge (non-Bt) were compared in two scenarios during each crop season. In the first scenario, no pest management was adopted. In the second scenario, Integrated Pest Management (IPM) was adopted only in the refuge fields, spraying the insecticide chlorantraniliprole (FMC Química do Brasil Ltda, Campinas, São Paulo, Brazil) when the defoliation level achieved the Economic threshold (ET) of 30% in the vegetative stage or 15% in the reproductive stage [31].

Once a week, the larvae scouting was performed at 4 spots per plot, by counting the number of larvae per meter with the beat cloth, and the percentage of defoliation was accessed by visual grading (from 0 to 100%) [32]. A sample of ten plants per plot were collected and inspected in laboratory under a microscope stereoscope for the presence of eggs. Each egg was identified by species level considering morphological characteristics. Eggs of *A. gemmatalis* are blue-greenish right after laid and then they become darker as the embryo develops. The shape is semi-spherical, and the chorion is crossed from the base to the top by nine to 10 well-defined ridges that reach the cells surrounding the micropyle, and between them, there are one or two short, less conspicuous ridges that are connected one to another by lateral bridges [33]. Eggs of *C. includens* vary from pale yellow to cream, their shape is hemispherical, slightly fattened in the top and base. The micropylar rosette has 6-10 petals, surrounded by two concentric rosettes, with gradually bigger petals on the outside border. They have conspicuous ribs and cross-ribs [34]. Eggs identified as *A. gemmatalis* or *C. includens* were counted and the total of eggs per moth species was recorded. Evaluations were weekly performed until the number of eggs were highly reduced at the reproductive soybean growth stage R6 for all the 6 experiments, during the three growing seasons.

2.3. Analysis

Data from the dual-choice oviposition preference between Bt and refuge at different soybean growth stages were submitted to "one sample t test" (Proc *t* test) to test whether the percentage of eggs laid on Bt was different from 50% [35]. Data from multi-choice oviposition preference between different soybean cultivars and between Bt and non-Bt plants under larvae injury were submitted to ANOVA (PROC GLM) followed by Tukey test (α=0.05). For both data, homogeneity of variance and normality of the response variables were checked using residual analysis of the linear model (PROC UNIVARIATE) [35].

The number of eggs data at each soybean growth stage from the oviposition preference field experiments were tested using ANOVA with repeated measures (PROC MIXED), as the same plot was evaluated every week. The model considered cultivar as fixed effect and growth stage as random effect. Homogeneity of variance and normality of the response variables were checked using residual analysis of the linear model (PROC UNIVARIATE). The data of *A. gemmatalis* oviposition from the season 2018-19 of the IPM experiment, 2019-20 and 2020-21 of the experiments without pest control and IPM experiments were $\sqrt{x+1}$ transformed. Data of *C. includens* oviposition from the experiments without pest control of the seasons 2019-20 and 2020-21 were $\sqrt{x+1}$ transformed and oviposition data from the IPM of the 2018-10 crop season experiment were $\sqrt{X}$ transformed. The total number of eggs along the experiments of each cultivar (Bt and non-Bt) and species were submitted to ANOVA (PROC GLM). Means of defoliation percentage of each cultivar were submitted to ANOVA with repeated measures (PROC MIXED), as the same plot was evaluated every week and then compared by the Tukey (PROC GLM), at seasons 2019-2020 and 2020-2021. Defoliation data from the experiments without pest control and IPM of the season 2020-21 were $\sqrt{X}$ transformed. In the season 2018-2019, the defoliation between Bt and non-Bt were compared by Mann-Whitney (PROC NPAR1WAY) within growth stage, because the residue did not attend the normality assumptions.

3. RESULTS

3.1. Semi-field experiments

3.1.1. Dual oviposition preference choice between Bt and refuge at different crop growth stages

There was no significant difference in the *A. gemmatalis* oviposition between Bt and refuge in plants sowed at the same day ($P>0.05$). When refuge field was planted 5 days ($P<0.05$) or 10 days ($P<0.05$) later the oviposition of *A. gemmatalis* expressed in percentage of eggs was significantly higher in Bt soybean plants than in refuge (Fig. 1). On the other hand, the oviposition of *C. includens* on Bt soybean of refuge was not significantly different, independently of the phenological stage of the plants (Fig. 2).

3.1.2. Multi-choice oviposition preference between Bt and non-Bt soybean cultivars

Anticarsia gemmatalis moths showed no oviposition preference towards any soybean cultivar that were tested ($P>0.05$). Similar results were observed for *C. includens,* with no oviposition preference for the three soybean cultivars under study ($P>0.05$) (Table 3).

3.1.3. Multi-choice oviposition preference between injured and non-injured soybean plants

Oviposition between the non-injured and injured soybean plants were not significantly different when exposed to moths of *A. gemmatalis* from laboratory population (P>0.05). Although the experiment performed in February 2019 (n=3) with the field population did not indicate any difference between treatments (P>0.05), in the experiment performed in April 2019 (n=5), a higher percentage of eggs, approximately 40%, was observed in non-injured Bt compared to injured non-Bt (P<0.05). *Chrysodeixis includens* did not show oviposition preference towards any treatment during the two experiments (P>0.05) (Table 1).

3.2. Field experiments

3.2.1. Oviposition preference without pest management in the refuge

During the execution of the experiments, in the three crop seasons, natural infestation of *A. gemmatalis* and *C. includens* were detected in experimental fields. Percentage of defoliation was significantly higher in non-Bt plots from the growth stages, V6, R2, and R3 until the end of the evaluation period, during the 2018-19, 2019-20, and 2020-21 crop seasons, respectively (Fig. 3). Oviposition of *A. gemmatalis* varied depending on the soybean growth stage during the first crop season (P<0.05), as the number of eggs laid on Bt soybean was higher than non-Bt at the V6, R4, R5.2 growth stages. In addition, the accumulated number of eggs throughout the evaluation period was higher in Bt than non-Bt experimental fields (P<0.05) (Fig. 3A). In the 2019-20 crop season, only at R4, there was a significantly higher number of eggs in Bt than non-Bt experimental fields (P<0.05), but no significant difference was observed in the total number of eggs (P>0.05) (Fig. 3B). In the third crop season of the study, there was a significantly higher number of eggs laid on Bt soybean, at the R5.4 and R5.5 growth stages (P<0.05). The total number of eggs follows the same patter during this crop season (P<0.05) (Fig. 3C). Oviposition of *C. includens* on Bt and non-Bt soybean experimental plots, during the three growth seasons under study did not statistically differ (Fig. 3).

3.2.2. Oviposition preference with IPM adoption in the refuge

During the three crop seasons under study, the defoliation in non-Bt plants were higher than Bt plants, achieving the action threshold of 15% defoliation at R3 growth stage (2018-19 and 2019-20 crops seasons) and R5.1 (2020-21 crop season) (Fig. 4). Insecticide was applied aiming to manage the main lepidopteran defoliators *A. gemmatalis*, *C. includens,* and *S. frugiperda.* The number of eggs laid by *A. gemmatalis* was not different on Bt and non-Bt experimental areas, within the same growth stage at all three crop seasons. However, the accumulated number of eggs along the evaluation period was higher in Bt than non-Bt field during 2018-19 (P<0.05) and 2020-21 (P<0.05) crop seasons (Fig. 4A-C). The total number of *C. includens* eggs during the crop seasons was not different on Bt and non-Bt fields during the three crop seasons, as well as at each growth stage in the 2018-19 and 2020-21 crop seasons (Fig. 4).

4. DISCUSSION

The results obtained from this study are the first information on the oviposition behavior of primary lepidopteran defoliators, *A. gemmatalis* and *C. includens* in Bt soybean and the structured refuge. Dual choice semi-field experiments clearly indicated that the current recommendation of planting the refuge at the same time that Bt field, does not change the oviposition pattern of the target lepidopteran pests by Bt technology in soybean. However, a 5 day-delay on planting the refuge resulted in a higher oviposition in Bt plants by *A. gemmatalis.* This preference towards more mature leaves seems to be shared with other lepidopteran species, such as *T. ni* [26], *C. medinalis* [25], and

O. nubilalis (Lepidoptera: Crambidae) [27]. This moth preference for older plants might be attributed to a lower density and length of glandular trichomes observed on leaves of older plants in comparison to younger soybean leaves, as these trichomes act as oviposition repellent to the moth [26], and mechanical defense to larval feeding [36], as observed for *T. ni*. Nevertheless, the same different plant growth stages tested for *A. gemmatalis* had no influence on *C. includens* oviposition choice, which means, the impact on a late refuge planting would not cause oviposition preference towards Bt, and so adults of this species would randomly oviposit between Bt and refuge fields. This difference observed between *A. gemmatalis* and *C. includens* oviposition preference might be due to high randomly plant-to-plant movement of the larvae observed in *C. includens* [37], which means, the moth does not need to be selective for the host site oviposition, as the larvae will disperse in the field. In addition, *C. includens* was observed to oviposit more frequently in the lower part of the soybean plant while *A. gemmatalis* in upper part. Thus, *C. includens* would be less exposed to the trichomes in general, as the lower part has older leaves, where a lower density of trichomes is present, whereas, the upper part of the soybean plant has the youngest leaves, where a higher density of trichomes is present [26]. Thus, it is reasonable that *A. gemmatalis* would show a preferable oviposition behavior for older plants when offered a choice like in the dual oviposition choice experiment. However, it is important to consider that aiming the maintenance of Bt soybean technology, it is crucial to consider the worst case scenario. Therefore, the results of this experiment validate nowadays recommendation of sowing both Bt and non-Bt (refuge) areas at the same date.

No preference on the oviposition of *A. gemmatalis* and *C. includens* on Bt and non-Bt cultivars at V6 growth stage was observed in the present study. The results clearly confirms that the adoption of structured refuge, and its consequent role as source of unselected populations of both pests is not compromised by the inexistence of isogenic lines of Bt and non-Bt in soybean. However, it is important to highlight that the comparison was done between Bt cultivars e non-Bt cultivar of 6.1 and the non-Bt was 6.2 relative maturity group [38]. Those adopted procedures followed recommendation of selecting a cultivar of the similar maturity group of the Bt cultivar as a refuge and planting both cultivars (Bt and refuge area) at the same time [28].

Experiments performed to investigate the impact of plants under defoliation could compromise moth oviposition choice. Previously, the impact of the defoliation in the attractiveness for egg deposition was investigated for *S. frugiperda* in Bt and non-Bt maize in Cuba, based in the hypothesis that plant volatiles emitted when the plant is under larvae feeding could deter oviposition by the moths, and they found an oviposition difference related to the injury, as the more injured the plant the less eggs were observed, which was the case of the refuge plants [39]. In contrast, Brazilian populations of *S. frugiperda* from two different ecoregions, did not show preference among injured and non-injured plants [40]. These contrasting results were attributed to the different landscape, methodology to obtain the results and level of infestation. In the present study, the impact of defoliation of *A. gemmatalis* and *C. includens* was first tested under controlled conditions in semi-field experiments. Moths of *A. gemmatalis* from laboratory colony did not differ between Bt and non-Bt plants with or without defoliation from both species. This result was expected as this population has been reared in artificial diet over 10 years, and has not been exposed to plant host, which might have decreased its ability to recognize plant volatiles. Therefore, a field-derived colony with population originated from soybean producing area in Mato Grosso State, Brazil, was used in the experiments. Only in the experiment performed in April 2019, a difference of percentage of eggs was observed, as more eggs were laid in the Bt than the injured non-Bt cultivar. Nonetheless, *C. includens* did not show oviposition preference neither on Bt or non-Bt plants. This species is known by its polyphagous behavior, with 73 host plants from 29 families [41]. The oviposition behavior in polyphagous pests is typically non-selective [42] and the non-preferred host site observed on this study, is consistent with a highly polyphagous and larval mobility species such as *C. includens*, as previously mentioned.

In order to further investigate the field oviposition behavior of *A. gemmatalis* and *C. includens*, experiments were carried out for three crop seasons (from 2019 to 2021) in Londrina County, north of Parana State, which is the second soybean producer among the States in Brazil [24]. At the soybean growth stages, V6, R4, and R5.2 (2019-2020 crop season), R4 (2019-2020 crop season), R5.4 and R5.5 (crop season 2020-2021), the number of *A. gemmatalis* eggs was higher in Bt experimental areas, and the total number of eggs laid along the evaluation period was also higher in Bt plants during 2018-2019 and 2020-2021 crop seasons. These results demonstrate an oviposition preference for Bt plants over refuge when no insecticide is applied. On the other hand, when IPM was adopted, no difference between Bt and refuge was observed within the same soybean growth stage at all the three crop seasons, only the total number of *A. gemmatalis* eggs along the evaluation period was higher in Bt than non-Bt soybean plants. Although *A. gemmatalis* has a high number of plant hosts, the larvae preferentially feed on plants of the Fabaceae family, as soybean [43], which is consistent with the discrimination of the oviposition site by the moths. This is the first report of oviposition preference to Bt soybean of *A. gemmatalis*, when refuge is used as IRM practice and is a clear indication that refuge area must be carried out under IPM practices. Therefore, pest outbreaks should be controlled whenever economic thresholds are reached or surpassed, thus IPM and IRM are complementary, which means, using Bt technology still requires an IPM strategies either for monitoring resistance to Bt toxins in field or monitoring the pests that are not Bt target and needs an adequate management [44].

Considering the high efficacy of Cry1Ac at controlling this species for 8 years already [4], maintaining that is essential to prevent yield losses in soybean. Therefore, managing the refuge with IPM appears to be an important practice that might reduce the oviposition preference for Bt, which means, the oviposition by the adults will be random, and larvae exposure to Bt will be regular.

Although, no oviposition preference was observed at any refuge situations for *C. includens*, preference behavior was detected in *A. gemmatalis* in semi-field and field experiments. Therefore, in order to preserve the technology, it is necessary to consider the worse scenario, which means, the current recommendation to implement refuges for Bt soybean, considering the synchronization of planting date for Bt and refuge fields, non-influence of the cultivar adopted for the refuge area, and adoption of IPM in refuge based on economic threshold are necessary in order to decrease level of defoliation of *A. gemmatalis* and guarantee the refuge effectiveness.

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Table 1 - Oviposition of *Anticarsia gemmatalis* and *Chrysodeixis includens* in Bt and non-Bt plants with and without injury by larvae in multi-choice semi-field preference experiments. Londrina, PR, Brazil (23° 11' 27.4" S 51° 10' 20.2 W").

Experiment date	Species/ population	Treatment	Soybean growth stage	Mean of percentage of eggs ± standard error (number of eggs)		Statistics		
						CV[1] (%)	F	*P*
February 2018	*A. gemmatalis*/ Laboratory (n[2]=3)	Bt	R1	37.21 ±1.67 (544.67 ±92.83)	a[3]	32.23	0.30	0.7584
		Non-Bt		31.02 ±6.76 (453.33 ±138.81)	a			
		Injured non-Bt		31.77 ±5.33 (457.67 ±99.25)	a			
February 2019	*A. gemmatalis*/ Field (n=3)	Bt	R2	42.73 ±0.98 (132.00 ±35.00)	a	39.78	1.13	0.4083
		Non-Bt		28.68 ±8.10 (89.00 ±29.30)	a			
		Injured non-Bt		28.58 ±7.12 (88.33 ±35.36)	a			
April 2019	*A. gemmatalis*/ Field (n=5)	Bt	R3	39.54 ±2.89 (764.40 ±87.09)	a	20.61	4.44	0.0504
		Non-Bt		33.84 ±2.88 (648.40 ±69.46)	ab			
		Injured non-Bt		26.62 ±1.50 (521.20 ±69.34)	b			
April 2019	*C. includens*/ Field (n=3)	Bt	R2	42.52 ±4.92 (669.70 ±119.84)	a	30.17	2.51	0.1964
		Non-Bt		33.37 ±5.70 (532.0 ±145.34)	a			
		Injured non-Bt		24.11 ±3.27 (409.7 ±133.37)	a			
January 2020	*C. includens*/ Field (n=3)	Bt	R5.1	29.77 ±2.82 (641.7 ±88.68)	a	29.78	0.30	0.7584
		Non-Bt		34.67 ±6.26 (746.3 ±150.86)	a			
		Injured non-Bt		35.57 ±4.27 (757.7 ±80.47)	a			

[1] Coefficient of variation. [2]Number of replicates. [3] Means (±standard error) followed by the same letter in the column within the same experiment do not differ by Tukey test ($P \leq 0.05$).

Table 2 - Information of the field oviposition preference experiments of *Anticarsia gemmatalis* and *Chrysodeixis includens* in Bt and non-Bt soybean cultivars during 3 seasons. Londrina, Paraná, Brazil (23° 11' 27.4" S 51° 10' 20.2 W").

	Season		
	2018-2019	2019-2020	2020-2021
Bt cultivar (Relative maturity group)	DM61i59 (6.1)	BRS1010IPRO (6.1)	BRS1010IPRO (6.1)
Non-Bt cultivar (Relative maturity group)	BRS413RR (6.2)	BRS413RR (6.2)	BRS413RR (6.2)
Sowing date	11/7/2018	11/18/2019	11/17/2020
First evaluation date	12/5/2018	12/19/2019	12/17/2020
Last evaluation date	02/25/2019	03/02/2020	02/24/2021
Soybean growth stages evaluated	V2, V3, V4, V6, R1, R2, R3, R4, R5.2, R5.3, R5.5, R6	V5, R1, R2, R3, R4, R5.1, R5.2, R5.3, R5.4, R5.5, R6	V5, V6, R1, R2, R3, R4, R5.1, R5.3, R5.4, R5.5, R6

Table 3 - Oviposition of *Anticarsia gemmatalis* and *Chrysodeixis includens* in different soybean cultivars in multi-choice preference tests carried out in semi-field conditions in January 2021. Londrina, PR, Brazil (23° 11' 27.4" S 51° 10' 20.2 W").

Species	Soybean cultivar	Mean of egg percentage ± standard error (number of eggs)		Statistics		
				CV[1] (%)	F	*P*
	BRS 413	31.15 ± 2.16 (156.75 ± 35.19)	a[2]			
A. gemmatalis	DM61i59 IPRO	31.25 ± 6.11 (170 ± 52.71)	a	37.58	0.35	0.7202
	BRS 1010IPRO	37.59 ± 6.04 (176.50 ± 29.15)	a			
	BRS 413	24.19 ± 3.12 (369.50 ± 54.37)	a			
C. includens	DM61i59 IPRO	35.53 ± 4.85 (570.75 ± 138.46)	a	29.80	2.77	0.1404
	BRS 1010IPRO	40.28 ± 4.02 (623.00 ± 116.67)	a			

[1]Coefficient of variation. [2]Means (±standard error), followed by the same letter in the column for each Lepidoptera species do not differ by Tukey test ($P < 0.05$). Number of replicates: 4.

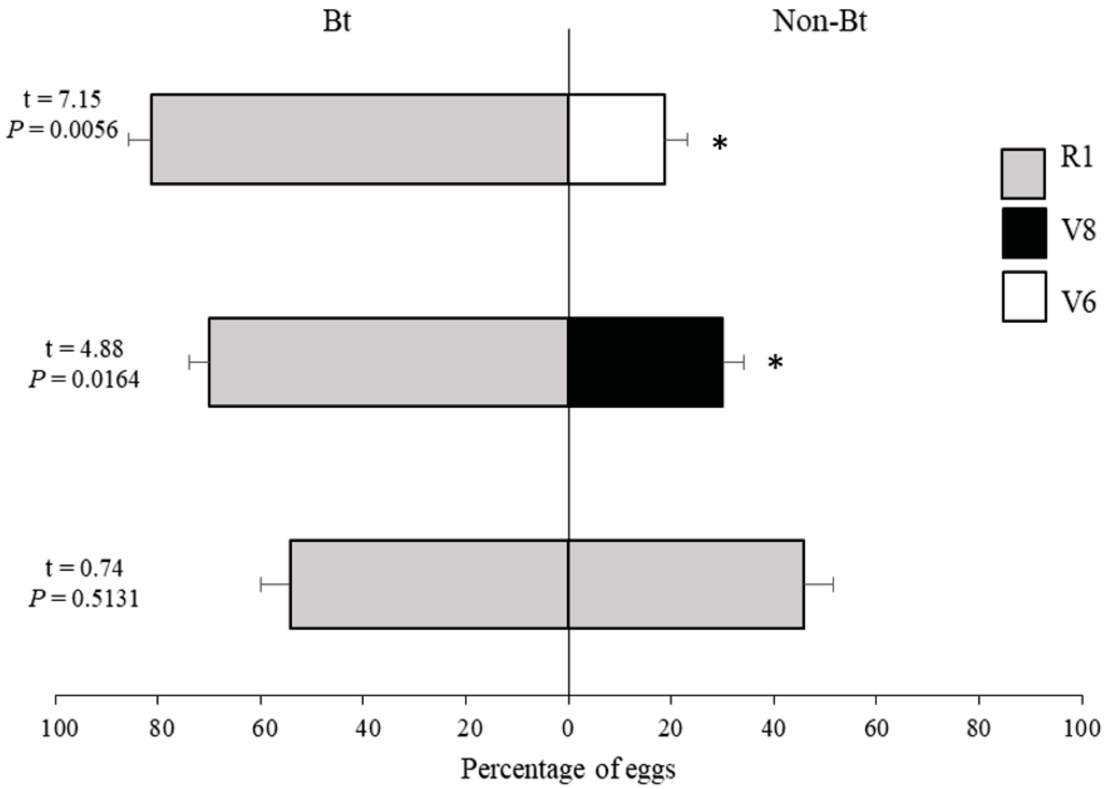

Figure 1 - Percentage (mean ± standard error) of *Anticarsia gemmatalis* oviposition in Bt and refuge (non-Bt) compared by the one sample t test (n=4, α=0.05), in a dual choice preference test. Refuge planted 0, 5 and 10 days after Bt. Bar colors correspond to soybean growth stage at the oviposition preference test.[1] *Percentage of eggs in Bt plants was significantly higher than 50% (P<0.05).

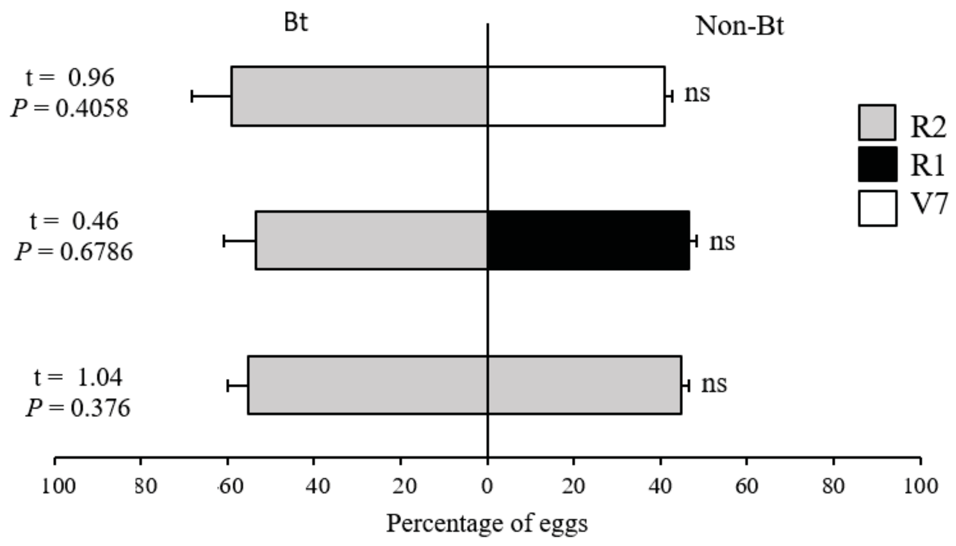

Figure 2 - Percentage (mean ± standard error) of *Chrysodeixis includens* oviposition in Bt and refuge (non-Bt) soybean plants 24h after moth releasing compared by the one sample t test (n=4, α=0.05), in a dual choice preference test. Refuge planted 0, 5 and 10 days after Bt. Bar colors correspond to soybean growth stage at the oviposition preference test.[1] ns: Percentage of eggs in Bt plants was not significantly higher than 50% (P>0.05).

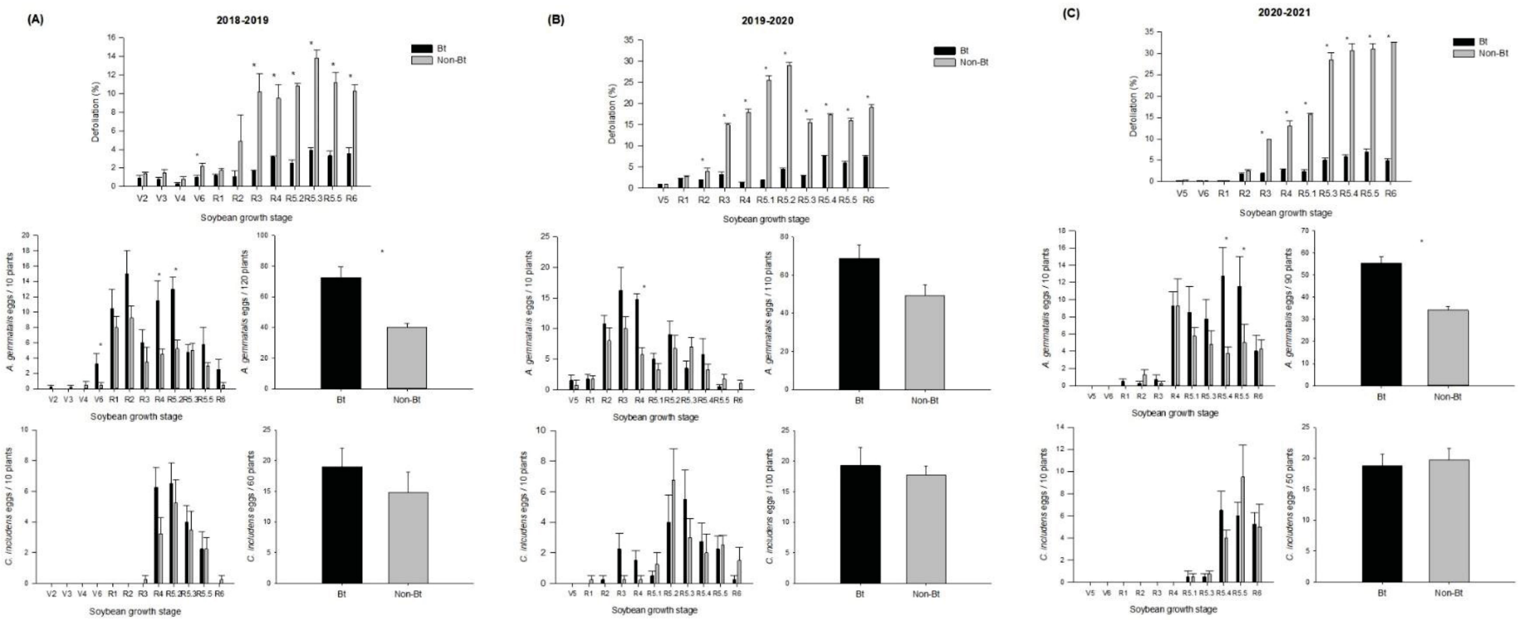

Figure 3 - Natural infestation of *Anticarsia gemmatalis* and *Chrysodeixis includens* **without pest management**. Percentage of defoliation (on the top), number of eggs per soybean growth stage (on the left) and the sum of eggs laid throughout the season (on the right) at soybean crop seasons **(A)** 2018-19, **(B)** 2019-20 and **(C)** 2020-21. Oviposition data of both species from seasons 2019-20 and 2020-21 were $\sqrt{x+1}$ transformed. Defoliation data of the season 2020-21 were $\sqrt{X}$ transformed *Bt and non-Bt plots are significantly different by Tukey or Mann-Whitney test (only the defoliation in 2018-19) within the same growth stage.

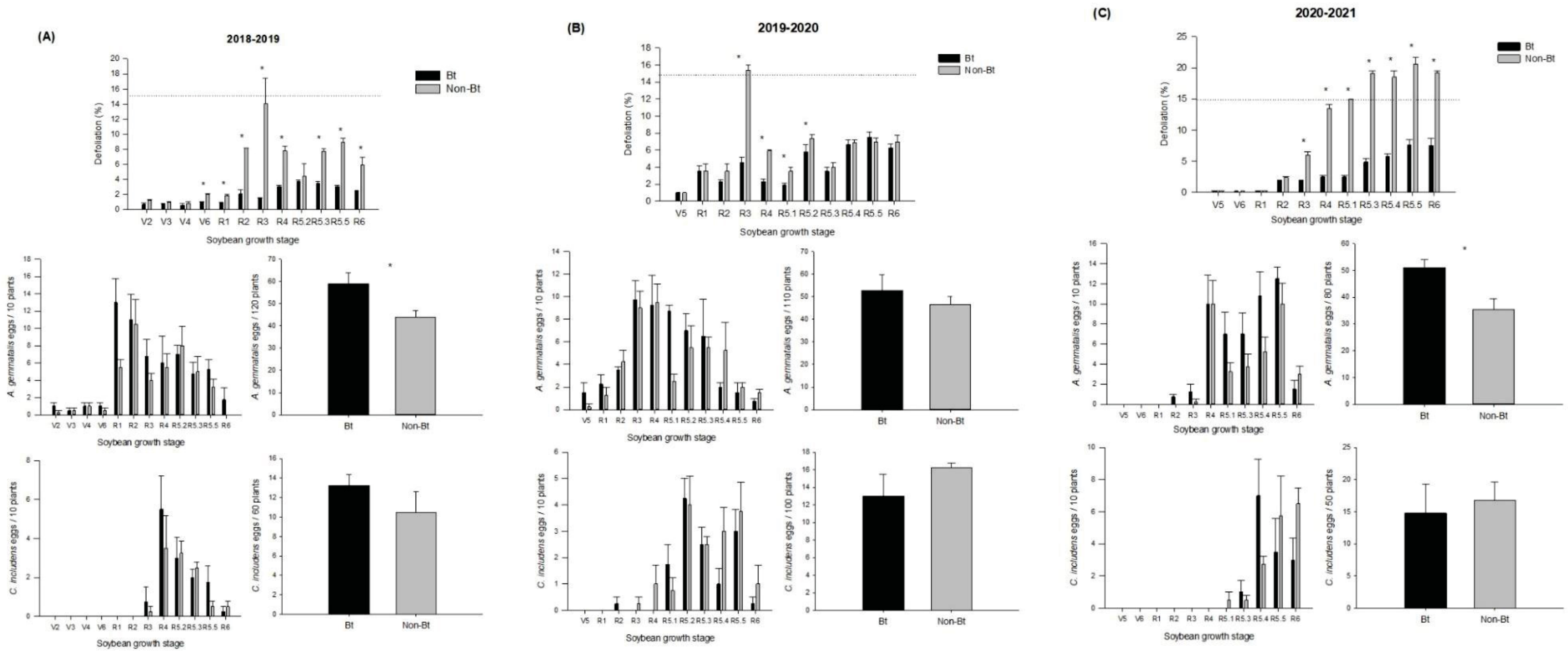

Figure 4 - Natural infestation of *Anticarsia gemmatalis* and *Chrysodeixis includens* adopting **Integrated Pest Management (IPM).** Percentage of defoliation (on the top). Economic threshold for insecticide spraying of 15% defoliation: dotted lines. Number of eggs per soybean growth stage (on the left) and the sum of eggs laid throughout the season (on the right) at soybean crop seasons **(A)** 2018-19, **(B)** 2019-20 and **(C)** 2020-21. . Oviposition data of *A. gemmatalis* from the three seasons were $\sqrt{x+1}$ transformed. Oviposition data of *C. includens* from season 2018-219 were $\sqrt{X}$ transformed. Defoliation data of the season 2020-21 were $\sqrt{X}$ transformed.*Bt and non-Bt plots significantly different by Tukey or Mann-Whitney test (only the defoliation in 2018-19) within the same growth stage.

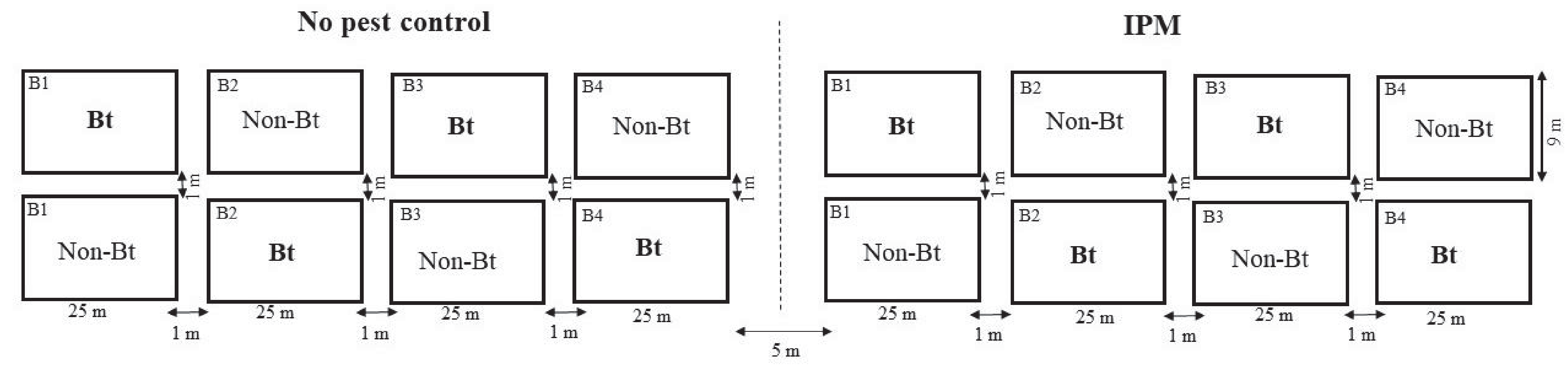

Figure S1- Experimental scheme of the field oviposition preference of *A. gemmatalis* and *C. includens* in Bt and non-Bt soybean performed at 2018-19, 2019-20 and 2020-21 crop seasons. No pest control: no insecticide spraying. IPM: insecticide sprayed based on Economic threshold of 30% defoliation (vegetative stage) or 15% defoliation (reproductive stage). **Bt:** Cultivars DM61i59 IPRO (2018-19) and BRS 1010 IPRO RR2 (2019-20 e 2020-21). **Non-Bt:** Cultivar BRS 413 RR (all crop seasons).

CHAPTER 3: FLIGHT RANGE OF *ANTICARSIA GEMMATALIS* AND *CHRYSODEIXIS INCLUDENS* IN SOYBEAN FIELDS: BASIS FOR THE REFUGE STRATEGY ESTABLISHMENT

ABSTRACT

Bt soybean adoption has increased in the last crops seasons, and Brazil is responsible for the largest cultivated area. Insect resistance management strategies are necessary to delay the development of resistance to the Bt toxin in the target pests, such as *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae) and *Chrysodeixis includens* Walker (Lepidoptera: Noctuidae). One of them is planting refuges close enough to Bt areas to guarantee that rare resistant individuals will be able to fly and mate with susceptible ones from the refuge area. Limited information is available to confirm if the current maximum 800 meters recommended corresponds to the flight range of these pests. We performed mark-release-recapture experiments with moths of both species during 2019-2020 and 2020-2021 crop seasons in a soybean production area. Based on the recapture of marked moths of *A. gemmatalis*, dispersal capacity of this species flight ranges from 50 to 1000 meters, and 100% of recaptured female moths indicated mated status. Marked *C. includens* moths were recaptured in the range of 50 to 500 meters from the released point, and all females were mated. Wild moths of both species, as well as *Rachiplusia nu* Guenée (Lepidoptera: Noctuidae), were also trapped during the study. Our results support the current refuge distance recommendation for *A. gemmatalis*. The high number of mated females of both species near to the release point of the marked insects indicate that this mating behavior could compromise the assortative mating assumption, which negatively affects the refuge effectiveness by increasing the possibility of increase of resistant alleles in the population of both target species.

Keywords: Cry1Ac, resistance, IRM, mark-release-recapture, behavior, refuge design

1. INTRODUCTION

The development of genetically modified crops expressing insecticidal proteins from *Bacillus thuringiensis* (Bt) provided a valuable biotechnology strategy for pest management (Brookes and Barfoot, 2018). This pest management strategy was widely and fast adopted, as the global planted area increased 113 times in the last 23 years, achieving 191.7 million hectares in 2018. The USA leads the planted area with Bt crops (75 million hectares), followed by Brazil (51.3 million hectares), with soybean, maize, and cotton as the main crops (ISAAA, 2018). The first Bt soybean generation of cultivars, expressing Cry1Ac protein, proved to be effective against important soybean pests, such as *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae), *Chrysodeixis includens* (Walker), *Chloridea virescens* (Fabricius), and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Bernardi et al., 2014, 2012; Dourado et al., 2016) turning into an important tool for the soybean Integrated Pest

Management (IPM) (Bueno et al., 2021). Particularly to Bt soybeans, Brazil was the first country to adopt Bt technology in soybean, since 2013 (James, 2013), followed later by other countries from South America and Asian countries (ISAAA, 2020).

Benefits provided by Bt crops are the reduction of yield losses (Sanglestsawai et al., 2014), and the decrease in insecticide adoption targeting lepidopteran pests (Brookes and Barfoot, 2020). In addition, the specific mode of action of Bt traits promotes the preservation of beneficial insects (Romeis et al., 2019). However, the high dose expression of Bt toxins in the crop and long term exposition of pests during the crop season increase the risk of selection of field resistant populations, which can compromise the technology performance (Tabashnik et al., 2014). The event MON 87701 x MON 89788, expressing Cry1Ac toxin, has provided high performance in the management of the two main defoliator species, *A. gemmatalis* and *C. includens*. However, since the 2019-2020 crop season, outbreaks of *Rachiplusia nu* Guenée (Lepidoptera: Noctuidae) have been reported in Bt soybean in the north portion of Paraná State and in the center of São Paulo State, and *Crosidosema aporema* Walsingham (Lepidoptera: Tortricidae) in the central-north portion of Paraná State, in the southern São Paulo and around the Distrito Federal (Bueno and Sosa-Gómez, 2021; Nardon et al., 2021, Horikoshi et al., 2021b).

Insect Resistant Management (IRM) aims to delay the evolution of resistance of insects targeted by Bt crops. Adopting of refuges is one of the IRM strategies, which consists of planting a non-Bt field near the Bt crop. This non-Bt field provides a source of susceptible individuals to Bt traits, that is expected to randomly mate with possible resistant individuals that survive from the Bt crop. This random mating results in heterozygotes insects to resistance, which are expected to be susceptible, based on the assumption that the resistance is a genetic recessive trait in target pests (Gould, 1994). However, the hypothesis of random mating based on the dispersal capacity of target pest still lack scientific data. The dispersion capacity of *C. includens*, also known as soybean looper, has never been documented in the literature. In the same way, the dispersal capacity of *A. gemmatalis*, previously reported as 900 meters from released marked insects has been reported only in soybean cultivated with sugarcane (Caixeta, 2014), which is not representative crop system adopted in large areas of soybean in Brazil, and around the world. The crop environment can affect the moth biological traits, including their flight range (Qureshi et al., 2005). Currently, the recommendation of refuge in soybean for Brazilian growers is that the maximum distance between any plant from the Bt soybean field should not be further than 800 meters from the refuge (IRAC Brazil, 2018). This general recommendation is based on previous studies with *Ostrinia nubilalis*

Hübner (Lepidoptera: Pyralidae) in the U.S. (Hunt et al., 2001; Qureshi et al., 2005) and *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae) (Vilarinho et al., 2011), which has been recommended by the U.S. Environmental Protection Agency (USEPA) (USEPA, 2001). The maximum distance of a plant of Bt maize to the non-Bt plant from the refuge must be approximately 800 meters (half-mile) (US EPA, 2018). However, this recommendation should not be adopted as one-size-fit-all, since there are other target lepidopteran species to Bt technology besides *O. nubilalis* and *S. frugiperda*. Additionally, the differential dispersion capacity of species target by Bt crops, region-specific information of the crop system adopted in each region should be considered when designing refuge recommendations. Therefore, this study was conduct aiming to document the dispersion capacity of two economic pests, *A. gemmatalis* and *C. includens*, in soybean, target by the Bt technology. The main contribution of the study was to validate refuge recommendations for Bt soybean, the maximum distance of 800 meters between the refuge and Bt crop.

2. MATERIAL AND METHODS

2.1.1. Insect colonies

Approximately 1000 larvae of *A. gemmatalis* were collected in a non-Bt soybean field in December 2018, in Campo Verde, Mato Grosso. They were transferred to the insect rearing laboratory at Embrapa Soja (Londrina, Parana) and reared in artificial diet (Greene et al., 1973) until pupation. Pupae were placed inside transparent acrylic cages (Criartshop, Londrina, Paraná, Brazil) (32 x 45 x 30 cm) for adult emergence, mating, and egg collection from the sulfite paper (Chamex®, Mogi-Guaçu, São Paulo, Brazil) placed in the inner walls. Eggs were then placed inside plastic cups (200 ml) (Copaza®, Içara, SC, Brazil) for larva hatching until the 3rd instar, when 3 larvae were transfered to one plastic cup (50 ml) with artificial diet. The insect colony was kept in the laboratory until used in the experiment, at the 12th and 24th generation, in 2019-2020 and 2020-2021 seasons, respectively.

Eggs of *C. includens* were purchased from PROMIP (Piracicaba, São Paulo, Brazil) and once they hatched, the neonates were placed inside plastic cups (Copaza®, Içara, Santa

Catarina, Brazil) filled with a small amount of artificial diet. The 3rd instar larvae were transferred to plastic cups (50 ml) with the same artificial diet previously described, in a total of three larvae/cup, and kept in the cups until pupation. The moths were used in the experiments performed during 2019-2020 and 2020-2021 crop seasons.

2.1.2. Insect marking, release, and recapture

Close to adult emergence, the pupae of both species were placed inside plastic boxes, 11 x 11 x 3.5 x cm (Gerbox®, São José dos Pinhais, Paraná, Brazil), and covered with polystyrene balls impregnated with 1162R Luminous Powder-Red (BioQuip Products, Inc., California, USA), a fluorescent powder under black light (Taschibra, Indaial, Santa Catarina, Brazil). Moths emerging inside the plastic boxes acquired the powder in their bodies allowing their differentiation from the wild moths in the field.

The cages were daily checked for emerged moths, and at the beginning of the day the cages with the moths were taken to the release point, in the field. All the moths released were younger than 24 hours old, and the moth releasing was performed by removing the cage lids, allowing the moths to disperse in the field. The release point was in a commercial soybean field (23°7'37.66"S 51° 1'10.16"W), located in the Fazenda Santa Maria, in Sertanópolis, Paraná. The field was 30 km away from Embrapa Soja, where the insects were kept in laboratory.

Commercial ball-funnel type traps containing feeding lures, Lurex®, both from Isca (Isca Tecnologias Ltda, Rio Grande do Sul, Brazil), were placed every 100 meters from the release point up to 1000 meters towards four directions: northeast, southeast, southwest and northwest. The total traps placed were 40 and 44, in the 2019-2020 season and 2020-2021 crop seasons, respectively (Fig. 1a), since 4 points were added at 50 meters from the release point towards the four directions (Fig. 1b), in the 2020-2021 crop season. In the 2020-21 season, pheromone trapping with sex lure Bio Pseudoplusia® (Biocontrole, São Paulo, Brazil) were placed at the same points of the ball-funnel type traps as an attempt to increase the recapture of *C. includens*.

The ball-funnel type traps were daily inspected and replaced when there were trapped moths in the net sac. The same procedure was done for the sticky liner of delta traps with caught moths in the 2020-2021, with replacing of the liner. For both trap types, bottoms and sticky liners were identified with a label containing the position number, including direction and distance from the released point. Although trapped moths caught in the delta traps were dead, those caught in the ball-funnel were often alive, thus the net sac was placed in the refrigerator for a few minutes to kill the insects in order to facilitate the species identification and the recognition of marker presence. Under UV black light, the samples were carefully examined, and fluorescent moths were separated by the wild moths. Marked and feral moths were frozen for later sex identification and spermatophore counting of mated females.

2.1.3. Analysis

Data collected from 2019-2020 and 2020-2021 crop seasons were presented in graphs with the numbers of females, males and the total of marked and wild moths captured at each distance towards the four directions.

3. RESULTS

3.1. Recapture of marked moths

The percentage of *A. gemmatalis* marked moths recapture during the 2019-2020 crop season experiment was 0.55% in the first release (Table 1). From the 14 moths recaptured, seven were males and seven females, which have mated at least once (Fig. 2a). Half of the caught marked moths was recaptured near the release point, with 43% of the marked moths at 100 m, and 7% at 200 m. The other half of the marked moths was recaptured further from the release point, with 22%, 14%, 7%, and 7% of the recaptured moths trapped at 400, 500, 700, and 1000 m, respectively (Fig. 2a). In the 2020-2021 crop season experiment, the percentage of *A. gemmatalis* recapture was lower than the previous year and only 0.11% of the marked moths, corresponding four females were recovered (Table 1). One female was recaptured in the closest trap to the release point, 50 m, and the other three females were recovered at 200 m. The four females had mated once (Fig. 2b).

The recapture of *C. includens* marked moths was lower than *A. gemmatalis* marked moths (Table 1). One mated female was recovered at 100 m, and the other female at 500 m (Fig. 3a). In the 2020-2021 crop season, only one marked male was recaptured in the delta trap at 200 m from the release point. No females were recaptured in the 2020-2021 crop season (Fig. 3b).

3.2. Capture of wild moths

Wild *A. gemmatalis* moths were captured by all the ball-funnel type traps during the 2019-2020 crop season experiment, totaling 959 moths caught along the trapping period (from 12/18/2019 to 01/07/2020). The highest number of captured moths were in traps positioned along the southeast direction (304), followed by southwest (249), northeast (209), and northwest (197) (Fig. 4a). At any direction, most moths captured were males (Fig. 4c). From the total of 307 females, 38 (12.38%) had not mated, and the majority, 163 (53.09%) had mated once. One hundred six caught females had mated more than once, with 79, 25, and 2 moths with 2, 3, and 4 spermatophores, respectively (Table 2).

During the 2020-2021 crop season experiment, a lower number of wild *A. gemmatalis* were captured, 65 moths total from 12/17/2020 to 01/15/2021. Similarly, to the results from the previous crop season, the highest number of moths were captured along the southeast direction (25), followed by southwest (21), northeast (12) and northwest (7) (Fig. 4b). The same pattern of more captured males than females were, except for the northeast, where around 70% of the moths were females (Fig. 4d). From a total of 17 females captured in the traps, almost all of them, 15 females (88.24%) had mated only once, and 2 females mated twice (Table 2).

Soybean looper wild moths were captured, totaling 38 moths, being 13 in the southeast direction, 11 at the southwest, 10 in the northwest, and 4 at the northeast (Fig. 5a). Unlike *A. gemmatalis*, the proportion of females were slightly higher than males, except for the southwest direction (Fig. 5c). The total of 18 females were caught, and 7 female moths (38.89%) had not mated, 4 females mated once, and other 4 had mated twice. Three captured female moths indicated mating, 3, 4 e 5 times, respectively (Table 2).

In the 2020-2021 crop season, approximately the same amount of *C. includens* were caught, in a total of 39 moths, being 18 in southwest, 11 at northeast, 6 at southeast, and 4 at northwest from the release direction (Fig. 5b). Males were predominant, with more than 75% of the total captured moths, in all the directions (Fig. 5d). Only 5 females were captured during this season, from which 2 (40%) were not mated, and the other 3 females indicated mating 1, 4 and 5 times, respectively (Table 2).

Another lepidopteran species that was cross-attracted during the trapping in both crop seasons was *R. nu*, also known as sunflower looper. The total of 109 *R. nu* moths were caught in the 2019-2020 crop season at the southwest (47), southeast (43), northeast (10), and northwest (9) directions from the release point (Fig. 6a). The ratio of females and males were around 50% each (Fig. 6c). From the total of 60 females, 9 (15%) had not mated, but the majority, 23 (38.33%) mated only once, 16 (26.67%) mated twice, 3 (13.33%) three times, 2 females mated 4 times, and other 2 females mated 5 times (Table 2).

In the 2020-2021 crop season, a similar number of *R. nu* was captured, 113 moths, at the northwest (37), southwest (28), northeast (28) and southeast (20) directions from release point (Fig. 6b). Unlike the previous year, the male proportion was much higher than females (Fig. 6d). Only 10 females were captured, from which 50% had mated once, 20% (2 females) mated twice, and the 3 other females, mated 3, 4 and 7 times each moth, the later was the maximum number of spermatophores observed in a moth of all the 3 species (Table 2).

4. DISCUSSION

Primary defoliators have successfully been managed by Bt soybean, MON 87701 x MON 89788 event, expressing Cry1Ac, which is a high-dose event for *A. gemmatalis* and near-high-dose for *C. includens* (Bernardi et al., 2012). No resistant populations of these two target species have been reported after eight years of the commercial release of Bt soybean. However, it becomes essential to deeper understand the interaction of IRM practices with these species in order to maintain the efficacy of the management tool in soybean production.

The trapping method used in this study demonstrated to be appropriated to capture wild moths of *A. gemmatalis*, *C. includens* and *R. nu* especially in the 2019-2020 crop season, when 959, 38 and 109 moths of each species were captured within 21 days, respectively. The releasing technique chosen was based on tests, where we first try to leave the pupa in the field with subsequent adult emergence. However, the study was performed during rainy season (Pereira et al., 2008), and a negative effect on the pupal infestation in field compromise the method. The alternative option adopted was to transfer emerged moths every morning to the field.

Although mark-release recapture experiments have been used for several Lepidoptera species, to document the dispersal capacity, the moth recapture rate is variable between experiments and species (Caixeta, 2010, 2014; Qureshi et al., 2006; Tavares et al., 2019; Vilarinho et al., 2011). In this study, recapture of *A. gemmatalis* moths was 0.55% and 0.11% in 2019-2020 and 2020-2021 crop seasons, respectively. During the first moth release in 2019-2020 crop season, the soybean cultivars were approximately in R2 growth stage and 40 cm, height. The accumulated rain between the period of the first and last marked moth releases was 76 mm (measured by pluviometer installed in the area). In this condition, the highest number of moths recapture was 14 moths, from 100 to 10000 meters far from the release point. Fifty percent of the marked moths (7 individuals) in the 2019-2020 crop season were caught up to 200 meters from the release point, and 100% were caught up to this same distance in 2020-2021 crop season. This distance of recaptured moths is lower than the recommended maximum distance between Bt crop and refuge area. However, 50% of the *A. gemmatalis* marked moths from the first release was recaptured at distances between 400 and 1000 meters. The rate of *C. includens* recapture was lower, with 0.02% and 0.07%, during the 2019-2020 and 2020-21 crop seasons, respectively. Recapture distances varied between 100 and 500 meters, and this are the first reported of distance dispersal capacity of *C. includens.*

Dispersal capacity of lepidopteran pests associated with corn has also reported a higher percentage of moths recaptured closer distances from the release point. For example, 99% of *O. nubilalis* was recaptured at 350 meters from the release point in a mark-release study performed in Kansas, USA (Qureshi et al., 2005). However, in another study in Iowa, USA, high distances of 23 to 49 km from the release point were recorded for the same species (Showers et al., 2001). This contrasting data shows that environmental conditions play a role in the moth dispersion. Also, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) egg albumin marked moths were mostly recaptured at 150 m from the releasing point, but some individuals were recovered as far as 1600 meters (Tavares et al., 2019).

Mating status of moths is an important information, providing reproductive behavior while dispersing in the agricultural landscape. The knowledge of how far the moths disperse before they mate is crucial, as it affects the expected random mating between resistant individual that emerge from the Bt area with susceptible individuals from the refuge, and consequently decrease of allelic frequency for resistance in the population (Fitt et al., 2004). The results showed that even at very small distances, such 100 meters from the release point, females of *A. gemmatalis* and *C. includens*, indicated mated status, by the presence of at least one spermatophore. These findings show that the females of these species may find a male to mate before they disperse from the adult emergence area. In a laboratory study, *A. gemmatalis* was documented ovipositing before taking long flights, although they would still oviposit after flying longer distances (WALES et al., 1985). Such mating behavior, possibly around the adult emergence area and before moths engaging in dispersion negatively affects the random mating assumptions for the refuge effectiveness, since homozygote insects for resistance from the Bt area may mate prior to dispersion. The risk of selection for resistance population of *C. includens* from Bt soybean crop may be minimized if males of this species are prompted to fly higher distances reaching the refuge area as reported in tethered flight of males, whose flight was up to 15 km (Caixeta, 2014). In the present study, one marked male of *C. includens* was recovered 200 m from the release point. In the case of *A. gemmatalis* one marked male was recovered 700 m from the release point, and future studies should validated the male dispersion capacity of these species.

The dispersion distance of the wild (non-marked) moths of *A. gemmatalis* and *C. includens* trapped during this study cannot precisely be estimated. However, the higher number of the wild moths of these species were from traps placed towards southeast (SE) and southwest (SW) directions, which were close to the refuge (non-Bt) fields. We hypothesized that these wild moths dispersed from the non-Bt soybean field, in both crop seasons. This observation might be in accordance with our data of the marked recaptured moths, where most of the moths remained near the place they were released.

Wild moths of *R. nu* were captured in higher amounts in the northwest direction in 2020-2021 crop season, which traps were placed between two Bt fields. The occurrence of *R. nu* in Bt soybean (Bueno and Sosa-Gómez, 2021; Nardon et al., 2021) in Brazil leads to hypotized that these moths were originated from experimental Bt soybean fields.

The mating status of wild moths of the *A. gemmatalis*, *C. includens*, and *R. nu* captured in the trapping during this study was also assessed. Both unmated and mated females were captured in the traps. Mating frequency of *A. gemmatalis* moths ranged from one to four times, while *C. includens* were found to mate one to five times, and *R. nu* was found with up to seven spermatophores in one female. Wild moths of *A. gemmatalis* and *R. nu* indicated to mate at least one time, independently of the distance and direction of trapping. In the case of *C. includens*, the wild moths captured during the trapping indicated 40% of not mated females.

This is the first study documenting dispersal of both species, *A. gemmatalis* and *C. includens*, performed in fields cultivated with large fields of Bt-soybean and refuge area, in 274 hectares and 193 hectares, respectively. The maximum dispersal distance of *A. gemmatalis* female was recorded 1000 m far from the released point of marked insects, which is further of the 800 m distance recommended (IRAC Brazil, 2018), when planting structured refuge. On the other hand, the number of spermatophores observed in the females trapped up to 500 m, demonstrates a probability of females to mate before they disperse, which may jeopardize the refuge strategy, with resistant insects from Bt field dispersing when already mated.

Based on the results obtained for *C. includens* dispersal, the maximum distance of trapped marked insects was 500 meters, lower than the recommendation of refuge area for Bt soybean. The low dispersion capacity combined with the mating occurring around the releasing point of marked moths indicate that *C. includens* should be focused when designing and recommending IRM strategies in Bt soybean. This priority is based on this pest economic impact and host plant range (Baldin et al., 2014; Moscardi et al., 2012; Specht et al., 2019), and reports of insecticide resistance (Stacke et al., 2019). Thus, the near-high-dose of Cry1Ac soybean (Bernardi et al., 2012) is a value management tactic for this defoliator, which cases of field evolved resistance has not been reported in soybean production. In addition, infestations of *R. nu* have been reported in Bt soybean (Bueno and Sosa-Gómez, 2021; Nardon et al., 2021). Since morphological differentiation of this species from *C. includens,* based on larva and wings of adults are challenging (Herzog, 1980; Shaw, T.J. et al., 2021), resistance monitoring programs should focus, on genitalia dissections or DNA analysis to differentiate the infestation of species.

Although *R. nu* is not listed as a target pest of Cry1Ac Bt soybean, a CL_{50} of 0.70 µg.mL^{-1} Cry1Ac toxin for one population from the southern Brazil, Rio Grande do Sul (Yano et al., 2012), which raises a concern on a field resistance evolving to Cry1Ac allowing an increase on populations of this species into soybean growing areas. The second generation of Bt soybean, with pyramided events have shown efficacy on the management of this species in preliminary studies, thus maintain structured refuges for *R. nu* in this second generation soybean is crucial (Bueno and Sosa-Gómez, 2021), as well as, understand its flight capacity to better design structured refuges.

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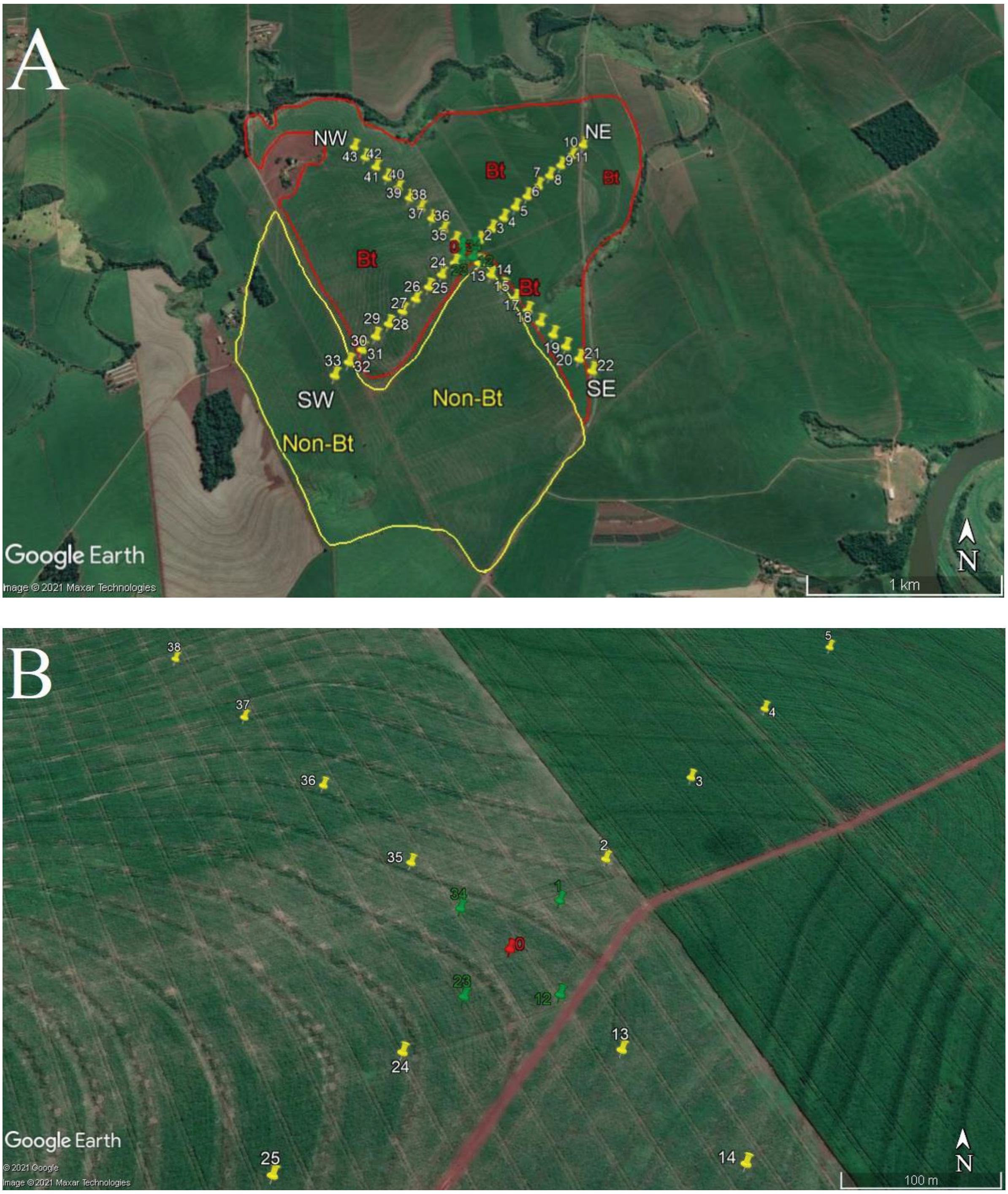

Figure 1 – **(A)** Release point and traps positions in the soybean commercial field **(B)** Highlight on the release point and nearby traps at 2020-2021 season. Point 0: release point (red); Points in yellow are 100 m distant from each other towards the four directions (only in 2019-2020 season); Points in green (1, 12, 23, 34) are 50 meters from the releasing point (only in 2020-2021 season). Sertanópolis, Paraná, Brazil.

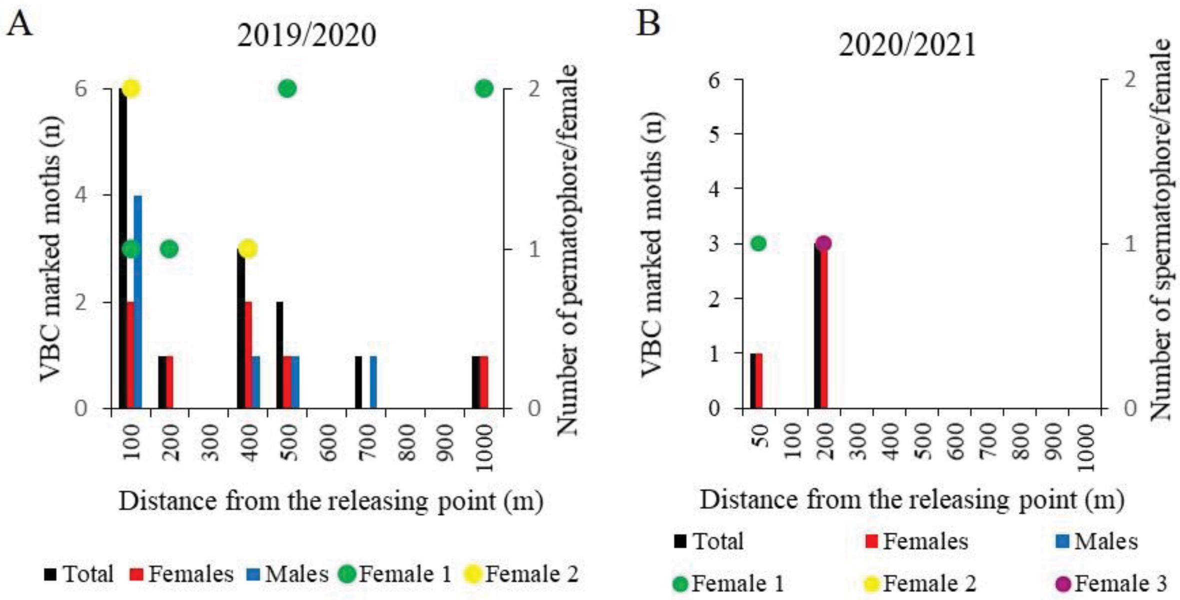

Figure 2 – Number of marked *Anticarsia gemmatalis* moths recaptured in each trapping distance (bars - left axis), and the number of spermatophores of the mated females (circles - right axis) at **(A)** 2019-2020 and **(B)** 2020-2021 crop seasons.

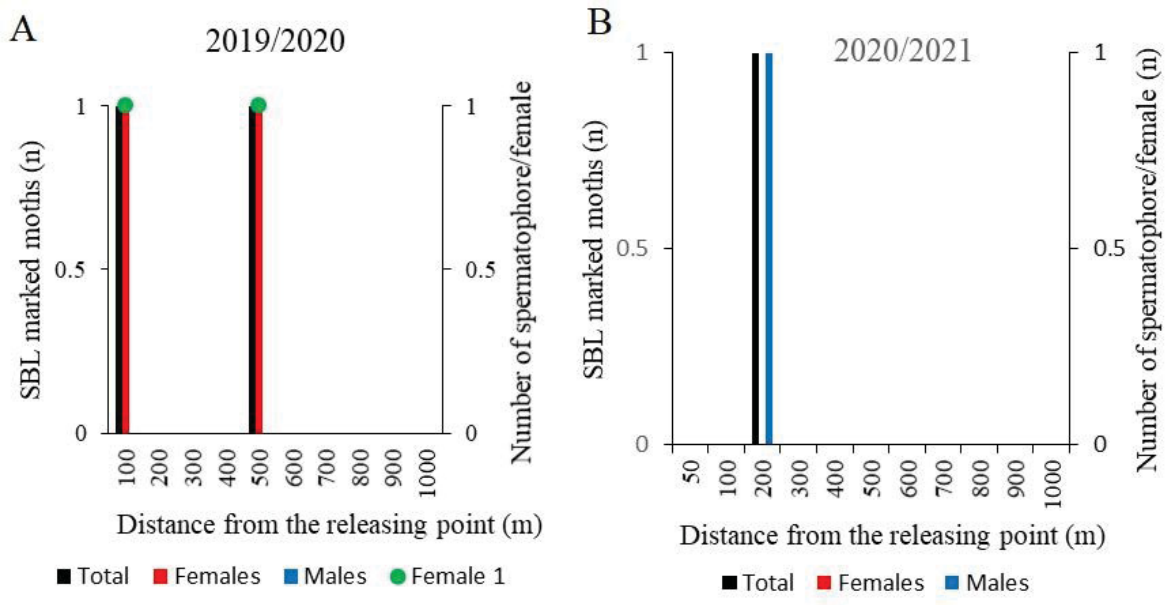

Figure 3 – Number of *Chrysodeixis includens* moths recaptured in each trapping distance (bars - left axis) and the number of spermatophores of the mated females (circles - right axis) at **(A)** 2019-2020 and **(B)** 2020-2021 crop seasons.

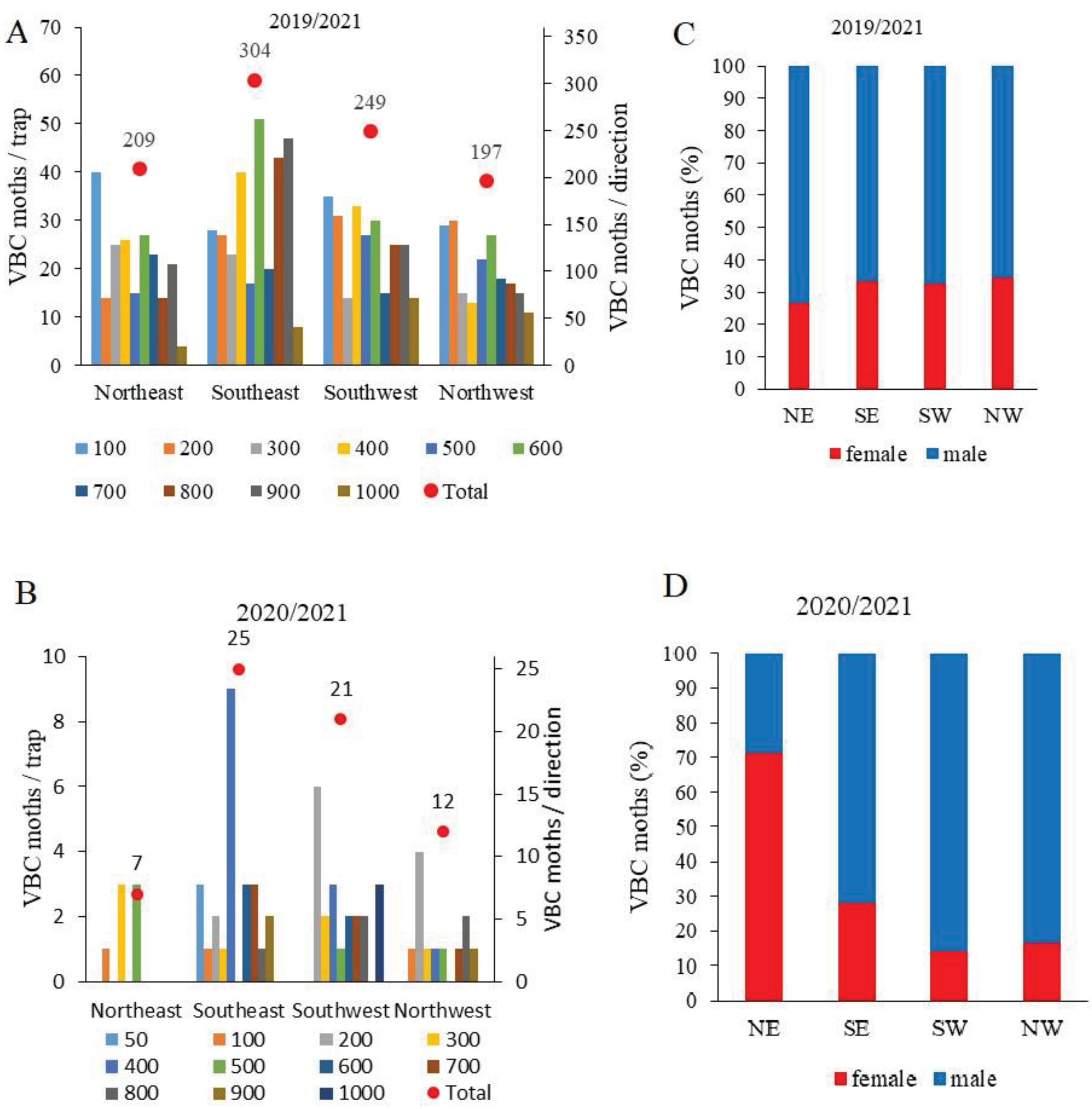

Figure 4 – Wild *Anticarsia gemmatalis* moths captured at each trapping point the four cardinal directions (bars - left axis), and the total moths captured per cardinal direction (red circles - right axis) in 2019/20 **(A)** and 2020-2021 **(B)**. Proportion of females and males captured at each cardinal direction in the 2019-2020 **(C)** and 2020-2021 **(D)**. NE: northeast, SE: southeast, SW: southwest, NW: northwest.

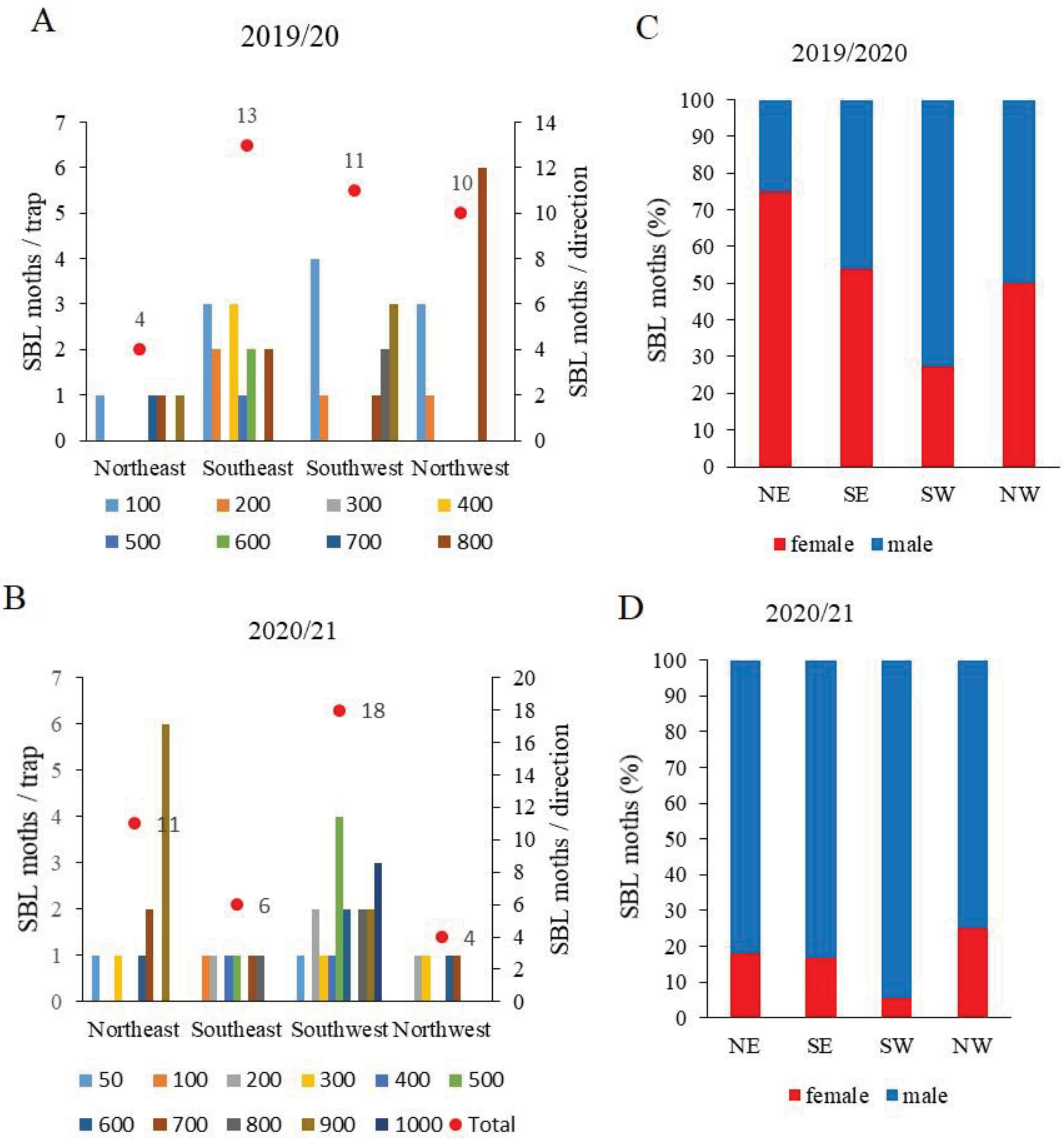

Figure 5 – Wild *Chrysodeixis includens* moths captured at each trapping point the four cardinal directions (bars - left axis), and the total moths captured per cardinal direction (red circles - right axis) in 2019/20 **(A)** and 2020-2021 **(B)**. Proportion of females and males captured at each cardinal direction in the 2019-2020 **(C)** and 2020-2021 **(D)**. NE: northeast, SE: southeast, SW: southwest, NW: northwest.

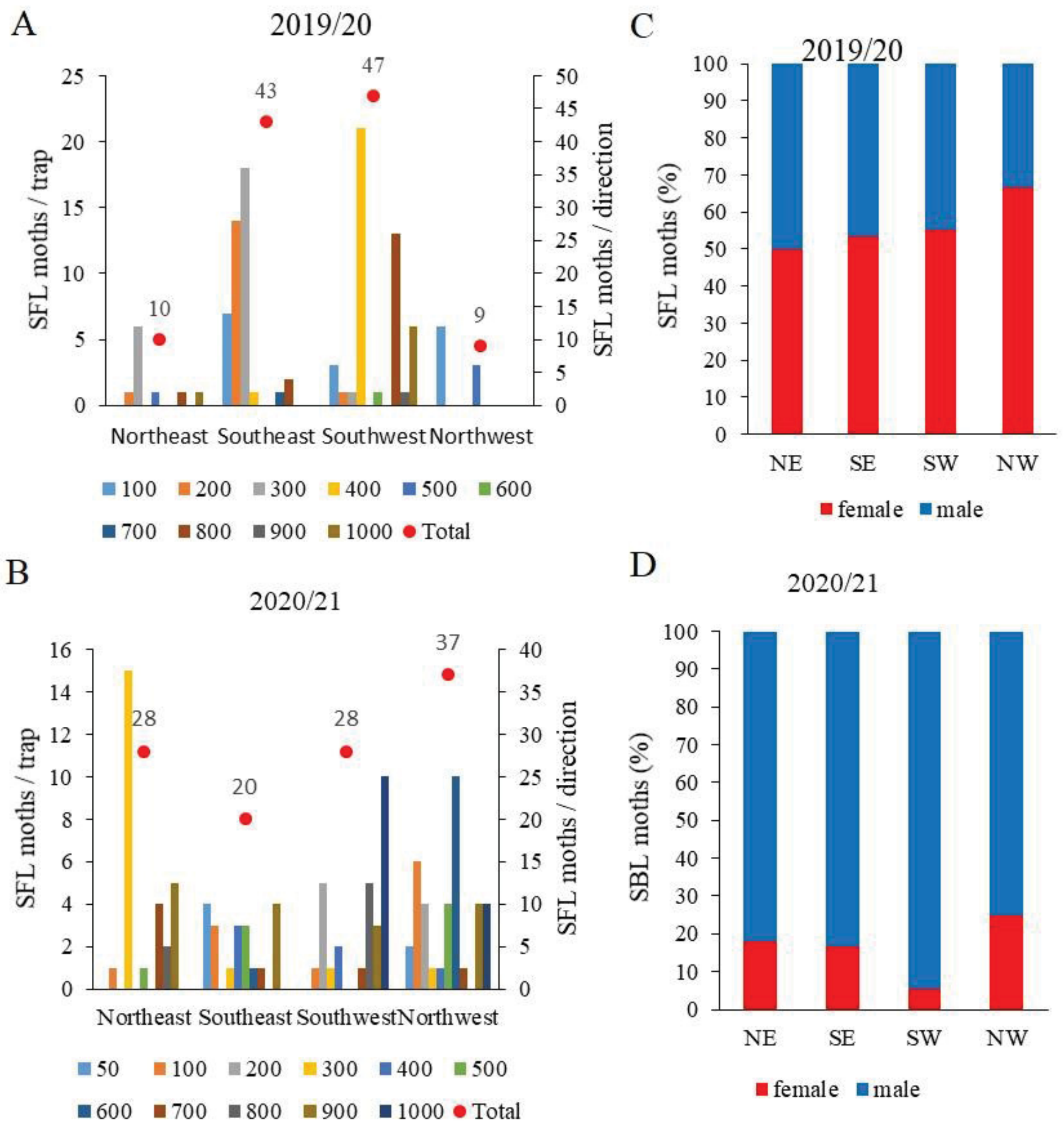

Figure 6 – Wild *Rachiplusia nu* moths captured at each trapping point the four cardinal directions (bars - left axis), and the total moths captured per cardinal direction (red circles - right axis) in 2019-2020 **(A)** and 2020-2021 **(B)**. Proportion of females and males captured at each cardinal direction in the 2019-2020 **(C)** and 2020-2021 **(D)**. NE: northeast, SE: southeast, SW: southwest, NW: northwest.

Table 1 – Release and recapture of *A. gemmatalis* (VBC) and *C. includens* (SBL) marked moths in the 2019-2020 and 2020-2021 seasons.

Species	Season	Soybean growth stage	Release period	Evaluation period	Moth number	
					Released	Recaptured N (%)
VBC	2019-2020	R2	12/17/2019 to 12/21/2019	12/18/2019 to 1/7/2021	2530	14 (0,55%)
	2020-2021	V4	12/22/2020 to 01/02/2021	12/23/2020 to 01/15/2021	3600	4 (0,11%)
SBL	2019-2020	R2	26/12/2019 to 31/12/2019	12/27/2019 to 1/7/2021	8400	2 (0,02%)
	2020-2021	V4	16/12/2020 to 12/22/2020	12/17/2020 to 01/15/2021	1500	1 (0,07%)

Table 2 – Number of mating per female of wild females of *Anticarsia gemmatalis*, *Chrysodeixis includens* and *Rachiplusia nu* trapped during 2019-2020 and 2020-2021 crop seasons.

		Number of spermatophores								
Moth species	**Season**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Total females**
A. gemmatalis	2019-20	38	163	79	25	2	-	-	-	307
	2020-21	-	15	2	-	-	-	-	-	17
C. includens	2019-20	7	4	4	1	1	1	-	-	18
	2020-21	2	1	-	-	1	1	-	-	5
R. nu	2019-20	9	23	16	8	2	2	-	-	60
	2020-21	5	2	1	1	-	-	-	1	10

FINAL CONSIDERATIONS

Many studies have addressed the field resistance to Bt crops in target species. The IRM approaches are based on genetic factors related to resistance heritage such as the resistance dominance and initial resistance alleles in the populations. However, features concerned to the pest biology and behavior are limited understood, and play an important role in the success of an IRM program. Regarding refuge as one of the IRM strategies to maintain susceptible genotypes, it is important to understand the influence of having two soybean genotypes planted at the same time in the dispersal of the target pest. For eight years, the Bt soybean, event MON 87701 x MON 89788 expressing Cry1Ac toxin has been providing a high performance in the management of the main soybean defoliators, *A. gemmatalis* and *C. includens* and information to improve IRM and promote the long term performance of this technology are critical..

This study supports the refuge recommendation in Bt soybean as part of the IRM high-dose refuge strategy to *A. gemmatalis* and *C. includens*. The results here validate the importance of selection of similar non-Bt cultivar to the Bt cultivar, and plants from Bt soybean and correspondent structured refuge being planted as closest as possible in time in order to guarantee synchronization of phenological stages of the fields and avoid oviposition preference. In addition, the management of the target pests in the refuge area, an IPM approach by the adoption of thresholds will also decrease defoliation and effect in egg deposition. Since Bt adoption, the risk aversion from the farmers has increased, due to the high efficacy of the technology to manage the main pests (Paula-Moraes et al., 2017), thus the farmers are less tolerant for plant injury, and therefore do not contribute to refuge implementation, and when it is adopted, the IPM tactics are not applied. Altogether offers a threat for properly management of pests in a region area.

Most of the marked moths in the present study was recaptured in less than 800-meter distance from release point, which is the actual recommendation or refuge in Bt soybean. In addition, females recaptured near the releasing point (up to 200 meters) were mated, which raises a concern that mating will tend to happen before moths dispersal, between females and males emerging from the same plots.

In conclusion, the adoption of refuge for the target pests *A. gemmatalis* and *C. includens* in Bt soybean is essential to keep this pests under control. In Brazil, the refuge should be adopted according with IRAC recommendation. In addition, as the occurrence of *Rachiplusia nu* has been increasing in Bt soybean fields, and studies on the performance of the Bt technology managing populations of this species, information regarding its behavior and biology are priorities in future researches to support adjustments in the IRM for Bt soybean.

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