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Effect of dual biotic stress on plant volatile synomones used by an egg parasitoid

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DEDICATION

To the soul of my uncle ***Faouzi*** *who always supported me with great love and affection. And whom I miss enormously.*

To my ***dear parents*** *who have always been here for me, and gave me a magnificent model of labor and perseverance.*

Your gracious presence and your boundless love have never ceased to give me the power and the courage to face gaily the life.
God bless you.

To my grandmothers, my Grandmother ***Kaboura*** *, uncle* ***Mourad****, my aunt* ***Souhaya***

They will find here the fruits of their sacrifice and witness of my recognition.

To my dear ***brother*** *and* ***sisters***
Thanks for being the happiness of my life.

To my dear ***Friends***
For their support and love.

With all my best wishes
Rihem

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Chapter 1

Chapter 1
Plant volatile synomones used by parasitoids: Multi-trophic perspective

Abstract

Plants respond to arthropod herbivory with the induction of volatiles called herbivore-induced plant volatiles (HIPVs). These volatiles appear to be important sources of information that attract parasitoids. Parasitic wasps foraging decisions are often affected by community characteristics such as community diversity and complexity. As part of a complex habitat, the presence of unsuitable hosts (non-host) may affect foraging behavior of parasitoids. In this chapter, we outline the importance of the presence of unsuitable herbivores on the behavioral responses of parasitoids. First we review the foraging behavior of parasitoid in tritrophic interaction. Then we focus on foraging behavior by parasitoids in multiherbivore communities either with the presence of non-host in above- ground or in below- ground part.

Key-words: Parasitoids, HIPVs, non-host, above- ground, below-ground.

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1.1 Introduction

Plants are the key components of the majority of food webs on the Earth (Schoonhoven *et al*., 2005). The interactions between plants and insect herbivores play a major role in ecological interactions in nature, the broadness of the field is enormous and the ongoing theory development reveals a lot of attention (Johnson *et al*., 2011). Plants are far from passive victims of their attackers and they have evolved a wide spectrum of strategies to defend themselves against their various attackers (Heil and Ton, 2008). These defense strategies can be classified as direct and indirect that can be either constitutive (e.g. always expressed) or inducible (e.g. appear only in need) (Agrawal and Heil, 2012). Direct defenses have a direct negative impact on the development and behavior of herbivore via physical barriers, such as spines, thorns, trichomes, and waxes; or via chemical compounds producing toxins, anti-digestive and anti-nutritive compounds. In addition, plants also benefit from indirect defenses through the recruitment of natural enemies (i.e. parasitoids or predators) of herbivores that actively reduce the number of herbivores. Attraction of natural enemies can be achieved by the synthesis and emission of specific Volatile Organic Compounds (VOCs) called Herbivore Induced Plant Volatiles (HIPVs) (Price *et al*., 1980; Kessler and Baldwin, 2001; Dicke and Baldwin, 2010). More recently, studies have also showed that plants can respond to herbivore oviposition by releasing Oviposition-Induced Plant Volatiles (OIPVs) which can recruit egg parasitoids of insect herbivores (reviewed by Hilker and Meiners, 2010) even if, in some case studies, a combination of oviposition and feeding activity of the herbivore host is required to trigger attraction (reviewed by Colazza *et al*., 2010; Conti and Colazza., 2012).

Historically, studies on HIPVs have been largely focused on tritrophic interactions involving plant, above ground herbivore and natural enemy in a tightly controlled laboratory environment. This approach overlooked that plant growing in agro-ecosystems exchange information with other neighboring plants and they are normally under simultaneously or sequentially attack by several species of insect herbivores that could damage both above and below-ground plant tissues, mainly leaves and roots. Furthermore, plants may interact with soil-borne beneficial microbes and abiotic factors (Gouinguené and Turlings, 2002; Pieterse and Dicke, 2007; Dicke, 2009; Holopainen and Gershenzon, 2010; Pineda *et al*., 2010; Pierik *et al*., 2014).

Since the last decade, several studies have attempted to fill this gap by analyzing the chemical composition of volatile blends of plants, and investigating the foraging behavior of carnivores, upon multiple- infestation (Shiojiri *et al.* 2001; Cardoza *et al*., 2002; Rodriguez-Saona *et al.*, 2003; Rostás *et al.*, 2006; Moayeri *et al.*, 2007; Rasmann and Turlings, 2007; Soler *et al.*, 2007a, b; Zhang *et al*., 2013; Ponzio *et al*., 2014). This chapter will review the role of plant volatile synomones in the recruitment of parasitoids under tritrophic and multitrophic interactions.

1.2 Plant Synomones induced by Feeding and/or Oviposition

Over 30 years ago, it was demonstrated for the first time that the release of specific volatiles by herbivore infested plant called Herbivore-induced plant volatiles (HIPVs) attract the natural enemies of the attacking herbivores (Dicke and Sabelis, 1988; Turlings *et al*., 1990). Only 10 years ago studies showed also that plant infested with herbivore eggs can emit oviposition- induced plant volatiles(OVIPs) that attract egg parasitoids (Meiners and Hilker , 2000 ; Hilker *et al*., 2002a,b; Mumm and Hilker, 2005). In some cases a combination of egg deposition and feeding of the adults are necessary to attract egg parasitoids (Colazza *et al.,* 2004a). These plant volatiles are classified as synomones because they can benefit both the emitting plant as well as the responding natural enemy (Vet *et al*., 1991). Several functions are allocated to HIPVs; in addition to attracting natural enemies of herbivores, induced plant volatiles can also act as feeding and/or oviposition deterrents to the attacking herbivores, thus can be considered as key components of direct and indirect defense systems (Kessler and Baldwin, 2001; Arimura *et al*.,2009). Furthermore, HIPVs can mediate plant-plant interaction by inducing the expression of defense genes and emission of volatiles on the neighboring undamaged plant, thus increasing their attractiveness to carnivores and decreasing their susceptibility to the damaging herbivores (Arimura *et al*., 2000; Engelberth *et al*., 2004; Baldwin *et al*., 2006; Heil and Karban, 2010).

HIPVs often consist of a blend of various Volatile Organic Compounds (VOCs) mostly belonging to terpenoids, phenylpropanoids/benzenoids and fatty acid derivates, upon herbivore attack, some of which induce quantitative change in constitutive emission while other are synthesized de *novo* (Paré and Tumlinson, 1999; Turling *et al.*, 1998 ; Dudareva *et al*.,2006). The emitted HIPVs are generally induced by elicitors present in the herbivore saliva

or in oral secretion (De Moraes *et al*., 1987; Truitt and Pare, 2004; Truitt *et al*., 2004; Schmelz *et al*., 2006). Different biosynthesis pathways can be involved, such as the octadecanoid pathway, with the central role of the phytohormone jasmonic acid (JA); the shikimate pathway, with the central role of the phytohormone salicylic acid (SA); and the ethylene (ET) pathway (Kessler and Baldwin, 2002; Van Poecke and Dicke, 2002; Dicke *et al*., 2003; Zhang *et al.,* 2013). The HIPVs are emitted not only from the damaged parts, but also from undamaged parts of the plant, increasing the detectability of the signal (**Fig.2**) (Dicke *et al*., 2009). Apart from the aerial parts, roots of plants also emit HIPVs in response to the infestation by below-ground insect pests, which attract natural enemies of the infesting herbivore (**Fig.2**) (van Tol *et al*., 2001; Rasmann *et al*., 2005).

HIPVs are highly produced by the plants. The blend of volatiles may vary, both quantitatively and qualitatively, with plant species, herbivores species, the nature of damage (feeding or /and oviposition), the age, developmental stage and herbivore density (Hilker and Meiners, 2002; Hilker *et al.*, 2002a, b; Colazza *et al*., 2004 a; Hilker and Meiners, 2011, McCormick *et al*., 2012). All these feature makes HIPVs reliable indicators of the identity of the feeding herbivores for foraging carnivores (De Moraes *et al.*, 1998; Takabayashi *et al.*, 1995; Dicke, 1999; Shiojiri *et al*., 2010; McCormick *et al*., 2012). According to literature, HIPV profiles can change if multiple herbivore species are feeding on a plant. This change have been found to be mainly quantitative (de Boer *et al*., 2008; Dicke *et al*., 2009; Moayeri *et al*., 2007; Rodriguez-Saona *et al*., 2003; Shiojiri *et al*., 2001; van Poecke *et al*., 2002; Zhang *et al*., 2009) (Table 1 and 2). Similar to above-ground interactions, below-ground can be specific at both the plant and herbivore levels (Dudareva *et al*., 2006; Rasman and Turling, 2007).

Several investigations have demonstrated that indirect plant defences constitute a widespread ecological phenomenon (Dicke and Baldwin, 2010). However, the majority of the studies have been carried out in linear tritrophic systems consisting of one species each of the plant, herbivore host, and the associated predator/parasitoid. In nature, plants often suffer multiple biotic or abiotic stresses, a scenario that may interfere with the recruitment of natural enemies (Dicke *et al.*, 2009). Therefore, indirect plant defences under multiple herbivory deserve also a better understanding, as plant responses may be shaped by a whole community of interacting herbivores than by single pair wise plant-insect interactions (Agrawal *et al.*, 2006; Dicke and Baldwin, 2010; Poelman and Dicke, 2014).

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In this scenario, it would be particularly interesting to investigate how a plant responds to multiple herbivore species attack with consequences for the parasitoids' foraging behavior.

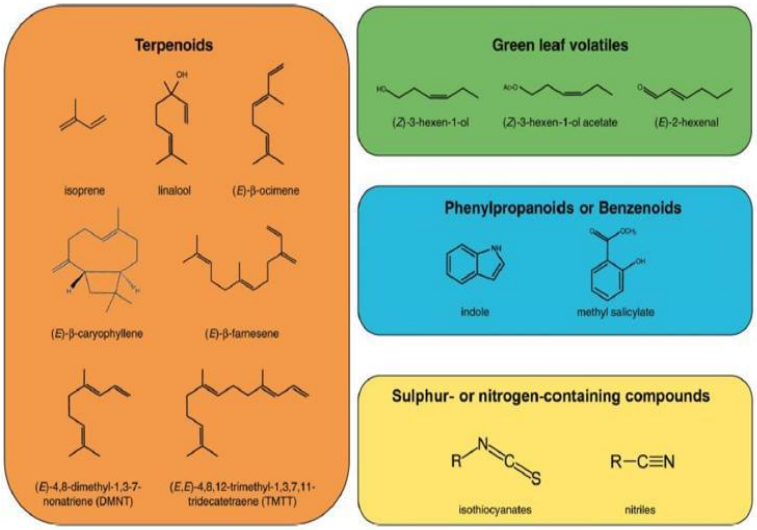

Figure 1. Major volatile compounds (groups) produced by plants. (Mumm and Dicke, 2010).

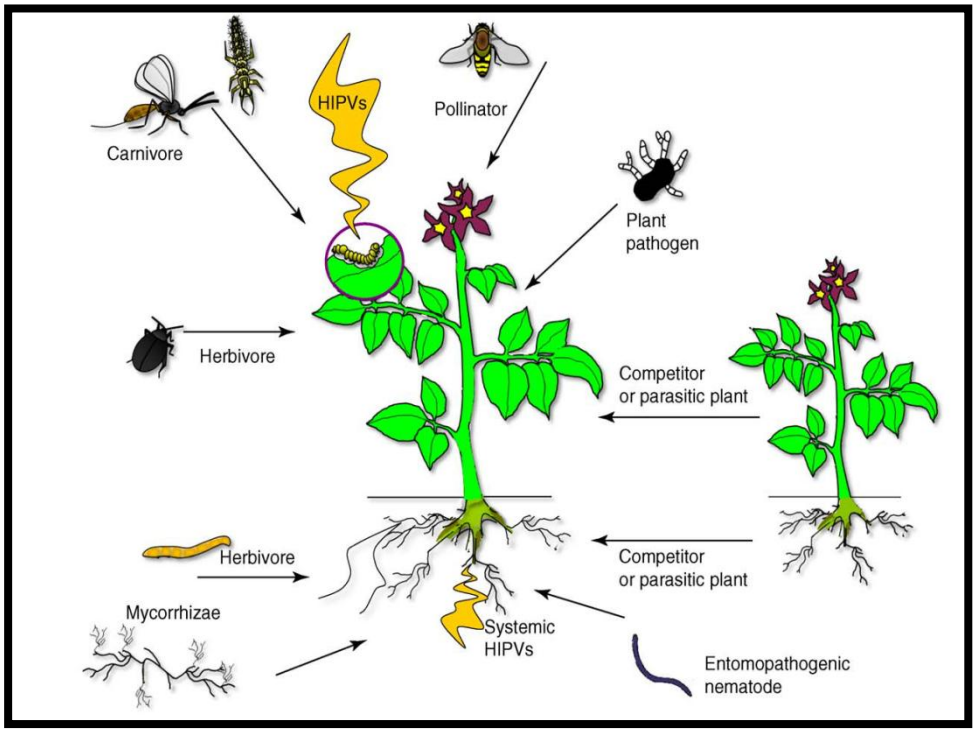

Figure 2: Systemically Herbivore-induced plant volatile emission from plant, locally, attacked by herbivores (Dicke and Baldwin, 2010)

1.3 Foraging of parasitoids in tritrophic interaction

One of the most important groups of natural enemies of herbivorous insect is parasitoids wasps (parasitoids). They are consumer in the food web and play a vital role in a multitrophic interaction context in natural communities (Pedersen and Mills, 2004). Many of their hosts are crops pests, which makes parasitoids important organisms for their use in biological pest control, contributing to enormous saving in agriculture (Simpson *et al*., 2011a). Unlike predators that may need to feed on several preys to reach maturity, the resources for parasitoid development are finite and are packed into a single host. Consequently, parasitoids are under rigorous selection pressure to optimize use and disposal of these limited host resources (reviewed by Harvey 2005). Therefore, female parasitoids need to find suitable hosts for reproduction otherwise their genes will not be passed on to future generations. Generally, a female parasitoid must find hosts at a stage suitable for parasitism. The host selection process involves a sequence of phases mediated by physical and chemical stimuli from the host, the substrate, and/or associated organisms, eventually leading to successful parasitism (Vinson, 1985; Godfray, 1994). Because parasitoid foraging time is limited and the potential cues available are numerous, a parasitoid faces the need to optimize exploitation of available cues and discriminate those most reliable in indicating the presence of a suitable host (Vet and Dicke, 1992, Hilker and McNeil, 2007). However, the location and recognition of a suitable host is a complex process, especially for egg parasitoids, because of major constraints due to the small sizes of both the host and the parasitoid itself.

Eggs are usually unapparent, especially when they are small, dispersed in the habitat, and concealed in plant tissue. As such, cues that are directly related to the presence of eggs may have low detectability, but high reliability (Vet and Dicke, 1992, Vinson, 1994, Vet *et al*., 1995). Additionally, suitable host eggs are generally available for only a short time due to their rapid development (Vinson, 1998). Therefore, egg parasitoids have developed specialized strategies to overcome the reliability-detectability dilemma in order to efficiently parasitize host eggs. Successful parasitism is accomplished through the combined exploitation of cues that are directly and indirectly related to host eggs (Vinson, 1998, Vet and Dicke, 1992, Fatouros *et al*., 2008, Colazza *et al*., 2010).

First, egg parasitoids may detect volatiles from non-target instars of the host that is, adults or juveniles, to reach the vicinity of the host eggs (sensu "infochemical detour" Vet and Dicke, 1992) eventually enabling them to pin-point eggs using additional long- and/or short-range cues. A particular and interesting example of such detour behavior of egg parasitoids is phoresy on adult host females; via this strategy, not only relevant cues are more detectable, but the adult itself is also exploited by the parasitoid as a vehicle to arrive at host eggs (Clausen, 1976, Huigens *et al*., 2010).

Second, egg parasitoids may exploit Herbivore induced synomones called also, herbivore induced plant volatiles (HIPVs), which are emitted in large quantity from plant upon herbivore attack, and are, therefore, easily detectable by foraging parasitoids but not necessarily highly reliable (Fatours *et al*.,2008). Indeed, numerous studies documented the key role of these volatiles as reliable long range cues for naturals enemies of insect herbivores, therefore HIPVs are of crucial importance for foraging carnivores (see reviews, Fatours *et al*., 2008, Dicke and Baldwin , 2010, Hare, 2011, Kessler and Heil, 2011). More recently ,studies has also showed that some egg parasitoids are capable of exploiting plant chemicals emitted as a result of egg deposition called oviposition- induced plant volatiles (OIPVs) , thus rendering such highly detectable cues also highly reliable (reviewed by Hilker and Meiners, 2011, Hilker and Fatours, 2015). Depending on the herbivore species, OIPV emission occurs without plant wounding or it can be associated with plant damage caused by the herbivore's activities during/before oviposition (reviewed by Colazza *et al*.,2010; Conti *et al*.,2012). Finally, egg parasitoids have been observed to associate, through learning, highly detectable but less reliable cues with the presence of suitable hosts, thus increasing reliability of such cues in experienced wasp females (Peri *et al.*, 2006, Dauphin *et al*., 2009).

1.4 Foraging behavior by parasitoids in multi-herbivore communities

The host searching behavior of various parasitoids has been studied extensively in tritrophic systems consisting of a single food chain of plant, herbivore and parasitoid species (Vet and Dicke, 1992; Heil, 2008). In (agro) ecosystems, however, parasitoids forage in a complex habitat consisting of a diverse community (Dicke *et al*., 2009). Only in the last decade experimental studies have addressed parasitoid foraging behavior in more natural, complex habitats.

Results from these studies have shown that predictions on parasitoid foraging in simple tritrophic communities should be nuanced for foraging behavior of parasitoids in more complex habitats (e.g. Rodriguez-Saona *et al*., 2005; Bukovinszky *et al.,* 2012). One of the factors of a complex habitat is the presence of a community of other herbivores that may be unsuitable host species (here called non-host herbivores). The presence of non-host herbivores in the habitat in which parasitoids search for hosts has been shown to have a strong effect on parasitoid foraging behavior (Rodriguez- Saona *et al*., 2005; Dicke *et al*., 2009). These non-host herbivores may either be present on neighbouring plants or share the same plant with host herbivores of a parasitoid (de Rjik *et al*., 2013). The shared food plant may be attacked simultaneously or sequentially (Vos *et al.,* 2001; Poelman *et al*., 2010) and, on the shared food plant, the herbivores may feed on a single plant organ or may feed spatially separated on different plant organs above- as well as below-ground (Van Dam and Heil, 2011).

As a result, the presence of non-host herbivores can affect parasitoid foraging behavior on several levels, from finding the plant to locating the host on the food-plant and deciding whether or not to parasitize the host, each decision phase being an important attribute of parasitoid fitness (McArthur and Planka, 1966; Van Alphen *et al*., 2003). In host location, the presence of non-host herbivores may affect the parasitoid in two phases. First, like several other biotic and abiotic factors, non-hosts may influence the ability or efficiency of parasitoids to locate patches of host-infested plants from a distance (Gouinguené and Turlings 2002; Dicke *et al*., 2009). From long distance, parasitoids exploit HIPVs to locate their host (Vet and Dicke, 1992; Heil, 2008). In more complex habitats, HIPV cues of host presence are surrounded by noise of volatiles induced by unsuitable hosts (Dicke *et al*., 2009).

What is clear from literature to date is that the effect of non-hosts on parasitoid foraging decisions may be determined by the host range specialization (generalist, specialist) of parasitoids. Moreover, a growing body of literature suggests that, under multiple herbivore attack, the emission of induced volatile blends can be altered in a specific manner that is dependent on the herbivorous insect feeding guild (biting–chewing or piercing–sucking), plant organ attacked (root damage or leaf damage), herbivore density, order of colonization and time lag between the arrivals of the different attackers (de Rijk *et al*., 2013 and references therein). Parasitoid recruitment as a consequence of altered volatile emissions is likely disrupted when plants are simultaneously exposed to herbivore species which induce different defense pathways (Zhang *et al*., 2009, 2013).

This disruptive effect can be mediated by cross-talk between the main plant defense signaling pathways, namely, the jasmonic acid (JA) and salicylic acid (SA) pathways (Pieterse *et al*., 2009, 2012). In the sections that follow we will study the influence of non-host herbivore presence either in above or / below- ground plant part on parasitoids behavior.

1.4.1 The effect of non-host herbivores attacking above-ground plant organs

In herbivore-rich arthropod communities several herbivores share the same host plant simultaneously or sequentially. Two or more herbivores can indirectly interact with each other through direct plant defense traits. However, there are ample possibilities for interaction between attackers through indirect defense too (Dicke *et al*., 2009).

The specificity of the effect of a non-host herbivore species on a plant while feeding together with a host herbivore may be found in elicitation of different signal transduction pathways by both herbivore species within the plant. Through crosstalk, signal transduction pathways that are induced by either herbivore species can interact, which thereby may alter the volatile profiles emitted by the dual infested plant compared to singly infested plants (Dicke *et al*., 2009). In fact, the JA signaling pathway (induced by leaf chewers and by insect oviposition) and the SA signaling pathways (induced by phloem feeders) are often found to act antagonistically (Dicke *et al.*, 2009; Pieterse *et al*., 2009; Thaler *et al*., 2012), although exceptions do exist (Heidel and Baldwin, 2004; De Vos *et al*., 2005). As herbivores from different feeding guilds generally induce different defense signaling pathways (Howe and Jander, 2008), simultaneous feeding by non-hosts from other feeding guilds than the host could affect the biosynthesis and release of HIPVs (Schwartzberg *et al*., 2011; Zhang *et al*., 2013) and in this way the host-searching efficiency of parasitoids (Dicke *et al*., 2009).

According to various studies, the effect of multiple herbivore attacks belonging to different feeding guilds on natural enemies foraging success could be positive, negative or neutral (Tab.1); Mumm and Dicke ,2010, Zhang *et al*.,2013).

In some cases, the attack by dual herbivore species respectively affecting JA and SA pathways led to a decrease in carnivore attraction compared to single species herbivory. For instance, when lima bean plants were infested with spider mites *Tetranychus urticae* (inducing the JA pathway) and silver-leaf whitefly *Bemisia tabaci* (inducing SA pathway), predatory mites preferred plants damaged only by its host over dually damaged plant.

This attenuation effect was the consequence of reduction in (*E*)-β-ocimene in dually infested plants (Zhang *et al*., 2009). So far, in all the other studies using herbivores of different guilds, natural enemies were either equally attracted to dual- and single-herbivore infested plant, or preferred dual above-ground infested plants over single infested plant (Tab.1) (Rodriguez-Saona *et al* ., 2005; Agbogba and Powell, 2007; Moayeri *et al*., 2007; Erb *et al*., 2010).

Also within feeding guilds, different species of non-hosts may differentially affect parasitoid responses to HIPVs. The parasitoid *C.glomerata* , for example, respond differently to two non-host caterpillars, *P.xylostella,* and the cabbage moth, *Mamestra brassicae,*, when discriminating between plants infested with a non-host and plants infested with both host (*P.rapae*) and non-host. With the non-host *M. brassicae,* the parasitoid prefers dual infestations over non-host infestations (Bukovinszky *et al*., 2012), whereas with the non-host *P. xylostella* the parasitoid does not discriminate between dual and non-host infestations (Vos *et al*., 2001). This may imply that, within the same feeding guild, some non-host species feeding simultaneously with the host make the plant more attractive to parasitoids (also observed by Rodriguez- Saona *et al*., 2005) while other non-host species do not.

Not only the nature of herbivore species play a role in multi-trophic interactions, but also other factors enhance the level of specificity in plant odours emitted in response to multiple herbivory. Effects of multiple herbivory on HIPV emissions may be plant species or plant genotype specific. For example, combined infestation of lima bean, *Phaseolus lunatus*, plants by the spider mite, *Tetranychus urticae*, and the beet armyworm caterpillar, *Spodoptera exigua*, resulted in the majority of compounds being more strongly induced than with either herbivore alone, while in cucumber, *Cucumis sativus*, it resulted in two compounds being emitted in lower amounts from dual-infested plants than the sum of the amounts emitted by plants treated with a single infestation of either herbivore. This suggests that the effects of dual infestation are driven by different mechanisms in lima bean and cucumber plants (De Boer *et al*., 2008). However, for both plants, the effect of dual infestation versus single infestations on the predator response was similar as the natural enemy was more attracted to volatiles from dual-infested plants (De Boer *et al*., 2008).

Observed effects of non-hosts may also be dependent on the density of host and non-host herbivores attacking the plant (Zhang *et al*., 2009). At least for certain species, non-host presence negatively interferes with host attractiveness only when non-host density is above a certain threshold (Zhang *et al.*, 2009, 2013; Yamamoto *et al*., 2011). In addition, the developmental stage of non-hosts may determine the attractiveness of a dual infestation.

A single second-instar non-host caterpillar, for example, did not affect the attractiveness of dual infested plants to parasitic wasps, while a single fifth-instar non-host caterpillar negatively affected the attractiveness of these plants. Yet, the exact factors that caused the change in attractiveness need to be identified (Yamamoto *et al*., 2011).

Hosts and non-hosts usually do not arrive simultaneously on the plant. The order of arrival could also affect plant responses in terms of interfering with signaling pathways (Dicke *et al*., 2009) and can affect plant-mediated interactions between herbivores (Kessler and Baldwin, 2004; Erb *et al*., 2011). Interference among signaling pathways may affect HIPV emission and this may result in altered parasitoid searching behavior (Dicke *et al*., 2009). In at least one system, however, non-prey infestation interferes with the attraction of a carnivore irrespective of the order of infestation (Zhang *et al*., 2009). From the available literature; we did not find any general patterns regarding parasitoid response to HIPVs affected by several aspects of the plant/host/non-host/parasitoid complex. However, we conclude that specificity of plant responses to herbivory is the main driver of specificity in parasitoid responses to situations of non-hosts inducing emission of plant volatiles.

Above mentioned studies deal it with HIPVs in multi-herbivores perspectives. A very recently growing body of literature suggests that, under multiple herbivore attack, the emission of OIPVs also can be altered depending on several aspects of the non-host herbivore attack such as insect feeding guild (Cusumano *et al*., 2015), plant organ attacked (Moujahed *et al*., 2014), herbivore density (Ponzio *et al*., accepted) and lack of plant-insect co-evolution (Cusumano *et al*., 2015). Consequently, depending on the interplay of the plant-insect interactions, indirect egg-induced plant defences could be disrupted or withstand non-host herbivore interference. Therefore, oviposition-induced plant defences under multiple herbivory deserves a better understanding.

1.4.2 The effect of non-host herbivore attacking below-ground plant organs

Over the past three decades attention has been paid to interactions between above-ground and below-ground insect herbivores sharing the same host plant (Tab.2). It is now well known that herbivore insects can indirectly interact even when they are spatially or temporally separated from other herbivores associated with the same host plant (Gange and Brown, 1989; Masters, 1995; Masters and Brown, 1997; Gange, 2001; Masters *et al*., 2001).

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Pioneering studies from the early 1990s revealed that root herbivory can shape plant life history, plant performance and fecundity, and can have a significant impact on interactions between plants and above-ground insect herbivores (Gange and Brown, 1989, Moran and Whitham, 1990, Masters *et al*., 1993; Gange ,2001; Bezemer *et al*., 2002, 2003; Blossey *et al.*, 2003).

Plant response to root feeders can even cascade up the above-ground trophic chain and it can affect higher trophic levels including parasitoids (third trophic level), and even hyper parasitoids (fourth trophic level) (van der Putten *et al*., 2001, Bezemer and van Dam ,2005, Soler *et al*., 2005, Rasmann and Turlings, 2007, Soler *et al.*, 2007 a,b ; Erb *et al*., 2009, Soler *et al*., 2013). This has opened up new challenges for the study of multi-trophic plant–insect interactions. Just over a decade ago, a flow of research started to give attention to the potential effects of root herbivores on foraging behavior of a parasitoid of an above-ground herbivore and the mechanisms mediating these interactions. To date, these rare above-below ground studies do not lend themselves to simple generalizations (Master *et al*., 2001; Poveda *et al*., 2005; Rasmann and Turlings, 2007; Soler *et al*., 2007a, 2013). Masters *et al.* (2001) were the first to study this interaction revealing a positive effect of root herbivory on the recruitment of parasitoids above-ground: the population abundances of both tephritids and parasitoids were greater on thistle plants subjected to root herbivory. However, this seemed to be correlated to the higher number of herbivores on plants and does not necessarily imply a change in signal emission. Similarly, *Sinapis arvenis* plant concurrently exposed to above and below- ground herbivores were more attracted to aphid parasitoids than conspecific root-undamaged plants. However, the authors did not investigate the possible mechanisms mediating these interactions (Poveda *et al*., 2005).

Since the parasitoid host searching is primarily guided by volatile cues emitted from host-infested plant (Turlings *et al*., 1990, Vet and Dicke, 1992), HIPVs were the primary mediating cues to be tested. There is empirical evidence indicating that volatile blends emitted by plants exposed both to foliar-feeding insects and to root-feeding insects quantitatively and qualitatively differ from blends emitted by plants exposed to each herbivore alone (Rasmann and Turlings, 2007, Soler *et al*., 2007a, Pierre *et al*., 2011). For instance, Pierre *et al*. (2011) showed that HIPVs emitted by *Brassica napus* plants simultaneously infested by a root- and a leaf-feeding insect, *Delia radicum* and *P. brassicae*, respectively, differed from volatiles emitted when the plants were infested by a single shoot herbivore only.

In similar system, Soler *et al*. (2007a), showed that volatile blends of *B. nigra* plants exposed to *P. brassicae*, a leaf-chewing host of the parasitoid *C. glomerata*, were characterized by high levels of volatile compounds that are reported to act as insect attractants, such as β-farnesene and dimethylnonatriene. In contrast, plants exposed to *D. radicum*, a root-feeding insect, were characterized by high amounts of sulphides such as dimethyl disulphide and dimethyl trisulphide, which often act as repellents and/or toxins to insects.

Plants co-infested by both herbivore species showed a relatively high level of these repellents and a low level of the attractants compared with conspecific plants with only the above-ground herbivore. *Cotesia glomerata* females were significantly less attracted to plants with hosts that were also infested with root herbivores, and this reduced preference was correlated with the distinct volatile blend that characterized this plant host complex. Similarly, in maize plants (*Zea mays*), the emission of the principal attractant, (*E*) β-caryophyllene, was lower when leaf- and root-chewing herbivores infested the plant compared with the single infestations. Female *C. marginiventris* parasitoids also preferred host-infested plants over plants infested with both hosts and root herbivores (Rasmann and Turlings 2007). Considering that foraging efficiency in parasitoids is directly linked with their reproductive success, Soler *et al*. (2013) proposed the "below-ground root-feeding insect avoidance" hypothesis. This hypothesis suggest that female parasitoids of leaf chewers, whose performance is reduced when feeding on plants previously attacked by root-feeding insects, preferentially oviposit in herbivorous hosts feeding on root-uninfested plants. The authors also provide evidence that changes in the plant-volatile blend induced by root feeding insects may alert the above-ground parasitoids about the presence of the root herbivores on the host plant, which has potentially negative consequences for offspring fitness of the parasitoid. Apart the shared host plant, proof has shown that root herbivores can also influence above-ground host-parasitoid interactions via changes in the 'attractiveness' of surrounding conspecific plants (Soler *et al* .,2007 b).

Other factors can also play an important role in shaping the nature of parasitoids response in a multi-herbivore scenario. The density and the developmental stage of below-ground herbivore, which both are related to the amount of the inflicted damage, are factors determining the nature of response. For instance, the parasitoid wasps, *C. glomerata* foraging for above-ground hosts (*P. brassicae*) only avoided *B. nigra* plants when they were infested by final instars of a root fly larvae (*Delia radicum*), not those plants with younger instars larvae (Soler *et al*., 2007a).

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Parasitoid foraging behavior also can strongly change with experience (Turlings *et al*., 1990; Bukovinsky *et al.*, 2007). The responses of adults of the generalist parasitoid, *C. marginiventris*, to the volatiles produced by maize when attacked by larvae of *Spodoptera littoralis* above-ground and/or larvae of the western corn rootworm, *Diabrotica virgifera*, below-ground varied with the level of experience, or training, of the parasitoid prior to bioassay. Naive adults, or those allowed to oviposit while exposed to the volatile blends of maize induced only by *S. littoralis*, preferred the HIPV blends induced by *S. littoralis* in subsequent tests, whereas wasps allowed to oviposit while exposed to the HIPV blends of maize induced by both herbivore species preferred the HIPV blends of maize induced by both species subsequently (Rasmann and Turlings, 2007). These results point out the necessity of carefully considering and controlling for the type of pre-assay experiences of natural enemies in evaluating their responses to HIPV blends of a particular plant species when attacked by different combinations of herbivore species. In particular, it may be premature to assume that host location by natural enemies is impeded on plants damaged by multiple herbivore species without specifically testing a group of natural enemies that had prior experience with their hosts on plants that were damaged by multiple herbivore species. However, the role of parasitoid learning in dealing with natural variation in plant (and host) quality and plant volatiles induced by root herbivory remains largely unstudied.

So far previous studies suggest that multiple herbivores on a single plant can interact in much more complex and indirect ways than has been considered. These studies provide evidence that female parasitoids can exploit qualitative and quantitative characteristics of the surrounding environment, triggered by a non-host herbivore, to maximize their searching efficiency (Dicke and Van Loon., 2000; Dicke *et al*., 2009). More research is needed to disentangle these complex interactions mediated via changes in the plant volatile blend. Actually, to implement efficient control strategies more data are required about the response of parasitoids perceiving cues emitted by plants under multiple infestations.

1.5 Research objectives and outline of the thesis

In the last 5-10 years, there were a growing number of reports in the North of Europe, such as France and Belgium, indicating that *N. viridula* is expanding its distribution northwards in all probability as a consequence of global warming. In the same time, it was observed that *N. viridula* damages are enhanced and that winter survival is higher and reproductive activity starts earlier in the season.
A project aiming to make the 'smell' of Fava bean more attractive for *T. basalis*, the egg parasitoid of *N. viridula*, responds to growing demand of sustainable technologies for pest management. Furthermore, egg-parasitoids are often favored for biological control deployment because they attack the pests before their hosts molt to the crop-feeding stages and thus have a high potential for preventing damages to the crops.

Actually, the efficacy of insect parasitoids to control a population of target pests can be improved through an accurate understanding of important behavior al and chemical features related to their ability to discover their hosts and to attack them. In this context , this thesis that study the "Role of VOCs emitted by legume plants under biotic stress (*N .viridula* and *S. lineatus*) in the recruitment of egg parasitoids" represents an important step to enhance our understanding of the mechanisms of successful control of pest populations by insect egg parasitoids. Within sustainable crop management regimes, VOCs can be synthetically produced and used in field conditions to attract natural enemies of herbivores showing the potential application of these substances for pest suppression. In conclusion, the acquisition of the above information along with what is already known about the chemical communication between plant/herbivore/parasitoid will provide more insight into the co-evolution of multi-trophic systems. My thesis is covering one objective of an European project called "Going to the root of plant productivity: how the rhizosphere interact with the aboveground armament for indirect and direct defense against abiotic and biotic stressors (PRO-ROOT)". This project is funded by "*Ministero dell', Istruzione, dell', Università e della Ricerca* (MIUR)".

In Italy, *V.faba* plants are commonly attacked by both *N. viridula* and *S. lineatus*, with adults of both species attacking above-ground plant parts early in growing season, whereas both above-and below-ground attacks occur later as the developing weevil larvae feed on roots (Cusumano and Salerno, personal observations). To locate *N. viridula* eggs in such complex

environments that undergo temporal changes in infestation by both hosts and non-hosts, and corresponding changes in plant-derived odor cues, *T.basalis* females could rely on learning abilities. In these circumstances, plasticity in a parasitoid's response would be adaptive and learning could provide valuable flexibility (Peri *et al*., 2006; Fatouros *et al*., 2008; Colazza *et al*., 2010; Cusumano *et al*., 2012). Thus, the first objective of this thesis was to investigate the effects of *S. lineatus* adult or larval attack on the attraction of naïve and experienced *T. basalis* females to *V. faba* plants that were concurrently attacked by the parasitoid's host, N.*viridula*. Bioassays were conducted using above-ground, below- ground and above-below ground infested plants (Chapter 2). The second objective was to evaluate plant volatile under different experimental conditions. Identification of plant volatiles synomones induced in the multitrophic system *V.faba-N.viridula-S.lineatus* (chapter 3). The third objective was to investigate the molecular response of *V.faba* plant to different activities of *N.viridula* (oviposition, feeding and ovipostion) to gain new insight into the mechanisms of egg parasitoid attraction. Also behavioral response of naïve *T.basalis* towards different treated *V.faba* plants was evaluated (Chapter 4).

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Table 1: Effects of non-host/non-prey presence in above-ground part on HIPVs production and on parasitoids /predators preference: Non-host/ Non prey herbivore species.

Host: The organism that harbors a parasite, typically providing nourishment and shelter. **Prey**: The organism that is attacked by predators; Non-host/Non-prey: the organisms that is unsuitable for parasitoids and predators respectively.

System studied				Results	References
Parasitoid / predators species	**Herbivores species**		**Plant species**		
	Host/prey	**Non-host/non-prey**			
Predatory mirid *Macrolophus caliginosus*	Spider mites *Tetranychus urtica*	Aphid *Mizus persicae*	Pepper (*Capsicum annuum* L.)	-The predator showed a stronger response to volatiles emitted from dual infested plant than to those emitted from single infested plant, irrespective of the species. -The amount of VOC emitted from pepper infested by both herbivores was significantly higher than from pepper infested by a single herbivore.	Moayeri *et al*., 2007
Predatory mite *Phytoseiulus Persimilis*	Spider mite *T.urticae*	Caterpillar *Spodoptera Exigua*	Lima bean (*Phaseoluslunatus*) and Cucumber (*Cucumissativus*)	-Predatory mite preferred HIPVs from both species of plant infested with both herbivores over plants infested with either species singly – Quantitative changes in HIPV emission of doubly infested plants, compared to single infested plant	De Boer *et al*., 2008
Predatory mite *P.persimilis*	Spider mite *T.urticae*	Whitefly *Bemisia Tabaci*	Lima bean (*P.lunatus*)	-Predatory mite preferred host damaged plant over dual infested one. –Change in attractiveness was due to a reduction in (E)–ocimene emission from double infested plants.	Zhang *et al.*,2009
Ectoparasitoids *Diadegma semiclausum* *D. fenestrale*	Caterpillar *P.xylostella*	Caterpillar *P. brassicae*	Brassicaceous plant species: - Wild cabbage(*B. oleracea)* -white mustard (*Sinapis alba*) -Feral *Brassica* strain	When offered a choice between HIPV induced by hosts and non-hosts feeding on *B.oleracea*, both parasitoid species preferred host-induced volatiles, but they could not distinguish volatile blends induced by hosts and non-hosts when the caterpillars had been feeding on feral *Brassica* or *S. alba*.	Gols *et al*.,2012
Ectoparasitoids *D. semiclausum*	Caterpillar *P. xylostella*	Whitefly *B. tabaci*	Arabidopsis *(Arabidopsis thaliana)*	-Female of *D. semiclausum* showed a significant preference for the volatile blend from *P. xylostella*-infested plant over that from plants infested with *P. xylostella* plus *B. tabaci.* -Chemical analysis of plant volatiles showed that the composition of the blend emitted in response to the caterpillars was significantly altered by co-infestation with whiteflies.	Zhang *et al*., 2013
Endoparasitoid *Cotesia glomerata*	Caterpillar *Pieris rapae*	Caterpillar *Plutella Xylostella*	Cabbage (*Brassica oleracea*)	-Parasitoid was unable to discriminate between leaves infested with their host and the ones infested with the non-host.	Vos *et al*., 2001

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Endoparasitoid *C. glomerata*	Caterpillar *P. rapae*	Caterpillar *P. xylostella*	Cabbage (*B.oleracea*) and Japanese radis (*Raphanus sativus*)	-*C.plutellae* preferred host infested plants over the non host*; C.plutellae* preferred the HIPV blends induced by its host over both species. –*C.glomerata* preferred plants infested by both host and non-host larvae; quantitative differences in HIPVs emitted were found between different situations.	Shiojiri *et al*., 2000, 2001
Endoparasitoid *C. plutellae*	Caterpillar *P. xylostella*	Caterpillar *P. rapae*			
Endoparasitoid *Aphidius ervi*	Aphid *Acyrthosiphon pisum*	Aphid *Megoura viciae*	Broad bean *(Vicia faba)*	-The presence of non-host reduces the searching efficiency of the parasitoid.	Van Veen *et al*., 2005
Endoparasitoid *Cotesia marginiventris*	Caterpillar *Spodoptera exigua*	Aphid *Macrosiphum Euphorbiae*	Tomato (*Lycopersicon esculentum)*	-Plants infested by both non-host and host were preferred over healthy plants.	Rodriguez-Saona *et al*., 2005
Endoparasitoid *Diaeretiella rapae*	Aphid *Myzus Persicae*	Caterpillar *Plutella xylostella*	Cabbage (*Brassica chinensis*)	-*D. rapae* had an equal preference for host plant, and plant infested with both aphid and caterpillar.	Agbogba and Powell, 2007
Endoparasitoid *C. rubecula* and *C.glomerata*	Caterpillar *P.rapae*	Caterpillar *M. brassicae*	Cabbage (*B.oleracea cv capitata)*	-*C. glomerata* parasitized more efficiently than *C. rubecula* in complex situations; – After a learning experience *C. rubecula* distinguished between non-host and host, whereas *C.glomerata* wasn't .	Efremova, 2009
Endoparasitoid *C. marginiventris*	Caterpillar *S. Littoralis*	Leafhopper *Euscelidius Variegatus*	Maize (*Zea mays*, var. Delprim)	– *C. marginiventris* preferred host infested plants over non-host and healthy plants; – *C. marginiventris* did not distinguish between host only and dual infested plants.	Erb *et al*., 2010
Endoparasitoids *C. glomerata*	Caterpillar *P.rapae*	Caterpillar *M.Brassicae*	Cabbage (*B.oleracea* cv Capitata)	-A mixture of herbivores was more attractive than *P. rapae* or *M. brassicae* alone for *C. glomerata;* -Parasitoids were equally attracted towards host and non host infested plants - The efficiency of parasitation was reduced by non-host presence	Bukovinszky *et al*.,2012
Endoparasitoid *C. rubecula*	Caterpillar *P. rapae* *P.brassicae*	Caterpillar *M.brassicae*	Cabbage (*B.oleracea)*	-*C. rubecula* was equally attracted to *P. brassicae* and *P. rapae.* -*C. rubecula* did also not show a preference between plants infested only by the host *P.brassicae* and plant infested by both host and non-host infested plants.	Pepping, 2011
Endoparasitoid *C. glomerata*	Caterpillar *P. brassicae*	*Aphid* *Brevicoryne brassicae*	Black mustard *(Brassica nigra)*	-Wasp foraging behavior was unaffected by the simultaneous presence of a non-host attacker. Analysis of the plant volatiles shows that, dually attacked plants could not be separated from those with only caterpillars.	Ponzio *et al*.,2014

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Egg parasitoids (*Trichogramma brassicae)* *(T. evanescens*)	Eggs *P. brassicae*	Caterpillar *P. brassicae* Aphid *B. brassicae* eggs and caterpillars *S. exigua*	Black mustard *(B. nigra)*	- *P. brassicae* and *S. exigua*, but not *B. brassicae*, can disrupt the attraction of *Trichogramma* species toward *P. brassicae* egg-induced volatiles.	Cusumano *et al.*, 2015
None	Whitefly *Bemisia tabaci*	Caterpillar *S.exigua*	Cotton (*Gossypium hirsutum*)	-Volatile emission in dual infested plant was significantly less than for plants infested with *S.exigua* alone.	Rodriguez-saona *et al.*, 2003
None	Aphid *A. Pisum*	Caterpillar *S. exigua*	Broad bean *(Vicia faba)*	-Several expected caterpillar induced VOCs are reduced.	Schwartzbzerg *et al*.,2011

Table2. Description of studies in which the effects of non-host/non prey presence in below-ground part on HIPVs production and on parasitoids /predators preference were investigated.

System studied				Results	References
Parasitoids /predators	**Herbivore species**		**Plant species**		
	Host/prey Above –ground	**Non host/Non prey Below –ground**			
Parasitoids *Pteromalus Elevates* and *Torymus chloromerus*	Fruit fly *Terellia ruficauda*	*Phyllopertha horticula*L., *Otiorhynchus sulcatus*(Fabricius) and *Tipula oleracea* L.	Marsh thistle, *(Cirsium palustre* L.)	-Above-grounds herbivore preferentially feeding on thistles whose roots had been attacked. -Parasitoids prefer above-belowground attacked plants.	Master *et al.*,2001
Parasitoids Aphids parsitoids	Aphids *Brevicoryne brassicae lipaphis erysimi Mizus persicae Macrosiphon euphorbiae*	Wireworms (*Agriotes* sp.) Earthworms *Octolasiontyrtaeum*	Wild mustard *(Sinapis arvensis)*	-The root herbivory increase the aphids colonization .The number of parasitoids increased as the number of aphids increased	Poveda *et al*.,2005
Parasitoids *C. marginiventris* Nematode *Heterorhabditis megidis*	Caterpillar *Spodoptera littoralis*	Cornrootworm *Diabrotica virgifera* virgifera	Maize plant *(Zea mays)*	-The emission of the principal root attractant was reduced due to double infestation, which impacts on the behavior of respective natural enemies. -However this was not evident for the leaf volatiles. The parasitoid showed an ability to learn the differences in odor emissions and increased its response to the odor of a doubly infested plant after experiencing this odor during an encounter with hosts.	Rasman and Turling ,2007
Parasitoids *Cotesia glomerata*	Caterpillar *Pieris brassicae*	Root fly *Delia radicum*	Black mustard *(Brassica nigra)*	-Parasitoids prefer to search for hosts on plants without root herbivores. -Plants exposed to root herbivory were shown to emit a volatile blend reported to be highly toxic for insects.	Soler *et al*.,2007 a

Plant volatile synomones used by parasitoids

Parasitoid *Microplitis croceipes*	*Caterpillar* *Helioco verpazea*	*Root- Knot nematode* *Meloidogyne incognita*	Cotton (*Gossypium* spp.)	-Increased levels of HIPVs were recorded when cotton plants were exposed to the leaf-chewing insect the root-knot nematode compared with plants that were only exposed to the leaf chewer.	Olson *et al*., 2008
None	Caterpillar *Pieris brassicae*	*Root fly* *Delia radicum*	*Rapeseed* *(Brassica napus)*	-VOCs emitted by *Brassica napus* plants simultaneously infested by both herbivores differed from volatiles emitted when the plants were infested by a single shoot herbivore only.	Pierre *et al*., 2011

1.6 **Study system**

The purpose of this thesis is to study the effect of multiple herbivore attacks on above and/or below- grounds plant attacks organs in terms of VOC emissions and recruitment of egg parasitoids. The model system used for the experiments consists of the legume plant species, *Vicia faba* L. (Fabales: Fabaceae -first trophic level), the above-ground herbivore species, the host *Nezara viridula* (L.) (Heteroptera: Pentatomidae - Second trophic level), the above-below ground herbivore species, the non-host *Sitona lineatus*(L) (Coleoptera: Curculionidae - Second trophic level) and the egg parasitoid of *N. viridula*, *Trissolcus basalis* (Wollaston) (Hymenoptera: Platygastridae - third trophic level) (**Fig.3**).

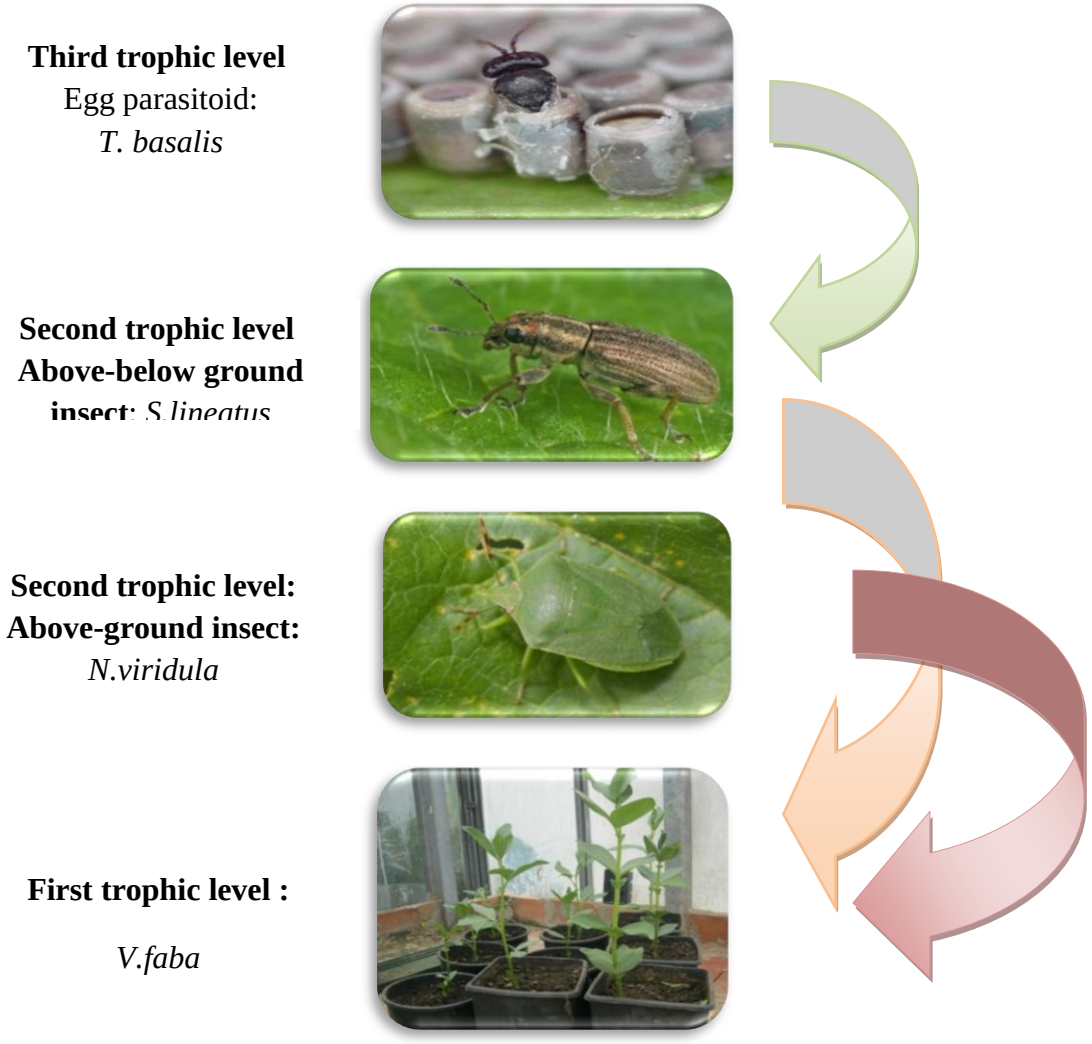

Figure3: The model system

Plant volatile synomones used by parasitoids

First trophic level: *Vicia faba*

The faba bean is an ancient crop, being a major food source for Mediterranean countries due to the high nutritional value of its seeds, which are rich in protein and starch. *Vicia faba* is grown widely under a range of climatic conditions from temperate to subtropical and it hosts a wide variety of regional, native and exotic cosmopolitan insect pests (over 70 spp.), that collectively cause damage at all stages of plant development (Stoddard *et al*., 2010). In the Mediterranean basin, *V. faba* plants are commonly attacked by *N. viridula* as well as by *S. lineatus*.

Previous studies have showed that *V. faba* plants emit HIPVs in response to damage caused by different insects such as *Acyrthosiphon pisum* Harris (Homoptera: Aphididae) (Du *et al*., 1998), *N. viridula* (Colazza *et al*., 2004a), *Aphis fabae* Scopoli (Hemiptera: Aphididae) (Webster *et al*., 2008) and *Lygus rugulipennis* Poppius (Heteroptera: Miridae) (Frati *et al*., 2009). These blends of volatiles differ in composition from those released by undamaged or mechanically damaged plants (Du *et al*., 1998; Angelopoulos *et al*., 1999). In our system there is evidence of the induction of volatile synomones. In fact it is know that feeding and oviposition on *V. faba* plants by *N.viridula* induce a significant increase of some VOCs, mainly (*E*)-ß-caryophyllene, that attract the parasitoid *T. basalis* (Colazza *et al.*, 2004a,b).

Second trophic level: *Nezara viridula* (L.)

The southern green stink bug (SGSB) or green vegetable bug is a cosmopolitan insect herbivore probably native to the Ethiopian region (Jones 1988). It is one of the most important pentatomidae in many temperate and tropical regions of the world (Todd, 1989; McPherson and McPherson, 2000). The bug is a highly polyphagous feeder, attacking many important food crops (Panizzi, 2000). It host range encompasses over 30 families of dicotyledonous plants and a number of monocots, with preference for leguminosae and solanaceae such as soybean, tomato, beans (Todd, 1989). Females deposit pale yellow eggs in large masses predominantly on the undersides of leaves without causing any apparent physical damage to the plant. *Nezara viridula*, like other stink bug species, develops through five nymphal instars. This pest is typically either bivoltine or multivoltine(to have more than 2 generation per year) (Panizzi, 1997) and possesses piercing-sucking mouthparts which destroy only a few cells and causing a minimum mechanical damage, however the main damage is caused by the insect saliva which reduce the crop quality (Miles, 1972).

It may attack all parts of a plant, including stems and leaf veins, but the bugs feed mostly on fruiting structures and growing shoots, often resulting in either direct loss or unmarketable product (Panizzi and Slansky, 1991). In Italy, its economic importance is related to vegetable crops (Colazza and Bin, 1995).

In integrated pest management, this insect is controlled mainly by the use of insecticides, but biological control is a promising strategy that can be used as alternative of chemical control (Phyllis *et al*., 2007). Example of biological control of *N. viridula* is the use of sterile-insect technique that might have application to prevent reproduction, however, the high cost of the technique and the fact that adults as well as nymphal stages can cause crop damage, currently limits the use of this method (Knight and Gurr, 2006). The entomopathogenic fungi have greater potential as biopesticides for sucking pests such as *N. viridula*. However, there is reducing in the efficiency of this method where the bugs are present in low tolerance crops (Sosa-Gómez and Moscardi, 1998).

Trap crops are used to prevent the pest from reaching the crop and to concentrate herbivores in a certain part of the field where it can be strategically destroyed (Knight and Gurr, 2006). A border planting of white mustard (*Sinapsis albus*) was used as a trap crop with organic sweet corn (*Zea mays*) in New Zealand. *N. viridula* population densities were much higher in the mustard plots (8–12 insects/m^2) than in the sweet corn (<1 insects/m^2) (Rea *et al*., 2002). These data show that trap crops are a potentially useful tactic for an integrated management of *N. viridula* but only if herbivore attracted in the trap crop will be removed from the environment, to prevent its spreading into adjacent or main crops (McPherson and Newsom, 1984).

Second trophic level: *Sitona lineatus*

The pea leaf weevil is a serious pest of field pea, *Pisum sativum* L., and broad bean, *V. faba*, in Europe, Africa and North America (Jackson, 1920; Hoebeke and Wheeler, 1985). The insect has annual life cycle; in autumn, adults migrate to shelter belts where they consume foliage of secondary leguminous hosts like alfalfa (*Medicago sativa* L., Fabales: Fabaceae) before overwintering (Jackson, 1920; Schotzko and O'Keeffe, 1988). In early spring, adults migrate to their primary hosts, pea and bean crops and feed on seedlings causing U-shaped notches (Stein, 1972; Fisher and O'Keeffe, 1979; Hamon *et al*., 1987; Landon *et al*., 1995). After mating, females oviposit over the soil surface. Then larvae penetrate inside root nodules and feed upon the nitrogen-fixing bacteria, *Rhizobium leguminosarum* Frank (Rhizobiales:

Rhizobiaceae) (Jackson, 1920; Johnson and O'Keeffe, 1981; Hoebeke and Wheeler, 1985), reducing nitrogen fixation (Cárcamo and Vankosky, 2011). Thus both adults and larvae can reduce yield, but the damage caused by a reduced photosynthetic area is probably less than the damage caused by reduced nitrogen fixation (Cantot *et al*., 1989). Larval damage to root nodules can range from 40% to 98% of nodules, which may cause yellowing leaves, typical of nitrogen deficiency (El-Dessouki, 1971; Cantot, 1986). Moreover, larval feeding reduces seed protein content, especially in nutrient-poor soils, as well as the amount of nitrogen returned to the soil (Dore and Meynard, 1995; Corre-Hellou and Crozat, 2005).

Third trophic level: *Trissolcus basalis*

Trissolcus basalis is one of the most important and widely distributed natural enemies of the Southern Green Stink Bug. It is a solitary egg parasitoid that can successfully develop on several other pentatomid species (Jones, 1988; Colazza and Bin, 1995). It was first used to control *N. viridula* in Egypt and Australia (1933), and then later in the Antilles (1952 and 1953), South Africa (1980), Brazil (1980) and the USA (1979 and 1981) (Clarke, 1990). The host location strategies adopted by *T. basalis*, have been extensively explored, showing that females are able to exploit volatile kairomones from virgin males and preovipositing females of *N. viridula*, and contact kairomones in the host footprints (Colazza *et al*., 1999; 2004a; Peri *et al*., 2006). Previous investigations under tritrophic conditions have shown that the broad bean plant responds to *N. viridula* feeding and oviposition damages by emitting volatile synomones that recruit naïve *T. basalis* females. Specifically, it was found that the sesquiterpene (*E*)-ß-caryophyllene plays a key role in the attraction of *T. basalis.* Such attraction was shown to be systemically induced and time specific, because feeding- damaged leaves bearing *N. viridula* eggs also attracted the parasitoid until the eggs were 72–96 old (Colazza *et al*.,2004a,b).

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Chapter 2

Chapter 2
Egg parasitoid attraction toward induced plant volatiles is disrupted by a non-host herbivore attacking above or belowground plant organs.

Abstract

Plants respond to insect oviposition by emission of oviposition-induced plant volatiles (OIPVs) which can recruit egg parasitoids of the attacking herbivore. To date, studies demonstrating egg parasitoid attraction to OIPVs have been carried out in tritrophic systems consisting of one species each of plant, herbivore host, and the associated egg parasitoid. Less attention has been given to plants experiencing multiple attacks by host and non-host herbivores that potentially could interfere with the recruitment of egg parasitoids as a result of modifications to the OIPV blend. Egg parasitoid attraction could also be influenced by the temporal dynamics of multiple infestations, when the same non-host herbivore damages different organs of the same plant species. In this scenario we investigated the responses of egg parasitoids to feeding and oviposition damage using a model system consisting of *Vicia faba*, the above-ground insect herbivore *Nezara viridula*, the above- and below-ground insect herbivore *Sitona lineatus*, and *Trissolcus basalis*, a natural enemy of *N. viridula*. We demonstrated that the non-host *S. lineatus* disrupts wasp attraction toward plant volatiles induced by the host *N. viridula*. Interestingly, *V. faba* damage inflicted by either adults (i.e., leaf-feeding) or larvae (i.e., root-feeding) of *S. lineatus*, had a similar disruptive effect on *T. basalis* host location, suggesting that a common interference mechanism might be involved. Neither naïve wasps nor wasps with previous oviposition experience were attracted to plant volatiles induced by *N. viridula* when *V. faba* plants were concurrently infested with *S. lineatus* adults or larvae. Analysis of the volatile blends among healthy plants and above-ground treatments show significant differences in terms of whole volatile emissions. Our results demonstrate that induced plant responses caused by a non-host herbivore can disrupt the attraction of an egg parasitoid to a plant that is also infested with its hosts.

Key-words: *Trissolcus basalis, Sitona lineatus, Nezara viridula, Vicia faba*, indirect plant defenses, multi-trophic interactions, chemical ecology

Egg parasitoid attraction toward induced plant volatiles

2.1 Introduction

Parasitoids adopt specialized strategies to efficiently locate and parasitize their herbivorous hosts. Host-seeking females may exploit a plethora of cues, among which volatile organic compounds (VOCs) emitted by the plant as a consequence of herbivore attack, and therefore called herbivore induced plant volatiles (HIPVs), often play a key role (Kessler and Baldwin, 2001; Dicke, 2009). From the plant's side, such a strategy developed as a consequence of plant-herbivore-parasitoid coevolution should be considered as an indirect defense against insect herbivores. It is known that plants defend themselves against herbivores either directly, through negative effects on herbivore performance, or indirectly, by recruiting natural enemies of the herbivore through synthesis and release of HIPVs (Kessler and Baldwin, 2001; Schoonhoven *et al*., 2005; Dicke, 2009; Ode, 2013). Numerous studies have documented the role of HIPVs as easily detectable and reliable host location cues for natural enemies of insect herbivores (Dicke and Baldwin, 2010; Kessler and Heil, 2011; Meiners and Peri, 2013).
More recently, it has also been demonstrated that egg parasitoids exploit oviposition-induced plant volatiles (OIPVs; reviewed by Hilker and Meiners, 2010). Specifically, insect oviposition up-regulates plant defensive responses via the salicylic acid signal-transduction pathway (Bruessow *et al*., 2010; Reymond, 2013). Host eggs are unapparent, thus OIPVs provide female egg parasitoids highly detectable and reliable information on the presence of host eggs. Although OIPVs can recruit egg parasitoids of insect herbivores in some case studies, a combination of oviposition and feeding activity of the herbivore host is required to trigger attraction (reviewed by Colazza *et al*., 2010; Conti and Colazza, 2012).

Under natural conditions, plants often are attacked by multiple herbivore species, a scenario which potentially could interfere with the attraction of natural enemies as a result of modifications to the HIPVs blend (Soler *et al*., 2013). A growing body of evidence shows that communities and processes are intrinsically linked, and that they have important implications for community structure and ecosystem functioning (Dicke, 2009; Stam *et al*., 2014). These interactions may vary in terms of complexity and may involve organisms from several trophic levels and feeding guilds. Because we can expect plants to be adapted only to events that are common over evolutionary time spans, studies to elucidate these interactions preferably should be carried out at realistic densities and natural temporal sequences at which the various associations are established. To date, the land-mark studies demonstrating egg parasitoid attraction to OIPVs have been carried out in tritrophic systems consisting of one species each

of plant, host herbivore, and egg parasitoid (Hilker and Meiners, 2002; Colazza *et al*., 2004a,b; Fatouros *et al*., 2012). Less attention has been given to plants experiencing multiple attacks by host and non-host herbivores that potentially could interfere with the attraction of egg parasitoids as a result of modifications of the OIPV blend. The interference effect on egg parasitoids could be affected by the non-host herbivore identity, feeding guild and impact on above- or below-ground plant tissues, as demonstrated for larval parasitoids (Rasmann and Turlings, 2007; Soler *et al*., 2007, 2013; Erb *et al*., 2010; Ponzio *et al*., 2014). We hypothesized that egg parasitoid attraction to induced plant volatiles could be influenced by temporal dynamics of multiple infestations, especially when the same non-host herbivore damages different organs, e.g. above- and below-ground, of the same plant species during the course of the growing season. We are not aware of any previous studies that have investigated the potential disrupting effect of a non-host herbivore, attacking either above or below-ground plant organs, on attraction of egg parasitoids to volatiles produced by plants that are also infested with their typical hosts.

Therefore, in this paper, we investigate responses of the egg parasitoid *Trissolcus basalis* (Wollaston) to induced plant volatiles using the model system broad bean plants, *Vicia faba* L., infested with its typical host *Nezara viridula* (L.), and also infested with *Sitona lineatus* (L.), an above- and below-ground herbivore that is not a host for *T. basalis*. Previous investigations under tritrophic conditions have shown that the broad bean plant responds to *N.viridula* feeding and oviposition damage by emitting plant volatiles that recruit naïve *T. basalis* females. Such attraction was shown to be systemically induced and time specific, because feeding damaged leaves bearing *N. viridula* eggs attracted the parasitoid until the eggs were 72–96 h old (Colazza *et al*., 2004a,b). In agroecosystems, broad bean plants can be attacked by over 50 herbivore species including aphids, leafhoppers, true bugs, thrips, moths, leafminers, and beetles (Bardner, 1983; van Emden *et al*., 1988; Nuessly *et al*., 2004). Among them, different life stages of weevils in the genus *Sitona* are known to attack different organs of the same plant throughout the growing season, and thus act as above- and below-ground herbivores. For example, adults of the pea weevil, *S.lineatus*, feed on foliage whereas larvae feed upon nitrogen-fixing bacteria *Rhizobium leguminosarum* Frank associated with rootlets and roots (Johnson and O'Keeffe, 1981; Hoebeke and Wheeler, 1985; Corre-Hellou and Crozat, 2005).

In Italy, *V. faba* plants are commonly attacked by both *N. viridula* and *S. lineatus*,with adults of both species attacking above-ground plant parts early in growing season, whereas both above- and below-ground attacks occur later as the developing weevil larvae feed on the roots (Cusumano and Salerno, personal observations). To locate *N. viridula* eggs in such complex environments that undergo temporal changes in infestation by both hosts and non-hosts, and corresponding changes in plant-derived odor cues, *T. basalis* females could rely on learning abilities. In these circumstances, plasticity in a parasitoid's response would be adaptive and learning could provide valuable flexibility (Peri *et al*., 2006; Fatouros *et al*., 2008; Colazza *et al*., 2010; Cusumano *et al*., 2012). Although it is well known that experience can strongly influence parasitoid foraging behavior, the role of previous experience in egg parasitoids foraging in a multitrophic system has not been addressed. Thus, the aim of this paper was to investigate the effects of *S.lineatus* attack on the attraction of naïve and experienced females of the wasp *T. basalis* to *V. faba* plants that were being simultaneously attacked by the parasitoid's host, *N. viridula*. Experiments were conducted with plants that were infested with insects above-ground, below-ground and both above and below- ground. The emission of plant volatiles also was evaluated in response to above-ground attacks under different experimental conditions.

2.2 Material and methods

2.2.1 Plant growing

Seeds of broad bean plants (*V. faba* cv. Superaguadulce) were immersed for 24 h in a slurry of water and soil (1:4) to favor root nodulation. The seeds then were individually planted in plastic pots (9 × 9 × 13 cm) filled with a mixture of agriperlite (Superlite, Gyproc Saint-Gobain, PPC Italia, Italy), vermiculite (Silver, Gyproc Saint-Gobain, PPC Italia, Italy), and sand (1:1:1) and grown in a climate controlled chamber (24 ± 2◦C, 45 ± 10% RH, 12 h:12 h L:D). Plants were watered daily and, from 1 week post-germination, fertilized with an aqueous solution (1.4 g/l) of fertilizer (5-15-45, N-P-K, Plantfol, Valagro, Italy). In the case of "*above-ground treatments*" (see below), 18–20 days old broad bean plants, with approximately six fully expanded leaves, were used. For the "*below-ground treatments*" and "*above- + below-ground treatments*," 15 days old plants were infested with *S. lineatus* eggs, left to grow to allow development of *S. lineatus* larvae on the root nodules, and then exposed to *N. viridula* (after 12 days) and/or tested (after 15 days; see below).

2.2.2 Insect rearing

The *N. viridula* colony established from material collected in cultivated and uncultivated fields around Perugia and Palermo (Italy), was reared under controlled conditions (24 ± 2◦C; 70 ± 5% RH; 16 h:8 h L:D) in wooden cages (50 × 30 × 35 cm) with mesh covered holes (5 cm diameter) for ventilation. Bugs were fed with a diet of sunflower seeds and seasonal fresh vegetables. Food was changed every 2–3 days, and separate cages were used for nymphs and adults. Egg masses were collected daily and used to maintain cultures of both *N. viridula* and *T. basalis.* The *N. viridula* colony was supplemented regularly with field-collected bugs. *Sitona lineatus* adults were collected from *V. faba* fields around Perugia and Palermo and maintained in a climate-controlled chamber (8 ± 2◦C; 70 ± 5% RH; 16 h:8 h L:D). The colony was reared in plastic food containers (30 × 19.5 × 25 cm) with 5 cm diameter mesh-covered holes. Adults were fed with vegetative parts of *V. faba* changed once a week and eggs were collected daily. Eggs were kept in Petri dish with the bottom covered by a filter paper disk moistened with distilled water. The Petri dish was sealed with Parafilm® and maintained under controlled conditions (24±2◦C; 70 ± 5% RH; 16 h:8 h L:D). The colony of *T. basalis* was originally established from wasps emerging from *N. viridula* egg masses, located in wild and uncultivated fields around Perugia and Palermo. The parasitoid was reared on *N. viridula* egg masses that were glued on paper strips. Wasps were maintained in 85 ml glass tubes, fed with a honey water solution and kept in controlled environment room under the same rearing conditions of *N. viridula*. After emergence, male and female wasps were kept together to allow mating. For all bioassays, naïve or experienced (with oviposition experience on host eggs) 2–4 days old females were used. Naïve females were individually isolated in small vials 1 h before bioassays and then transferred to the bioassay room to be acclimatized. Experienced wasps were obtained with the following protocol: a *V. faba* leaf bearing a 24 h old *N. viridula* egg mass was placed in a circular arena (Ø = 1.8 cm; *h* = 0.5 cm), and then a single naïve *T. basalis* female was released in the arena to allow oviposition. After 10–15 min, experienced wasps (i.e., those that had parasitized one *N. viridula* egg) were recaptured and kept isolated in a small vial with a drop of honey-water solution for 24 h under controlled conditions (24 ± 2◦C; 70 ± 5% RH; 16 h:8 h L:D) before being transferred to the bioassay room to be acclimatized for the next bioassay.

2.2.3 Plant treatments

Plants were left untreated as controls, or subjected to the following treatments (**Fig. 1**):

Above-ground damage

(a)*Nezara viridula* feeding and oviposition obtained by exposing individual plants to three *N. viridula* gravid females for 24 h.

(b) *Sitona lineatus* leaf-feeding obtained by exposing three leaves of a plant to 15 *S. lineatus* adults (five adults/one leaf) for 24 h using a "clip cage"; the latter consisted of two modified plastic Petri dishes (Ø = 10 cm; *h* = 1 cm), each with a mesh covered hole in the bottom and the rim covered by a small sponge ring.

(c)*Nezara viridula* feeding and oviposition and *S. lineatus* adult leaf-feeding, obtained as described above by exposing the same plant first to *N. viridula* and then after 1 day to *S. lineatus* adults; attacks by the two species on the same leaves were avoided.

(d) Mechanical damage simulating *S. lineatus* leaf damage [leaf area removed by 15 adults in 24 h : 161.1 ± 27.45 mm2 (mean ± SE)], obtained by removing with scissors six triangular sections per leaf from three leaves (total leaf area removed: 132.88 ± 10.90 mm2).

(e)Mechanical damage combined with *N. viridula* feeding and oviposition. Plants were first exposed to *N. viridula* and, after 1 day, were damaged mechanically as described above; mechanical damage on the leaves carrying a *N. viridula* egg mass was avoided.

Below-ground damage

(f) *Sitona lineatus* larvae feeding on root nodules, obtained by infesting individual plants with 30 *S. lineatus* eggs ready to hatch (6–7 days old). With the aid of a fine paintbrush, eggs were gently put inside a dimple made *ad hoc* on the plant substrate and then they were covered with the same substrate. These treated plants were tested 15 days after inoculation with eggs in order to allow larval feeding damage to the root nodules.

Above- + below-ground damage

(g) To get plants damaged with a combination of *N. viridula* feeding and oviposition and *S. lineatus* larval damage to root nodules, test plants were first infested with *S. lineatus* eggs as described above and, 12 days after inoculation, they were exposed to *N. viridula* as previously described.

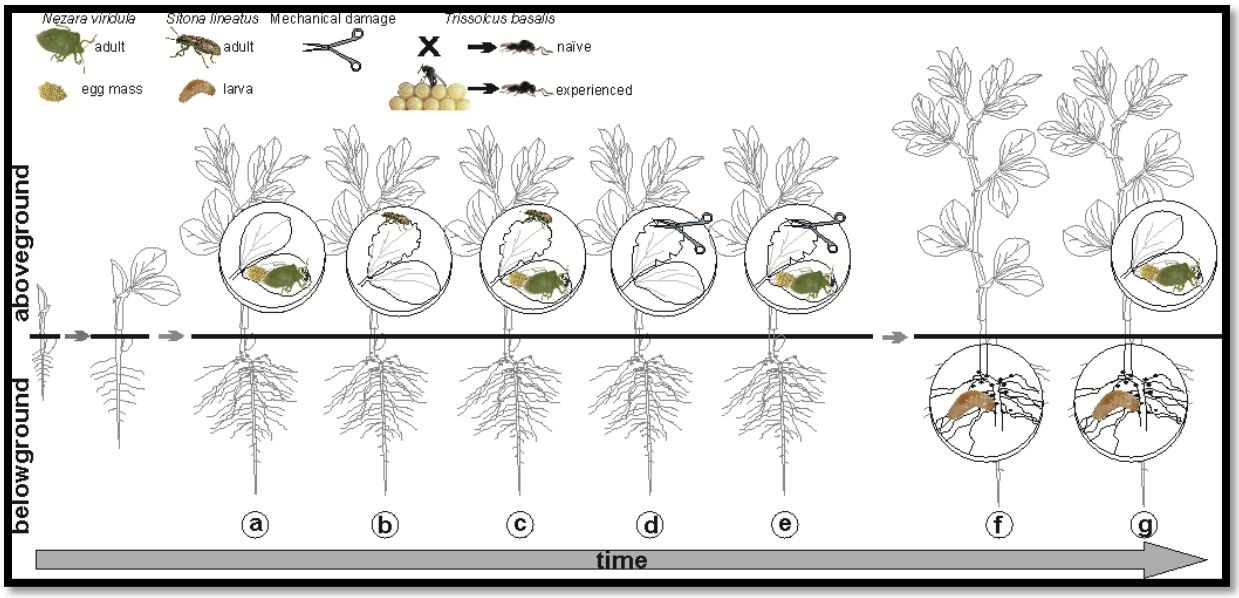

Figure 1 |Visual summary of the main plant treatments. Above-ground *Nezara viridula* feeding and oviposition (a); *Sitona lineatus* adult leaf-feeding (b); *N. viridula* feeding and oviposition and *S. lineatus* adult leaf-feeding (c); mechanical damage (d); *N. viridula* feeding and oviposition and mechanical damage (e); below-ground (*S. lineatus* larvae root-nodules feeding) (f) and above + below-ground treatment (*N. viridula* feeding and oviposition and *S. lineatus* larvae root-nodules feeding) (g) and temporal dynamics of multi-trophic infestations on *Vicia faba.*

2.2.4 Y-tube olfactometer bioassays

Wasps' responses to volatile chemicals from differently treated *V.faba* plants were investigated with a dual choice Y-tube olfactometer made from a polycarbonate body (stem 9 cm; arms +8 cm at 130◦ angle; ID 1.5 cm) sandwiched between two glass plates. A stream of clean air (medical-grade compressed air, N2:O2 80:20), humidified by bubbling through a water jar, was regulated in each arm by a flowmeter at about 0.4 l min−1. The device was illuminated from above by two 22-W cool white fluorescent tubes, and from below by an infrared source (homogeneous emission of wavelengths at 950 nm provided by 108 LEDs). Before entering the olfactometer arms, each air stream passed through a cylindrical glass chamber (Ø = 12 cm; h = 52 cm) with an O-ring sealed middle joint, containing a treated plant as odor source. The stimuli were randomly assigned at the beginning of the bioassays and were reversed after testing five parasitoid females. At every switch, the whole system was changed with cleaned parts. At the end of the bioassays the polycarbonate olfactometer and all glass parts were cleaned with water and detergent. The glass parts were then cleaned with acetone and baked overnight at 180◦C. Wasp females were singly introduced into the Y-tube olfactometer at the entrance of the stem and allowed to move freely for 10 min.

Their behavior was recorded using a monochrome CCD video camera (Sony SSC M370 CE) fitted with a 12.5–75 mm/F 1.8 zoom lens. The camera lens was covered with an infrared pass filter (Kodak Wratten filter 87 Å) to remove visible wavelengths. Analog video signals from the camera were digitized by a video frame grabber (Canopus® ADVC 110, Grass Valley CA, USA). Digitized data were processed by XBug, a video tracking and motion analysis software (Colazza *et al*., 1999). Wasp response was measured in terms of residence time, i.e., the time spent by the wasps in each arm during the entire bioassay. The Y tube olfactometer bioassays were carried out as paired choices, in which odor sources were always tested *versus* healthy plants used as control. Test odor sources included plants subjected to *above-ground, below-ground* and *above- + below-ground* treatments, described in the previous section. For each treatment, bioassays were conducted using either naïve or experienced wasp females. In the bioassays with *above-ground* and *below- + aboveground* treatments, the roots of each test plant were checked after the bioassay under a stereomicroscope to assess the presence of *S. lineatus* larvae and damaged root nodules. When no larvae were detected the data were discarded. About 40 replicates were conducted for each treatment. Bioassays were conducted from 09:00 to 13:00 h under controlled conditions (26 ± 1◦C; 50 ± 5% R.H.).

2.2.5 Collection of plant volatiles

A cylindrical glass chamber (Ø = 9 cm ID; h = 29 cm) was used to collect headspace volatiles from above-ground treated plants and healthy plant controls (n = 5 for each). Before each collection, the glass chamber was washed with water and detergent, rinsed with acetone, and baked overnight at 180◦C. Singly potted plants were placed in each aeration chamber, separated from the pot and soil with two semi-circular Teflon plates to reduce contamination from soil odors. Air, purified by passage through an activated charcoal filter, was pumped into the chamber at 900 ml/min, with 600 ml/min being pulled through a glass tube filled with Porapak Q (Sigma Aldrich; 60 mg, 80–100 mesh), which was pre-cleaned with hexane and then heat conditioned for at least 2 h in a stream of nitrogen (100 ml/min) at 130◦C. Volatiles were collected for 24 h, then traps were eluted with 700 µl of hexane, and the resulting extracts were concentrated to 100 µl under a gentle nitrogen stream. Extracts were stored at −20◦C in glass vials with Teflon cap liners until used for gas chromatography (GC) analyses. For each plant used in the volatile capture, the total leaf area was measured.

2.2.6 Chemical analysis

Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard 5890 GC system interfaced with an HP 5973 quadruple mass spectrometer. For each sample (n = 5), 1 µl of extract was injected onto a HP5-MS column (5% diphenyl–95% dimethylpolysiloxane 30 m × 0.2 mm, 0.25-µm film, J&W Scientific, Folsom CA, USA) in splitless mode. Injector and detector temperatures were 260◦C and 280◦C respectively. Helium was used as the carrier gas. The GC oven temperature program was 40◦C for 5 min, then increased by 10◦C/min to 250◦C. Electron impact ionization spectra were obtained at 70 eV, recording mass spectra from 40 to 550 amu. Peak area of each detected compound was calculated and related to the total leaf area of the plant. The purpose of this chemical analysis was to investigate if the composition of the *V. faba* volatile blend varied according to the treatments; consequently no chemical characterization of the detected compounds was carried out.

2.2.7 Statistical analysis

For the bioassays, the time spent by wasp females in each arm was statistically compared by parametric paired *t*-tests for dependent samples and data were analyzed using the STATISTICA7 software (StatSoft, 2001). Data from analysis of volatiles extracts were analyzed by multivariate analysis using projection to latent structures discriminate analysis (PLS-DA) using the SIMCA-P+ 12.0 software program (Umetrics AB, Umeå, Sweden). This projection method determines if samples belonging to the different treatment groups can be separated on the basis of quantitative and qualitative differences in their volatile blends. The results of the analysis are visualized in score plots, which reveal the sample structure according to model components and loading plots, which display the contribution of the variables to these components as well as the relationships among the variables.

2.3 Results

2.3.1 Y-tube olfactometer bioassays

Above-ground treatments

Naïve *T. basalis* females (**Fig. 2**) were significantly attracted to volatiles emitted by plants damaged by *N. viridula* feeding and oviposition (t = 4.75; df = 33; $p < 0.001$), and by plants damaged by leaf-feeding by *S. lineatus* adults (t =−2.13; df =37; p=0.040) compared to undamaged control plants. Although, a sensitivity of *S. lineatus* treated-plants to clip cages, not present in controls, cannot be excluded. However, these differences disappeared when plants were damaged by both *N. viridula* feeding and oviposition, and *S. lineatus* leaf-feeding, with these plants being equally attractive to controls (t = −0.87; df = 40; p = 0.389). The simple mechanical damage did not stimulate a significant response from naïve wasps compared to undamaged plants (t = 0.90; df = 34; p = 0.375), whereas the combination of *N. viridula* feeding and oviposition plus mechanical damage did (t = −3.50; df = 42; p = 0.001).

Experienced female wasps (**Fig. 3**) also showed a significant preference for volatiles released by plants with *N. viridula* feeding and oviposition compared to controls (t =−2.4; df = 30; p=0.022).However, in contrast to naïve wasps, experienced wasps preferred the odors of undamaged plants to the odors from plants damaged by *S. lineatus* adult leaf-feeding (t = −2.33; df = 29; p = 0.027). No significant choice was displayed by experienced females when presented with the other above-ground treatments (*N. viridula* feeding and oviposition and *S. lineatus* adult leaf feeding: t = −1.12; df = 39; p = 0.27; mechanical damage: (t =−110.90; df=34; p=0.28; *N. viridula* feeding and oviposition and mechanical damage: t =−0.95; df = 36; p = 0.35).

Below-ground treatments

Naïve wasps were significantly attracted to volatiles emitted by plants damaged by *S. lineatus* larvae feeding on root nodules (t = 2.11; df = 36, p = 0.042; **Fig. 2**) compared to controls, whereas experienced females did not discriminate between the treatment and control (t = 0.68; df = 29; p = 0.50; **Fig. 3**).

Above- + below-ground treatments

Naïve parasitoids did not discriminate between volatiles emitted by plants damaged by *N. viridula* feeding and oviposition plus *S. lineatus* larval damage to root nodules *vs.* healthy

plants (t = 1.31; df = 40; p = 0.20; **Fig. 2**). In contrast, experienced parasitoids significantly preferred volatiles released by healthy plants to volatiles emitted by plants with *N. viridula* feeding and oviposition plus *S. lineatus* larval feeding on root nodules (t = 2.06; df = 38; p = 0.046; **Fig. 3**).

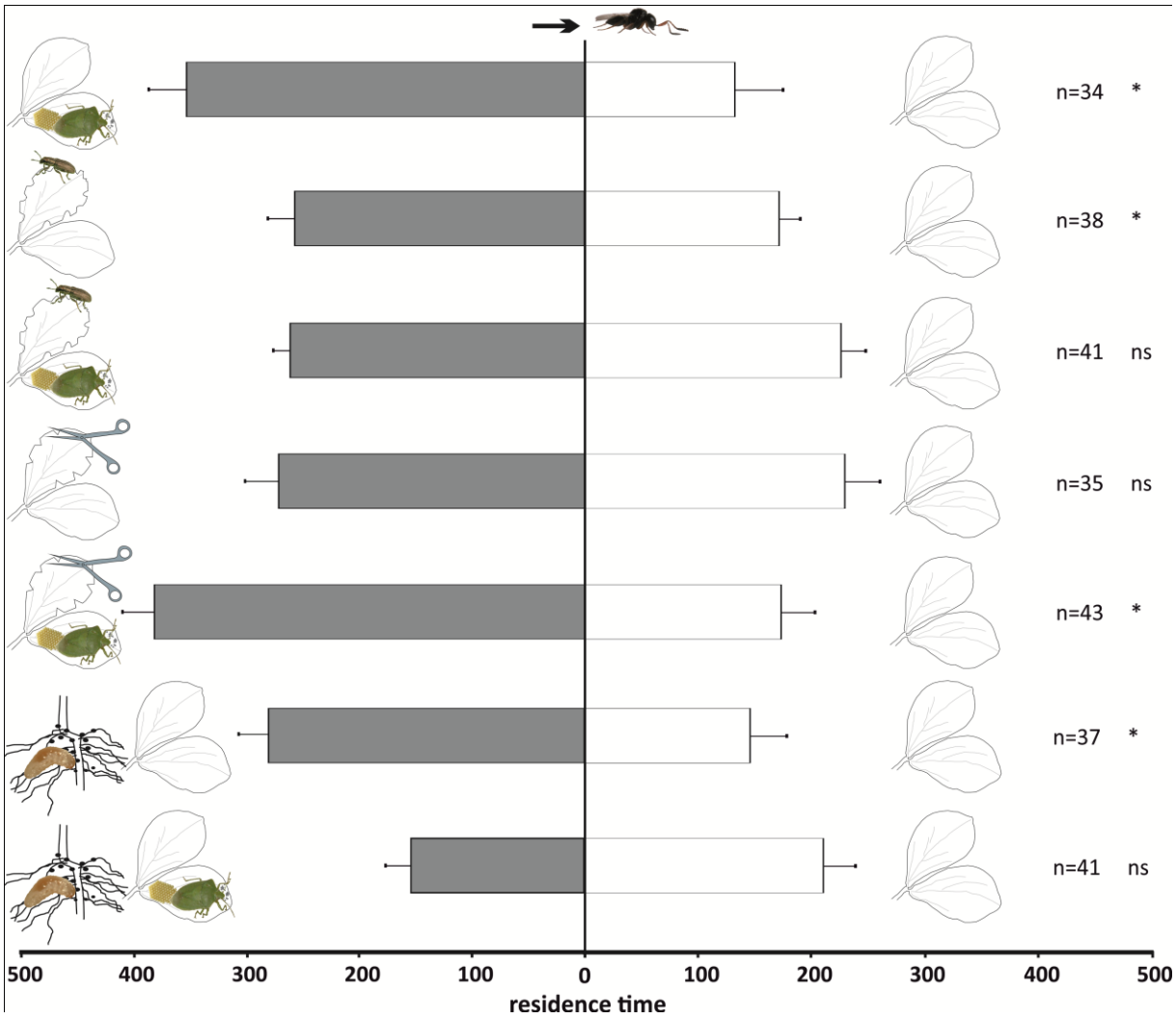

Figure 2: Response of naïve *Trissolcus basalis* females in a Y-tube olfactometer to volatiles from *V. faba* plants subjected to above-ground and below-ground treatments *versus* healthy plants. Plant treatments: *N. viridula* feeding and oviposition; *S. lineatus* adult leaf-feeding; *N. viridula* feeding and oviposition and *S. lineatus* adult leaf-feeding; mechanical damage; *N. viridula* feeding and oviposition and mechanical damage; *S. lineatus* larvae root-nodules feeding and above- + below-ground treatment (*N. viridula* feeding and oviposition and *S. lineatus* larvae root-nodules feeding). *n* = number of replicates. Bars represent mean (± SEM) of the time spent by wasp females in each arm over an observation period of 600 s (ns = not significant; * = $P < 0.05$).

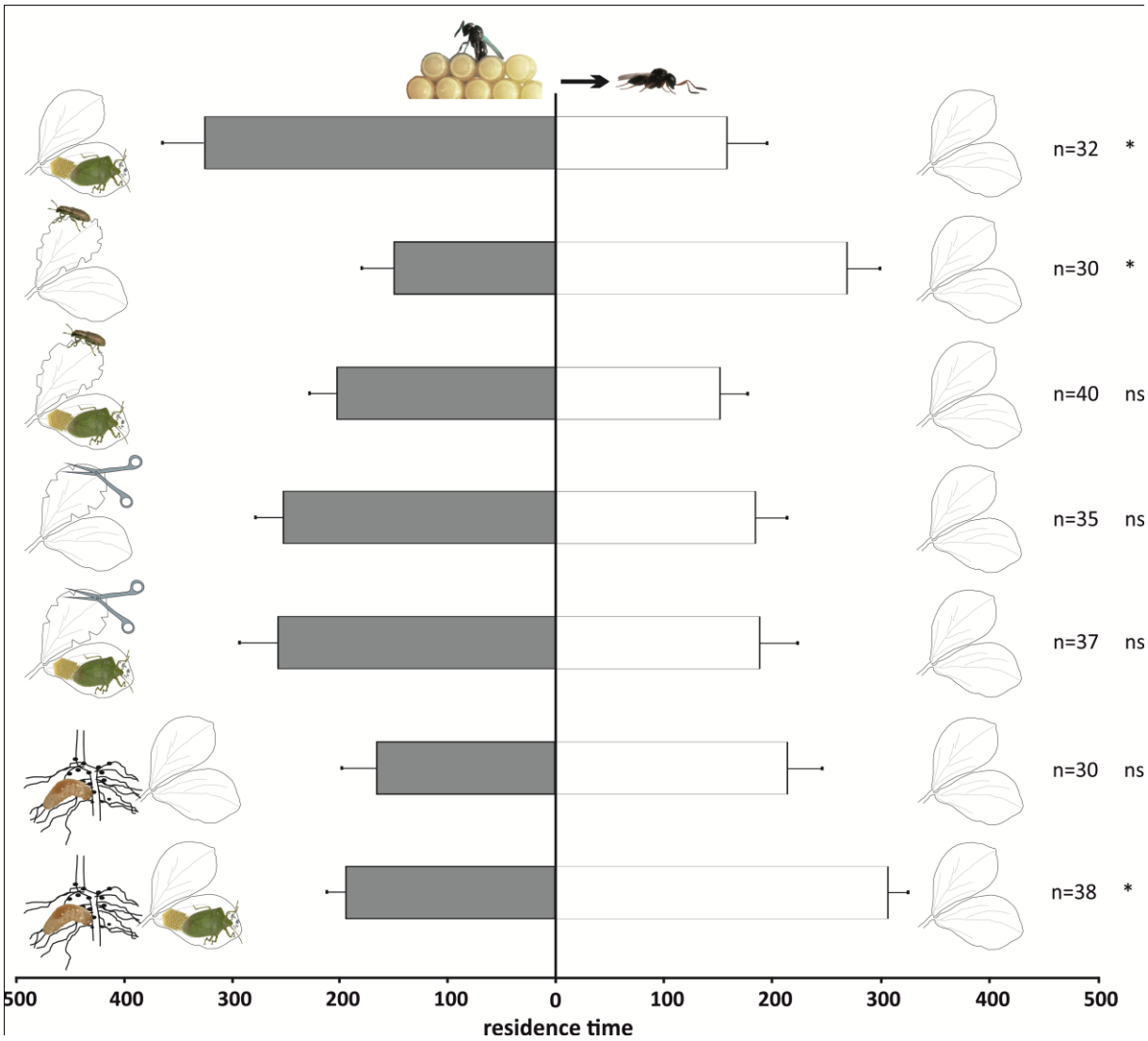

Figure 3: Response of experienced *T. basalis* females in a Y-tube olfactometer to volatiles from *V. faba* plants subjected to above-ground and below-ground treatments *versus* healthy plants. Plant treatments: *N. viridula* feeding and oviposition; *S. lineatus* adult leaf-feeding; *N. viridula* feeding and oviposition and *S. lineatus* adult leaf-feeding; mechanical damage; *N. viridula* feeding and oviposition and mechanical damage; *S. lineatus* larvae root-nodules feeding and above- + below-ground treatment (*N. viridula* feeding and oviposition and *S. lineatus* larvae root-nodules feeding). *n* = number of replicates. Bars represent mean (± SEM) of the time spent by wasp females in each arm over an observation period of 600 s (ns = not significant; * = $P < 0.05$).

2.3.2 Plant volatile analysis

Twelve compounds were detected in the analyses of odors collected from *V. faba* plants. A PLS-DA comparison including samples of healthy plants and all above-ground treatments resulted in a model with two significant principal components (PCs; $R2X = 0.219$; $R2Y = 0.135$; $Q2 = -0.095$; **Fig. 4A**). In particular, the PLSDA separated the plants subjected to *N. viridula* damage and the plants subjected to *N. viridula* + *S. lineatus* damage. Examination of the loading plot showed that a group of five compounds contributed the most to explaining the variation in the model (**Fig. 4B**). These compounds have the following retention time (min) and corresponding VIP values (variable importance for the projection): (1) = 17.58, 1.37; (2) = 4.34, 1.29; (3) = 8.41, 1.25; (4) = 10.28, 1.16; (5) = 22.34, 1.15.

2.4 Discussion

In this study we demonstrated that, under our experimental conditions, a non-host herbivore species that feeds on both above and below-ground plant parts can alter the responses of an egg parasitoid toward OIPVs. However, it is important to keep in mind that damage inflicted by non-host herbivores can vary in terms of intensity and duration, and that such factors can also affect plant responses as well as parasitoid foraging behavior (Ponzio *et al*., 2014). Previous investigations showed that the egg parasitoid *T. basalis* is attracted to OIPVs emitted by *V. faba* plants as a consequence of combination of egg deposition and feeding activity of the host *N. viridula* (Colazza *et al*., 2004a, b). However, the attraction of *T. basalis* to *V. faba* plants infested with *N. viridula* is eliminated when the plants are also attacked by *S. lineatus*, regardless of whether non-host infestation occurs on leaves or on roots. Studies on larval parasitoids have also demonstrated that below-ground herbivore species can disrupt infochemical networks. For example, Soler *et al*. (2007) provided evidence that the foraging behavior of *Cotesia glomerata* (L.), a larval parasitoid of *Pieris brassicae* (L.), can be affected by the below-ground herbivore *Delia radicum* (L.) through changes in the host plant (*Brassica nigra*) odor blend. Similarly, the congeneric *C. marginiventris* (Cresson) prefers odors emitted by host-infested plants over plants infested with both host and non-host root herbivores (Rasmann and Turlings, 2007). In the case of dual above-ground stresses, naïve *T. basalis* were still attracted to odors emitted by plants damaged with a combination of *N. viridula* feeding and oviposition and mechanical damage, but naïve wasps were not attracted to plants suffering dual above-ground herbivore attacks (*N. viridula* feeding and oviposition + *S. lineatus* adult feeding).

Egg parasitoid attraction toward induced plant volatiles

Plants are able to sense touch, feeding and oviposition activity of herbivore insects (Hilker and Meiners, 2010).However, even if it cannot be completely ruled out that plants can also sense the pressure induced by clip cages used in *S. lineatus* treatments, our preliminary results (Cusumano and Salerno personal observations) and previous investigations (Guerrieri *et al*., 1999) both suggest that clip cages have a negligible effect in the context of this study. The results discussed above suggest instead that *S. lineatus* oral secretions or damage patterns could be involved in altering the blend of induced volatiles. Indeed, many elicitors that plants use to activate indirect defense mechanisms have been identified in the oral secretions of insects that come in contact with plant tissues during feeding (Bonaventure, 2012). Oral secretions can also contain microorganisms that could potentially trigger plant responses (Zhu *et al*., 2014). The role of herbivore-associated microorganisms in plant defenses is an emerging and poorly understood area of plant insect interactions, making it difficult to speculate about whether microorganisms are involved in our study system. Further investigations should be conducted to screen for the presence of microorganisms in *S. lineatus* oral secretions. Among the others factors that could explain our results, we doubt that differential patterns of herbivory played a major role, since mechanical damage was performed by carefully mimicking the damage inflicted by *S. lineatus* adults. PLS-DA analysis of the odor blends from the different treatments supports our behavioral data, with significant changes to odor profiles of *V. faba* plants as a consequence of single or dual herbivore attack. In previous studies of dual above-ground herbivore attack, natural enemy attraction was either disrupted, unaffected, or even enhanced by dually infested plants, indicating that the effect of multiple herbivore attack on HIPVs emissions is variable (Shiojiri *et al*., 2001; Agbogba and Powell, 2007; Moayeri *et al*., 2007; de Boer et al., 2008; Erb *et al*., 2010; Bukovinszky *et al*., 2012; de Rijk *et al*., 2013; Ponzio *et al.*, 2014).

Interestingly, under our experimental conditions, *V. faba* damage inflicted either by *S. lineatus* adults or larvae had a similar disruptive effect on attraction of naïve *T. basalis*. This suggests that the damage caused by larvae and adults of *S. lineatus*, both of which are chewing insects even though they feed on different parts of the plant, may cause similar responses in the plant, which in turn may affect the feeding and oviposition activity of piercing-sucking insects. Considering previous studies on other model systems (Moran and Thompson,2001; Thaler, 2002; Kempema *et al*., 2007; Zarate *et al*., 2007; Smith *et al*., 2009; Ode, 2013; Reymond, 2013) one could hypothesize that the disrupting effect of *S. lineatus* on *T. basalis* attraction is due to cross-talk between JA and SA pathways.

Egg parasitoid attraction toward induced plant volatiles

So far, phytohormonal consequences of egg deposition have been investigated only in model systems of lepidopteran herbivores associated with brassicaceous plants, whereas nothing is known in other systems. Consequently, to confirm our hypothesis, further research on phytohormonal signaling pathways in response to feeding and oviposition activities of piercing-sucking insects is required.

In our study, an oviposition experience affected the response of *T. basalis* females to plant volatiles in a Y-tube olfactometer. It has often been suggested that learning can be adaptive for egg parasitoids when foraging for hosts in complex and dynamic environments (Fatouros *et al*., 2008; Colazza *et al*., 2010; Cusumano *et al*., 2012). Learning appears to be partially adaptive in our study as well, especially considering that volatiles induced in *V. faba* plants infested only with *S. lineatus* adults or larvae attracted naïve *T. basalis* females. For the parasitoids, attraction to *S. lineatus* induced volatiles may be costly in terms of reproduction, because they would waste time searching on plants where there were no hosts present. This negative effect could be particularly severe for *T. basalis* given that it occurs twice during the*V. faba* growing season, considering the life history traits of univoltine species like *S. lineatus*. In fact, above-ground attacks occur early in the growing season whereas below-ground attacks occur later when the developing larvae feed on the roots. Such temporal dynamics of non-host herbivore infestation could considerably extend the temporal window of disturbance and consequently decrease the efficiency of host location by *T. basalis*. Oviposition experience on *N. viridula* eggs laid on *V. faba* leaves changed the behavioral responses of parasitoids so that they were no longer attracted to plants infested with *S. lineatus* adults or larvae, nor were they attracted to plants infested with both *N. viridula* and *S. lineatus*. Furthermore, the response showed by experienced *T. basalis* was not straight forward because wasps were not attracted to mechanically damaged plants that were also infested with *N. viridula*. Overall, the divergence of *T. basalis* behavior between naïve and experienced wasps suggests that the wasps are using associative learning to optimize their foraging efficiency (Steidle and Van Loon, 2003; Hoedjes *et al*., 2011; Gols *et al*., 2012). The role of learning in multitrophic systems has been investigated in few other case studies; for instance, the larval parasitoid *C. marginiventris* preferred HIPVs induced by its host *Spodoptera littoralis* (Boisduval), but after oviposition experience while exposed to maize plants infested with the host *S. littoralis* and the non–host *Diabrotica virgifera virgifera* (Leconte), the parasitoid preferred HIPV blends of maize induced by both herbivores (Rasmann and Turlings, 2007). All these results emphasize the need to control the pre-assay

experience of egg parasitoids when evaluating their responses to induced plant volatiles, when the plants are being attacked by different combinations of herbivore species. In summary, the present study investigated the effects of an above- and below-ground non-host herbivore on attraction of an egg parasitoid to plant volatiles induced by feeding and oviposition of its host. Our results demonstrated that attraction of this wasp was disrupted by both larvae and adults of *S. lineatus* when foraging for *N. viridula* eggs laid on *V. faba* plants. Further studies will focus on the identification of the volatile compounds emitted by *V. faba* plants that are attacked individually or concurrently by *N. viridula* and *S. lineatus* in order to identify the blend of compounds that play a role in egg parasitoid recruitment, and how that blend is altered or disrupted by *S. lineatus* feeding.

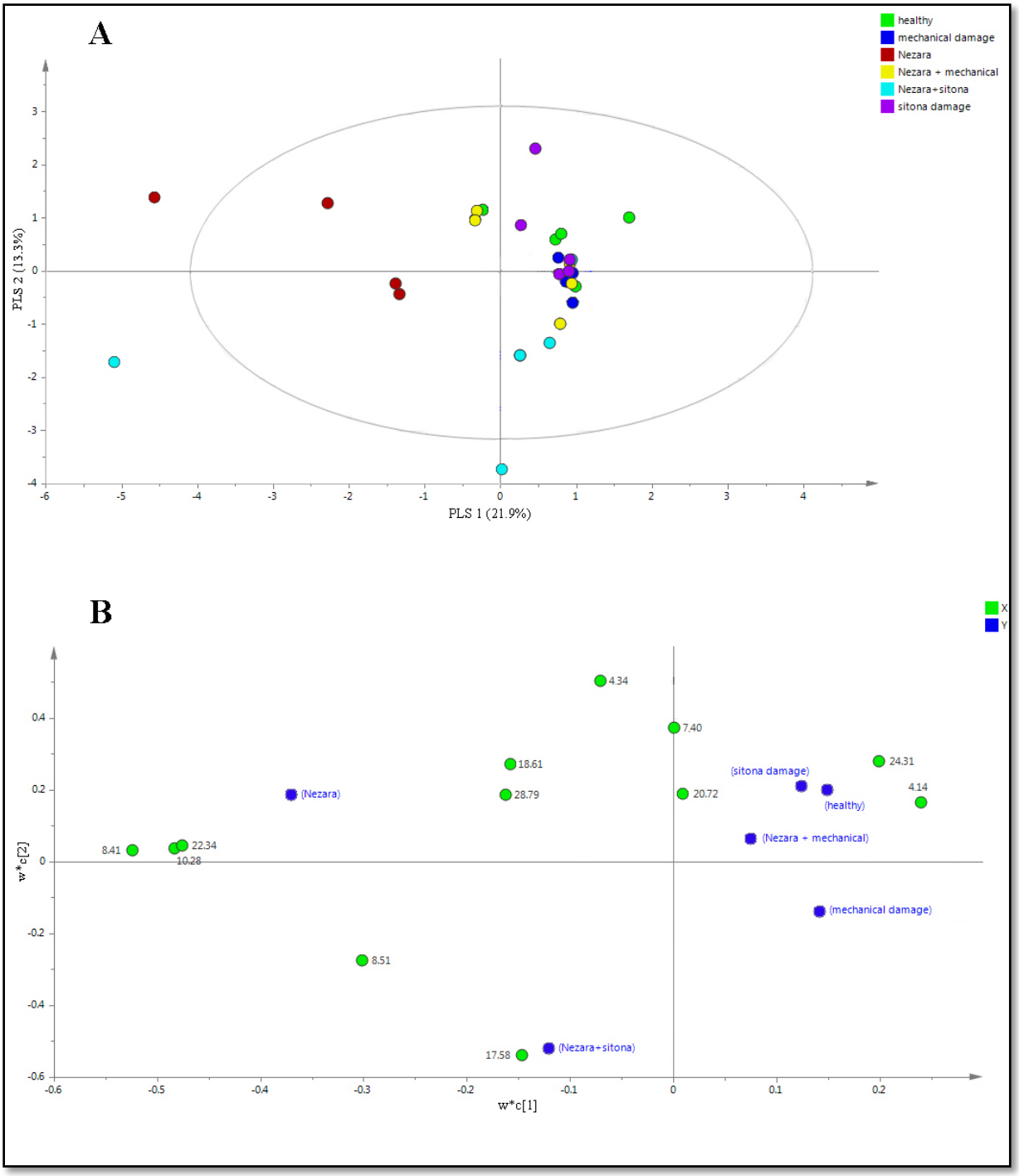

Figure 4 | Projection to latent structures discriminant analysis (PLS-DA) comparison of the volatile compounds emitted by individual *V. Faba* plants. (A) Score plot of the samples, with the percentage of explained variation in parentheses. The PLS-DA resulted in a model with two significant principal components (PCs). The ellipse defines the Hotelling's T2 confidence region (95%). **(B)** Loading plot of the first two components of the PLS-DA, showing the contribution of each of the compounds toward the model. Numbers refer to the retention time of volatile compounds.

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Chapter 3

Chapter 3
Identification of plant volatile synomones induced in the multi-trophic system *Vicia faba-Nezara viridula-Sitona lineatus*

Abstract

It is well established that plants infested with a single herbivore species can attract specific natural enemies through the emission of herbivore-induced volatiles. However, in chemical point of view, it is less clear what happens when plants are simultaneously attacked by more than one herbivore species. In this scenario, we analyzed volatile emissions of broad bean plant upon multi-species herbivory by *Nezara viridula* feeding and oviposition and *Sitona lineatus* (above and below-ground attack) in comparison to single-species herbivory. Moreover, *Trissolcus basalis* response to fractions of *N. viridula* feeding and oviposition headspace extracts were investigated with a dual choice Y-tube olfactometer. A total of 125 different volatile compounds were detected across all treatments in the headspace of *V. faba* plant. Since the concentration of compounds in our extracts was below the detection threshold level for GC-FID so it was difficult to identify the obtained peaks in GC-MS. The low concentration of compound also explains the lack of response for the parasitoids in bioassays. Using SPME enabled the identification of 15 compounds associated with *N.viridula*-infested *V. faba* plants. These results indicate that SPME is promising technique that could be used in the future to identify volatiles.

Key-words: *Vicia faba*, *Nezara viridula, Sitona lineatus* , GC-MS, GC-FID

Identification of plant volatile synomones

3.1 Introduction

It is well known that plants respond to herbivore feeding and egg deposition by production mixtures of volatiles called herbivore induced plant volatile (HIPVs) that not only differ in the total abundance of volatiles released, but more importantly, also in the composition of the volatile blend (Kessler and Baldwin, 2001; Dicke, 2009). The change in composition can be quantitative, i.e., different ratios of the same components, or qualitative, i.e., by the release of compounds that do not occur in the blend emitted by the intact plant (Dicke *et al*., 2003).

Odor blends emitted by herbivore–infested plants are complex mixtures that are often composed of more than 200 different compounds, many of which occur only as minor constituents (Dicke and van Loon 2000; Dudareva *et al*., 2006).Despite the enormous diversity of existing volatile compounds that are released after herbivory , they can be divided into three major classes-namely terpenoids (isoprenoids), fatty acid derivatives and phenylpropanoids or benzenoids (Dudareva *et al*.,2006 ; Arimura *et al*.,2009). There is indeed ample behavioural evidence that carnivores selectively exploit HIPVs during the location of their herbivorous hosts or prey and this includes field studies (See reviews, Fatouros *et al*., 2008, Dicke and Baldwin, 2010, Hare, 2011, Kessler and Heil, 2011). However, this indirect chemical information is often more variable than information from the prey itself. Variation in the composition of HIPVs can be related to plant species and cultivars, herbivore species, multiple infestation by another herbivore species or pathogen, and abiotic factors (e.g., de Boer *et al*., 2008, Hilker and Meiners, 2002; Colazza *et al*., 2004 a, Dicke *et al.*, 2009, Zhang *et al*., 2009, Holopainen and Gershenzon, 2010, Hilker and Meiners, 2011, McCormick *et al*., 2012). For foraging carnivores, it is especially important to attend the differences or variation in volatile blends that are associated with herbivore species because the herbivores may differ in their suitability as hosts or preys.

Most studies demonstrating the attraction of natural enemies to HIPVs have done this when the plant is attacked by single herbivore species. However, in nature, complex multitrophic interactions with simultaneous or sequential attack are the norm. Interplay between attackers can have strong implications for the interaction between plants and natural enemies of their associated herbivores, via modifications of the emitted volatile blend (Dicke *et al*., 2009; Ponzio *et al*., 2013). It is difficult to predict whether or not changes in HIPV blends upon multi-species herbivory affect attraction of natural enemies, and in what direction. Since the last decade, several studies have attempted to fill this gap by analyzing the chemical

composition of volatile blends of plants, and investigating the foraging behavior of carnivores, upon multiple infestation (Shiojiri *et al*.,2001; Cardoza *et al*.,2002;Rodriguez-Saona *et al*.,2003;Rostas *et al*.,2006;Moayeri *et al*.,2007;Rasmann and Turlings,2007;Soler *et al*.,2007;Zhang *et al*.,2013; Ponzio *et al*.,2014). For instance, Ponzio *et al*. 2014 showed that the parasitoid wasp *Cotesia glomerata* foraging behavior was equally attracted to *Brassica nigra* plants infested with the host *Pieris brassicae* and to the *B.nigra* plant infested by both *P. brassicae* and non-host *Brevicoryne brassicae* aphids. Analysis of the volatile emissions showed that dually attacked plants could not be separated from those with only caterpillars. In contrary, within our system, in previous work Moujahed *et al*. 2014, demonstrated that attraction of *T. basalis* was disrupted by both larvae and adults of the non-host *S. lineatus* when foraging for *N. viridula* eggs laid on *V. faba* plants. The aim of the present study was the identification of the volatile compounds emitted by *V. faba* plants that are attacked individually or concurrently by *N. viridula* and *S. lineatus* (above/below- ground) in order to identify the blend of compounds that play a role in egg parasitoid recruitment, and how that blend is altered or disrupted when concurrent feeding by *S. lineatus* occurred in the plant.

3.2. Material and methods

3.2.1 Plant growing

Seeds of broad bean plants (*V. faba* cv. Superaguadulce) were immersed for 24h in slurry of water and soil (1:4) to favor root nodulation. The seeds then were individually planted in plastic pots (9x9x13 cm) filled with a mixture of agriperlite (Superlite, Gyproc Saint-Gobain, PPC Italia, Italy), vermiculite (Silver, Gyproc Saint-Gobain, PPC Italia, Italy) and sand (1:1:1) and grown in a climate controlled chamber (24±2°C, 45±10% RH, 12h:12h L:D). Plants were watered daily and, from one week post-germination, fertilized with an aqueous solution (1.4g/l) of fertilizer (5-15-45, N-P-K, Plantfol, Valagro, Italy). In the case of "*above-ground treatments*" (see below), 18-20 day old broad bean plants, with approximately six fully expanded leaves, were used. For the "*below-ground treatments*" and "*below- + above-ground treatments*", 15 day old plants were infested with *S. lineatus* eggs, left to grow to allow development of *S. lineatus* larvae on the root nodules, and then exposed to *N. viridula* (after 12 day) and/or tested (after 15 d) (see below).

3.2.2 Insect rearing

The *N. viridula* colony established from material collected in cultivated and uncultivated fields around Perugia and Palermo (Italy), was reared under controlled conditions (24±2°C, 70±5% RH, 16h:8h L:D) in wooden cages (50x30x35 cm) with mesh covered holes (5 cm diameter) for ventilation. Bugs were fed with a diet of sunflower seeds and seasonal fresh vegetables. Food was changed every 2–3 d, and separate cages were used for nymphs and adults. Egg masses were collected daily and used to maintain cultures of *N. viridula*.

Sitona lineatus adults were collected from *V. faba* fields around Perugia and Palermo and maintained in a climate-controlled chamber (8±2°C, 70±5% RH, 16h: 8h L: D). The colony was reared in plastic food containers (30x19.5x25 cm) with 5cm diameter mesh-covered holes. Adults were fed with vegetative parts of *V. faba* changed once a week and eggs were collected daily. Eggs were kept in Petri dish with the bottom covered by a filter paper disk moistened with distilled water. The Petri dish was sealed with Parafilm® and maintained under controlled conditions (24±2°C, 70±5% RH, 16h: 8h L: D).

The colony of *T. basalis* was originally established from wasps emerging from naturally and/or sentinels *N. viridula* egg masses, located in wild and uncultivated fields around Perugia and Palermo. The parasitoid was reared on *N. viridula* egg masses that were glued on paper strips. Wasps were maintained in 85ml glass tubes, fed with a honey-water solution and kept in controlled environment room under the same rearing conditions of *N. viridula*. After emergence, male and female wasps were kept together to allow mating. For all bioassays, naïve 2-4 d old females were used. Naïve females were individually isolated in small vials 1 hr before bioassays and then transferred to the bioassay room to be acclimatized.

3.2.3 Plant treatments

A) Plants dedicated to dynamic headspace sampling

Plants were left untreated as controls, or subjected to the following treatments (**Fig. 1**).

Above-ground damage

a: *N. viridula* feeding and oviposition obtained by exposing individual plants to 3 *N. viridula* gravid females for 24h.

b: *S. lineatus* leaf-feeding obtained by exposing three leaves of a plant to 15 *S. lineatus* adults (5adults/1 leaf) for 24h using a “clip cage”; the latter consisted of two modified plastic Petri

dishes (Ø=10 cm; h=1 cm), each with a mesh-covered hole in the bottom and the rim covered by a small sponge ring.

c: *N. viridula* feeding and oviposition plus *S. lineatus* leaf-feeding, obtained as described above by exposing the same plant first to *N. viridula* and then after 1 d to *S. lineatus* adults; attacks by the two species on the same leaves were avoided.

Below- ground damage

d: *S. lineatus* larvae feeding on root nodules, obtained by infesting individual plants with 30 *S.lineatus* eggs ready to hatch (6-7 d old). With the aid of a fine paintbrush, eggs were gently put inside a dimple made ad hoc on the plant substrate and then they were covered with the same substrate. These treated plants were tested 15 d after inoculation with eggs in order to allow larval feeding damage to the root nodules.

Above + below-ground damage.

e: To get plants damaged with a combination of *N. viridula* feeding and oviposition and *S. lineatus*larval damage to root nodules, test plants were first infested with *S. lineatus* eggs as described above and, 12 d after inoculation, they were exposed to *N. viridula* as previously described.

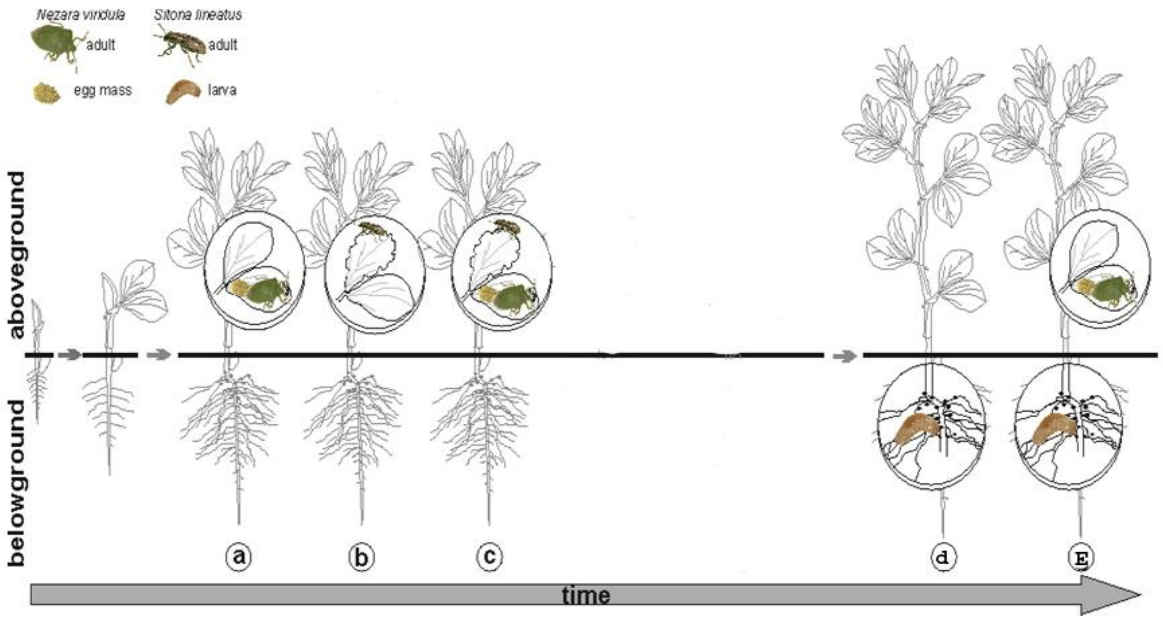

Figure1: Visual summary of the main plant treatments. Above-ground *Nezara viridula* feeding and oviposition (a); *Sitona lineatus* adult leaf-feeding (b); *N. viridula* feeding and oviposition and *S. lineatus* adult leaf-feeding (c).Below-ground (*S. lineatus* larvae root-nodules feeding) (d) and above + below-ground treatment (*N. viridula* feeding and oviposition and *S.lineatus* larvae root-nodules feeding) (E) and temporal dynamics of multi-trophic infestations on *Vicia faba.*

B) Plants dedicated to static headspace sampling

Plants were either left healthy or treated with *N.viridula* feeding obtained by exposing individual plants to 3 *N.viridula* females for 24 h.

3.2.4 Collection of plant volatiles

Dynamic headspace/adsorbent traps

A cylindrical glass chamber (Ø=9 cm ID; h=29 cm) was used to collect headspace volatiles from empty pots, treated plants and healthy plants (n = 6 for each) (**Fig.2**). Before each collection, the glass chamber was washed with water and detergent, rinsed with acetone, and baked overnight at 180º C. Singly-potted plants were placed in each aeration chamber, separated from the pot and soil with aluminum foil to reduce contamination from soil odors. Air, purified by passage through an activated charcoal filter, was pumped into the chamber at 900 ml/min, with 600 ml/minbeing pulled through a glass tube filled with Porapak Q (Sigma Aldrich; 60 mg, 80-100 mesh), which was pre-cleaned with hexane and then heat conditioned for at least 2 h in a stream of nitrogen (100 ml/min) at 130°C. Volatiles were collected for 24h, and then traps were eluted with 700 µl of dichloromethane containing dodecane as internal standard (10ng/µl).The resulting extracts were divided in two equal proportions, 50 % for extract A and 50% for extract B. Extract A was attributed to chemical analysis while extract B was selected to bioassays. Extracts were stored at -20°C in glass vials with Teflon cap liners until used for the different analyses.

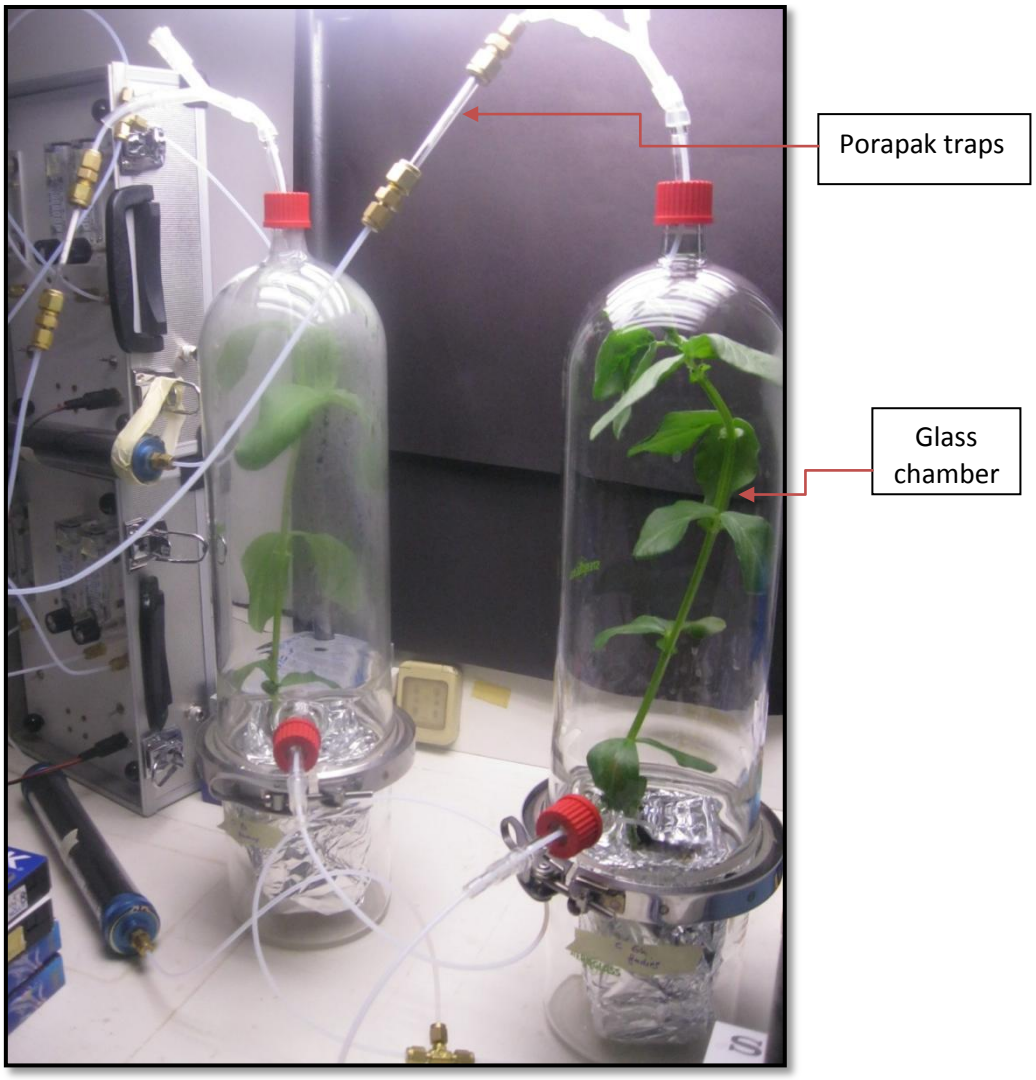

Figure 2: Dynamic collection system

Static headspace /Solide phase microextraction (HS/SPME)

Teflon bags were used, 30 min before the collection, to cover the treated plants (n=4, each). Volatiles from each sample were collected by means of the SPME technique. SPME devices coated with Polydimethylsiloxane/divinylbenzene/carboxen (PDMS/DVB/CAR) were used to sample the headspace of all the samples (Fig.3). Then, the SPME fiber was exposed to the headspace for 30 min at 25 °C. Once sampling was finished, the fiber was then retracted into the needle and immediately transferred into GC/MS injector for desorption and analysis of volatiles.

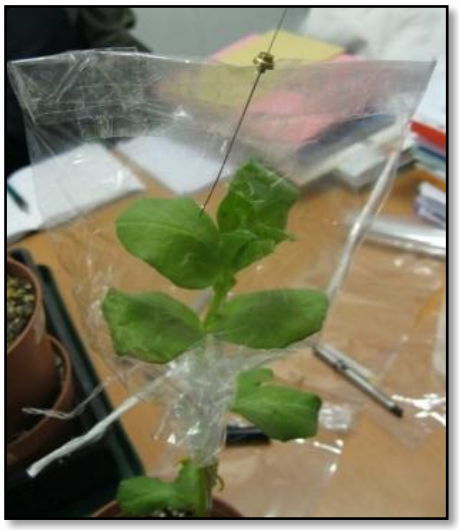

Figure 3: Static collection system

3.2.5 Chemical VOC analyses

The plant volatile compounds collected through dynamic headspace were analyzed using a combination of capillary GC-FID and GC-MS techniques. GC-FID and GC-MS were monitored in parallel on all samples to allow an accurate quantification and identification, respectively. While the VOCs trapped via HS/SPME were only analyzed by GC-MS technique.

GC-FID analysis

GC-FID was carried out on a 5973N GC system. A 3 µl, from the extract A, for each sample was injected onto HP-5MSColumn (30m×0.25m×0.25µm) in splitless mode, with a flame ionization detector (FID). Helium was used as the carrier gas. Injector and detector temperatures were 260 °C and 280°C respectively. Helium was used as the carrier gas. The GC oven was programmed from 40°C for 3 min, and then increased by 8°C/min to 250°C. Volatiles were separated by GC and then quantified using the peak area method. Quantification of identified compounds was based on comparison with a set of authentic reference compounds (Limonene, 2-hexanol, alpha-pinene, beta-caryophyllene, beta-pinene, cis-hexanol, hexane, terpinene) injected under identical condition.

Gas chromatography–mass spectrometry analysis (GC-MS)

GC-MS analyses were performed on a 6890 N GC system interfaced with an HP-5973 quadruple mass spectrometer. For each sample (n=6), 3µl of extract was injected onto a HP5-MS column (5% diphenyl–95% dimethylpolysiloxane 30 m×0.2 mm, 0.25-µm film) in splitless mode. Injector and detector temperatures were 260 °C and280°C respectively. Helium was used as the carrier gas. The GC oven temperature program was40°C for 5 min, then increased by 10°C/min to 250°C. Electron impact ionization spectra were obtained at 70 eV, recording mass spectra from 40-550 amu. Identification of compounds was based on comparison of mass spectra with those in the NIST mass spectral library 2005 and experimentally obtained linear retention indices (RI) were also used as additional criterion for confirming the identity of compounds. Peak area of each detected compound was calculated and related to the total leaf area of the plant.

32.6 Bioassay Procedure

Wasps' response to fractions of headspace extracts were investigated with a dual choice Y-tube olfactometer made from a polycarbonate body (stem 9 cm; arms 8 cm at 130° angle; ID 1.5 cm) sandwiched between two glass plates. A stream of clean air (medical-grade compressed air, N2:O2 80:20), humidified by bubbling through a water jar, was regulated in eacharm by a flowmeter at about 0.4 l min^{-1}. The device was illuminated from above by two 22-W cool white fluorescent tubes, and from below by an infrared source (homogeneous emission of wavelengths at 950 nm provided by 108 LEDs). Before entering the olfactometer arms, a volume of 200 µl from headspace extracts and an equal volume from dichloromethane (control) were adsorbed over a 1.2–cm piece of filter paper each then placed in small cylindrical glass vial. Each individual parasitoid was introduced into the Y-tube at the entrance ofthe stem and thus had a choice between the test and control.

New filter papers with the extracts and dichloromethane were used for about 10 parasitoids. The position of the arms containing the treatment and control odors was reversed to avoid position bias after every 10 individuals had been tested. Each parasitoid spent 10 min in the olfactometer. At every switch, the whole system was changed with cleaned parts.

Their behavior was recorded using a monochrome CCD video camera (Sony SSC M370 CE) fitted with a 12.5–75 mm/F 1.8 zoom lens. The camera lens was covered with an infrared pass filter (Kodak Wratten filter 87 Å) to remove visible wavelengths.

Analog video signals from the camera were digitized by a video frame grabber (Canopus® ADVC 110, Grass Valley CA, USA). Digitized data were processed by XBug, a video tracking and motion analysis software (Colazza *et al*., 1999). Wasp response was measured in terms of residence time, *i.e*. the time spent by the wasps in each arm during the entire bioassay. At the end of the bioassays the polycarbonate olfactometer and all glass parts were cleaned with water and detergent. The glass parts were then cleaned with acetone and baked overnight at 180°C. Bioassays were conducted from ~09:00h to 13:00h under controlled conditions (26 ±1° 218 C, 50±5% R.H.).Preliminary tests had shown that *T.basalis* had no preference for solvent control (dichloromethane) and an extract composed with a solvent and internal standard. This confirmed that Y-tube olfactometer investigations could be used for further behavioral experiments.

3.2.7 Data analysis

Data collected from GC-FID were analyzed by Graph pad prism 5.1.01. Volatiles extracts from SPME were analyzed by cluster analysis using MVSP 3.21, Kovach Computing Systems. For the bioassays, the time spent by wasp females in each arm was statistically compared by parametric paired *t*-tests for dependent samples and data were analyzed using the STATISTICA7 software (StatSoft, 2001).

3.3 Results

3.3.1 Chemical VOC analysis

A total of 125 different volatile compounds were detected across all treatments in the headspace of *V. faba* plant. The concentration of compounds in our extracts was below the detection threshold level for GC-FID so it was difficult to identify the obtained Peaks in GC-MS (**Fig. 4**). However, analyses by SPME enabled the identification of 15 compounds belonging to different families (Table. 1) such as monoterpenes, aromatic hydrocarbons, and alcohols. By examining cluster from SPME analysis it is obvious that groups of *N. viridula* samples are similar to each other but different from healthy plants (**Fig.5**).

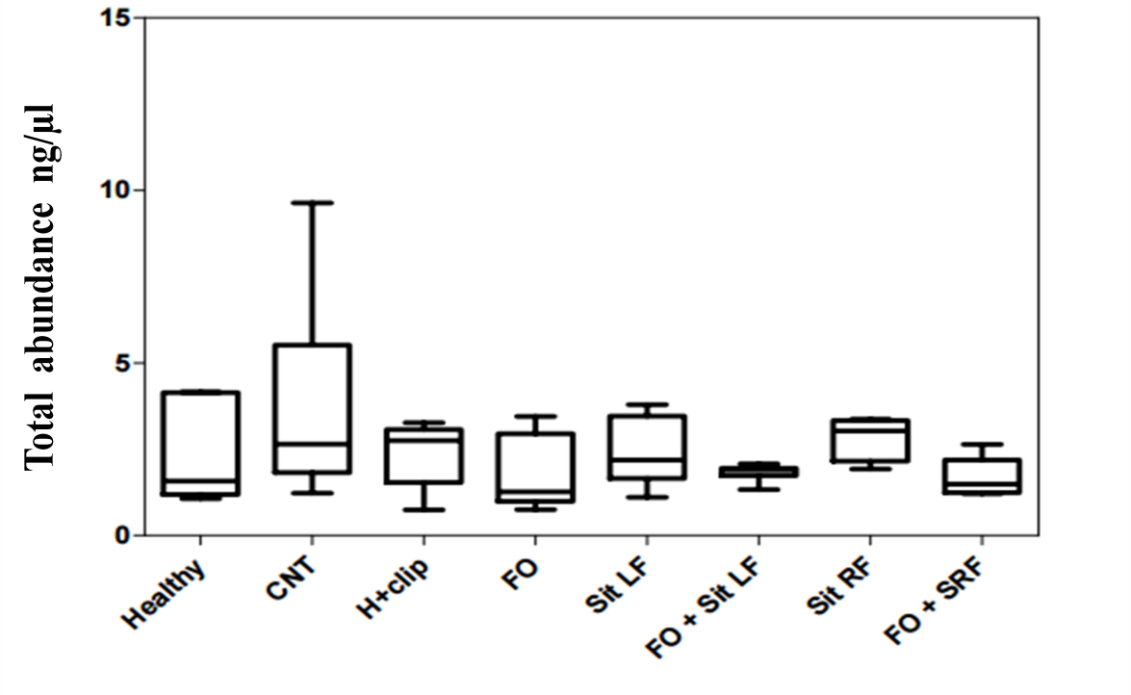

Figure 4: Graph total abundance per treatment

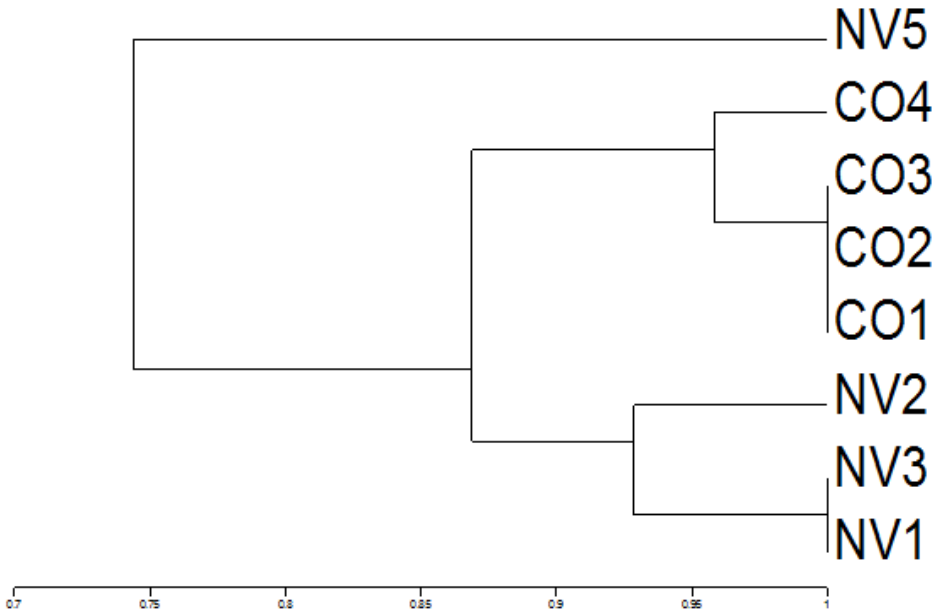

Figure 5: Dendrogram of cluster analysis performed on 8 samples (Simspon's-coefficient); NV1: *Nezara viridula*, CO: Control

Identification of plant volatile synomones

Table 1. Volatiles collected from different treated broad bean plants through SPME technique RI based on identified compound retention times calculated from a linear equation between each pair of straight chain alcanes (C5−C14).

Peak	Retention time	Retention index (RI)	Identified compounds	Family
1	4.59	550	Pentane,2-methyl	Alkane
2	5.177	565	Hexane	Alkane
3	5.804	558	Butanol	Alcohol
4	6.5	621	Butanal,2-methyl	Aldehyde
5	8.213	699	Toluene	Aromatic hydrocarbon
6	8.7	803	2-Hexenal,E	Aldehyde
7	8.9	725	Hexanal	Aldehyde
8	11.3	801	Cis hex-3-enol	Alcohol
9	11.76	818	Hexanol	Alcohol
10	12	883	Para-xylene	Aromatic hydrocarbon
11	13.7	934	Alpha-pinene	Monoterpene
12	14.325	948	Alpha-Thujene	Monoterpene
13	15.2	976	Beta-pinene	Monoterpene
14	15.458	990	3-hexen-1-ol,acetate,(z)-	Alcohol
15	15.86	999	Sabinene	Monoterpene
16	16.17	1009	Delta-3-carene	Monoterpene
17	16.4	1017	Paracymene	Monoterpene
18	16.7	1023	Limonene	Monoterpene
19	17.368	1047	Alpha terpinene	Monoterpene
20	17.468	1050	Meta-Cymene	Monoterpene
21	19.803	1123	Terpinolene	Monoterpene
22	20.943	1754	Benzoic acid	Aromatic carboxylic acid

3.3.2. Responses to volatile extracts in the olfactometer

There was no significant difference between extract composed of solvent and internal standard (Test) and the one with only solvent (control) (*t*= 0, 6410; df=2; p=0,587; **Fig. 6**). This result allows us to continue the further experiment to test the attractiveness of *T. basalis* towards headspace volatile extracts of *N.viridula* feeding and oviposition.

No significant choice was displayed by naïve *T. basalis* when presented with the extract from plant damaged with *N.viridula* feeding and oviposition versus solvent (*t*= -1,329; df=6; p= 0.234; **Fig. 7**).

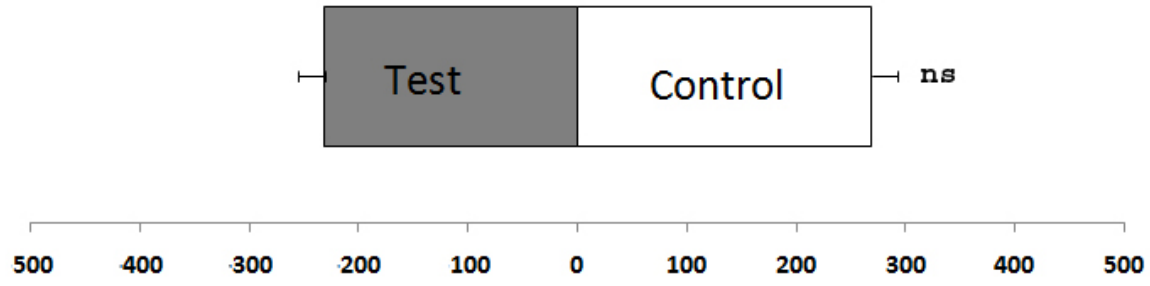

Figure 6: Response of naïve *Trissolcus basalis* females in a Y-tube olfactometer to extract composed of solvent and internal standard (Test) versus solvent (Control).

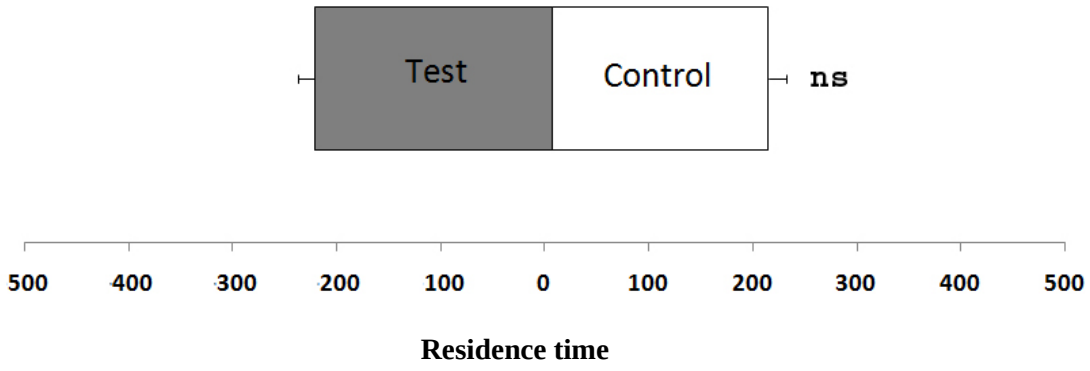

Figure 7: Responses of naïve *Trissolcus basalis* in a Y- tube olfactometer to headspace volatile extracts of *N.viridula* feeding and ovipostion (Test) versus a solvent (Control).

3.4 Discussion

It is obvious that herbivore-inflicted injury induced *V. faba* plants to release volatile belonging to different family groups including green leaf volatiles (GLVs) and terpenoids (Table.2) and it is well known that parasitoids and predators used these volatiles as major cues to locate their hosts (Dicke *et al*., 1990; Kessler and Baldwin, 2001; Colazza *et al*., 2004a; de Boer and Dicke, 2004; Mumm and Hilker, 2005). In this context, previous investigations showed that egg parasitoids *T. basalis* is attracted to OIPVs emitted by *V. faba* plants as a consequence of combination of egg deposition and feeding activity of host *N. viridula* (Colazza *et al*.,2004 a, b, Moujahed *et al*.,2014). The blend containing (*E*)-*β*-caryophyllene was the responsible of *T. basalis* attraction (Colazza *et al*., 2004a) (Table.2). However, the attraction of *T. basalis* to *V. faba* plants infested with *N. viridula* is eliminated when the plants are also attacked by *S. lineatus*, regardless of whether non-host infestation occurs on leaves or on roots. In chemical point of view, in system with *Vicia faba-Nezara viridula-Sitona lineatus*, we were not able to obtain interpretable results through GC-FID and GC-MS because of the insufficient concentrations of compounds in our extract which could explain the lack of response for the parasitoids in bioassays. Moreover, the blend from *V. faba* extract seemed to undergo a degradation process as the use of treated filter paper discs increased over time. So in further investigations it is better to use a new filter paper for each tested parasitoid. The low compound concentration is maybe due to the storage and shipment condition of the extract. In fact, the collection of plant volatile and GC-MS analysis were preformed between different laboratories in Italy and France respectively. Therefore, there was a time lag between the collection and analysis of extracts which were first stored for a long time to -4 ° C then shipped to France. So the long storage time and shipment could result in evaporation and loss of the most volatile metabolites. The choice of the effective collection technique (headspace or static) as well as the choice of the right analytical methods could also influence the chemical analysis. A traditional form of analysis for plant volatile is desorption of the compound of interest from the sorbent to the solvent followed by analysis of the solution. This form was applied in several works include ours (Colazza *et al*.,2004b ; Webster *et al*., 2008; Schwartzberg *et al*.,2011 ; Moujahed *et al*.,2014).Nevertheless a good extraction by this form will depend of the affinity of the molecules with the solvent; therefore all solvent will definitely not give the same results. So it is important to choose the right solvent. As alternative there is the thermal desorption technique that could be applied instead of solvent desorption. Instead of liquid extraction of the sorbent, the sampling tube is heated and the

absorbable compounds are purged directly into analytical instrument. Thermal desorption of VOCs eliminates the need for solvents that may contain impurities which will interfere with sample analysis. However, by desorbing the entire sample into the injector, no repeated injections of the sample are possible, (Ramirez *et al*., 2010). Thermal desorption is based on collecting the compounds of interest from solid sorbent collection devices and then heating this sorbent in a flow of gas to release the compounds and concentrate them into a smaller volume. A wide variety of sampling configuration are used for thermal desorption, depending on the application. One of the most popular one is solid phase microextraction (SPME). As confirmed from other studies, (Kicel and Wolbis, 2009; Oomah *et al*., 2014) (Table.2), the sensitivity of latter technique was also proved in our work. Moreover, we were able to determine that SPME could be used to identify compound associated *N. viridula*-infested *V.faba* plants. Unfortunately the SPME method does not give the opportunity to produce solvent extracts to perform bioassays. One possibility could be to purchase synthetic mixtures including the identified compounds (alone or in mixtures with different ratios) for the bioassays (Alessandro and Turlings, 2005; Gadino *et al*., 2011). Unfortunately I did not have enough time to continue experiments with SPME and bioassays during my Phd but that could be a best option in the future.

Table 2. Volatiles collected from different plants belonging to Fabaceae

Plant	Compounds	Family	Collection volatile	Volatile analysis	Insects	References
V.faba (Fabaceae)	(*E*)- β- caryophyllene	Terpenoids	Traps : charcoal Solvent: Dicloromethane	GC-FID	*N.viridula* feeding and oviposition	Colazza *et al*., 2004b
	(*E; E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT)					
	(E-E)-α-Farnesene	Ester				
	(Z)-3-hexenyl-acetate					
	Myrcene	Aldehyde				
	Linaool					
	Hexanal	Aldehyde				
	Z-(3) Hexenol	Alcohol				
V.faba (Fabaceae)	(E)-2-hexenal	Aldehyde (GLV)	Traps :Porapack	GC-EAD	*Aphis fabae*	Webster *et al.*, 2008
	(Z)-3-hexen-1-ol	Alcohol (GLV)				
	1-hexanol	Alcohol (GLV)				
	Benzaldehyde	Aldehyde				
	6-methyl-5-hepten-2-one	Terpenoid				
	Octanal	Aldehyde				
	(Z)-3-hexen-1-yl acetate	Ester				
	(R)-linalool	Monoterpene				
	methyl salicylate	Ester (GLV)				
	Decanal	Aldehyde				
	Undecanal	Aldehyde				
	(E)-caryophyllene	Sesquiterpenes				
	(E)-b-farnesene	Sesquiterpenes				
	(S)-germacrene D	Sesquiterpenes				
	(E, E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	Homoterpene				
V.faba (Fabaceae)	E-2-hexenal	Aldehyde (GLV)	Traps : Super Q	GC-FID GC-MS	Acyrthosiphon Pisum: *Spodoptera exigua*	Schwartzberg *et al*.,2011
	Z-3-hexen-1-ol	Alcohol (GLV)				
	E-2-hexen-1-ol	Alcohol(GLV)				
	Benzaldehyde/α-inene	Aldehyde				
	Z-3-hexenyl acetate	Ester				
	E-ß-ocimene	Monoterpene				
	DMNT	Terpenoid				
	ß-caryophyllene	Terpenoid				
	E-ß-farensene	Terpenoid				
	n-Pentadecane	Alkane				
	TMTT *E; E*)-4,8,12-trimethyl- 1,3,7,11-tridecatetraene,	Terpenoid				
	6-methyl-5-hepten-2-one					

Identification of plant volatile synomones

V.faba (Fabaceae)	Toluene	Aromatic hydrocarbons	HP/SPME Volatile were extracted by exposing a fiber coated with a 50/30 µm (DVB/CAR/PDMS) at 50° C for 1 hour	GC-MS		Oomah *et al*., 2014
	Ethylbenzene					
	p-Xylene					
	Isopropylbenzene					
	Vinyl benzene					
	Pentanal	Aldehydes				
	Hexanal					
	Heptanal					
	Octanal					
	(*E*)-2-Heptenal	Alkane				
	Nonanal					
	(*E*)-2-Octenal					
	Decanal					
	Benzaldehyde					
	Heptane					
	Octane					
	Nonane					
	Undecane					
	Dodecane					
	Tridecane					
	3-Ethyl-2-methyl-1,3-Hexadiene	Alkenes				
	Sesquiterpene (unidentified)					
	1-Pentanol	Alcohol				
	1-Hexanol					
	1-Octen-3-ol					
	1-Heptanol					
	2-Ethyl-1-hexanol					
	1-Octanol					
	2-Octen-1-ol					

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Chapter 4

Chapter 4
Molecular investigation of host induced plant responses in the tri-trophic system *Vicia faba – Nezara viridula- Trissolcus basalis*

Abstract

Plants activate direct and indirect defenses in response to insect damage. At the molecular level, herbivores trigger massive transcriptional changes that are mainly controlled by the jasmonic acid (JA) and salicylic acid (SA) pathway. This study investigated for the first time the molecular response of *Vicia faba* plant to different activities of the piercing sucking insect *Nezara viridula.* Also behavioral response of naïve *Trissolcus basalis* towards different treated *V. faba* plants was evaluated. We found a systemic activation of SA in the presence of *N. viridula* footprints and oviposition. Contrary there was no activation of JA pathway. However additional molecular analysis is in course to verify this aspect. Behavior test confirmed that *T. basalis* attracted to OIPVs emitted by *V. faba* plants as a consequence of oviposition and feeding activity of the host *N. viridula.*

Key –words: *Vicia faba , Nezara viridula , Trissolcus basalis* , JA , SA , OVIPs.

Molecular investigation

4.1 Introduction

Plants have developed various mechanisms to defend themselves against herbivorous insects (Howe and Jander, 2008). In addition to nonspecific, constitutively expressed physical and chemical barriers (e.g. trichomes, thick cell walls, adverse secondary metabolites), plants employ specific induced defenses in response to insect feeding or even egg laying (Hilker and Meiners, 2010, 2011).

In contrast to feeding, insect egg laying causes minimal damage to plants, dependent on the egg laying behavior of herbivorous insects, which can be quite distinct in different species (Hilker and Meiners, 2006). Direct defenses against insect eggs have been reported for crop and herbaceous species, including the production of ovicidal substances (Seino *et al*., 1996), growth of neoplasms (Doss *et al*., 2000), development of necrotic zones (Shapiro and Devay, 1987). Indirect defense against insect egg laying comprises induced changes of plant volatile emissions (oviposition–induced synomones) or modifications of the plant surface chemistry,which results in attracting or arresting egg parasitoids and in turn killing the eggs of the herbivores (Hilker and Meiners,2002, Fatours *et al*.,2005).

The first studies demonstrating the existence of oviposition-induced synomones were carried out on perennial plants. *Ulmus minor* Mill. and *Pinus sylvestris* L., respond to oviposition by their herbivores, *Xanthogaleruca luteola* Muller Coleoptera: Chrysomelidae) and *Diprionpini*(L.) (Hymenoptera: Diprionidae), respectively, by emitting volatiles that attract specialist egg parasitoids of these herbivores, *Oomyzus gallerucae* (Fonscolombe) (Hymenoptera: Eulophidae) and *Chrysonotomyia ruforum* (Krausse) (Hymenoptera: Eulophidae) (for review see Hilker and Meiners, 2002). A following study concerned the annual plants *Vicia faba* L. and *Phaseolus vulgaris* L., which, under the combined feeding and oviposition activity of a piercing/sucking herbivore, *Nezara viridula* (L.) (Heteroptera: Pentatomidae), emit volatiles that attract the egg parasitoid *Trissolcusbasalis*(Wollaston) (Hymenoptera: Scelionidae) (Colazza *et al*., 2004 a, b). Inducible defenses might start with plant perception of insect attack. Compounds released onto the leaves by the female insect with her eggs (e.g. oviduct secretion or accessory gland secretion used to glue the eggs on the leaf tissue) or substances released into plant wounds during feeding (saliva- or regurgitate-derived compounds) most likely convey the information indicating an “insect attack”, and so trigger a cascade of plant reactions. These are followed by downstream signaling pathways that mediate specific gene expression, leading to the biosynthesis of metabolites which are responsible for the direct and indirect defenses (Eisner *et al*., 2002; Bruce *et al*., 2010).

Molecular investigation

Two phytohormones, jasmonic acid (JA) and salicylic acid (SA) are known to play a role in mediating plant responses to insect egg deposition (reviewd by Hilker and Meiners 2010, 2011; Reymond, 2013; Hilker and Fatours, 2015). JA is involved in egg-induced responses of very different plant species. Enhanced levels of JA or induction of transcription of JA-responsive defensive genes seems to be independent of the mode of egg deposition on the plant. JA is involved in plant responses to eggs laid on unwounded leaves (e.g., eggs of the moth *Helicoverpa zea* on tomato; Kim *et al*., 2012), eggs laid on leaf tissue that experienced ovipositional wounding (e.g., by sawflies on pine; Hilker *et al*., 2002), or feeding damage by gravid females (e.g., leaf beetles on elm; Buchel *et al*., 2012, Babst *et al*., 2009; Planthoppers on rice; Lou *et al*., 2005, 2006; Tong *et al*., 2012). In addition to JA, SA plays a major role in egg-induced plant responses. It accumulates beneath the eggs of *Pieris Brassicae,* laid on *Arabidopsis thaliana* leaves (Bruessow *et al*., 2010). Furthermore, expression of several SA responsive genes is inducible by *P. brassicae* and *P. rapae* egg deposition; expression of *PR1* is significantly enhanced in leaf tissue beneath the eggs and in close proximity to them (Little *et al*., 2007). Similarly, *P. rapae* egg deposition enhanced expression of *PR1* in *Brassica nigra* plants, but only when an HR-like necrosis was visible (Fatouros *et al*., 2014).

During the last years there was a progress in the understanding of how plant perceive insect damage (feeding and/or egg deposition) on leaf and induces a defense response, with some emphasis on the molecular events underlying these processes (reviewd by Bruessow *et al*., 2010; Reymond, 2013). As described above, several tritrophic systems were well studied. The present Ph.D. thesis examines the model system entailing *V. faba* L., the piercing/sucking herbivore *N. viridula* (L.) (Heteroptera : Pentatomidae), and the egg parasitoid *T. basalis*(Wollaston) (Hymenoptera: Scelionidae). Previous investigations showed that feeding and oviposition by *N.viridula* induce *V.faba* to produce oviposition-induced plant synomones (OVIPs) that attract female *T.basalis.* Furthermore, the induced volatiles were released both locally (the leaf bearing a deposited egg mass) and systemically (leaves above the attacked leaf) (Colazza *et al*., 2004 a,b). Plants with *N. viridula* feeding and oviposition show an enhanced emission of terpenoids, including (E)-β-caryophyllene which increases significantly only when oviposition and feeding are present. The chemical fraction containing (E)-β-caryophyllene attracts *T. basalis* females.Based on this knowledge, we investigated the molecular response of *V.faba* plant to different activities of *N.viridula* (oviposition, feeding and release of chemical traces) to gain new insight into the mechanisms of egg parasitoid attraction.

Also behavioral response of naïve *T. basalis* towards different treated *V. faba* plants was evaluated. Emphasis was placed on the identification and expression of genes responsible for salicylate and jasmonate pathways.

4.2 Material and methods

4.2.1 Plants

Seeds of broad bean plants (*V. faba* cv. Superaguadulce) were immersed for 24 h in slurry of water and soil (1:4) to favour root nodulation. The seeds were then individually planted in plastic pots (9 × 9 × 13 cm) filled with a mixture of agriperlite (Superlite, Gyproc Saint-Gobain, PPC Italia, Italy), vermiculite (Silver, Gyproc Saint-Gobain, PPC Italia, Italy), and sand (1:1:1) and grown in a climate controlled chamber (24 ± 2°C, 45 ± 10% RH, 12 h:12 h L:D). Plants were watered daily and, from 1 week post-germination, nourished with an aqueous solution (1.4 g/l) of fertilizer (5-15-45, N-P-K, Plantfol, Valagro, Italy). For the experiments, 18–20 days old broad bean plants, with approximately six fully expanded leaves, were used.

4.2.2 Insects rearing

The *N. viridula* colony, established from material collected in cultivated and uncultivated fields around Perugia and Palermo (Italy), was reared under controlled conditions (24 ± 2°C; 70 ± 5% RH; 16 h:8 h L:D) in wooden cages (50 × 30 × 35 cm) with mesh-covered holes (5 cm diameter) for ventilation. Bugs were fed with a diet of sunflower seeds and seasonal fresh vegetables. Food was changed every 2–3 days, and separate cages were used for nymphs and adults. Egg masses were collected daily and used to maintain cultures for both *N. viridula* and *T. basalis*. The *N. viridula* colony was supplemented regularly with field-collected bugs.

The colony of *T. basalis* was originally established from wasps emerging from *N. viridula* egg masses, located in wild and uncultivated fields around Perugia. The parasitoid was reared on *N. viridula* egg masses that were glued on paper strips. Wasps were maintained in 85 ml glass tubes, fed with a honey-water solution and kept in controlled environment room under the same rearing conditions of *N. viridula*. After emergence, male and female wasps were kept together to allow mating. For all bioassays, 2–4 days old females were used. Females were individually isolated in small vials 1 h before bioassays and then transferred to the bioassay room to be acclimatized.

4.2.3 Plant treatments

Plants were subjected to the following treatments for the duration of 24h.

a) *N. viridula* feeding and oviposition, obtained by exposing thelower surface of the 3rdleaf of*V.faba* plant to one *N. viridula* gravid female using a clip cage.

(Clip cage consists in a 3.8 cm diameter x 1.0 cm height modified petri dish with the rim covered by a sponge ringand the bottom provided with a mesh-covered hole andsupported by a hairpin attached to a wooden tutor inserted into the soil).

b) *N. viridula* feeding, obtained as it was described in a) except that here the female only fed on the leaf.
c) *N. viridula*footprints and oviposition, obtained as in a) but the female styletshad been carefully removed with scissors prior to the experiment to prevent feeding activity.
d) *N.viridula* footprints, obtained as in c), but the female only walked on the leaf and did not oviposit.
e) *N.viridula*eggs, obtained by gently placing, on the lower side of the 3rd leaf of *V.faba* to 40 *N. viridula* eggsthat had been previously collectedfrom the ovary of a dissected gravid female.
f) Control plant, obtained by clipping an untreated 3rd leaf with a clip cage.

After the treatment period,plants were kept for 24h in climatic chamber then subjected either to behavioral observations or to molecular investigations. In order to evaluate the induction of defense genes is only locally or also systemically, the treated leaf (2nd node leaf), an untreated leaf (3rd node leaf) and the roots were excised from the plant, rapidly frozen using liquid nitrogen and stored at -80°C until analysis **(Fig. 1).**

4.2.4 Behavioral observations

The female parasitoid responses to volatile chemicals from differently treated *V. faba* plants were investigated with a dual choice Y-tube olfactometer made from a polycarbonate body (stem 9 cm; arms 8 cm at 130° angle; ID 1.5 cm) sandwiched between two glass plates. A stream of clean air (medical-grade compressed air, N2:O2 80:20), humidified by bubbling through a water jar, was regulated in each arm by a flowmeter at about 0.4 l min-1. The device was illuminated from above by two 22-W cool white fluorescent tubes, and from below by an infrared source (homogeneous emission of wavelengths at 950 nm provided by 108 LEDs).

Before entering the olfactometer arms, each air stream passed through a cylindrical glass chamber (Ø = 12 cm; h = 52 cm) with an O-ring sealed middle joint, containing a treated plant as odor source. The stimuli were randomly assigned at the beginning of the bioassays and were reversed after testing five parasitoid females. At every switch, the whole system was changed with cleaned parts. At the end of the bioassays the polycarbonate olfactometer and all glass parts were cleaned with water and detergent. The glass parts were then cleaned with acetone and baked overnight at 180°C. Wasp females were singly introduced into the Y-tube olfactometer at the entrance of the stem and allowed to move freely for 10 min. Their behavior was recorded using a monochrome CCD video camera (Sony SSC M370 CE) fitted with a 12.5–75 mm/F 1.8 zoom lens. The camera lens was covered with an infrared pass filter (Kodak Wratten filter 87 Å) to remove visible wavelengths. Analog video signals from the camera were digitized by a video frame grabber (Canopus® ADVC 110, Grass Valley CA, USA). Digitized data were processed by XBug, a video tracking and motion analysis system. Wasp response was measured in terms of residence time, i.e., the time spent by the wasps in each arm during the entire bioassay. The Y-tube olfactometer bioassays were carried out as paired choices, in which odor sources were always tested versus clean plants used as control. Test odor sources included plants subjected to the treatments a), b), d), e), f) reported in the previous section. About 40 replicates were conducted for each treatment. Bioassays were conducted from 09:00 to 13:00 h under controlled conditions (26 ± 1°C; 50 ± 5% R.H.). The time spent by wasp females in each arm was statistically compared by parametric paired *t*-tests for dependent samples and data were analysed using the STATISTICA7 software (StatSoft, 2001).

4.2.5 Molecular investigation

RNA extraction,cDNA synthesis and quantitative RT-PCR

Total RNA of broad bean leaves and roots was extracted using Invisorb Spin Plant Mini kit (Invitek, US) (**Fig. 1**). DNaseI treatment was applied to remove genomic DNA. The integrity of total RNA was checked by gel electrophoresis, and the concentration was determined with a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, US). About 10ng total RNA was used to synthesize the cDNA using iScriptc DNA Synthesis Kit (Bio-Rad Laboratories Inc., US) according to the manufacturer's instructions. Expression of defense genes was conducted using SsoFast™ EvaGreen® Supermixes (Bio-Rad Laboratories Inc., US) in 20 µl reaction. The following primer sequences were obtained from the literature (Gutierrez *et al*., 2011; Cheng *et al*., 2012) or designed from sequences in GenBank

(http://www.ncbi.nlm.nih.gov/genbank/) using Primer3 v. 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/): pathogenesis-related protein 1 (PR1): forward 5'-TCACCACAAGACTACCTCAACA-3', reverse 5'-ATGGACCCTTTGAGTGTACCAT-3'; ethylene- and jasmonate-responsive plant defensin (PDF1.2): forward 5'-GGCGTTATTAGGCCGCTGTA-3', reverse 5'-AGCCGTGACAATCACCACCT-3'; elongation factor 1 (EF1): forward 5'-TTCTGGTTTTGAGGGTGACAAC-3', reverse 5'-AAACATCTTGCAATGGAAGCCT-3';cyclophilin (CYP2):forward 5'-TGCCGATGTCACTCCCAGAA-3', reverse 5'-CAGCGAACTTGGAACCGTAGA-3'). Primers were used in 400 nM reaction concentration each. Amplification was performed for 40 cycles at an annealing temperature of 62°C in the CFX-96 real-time PCR detection system (Bio-Rad, US). Three to four biological replicates were performed. Threshold cycles were used to quantify the normalized relative gene expression (NRQ) as in Hellemans *et al*. (2007).Data were log+1 transformed and analysed by means of ANOVA followed by Dunnett method for multiple comparisons.

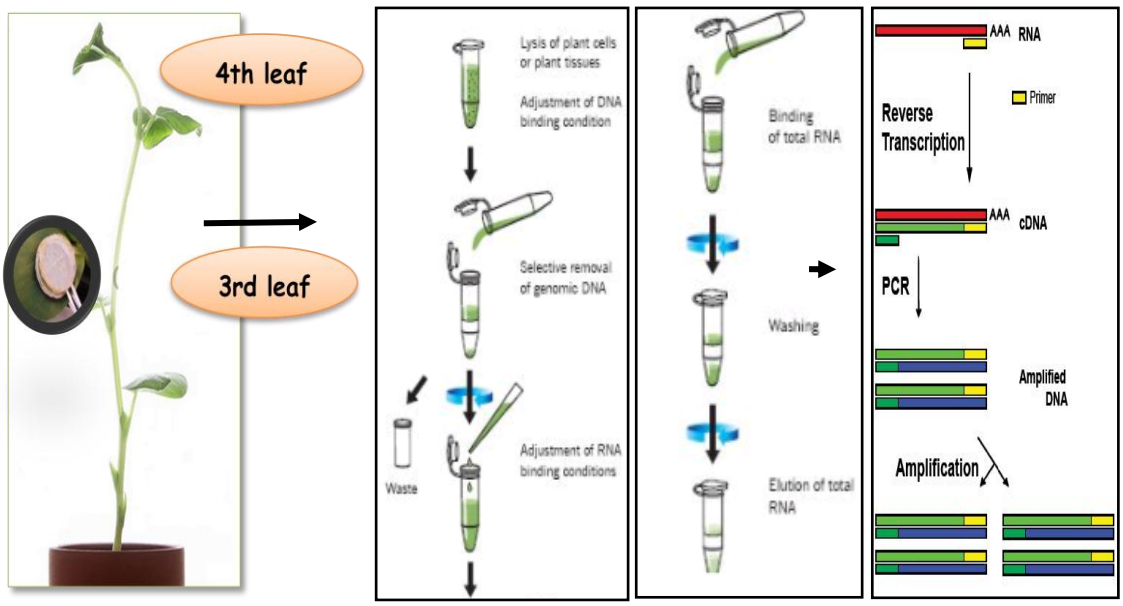

Figure 1: Total RNA extraction and cDNA synthesis

4.3 Results

4.3.1 Behavioral observations

T. basalis females (**Fig**. 2) were significantly attracted to volatiles emitted by plants with *N. viridula* feeding, oviposition and footprints (t = -2.09; df = 45; p = 0.042) compared to undamaged control plants. The volatiles emitted by plants with *N. viridula* feeding and footprints did not stimulate a significant response in *T. basalis* (t = -0.10; df = 35; p = 0.920), neither the presence of *N. viridula* eggs alone (t = 1.84; df = 39; p = 0.074). *T. basalis* preferred control plants over *N. viridula* footprints (t = 4.72; df = 40; p < 0.001).

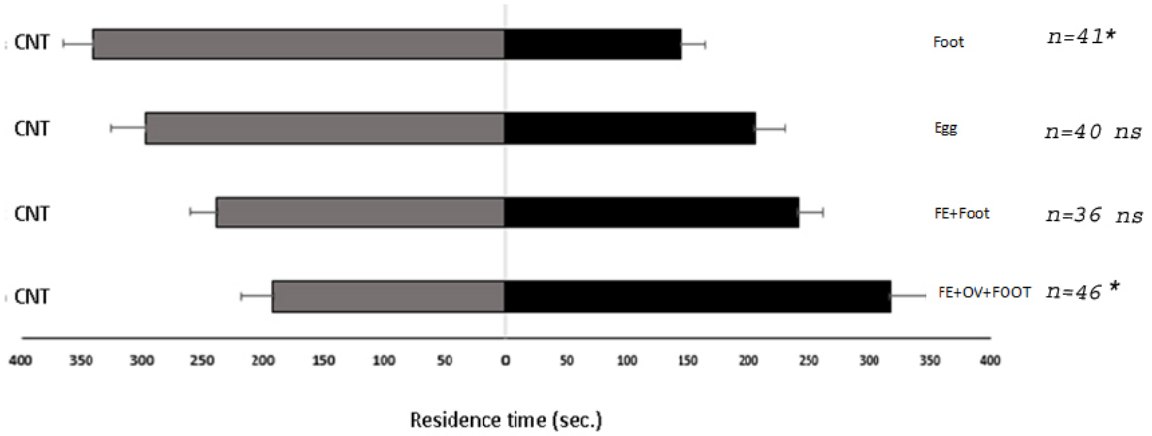

Figure 2: Response of *T. basalis* females in a Y-tube olfactometer to volatiles from *V. faba* plants subjected to different treatments versus healthy plants. Plant treatments: FOOT = *N. viridula* footprints; EGG = *N.viridula* ovarian eggs; FE+FOOT= *N. viridula* feeding and footprints; FE+OV+FOOT = *N. viridula* feeding and oviposition and footprints. Bars represent mean (± SEM) of the time spent by wasp females in each arm over an observation period of 600 s (compared with the control: * = P < 0.05; ns = not significant).

4.3.2 Molecular analysis

Expression of PR1 (**Fig. 3**) in plants exposed to *N. viridula* oviposition and footprints was significantly higher compared with the control both locally, in the 3rd leaf (P = 0.005, ANOVA followed by Dunnett's method for multiple comparisons), and systemically in the 4th leaf (P = 0.016, ANOVA followed by Dunnett's method for multiple comparisons) and in the roots (P = 0.034, ANOVA followed by Dunnett's method for multiple comparisons).Plants exposed to *N. viridula* eggs exhibited a higher expression of PR1 in the roots (P = 0.035, ANOVA followed by Dunnett's method for multiple comparisons) but not in 3rd or 4th leaf (P > 0.161, ANOVA followed by Dunnett's method for multiple comparisons).

Molecular investigation

Expression of PDF1.2 (**Fig. 4**) in plants exposed to *N. viridula* footprints and oviposition was not significantly different neither locally or systemically (P = 0.090 and P = 0.143, for 3rd leaf and 4th leaf respectively, ANOVA followed by Dunnett's method for multiple comparisons).

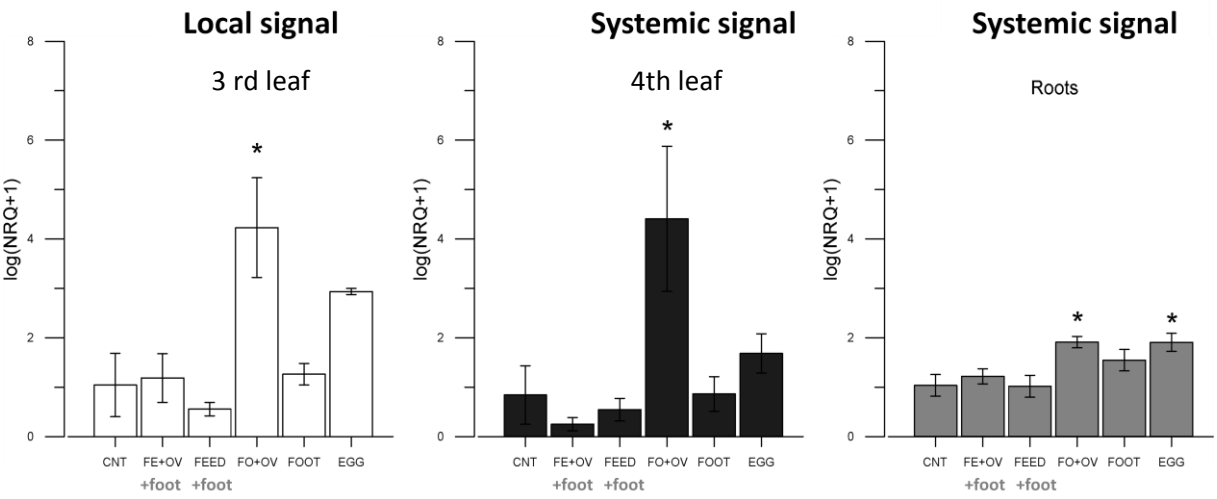

Figure 3: Expression of the defense-related gene PR1 in *V. faba* treated leaves, untreated leaves and roots at the different experimental conditions. Plant treatments: CNT = Control; FE+OV+FOOT = *N. viridula* feeding, oviposition and footprints; FEED+ FOOT= *N. viridula* feeding and footprints; FO+OV = *N. viridula* footprints and oviposition; FOOT = *N. viridula* footprints; EGG= *N. viridula* ovarian eggs. Bars represent means (±SEM) of the log-transformed normalized relative gene expression (NRQ)(* = P<0.05 compared with the control treatment).

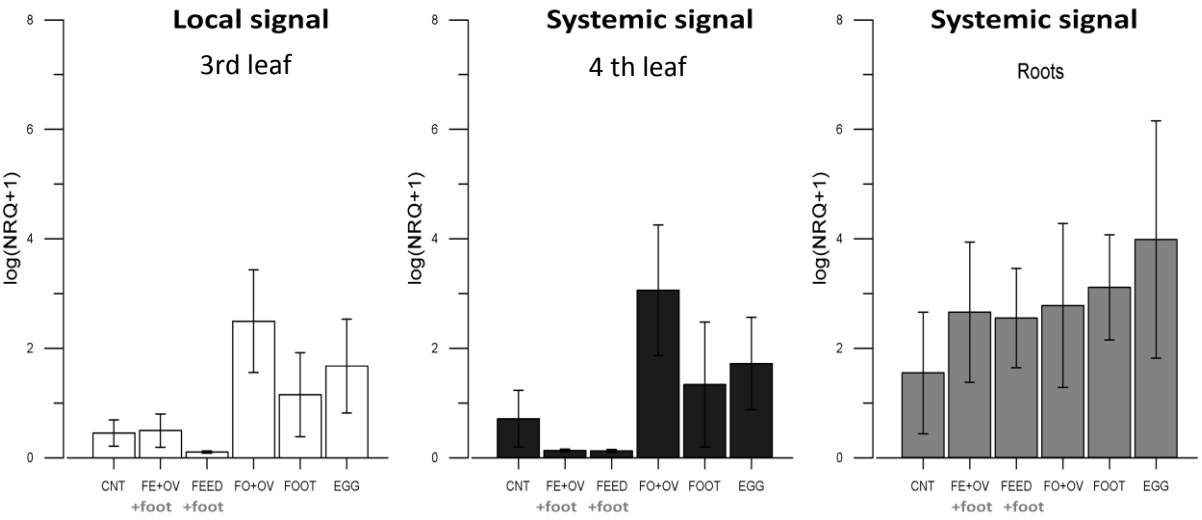

Figure 4: Expression of the defense-related gene PDF1.2 in *V. faba* treated leaves, untreated leaves and roots at the different experimental conditions. Plant treatments: CNT = Control; FE+OV+FOOT = *N. viridula* feeding, oviposition and footprints; FEED+FOOT= *N. viridula* feeding and footprints; FO+OV = *N. viridula* footprints and oviposition; FOOT = *N. viridula* footprints; EGG= *N. viridula* ovarian eggs. Bars represent means (±SEM) of the log-transformed normalized relative gene expression (NRQ) (* = P<0.05 compared with the control treatment).

4.4 Discussion

Plant defense against herbivore attack involves many signal transduction pathways that are mediated by a network of phytohormones. Most of the plant defense responses against insects are activated by signal transduction pathways mediated by JA, SA, and ethylene (Gill *et al*., 2010, Shivaji *et al*., 2010). Specific sets of defense related genes are activated by these pathways upon insect feeding or egg deposition. These hormones may act individually, synergistically or antagonistically, depending on the attacker (War *et al*., 2012). Our results showed a significant increase of PR1 gene expression in the presence of *N. viridula* footprints and oviposition, which indicates the activation of salicylate pathway in the damaged leaf (3rd leaf), apical leaf and roots. Therefore the activation is systemic. This result confirms the general thought that SA mediate defense against piercing-sucking insects (Pieterse and Dicke, 2007).Moreover, our findings proved the major role of SA in egg-induced plant responses (Little *et al*., 2007; Bruessow *et al*., 2010; Fatouros *et al*., 2014). The systemic activation of SA confirms results from several plant–insect damage systems, where plants respond not only locally at the site of feeding and/or egg deposition, but also systemically at damage-free sites (e.g., Colazza, 2004a; Chiappini *et al*., 2012, Fatouros *et al*., 2012).

We found also that artificially applied eggs induced PR1 expression, but this was only detected in the roots. This indicates that the artificial application of herbivore eggs can mimic natural egg deposition, and comes to confirm other studies, as Bruessow *et al*. (2010) and Darimont *et al*. (2013) found that plant treatments with egg extracts of *P. brassicae* and *S. littoralis* induced expression of PR1.

Our results with artificially placed eggs showing PR1 expression only in the roots, indicate that time course experiments are necessary to evaluate timing of expression, i.e. the exact time of gene activation in the different plant portions (leaves and roots).Time-course experiments may also clarify why we did not find PR1 expression in the case of plants treated with oviposition, footprints and feeding. Contrary to PR1 gene, there was no expression of PDF1.2 in the different damaged plants. This suggests that there is no activation of the JA pathway. Our result is consistent with other observations showing that the JA pathway seems to be prominent in cases where oviposition is accompanied by wounding of the leaf, whereas the SA pathway is involved when eggs are only deposited onto the surface without any apparent damage (Hilker and Fatouros, 2015).

However, in our study, there was a borderline increase of PDF1.2 gene expression in the case of *N.viridula* oviposition and footprints, suggesting that, although not significant, an activation of the jasmonate pathway is possible. Additional molecular analysis is in course to verify if a JA pathway can be activated by *N.viridula* activities.

From the behavioral point of view, *T. basalis* was only attracted to plants with feeding and footprints and oviposition, suggesting that the parasitoid exploits a complex of specific odor cues to locate its host. These results confirm previous investigations showing that the egg parasitoid *T. basalis* is attracted to OIPVs emitted by *V. faba* plants as a consequence of combination of egg deposition and feeding activity of the host *N. viridula* (Colazza *et al*., 2004a,b; Moujahed *et al*., 2014).

Additional experiments are in progress to shed light on the mechanisms of plant defense responses to oviposition by pentatomid bugs. The acquired knowledge on tri-trophic systems would be basic for designing multi-trophic experiments and for evaluation of possible applications in IPM

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Chapter 5

Chapter 5
Concluding Remarks

Research on the interaction between plants, herbivores, and their natural enemies, the field of multitrophic interaction is fast, developing research area that is tacking major new challenge. The study of plant defense is central to multitrophic theory. Plants can defend themselves directly against herbivores, but also indirectly by emitting volatiles that attract parasitoids and other natural enemies. Knowledge of the mechanisms underlying the induction of these HIPVs, and of the response of the parasitoids, is progressing rapidly. The presence of non-host can affect the reliability of plant information and these HIPVs can influence parasitoid community persistence and stability. To understand the functioning of multitrophic system. Information is needed to know how parasitoids deal with such complexity and identity the mechanisms involved.

In this dissertation, we investigated the potential disrupting effect of a non-host herbivore (*S.lineatus*), attacking either above or below-ground plant organs, on attraction of egg parasitoids (*T. basalis*) to volatiles produced by *V.faba* plants that are also infested with their typical hosts (*N. viridula*). Our results demonstrated that attraction of this wasp was disrupted by both larvae and adults of *S. lineatus* when foraging for *N. viridula* eggs laid on *V. faba* plants. From a chemical point of view, PLS-DA analysis of the odor blends from the different treatments supports the behavioral data, with significant changes to odor profiles of *V. faba* plants as a consequence of single or dual herbivore attack (See Chapter 2). We are not aware about any other study deal it with the potential disrupting effect of a non-host herbivore, attacking either above or below-ground plant organs, on attraction of egg parasitoids to volatiles produced by plants that are also infested with their typical hosts. We conducted further chemical analysis to identify volatile compounds emitted by *V. faba* plants that are attacked individually or concurrently by *N. viridula* and *S. lineatus* in order to identify the blend of compounds that play a role in egg parasitoid recruitment, and how that blend is altered or disrupted by *S. lineatus* feeding. Due to technical issue related to volatile collection system used, we were not able to obtain interpretable results. Therefore we were not able to identify the compounds (See Chapter 3). Hence, in the future other chemical tests are required using different extraction system.

Concluding Remarks

Finally, to understand better the disruption effect of larvae or adult *S. lineatus* on *T. basalis* attraction we referred to the well known cross-talk between JA and SA pathways. It is known that herbivores from different feeding guilds generally induce different defense signaling pathways (Howe and Jander 2008), simultaneous feeding by non-host from other feeding guilds than the host could affect the biosynthesis and release of HIPVs (Schwartzberg *et al*., 2011; Zhang *et al*., 2013) and in this way the host-searching efficiency of parasitoids (Dicke *et al*., 2009). The leaf chewer-responsive JA and the phloem feeder and oviposition-responsive SA signaling pathways are often found to act antagonistically. To confirm our hypothesis, we started by the investigation on phytohormonal signaling pathway in response to feeding and oviposition activities of *N.viridula*. Preliminary results indicated systemic activation of (SA) in response of *N. viridula* footprints and oviposition. However, ther was no activation of JA pathway. Thus, additional molecular analysis is in course to verify this aspect. Concerning the behavior test they confirmed that *T. basalis* attracted to OIPVs emitted by *V. faba* plants as a consequence of oviposition and feeding activity of the host *N. viridula* (See chapter 4).

5.1 Future perspective

Although, our study demonstrated that *V. faba* plant simultaneously attacked by *N. viridula* and *S.lineatus* interfere with the attraction of *T. basalis* as result of HIPVs blend modification, additional chemical analysis are required to identify the blend of compound that play a role in egg parasitoid recruitment and how that blend is altered or disrupted by *S. lineatus* feeding. Since the interference effect on egg parasitoids could be also affected by the density of non-host herbivore (Zhang *et al*., 2009), this aspect could be studied in the future.

Even if we started to examine the molecular signal transduction pathways activated after *N. viridula* feeding and oviposition, it will be interesting to study the crosstalk during dual herbivore attack (the host *N. viridula* feeding and oviposition and the non-host *S. lineatus* feeding). Furthermore, we need to identify the importance of effects caused by non-host herbivore over other factors such as surrounding vegetation that may affect parasitoid foraging in the field. So, it will be important to extend our research and to move from laboratory to field study. Hence, our results could be a good start for a more complex investigation of our studied system.

Concluding Remarks

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