Article

Side Effects of Single-Transgene or Pyramided Genetically Modified Maize on the Generalist Endoparasitoid *Palmistichus elaeisis* (Hymenoptera: Eulophidae)

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Abstract: The fall armyworm, *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae), is an agricultural pest native to tropical and subtropical regions of the Americas, with considerable potential for global invasion and seasonal migration. Although genetically modified (GM) plants have shown positive impacts on the economy and the environment as they synthesize proteins that act as natural insecticides and are primarily intended to protect the crops from damage by specific pests, potential effects of Bt toxins on non-target organisms can occur. This experiment aimed to evaluate the potential impacts on the parasitoid *Palmistichus elaeisis* (Hymenoptera: Eulophidae), using the pupae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) as hosts, which were fed with transgenic maize genotypes such as Herculex®, expressing Cry1F protein, and PowerCore®, expressing Cry1F, Cry1A.105, and Cry2Ab2 proteins, or their non-transgenic isohybrid. The experiment was conducted in a completely randomized design with ten replicates. *Spodoptera frugiperda* larvae were fed *ad libitum* with transgenic and non-transgenic maize until the pupal stage and then individually exposed to six *P. elaeisis* females for 78 h. The total number of adults, emerged males, tibia size, cephalic capsule size, and parasitoid body size were not influenced by host feeding. However, the number of emerged females from the Herculex® and PowerCore® treatments was lower than that for the isohybrid treatment. The sex ratio and longevity of parasitoids emerging from PowerCore® were 1.05 and 1.26 times lower, respectively, than that of those from the isohybrid. Furthermore, the number of dead *P. elaeisis* within the host pupa was 10.56 times higher in the PowerCore® genotype. Combining Bt proteins within pyramided genotypes should cause minimal impacts and promote the conservation and integration of beneficial insects. The results of this study provide helpful information for developing effective and compatible integrated pest management (IPM) strategies.

Keywords: *Bacillus thuringiensis*; biological control; Cry proteins; non-target organisms

1. Introduction

The fall armyworm, *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae), is an agricultural pest native to tropical and subtropical regions of the Americas [1], with considerable potential for global invasion and seasonal migration [2]. This insect is distributed from the United States, Mexico, and Canada in North America to countries such as Costa Rica, Honduras, and Guatemala in Central America and extending through Brazil,

Argentina, Colombia, Peru, Venezuela, and Ecuador in South America, as well as some Caribbean islands. However, over time, this pest has spread globally, being documented in several regions outside the Americas, including vast areas of Africa, as well as in Asia and the Pacific Island nations, such as Australia and Papua New Guinea. *Spodoptera frugiperda* is also classified as one of the main quarantine pests for the European continent. Its global spread represents a severe concern to agriculture, as this pest can cause substantial damage to a wide range of crops under different altitude and latitude conditions. Its remarkable adaptability to diverse climates and its ability to reproduce quickly make *S. frugiperda* a significant threat to food security in many regions of the world. It feeds on at least 186 host plants and is one of the major pests that affect important crops such as *Gossypium hirsutum* L. (Malvaceae), *Oryza sativa* L. (Poaceae), and *Zea mays* L. (Poaceae) [3].

The Green Revolution, at its height in the 1960s and 1970s, aimed to expand global agricultural production by introducing high-yield crop varieties, genetic improvement, advanced agricultural technologies, and intensive management practices. Although it has had beneficial impacts on food production and contributed to the fight against hunger in many parts of the world, it has also increased the use of insecticides, resulting in an increasing dependence of farmers on chemicals, including insecticides, as part of their food system's pest control. This scenario sometimes triggered cycles of continuous insecticide applications to maintain control over pests. Chemical pest control is the most widely used method worldwide, and several studies have aimed to develop more selective insecticidal molecules to minimize the impact on natural enemies [4].

The first transgenic organisms were designed in the 1970s and 1980s, but it was only in the 1990s that transgenic foods began to be widely commercialized. They are the products of decades of research and development in agricultural biotechnology, which involved transferring genes from one species to another to impart desired traits such as pest resistance, herbicide tolerance, increased shelf life, and improved nutritional value. Following the commercialization of genetically modified (GM) plants that produce insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* Berliner (Bt), the use of synthetic insecticides has decreased [5]. The Bt bacterium is found in several ecological niches, exhibiting entomo-pesticide attributes due to the synthesis of soluble proteins (Cry and Vip) throughout distinct growth phases [6,7]. After ingesting these proteins, vulnerable insect species suffer substantial damage to their midgut cells, often dying from septicemia.

The responsible use of Bt crops in agriculture can contribute to more efficient and targeted pest control [8], assisting in the preservation of natural enemies [9,10]. Field results from efficacy trials conducted over three years in key commercial corn-producing regions in Brazil revealed that Bt technology, expressing the proteins Cry1F + Cry1A.105 + Cry2Ab2 + Vip3Aa20, delivered substantial protection against the pest *S. frugiperda* in several maize varieties [8]. The strategy of pyramiding toxic proteins with distinct modes of action has been increasingly recognized as a viable approach for mitigating the emergence of resistance in targeted insect populations [6,7]. Thus, transgenic plants expressing insecticidal proteins have brought advancements to cultivation and integrated pest management (IPM) [11].

Biological control is also essential in promoting sustainability in agriculture, mitigating environmental impacts, and promoting more balanced and ecologically responsible agricultural practices. This pest management technique is based on using living organisms, such as predators, parasitoids, and microorganisms, to control agricultural pests. Biological control is a cornerstone of sustainable agriculture, as it reduces the need for insecticides, mitigates environmental pollution, minimizes risks to human health and non-target wildlife, conserves biodiversity, strengthens food security, and increases consumers' confidence concerning agricultural products. *Palmistichus elaeisis* Delvare and LaSalle (Hymenoptera: Eulophidae) is a gregarious endoparasitoid wasp native to the Neotropics, which develops in lepidopteran species of economic importance, such as *Anticarsia gemmatalis* Hübner (Noctuidae) [12], *Diatraea saccharalis* Fabricius (Crambidae), *Heliothis virescens* F. (Noctuidae), and *S. frugiperda* [13], showing great potential as a biological control agent. Female *P. elaeisis*

lays eggs on host pupae, and after hatching, their parasitic larvae feed on the host's organs and tissues [14].

Although GM plants benefit the economy and the environment by these proteins that act as natural insecticides and are primarily intended to protect the crops from damage by specific pests [15], potential effects of Bt toxins on non-target organisms can occur, especially as these toxins move through different trophic levels [16,17]. The flow of Cry proteins in trophic levels has been reported in various insects [18–20], including *S. frugiperda* that fed on maize leaves expressing Cry1F or Cry1A.105 and Cry2Ab2, with the accumulation and transfer of proteins to the predator *Podisus nigrispinus* (Dallas, 1851) (Hemiptera: Pentatomidae) [16]. The parasitoids are potentially exposed to Bt proteins when feeding on their hosts. Similar to predators, the concentration of Bt protein in the host, as well as the feeding habit of the parasitoid, influences exposure [10].

For use in IPM, natural enemies must be compatible with other control methods [20]. Compatibility between control methods refers to the harmony and ability of different pest management strategies to work together efficiently. In this context, this study aimed to evaluate the possible effects of the Cry1F protein and the combination of the Cry1F, Cry1A.105, and Cry2Ab2 proteins on the parasitoid *P. elaeisis*, using *S. frugiperda* pupae as hosts that developed from larvae fed with transgenic maize.

2. Materials and Methods

The hybrids isohybrid (Dow AgroSciences, São Paulo, SP, Brazil) non-Bt isogenic maize of the same genetic background used as control, Herculex® (TC1507, Dow AgroSciences LLC, Indianapolis, IN, USA) transgenic maize coding for protein Cry1F, and PowerCore® (MON 89034 × TC1507 × NK603, Monsanto Technology LLC and Dow AgroSciences LLC, Indianapolis, IN, USA) transgenic pyramidal maize coding for proteins Cry1F, Cry1A.105, and Cry2Ab2 were used in this experiment. The maize was kept in a greenhouse in 8 L pots. The cultivation was carried out according to the recommendations for Brazil [21] without applying insecticides, fungicides, and herbicides.

2.1. Insects

The larvae of *S. frugiperda* were reared separately in polyethylene containers (15 × 9 cm) maintained at 25 ± 2 °C, 75 ± 5% relative humidity (RH), under a 12:12 h light/dark (L/D) photoperiod. The larvae were fed an artificial diet composed of 6.2 g agar, 15.2 g brewer's yeast, 23.7 g wheat germ, 50 g beans, 15.3 g ascorbic acid, 0.5 g sorbic acid, 1 g methylparaben, and 1.2 mL of antifungal solution (41.8% propionic acid and 4.2% phosphoric acid) [1].

The pupae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were used to mass rear *P. elaeisis*. *Tenebrio molitor* individuals were kept in plastic boxes (60 × 40 × 12 cm) at 25 ± 1 °C, 75 ± 5% RH, and a 12:12 h L/D photoperiod. The larvae and adults were fed *ad libitum* with wheat bran, pieces of stem from *Saccharum officinarum* L. (Poaceae), and *Sechium edule* Swartz (Cucurbitaceae) until pupation.

Adults of *P. elaeisis* were placed in glass tubes (14 × 2.2 cm) containing *T. molitor* pupae and sealed with a cotton plug. After parasitism, newly emerged individuals (24 h old) were placed in glass tubes (14 × 2.2 cm), maintained at 25 ± 2 °C, 70 ± 10% RH, and a 12:12 h L/D photoperiod, and were provided with *ad libitum* honey for feed.

2.2. Bioassays

The experiment was completely randomized with three treatments: (i) isohybrid, (ii) Herculex®, and (iii) PowerCore®. Ten replications were used, each one including six females of *P. elaeisis* and one pupa of *S. frugiperda* originating from one of the described treatments.

The fifth-instar larvae of *S. frugiperda* were individually placed in transparent plastic containers (500 mL capacity) and fed *ad libitum* until the pupal stage with leaves from isohybrid, Herculex®, or PowerCore® maize plants at the V3 vegetative stage. The leaf

material was renewed every 24 h. The pupae were weighed and selected using a precision balance to ensure they had an approximate mean weight.

Six females of *P. elaeisis* (24 h old) were placed in glass tubes (14 × 2.2 cm) covered with a cotton ball containing a drop of honey and a pupae of *S. frugiperda* (48 h old) originating from one of the isohybrid, Herculex®, or PowerCore® treatments. After 78 h, the pupae were individually placed in transparent plastic containers with lids (250 mL capacity) and placed in a climate-controlled room (25 ± 2 °C and a 12:12 h L/D photoperiod) until emergence.

Six newly emerged females of *P. elaeisis* (F1) from each treatment were placed in glass tubes (14 × 2.2 cm) covered with a cotton ball and containing a drop of honey for longevity assessment (days). Other parasitoids were assessed for the number of emerged individuals, males, females, and sex ratio.

The morphometry of one female of *P. elaeisis* from each replication was determined using a camera attached to a stereomicroscope and Optika Vision Lite 2.1 software. The body size (mm), measured from the median line of the head to the abdomen; the posterior tibia size (mm); and the cephalic capsule size (mm), measured from the median line of the eyes were obtained. After the death of all the parasitoids, the pupae were dissected to determine the percentage of non-emerged *P. elaeisis*.

2.3. Protein Detection

The detection of transgenic proteins was performed using the Envirologix QuickStix™ kit for Cry1F and Cry2A. EnviroLogix QuickStix™ kits (Envirologix Inc., Portland, OR, USA) are rapid testing devices that enable the detection of specific elements in biological or environmental samples, such as genetically modified organisms (GMOs).

Three pupae from the isohybrid, Herculex®, or PowerCore® treatments were macerated in Eppendorf® tubes containing 0.5 mL of extraction buffer (SEB4). The tubes were closed and shaken for 30 s. They were later placed in a holder and an EnviroLogix QuickStix™ Cry1F strip for Herculex® samples and a Cry2A strip for PowerCore® were added.

The strips were kept inside each sample for five minutes to read the results. QuickStix contains specific reagents and antibodies that react with the element to be detected, such as a GMO protein. When the sample is applied to the device, it moves along the QuickStix, and the reagents react with the specific target, generating a response. In samples with Cry1F or Cry1A.105 and Cry2Ab2 proteins, a second line (test line) was detected in the region between the control line and the lower end of the strip. For negative samples, the strip only developed the control line.

2.4. Statistical Analysis

The experiment was completely randomized with three treatments and ten replications, including in each six females of *P. elaeisis* (sampling effort: 180 parasitoids). The total number and the number of females who emerged, the longevity (days), and the weight of pupae (g) were subjected to an analysis of variance using the F test, and the measurements of size (mm), number of males, sex ratio, and percentage of deaths were subjected to a chi-square test. The comparison of means was carried out using the Kruskal–Wallis and Scott-Knott tests at 5% significance using the statistical program R Studio version 4.3.1.

3. Results

The immunochromatographic test with EnviroLogix QuickStix™ strips for Cry1F and Cry2A was negative for Cry proteins in the pupae of the isohybrid treatment. In pupae from the Herculex® and PowerCore® treatments, the strips for the Cry1F and Cry2A proteins were positive.

The total number of *P. elaeisis* (52.70 ± 17.30 to 112.40 ± 17.25) and the number of emerged males (3.00 ± 1.14 to 4.90 ± 2.02), as well as the size of the body (1.68 ± 0.12 to 1.89 ± 0.04), the tibia (0.56 ± 0.02 to 0.60 ± 0.01), and head capsule (0.49 ± 0.02 to 0.52 ± 0.02), did not differ between treatments. However, the number of females who

emerged was higher in isohybrid (108.70 $\pm$ 16.69) and did not differ for Herculex[®] (59.30 $\pm$ 19.68) and PowerCore[®] (47.80 $\pm$ 15.48) (Figures 1 and 2). The sex ratio was 5.20% lower in PowerCore[®] (0.91 $\pm$ 0.01) when compared to isohybrid (0.96 $\pm$ 0.01). The Herculex[®] treatment (0.95 $\pm$ 0.01) did not differ from the PowerCore[®] and isohybrid treatments (Figure 3).
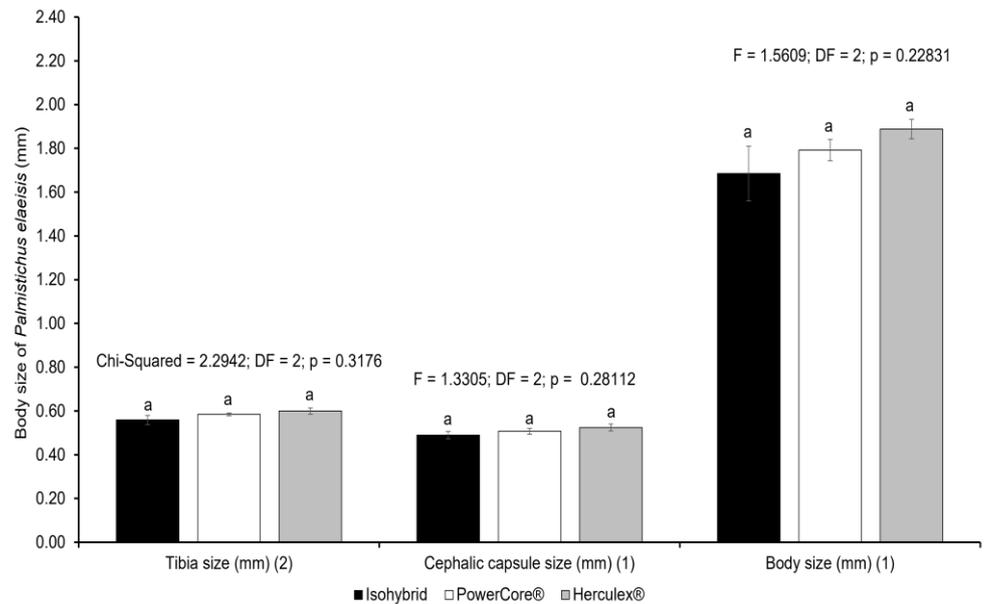

Figure 1. Size of the tibia, cephalic capsule, and body (mean $\pm$ standard error) of *Palmistichus elaeisis* (Hymenoptera: Eulophidae) in pupae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) fed with three maize genotypes: isohybrid, Herculex[®], or PowerCore[®]. Similar letters arranged on the bars do not differ statistically from each other based on the respective sample comparison test. (1) Parametric test: F test at 5% significance. (2) Non-parametric test: Kruskal–Wallis test at 5% significance.
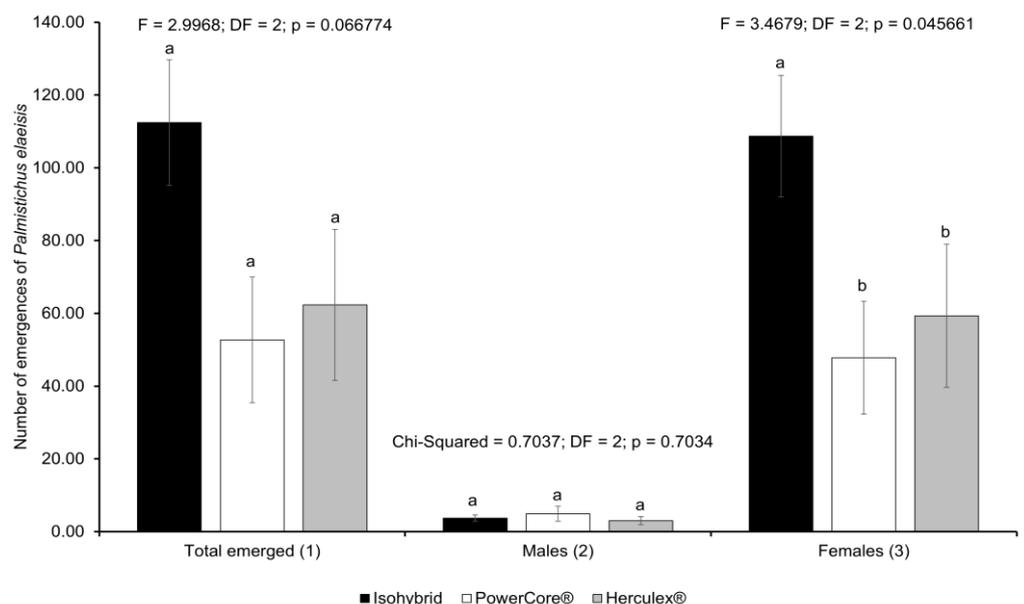

Figure 2. Total number of emerged males and females (mean $\pm$ standard error) of *Palmistichus elaeisis* (Hymenoptera: Eulophidae) in pupae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) fed with three maize genotypes: isohybrid, Herculex[®], or PowerCore[®]. Similar letters arranged on the bars do not differ statistically from each other based on the respective sample comparison test. (1) Parametric test: F test at 5% significance. (2) Non-parametric test: Kruskal–Wallis test at 5% significance. (3) Parametric test: Scott-Knott test at 5% significance.
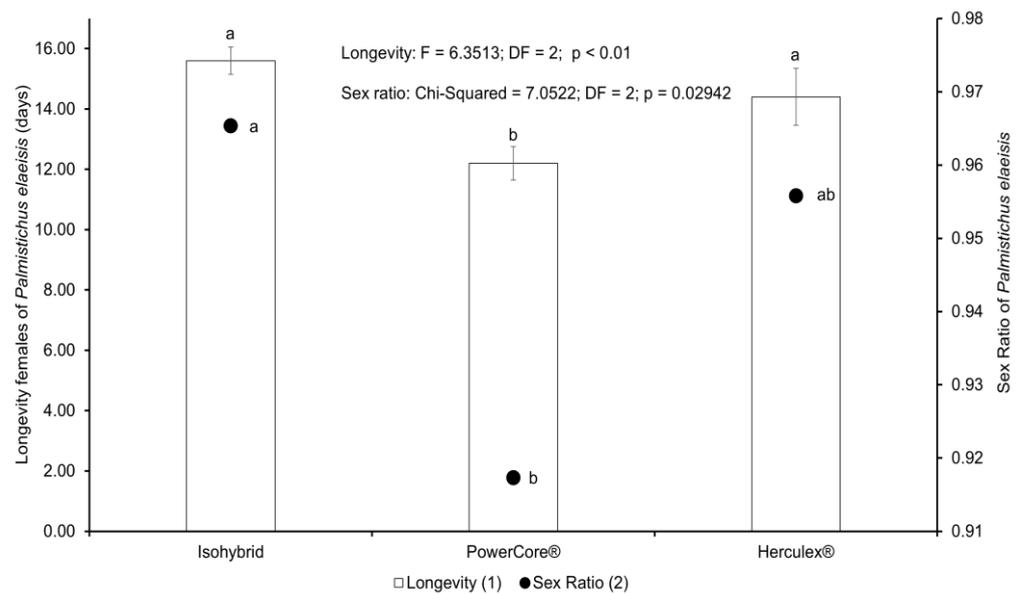
Figure 3. Female longevity and sex ratio (mean $\pm$ standard error) of *Palmistichus elaeisis* (Hymenoptera: Eulophidae) in pupae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) fed with three maize genotypes: isohybrid, Herculex®, or PowerCore®. Similar letters arranged on the bars do not differ statistically from each other based on the respective sample comparison test. (1) Parametric test: Scott-Knott test at 5% significance. (2) Non-parametric test: Kruskal–Wallis test at 5% significance.

The Longevity was 21.79% lower in the PowerCore® treatment (12.20 $\pm$ 0.55) when compared to the isohybrid one (15.60 $\pm$ 0.45). For the isohybrid and Herculex® treatments (14.40 $\pm$ 0.94), longevity did not differ.

The pupae weights before parasitism had a total average of 0.22 $\pm$ 0.01 g. The percentage of *P. elaeisis* not emerging was lower in the isohybrid treatment and higher in the PowerCore®, 3.20 $\pm$ 1.91 and 33.68 $\pm$ 12.56, respectively. In the Herculex® treatment (24.35 $\pm$ 12.72), the percentage of non-emerged was the same as in the other treatments (Table 1).

Table 1. Weight (mean $\pm$ standard error) of pupae and percentage of dead parasitoids (mean $\pm$ standard error) of *Palmistichus elaeisis* (Hymenoptera: Eulophidae) in pupae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) fed in the larval stage with three maize genotypes: isohybrid, Herculex®, or PowerCore®.

Treatment	Pupa Weight [1] (g)	Percentage of Deaths [2] (%)
Isohybrid	0.23 $\pm$ 0.0097 a	3.20 $\pm$ 1.9187 b
Herculex®	0.22 $\pm$ 0.0133 a	24.35 $\pm$ 12.7232 ab
PowerCore®	0.22 $\pm$ 0.0122 a	33.68 $\pm$ 12.5673 a
F = 0.17556; GL = 2; p = 0.83994		Chi-square = 6.2773; GL = 2; p = 0.04334

Letters similar to each other in the column do not differ statistically based on the respective sample comparison test. [1] Parametric test: F test at 5% significance. [2] Non-parametric test: Kruskal–Wallis test at 5% significance.

4. Discussion

The combined utilization of Bt crops and parasitoids emerges as a promising integrated pest management (IPM) strategy, owing to its cost effectiveness, high efficiency, potential to address insect resistance, and minimal environmental impact. These two complementary approaches work in synergy to regulate pest populations, with Bt crops reducing the initial pest numbers and facilitating the subsequent control by parasitoids. Nevertheless, the potential impacts of Cry proteins on the parasitoid *P. elaeisis* remain relatively understudied, especially the sublethal effects.

In this experiment, exposure of *S. frugiperda* larvae to Bt via ingestion resulted in the detection of the Cry proteins in the pupal stage. The physiological mechanism that allows the passage of Cry proteins out of the insect gut lumen remains unclear [22]. However, one possible route is the movement of proteins from the hemolymph to the fat body by pinocytosis, when the cell membrane forms small vesicles to engulf and internalize small molecules in the extracellular environment [23], allowing insects to store proteins throughout the larval stage as a reserve for new adult tissues [23–26]. The Cry1F protein was also detected in *S. frugiperda* eggs fed until pupation with Bt maize leaves [19]. Studies on the flow of Cry proteins in the food chain confirm that most herbivores ingest these proteins and transmit them to their natural enemies [17,19]. Newly hatched adults of the parasitoid *Anagrus nilaparvatae* Pang et Wang (Hymenoptera: Mymanidae) presented the Cry1Ab protein when raised on eggs of the planthopper *Nilaparvata lugens* Stal (Hemiptera: Delphacidae) that fed on Bt rice [25]. However, the impacts on natural enemies may vary depending on the specificity of the Bt proteins used, the characteristics of the transgenic plant, and the biology of the natural enemies. Furthermore, many studies have been carried out to evaluate the risks and benefits associated with the use of Bt plants, with a focus on minimizing negative impacts on natural enemies and preserving these biological control agents. The selection of Bt plants and the implementation of pest management practices that consider the conservation of natural enemies are important strategies to minimize the potential negative impacts and maximize the benefits of using transgenic Bt crops.

The similarity of the total number and number of emerged males, body size, tibia, and head capsule of *P. elaeisis* in hosts in the presence or absence of Bt proteins confirms other reports in which the ingestion of Bt proteins reduced the hosts' aptitude for parasitism, but survivors developed normally [27]. The development, parasitism, survival, sex ratio, longevity, or fecundity of *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) when they parasitized *S. frugiperda* fed on Cry1F maize were not affected [27]. Other tritrophic studies also demonstrate that the parasitoids *C. marginiventris* and *Copidosoma floridanum* Ashmead (Hymenoptera: Encyrtidae) are not sensitive to the Cry1Ac and Cry2Ab proteins [28], combining synergistic effects to regulate pest populations.

Some Hymenoptera have arrhenotokous parthenogenesis, wherein males develop from unfertilized eggs and females from fertilized ones [29]. Therefore, the reduction in the number of females emerging from the Herculex® and PowerCore® treatments may be due to lower sperm production or male sterility, as already observed in *P. elaeisis* and other parasitoids exposed to insecticides [20,30]. The low number of females in the *P. elaeisis* population is undesirable, as they are responsible for pest control and reproduction of the species, which decreases the population and parasitism rates [30]. Utilizing parasitoid species primarily reproducing through thelytokous parthenogenesis, wherein unfertilized eggs give rise to new females, can help mitigate these effects of the Herculex® and PowerCore® genotypes.

Pyramidal Bt plants have been used in many crops, including corn, cotton, and soybeans, which are often targeted by a wide range of insect pests. These crops provide farmers with an effective and economical way to manage pest populations while decreasing the ecological footprint associated with pest control. Adopting pyramidal plants is a fundamental strategy in agricultural biotechnology, particularly in response to concerns regarding insect resistance. When a single Bt protein is extensively employed, insects can develop resistance. However, by integrating multiple Bt proteins with distinct modes of action, the likelihood of insect pests developing resistance is minimized due to the reduced probability of an insect simultaneously developing resistance to multiple Bt proteins. The development of resistance to transgenic plants is a significant challenge, and it is essential to take proactive measures to slow this process and preserve the value of Bt technologies for pest control. On the other hand, different Cry proteins in pyramidal Bt plants can interact synergistically, leading to unexpected effects in non-target species [31]. In this study, exposure to more Cry proteins expressed in the PowerCore® genotype reduced the production of female *P. elaeisis*, reducing longevity and the percentage of parasitoids that

emerged. When the Bt plant has more Cry proteins, the reduction in the nutritional quality of the prey or host is accentuated [32–35]. The survival of *P. elaeisis* emerging from hosts sprayed with Bt-based insecticides decreases over generations and can reduce parasitoid populations in the field. A decline in parasitoid populations can increase pest numbers, heightening the risk of crop damage and subsequent yield losses, leading to economic hardships for farmers. Previous studies report increased larval development time, lower emergence rates, reduced longevity, and fecundity in parasitoids after contact with Bt toxins through artificial diets or transgenic plants [26,27,35–37]. The outcomes of these studies exhibit variations based on the organisms under investigation, the methodologies employed, and the specific proteins and their combinations within pyramided plants.

In the context of reducing synthetic insecticides, preserving natural enemies to contain primary and secondary pests not controlled by Bt crops is an essential aspect of IPM [38]. Furthermore, natural enemies may delay the evolution of resistance to Bt plants in the target pest [39]. However, Bt proteins or their associations can affect non-target organisms, making assessing the ecotoxicological risk in these systems essential. Contrasting results between risk assessment studies of transgenic crops highlight the seriousness of developing practical methodological approaches and studies of different models.

5. Conclusions

Spodoptera frugiperda, commonly known in Brazil as the fall armyworm, is a species of insect pest that is widely distributed and affects numerous crops of economic importance. It is native to the Americas, with recent dispersion to countries in Africa, Asia, and Oceania. During the larval stage, it feeds voraciously on the leaves and reproductive structures of plants, which can result in economic losses and food insecurity. Control methods include the use of insecticides, the planting of transgenic varieties, biological control with parasitoids, and other integrated pest management (IPM) practices that aim to combine several strategies to reduce the impact.

Palmistichus elaeisis parasitism in *S. frugiperda* pupae after feeding on transgenic maize reduced the number of emerged females, and the combination of Cry proteins in the PowerCore® event affected the sex ratio and longevity and increased the number of non-emerged individuals.

Reducing the parasitoid population in agricultural fields can have detrimental effects on pest management and the overall health of agroecosystems. Parasitoids play a role in the natural regulation of insect populations, and their decline can result in direct consequences such as crop yield losses, increased use of synthetic insecticides, the proliferation of insects resistant to Bt technology, and ecological imbalances.

Combining Bt proteins within pyramided genotypes should cause minimal impacts and promote the conservation and integration of beneficial insects. The reduction in parasitoids in the context of biological control carries implications for both agriculture and ecosystems. Therefore, advancements in agricultural biotechnology should prioritize approaches that support the preservation and effective utilization of these indispensable biological control agents.

The results of this study provide helpful information for developing effective and compatible IPM strategies.

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