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Efficiency of *Trichogramma pretiosum* pupa release in soil and on plants: effects of predator protection

Eficiência de liberação de pupas de *Trichogramma pretiosum* em solo e em plantas: efeitos da proteção contra predadores

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ABSTRACT

The development of effective techniques for the release of *Trichogramma pretiosum* parasitoids in the control of lepidopteran eggs is crucial for improving pest management in agricultural crops. This study aims to investigate the predation of parasitoid-infested eggs using different release

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methods and to evaluate the efficacy of the loose pupae release technique in the soil. Three bioassays were conducted using paper cards containing parasitized eggs, comparing unprotected cards, cards protected in capsules with exit holes of different sizes (4 mm² and 0.25 mm²), and the release of loose pupae in the soil. The parasitism efficiency on *Anagasta kuehniella* eggs was also assessed. Results indicated that predation of eggs parasitized by T. *pretiosum* was significantly higher in the soil compared to the leaves of sugarcane and maize. Among the release methods, unprotected cards showed a much higher predation rate than protected cards in capsules, reaching 100% predation in the soil within a few days. Sugarcane areas exhibited higher predation rates than maize areas. Capsules with larger holes (4 mm²) allowed greater predation of parasitized eggs compared to capsules with smaller holes (0.25 mm²). The loose pupae release technique in the soil proved effective for controlling lepidopteran eggs, with higher pupae densities resulting in a significant increase in parasitism. This study suggests that the loose pupae release technique is a promising approach for biological pest control, emphasizing the importance of selecting the appropriate release method to maximize control efficacy.
KEYWORDS: Release technique, Biological control, Hymenoptera, Parasitoid.

RESUMO

O desenvolvimento de técnicas eficazes para a liberação do parasitoide Trichogramma pretiosum no controle de ovos de lepidópteros é essencial para melhorar a gestão de pragas em culturas agrícolas. Este estudo visa investigar a predação de ovos parasitados por T. pretiosum utilizando diferentes métodos de liberação e avaliar a eficácia da técnica de liberação de pupas soltas no solo. Foram conduzidos três bioensaios com cartões de papel contendo ovos parasitados, comparando cartões não protegidos, cartões protegidos em cápsulas com orifícios de saída de diferentes tamanhos (4 mm² e 0,25 mm²), e a liberação de pupas soltas no solo. Também foi avaliada a eficiência do parasitismo em ovos de Anagasta kuehniella. Os resultados indicaram que a predação de ovos parasitados por T. pretiosum foi significativamente maior no solo comparado às folhas de cana-de-açúcar e milho. Entre os métodos de liberação, os cartões não protegidos apresentaram uma taxa de predação muito superior aos cartões protegidos em cápsulas, alcançando 100% de predação no solo em poucos dias. As áreas de cana-de-açúcar mostraram maior predação em comparação às áreas de milho. Os orifícios maiores (4 mm²) nas cápsulas possibilitaram uma maior predação dos ovos parasitados em relação aos orifícios menores (0,25 mm²). A técnica de liberação de pupas soltas no solo mostrou-se eficaz para o controle de ovos de lepidópteros, com uma maior densidade de pupas, resultando em um aumento significativo no parasitismo. Este estudo sugere que a técnica de liberação de pupas soltas é uma abordagem promissora para o controle biológico de pragas, destacando a importância da escolha adequada do método de liberação para maximizar a eficácia do controle.
***PALAVRAS-CHAVE**: Técnica de liberação, Controle biológico, Hymenoptera, Parasitoide.*

INTRODUCTION

Brazil is a leader in tropical agriculture and faces several challenges to also become a leader in the use of biological control in tropical regions (Parra and Coelho, 2019). Among the various challenges identified by Parra (2014), including the “culture” of farmers accustomed to using chemical products, there are also problems related to the large size of cultivated areas.

This control tactic gains strength as farmers and consumers become more demanding, phytosanitary barriers imposed by food importing countries become more stringent, and more pests develop resistance to insecticides (Parra and Coelho, 2019).

Among natural pest enemies, parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) are widely recognized as important biological control agents of lepidopteran pests worldwide, in a variety of crops and forest areas, due to the ease of their breeding in pest eggs, the use of alternative hosts, and their aggressiveness in parasitizing pest insect eggs (Parra, 1997; Haji et al., 1998; Botelho, 1997).

Trichogramma is used in biological control programs in 49 countries, treating more than 21 million hectares per year with inundative releases of this parasitoid (Hassan, 1993). In Brazil, the most commonly used natural enemy in various crops is *Trichogramma* spp. (Parra and Coelho, 2019). According to Parra and Coelho (2019), the species *Trichogramma galloi* Zucchi is used to parasitize *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae) in 2,000,000 hectares of sugarcane, while *Trichogramma pretiosum* (Riley), which has the largest host range among those produced on a large scale in the country, parasitizes *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae) in 250,000 hectares of soybean and maize, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in 1,500 hectares of tomato, and *Lasiothyris luminosa* (Razowski and Becker) (Lepidoptera: Tortricidae) in 1,000 hectares of grape crops.

The success of a biological control program can be impaired by the release technique adopted (Pinto and Parra, 2002). There are issues with how natural enemies are released (drones are currently being used), as well as problems related to sampling to determine the appropriate time to release the control organisms. Methods of remote sensing by reflectance have been investigated (Nansen et al., 2013).

Pinto et al. (2003) found that releasing *T. galloi* in sugarcane fields for the control of *D. saccharalis* is more effective when the parasitoid is introduced as pupae and allowed to emerge in the field, compared to adult releases. However, Pinto (1999) found that the predation of *D. saccharalis* eggs in the field is very high, reaching 100% in many areas, which can also affect the success of pupal releases of *Trichogramma*. For the release of *Trichogramma* in pupal form, the author suggested that parasitized eggs need to be packed in capsules to protect them against rain and predators.

Conversely, Mills et al. (2000) found that the release of loose *Trichogramma* pupae on the canopy is a viable and efficient technique for controlling *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) in apple and pear orchards in the United States. The authors noted that predation is probably minimized because parasitized eggs are distributed over the leaves of the tree canopy, where predation is lower. Rajendran and Mohammed (1998) also found better efficiency in distributing parasitized eggs in sugarcane fields compared to conventional techniques, where the eggs are attached to cards and protected.

Although the authors mentioned that *Trichogramma* pupae predation may be decreased when using the loose pupal distribution technique, no studies have been conducted to evaluate this.

Thus, this study aimed to verify the predation of *T. pretiosum* (parasitized eggs) under different release techniques and evaluate the efficiency of parasitism by the loose pupal distribution.

MATERIALS AND METHODS

Insects

Colonies of the two species of insects (*T. pretiosum* and *Anagasta kuehniella*) used in the experiments were kept in the laboratory under controlled conditions at a temperature of 25 ± 1 °C, relative humidity (RH) of 70 ± 10%, and a photoperiod of 12 hours of light and 12 hours of darkness. *T. pretiosum* were obtained from BUG Biological Agents Company (BUG), Piracicaba, SP, Brazil, and were reared on *A. kuehniella* eggs, maintained according to the method described by Parra (2010).

The *T. pretiosum* colony consisted only of females, which reproduced by thelytokous parthenogenesis (Pinto and Stouthamer, 1994).

Predation of *T. pretiosum* Pupae in Unprotected and Protected Capsules

The bioassay was installed on October 5th, 2015, in an experimental sugarcane area using the variety SP89-1115 (CP73-1547) with 13 months of development, and in an experimental maize area using the hybrid DAS CO32 (short season), 17 days after sowing, at the Moura Lacerda University, Ribeirão Preto, SP, Brazil. One hundred unprotected 4 cm² cards and 100 protected cards (developed by BUG Biological Agents) were randomly scattered across the soil between the sugarcane fields (Figures 1A and 1B). The cards were placed in cardboard capsules with four 4 mm² side holes.

Figure 1 - Cards containing *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* in the pupal phase, which are unprotected (A) and protected (B), as developed by BUG Biological Agents.

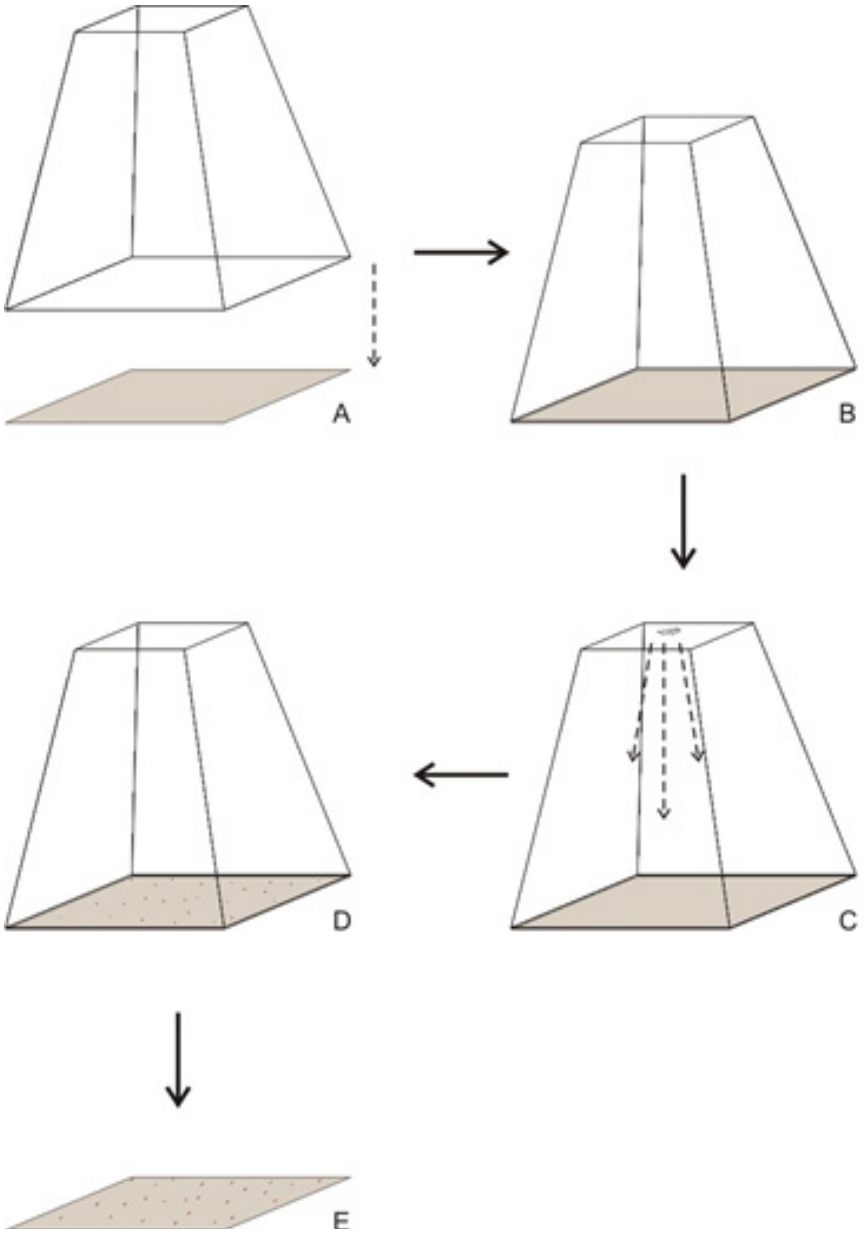
The cards contained *A. kuehniella* eggs parasitized by *T. pretiosum* in the pre-pupal phase. The same procedure was repeated with the cards spread between the leaves of sugarcane and maize plants. Twenty-four hours after the release, 10 cards were collected daily from each site to count the number of preyed eggs in the laboratory.

Predation of *T. pretiosum* Pupae in Capsules with Two Orifice Sizes

The bioassay was installed in a maize crop on January 22, 2017, of an unknown variety, in the reproductive period, at the Entomology and Acarology Department (ESALQ), University of São Paulo, Piracicaba, SP, Brazil. Capsules developed by BUG were evaluated with two holes (opposite sides) of 2 x 2 mm (4 mm²) and a modification with two holes of 0.5 x 0.5 mm (0.25 mm²) (Figure 2).

The pack developed by BUG contained parasitized eggs attached to one side of the capsule (about 2,000), with side holes, but only two were kept for testing. The others were closed with paper-mache, made without glue. For the smaller hole capsules, all larger holes were closed with paper-mache. The smaller ones were made with a sewing needle, with the perforations made from the inside out to make entry difficult for predators. Ninety-nine capsules were randomly placed in a 100 m² maize crop area. After 6, 24, and 72 hours, 33 capsules were taken from each field treatment and evaluated for predation in the laboratory.

Figure 2 - Sequence of cardboard preparation with *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* for predation bioassay. (A) Overlap of isolation chamber over Arabic gum cardboard. (B) Camera Dock. (C) Release of previously quantified eggs with force. (D) Collage of eggs. (E) Location of eggs on the side-marked cardboard.



Predation of *T. pretiosum* Pupae and Parasitism Efficiency of A. kuehniella Eggs in the Release of Loose Pupae Technique in Soil

The bioassay was installed on October 21st, 2017, in an experimental sugarcane area using the SP89-1115 variety (ratoon-cane), with 14 months of development. The following treatments were conducted: (1) *A. kuehniella* eggs parasitized by *T. pretiosum*, at a density of 5 per square meter (equivalent to 50,000 ha^{-1}), pasted on a beige cardboard; (2) at a density of 15 m^{-2}; (3) at a density of 25 m^{-2}; (4) at a density of 35 m^{-2}; (5) at a density of 45 m^{-2}, and (6) one capsule in 400 m^2 containing protected *T. pretiosum* pupae (Bug) (equivalent to 50,000 ha^{-1}). It is worth noting that the recommendation for *T. galloi* release for the control of *D. saccharalis* in sugarcane is 50,000 ha^{-1} (Pinto et al., 2006).

The parasitized eggs, three days before emergence, were quantified under a stereoscopic microscope at 20x magnification. These eggs were laid on a 1 x 1 m beige cardboard covered with

a thin layer of gum arabic diluted 50% in water. For the release of these eggs, a wooden chamber (Figure 2) was used, which was placed on the cardboard to prevent egg loss.

After drying, the glued eggs were located and identified using a colored pen. The cardboards were attached to the ground, in the middle of the sugarcane field, between the rows, with stones at the edges to prevent them from being torn away by the wind. The capsules were randomly placed on the soil between the rows of sugarcane. After the installation of the bioassay, the eggs were evaluated daily until 90% of the eggs were preyed upon or until the fourth day of evaluation.

The treatments where pupae were released loose were repeated seven times, and the treatment where pupae were released inside capsules were repeated 30 times in a completely randomized design. With the support of a 10x magnifying glass, the eggs were observed on the cardboard for predation verification. As for the capsules, 10 were removed from the field, opened, and with the support of a checkered transparency (0.25 x 0.25 cm each square), the number of predated squares was counted.

Subsequently, the parasitism efficiency of different soil pupae densities was evaluated in a new bioassay. The treatments were: (1) spread of 5 *A. kuehniella* eggs parasitized by *T. pretiosum* m^{-2}; (2) 10 eggs m^{-2}; (3) 15 m^{-2}; (4) 25 m^{-2}; (5) 35 m^{-2}; and (6) Control (without release).

The spread of parasitized eggs was made in an area of 25 m^2, leaving 50 m of border between repetitions. In the places where the eggs were distributed, 10 cartons of 10 x 0.5 cm cardboard were placed, with new and sterile *A. kuehniella* eggs at the tip (0.5 x 0.5 cm), totaling about 40 eggs per repetition. The cards were randomly attached with a stapler to the sugarcane leaves, and after 8 hours of exposure to parasitism, the cards were removed and taken to the laboratory, where they were placed in flat-bottomed glass tubes (8 x 2.5 cm) and kept in a climate chamber at 28 ± 1°C, 70 ± 10 % RH, and 14 hours of photophase. The next day, new cards were placed at the assay site and kept there again for 8 hours.

Statistical analysis

The average percentage of predated eggs and the average number of parasitized eggs were calculated. The averages of the first two trials were compared using the t-test at the 5% significance level. The averages calculated in the test of loose pupae in the soil were subjected to polynomial regression analysis at the 5% significance level to verify correlations. In the same test, the data were subjected to the homoscedasticity test to determine the best form of analysis, and the means were compared with each other using Tukey's test ($p \leq 0.05$). All statistical calculations were performed using R program (R Core Team, 2020).

RESULTS AND DISCUSSION

Predation of *T. pretiosum* Pupae in Unprotected and Protected Capsules

Significant differences were observed between treatments regarding the average egg predation up to the third day after release (Figure 3).

One day after release, the predation of eggs in unprotected cartons reached 100%, significantly differing from the protected cartons in capsules, which showed around 60% predation. By the fourth day after release, predation in the protected capsules reached nearly 100%, with significant differences between treatments (Figure 3).

In sugarcane leaves, significant differences in average predation of parasitized eggs were also observed. One day after release, the average predation percentage was significantly higher in the unprotected packs (60%) compared to the protected packs (less than 5%) (Figure 4).

Figure 3 - Average predation percentage of *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* glued on unprotected cards or protected by a capsule, released on sugarcane soil. Bars with different letters differ from each other, within each treatment, t test (p≤0,05).

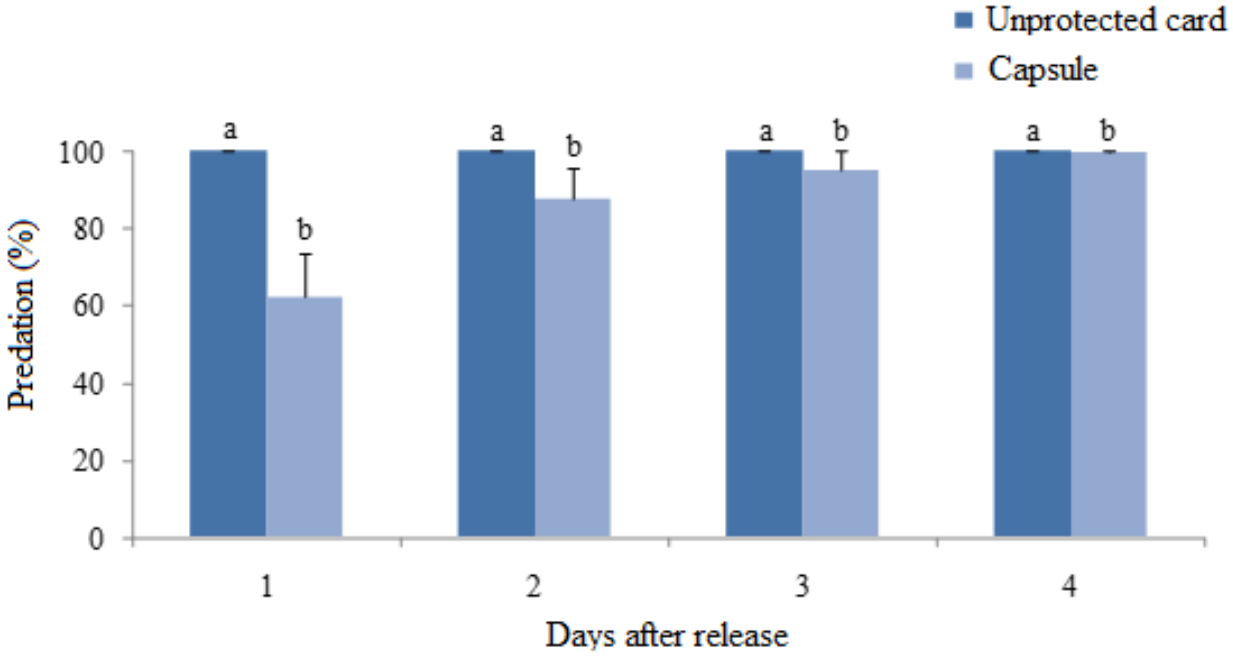
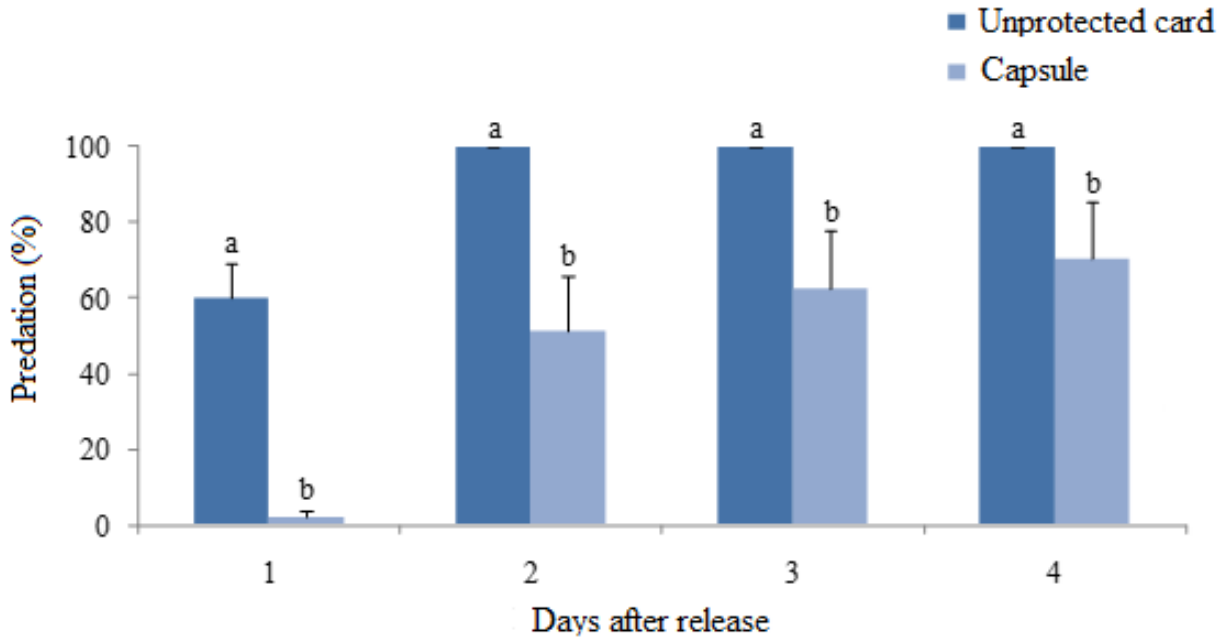


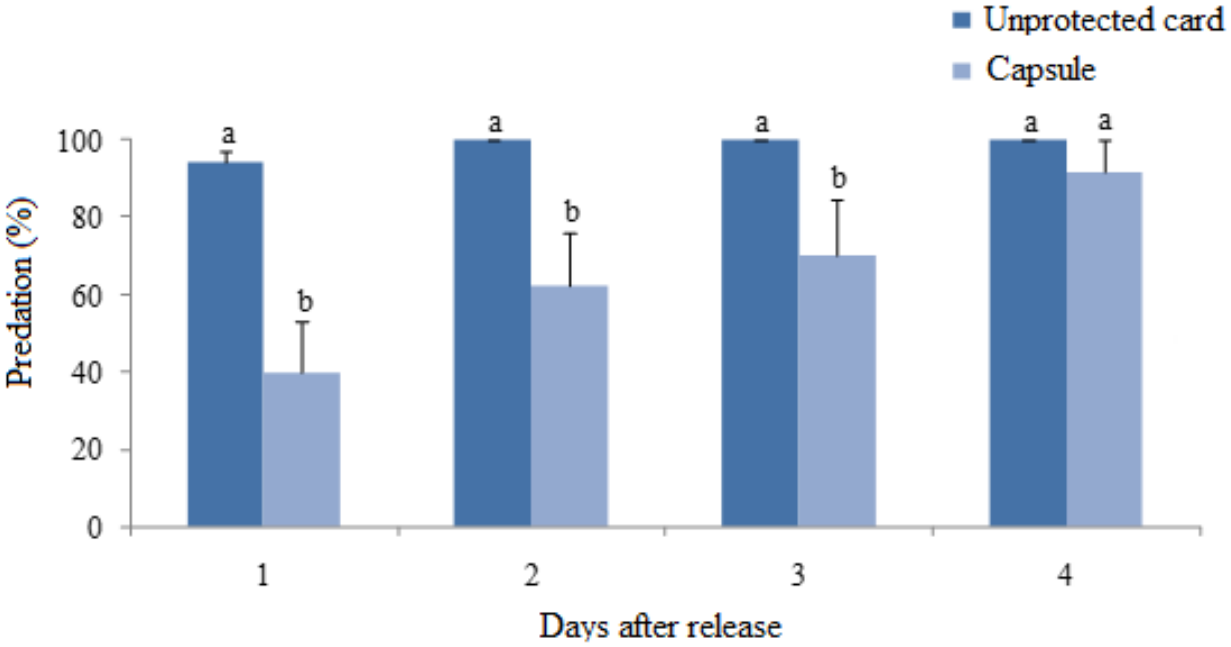
Figure 4 - Average predation percentage of *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* glued on unprotected cards or protected by a capsule, released on on the leaves of sugarcane plants. Bars with different letters differ from each other, within each treatment, t test (p≤0,05).



By the second day, predation in unprotected card treatments reached 100%, with significant differences from all other treatments at subsequent evaluation times. At the final evaluation, four days after release, the predation of parasitized eggs in the protected card treatment reached approximately 80%.

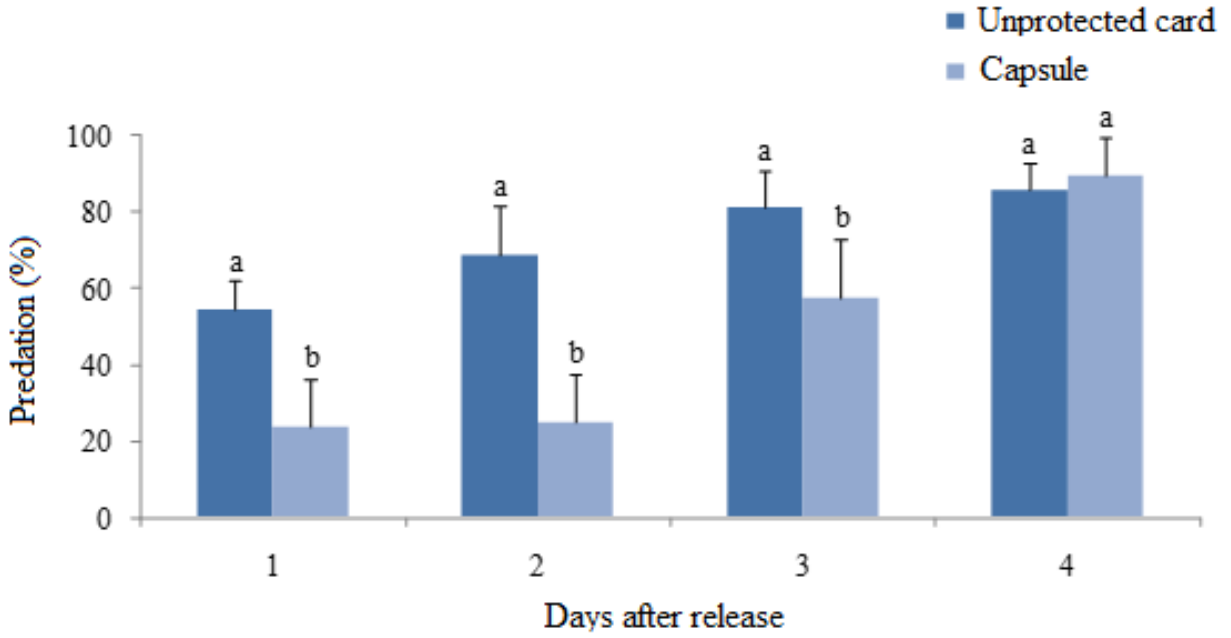
Similar results were observed in the maize crop. On the first day after release, predation of eggs in unprotected cartons reached almost 100%, significantly differing from the protected capsules, which showed less than 40% predation (Figure 5).

Figure 5 - Average predation percentage of *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* glued on unprotected cards or protected by a capsule, released on maize soil. Bars with different letters differ from each other, within each treatment, t test (p≤0,05).



By the fourth evaluation, predation in the capsule treatment reached 91.5% in the soil. For maize leaves, the average predation percentage in unprotected cartons was 54.6% one day after release, significantly differing from the protected cartons (23.9%) (Figure 6).

Figure 6 - Average predation percentage of *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* glued on unprotected cards or protected by a capsule, released on on the leaves of maize plants. Bars with different letters differ from each other, within each treatment, t test (p≤0,05).



By day four, no significant differences in predation rates were observed between treatments. These results suggest that *T. pretiosum* releases in the larval or pupal phases should be protected (e.g., using capsules) to mitigate predator impact. The predation of parasitized eggs in unprotected

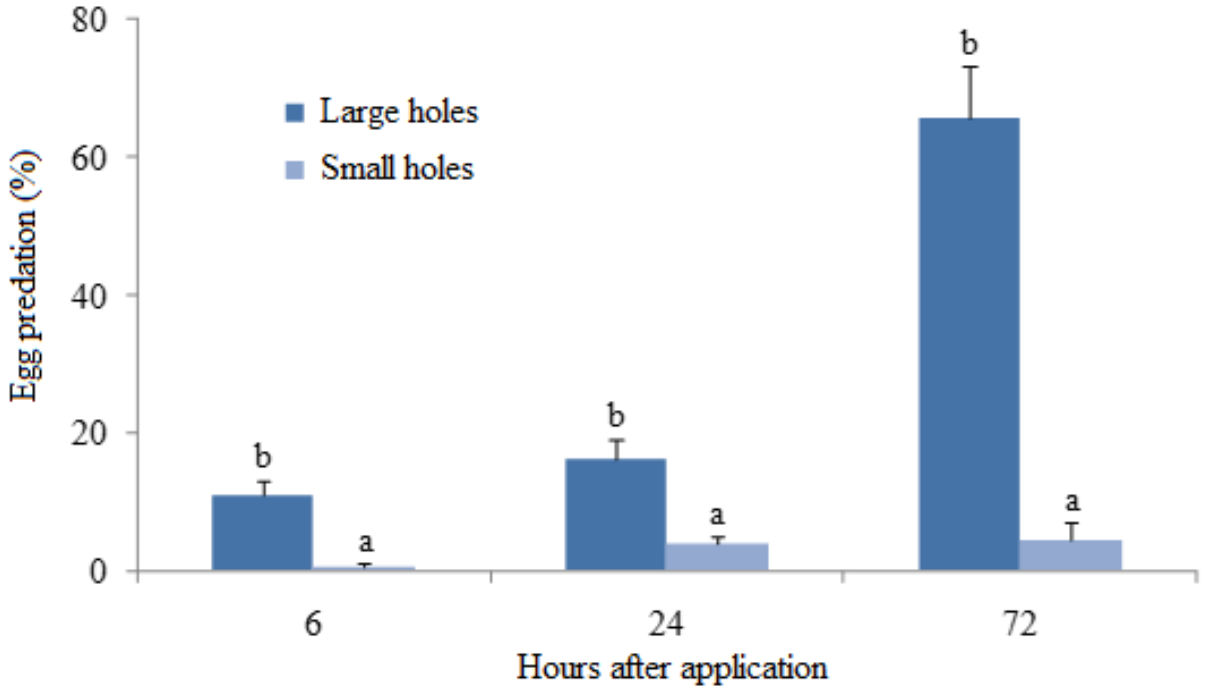
pouches was nearly 100% on the first day after release, except in maize plants, where full predation took about five days.

Eggs placed on soil were more heavily predated than those on plants, primarily by ants and orthopterans. In protected cards placed in the sugarcane soil, predation exceeded 50% one day after release, whereas predation did not exceed 5% when these cards were placed on plants. This indicates that protected capsules should be used when releasing *T. pretiosum*. Additionally, predation was higher in the sugarcane area compared to the maize area. Despite differences in techniques, protected cards experienced high predation due to predator entry through the exit holes.

Predation of *T. pretiosum* Pupae in Capsules with Two Orifice Sizes

Significant differences were found between treatments at all times of exposure, with higher average predation percentages observed in capsules with larger holes (11.02 ± 2.00%, 16.29 ± 2.83%, and 65.67 ± 7.37% for 6, 24, and 72 hours, respectively) compared to those with smaller holes (0.61 ± 0.61%, 3.98 ± 0.94%, and 4.37 ± 2.66%, respectively) (Figure 7).

Figure 7 - Average predation percentage of *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* after 6, 24 and 72 hours in the field on maize soil. Bars with different letters differ from each other, within each treatment, t test (p≤0,05).

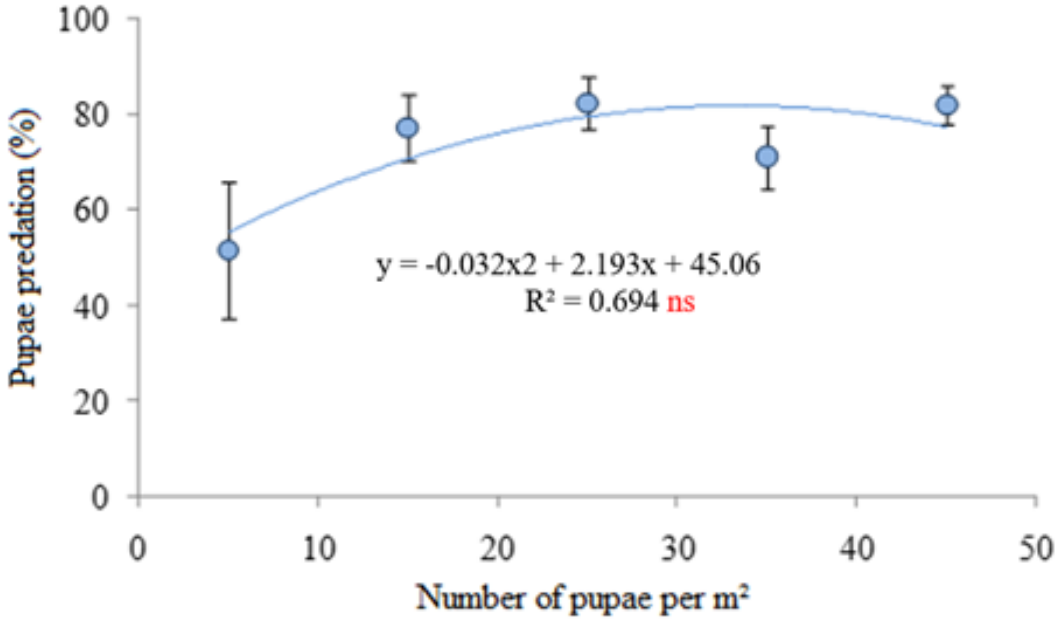


Predation in unprotected capsules was high but did not reach 100% until the third day after release. The size of the capsule's exit port may influence parasitism efficiency, as larger dimensions and longer exposure times resulted in higher predation rates.

Predation of *T. pretiosum* Pupae and Parasitism Efficiency of A. kuehniella Eggs in the Loose Pupae Release Technique in Soil

The predation of *T. pretiosum* pupae contained within parasitized *A. kuehniella* eggs, which were released randomly on the sugarcane soil, was evaluated. No significant differences were found between treatments regarding the average predation percentage of pupae. Additionally, there was no significant correlation between the average predation percentage and the density of loose pupae in the soil (Figure 8).

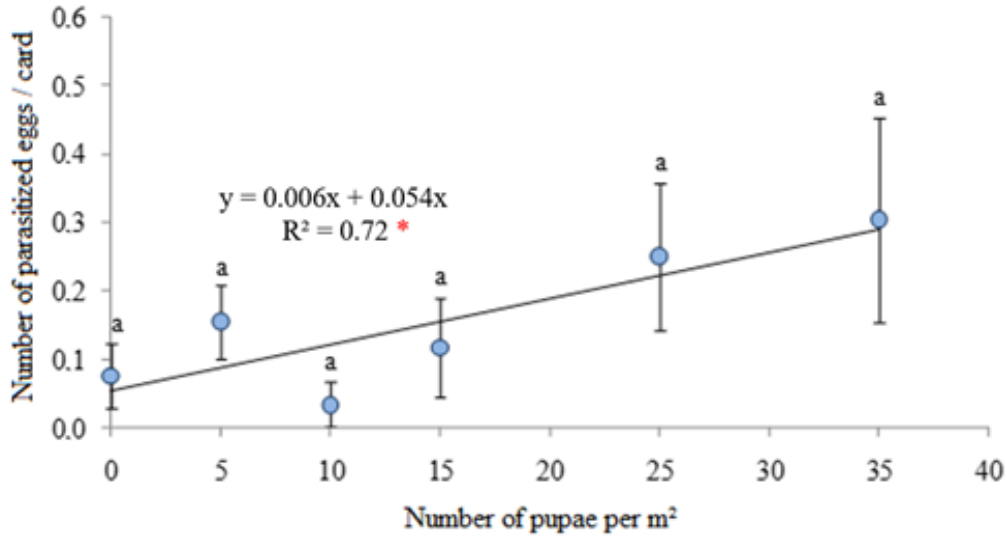
Figure 8 - Average predation of *Trichogramma pretiosum* pupae at different densities on sugarcane soil, and second degree polynomial regression curve, with regression equation between parameters and coefficient determination. ns, not significant (p> 0.05).



The average predation percentage in capsules was 80.44 ± 11.95%, similar to densities of 15 to 45 loose pupae per square meter of soil (Figure 8).

One day after the release of loose *T. pretiosum* pupae in the soil, there were no significant differences between treatments regarding the average number of parasitized eggs. However, a linear, positive, and significant correlation was found between the density of pupae and parasitism efficiency (Figure 9).

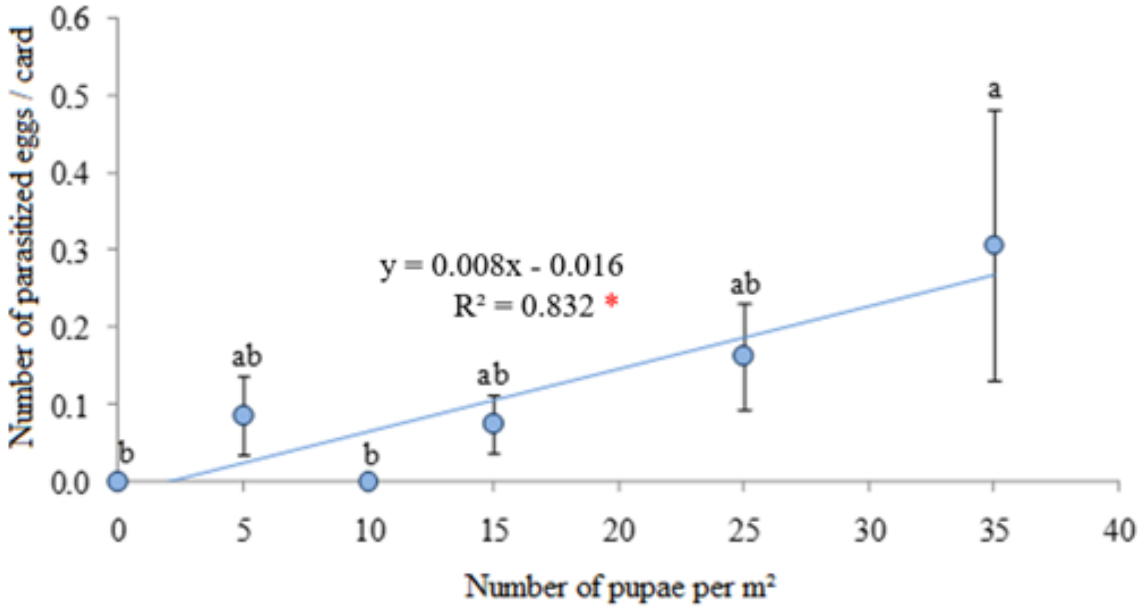
Figure 9 - Average number of *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* released at different densities on sugarcane soil and linear regression line between parameters, with regression equation and coefficient of determination (followed by *, which indicates that there was significant correlation between the points and the straight at 5% level) one day after the release. Points followed by the same letter did not differ from each other by Tukey's test (p≤0.05).



In other words, higher pupae densities in the soil resulted in greater parasitism of *A. kuehniella* eggs on the leaves. After two days, there was a statistical difference between treatments, and a

significant linear correlation between parameters was observed. The treatment with 35 pupae m^{-2} showed significantly more parasitized eggs than the treatment with 10 pupae m^{-2} and the control (Figure 10). Higher pupae densities resulted in higher average parasitism percentages (Figure 10).

Figure 10 - Average number of *Anagasta kuehniella* eggs parasitized by *Trichogramma pretiosum* released at different densities in sugarcane soil and linear regression line between parameters, with regression equation and coefficient of determination (followed by *, which indicates that there was significant correlation between the points and the straight at 5% level) two days after the release. Points followed by the same letter did not differ from each other by the Tukey's test (p≤0.05).



The results of this study partially align with those of Pinto (1999), who observed lower predation of *D. saccharalis* eggs at lower densities in the field. However, no significant correlation was found in this trial. Pinto (1999) evaluated egg predation on sugarcane leaves, which was also lower in this study. This suggests that protected capsules should be used when releasing parasitoids on plants.

The results also partially agree with Broglio-Micheletti et al. (2007), who found higher parasitism with increased parasitoid density. However, their study noted that parasitism increased up to a certain density and then decreased, a trend not observed in this work. Broglio-Micheletti et al. (2007) released emerged adult parasitoids, whereas this study used a different approach.

Mills et al. (2000) demonstrated effective parasitism of *Trichogramma* platneri for controlling *C. pomonella* in apple and pear orchards when 80,937 parasitoids were released per hectare. Above this density, parasitism did not increase, a finding consistent with the current study. This research marks the beginning of a new line of investigation aimed at refining this technique in Brazil, where predation levels are higher due to greater predator diversity.

According to Basso et al. (2020), the aerial application of *Trichogramma* wasps using drones achieved results comparable to or better than terrestrial methods, even with higher doses and 100 release capsules per hectare. This effectiveness was observed despite significant predation of *E. kuehniella* eggs parasitized by *T. pretiosum*, likely due to ants present in the crops. Egg survival rates were significantly lower when placed close to the ground - only 20% after 24 hours and 6% after 48 hours - compared to 62% and 44% at foliage level, respectively.

Additionally, Pratissoli et al. (2024) demonstrated that *Trichogramma pretiosum* exhibited a dispersal capacity of up to 14.21 m within 72 hours under protected strawberry cropping systems,

effectively covering an area of approximately 17 m² when released from a centralized point inside agricultural tunnels. These findings suggest that a release pattern with capsule spacing not exceeding 14 m can ensure adequate field coverage. This complements the results of the present study, which indicate a linear increase in parasitism with pupae density (up to 35 pupae/m²) and no evidence of saturation.

Drone application offers logistical advantages, such as avoiding weather-related delays and not depending on immediate access to the crop post-rain, which are common limitations with terrestrial methods. This approach has been prioritized in Brazil's biological control programs, highlighting its potential for improving pest management efficiency.

The technique of releasing *T. pretiosum* pupae on soil is effective for controlling lepidopteran eggs. However, predation of *T. pretiosum* parasitized by *A. kuehniella* eggs is higher in soil compared to leaves, with higher pupae densities leading to greater parasitism. Predation was higher in unprotected release cards compared to protected cards (capsules).

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