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RESEARCH ARTICLE

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Susceptibility of the egg parasitoid *Trichogramma achaeae* (Hymenoptera: Trichogrammatidae) to selected insecticides used in tomato greenhouses

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Abstract

The South American tomato moth *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a pest species of great economic importance in tomatoes, both in greenhouses and in open-air crops. This importance has increased in recent years because it has been introduced in many countries in Europe, Africa, and Asia. Insecticides different active ingredients and biological control agents are being used in the control of this pest species. This implies the need to make both groups compatible within IPM programmes. Therefore, the objective of this work was to study the compatibility between different insecticides and the use of the egg parasitoid *Trichogramma achaeae* Nagaraja and Nagakartti (Hymenoptera: Trichogrammatidae). Three groups of trials were carried out under laboratory and greenhouse conditions. Ten insecticides with the following active ingredient were evaluated: abamectin, azadirachtin, *Bacillus thuringiensis*, chlorantraniliprole, emamectin, flubendiamide, indoxacarb, methomyl, spinosad, and spiromesifen. In the results, three groups of insecticides were established based on their compatibility with the use of biological control: The first group (abamectin, *B. thurigiensis*, flubendiamide, indoxacarb and spiromesifen) showed a high degree of compatibility with egg parasitoid releases. The second group (azadirachtin and chlorantraniliprole, and methomyl) presented compatibility problems. Finally, the last group (emamectin, methomyl, and spinosad) did not apper to be compatible. The results found will allow a better application of IPM programmes in tomato crops for the control of this pest species.

Additional keywords: biological control; IPM; ecotoxicology; South American tomato moth; parasitoid; insecticides; side effects.

Abbreviations used: a.i. (active ingredient); *Bt* (*Bacillus thurigiensis*); *E* (percentage reduction of the evaluation parameters with respect to the control); EC (emulsifiable concentrate); GLM (general linear model); GZLM (generalized linear model); HSD (honestly significant difference); IOBC (International Organization for Biological and Integrated Control); IPM (Integrated Pest Management); IRAC (Insecticide Resistance Action Committee); RH (relative humidity); SC (suspension concentrate); SG (soluble granule); UVL (ultraviolet light); WG (water dispersible granules); WP (wettable powder).

Authors' contributions: TC designed the study. JGM carried out the laboratory trials. FJFM and JRG carried out the greenhouse trials. TC and JRG analyzed the data, intellectually reviewed the content and collaborated in writing the article. All authors read and approved the final manuscript.

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Introduction

In Europe and the United States, the environmental cost associated with the use of chemical pesticides is considered too high. Thus, there is a general movement towards environmentally safer control and production (Dent, 2000). This has been transcribed into the European Union's legislative policies with the aim of reducing the use of pesticides, removing large quantities of products, and giving renewed importance to Integrated Pest Management (IPM) (Lefebvre *et al.*, 2015). The adoption of IPM in all member states in 2014 is the main pillar of the EU strategy to mitigate the negative impact of rapid removal of chemical pesticides from food production (Clark & Hillocks, 2014).

According to the Food and Agriculture Organization (FAO, 1966), IPM means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides

and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agroecosystems and encourages natural pest control mechanisms.

The South American tomato moth *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the main tomato pests in South American countries (Guedes & Picanço, 2012). In addition, since its accidental introduction in 2006, this species has become a pest of great economic importance in the countries of the Mediterranean area and many others of Europe and Asia (Desneux *et al.*, 2011; Campos *et al.*, 2017; Biondi *et al.*, 2018). In Spain, *Tu. absoluta* rapidly became a serious impediment to biological control programmes in tomato production greenhouses, requiring applications of more than 15 different insecticidal substances directed specifically towards *Tu. absoluta* (Desneux *et al.*, 2011). The damage is caused by larval feeding mainly on leaves and fruits, but the pest can also attack stems, buds and flowers causing severe crop losses that can reach 100% if no control measures are taken. The dynamics of populations of *Tu. absoluta* and their consequent damage differ depending on their presence in greenhouse or outdoor crops, date of transplantation, etc., creating significant challenges for the development and successful application of biocontrol methods (Cabello, 2009), and the results are not always satisfactory due to the overlap of *Tu. absoluta* generations and the continuous re-infestations in the crops, which motivates the need for the application of several treatments per crop cycle to adapt the population levels to the capacity of control by natural enemies.

At present, pest control of *Tu. absoluta* is based on biological control, chemical control or a combination of both, although the most common method of control is based on the intensive use of insecticides and this constitutes the first tool in newly invaded areas (Bielza, 2010; Campos *et al.*, 2017; Biondi *et al.*, 2018).

Parasitic insects of the *Trichogramma* genus have been widely used during the 20th century to control lepidopteran pests in maize and sugarcane crops, and subsequently extended to control many other pests in many different crops; the list of crops continues to increase (Smith, 1996; van Lenteren *et al.*, 2018). *Trichogramma*, with approximately 200 species described, is the best-known genus in the family due to its use in the biological control of pest species in agriculture of which more than 25 species are used in pest control in 34 crops across 30 countries (Pinto & Stouthamer, 1994; Querino *et al.*, 2010; van Lenteren *et al.*, 2018). Trichogrammatids can therefore play a vital role in pest control programmes by destroying the first developmental state (egg) of pest, limiting the use of pesticides and contributing to the prevention of environmental contamination (Kumar *et al.*, 2013).

The establishment and subsequent commercialization of the parasitoid *Trichogramma achaeae* Nagaraja and Nagakartti (Hymenoptera: Trichogrammatoidea) has been an important advance in the control of the pest in Spain (Cabello *et al.*, 2009, 2012; Vila & Cabello, 2014). In addition, *T. achaeae* has been or is being used in Europe in the following countries: Germany, Belgium, Spain, France, Greece, the Netherlands, Romania, and Portugal, against several species of Lepidoptera in more than 15 crops both horticultural and ornamental (Leppla *et al.*, 2017; Vila, 2017 *pers. com.*; van Lenteren *et al.*, 2018).

Studying the side effects of insecticides on natural enemies is necessary to minimize any adverse impacts within the IPM programmes (Goulart *et al.*, 2012). The integration of biological and chemical control tactics requires a thorough understanding of how pesticides affect biological control organisms (Brunner *et al.*, 2001). Prior to the release of Trichogrammatids in an IPM system, it is essential to know their compatibility with other pest control methods, including the use of chemical pesticides. Such information will assist in the timing of parasitoid releases regarding the application of chemical pesticides (Jalali *et al.*, 2016).

Currently, the strategy used in IPM programmes in the control of *Tu. absoluta* in Spanish southeast tomato greenhouses consists of the early inoculation of the omnivorous predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) in combination with inundative or inoculative releases of *T. achaeae* (Cabello *et al.*, 2009, 2012; Desneux *et al.*, 2011; Vila & Cabello, 2014).

The objective of this work was to establish the side effects of 10 insecticides commonly used in the chemical control of *Tu. absoluta* on the parasitoid *T. achaeae*. The selected materials are abamectin, azadirachtin, *Bacillus thurigiensis*, emamectin, flubendiamide, indoxacarb, methomyl, chlorantraniliprole, spinosad and spiromesifen. All are included in the list of substances authorized in the South zone of Annex I of the EC Regulation (EC, 2009) that covers the substances allowed in the members states of the EU.

Material and methods

Insects

A colony of *T. achaeae* was obtained from wild populations and reared in the entomology laboratory of Almeria University according to the method described

by Cabello (1985) and maintained in a climatic chamber at 25 ± 1ºC, 70 ± 10% RH and a 16:8 h light: dark photoperiod. The wasps were reared on UVL-sterilized eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). A weekly egg supply of *E. kuehniella* was obtained from a commercial supplier (Agrobio S.L, Almería, Spain). The eggs were glued with distilled water onto paper cards (273 cm^2) and exposed to adult *T. achaeae* in 1 L plastic pots sealed with a fine nylon mesh. After 24 h of exposure, the cards were transferred to new plastic pots, where they were held until adult emergence. Adult *T. achaeae* were provided honey as droplets smeared on the inside wall of the pots.

Insecticides

Ten commercial formulations, with different insecticide active ingredients (AIs) were used, as listed in Table 1. These compounds were selected because of their current and main use in the chemical management of *Tu. absoluta* in the Mediterranean area, and because they represented a variety of chemical groups used in insecticide resistance management. The doses tested were the maximum authorized or recommended by the manufacturer in greenhouse tomato culture. Application rates of the insecticide formulations used were prepared by diluting the products in distilled water according to the manufacturer's instructions.

Insecticide application on pupae and sublethal effects

The insecticide formulations were applied to the pupa stage inside the host egg. Ten separate trials were carried out with each insecticide a.i. (abamectin, azadirachtin, *B. thuringiensis*, chlorantraniliprole, emamectin, flubendiamide, indoxacarb, methomyl, spinosad, and spiromesifen) plus a control (water). Following Hassan's (1998) recommendations, each insecticide formulation was tested at its maximum recommended field dose (Table 1). Each trial was carried out in two steps as follows: the first step was to evaluate the effects of each a.i. compared to the control (water) on the pupal survival of *T. achaeae*; the second step, consecutive to the previous one, was to evaluate the effects of the same a.i. on adult longevity and fertility when applied at the pupa stage. In first the steps of each trial, four card disks (17.5 mm diameter each) with over 300 parasitized eggs (containing parasitoid pupae, less than 3 days for adult emergence at 25 °C and a 16:8 h light: dark photoperiod) were treated by spraying with a Potter tower sprayer (Burkard®, Uxbridge, UK) (working pressure: 0.76 atmosphere) for 2-2.5 seconds (time required to apply a product quantity of 0.285 mL/cm^2 close to the recommended optimum in the field). Additionally, distilled water was applied as described above and to a similar number of card disks with parasite eggs as a control in each trial. After treatments, the cards were kept over filter paper at room temperature until the excess liquid had drained. They were then transferred to emergency tubes and kept in a climatic chamber with controlled conditions. Emergence was evaluated by counting parasitized hosts that presented holes due to the emergence of adult offspring from them. In the second step of each trial, to evaluate the side effects of the insecticides on the longevity and fecundity of adults, subsequent bioassays were performed. Thirty couples (♀/♂) of adults emerged from the treated pupae, and 30 couples of adults emerged from the control (water) were isolated in glass vials. Each couple was offered 50 UVL-sterilized eggs of *E. kuehniella*, glued with water to a card (5 × 0.9 cm), every 3 days (days: 0, 3, 6, 9, and 12). Isolated couples *T. achaeae* were fed honey, and their survival was assessed daily until death. Host eggs that changed colour to black were tallied as parasitized; all others were counted as non-parasitized (Rodriguez *et al.*, 1994). After female oviposition, the cards were transferred to new glass vials. All trials were conducted in a climatic cabinet (ICP 600, Memmert®, Memmert GmbH+Co. KG, Schwabach, Germany) at chamber conditions.

Experimental design and data analysis

The experimental design in each trial was completely randomized with a single factor at two levels (insecticide and control). The data (pupal survival, longevity of females and males, and female fecundity) obtained were analysed using general linear models (GLMs) and mean values for each insecticide were compared using Tukey's HSD test (at $p = 0.05$) with respect to the respective control (water). Additionally, in each trial for the pupal survival value, the number of replications was 4 (each with 300 parasitized eggs) and for the longevity and fertility values of adults the number of replications was 30 couples (1♀+1♂). Subsequently, the percentage reduction in emergence from parasitized eggs, adult longevity and percentage of parasitism relative to the control was evaluated by the following equation: $E(\%) = [1 - (Q / q) \times 100]$, where E is the percentage of reduction of the capacity of the biological parameter in question, Q is the average value of the parameter to be analysed for the insecticide, and q represents the mean value of the parameter obtained in the control (water). Based on the results obtained in this study, each insecticide was classified according to the IOBC criteria for laboratory tests: class 1 = harmless

Table 1. General information on tested insecticide formulations: active ingredient/ commercial name, manufacturer/ distributor, mode of action, chemical group, concentration of spray mixtures, formulation type, tested rate, and EU countries in which a.i. is authorised.

Active Ingredients (AI)/ Trade name	Supplier	Mode of action[1]	Chemical group	Concentration	Formulation[2]	Dose[3]	AI authorised in EU countries[4]
Abamectin /Vertimec®	Syngenta	Glutamate-gated chloride channel (GluCl) allosteric modulators	Avermectins, Milbemycins	1.8%	EC	0.01 mL/L	AT, BE, BG, CY, CZ, DE, EE, EL, ES, FI, FR, HR, HU, IE, IT, LT, LU, LV, MT, NL, PL, PT, RO, SE, SI, SK, UK
Azadirachtin /Align®	Sipcam Inagra	Compounds of unknown or uncertain MoA	Azadirachtin	3.2%	EC	1.5 mL/L	AT, BE, BG, CY, CZ, DE, DK, EE, EL, ES, FR, HU, IT, LT, LU, LV, NL, PT, SE, SI, SK, UK
Bacillus thuringiensis /Dipel®	Valent Biosciences Corporation	Microbial disruptors of insect midgut membranes	*Bacillus thuringiensis* and the insecticidal proteins they produce	16%	WP	0.5 g/L	AT, BE, BG, CY, DE, DK, EL, ES, FI, FR, IT, LU, NL, PL, PT, SE, SI, UK
Chlorantraniliprole / Altacor®	Du Pont	Ryanodine receptor modulators	Diamides	35%	WG	0.1 g/L	AT, BE, BG, CY, CZ, DE, EL, ES, FR, HR, HU, IE, IT, LU, MT, NL, PL, PT, RO, SI, SK, UK
Emamectin (benzoate) /Affirm®	Syngenta	Glutamate-gated chloride channel (GluCl) allosteric modulators	Avermectins, Milbemycins	0.855%	SG	1.5 g/L	BE, BG, CY, EL, ES, FR, HR, HU, IT, NL, PL, PT, RO, SI, SK
Flubendiamide /Fenos®	Bayer CropScience	Ryanodine receptor modulators	Diamides	24%	WG	0.25 g/L	CY, DK, NL
Indoxacarb /Steward®	Du Pont	Voltage-dependent sodium channel blockers	Indoxacarb	30%	WG	0.126 g/L	AT, BE, BG, CY, CZ, DE, EE, EL, ES, FI, FR, HR, HU, IE, IT, LT, LU, LV, MT, NL, PL, PT, RO, SE, SI, SK, UK
Methomyl /Lannate®	Du Pont	Acetylcholinest-erase (AChE) inhibitors	Carbamates	25%	WP	1.25 mL/L	BG, CY, EL, ES, HU, IT, MT, PT, RO
Spinosad /Spintor®	Dow AgroSciences	Nicotinic acetylcholine receptor (nAChR) allosteric activators	Spinosyns	48%	SC	0.25 mL/L	AT, BE, BG, CY, CZ, DE, EL, ES, FR, HR, HU, IE, IT, LU, MT, NL, PL, PT, RO, SE, SI, SK, UK
Spiromesifen /Oberon®	Bayer CropSciences	Inhibitors of acetyl CoA carboxylase	Tetronic and Tetramic acid derivatives	24%	SC	0.6 mL/L	BE, CY, EL, ES, FR, HU, IT, LU, MT, NL, PT

[1]Mode of action: IRAC (2016). [2]Formulation: EC: emulsifiable concentrate, WP: wettable powder, WG: water dispersible granules, SG: soluble granule, SC: suspension concentrate. [3]The dose used was the highest authorized for application in tomato crops. [4]Authorised EU countries at the date of 03.04.2019: AT: Austria, BE: Belgium, BG: Bulgaria, CY: Cyprus, CZ: Czechia, DE: Germany, DK: Denmark, EE: Estonia, EL: Greece, ES: Spain, FI: Finland, FR: France, HR: Croatia, HU: Hungary, IE: Ireland, IT: Italy, LT: Lithuania, LU: Luxembourg, LV: Latvia, MT: Malta, NL: Netherlands, PL: Poland, PT: Portugal, RO: Romania, SE: Sweden, SI: Slovenia, SK: Slovakia, UK: United Kingdom.

(E < 30% reduction of emergence, longevity, or fecundity), class 2 = slightly toxic (30% ≤ E ≤ 79% reduction), class 3 = moderately toxic (80% < E ≤ 99% reduction), and class 4 = toxic (E > 99% reduction) (Hassan *et al.*, 1991; Jepson, 1998; Sterk *et al.*, 1999; Amano & Haseeb, 2001). All statistical analyses were carried out using the SPSS software, version 23 (IBM, 2014).

Insecticide application on adults

Evaluation followed the method prescribed by IOBC for selectivity tests with parasitoids of the genus *Trichogramma* (Hassan, 1998; EPPO, 1999; Hassan *et al.*, 2000). Four trials were carried out. For each bioassay, *T. achaeae* was exposed to fresh and dried residues of insecticide formulation sprayed on

2 mm thick glass plates measuring 13 cm × 13 cm. The products were sprayed using a Potter tower sprayer under the same conditions indicated in the previous section. After spraying, the plates were kept in the shade for approximately 3 h to dry, forming a dry insecticide film. The surfaces of the two glass plates with the dry insecticide film were used as the internal back and the top of the cage.

Each cage (equal to those described by Hassan *et al.*, 2000) was made of an aluminum frame measuring 13 cm (length) × 13 cm (width) × 1.5 cm (height). Single-coated adhesive foam tape, 1.5 cm wide, was fixed on the aluminum frame to hold the glass plates. Six ventilation holes (~ 1 cm in diameter) were drilled into three sides of the aluminum frame. The holes were covered with thin, black muslin fabric glued onto the frame with adhesive foam tape to promote ventilation. The fourth side of the aluminum frame had two openings. The first opening was 3.5 cm wide × 1 cm high and was used to transfer the eggs to be parasitized and food for the parasitoids into the cage; the second opening was a 1 cm diameter hole to allow for the release of the parasitoids inside the cage. These two openings were closed from the outside with black cardboard and were opened only to place the cards with eggs and the parasitoids into the cages.

To prevent the escape of parasitoids to the margins of the glass plate, the external surfaces (untreated) were covered with black cardboard (7 cm × 7 cm). Because the parasitoids were attracted to light, they were active on the glass surface exposed to light and thus more exposed to the insecticides being tested. Afterwards, the glass plates were fixed to the aluminum frame with four rubber bands.

Approximately 2500 parasitized eggs with a time for the emergence of parasitoid adults of less than 24 h and were placed in a corner in each frame. As food sources, they were given a piece of non-absorbent paper (6 × 1.5 cm) with 6 thin lines of honey. Through the opening of the frame, a strip of paperboard (3 × 10 cm) was supplied with approximately 3000 eggs, which were replaced at 24, 72, and 96 h. The replaced paperboard was placed in a plastic container and evolved in a chamber under chamber conditions. Once all the parasitized eggs (more than 5 days) were evolved and showed black colour they were photographed, and digital measurement of the surface occupied by parasitized eggs was performed by image processing using Photoshop® CS6 software (Adobe System Software Ltd, Ireland) and Fiji software (Schindelin *et al.*, 2012). Previously the average surface (in pixels^2) equivalent for an egg was calculated. To avoid and prevent the accumulation of toxic gases, the frames were placed in a closed structure equipped with an air extractor (flow rate = 98 m^3/h) which created a continuous air flow during the experiments. All trials were conducted for 24 h in a climatic cabinet (model ICP 600, Memmert GmbH+Co. KG, Schwabach, Germany) (25 ºC ± 1, RH: 75-85% and 16:8 h light: dark photoperiod).

Experimental design and data analysis

In each trial of insecticide application on adults, the design was completely randomized with a single factor at four levels (trial 1, a.i.: chlorantraniliprole, flubendiamide, indoxacarb, and control; 2, a.i.: abamectine, azadirachtin, spinosad, and control; and 3, a.i.: *B. thuringiensis*, emamectin, spiromesifen, and control) o at two levels (trial 4, a.i.: methomyl and control) and three replicates per treatment. The data (percentage of parasitism) obtained in the different trials were analyzed by GLMs and their means compared with Tukey's HSD test (p = 0.05) with SPSS software, version 23 (IBM, 2014). Subsequently, the number of parasitism reductions was evaluated in relation to the control (water). This was calculated with equation (E) and classified according to the IOBC scale as indicated above for laboratory tests.

Greenhouse evaluation

To evaluate the effect of the application of the different insecticides under conditions of tomato greenhouse production, in a previous trial, we tried to use the methodology proposed by EPPO (1999) and Hassan *et al.* (2000) for *T. cacoeciae* using parasitized sentinel eggs (*E. kuehniella* eggs stuck on a piece of green cardboard) as a *Trichogramma* activity measurement but it was verified for *T. achaeae* that the number of parasitized sentinel eggs does not well reflect the actual activity of this species. This has also been previously cited by Cabello *et al.* (2010) and Sanchez *et al.* (2014). As an alternative, the release-recapture method was used. We used yellow sticky traps because this method has demonstrated efficacy monitoring the activity of others *Trichogramma* species (Romeis *et al.*, 1998; Chapman *et al.*, 2009), and it has been proposed by Yong & Hoffman (2006) and Cabello *et al.* (2010) for assessing *Trichogramma* adult activity.

Using this methodology, four trials with different insecticides were carried out in four Almería-type commercial greenhouses with tomato crops located at different locations in the province of Almería, Spain. In all of them, the crop plant height was greater than 1.40 m, and no prior chemical control had been carried out. In every greenhouse, the insecticide applications were carried out by a backpack sprayer equipped (Maruyama®, model MS073D). Also, in each trial, the equipment was

pre-calibrated, in relation to the application time per plot, for an application rate of 1500 L/ha. The products used, and the tested doses are listed in Table 1. Inside the greenhouses, the different blocks and plots were delimited with a plastic sheet to avoid drift. Where it was not possible to place the plastic sheet two guard lines were left (4.5 m separation) for each treatment.

Experimental design and data analysis

In each of the four trials (greenhouses), the experimental design employed random blocks (four) arranged inside the surface of each greenhouse. Each plot had an area of 120 m^2. The number of tested insecticides was different according to the greenhouse trial (Trial 1, a.i.: abamectin, azadirachtin, *B. thuringiensis*, spinosad, and spiromesifen; Trial 2, a.i.: chlorantraniliprole, indoxacarb, and methomyl; Trial 3, a.i.: flubendiamide and spiromesifen. Trial 4, a.i.: emamectin); with doses as indicated in Table 1. In addition, in each greenhouse a control (check) was sprayed only with water. In each plot of the four tests, 9 yellow sticky traps (2 × 2 cm) were arranged, according to the arrangement shown in Fig. 1, 24 h prior to the insecticide applications. Later, these sticky traps were visited at 3, 6, 9 and 12 days after treatments, and the number of adults of *T. achaeae* captured per treatment plot was evaluated. These data were analysed by generalized lineal models (GZLMs). Then, the mean values were analysed by a pairwise multiple comparison procedure (Wald test) (Aruna & Aruna, 2015). For this, we used the SPSS software, vers. 23 (IBM, 2014). Subsequently, the parasitism reduction was evaluated in relation to the control (*E*). This was calculated with the equation and classified according to the IOBC scale for field trials (Hassan *et al.*, 1991; Jepson, 1998; Amano & Haseeb, 2001): class 1 = harmless ($E < 25\%$), class 2 = slightly harmful ($25\% \leq E \leq 50\%$), class 3 = moderately harmful ($51\% \leq E \leq 75\%$), and class 4 = harmful ($E > 75\%$).

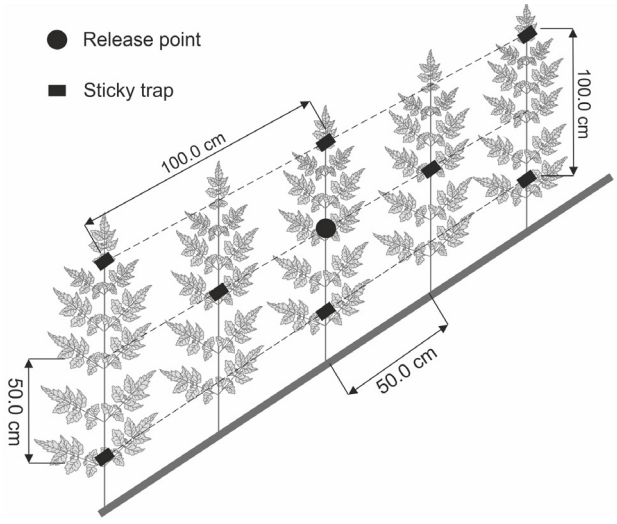

Figure 1. Chromatic trap distribution in tomato plants for *Trichogramma achaeae* (Hymenoptera: Trichogrammatidae) greenhouse dispersal study after insecticide applications.

Results

Insecticide application on pupae and sublethal effects

The side effects of the different insecticides on pupal survival, longevity, and fecundity of *T. achaeae* after application of insecticide formulations to the pupal stage are shown in Table 2.

The pupal survival decreased significantly, with respect to controls, after treatment with six AIs (azadirachtin, chlorantraniliprole, emamectin, indoxacarb, methomyl and spinosad) (Table 2). These values less than 30% (IOBC class 1-harmless) for the AIs: abamectin (E = 1.3%), azadirachtin (E = 20.77%), *B. thuriendiensis* (E = 5,58%), emamectin (E = 19.78%), flubendiamide (E = 12,63%), indoxacarb (E = 16.38%) and spiromesifen (E = 0). In turn, the decrease in pupal survival was between 30 and 50% (IOBC class 2-slightly toxic) for the AIs: chlorantraniliprole (E = 33.72%) and methomyl (E = 41.92%). Only one, AI spinosad (E = 79.16%) showed a greater decrease in the pupal survival (IOBC class 3-harmless) (Fig. 2).

The longevity of female that emerged from pupae treated with insecticides decreased significantly with respect to controls in four AIs treatments (emamectin, flubendiamide, indoxacarb and spiromesifen) (Table 2). All of the AIs, except for the first one, show decreases in female longevity under 30% (IOBC class 1-harmless); emamectin showed a greater reduction (E = 34.20%) (IOBC class 2-slightly toxic) (Fig. 2). In turn, the deleterious effects of treatments on male longevity were not significant, with the exception of the AI emamectin (E = 37.50%) (IOBC class 2-slightly harmful (Fig. 2).

Finally, when the applications were carried out in the pupal stage, significant reductions were found in the fecundity of females for six AIs (abamectin, azadirachtin, chlorantraniliprole, emamectin, flubendiamide and indoxacarb) (Table 2). For these, only the AI azadirachtin (E = 31.55%) presented a decrease in fertility greater than 30% (IOBC class 2-slightly harmfull). For the rest, the value of E is located within IOBC class 1 (Fig. 2).

Analysis of the effects of the AI spinosad treatment on the F_1 generation was not performed, as the number

Table 2. Mean values (± SE) of adult emergence of the F_0 generation, and longevity and fecundity of adults of the F_1 generation of *Trichogramma achaeae* (Hymenoptera: Trichogrammatidae) after application of insecticide formulations to parasitized eggs of *Ephestia kuehniella* (Lepidoptera: Pyralidae) when the immature parasitoid was in the pupal stage and under laboratory conditions

Treatment	Pupal survival (%)[1]		Longevity of adults (days)[1]				Fecundity of females (no. eggs/♀)[1]	
			Female		Male			
	a.i.	Control	a.i.	Control	a.i.	Control	a.i.	Control
Abamectin	68.30±8.55	69.20±8.64	9.13±2.00	9.47±1.61	7.03±1.67a	6.18±1.66b	43.90±10.74a	52.43±9.05b
	$F_{1,6}$=0.02, NS		$F_{1,58}$=0.5, NS		$F_{1,58}$=4.1, NS		$F_{1,58}$=11.1, p=0.002	
Azadirachtin	59.33±6.93a	74.88±6.29b	9.90±1.09	10.33±1.46	5.97±1.19	6.13±1.14	45.40±10.84a	66.33±11.13b
	$F_{1,6}$=9.2, p=0.023		$F_{1,58}$=0.8, NS		$F_{1,58}$=0.3, NS		$F_{1,58}$=54.5, $p < 0.0001$	
B. thuringiensis	77.49±6.29	79.54±7.74	11.00±1.51	11.92±0.81	8.67±1.83	9.02±1.79	65.07±8.10	67.57±6.88
	$F_{1,6}$=2.5, NS		$F_{1,58}$=0.1, NS		$F_{1,58}$=0.6, NS		$F_{1,58}$=1.7, NS	
Chlorantraniliprole	50.56±3.36a	76.28±7.53b	9.60±1.43	9.72±1.47	4.90±1.49	4.93±1.70	53.20±14.78a	60.73±14.15b
	$F_{1,6}$=38.9, p=0.001		$F_{1,58}$=0.097, NS		$F_{1,58}$=0.01, NS		$F_{1,58}$=4.1, p=0.048	
Emamectin	60.56±3.46a	75.49±5.42b	6.10±1.27a	9.27±1.20b	3.30±1.30a	5.28±0.96b	42.27±12.36a	59.37±9.61b
	$F_{1,6}$=21.6, p=0.004		$F_{1,58}$=97.8, $p < 0.0001$		$F_{1,58}$=36.6, $p < 0.0001$		$F_{1,58}$=35.8, $p < 0.0001$	
Flubendiamide	66.82±5.04	76.48±5.83	7.93±1.57a	10.43±1.65b	6.30±1.47	6.08±0.97	50.47±9.88a	66.93±9.21b
	$F_{1,6}$=4.6, NS		$F_{1,58}$=36.0, $p < 0.0001$		$F_{1,58}$=0.457, NS		$F_{1,58}$=44.6, $p < 0.0001$	
Indoxacarb	63.20±5.28a	75.58±6.38b	9.93±2.00a	10.93±1.29b	5.33±1.45	5.47±1.17	48.43±10.11a	63.33±9.90b
	$F_{1,6}$=7.3 p=0.036		$F_{1,58}$=4.9, p=0.03		$F_{1,58}$=0.2, NS		$F_{1,58}$=33.3, $p < 0.0001$	
Methomyl	43.37±5.90a	74.68±6.49b	10.27±1.80	10.42±1.50	6.53±1.11	6.73±1.44	61.93±10.32	60.80±11.28
	$F_{1,6}$=42.8, p=0.001		$F_{1,58}$=0.1, NS		$F_{1,58}$=0.01, NS		$F_{1,58}$=0.2, NS	
Spinosad	14.32±5.49a	68.70±7.65b						
	$F_{1,6}$=133.5, $p < 0.0001$							
Spiromesifen	78.33±6.84	76.49±6.41	10.83±1.76a	9.33±1.73b	4.90±1.56	5.50±1.63	59.20±9.13	63.30±10.36
	$F_{1,6}$=2.1, NS		$F_{1,58}$=11.1, p=0.002		$F_{1,58}$=2.1, p=NS		$F_{1,58}$=2.7, NS	

[1]Different letters indicate significant differences verified by GLM and Tuky's HSD test at $p < 0.05$. NS = not significant.

of offspring females was very low, and they died in less than 24-48 h. It should be noted that the pupal survival trial for this AI was repeated up to 3 times.

Insecticide application on adults

The effect on parasitism by *T. achaeae* females, when they were exposed to the residue of freshly sprayed insecticides is shown in Table 3. A statistically significant decrease was found in relation to the control (water) for four AIs: azadirachtin (E = 33.89%), emamectin (E = 75.19%), methomyl (E = 80.29%), and spinosad (E = 69.38%). This allows the classification of the AI methomyl into the moderately harmful IOBC class 3, and the other three AIs into the slightly harmful IOBC class 2.

Greenhouse evaluation

The results obtained in the four commercial greenhouse trials are shown in Table 4. Three AIs showed statistically significant decreases with respect to the water control (check): emamectin, methomyl, and spinosad. The AI emamectin showed an E = 40.00% (IOBC class 2-slightly harmful), and the others IA methomyl (E = 51.97%) and IA spinosad (E = 52.30%) were grouped into the IOBC class 3-moderately harmful.

Discussion

In Europe, *T. achaeae* has been shown to be a suitable biological control agent against *Tu. absoluta*, as mentioned above. However, the control of this pest is difficult to manage alone, with the simultaneous use of natural enemies or insecticides being necessary for a satisfactory pest control (Campos *et al.*, 2017). This is more pronounced at present; thus, the incidence of the pest has increased in tomato crops in Spain and Europe in recent years, especially in greenhouse crops (Vila, 2018, *pers. com.*), possibly motivated by problems of resistance to the AIs currently used in these crops. Thus, Roditakis *et al.* (2018) cited several cases of resistance to emamectin, spinosad, indoxacarb,

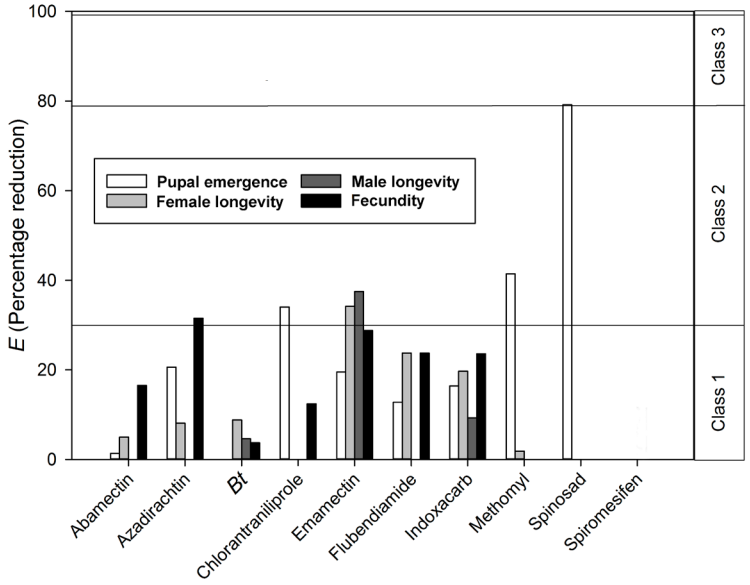

Figure 2. Reduction of adult emergence of F_0 generation, longevity and fecundity of adults of the F_1 generation of *Trichogramma achaeae* (Hymenoptera: Trichogrammatidae) after application of insecticide formulations to parasitized eggs of *Ephestia kuehniella* (Lepidoptera: Pyralidae) when the immature parasitoid was in the pupal stage and under laboratory conditions. Class of toxicity according to the IOBC, where: 1-harmless, $E < 30\%$; 2-slightly harmful, $30 \leq E \leq 79\%$; 3-moderately harmful, $80 \leq E \leq 99\%$; and 4-harmful, $E > 99\%$.

and chlorantraniliprole in that geographic area. In this sense, it has been mentioned that multiple sublethal effects, sometimes counterintuitive ones, on natural enemies have been reported for modern slower-acting insecticides and/or biopesticides. This highlights the need to revise the labelling of these products to indicate their compatibility with sustainable IPM programmes (Biondi *et al.*, 2018).

Table 3. Parasitism (mean±SE) of *Ephestia kuehniella* eggs (Lepidoptera: Pyralidae) by *Trichogramma achaeae* (Hymenoptera: Trichogrammatidae) when parasitoid females were exposed to residues of insecticide formulations, under laboratory conditions, for four trials.

Trial	Treatment	Parasitism (%)[1]		*E*[2]		C[3]
1	Chlorantraniliprole	87.83±8.78 b	$F_{3,8} = 10.8$	0.00	1	Harmless
	Flubendiamide	59.81±9.56 a	$p = 0.004$	20.69	1	Harmless
	Indoxacarb	63.53±2.69 a		15.75	1	Harmless
	Control	75.41±2.34 ab				
2	Abamectine	63.79±8.65 ab	$F_{3,8} = 48.2$	16.79	1	Harmless
	Azadirachtin	50.68±3.23 b	$p < 0.001$	33.89	2	Slightly harmful
	Spinosad	23.47±6.04 c		69.38	2	Slightly harmful
	Control	76.66±2.67 a				
3	*B. thuringiensis*	72.57±11.95 a	$F_{3,8} = 26.5$	11.04	1	Harmless
	Emamectin	20.24±5.74 b	$p < 0.001$	75.19	2	Slightly harmful
	Spiromesifen	68.04±9.40 a		16.60	1	Harmless
	Control	81.58±12.85 a				
4	Methomyl	15.38±5.18 a	$F_{1,4} = 39.3$	80.29	3	Moderately harmful
	Control	78.04±11.76 b	$p < 0.001$			

[1]In each trial, different letters indicate significant differences verified by GLM and Tukey's HSD at $p < 0.05$; [2]Reduction in parasitism (%); [3]IOBC Classes: 1, harmless ($E < 30\%$); 2, slightly harmful ($30 \leq E \leq 79\%$); 3, moderately harmful ($80 \leq E \leq 99\%$); 4, harmful ($E > 99\%$).

Table 4. Total number (mean±SE) of *Trichogramma achaeae* adult parasitoid (Hymenoptera: Trichogrammatidae) caught on yellow sticky traps (release-recapture method), per experimental plot (9 traps/plot), after application of insecticide formulations in four trials carried out under commercial greenhouse conditions.

Trial	Treatment	Adults caught[1]	Omnibus test (χ^2 likelihood ratio)	E[2]		C[3]
1	Abamectin	87.25±4.67	$\chi^2 = 132.611$ df = 5 $p < 0.001$	5.42	1	Harmless
	Azadirachtin	109.75±5.24 *		0	1	Harmless
	B. thuringiensis	97.25±4.93		0	1	Harmless
	Spinosad	44.00±3.32 *		52.30	3	Moderately harmful
	Spiromesifen	91.00±4.77		1.36	1	Harmless
	Control	92.25±4.80				
2	Chlorantraniliprole	44.50±3.35	$\chi^2 = 48.840$ df = 3 $p < 0.001$	0	1	Harmless
	Indoxacarb	34.75±2.95		8.55	1	Harmless
	Methomyl	18.25±2.14 *		51.97	3	Moderately harmful
	Control	38.00±3.08				
3	Flubendiamide	57.50±3.79	$\chi^2 = 0.609$ df = 2 NS	6.50	1	Harmless
	Spiromesifen	58.50±3.82		4.88	1	Harmless
	Control	61.50±3.92				
4	Emamectin	66.00±5.75 *	$\chi^2 = 22.235$ df = 1 $p < 0.001$	40.00	2	Slightly harmful
	Control	110.00±7.42				

[1]For each trial, treatments differing significantly (GZLM analyses and pairwise multiple comparison procedure and Wald test) at $p < 0.01$) from the water control are indicated by an asterisk (*); [2]reduction in parasitism (%); [3]IOBC class 1 = harmless ($E < 25\%$), class 2 = slightly harmful ($25\% \leq E \leq 50\%$), class 3 = moderately harmful ($51\% \leq E \leq 75\%$), and class 4 = harmful ($E > 75\%$).

In our work we have shown a broad picture of the side effects of the AIs used in the chemical control of *Tu. absoluta* on the parasitoid *T. achaeae*.

First, the group of AIs, abamectin, *B. thurigiensis*, flubendiamide, indoxacarb, and spiromesifen did not present side effects, or these were negligible, on *T. achaeae* for all the trials carried out under laboratory or greenhouse conditions (IOBC class 1).

It should be noted that in other studies with the AI abamectin, discrepant results have been found for other *Trichogramma* species. Thus, Brunner *et al.* (2001) found a high mortality of adults (56-60%) for *T. platneri* Nagakartti, when they were exposed to leaf residues less than 3 days old. Additionally, Carvalho *et al.* (2003) found side effects on adult emergence and the longevity and fecundity of adults when this AI was applied on the pupal stage. This was corroborated by Moura *et al.* (2006) for the same species and AI. On the other hand, Consoli *et al.* (1998) and Nornberg *et al.* (2009) reported that this AI had no side effects for *T. pretiosum* Riley when it was applied in the protected life stage (pupa). Our results seem to be intermediate those previously mentioned; thus, side effects were observed in female fecundity (E = 16.27%, IOBC class 1) (Fig. 2), parasitism (E = 16.79%, IOBC class 1) (Table 3) (both IOBC class 1); and adult activity in the greenhouse trial (E = 5.42%, IOBC class 1) (Table 4). Perhaps the differences noted above may be due to different degrees of susceptibility; this has been reported for different populations of *T. pretiosum* by Vianna *et al.* (2009). For this AI, we can highlight what was indicated by Gentz *et al.* (2010) that despite significant toxicity to several non-target species, abamectin was once considered suitable for use with many beneficial insects due to its short environmental persistence.

In relation to the AI *B. thurigiensis*, several authors agree that side effects have not been found in *T. achaeae* (Saelices *et al.*, 2012; Fontes *et al.*, 2018), and in other species of the same genus: *T. dendrolimi* (Matsumura), *T. pretiosum, T. bourarachae* Pintureaeu and Babault, *T. cacoeciae* Marchal, and *T. evanescens* Westwood (Takada *et al.*, 2001; Vianna *et al.*, 2009; Ksentini *et al.*, 2010). Our results (Fig. 2; Tables 3 and 4) corroborate the above.

Our results for the AI flubendiamide indicate that there were significant side effects on female longevity (E = 23.97%) and female fecundity (E = 24.59%), when the application was made on the pupal stage (Fig. 2), as well as in the exposed phase of the parasitoid (adult) (E = 20.69%); all of them were within IOBC class 1. However, in the greenhouse trial, these side effects were lower (E = 6.5%, IOBC class 1) (Table 4). In addition,

the compatibility between the AI flubendiamide and *T. achaeae* found in this work corroborates the results found with other species of the same genus, such as *T. chilonis* Ishi and *T. pretiosum*. Thus, side effects were not found on adults (Sattar *et al.*, 2011; Martins *et al.*, 2011) or on the developmental stages of *T. pretiosum* (Carvalho *et al.*, 2005). The same results have been reported for *T. atopovirilia* Oatman and Platner (Rezende *et al.*, 2005), that is, in the latter case with a different methodology from the one used in the present work.

Similar effects, to those previously indicated for the AI flubendiamide, were found in our work for the AI indoxacarb, both in laboratory and greenhouse trials (Fig 2, Table 4). The values found were less than a 30% reduction in pupal survival (E = 16.38%), female longevity (E = 9.15%), female fecundity (E = 23.53%) (Fig. 2), and parasitism (E = 15.75%) with respect to the control for laboratory trials (Table 3). In the greenhouse trial, the AI indoxacarb presented (E = 8.55%) harmless side effects, grouping into IOBC class 1. Similar side effects have been found by Scholz & Zalucki (2000) for *T. pretiosum* and Hewa-Kapuge *et al.* (2003) for *T.* sp. nr *brassicae* under laboratory and field conditions. Only, Sattar *et al.* (2011) found a slightly harmful effect (IOBC class 2) on the adult emergence and female fecundity of *T. chilonis.*

Finally, in relation to the first five AIs indicated at the beginning of this discussion, the AI spiromesifen did not present harmful effects on the biological parameters analysed in the laboratory trial (Tables 2 and 3). The same result was obtained in the greenhouse trial (Table 4). This AI is highly compatible with *T. achaeae*. The same results have been cited by Kavitha *et al.* (2006) for *T. chilonis*.

Second, there is another group of two AIs that presented slightly harmful side effects (IOBC class 2): azadirachtin and chlorantraniliprole, in laboratory trials, but that, under greenhouse conditions, showed no side effects (IOBC class 1-harmless).

Additionally, the AI azadirachtin had side effects on pupal survival (E =20.77%) (IOBC class 1) (Fig. 2). Similar side effects on adult emergence have been cited for *T. cacoeciae* for this AI (Saber *et al.*, 2004). It should also be noted that this AI presented side effects on female fecundity (E = 31.55%) (IOBC class 2) (Fig. 2). However, in the greenhouse trial no side effects were found on parasitoid activity (IOBC class 1) (Table 4).

The AI chlorantraniliprole, in tests carried out with predatory species, presents different degrees of toxicity; from very high in some species of Coccinelids and Chrysopids, to no side effects in other species (Stanley & Preetha, 2016). In relation to the species of the genus *Trichogramma*, the first studies have indicated that this AI is safe for *T. chilonis*, *T. galloi* (Zucchi), and *T. pretiosum* (Preetha *et al.*, 2009; Brugger *et al.*, 2010; Oliveira *et al.*, 2013). Additionally, it does not affect the emergence of *T. chilonis* and *T. pretiosum* adults (Brugger *et al.*, 2010). However, our laboratory data differ from those found in these species; thus, pupal survival (E = 33.7%) (IOBC Class 2) and, to a lesser extent, female fertility (E = 12.40%) (IOBC class 1) were affected by this AI (Fig. 2). Our results in relation to *T. achaeae* female fertility agree with those cited by Fontes *et al.* (2018). Despite these effects in the laboratory, no side effects were found under greenhouse conditions (IOBC class 1) (Table 4).

For the two insecticide groups discussed above, abamectin, azadirachtin, *B. thuringiensis*, chlorantraniliprole, flubendiamide, indoxacarb, and spiromesifen, can be considered very compatible with the use of the parasitoid *T. achaeae*.

On the other hand, there is a third group of AIs: emamectin, methomyl, and spinosad, which presented side effects both in the laboratory and in greenhouse trials.

The AI emamectin showed side effects on the longevity of females and males (IOBC class 2) and, to a lesser extent, in the survival of pupae and fecundity of females (IOBC class 1) in the laboratory tests (Fig. 2). The decrease in fecundity of females is lower than that cited for the same species and AI by Fontes *et al.* (2018) (IOBC class 2). Additionally, the reduction of the values of parasitism when the females were exposed to the fresh residue of the AI (IOBC class 2) (Table 3) was lower than that cited, also for the same species and AI, by Saelices *et al.* (2012). Similar results have been cited for this AI in relation to the *Trichogramma* species *T. chilonis* and *T.* sp. nr *brassicae* (Hewa-Kapuge *et al.*, 2003; Sattar *et al.*, 2011). The detrimental effects of the AI emamectin in *T. achaeae* are also shown in greenhouse trials (IOBC class 2) (Table 4).

The AI methomyl has shown an important side effect on *T. achaeae* pupae. It represented a reduction in adult emergence (E = 41.92%) (IOBC class 2-slightly harmful) (Fig. 2). In contrast, there were no such effects on adult longevity and female fecundity (IOBC class 1) (Fig. 2). In turn, this insecticide shows an important side effect on parasitism (E = 80.29%) (IOBC class 3-moderately harmful) (Table 3). This value is very similar to that found by Fontes *et al.* (2018) for the same species. Similar results have been reported for other species of *Trichogramma* (Bull & House, 1983; Hassan *et al.*, 1987; Scholz & Zalucki, 2000; Takada *et al.*, 2001; Bueno *et al.*, 2008). In the greenhouse trial, it was also shown to be moderately harmful (IOBC class 3) (Table 4). This corroborates the results found by Tipping & Burbutis (1983) and Campbell *et al.* (1991)

for *T. nubilale* Ertle and Davis, *T. exiguum*, *T. minutum* and *T. pretiosum*.

Finally, the AI spinosad had significant side effects for *T. achaeae*. This AI decreased pupal survival (*E* = 79.16%) (IOBC class 3-moderately harmful) (Fig. 2) and parasitism (*E* = 80.29%) (IOBC class 3-moderately harmful) (Fig. 2, Table 3). These results corroborate those found by Fontes *et al.* (2018) for this AI in the same species. At the same time, the toxicity of the AI spinosad in other species of *Trichogramma* has been studied by several authors (Suh *et al.*, 2000; Consoli *et al.*, 2001; Maia *et al.*, 2010; Liu & Zhang, 2012; Saljoqi *et al.*, 2012; Costa *et al.*, 2014), who have cited side effects in some of the biological parameters of *Trichogramma* spp. In our work, the AI spinosad also had side effects for *T. achaeae* in the greenhouse trial (*E* = 51.97) (IOBC class 3-moderately harmful) (Table 4). This insecticide has traditionally been included in IPM programmes because of its low toxicity to mammals and birds, its slight or moderate toxicity to aquatic organisms, and its relative harmless effect for a wide range of natural enemies (Gentz *et al.*, 2010). However, William *et al.* (2003) have cited that this AI shows a significant side effect on the Hymenopteran parasitoid complex, both in the field and laboratory trials.

According to the values indicated above for this third group of AIs: emamectin, methomyl, and spinosad, we must mention that their use is not compatible with the release of *T. achaeae* in tomato crops.

Recently it has been mentioned that pesticide risk assessments for entomophagous species are being performed by categorizing pesticides based on mortality in laboratory and semi-field trials and reduction in field studies. Testing the pesticides under field recommended concentrations at laboratory conditions does not exactly reveal how the pesticides behave in complex field conditions (Stanley & Preetha, 2016). This last point has also been previously indicated by other authors (*e.g.*, Stark *et al.*, 1995).

Therefore, in the present work the tests were carried out in laboratory and field conditions indicated by the IOBC WPRS methodology, without ruling out any AI in the different stages of the sequential testing scheme methodology.

In this sense, we must indicate, on the one hand, that a very good correlation has been found between the results of laboratory tests and those carried out under greenhouse conditions. Thus, the AIs emamectin, methomyl, and spinosad presented the highest side effects, and in more tests, under laboratory conditions, they also had the highest side effects under greenhouse conditions.

On the other hand, based on the results found, we consider that the new methodology used in the evaluation of adult activity of *T. achaeae* could be more feasible under field conditions and provide reliable results to evaluate the secondary effects these conditions. This is compared to the methodology recommended by the IOBC WPRS (Hassan, 1985; EPPO, 1999) for field tests for the evaluation of parasitism in sentinel eggs and those that could be extended to other species of *Trichogramma*.

Based on the results found in this work, we can conclude that there is an important group of insecticide formulations, especially those of the new generation, which present a high degree of compatibility with the use of the egg parasitoid *T. achaeae*. This allows a better adaptation of the use of both control systems of *Tu. absoluta* in tomato, both in greenhouses and open-air crops. This aligns with the recommendations indicated by Campos *et al.* (2017) for better control of this pest species.

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