UNIVERSIDADE FEDERAL DE LAVRAS

LUIS CLEPF PASSOS

SIDE EFFECTS OF INSECTICIDES IN TOMATO CROPS ON THE PREDATORY MIRIDS *Macrolophus basicornis* AND *Nesidiocoris tenuis*

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(Advisor)

Dr. Antonio Biondi
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GENERAL ABSTRACT

The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is probably the main tomato pest in several countries around the world. Caterpillars of this species feed on all aerial parts of tomato plants and can cause up to 100% yield loss if not controlled. Chemical control with synthetic insecticides is the main tactic employed in most of the cases, however there are several problems associated with this practice, such as selection of *T. absoluta* resistant populations. For this reason, other control measures should be adopted to reduce *T. absoluta* populations. The biological control with predatory hemipterans is a well succeeded strategy in several Europeans countries, most frequently with the mirid *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae). In Brazil the species *Macrolophus basicornis* (Stal) (Hemiptera: Miridae) is being studied, and was observed that it can prey upon *T. absoluta* eggs and small larvae. However, for these species effectively exerce their biological control, insecticides must be employed carefully. Therefore, the main objectives of this study were: (I) evaluate lethal and sublethal effects of insecticides recommended to control *T. absoluta* in Brazil on *M. basicornis*, (II) determinate sublethal concentrations of insecticides and plant essential oils to *N. tenuis*, and (III) evaluate the effect of sublethal concentrations (LC_1, LC_{10}, LC_{30}) of insecticides and plant essential oils on *N. tenuis* fecundity and orientation. In the experiments of the present thesis the predators *M. basicornis* and *N. tenuis* were exposed to insecticide compounds in laboratory conditions. Nymphs of the South American species (*M. basicornis*) were exposed to full doses of insecticides considered safe to natural enemies (teflubenzuron, methoxifenozide and chlorantraniliprole), plus a positive control (abamectin) by residual contact on tomato leafles. Only abamectin caused high nymph mortality, however the other compounds caused sublethal effects, such as reduction in adult size and increase of development time, although adult survival and progeny were not affected. Among them, chlorantraniliprole was considered the safest insecticide for *M. basicornis*. For the European species (*N. tenuis*), adult females were directly treated with sublethal concentrations of three neurotoxic insecticides (lambda-cyhalothrin, chlorpyrifos and spinosad) and four plant essential oils encapsulated in nanoemulsions (anice, lavender, garlic and fennel). First, a concentration-mortality relation was stablished for all compounds and *N. tenuis* females, with this was possible to estimate sublethal concentrations. *Nesidiocoris tenuis* females were exposed to three sublethal concentrations of each compound (LC_1, LC_{10} and LC_{30}) and their effect was assessed on their fecundity and orientation. The fecundity of females was affected by sublethal concentrations of the compounds. Moreover, some of the compounds, even in very low concentrations, affected the orientation insects and also the response time to make a choice. The insecticides and essential oils must be used carefully.

Keywords: Ecotoxicology. Risk assessment. Predatory mirid. Biopesticide. Physiological selectivity.

RESUMO GERAL

A traça-do-tomateiro, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) é a principal praga da cultura do tomateiro em diversos países do mundo. As lagartas se alimentam de toda a parte aérea das plantas de tomateiro e podem causar até 100% de perda de produção se não forem controladas. O controle químico com inseticidas sintéticos é a principal tática empregada na maioria dos casos, porém existem vários problemas associados a essa prática, como a seleção de populações resistentes. Assim, outras medidas de controle devem ser adotadas para reduzir populações de *T. absoluta*. O controle biológico com mirídeos predadores é uma estratégia bem-sucedida em vários países europeus, utilizando principalmente a espécie *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae). No Brasil, o mirídeo *Macrolophus basicornis* (Stal) (Hemiptera: Miridae) está sendo estudado, e observou-se que pode predar ovos e larvas de primeiros instares de *T. absoluta*. No entanto, para que essas espécies exerçam efetivamente seu controle biológico, inseticidas devem ser empregados com cuidado. Desta forma, os objetivos deste estudo foram: (I) avaliar os efeitos letais e subletais de inseticidas recomendados para controlar *T. absoluta* no Brasil sobre o predador *M. basicornis*, (II) determinar concentrações subletais de inseticidas e óleos essenciais de plantas para *N. tenuis*, e (III) avaliar o efeito das concentrações subletais (LC_1, LC_{10} e LC_{30}) de inseticidas e óleos essenciais de plantas na fecundidade e orientação de *N. tenuis*. Os predadores *M. basicornis* e *N. tenuis* foram expostos a compostos inseticidas em condições de laboratório. Ninfas de *M. basicornis* foram expostas via contato residual à máxima dosagem recomendada de inseticidas considerados seguros a inimigos naturais na cultura do tomateiro (teflubenzuron, metoxifenozida e clorantraniliprole), além de um controle positivo (abamectina). Somente a abamectina causou alta mortalidade sobre ninfas, porém os outros compostos causaram efeitos subletais, como redução no tamanho do adulto e aumento do tempo de desenvolvimento. Entre eles, o clorantraniliprole foi considerado o inseticida mais seguro para *M. basicornis*. Para *N. tenuis*, as fêmeas adultas foram tratadas diretamente com concentrações subletais de três inseticidas neurotóxicos (lambda-cialotrina, clorpirifós e espinosade) e quatro óleos essenciais de plantas encapsulados em nanoemulsões (anis, lavanda, alho e erva-doce). Primeiro, foi estabelecida uma relação concentração-mortalidade para todos os compostos e fêmeas de *N. tenuis*, sendo possível estimar as concentrações subletais. Com isso, as fêmeas de *N. tenuis* foram expostas a três concentrações subletais de cada composto (LC_1, LC_{10} e LC_{30}) e seu efeito foi avaliado sobre a fecundidade e orientação do predador. A fecundidade das fêmeas foi afetada por todas as concentrações subletais dos compostos. Além disso, alguns dos compostos, mesmo em concentrações muito baixas, afetaram a orientação dos insetos e também seu tempo de resposta. Os inseticidas e óleos essenciais devem ser usados com cuidado.

Palavras-chave: Ecotoxicologia. Avaliação de risco. Mirídeo predador. Biopesticidas. Seletividade fisiológica.

SUMMARY

PART I – GENERAL INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a crop grown in almost all world, in nearly 5 million hectares, generating a production of more than 180 million tons of fruits in 2018. Nearly 60% of world's production is concentrated in Asia, mainly on China, India and Turkey, which are the three major tomato producers, while Brazil occupies the tenth position (FAO, 2019). In Brazil, almost half of the country's production is concentrated in the states of Goiás, São Paulo and Minas Gerais (IBGE, 2019). One of the main issues that compromises higher tomato productions is the incidence of phytophagous insects during the whole crop cycle, reducing yields and increasing managements costs.

Nowadays, the South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is considered a worldwide threat for tomato production (CAMPOS et al., 2017). This cosmopolitan pest is originated from South America, but in 2006 was first reported in Europe, and in 2020 is present in several tomato producer countries in Europe, Africa and Asia, causing severe yield losses (DESNEUX et al., 2010; SYLLA et al., 2017; BIONDI et al., 2018; MANSOUR et al., 2018). To reduce populations of *T. absoluta* and other phytophagous insects in tomato crops chemical control with synthetic insecticides is usually the primary tactic employed (GUEDES & PICANÇO, 2012). However, besides their toxicity, these substances are often used excessively and incorrectly, without any kind of pest sampling and rotation of active ingredients, resulting in problems such as selection of resistant pest populations, secondary pests outbreaks and deleterious effects in beneficial organisms (YU, 2008; GUEDES & SIQUEIRA, 2012; HADDI et al., 2012; RODITAKIS et al., 2015). For these reasons, other control tactics must be incorporated in integrated pest management (IPM) programs to enhance pest control. The biological control is a very important tool in pest control, since it mitigates the problems mentioned above (VAN LENTEREN et al., 2012, 2018).

After *T. absoluta* invasion in Europe, researchers and farmers observed that eggs and first instar larvae of this lepidopteran are preyed by predatory mirids used to control other tomato pests (URBANEJA et al., 2009; MOLLÁ et al., 2011; BIONDI et al., 2018). The species are *Nesidiocoris tenuis* (Reuter) and *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae), that were already being conserved and released in tomato crops to control populations of pests such as the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (CALVO et al., 2012; ZAPPALÀ et al., 2013). Therefore, IPM programs were developed focusing on the action

of the predatory mirids to control *T. absoluta*. With the good results obtained in Europe, researchers started to search native mirid species in Brazil, capable to prey upon *T. absoluta*, and three species were collected: *Engytatus varians* (Distant), *Campyloneuropsis infumatus* (Carvalho) and *Macrolophus basicornis* (Stal) (Hemiptera: Miridae) (Bueno et al., 2013). All of them feed on *T. absoluta* eggs and small larvae and other pests such as *B. tabaci*, thus have potential to be used in biological control programs, but among them *M. basiconirs* stands out for its higher prey capacity and positive funcional response according to prey density (SILVA et al., 2016; VAN LENTEREN et al., 2016, 2017).

In addition to predatory mirids, another promising approach in *T. absoluta* control is the use of plant essential oils with insecticide proprieties. These oils are secondary metabolites produced by plants, and some of them can exert toxic effects to insects, thus are used in IPM programs to reduce pest populations, including *T. absoluta* (CAMPOLO et al., 2017). Plant essential oils present generally low mammalian toxicity, low persistence in water and soil, and are relatively cost-efficient (ISMAN, 2000; BULLANGPOTI, 2017). For this reason, several insecticides based on plant essential oils are being developed and studied to potentially contribute to pest control in organic crops and also be incorporated in conventional IPM programs as an alternative or complement to synthetic insecticides, including in *T. absoluta* management programs (ISMAN 2006; REGNAULT-ROGER et al., 2012; CAMPOLO et al., 2018; GIUNTI et al., 2019). These products are generally considered safer than insecticides, but they can also affect natural enemies present in the crop, therefore their effects on beneficial insects must be evaluated (CHARLESTON et al., 2005; SOARES et al., 2019).

In order to biological control agents exert their pest control function, they must be compatible with chemical compounds and essential oils. For this, the effects of insecticide compounds on natural enemies must be evaluated in order to determine selective products, and with this integrate the control tactics and improve pest control. Selectivity is the capacity of an insecticide to control the pest with low or none effect on nontarget insects (BUENO et al., 2017; CARVALHO et al., 2019). Besides the mortality, insecticides can cause sublethal effects on natural enemies' biology and behavior, biasing their capacity to control pests (DESNEUX et al., 2007; BIONDI et al., 2012; MARTINOU et al., 2014). Hence, the selectivity of insecticides on natural enemies must be evaluated, in order to incorporate this integration in IPM programs (DEGRANDE et al., 2002).

Therefore, the main objectives of this study were: (I) evaluate lethal and sublethal effects of insecticides recommended to control *T. absoluta* in Brazil on *M. basicornis*, (II) determinate

sublethal concentrations of insecticides and plant essential oils to *N. tenuis*, and (III) evaluate the effect of sublethal concentrations (LC_1, LC_{10}, LC_{30}) of insecticides and plant essential oils on *N. tenuis* fecundity and orientation.

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PART II - THEORETICAL REFERENCE

1. PREDATORY MIRIDS: BIOECOLOGY AND APPLIED IMPORTANCE

The order Hemiptera contemplate very diverse insects, with different life and feeding habits. The majority feed on plants, often competing with humans for resources. These insects can cause direct plant damage by destroying or negatively affecting reproductive structures desired by humans, or forcing the plant to undertake energetically expensive costs to repair damages in vegetative structures (SCHAEFER & PANIZZI, 2000). Other hemipterans, however, can feed on other insects or smaller prey, and there are even species that feed on blood. In the family Miridae, many differences can be observed, with general and specific plant feeders, and general and specific feeders on other arthropods. Hence, many mirid species are efficient biological control agents, and several others have this potential (SCHAEFER & PANIZZI, 2000; WHEELER JR., 2001).

Most mirids are plant feeders, but many feed as scavengers or predators. Therefore, they have a great capacity to be used as biological control agents, especially because most of them have polyphagous habit and are classified as generalist predators (WHEELER JR., 2000a). Mirid generalist predators can feed on a vast array of arthropod preys, such as early instar whiteflies, mites, thrips and leafminers' eggs and neonate caterpillars (WHEELER JR., 2000b; CASTAÑÉ et al., 2011; CALVO et al., 2012; BOMPARD et al., 2013; JAWORSKY et al., 2013). Hence, some mirid species are used as biological control agents in many countries, by artificial releases or conservation of existent populations, regulating pest populations on crops through direct feeding (CASTAÑÉ et al., 2011; CALVO et al., 2012; URBANEJA et al., 2012; VAN LENTEREN et al., 2018b). Differently from insecticide applications, the preservation and release of natural enemies on the agroecossystem offers nearly no resistance pressure to the pest and less undesirable impacts on the environment and no risk of food contamination, and their use should be stimulated (VAN LENTEREN, 2009, 2012; VAN LENTEREN et al., 2018a).

The process by which bugs feed is involved with extraoral digestion, in a solid-to-liquid feeding method by attacking the nutrient-rich solid or semisolid organs and tissues of their prey (WHEELER JR., 2001). For this reason, their digestive trait does not present the complexities that characterize exclusive sap feeders, such as aphids and coccids (COHEN, 2000). In their feed process, hemipterans penetrate their stylets on the intracellular space of the food source

(plant sap, hemolymph or blood) performing mechanical breakdown (COHEN, 2000). After disruption, starts the extraoral digestion. Hemipteran's mouthparts contain two channels, one through which salivary fluid containing potent digestive enzymes is pumped into the organism, chemically dissolving tissues and cells, and the other through which the liquefied product are sucked back into the insect (SCHAEFER & PANIZZI, 2000; WHEELER JR., 2001).

The predatory mirids are zoophytophagus, which means that they can feed on both plants and prey at the same development stage (ALBAJES et al., 2006; CASTAÑÉ et al., 2011). Despite exploiting a wider range of food resources can be an advantage for the predators (SYMONDSON et al., 2002), predatory mirids can present a certain risk, since plant injury and consequent economic damage can occur in situations of high predator densities and low prey availability due to excessive feeding of tomato plants (including fruits) by mirids, in order to obtain nutrients (ALBAJES & ALOMAR, 2008; CASTAÑÉ et al., 2011). Injuries caused by the mirids can be resulted of mechanic damage caused by the stylets when penetrating plant tissue, and also as a result of a chemical process by which affected cells are destroyed by salivary enzymes (CASTAÑÉ et al., 2011). Damages include brownish lesions or rings on stems, petioles, leaf veins, and also foliar crinkling. Affected parts may become brittle and drop prematurely (WHEELER JR., 2000a; ARNÓ et al., 2010; CANSTAÑÉ et al., 2011; SISCARO et al., 2019). Damages are more intense if insects feed on plant vascular tissues, causing the assimilates not reach developing fruits (ARNÓ et al., 2010).

The acquisition of water seems to be the main reason why zoophytopaghous mirids feeds on plants. Certain quantity is required to maintain the insect's physiological status and to use during feeding process. Therefore, a certain amount of plant feeding is necessary and occurs regardless prey abundance. Moreover, predators do not produce visible injuries during all the time they spend feeding on plants, hence it is probable that feeding-associated damage is determined by the specific part of the plant attacked (CASTAÑÉ et al., 2011).

On the other hand, by being plant-feeders, these predators can stablish themselves on crops in early season (along with pests) and are able to survive in prey absence or shortage (GABARRA et al., 2004; CASTAÑÉ et al., 2011). Besides, there is a notorious advantage of being generalist, thus many species can be used to control both indigenous and exotic pests (SYMONDSON et al., 2002). For example, the invasive *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in Europe, of which pest management programs were developed based on conservation and release of the native mirids *Nesidiocoris tenuis* (Reuter) and *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) in tomato crops (URBANEJA et al., 2009;

DESNEUX et al., 2010; MOLLÁ et al., 2011; ZAPPALÀ et al., 2013; CAMPOS et al., 2017; BIONDI et al., 2018). Hence, the advantages offered by mirid predators tend to overcome the risk of crop damage (CASTAÑÉ et al., 2011). A low level of crop damage would not be an obstacle as long as generalist predators offer benefits in pest control and consequent reductions in insecticide sprays, therefore management programs must seek to minimize risks and maximize benefits (SYMONDSON et al., 2002; CASTAÑÉ et al., 2011).

The two species mentioned above, *N. tenuis* and *M. pygmaeus*, are good examples of generalist predators successfully used to control *T. absoluta* and *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in Europe (CALVO et al., 2012; VAN LENTEREN, 2012; BIONDI et al., 2016; BIONDI et al., 2018; VAN LENTEREN et al., 2018b). Both have their own advantages and disadvantages. *Macrolophus pygmaeus* is not as efficient as a predator compared to *N. tenuis* and is unable to stablish populations in tomato plants in absence of alternative prey other than *T. absoluta*, but is unlikely to cause plant damage (CASTAÑÉ et al., 2011; MOLLÁ et al., 2014). On the other hand, *N. tenuis* has a higher predation capacity of many tomato pests but can cause plant and fruit damage in prey absence and high populations (CASTAÑÉ et al., 2011; PÉREZ-HEDO & URBANEJA, 2016).

Although the use of these mirids may be considered risky in some cases, *M. pygmaeus* and *N. tenuis* are not only conserved, but widely released in several crop areas (CASTAÑÉ et al., 2011; BIONDI et al., 2018). The risk associated with its use is considered lower than the problems caused by pests, specially virus vectors, and also lower than disrupt IPM programs by using broad-specter insecticides to control pest such as *T. absoluta* (CANSTAÑÉ et al., 2011). Moreover, these zoophytophagous natural enemies can also contribute to control populations of other tomato pests, such as thrips and mites (CALVO et al., 2012; URBANEJA et al., 2012; BOMPARD et al., 2013; JAWORSKY et al., 2013).

With the success of IPM programs focusing on predatory mirids to control *T. absoluta* in Europe, researches started in Brazil aiming to determine native species with potential to control this pest in its native continent. Three mirid species were collected: *Engytatus varians* (Distant), *Campyloneuropsis infumatus* (Carvalho) and *Macrolophus basicornis* (Stal) (Hemiptera: Miridae) (BUENO et al., 2012). These species present similar characteristics with European mirids, such as wide prey range and the zoophytophagous habit, and were observed to prey upon *B. tabaci* nymphs and *T. absoluta* eggs and larvae, with better preliminary results and thus higher potential to be used as biological control agents for *M. basicornis* and *E. varians* (BUENO et al., 2013a, 2013b, 2017; VAN LENTEREN et al., 2016, 2017).

These Neotropical species can use tomato plants as oviposition substrate and are able to complete their development and build up populations in these plants, besides being able to walk on tomato plants, even with the presence of trichomes (BUENO et al., 2013, 2017, SILVA et al., 2016a). Additionally, they use tomato plant volatiles to locate *T. absoluta* damaged plants (SILVA et al., 2018), and nymphs and adults of these species appear to cause low damage to tomato seedling and fruits, even in conditions of high density and prey shortage (SILVA et al., 2016b; VAN LENTEREN et al., 2018b).

Both mirid species used in the bioassays (*M. basicornis* and *N. tenuis*) have similar charactheristics. Both belong to the Diciphynae subfamily, perform endophytic oviposition (females lay eggs inside the plant tissues, to protect from perdators and avoid water losses), preferentially in newer, tender parts of the plant, present 5 nymphal stages, and are absent of oscelli (WHEELER JR, 2001). Adults of *N. tenuis* are slightly smaller than *M. basicornis*, measuring approximately 3.0-3.3 mm (BHATT & PATEL, 2018), while the South-American species size ranges around 3.6-4.0 mm (FERREIRA & HENRY, 2011).

Macrolophus basicornis was first reported on tobacco plants, therefore this plant species is considered more adequate for rearing in laboratory because mirids can be easily seen and reliably counted and handeld in tobacco plants, although it can also suscefully reproduce and develop on tomato plants (SILVA et al., 2016a; BUENO et al., 2018). *Nesidiocoris tenuis* has a wider host range, mostly from Curcubitaceae and Solanaceae families, and can be reared in many plant species, such as tomato and sesame (BIONDI et al., 2016; BHATT & PATEL, 2018).

The optimal tememperature for both species is also similar, ranging around 25 °C (HUGHES et al., 2009; BUENO et al., 2013; BHATT & PATEL, 2018). In such conditions, *M. basicornis* can complete its development in approximately 38 days, the adults can prey more than 90 *T. absoluta* eggs per day, and females have a total fecundity ranging from 98 to 311 descendants, depending on the plant species (higher values for tobacoo plants) (BUENO et al., 2012, 2013, 2018). *Nesidiocoris tenuis* can occur in different countries and even different continents, so several differences can be observed regarding its biological parameters among different populations and also according to the host plants and prey offered (YANO et al., 2020). Differences can be observed, for example, in its preying capacity, ranging from approximately 30 (URBANEJA et al., 2009) to more than 100 eggs of *T. absoluta* per day (ARNÓ et al., 2009), development time ranging from 23 (HUGHES et al. 2009) to more than

45 days (BHATT & PATEL, 2018), and fecundity, with values ranging form 60 (SANCHEZ et al., 2009) to 97 eggs per female (YANO et al., 2020).

With all this information considered, is notorious that predatory mirids are important biological control agents, and must be studied to maximize their potential to control pests. The South American species are being evaluated, therefore still not commercialized, but preliminary results seems promising (SILVA et al., 2016a, 2016b; VAN LENTEREN et al., 2016, 2019). Currently used European species are also being studied, specially regarding the efficiency of pest control, association with other control methods and reduction in plant damage (CALVO et al., 2012; SISCARO et al., 2019; SOARES et al., 2019a).

2. MAIN MIRID PREY ON TOMATO

2.1 South American tomato pinworm

The South American tomato pinworm *T. absoluta* is considered the most important tomato pest in several countries (CAMPOS et al., 2017). It was first reported in Peru, in 1914, and posteriorly spread to other South American countries (GUEDES & PICANÇO, 2012). In 2006, *T. absoluta* was recorded in Spain and rapidly advanced to other European countries, and after was reported in many countries in Africa, Middle East and Asia, affecting more than 60% of world tomato production (DESNEUX et al., 2011; CAMPOS et al., 2017; SYLLA et al., 2017; XIAN et al., 2017; BIONDI et al., 2018).

Adults are small silver-grey moths (~10 mm of wingspread), which females can oviposit more than 50 eggs during their life span. The eggs are yellow, elliptical and placed individually on leaves, flowers and fruits. The larvae are green, with a brown spot on the back. As soon as they hatch from the eggs they penetrate tomato plants, preferentially on leaves, stems, sprouts and fruits, forming galleries (SILVA et al., 2013). After four larval instars, larvae exit from the galleries and pupate on the soil, forming a brown pupa, from which adults will emerge (VILLAS BÔAS et al., 2009). *Tuta absoluta* biological cycle ranges from 26 and 38 days, which means that may be consecutive generations in areas where tomato is grown during the whole year, favoring population increase and dispersion potential (DESNEUX et al., 2010; GUEDES & PICANÇO, 2012).

The damages caused by *T. absoluta* larvae can lead up to 100% losses in tomato yield without adoption of control measures (DESNEUX et al., 2010; TROPEA GARZIA et al., 2012; URBANEJA et al., 2012). Larvae can feed on almost all parts of tomato plants, mainly leaves,

stems and fruits at any development stage, thus causing direct and indirect damage (ARNO & GABARRA, 2011; BIONDI et al., 2018). Moreover, damage caused by larvae favors the entry of pathogens through open wounds in plant tissue and cause further losses (TROPEA GARZIA et al., 2012). Additionally, *T. absoluta* reproduces rapidly, damaging tomato plants during the whole crop cycle and can survive in alternative hosts (GUEDES & PICANÇO, 2012; BIONDI et al., 2018).

The use of synthetic insecticides remain the most employed tactic in *T. absoluta* control (GUEDES & PICANÇO, 2012; BIONDI et al., 2018). However, it is not always efficient, due to early establishment in field and resistance towards many chemical groups (GUEDES & SIQUEIRA, 2012; HADDI et al., 2012; RODITAKIS et al., 2015; BIONDI et al., 2018). Therefore, chemical groups must be rotated to reduce the resistance pressure (GUEDES & PICANÇO, 2012). Also, since larvae live inside the galleries, they are protected from the action of most contact insecticides (TERZIDIS et al., 2014). For all these reasons, it is evident that *T. absoluta* management cannot be exclusively dependent on insecticides, and strategies based on supplementary control tactics must be employed (TERZIDIS et al., 2014; BIONDI et al., 2018).

Besides chemical control, cultural tactics must also be adopted in order to reduce *T. absoluta* infestations, such as: elimination of crop remains and host plants, and also crop rotation. Cleaning boxes and other materials used in the harvest are also important measures (SILVA et al., 2013). Synthetic pheromones can also be used for both sampling and capture of adult males. Besides, researches with plant essential oils are ongoing, with good results achieved (MORENO et al., 2012; CAMPOLO et al., 2017; CAMPOLO et al., 2018). The use of resistant tomato varieties is also a very promising control method, however no resistant varieties are commercially available yet (BIONDI et al., 2018).

The biological control with generalist predators is a good management option when available, and is used in several countries (CAMPOS et al., 2017; ZAPPALÀ et al., 2017; BIONDI et al., 2018). In Brazil, the only mass-reared and commercialized natural enemy for *T. absoluta* control is the egg parasitoid *Trichogramma pretiosum* Riley, 1879 (Hymenoptera: Trichogrammatidae) (MAPA, 2019). Besides, the entomopathogenic bacteria *Bacillus thuringiensis* var. kurstaki is also used in *T. absoluta* management in several countries (URBANEJA et al., 2012; SILVA et al., 2013).

2.2 Whitefly

The whitefly *B. tabaci* is a small hemipteran (~ 1 mm length) that feeds on the phloem of several plant species, including vegetables, fruits, grains, and several spontaneous plants (BYRNE & BELLOWS, 1991; DE BARRO et al., 2011). Females lay eggs on the abaxial part of the leaves, and after egg hatching, the nymphs start to feed on sap. First instar nymphs can move across the plant, while the following three nymphal stages are motionless (BYRNE & BELLOWS, 1991). The immature stage lasts approximately 15 days. The female longevity is approximately 18 days, and each female lays an average of 110 eggs.

The damages caused by *B. tabaci* can be direct by feeding on plants, and indirect by the transmission of several phytopatogenic viruses, specially begomoviruses and criniviruses (JONES, 2003; MICHEREFF FILHO & INOUE-NAGATA, 2015). The viruses can be acquired by nymphs and adults by feeding on infected plants, and after transmitted to healthy plants by the injection of virus particles along with the saliva during feeding (SILVA et al., 2013).

The egg-adult period ranges from 20 to 25 days. Eggs and adults are more likely to be found on sprouts and new leaves, while nymphs are more common on more developed leaves. Direct damages can be seen on high infestations, resulting on weaknesses of the plant, deformation in fruits and uniformity of fruit maturation caused by the injection of toxins during whitefly feeding. Indirect damages are caused by virus transmissions and for the production of "honeyew" resulting of its feeding. This sugary liquid creates a substrate for fungal development, where a sooty mold grows and reduces the plant photosynthesis area (BYRNE & BELLOWS, 1991; SILVA et al., 2013).

Symptoms of a plant infected by begomoviruses include chlorosis between nerves (yellow mosaic pattern), leaf deformation and growth reduction. In Brazil, the most important viral agents are the tomato severe rugose virus (ToSRV) and tomato golden mosaic virus (TGMV), due their high distribution and predominance, but many other viruses can infect tomato plants causing similar symptoms. In the crinivirus group, only one species is related infesting tomato plants in Brazil, the tomato chlorosis virus (ToCV). The main symptoms are leaf roll and chlorosis on older leaves (similar to a nutritional deficiency). The damages caused by virus infections are higher in early infestations, reducing drastically the size and number of fruits per plant. Later infestation impact is less severe on the plant, and for this reason most farmers do not remove infected plants from the field, creating sources of inoculum to new plants. One singe adult whitefly infected with a virus is able to transmit it to several plants in

its life span, that can last up to 25 days (JONES, 2003; MICHEREFF FILHO & INOUE-NAGATA, 2015).

There are several biotypes in *B. tabaci* species, morphologically similar but with genetic variability between them, resulting in differences in host range, dispersion, capacity to transmit viruses and develop insecticide resistance (DE BARRO et al., 2011). In Brazil the most commonly find biotypes are A (NW-1 and NW-2) and B (MEAM-1), and in the past years the Q (MED) biotype, more commonly find in Europe, was reported in the south of the country (MICHEREFF FILHO & INOUE-NAGATA, 2015).

Many populations of whiteflies were reported to be resistant to different insecticide molecules. Therefore, the efficiency of chemical control applied isolated or incorrectly is very limited in whitefly control, and therefore in virus transmission. The integrated management is, therefore, the best approach to control this pest. The monitoring must be done during the whole crop cycle, to avoid both direct and indirect damage. Tomato varieties resistant to begomoviruses and crinivirus should be preferred (DE BARRO et al., 2011; MICHEREFF FILHO & INOUE-NAGATA, 2015).

Preventive tactics to control *B. tabaci* include: weed control around and inside the crop, elimination of remaining of old plants (with or without viruses), respect an interval (15 days) before plant the next tomato seedlings in the area, crop rotation with non-host plants, grow tomato in greenhouses covered by anti-aphid mesh, use yellow adhesive traps to mass collection and monitoring, prefer to use plants of varieties resistant to the viruses (SILVA et al., 2013). Biological control agents include the nymph parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) and the pathogenic fungi *Beauveria bassiana* (FLINT & DREISTADT, 1998; SILVA et al., 2013).

2.3 Thrips

Several thrips species are known to feed on tomato plants. The most common species in tomato crops are *Thrips tabaci* (Lindeman, 1888); *Thrips palmi* Karny, 1925; *Frankinella schultzei* (Trybom, 1920), and *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae). These are very small insects (approximately 1mm length and 2mm wingspan), which makes them difficult to be seen by naked eye. Adults' color varies between black, yellow and brown, with relatively long wings. Thrips prefer to oviposit in tender parts of the plant, such as flowers and sprouts, and each female can oviposit more than 100 eggs in its life span,

usually where the insects will stay and feed upon. The egg hatches after 4 days, generating nymphs that will pass through two larval, active feeding stages and two inactive stages (pre-pupae and pupae). Nymphal period last up to 12 days, depending on climatic conditions. Adults live approximately 30 days. Both nymphs and adults scratch and puncture plant tissues and feed on sap. They can directly damage plants by feeding, specially the fruit, compromising the sale. Leaves and flowers can also be attacked, with damages similar to silver stripes, causing the leaves to get dry and damaging sprouts, with consequent effect on fruit development (SILVA et al., 2013; LIMA, 2015; DE MOURA et al., 2014; MONTEIRO & LIMA, 2015).

Besides direct damage, several thrips species are known to transmit plant viruses, mainly from the Tospovirus genera. Infected plants exhibit bronzing of the upper sides of young leaves, which later develop to necrotic spots. The apical tip may dieback, and the fruits present chlorotic rings (ULLMAN et al., 1992). The insect can acquire the virus after feeding upon infected plants, and inoculate the virus on healthy plants in the feeding process. Once infected at the larval stage, adults continue to transmit tropoviruses during their whole life span, in the feeding act (JONES, 2005). The viruses can be transmitted during the whole crop cycle, but the first 60 days are the most critical because plant can die after being infected (JONES, 2005; SILVA et al., 2013).

Due to the capacity of thrips to transmit plant diseases, the economic threshold for this species is very low. For this reason, the prevention is probably the most important control method, thus seedlings must be produced in protected and thrips-free areas. Besides, removing alternative hosts (mainly Solanaceous) and use tomato varieties resistant to the viruses are also important control methods (SILVA et al., 2013). Chemical control is often not effective to control thrips, because the eggs stay inside the vegetal tissue, and pupae go to the soil, nevertheless neonicotinoids are the most recommended products due to their systemic activity. The insecticides can be sprayed in the plant base, by drench, or in grains during the planting (SILVA et al., 2013; MONTEIRO & LIMA, 2015).

2.4 Mites

Mites are very small arachnids (0.2-0.3 mm) that can be present during the whole tomato cycle. They damage plants directly by scratching leaves to get to the sap, and posteriorly the leaves fall and the plant can die. Mites are favored by dry and hot weather, and conditions such as water stress and dust excess can lead to population outbreaks (FLINT & DREISTADT, 1998).

In Brazil, two species are the most common in tomato: *Tetranychus urticae* (Koch, 1836) (Acari: Tetranychidae) and *Aculops lycopersici* (Massee, 1937) (Acari: Eriophyidae). The females of *T. urticae* lay spherical yellow eggs in the web, in the adaxial part of leaves. There is a clear dimorphism between males and females, being the females larger and with one dark spot in each side of its body. For this reason, its common name is two-spotted spider mite. Severe infestations can cause leaf dry, and this mite can survive and go from one plant to another if tomato crops are planted in sequence. The infestation development starts in the leaves' abaxial part, but can also goes to the adaxial part when the infestation level is high (SILVA et al., 2013).

The species *A. lycopersici* has a vermiform shape, white color and is almost invisible to naked eye. It develops on leaves and stems, and females lays eggs on leaf nerves and in the base of trichomes. The wind is its main dispersion agent. This species is also know as tomato russet mite, because the infestation results in a damage similar to rust in leaves and fruits. If the attack occurs prior to fructification leaves can dry and fall, thus plants cannot develop and die. Otherwise, attacks during fructification can compromise fruit development, turning its surfaces harsh, russet, and burned by the sun due to leaves' fall (SILVA et al., 2013).

Several acaricides are recommended to control phytophagous mites. Moreover, predatory mites are mass-produced and released to control the pests. Most of them are from the Phytoseiidae family, and probably the most important species are *Neoseiulus californicus* (McGregor, 1954) and *Phytoseiulus macropilis* (Banks, 1905). This species is able to prey several phytophagous mites, and also feed on polen, thus persisting in the crop even in low prey densities (FLINT & DREISTADT, 1998).

3. INSECTICIDE TOXICOLOGY

3.1 General concepts

According to the Enviromental Protection Agency (EPA), a pesticide is any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating pests, including insects, nematodes, weeds, fungus etc., and in this sense insecticides are a group of substances designed to act specifically upon pest insects (YU, 2008). With the increase in the global population, vegetable yields must increase, and for this purpose damage caused by pests must be mitigated. Approximately one third of food crops are destroyed by pests during growth, harvest and storage (WARE & WHITCARE, 2004). To reduce these damages, pesticides plays

a major role in pest control, increasing yields, reducing hand labor, preventing storage losses and providing a more stable product (YU, 2008).

However, the use of insecticides alone is not a feasible strategy. The abuse of such substances can affect the beneficial organisms present in the crop (i.e. pollinators, predators, parasitoids, decomposers, among others), leave residues in vegetables and select resistant pest populations (; BIONDI et al., 2012; BAJWA & SANDHU, 2014; CAMPOS et al., 2014; RODITAKIS et al., 2015; SOARES et al., 2019b), besides le. Pesticide resistance is one of the greatest challenges facing modern agricultural production (YU, 2008). The integrated pest management (IPM) concept was developed as a response to the incompatibility of pesticide and biological controls, with the emphasis on integrate the use of insecticides with the natural pest control provided by predators and parasitoids, by using products that affect pests and cause low or no effect on beneficial organisms (RIPPER, 1956; CROFT, 1990a; FOERSTER, 2002; BUENO et al., 2017; CARVALHO et al., 2019). Hence, impact of insecticides on beneficial organisms must also be taken into account, and for this purpose their toxicology must be evaluated.

Toxicology is defined as the study of harmful effects of chemical substances on living organisms (CHASIN & PEDROSO, 2003). The ecotoxicology is defined as the scientific study of adverse effects caused by chemical substances released in the environment on living organisms, mainly on populations and communities of an ecosystem (FERNICOLA et al., 2003). Ecotoxicology studies are often performed to assess the effect of insecticides in beneficial organisms present in the agroecossystem.

The most common way to express the toxicity of a compound is when the toxic particle reaches the target, generating perturbations on cellular structure or function. The intensity of the toxic effect depends on the concentration and permanence of the toxicant on the action site. Occasionally, the "final" toxicant is a metabolite from the initial compound, generated inside the organism by biotransformation (PAOLIELLO & SILVA, 2003).

In order to a chemical compound exert its toxic effect on an organism, two aspects must be considered: absorption by the organism, reaching a target site, and the cellular effects mediated by chemical agents on organs and cells (PAOLIELLO & SILVA, 2003). The first aspect is referred as toxicokinetics, which is the field of toxicology that aims to discover the behavior of the toxic compound after its contact with the organism, understanding its processes of absorption, distribution and accumulation on tissues, biotransformation and elimination (AZEVEDO & LIMA, 2003). The second aspect, referred as toxicodynamic, is the action of

chemical agents on target site (usually endogenous molecules such as receptors, enzymes, proteins and lipids) (RENWICK, 1999; PAOLIELLO & SILVA, 2003).

3.2 Factors affecting the action of insecticides

Effects of insecticides are nearly all systemic, which is, demands absorption and distribution of the toxic agent from its entrance point to the target site where deleterious effects will be exerted by biochemical reactions (CROFT, 1990b). Effects can be morphologic (changes in tissue and organ morphology, usually irreversible, such as lysis and necrosis) or functional (usually reversible and detected after exposition to low doses) (CHASIN & AZEVEDO, 2003). The toxicity expressed depends on the chemical substance (molecule proprieties, intrinsic structure, dose, behavior and destiny of the compost on the organism) and toxicokinetics properties (MCKINNEY, 1981; FERNICOLA et al., 2003).

According to Paoliello & Silva (2003), the factors that affect the success of the toxic compound to reach the target site are:

1)   Absorption – transference of a chemical agent from the exposition spot to the systemic circulation;

2)   Distribution – once inside the organism, compounds follow to the distribution phase, reaching extracellular spaces, and penetrating cells. Lipophilic compounds can enter easily in the cells, by diffusion. On the other hand, hydrophilic compounds are more restrict to the extracellular space, unless there is a specialized transport mechanism in the membrane;

3)   Excretion – physical process, aiming the removal of the toxicant agent from the organism;

4)   Toxification or metabolic activation – the biotransformation of chemical compounds to more toxic products. In this process, the toxicant has its reactivity enhanced after the formation of metabolites (electrophiles, free radicals and nucleophiles). In contrast, the biotransformation process that eliminates the final toxicant or avoids its action is called detoxification.

In order to be effective, insecticides must penetrate into insect organism. The main exposition route is through contact with body surface, although other routes are also used. Nevertheless, most insecticides are contact poisons, thus their efficiency depends on its ability to penetrate through the insect cuticle to reach its target site (YU, 2008).

To successfully penetrate insect tissues, molecules must overcome biological barriers, represented by cellular membranes (AZEVEDO & LIMA, 2003). The cuticle of insects is

considered a two-phase system. The first phase consists of a waxy, lipophilic layer (epicuticle), and beneath it there is the second layer (procuticle), a hydrophilic chitin-protein complex containing considerable quantities of water. Since most insecticides are nonpolar, the epicuticle represents an advantage for their contact action, but in some cases it can be a rather effective barrier (YU, 2008).

According to Azevedo & Lima (2003), Chasin & Azevedo (2003) and Yu (2008), the passage through membranes depends on several factors related to characteristics from both membrane and substance, such as:

1)   Polarity of insecticides – The tendency to and insecticide, lipid or water soluble, to move through the cuticle depends on whether it can pass through the two-phase system. This movement is dependent on the oil-water partition coefficient of insecticide, nature of the surfactant or solvent and the nature of the cuticle itself;

2)   Oil-water partition coefficient (Kow) – is a ratio of the partition of a substance in the lipid fraction and its partition on water. It refers to the tendency of a chemical to distribute itself between lipid and aqueous phases when both are present. Higher values indicate high affinity to the lipid part, and values close or below zero indicate affinity to water, thus grater solubility;

3)   Cuticular composition – epicuticles of some insects may be composed by softer waxes than others, thus it may dissolve insecticides more rapidly, increasing the uptake. This can be an advantage or disadvantage, depending on the lipid solubility of the insecticide. For example, high solubility would tend to trap such compounds at this point and hence tend to protect the insect from such insecticides. The same can be said of the procuticle, which may be rich in lipid. Soft epicuticle and lipid-rich procuticle could provide an easy route of diffusion for insecticides with more balanced oil-water partitioning characteristics;

4)   Ionization degree (pKa) - A higher proportion of ionized particles hardly penetrates into lipid membranes for having low lypophilicty, or for being unable to penetrate the membrane pores;

5)   Particle size and charge – bigger particles can have their passage compromised in the membrane pores, and do not penetrate insect body. The same can happen with some cations, due the positive charges that can be present in membranes, while anions can penetrate easier;

6) Metabolism of insecticides in the cuticle – there are detoxification enzymes present in the cuticle (mainly cytochrome P450 monooxygenases) that can help in the degradation of the insecticides.

Once inside insect organism, insecticide molecules must reach their target site. To protect themselves against toxins, insects have evolved detoxification mechanisms, that allow them to survive insecticides, and the level and type differ greatly, resulting in differing toxicity among different stages, populations and species (YU, 2008).

The detoxification process can be divided in two phases: phase I (primary) – oxidation, hydrolysis and reduction, and the metabolites are sometimes polar enough to be excreted, but are usually further converted by phase II reactions; phase II (secondary) – conjugation with sugars, amino acids, glutathione, phosphate etc., and subsequently excreted. These reactions are responsible to reduce biological activity of a toxicant and to decrease their lipophilicity, so that ultimately they can be excreted (YU, 2008). The first stage, the oxidative reactions, considered the most important, and are carried out mainly by a group of enzymes called cytochrome P450 monooxygenases that can perform several reactions in insecticide metabolism. Most of the detoxifying enzymes appear to be quite nonspecific in their substrate requirements, requiring only lipid solubility and susceptible functional groups in the substrate molecules (YU, 2008).

Regarding the response of organisms to toxic compounds, many factors take part. The harmful effects can change according to different species, and even in the same species differences can be observed in different sexes, ages and populations (FOERSTER, 2002; FERNICOLA et al., 2003; YU, 2008). Most differences are conferred by genetic characteristics, usually expressed by physiological and anatomic differences (affecting the absorption and target site) and the presence or absence of certain enzymes, changing the metabolism. For this reason, ecotoxicology studies must be carried with uniform populations, reducing genetic differences (CHASIN & AZEVEDO, 2003).

The kind of compost and the concentration available to get in contact with an organism depend on what happen to the compost before the contact. The main abiotic factors related to degradation of chemicals are: hydrolysis (alteration in the structure by reaction with water); oxidation (modification involving transference of electrons from the chemical to an oxidating receptor); reduction (transference of electrons from a reductor agent to the chemical); and photolysis (transformation caused by interaction of solar radiation) and degradation by temperature (WALKER, 2001; CHASIN & PEDROSO, 2003; FERNICOLA et al., 2003).

Biotic and abiotic reactions can change physical and chemical proprieties of insecticides, such as oxidation state, lipophylicity and volatility (KENDALL et al., 2001; LIAO et al., 2016). For instance, natural degradation of insecticides in field can reduce their efficiency (LIAO et al., 2016; MAHAPATRA et al., 2017). Therefore, in the evaluation of toxic effects of an insecticide on natural enemies, is important to start on laboratory conditions, where exposition conditions are more intense for the organism and the degradation of insecticides is lower (VAN DE VEIRE et al., 2002).

3.3 Selectivity of pesticides

Pest control is based on the probability that species differ greatly in susceptibility to toxicants and that it is possible to show selective toxicity among species, i.e. controlling one without harming others in the same environment (YU, 2008). The main purpose of chemical insecticides is to control pests, but many have broad-spectrum effects and can be harmful to nontarget species (CROFT, 1990a; FOERSTER, 2002). Even products considered biorational, based on natural products or more host (pest)-specific can cause side effects (BIONDI et al., 2013; PASSOS et al., 2017; SOARES et al., 2019a). Therefore, IPM is based on an understanding of the necessary interrelatedness of pesticides and natural enemies, exploiting the more subtle interaction between biological and chemical pest control agents in the development of selective insecticides (CROFT, 1990a; PARRA, 2014).

Predators and parasitoids are important naturally occurring biological control agents of insect and mite pests in crop ecosystems. Pesticides can be disruptive to trophic relations involving beneficial species, hence plant pest populations may increase to more damaging levels than occurred before treatment. Because of basic physiologic similarities between pest and natural enemies, pesticides often inflict severe mortality on both groups, while some differences regarding cuticle composition, presence/absence of detoxifiers enzymes or other metabolic processes can result in different degrees of toxicity (CROFT, 1990a; DEGRANDE et al., 2002). If we can use selective insecticides that are effective against pest species but relatively safe for beneficial insects, we would preserve these natural enemies and hence reduce insecticide usage (YU, 2008).

According to Ripper (1956), selectivity can be defined as the capacity of a product to control the target pest (plant-feeding arthropods), causing the least possible impact on beneficial organisms such as predators, parasitoids and pollinators, and can be divided into physiological and ecological.

The physiological selectivity refers to greater activity of a pesticide on a pest than on a natural enemy when direct contact on both species has occurred. It is related to the chemical nature of the insecticide and its relation to both pests and beneficial insects (RIPPER, 1956; BUENO et al., 2017; CARVALHO et al., 2019). The processes of absorption, penetration, transport, activation and degradation of insecticides in each insect species may cause a product to be more toxic to the pest than to its natural enemy in a situation in which both have contact with the compound or its residues (CROFT & MULLIN, 1990; DEGRANDE et al., 2002). Other processes can also take part, such as sequestration, excretion and selective metabolism, including the level of insecticide-detoxifying enzyme activity and target site insensitivity (CROFT, 1990c; CROFT & MULLIN, 1990; YU, 2008).

The ecological selectivity, on the other hand, is not related with the insecticide itself but the ways it is applied, aiming to minimize the exposure of natural enemies. It occurs due to behavioral differences between pests, natural enemies and pollinators, causing the chemical to have contact only with pests. The efforts to achieve the ecological selectivity must be concentrated to reduce as much as possible the exposure of natural enemies to insecticides in comparison to pests, hence is necessary to know the bioecology of pest and beneficial arthropods present in the agroecosystem (feeding behavior, life story, movement, spatial distribution, mating etc.). The operational measures include manipulation of pesticide formulation, timing of application, method of application and spatial distribution of treatment. (RIPPER, 1956, STERN et al., 1959; CROFT, 1990d; DEGRANDE et al., 2002; BUENO et al., 2017; CARVALHO et al., 2019).

The physiological selectivity tends to be preferred because special monitoring and application techniques must be implemented in ecological selectivity (CROFT, 1990d). Conversely, because they can be applied more liberally, physiologically selective insecticides may exert greater selection pressure on pests, increasing the development of pesticide resistance (RODITAKIS et al., 2015; BUENO et al., 2017). Hence, both selectivities must be emphasized to offer the most benefit and stability to IPM systems.

3.4 Evaluation of toxicity: mortality and sublethal effects

The purpose of toxicity evaluation is to provide evidences available regarding the potential of a toxicant agent to cause adverse effects to exposed individuals and provide (if possible) an estimation of relation between the extension of exposition and the increase or severity of adverse effect (CHASIN & AZEVEDO, 2003).

Toxic effect can be grouped in many forms. The effect can be immediate or retarded; reversible (knock-down) or irreversible; somatic (affects one or more functions of vegetative life) or germinal (perturbations in reproductive functions and integrity of descendants), and all these factors must be observed. The exposition route is also important in toxicity evaluation, because the effect of an insecticide can vary according to the contact route. For example, some products cause a higher detrimental effect if ingested, while others are more easily absorbed by contact (CHASIN & AZEVEDO, 2003). There are many methods of testing insecticides, i.e. topical, injection, dipping, contact (residual), fumigation and feeding (YU, 2008; MOSCARDINI et al., 2013; GONTIJO et al., 2018, PASSOS et al., 2018; SOARES et al., 2019b). In real crop conditions, all modes of uptake may act collectively to induce mortality or sublethal responses in predators and parasitoids (CROFT, 1990b). Hence, ideal ecotoxicology studies should prefer the most probable route (or routes) that the insect will be exposed to insecticides in field conditions.

In toxicology studies, many factors can become variability sources. First, conditions must be uniform and controlled (rearing temperature, humidity, illumination, and food supply) (YU, 2008). Tested insects must be originated from a uniform population, with reduced genetic differences (CHASIN & AZEVEDO, 2003). The development stages must be similar, since differences can be observed in the susceptibility of different life stages (VAN DE VEIRE et al., 2002; PASSOS et al., 2017). Even considering only adults their age must be the same, because their susceptibility may be affected by changing habits of feeding, sexual maturity and eventually aging. The sex must also be standardized. In general, females are more tolerant to insecticides than males. In most species females are larger, and their higher body weight can explain this tolerance (YU, 2008).

Toxic interactions of an insecticide with a biological system are dose dependent. The toxicity of an insecticide to an organism can be expressed in median lethal dose (LD_{50}), which is the dose lethal to 50% of the population of the organism (expressed in mg of the insecticide per weight unit). However, to a more practical approach the median lethal concentration (LC_{50}), which is the concentration of the insecticide in the external media that will kill half of the insect population, is used to express the toxicity. The LC_{50} is preferred than the LD_{50} because in most situations the exact dose initially given to the insect cannot be determined, specially considering field conditions (YU, 2008).

Dose-response indicates the relation between concentration of a substance and the magnitude of an adverse effect to an individual and the incidence rate of this effect on a

population. The index that expresses median acute toxicity (LC_{50}) is an estimated value that represents the best estimate of necessary dose to induce death in 50% of insects. By comparing the LC_{50} of two compounds, for the same species and conditions, it is possible to classify one substance more toxic than the other if the LC_{50} is lower. Therefore, the necessary dose to induce similar effect is one possible criteria to describe the relative toxicity of two chemical agents (CHASIN & AZEVEDO, 2003).

Besides the dose-response, another approach in ecotoxicology studies is to test the maximum recommended dose of insecticides on beneficial insects in laboratory (BIONDI et al., 2012; MARTINOU et al., 2014; PASSOS et al., 2017, 2018; SOARES et al., 2019b). If the insecticides are selective in this condition, they can be used in field conditions; otherwise they must be tested in greenhouse and field conditions to confirm its safety (VAN DE VEIRE et al., 2002).

The mortality caused by insecticides on natural enemies is an important parameter to be considered in ecotoxicology studies and exported to IPM programs. However, insecticides can also induce sublethal effects on surviving insects (CROFT, 1990a; DESNEUX et al., 2007). Initially, the lethal effect was the only parameter evaluated to arthropods, mainly aiming on local pest control. The evaluation of sublethal effects has also started on pests, and later on natural enemies and other nontarget species (CROFT & MESSING, 1990).

The severity and duration of sublethal effects in natural enemies caused by a compound is a result of many chemical, ecological and physiological processes. Biorational insecticides (growth regulators, chitin synthesis inhibitors, antifeedans and microbial pest control agents) usually cause lower mortality than conventional insecticides, and are more likely to have an effect over a longer time period, acting slowly and in a more subitle, sublethal ways (CROFT & MESSING, 1990). Some sublethal effects (specially behavioral) are reversible, so that unless observations are made during a specific time interval, the effects may go unnoticed (CROFT & MESSING, 1990; CHASIN & AZEVEDO, 2003).

The sublethal effects caused by insecticides can be observed on biological and behavioral traits of insects. The effects on the biology can be alterations on size, fecundity, egg hatch, oviposition, longevity, development rate, sex ratio etc. (BIONDI et al., 2012; MOSCARDINI et al., 2013; GONTIJO et al., 2017; PASSOS et al., 2018; SOARES et al., 2019a). Biological parameters tend to be the most sensitive characteristics to sublethal effects, and are the most important in terms of population dynamics (CROFT & MESSING, 1990). Behavioral effects are alterations in mobility, searching behavior, olfactory response, feeding,

oviposition (DESNEUX et al., 2007; CORDEIRO et al., 2010; PEREIRA et al., 2014; GONTIJO et al., 2017). Since the preying and parasitism rates can be affected, pesticides can severely reduce or eliminate the pest-regulating ability of natural enemies (CROFT & MESSING, 1990; DESNEUX et al., 2007). Therefore, the sublethal effects of pesticides on natural enemies must be assessed prior recommendation in IPM programs, otherwise their toxicity can be underestimated and compromise the pest management.

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PART III - ARTICLES

ARTICLE 1

Lethal, sublethal and transgenerational effects of insecticides on *Macrolophus basicornis*, predator of *Tuta absoluta*

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Lethal, sublethal and transgenerational effects of insecticides on *Macrolophus basicornis*, predator of *Tuta absoluta*

ABSTRACT

The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a key pest in tomato crops worldwide. In Europe, Integrated Pest Management (IPM) programs of this pest focus on releases of generalist mirid predators. In Brazil, *Macrolophus basicornis* (Stal) (Hemiptera: Miridae) is a potential biological control agent of *T. absoluta*. However, the compatibility of this predator with insecticides applied on tomato crops must be evaluated. Therefore, the non-target effects of four insecticides (abamectin, chlorantraniliprole, teflubenzuron and methoxyfenozide) were evaluated on the predator *M. basicornis*. Fourth instar nymphs were exposed to treated tomato leaflets, and their mortality was evaluated over 72h. In those treatments with low mortalities, sublethal effects on developed adults were evaluated (hind tibia length and adult survival). Moreover, the effects on progeny (nymphs/female, development time and hind tibia length) were evaluated in three oviposition dates. Abamectin was very toxic to nymphs, while methoxyfenozide, teflubenzuron and chlorantraniliprole caused low mortalities. Adult survival was not affected by the insecticides, however the growth regulators (teflubenzuron and methoxyfenozide) reduced the tibia length of females during the nymphal stage. The same reduction was observed for female progeny. The number of nymphs originated by exposed females was similar to the untreated control in all treatments. However, development time of *M. basicornis* progeny increased with the oviposition date, and treatments differed from control only in the last oviposition date. The results of this research indicate that, among the tested chemicals, chlorantraniliprole is the safest insecticide to *M. basicornis*.

Keywords: Ecotoxicology, Risk assessment, Integrated Pest Management, Predatory mirid, Pesticides.

INTRODUCTION

The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a major pest on tomato crops around the world (Campos et al. 2017, Biondi et al. 2018). It was first reported in Peru, on the early 20th century, and posteriorly was related causing damage on tomato plants in other South American countries, such as Argentina, Chile, Colombia and Brazil (Guedes & Picanço 2012). In 2006, *T. absoluta* was recorded for the first time outside South America, in Spain, and quickly spread to other European countries (Desneux et al. 2011). Nowadays, *T. absoluta* is present in many countries in Europe, Africa, Middle East and Asia, affecting nearly 60% of all cultivated tomato in the world (Haddi et al. 2012, Campos et al. 2017, Haddi et al. 2017, Sankarganesh et al. 2017, Sylla et al. 2017, Xian et al. 2017, Biondi et al. 2018). The caterpillar feeds on several parts of tomato plants, such as leaves, stems and fruits, causing direct and indirect damage that could result in 100% yield loss if no control measures are implemented (Desneux et al. 2010, Tropea Garzia et al. 2012, Urbaneja et al. 2012).

In many countries, chemical control using synthetic insecticides remain the most employed tactic in *T. absoluta* management (Guedes & Picanço 2012, Biondi et al. 2018). However, achieved results are not always satisfactory, mainly due to early establishment of *T. absoluta* populations in field (Biondi et al. 2018) and development of resistance towards conventional insecticides (Silva et al. 2011, Guedes & Siqueira 2012, Campos et al. 2014, Terzidis et al. 2014, Roditakis et al. 2015). Moreover, the excessive and indiscriminate use of insecticides can negatively affect natural enemies present in tomato crops (Biondi et al. 2012, Biondi et al. 2013, Pereira et al. 2014, Pérez-Aguilar et al. 2018). Therefore, more sustainable control measures must be employed in integrated pest management (IPM) programs to reduce *T. absoluta* populations at acceptable levels in tomato crops. In this context, the biological control with generalist predators seems to be a promising alternative management option (Zappalà et al. 2017, Campos et al. 2017).

Since the *T. absoluta* invasion in Europe, IPM programs based on conservation and augmentation of predatory generalist mirids are employed in tomato crops (Desneux et al. 2010, Biondi et al. 2018). The most used species have been *Macrolophus pygmaeus* Rambur and *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae), both native to Mediterranean basin (Mollá et al. 2011, Urbaneja et al. 2012, Zappalà et al. 2013, Biondi et al. 2016, Campos et al. 2017, Naselli et al. 2017). Besides feeding on *T. absoluta* eggs and larvae, these zoophytophagous natural enemies can also regulate populations of other tomato pests, such as aphids, whiteflies

and mites (Calvo et al. 2012, Urbaneja et al. 2012, Bompard et al. 2013, Jaworsky et al. 2013, Jaworsky et al. 2015).

Seen as a success in Europe, researches started in Brazil searching native mirids with potential to control *T. absoluta* (Bueno et al. 2013). Among the reported species, *Macrolophus basicornis* Stal (Hemiptera: Miridae) stands out as the most promising biological control agent of *T. absoluta* in Brazil. This predator has a high walking capacity on tomato plants, is capable to prey more than 90 *T. absoluta* eggs per day, and, similarly as the European species, has the zoophytophagous habit that keeps them in plants when prey populations are scarce (Bueno et al. 2012, Bueno et al. 2013). Despite all these beneficial traits, releases of *M. basicornis* in tomato crops may be compromised by the intense spraying of insecticides, that can reach up to 30 applications per cycle in Brazilian commercial fields (Guedes & Picanço 2012).

The effects of insecticides on *T. absoluta* natural enemies can be different according to the species evaluated and the active ingredient of each insecticide. Besides the lethal effects caused by insecticides, natural enemies can suffer sublethal effects after exposure to chemical compounds, affecting biological parameters such as fecundity, development time, olfactory response and prey capacity (Desneux et al. 2007, Moscardini et al. 2012, Biondi et al. 2013, Martinou et al. 2014, Pereira et al. 2014, Abbes et al. 2015, Michalko & Kosulic 2016, Bueno et al. 2017). Early studies evaluated the effect of insecticides on *M. basicornis* (Wanumen et al. 2016, Passos et al. 2017), focusing on the mortality caused by the compounds in different predator's life stages under laboratory and greenhouse conditions. However, studies regarding sublethal effects of chemicals on *M. basicornis* are still scarce. Thus, the objective of this study was to evaluate to evaluate lethal, sublethal and transgenerational effects of four insecticides, commonly used for the control of *T. absoluta*, on various biological traits of *M. basicornis*.

MATERIALS & METHODS
Insects

Insects used in bioassays were obtained from a rearing established in the Ecotoxicology Laboratory, in the Federal University of Lavras, Brazil. The rearing began in 2012, with approximately 200 *M. basicornis* adults collected from open field tobacco plants (*Nicotiana tabacum* L.) in Lavras (21° 8.5960' S, 045° 3.4660' W) in the state of Minas Gerais, Brazil. After their capture, insects were identified and transferred to acrylic cages (60 × 30 × 30 cm) containing *N. tabacum* (cv. TNN) plants, and were kept in laboratory conditions (25 ± 2 °C, 70

± 10% relative humidity, 12L:12D). Periodically, new adults (originated from subsequent capture campaigns) were added to the rearing. In order to obtain nymphs to start the experiments, ~200 *M. basicornis* adults were kept in acrylic cages ($60 \times 30 \times 30$ cm) with a N. tabacum (cv. TNN) plant for oviposition. *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs were offered ad libitum. After 48h, adults were removed from the cage, and the plant with *M. basicornis* eggs was kept in laboratory conditions (as described above), until nymphs reach the fourth instar.

Insecticides

Insecticides were evaluated at their highest recommended concentrations to control T. absoluta in tomato crops (MAPA 2017). The active ingredients (followed by brand name, manufacturer, mode of action according to IRAC (2018) and dosage employed (g a.i./L)) were: teflubenzuron (Nomolt 150®, BASF, inhibitor of chitin biosynthesis Type 0, 0.0375), chlorantraniliprole (Prêmio®, DuPont, ryanodine receptor modulator, 0.0276), methoxyfenozide (Intrepid 240®, Dow AgroSciences, ecdysone receptor agonist, 0.12), and abamectin (Abamectin Nortox®, Nortox, glutamate-gated chloride channel allosteric modulator, 0.0135). Abamectin was harmful to M. basicornis nymphs in a previous experiment (Passos et al. 2017), therefore it was considered a positive control. Distilled water was used as negative control.

Experiment I – Contact toxicity to M. basicornis nymphs and effect on adults' survival and growth

Macrolophus basicornis nymphs were exposed to tomato leaflets (cv. Santa Clara) treated with insecticides. For this, tomato plants were grown in greenhouse conditions (25 ± 4 °C, $60 \pm 10\%$ relative humidity, natural photoperiod) without any prior chemical applications. After reaching a stage with approximately four fully expanded leaves, tomato plants had their leaflets excised. The petioles of leaflets were inserted in Eppendorf® tubes (1.5 mL) containing an agar-H_2O solution (1%) to maintain their turgidity. The leaflets were submitted to insecticide spraying in a Potter precision tower (Burkard Scientific Co., Uxbridge, UK, pressure: 100 kPa, application volume: 1.5 ± 0.5 mL/cm^2). After drying under laboratory conditions (1h), one leaflet was placed in a Petri dish (10 cm diameter, 2 cm height) along with 10 fourth instar

nymphs per repetition. Each Petri dish was closed in its upper gap with voile fabric, to ensure ventilation and to prevent nymphs from escaping. *Ephestia kuehniella* eggs were offered ad libitum as a food source. Thereafter, mortality of nymphs was recorded daily during 72h. The bioassay was performed with seven repetitions per treatment, each one containing ten M. basicornis nymphs.

In treatments which mortality was less than 25%, surviving nymphs were individualized in glass test tubes (1.5 cm diameter, 9 cm height) with an untreated tomato leaflet in a Eppendorf® tube with *E. kuehniella* eggs (as described above). After reaching adulthood, insects were maintained in the tubes, and the survival of *M. basicornis* adults was recorded daily until the death of the last insect. During the whole bioassay, leaflets were replaced approximately each 5 days (before they started to dry) and *E. kuehniella* eggs (0.2 g) were added in this occasion. After the adults' death, 20 males and 20 females were randomly selected per treatment to assess potential effects on insect growth, based on the hind tibia length. The posterior leg on the right side of insects' body was removed, and the tibia was measured with a digital high precision caliper rule (MTX®) using a stereoscopic microscope (40×).

Experiment II – Effect of insecticides on *M. basicornis* progeny

This experiment was performed with treatments that allowed more than 75% survival in the experiment I. Ten couples of *M. basicornis* were formed with maximum of 24h of difference in adult emergence. Each couple was placed in a 400 mL plastic cup (5 cm height) containing an untreated tomato leaflet in an Eppendorf® tube and *E. kuehniella* eggs. Leaflets were replaced approximately every 5 days (before they started to dry) and *E. kuehniella* eggs (0.4 g) were added. In three dates after couple formation (seven, fourteen, and twenty-one days after couple formation), each couple was taken from the cups and transferred to 700 mL cups containing a tomato seedling (cv. Santa Clara), approximately 10 cm height, where they remained 24h for oviposition. The cups were sealed in their upper gaps with voile fabric, to ensure ventilation and to prevent adults from escaping. After 24h, the couples were taken back to 400 mL cups. Tomato seedlings were maintained in laboratory conditions until nymphs' eclosion. In each date, the number of nymphs per female and the development time were evaluated. As described in the experiment I, the hind tibia length of the progeny (20 females and 20 males per treatment) was also measured.

Data analyses

All data were submitted to homoscedasticity (Bartlett 1937) and normality (Shapiro & Wilk 1965) tests. Because these assumptions were not verified in any situation, generalized linear models (GLM's) were fitted. Nymph mortality data were analyzed in a GLM using the quasi-Binomial family. For tibia length data, a GLM analysis (quasi-Poisson family) with two factors (treatments and generation) was fitted, with potential interactions between them. The adult survival data was submitted to a survival analysis using the Kaplan-Meier estimator, and treatments were compared by Log-Rank test at $p = 0.05$. Regarding the effect of pesticides on the progeny in three different dates (nymphs per female and development time), a GLM analysis (quasi- Poisson family) was carried out using two factors (treatments and oviposition time) with potential interactions between them. In all GLM's analyses in this experiment, means were separated by pairwise comparison. Statistical analyses were performed using the statistical software R (R Core Team 2018). Graphs were assembled in the software SigmaPlot v. 11.0 (Systat 2008).

RESULTS

Contact toxicity to *M. basicornis* nymphs and effect on adults' survival and growth

Insecticides caused different mortality levels to *M. basicornis* nymphs (F = 29.74, df = 4, p < 0.05) (Fig. 1). Abamectin caused the highest lethal effect, with more than 70% mortality. Methoxyfenozide caused the second highest mortality, although it was under 25%. Teflubenzuron and chlorantraniliprole caused the lowest mortalities and were similar to control. Therefore, sublethal effects of methoxyfenozide, teflubenzuron and chlorantraniliprole were evaluated on surviving predators.

The contact with insecticides in nymphal stage did not affect the survival of *M. basicornis* adults (Fig. 2). Survival curves of females and males were similar to control treatment. However, insecticides affected insects' hind tibia length (statistical values are summarized in Table 1). A reduction in tibia length was observed in *M. basicornis* females exposed to growth regulators (teflubenzuron and methoxyfenozide) in the nymphal stage, while no effect was observed to males (Table 2).

Effect of insecticides on *M. basicornis* progeny

Statistical values of nymphs per female in three different oviposition dates are summarized in Table 3A. There was no effect of insecticides on this trait (Fig. 3). Regarding the development time (Table 3B), there was a significant effect among insecticides and the oviposition dates. Moreover, an interaction occurred between these two factors, indicating that insecticides effect changed according to the oviposition date (Fig. 4). In all treatments (insecticides and control), development time of predators increased along with oviposition date. In the first oviposition date (7 days after mating), methoxyfenozide prolonged the development time compared with chlorantraniliprole. In the second oviposition date (14 days after mating), there was no difference between treatments. In the last date (21 days after mating), chlorantraniliprole and teflubenzuron increased development time in comparison to control treatment. Methoxyfenozide was not included in this analysis due to small number of remaining insects originated from this oviposition date. In all treatments, development time of *M. basicornis* progeny increased with the oviposition date.

Regarding the hind tibia length of the progeny, the IGRs affected the growth of females of the subsequent generation (Table 2). No effect in tibia length was observed for M. basicornis males of the subsequent generation.

DISCUSSION

The tested insecticides caused lethal and sublethal effects on *M. basicornis*. Although abamectin was the only product that caused high mortality to nymphs after 72h, the other products caused sublethal effects on exposed insects or in the offspring. The high mortality caused by abamectin to *M. basicornis* confirms the deleterious effects of this pesticide to predatory hemipterans (Biondi et al. 2012, Morcardini et al. 2013, Passsos et al. 2017). Therefore, the use of abamectin in areas where *M. bascornis* is naturally or artificially present does not represent a suitable strategy.

Although no detrimental effects caused by the insect growth regulators (IGR's) teflubenzuron and methoxyfenozide were observed on either *M. basicornis* adult survival or number of nymphs per female, adult females size was affected. Hind tibia length is considered an indicative of insect size and is often used to measure growth alterations in natural enemies such as parasitoids (Charleston et al. 2005, Soller et al. 2007) and predatory mirids (Castané et al. 2006). Sublethal effects of insecticides in tibia length, and consequently on predator's size, can affect fitness and mating, reducing its efficiency as a biological control agents (Desneux et

al. 2007). Furthermore, reduction in the leg size might also affect to *M. basicornis* walking capacity in tomato plants. It is an important factor because these predators must walk on tomato plants to find its prey (Wheeler & Krimmel 2015), and this plant species has trichomes that impair the mobility of other predators, such as the anthocorid *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) (Bueno et al. 2012). Since *M. basicornis* nymphs were exposed to these active substances, their growth might have been affected, causing adult females to be smaller than untreated insects. Moreover, the two IGRs reduced the size of females of the following generation. This could be attributed to possible malformations in the reproductive system or transovarial effects caused by IGRs (Rugno et al. 2016). Once IGRs act mainly on immature stages (Carlson et al. 2001, Sun et al. 2015), the effect would be less significant on adults than nymphs, although this hypothesis should be evaluated specifically.

Methoxyfenozide has high affinity with Lepidoptera ecdysone receptor complex and could be considered safe for non-target organisms (Carlson et al. 2001, Giolo et al. 2009, Colomer et al. 2011). For example, this product caused low mortality to *O. insidiosus* adults (Rodriguez-Saona et al. 2016). However, several sublethal effects of methoxyfenozide were reported on natural enemies, affecting mainly reproductive processes, and the same situation was observed in *M. basicornis* in the present research, generating smaller descendants. When applied on larval stage of the predator *Ceraeochrysa cincta* (Schneider) (Neuroptera: Chrysopidae), methoxyfenozide significantly reduced the fecundity (eggs per female) and longevity of adults, besides increasing the number of deformed eggs (Rugno et al. 2016). Moreover, methoxyfenozide considerably reduced the net reproductive rate (Ro), intrinsic rate of increase (r) and finite rate of increase (k) of C. cincta, affecting its population growth and the efficiency as a biological control agent. The fecundity of the predator *Deraeocoris brevis* (Uhler) (Hemiptera: Miridae), whose parental generation was exposed to methoxifenozide, was also affected (Kim et al. 2006). Although methoxifenozide caused no lethal effect on adult lepidopterans, effects on fecundity and egg hatching were reported (Trisyono & Chippendale 1997, Sun & Barret 1999, Sun et al. 2000).

Teflubenzuron showed similar results as methoxifenozide toward *M. basicornis*. Despite the safety of chitin synthesis inhibitors (such as teflubenzuron) to non-target organisms (Sun et al. 2015), studies showed sublethal effects of these insecticides on natural enemies. Teflubenzuron reduced the feeding activity of the predator *O. insidiosus* (Gorri et al. 2015) and its residues negatively affected the predation rate of *Orius laevigatus* (Fieber) (Hemiptera:

Anthocoridae) (Scott Brown et al. 2003). Regarding phytophagous insects, teflubenzuron caused several sublethal effects to *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) larvae, such as reduction in size and weight, inhibition of foliar consumption and compromised the ecdysis process (Meng et al. 2018). The size of *M. basicornis* adult females from the F0 generation in the present research might have been affected in a similar way. Reduction on females' hind tibia size in F1 generation might be resultant from transovarian effects caused by teflubenzuron. Transovarian effects of lufenuron, another chitin synthesis inhibitor, to the parasitoid *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) were reported in previous studies (Pratissoli et al. 2004, Bueno et al. 2008).

Among the evaluated insecticides, chlorantraniliprole proved to be the less toxic compound for *M. basicornis*. Despite the development time increase of the progeny in third oviposition date, chlorantraniliprole did not show any other side effect on this predator. Results of this research corroborate with literature, which revealed low effect of chlorantraniliprole to other mirid predators. According to Castro et al. (2013), this insecticide was selective for *Podisus nigrispinus* (Dallas) and *Supputius cincticeps* (Stal) (Hemiptera: Pentatomidae). Chlorantraniliprole caused low effect on the predator *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae), reducing time spent on plant feeding, but no other lethal or sublethal effect was observed (Martinou et al. 2014).

Insecticides could cause lethal and sublethal effects on insects that are directly exposed (short-term) or impacting subsequent generations (long-term), with potential effects on their populations (Guo et al. 2013, Costa et al. 2014). Although teflubenzuron, methoxyfenozide and chlorantraniliprole caused low effects on mortality and survival of predators, sublethal effects were observed on offspring development time in F1 generation. According to Guo et al. (2013), chlorantraniliprole is able to reduce the fecundity of *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) females by up to 42%. Reports discussing also the reduced fecundity caused by insect growth regulators in *E. kuehniella*, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and *Lobesia botrana* Denis & Schiffermüller (Lepidoptera: Tortricidae) was correlated with physiological and morphological disturbances in males and females (Marco & Vinuela 1994, Saenz-De-Cabezon et al. 2006).

Although effects on the subsequent generations of predators are still scarce, transgenerational effects induced by pesticides in the present study might have delayed the

development time and caused reduction in tibia length. Similar alterations were related in other researches (Wang et al. 2017). According to Xiao et al. (2015), the insecticide pirimicarb increased the development time of the aphid *Rhopalosiphum padi* (Linnaeus) (Hemiptera: Aphididae), besides affecting the fecundity and demographic parameters. Müller et al. (2017) related similar transgenerational effects of the insecticide lambda-cyhalothrin on the progeny of the leaf beetle *Phaedon cochleariae* Fabricius (Coleoptera: Chrysomelidae). This compound increased the progeny development time and affected females' growth, expressed in antennae asymmetry. According to authors, enzymes involved into detoxification processes, such as cytochrome P450 monooxygenases, are also related to development and reproduction, and a higher expense in insecticide catabolism may impair insect growth and investment on offspring (Feyereisen 1999, Müller et al. 2017). Maternal insecticide transfer and epigenetic mechanisms based on heritable changes in the expression of these enzymes are also considered possible causes of these sublethal effects on the subsequent generation (Collotta et al. 2013, Müller et al. 2017).

There was also a delay in development time according to oviposition date in all treatments, probably due to a reduction in the maternal investment per egg in later ages, as reported by Giron & Casas (2002). In their experiment with the parasitoid *Eupelmus vuilletti* (Crawford) (Hymenoptera: Eupelmidae) there was a reduction in egg's nutritional content over the course of mother's life, with possible effects on the progeny. The same situation might have occurred to *M. basicornis*. Therefore, individuals from eggs with lower nutrient content took longer time to reach adulthood. The reduction of reproduction investment according to mother's age could also explain the higher number of *M. basicornis* descendants originated from the first oviposition date compared with the other two dates.

In conclusion, chlorantraniliprole was shown to be, among the tested chemicals, the safest insecticide to *M. basicornis*. Despite the low mortality caused by the IGRs teflubenzuron and methoxyfenozide to *M. basicornis* nymphs and the lack of side effects on adult survival, the active substances affected the growth of exposed individuals and the size of insects of the subsequent generation. These effects might affect their efficiency as biological control agents. Greenhouse and field trials should be performed to confirm pesticides' effects on *M. basicornis* in realistic cropping conditions. Furthermore, potential changes caused by these active substances on beneficial traits of *M. basicornis* (i.e. predatory capacity, flight activity, response to plant volatiles) should also be assessed in order to obtain more information about how these

insecticides affect predatory mirids, and how natural enemies and pesticides can be combined together efficiently in IPM (e.g. Desneux et al. 2005).

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Table 1. Statistical results of two-way GLM (quasi-Poisson distribution) with two factors (insecticides and generation), plus interaction between them, for tibia length of *M. basicornis* males and females.

|	*A*: Females			*B*: Males		
	F	*df*	*p*	*F*	*df*	*p*
Treatments (*T*)	20.09	3	< 0.05	1.71	3	0.17
Generation (*G*)	2.08	1	0.15	0.26	1	0.61
T x *G*	2.47	3	0.07	0.32	3	0.81

Table 2. Tibia length (mean ± SE) of *M. basicornis* adults exposed to insecticides in nymphal stage (F_0 generation) and adults of the subsequent generation (F_1).

|	Tibia length (mm)			
Treatment	Males		Females	
	F_0	F_1	F_0	F_1
Control	1.98 ± 0.04	1.97 ± 0.02	1.99 ± 0.02 a	1.95 ± 0.03 a
Chlorantraniliprole	1.95 ± 0.04	1.92 ± 0.02	1.94 ± 0.03 ab	1.87 ± 0.03 ab
Teflubenzuron	1.89 ± 0.05	1.92 ± 0.02	1.88 ± 0.02 b	1.84 ± 0.02 b
Methoxyfenozide	1.93 ± 0.02	1.90 ± 0.03	1.75 ± 0.03 c	1.81 ± 0.02 b

Treatments followed by different letters in the same column are significantly different (quasi-Poisson GLM, pairwise comparison, $p < 0.05$).

Table 3. Statistical results of two-way GLM (quasi-Poisson distribution) with two factors (insecticides and oviposition dates) plus interaction between them for number of nymphs per female and development time.

|	*A*: Nymphs per female			*B*: Development time		
	F	*df*	*p*	*F*	*df*	*p*
Treatments (*T*)	0.77	3	0.52	4.64	3	< 0.01
Oviposition dates (*O*)	39.99	2	< 0.01	38.77	2	< 0.01
T x *O*	0.13	6	0.93	3.03	6	< 0.01
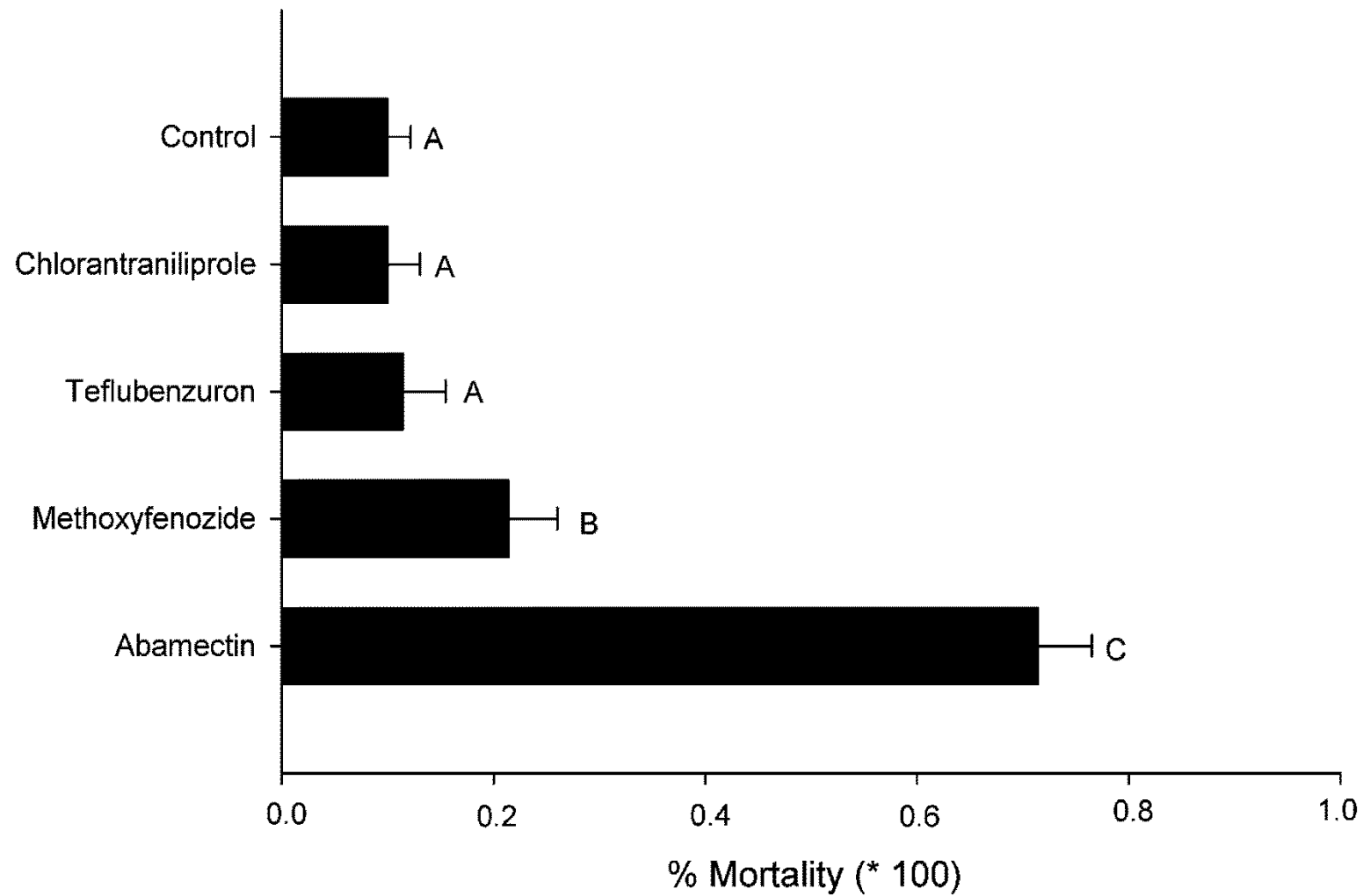
Fig. 1. Mortality (mean ± SE) caused by insecticides to *M. basicornis* fourth instar nymphs after 72h in treated tomato leaflets in laboratory. Treatments followed by different letters are significantly different (quasi-Binomial GLM, pairwise comparison, $p < 0.05$).
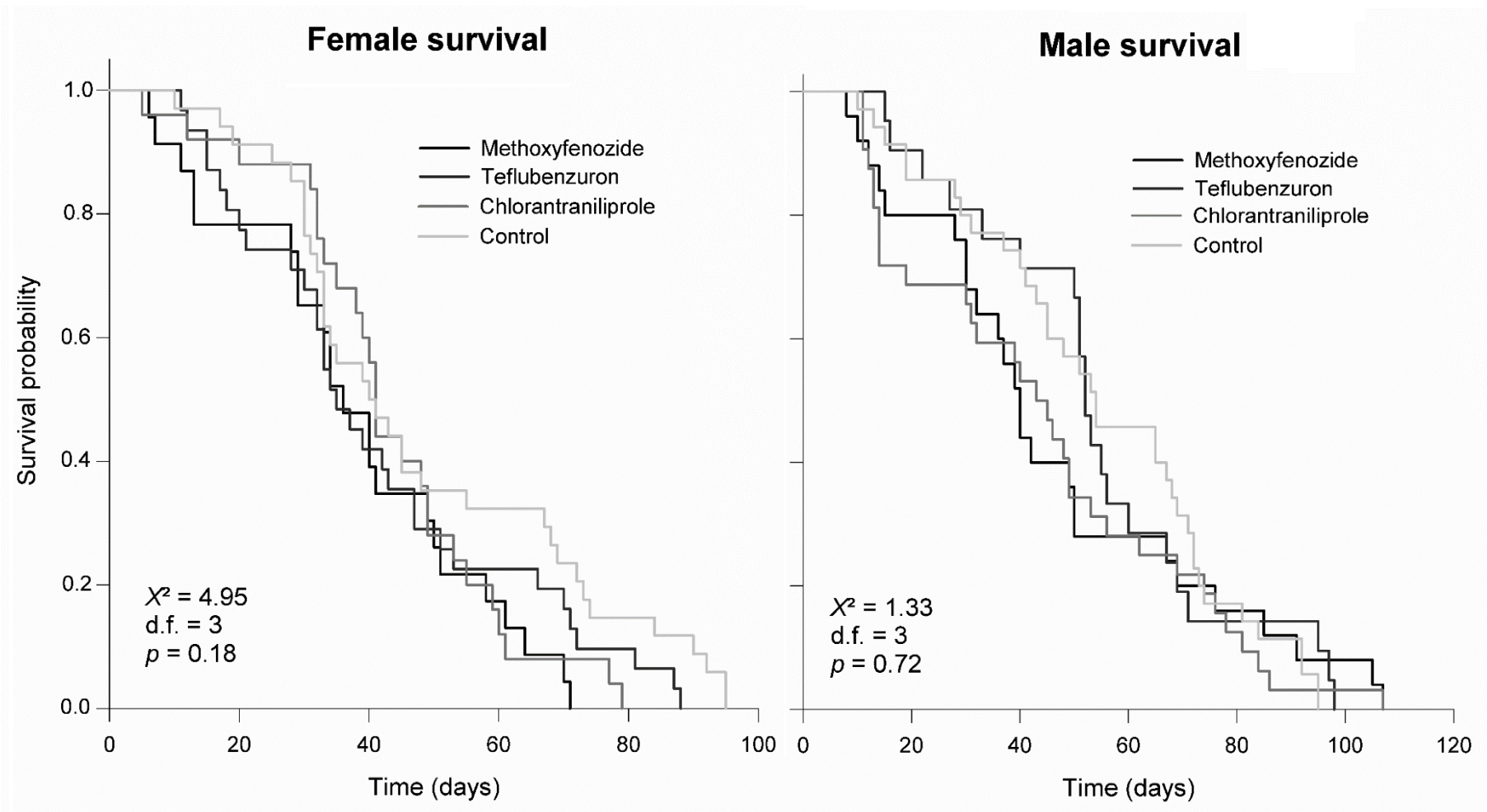
Fig. 2. Survival curves of *M. basicornis* females and males exposed to treated tomato leaflets in fourth nymphal stage (Log-Rank test, *p* < 0.05).
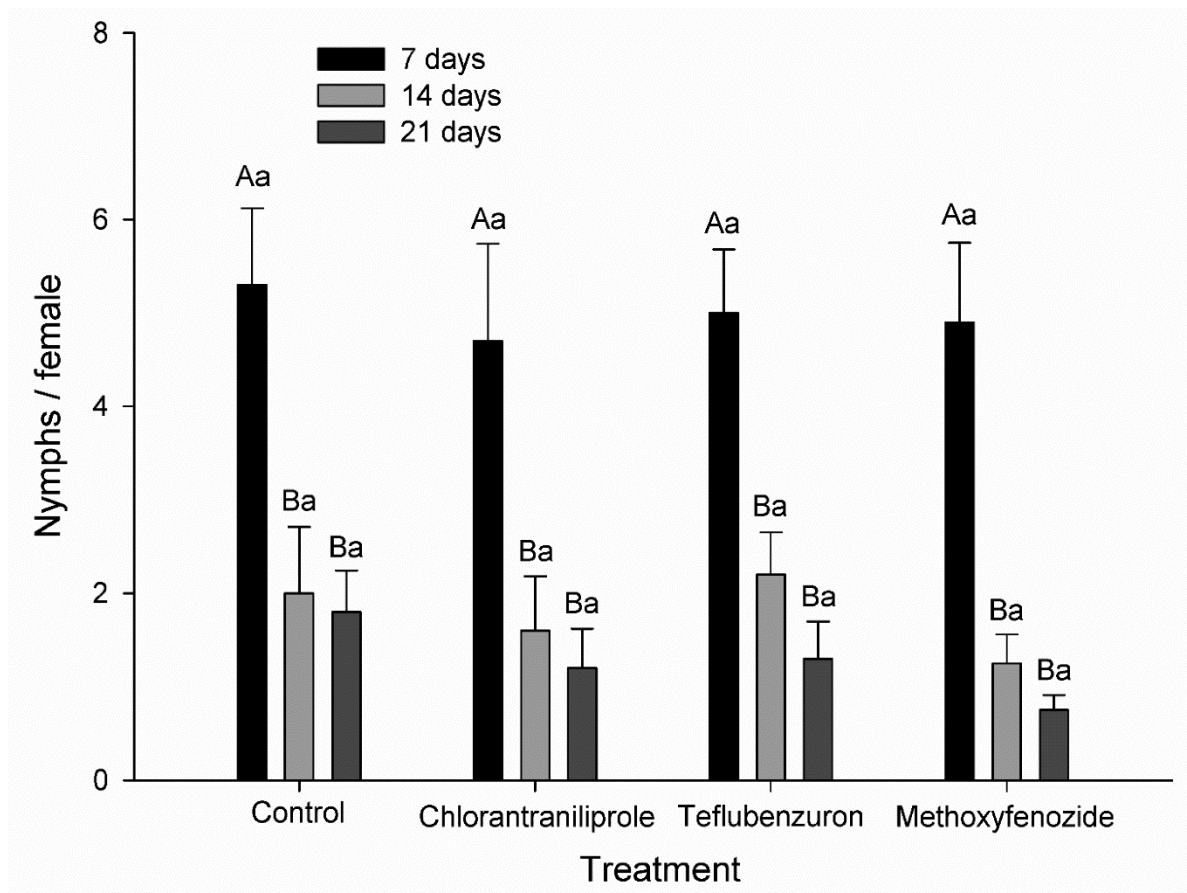
Fig. 3. Number of *M. basicornis* nymphs (mean ± SE) originating from mated females exposed to insecticides in nymphal stage, in three oviposition times (7, 14 and 21 days after mating). Different capital letters indicate difference in time for a treatment, and different lower case letters indicate difference among treatments in a same oviposition time (quasi-Poisson GLM, pairwise comparison, $p < 0.05$).
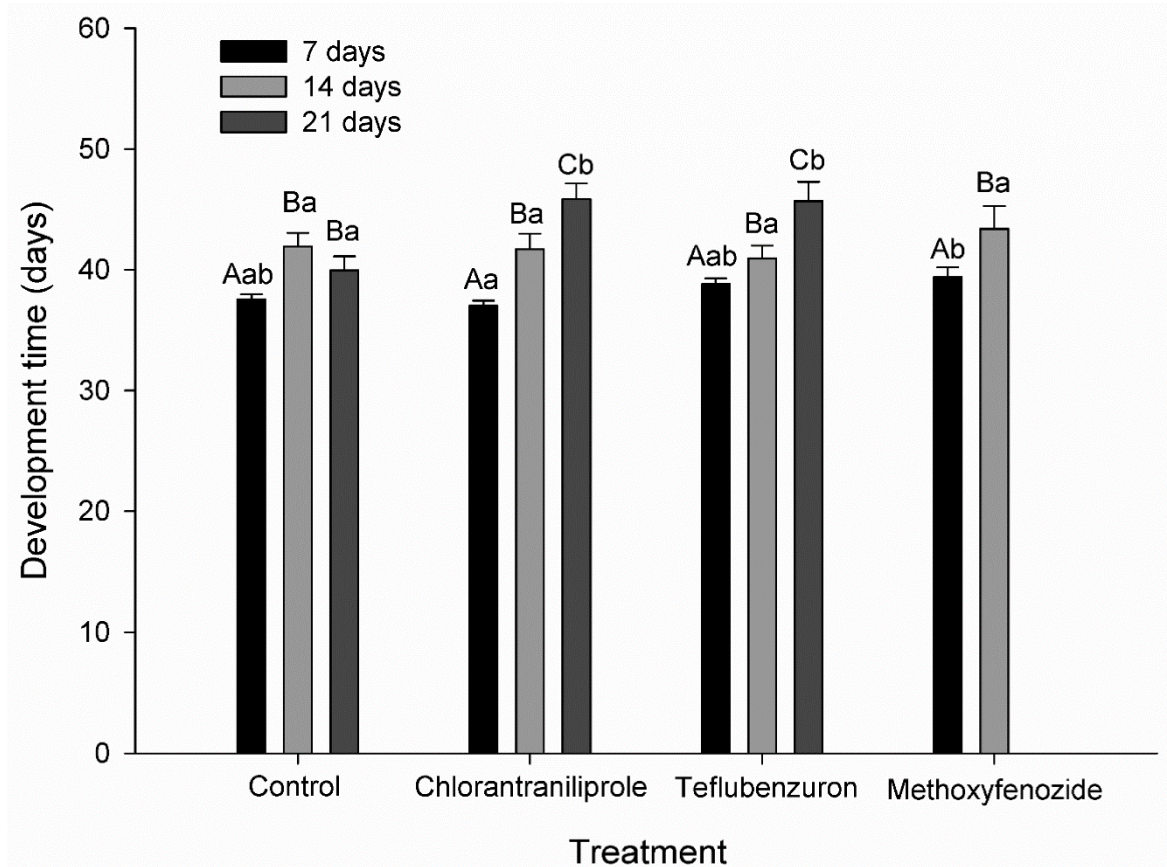
Fig. 4. Development time (days, mean ± SE) of *M. basicornis* originating from insects exposed to insecticides in nymphal stage, in three oviposition times (7, 14 and 21 days after mating). Different capital letters indicate difference in time for a treatment, and different lower case letters indicate difference among treatments in a same oviposition time (quasi-Poisson GLM, pairwise comparison, $p < 0.05$).

ARTICLE 2

Behavioral and biological effects of sublethal concentrations of neurotoxic insecticides on the omnivorous predator *Nesidiocoris tenuis* (Hemiptera: Miridae)

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Behavioral and biological effects of sublethal concentrations of neurotoxic insecticides on the omnivorous predator *Nesidiocoris tenuis* (Hemiptera: Miridae)

Highlights

Concentration-mortality analyses were performed to *N. tenuis* with three insecticides

All insecticides and sublethal concentrations reduced the females progeny

Every sublethal concentrations of chlorpyrifos affected *N. tenuis* behavior

Spinosad and lambda-cyhalothrin affected *N. tenuis* behavior at LC_{30}

Chlorpyrifos and lambda-cyhalothrin increased *N. tenuis* response time

Abstract

Tuta absoluta (Lepidoptera: Gelechiidae) is a very harmful pest in tomato crops in many countries. Chemical control is the main method employed to reduce *T. absoluta* populations in tomato crops, however it is not always efficient, therefore other control strategies can be incorporated in its management, such as releases of the predator *Nesidiocoris tenuis* (Hemiptera: Miridae). Aiming to understand the effects of sublethal concentrations of neurotoxic insecticides on that generalist predator, and the integration between chemical and biological control tactics in the management of *T. absoluta*, the objective of this study was to evaluate the toxicity of two insecticides used to control this pest (lambda-cyalothrin and spinosad), plus a positive control (chlorpyrifos), on the predator *N. tenuis*. Concentration-mortality trials were performed for each one of these compounds to *N. tenuis* females, and sublethal concentrations were estimated. Three sublethal concentrations (LC_1, LC_{10} and LC_{30}) were selected, and their effects were evaluated on *N. tenuis* orientation and reproduction. The fecundity of *N. tenuis* females was reduced by all insecticides in all concentrations. The orientation of *N. tenuis* females was compromised by chlorpyrifos at all concentrations, and by lambda-cyhalothrin and spinosad at LC_{30}. The response time of *N. tenuis* females was also affected by chlorpyrifos (LC_{10} and LC_{30}) and lambda-cyhalothrin (all concentrations). The results indicate that these insecticides should be avoided when *N. tenuis* is present in the crop, or could be considered to reduce their populations to limit plant damage.

Keywords: ecotoxicology; non-target effects; predatory mirid; pesticides; integrated pest management

1. Introduction

The use of predatory mirids is widely diffused in European countries for the biological control in many greenhouse crops. These insects are zoophytophagous, generalist predators, with the capacity to control populations of several arthropod pests (Calvo et al., 2012; Van Lenteren, 2012; Jaworsky et al., 2013; Biondi et al., 2018). The most used species is *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae). This mirid can occur naturally or be augmentative released in greenhouses in the Mediterranean Basin (Campos et al., 2017; Ferracini et al., 2019). Predatory mirids exhibits a highly predaceous capacity, and the ability to survive in plants when prey is scarce, although it can cause plant damage at high populations (Castañé et al., 2011; Mollá et al., 2014; Siscaro et al., 2019). This mirid is used in tomato crops as a biocontrol agent of many pests (e.g. aphids, whiteflies and leafminers) (Calvo et al., 2012; Urbaneja et al., 2012; Zappalà et al., 2013; Jaworski et al., 2015; Biondi et al., 2018).

One of the main purposes of *N. tenuis* use in biological control programs is to reduce populations of the South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). This pest represents a threat to tomato production in many countries (Campos et al., 2017; Biondi et al., 2018). Until 2006 this species was restricted to South America region, however after its first report in Spain *T. absoluta* rapidly spread to other European countries, and nowadays is also related damaging tomato crops in Asia and Africa (Desneux et al., 2010; Guedes and Picanço, 2012; Campos et al., 2017; Sylla et al., 2017; Biondi et al., 2018; Mansour et al., 2018). *Tuta absoluta* larvae feed on tomato leaves, sprouts and fruits, resulting in direct and indirect damage that can reach up to 100% yield losses if not controlled (Desneux et al., 2010; Urbaneja et al., 2012).

The management of this invasive pest usually is a difficult task. It has a wide host range, capacity of adapting to different environmental conditions, fast development and high capacity to develop resistance toward conventional insecticides (Guedes and Picanço, 2012; Guedes and Siqueira, 2012; Tropea Garzia et al., 2012; Campos et al., 2014, 2015; Terzidis et al., 2014; Roditakis et al., 2015). Nevertheless, chemical control relied on synthetic insecticides remains one of the most employed tactics to control *T. absoluta* in the world (Campos et al., 2017; Biondi et al., 2018), which can result in adverse effects on beneficial arthropods present in tomato crops (e.g. pollinators and natural enemies) (Desneux et al., 2007, Biondi et al., 2012a; Passos et al., 2018; Soares et al., 2019).

For these reasons, the biological control provided by *N. tenuis* is highly desirable. Unfortunately, most insecticides available for *T. absoluta* control are neurotoxic, i.e. act upon insects' nervous system, and are usually harmful to non-target species (Desneux et al., 2007; Guedes and Picanço, 2012; Casida and Durkin, 2013; Fernandes et al., 2016). In order to achieve a more efficient *T. absoluta* control, the other control measures employed (i.e. insecticide applications) should be compatible with *N. tenuis* in integrated pest management (IPM) programs, optimizing its biocontrol services. For this, the evaluation of insecticides on natural enemies is an important approach to enhance pest control, by integrating chemical and biological tactics in the management programs (Bueno et al., 2017; Carvalho et al., 2019). Therefore, the aim of this study was to assess the lethal effect caused by three insecticides on the predator *N. tenuis*, and evaluate potential behavioral and physiological effects caused by sublethal concentrations of these compounds on *N. tenuis* olfactory response and fecundity.

2. Materials and Methods

2.1 Insects

Nesidiocoris tenuis used in these bioassays were obtained from a rearing in the Department of Agri-food and Environmental Systems Management (DiGeSA), in the University of Catania (Catania - Italy), where also all following experiments were developed. Periodic collections were also performed in untreated tomato greenhouses in Fiumifreddo - Italy, and after identification insects were added to the rearing. Adults of *N. tenuis* (~150 individuals) were kept in entomological cages (32 x 40 x 70 cm) covered by tulle mesh and containing sesame (*Sesamum indicum* L.) plants (~30 cm ht) as water and oviposition sources. The mixture of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs and *Artemia* spp. cysts (Entofood® Koppert, the Netherlands) were offered to predators as food source. Adults were kept in the plants for three days, and subsequently they were collected and transferred to new entomologic cages as a described above. The sesamum plants containing *N. tenuis* eggs were kept in the cages, and originated insects were maintained until they reach adulthood, when were used in the bioassays. New plants and Entofood® were added in each cage when necessary. The rearing was maintained in laboratory conditions (25 ± 1 ºC, 55 ± 5 % RH, and 14L:10D h photoperiod).

2.2 Insecticides

In order to observe the physiological and behavioral effects on *N.* tenuis, three neurotoxic insecticides were evaluated in this study. Two are recommended to *T. absuluta* control, which are (followed by their brand name and chemical group): lambda-cyalothrin, Karate Zeon®, Na^+ channel modulator; and spinosad, Laser®, nicotinic

acetylcholine receptor allosteric modulator. Lambda-cyhalothrin is a synthetic insecticide often used to control *T. absoluta* in conventional crops, while spinosad is a natural insecticide, therefore is able to be used in both conventional and organic crops. These insecticides were selected due to their frequent use in tomato crops to control *T. absoluta* and other pests. Besides, the insecticide chlorpyrifos (Dursban®, acetylcholinesterase inhibitor) was used as a positive control. Stock solutions were prepared with the dilution of insecticides in tap water, according the manufacturers recommendation.

2.3 Insecticides' toxicity to N. tenuis

In this bioassay we evaluated the lethal effect of insecticides upon *N. tenuis*. Newly emerged females (~2 days old) were exposed to different concentrations of the insecticides. The maximum recommended dosage for tomato crop was the highest concentration used, and subsequently 5 to 6 serial dilutions were made for each insecticide. The dilutions were based on preliminary observations to comprehend the interval of significant lethal effect upon *N. tenuis* females. In addition, a negative control treatment (tap water) was used for all treatments.

In this experiment was developed a methodology that allowed topical application of the treatments on adult mirids. Thus, *N. tenuis* females were separated in groups of 5 individuals and kept inside conical plastic tubes (Falcon® - 50 mL). The tubes were maintained inside a thermic box containing ice during 3h, in order to reduce the insects' activity. Thereafter, the insects were placed in a plastic cup (100 mL) covered with an absorbent paper on the inside, where *N. tenuis* females were topically treated with compounds by a hand-sprayer (50 mL). The paper was added to prevent the formation of drops after spraying and consequent attachment of insects to the plastic, and it was

changed after every repetition. After spraying, each group of *N. tenuis* females were transferred to an acrylic pot (5.5 cm diameter $\times$ 3 cm height), along with a zucchini (*Cucurbita pepo* L.) leaf disc and Entofood®. Each pot containing 5 females was considered a repetition. Mortality caused by the insecticides on *N. tenuis* females was evaluated after 48h. It was performed 8 repetitions for each concentration and insecticide (3 treatments x 5 concentrations).

2.4 Effects of sublethal concentrations of insecticides on N. tenuis *fecundity*

Based on the results of the previous bioassay, *N. tenuis* males and females (2-3 d old) were exposed to sublethal concentrations of insecticides (LC_1, LC_{10} and LC_{30}). The given sublethal concentrations were chosen in order to expose the predators to very low concentrations that can occur in field conditions after natural degradation (LC_1 and LC_{10}) and to a concentration in the superior threshold to an insecticide to be considered safe according to the International Organization of Biological Control (IOBC) parameters (LC_{30}) (Van de Veire et al., 2002).

Adults were treated with insecticides sublethal concentrations using a hand sprayer, as described in the "Insecticides' toxicity to *N. tenuis*" section. Treated couples were kept in a plastic cup (400 mL) containing a green bean pod as water source and oviposition substrate, and *E. kuehniella* eggs (1g) as food supply. Each *N. tenuis* couple was kept in a cup during three days. Cups containing green bean pods with *N. tenuis* eggs were maintained in laboratory conditions, and the number of emerged nymphs was recorded until 20 days after adults' removal. For each treatment and concentration 25 couples were evaluated.

2.5 Effects of sublethal concentrations of insecticides on N. tenuis olfactory response

The effects of sublethal concentrations (LC_1, LC_{10} and LC_{30}) were evaluated on *N. tenuis* olfactory response. Adult females (2-3 days old) were treated with the sublethal concentrations, as a described in the "Insecticides' toxicity to *N. tenuis*" section. After 24h, the females were individually transferred to a Y-shape olfactometer (main arm and lateral arms 15 cm long and 4 cm internal diameter). The odor sources used were pure air and a sesame plant (~ 20 cm height). This plant species was chosen based on previous studies (Biondi et al., 2016; Naselli et al., 2017), in which the authors observed that sesame is a highly attractive host plant to *N. tenuis* (even more than tomato plants) and can even be used as a trap plant in crops. The sesame plant was placed in one of the glass pots connected to the distal arms of the olfactometer. A vacuum pump connected to the glass pots generated airflow into the system, conducting air through the olfactometer's lateral arms and reaching the main arm. The olfactometer was placed vertically on the bench surface and *N. tenuis* females were placed individually on the central arm.

The choice of each insect was considered after it crossed the line that delimits half of the corresponding lateral arm. Each predator was observed for 5 minutes at a maximum, and if no choice is made after that period it was considered "no-choice" and discarded. After every two replicates, the olfactometer was inverted in order to reduce environmental interference in the insect response. For each treatment and concentration, 30 choices were considered. The response time for insects that made a choice was also recorded.

2.6 Statistics

To determinate the baseline toxicity of the insecticides and the sublethal concentrations, a log-probit regression model was performed for all insecticides. The preference data were analyzed using a chi-squared goodness-of-fit, in order to determine if the females' attraction to sesame plants were different from a 50:50 distribution (a significant attraction towards sesame plants was expected for untreated insects).

The data regarding response time for the insects make a choice and fecundity were tested for normality (Shapiro & Wilk 1965) and homocedaticity (Bartlett, 1937), however these assumptions were not attended. Therefore, these data were fitted to generalized linear models (GLMs), and potential interactions between treatments and concentrations were tested. The models were fitted using the Poisson family for fecundity and Negative Binomial family for response time (Poisson and quasi-Poisson families were first tested, but the overdispersion observed biased the analyses, therefore Negative Binomial was used in this case). The interaction was not significant for any of the factors, therefore they were analyzed separately. Means were separated by a post-hoc Tukey HSD test ($P < 0.05$). Probit analyses were performed in the statistical program SPSS v. 21.0 (IBM, SPSS Statistics), while the analyses related with the fecundity and olfactory response bioassays were performed using R (R Core Team, 2019). Charts were assembled on SigmaPlot v.11.0 (Systat, 2008) and Microsoft Excel (2013).

3. Results

3.1 Insecticides' toxicity to N. tenuis

The Probit models were fitted to observed data for all treatments (no significance differences between observed and the expected data), validating the sublethal

concentrations for all insecticides (Table 1). Lambda-cyhalothrin was the insecticide with lowest LC_1, LC_{10} and LC_{30}, therefore was the most lethal insecticide for *N. tenuis* females. Furthermore, we could observe that *N. tenuis* females were highly susceptible to all insecticides, with LC_{30} values much lower than their field rate (5.27%, 7.42% and 8.46% of the field rate for lambda-cyhalothrin, chlorpyrifos and spinosad, respectively).

3.2 Effects of sublethal concentrations of insecticides on N. tenuis fecundity

There was no interaction between treatments and concentrations (χ^2 = 12.023, *d.f.* = 6, *p* = 0.061), therefore the data was evaluated separately. The fecundity of treated females was reduced by all insecticides, at all evaluated concentrations (LC_1: χ^2 = 64.642, *d.f.* = 3, *p* < 0.001; LC_{10}: χ^2 = 73.707, *d.f.* = 3, *p* < 0.001; LC_{30}: χ^2 = 118.560, *d.f.* = 3, *p* < 0.001). The reduction was higher for chlorpyrifos at LC_{30} (χ^2 = 9.939, *d.f.* = 2, *p* = 0.007), while no differences were observed among concentrations for lambda-cyhalothrin (χ^2 = 2.659, *d.f.* = 2, *p* = 0.265), spinosad (χ^2 = 1.008, *d.f.* = 2, *p* = 0.604) and the control treatment (χ^2 = 0.427, *d.f.* = 2, *p* = 0.808) (Figure 1).

3.3 Effects of sublethal concentrations of insecticides on N. tenuis olfactory response

The preference of *N. tenuis* females to sesame plants instead of pure air was not affected by lambda-cyhalothrin and spinosad at LC_1 and LC_{10}, however the choices of insects treated with chlorpyrifos did not differed between sesame and air for these two concentrations. At LC_{30}, all insecticides affected *N. tenuis* orientation, resulting in no difference between the proportion of choices to sesame and pure air (Figure 2).

There was no interaction between treatments and concentrations for the response time ($\chi^2 = 9.066$, *d.f.* = 6, *p* = 0.170), therefore the data was evaluated separately. Differences in the response time were observed in all concentrations (LC_1: $\chi^2 = 9.358$, *d.f.* = 3, *p* < 0.024; LC_{10}: $\chi^2 = 22.566$, *d.f.* = 3, *p* < 0.001; LC_{30}: $\chi^2 = 33.291$, *d.f.* = 3, *p* < 0.001). Insects treated with all sublethal concentrations of lambda-cyhalothrin spent more time to make a choice in comparison with the control treatment. The same was observed for insects treated with chlorpyrifos at LC_{10} and LC_{30}. Spinosad was similar to control in all concentrations. No differences were observed among concentrations for any of the treatments (control: $\chi^2 = 0.508$, *d.f.* = 2, *p* = 0.777; lambda-cyhalothrin: $\chi^2 = 0.634$, *d.f.* = 2, *p* = 0.729; chlorpyrifos: $\chi^2 = 4.981$, *d.f.* = 2, *p* = 0.083; spinosad: $\chi^2 = 3.589$, *d.f.* = 2, *p* = 0.166) (Figure 3).

4. Discussion

The integration between chemical and biological tactics in IPM was first proposed by Stern et al. (1959), and is desired to achieve satisfactory results in pest control. Strategies include time and space separation between insecticide treatment and the natural enemies' activity (ecological selectivity) and preference for insecticides that are safe to natural enemies at the same time toxic to the pests (physiological selectivity) (Carvalho et al., 2019). However, broad-spectrum insecticides are still used in pest management, which can reduce natural enemies' populations, and consequently to compromise pest control. Neurotoxic insecticides are the most used compounds due to their rapid lethal effect, ceasing crop damage by pests in short time (Yu, 2008; Casida and Durkin, 2013). The deleterious effects on natural enemies can be severe, therefore these effects should be mitigated by selecting safer insecticides. Additionally, it is important to evaluate also

sublethal concentrations of insecticides on beneficial arthropods. Due to natural degradation in field, non-target insects can be exposed to sublethal concentrations, even after spraying of recommended concentrations (Eijaza et al., 2015). Lower concentrations causing low mortality can reveal a hidden toxicity, expressed in alterations in biological and behavioral effects (Desneux et al., 2004; 2007; Biondi et al., 2012a; Ricupero et al., 2020).

Probit models are often used to estimate concentration-mortality of pesticides to pests and natural enemies, in order to determine efficient and safe compounds, respectively (Tan et al., 2012; Wang et al., 2016; Fernandes et al., 2016; Wang et al., 2017; Ricupero et al., 2020). In our observation, spinosad was the least toxic insecticide at LC_1 and LC_{10}, however at LC_{30} this insecticide was more toxic than chlorpyrifos. The highest slope was obtained in the spinosad treatment, which could indicate that a slight increase in insecticide concentration will lead to high mortality (Vojoudi et al., 2011). Still, lambda-cyhalothrin was the most toxic insecticide at all concentrations, even more than chlorpyrifos, which was initially expected to cause the highest mortality.

Lambda-cyhalothrin is a type II pyrethroid, highly effective against a broad spectrum of insect and acarine pests. Pyrethroids acts to disrupt nerve conduction and their primary site of action is the voltage-gated sodium channels, impairing nervous activity (Khambay and Jewess, 2010). While the insecticide is acting, the membrane potential is above excitation threshold by the permanent opening of sodium channels, hence the stimulus conduction is blocked (Casida and Durkin, 2013). In fact, less than 1% of sodium channels have to be affected by pyrethroids to induce poisoning (Narahashi et al., 1992), which could explain that even low concentrations of lambda-cyhalothrin affected *N. tenuis* fecundity and response time in our experiments. Desneux et al. (2004) also observed that sublethal doses of lambda-cyhalothrin affected the orientation behavior

(LD$_{0.1}$) and reproduction (LD$_{20}$) of the parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae). The parasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) exhibited reduction in parasitism and longevity after treatment with sublethal concentrations of lambda-cyhalothrin, although progeny was not affected (D'Ávila et al., 2018). Adverse effects on *N. tenuis* fecundity and behavior were reported caused by lambda-cyhalothrin (Soares et al., 2019) and other pyrethroids (Wanumen et al., 2016; Madbouni et al., 2017).

Chlorpyrifos acts primarily by inhibiting the action of the enzyme acetylcholinesterase (AChE) in the central and peripheral nervous system, which causes acetylcholine (ACh) to accumulate in the synapses, overstimulating cholinergic receptors, disrupting nerve function (Mileson et al., 1998; Casida and Durkin, 2013). One of the symptoms associated to chlorpyrifos intoxication is incoordination (Gholamzadeh-Chitgar et al., 2015), as was observed in the present study. *Nesidiocoris tenuis* females were not able to orient toward a host plant after treatment with all three sublethal concentrations. The response time also increased after treatment with LC$_{10}$ and LC$_{30}$. Similar situation was observed for *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), which chlorpyrifos at LC$_{50}$ increased the time spent before the first probing event and first phloem activity, besides affecting physiological and behavioral traits (He et al., 2013). This insecticide was toxic to hemipteran predators *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) and *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae), which at LC$_{30}$ concentration several sublethal effects were observed, such as reduction in fecundity, predation rate, enzyme activity and alterations in behavior and life table parameters (Gholamzadeh-Chitgar et al., 2015; Sharifian et al., 2017). Fernandes et al. (2016) also observed negative effects on reproduction and predation rate after chlorpyrifos exposition at LC$_{20}$ for the predators *Cycloneda sanguinea* (L.)

(Coleoptera: Coccinellidae), *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) and *Chauliognathus flavipes* Fabricius (Coleoptera: Cantharidae).

Spinosad is produced by the actinomycete *Saccharopolyspora spinosa* through the fermentation of derived macrocyclic lactone. This chemical compound is often considered as environmentally safe, presenting rapid environmental degradation and relatively low risk to non-target insects. Thus, spinosad is even recommended in IPM programs as an alternative to board-spectrum insecticides (Crouse et al., 2001; Salgado and Sparks, 2010; Biondi et al., 2012b). However, the safety classification of spinosad is not consensual. Even though spinosad is a natural insecticide, it still has neurotoxic action, thus can potentially cause sublethal effects on biological and behavioral traits of beneficial insects (i.e. feeding, oviposition, navigation and orientation) (Sparks et al., 2001; Desneux et al., 2007; Biondi et al., 2012b; Casida and Durkin, 2013). D'Ávila et al. (2018) observed that spinosad was even more toxic to the parasitoid *A. colemani* than lambda-cyhalothrin, demonstrating that spinosad, despite being a natural insecticide, is not always the safer option to be associated with natural enemies, as other authors have reported (Arnó and Gabarra, 2011; Biondi et al., 2012a; Barbosa et al., 2015). These studies corroborate with the results obtained in this research, in which all sublethal concentrations of spinosad severely reduced *N. tenuis* fecundity, and caused effects on females' olfactory response at LC_{30}.

Nesidiocoris tenuis untreated females are highly attracted to sesame plant volatiles, as previously observed by Naselli et al. (2017). Moreover, the orientation behavior of *N. tenuis* females was altered by the three chlorpyrifos sublethal concentrations, and for spinosad and lambda-cyhalothrin at LC_{30}. Due to their neurotoxic action, all the three insecticides can affect the capacity of the nervous system to react to external stimulus (Desneux et al., 2004, 2007; Biondi et al., 2012b; Casida and Durkin,

2013; Gholamzadeh-Chitgar et al., 2015). Thus, beside the observed misorientation, predators' females also presented delay in the response time after treatment with lambda-cyhalothrin (all concentrations) and chlorpyrifos (LC_{10} and LC_{30}).

Several plant hosts (including sesame) are suitable to *N. tenuis* biological development. These plants could serve as water and oviposition source, and is also where *N. tenuis* prey can be found (Castané et al., 2011; Biondi et al., 2016). For this reason, disrupting predators' capacity to locate host plants directly influence their survival and success as biological control agents. Therefore, the insecticides must be employed carefully. The misorientation caused by sublethal doses of insecticides could also compromise the *N. tenuis* capacity to locate plants infested with herbivorous prey, although it must be studied. The reduction on predator ability was observed to *Cyrtorhinus lividipennis* Reuter (Hemiptera: Miridae) after deltamethrin exposure (Zhang et al., 2015).

The level of activity of insecticides is determined by penetration, metabolism within the insect, and requirements at the target site (Yu, 2008). The mirids evaluated in this research are susceptible to all three insecticides, most likely because this population has never experienced any prior contact to these compounds. However, under selection from repeated sprays of insecticides, insects possessing biochemical mechanisms able to rapidly detoxify insecticides, or that are less sensitive to them, can be favored by surviving to concentrations that would kill sensitive individuals (Yu, 2008, Khambay and Jewess, 2010; Casida and Durkin, 2013). Despite not as often reported as pests, resistant populations of natural enemies can be selected by frequent insecticide applications. The lack of cases reported are explained by the lack of specific studies, lack in the continuity in selection pressure over the same population (migration to other plants after harvest of vegetable crop) and diluting resistance due to frequent mass-release of non-resistant

populations (Bielza, 2016; Wu et al., 2018). Besides the toxic effects of insecticides itself, it is difficult to most natural enemies to develop resistance towards insecticides due to the lack of prey after plant treatment (Tabashnik and Johnson 1999). However, zoophytophagous insects can supply themselves upon plants when prey is scarce (Castañé et al., 2011). Thus, predatory mirids, compared to specialized predators, should develop resistance faster.

5. Conclusions

The baseline toxicity showed that the LC_{30} of all insecticides to *N. tenuis* were much lower than their maximum recommended field rate ($8.446 \cdot 10^{-2}$, $5.274 \cdot 10^{-2}$ and $7.247 \cdot 10^{-2}$ for spinosad, lambda-cyhalothrin and chlorpyrifos, respectively). These finds imply that the insecticides studied are highly toxic to *N. tenuis*. Even at LC_1 and LC_{10} the fecundity of *N. tenuis* females was compromised by all insecticides. In addition, it was also observed sublethal effects on predators' orientation. We concluded that all three insecticides are noxious to *N. tenuis*, although field trials must be carried to confirm their toxicity.

On the other hand, at high densities and prey shortage *Nesidiocoris tenuis* populations can excessively feed on tomato plants, resulting in damages and economic losses (Castañé et al., 2011; Pérez-hedo and Urbaneja, 2016; Siscaro et al., 2019). In this case, chemical control measures should be applied to reduce the number of *N. tenuis* individuals on commercial crops. We observed that this predator had their survival, physiological and behavioral characteristics compromised by low insecticides concentrations. Hence, lambda-cyhalothrin and spinosad can be considered to reduce damages caused by *N. tenuis* on tomato crops. The same cannot be applied to chlorpyrifos,

which was used as a positive control in this study, and was recently prohibited in the European market.

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Credit authorship contribution statement

Luis C. Passos: Conceptualization, Methodology, Execution of experiments, Data collect, Statistical analysis, Investigation, Writing – original draft. Michele Ricupero: Conceptualization, Methodology, Execution of experiments, Data collect. Antonio Gugliuzzo: Execution of experiments, Data collect. Marianne A. Soares: Statistical analysis, Writing - review & editing. Nicolas Desneux: Writing - review & editing. Geraldo Carvalho: Writing - review & editing. Antonio Biondi: Conceptualization, Methodology, Supervision, Project administration, Writing - review & editing. Lucia Zappalà: Writing - review & editing.

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Table 1. Baseline toxicity of three insecticides to *Nesidiocoris tenuis* females 48h after treatment.

Insecticide	Field rate (ppm)	Slope ± SE	$\chi 2$ (df)	P	Lethal concentration (ppm)	95% Confidence limits (ppm)	LC/FR
Spinosad	0.3315	1.974±0.260	33.355 (31)	0.353	$LC_1 = 3.37 \cdot 10^{-3}$	$1.35 \cdot 10^{-3}$ - $5.88 \cdot 10^{-3}$	$1.017 \cdot 10^{-2}$
					$LC_{10} = 0.011$	0.007 - 0.016	$3.318 \cdot 10^{-2}$
					$LC_{30} = 0.028$	0.020 - 0.036	$8.446 \cdot 10^{-2}$
Lambda-cyhalothrin	0.2844	1.301±0.201	42.901 (36)	0.201	$LC_1 = 6.39 \cdot 10^{-4}$	$1.10 \cdot 10^{-4}$ - $1.68 \cdot 10^{-3}$	$2.247 \cdot 10^{-3}$
					$LC_{10} = 0.004$	0.001 - 0.007	$1.406 \cdot 10^{-2}$
					$LC_{30} = 0.015$	0.009 - 0.022	$5.274 \cdot 10^{-2}$
Chlorpyrifos	0.9797	0.948±0.202	35.563 (33)	0.349	$LC_1 = 8.87 \cdot 10^{-4}$	$2.30 \cdot 10^{-4}$ - $4.09 \cdot 10^{-4}$	$9.054 \cdot 10^{-4}$
					$LC_{10} = 0.011$	0.002 - 0.026	$1.123 \cdot 10^{-2}$
					$LC_{30} = 0.071$	0.033 - 0.114	$7.247 \cdot 10^{-2}$

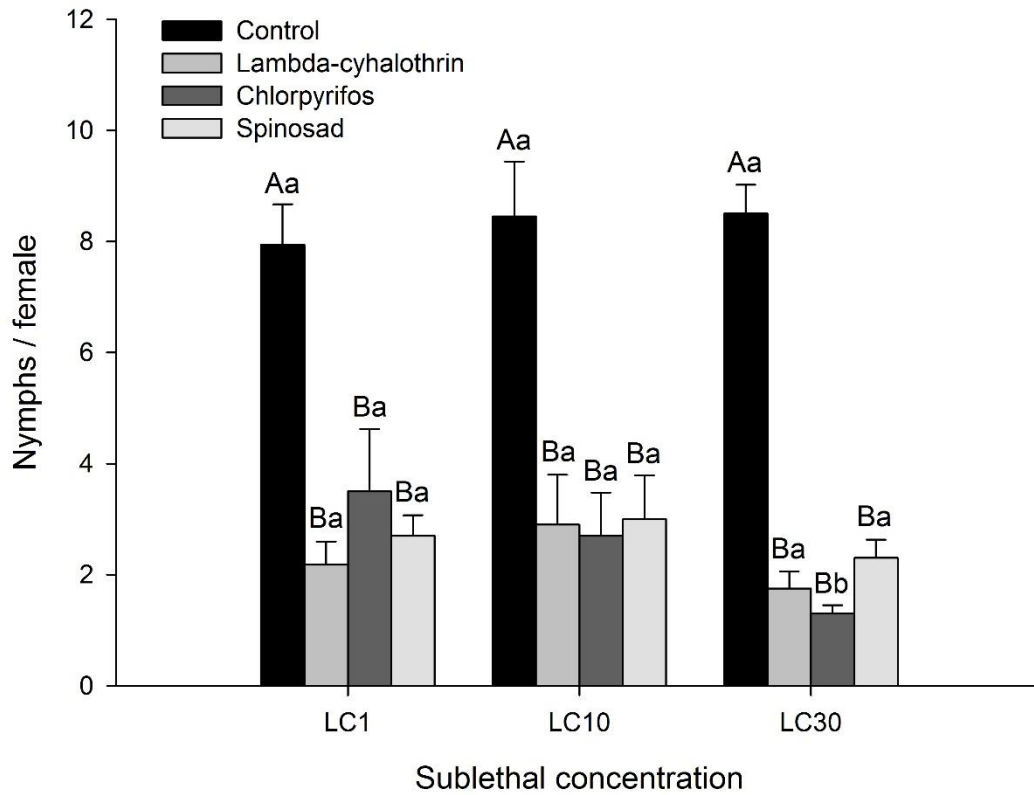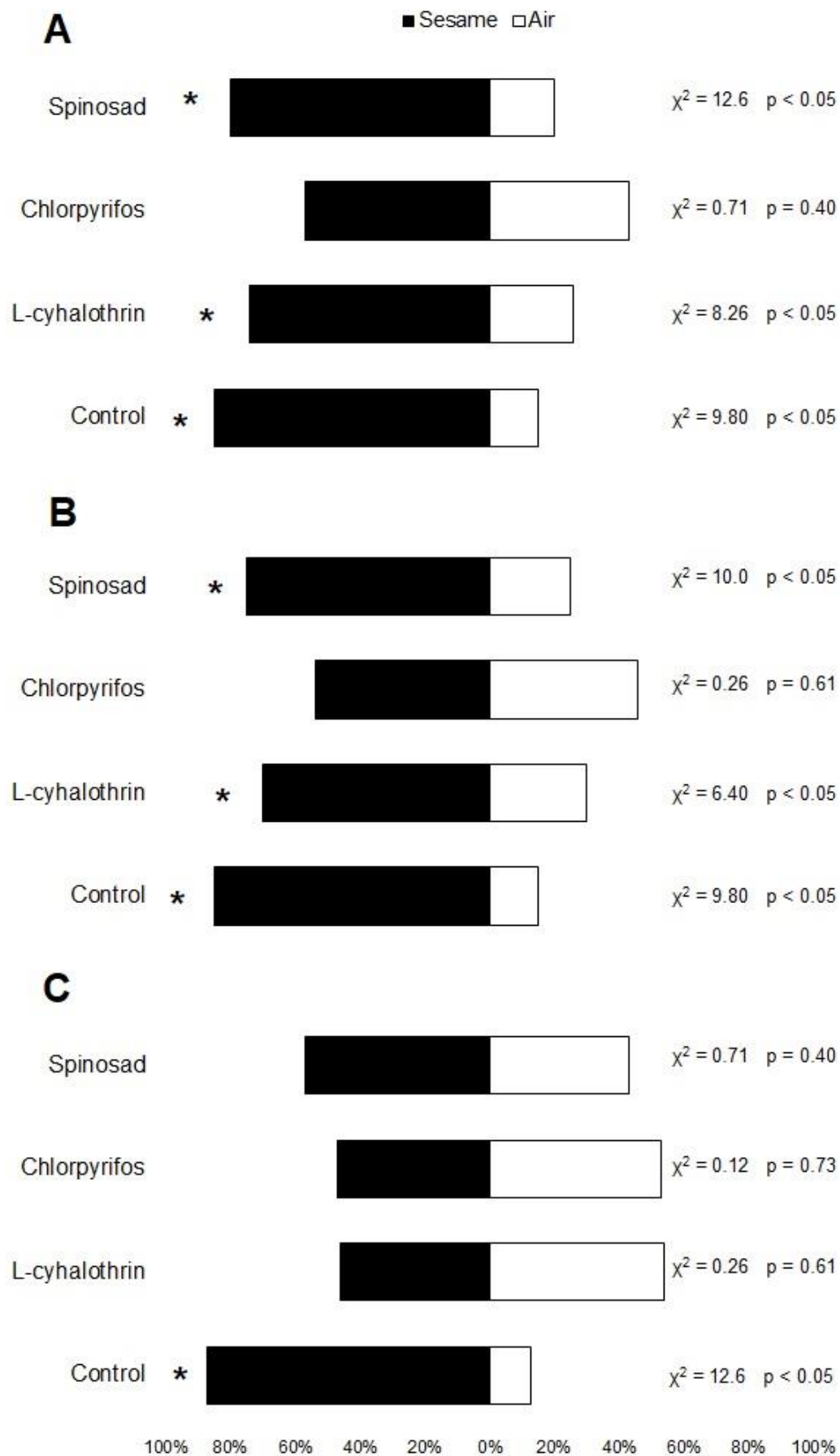LC/FR: ratio between the lethal concentration and field rate.

Figure 1. Fecundity of *Nesidiocoris tenuis* females after treatment with three insecticides in three sublethal concentrations. Different capital letters indicate differences among treatments in a concentration, while different lower case letters indicate differences in the concentrations for a treatment (GLM – Poisson distribution, Tukey HSD test, $p < 0.05$).

Figure 2. Response of *Nesidiocoris tenuis* females treated with three insecticides in three sublethal concentrations, LC_1 (A), LC_{10} (B) and LC_{30} (C) towards the volatiles produced by a *Sesamum indicum* plant. Asterisks indicate differences in the attraction proportion towards *S. indicum* plant and blank according to the likelihood chi-squared ($p < 0.05$).

A

Sesame ☐Air

Spinosad *		$\chi^2 = 12.6$ $p < 0.05$
Chlorpyrifos		$\chi^2 = 0.71$ $p = 0.40$
L-cyhalothrin *		$\chi^2 = 8.26$ $p < 0.05$
Control *		$\chi^2 = 9.80$ $p < 0.05$

B

Spinosad *		$\chi^2 = 10.0$ $p < 0.05$
Chlorpyrifos		$\chi^2 = 0.26$ $p = 0.61$
L-cyhalothrin *		$\chi^2 = 6.40$ $p < 0.05$
Control *		$\chi^2 = 9.80$ $p < 0.05$

C

Spinosad		$\chi^2 = 0.71$ $p = 0.40$
Chlorpyrifos		$\chi^2 = 0.12$ $p = 0.73$
L-cyhalothrin		$\chi^2 = 0.26$ $p = 0.61$
Control *		$\chi^2 = 12.6$ $p < 0.05$

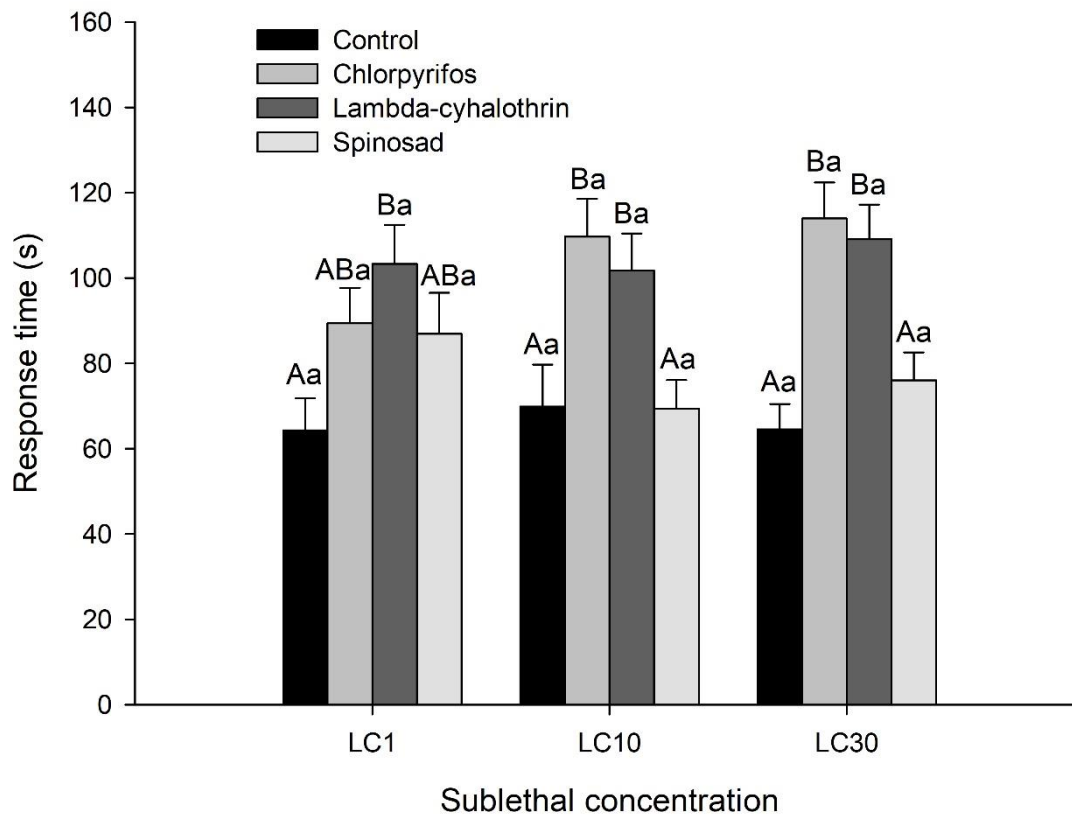100% 80% 60% 40% 20% 0% 20% 40% 60% 80% 100%

Figure 3. Mean response time (s) to *Nesidiocoris tenuis* females make a choice towards a *S. indicum* plant or pure air after treatment with three insecticides at three sublethal concentrations. Different capital letters indicate differences among treatments in a concentration, while different lower case letters indicate differences in the concentrations for a treatment (GLM – Negative Binomial distribution, Tukey HSD test, $p < 0.05$).

ARTICLE 3

Nanoemulsions containing plant essential oils with bioactivity to *Tuta absoluta* can affect the zoophytophagous predator *Nesidiocoris tenuis*?

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Nanoemulsions containing plant essential oils with bioactivity to *Tuta absoluta* can affect the zoophytophagous predator *Nesidiocoris tenuis*?

Abstract

Plant essential oils (PEOs) are secondary metabolites produced by plants and have many functions, including defense against herbivores. Due to their insecticide activity, PEOs can be used as bioinsecticides to protect crops from phytophagous insects, specially those difficultly managed with insecticides. For instance, the South American tomato pinworm, *Tuta absoluta* (Lepidoptera: Gelechiidae), a tomato pest present in most tomato producer countries, with several resistant populations reported. Therefore, PEOs have potential to be incorporated in *T. absoluta* control. Moreover, the predatory bug *Nesidiocoris tenuis* (Hemiptera: Miridae) is used as a biological control agent of *T. absoluta* in many countries. In order to achieve a more efficient control, the aim of this study is assess lethal and sublethal of PEOs encapsulated in nanoemulsions on the predator *N. tenuis*. For this, probit analyses were performed to evaluate PEOs baseline toxicity, and with this was possible to stablish sublethal concentrations (LC_1, LC_{10} and LC_{30}). Posteriorly, *N. tenuis* adults were treated with the three sublethal doses, and their effects was evaluated on *N. tenuis* fecundity and orientation. The fecundity of *N. tenuis* females was reduced by all oils, at all concentrations, with a more significant reduction at LC_{30} in comparison to other concentrations. The attraction of *N. tenuis* females towards an attractive source (*Sesamum indicum*) was affected by garlic, lavender and fennel oils in all concentrations, while anise only affected their orientation at LC_{30}. The response time to *N. tenuis* make a choice towards an odor source was also affected by garlic oil in all concentrations and for fennel and lavender oils at LC_{30}. The results of this study provide relevant information to incorporate new technologies into *T. absoluta* management.

1. Introduction

Conventional pest management is often relied on synthetic insecticides. The continuous use of such compounds can result in several problems, i.e. environmental contamination, adverse effects on nontarget organisms, and selection of resistant pest populations. As alternative, natural insecticides (originated from organic sources) are developed to be used in pest control, and some of them are based on plant derived products, i.e. plant essential oils (PEOs) (Regnault-Roger et al., 2012; Isman, 2016; Rathore, 2017). These essential oils can be present in several parts of plants and are constituted by volatile and semi-volatile compounds, products of secondary metabolism of plants of several botanic families, that act as defense mechanisms against herbivores and pathogens, therefore can present insecticide activity (Walling, 2000; Miresmailli and Isman, 2014). Moreover, PEOs present generally low mammalian toxicity, low persistence in water and soil, and are relatively cost-efficient (Isman, 2000; Bullangpoti, 2017). For this reason, several insecticides based on PEOs are being developed and studied to potentially contribute to pest control in organic crops and also be incorporated in conventional IPM programs as an alternative or complement to synthetic insecticides (Isman 2006; Regnault-Roger et al., 2012; Giunti et al., 2019).

The interest on such substances has increased in the past years, in order to incorporate more eco-friendly tactics in pest control, and also due to the difficulty of pest control, resulted of many cases of pest populations resistant to synthetic insecticides. This is the case of the South-American tomato pinworm, *Tuta absoluta* (Meyrick)

(Lepidoptera: Gelechiidae), a highly invasive pest, whose cases of resistant populations were reported in many countries (Biondi et al., 2018; Guedes and Picanço 2012; Tropea Garzia et al. 2012; Campos et al., 2014; Roditakis et al., 2015). PEOs have potential to control this pest (Campolo et al, 2018; Soares et al., 2019). Additionally, releases of natural enemies is also a tactic employed to reduce *T. absoluta* populations in many countries. Among the used species of natural enemies, the predatory bug *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) is one of the most efficient biological control agents against *T. absoluta*, and therefore one of the most used (Campos et al., 2017; Biondi et al., 2018).

Despite the benefits offered by PEOs, there are some drawbacks involved in its use for pest control due to their chemical characteristics, such as low solubility on water, high volatility and fast environmental degradation (Moretti et al., 2002; Regnault-Roger et al., 2012). Therefore, in order to produce a better quality insecticide based on PEOs, these characteristics, along with improvement of stability in storage and transport, increase the residual action of active ingredients and reduce phytotoxicity, must be improved (Isman, 2016). Facing this scenario, researches enlightened the possibility of essential oils encapsulation in nanoparticles, guaranteeing a slow and constant release of the active ingredients, reducing their volatility, minimizing negative effects on nontarget organisms, improving their water solubility, stability and efficacy to be used in pest control (Gogos et al., 2012; Kah et al., 2013, de Oliveira et al., 2014; Campolo et al., 2017; Giunti et al., 2019).

The association between PEOs and the predator *N. tenuis* can be an interesting strategy to improve *T. absoluta* management, however the insecticide effects of PEOs can also be harmful to the predator by causing lethal and sublethal effects, reducing their populations and compromising their capacity to control *T. absoluta* (Desneux et al., 2007;

Biondi et al., 2012; Soares et al., 2019). In fact, PEOs are considered broad-spectrum compounds, which can cause insects' mortality and secondary effects (i.e. antifeedant, repellent, oviposition deterrent and growth regulatory) (Rathore, 2017; Isman and Tak, 2017). Most of the studies on PEOs is focused on bioactivity to pests, therefore there are not many studies regarding the effects of such substances on biological control agents (Miresmailli and Isman 2006; Isman, 2016). In order to incorporate more eco-friendly tactics in *T. absoluta* management programs, the evaluation of the toxicity of PEOs on biocontrol agents is fundamental approach to enhance the control of this pest. Therefore, the aim of this study was to assess the lethal effect caused by different doses of four plant essential oils on the predator *N. tenuis*, and evaluate behavioral and physiological effects caused by sublethal concentrations of these compounds.

2. Material and Methods

2.1 Insects

Nesidiocoris tenuis used in these bioassays were obtained from a rearing in the Department of Agri-food and Environmental Systems Management (DiGeSA), in the University of Catania (Catania - Italy), where all following experiments were also developed. Periodic collections were also performed in untreated tomato greenhouses in Fiumifreddo - Italy, and after identification insects were added to the rearing. Adults of *N. tenuis* (~150 individuals) were kept in entomological cages (32 x 40 x 70 cm) covered by tulle mesh and containing sesame (*Sesamum indicum* L.) plants (~30 cm ht) as water and oviposition sources. The mixture of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs and *Artemia* spp. cysts (Entofood® Koppert, the Netherlands) were offered to predators as food source. Adults were kept in the plants for three days, and

subsequently they were collected and transferred to new entomologic cages as a described above. The sesamum plants containing *N. tenuis* eggs were kept in the cages, and originated insects were maintained until they reach adulthood, when were used in the bioassays. New plants and Entofood® were added in each cage when necessary. The rearing was maintained in laboratory conditions (25 ± 1 ºC, 55 ± 5 % RH, and 14L:10D h photoperiod).

2.2 Essential oils composition and preparation of nanoemulsions

The PEOs used in this bioassay were previously evaluated against *T. absoluta* and showed good insecticide activity to this pest, therefore were selected to have their effects evaluated on the predator *N. tenuis*, aiming to integrate these control tactics in *T. absoluta* management (Campolo et al., 2018). The compounds evaluated in this experiment are plant essential oils provided by Esperis s.p.a. (Milano, Italy) extracted with cold press technique from the following plant species: anise (*Pimpinella anisum* L. -Apiaceae), fennel (*Foeniculum vulgare* Mill. - Apiaceae), lavender (*Lavandula angustifolia* Miler. - Lamiaceae) and garlic (*Allium sativum* L. - Liliaceae). The oils were characterized by chromatography–mass spectrometry and flame ionization detector, and are available in Campolo et al. (2018). The results showed that anise oil presented 8 compounds in its constitution, with a higher proportion of anethole (89.96%), followed by estragole (4.03%). For fennel, 14 compounds were observed, with greater proportions of cis-anethole (42.33%), limonene (32.33%) and fenchone (10.35%). The garlic oil consisted of more than 80 volatile compounds, from which 90% were sulfurized groups, mostly diallyl disulfide (33.58%), diallyl trisulfide (21.23%) and diallyl tetrasulfide (14.05%). Lavender oil consisted of 12 compounds, which main fractions were linalool (41.13%),

linalyle formiate (36.05%) and camphor (7.20%). Nanoemulsions were prepared by the self-emulsifying sonication process and included 15% of each essential oil, the 5% of a tensioactive and 80% of distilled water. The final formulations showed nanometric scale dimensions (113-146 nm) and good stability over time (average ζ: -15.4 mV) (Campolo et al., 2018).

2.3 Baseline toxicity of essential oils to N. tenuis females

Based on the toxicity of the essential oils on *T. absoluta* (Campolo et al., 2018), newly emerged *N. tenuis* females (2-3 d old) were treated with different concentrations of these compounds. Stock solutions containing 15% of each essential oil were considered the maximum concentration, and based on preliminary observations of insect mortalities, *N. tenuis* females were submitted to treatment with 5 to 6 serial dilutions, plus a control treatment (water).

For insect treatment, *N. tenuis* females were separated in groups of 5 individuals in Falcon tubes (50 mL), and the tubes were maintained in a thermic box containing ice during 3h (the tubes did not touch the ice directly). With this was possible to in order to reduce the insects' activity, allowing them to be easily directly sprayed. After this period, each group of five insect was individually placed in a plastic cup (100 mL) and immediately sprayed with the oil solutions by a hand-sprayer. An absorbent paper covered the inside of the cup to prevent the formation of drops after spraying and consequent attachment of insects to the plastic, and after treatment of each group of five insects the paper was changed. After treatment, each group of *N. tenuis* females were transferred to an acrylic pot (5.5 cm diameter, 3 cm ht) containing a mixture of *E. kuehniella* eggs and *Artemia* spp. cysts (Entofood® Koppert, the Netherlands) as food

source and a zucchini (*Cucurbita pepo* L.) leaf disc (3 cm diameter) as water source. Each pot was considered a repetition, and the number dead females was recorded after 48h. For each oil and concentration, 8 repetitions were performed.

2.4 Sublethal effects of essential oils on N. tenuis fecundity

According to the results of the baseline toxicity, *N. tenuis* couples were treated with three sublethal concentrations of the essential oils (LC_1, LC_{10} and LC_{30}). The sublethal concentrations were chosen in order to assess the potential effects of very low concentrations of the essential oils to the predators (LC_1 and LC_{10}) and to a maximum concentration in which a given insecticide compound can be considered safe according to the International Organization of Biological Control (IOBC) standards (LC_{30}) (Van de Veire et al., 2002).

Recently emerged adults (2-3 d old) were treated with the sublethal concentrations, by the same process described above, and after were transferred to a plastic cup (400 mL) containing *E. kuehniella* eggs (1g) as food supply, and a green bean as water source and oviposition substrate. Each couple remained on the cup for three days. After this period, insects were removed. The green beans containing *N. tenuis* eggs were maintained in the cups, in laboratory conditions, and the number of emerged nymphs was recorded until 20 days after adults' removal. Twenty-five couples were evaluated in each treatment and concentration.

2.5 Sublethal effects of essential oils on N. tenuis orientation

The same sublethal concentrations had their effects evaluated on *N. tenuis* females' orientation towards a host plant (sesame, *Sesamum indicum* L.). Adult females (2-3 days old) were submitted to treatment to essential oils at LC_1, LC_{10} and LC_{30} in the same process described above. After 24h, insects were individually transferred to a Y-tube olfactometer (main arm and lateral arms 15 cm long and 4 cm internal diameter), placed vertically on the bench surface and attached by its lateral arms to two glass pots from which the air flow generated by a vacuum pump go through, carrying odor sources to the main arm. The odor sources used were pure air X *S. indicum* plant (20 cm height). The sesame plant was chosen based on previous studies (Biondi et al., 2016; Naselli et al., 2017), in which the authors observed that sesame is a highly attractive host plant to *N. tenuis* and can be used as a trap plant in crops. Each *Nesidiocoris tenuis* female was placed in the base of the central arm, and its choice was considered after it crossed the line that delimits half of the corresponding lateral arm. In order to reduce external interference, the olfactometer was inverted after each two replicates. For each treatment and concentration, 30 choices were considered to compare the proportions. The maximum evaluation time was 5 minutes, and if the insect did not choose a side in this period it was considered "no-choice" and discarded. For the insects who made a choice, the response time was also recorded.

2.6 Statistics

A log-Probit regression model was performed for each essential oil to determinate their baseline toxicity and estimate the sublethal concentrations. The preference data were analyzed using a chi-squared goodness-of-fit to determine if the females' orientation towards sesame plants diverge from a 50:50 distribution (a significant attraction towards

sesame plants was expected for untreated insects). Data regarding the response time for the insects make a choice and fecundity (Shapiro and Wilk, 1965) and homocedaticity (Bartlett, 1937), however these assumptions were not attended for any factor. Thus, these data were fitted to generalized linear models (GLMs), first testing interactions between treatments and concentrations, and if no interactions were observed the factors were analyzed separately. The models were fitted using the Poisson family for fecundity and Negative Binomial family for response time. Means were separated by a post-hoc Tukey HSD test. Probit analyses were performed in the statistical program SPSS v. 21.0 (IBM, SPSS Statistics), while the other analyses related with the fecundity and olfactory response bioassays were performed on R (R Core Team, 2019). Charts were assembled on SigmaPlot v.11.0 (Systat, 2008) and Microsoft Excel (2013).

3. Results

3.1 Baseline toxicity of essential oils to N. tenuis females

The concentration-mortality curves were fitted to observed data for all treatments, validating the sublethal concentrations for all essential oils (Table 1). The lowest sublethal concentrations were estimated for garlic, while the highest LC_1 and LC_{10} were estimated for anise and the highest LC_{30} for fennel.

3.2 Sublethal effects of essential oils on N. tenuis fecundity

The fecundity of *N. tenuis* females was reduced by all essential oils at all concentrations, with significant interaction between treatments and concentrations ($\chi^2 =$

22.25, $d.f. = 8$, $p < 0.01$), indicating that higher reduction was observer at concentration increase for all essential oils. For all PEOs, a more significant reduction occurred at LC_{30} (Figure 1).

3.3 Sublethal effects of essential oils on N. tenuis orientation

The contact with all sublethal concentrations of lavender, fennel and garlic oils affected the attraction of *N. tenuis* females to sesame plants. The orientation of insects treated with anise oil was not affected at LC_1 and LC_{10}, however it was compromised at LC_{30} (Figure 2). There was no interaction between treatments and concentrations for response time to make a choice ($\chi^2 = 9.39$, $d.f. = 8$, $p = 0.31$), therefore factors were analyzed separately. The response time was affected by the PEOs. At LC_1 ($\chi^2 = 16.29$, $d.f. = 4$, $p < 0.01$) and LC_{10} ($\chi^2 = 12.23$, $d.f. = 4$, $p = 0.02$) *N. tenuis* females treated with garlic oil significantly took more time to make a choice. At LC_{30}, all oils increased the time spent by insects to choose a side, except anise ($\chi^2 = 28.92$, $d.f. = 4$, $p < 0.01$). Regarding the oils, there was no increase in the response time according to the concentrations for anise ($\chi^2 = 2.25$, $d.f. = 2$, $p = 0.32$), garlic ($\chi^2 = 1.24$, $d.f. = 2$, $p = 0.54$) and fennel ($\chi^2 = 4.64$, $d.f. = 2$, $p = 0.10$), however for lavender the response time was higher at LC_{30} in comparison with LC_1 ($\chi^2 = 6.04$, $d.f. = 2$, $p = 0.04$) (Figure 2).

4. Discussion

All essential oils evaluated caused, at some extent, toxicity to the predator *N. tenuis*, expressed in behavioral and physiological alterations. The toxicity was certainly caused by the compounds present in the PEOs. Most PEOs are blends of plant secondary

metabolites, mainly monoterpenoids and sesquiterpenoids, and in a lesser extent, phenylpropanoids (Isman, 2006, 2016; Regnault-Roger et al., 2012). The constitution of PEOs can be very complex, reaching up to 200 chemical components, but usually there are few major constituents that hold a higher proportion in the oils (Isman, 2016; Rathore, 2017). PEOs composition can be very diverse among plant species (Regnault-Roger et al., 1993, 2012), and even in the same species differences in the proportion of components can occur due to differences in circadian rhythm, development stage, climate and nutrition, which makes it difficult to have a standardized product (Clark and Menary, 1981; Müller-Riebau et al., 1997; Raguso and Pichersky, 1999; Bullangpoti, 2017).

Monoterpenes are considered the principal group that exert bioactivity against insects, and can penetrate insect body through contact, fumigation and ingestion (Prates et al., 1998; Pavela, 2015; Giunti et al., 2019), in fact the lipophilic nature of PEOs favors its penetration through lipid layers in insect cuticle (Regnault-Roger et al., 2012). These substances act on neurotoxic activity due to several mechanisms, disrupting insects' cellular activities and biological processes (Regnault-Roger et al., 2012; Isman and Tak, 2017). Monoterpenes can act as antagonists of the neuromodulator octopamine, causing breakdown in the nervous system and affecting several behavioral and physiological processes, although the precise mode of action is still not completely defined (Enam, 2005; Price and Berry, 2006; Tripathi et al., 2009; Isman and Tak, 2017; Rathore, 2017). Besides, monoterpenes can also act inhibiting acetylcholinesterase, with a reversible competitive inhibition occupying the hydrophobic site of the enzyme's active site (López and Pascual-Villalobos, 2010). Therefore, PEOs can disrupt insect metabolism, resulting in negative physiological and behavioral effects (Regnault-Roger et al., 2012).

Among the PEOs evaluated in this experiment, lavender is the oil with higher proportions of monoterpenes (approximately 90%), which was more likely to cause

toxicity to *N. tenuis*. The toxicity of this oil was also observed on larvae of *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) (Shalabi et al., 2016). Erland et al. (2015) observed toxic effect of lavender oil on the invasive *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), and linalool exhibited insecticidal activity against this pest when used alone. This monoterpene is a recognized inhibitor of acetylcholinesterase (Ryan and Byrne, 1988), and counted with more than 40% of the composition of the lavender oil used in the present experiment, which could explain the high toxicity to *N. tenuis* females.

The fennel essential oil also possesses a considerable proportion of monoterpenes (approximately 50%), mainly limonene and fenchone. This plant is recognized for its insecticide effects against many insects, such as *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), *Culex quinquefasciatus* Say (Diptera: Culicidae) larvae and *Musca domestica* Linnaeus (Diptera: Muscidae) adults (Pavela et al., 2016) and the aphids *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) (Digilio et al., 2008). Indeed, Pavela (2018) verified significant insecticide effect of fennel oil on *M. persicae*, and also safety to the predaror *Harmonia axyridis* (Palias) (Coleoptera: Coccinellidae). Chaieb et al. (2018) observed insecticidal effect of limonene, which as a composes part of fennel oil.

The most present compound in fennel essential oil, however, was cis-anethole, a phenylpropanoid, that can also cause insecticide effect. Phenylpropanoids can neutralize insect defense mechanisms, i.e. P450, glutathione-S-transferases, and esterases (Jankowska et al., 2018). Additionally, the anise essential oil counted with nearly 95% of phenylpropanoids in its composition (anethole and estragole). The main constituents of anise oil can exert deleterious effects on insects. Hashem et al. (2018) observed that anise oil increased the mortality of *Triboluim castaneum* (Herbst) (Coleoptera: Tenebrionidae)

and progeny reduction with increasing concentrations, plus irritations and serious damages to various body parts, including cells of the midgut, and the lipophilicity aids the penetration through the cuticle.

Differently from the previous oils, the garlic essential oil has a most particular composition, with more than 90% of organosulfides. Garlic oil is known for its insecticide activities (Regnault-Roger, 1997), and is recognized and used as insecticide in some countries such as United States, Mexico and Colombia (Isman, 2015). Several studies reported the toxicity of garlic oil and its compounds on several pests at different development stages and sublethal effects including oviposition inhibition (Ho et al., 1996; Meriga et al., 2012; Yang et al., 2012; Plata-Rueda et al., 2017).

Although the toxic effect is often related to the compound or compounds that are present in higher proportions, the toxicity of PEOs should not be exclusively attributed to a single or even the major constituents, because synergic interactions can occur among constituents (even with compounds that are not bioactive by themselves), therefore this is the most feasible reason why PEOs cause toxicity, specially for PEOs with more complex constitutions (Isman, 2016; Rathore, 2017; Isman and Tak, 2017). For example, it was observed that 1,8-cineole facilitates the penetration of camphor on insects' tegument (Tak and Isman, 2015). Despite the individual components of PEOs are better known, the effects of their mixture in essential oils is more difficult to assess (Regnault-Roger et al., 2012). Most PEOs can potentially cause behavioral and physiological effects on insects at a given dose, however behavioral effects are more likely to be observed at lower concentrations than necessary to produce acute toxicity and secondary effects on physiology (Isman and Tak, 2017). Due to topical treatment, the oils probably penetrated *N. tenuis* integument, causing both physiological and behavioral effects at low concentrations, as was observed on fecundity, orientation and response time.

Healthy insects can detect fragrant and chemosensory-active compounds such as plant volatile compounds through odorant binding proteins and chemosensory proteins, located on the periphery of sensory receptors, with the function to capture and transport molecular stimuli (Picimbon, 2005; Regnault-Roger et al., 2012). In the experiment, untreated *N. tenuis* females were highly attracted to sesame plants, as reported by Naselli et al. (2017). After treatment with the PEOs, even at very low concentrations, the capacity of *N. tenuis* females to locate a host plant was compromised. The neurotoxic effects of PEOs might have affected the capacity of the predator to guide themselves towards the stimuli. Moreover, the direct treatment with the oils might have caused confusion to the insect, because many compounds can be occupying the proteins responsible for perception.

Despite their benefits, PEOs are notably less effective than most conventional pesticides, which demands higher rates and frequency of application (Bullangpoti, 2017). The persistence of PEOs (the time that the product remains biologically active against pests after application) is often considered low, although field trials showed that one single spray can provide up to three weeks of protection, presumably for the repellent effect of residual concentrations (Isman et al., 2011). PEOs originally have low persistence in the environment due to their high volatility (Hu and Coats, 2008; Rathore, 2017) but encapsulation in nanoemultions reduce their volatility by improving their stability, also in storage and transport, reducing the susceptibility to oxidation (Isman, 2016). Thus, increase the residual life of such compounds is highly desirable for pest control, and in this sense good results were observed after nanoencapsulation (de Oliveira et al., 2014). Moreover, the nanoencapsulation may also help to prevent the phytotoxicity that can be caused by PEOs depending on the concentration, plant species and oil composition (Isman, 2016; Rathore, 2017).

However, for mirid predators such as *N. tenuis* that has a zoophytopaghous habit and depends on plants to survive, the increased persistence provided by nanoencapsulation can be harmful, since the deleterious effects of PEOs can be prolonged, and several effects were observed even at low concentrations. The contact with sublethal concentrations is likely to happen in field conditions even when higher doses are used, due to natural degradation of the compounds (Eijaza et al., 2015). This can negatively affect their survival, development and their efficiency as biological control agents (Desneux et al., 2007; Soares et al., 2019). It should be taken into account before use these emulsions where the predation exerted by *N. tenuis* is desirable, however this predator, at high densities and prey scarcity, can injury tomato plants and cause economic damages (Siscaro et al., 2019). In situations where *N. tenuis* populations must be reduced, the encapsulated PEOs can be used as a management strategy to mitigate plant damage.

The desire of consumers for eco-friendly products and the awareness for pesticide residuals in food and environment makes this market grow fastly, being one of the main strategies to control pests in organic crops (Regnault-Roger et al., 2012). Besides, due to resistance problems of conventional insecticides towards several agriculture pests, PEOs may also be incorporated into conventional IPM programs (Isman, 2016). The complexity of compounds in PEOs is beneficial to ease the resistance evolution in comparison to a single compound, since the blend of organic compounds can have diverse physiological effects within insects (Feng and Isman, 1995; Regnault-Roger et al., 2012; Rathore, 2017). Moreover, there are numerous plants that produce essential oils with potential pesticide effect, thus researches must be continued in order to determine new PEOs for blending and/or constituents to achieve higher pesticide efficacy, and also improve the formulation to extend the residual bioactivity (Isman, 2016).

5. Conclusion

The nanoformulations can be efficient to delay the degradation and improve pest control, but the maintenance of the effect can be harmful to natural enemies, that become exposed to the PEOs for more time. In the present experiment, we observed that *N. tenuis* is susceptible to all evaluated PEOs and small concentrations of these compounds reduced the predator's fecundity and affected its orientation, which might impair their capacity to efficiently exert biological control. Therefore, these substances must be used carefully when the predator *N. tenuis* is present in the crop.

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Declaration of competing interest

The authors declare no conflict of interests.

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Table 1. Baseline toxicity of four plant essential oils encapsulated in nanoemultions to *Nesidiocoris tenuis* females 48h after treatment.

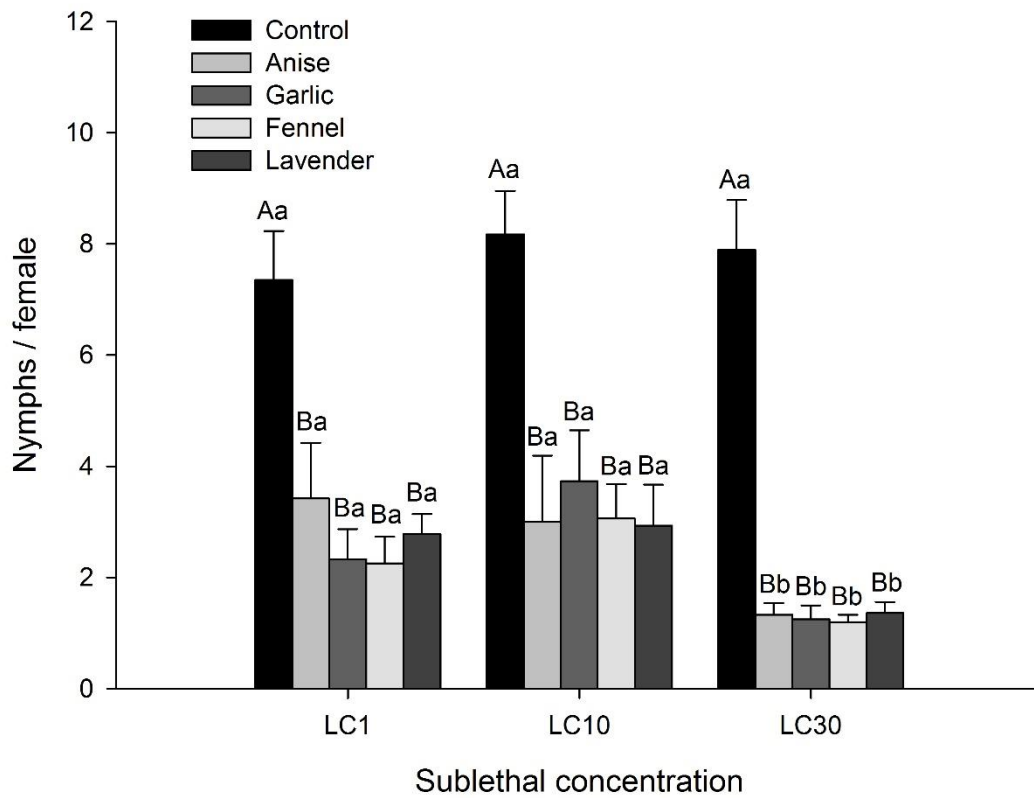| Plant	Oil percentage (%) in the full dose	Slope ± SE	$\chi 2$ (df)	*p*	Lethal concentration (oil %)	95% Confidence limits (oil %)
Anise	15	2.058±0.288	57.649 (46)	0.116	$LC_1 = 0.606$	0.212 - 1.065
					$LC_{10} = 1.949$	1.127 - 2.693
					$LC_{30} = 4.547$	3.450 - 5.772
Fennel	15	1.000±0.220	61.942 (47)	0.071	$LC_1 = 0.081$	0.001 – 0.354
					$LC_{10} = 0.903$	0.124 - 1.862
					$LC_{30} = 5.166$	2.931 - 8.673
Garlic	15	1.368±0.194	62.340 (47)	0.066	$LC_1 = 0.065$	0.011 - 0.159
					$LC_{10} = 0.375$	0.149 - 0.628
					$LC_{30} = 1.340$	0.856 - 1.899
Lavender	15	2.271±0.282	48.430 (38)	0.120	$LC_1 = 0.443$	0.176 - 0.755
					$LC_{10} = 1.277$	0.747 - 1.785
					$LC_{30} = 2.751$	2.013 - 3.516

Figure 1. Fecundity of *Nesidiocoris tenuis* females after treatment with four essential oils in three sublethal concentrations. Different capital letters indicate differences among treatments in a concentration, while different lower case letters indicate differences in the concentrations for a treatment (GLM – Poisson distribution, Tukey HSD test, p < 0.05).

Figure 2. Response of *Nesidiocoris tenuis* females treated with four plant essential oils in three sublethal concentrations (A, B and C) towards the volatiles produced by a *Sesamum indicum* plant. Asterisks indicate differences in the attraction proportion towards *S. indicum* plant and blank according to the likelihood chi-squared (p < 0.05).
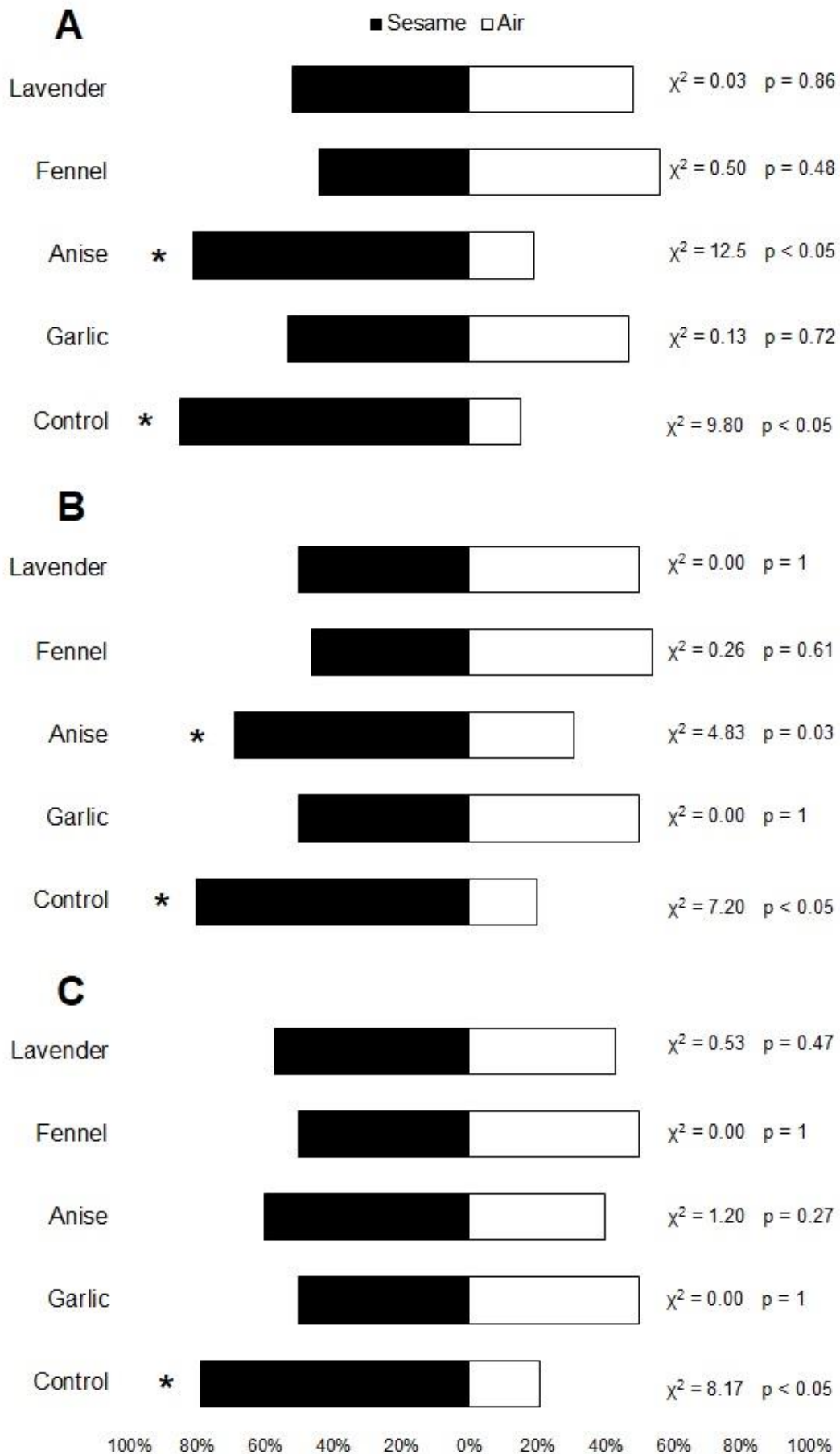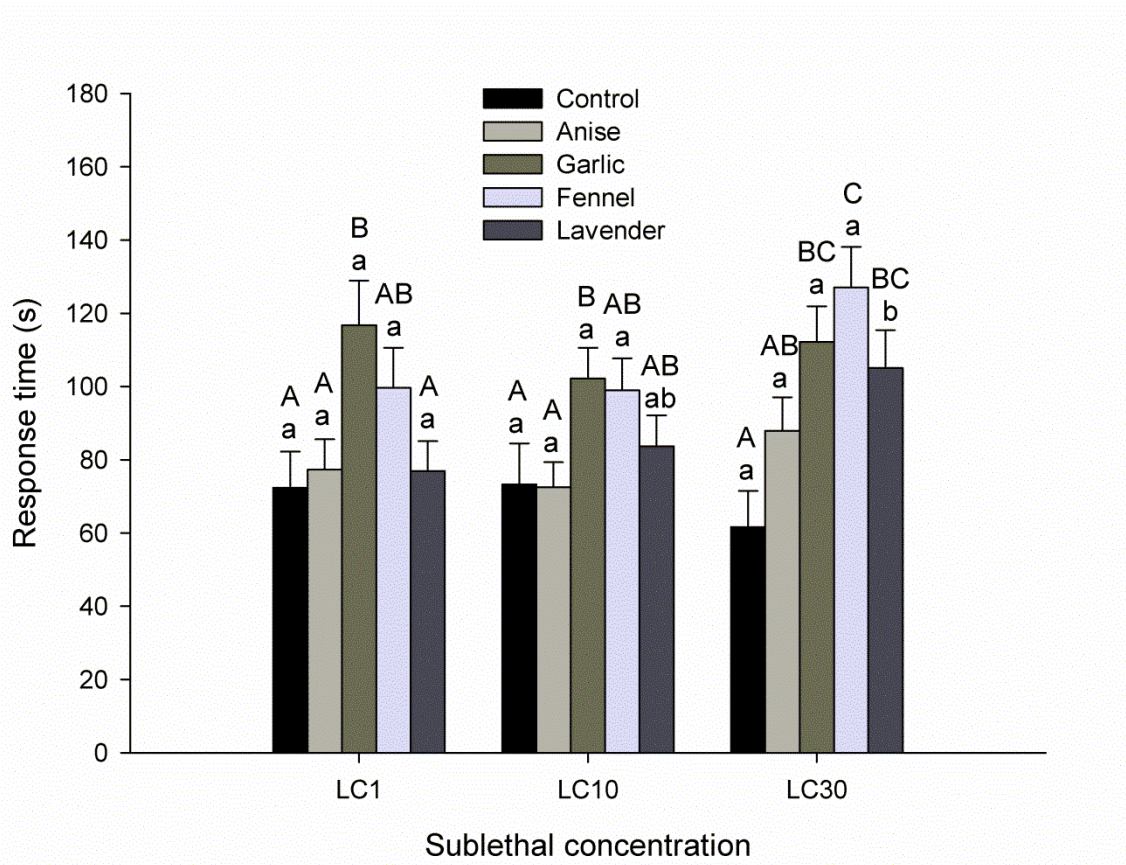
Figure 3. Mean response time (s) to *Nesidiocoris tenuis* females make a choice towards a *S. indicum* plant or pure air after treatment with four plant essential oils at three sublethal concentrations. Different capital letters indicate differences among treatments in a concentration, while different lower case letters indicate differences in the concentrations for a treatment (GLM – Negative Binomial distribution, Tukey HSD test, $p < 0.05$).

FINAL CONSIDERATIONS

The results obtained in this study provide information that can be used to improve *T. absoluta* management programs, by the association of natural enemies, insecticides and plant essential oils. The South American mirid *M. basicornis* has a great potential to be used as a biological control agent in *T. absoluta* management programs, however it can suffer sublethal effects of insecticides used to control *T.* absoluta, even some that are considered selective for natural enemies. Among all evaluated insecticides, chlorantraniliprole caused less deleterious effects, thus was considered the safest insecticide for this predator. The other insecticides must be avoided in tomato crops where *M. basicornis* is present.

Nesidiocoris tenuis is already being used in IPM programs in many countries, although in some situations insecticides still must be used to complement *T. absoluta* control. Females of *N. tenuis* experienced disorientation after contact with sublethal doses of evaluated insecticides. Chlorpyrifos biased insect choice in all concentrations (LC_1, LC_{10} and LC_{30}) while lambda-cyhalothrin and spinosad only affected the choice in the highest concentration. The time elapsed to insects make a choice was also affected by lambda-cyhalothrin (all concentrations) and chlorpyrifos (LC_{10} and LC_{30}). The plant essential oils also affected *N. tenuis* females' orientation, even in very low concentrations. Lavender, garlic and fennel biased insect choice in all concentrations tested (LC_1, LC_{10} and LC_{30}), while anice effect was deleterious only in the higher concentration. The fecundity of *N. tenuis* females was reduced when exposed to sublethal doses of insecticides and essential oils. Therefore, both insecticides and essential oils must be used carefully in tomato crops where *N. tenuis* is released or maintained, specially avoiding to spray the oils directly on the insects. Nevertheless, field studies must be performed to confirm the laboratory results.