University of São Paulo
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Selectivity of insecticides with two active ingredients on the parasitoid *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae)

Ana Clara Ribeiro de Paiva

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

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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1. Manejo integrado de pragas 2. Toxicidade 3. Controle bológico 4. Pesticida I. Título

DEDICATORY

I dedicate this thesis to my parents, Angela and Vete, that were always giving the necessay support. And to my fiancé, Fernando, without whom this work and days in the lab would be much more difficult.

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I would like to thank…

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CONTENTS

RESUMO

Seletividade de inseticidas formulados com dois ingredientes ativos sobre o parasitoide *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae)

Os resultados obtidos em testes de seletividade, propostos pela IOBC/WPRS, permitem o uso integrado do controle químico com o biológico. No entanto, é importante avaliar, além do efeito letal, outros parâmetros que possam comprometer a eficiência do inimigo natural. Baseado nessa necessidade e, na importância do parasitoide *Trichogramma pretiosum* Riley em programas de controle biológico no mundo todo, objetivou-se com esse estudo avaliar o efeito de inseticidas formulados com dois ingredientes ativos em diferentes estágios de vida desse parasitoide, seguindo as recomendações da IOBC/WPRS, considerando, também, os efeitos subletais. Para isso, os testes a seguir foram conduzidos em duas gerações do parasitoide: (i) efeito de inseticidas sobre pupas imersas em calda inseticida, (ii) efeito de inseticidas pulverizados sobre pupa (iii) efeito de inseticidas em adultos em contato com o resíduo em ovos de *Ephestia kuehniella*, (iv) efeito de inseticidas em adultos em contato com o resíduo em folhas de soja, (v) efeito dos inseticidas na capacidade de voo quando pupas e adultos foram expostas ao resíduo do inseticida, e (vi) duração da atividade nociva dos inseticidas. Os 7 inseticidas testados afetaram, de alguma maneira, pelo menos um dos parâmetros avaliados em todos os testes, mostrando a importância da cautela no uso de inseticidas com dois ingredientes ativos em programas de MIP. Quando a duração da atividade nociva desses inseticidas foi avaliada, 6 dos 7 inseticidas testados foram classificados como vida-curta, e somente a mistura ciproconazol + tiametoxam foi classificado como moderadamente persistente.

Palavras-chave: Manejo integrado de pragas, Toxicidade, Controle Biológico, Pesticida

ABSTRACT

Selectivity of insecticides with two active ingredients on the parasitoid *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae)

The integrated use of chemical and biological control is possible based on the Selectivity tests, as proposed by the IOBC/WPRS. However, it is also important to evaluate further than the lethal effect, as proposed by this organization, assessing other parameters that can alter the efficacy of a natural enemy in the field. Based on this necessity and the importance of the parasitoid wasp *Trichogramma pretiosum* on biological control programs worldwide, the objective of this study was to evaluate the effects of ready-mix insecticides on different life stages of the parasitoid, following the IOBC/WPRS recommendations, and also considering the sublethal effects. Therefore, the following tests were performed on two generations of the parasitoid: (i) effects of insecticides on pupae immersed in insecticide solution (ii) effects of insecticides on pupa sprayed with insecticide solution (iii) effect sof insecticdes on adults in contact with dried-residues on *Ephestia kuehniella* eggs (iv) effects of insecticdes on adults in contact with dried-residues on soyben leaves (v) effects of insecticide on flight capacity when pupae and adults were exposed to the dried-residue (vi) duration of insecticdes' harmful activity. All the insecticides tested affected somehow at least one of the parameters evaluated in all the tests, showing how important it is to carefully use ready-mix insecticides on IPM programs. Regarding the duration of the harmful activity, six out of the seven insecticides tested were classified as short-lived, and only cyproconazole + thiamethoxam was classified as slightly persistent.

Keywords: Integrated pest management, Toxicity, Biological control, Pesticide

1. INTRODUCTION

One of the main challenges of researchers, agronomists, and growers around the world is to find a method to use pesticides in a sustainable and ecological way, in accordance with Integrated Pest Management (IPM) principles. One way to start it is by knowing the effects of the insecticides on biological control agents, since it supports the combined use of biological and chemical controls (CROFT, 1990, JOHNSON AND TABASHNIK, 1999, WILLIAMS AND PRICE, 2004, BUENO et al., 2017, CARVALHO et al., 2019).

The Brazilian agricultural landscape is composed mainly by monocultures in a tropical climate. Thus, pesticides are indispensable and play an important role in our production, since the control of pests depends largely on them. But the use of pesticides has to be done in a proper way, and for these means, more selective insecticides are being required (BUENO AND BUENO, 2012, CHENG et al., 2018). Moreover, if a non-selective pesticide is used, it is possible to have problems not only with the natural enemies released, but also with the ones that were in the area previously to the spraying.

Parasitoids can be exposed to pesticides by two different routes, directly during the spraying, or indirectly, through contaminated host insects or by consuming nectar from plants treated with insecticides (LONGLEY AND JEPSON, 1996). This exposure can prejudice the efficacy of the parasitoid in innumerous manners. And, reducing the activity of a biological control agent, pest resurgence and secondary pest outbreaks are some of the problems that we will need to face (FERNANDES et al., 2010).

In the last few years, insecticides with new compounds and different mode-of-actions have been released to mitigate the problems caused by the overuse of insecticides and, to control chewing and sucking pests at the same time. Furthermore, the companies started to mix different active ingredients in the same formulation (LANTEIGNE et al., 2015, REDDY et al., 2018).

Some of these new active ingredients were released commercially as innocuous to natural enemies, but some studies have shown that this is not always true (BARBOSA et al., 2017, PAIVA et al., 2018, CHENG et al., 2018, KOCH et al., 2019). Moreover, although some studies about the effects of these compounds have been done, it is also important to study how they will act as a ready mixture (BLÜMEL et al., 2001).

Regarding the methodologies for such studies, the International Organization for Biological and Integrated Control/West Palearctic Regional Section (IOBC/WPRS) recommends a combination of tests in sequence to determine whether a pesticide is selective or not to a natural enemy (GRUTZMACHER et al., 2004). Based on these recommendations, all the tests should be performed with the highest field concentration, and the classification of each product is done by calculating the percentage of mortality and reduction on the benefical capacity of the parasitoid after the exposition.

The procedure starts with laboratory tests, where the most susceptible stages (e.g. adult parasitoids and larval predators) and the least susceptible ones (e.g. parasitoids inside the hosts) are exposed to the pesticide. There are several methods to expose the natural enemy to the pesticides, including, but not limited to, dry residue on glass, leaf, eggs, direct treatment, among other (HASSAN, 1997, HASSAN et al., 1998, GRUTZMACHER et al., 2004).

Following the IOBC/WPRS standards, only the pesticides that were not classified as harmless continue to be evaluated. Then, the persistence test is conducted by the exposure of the parasitoid adults to pesticide residues applied on plants 3, 7, 10, 14, 21, and 31 days after treatment, testing the duration of the harmful activity (HASSAN et al., 1998, GRUTZMACHER et al., 2004, CHOWDHURY et al., 2015).

After, the Organization indicate the conduction of a semi-field bioassay, carried under true field conditions. And, finishing the test sequence, the field test in which the crops are directly sprayed with the product and the natural enemies are released (HASSAN et al., 1998).

Considering the complexity of insects' biology, the classification of the pesticide based only on the mortality cannot show the real effect of the pesticide on the natural enemy. Therefore, it is important to highlight the necessity of sublethal effects study. The sublethal effects may affect the general physiology, biochemistry, neurophysiology, development, adult longevity, immunology, fecundity and/or sex ratio. Also, behavioral effects on the mobility, navigation/orientation, feeding, oviposition and or learning performance (DESNEUX et al., 2007).

The easiness to rear the parasitoids from the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) in large scale in laboratory conditions is the main reason ~~of~~ for their success as biological control agents (FLANDERS, 1927, PARRA, 2010). There are more than 200 species of the genus *Trichogramma* in the world, and 29 are registered in Brazil (QUERINO, ZUCCHI, 2019). The most common species used on biological control in Brazil are *Trichogramma galloi* Zucchi and *Trichogramma pretiosum* Riley (PARRA et al., 2014). In the past years, the last one has been used in more than 250.000 ha of soybean to control *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae) and *Chrysodeixis includens* Walker (Lepidoptera: Noctuidae) (PARRA et al., 2015).

Among soybean pests, we can mention some caterpillars that can be controlled by *T. pretiosum*, such as *H. armigera*, *C. includens*, *Anticarsia gemmatalis* Hubner (Lepidoptera: Erebidae), and *Chloridea virescens* Fabricius (Lepidotera: Noctuidae) (FATHIPOUR AND SEDARATIAN, 2013; BUENO et al., 2012, ANDRADE et al., 2011). Besides, this parasitoid presents a big advantage in the control of cartepilars provided by them, which is the fact that they avoid the feeding damage. This is due to the control being achieved in the pest stage prior to their feeding. Another advantage of their application is the development of the immature stages under the host chorion protection (BUENO et al., 2008, CARMO et al., 2010), acting as a protection to the parasitoid.

Based on this, the objective of this study was to evaluate the effects of seven ready-mix insecticides on different stages of the parasitoid *T. pretiosum*, following the IOBC/WPRS recommendations, but also considering the sublethal effects. These data could be used as a contribution to the implementation of IPM in soybean fields.

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2. DO READY-MIX INSECTICIDES CAUSE LETHAL AND SUBLETHAL EFFECTS ON *Trichogramma pretiosum* PUPA?

Abstract

The correct use of selective insecticides aids farmers in maintaining pest populations below the economic threshold level. The integrated use of biological and chemical control is only possible if the effects of insecticides on natural enemies are studied. Although the IOBC/WPRS standards allow us to compare these studies worldwide, the methods used are sometimes inconsistent.This study determined the effects of ready-mix insecticides applied on pupae of *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) and compared the effects on emergence of two different methods of exposing *T. pretiosum* pupae to insecticides: immersed or sprayed using a Potter tower. Both methods gave the same results, indicating that they can be compared. Moreover, it is important to go beyond IOBC/WPRS classification and study the effects of pesticides on different biological param- eters of natural enemies. This additional step may increase the likelihood of successful integration of biological and chemical control. Based on the emergence reduction, chlorantraniliprole + lambda-cyhalothrin, abamectin + chlorantraniliprole, and alpha-cypermethrin + teflubenzuron were classified as innocuous (class 1). Cypermethrin + profenofos and cyproconazole + thiamethoxam were classified as slightly harmful (class 2). Methanol + methomyl and lufenuron + profenofos were classified as harmful (class 4). Abamectin + chlorantraniliprole, although classified as innocuous, reduced the parasitism, longevity, and flight capability of the adult parasitoids. None of these insecticides altered the emergence and sex ratio of the second generation.

Keywords: Biological control; Integrated pest management; Selectivity; Parasitoid

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2.1. Introduction

One of the major problems in agriculture is arthropod pest outbreaks. The best way to reduce their population to a noneconomic-injury level is through a combination of control methods, as in Integrated Pest Management (IPM). It is important to guarantee that the control methods, when used together, do not compromise the effectiveness of each other (Oliveira et al. 2013, Torres and Bueno 2018, Feltrin-Campos et al. 2019). Therefore, an important step for integrating biological and chemical control is the evaluation of the pesticide selectivity.

In order to mitigate resistance problems and also to control chewing and sucking pests simultaneously, manufacturers purposely add certain chemicals to pesticide formulations as mixtures (Lanteigne et al. 2015, Reddy et al. 2018). Regarding their effects on nontarget organisms, these mixtures may contain compounds previously classified as harmless or harmful, making it important to study their effects when applied as a mixture (Blümel et al. 2001).

For determining the selectivity of a chemical product, the International Organization for Biological Control/ West Palearctic Regional Section (IOBC/WPRS) suggests a sequence of tests that starts with laboratory conditions, proceeds to semifield conditions, and finishes with field assays (Hassan 1989, Rowland et al. 1991). For the laboratory tests, it is also important to expose the least susceptible stage, i.e., the pupae inside the host egg, in the case of a parasitoid (Hassan 1998, Grützmacher et al. 2004).

Trichogramma pretiosum (Riley, 1879) (Hymenoptera: Trichogrammatidae) is a generalist egg parasitoid of Lepidopteran, parasitizing over 240 species (Pinto 1998, Chailleux et al. 2013). Thus, being an important and efficient agent for soybean biological-control programs. This parasitoid can control the eggs of *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae) (Bueno et al. 2010), *Heliothis* spp. (Lepidoptera: Noctuidae) (King et al. 1986), *Chrysodeixis includens* (Walker, 1858) (Lepidoptera: Noctuidae) (Querino et al. 2016), *Helicoverpa armigera* (Hubner, 1808) (Lepidoptera: Noctuidae) (Ballal and Singh 2003).

Several studies have tested the effects of insecticides on pupae of Trichogramma species, some by dipping the host eggs containing the pupae (Consoli et al. 1998, Carvalho et al. 2003, Moura et al. 2005, Chen et al. 2013, Paiva et al. 2018), and some by spraying the host eggs (Momanyi et al. 2012, Costa et al. 2014, Grande et al. 2018). However, none of these studies compared these different methods, dipping versus spraying. Moreover, only a few studies have assessed the transgenerational effects (Carvalho et al. 2003, Costa et al. 2014, Grande et al. 2018, Paiva et al. 2018).

A bioassay method must be easily replicable, considering aspects 2.15 such as better use of lab facility and feasibility of use with different types of insecticides and species of parasitoids. The method must be easy to set up, with minimal cost, and above all, provide consistent results (Williams and Price 2004).

There are different methods used in studies by various researchers worldwide, to classify the selectivity of pesticides according to the IOBC/WPRS standards. Hence, the purpose of this study was to compare the reduction in the parasitoid emergence using two different methods of treatment of *T. pretiosum* pupae to insecticides, immersed versus sprayed. Likewise, we determined whether the treatment method influenced the result of laboratory selectivity tests. We also assessed lethal and sublethal effects of ready-mix formulations, until the second generation, when *Ephestia kuehniella* (Zeller, 1879) (Lepidoptera: Pyralidae) containing *T. pretiosum* pupae were immersed in insecticide solution. Previous studies have shown that the individual pesticides have deleterious effects on this parasitoid (Suh et al. 2000, Moura et al. 2005, Grande et al. 2018, Paiva et al. 2018), but the effects of most ready-mix formulations have not been determined.

2.2. Methods

2.2.1. Rearing and maintenance of *Trichogramma pretiosum*

The colony of the parasitoid *T. pretiosum* was maintained in the Integrated Pest Management Laboratory at the Department of Entomology and Acarology, 'Luiz de Queiroz' College of Agriculture, University of São Paulo (ESALQ/ USP), as described by Parra, 1997. The eggs of *E. kuehniella* used in the rearing and bioassays were provided by Koppert Brasil, and sterilized as proposed by Stein and Parra (1987).

The colony was maintained, and the bioassays were carried out at a temperature of 25 ± 2°C, relative humidity of 70 ± 10%, and a photoperiod of 14:10 (L:D) h.

2.2.2. Insecticides tested

The insecticides used in the bioassays were ready-mix products recommended for the control of lepidopteran pests in soybean crops in Brazil. They were used at the highest concentration recommended by the company, diluted in distilled water to a spray volume of 200 liters/ha (Table 1). Distilled water was used for the control treatment.

The products were mixtures of two insecticides' active ingredients, except one that had a fungicide active ingredient (cyproconazole) in the mixture. This product was chosen because it is among the products most often used by soybean growers in the Cerrado region, central Brazil.

Table 1. Ready-mix insecticides formulated with two active ingredients used in the toxicity to *Trichogramma pretiosum* bioassays.

Trade name	Company	Active ingredient	Chemical group	Mode of action[5]	Concentration	
					AI[1]	CP[2]
Ampligo	Syngenta	chlorantraniliprole + lambda-cyhalothrin	diamide + pyrethroids	Ryanodine receptor modulators + sodium channel modulators	100+50	75[3]
Bazuka	Rotam	methanol + methomyl	aliphatic alcohol + oxime methylcarbamate	+ acetylcholinesterase inhibitors	383.5+216	1990[3]
Voliam Targo	Syngenta	abamectin + chlorantraniliprole	avermectin + anthranilamide	Glutamate-gated chloride channel allosteric modulator + Ryanodine receptor modulators	18+45	200[3]
Curyom 550 EC	Syngenta	lufenuron + profenofos	benzoylurea + organophosphate	Inhibitor of chitin biosynthesis (type 0) + acetylcholinesterase inhibitors	50+500	400[3]
Imunit	Basf	alpha-cypermethrin + teflubenzuron	pyrethroids + benzoylurea	Sodium channel modulators + Inhibitor of chitin biosynthesis (type 0)	75+75	200[3]
Polytrin 400/40 CE	Syngenta	cypermethrin + profenofos	pyrethroids + organophosphate	Sodium channel modulators + acetylcholinesterase inhibitors	40+400	120[3]
Verdadero 600 WG	Syngenta	cyproconazole + thiamethoxam	triazole+ neonicotinoid	Fungicide + nicotinic acetylcholine receptor competitive modulator	300+300	200[4]

1 Active ingredient concentration g L^{-1}. 2 Commercial dose. 3 mL ha^{-1}. 4 kg ha^{-1}. 5 Mode of action according to Insecticide Resistance Action Committee classification (IRAC, 2019).

2.2.3. Effect of the insecticides on *Trichogramma pretiosum* pupae - immersed

Cards with *E. kuehniella* eggs were offered to *T. pretiosum* females to be parasitized for 24 h. Each card represented a replicate. Next, the females (F_0) were discarded and the cards were maintained in glass tubes (85 × 10 mm) under controlled conditions as described earlier. The first assay, considering the emergence reduction was carried out in a completely randomized design, with 30 replicates of eight treatments (*n* = 240). While the second assay, evaluating the effect on F_1 parasitism capacity (number of eggs parasitized by each female during its life spam), sex ratio and the emergence of F_2 was also carried out in a completely randomized design, but with 15 replicates of seven treatments (*n* = 105), each card represented one replicate again. The number of treatments was reduced because methanol + methomyl caused high mortality in the first assay, which did not allow evaluation of sublethal effects.

The number of black eggs (parasitized) was assessed 168 h after the parasitism, when all the parasitoids had reached the pupal stage (Moura et al. 2005). After 24 h, the cards were immersed for 5 s in the insecticide solution and kept at room temperature until the residues dried.

For each treatment, 30 cards, with at least 10 parasitized eggs, were used. The cards were kept in glass tubes and checked daily to assess parasitoid emergence. The sex was also recorded for all the emerged adults, based on the adults' antennae characteristics (Bowen and Stern 1966), and the sex ratio (SR) was calculated using the formula: SR = [number of females / (number of males + number of females)].

The number of black eggs with an emergence orifice was evaluated 4 d after emergence. The percentage of emergence from the first generation (F_1) was calculated based on the proportion of the number of eggs with an emergence orifice to the number of parasitized eggs (black eggs) (Degrande and Gomez 1990).

The percentage of reduction in emergence was calculated using the formula: R = ((P/p)) × 100), in which R is the percentage of reduction in emergence, P is the mean value of emergence for the insecticide, and p is the mean emergence of the control treatment (Rocha and Carvalho 2004).

Considering the worldwide collaboration and importance of IOBC/WPRS, it is desirable to classify the insecticides based on this organization's standards. Therefore, the insecticides were classified according to the following classes: class 1 = innocuous

(<30% reduction), class 2 = slightly harmful (30–80% reduction), class 3 = moderately harmful (>80–99% reduction), and class 4 = harmful (>99% reduction in emergence percentage) (Hassan 1997).

To evaluate the sublethal effects of the insecticides on the parasitoid, 15 females from the F_1 generation in each treatment were iso- lated and offered cards with *E. kuehniella* eggs for parasitism. The cards with eggs were renewed every 24 h until the female died. All the cards were kept under the same conditions, as described above in item 'Rearing and maintenance of *Trichogramma pretiosum*'. Parasitism capacity and longevity were assessed. The emergence percentage of the second generation (F2) was also recorded.

2.2.4. Effect of the insecticides on *Trichogramma pretiosum* pupae - sprayed

IOBC/WPRS recommends spraying the cards using a Potter Tower (Burkard Scientific, Uxbridge, United Kingdom) with pressure adjusted to 0.7 kg cm^{-2}, corresponding to a deposit of 1.8 ± 0.1 mg cm^{-2} (Beloti et al. 2015). The bioassay was conducted exactly as the one aforementioned, when the host eggs were immersed. However, instead of immersing the cards in the pesticide solution, the cards were sprayed in the Potter Tower as described. These cards were monitored according to the immersing procedure and recorded until the emergence of the F_1. This allowed a direct comparison between the methods, to determine their influence on the parameters assessed (mortality, parasitism, emergence).

This assay was carried out in a completely randomized design, with 30 replicates of eight treatments (n = 240).

2.2.5. Sublethal effect of insecticides, evaluated using the flight test

Considering the importance of dispersal for parasitoids, the least deleterious insecticides from the previous bioassays were evaluated to determine their effect on the flight capability of *T. pretiosum*. The cards were treated as described in the bioassay 'Effect of the insecticides on *Trichogramma pretiosum* pupae—immersed', and kept in glass tubes in the test unit (Prezotti et al. 2002) (Fig. 1).

The test unit, based on the ESALQ model (Prezotti et al. 2002), consisted of a PVC cylinder, 18 cm tall and 11 cm in diameter, lined with black paper. A Petri dish covered with entomological glue was placed on the top of the cylinder. The same glue was used on the paper inside the tube, in a 1-cm ring 3.5 cm from the bottom, to catch the parasitoids that attempted to walk on the paper. The cylinder was placed over a Petri dish lined with black paper. The tube with the parasitized eggs was placed inside the cylinder, to allow the parasitoids to extend their wings before flying. After 72 h, the numbers of parasitoids on the top Petri dish (flying) and the bottom Petri dish, and on the glue ring on the walls (walking) were recorded.

This assay was carried out in a completely randomized design, with four repetitions of five treatments (n = 20). The treatments were chlorantraniliprole + lambda-cyhalothrin, abamectin + chlorantraniliprole, alpha-cypermethrin + teflubenzuron, cyproconazole + thiamethoxam, and distilled water, used as control.

Each replicate consisted of one test unit and approximately 100 *T. pretiosum* parasitized eggs. The percentage of adults on each site was calculated based on the total number of insects found.

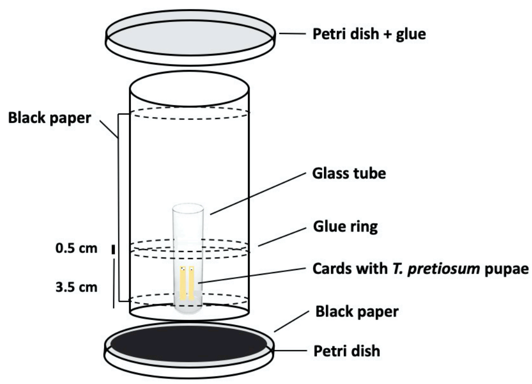

Figure 1. Test-unit based on ESALQ model, used to record the flight capability of *Trichogramma pretiosum*, adapted from Prezotti et al. (2002).

2.2.6. Statistical analysis

The data for parasitism capacity, longevity (days), emergence percentage (ratio of eggs with an emergence orifice to the total parasitized eggs), and sex ratio (ratio of females to the total population) were tested for normality, using the Shapiro–Wilki test.

Since the residuals did not follow a normal distribution, they were submitted to the nonparametric test of Kruskal–Wallis and compared by a Tukey test. The comparison of the two treatment methods, using emergence and sex-ratio data, was conducted using a generalized linear model, family quasibinomial. These analyses were done in the extension XLSTAT 2019 from Microsoft Excel (Addinsoft 2019).

Flight test data (number of insects found in each position) were also tested for normality. Then, a two-way analysis of variance was performed, with treatment and place the two factors, using the software SAS v.9.4 (SAS 2019).

2.3. Results and discussion

2.3.1. Effect of insecticides on *Trichogramma pretiosum pupae*

Considering our objective of comparing two different ways to treat the host eggs containing the pupae, sprayed and immersed, no difference in the emergence was found (all two-tailed tests resulted in $P > 0.05$). The same held true for the sex ratio. Thus, we suggest that both methods, sprayed and immersed cards, are comparable and might be used as a standard.

The IOBC/WPRS standardizes classes but not the insecticide exposure route for natural enemies, neither the method of application of the insecticide. Based on our results, it will be easier to compare studies when both of these different methods are used.

Among the seven insecticides tested, only three were classified as innocuous: chlorantraniliprole + lambda-cyhalothrin, abamectin + chlorantraniliprole and alpha-cypermethrin + teflubenzuron. Cypermethrin + profenofos and cyproconazole + thiamethoxam were classified as slightly harmful. Methanol + methomyl and lufenuron + profenofos were classified as harmful, causing more than 99% reduction in parasitoid emergence (Table 2).

Table 2. Effect of ready-mix insecticides on the emergence of *Trichogramma pretiosum*, by the immersion or spraying of the host *Ephestia kuehniella* eggs containing the pupal stage of the parasitoid (7 d after parasitism) (n = 30) (mean ± standard error).

Treatment	Sprayed			Immersed		
	Emergence (%)[1]	Reduction in Emergence (%)[2]	IOBC/WPRS Class[3]	Emergence (%)	Reduction in Emergence (%)	IOBC/WPRS Class
Control	88.89 ± 0.65 a	–	–	90.38 ± 0.49 a	–	–
chlorantraniliprole+ lambda-cyhalothrin	81.56 ± 1.18 ab	8.24	1	92.06 ± 0.65 a	0.00	1
methanol + methomyl	0.00 ± 0.00 e	100.00	4	0.00 ± 0.00 e	100.00	4
lufenuron+ profenofos	0.35 ± 0.12 d	99.61	4	4.82 ± 0.49 d	94.66	4
abamectin+ chlorantaniliprole	65.27 ± 2.97 b	26.57	1	74.88 ± 1.12 b	17.15	1
alpha-cypermethrin+ teflubenzuron	75.17 ± 1.09 b	15.44	1	90.76 ± 0.66 a	0.00	1
cypermethrin+ profenofos	28.59 ± 2.22 c	67.83	2	28.64 ± 2.05 c	68.31	2
cyproconazole +thiamethoxam	42.21 ± 2.16 c	52.52	2	62.97 ± 3.09 b	30.32	2

[1]Means followed by the same letter do not differ according to Tukey test ($p < 0.05$).
[2]Reduction in emergence calculated through the formula R = (1(P/p)) x 100), in which R is the percentage of reduction in emergence, P is the mean value of emergence for the insecticide, and p is the mean emergence in the control treatment.
[3]IOBC/WPRS classes: class 1 = innocuous (< 30% reduction), class 2 = slightly harmful (30–80% reduction), class 3 = moderately harmful (> 80–99% reduction) and class 4 = harmful (> 99% reduction in emergence percentage) (Hassan, 1997).

Two of the insecticides tested contain the active ingredient profenofos. Nevertheless, based on the reduction in emergence, cypermethrin + profenofos was classified as slightly harmful, and lufenuron + profenofos as harmful (Table 2). This difference in the results is easily explained by the difference in doses and the presence of the other insecticide in each mixture. In our study, cypermethrin + profenofos had a profenofos concentration more than four times higher than in lufenuron + profenofos. These results indicate the high toxicity of this organophosphate in high concentrations.

The combination of a pyrethroid and an organophosphate is expected to be toxic. In contrast, the present results with cypermethrin + profenofos were classified as slightly harmful (class 2) (Table 2). Momanyi et al. (2012) tested the same formulation, cypermethrin + profenofos (500 g A.I./liter), on *Trichogrammatoidea* sp. nr. *lutea* girault (Girauld), describing it as harmful to preimaginal development and causing almost total reduction of emergence. When Suh et al. (2000) evaluated the effects of these two active ingredients separately on *T. exiguum* Pinto & Platner, 1978 (Hymenoptera: Trichogrammatidae), both profenofos (11 g A.I./ liter) and cypermethrin (1.20 g A.I./liter) were classified as harmful based on the IOBC/WPRS, causing a reduction in emergence of 100 and 98%, respectively. These differences in results could be explained by the different species assessed and the likelihood of the ready-mix formulation changing the effect of the insecticide on the parasitoid.

Only one of the ready-mix insecticides tested here, cyproconazole + thiamethoxam, has a fungicide, cyproconazole, as an active ingredient, and was classified as slightly harmful. When Bueno et al. (2008) tested the effects of this fungicide mixed with azoxystrobin on *T. pretiosum*, the emergence was higher than 90%. Khan and Ruberson (2017) also tested a fungicide from the same group, triazoles, and found little to no adverse effect on *T. pretiosum* pupae.

In this study, the insecticide combined with the fungicide is a neonicotinoid, thiamethoxam. Costa et al. (2014) tested the effects of this active-ingredient on the same development stage of *T. galloi*, and classified it as harmless. The different results could be explained by the combination of the active-ingredients, as well as the species used in the bioassays. Therefore, further studies are necessary to confirm whether the fungicide and the mixes actually affects the parasitoid.

The classification methanol + methomyl as harmful obtained (Table 2) in our study corroborates Bueno et al. (2008), where insecticides with methomyl as the only active ingredient were classified as slightly harmful to *T. pretiosum*.

Although the combination of abamectin + chlorantraniliprole was classified as innocuous, parasitism capacity (K = 53.64, df = 6, $P < 0.0001$) and the longevity (K = 43.75, df = 6, $P < 0.0001$) were significantly reduced in insects exposed to this insecticide (Fig. 2). Hence, although this combination did not influence the emergence, it did alter the parasitoids' longevity, which is a parameter commonly used as an index of wasp quality (Marston and Ertle 1973, Waage and Ming 1984). Similar results were observed by Cônsoli et al. (1998) and Carvalho et al. (2003) for abamectin alone.

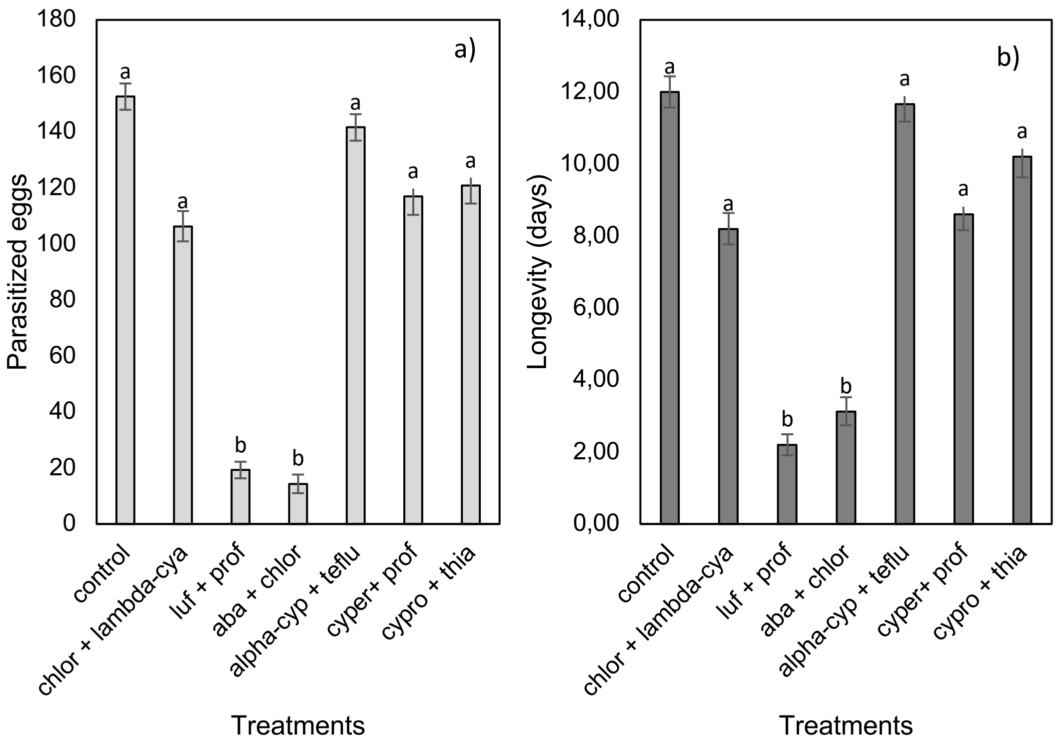

Figure 2. Parasitism capacity (a) and longevity (± standard error) (b) of *Trichogramma pretiosum* females (n=15) emerged from pupae exposed to pesticides (F_1). chlor = chlorantraniliprole; lambda-cya = lambda-cyhalothrin; luf = lufenuron; prof = profenofos; aba = abamectin; alpha-cyp = alpha-cypermethrin; teflu = teflubenzuron; cyper = cypermethrin; cypro = cyproconazole; thia = thiamethoxam.

Regarding sex ratio, no differences were found among the treatments in any of the methodologies tested. Carvalho et al. (2003) and Parra (1994) tested lambda-cyhalothrin, teflubenzuron and abamectin individually, and none affected the sex ratio, similar to this study, showing that, even when these active ingredients are combined with other active ingredients they do not affect the sex ratio.

Considering the preselection of offspring sex by females, and the fact that, in this assay, all the females were primarily exposed to the same conditions, no difference was expected in the sex ratio of the generation emerged after the treatments and control. A difference in the sex ratio would be expected if the insecticides were more harmful to females than to males, or vice versa, which did not occur in this case.

2.3.2. Sublethal effect of insecticides, evaluated using the flight test

The flight capability of parasitoids is not commonly observed in tests of the effects of insecticides on these natural enemies. Nevertheless, it is essential to analyze this parameter because females that cannot fly will not find a host and provide effective pest control. This is the first study to report on wasp flight capability as a sublethal effect of pesticide exposure in laboratory tests.

The locations where the insects were found differed significantly. Most of the insects were found at the top, which means that they flew. Some of the insects were found on the side of the test-unit (on the ring) and a minority was found at the bottom, indicating that they walked. Comparing the effect of the insecticides based on the position of the parasitoid adults in the test-unit, the only difference was a higher percentage of insects found at the bottom in the treatment with abamectin + chlorantraniliprole (19.50%) (Table 3). Regarding the number of adults on the side and top of each unit, no difference among the treatments were found.

None of the insecticides tested caused sublethal effects able to impair the parasitoid's ability to fly, since most (67–87%) of the adults were found at the top of the test-unit. The percentage of insects found at the top of the test-unit did not differ significantly in any of the treatments (Table 3).

Table 3. Effect on fligh capacity of *Trichogramma pretiosum* adults (n = 100) were found in the flight test.

Treatment/position	Bottom (%)	Glue ring (%)	Top (%)
Control	0.68 a[1]	12.80 ns	86.50 ns
chlorantraniliprole + lambda-cyhalothrin	1.46 a	12.70	85.80
abamectin + chlorantraniliprole	19.50 b	13.90	66.50
alpha-cypermethrin + teflubenzuron	6.82 a	7.00	86.20
cyproconazole + thiamethoxam	7.38 a	8.48	84.10

[1]Means followed by the same letter do not differ according to Tukey test ($p < 0.05$), comparing the treatments in each location (bottom, side and top).

Based on our results, researchers will be able to apply the most practicable methodology for each condition and compare the results whether the cards containing the parasitized eggs are immersed or sprayed. With respect to the lethal and sublethal effects, among the insecticides tested, only chlorantraniliprole + lambda-cyhalothrin, alpha-cypermethrin + teflubenzuron can be considered selective for the pupal stage of *T. pretiosum*, being liable to be used for IPM. However, we emphasize that more studies should be done with other development stages of the parasitoid, and under semifield and field conditions.

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3. LETHAL, SUBLETHAL AND PERSISTENCE EFFECTS OF SEVEN READY-MIX INSECTICIDES ON *Trichogramma pretiosum* RILEY (HYMENOPTERA: TRICHOGRAMMATIDAE)

Abstract

Selectivity studies act as allies in integrated pest management, allowing the combined use of biological and chemical control. To achieve consistent results, it is important to evaluate not only the lethal effects of insecticides on natural enemies, but also the sublethal effects on more than one generation, and the duration of insecticides' harmful activity on adults. This study evaluated the acute toxicity, sublethal effects (parasitism capacity, longevity, emergence of F_1 and F_2 generations, and sex ratio), and the persistence of seven ready-mix insecticides on the parasitoid *Trichogramma pretiosum*. The effects of the insecticides classified as harmless and slightly harmful on the parasitoids' flight capacity were also evaluated. Following the IOBC/WPRS standards, when the adults were exposed to treated leaves, only abamectin + chlorantraniliprole was classified as innocuous, with no deleterious effect. When adult wasps were exposed to the residue on cards containing eggs of *Ephestia kuehniella*, all the insecticides caused at least one deleterious sublethal effect, even the insecticides classified as innocuous. Regarding persistence, only cyproconazole + thiamethoxam was classified as slightly persistent, while all other insecticides were classified as short-lived. Sublethal effects were observed only until 5 days after spraying for chlorantraniliprole + lambda-cyhalothrin and alpha-cypermethrin + teflubenzuron.

Keywords: Biological control; Integrated pest management; Selectivity; Parasitoid

Highlights

- The effects of ready-mix insecticides on *Trichogramma pretiosum* were assessed.
- A mixture of two active ingredients increases the toxicity.
- Insecticides that cause low mortality can still have sublethal effects.
- Insecticides containing pyrethroids showed repellency properties.

3.1. Introduction

A selective pesticide is one that controls the target pest but has less impact on non-target organisms (Degrande and Gomez, 1990; Yamamoto et al., 1992; Bueno et al., 2017; Carvalho et al., 2019). Ripper et al. (1951) described two types of selectivity, physiological, based on the physiological differences between the organisms; and ecological, based on behavioral and habitat differences.

Protocols developed by the International Organization for Biological and Integrated Control of Noxious Animals and Plants/ West Palearctic Regional Section (IOBC/WPRS) aim to standardize selectivity trials. These tests follow a sequence, from the laboratory, to semi-field, and then to field studies (Hassan, 1997). The products tested are classified according to the mortality of the natural enemy being investigated. Although this information is essential, we cannot undervalue the importance of the sublethal effects and the potential ecological consequences from exposure to insecticides (Guedes et al., 2016).

Regarding sublethal effects of insecticides on natural enemies, most studies have shown that insecticides can alter the insects' physiology and behavior (Desneux et al., 2007). Direct and indirect consequences of insecticide application include reduction of host numbers, decrease in host quality (Cloyd and Bethka, 2010), and contamination of floral and extrafloral nectar, causing mortality of nectar-feeding natural enemies (Lord et al., 1968; Stapel et al., 2000). Some insecticides may have a repellent effect, preventing parasitoids or predators from entering a crop field (Guedes et al., 2016).

All these negative impacts pose a risk to pest-management programs and can have consequences, such as outbreaks of secondary pests, resurgence of pests, and selection of resistant pests, in addition to environmental degradation (Fathipour and Sedaratian, 2013). Nowadays, besides the overuse of pesticides and all other problems mentioned, pesticide companies are producing ready-mix formulations, with two or more active ingredients combined in the same commercial product (Regupathy et al., 2004). Even though these mixtures may be helpful in pest control, their effects on the natural enemies are still unknown.

Parasitoid wasps of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) are widely studied in selectivity tests, since these natural enemies are most often used in biological-control programs worldwide (Giolo et al., 2005; Parra et al., 2015). In Brazil alone, the parasitoid wasp *Trichogramma pretiosum* Riley is used on over 250 000 ha to control *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) in soybean (Parra et al., 2015).

Considering the necessity of evaluating the effects of insecticides on natural enemies, and the few studies that have assessed ready-mix insecticides, this study aimed to evaluate the acute toxicity and sublethal effects of seven ready-mix insecticides on the parasitoid *T. pretiosum.* The wasps were exposed to the insecticides by: (1) contact with soybean leaves dipped in insecticide solution; (2) contact with cards containing eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) dipped in insecticide solution; and (3) contact with soybean leaves from previously sprayed plants (persistence tests). We also compared the different routes of exposure, the persistence when plants were maintained in a greenhouse and when they were maintained in an open field, and the effects of the harmless insecticides on the flight capacity of the wasps.

3.2. Material and Methods

The colony of the parasitoid *T. pretiosum* was maintained and the bioassays conducted in the Integrated Pest Management Laboratory of the Department of Entomology and Acarology, "Luiz de Queiroz" College of Agriculture, University of São Paulo (ESALQ/ USP). Ambient conditions were controlled at 25 ± 2 ˚C, relative humidity 70 ± 10%, and photoperiod of 14:10 (light:dark).

All *E. kuehniella* eggs used in the bioassays were provided by Koppert® Brazil and sterilized with UV light as proposed by Stein and Parra (1987) and Parra (1997).

3.2.1. Parasitoid colony

The egg parasitoid *T. pretiosum* was reared using *E. kuehniella* eggs. The sterilized eggs of the alternative host were attached an area of 0.5 cm^2, in a paper cards (8 cm L × 2 cm W) with double-sided tape (3M®, Sumaré, Brazil).

Each card was placed in a glass tube (8.5 cm high × 2.5 cm diameter) containing newly emerged *T. pretiosum* adults. A droplet of pure honey was provided as food. After 24 h, the card was removed and maintained in controlled temperature until a new generation of adults emerged.

3.2.2. Insecticides

The insecticides selected were ready-mix commercial products that are commonly used to control lepidopteran pests in soybean fields in Brazil. They were tested in the following concentrations: chlorantraniliprole + lambda-cyhalothrin

(Ampligo®, 10.0% + 5.0%, EC, Syngenta) 0.00375 + 0.0019 g a. i. L^{-1}; methanol + methomyl (Bazuka 216 SL®, 38.4% + 21.6%, SL, Rotam) 2.14 + 3.81 g a. i. L^{-1}; lufenuron + profenofos (Curyom 550 EC®, 64% + 50%, EC, Syngenta) 0.1 + 1.0 g a. i. L^{-1}; abamectin + chlorantraniliprole (Voliam Targo®, 1.8% + 4.5%, SC, Syngenta) 0.018 + 0.045 g a. i. L^{-1}; alpha-cypermethrin + teflubenzuron (Imunit®, 7.5% + 7.5%, SC, BASF) 0.075 + 0.075 g a. i. L^{-1}; cypermethrin + profenofos (Polytrin®, 4% + 40%, EC, Syngenta) 0.024 + 0.24 g a. i. L^{-1}; and cyproconazole + thiamethoxam (Verdadero 600 WG®, 30% + 30%, WG, Syngenta) 0.3 + 0.3 g a. i. L^{-1}. All the concentrations were the highest field rate recommended for soybean, assuming a spray volume of 200 L per hectare. The products were diluted in distilled water, which was also used as negative control.

3.2.3. Effects of ready-mix insecticides on two generations of *Trichogramma pretiosum* in contact with cards dipped in insecticide solution

Cards containing approximately 250 *E. kuehniella* eggs were dipped for 5 s in insecticide solutions and kept at room temperature until they dried. Each card was placed in a glass tube (85 mm × 10 mm) containing one *T. pretiosum* female. Each tube was considered a repetition, and the bioassay was conducted in a completely randomized design, with 30 repetitions and 8 treatments (n = 240).

Every 24 h, a new, untreated card with *E. kuehniella* eggs was offered to the surviving females, until their death. Each card removed was placed in a new glass tube, and checked within 7 days, when all the parasitoids were at the pupal stage (Cônsoli et al., 1999). Using a stereoscopic microscope (40× magnification), the number of black (parasitized) eggs was counted. These data were used to assess the parasitism capacity and longevity of the F_0 generation.

The reduction of the parasitism capacity was calculated based on the formula R = (1–(P/p))*100, where: “R” corresponds to the percentage of reduction in the parasitism capacity, “P” to the mean parasitism in each treatment, and “p” to the mean parasitism in the control treatment (Rocha and Carvalho, 2004). All insecticides tested were also classified based on IOBC/WPRS categories: Class 1: Innocuous (reduction

< 30%); Class 2: slightly harmful (30 ≥ reduction ≤ 80%); Class 3: moderately harmful (80% > reduction ≤ 99%); and Class 4: harmful (reduction > 99%) (Hassan, 1997).

The assessment of the next generation's emergence started 7 days after the test was installed and was performed each day for 7 days. The F_1 (first) generation percentage of emergence was calculated considering the ratio between the number of black (parasitized) eggs and the number of eggs with a visible hatching orifice (Degrande and Gomez, 1990).

To assess the effects of insecticides on the F_1 generation sex ratio, all the insects emerged from the eggs with insecticide residue were sexed, based on the adults' antennae characteristics (Bowen and Stern, 1966). With this information, the sex ratio was calculated using the formula: [number of females / (number of males + number of females)].

Then, 15 females from each treatment (when possible, according to the number of surviving wasps) were placed in individual glass tubes (85 mm h × 10 mm diam.). The same characteristics assessed in the F_0 generation were checked in the F_1 generation, until the emergence of F_2 (second generation).

The results for parasitism from the maternal generation (F_0) and first generation (F_1) emergence were evaluated considering two periods. First, only the first 24 h, which was the period when the offered eggs were treated; and second, the period of each female's life span, when the offered eggs were not treated, assessing the total number of eggs parasitized and total progeny emergence.

3.2.4. Effects of ready-mix insecticides on *Trichogramma pretiosum* in contact with soybean leaves sprayed with insecticide solution

To evaluate the acute toxicity of insecticides on *T. pretiosum* in semi-field conditions, soybean leaves (variety BRS 232) were collected from plants grown in pots (10 L) in a greenhouse. The leaves were taken to the laboratory and cut into discs (4.0 cm diam.). Then, each disc was sprayed on both sides with 2 mL of insecticide solution, using a Potter Tower (Burkard Scientific, Uxbridge, UK) as recommended by IOBC/WPRS, with the pressure adjusted to 0.7 kg cm^{-2}, corresponding to a deposit of 1.8 ± 0.1 mg cm^{-2} (Beloti et al., 2015). Each tube containing a disc and one *T. pretiosum* female was considered a repetition. The bioassay was carried out in a completely randomized design, with 30 repetitions and 8 treatments (n = 240).

After the discs were sprayed, they were kept at room temperature until the insecticide solution on the surface dried completely. This was checked carefully, to prevent the wasps from exposure to gas emitted by the insecticides. After the leaf dried, some eggs of *E. kuehniella* were arranged isolated on its surface, using a wet brush, to ensure that the insect contacted the insecticide residue. Each disc was placed in a glass tube (8.5 cm H × 2.5 cm diam.) containing one *T. pretiosum* female.

The parasitoid mortality was assessed 24 h after the insects were exposed to the treated leaves. Each surviving female was offered one card (0.5 H × 0.5 cm W) containing approximately 250 *E. kuehniella* eggs. The card was replaced every 24 h until the wasp died.

The number of black (parasitized) eggs was counted using a stereoscopic microscope (40× magnification). Based on these data, the parasitism capacity and longevity of the F0 wasps were assessed.

Considering the mortality assessed within 24 h of exposure, the Corrected Mortality (Mc) was calculated, using Abbot's formula (Abbott, 1925). The insecticides were classified based on mortality, according to IOBC/WPRS classes: Class 1: innocuous (Mc < 25%); Class 2: slightly harmful (25 ≥ Mc ≤ 50%); Class 3: moderately harmful (50 > Mc ≤ 75%); Class 4: harmful (Mc > 75%) (Van de Veire et al., 2002).

Assessment of the next generation's emergence started 7 days after the test was installed and was performed each day for 7 days. The F_1 generation percentage of emergence was calculated based on the ratio between the number of black (parasitized) eggs and the number of eggs with a visible hatching orifice (Degrande and Gomez, 1990).

To assess the effects of insecticides on the F_1 generation sex ratio, all the insects emerged from the eggs with insecticide residue were sexed, based on the adults' antennae characteristics (Bowen and Stern, 1966). The sex ratio was calculated using the formula: [number of females / (number of males + number of females)].

Then, 15 females from each treatment (when possible, if enough wasps survived) were placed in individual glass tubes (85 mm H × 10 mm diam.). The same characteristics assessed in the F_0 generation were checked in the F_1 generation, until the emergence of F_2 (second generation).

3.2.5. Duration of harmful activity of insecticides to *Trichogramma pretiosum*

To implement the persistence test, evaluating the residual action of insecticides on the parasitoid *T. pretiosum*, conventional soybean plants (variety BRS 232) were grown in plastic pots (10 L) and kept either in a greenhouse or under field conditions. These plants were sprayed until the runoff point, using a hand sprayer.

Leaves were collected 5, 15, and 30 days after the spraying (DAS) and taken to the laboratory, where the parasitoids were exposed to the treated leaves, following the same method described in item 3.2.4. The tests with plants maintained in the open field, conduced using the same methodology, were discontinued after 5 days because the insecticides caused less than 30% mortality, indicating that further evaluations would not be necessary, according to IOBC/WPRS recommendations. For each treatment, 30 repetitions were used, each consisting of one 24 h-old female and one treated disc. The bioassay was carried out in a completely randomized design (n = 240).

Parasitoid mortality was assessed 24 h after the insects were exposed to the treated discs. For each surviving female, one card (0.5 cm L × 0.5 cm W) containing approximately 250 *E. kuehniella* eggs was offered. This card was replaced every 24 h until the wasp died.

The number of black (parasitized) eggs was counted, using a stereoscopic microscope (40× magnification). These data were used to assess the parasitism capacity and longevity of the F0 generation.

Assessment of the next generation's emergence started 7 days after the test was installed and was performed each day for 7 days. The F_1 generation percentage of emergence was calculated from the ratio between the number of black (parasitized) eggs and the number of eggs with a visible hatching orifice (Degrande and Gomez, 1990).

To assess the effects of the insecticides on the F_1 generation, the sex of all the emerged insects from the eggs with insecticide residue was checked based on the adults' antennae characteristics (Bowen and Stern, 1966). The sex ratio was calculated using the formula: [number of females / (number of males + number of females)].

Although IOBC/WPRS recommends stopping the evaluation when the Corrected Mortality is lower than 30%, we decided to continue evaluating in the succeeding periods, to examine sublethal effects. In this study, the evaluation of the insecticides effect only stopped if no difference was found in the sublethal effects.

The insecticides that reduced the females' survival by less than 30% compared to the control treatment were classified based on the persistence scale, according to IOBC/WPRS classes: Class 1: short-lived (< 5 days); Class 2: slightly persistent (5–15 days); Class 3: moderately persistent (16–30 days); Class 4: persistent (> 30 days) (Van de Veire et al., 2002).

3.2.6. Sublethal effects of insecticides on flight capacity

To evaluate the effects of insecticides on the wasps' flight capacity, only the insecticides that were considered innocuous (Class 1) or slightly harmful (Class 2) in the test described in section 3.2.3 were tested. Based on this, the treatments were: abamectin + chlorantraniliprole, lufenuron + profenofos, cypermethrin + profenofos, and cyproconazole + thiamethoxam, with distilled water as the control. This assay was carried out in a completely randomized design, with 4 repetitions of 5 treatments (n = 20).

Cards containing *E. kuehniella* eggs were dipped in the insecticide solution as described in item 2.3. After 7 days, the number of parasitized (black) eggs was counted, using a stereoscopic microscope (40× magnification). Then, approximately 100 parasitized eggs were placed in each test unit (Prezotti et al., 2002) (Figure 3) as described by Paiva et al. (2020 in press).

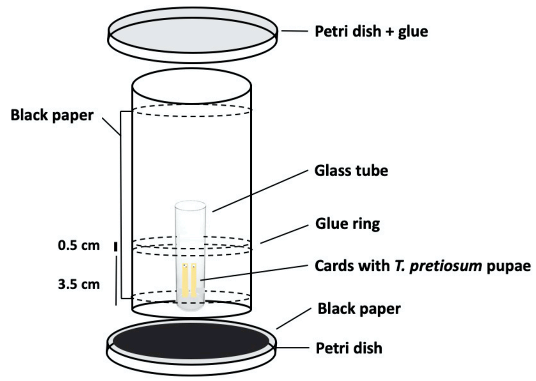

Figure 3. Flight test unit used to evaluate the sublethal effects of insecticides on the flight ability of parasitoids. ESALQ model, proposed by Prezotti et al. (2002). (Paiva et al., 2020) – modified based on Prezotti et al. (2002).

The parasitoids emerged from the treated eggs could move inside the test unit. The evaluation was conducted 96 h after the emergence started, to guarantee that all the parasitoids were emerged. The numbers of adults glued on the upper Petri dish (flying), on the bottom dish (not flying), and on the walls (not flying) were counted. The percentage of adults on each part of the test unit was calculated, based on the total number of emerged insects found.

3.2.7. Statistical analysis

The data for parasitism capacity (number of eggs parasitized) and longevity (days) were submitted to the non-parametric test of Kruskal-Wallis and compared by a Tukey test. The emergence percentage (ratio of eggs with an emergence orifice to the total number of eggs parasitized) and sex ratio were analyzed using a generalized linear model with quasi-binomial distribution, followed by post-hoc Tukey test. The quality adjustment for the GLM analyses was determined using half-normal graphs, with a simulation envelope (Hinde and Demétrio, 1998). These analyses were performed using the extension XLSTAT 2019 from Microsoft Excel (Addinsoft, 2019) and the statistical software “RStudio”, version 1.1.456 (R Development Core Team, 2019).

Flight test data (number of insects found in each position) were tested for normality. Then, a two-way analysis of variance was performed, with treatment and place the two factors, using the extension XLSTAT 2019 from Microsoft Excel (Addinsoft, 2019).

3.3. Results

3.3.1. Effects of ready-mix insecticides on two generations of *Trichogramma pretiosum* in contact with cards dipped in the insecticide solution

When the insecticides were classified, according to IOBC/WPRS criteria, based on the reduction in parasitism, cypermethrin + profenofos and lufenuron + profenofos were slightly harmful. Chlorantraniliprole + lambda-cyhalothrin, methanol + methomyl, and alpha-cypermethrin + teflubenzuron were moderately harmful; and only cyproconazole + thiamethoxam and abamectin + chlorantraniliprole were innocuous (Table 4).

Regarding the parasitism capacity, assessed as the number of eggs parasitized during the wasp's life span, methanol + methomyl, lufenuron + profenofos, abamectin + chlorantraniliprole, and cyproconazole + thiamethoxam caused a significant reduction in this parameter compared to the control treatment (χ^2 = 126.49, df = 7, 232 and P < 0.001). These same pesticides caused a reduction in the longevity of females that had contact with the dried residue (χ^2 = 130.99, df = 7, 232 and P < 0.001) (Table 4).

The only insecticide that did not significantly affect the F_1 generation emergence from treated eggs was chlorantraniliprole + lambda-cyhalothrin (χ^2 = 636.21, df = 7, 158 and P < 0.001). Also, this insecticide, as well as alpha-cypermethrin + teflubenzuron, did not affect the total emergence (χ^2 = 1213.7, df = 7, 204 and P < 0.001) (Table 4).

The other parameters evaluated for the F_1 generation: sex ratio (χ^2 = 20.39, df = 6, 111 and P = 0.09), total parasitism (χ^2 = 2.47, df = 6, 98 and P = 0.87) and longevity (χ^2 = 5.27, df = 6, 94 and P = 0.51) were not affected by any of the pesticides tested. The F_2 generation emergence was only significantly affected by lufenuron + profenofos, which caused a reduction (χ^2 = 114.66, df = 6, 85 and P < 0.001) (Table 4).

Table 4. Lethal and sublethal effect of ready-mix insecticides when adults of *Trichogramma pretiosum* were placed in contact with eggs of *Ephestia kuehniella* treated with an insecticide solution. (*Continues)*

Treatment	Generation F_0						Generation F_1	
	M_c (%)[1]	24-h Parasitism (number of black eggs)[2]	Parasitism Reduction 24h (%)	IOBC/ WPRS Class[3]	Total Parasitism (number of black eggs)[2]	Longevity (days)[2]	24h Emergence (%)[4]	Total Emergence (%)[4]
Control	-	16.60 ± 2.12 a	-	-	69.40 ± 7.35 a	6.57 ± 0.73 a	92.72 ± 1.53 a	93.45 ± 0.75 a
Chlorantraniliprole + lambda-cyhalothrin	0	1.10 ± 0.22 d	93.37	3	79.70 ± 7.11 a	8.13 ± 0.80 a	67.65 ± 10.15 abc	94.14 ± 1.43 a
Methanol + methomyl	100	0.94 ± 0.21 d	94.38	3	0.93 ± 0.21 c	1.00 ± 0.00 c	-	-
Abamectin + chlorantraniliprole	40.74	12.07 ± 1.43 a	27.31	1	17.73 ± 2.53 b	2.10 ± 0.31 b	40.69 ± 3.40 d	48.68 ± 4.76 d
Lufenuron + profenofos	33.4	6.40 ± 1.24 bc	61.45	2	18.00 ± 4.53 b	2.43 ± 0.43 b	11.52 ± 3.45 e	28.11 ± 6.78 d
Alpha-cypermethrin + teflubenzuron	0	0.93 ± 0.27 d	94.38	3	82.40 ± 9.75 a	8.03 ± 0.81 a	82.33 ± 5.21 cd	92.47 ± 3.54 a
Cypermethrin + profenofos	0	4.73 ± 0.90 c	71.49	2	70.33 ± 7.41 a	7.13 ± 0.80 a	43.59 ± 10.88 bc	94.74 ± 0.97 b
Cyproconazole + thiamethoxam	11.2	12.20 ± 1.72 ab	26.51	1	33.77 ± 6.08 b	3.70 ± 0.53 b	80.41 ± 4.39 b	87.48 ± 1.91c

[1]Corrected Mortality calculated using Abbot's formula (Abbott, 1925).
[2]Data followed by the same letter do not differ statistically (Kruskal-Wallis test, followed by *post-hoc* Tukey test, P < 0.05).
[3]Toxicity classes proposed by the IOBC/WPRS for pesticide selectivity studies on natural enemies, based on reduction on parasitism: Class 1 = Innocuous (< 30% reduction), Class 2 = slightly harmful (30–80% reduction), Class 3 = moderately harmful (80%–99% reduction) and Class 4 = harmful (> 99% emergence reduction) (Hassan, 1997).[4]Data followed by the same letter do not differ statistically (GLM with quasi-binomial distribution, followed by *post-hoc* Tukey test, P < 0.05).

Table 4. *(continued)* Lethal and sublethal effects of ready-mix insecticides when adults of *Trichogramma pretiosum* were placed in contact with eggs of *Ephestia kuehniella* treated with an insecticide solution.

Treatment	F_1 Generation			F_2 Generation
	Sex Ratio[4]	Total Parasitism (number of black eggs)[5]	Longevity (days)[5]	Emergence (%)[4]
Control	0.65 ± 0.04 ns	102.93 ± 10.52 ns	8.47 ± 1.12 ns	98.79 ± 0.31 a
Chlorantraniliprole + lambda-cyhalothrin	0.81 ± 0.08	90.80 ± 15.53	6.07 ± 1.18	98.49 ± 0.33 a
Methanol + methomyl	-	-	-	-
Abamectin + chlorantraniliprole	0.50 ± 0.07	73.47 ± 17.50	7.07 ± 1.40	97.66 ± 0.95 ab
Lufenuron + profenofos	0.50 ± 0.16	83.73 ± 14.38	6.43 ± 0.90	92.94 ± 3.70 b
Alpha-cypermethrin + teflubenzuron	0.88 ± 0.1	76.93 ± 13.37	6.42 ± 0.85	98.47 ± 0.70 ab
Cypermethrin + profenofos	0.69 ± 0.07	86.27 ± 11.83	5.67 ± 0.80	97.72 ± 0.68 ab
Cyproconazole + thiamethoxam	0.58 ± 0.06	80.47 ± 11.53	6.73 ± 0.81	97.09 ± 0.82 ab

[4]Data followed by the same letter do not differ statistically (Kruskal-Wallis test, followed by *post-hoc* Tukey test, P < 0.05).
[5]Data followed by the same letter do not differ statistically (GLM with quasi-binomial distribution, followed by *post-hoc* Tukey test, P < 0.05).

3.3.2. Effects of ready-mix insecticides on *Trichogramma pretiosum* in contact with soybean leaves sprayed with insecticide solution

Considering the IOBC/WPRS classification based on Corrected Mortality, when the females were exposed to dried residue on soybean leaves, cypermethrin + profenofos was classified as slightly harmful (Class 2), lufenuron + profenofos and cyproconazole + thiamethoxam as moderately harmful (Class 3), and methanol + methomyl as harmful (Class 4) (Table 5).

All these insecticides, which were not classified as innocuous, caused a reduction in the females' longevity (χ^2 = 91.16, df = 7, 232 and P < 0.001). Besides reducing the wasp longevity, lufenuron + profenofos also caused a reduction in the F0 generation's parasitism capacity (χ^2 = 17.26, df = 6, 138 and P < 0.001) (Table 5).

When the effects of the insecticides were evaluated for the F_1 generation, only chlorantraniliprole + lambda-cyhalothrin caused a reduction in parasitism (χ^2 = 14.05, df = 6, 98 and P = 0.02). This same insecticide, along with alpha-cypermethrin + teflubenzuron, caused a reduction in wasp longevity (χ^2 = 24.28, df = 6, 98 and P < 0.001) (Table 5). The parasitoid emergence was not affected in the F1 generation (χ2 = 16.52, df = 6, 98 and P = 0.56) or in the F_2 generation (χ^2 = 29.94, df = 6, 101 and P = 0.06). The sex ratio of the F_1 generation was not affected by any of the insecticides (χ^2 = 45.92, df = 6, 98 and P = 0.28) (Table 5).

Table 5. Lethal and sublethal effects of ready-mix insecticides when adults of *Trichogramma pretiosum* were placed in contact with soybean leaves treated with an insecticide solution.

Treatment	Generation F_0				Generation F_1				Generation F_2
	M_c (%)[1]	IOBC/ WPRS Class[2]	Parasitism (number of black eggs)[3]	Longevity (days)[3]	Emergence (%)[4]	Sex Ratio[4]	Parasitism (number of black eggs)[3]	Longevity (days)[3]	Emergence (%)[4]
Control		-	63.96 ± 10.77 ab	6.76 ± 0.99 ab	95.72 ± 1.41 ns	0.58 ± 0.06 ns	107.8 ± 11.75 a	7.13 ± 0.89 a	92.02 ± 0.94 ns
Chlorantraniliprole + lambda-cyhalothrin	0.00	1	71.32 ± 10.02 a	8.56 ± 0.87 a	90.75 ± 3.59	0.66 ± 0.09	56.86 ± 10.50 b	2.73 ± 0.51 c	93.97 ± 1.45
Methanol + methomyl	100.0	4	-	1.00 ± 0.00 e	-	-	-	-	-
Abamectin + chlorantraniliprole	7.41	1	34.36 ± 7.19 bc	4.83 ± 0.64 b	86.14 ± 7.07	0.67 ± 0.07	92.33 ± 12.52 a	7.13 ± 1.09 ab	92.18 ± 1.31
Lufenuron + profenofos	51.85	3	16.92 ± 3.55 c	1.6 ± 0.14 d	94.77 ± 1.18	0.58 ± 0.09	112.86 ± 15.11 a	8.53 ± 1.24 a	88.82 ± 0.66
Alpha-cypermethrin + teflubenzuron	14.81	1	51.09 ± 8.90 abc	6.73 ± 1.02 b	91.56 ± 4.87	0.72 ± 0.07	86.13 ± 13.17 ab	4.66 ± 0.77 bc	93.00 ± 1.35
Cypermethrin + profenofos	40.74	2	50.00 ± 11.21 abc	4.00 ± 0.74 c	94.87 ± 1.70	0.76 ± 0.04	87.66 ± 13.37 ab	6.26 ± 0.87 ab	90.00 ± 1.73
Cyproconazole + thiamethoxam	51.85	3	48.54 ± 15.38 abc	3.60 ± 0.87 cd	85.99 ± 8.85	0.78 ± 0.07	89.06 ± 10.56 a	5.40 ± 0.75 ab	89.32 ± 1.75

[1]Corrected mortality calculated using Abbot's formula (Abbott, 1925).
[2] Toxicity classes proposed by the IOBC/WPRS for pesticide selectivity studies on natural enemies: Class 1, M_c < 25% (innocuous); Class 2, 25 ≥ M_c ≤ 50% (slightly harmful); Class 3, 50 > M_c ≤ 75% (moderately harmful); Class 4, M_c > 75% (harmful) (Van de Veire et al., 2002).
[3]Data followed by the same letter do not differ statistically (Kruskal-Wallis test, followed by *post-hoc* Tukey test, P < 0.05).
[4]No statistical difference (GLM with quasi-binomial distribution, followed by *post-hoc* Tukey test, P < 0.05).

3.3.3. Duration of harmful activity of insecticides to *Trichogramma pretiosum*

When the parasitoids were exposed to dried residue on leaves collected over time from plants kept outside the greenhouse, no insecticide caused mortality higher than 30% at 5 days after spraying (DAS). On the other hand, when the soybean pots were maintained in the greenhouse, cyproconazole + thiamethoxam was the only pesticide that caused more than 30% mortality 5 DAS, and was therefore classified as slightly persistent following the IOBC/WPRS classification.

Regarding the parasitism by females that had contact with the dried residue 5 DAS, chlorantraniliprole + lambda-cyhalothrin, lufenuron + profenofos, and alpha-cypermethrin + teflubenzuron had a significant impact, causing a reduction (χ^2 = 20.37, df = 7, 196 and *P* < 0.001). However, parasitism was not affected by any insecticide when the females were exposed to dried residue 15 DAS (χ^2 = 7.18, df = 7, 222 and *P* = 0.41). Regarding the F_1 emergence, there was no effect from the treatments 5 DAS (χ^2 = 21.39, df = 7, 177 and *P* = 0.59) or at 15 DAS. Although abamectin + chlorantraniliprole caused a reduction in emergence, this reduction did not differ significantly from the control treatment (χ^2 = 42.02, df = 7, 219 and *P* = 0.02).

The sex ratio of the adults emerged was not affected by any of the insecticides 5 DAS (χ^2 = 49.12, df = 7, 130 and *P* = 0.43). However, at 15 DAS there was a slight difference in this parameter, and chlorantraniliprole + lambda-cyhalothrin caused a higher proportion of females compared to the control treatment (χ^2 = 133.09, df = 7, 180 and *P* = 0.02).

3.3.4. Sublethal effects of insecticides on flight capacity

The results of the flight test to assess the sublethal effects of insecticides on the wasps' ability to fly indicated no significant difference between the control treatments and the insecticides. The locations where the insects were found did differ statistically, with more insects at the top of the container (F = 202.16, df = 4, 74 and *P* < 0.001). These results showed that the insecticides did not harm the wasps' ability to fly (Figure 4).

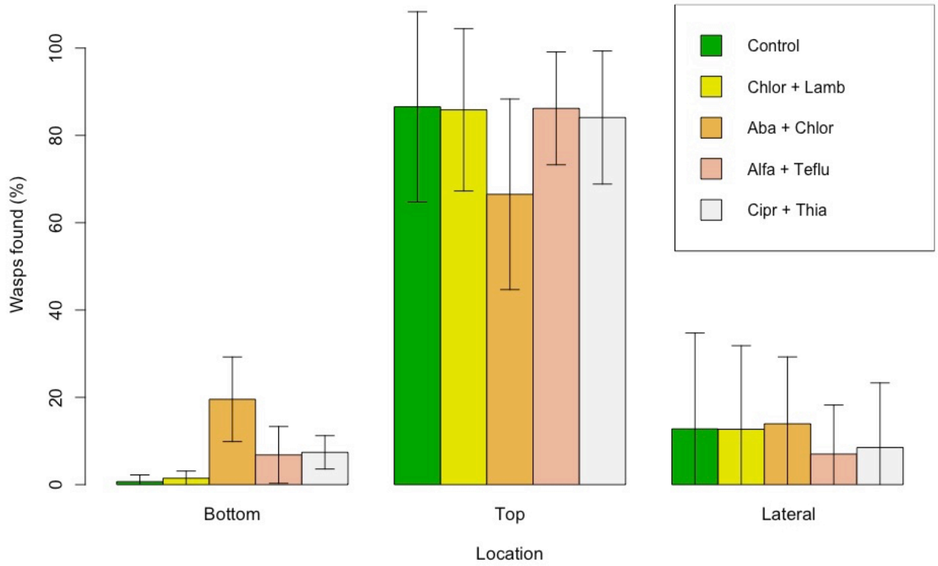

Figure 4. Percentage of wasps found in each location in the flight test. Legend: Chlor + Lamb = Chlorantraniliprole + Lambda-cyhalothrin; Aba + Chlor = Abamectin + Chlorantraniliprole; Alpha + Teflu = Alpha-cypermethrin + Teflubenzuron; Cipr + Thia = Ciproconazole + Thiamethoxam.

3.4. Discussion

A pesticide has effects on a natural enemy beyond the mortality. It is also important to evaluate other biological attributes of an insect, such as longevity, fecundity, fertility, and parasitism/predation capacity (Desneux et al., 2007). This should be done to guarantee that the pesticide will not lower the efficiency of biological-control agents used as part of integrated pest-management programs.

In the assay with cards dipped in insecticide solution, the three ready-mix pesticides with a pyrethroid in their composition caused a reduction in parasitism in the first 24 h. However, the Corrected Mortality of females that had been exposed to the dried residues of these insecticides was 0%. Moreover, no effect was found on the total parasitism or the longevity. This group of results reflects the repellent effect of pyrethroids on *T. pretiosum*, which leads the females to avoid the dried insecticide residue and therefore not parasitize the treated eggs. This effect was previously reported by Carvalho et al. (2001) and Paiva et al. (2018).

The mortality of females that contacted the dried residue on the leaves was highest for alpha-cypermethrin + teflubenzuron and cypermethrin + profenofos. All the pyrethroids tested caused some sublethal effects on the females' longevity and parasitism. This could be explained by the level of penetration and/or translocation of the insecticide in the different substrates (Guedes et al., 1992), and/or by the female's capacity to recognize a particular substance and avoid contact with it. Further studies are necessary to more thoroughly investigate the reason for this difference.

Insect Growth Regulators (IGRs), such as lufenuron and teflubezuron, can inhibit the formation of imaginal organs, cause malformation of the ovaries, and reduce the number of viable eggs (Desneux et al., 2007; Haseeb and Amano, 2002; Schneider et al., 2004). This could explain the reduction in parasitism caused by lufenuron + profenofos. Lufenuron + profenofos reduced the longevity of females, similarly to the findings for lufenuron by Carvalho et al. (2010). Similar results for parasitism reduction were found by Sattar et al. (2011) for *T. chilonis* and by Bastos et al. (2006) for *T. pretiosum*. These latter authors also found that profenofos caused a reduction in parasitism.

Methomyl belongs to the group of carbamates, which along with organophosphates are harmful to insects because of their ability to inactivate acetylcholinesterase. Wang et al. (2014), comparing the toxicity of different chemical groups to *Trichogramma evanescens* Westwood, found that these two groups showed the highest intrinsic toxicity. Similarly, Hajjar and Al-Masoud (2018) and Hassan (1998) found that methomyl caused 99% mortality in the parasitoid *Trichogramma cacoeciae* Marchal.

Chlorantraniliprole is a member of the diamides chemical subgroup. These pesticides have mainly oral toxicity, which could explain the low mortality in females that contacted the dried residue (Stecca et al., 2014). These results led the two products containing this active ingredient to be classified as innocuous. Importantly, although we found no significant effect on the generation that had contact with the residues on the leaves, only chlorantraniliprole + lambda-cyhalothrin affected the parasitism and longevity of first-generation females, showing a transgenerational effect.

The other ready-mix product with chlorantraniliprole in its composition was abamectin + chlorantraniliprole, which was also classified as innocuous for both routes of exposure. However, when the females were exposed to the dried residue on the cards, this pesticide caused significant effects on the total parasitism, longevity and emergence percentage in the first 24 h and in the total life span. The action of chlorantraniliprole by ingestion (Stecca et al., 2014) could explain these effects, since the female feeds on the egg exudates after the parasitism.

In contrast, Matioli et al. (2019) found that the insecticide mixture lambda-cyhalothrin + chlorantraniliprole should be classified as harmful to *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae), and were therefore unable to evaluate th sublethal effects on the parasitoid. This difference could be explained by the concentration of active ingredients in the ready-mix product that they used (33.5 + 67 mg L–1), as well as by the biological and physiological differences between the species tested.

Among the ready-mix insecticides tested here, only cyproconazole + thiamethoxam contains a fungicide. Hassan (1998) tested this fungicide, cyproconazole, on *T. cacoeciae* and classified it as innocuous. Magano et al. (2015) tested cyproconazole on *T. pretiosum* and found a similar outcome. The classification of “moderately harmful” obtained here could be explained by the mixture with thiamethoxam, a neonicotinoid insecticide, which acts as an agonist of acetylcholine receptors in the insect central nervous system, causing first paralysis and eventually death (IRAC, 2020; Tomizawa and Casida, 2005). Besides the acute toxicity, neonicotinoids may have sublethal effects on reproduction, behavior, fecundity and longevity (Croft, 1990). The reduction in longevity caused by thiamethoxam observed here was also reported by Paiva et al. (2018).

Evaluation of the duration of the harmful activity of an insecticide allows a biological control agent to be released at a time when it is least harmful to the agent. This is only possible when other parameters in addition to mortality are evaluated, guaranteeing that the agent can perform effectively. The ready-mix pesticides tested in this study, caused some harmful effects on our subject parasitoid, *T. pretiosum*, at 15 DAS.

Pasini et al. (2017) studied the persistence of cypermethrin (0.008% a.i.), lambda-cyhalothrin (0.003%) and methomyl (0.064%), in single formulations, on *T. pretiosum*, and classified all these active ingredients as class 3, reducing the parasitism for more than 24 days; whereas in our study, the same insecticides were classified as short-lived. The exposure route might account for this difference, since Pasini et al. (2017) released the parasitoids in cages inside a greenhouse. The difference might also stem from the formulations used, since in the present study these active ingredients were tested in ready-mixes and in lower concentrations.

Flight capacity is not commonly assessed, and few studies could be found to compare with the present results. Here, the locations where the wasps that emerged from the treated eggs differed significantly, with most of the wasps found in the top dish. In this dish, the number of wasps was statistically equal among all the treatments. These results showed that the flight capacity was not affected by the insecticides, similar to findings by Paiva et al. (2020 in press), who assessed the effects of other insecticides applied to *T. pretiosum* pupae.

The results of the present study provide useful information for the implementation of IPM programs that combine biological and chemical control. These findings are also useful to improve conservative biological control. However, these studies require continuous updating, since the numbers of chemicals and their combinations as well as non-target organisms to consider are continually increasing. Also, field studies are needed to complement the results obtained in the laboratory and semi-field tests.

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4. FINAL CONSIDERATIONS

The main concern in selectivity tests based on the IOBC/WPRS classification is whether the classes, by themselves, are capable of describing the toxicity of an insecticide to a natural enemy. Based on this, the researchers started evaluating the sublethal effects of the products, focused on parameters that could alter the efficacy of the natural enemy studied.

When it comes to ready-mix insecticides, there are two active ingredients acting in different ways on the insect, then the deleterious effects become more recurrent, as happened in this work. It is difficult to determine if these effects happened because of a synergy or an agonistic relation between the two active ingredients.

Considering the seven tested insecticides, all affected somehow the parameters evaluated in, at least one of the tests (effect on pupa, effect on adult exposed to the treated leaf, effect on adult exposed to the treated *E. kuehniella* eggs and persistence (Table 6).

The only two insecticides that were not classified as moderately harmful (class 3) or harmful (class 4), were abamectin + chlorantraniprole and cypermethrin + profenofos. Nevertheless, they caused effects on parasitism, longevity and/or emergence.

Despite these negative effects, considering the duration of harmful activivty of all the seven tested insecticides, it is important to highlight that none of them caused deleterious effects for more the 5 days. Therefore, they can be classified as short-lived insecticides, allowing their joint use with *T. pretiosum* in IPM programs. as long the parasitoid is released 5 days after they were sprayed. We also highlight the importance of new studies evaluating their effect on different species of natural enemies, which might provide dipper knowledge about this kind of agrochemical.

Table 6. Effect of ready-mix insecticides on different life-stages and routes of exposition on the parasitoid *Trichogramma pretiosum*

Chemical product	Pupae			Adult - Sprayed leaf				Adult - Dipped card				Persistence		
	IOBC/WPRS Class	F1	F2	IOBC/WPRS Class	F0	F1	F2	IOBC/WPRS Class	F0	F1	F2	IOBC/WPRS Class	5 DAS	15 DAS
Chlorantraniliprole + lambda-cyhalothrin	1	No	No	1	No	Par.	No	3	Par. (24h)	No	No	1	Par.	No
Methanol + methomyl	4			4	Yes			3				1	No	No
Abamectin + chlorantraniliprole	1	Par.[1] Long.[2]	No	1	No	No	No	1	Par. (total) Long.	Emerg.[3] (24h) Emerg. (total)	No	1	No	No
Lufenuron + profenofos	4	Par. Long.	No	3	Par. Long.	No	No	2	Par. (24h) Par. (total) Long.	Emerg. (24h) Emerg. (total)	Yes	1	Par.	No
Alpha-cypermethrin + teflubenzuron	1	No	No	1	No	Long.	No	3	Par. (24h)	Emerg. (24h)	No	1	Par.	Emerg.
Cypermethrin + profenofos	2	No	No	2	Long.	No	No	2	Par. (24h)	Emerg. (24h) Emerg. (total)	No	1	No	No
Cyproconazole + thiamethoxam	2	No	No	3	Long.	No	No	1	Par. (total)	Emerg. (24h) Emerg. (total)	No	2	No	No

[1]Par = Parasitism; [2]Long = Longevity; [3]Emerg. = Emergence