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Food web interactions in an ecological community model: Tomato plant, *Tuta absoluta* and its natural enemies

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Abstract

The introduction of an invasive pest in a new area often disrupts the ecological stability existing in a consolidated agricultural system. In this case, the knowledge of the mechanisms of interaction occurring among the species characterizing the food web becomes crucial. In my PhD research activity, I investigated the multitrophic interactions occurring in the ecological model of Tomato, Tuta absoluta *and its natural enemies, referring particularly to the omnivorus predator* Nesidiocoris tenuis. *I conducted behavioral observations on potential intraguild interactions occurring between* N. tenuis *and the two* T. absoluta *parasitoids* Necremnus tutae *and* Bracon nigricans. *Direct behavioral observations on the mirid were carried out under a microscope supplying* T. absoluta *larvae parasitized by each of the two parasitoids. Another set of trials was conducted in microcosms to assess the effects of the mirid on the development of both parasitoids. Furthermore, to study the indirect plant responses triggered by the feeding behavior of* N. tenuis *on tomato plant, we assessed the attraction of the whitefly* Bemisia tabaci *and its parasitoid* Encarsia formosa *toward punctured plants by different* N. tenuis *instars.These data, obtained in olfactory studies, were also related to the analysis of the gene expression involved in the codification for the main phytohormones which lead plant defense and to the analysis of the levels of these phytohormones in healthy and punctured tomato plants. Finally, further studies on the plant-insect interactions were conducted comparing the different attraction of* N. tenuis *for healthy tomato, the potential companion plants* Sesamum

indicum *and* Dittrichia viscosa *plants as well as tomato plants infested by* T. absoluta *eggs and larvae. At the same time gas chromatographic analysis was carried out in order to obtain the volatile profile of each plant to be related with the olfactory studies.*

These three lines of research provided useful data in the understanding and in the management of the complex interactions in the chosen model. The key role played by the plant in directing these interactions and the competitiveness shown by the zoophytophagous mirid N. tenuis *were highlighted. Data evidenced that this omnivorous predator showed to be able to compete with the two parasitoids* B. nigricans *and* N. tutae *performing intraguild interferences such as Kleptoparasitism and Intraguild Predation on them. All the feeding instars of* N. tenuis *were able to induce indirect plant defense in tomato through the activation of the Jasmonic acid pathway responsible for* E. formosa *attraction and the abscissic acid pathway involved in* B. tabaci *rejection. Finally in our study with alternative plants, the capability of the mirid to exploit plant biodiversity showing a positive orientation behavior toward potential companion plants of tomato such as sesame emerged; furthermore in these studies we identified volatile compounds emitted by plants potentially responsible for this attraction activity.*

Sommario

L’introduzione di specie invasive in nuovi areali porta alla rottura degli equilibri ecologici che contraddistinguono un consolidato agro-ecosistema. In tali casi, la conoscenza dei meccanismi di interazione tra le varie specie che compongono la rete trofica diventa cruciale. Nel mio lavoro di ricerca, nel corso dei tre anni di dottorato, mi sono occupato dello studio di queste interazioni multi trofiche che si verificano nel modello di studio caratterizzato dal sistema Pomodoro, Tuta absoluta *e i suoi nemici naturali, riferendomi in particolar modo all’attività del predatore onnivoro* Nesidiocoris tenuis. *Il lavoro è stato condotto svolgendo osservazioni comportamentali sui potenziali fenomeni di “intraguild interactions” che si possono verificare fra* Nesidiocoris tenuis *e i due parassitoidi di* T. absoluta Necremnus tutae *e* Bracon nigricans. *Il miride predatore è stato osservato direttamente al microscopio esponendo larve di* T. absoluta *parassitizzate da ognuno dei due parassitoidi; un altro gruppo di osservazioni sono state condotte in microcosmi al fine di valutare gli effetti del miride sullo sviluppo dei due parassitoidi. Inoltre, al fine di valutare le risposte di difesa indiretta, indotte nelle piante di pomodoro dall’alimentazione da parte di* N. tenuis, *è stata testata la preferenza dell’aleirodide* Bemisia tabaci *e del suo parassitoide* Encarsia formosa *nei confronti delle piante punte da diversi stadi di* N. tenuis. *I dati ottenuti all’olfattometro sono stati correlati con l’analisi dei geni coinvolti nella codifica dei principali fitormoni responsabili dell’attivazione della risposta di difesa nelle piante e con l’analisi dei livelli di questi fitormoni nelle piante di*

pomodoro sane e punte da N. tenuis*. Infine ulteriori studi sono stati condotti sulle interazioni pianta-insetto comparando il differente grado di attrazione di* N. tenuis *per piante sane di pomodoro, le potenziali piante alternative* Sesamum indicum *e* Dittrichia viscosa*, nonché per piante di pomodoro infestate con uova o larve di* T. absoluta*. Parallelamente sono state condotte delle analisi al gas cromatografo al fine di ottenere un profilo di volatili per ciascuna pianta, da poter correlare con i risultati all'olfattometro.*

I tre filoni di ricerca ci hanno permesso di ottenere dei dati utili per la comprensione e gestione delle complesse interazioni ecologiche sul nostro modello di studio. In particolare è emerso il ruolo chiave delle piante nel dirigere queste interazioni e la competitività dimostrata dal miride zoofitofago N. tenuis*. Infatti, questo predatore onnivoro si è dimostrato capace di competere con i due parassitoidi* B. nigricans *e* N. tutae *svolgendo delle interferenze ecologiche quali il cleptoparassitismo e la predazione degli stadi larvali dei parassitoidi. Riguardo le altre indagini svolte, è stato dimostrato che tutti gli stadi di sviluppo di* N. tenuis *sono in grado di indurre risposte di difesa nelle piante di pomodoro attraverso l'attivazione della via metabolica dell'acido jasmonico responsabile dell'attrazione di* E. formosa *e della via metabolica dell'acido abscissico che svolge un ruolo nella repellenza nei confronti di* B. tabaci*. Infine, nello studio svolto con le potenziali piante alternative al pomodoro è emersa la capacità del miride di sfruttare la biodiversità colturale, mostrando un'attrazione nei confronti del sesamo; inoltre in questi studi sono stati* *identificati alcuni volatili potenzialmente responsabili dell'attività attrattiva delle piante testate.*

1. Introduction

In the last years, the interest in organically produced food has increased among consumers; this is mainly due to the negative effects of conventional agricultural practices on the environment and on human health (Pacini et al., 2003). Therefore, the economic importance of organic farming is increasing as recent surveys demonstrate (Pacini et al., 2003; Willer and Yussefi 2006; Lin et al., 2016).

Despite the progress of organic agriculture, there has been a lack of research-based information to address the need for a better understanding of the mechanisms operating in organic farming systems, including plant pest interactions (Zehnder *et al.*, 2007).

The knowledge of these mechanisms becomes crucial when the general stability is disrupted by the introduction of new exotic pests in agricultural systems. This often leads to a greater number of pesticide applications with environmental, safety and economic consequences. Therefore, acquiring knowledge on the natural limiting factors may be a crucial point in the implementation of sustainable pest management strategy.

Applied ecology surveys in agricultural systems are mainly based on the study of the interactions between one antagonist species and one phytophagous insect. More recently several studies have considered all the complex associated in a multitrophic approach, also focusing on the key role played by plants in the interactions by means of the volatile compounds emitted (Heil, 2007).

Multitrophic interaction studies aim at deepening molecular, physiological and behavioral mechanisms involved in the interactions between plants, herbivores and their natural enemies in order to explain, at higher levels, complex processes such as population dynamics and the structure of ecological communities (Vet and Godfray, 2008).

In the biological system we studied, a particular role, is played by the generalist natural enemies which are largely recovered in the newly invaded areas; most of them are generalist idiobiont parasitoids causing low levels of parasitism in open fields (Zappalà et al. 2012a) and other belong to the group of generalist omnivorous predators, that are able to interact at the same time at two different levels of the food chain feeding both on plants and on prey.

1.1. The biological model system: Tuta absoluta *and its indigenous natural enemies in the Mediterranean area*

The South American tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) originating from the Neotropical area, since its introduction in Europe has become one of the most harmful pest of tomato in the whole Mediterranean basin (Urbaneja et al., 2007; Desneux et al. 2010, 2011, Tropea Garzia et al., 2012).
In the newly invaded areas several generalist natural enemies have been reported feeding on *T. absoluta* (Zappalà et al., 2012a; Abbes et al., 2014; Gabarra et al., 2014).
When a new organism is introduced in a new area, generally there can be few indigenous potential natural enemies and they need time to get adapted and effectively control the exotic species given that enemies have greater impact on native than on exotic species (Keane and Crawley, 2002). Some of these exotic species have been recently introduced in Mediterranean area such us *Drosophila suzukii* (Matsumura), *Halyomorpha halys* (Stal) and the above cited *T. absoluta* (Desneux et al. 2010; Asplen et al., 2015; Cesari et al., 2015).
Most of the indigenous natural enemies that may control new exotic pests are generalist and in the case of *T. absoluta* generalist predators and parasitoids have been early collected on tomato crops. The study of some of them is still in progress and others have been employed in biological control programs (Desneux et al., 2010; Gabarra and Arnò, 2010; Urbaneja et al., 2012; Zappalà et al., 2012a; 2013). Among them, a multifunctional role was attributed to *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae), given that it is able to effectively control whiteflies, thrips, leaf

miners, aphids, mites, and lepidopterans (Vacante and Tropea Garzia 1994; Biondi et al., 2013a; Pérez-Hedo and Urbaneja 2015). In Sicily other two interesting natural enemies of *T. absoluta* have been found. Both are ectophagous generalist parasitoids: the eulophid *Necremnus tutae* (Ribes & Bernardo) and the Braconid *Bracon nigricans* (Szépligeti) (Biondi et al. 2013b; Gebiola et al. 2015).

1.1.1 The South American tomato leaf miner: Tuta absoluta

Tuta absoluta is a lepidopteran insect belonging to Gelechiidae family; this moth develops through 4 development stages: egg, larva, pupa and adult (Desneux et al., 2010). The eggs are laid by the females on the stems and on both sides of the leaves, they are yellow 0.2 mm wide and 0.4 mm in length (Urbaneja et al., 2007; Desneux et al. 2010, Tropea Garzia et al., 2012). Young larvae hatching from eggs penetrate into leaves, stems or fruits and they develop through four instars; their size is between 1.6 (first instar) and 8 mm (fourth instar), their color ranges from yellow at the early instars to green with reddish dorsal part at the 4th instar (Urbaneja et al., 2007; Desneux et al. 2010; Tropea Garzia et al., 2012). The mature larvae usually drop to the soil to pupate forming a brown-greenish pupa; the adults are 6-7 mm in length and they have crepuscular and nocturnal habits (Urbaneja et al., 2007, Tropea Garzia et al., 2012).

The tomato borer is native to South America, probably in the areas of Peru and Chile (Siqueira et al., 2000). This moth shows a high reproductive potential, with 10-12 generations per year and a lifecycle of 30 days with 24°C of temperature

(Barrientos et al., 1998; Pereyra and Sanchez, 2006; EPPO, 2005; Vercher et al., 2010).

The species attacks few plants such as the cultivated plants: *Solanum lycopersicum* L., *Solanum tuberosum* L., *Solanum melongena* L., *Solanum muricatum* Aiton, and the weeds: *Solanum nigrum* L., *Solanum lyratum* Thunberg, *Lycopersicon hirsutum* Humb. & Bonpl. and *Datura stramonium* L. (EPPO, 2005; Desneux et al. 2010; Tropea Garzia et al., 2012).

All the larval instars of the tomato leafminer can inflict damages to leaves, stems and fruit both green and ripe, while oviposition is possible on leaves, stems and unripe fruits (Monserrat 2009; Desneux et al. 2010; Tropea Garzia et al., 2012).

The pest from its arrival in Europe in 2006 spread quickly in the whole Mediterranean basin arriving in Africa and Asia (Desneux et al., 2011, Tropea Garzia et al., 2012) reaching India in 2015 (Kalleshwaraswamy, 2015).

The reasons of this fast spread are several, first of all the high reproductive potential of the species (Uchoa-Fernandes et al. 1995; Estay 2000; Vercher et al. 2010), furthermore physical diffusion of the species operated by human activities through trading of tomato fruits (Tropea Garzia et al., 2012) and finally the reduced effectiveness of the indigenous natural enemies in controlling this pest in the newly invaded areas (Zappalà et al., 2012a, 2013).

Moreover, the management of the pest in the field is complicated by the tendency of the pest to develop insecticide resistance toward (Siqueira et al., 2000; Lietti et al., 2005; Haddi et al., 2012; Campos et al., 2015; Roditakis et al., 2015) and by the lack of knowledge about the ecological mechanisms operating in farming systems, which mostly involve the relationships occurring in tritrophic (plant – pest – natural enemies) interactions (Heil 2007; Zehnder *et al.*, 2007).

1.1.2 The generalist eulophid: Necremnus tutae

Necremnus tutae (Ribes & Bernardo) has been recently described by Gebiola et al. (2015) as a species belonging to the entity group of *Necremnus artynes* (Walker). As *N. artynes* it has been initially reported and widely recovered in Italy, Spain, France and Tunisia parasitizing *T. absoluta* larvae (Desneux et al., 2010; Ferracini et al., 2012, Zappalà et al., 2012a, 2013; Abbes et al., 2014; Gabarra et al., 2014) until its correct identification (Gebiola et al., 2015). Also this species has been suggested as potential biological control agent (Urbaneja et al., 2012) especially due to its great rate of host feeding (Ferracini et al., 2012). This Eulophid is the most effective example of an indigenous generalist larval parasitoid adapted to the new invasive pest *T. absoluta* (Urbaneja et al., 2012, Ferracini et al., 2012, Zappalà et al., 2012a; 2013) and some European biocontrol companies have already started the mass rearing and field releases of this species (Urbaneja et al., 2012; Calvo et al., 2013).

Necremnus tutae is a Mediterranean species known as *T. absoluta* parasitoid, while the native host is unknown (Gebiola et al. 2015). The females are 1.7 – 3 mm in length, the head is dark green and the body metallic green with bronze reflection. Males are similar to females except in structure of antennae (ramified in the males), gaster, hyaline wings and for the smaller size (1.2 – 1.9 mm in length) (Gebiola et al. 2015).

The females of *N. tutae* have a longevity of 15 days and they are able to kill and/or parasitize, on average, 4 *T. absoluta* larvae per day per female (Chailleux et al., 2014); the parasitoid can perform a high rate of host feeding (Ferracini et al., 2012, Chailleux et al., 2014, Calvo et al., 2016). The Eulophid can parasitize larvae of *T. absoluta* from the second to fourth instar (Chailleux et al., 2014, Calvo et al., 2016). Larval age at parasitization timeinfluences also the size of emerging adults that can have a huge variation among the population (Chailleux et al., 2014).

1.1.3 The generalist braconid: Bracon nigricans

Bracon nigricans (Szépligeti) is a generalist larval ectoparasitoid of different Lepidoptera recovered for the first time on *T. absoluta* in Sicily in 2010 (Biondi et al., 2013a), in Jordan (Al-Jboory et al., 2012) and in Spain (Gabarra et al., 2014). Its presence was documented in Sardinia on the Gelechiidae *Phtorimaea operculella* Zeller (Ortu and Floris, 1989). The braconid is a Palaearctic species, recorded as generalist larval ectoparasitoid of some Lepidoptera and one coleopteran species (Zappalà et al., 2012a; 2013). Some studies have deepened the biology and behavioral traits of the parasitoid that attacks especially the 4th larval instar of the tomato leafminer, indicating *B. nigricans* as a potential biological control agent of *T. absoluta* in the newly invaded Palaeartic regions (Biondi et al. 2013). The parasitoid females have an average longevity of 40 days and they can produce an offspring of 30 individuals throughout their life. The juvenile survival rate is low on *T. absoluta* (37%). Furthermore, *B. nigricans* females are able to directly kill in addition to those parasitized (host killing) an average of 2 *T. absoluta* larvae per parasitoid female per day during all the female survival period. As consequence, the total rate of mortality of *T. absoluta* 3rd – 4th instar larvae is 70% (30% due to parasitization and 40% to host killing) (Biondi et al. 2013).

1.1.4 The zoophytophagous predator: Nesidiocoris tenuis

Nesidiocoris tenuis (Reuter) (Hemiptera: Miridae) was the first predator recovered on *T. absoluta* in the Mediterranean basin (Urbaneja et al., 2009, Zappalà et al., 2012b). This zoophytophagous predator is able to control several key arthropod pests such as thrips, whiteflies, spider mites, aphids, leafminers and lepidopterans (Calvo et al., 2012; Perez-Hedo et al., 2015). For these reasons, the mirid is released in biological control programs to control the tomato leafminer and other target pests in the Mediterranean basin (Calvo et al. 2012; Zappalà et al. 2012b).

Recently, an increasing interest in mirid predators is emerging both in biological control programs and in ecological surveys, especially due to their great adaptation and great success compared to other more specialized natural enemies (Calvo et al., 2009; Wheeler and Krimmel, 2015). Some of these zoophytopagous predators, such as *N. tenuis*, may, however, induce damage to the crops by feeding on young shoots causing necrotic rings, flower and small fruit abortion. In some environmental conditions (high temperatures, scarcity of preys), they may become, therefore, detrimental rather than beneficial (Sanchez and

Lacasa, 2008; Wheeler and Krimmel, 2015). Other authors have also demonstrated the capability of mirid predators to perform intraguild interactions interfering with other natural enemies (Moreno Ripoll et al., 2012; Moreno Ripoll et al., 2013; Chailleux et al., 2014).

However, apart from the predaceous beneficial effects, the feeding activity of *N. tenuis* on tomato plants was also proven to activate plant defense through hormonal pathways, which made tomato plants less attractive to herbivores and more attractive toward natural enemies (Pérez-Hedo et al., 2015; Naselli et al., 2016). These results on plant response might be an additional explanation for the great success achieved by *N. tenuis* as a key biocontrol agent in tomato crops.

1.2 Trophic interactions in biological control

In the modern conception of pest biological control, ecological strategies based on the knowledge and manipulation of trophic interaction through the three level of food chain are more and more applied (Knight et al., 2005; Zehnder et al., 2007). These interactions, may influence or affect biological control, more frequently, at the level of the relation between natural enemy and herbivore. Furthermore, some recent studies investigated intraguild interferences occurring between natural enemies (Denno and Finke 2006) and the ability of plants to communicate with carnivore consumers at different levels of the food chain (Alba et al., 2012). The knowledge of these interactions becomes more relevant in the case of the invasive species (such as *T. absoluta*), for which a long lasting process of co-evolution with the other components of the system has not occurred, and in the case of zoophytophagous species (such as *N. tenuis*), that are able to interact at two different levels of the food chain (feeding both on plants and on pests).

Furthermore, the ecological success of an invasive phytophagous species is also related to the efficacy of its natural enemies. The reasons of the success of a natural enemy are more frequently searched in the biological traits of the beneficial or in its direct behavior against the target pest. However, considering complexities of food webs among arthropod communities, other elements are worth being investigated, with the aim to improve biological control services.

1.2.1 Intraguild interactions occurring among natural enemies

Some ecological studies have highlighted the importance of the competition occurring among higher level consumers (natural enemies) that share the same prey or host; actually, these so called intraguild interactions phenomena (IGI) can influence the efficacy of biological control (Rosenheim et al., 1995). These kind of competitions may be the main causes of negative impact of exotic natural enemies against native ones (Burgio et al., 2002).

The interactions between natural enemies sharing the same prey/host does not necessarily imply negative consequences for the biological control of a target herbivore (Moreno Ripoll et al., 2013); for instance, among aphid communities the joint action of predators and the parasitoid *Aphidius ervi* (Haliday) (Hymenoptera: Braconidae) increase the control of the aphid *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae), even if the predator preys actively on the parasitoid (Snyder and Ives 2001). The effects of interactions among natural enemies on pest population dynamics has been investigated for example in the case of the two mirids *N. tenuis* and *Macrolophus pygmaeus* Rambur by Moreno Ripoll et al. (2013), who proved that their coexistence could affect the control of *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae) although Intra Guild Predation (IGP) between the two mirids did not occur. In the same paper the authors applied molecular techniques to detect the DNA of the parasitoid in the gut content of the mirids, in order to assess, in field conditions, IGP between the two mirid predators and the endoparasitoid *Eretmocerus mundus* (Mercet) (Hymenoptera: Aphelinidae). This method is a reliable way to conduct these studies in open field ecological surveys.

Many cases of intraguild interferences have been reported on aphids, whiteflies and spider mites communities (Rosenheim et al., 1995; Frago and Godfray, 2014; Moreno Ripoll et al., 2013). These interferences are often direct and they refer to asymmetrical predation of one species (intraguild predator) against another species (intraguild prey) (Fig. 1a), this type of predation is common between a predator and parasitoids (especially immature stages of parasitoids) that share the same food source (Frago and Godfray, 2014; Gkounti et al., 2014). In some other cases the interferences can be indirect and consist in various types of not consumptive competitions between natural enemies, such as predator avoidance behaviors (Lang, 2003; Frago and Godfray, 2014) or Kleptoparasitism (Fig. 1b): this latter occurs when a predator steals food source already procured by competitors (Chailleux et al., 2014).

Some authors tried to develop models in order to predict the effect on an assemblage of natural enemies that practice IGP. These studies demonstrated that the coexistence of two consumers on a single resource occurs only if the intraguild prey species is more efficient than the intraguild predator at suppressing the given pest (Borer et al., 2007).

Intraguild predation in some insect taxa may be related with their evolution history, for instance, the propensity to IGP in heteropteran insects may be due to their non-specialized features (Eubanks et al., 2003; Wheeler and Krimmel, 2015). Indeed, some groups of heteropterans (mirids) are characterized by their zoophytophagy and they have intermediate traits of salivary glands and piercing-sucking mouthparts between herbivores and predators (Eubanks et al., 2003). These not defined morphological traits may lead to cryptic behavioral strategies, such as for example scavenging, that is often present in zoophytophagous heteropterans and that may be considered as a primordial form of predation (Sugiura and Yamazaki, 2006; Wheeler and Krimmel, 2015). In the evolution process, the transition from scavenging to predation could then have passed through intraguild interaction phenomena (Eubanks et al., 2003; Foltan et al., 2005; Sugiura and Yamazaki, 2006; Wheeler and Krimmel, 2015), given that generalist predators do not discriminate between parasitized and healthy prey (Rosenheim et al., 1995; Gkounti et al., 2014).

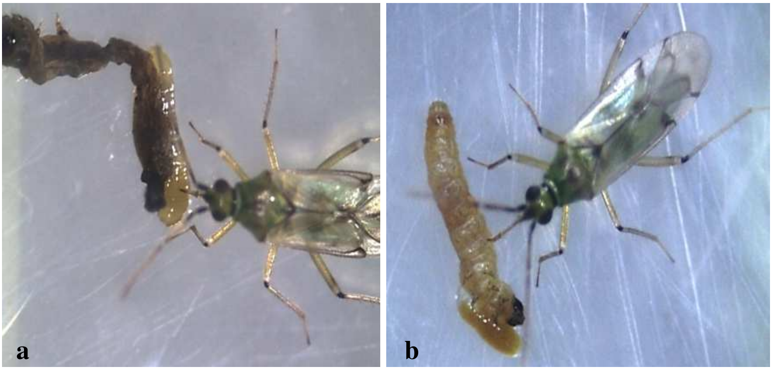

Fig. 1 a) ***Nesidiocoris tenuis*** **performing Intraguild Predation on a** ***Necremnus tutae*** **larva; b)** ***N. tenuis*** **feeding on a parasitized** ***Tuta absoluta*** **larva**

1.2.2 The role of plants in the plant-beneficial insects interactions

It is well known that plants are able to defend themselves from pathogens, arthropods and, in a wider sense, from stressing conditions, activating a cascade of biochemical events that lead direct and indirect defense dynamics.

Indirect defense consists in the production of volatile organic compounds (VOCs), performing a function in the attraction of arthropod natural enemies and repellence of pests (Fig. 2) (Thaler et al., 2002; De Vos et al., 2005; Heil, 2007; Ponzio et al., 2013). This issue has been investigated especially in terms of herbivore induced plant volatiles (HIPVs) (Dicke & Sabelis 1988). Some studies highlight how the HIPV blend is present, in nature, among a multitude of volatiles from other plants, which can influence the responses of insects to HIPVs (Dicke and Baldwin, 2010). Another issue recently investigated is the response of plants to multiple herbivorous attack; Dicke et al. (2009) discussed the preference for the HIPV blends emitted by plants infested by two herbivore species over the blends emitted by plants infested by herbivore species separately. These researches also show the role of the environmental background odor in the attraction of insects and put the plants (target and not target) in the center of these communications across food web (Dicke, 2009).

In order to exploit these capabilities some authors have recently carried out studies to assess the preference of the zoophytophagous *N. tenuis* toward the alternative plants *Dittrichia viscosa* L. (Asteraceae) and *Sesamum indicum* L. (Pedaliaceae) that could become useful banker plants to be introduced in greenhouse conditions and to be used in the management of this zoophytophagous mirid in protected crops (Biondi et al. 2016).

1.2.2.1 Plant signals to communicate with insects

We use the term *plant signals* to refer to all the chemical tools used by plants to communicate with the surrounding environment. In particular, we will consider the communications involving plant and insects.

The plants are able to receive signals, coming from insect such as insect elicitors (Bonaventure et al., 2011) or caused by insect activity such as feeding and/or oviposition (De Vos et al., 2005; Bruessow et al., 2010; Anastasaki et al., 2015; De Backer et al., 2015).

At the same time plants emit VOCs to communicate with insects and their nature also depends on the type of organisms that caused VOCs emission (Dicke 2009; Dicke and Baldwin 2010; Fatouros et al., 2012). This implies that the blend of scents emitted by plants, responding to herbivorous attack, is also affected by herbivore-associated elicitors (HAEs), such as fatty acid–amino acid conjugates (FACs): molecules present in the oral secretions or saliva of lepidopteran larvae and of other insects (Bonaventure et al., 2011) or elicitors present in eggs or egg-cement (De Puysseleyr et al., 2011; Fatouros et al., 2012). Indeed, many plants can distinguish between insect attacks and mechanical damage using these insect signals (Bonaventure et al., 2011). The HAEs involved in this recognition belong to different chemical classes, such as β – glucosidase, sulfur-containing fatty acids, modified forms of lipids (e.g. fatty acid–amino acid conjugates (FACs), cell walls fragments (e.g. pectins and oligogalacturonides) or peptides released from plant proteins digested by pests (Doares et al., 1995; Mattiacci et al., 1995; Alborn et al., 1997; Bergey et al., 1999; Alborn et al., 2007). As a consequence of these signals, plants react activating transcriptome changes of genes involved in the biosynthesis of phytohormones, responsible of direct and indirect defense. The main phytohormones which are involved in these dynamics, as well as in plant growth (Santino et al., 2013), are jasmonates (JA), salicylic acid (SA), and abscisic acid (ABA) (Flors et al., 2008; Ton et al., 2009; Dicke and van Loon, 2014). Distinct feeding insect behavior can trigger different hormone-related signaling pathways; for instance, it is known that sap sucking insects (especially phloem feeders) induce mainly SA-mediated resistance pathways, while chewing insects trigger predominantly JA signaling (Heil, 2007; Coppola et al., 2013; Ponzio et al., 2013). Instead, ABA has been reported both as negative and positive signal for defense, respectively against biotrophic and necrotrophic pathogens (Bari and Jones, 2009). ABA phytohormone seems to be involved in cross talk with both SA and JA signaling pathways; moreover, ABA can affect transcription factors that lead the expression of JA-dependent responses (Flors et al., 2008; Forcat et al., 2008; Bari and Jones, 2009; Santino et al., 2013).

This attitude represents the main evolutionarily conserved mechanism to regulate the expression of direct and indirect defenses toward a wide variety of biotic stressors (Howe and Jander, 2008). Indirect defense especially culminates in the emission of VOCs that attract natural enemies and repel herbivorous insects (Thaler et al., 2002; De Vos et al., 2005; Heil, 2007; Ponzio et al., 2013).

These compounds share the same properties to be easily widespread through air but they belong to different chemical classes.

Composition of VOCs blend can differ depending on plant species, herbivorous species and abiotic conditions (Allmann and Baldwin 2010). The best studied class of plant volatiles attracting natural enemies belong to the group of isoprene-derived terpenoids and fatty acid-derived green leaf volatiles (Dicke and Sabelis 1988, Besser et al., 2009; Allmann and Baldwin 2010, Hassan et al. 2015). These two groups of compounds work differently in the attraction of carnivore insects (Allmann and Baldwin 2010). Terpenoid compounds are released more slowly by plants and on average after one day from herbivorous attack, while Green Leaf Volatiles (GLV), which consist in aldehydes, alcohols and their esters, are released passively from wounded leaves (Dickens, 1999; Kessler and Baldwin, 2001; Allmann and Baldwin 2010; Hassan et al. 2015). For these reasons terpenoids are more related with long distance attraction of insect carnivores, while, GLV represent a rapid but nonspecific information for insects (Dickens, 1999; Kessler and Baldwin, 2001; Allmann and Baldwin 2010; Hassan et al. 2015).

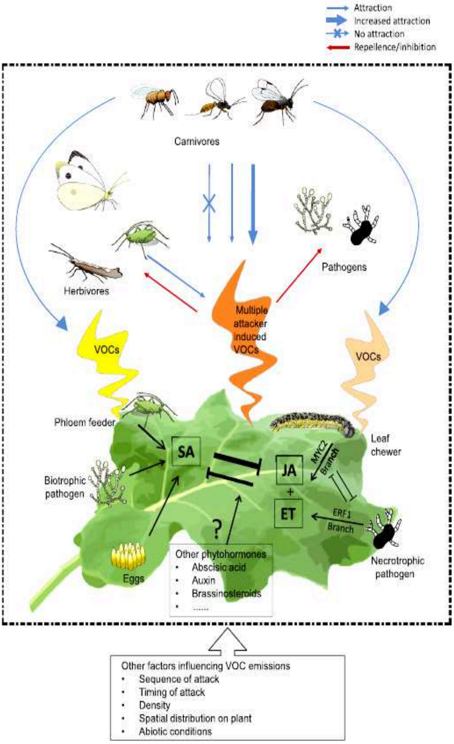

Fig. 2 Volatile organic Compounds (VOCs) emitted by plant as response to different attacks. *Adapted from Ponzio et al., 2013 Funct Ecol*

1.2.2.2 Interactions between tomato plant and insects in Tuta absoluta *food web*

Tomato plants and several species of the solanaceous family are rich in secondary metabolites which are produced by these plants to ensure defense activity towards herbivorous insects thanks to their repellence and insecticide properties. Furthermore, these compounds have a key role in indirect defense as attractant for beneficial insects (Birkett et al., 2004; Bleeker et al., 2009). In tomato plants, many of these compounds are produced and stored in specific structures, performing an important role in plant defenses, called glandular trichomes (Besser et al., 2009, Kang et al., 2010). The set of secondary metabolites produced by tomato plants in glandular trichomes includes a lot of chemicals interacting positively or negatively with insects. The main compounds are acyl sugars, methyl ketones, alkaloids and especially terpenes (Kang et al., 2010). Despite the large amount of defense mechanisms of tomato (Orozco-Cardenas et al. 2001), many organisms, adapted to overcome these defenses, can develop on this crop (Letourneau and Goldstein, 2001; Inbar and Gerling, 2008).

In its areas of origin of Central and South Americatomato co-evolved with a wide group of pests and there are several studies highlighting the great cohort of herbivores and beneficials associated with tomato plants in those areas (Letourneau and Goldstein, 2001; Inbar and Gerling, 2008). Among these organisms, there is the South American tomato leafminer *T. absoluta,* that is one of the most harmful pests of tomato both in its native area and especially in the newly invaded areas (Urbaneja et al., 2007; Desneux et al. 2010, 2011, Tropea Garzia et al., 2012). The species owes its harmfulness to its reproductive potential but probably also to its ecological strategies, based on the favorable interactions existing with and among its natural enemies and with plants. In particular, this last relationship has not been enough investigated, as cause of the great harmfulness of *T. absoluta*.

Few studies demonstrate that *T. absoluta* feeding or ovipositing on tomato plants may cause the induction of plant defenses making plants attractive to predators when they are infested (Lins et al., 2014; Anastasaki et al. 2015; De Backer et al. 2015). In particular, while the trials carried out by Lins et al. (2014) showed that oviposition did not cause attractive effects toward *N. tenuis* and *M. pygmaeus*, Anastasaki et al. (2015) found that the volatile profile of tomato plants infested with *T. absoluta* eggs changed, but only 3 days after oviposition. Moreover, Lins et al. (2014) showed that the terpene β-phellandrene increased significantly in a treatment infested with 20 larvae/plant , while the same compound, interestingly, decreased in the work carried out by Anastasaki et al. (2015) on tomato plants infested with *T. absoluta* eggs compared to healthy plants. This opposite induction could confirm the hypothesis that *T. absoluta* can manipulate tomato defense mechanisms through oviposition as shown in other models (Bruessow et al., 2010).

This richness of volatile compounds may explain the diversity of species developing on tomato plants also in terms of beneficial insects attracted to this crop.

Many of the studies cited above deal with the relations occurring between tomato and *T. absoluta* and the effect of tomato plant on natural enemies behavior (Lins et al. 2014;

Anastasaki et al. 2015; De Backer et al. 2015). In particular, the attraction performed by tomato plants infested with *T. absoluta* larvae toward the mirids *N. tenuis* and *M. pygmaeus* was proved (Lins et al. 2014; De Backer et al. 2015), while no other models, such as *T. absoluta* parasitoids, have been investigated in terms of interactions with tomato plants.

Another interesting issue, worth being investigated, is the interaction between beneficial insects and tomato plant, i.e. the defense response induced by the zoophytophagous predator *Nesidiocoris tenuis* in tomato plants (Pérez-Hedo et al., 2015; Naselli et al., 2016). Recent investigations demonstrated that the feeding activity of *N. tenuis* on tomato plants can activate JA and ABA pathway defenses that cause the attraction of the parasitoid of whitefly *Encarsia formosa* and the repellence toward its host *Bemisia tabaci* (Pérez-Hedo et al., 2015; Naselli et al., 2016).

1.3 Aims

Within this framework, the main goal of my doctoral research was to investigate the ecological factors involved in the success of a newly introduced organism on a domesticated crop considering different level interactions.

In order to study these aspects, a simplified biological model, represented by a crop plant, its pest and its natural enemies, has been developed in the laboratory.

Particularly we wondered how tomato plants and the antagonistic complex associated with *T. absoluta* interact and how the phenomena associated with their interaction could be better understood, to enhance biological control services.

Furthermore, my research intended to complete the ten- year studies carried out in Mediterranean basin on the ecological reasons of the reduced natural control of *T. absoluta* in this area. An other aim was to deepen the knowledge on the multitrophic interaction mechanisms that should allow the sustainable management of the invasive South American tomato pinworm and of its indigenous natural enemies.

To pursue these objectives different phases and levels of investigation have been pointed out; these were implemented in the course of my researches.

The first step was the study of the mechanisms of interaction occurring between the herbivorous insect pest *T. absoluta* and some natural enemies recovered in the new introduction areas through ethological observations and experiments carried out using microcosms under laboratory conditions, with the final aim to investigate if some competition between natural enemies may disrupt biological control of *T. absoluta*.

The second phase was the study of the effects of *N. tenuis* feeding on tomato plant, with the aim to investigate the defense responses induced in tomato by the mirid. These effects were investigated through the analysis of genes and phytohormones involved in defense response and by means of behavioral surveys with olfactometer involving "induced plants", *B. tabaci* and its parasitoid *E. formosa*.

The third line of research was aimed at deepening the effects of tomato plant and potential companion plants on the attraction of the predator *N. tenuis*. In this case, our purpose was to investigate the biochemical bases of this attraction analyzing VOC profiles of plants and correlating these results with olfactory studies.

The general objective of the research (i.e. to obtain useful elements elucidating multitrophic interactions within *T. absoluta* model) was then extended to the applied aim in order to set up conservation biological control practices to protect and enhance specific natural enemies or plant resilience to reduce the effect of pests.

CHAPTER II

Insights on food webs associated with the South American Tomato Pinworm in Europe

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Insights on food webs associated with the South American Tomato Pinworm in Europe

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Abstract

BACKGROUND: Complexity of both natural and managed ecosystems involves various forms of interaction among organisms. Two or more species that exploit the same resource can engage in competitive behaviors, usually referred to as intra-guild interactions. These can be direct, i.e. one species feeds directly upon the competitor (intra-guild predation) or indirect, e.g. when the dominant organism competes for a food source which another organism is feeding on (kleptoparasitism). We investigated the potential for such interactions in a biological model composed by the South American tomato pinworm, *Tuta absoluta*, and three of its newly associated natural enemies: the zoophytophagous predator *Nesidiocoris tenuis*, and two idiobiont ectoparasitoids, *Bracon nigricans*, and *Necremnus tutae*. RESULTS: *Nesidiocoris tenuis* showed (i) to scavenge on parasitized *T. absoluta* larvae, and (ii) to directly attack and feed on larvae of both parasitoid species, although at a higher percentage in the case of *N. tutae*. In the presence of the host plant the predator reduced the emergence of both *B. nigricans* and *N. tutae* adults significantly. CONCLUSION: This study stresses the ecological success of a generalist predator over indigenous parasitoids attacking an invasive pest. Besides, these findings provide potential elements to better design biological control programs against *T. absoluta*.

Keywords: Intraguild interactions, Mirid predator, *Bracon nigricans, Necremnus tutae,* Tomato borer, trophic networks

1. INTRODUCTION

Among the various factors that can influence the spread and the invasion speed of an exotic pest, the efficiency of the complex of fortuitous natural enemies can play a key role. Commonly, the most investigated aspects to evaluate the efficacy of these beneficials are the basic biological traits and their ecological services in controlling the pest. However, the resulting food webs are not so simple, and recently several studies focused on the competition occurring among higher level consumers that share the same prey or host.[1,2] Indeed, these intraguild interactions (IGI) can influence the efficacy of biological control agents.[3,4] For this reason, some authors developed theoretical models aimed at predicting the consequences of the activity of natural enemies with intraguild predation (IGP) attitude on the natural enemy communities. These studies mainly support the idea that coexistence of two consumers on a single resource occurs only if the intraguild prey species is more efficient than the intraguild predator at suppressing the given pest.[5] Many cases of IGI have been reported for aphids, whiteflies and spider mite communities.[3,6,7] These interferences are often direct and they refer to asymmetrical predation of one species (intraguild predator) on another species (intraguild prey); this type of interference (unidirectional) is common from predator towards parasitoids (especially immature stages) that share the same food source.[6,8] In some other cases the interferences can be indirect and consist in various types of non-consumptive competitions between natural enemies,[9,10,6,11] such as predator avoidance behaviors or kleptoparasitism (KLP), this latter occurring when a predator steals food source already exploited by competitors.[12]

Intraguild predation between predators and parasitoids often occurs because generalist predators do not discriminate between parasitized and unparasitized prey.[3,13,8] This behavior is enhanced when the generalist predator practices scavenging, feeding on dead prey.[14,15] Some beneficial arthropods have developed morphological and behavioral strategies to limit IGP phenomena, e.g. parasitoids that cause the formation of mummies on parasitized aphids as a strategy to avoid the consumption of parasitized host by generalist predators, selfparasitism or hyperparasitoids.[3,16,17] Examples of behavioral strategies consist in the avoidance by parasitoids (intraguild prey) of the patches visited by a potential intraguild predator.[6]

The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera:Gelechiidae), was recorded for the first time in Europe in 2006, in Eastern Spain. After a fast invasion this pest caused heavy losses on tomato crops in the whole Mediterranean basin, Middle East, Central Europe and Subsaharian Africa.[18,19,20,21] In these areas, several native natural enemies feeding on *T. absoluta* have been reported.[22,23] Among these, *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) was the first predator species recovered.[24,25] This zoophytophagous generalist predator is known as a control agent of several pests such as thrips, whiteflies, spider mites, aphids, leafminers and moths.[26,27] Moreover, *N. tenuis* has proved to trigger plant defense mechanisms through its feeding activity, giving a further contribution in reducing pest development.[28,29] Recently, an increasing interest on mirid generalist predators is emerging for both ecological and applied reasons, especially due to their great adaptation and widespread success compared to other more specialized natural enemies.[30,15] Despite their efficacy as biocontrol agents, some of these zoophytopagous predators, such as *N. tenuis*, may cause damage to the crops, due to their feeding activity on young shoots, flowers and fruits, becoming, in some environmental conditions, detrimental rather than beneficial.[31,15,32] Besides, negative intraguild interactions between mirid predators and other natural enemies have been reported.[33,7,12,13]

Bracon nigricans (Szépligeti) (Hymenoptera: Braconidae) is a generalist larval ectoparasitoid of Lepidoptera species recovered for the first time on *T. absoluta* in Italy in 2010 and afterwards in other Western Palaearctic countries.[34,22] The biological and behavioral traits of the parasitoid, that attacks mainly 4th instar *T. absoluta* larvae, indicate it as a potential biological control agent of the borer in the newly invaded Palaearctic regions.[35] *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) has been recently described by Gebiola et al. as an entity belonging to the species group of

Necremnus artynes (Walker) that has been widely recovered in Italy, Spain, France and Tunisia feeding on *T. absoluta*.[36,25,22,23] The biological control applications of this species have been recently debated because of contrasting results in laboratory conditions, with high rates of host killing, and in protected tomato crops, where relatively low parasitization rates were recorded.[37,34,38]

In this framework, the present study aims (i) to investigate if potential asymmetrical intraguild interaction between the mirid *N. tenuis* and the two parasitoids *N. tutae* and *B. nigricans* may occur and (ii) to further characterize the mirid feeding behavior in a multitrophic system. The outcomes of the competitive behaviors of the three natural enemies are discussed in an ecological perspective as well as for their applied consequences in terms of biocontrol efficacy of the invasive pest *T. absoluta*.

2. EXPERIMENTAL METHODS

2.1. Insect rearing

Tuta absoluta, *N. tenuis*, *B. nigricans* and *N. tutae* colonies were reared at the laboratories of the department of Agriculture, Food and Environment of the University of Catania (Italy). The colonies of *T. absoluta* and *N. tenuis* were started from specimens collected in tomato crops in southeastern Sicily (Ragusa and Siracusa provinces, Italy), and they were reared under laboratory conditions (25 ± 1˚C, 50 ± 10 % RH and 16:8 L:D) following the procedures described by Zappalà et al. and Biondi et al.[34,32] *Nesidiocoris tenuis* females used in the experiments were starved for 48 h in plastic transparent tubes (ø 15 mm, h 60 mm) plugged with wet cotton to supply water.

Bracon nigricans and *N. tutae* colonies originated from field collections in Catania and Ragusa province (Sicily, Italy). Both parasitoid species were reared following the methodology described in Biondi et al.[35] To obtain newly emerged adults, 150-mm Petri dishes ventilated by a 4-cm^2 opening covered with a fine mesh net were used. Five females and ten males were introduced inside the Petri dish containing tomato leaves infested by *T. absoluta* 3rd-4th and 2nd-3rd instar larvae for *B. nigricans* and *N. tutae* respectively. Honey droplets were provided as food source for the adult parasitoids. Thirty larvae were supplied (6 larvae/female) for 3 days, then the parasitoids were provided new hosts. Once emerged, the adult parasitoids to be used for the experiments were isolated using the same rearing arena and they were experienced on *T. absoluta* one week exposing the adults to T. absoluta larvae before starting the experiments. All the parasitoid females were used 7-10 days after emergence. The immature parasitoids were collected from parasitized larvae of *T. absoluta* taken from the rearing; the mines were carefully opened with the help of a cutter and a pair of tweezers.

2.2 Experimental setup

All the experiments were carried out using two different insect species combinations: (i) *T. absoluta, N. tenuis* and *B. nigricans*, (ii) *T. absoluta*, *N. tenuis* and *N. tutae.* For each combination, behavioral and microcosm trials were carried out, replicating the same experiment for both food-web models (*B. nigricans* and *N. tutae*). All the experiments were conducted at 25 ± 1°C, 50 ± 10 % RH and 16:8 L:D.

2.3 Behavioral trials

Direct behavioral observations were performed in a 2.5 cm-diameter arena, observing *N. tenuis* activity on parasitized larvae of the tomato pinworm under a stereomicroscope (EZ4® Leica), during 30 minutes in a no-choice experiment. The aim of the trial was to assess whether starved females of the mirid were able to feed on parasitized larvae of *T. absoluta* (IGI - KLP) or to feed directly upon the parasitoid instars performing IGP. One parasitized larva of *T. absoluta* was exposed to one *N. tenuis* female comparing the following treatments: i) parasitized *T. absoluta* larva bearing one egg of the parasitoid (either *B. nigricans* or *N. tutae*); ii) parasitized *T. absoluta* larva bearing one larva of the parasitoid (either *B. nigricans* or *N. tutae*). The number of KLP and IGP events were recorded. Twenty replicates for each treatment were performed.

2.4 Microcosm trials

The indirect observations were conducted reproducing in the lab the tri-trophic system including, in addition to each of the two models described above, the tomato plant. The experimental arena consisted in a plastic cup (400 cm^3), superposed to another one serving as water supply for a tomato sprout infested by 10 *T. absoluta* larvae of 2nd – 3rd or 4th instar, suitable, respectively, for *N. tutae* or *B. nigricans*.[39] In the same arena, one female and one male of either *B. nigricans* or *N. tutae* were released. After 72 hours they were removed and one *N. tenuis* female, starved for 48 hours, was released and left in the arena for 48 hours. Four treatments, each replicated 16 times for each model, were compared: i) *T. absoluta* control (only the herbivore); ii) Parasitoid control (the herbivore + one of the two parasitoid species); iii) *N. tenuis* control (the herbivore + the omnivorous predator); iv) *T. absoluta* + Parasitoid + *N.*

tenuis (the herbivore + one of the two parasitoid species + the omnivorous predator). The number of *T. absoluta* and parasitoid adults emerged in each treatment was recorded in the following three weeks after the release.

2.5 Data analysis

Data on the frequencies of KLP and IGP generated in the behavioral bioassays were analyzed using a Generalized Linear Model (GLM), where the three indipendent factors were: the *parasitoid species*, the *parasitoid stage* (egg or larvae) and their *interaction*. Whereas, in microcosm trials, the data (number of parasitoid emergences and percentages of *T. absoluta* mortality), were first tested for normality and homogeneity of variance using the Kolmogorov-Smirnov D test and the Cochran test, respectively, and were transformed when necessary. Then factorial ANOVAs were carried out considering as independent factors the parasitoid presence, predator presence, parasitoid species and all their two-way interactions. Tukey HSD post-hoc analyses were conducted for multiple comparisons of the means.

3. RESULTS

3.1 Behavioral trials

In direct behavioral observations the attitude of *N. tenuis* to feed on larvae of *T. absoluta* parasitized by *B. nigricans* or *N. tutae* was widely recorded. Parasitized caterpillars bearing parasitoid larvae were fed on by mirids in 55% of the observations in the *B. nigricans* treatment and in 75% of the observations in the *N. tutae* treatment (Figs. 1 and 2). Parasitized caterpillars bearing parasitoid eggs were fed on by mirids in 50% of the observations in the *B. nigricans* treatment, but only 30% of the observations in the *N. tutae* treatment (Figs. 1 and 2). We also observed intraguild predation of parasitoid larvae: 10% of *B. nigricans* and 40%

of *N. tutae* larvae were fed on by mirids. Conversely, the mirid was never observed preying on any parasitoid egg (Figs 1, 2). Analyzing these results with GLM, it emerged that the factors "parasitoid species" ($F_{1,79}$= 7.364; P=0.008) and "parasitoid stage" ($F_{1,79}$= 29.457; P<0.001), but not their interaction ($F_{1,79}$= 0.295; P=0.589), significantly influenced the KLP frequency. Referring to IGP, the factors parasitoid species ($F_{1,79}$= 8.941; P=0.004) and parasitoid instars ($F_{1,79}$= 20.118; P<0.001) significantly influenced the IGP frequency. The interaction of these two factors was also significant ($F_{1,79}$= 8.941; P=0.004).

3.2 Microcosm trials

The experiments conducted in microcosms showed that *N. tenuis* influenced the juvenile development of both *B. nigricans* and *N. tutae.* The emergence of adult parasitoids without the release of *N. tenuis* was 1.88/cup for *B. nigricans* and 1.81/cup for *N. tutae*, while the rate of emergence was 0.88/cup for *B. nigricans* and 0.63/cup for *N. tutae* when *N. tenuis* was released (Fig. 3). The differences in the rates of parasitoid emergence between the treatment with only the parasitoid (parasitoid control) and the treatments where one of each parasitoids and the mirid were released (Intraguild treatment), were significant. Indeed, the *predator presence* affected parasitoid emergence ($F_{1,76}$= 20.671, P<0.001) significantly. Besides, the *parasitoid species* had no effects on emergence values both in parasitoid control treatments, ($F_{1,76}$= 0.047, P=0.829), and in the intraguild treatments (Fig. 3), i.e. the *predator presence* affected parasitoid emergence regardless of the *parasitoid species* (*predator presence X parasitoid species*: $F_{1,76}$= 0.187, P=0.666).

The experimental mortality in *T. absoluta* control treatment (only the herbivore) averaged 38% and 21% in *N. tutae* and *B. nigricans* trials, respectively. Overall, the host/prey mortality varied significantly depending on the *parasitoid species* ($F_{1,120}$= 13.116, P<0.001), being always higher in the trials with *N. tutae* (Fig. 4). Furthermore, the *parasitoid presence* factor ($F_{1,120}$= 12.697, P=0.001), but not its interaction with the *parasitoid species* ($F_{1,120}$= 0.491, P=0.485), influenced *T. absoluta* survival significantly; i.e., both parasitoids increased the mortality of *T. absoluta* larvae (Fig. 4). By contrast, the *predator presence* factor per se did not affect *T. absoluta* mortality ($F_{1,120}$= 0.768, P=0.383) (Fig. 4). From the analysis of predator effects emerged that the presence of the predator per se did not affected *T. absoluta* mortality indifferently from the instar of larvae ($F_{1,120}$= 1.106, P=0.295), furthermore *T. absoluta* mortalities were not affected by the joint presence of the predator and parasitoid ($F_{1,120}$= 0.167, P=0.683) (Fig. 4).

4. DISCUSSION AND CONCLUSIONS

The data obtained from the direct behavioral observations highlight the prevailing scavenging behavior (necrophagy) of *N. tenuis*. Moreover, we found that *N. tenuis* may alternatively perform IGP on larvae of ecto-parasitoids, probably due to its generalist behavior, confirming potential intraguild interference as a feature typical of polyphagous predator communities.[40,9]

The mirid predator preferred decayed prey (already killed and partially consumed by parasitoid larvae) rather than live ones. This opportunistic behavior may be explained with the trophic strategy of mirids that digest prey extraorally,[15] thus, consuming decayed prey, they obtain already decomposed nutrients ready to be acquired. The role of scavenging and polyphagy in heteropteran insects may be explained as an evolutionary transition from herbivory to carnivory. Actually, some recent studies demonstrate that mirids visit more frequently host plants (whereon they feed) which are able to attract and kill, through sticky exudates, a wide range of insects which are used as nitrogen source.[14,15] The tendency to perform necrophagy affected, in our trials, the development of both parasitoids. Indeed, kleptoparasitism carried out by the mirid (Figs 1 and 2), reduced the performance of both ectoparasitoids. However, while in the work by Chailleux et al. the mirid *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) was never observed preying on juveniles of the ectoparasitoid *Stenomesius japonicus* (Ashmead) (Hymenoptera: Eulophidae),[12] in our experiment *N. tenuis* reduced the emergence of the parasitoids, particularly *N. tutae*, also due to IGP. The differences in IGP carried out on *B. nigricans* (lower) and *N. tutae* (higher) may be explained with the higher motility and size of the braconid larva compared to the eulophid one. Furthermore, *N. tenuis* did not perform any predation on the tested ectoparasitoid eggs, differently from what we expected considering that the mirid prefers immobile prey such as, for example, eggs of moths or nymphs of whiteflies.[24,41,42] This may be due to an avoidance behavior of the parasitoid female that marks the egg with deterrent chemicals,[43,44] which may decrease or disappear in developing larvae. Further specific investigations are thus needed to confirm or reject this hypothesis. In similar intraguild studies, *N. tenuis* was observed performing food web interferences in other biological systems. Moreno-Ripoll et al. detected IGP between the predators *N. tenuis* and *M. pygmaeus* toward the endophagous parasitoid *Eretmocerus mundus* (Mercet) feeding on *Bemisia tabaci* (Gennadius) (Hemiptera; Aleyrodidae);[7] Cabello et al. showed that *N. tenuis* interfered with the egg-parasitoid *Trichogramma achaeae* Nagaraja and Nagarkatti,[46] feeding on parasitized *T. absoluta* eggs. In other studies, IGP between *N. tenuis* and *M. pygmaeus* was observed.[33,45] Thus, our work confirms the results obtained in other studies on the intraguild predation behavior of the zoophytophagous mirid, although we highlight for the first time the evidence that *N. tenuis* may carry out IGP upon an ectoparasitoid feeding on *T. absoluta*.

The results obtained may be useful in better designing biological control programs involving together a predator and a parasitoid, setting up release strategies available to avoid the possible detrimental effect due to the joint use of two different natural enemies on the biological control of the target pest. Particularly, comparing the two different parasitoid models (*B. nigricans* and *N. tutae*) we can speculate that, despite *B. nigricans* appears less efficient compared to *N. tutae* in controlling *T. absoluta,* the eulophid wasp is potentially more exposed to IGI phenomena. This is prompted by i) our data on parasitoid emergence in microcosm; ii) the higher rate of IGP carried out by *N. tenuis* on *N. tutae* compared to *B. nigricans*; iii) the susceptible pest instar shared by *N. tenuis* and *N. tutae;* iv) the location in the upper part of the plant common to *N. tenuis* and *N. tutae* (*B. nigricans* attacks larger larvae commonly located in fully developed leaves in the lower portion of the plant); v) the smaller size of larvae attacked by *N. tutae* which may not provide enough food to support 2 consumers. Nevertheless, despite intraguild interference phenomena may occur, we can predict a possible coexistence of *N. tenuis* and *N. tutae* under field conditions. Indeed, *N. tutae* (IGP prey) appears more efficient than *N. tenuis* (IGP predator) at suppressing the basal resource (*T. absoluta* young larvae).[5]

Besides, further investigations are needed to verify whether the joint use of *N. tenuis* and *N. tutae*, given the high level of host feeding performed by the eulophid parasitoid and *N. tenuis* tendency to scavenge,[37] may give a support in the settlement of the mirid under real field conditions. Moreover, field experiments carried out involving molecular methods,[47,2] may be helpful for gaining more evidences on such trophic interactions and to confirm or reject our laboratory results and deriving hypotheses.

Author contributions

Conceived and designed the experiments: MN AB ND GS LZ. Performed the experiments: MN. Analyzed the data: MN. Contributed reagents/materials/analysis tools: GS LZ. Wrote the paper: MN LZ. Provided access to the facilities: AR GS LZ. Revised the manuscript: MN AB LZ GS. Reviewed the final manuscript: MN AB GTG ND AR GS LZ.

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Figure legends

Fig. 1. ***Kleptoparasitism performed by Nesidiocoris tenuis*** **on** ***Necremnus tutae and Bracon nigricans*****.** Frequencies of feeding events on parasitized larvae of *T. absoluta.* Data are expressed as mean percentage of the cases in which KLP occurred in direct observations(± SE). Different letters indicate significant differences (*P*<0.05) between parasitoid species .

Fig. 2. ***Intraguild Predation performed by Nesidiocoris tenuis*** **on** ***Necremnus tutae and Bracon nigricans*****.** Frequencies of feeding events performed by *N. tenuis* on *parasitoid* immature instars. Data are expressed as mean percentage of the cases in which IGP occurred in direct observations (± SE). Different letters indicate significant differences (*P*<0.05) between parasitoid species.

Fig. 3. ***Parasitoid emergence under Intraguild Interaction risk*****.** Mean numbers (± SE) of emerged

parasitoids (*N. tutae* and *B. nigricans*) per each microcosm. Data refer to (i) *parasitoid control*, i.e., a treatment including only the parasitoid of one of the two species (dark bars), and to (ii) *Intraguild treatment* including the parasitoid and the predator, *N. tenuis* (white bars). Within each parasitoid species, different letters indicate significant differences (*P*<0.05) between treatments using factorial ANOVA.

Fig. 4. Tuta absoluta *biological control levels under attack by different combinations of natural enemies.* Mean percentages (± SE) of *Tuta absoluta* mortality obtained in microcosm trials, data refer to four treatments: i) only the parasitoid, ii) only *N. tenuis*, iii) one of the two parasitoid species and *N. tenuis,* iv) *T. absoluta* control (for *N. tutae* and for *B. nigricans*). Dark bars refer to *N. tutae* and white bars to *B. nigricans* microcosm trials. Within each parasitoid species, columns bearing different letters (upper case: *N. tutae*; lower case: *B. nigricans*) are significantly

different (Tukey HSD post hoc test for multiple comparisons at *P*<0.05).

Fig. 1

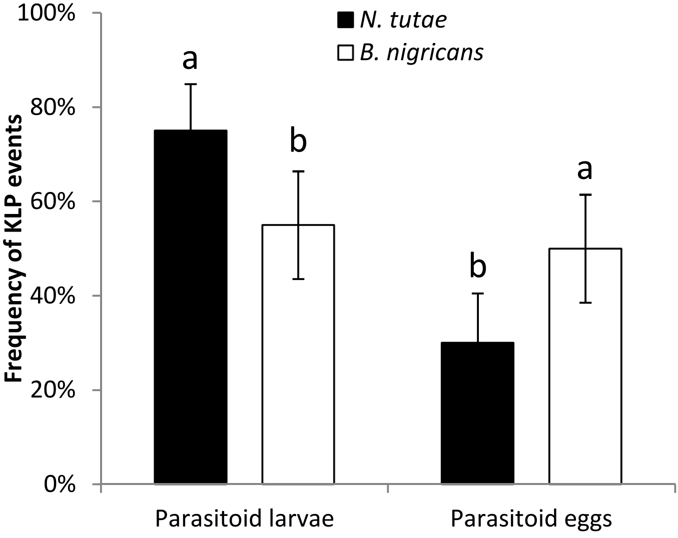


Fig. 2

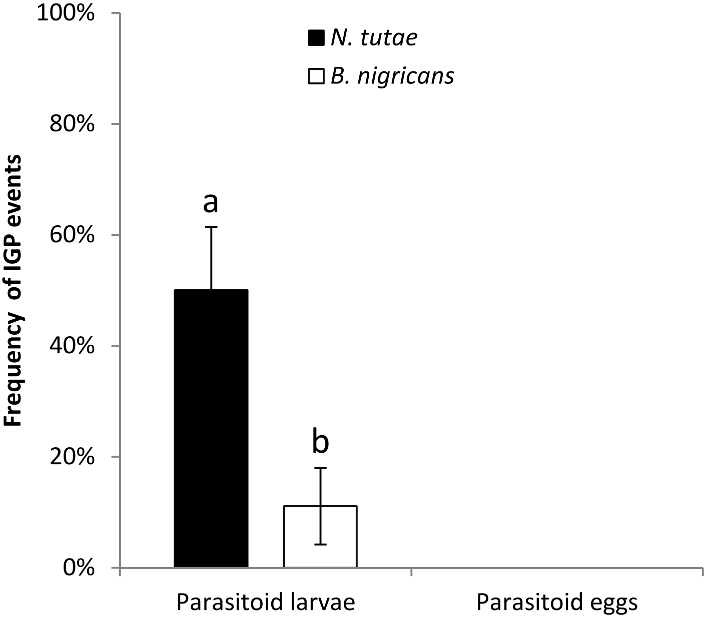

100%
80%
60%
40%
20%
0%
Frequency of IGP events
■ *N. tutae*
□ *B. nigricans*
a
b
Parasitoid larvae
Parasitoid eggs

Fig. 3

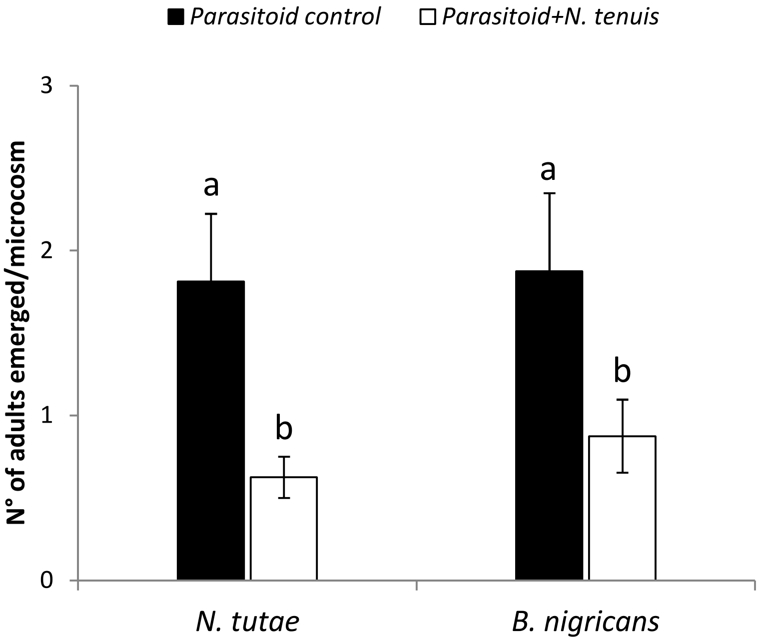
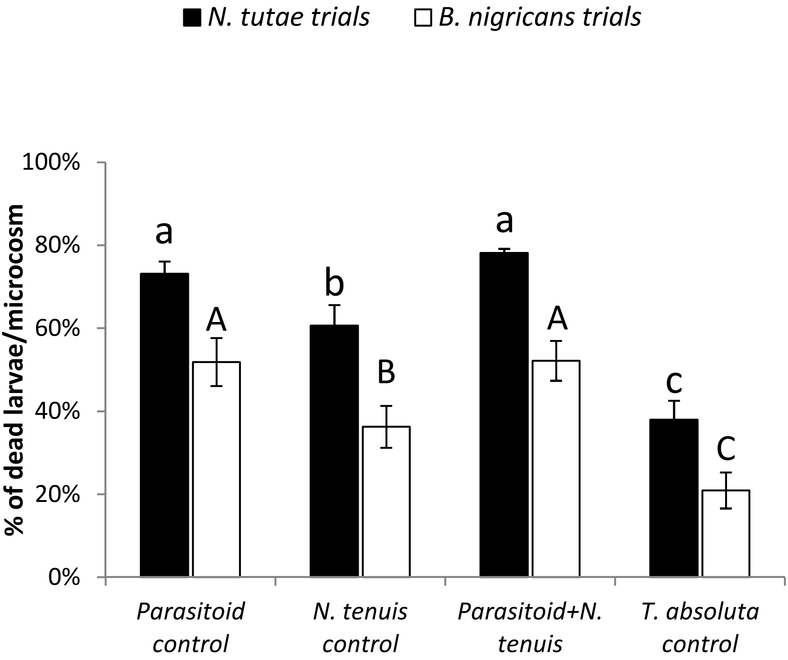

■ Parasitoid control
□ Parasitoid+N. tenuis
N° of adults emerged/microcosm
3
2
1
0
a
b
a
b
N. tutae
B. nigricans

Fig. 4


■ N. tutae trials
□ B. nigricans trials
100%
80%
60%
40%
20%
0%
% of dead larvae/microcosm
a
A
b
B
a
A
c
C
Parasitoid control
N. tenuis control
Parasitoid+N. tenuis
T. absoluta control

CHAPTER III

Stage-Related Defense Response Induction in Tomato Plants by *Nesidiocoris tenuis*

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Article

Stage-Related Defense Response Induction in Tomato Plants by *Nesidiocoris tenuis*

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Abstract: The beneficial effects of direct predation by zoophytophagous biological control agents (BCAs), such as the mirid bug *Nesidiocoris tenuis*, are well-known. However, the benefits of zoophytophagous BCAs' relation with host plants, via induction of plant defensive responses, have not been investigated until recently. To date, only the females of certain zoophytophagous BCAs have been demonstrated to induce defensive plant responses in tomato plants. The aim of this work was to determine whether nymphs, adult females, and adult males of *N. tenuis* are able to induce defense responses in tomato plants. Compared to undamaged tomato plants (*i.e.*, not exposed to the mirid), plants on which young or mature nymphs, or adult males or females of *N. tenuis* fed and developed were less attractive to the whitefly *Bemisia tabaci,* but were more attractive to the parasitoid *Encarsia formosa*. Female-exposed plants were more repellent to *B. tabaci* and more attractive to *E. formosa* than were male-exposed plants. When comparing young- and mature-nymph-exposed plants, the same level of repellence was obtained for *B. tabaci,* but mature-nymph-exposed plants were more attractive to *E. formosa*. The repellent effect is attributed to the signaling pathway of abscisic acid, which is upregulated in *N. tenuis*-exposed plants, whereas the parasitoid attraction was attributed to the activation of the jasmonic acid signaling pathway. Our results demonstrate that all motile stages of *N. tenuis* can trigger defensive responses in tomato plants, although these responses may be slightly different depending on the stage considered.

Keywords: *Bemisia tabaci; Encarsia formosa;* tomato; induced plant response; indirect defense; phytohormones

1. Introduction

Plants are able to defend themselves from arthropods, pathogens and, in general, from biotic and abiotic stress conditions [1–3]. To this end, plants activate a cascade of events that include transcriptome changes of some of the genes involved in the biosynthesis of phytohormones that lead—directly and indirectly—to defensive responses [4,5]. The main phytohormones responsible for these responses are jasmonic, salicylic, abscisic acids, and ethylene (JA, SA, ABA, and ET respectively) [1–3,6–8]. Depending on the herbivore’s feeding habits (chewing, phloem, or cell content feeders), different hormone-related signaling pathways are triggered [9–13]. For instance, it is known that insects with piercing-sucking mouthparts (especially phloem feeders like most of the Hemiptera) mostly induce the SA-mediated resistance pathway, whereas insects with chewing mouthparts predominantly trigger the JA pathway [14–16]. However, JA may also be induced by cell content feeders such as thrips (Thysanoptera: Thripidae) and spider mites (Acari: Tetranychidae) [8].

Zoophytophagous arthropods—which feed both on other arthropod as prey and on plants during the same developmental stages—can also activate the same defense mechanisms as strict herbivores [17–21]. It is well-known that zoophytophagy provides adaptive advantages, such as the ecological flexibility to consume both prey and plants, thereby allowing the survival of these predators on plants when prey is scarce [22–24]. This is the case for *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae), a widely used biological control agent (BCA) which has been extremely effective in controlling some key tomato pests, including the tobacco whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) and the invasive South American pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) [25–27]. Apart from this predation-dependent beneficial effect, the activity of *N. tenuis* females (feeding and/or oviposition) on tomato plants activates the ABA and JA pathways, which make tomato plants less attractive to phytophagous *B. tabaci* and more attractive to the whitefly parasitoid *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae), respectively [21]. In addition, herbivore-induced plant volatiles (HIPVs) from *N. tenuis*-exposed plants can induce plant defenses in neighboring, undamaged (not exposed to the mirids) plants via JA, which result in the attraction of parasitoids [21]. These effects on plant defensive responses might be a reasonable explanation of the achievement reached by *N. tenuis* in integrated pest management programs in tomatoes.

Pérez-Hedo, *et al.* [20] showed that the females of three different zoophytophagous BCAs (*N. tenuis, Macrolophus pygmaeus* Rambur, and *Dicyphus maroccanus* Wagner) differ in their ability to induce defensive responses in tomato plants, resulting in varying degrees of attractiveness of the plants to pests and natural enemies. In the case of tomato plants exposed to and therefore presumably punctured by *N. tenuis*, these plants were less attractive to the whitefly *B. tabaci* and to the lepidopteran *T. absoluta*. In contrast, tomato plants exposed to *M. pygmaeus* and *D. maroccanus* were not able to repel *B. tabaci* and, more interestingly, became more attractive to *T. absoluta*. Pappas, *et al.* [28] showed that tomato plants exposed to adult females, fifth instar nymphs, and young virgin females of *M. pygmaeus* were able to induce plant resistance against the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae). In tomato plants infested by *T. urticae*, the number of *T. urticae* eggs laid was lower when these tomato plants had been exposed previously to *M. pygmaeus* [28].

To date, it has been shown only that the feeding and oviposition activities of *N. tenuis* adult females induce defensive plant responses in tomato plants [20,21]. Nevertheless, under field conditions, it is usually common to find a mix of instars and/or stages of this and other mirids [29]. To know whether nymphal instars and males are also able to induce defensive plant responses, in this work we evaluated the response induced by the feeding (plus oviposition in the case of adult females) activity of different instars/stages of *N. tenuis* compared to undamaged plants. This response was assessed by means of behavioral bioassays in a Y-tube olfactometer using adults of the herbivore *B. tabaci* and of the entomophagous parasitoid *E. formosa*. In addition, ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) and quantitative gene expression of selected phytohormones and genes, were analyzed.

2. Results

2.1. Olfactory Responses Induced by N. tenuis*-Punctured Plant*

When plants exposed to young (NI and NII) or mature nymphs (NIV and NV) of *N. tenuis* were compared to undamaged plants (*i.e.*, not exposed to the mirid), a clear preference of *B. tabaci* for undamaged plants was observed ($\chi^2 = 5.000$, $p = 0.0253$ and $\chi^2 = 7.200$, $p = 0.0073$, respectively, Figures 1a and 2a).

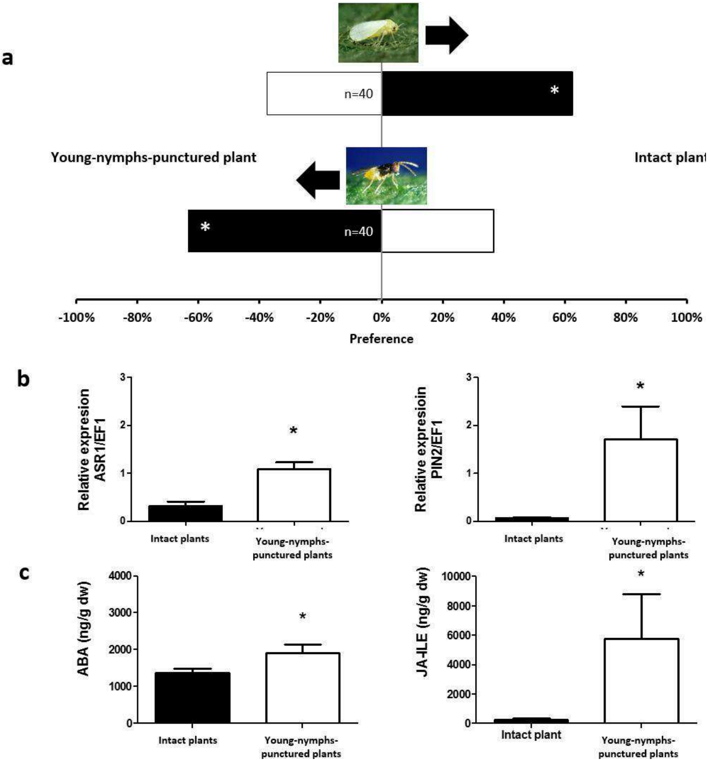

Figure 1. Plant responses induced by young *N. tenuis* nymphs. (**a**) Response of *B. tabaci* and *E. formosa* females when they were given the choice between intact tomato plants and punctured tomato plants in a Y-tube olfactometer. Significant differences using χ^2 test, $p < 0.05$ are marked with (*); (**b**) Expression of the defensive genes *ASR1* and *PIN2* (target genes induced by the phytohormones abscisic acid (ABA) and jasmonic acid-isoleucine (JA-ILE), respectively). Data are presented as the mean of the ratio between the concentration of the gene transcripts and that of the constitutive elongation factor 1 (*EF1*) gene. Significant differences were obtained by

comparing punctured plants to intact plants. Results from a one-tailed *t*-test are marked with (*) ($p < 0.05$); (**c**) ABA and JA-ILE levels in the apical part of tomato plants. Each of the presented results is the mean of the hormone concentration (ng/g) of five independent analyses ± SE ($n = 5$). Significant differences were obtained by comparing punctured plants to intact plants. Results from a one-tailed *t*-test are marked with (*) ($p < 0.05$).

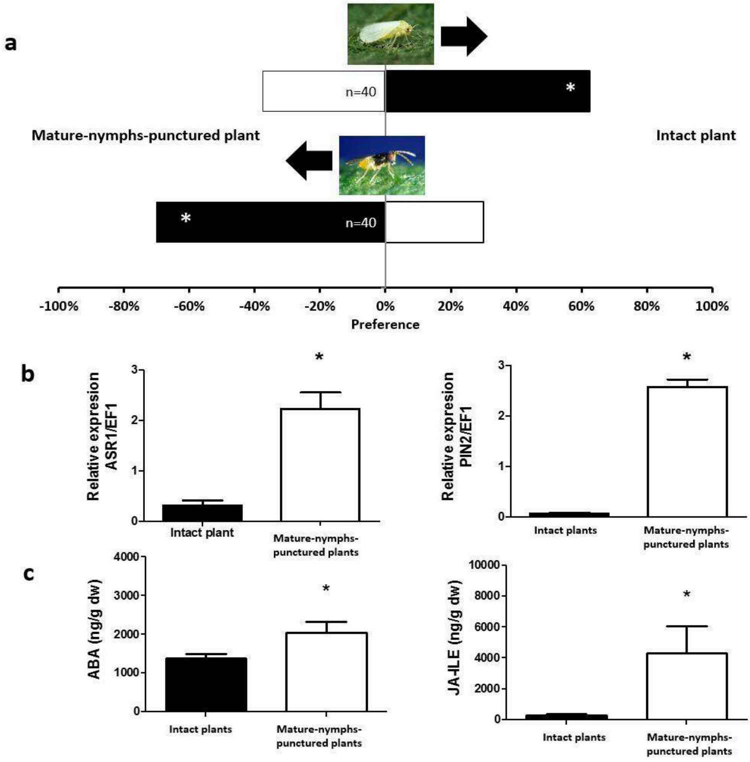

Figure 2. Plant responses induced by mature *N. tenuis* nymphs. (**a**) Response of *B. tabaci* and *E. formosa* females when

were given the choice between intact tomato plants and punctured tomato plants in a Y-tube olfactometer. Significant differences using χ^2 test, $p < 0.05$ are marked with (*); (**b**) Expression of the defensive genes *ASR1* and *PIN2* (target genes induced by the phytohormones ABA and JA-ILE, respectively). Data are presented as the mean of the ratio between the concentration of the gene transcripts and that of the constitutive *EF1* gene. Significant differences were obtained by comparing punctured plants to intact plants. Results from one tailed *t*-test are marked with (*) ($p < 0.05$); (**c**) ABA and JA-ILE levels in the apical part of tomato plants. Each of the presented results is the mean of the hormone concentration (ng/g) of five independent analyses ± SE ($n = 5$). Significant differences were obtained by comparing punctured plants to intact plants. Results from a one tailed *t*-test are marked with (*) ($p < 0.05$).

Similarly, undamaged tomato plants were more attractive for *B. tabaci* than tomato plants exposed to either adult females or males of *N. tenuis* ($\chi^2 = 9.800$, $p = 0.0017$ and $\chi^2 = 3.951$, $p = 0.0468$, respectively, Figures 3a and 4a). Unlike *B. tabaci,* all plants exposed to the mirids were found to be more attractive to the parasitoid *E. formosa* than to undamaged plants ($\chi^2 = 4.267$, $p = 0.0389$ for young nymphs—Figure 1a; $\chi^2 = 9.600$, $p = 0.0019$ for mature nymphs—Figure 2a; $\chi^2 = 6.898$, $p = 0.0086$ for adult males—Figure 3a; and $\chi^2 = 11.640$, $p = 0.0006$ for adult females—Figure 4a).

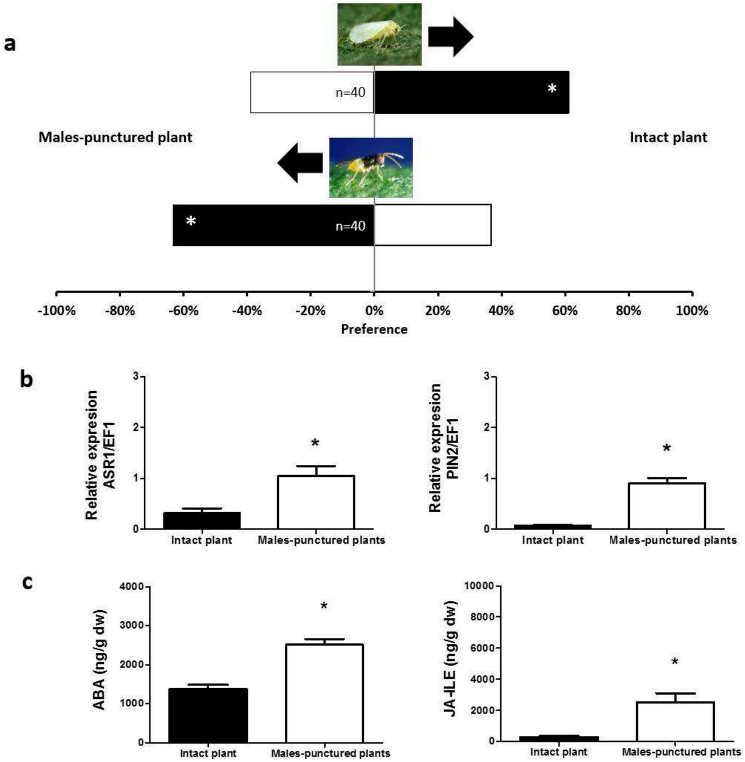

Figure 3. Plant responses induced by *N. tenuis* males. (**a**) Response of *B. tabaci* and *E. formosa* females when were given the choice between intact tomato plants and punctured tomato plants in a Y-tube olfactometer. Significant differences using χ^2 test, $p < 0.05$ are marked with (*); (**b**) Expression of the defensive genes *ASR1* and *PIN2* (target genes induced by the phytohormones ABA and JA-ILE, respectively). Data are presented as the mean of the ratio between the concentration of the gene transcripts and that of the constitutive *EF1* gene. Significant differences were obtained by comparing punctured plants to intact plants. Results from a one-tailed *t*-test are

marked with (*) ($p < 0.05$); (**c**) ABA and JA-ILE levels in the apical part of tomato plants. Each of the presented results is the mean of the hormone concentration (ng/g) of five independent analyses ± SE ($n = 5$). Significant differences were obtained by comparing punctured plants to intact plants. Results from a one-tailed *t*-test are marked with (*) ($p < 0.05$).

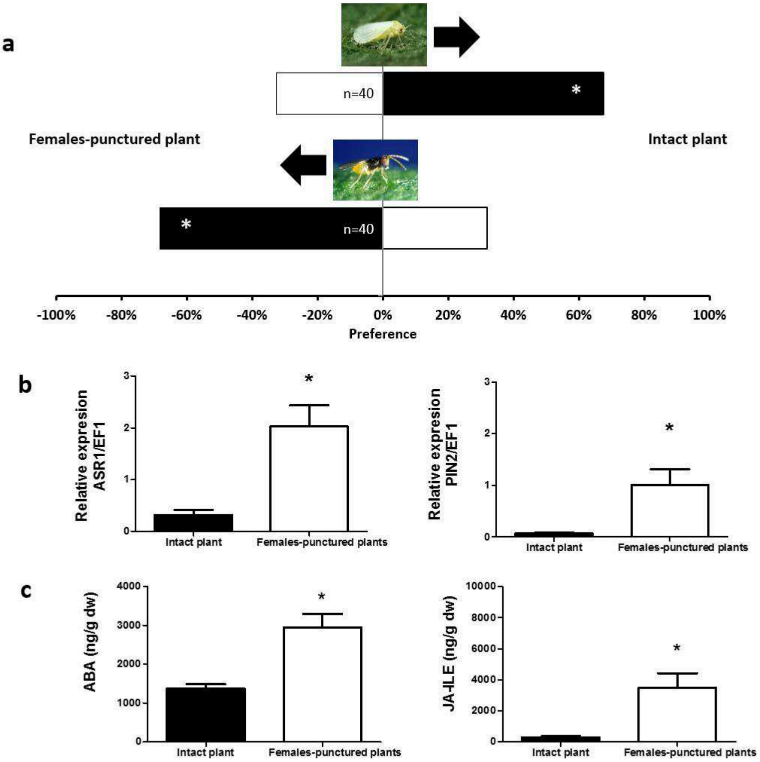

Figure 4. Plant responses induced by *N. tenuis* females. (**a**) Response of *B. tabaci* and *E. formosa* females when were given the choice between intact tomato plants and punctured tomato

plants in a Y-tube olfactometer. Significant differences using χ^2 test, $p < 0.05$ are marked with (*); (**b**) Expression of the defensive genes *ASR1* and *PIN2* (target genes induced by the phytohormones ABA and JA-ILE, respectively). Data are presented as the mean of the ratio between the concentration of the gene transcripts and that of the constitutive *EF1* gene. Significant differences were obtained by comparing punctured plants to intact plants. Results from a one-tailed *t*-test are marked with (*) ($p < 0.05$); (**c**) ABA and JA-ILE levels in the apical part of tomato plants. Each of the presented results is the mean of the hormone concentration (ng/g) of five independent analyses ± SE ($n = 5$). Significant differences were obtained by comparing punctured plants to intact plants. Results from a one-tailed *t*-test are marked with (*) ($p < 0.05$).

When adult-female-exposed plants were compared with plants exposed to adult males, the former were more repellent to *B. tabaci* ($\chi^2 = 5.000$, $p = 0.0253$) and more attractive to *E. formosa* ($\chi^2 = 10.90$, $p = 0.0010$, Figure 5b) than those exposed to adult males. However, when plants exposed to either young or mature nymphs were exposed to *B. tabaci*, no significant differences were detected ($\chi^2 = 1.852$, $p = 0.1736$). However, plants exposed to mature nymphs were more attractive to the parasitoid *E. formosa* than those exposed to young nymphs ($\chi^2 = 6.667$; $p = 0.0098$, Figure 5a).

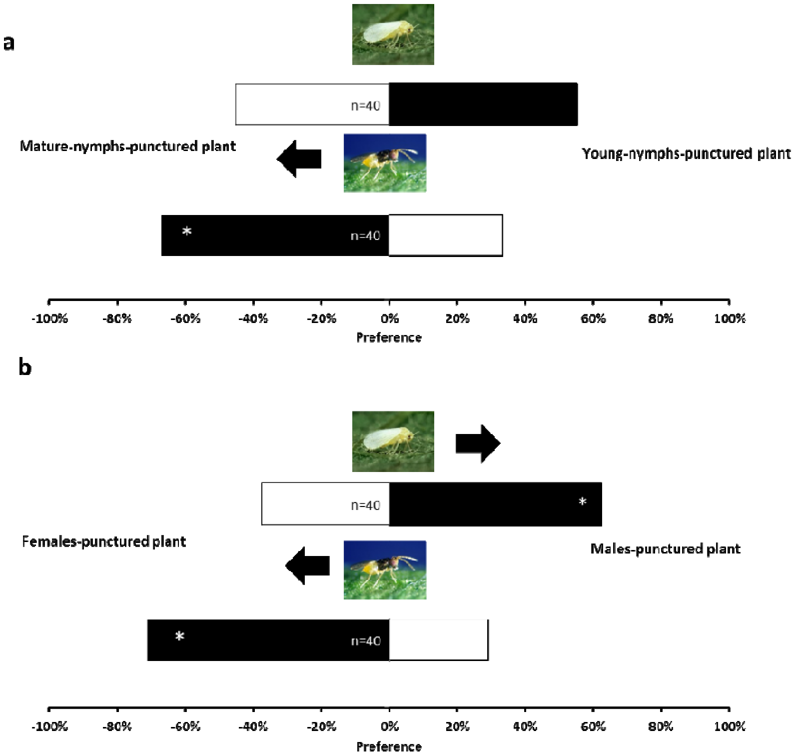

Figure 5. Response of *B. tabaci* and *E. formosa* females in a Y-tube olfactometer. (**a**) Comparison between mature-nymph-punctured tomato plants with young-nymph-punctured tomato plants; (**b**) Comparison between female-punctured tomato plants with male-punctured tomato plants. Significant differences using χ^2 test, $p < 0.05$ are marked with (*).

2.2. *Phytohormones Analysis and Plant Gene Expression*

In the apical part of all plants exposed to *N. tenuis*, the endogenous levels of ABA and of the bioactive phytohormone JA-Isoleucine (JA–ILE) were higher than in the apical part of undamaged plants (ABA: $t = 1.992$, $p = 0.0359$ for young nymphs—Figure 1c; $t = 2.206$, $p = 0.0260$ for mature nymphs—Figure 2c; $t = 6.065$, $p < 0.0001$ for males—Figure 3c; $t = 4.298$, $p = 0.0008$ for females—Figure 4c; and jasmonic acid-isoleucine (JA-ILE): $t = 1.815$, $p = 0.0498$ for young nymphs—Figure 1c; $t = 2.153$, $p = 0.0272$ for mature nymphs—Figure 2c; $t = 3.711$, $p = 0.0020$ for males—Figure 3c; $t = 3.344$, $p = 0.0037$ for females—Figure 4c). Consistent with these observations, the analysis of the relative expression of genes involved in indirect defense showed transcriptional differences between *N. tenuis*-exposed plants and undamaged plants. The *ASR1* gene (a marker for ABA) and the *PIN2* gene (a marker for JA) were upregulated in all *N. tenuis*-exposed plants compared to undamaged plants (*ASR1*: $t = 4.276$, $p = 0.0010$ for young nymphs—Figure 1b; $t = 5.227$, $p = 0.0003$ for mature nymphs—Figure 2b; $t = 3.239$, $p = 0.0059$ for males—Figure 3b; $t = 3.730$, $p = 0.0023$ for females—Figure 4b; and *PIN2*: $t = 2.374$, $p = 0.0225$ for young nymphs—Figure 1b; $t = 15.65$, $p < 0.0001$ for mature nymphs—Figure 2b; $t = 8.185$, $p < 0.0001$ for males—Figure 3b; $t = 3.033$, $p = 0.0081$ for females—Figure 4b).

3. Discussion

Our results are the first evidence that all motile stages of *N. tenuis* can induce defensive responses in tomato plants. All motile stages can damage plants by feeding on them, whereas adult *N. tenuis* females may also cause damage through oviposition. Our results also demonstrate the direct relationship between *N. tenuis'* plant feeding and defense induction in tomato. Specifically, the feeding on tomato plants by *N. tenuis* NI, NII, NIV, NV, adult males, and adult females all resulted in reduced attractiveness for *B. tabaci* and enhanced attractiveness for *E. formosa* relative to undamaged plants.

Olfactometer results confirm the positive correlation between ABA concentrations and induced repellence to whiteflies as well as between JA and induced attraction of *E. formosa* [20,21]. Interestingly, the stronger behavioral response observed in plants exposed to *N. tenuis* adult females in the olfactometer could be attributed to chemicals either emitted or triggered by *N. tenuis* eggs inserted into the plant tissues, as occurred in other phytophagous species [19,31]. Therefore, the presence of *N. tenuis* eggs on a plant could cause a synergistic effect with feeding, resulting in enhanced repellence for *B. tabaci* and enhanced attraction of *E. formosa* in olfactory bioassays. In sum, the dual activity of females (*i.e.,* feeding and oviposition) could explain these results. However, further research is needed. Indeed, volatile organic compounds (VOCs) emitted by plants in response to herbivore attack—either by feeding and/or by endo/esophytic oviposition—are known to repel further herbivore attacks [30,31]. Thus, the higher intensity of the effect produced by *N. tenuis* adult females on exposed plants might explain the stronger attraction of *E. formosa,* as previously demonstrated in different biological models [32]. Therefore, the potential presence of egg elicitors has to be better investigated. Another issue is the absence of specific defense responses by the plant to the mirid eggs, but this could be explained by the high degree of adaptation of these mirids to plants, confirming their mutualistic relations and suggesting a co-evolutionary approach to understanding these interactions.

Furthermore, the results suggest that the joint use of different instars of *N. tenuis* under greenhouse conditions is a better implementation strategy with this mirid, because the simultaneous presence of different cohorts avoids strong hormonal fluctuations in tomato plants, thereby reducing the negative impact on harvest.

Abiotic stresses, such as water stress or desiccation, induce the activation of the ABA pathway [33–35]. Nevertheless, ABA has been reported both as an inducer of plant defense response to necrotrophic pathogens and as an inhibitor of biotrophic pathogens [8]. Apart from the work of Pérez-Hedo, *et al.* [21], where high levels of ABA in tomato plants were shown to repel the whitefly *B. tabaci*, little information is available in relation to the effects of ABA on arthropods. JA is known for inducing direct and indirect plant defense responses against arthropods, this phytohormone works together with ET in orchestrating these responses [1,8]. The direct defense consists of the production of secondary metabolites, such as proteinase inhibitors, that inhibit the development of insects on activated plants [36,37], whereas indirect defense has been recently observed in trophic interaction studies. Particularly, this latter phenomenon is due to the production and release of VOCs. The synthesis and emission of VOCs is triggered by JA synthesis and mediates the attraction or rejection of beneficial and phytophagous species, respectively [10,36,38,39]. All the above-mentioned phytohormonal activity for indirect plant defense—which is induced by herbivores—culminates in the production and release of HIPVs [40]. These HIPVs are the signals for plant–plant and plant–arthropod communication, and result in the attraction of natural enemies and the repellence of herbivores, demonstrating the key role played by plants in orchestrating tritrophic interactions [30]. Future research may be oriented toward the extraction and characterization of VOCs involved in repelling herbivores and attracting natural enemies. Such research would lead to a better understanding of the phenomenon and help researchers to acquire new elements for future practical applications.

Our results highlight that in those crops where the release and subsequent conservation of *N. tenuis* is common practice (e.g., more than 80% of tomato greenhouses in southeastern Spain [41]), the persistence of this mirid on the plant throughout the growing season is an extra benefit in protecting them against pests.

In southeastern Spain, it is common practice to release *N. tenuis* adults on to the seedlings at a ratio of 0.5–1 adults per plant, and this release occurs approximately seven days before transplanting [25]. During this period, these adults—which are fed with eggs of the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)—lay eggs on the tomato plants such that when these plants are transplanted to the greenhouse, they already carry eggs of *N. tenuis*. Nevertheless, this is not the only benefit of this pre-plant release. During this seedling period, *N. tenuis* also feeds on the tomato plants, thereby activating the defenses of these plants [21]. Recently, Pappas, *et al.* [42], using *M. pygmaeus* as a model, proposed the term "plant vaccination" to describe this type of defensive activation induced in seedlings, because tomato plants reaching the greenhouse are "ready-to-defend" against herbivory. In the case of *M. pygmaeus*, Pappas, *et al.* [28] showed that this induction can last up to two weeks after the tomato plant comes in contact with the mirid. We have confirmed that this induction period is similar or longer for *N. tenuis* (same authors, unpublished data) such that the tomato plant would be "vaccinated" in the nursery, and this effect would last until the newly emerged nymphs start to feed on the plant, possibly renewing this defensive induction (as our results suggest). This effect on plants by the mirid could potentially counterbalance its potential damage on fruit [43]. The vaccination effect should be verified through field evaluations to confirm that plant activation is possible throughout the seedling, establishment, and conservation periods of *N. tenuis* in the crop.

4. Materials and Methods

4.1. Plants and Insects

Tomato plants *Solanum lycopersicum* cv. Optima (Seminis Vegetable Seeds, Inc.; Almería, Spain) were sown in soil, and two weeks after germination, seedlings were individually transferred to pots (8 × 8 × 8 cm). Plants were maintained undisturbed at 25 ± 2 °C, while relative humidity and photoperiod were held constant at 65% ± 5% RH and 14:10 h (Light:Dark). Pesticide-free tomato plants were used for the experiments at seven weeks of age (approximately 20 cm high).

B. tabaci, *E. formosa,* and all instars and stages of *N. tenuis* were provided directly by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain). Young nymphs consisted of a proportional mix of NI and NII, whereas mature nymphs corresponded to a proportional mix of NIV and NV. Adult females and males of *N. tenuis* were less than 4 days old, whereas adult females of *B. tabaci* and *E. formosa* were less than 2 days old. To obtain *N. tenuis*-punctured plants, four undamaged tomato plants were enclosed for 24 h in a 60 × 60 × 60 cm plastic cage (BugDorm-2; Mega View Science Co. Ltd.; Taichung, Taiwan) and exposed to 80 *N. tenuis* of the corresponding nymphal instar or adult sex (20 individuals per plant). All motile individuals were removed from plants before the experiment.

4.2. Y-Tube Bioassays

The olfactory preference of *B. tabaci* and *E. formosa* for different scent sources was tested using a Y-tube olfactometer (Analytical Research Systems, Gainesville, FL, USA). This Y-tube consisted of a Y-shaped glass tube (2.4 cm in diameter with a base of 13.5 cm in length) which was connected to two identical 5 L glass jars via plastic tubes. Each jar was connected to an air pump that produced a unidirectional airflow at a rate of 150 mL/min and contained a tested odor source (tomato plant). The experiments were conducted at 23 ± 2 °C, 60% ± 10% RH, and a light intensity of 2516 lux [44].

The first set of observations was conducted comparing the olfactory preference of *B. tabaci* and *E. formosa* adult females for each treatment of *N. tenuis*-punctured plants (young nymphs—NI and NII, mature nymphs—NIV and NV, adult males and females) relative to undamaged plants, while another series of observations was carried out comparing adult-male- *versus* adult-female-exposed plants and young-nymph- *versus* mature-nymph-exposed plants. The choice of each *B. tabaci* and *E. formosa* adult female was recorded when the insect walked a distance of 3 cm in the chosen arm; in the case that a female did not make a choice after 15 min, they were excluded from the analysis. Each individual was used only once. After testing five individuals, odor sources were interchanged to avoid any influence of asymmetries in the setup. Thirty to forty valid replicates were performed for each treatment.

4.3. Phytohormone Analysis

Apical parts of punctured tomato plants were exposed to *N. tenuis* at different instar stages for 24 h, and samples from undamaged plants were stored at −80 °C and analyzed to compare phytohormone concentrations. The apical part was considered to be the first 5 cm of the plant formed by the apical stem and young leaves. The phytohormone profile was analyzed using ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) [6,21,45]. This method can detect the concentrations of the two phytohormones involved in the tomato plant defensive responses: ABA and JA–Ile.

4.4. Quantification of Plant Gene Expression

The apical parts of the plants were used to quantify the gene expression of *ASR1* (ABA stress ripening protein 1)—a marker gene for ABA—and *PIN2* (proteinase inhibitors 2), a marker gene for JA. Immediately after collection, apical samples were ground in liquid nitrogen and a portion of them served for RNA extraction. Plant RNA Kit (Omega Bio-TekInc, Doraville, GA, USA) was used to extract total RNA (1.5 µg), and RNase-free DNase (Promega Corporation, Madison, WI, USA) was employed to eliminate genomic DNA contamination. Reverse transcription, primers, and the PCR SYBR reaction were carried out as previously described by Pérez-Hedo, *et al.* [21]. Quantitative PCR was performed with the Smart Cycler II (Cepheid, Sunnyvale, CA, USA) sequence detector using standard PCR conditions. Expression of *EF1* (Elongation factor 1) was used for normalization as housekeeping gene. Table 1 describes the sequences of the gene-specific primers used.

4.5. Data Analyses

Data sets obtained from the Y-tube olfactometer observations were analyzed using a chi-square (χ^2) test in order to evaluate whether the response of insects to different scent sources deviated from a null model, where odor sources were chosen with equal frequency. Data concerning gene expression levels and phytohormone analyses between different instars of *N. tenuis*-exposed plants and undamaged plants were compared by one tailed *t*-test ($p < 0.05$).

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Author Contributions: A.U. and M.P.-H. designed the research and, together with M.N., analyzed the data. All authors performed research and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER IV

Olfactory response of the zoophytophagous mirid *Nesidiocoris tenuis* to tomato and alternative host plants

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Olfactory response of the zoophytophagous mirid *Nesidiocoris tenuis* to tomato and alternative host plants

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Abstract

We assessed the potential of tomato (*Solanum lycopersicum*) and two alternative plants, sesame (*Sesamum indicum*) and *Dittrichia viscosa*, to attract the zoophytophagous bug *Nesidiocoris tenuis* (Hemiptera: Miridae). A Y-tube-olfactometer was used to test the mirid olfactory preference in dual-choice bioassays testing the volatiles emitted by healthy plants of tomato *S. indicum* and *D. viscosa,* and by tomato plants infested by eggs and larvae of *Tuta absoluta* (Lepidoptera: Gelechiidae). Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS) was used for characterization of volatile compounds emitted by the plants. *Sesamum indicum* was the most attractive plant; the *T. absoluta* infestation did not cause a significant *N. tenuis* attraction. We identified fifty-seven volatiles belonging to

the classes of C_{13}norisoprenoids, aliphatic and aromatic aldehydes, esters, alcohols, hydrocarbons, monoterpene and sesquiterpene hydrocarbons and their oxygenated compounds. Sesame plants emitted the lowest amount of monoterpene hydrocarbons and a higher rate of oxygenated terpenes. Green Leaf Volatiles (GLVs), known for attracting mirids, were constitutively more present in sesame plants. Whereas, tomato plants infested by *T. absoluta* larvae showed the highest levels of monoterpene hydrocarbons. The potential biochemical bases of the attraction of an omnivorous predator toward shelter plants are discussed in the frameworks of the mirid ecology and the tomato integrated pest management (IPM).

Keywords: Plant volatiles; HS-SPME-GC-MS; Olfactometer; Generalist predator; Functional biodiversity; Banker plant

Introduction

Plants are able to attract natural enemies through the constitutive or induced production of volatile organic compounds (VOCs). Some plant species exhibit more than others the ability to “communicate” with their consumer or beneficial insects. Herbivore associated elicitors or elicitors induced by the wounds caused by insects allow plants to respond to insect attacks (Bonaventure et al. 2011). Plants react activating a cascade of events that lead to direct and indirect defense dynamics. These include the transcriptome changes of genes involved in the biosynthesis of phytohormones with the emission of volatile organic compounds (VOCs) that attract natural enemies and repel herbivorous insects (Thaler et al. 2002; De Vos et al. 2005; Ponzio et al. 2013; Pérez-Hedo et al. 2015). The production of VOCs, functioning as attractant for arthropod natural enemies, has been investigated especially in terms of herbivore induced plant volatiles (HIPVs), most of which belong to terpenoids, fatty acid derivatives, phenyl propanoids and benzenoids (Dicke and Sabelis 1988). Also, other researches showed the role of the environmental volatile background (HIPV blend naturally present) in insect responses, putting all plants in the center of these food web community relationships (Dicke 2009; Dicke and Baldwin 2010).

The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera:Gelechiidae), since its introduction in Europe from South America, has become one of the most harmful pests of tomato in all the newly invaded areas (Desneux et al. 2010). In the Mediterranean basin, it has become a serious agricultural threat to tomato production in both greenhouse and outdoor tomato crops (Tropea Garzia et al. 2012). Moreover, it recently showed to be a serious threat for Central and North European tomato protected crops (van Damme et al. 2015). Soon after its arrival, an extensive use of insecticides led to the development of insecticide resistance phenomena (Campos et al. 2015; Roditakis et al. 2015), and to several side effects on non-target arthropods (Biondi et al. 2012, 2013a; Abbes et al. 2015). Thus, various generalist natural enemies feeding and developing on this new host/prey have been studied and exploited as sustainable control tools (Bompard et al. 2013; Zappalà et al. 2013). Among them, a multifaceted role is given to the omnivorous bug *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae), because it is able to spontaneously or artificially control several tomato pests, such as whiteflies, thrips, leaf miners, aphids, mites, and lepidopterans (Zappalà et al. 2012a; Biondi et al. 2013b; Pérez-Hedo and

Urbaneja 2015). The ability of this bug species to use the volatiles from *T. absoluta*-infested tomato plants has been recently shown (Lins et al. 2014). Nevertheless, *N. tenuis* can turn into a pest being responsible for damages on tomato plants through its feeding activity that causes necrotic rings, flower and small fruit abortion, reduced growth of stems and abscission of organs (Sánchez and Lacasa 2008; Castañé et al. 2011).

Alternative non-crop plants could be employed in biological and integrated pest control tomato schemes as either banker plants, to promote the predator presence and installation within or near the crop, and/or as trap plants to disrupt the presence, and thus the damage, of the mirid in the crops when prey populations are scarce (Parolin et al. 2014). *Dittrichia* (=*Inula*) *viscosa* L. (W. Greuter) is native to the Mediterranean area and it is well known as medicinal plant and for its richness in terpenoid compounds (Pérez-Alonso et al. 1996) and as a natural refuge for mirid bugs (Alomar et al. 2002; Perdikis et al. 2007; Cano et al. 2009). Sesame, *Sesamum indicum* (L.) (Pedaliaceae), is native to India, it is cultivated worldwide and it can successfully support the *N. tenuis* development and reproduction (Nakaishi et al. 2011; Biondi et al. 2016). In a recent laboratory study, Biondi et al. (2016) assessed the oviposition preference of this zoophytophagous mirid among tomato and these two alternative plants. *Sesamum indicum* was proven to be a favorite host for both feeding and oviposition. However, in that cage experiment the potential long-distance attractiveness of the volatiles from these host plants was not assessed.

In this context, we aimed at characterizing the volatile emissions from healthy tomato, *S. indicum* and *D.*

viscosa plants and tomato plants infested by *T. absoluta* eggs and larvae using the headspace solid-phase microextraction (HS-SPME) technique combined with gas chromatography-mass spectrometry (GC-MS). Moreover, the potential for attracting *N. tenuis* females of the raw volatiles emitted by these plants was studied in dual-choice bioassays using a Y-tube olfactometer.

Materials and Methods

Plants and insects

Seedlings of tomato (*Solanum lycopersicum*, cv. Marmande), sesame (*Sesamum indicum*, cv. T-85 Humera) and *D. viscosa* (from seeds of wild plants collected in Catania, Italy) were grown under greenhouse conditions (23 ± 10°C, 60± 20 % RH and 14:10h L:D) at the department of Agriculture, Food and Environment of the University of Catania. Plants were grown without any pesticide application, into pots (7 x 7 x 6.5 cm) filled with commercial soil (Gramoflor ® GmbH & Co. KG – Germany) and watered as needed. Plants were used for the experiments at six weeks of age, approximately 20 cm high.

The *T. absoluta* and *N. tenuis* laboratory colonies were obtained starting from tomato samples collected in various commercial tomato crops located in the Ragusa and

Siracusa provinces (Italy) in 2011 (Zappalà et al. 2012b). New field-collected specimens were regularly introduced into the rearing cages (32 x 40 x 70 cm) maintained at constant environmental conditions (22 ± 2°C, 50± 10 % RH and 14:10h L:D). One/two-months old tomato plants were used to rear the moth (see Biondi et al. 2013c for a detailed description) and as feeding and oviposition substrate for the mirid rearing. *Ephestia kuehniella* eggs mixed with *Artemia* sp. cystis (Entofood®, Koppert, The Netherlands) were provided *ad libitum* on plant leaves as factitious prey (Mollá et al. 2014).

Plants infested by *T. absoluta* eggs to be used in the bioassays were obtained releasing 10 adults/plant into a rearing cage for 48 hours, this resulted in an oviposition of approximately 50 eggs per tomato plant. Eight second instars larvae were used to infest each tomato plant 48 hours prior to the experiments.

Y-tube olfactory bioassays

The olfactory preference of *N. tenuis* females for different sources of scent was tested via dual-choice experiments using a Y-tube olfactometer. The device consisted of a Y-shaped glass tube of 1.5 cm in diameter, with an entry arm 18.5 cm long and two side arms of 18.5 cm in length and 70° angle; the Y-tube was vertically oriented as suggested for behavioral studies on Dicyphinae subfamily (Ingegno et al. 2011, 2013). The Y-tube was connected to an air pump (Ferplast – Airfizz®) that produced a unidirectional airflow regulated at 150 ml/min. Before entering the Y – tube, the airflow passed through a water filter. The olfactometer was kept in a dark room whereas the choosing arena was illuminated with two 22-W cool white fluorescent circular tubes, located 0.8 m above the device (Biondi et al. 2015). At least 40 replicates (a female that made a choice) for each choice test were carried out; every two replicates the tube was turned to prevent orientation errors. The bioassays were conducted between 9:00 and 18:00, at 23 ± 2°C, 60 ±10% RH. Three/five-day old *N. tenuis* mated females were starved for 48h into transparent vials (6 cm height and 1.5 cm diameter) plugged by a wet cotton as water supply. The choice of each tested *N. tenuis* female was considered as such when the insect reached the end of a given arm; females that didn't make any choice after 10 min were excluded from the data analysis.

The first set of dual-choice observations was carried out comparing the olfactory preference of *N. tenuis* toward the constitutive volatiles of healthy tomato, *S. indicum* and *D. viscosa* plants. For this, six combinations of odour sources were tested, i.e., all the possible combinations of two of three plants and of each plant combined with clean air as control. Then, to test the potential role of *T. absoluta*-induced volatiles in tomato plants, we compared the attractiveness of egg-infested and larvae-infested tomato plants compared to clean air and healthy tomato, *S. indicum* and *D. viscosa* plants.

Headspace solid phase microextraction (SPME) of emitted volatiles

A headspace solid-phase microextraction (HS-SPME) method combined with gas chromatography-mass spectrometry (GC-MS) was developed for the extraction, analysis and quantification of the plant emitted volatiles. The collection of headspace volatiles of the plant samples used for the Y-tube bioassays was carried out through the SPME technique in a static environment. Plants of the same batch as those employed in olfactory bioassays were placed in glass collection chambers (25 cm height and 15 cm diameter) with the lid equipped with a silicone septa for the insertion of an SPME fiber. Extraction was performed in the headspace glass collection chamber using a commercially available fiber housed in its manual holder (Supelco, Bellefonte, PA, USA). The chamber was maintained at 23 ± 2°C. All extractions were carried out using a DVB/CAR/PDMS fiber, of 50/30 µm film thickness (Supelco, Bellefonte, PA, USA). Prior to the analysis, fiber was conditioned according to the manufacturer's instructions by heating it in the injection port of the gas chromatograph (GC) at the recommended temperature. The samples were equilibrated for 1 h and after the fiber was inserted and exposed for 20 min, 1 h, 10 h and 24 h to determine the appropriate time for collecting the volatiles.

Because of extensive evapo-transpiration of the plants, the fiber was left in the chamber for 1 h. After the sampling, the SPME fiber was introduced onto the splitless injector of the GC-MS using the conditions reported below. The fiber was kept in the injector for 3 min for thermal desorption of the analytes onto the capillary GC column. The splitless injector port was maintained at 260 °C. Four replicated analyses for each sample were performed.

Volatiles Analysis

A Varian 3800 gas chromatograph directly interfaced with a Varian 2000 ion trap mass spectrometer (Varian Spa, Milan, Italy) was used (GC-MS). The conditions were as follows: injector temperature 260°C; injection mode, splitless; capillary column, CP-Wax 52 CB, 60 m, 0.25 mm i.d., 0.25 µm film thickness (Chrompack Italy, s.r.l. Milan, Italy); oven temperature, 40 °C with an increase up to 200 °C at a rate of 3 °C/min and to 240 °C at 10 °C/min; carrier gas, helium at a constant pressure of 10 psi; transfer line temperature, 250 °C; acquisition range, 40–200 m/z; scan rate, 1 scan s^{-1}. Data was acquired in Total Ion Current (TIC). Each component was identified using mass spectral data, NIST'14 (NIST/EPA/NIH Mass Spectra Library, version 2.0, USA), FFNSC (Flavors and Fragrances of Natural and Synthetic Compounds, mass spectral database, version 2.0, Italy), Linear Retention Indices (LRI), literature data, and the injection of standards where available. Peaks were considered "identified" when their mass spectral fit values were at the default value of 700 or above; their LRI provided a low match window of ±10 index units respect to those from literature and their RT (Retention Time) matched to that of standards.

Quantitative Analysis

Quantitative results were obtained by using the method of standard additions. For the quantification of all the volatiles the standards were purchased from Sigma-Aldrich (Milan, Italy) of the highest purity available. To quantify volatiles, being standards not available, the calibration curve of the most similar carbon skeleton sesquiterpene was used. Stock solutions of individual standards were prepared by dissolving the appropriate amount of each compound in ethyl alcohol (95%) in order to obtain a final concentration of 0.4 mg/mL. The solutions were stored at -30 °C. Furthermore, five different concentrations of each stock solution were added to multiple aliquots of each sample. The sample alone was also analyzed. Quantification was based on a calibration curve generated by plotting detector response versus the amount spiked of each standard. The peak area of each component in total ion current chromatograms was determined during three replicates, and the average value was calculated.

Statistical analysis

Preference data, obtained through the Y-tube olfactometer bioassays, were analyzed using a chi-square goodness of fit test, in order to assess whether the response of insects to different source scents was significantly different from a 50:50 distribution. Females that did not make a choice were not considered. The results concerning analysis of volatile compounds were expressed as means and were subjected to one-way ANOVA followed by a Duncan's multiple range test.

Results

Response of N. tenuis *to plant volatiles*

The three plant species tested in the bioassays were significantly attractive for *N. tenuis* females when the predators were exposed to volatiles of tomato and clean air (χ^2_1 = 20.17, $P<0.0001$), *D. viscosa* and clean air (χ^2_1 = 14.73, $P=0.0001$), and *S. indicum* and clean air (χ^2_1= 10.47, $P=0.0012$) (Figure 1). Furthermore, starved females of *N. tenuis* preferred volatiles emitted by *S. indicum* when compared to tomato (χ^2_1= 20.00, $P<0.0001$), and *D. viscosa* (χ^2_1= 10.47, $P=0.0012$). The latter was not significantly more attractive toward *N. tenuis* compared to tomato (χ^2_1= 0.1818, $P=0.6698$).

Predator females were significantly attracted by the volatiles emitted by tomato plants infested with *T. absoluta* eggs (tomato + eggs and clean air: χ^2_1 = 25.51, $P<0.0001$)

and larvae (tomato + larvae and clean air: χ^2_1= 34.09, P<0.0001) only when compared to clean air. Between the two stages, *N. tenuis* was significantly attracted by tomato plants infested by *T. absoluta* larvae rather than those infested with eggs (χ^2_1= 10.00, P=0.0016).While, when the dual-choice was between healthy and infested tomato plants, the predators did not show any preference (tomato and tomato + eggs: χ^2_1 = 2.783, P=0.0953; tomato and tomato + larvae: χ^2_1= 1.064, P=0.3023). The volatiles emitted by *S. indicum* plants were more attractive compared to tomato plants infested by both instars (tomato + *T. absoluta* eggs and *S. indicum*: χ^2_1 = 17.39, P<0.0001; tomato + *T. absoluta* larvae and *S. indicum*: χ^2_1= 10.67, P=0.0011). No significant differences were found comparing the potential attractiveness of the volatiles emitted by infested tomato plants vs *D. viscosa* plants (tomato + *T. absoluta* eggs and

D. viscosa: χ^2_1= 2.08, P=0.149; tomato + *T. absoluta* larvae and *D. viscosa*: χ^2_1= 1.39, P=0.238).

Gas chromatographic volatile profile

Fifty seven compounds, such as exactly monoterpenes and sesquiterpenes and their oxygenated compounds, C_{13}-norisoprenoids, aliphatic aldehydes, esters, alcohols and hydrocarbons, were identified (see table 1 for details on substances and concentrations). Statistically significant differences in most of the volatile concentrations were observed among the plant samples. Overall, the volatile emission by tomato plants infested by *T. absoluta* larvae was quantitatively the richest in VOCs (8.33 µg/4L) followed by healthy tomato (5.79 µg/4L), tomato plants infested by *T. absoluta* eggs (5.38 µg/4L), *D. viscosa* (2.61 µg/4L) and *S. indicum* (2.4 µg/4L) (Table 1).

Among tomato plant volatiles hydrocarbon monoterpenes were quantitatively the most abundant class of compounds, followed by oxygenated monoterpenes with β-phellandrene, sabinene, terpenyl acetate and 4-ethyl acetophenone the main compounds, respectively. The amount of β-phellandrene and sabinene and most of the monoterpene hydrocarbons increased in the infested plants, mainly in those with the larvae. An opposite trend resulted for terpenyl acetate and 4-ethyl acetophenone,the amount of which decreased mainly in the egg-infested tomato plants. The monoterpenes, present in the tomato samples, were also identified in the other two alternative plants, but their amount resulted lower in *D. viscosa* and very often inferior to the limit of quantification in *S. indicum*. An opposite trend was found for 4-ethyl-acetophenone the amount of which resulted higher in *D. viscosa* and *S. indicum* plant emissions (Table 1).

The volatile emissions from *D. viscosa* plants had the highest number of sesquiterpenes, with β-elemene and D germacrene as the main compounds. β-cariophyllene was the only sesquiterpene identified in *S. indicum*. α-Humulene prevailed in tomato plants, it was not identified in sesame and *D. viscosa*. α- Cadinol, an oxygenated sesquiterpene, was identified only in *D. viscosa*. Sesame plant emissions had the highest amount of aliphatic compounds especially octanal and nonanal which are classified as Green Leaf Volatiles (GLV).

C_{13}Norisoprenoids were identified in all the plant samples. Their total amount was similar in all the plant volatile emissions except for *D. viscosa* that showed a very low amount. Some authors postulated that this kind of compounds such as β-ionone, pseudoionone and geranyl acetone are products of oxidative cleavage of carotenoids (Simkin et al. 2004). The differences among the emissions of the three plant species are presented in Figure 3 that compares the SPME-GC-MS chromatograms of the different plant samples. Tomato samples showed a more complex blend of volatiles and the highest amount of hydrocarbon monoterpenes. The most abundant substances in sesame samples were nonanal, terpenyl acetate and 4-ethyl acetophenone, which constituted more than 50 % of the total volatiles. In *D. viscosa*, nonanal was not detected, while terpenyl acetate and 4-ethyl acetophenone alone constituted around 70 % of the total volatiles.

Discussion

The omnivorous mirid bug *N. tenuis* showed a marked preference for the volatiles emitted by *S. indicum* over those emitted by *D. viscosa* and tomato plants, healthy and/or infested by *T. absoluta* larvae or eggs. The analyses of the volatiles highlighted a lower complexity of *S. indicum* compared to the other tested plants. Among the VOCs, the most representative classes in all tomato and *D. viscosa* plant samples were monoterpene and sesquiterpene hydrocarbons. These profiles resulted very similar to those obtained by Ingegno et al. (2016) analyzing the VOCs of tomato and *D. viscosa.* Furthermore, in our study, we obtained for *D. viscosa* a significant amount of oxygenated monoterpenes and sesquiterpenes while in *S. indicum* terpene hydrocarbons were very poorly represented, contrarily to oxygenated monoterpenes. In *S. indicum* the rate between oxygenated and terpene hydrocarbons reached the highest levels. Terpenes are important HIPVs involved in direct and indirect plant defense against herbivores (Bukovinszky et al. 2005; Dicke 2009; Gols et al 2011). They are synthesized by two pathways. Monoterpenes are usually produced through the Methylerythritol 4-phosphate (MEP) pathway that occurs in plastid and they are derived from geranyldiphosphate (GDP). Sesquiterpenes are produced through the mevalonic acid (MVA) pathway from farnesyldiphosphate (FDP) in cytosol (Akhila 2007). The gas chromatographic volatile profiles of tested plants may justify this *N. tenuis* orientation trend. Indeed, *S. indicum* demonstrated to have (i) a poorer composition in terpene hydrocarbons, that are known to have repellent properties toward insect pests (Nerio et al. 2010) (in this case phytophagous behavior may have prevailed); and (ii) a higher rate of oxygenated terpenes and GLV compounds, such as nonanal and other aliphatic compounds, that have been showed to play a role in the attraction of the predatory mirid bug *Geocoris pallens* (predatory behavior orientation) (Halitschke et al., 2008). As for the GLV, such as octanal and nonanal collected from *S. indicum*, it is known that these two compounds are used as standard in electroantennography (EAG) studies, for their activity in stimulating mirid antennae (Williams et al. 2010; Pan et al. 2015). GLVs are fatty acid-derived compounds formed by three processes, α-oxidation, β-oxidation and the lipoxygenase pathway. They are found ubiquitously in the plant kingdom at high concentrations (Schwab et al. 2008). At the same time, according to Agrawal and Klein (2000), the repellent compounds present in plants (tomato and *D. viscosa* in our case) could modulate the phytophagy of the mirid toward carnivory. We can thus speculate that, because of the dual herbivorous and carnivorous behavior of *N. tenuis*, the class of terpenoids may both attract and repel this insect. In particular, monoterpenes and sesquiterpenes hydrocarbons may inhibit the mirid, because of their anti-feeding role when the predator feeds upon the plants (Besser et al., 2009; Kang et al., 2010). Similarly, we hypothesized that oxygenated terpenes could attract *N. tenuis* toward those plants (*S. indicum*) where the percentage of total terpenoids was almost exclusively due to oxygenated terpenes with a poor amount of the hydrocarbons form, as highlighted in other different models (Wang and Kays, 2002). However, this hypothesis should be studied by screening the olfactory responses to the specific synthetic compounds, using the electroantennography (EAG) technique (Ingegno et al. 2016). Moreover, because of the plasticity of insect behavioral responses to different odour types according to their physiological status, e.g., age, feeding state, circadian rhythm, and mating status of insects, further studies should take these factors into account (Gadenne et al. 2016).

The differences in volatile chemicals between tomato, *D. viscosa* and sesame plants may be explained also by their different morphological traits.Indeed, the first two species are rich in glandular trichomes that are less represented in *S. indicum* (Besser et al., 2009; Kang et al., 2010). Glandular trichomes are plant structures rich in chemical compounds, such as terpenoids and adhesive substances, useful in plant defense against herbivores (Sugiura and Yamazaki, 2006; Wheeler and Krimmel, 2015). In the present study, we saw how the most attractive plant (sesame) is the poorest in repellent compounds, thus supporting the suitability of sesame as host plant for *N. tenuis* development (Ahirwar et al. 2010; Biondi et al. 2016).

Halitschke et al.(2008) proved that mirids, because of their generalist orientation, are attracted by individual chemical components of the blend of VOCs characterizing the profile of target plants. They stated, by contrast, that specialized parasitoids use the whole plant volatile bouquets to locate their hosts. Those studies may explain the preference of *N. tenuis*, in our trials, for *S. indicum* which is poor in monoterpene and sesquiterpene hydrocarbons, making the identification of the target plant easier. Moreover, this hypothesis is further supported by the study of McGregor and Gillespie (2004) on the predatory mirid *Dichyphus hesperus*. These authors conclude that some background odours may mask the scents coming from the target plant. Similarly, the higher volatile complexity of *D. viscosa* and tomato could inhibit the effect of the active volatiles present in these two species in comparison with sesame. By contrast, in a recent study, Ingegno et al. (2016) identified as the best hosts the plants with complex volatile profiles for the predatory mirids *Macrolophus melanotoma* (Costa) and *M. pygmaeus* (Rambur). In our study we can agree with this hypothesis when considering *N. tenuis* as a predator in tomato and *D. viscosa* plants that have a complex volatile profile. On the other hand, we suggest that the low amount and complexity of volatiles of *S. indicum* refers to another kind of suitability model in which the herbivore tendency of *N. tenuis* prevail. Furthermore, a recent study shows the positive influence of *S. indicum* in improving the biocontrol performances of the predatory mirid bug *Cyrtorhinus lividipennis* Reuter on rice pests (Zhu et al., 2014). Other studies report *N. tenuis* as sesame pest and highlight how the development of this zoophytophagous mirid is enhanced when it is reared on sesame rather than on other host plants (Ahirwar et al. 2010; Nakahishi et al. 2011). Recently, Biondi et al. (2016) demonstrated that sesame may be employed as *biocontrol plant* in order to improve biological control of tomato pests while reducing the damages on tomato, due to the herbivore attitude of *N. tenuis*.

When *T. absoluta*-infested tomato plants were tested, we found no significant differences in the preference for larvae-infested compared to healthy tomato plants, and for egg-infested compared to healthy ones. However, the predator was more attracted by tomato plants infested by *T. absoluta* larvae rather than eggs. The analysis of the volatile profiles of plants infested by *T. absoluta* larvae highlighted a significant increase in VOCs production, especially for the terpenes β-phellandrene and sabinene. This suggests that a plant response was induced by the activity of the larvae, despite this response was not perceived by *N. tenuis*. Our results obtained with infested plants differ slightly from other similar studies in which tomato plants infested with larvae of *T. absoluta* were more attractive than healthy plants, while infested plants with eggs had the same degree of attraction of healthy plants (Lins et al. 2014; De Backer et al. 2015). These differences may be due to the duration of exposure of the plant to the pest, indeed Lins et al. (2014) and De Backer et al. (2015) exposed tomato plants to the larvae of *T. absoluta* 72 h and 7 days before olfactory test, respectively.. Similarly, in studies on volatiles emitted by tomato plants infested with eggs of *T. absoluta* differences between infested and healthy plant emerged only 72 h after oviposition (Anastasaki et al. 2015), while Lins et al. (2014) in olfactometer did not get any differences comparing healthy tomato plants and plants used 48 h after oviposition, in agreement with our results. Potential result discordance can be simply due to the variable compositions and contents of volatile compounds among tomato varieties (Zhao et al 2012). Thus, it would be interesting screening commercial tomato varieties for their potential attraction toward natural enemies.

Despite this, significant differences emerged when we compared egg-infested plants to larvae-infested plants in olfactometer, these latter were more attractive only in this comparison. This may depend on the fact that, in our study, the plants infested with eggs emitted less attractive compounds than healthy plants, and plants infested with larvae, in total, released the highest amount of VOCs. This hypothesis is also confirmed by Fatouros et al. (2012) according to which eggs deposition reduces volatile emission in plants. Indeed, other studies demonstrate that eggs are able to suppress plant defense against larval instars and as consequence the volatile emission (Bruessow et al. 2010). Nevertheless, several studies demonstrate that mirids are attracted by tomato plants infested with *T. absoluta* larvae (Lins et al. 2014; De Backer et al. 2015). Interestingly, Lins et al. (2014) found that plants infested by whiteflies and *T. absoluta* simultaneously are the most attractive, suggesting that host selection via olfaction can be more complicated than expected. Thus, we can assume that there is an effect of the feeding activity of larvae in inducing the production of more attractive volatiles for *N. tenuis* in tomato plants. However, the effect is fairly moderate by *T. absoluta* eggs as they induce a reduction of plant volatiles, at least in the first phase of infestation. These data are confirmed also by the headspace volatile analysis that shows a decrease of terpenyl acetate and 4-ethyl acetophenone in tomato plants infested with eggs. We can conclude that, in the earliest phases of the infestation by *T. absoluta* on tomato (48 hours after oviposititon and 48 hours after larval feeding activity), the attraction toward *N. tenuis* is moderate and the attraction of the mirid from natural ecosystems could not be enhanced by the simple presence of the prey.

In this study, we provided experimental evidences, that the use of more attractive banker plants, such as sesame, may stimulate the presence of predators since the first phase of pest attack. This highlights the key role played by the weed diversity in attracting natural enemies of arthropods.

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Table 1. Mean (±SD) of the composition (µg/4L) of the headspace volatiles emitted by: i) tomato healthy plants, ii) tomato plants infested by *Tuta absoluta* eggs, iii) tomato plants infested by *T. absoluta* larvae*,* iv) *Dittrichia viscosa* plants, and by v) *Sesamum indicum* plants. Different letters within the same row represent significant differences at P <0.05 by Duncan's multiple range test; -: not detected; tr= traces;< 0.01 µg/4L.

Code	*Compounds*	***Tomato***			***D. viscosa***	***S. indicum***
		Healthy	**Egg**	**Larvae**		
	Terpenes					
2)	α-Pinene	0.07b±0.025	0.09b±0.025	0.20a±0.016	0,08b±0.011	-
3)	α-Tujene	-	-	-	0.01±0.002	-
5)	Camphene	0.02a±0.005	tr b	0.02a±0.001	0.01a±0.002	tr b
6)	β-Pinene	tr b	-	0.01b±0.002	0.04a±0.014	-
7)	Sabinene	0.81b±0.241	0.99b±0.243	1.77a±0.451	Trc	0.01c±0.003
9)	δ-3-Carene	tr b	tr b	0.01b±0.002	0.02a±0.004	-
10)	Myrcene	-	-	-	0.02±0.002	-
11)	α-Phellandrene	0.26a±0.029	0.34a±0.052	0.58a±0.147	tr b	tr b
12)	α-Terpinene	0.07a±0.014	0.06a±0.014	0.09a±0.019	0.01b±0.002	-
14)	Limonene	0.57a±0.0195	0.59a±0.53	0.95a±0.141	0.15b±0.037	0.02c±0.005
15)	ß-Phellandrene	1.24b±0.318	1.67b±0.376	2.40a±0.568	tr c	0.02c±0.004
17)	γ-Terpinene	0.01a±0.002	0.01a±0.002	0.02a±0.003	0.01a±0.002	-
18)	Terpinolene	0.02a±0.005	0.01a±0.003	0.02a±0.007	0.01b±0.001	tr c
	All	***3.07***	***3.76***	***6.07***	***0.36***	***0.05***
	Oxygenated terpenes					
16)	Eucalyptol	-	-	-	0.02±0.001	-
21)	(Z)-Rose oxide	tr	Tr	0.01±0.002	-	-
43)	Terpenyl acetate	1.07a±0.310	0.16b±0.006	0.90a±0.184	0.28b±0.016	0.41b±0.106
47)	p-Menth-ene-3-one (Piperitone)	-	0.01±0.002	0.01±0.003	0.01±0.002	-
50)	Cuminaldehyde	0.01b±0.002	0.02a±0.004	0.02a±0.002	-	-
53)	4-Ethyl acetophenone	0.68b±0.147	0.45b±0.038	0.48b±0.010	1.49a±0.206	0.91a±0.242
	All	***1.76***	***0.64***	***1.41***	***1.80***	***1.32***
	Sesquiterpenes hydrocarbons					
27)	α-Cubebene	0.01	0.02	0.01	0.01	-
28)	Ylangene	-	-	-	0.02	-
30)	Cycloisolongifolene	-	-	-	0.01	-
31)	(E)-α-Bergamotene	-	-	-	0.01	-
33)	β-Caryophyllene	0.03b	0.09a	0.05b	0.03b	0.05b
34)	β-elemene	-	Trb	-	0.08a	-
35)	Sesquiterpenen.i.	0.01	0.02	0.02	0.02	-
37)	Sesquiterpenen.i.	0.01	0.01	0.01	-	-
38)	Alloaromadendrene	-	-	-	Tr	-
39)	γ-Elemene	0.01	-	-	-	-
40)	(E)-β-Cubebene	-	-	-	0.01	-
41)	α-Humulene	0.24	0.27	0.15	-	-
42)	D germacrene	-	-	-	0.09±0.025	-
44)	β-Cadinene	-	-	-	Tr	-
45)	α-Selinene	-	-	-	0.01±0.002	-
46)	β-Selinene	-	-	-	0.01±0.001	-
48)	δ-Cadinene	-	-	-	0.01±0.001	-
49)	α-Curcumene	-	-	-	0.03±0.008	-
51)	(Z)-Calamenene	-	-	-	0.01±0.002	-
	All	***0.31***	***0.41***	***0.24***	***0.30***	***0.05***
	Oxygenated sesquiterpenes					
57)	α-Cadinol	-	-	-	0.07±0.027	-
	All	**-**	**-**	**-**	***0.07***	**-**
	C_{13}-norisoprenoids					
8)	4-Methyl-5-penten-2-one	0.10a±0.026	-	0.04a±0.006	tr b	-
22)	6- Methyl-5-hepten-2-one	0.11a±0.003	0.14a±0.035	0.13a±0.018	0.03b±0.002	0.10a±0.023
52)	Geranyl acetone	0.20a±0.031	0.23a±0.031	0.25a±0.062	-	0.14b±0.005
56)	β-Methyl-ionone	-	-	-	-	0.12±0.035
	All	***0.41***	***0.37***	***0.42***	***0.03***	***0.36***

	Aliphatic compounds					
	Aldehydes					
19)	Octanal	-	-	-	-	0.08±0.007
25)	Nonanal	0.19b±0.016	0.15b±0.035	0.15b±0.027	-	0.31a±0.038
	All	***0.19***	***0.15***	***0.15***	***-***	***0.39***
	Esters					
36)	Methylbenzoate	-	-	-	0.03±0.002	-
	All	-	-	-	***0.03***	-
	Alcohols					
20)	(E)-2-penten-1-ol	-	-	-	-	.02±0.009
23)	Diacetonealcohol	-	-	-	-	.03±0.004
24)	(Z)-3-hexen-1-ol	0.05±0.019	0.05±0.013	0.04±0.003	-	-
26)	Heptanol	-	-	-	-	.03±0.001
55)	Dodecanol	-	-	-	-	.07±0.023
	All	***0.05***	***0.05***	***0.04***	***-***	***0.15***
	Hydrocarbons					
1)	Decane	-	-	-	-	tr
4)	Toluene	-	-	-	-	tr
13)	Dodecane	-	-	-	-	tr
29)	Pentadecane	-	-	-	-	0.07±0.009
32)	Hexadecane	-	-	-	0.02±0.003	-
54)	Nonadecane	-	-	-	-	0.01±0.002
	All	***-***	***-***	***-***	***0.02***	***0.08***
	Total volatiles	***5.79***	***5.38***	***8.33***	***2.61***	***2.40***

Figures legends

Figure 1A. Percentages of *Nesidiocoris tenuis* female showing a response toward the volatile cues produced by healthy tomato, *Sesamum indicum* and *Dittrichia viscosa* plants. Per each test, asterisks indicate statistical significance of the likelihood chi-square, at confidence interval level of 95%; n is the number of insects that made a choice and NC is the number of insects that did not made a choice in the 10 minutes of observation.

Figure 1B. Percentages of *Nesidiocoris tenuis* female showing a response toward the volatile cues produced by healthy and infested tomato plants with eggs and larvae of *Tuta absoluta*, *Sesamum indicum* and *Dittrichia viscosa* plants. Per each test, asterisks indicate statistical significance of the likelihood chi-square, at confidence interval level of 95%; n is the number of insects that made a choice and NC is the number of insects that did not made a choice in the 10 minutes of observation.

Figure2. Chemical structure of the main volatile terpenes identified in the volatile emissions of the analyzed plants.

Figure3. Headspace Solid-Phase Microextraction coupled with Gas Chromatography–Mass Spectrometry (HS-SPME-GC-MS) chromatograms of volatile emissions in Tomato, *Dittrichia viscosa* and *Sesamum indicum* plants.

Fig 1 A

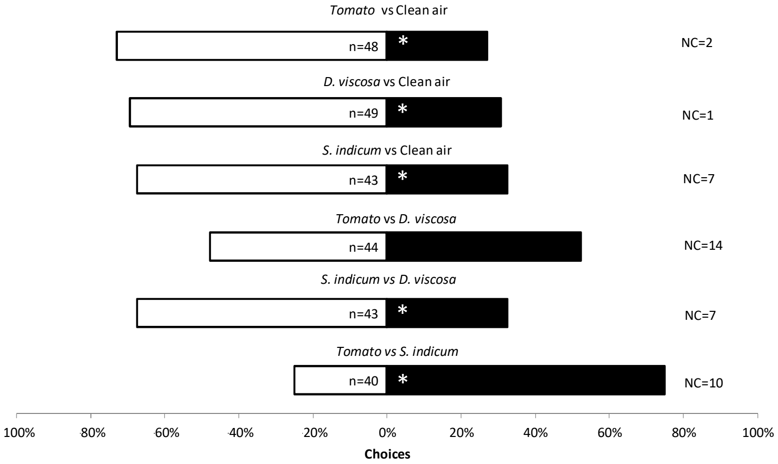

Tomato vs Clean air
n=48
*
NC=2
D. viscosa vs Clean air
n=49
*
NC=1
S. indicum vs Clean air
n=43
*
NC=7
Tomato vs *D. viscosa*
n=44
NC=14
S. indicum vs D. viscosa
n=43
*
NC=7
Tomato vs S. indicum
n=40
*
NC=10
100%
80%
60%
40%
20%
0%
20%
40%
60%
80%
100%
Choices

Fig 1 B

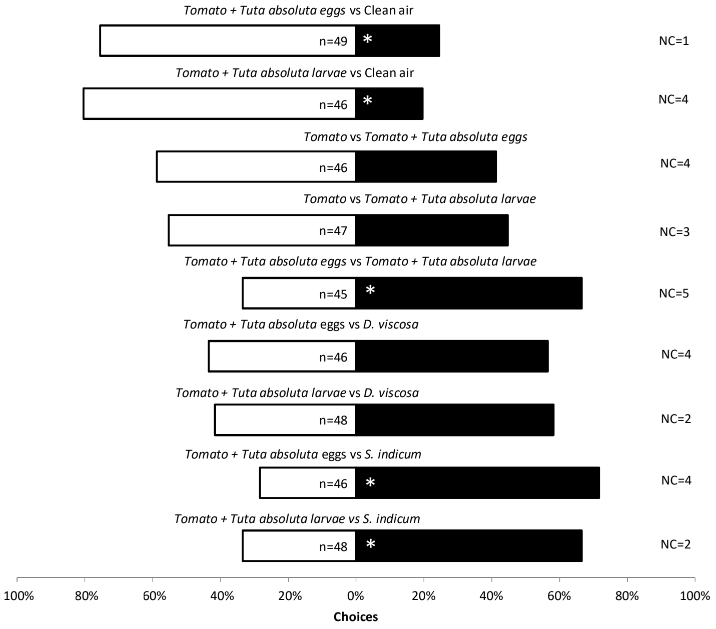

Tomato + Tuta absoluta eggs vs Clean air
n=49
*
NC=1
Tomato + Tuta absoluta larvae vs Clean air
n=46
*
NC=4
Tomato vs Tomato + Tuta absoluta eggs
n=46
NC=4
Tomato vs Tomato + Tuta absoluta larvae
n=47
NC=3
Tomato + Tuta absoluta eggs vs Tomato + Tuta absoluta larvae
n=45
*
NC=5
Tomato + Tuta absoluta eggs vs D. viscosa
n=46
NC=4
Tomato + Tuta absoluta larvae vs D. viscosa
n=48
NC=2
Tomato + Tuta absoluta eggs vs S. indicum
n=46
*
NC=4
Tomato + Tuta absoluta larvae vs S. indicum
n=48
*
NC=2
100%
80%
60%
40%
20%
0%
20%
40%
60%
80%
100%
Choices

Fig 2

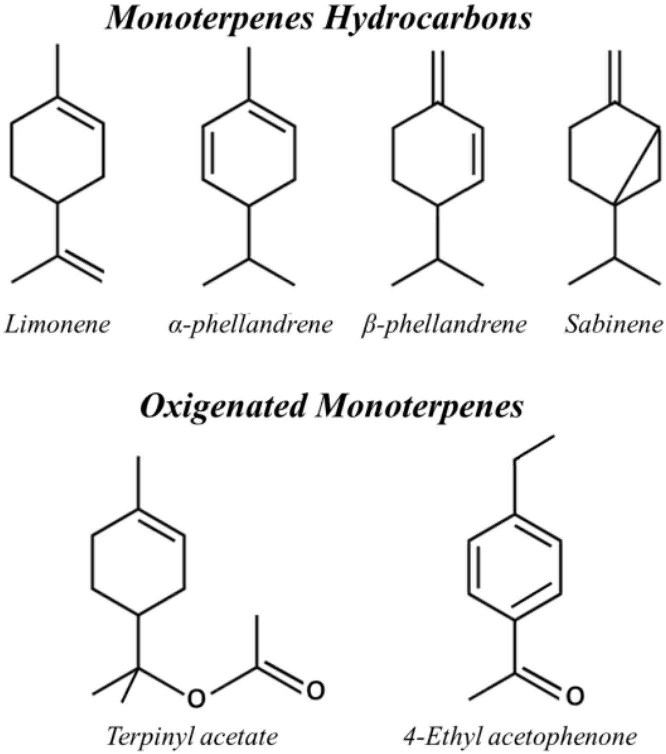
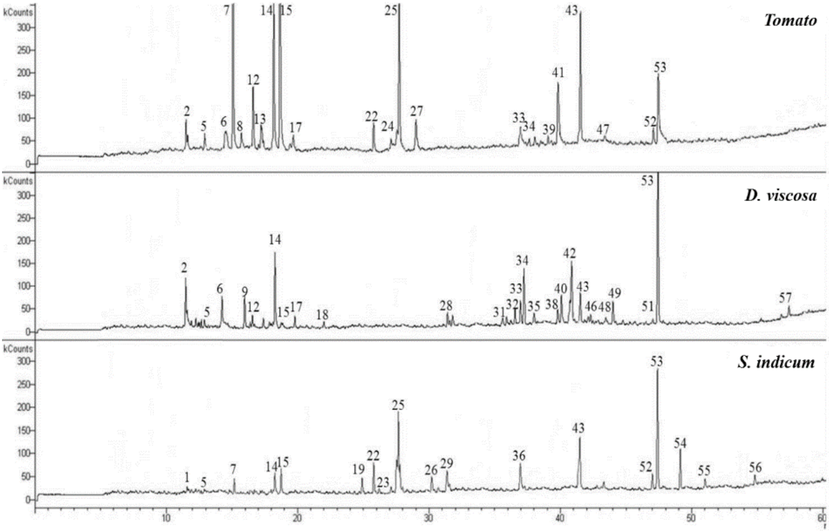

Monoterpenes Hydrocarbons
Limonene
α-phellandrene
β-phellandrene
Sabinene
Oxigenated Monoterpenes
O
O
Terpinyl acetate
O
4-Ethyl acetophenone

Fig 3


kCounts
300
250
200
150
100
50
0
Tomato
2
5
6
7
8
12
13
14
15
17
22
24
25
27
33
34
39
41
43
47
52
53
D. viscosa
2
5
6
9
12
14
15
17
18
28
31
32
33
34
35
38
40
42
43
46
48
49
51
53
57
S. indicum
1
5
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5. Discussion and conclusions

My doctoral studies aimed at better understanding multitrophic interactions existing in the food web involving the Neotropical invasive pest *Tuta absoluta* and its natural enemies in the Mediterranean basin. The experimental work, carried out on this biological system, highlighted the key role performed by the plant in orchestrating interactions in the whole multitrophic system and in particular with reference to zoophytophagous species, such as *N. tenuis*.

We acquired several elements that confirm the mirid opportunistic behavior: i) the tendency to scavenge on carrions, such as *T. absoluta* parasitized larvae, ii) the ability to perform IGP on parasitoids, increasing its competitiveness, iii) the capability of both nymphs and adults to induce plant defense against other organisms feeding on plant iv) the capability to detect the more suitable plants through their volatiles.

These behavioral traits could support the evolutionary hypothesis that the species belonging to the Miridae family represent a transitional model from herbivory toward carnivory and for this reason it is one of the most interesting group of insects to be studied in an ecological perspective.

Our studies confirm all the adaptive behavioral traits of *N. tenuis*, such as the ecological flexibility to consume both prey and plant that allows the establishment of this group of predators in the crop and their preservation when prey is scarce (Perdikis et al., 1999; Sanchez et al., 2004; Urbaneja et al., 2005).

Several ecological studies, which involve zoophytophagous predators, consider the effects of the interaction with plant on the effectiveness of these organisms as biocontrol agents

(Agrawal et al. 1999; Eubanks et al. 2003). These interactions with plants, often, have a positive effect on the suppression of a pest. Indeed, some studies highlight the effect of the secondary metabolites produced by plants and induced by omnivores, in the process of switching feeding from plant to prey (Agrawal et al. 1999). In fact, plants produce toxins that, if ingested by an omnivorous predator, may induce zoophagy. Omnivore mirids, are known to be associated with glandular plants, provided with particular organs (trichomes) where many defense compounds such as terpens, alkaloids and other metabolites are stored and act as direct defence tools against phytophagous species. Very often these glandular sticky trichomes capture and kill insects so mirids may feed on their carrions.This highlights a scavenging behavior by these mirids that may be one of the adapatative pathways toward carnivory mediated by plants (Sugiura and Yamazaki, 2006; Wheeler and Krimmel, 2015). Furthermore, scavenging related to intraguild predation may be a further evolutionary step leading to predatory behavior. Actually, mirids, such as *N. tenuis* and *M. pygmaeus*, demonstrated to prefer decayed prey and for that they consume already parasitized host performing kleptoparasitism (Chailleux et al. 2014; Naselli et al. 2016). Thus, as scavengers they have the possibility to come into contact with parasitized prey and as consequence with parasitoid living instars, and from these interactions the evolution towards predation could originate (Wheeler and Krimmel, 2015).

The competitiveness of *N. tenuis* is further proved by our observations on the plant responses to its feeding activity. Our results, indeed, demonstrate that all motile stages of *N.*

tenuis can trigger defensive responses in tomato plants against other organisms feeding on the plant such as *B. tabaci*. *Nesidiocoris tenuis* punctured tomato plants are repellent for *B. tabaci* and attractive for its parasitoid *E. formosa*.

Moreover, our studies evidenced the important role that companion plants may have in the management of pests and their natural enemies. Our olfactory bioassays demonstrated a preference of *N. tenuis* for healthy sesame plants even when compared with infested tomato plants, therefore showing a prevalent herbivorous behavior of the mirid. This further confirms the mirid trophic flexibility being able to switch from one feeding regime to the other.

Furthermore, the bioassays on infested *T. absoluta* tomato plants, showed a non significant attractivity of these plants toward the mirid. This can depend not only on the mirid tendency to herbivory but also on the manipulation of the plant response by the pest. This is also the case of some phloem-feeding insects, such as whiteflies, which are able to activate SA pathways in plant, in order to suppress the antagonist JA pathway and the related defense response in plant (Zarate et al. 2007; Walling, 2008). Other more comparable studies demonstrated the capability of lepidopteran eggs to manipulate plant defenses (Bruessow et al. 2010; Fatouros et al. 2012).

On the basis of our main achievements new research lines and extended applications can originate.

Some of these possibilities are to test single volatiles identified in our study through electroantennography (EAG) studies and in olfactometer in order to identify the compounds playing a leading role in attracting *N. tenuis*.

The resulting applications from these achievements could be the use in the field of baited traps using synthetic volatiles or to spray the substances on the crops to attract beneficials. Other interesting applications, deriving from these studies, could be the enhancement of plant defenses in order to reduce the effects of pest on crops.This issue needs further laboratory studies in order to check potential practices or substances that may enhance plant defenses. One potential application in this direction could be the use of plant extracts (botanicals) with defense induction properties (Kessler et al., 2006) in order to stimulate plant metabolism toward the production of metabolites involved in direct and indirect defenses, reducing the effects of pests and enhancing the attraction of natural enemies.

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