UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di Ricerca in Scienze Agrarie,Alimentari, Forestali e Ambientali (SAAF).
Dipartimento di Scienze Agrarie, Alimentari e Forestali
AGR/11 Entomologia generale e Applicata

Chemical ecology of *Bagrada hilaris* (Burmeister) (Heteroptera: Pentatomidae): intraspecific and interspecific chemical cues

IL DOTTORE
Mokhtar Abdulsattar Arif

IL COORDINATORE
Prof. Vincenzo Bagarello

IL TUTOR
Prof. Stefano Colazza

EVENTUALE CO TUTOR
Prof. Ezio Peri

CICLO XXXII.
2020

Dedication

I would like to dedicate this thesis:

To my great father and lovely mother.

To my lovely wife and dear sons Ibrahim, Ahmed and Arshad.

To my precious sisters and brothers.

To all my friends and colleagues.

Thanks for your support and encouragement. Mokhtar.

Acknowledgment

In foreword of this thesis, I would like to extend of my sincere thanks to my supervisor **Prof. Stefano Colazza** and co-supervisor **Prof. Ezio Peri** for their support, precious advice suggestions and guidance during all Ph.D. phases. A special appreciation also goes to **Prof. Vincenzo Bagarello** coordinator of the Ph. D course for his kindness support and assistance. Moreover, I would like to express my cordial thanks and great gratitude to my advisor **Dr. Salvatore Guarino** (Italian National Council of Research - IBBR) and his family for his support, precious advice suggestions and guidance for the achievement of this work.

I am grateful to **Dr. Hussain Soaud** (Advisor of the Iraqi Ministry of Agriculture and the general director of directorate of Plant Protection **Dr. Hamed Ali Hadwan** and **Dr. Abdulkareem J. Ali** / Director of NCPC-MOA (Iraq) for offering me help and support.

I am heartily thankful to **Prof. Jocelyn G. Millar** from Department of Entomology, University of California, Riverside, California, United States of America for his support and for his great help in the laboratory work. I would like to express my cordial thanks to the Mediterranean Agronomic Institute of Bari (**CIHEAM IAMB**) with cooperation with Project "High Studies Programme (Master and Ph.D.) for Iraqi officials at Italian Academic Institutions (**HiSPro**)" supported by the Iraqi Government and the Italian Ministry of Foreign Affairs (MAE). Sincere thanks and deep appreciation to **Prof. Abdulssatar A. Ali** and **Prof. Ibrahim Al- Jboory** for his unlimited support.

A special appreciation also to **Dr. Paolo Lo Bue** and **Dr. Claudio De Pasquale** and all the staff from SAAF for their kindness support and assistance. Furthermore, I would like also to express my deep sense of gratitude to **Dr. Paul Innocenzi** for helpful suggestions and for revising a part of this manuscript. Also many thank to **Rosario Cappadona** of the Cooperative of Caper Producers of Pantelleria and **Erik Vallini** and **Gabriele Lasagni** of Bonomo and Giglio Srl for their helpful assistance during the collection of insects in the field.

Finally, I would like to express my deep appreciation for the precious friends: **Ahmed zouhair**, **Mudhar Mohamed**, **Osama emad**. **Khaled Ibrahim**, **Ihab M. salman**, **Mithal Hadi** and all other colleagues for their friendly support and encouragement.

Table of Contents

Chapter 1

Chemical ecology of herbivore Heteroptera with focus on *Bagrada hilaris* (Burmeister) (Heteroptera: Pentatomidae)

Chapter 1

Chemical ecology of herbivore Heteroptera with focus on *Bagrada hilaris* (Burmeister) (Heteroptera: Pentatomidae)

Abstract

The chemical ecology of Heteroptera insects is determined by a wide array of chemical signals (semiochemicals) that drive their behavior at intra- and inter-specific level. Intraspecific semiochemicals are called pheromones, interspecific chemicals are named allelochemicals. In the case of stink bugs, sex-pheromones and aggregation pheromone are produced by adult males. Furthermore, phytophagous stink bugs exploit chemical cues emitted from plants to find a suitable food and oviposition source. The semiochemicals involved in this process are named kairomones and are generally formed by specific blend or key odorants emitted from host plant. The chemical ecology of the phytophagous Pentatomid species *Bagrada hilaris*, or Painted bug, native from Asia and invasive in the Americas is characterized by similarities and differences with the other stink bugs. In particular at intraspecific level is been observed that males volatiles attract females, and chemicals analyses showed that both adults produced a similar pattern of chemicals, with the only quantitative difference related to (*E*)-2-octenyl acetate, produced in higher amounts from males. However, the possible attractant role of this compound at intraspecific level is still to be assessed. Moreover at interspecific level, although *B. hilaris* is reported to be highly attracted to brassicas, few studies have attempted to elucidate the chemicals cues exploited in its host location process. In the specific, the chemicals cues exploited from this pest in the location of host plant at seedling stage, the stage more vulnerable and subjected to the attack from *B. hilaris* have still never been investigated.

Key words: Semiochemicals, Pheromones, Kairomones, Pentatomids, VOCs,

1.1 Introduction

Insects use chemical signals named infochemicals or semiochemicals (from the ancient Greek *semeion* = signal) for communication at intra- and interspecific level (Law and Regnier, 1971). Semiochemicals are classified, based on their function or effect, into two broad groups named pheromones and allelochemicals. Pheromones mediate interactions among individuals of the same species (intraspecific interaction) eliciting a short-term or immediate behavioral response and are divided into different categories such as sex pheromones, aggregation pheromones, alarm pheromones and trail pheromones (Law and Regnier, 1971; Howse et al. 2013; El-Shafie and Faleiro, 2017). Allelochemicals mediate interactions among different species individuals (interspecific interaction); they are classified into, 1) kairomones, cues that mediate interactions favoring the perceiver, 2) allomones, which are favorable for the emitter, 3) synomones, favoring both the emitter and the perceiver (Mizutani, 1999; Suckling et al. 2000; Howse et al. 2013; El-Shafie and Faleiro, 2017).

This chapter focuses on the semiochemicals involved in the chemical ecology of Heteropteran species also known as true bugs. For these species the intraspecific interactions are driven by aggregation, sex and alarm pheromones (Fucarino et al. 2004; Millar, 2005).

The identification of the sex and aggregation pheromones has particular importance in applied entomology for those Heteroptera species that are herbivore. Once identified and synthetized, these semiochemicals can in fact be used for monitoring and mass trapping purposes of these dangerous species. Several herbivore heteropteran species are in fact serious pests of a wide variety of crops and growers often rely on the use of insecticides to control them (Schaefer and Panizzi, 2000; Greene et al.2006; Leskey et al. 2012). The damages determined on the plants by these species are caused by their feeding habits with their characteristic piercing-sucking mouthparts and increased by their typical aggregation behavior.

Herbivorous Heteroptera exploit plant volatile organic compounds in the processes of host location and acceptance (see Chapter 3). The identification of such cues exploited by these dangerous pests is not only of great importance to better understand the chemical ecology of

these species but can be also relevant in consideration that characteristic host plant odors can be also exploited for field application as attractants.

1.2 Pheromones

Mating behavior of Heteroptera is divided into two distinct main phases: 1) long-range mate location that is mediated mainly by sex and/or aggregation pheromones, which consist of chemical volatiles such as alcohols, esters, and terpenoids (Borges et al. 1987; Aldrich, 1988), and 2) close range courtship behavior, mediated by short-range or contact pheromones as well as visual and/or acoustic stimuli (Borges et al. 1987; Lu et al. 2007).

Semiochemicals that act as sex or aggregation pheromones of true bugs can be produced by male, female or nymphs. Pheromone compounds have been identified in several species of Heteroptera. They can be emitted by females or males. For example, in some Miridae species, females release a pheromone that attracts males, such as the Mullein bug *Campylomma verbasci* (Meyer) whose females emit butyl butyrate and (*E*)-crotyl butyrate attracting males (Smith et al. 1991). Similarly, females of the plant bug *Phytocoris californicus* (Knight) emit a blend of hexyl acetate and (*E*-)2-octenyl acetate (Millar and Rice, 1998). In other Heteroptera families, males produce sex pheromones to attract females, for example the adult male of *Phthia picta* (Drury) (Coreidae) releases 5,9,17-trimethylheneicosane, which is strongly attractive to females (Soldi et al. 2012). In some cases, male bugs release an aggregation pheromone that attract both sexes such in the case of the Leaffooted bug *Leptoglossus clypealis* (Coreidae) (Wang and Millar, 2000). Moreover, nymphs also produce sex pheromone attracting females as in the case *Sehirus cinctus* (Palisot de Beauvois) (Cydnidae) (Kölliker et al. 2006, Weber et al. 2018).

In the Pentatomidae family, also known as stink bugs, male adults produce aggregation pheromones that induce aggregation behavior of adults and nymphs stages. In the case of herbivorous species, these aggregations on host plant of hundreds or thousands of individuals increase their dangerousness as often lead plant to death (Ludwig and Kok, 2001). This aggregation behavior has different advantages that Wertheim et al. (2005) summarize in four

general categories: (a) increasing the efficiency of resource use, (b) finding mates, (c) protection from natural enemies, and (d) protection from environmental conditions. Aggregation pheromones that have been identified in stink bug species are reported in Table 1.

Aggregation pheromones produced by adults and nymphs of stink bugs are observed in *Halyomorpha halys* (Stål) (Khrimian et al. 2014b), *Eysarcoris lewisi* (Distant) (Takita et al. 2008) and *Plautia stali* (Scott) (Moritya and Shiga, 1984). In the latter case for example, was observed that males and females responded to an aggregation pheromone produced by males, however no sexual behavior was observed after that aggregation is formed.

Aggregation behavior is particularly visible in young stink bug nymphs, as is advantageous for such vulnerable stages in the protection-avoidance from predators (Wertheim et al. 2005). For example, in *Nezara viridula* (L.) active defense by aggregated nymphs significantly can lower the per capita risk of predation (Lockwood and Story, 1986).

Several authors have proposed the presence of 4-oxo-(*E*)-2-decenal might be involved in the aggregation behavior of the first instars nymphs (Borges and Aldrich, 1992, Pavis et al. 1994, Fucarino et al. 2004). This compound has been identified from the dorsal abdominal glands (DAGs) of first instar nymphs for *Euschistus heros* (F.), *E. conspersus* (Uhler), *E. tristigmus* (Say), *Thyanta perditor* (F.), *T. pallidovirens* (Stål), *N. viridula* (L.), and *Chinavia aseada* (Rolston), and its potential role as an aggregation pheromone has been demonstrated for *N. viridula* L. (Pavis et al. 1994; Fucarino et al. 2004; Weber et al. 2018). Interestingly 4-oxo-(*E*)-2-alkenals have only been reported from the Heteroptera among insects (Weber et al. 2018).

In other cases, Pentatomids species are characterized by a sex pheromone emitted from males that attract females such as *Chinavia hilaris* (Say) and *Eysarcoris parvus* (Uhler) (Mcbrien et al. 2001; Alizadeh et al. 2002) (table 1).

1.2.1 Challenges in the identification of Heteroptera pheromones

Overall, pheromone chemistry of true bugs could be in many cases is very complex, and include several molecules structures (Staddon et al. 1994; Khrimian et al. 2014b). This complexity

makes the pheromone more difficult in the analysis and in its chemical synthesis. In spite of the difficulties in analytical and synthetic chemistry, many species are attracted to the mixtures containing unnatural ratios of active pheromone components and/or unnatural isomers of the pheromone components, thus giving the opportunity to synthesize less expensive mixtures of pheromone stereoisomers for use in the various management programs (Weber et al. 2018).

Table 1. List of Pentatomid species pheromone compounds.

Pentatomidae species	Pheromones (male produced)	Gender/stage Responders	Reference
Agroecus griseus (Dallas)	methyl 2,6,10-trimethyltridecanoate	f	(Fávaro et al. 2012)
Bagrada hilaris (Burmeister)	(*E*)-2-octenyl acetate	f	(Guarino et al. 2008)
Biprorulus bibax (Breddin)	3*R*,4*S*,1′*E*-3,4-bis(1′-butenyl) tetrahydro-2-furanol, linalool, farnesol, and nerolidol	m, f	(James, 1994; James et al. 1996)
Chinavia hilaris (Say)	(4*S*)-*cis*-*Z*-BAE and (4*S*)-*trans*-*Z*-BAE	f	(Mcbrien et al. 2001)
Chinavia impicticornis (Stål)	*cis*-(*Z*) –(4*S*)-BE and *trans*-(*Z*) –(4*S*)-BE	f	(Blassioli-Moraes et al. 2012)
Chinavia ubica (Rolston)	*cis*-(*Z*) –(4*S*)-BE and *trans*-(*Z*) –(4*S*)-BE	f	(Blassioli-Moraes et al. 2012)
Chlorochroa ligata (Say)	methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate, methyl (2*E*,6*E*)-farnesoate, and methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate	f	(Ho and Millar, 2001)
Chlorochroa sayi (Stål)	methyl geranate, methyl citronellate, and methyl (*E*)-6-2,3-dihydrofarnesoate	m, f	(Ho and Millar, 2001; Millar et al. 2010)
Chlorochroa uhleri (Stål)	methyl (*E*)-6-2,3-dihydrofarnesoate	m, f	(Ho and Millar, 2001; Millar et al. 2010)
Edessa meditabunda (Fabr)	methyl 4,8,12-trimethylpentadecanoate and methyl 4,8,12-trimethyltetradecanoate	f	(Zarbin et al. 2012)
Euschistus conspersus (Uhler)	methyl (2 *E*, 4 *Z*)-decadienoate	m, f, n	(Aldrich et al. 1991; Millar 2005)
Euschistus heros (Uhler)	Methyl 2,6,10-Trimethyldodecanoate and Methyl 2,6,10-Trimethyltridecanoate	f	(Zarbin et al. 2000; Millar 2005)
Euschistus servus (Say)	methyl (2 *E*, 4 *Z*)-decadienoate	m, f, n	(Aldrich et al. 1991; Millar 2005)
Euschistus tristigmus (Say)	methyl (2 *E*, 4 *Z*)-decadienoate	m, f, n	(Aldrich et al. 1991; Millar 2005)
Eysarcoris lewisi (Distant)	(*Z*)-2-methyl-6-(4-methylenebicyclo [3.1.0] hex-1-yl) hept-2-en-1-ol.	m, f, n	(Takita et al. 2008)
Eysarcoris parvus (Uhler)	(*Z*)-exo-α-bergamotenal	f	(Alizadeh et al. 2002)
Halyomorpha halys (Stål)	(3*S*,6*S*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol (3) and (3*R*,6*S*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol	m, f, n	(Khrimian et al. 2014b)

Murgantia histrionica (Hahn)	(3*S*,6*S*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol and (3*S*,6*S*,7*R*,10*R*)-10,11-epoxy-1-bisabolen-3-ol	m, f, n	(Khrimian et al. 2014a; Weber et al. 2014)
Nezara antennata (Scott)	(*Z*)-α-bisabolene (1-methyl-4-(1,5-dim ethyl-(*Z*)-1,4- hexadienyl)-cyclohexene), and *trans*- and *cis*-1,2-epoxides of (*Z*)-α-bisabolene	m, f	(Aldrich et al. 1993)
Nezara viridula (L.)	(*Z*)-α-bisabolene, trans- and *cis*-1,2-epoxides of (*Z*)-α-bisabolene, (*E*)-nerolidol, and *n*-nonadecane.	m, f, n	(Aldrich et al. 1987)
Oebalus poecilus (Dallas)	Zingiberenol, (1*S*,4*R*,1′*S*)-4-(1′,5′-Dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol	f	(de Oliveira et al. 2013)
Pallantia macunaima (Grazia)	(6*R*,10*S*)-Pallantione	f	(Fávaro et al. 2013)
Piezodorus guildinii (Westwood)	(7*R*)- (+)-β-sesquiphellandrene	f	(Borges et al. 2007)
Piezodorus hybneri (Gmelin)	β-sesquiphellandrene, (*R*)-15-hexadecanolide, and methyl 8-(*Z*)-hexadecenoate	m, f, n	(Leal et al. 1998; Endo et al. 2010)
Plautia stali (Scott)	methyl (*E*, *E*, *Z*)-2, 4, 6-decatrienoate	m, f	(Sugie et al. 1996)
Podisus maculiventris (Say)	(*E*)-2-Hexenal, a-terpineol, and benzyl alcohol	m, f, n	(Aldrich et al. 1984; Weber et al. 2018)
Thyanta pallidovirens (Stål)	methyl (*E*2, *Z*4, *Z*6)-decatrienoate (*E*2, *Z*4, *Z*6–10: COOMe), and the sesquiterpenes (+) α-curcumene, (−)-zingiberene, and (−) -β-sesquiphellandrene	f	(McBrien et al. 2002)
Thyanta perditor (F.)	methyl (2*E*,4*Z*,6*Z*)-decatrienoate (2*E*,4*Z*,6*Z*-10:COOMe)	f	(Moraes et al. 2005)
Tibraca limbativentris (Stål)	isomers of 1′S-zingiberenol	f	(Borges et al. 2006)

1.3 Kairomones

In order to locate a suitable host plant, phytophagous true bugs exploit several visual and olfactory stimuli that can bring the insect in the proximity of the plant where the herbivore feed and oviposit. Host searching behavior consists of several sequential steps that orientate the herbivorous bugs from a random dispersal movement to an orientate movement to the plant by walking, flying or crawling (Schoonhoven et al. 2005). This host searching behavior is influenced by several internal factors, such as insect feeding range (polyphagy/monophagy), developmental stage and oviposition behavior, and also influenced by external factor as plant stage and environmental conditions (Guarino et al. 2017a). As mentioned above, this process is mediated by visual and chemical stimuli, the latter having an important role in the long-range attraction

behavior. In specific, chemical volatiles emitted from the host plant can play a crucial role in this process acting as kairomones for the herbivore (Schoonhoven et al. 2005). Generally, the ability to find a suitable host plant and its acceptance can be determined by the perception from the insect of plant-specific volatiles or of specific blend of selected ubiquitous volatiles (Bruce et al. 2005). Polyphagous insects tend to exploit a great range of plant volatiles, while specialist insect herbivores should show more efficient forms of adaptation, since they are expected to use specific cues.

The majority of Heteropteran herbivores are polyphagous (Backus, 1988, Holopainen and Varis, 1991), some species exhibit narrower host plant ranges and very few of them are monophagous; as a consequence, it is likely that they might respond to a blend of plant volatiles rather than a plant-specific compound. The responses of Heteropteran herbivorous insects to volatiles released by their host plants have been shown in several species as described in table 2. For example, a study carried out by Rather et al. (2010) by using olfactometer bioassays techniques evidenced the positive response of the Pentatomid bug, *Eurydema pulchrum* (Westwood) to volatiles from different plant species such as *Brassica oleracea* L., *Raphanus sativus* L. and *Solanum lycopersium* L., in different forms (intact plant/macerated). Overall, in phytophagous Heteroptera the responsiveness of females to plant volatiles seem to be higher than to males and nymphs, probably as several of them lay the eggs on the host plant. For example, electroantennographic (EAG) analyses on the Miridae species, *Lygus lineolaris* (Palisot de Beauvois) and *Lygocoris pabulinus* L. showed that the responses of female bug antenna to green leaf volatiles and monoterpenes were more marked than the antennal responses of males (Chinta et al. 1994; Groot et al. 1999). Similarly, in other behavioral studies on *L. hesperus* (Knight) was evidenced that female bugs are more sensitive to volatiles from alfalfa, *Medicago sativa* L., than males (Williams III et al. 2010). However, in *L. hesperus*, nymphs show more responsiveness to odors emanating from a plant than adults (Blackmer et al. 2004); in addition, the response of this species to these volatiles can be increased when supported by visual cues such as specific light frequencies (Blackmer and Canas, 2005). Ecological habits of herbivorous Heteroptera such as the sites of feeding, oviposition and mating behavior also adapt the host selection process, since the majority of them lays their eggs directly on the host plant (Martínez

et al. 2013; Guarino et al. 2017a). This process follow the "mother know best" principle, as ovipositing directly on host plant allows young nymphs to have ready food and shelter after emergence (Wennström et al. 2010). Consequently, females have evolved a behavior that maximizes offspring performance and thus positively affects female fitness. For example, many species of Heteropteran herbivore are attracted to the plants especially in the stage of growing shoots and from the developing seeds or fruits. These stages are nutrient-rich and are consequently the favorite sites for feeding and oviposition activity (McPherson and McPherson, 2000; Olson et al. 2011). For example, the western boxelder bug, *Boisea rubrolineata* Barber (Rhopalidae) is attracted by phenyl acetonitrile, a compound produced by pollen-bearing staminate trees and pistillate trees with maturing seeds of its host *Acer negundo* L., suggesting that this pest can track and exploit the availability of nutrient- rich food sources by adapting its reproductive ecology to the phenology of its host (Schwarz et al. 2009).

Table 2. Heteroptera species responding to different plant volatiles (Kairomones).

	Insect sp.	Family	Kairomone	from	Ref
5	*Apolygus lucorum*. (Meyer-Dür)	Miridae	m-xylene, butyl acrylate, butyl propionate and butyl butyrate	18 flowering host plants ex: *Polygonum orientale* L. and *Ricinus communis* (L.)	(Pan et al. 2015)
6	*Lygus rugulipennis* (Poppius)	Miridae	y- terpinene, 1,8-cineole, sabinene, α-terpinene, β-phellandrene, p-cymene, α-pinene, β-pinene	Flowering sunflower *Helianthus annuus* (L.)	(Ondiaka et al. 2016)
2	*Lygus spp.*	Miridae	phenylacetaldehyde	Flowering plant in the mustard family *Lesquerella fendleri* (Gray)	(Blackmer and Byers, 2009)
1	*Macrolophus caliginosus.* (Wagner)	Miridae	α-zingiberene and dodecyl acetate	Sweet pepper *Capsicum annuum* (L.) infested with two-spotted spider mites, *Tetranychus urticae* (C. L. Koch), and green peach aphid, *Myzus persicae* (Sulzer)	(Moayeri et al. 2007)
3	*Stenotus rubrovittatus* (Matsumura)	Miridae	β-caryophyllene and β-elemene	Flowering rice panicles *Scirpus*	(Hori and Namatame,

				juncoides (Roxb).	2013)
7	*Trigonotylus caelestialium* (Kirkaldy)	Miridae	β-caryophyllene	Flowering Rice Panicles of *Oryza sativa* (L.)	(Fujii et al. 2010)
4	*Trigonotylus caelestialium* (Kirkaldy)	Miridae	β-caryophyllene, n-decanal, n-tridecene, methyl salicylate, geranyl acetone, methyl benzoate and β-elemene	Flowering Rice Panicles of *Oryza sativa* (L.)	(Hori and Enya, 2013)
8	*Antestiopsis thunbergii* (Gmelin).	Pentatomidae	anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin	Mature green berries volatiles of *Coffea arabica* (L.)	(Njihia et al. 2017)
10	*Bagrada hilaris* (Burmeister)	Pentatomidae	benzaldehyde, octanal, nonanal, and acetic acid	Cauliflower *Brassica oleracea* var botrytis (L.)	(Guarino et al. 2017b)
9	*Podisus maculiventris.* (Say)	Pentatomidae	hexan-1-ol, (*E*)-2-hexenal, and α-terpineol	Plant volatile	(Sant'Ana et al. 1999)
11	*Boisea rubrolineata.* (Barber)	Rhopalidae	phenylacetonitrile	pistillate trees *Acer saccharinum* (L.) and *Acer negundo* (L)	(Schwarz et al. 2009)
12	*Thaumastocoris peregrinus*, (Carpintero and Dellapé)	Thaumastocoridae	p-cymene	Leaves of *Eucalyptus spp.*	(Santadino et al. 2017)

1.4 *Bagrada hilaris* chemical ecology

Bagrada hilaris (Burmeister) also known as Painted bug, is a stink bug belonging to the family Pentatomidae. The Painted bug is a phytophagous insect, with a broad host range of 74 plants in 23 families (Anwar et al. 1973; Boopathi and Pathak, 2012; Huang et al. 2014; Palumbo et al. 2016). Although polyphagous, *B. hilaris* is mainly a pest of important crops belonging to the family of Brassicaceae. This pest is native to Asia and Africa (Ahuja et al. 2008; Reed et al. 2013; Palumbo et al. 2016). Around 40 years ago was reported in Europe, in the specific in the islands of Malta and Pantelleria (Italy), where it feeds on the caper bush *Capparis spinosa* L. (Carapezza, 1981, Colazza et al. 2004).

Starting from 2008 *B. hilaris* was accidentally introduced in the United States, Mexico, and more recently, South America (Palumbo et al. 2016) (Fig. 1). A recent study of Carvajal et al. (2019)

evidenced that *B. hilaris* has a high environmental adaptability to a wide array of climates ranging from the Mediterranean to the arid regions. Therefore, this pest might be potentially very dangerous in the new invading areas as it might take advantage of the rising temperature determined by global warming.

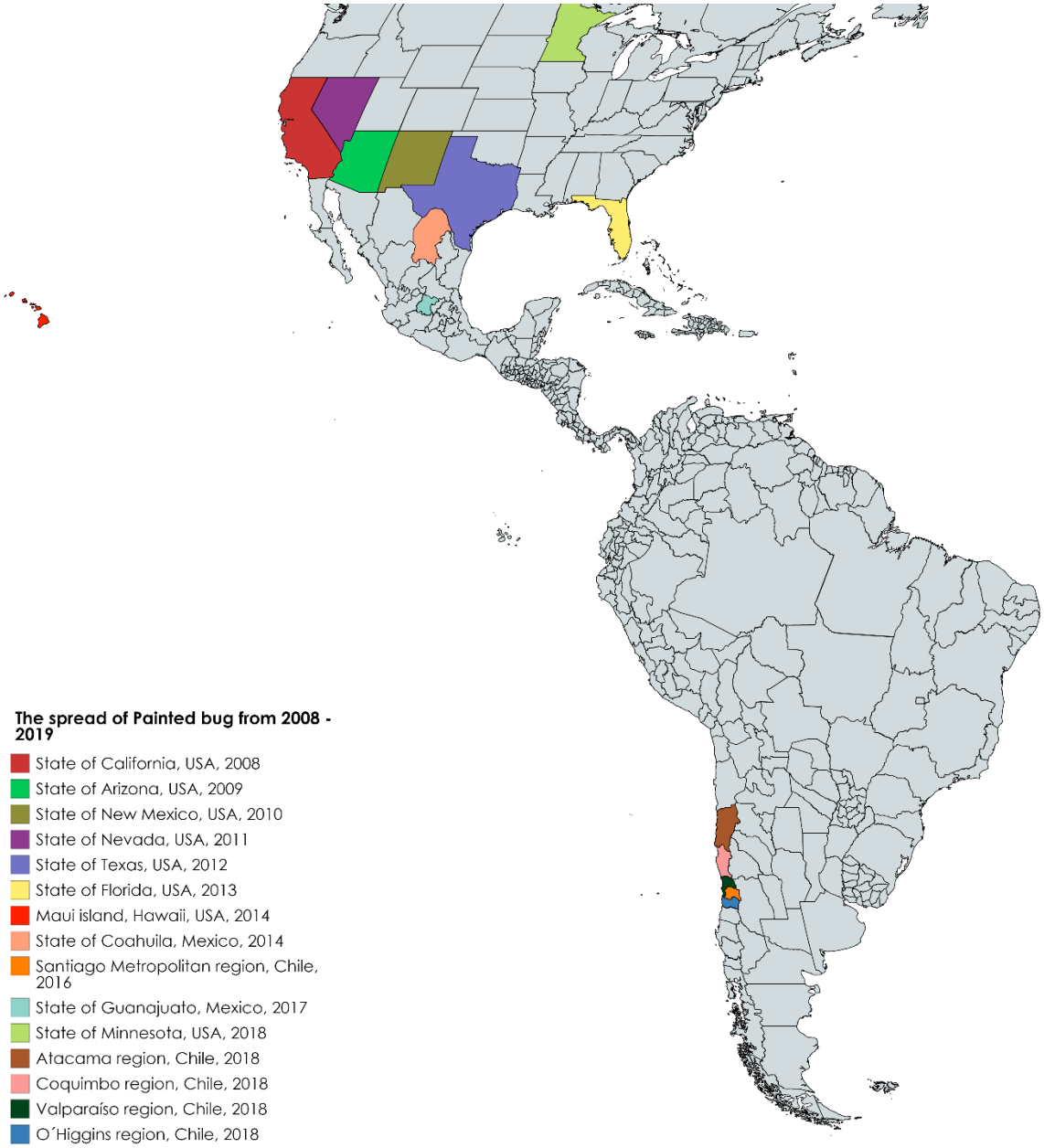

Figure 1. Distribution map of Painted bug from 2008 -2019 in the United States, Mexico and Chile.

1.5 Intraspecific interactions

Among the intraspecific interaction occurring in this species, the sexual behavior in terms of long-range mate location and close-range courtship of *B. hilaris* was studied from Guarino et al. (2008). In particular, long range mate location of *B. hilaris* seems to be mediated by a male-produced pheromone. In the specific, in the laboratory bioassays with vertical Y-shaped olfactometer, female bugs were attracted to odors from sexually mature males, while males were not attracted to odors from females. Moreover, males were not attracted to odors from other males nor females to odors from females. In addition, females were also attracted to extracts of odors from male bugs, indicating that the pheromone was not masked by defensive compounds. Chemical analyses by GC/MS of the odors from bugs showed that both males and females produced nonanal, decanal and (*E*)-2-octenyl acetate, with the last being more abundant in extracts from males (Guarino et al. 2008). This might suggest that females locate males from a distance partly or wholly by responding to (*E*)-2-octenyl acetate. This compound is also used as a sex and/or aggregation pheromone by other Heteropteran species for example, in the lygaeid *Geocoris punctipes* (Say), (*E*)-2-octenyl acetate is produced mainly from females and plays a role as sex pheromone (Marques et al. 2000). Furthermore, in the alydid *Leptocorisa chinensis* (Dallas), a blend of (*E*)-2-octenyl acetate and octanol is produced by both sexes, but it is only attractive to males (Leal et al. 1996). Moreover, nonanal and decanal, present in the Painted bug air collections, are commonly produced by other true bugs species such as *T. limbativentris* (Stal) and *Euschistus* spp., with defensive roles (Aldrich et al. 1995; Borges et al. 2006). To date attempts to develop this compound into a reliable monitoring tool have not yet been successful (Bundy et al. 2018), and need further studies on the real role of this compound in the chemical ecology of this species.

Once the two sexes of Painted bug are in proximity, before mating they display a unique close-range courtship behavior take place. This can be divided into three sequential steps: (a) the contact phase, in which male first contacts and antennates female; (b) the mount-antennation phase, in which the male mounts the female and antennates her antennae and genitalia; and (c)

the engagement phase, in which the male dismounts and couples his genitalia to those of the female in end-to-end fashion (Fig. 2) (Guarino et al. 2008; Palumbo et al. 2016).

This behavior might be mediated by contact chemical cues. In a study conducted by De Pasquale et al. (2007) on the *B. hilaris* male and female cuticular surfaces, were identified thirteen homologous cuticular *n*-alkanes (*n*C17-*n*C29) from both sex of Painted bug, however hydrocarbon profiles of both gender are qualitatively equal, but marked quantitative differences in sex-specific of some components, which were suggested to be involved in short- range courtship behavior (De Pasqual*e* et al. 2007).

Aggregation behavior

Mating behavior

Figure 2. Intraspecific interaction of *Bagrada hilaris*.

1.6 Interspecific interactions

Although host plant VOCs are known to be exploited by phytophagous Pentatomid species as host location cues (Rather et al. 2010), information about the VOCs that might serve as host cues for *B. hilaris* are limited (Guarino et al. 2017a). Recent electroantennographic studies showed that adults of this species perceive several plant VOCs, including octanal, nonanal, acetic acid, benzaldehyde, 3-butenyl isothiocyanate, and 4-pentenyl isothiocyanate (Fig. 3 and Fig. 4) (Palumbo et al. 2016; Arif, 2016; Guarino et al. 2017a). Among these chemicals, only

isothiocyanates are characteristic to *Brassica* spp., however, they are produced mainly upon damage to tissues rather than by undamaged plants (Mccormick et al. 2014; Chabaane et al. 2015; Guarino et al. 2017a). In field bioassays, *B. hilaris* were not consistently attracted by traps baited with various isothiocyanates, either singly or in blends (Palumbo et al. 2016), and to date, no consistently effective plant-derived attractants have been identified for this species (Bundy et al. 2018). Any search for candidate attractants for herbivore pests should consider not only the preferred host plant species and their main VOCs, but also other plant traits, which may be indicators of host quality as food and/or shelter. These characteristics may change during plant development, and as a consequence, the attraction of *B. hilaris* may change with the development of its hosts (Boege, 2005).

The Painted bug appears to strongly prefer host plants at the stage of newly emerged seedlings (Huang et al. 2014; Joseph et al. 2017), differently to other stink bug specialists of Brassicaceae, such as *Murgantia histrionica* (Hahn), which prefers to feed on plants with at least 4-5 pairs of true leaves (White and Brannon, 1933; Ludwig and Kok, 2001). The feeding damage of *B. hilaris* on cotyledons and young tissues results in significant reductions in leaf area, chlorophyll content, and dry weight, if it does not kill the seedling outright (Huang et al. 2014; Sánchez-Peña 2014).

This suggests that VOCs produced by seedlings of preferred *B. hilaris* host plants are likely to be particularly attractive for this species. The identities of host-produced attractants can be obtained by comparing the profiles of VOCs produced by more or less susceptible brassicaceous species and varieties. The identification of these VOCs could have several major benefits. First, it will lead to a better understanding of the ecology of this species, and the factors mediating its host selection behaviors. Second, given the demonstrated strong attraction of *B. hilaris* to *Brassica* seedlings, the attractant chemicals would be excellent candidates for development into tools for monitoring and attract-and-kill strategies.

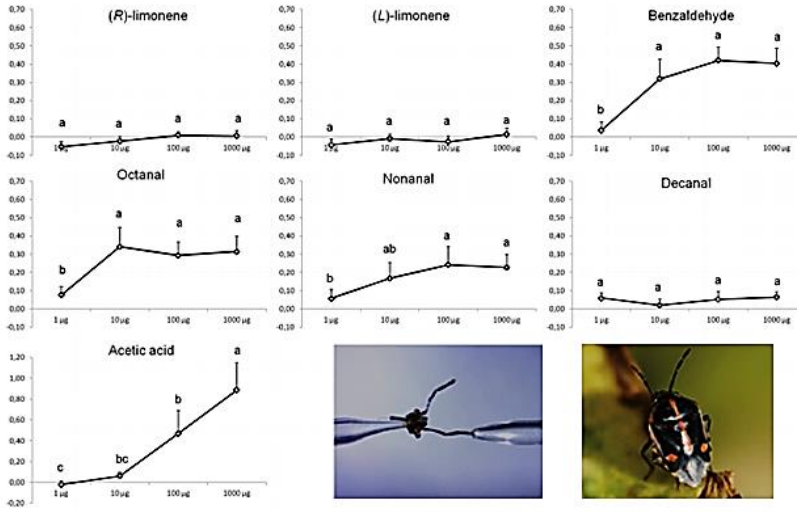

Figure 3. EAG dose–response curves of *B. hilaris* adults to VOC of *B. oleracea* var botrytis leaves. EAG amplitudes were adjusted to a control stimulus (hexane). Different letters indicate that values differ statistically at $P < 0.05$ (Repeated measures ANOVA, followed by Fisher LSD test) (Guarino et al. 2017b).

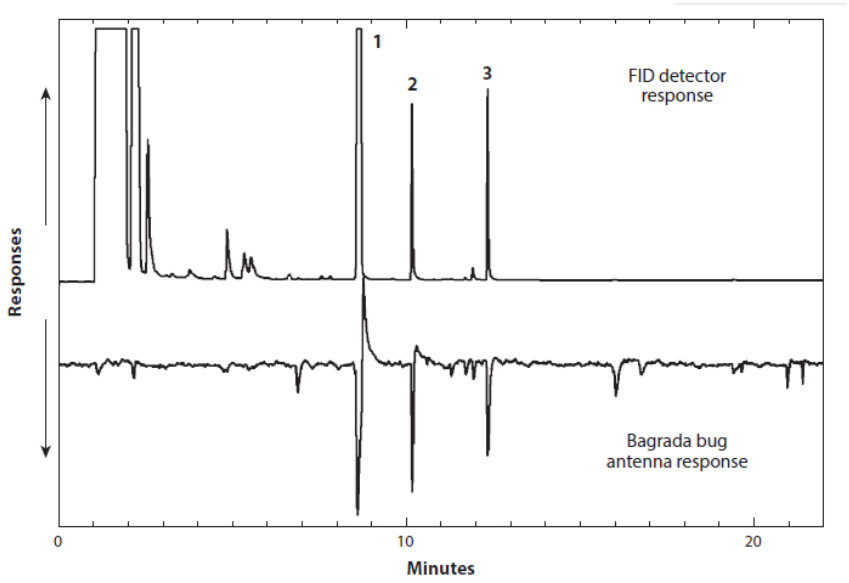

Figure 4. Analysis of volatiles from chopped sweet alyssum by coupled gas chromatography (GC)-electroantennography, using an antenna from a *Bagrada hilaris* female. The upper trace is the flame ionization detector (FID) response from the GC detector, and the lower, inverted trace is the antennal response. Peak 1: 3-butenyl isothiocyanate; peak 2: 4-pentenyl isothiocyanate; peak 3: isomer of peak 1 (Palumbo et al. 2016).

1.7 Objectives of the thesis

Overall, this thesis aims to investigate the chemical ecology of *B. hilaris* at intra- and interspecific level, with the aim to identify the main attractant semiochemicals that drive the behavior of this species. Specific objectives were:

1. To investigate in laboratory bioassays the role of (*E*)-2 octenyl acetate in the *B. hilaris* intraspecific chemical ecology, described in chapter 2.

2. Among possible host plants in the *Brassica* genus, to identify species that are more attractive at the seedling stage to *B. hilaris* nymphs and adults; to prepare active extracts of one or more attractive species and use bioassay-driven fractionation to characterize the attractant(s) present in extracts from the attractive species, described in chapter 3.

3. To investigate in laboratory and field bioassays, the possibility to use Brassicaceous seedlings of one or more attractive species as candidates for trap cropping *B. hilaris* individuals in caper field in the Pantelleria Island, described in chapter 4.

1.8 References

- Ahuja, B., Kalyan R., Ahuja, U., Singh, S., Sundria, M., Dhandapani, A. (2008). Integrated management strategy for Painted bug, *Bagrada hilris* (Burm.) inflicting injury at seedling stage of mustard (*Brassica juncea*) in arid western Rajasthan. *Pesticide Res. J*, 20, 48-51.

- Aldrich, J. (1988). Chemical ecology of the Heteroptera. *Annual Review of Entomology*, 33(1), 211-238.

- Aldrich, J. R., Hoffmann, M. P., Kochansky, J. P., Lusby, W. R., Eger, J. E., Payne, J. A. (1991). Identification and attractiveness of a major pheromone component for Nearctic *Euschistus spp*. stink bugs (Heteroptera: Pentatomidae). *Environmental Entomology*, 20(2), 477-483.

- Aldrich, J. R., Rosi, M. C., Bin, F. (1995). Behavioral correlates for minor volatile compounds from stink bugs (Heteroptera: Pentatomidae). *Journal of Chemical Ecology*, 21(12), 1907-1920.

- Aldrich, J., Kochansky, J., Lusby, W., Sexton, J. (1984). Semiochemicals from a predaceous stink bug, *Podisus maculiventris* (Hemiptera: Pentatomidae). *Journal of The Washington Academy of Sciences*, 39-46.

- Aldrich, J., Numata, H., Borges, M., Bin, F., Waite, G., Lusby, W. (1993). Artifacts and pheromone blends from *Nezara spp*. and other stink bugs (Heteroptera: Pentatomidae). *Zeitschrift für Naturforschung* C, 48(1-2), 73-79.

- Aldrich, J., Oliver, J., Lusby, W., Kochansky, J., Lockwood, J. (1987). Pheromone strains of the cosmopolitan pest, *Nezara viridula* (Heteroptera: Pentatomidae). *Journal of Experimental Zoology*, 244(1), 171-175.

- Alizadeh, B. H., Kuwahara, S., Leal, W. S., Men, H.-C. (2002). Synthesis of the racemate of (*Z*)-exo-α-bergamotenal, a pheromone component of the white-spotted spined bug, *Eysarcoris parvus* uhler. Bioscience, *biotechnology, and biochemistry*, 66(6), 1415-1418.

- Anwar, C. M., Irshad, M., Murtaza, M., Ghani, M. (1973). Pentatomids associated with Gramineae and their natural enemies in Pakistan. Technical Bulletin, *Commonwealth Institute of Biological Control* (16), 47-67.

- Arif, M. A. (2016). Host plant volatile compounds for the management of *Bagrada hilaris* Burmeister (Heteroptera: Pentatomidae). *Mediterranean Agronomic Institute of Bari,* (CIHEAM) Bari, Italy. (Master thesis).

- Backus, E. A. (1988). Sensory systems and behaviours which mediate hemipteran plant-feeding: a taxonomic overview. *Journal of Insect Physiology*, 34(3), 151-165.

- Blackmer, J. L., Byers J. A. (2009). *Lygus spp*. (Heteroptera: Miridae) host–plant interactions with *Lesquerella fendleri* (Brassicaceae), a new crop in the arid southwest. *Environmental Entomology*, 38(1), 159-167.

- Blackmer, J. L., Canas, L. A. (2005). Visual cues enhance the response of *Lygus hesperus* (Heteroptera: Miridae) to volatiles from host plants. *Environmental Entomology*, 34(6), 1524-1533.

- Blackmer, J. L., Rodriguez-Saona, C., Byers, J. A., Shope, K. L., Smith, J. P. (2004). Behavioral response of *Lygus hesperus* to conspecifics and headspace volatiles of alfalfa in a Y-tube olfactometer. *Journal of Chemical Ecology*, 30(8), 1547-1564.

- Blassioli-Moraes, M., Laumann, R., Oliveira, M., Woodcock, C., Mayon, P., Hooper, A., Pickett, J., Birkett, M., Borges, M. (2012). Sex pheromone communication in two sympatric Neotropical stink bug species *Chinavia ubica* and *Chinavia impicticornis*. *Journal of chemical ecology*, 38(7), 836-845.

- Boege, K. (2005). Influence of plant ontogeny on compensation to leaf damage. *American Journal of Botany*, 92(10), 1632-1640.

- Boopathi, T., Pathak, K. (2012). Seasonal abundance of insect pests of broccoli in North Eastern Hill Region of India. *Madras Agric*. J, 99(1-3), 125-127.

- Borges, M., Aldrich, J. R. (1992). Instar-specific defensive secretions of stink bugs (Heteroptera: Pentatomidae). *Experientia*, 48(9), 893-896.

- Borges, M., Birkett, M., Aldrich, J. R., Oliver, J. E., Chiba, M., Murata, Y., Laumann, R. A., Barrigossi, J. A., Pickett, J. A., Moraes, M. C. (2006). Sex attractant pheromone from the rice stalk stink bug, *Tibraca limbativentris* Stal. *Journal of chemical ecology*, 32(12), 2749.

- Borges, M., Jepson, P., Howse, P. (1987). Long-range mate location and close-range courtship behaviour of the green stink bug, *Nezara viridula* and its mediation by sex pheromones. *Entomologia Experimentalis et Applicata*, 44(3), 205-212.

- Borges, M., Millar, J. G., Laumann, R., Moraes, M. C. (2007). A male-produced sex pheromone from the neotropical redbanded stink bug, *Piezodorus guildinii* (W.). *Journal of Chemical Ecology*, 33(6), 1235.

- Bruce, T. J., Wadhams, L. J., Woodcock, C. M. (2005). Insect host location: a volatile situation. *Trends in plant science*, 10(6), 269-274.

- Bundy, C. S., Perring, T. M., Reed, D. A., Palumbo, J. C., Grasswitz, T. R., Jones, W. A. (2018) *Bagrada hilaris*. In: McPherson JE (ed) Invasive Stink Bugs and Related Species (Pentatomoidea): Biology, Higher Systematics,Semiochemistry, and Managemen, *CRC Press, Boca Raton*, *FL*, 205–242.

- Carapezza, A. (1981). Gli Eterotteri dell'isola di Pantelleria (Insecta, Heteroptera). *Il Naturalista siciliano*, 5, 73-91.

- Carvajal, M. A., Alaniz, A. J., Núñez-Hidalgo, I., González-Césped, C. (2019). Spatial global assessment of the pest *Bagrada hilaris* (Burmeister) (Heteroptera: Pentatomidae): current and future scenarios. *Pest management science*, *75*(3), 809-820.

- Chabaane, Y., Laplanche, D., Turlings, T. C., Desurmont, G. A. (2015). Impact of exotic insect herbivores on native tritrophic interactions: a case study of the African cotton leaf worm, Spodoptera littoralis and insects associated with the field mustard Brassica rapa. *Journal of Ecology*, 103(1), 109-117.

- Chinta, S., Dickens, J. C., Aldrich J. R. (1994). Olfactory reception of potential pheromones and plant odors by tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae*). Journal of Chemical Ecology*, 20(12), 3251-3267.

- Colazza, S., Guarino, S., Peri, E. (2004). *Bagrada hilaris* (Burmeister)(Heteroptera: Pentatomidae) a pest of capper in the island of Pantelleria [*Capparis spinosa* L.; Sicily]. *Informatore Fitopatologico* (Italy).

- de Oliveira, M. W. M., Borges, M., Andrade, C. K. Z., Laumann, R. A., Barrigossi, J. A. F., Blassioli-Moraes M. C. (2013). Zingiberenol, (1 S, 4 R, 1′ S)-4-(1′, 5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol, identified as the sex pheromone produced by males of the rice stink bug *Oebalus poecilus* (Heteroptera: Pentatomidae). *Journal of Agricultural and Food Chemistry*, 61(32), 7777-7785.

- De Pasquale, C., Guarino, S., Peri, E., Alonzo, G., Colazza S. (2007). Investigation of cuticular hydrocarbons from *Bagrada hilaris* genders by SPME/GC-MS. *Analytical and Bioanalytical Chemistry*, 389(4), 1259-1265.

- El-Shafie, H. A. F., Faleiro, J. R. (2017). Semiochemicals and their potential use in pest management. *Biological Control of Pest and Vector Insects*, 1.

- Endo, N., Sasaki, R., Muto, S. (2010). Pheromonal cross-attraction in true bugs (Heteroptera): attraction of *Piezodorus hybneri* (Pentatomidae) to its pheromone versus the pheromone of *Riptortus pedestris* (Alydidae). *Environmental Entomology*, 39(6), 1973-1979.

- Fávaro, C. F., Santos, T. B., Zarbin, P. H. (2012). Defensive compounds and male-produced sex pheromone of the stink bug, *Agroecus griseus. Journal of Chemical Ecology*, 38(9), 1124-1132.

- Fávaro, C. F., Soldi, R. A., Ando, T., Aldrich, J. R., Zarbin, P. H. (2013). (6 R, 10 S)-Pallantione: the first ketone identified as sex pheromone in stink bugs. *Organic Letters*, 15(8), 1822-1825.

- Fucarino, A., Millar, J. G., McElfresh, J. S., Colazza, S. (2004). Chemical and physical signals mediating conspecific and heterospecific aggregation behavior of first instar stink bugs. *Journal of Hemical Ecology*, 30(6), 1257-1269.

- Fujii, T., Hori, M., Matsuda, K. (2010). Attractants for rice leaf bug, *Trigonotylus caelestialium* (Kirkaldy), are emitted from flowering rice panicles. *Journal of Chemical Ecology*, 36(9), 999-1005.

- Greene, J. K., Bundy, C. S., Roberts, P. M., Leonard, B. R. (2006). Identification and management of common boll feeding bugs in cotton. *Clemson Extension, Clemson University Extension Bulletin* 158, 1–28.

- Groot, A. T., Timmer, R., Gort, G., Lelyveld, G. P., Drijfhout, F. P., Van Beek, T. A., Visser, J. H. (1999). Sex-related perception of insect and plant volatiles in *Lygocoris pabulinus*. *Journal of Chemical Ecology*, 25(10), 2357-2371.

- Guarino, S., De Pasquale, C., Peri, E., Alonzo, G., Colazza, S. (2008). Role of volatile and contact pheromones in the mating behaviour of *Bagrada hilaris* (Heteroptera: Pentatomidae). *European Journal of Entomology*, 105(4), 613.

- Guarino, S., Peri, E., Colazza, S. (2017a). Plant and stink bug interactions at different trophic levels. In *Stinkbugs* (pp. 180-199). CRC Press.

- Guarino, S., Peri, E., Colazza, S., Luchi, N., Michelozzi, M., Loreto, F. (2017b). Impact of the invasive Painted bug *Bagrada hilaris* on physiological traits of its host *Brassica oleracea* var botrytis. *Arthropod-Plant Interactions*, 11(5), 649-658.

- Ho, H.-Y., Millar J. G. (2001). Identification and synthesis of male-produced sex pheromone components of the stink bugs *Chlorochroa ligata* and *Chlorochroa uhleri*. *Journal of Chemical Ecology*, 27(10): 2067-2095.

- Holopainen, J., Varis, A. L. (1991). Host plants of the European tarnished plant bug *Lygus rugulipennis* Poppius (Het., Miridae). *Journal of Applied Entomology*, 111(1-5), 484-498.

- Hori, M., Enya, S. (2013). Attractiveness of synthetic volatile blends of flowering rice panicles to *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae). *Journal of Applied Entomology*, 137(1-2): 97-103.

- Hori, M., Namatame, M. (2013). Host plant volatiles responsible for the invasion of *Stenotus rubrovittatus* (Heteroptera: Miridae) into paddy fields. *Journal of Applied Entomology*, 137(5), 340-346.

- Howse, P., Stevens, J. M., Jones, O. T. (2013). Insect pheromones and their use in pest management. *Springer Science & Business Media*.

- Huang, T.-I., Reed, D. A., Perring, T. M., Palumbo, J. C. (2014). Host selection behavior of *Bagrada hilaris* (Hemiptera: Pentatomidae) on commercial cruciferous host plants. *Crop Protection*, 59: 7-13.

- James, D. G. (1994). The development of suppression tactics for *Biprorulus bibax* (Heteroptera: Pentatomidae) as part of an integrated pest management program in citrus in inland south-eastern Australia. *Bulletin of Entomological Research*, 84(1), 31-37.

- James, D. G., Heffer, R., Amaike, M. (1996). Field attraction of *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae) to synthetic aggregation pheromone and (*E*)-2-hexenal, a pentatomid defense chemical. *Journal of chemical ecology*, 22(9), 1697-1708.

- Joseph, S. V., Grettenberger, I. M., Godfrey, L. D., Zavala, N. (2017). Susceptibility of germinating cruciferous seeds to *Bagrada hilaris* (Hemiptera: Pentatomidae) feeding injury. *Arthropod-Plant Interactions*, 11(4), 577-590.

- Khrimian, A., Shirali, S., Vermillion, K. E., Siegler, M. A., Guzman, F., Chauhan, K., Aldrich, J. R., Weber, D. C. (2014a). Determination of the stereochemistry of the aggregation pheromone of harlequin bug, *Murgantia histrionica*. *Journal of Chemical Ecology*, 40(11-12), 1260-1268.

- Khrimian, A., Zhang, A., Weber, D. C., Ho, H.-Y., Aldrich, J. R., Vermillion, K. E., Siegler, M. A., Shirali, S., Guzman, F., Leskey, T. C. (2014b). Discovery of the aggregation pheromone of the brown marmorated stink bug (*Halyomorpha halys*) through the creation of stereoisomeric libraries of 1-bisabolen-3-ols. *Journal of Natural Products*, 77(7), 1708-1717.

- Law, J. H., Regnier, F. E. (1971). Pheromones. *Annual Review of Biochemistry*, 40(1), 533-548.

- Leal, W. S., Kuwahara, S., Shi, X., Higuchi, H., Marino, C. E., Ono, M., Meinwald, J. (1998). Male-released sex pheromone of the stink bug *Piezodorus hybneri*. *Journal of Chemical Ecology*, 24(11), 1817-1829.

- Leal, W. S., Ueda, Y., Ono, M. (1996). Attractant pheromone for male rice bug, *Leptocorisa chinensis*: semiochemicals produced by both male and female. *Journal of Chemical Ecology*, 22(8), 1429.

- Leskey, T. C., Lee, D. H., Short, B. D., Wright, S. E. (2012). Impact of insecticides on the invasive *Halyomorpha halys* (Hemiptera: Pentatomidae): analysis of insecticide lethality. *Journal of Economic Entomology*, 105(5), 1726-1735.

- Lockwood, J. A., Story, R. N. (1986). Adaptive functions of nymphal aggregation in the southern green stink bug, *Nezara viridula* (L.)(Hemiptera: Pentatomidae*). Environmental Entomology*, 15(3), 739-749.

- Lu, W., Wang, Q., Tian, M. Y., He, X. Z., Zeng, X. L., Zhong, Y. X. (2007). Mate location and recognition in *Glenea cantor* (Fabr.)(Coleoptera: Cerambycidae: Lamiinae): Roles of host plant health, female sex pheromone, and vision. *Environmental Entomology*, 36(4), 864-870.

- Ludwig, S. W., Kok, L. T. (2001). Harlequin bug, *Murgantia histrionica* (Hahn)(Heteroptera: Pentatomidae) development on three crucifers and feeding damage on broccoli. *Crop Protection*, 20(3), 247-251.

- Marques, F. D. A., McElfresh, J. S., Millar, J. G. (2000). Female-produced sex pheromone of the predatory bug *Geocoris punctipes*. *Journal of Chemical Ecology*, 26(12), 2843-2855.

- Martínez-Córdova, L. R., Campaña-Torres, A., Villarreal-Colmenares, H., Ezquerra-Brauer, J. M., Martínez-Porchas, M., Cortés-Jacinto, E. (2013). Evaluation of partial and total replacement of formulated feed by live insects, *Trichocorixa* sp.(Heteroptera: Corixidae) on the productive and nutritional response, and postharvest quality of shrimp, *Litopenaeus vannamei* (Boone 1931). *Aquaculture Nutrition*, 19(2), 218-226.

- Mcbrien, H. L., Millar, J. G., Gottlieb, L., Chen, X., Rice, R. E. (2001). Male-produced sex attractant pheromone of the green stink bug, *Acrosternum hilare* (Say). *Journal of Chemical Ecology*, 27(9), 1821-1839.

- McBrien, H., Millar, J., Rice, R., McElfresh, J., Cullen, E., Zalom, F. (2002). Sex attractant pheromone of the red-shouldered stink bug *Thyanta pallidovirens*: a pheromone blend with multiple redundant components*. Journal of Chemical Ecology*, 28(9), 1797-1818.

- Mccormick, C. A., Gershenzon, J., Unsicker, S. B. (2014). Little peaks with big effects: establishing the role of minor plant volatiles in plant–insect interactions. *Plant, cell & environment*, 37(8), 1836-1844.

- McPherson, J. E., McPherson, R. (2000). Stink bugs of economic importance in America north of Mexico. CRC Press.

- Millar, J. G. (2005). Pheromones of true bugs. In *The chemistry of pheromones and other semiochemicals II*. *Springer*, Berlin, Heidelberg. (pp. 37-84).

- Millar, J. G., McBrien, H. M., Steven, M. J. (2010). Field trials of aggregation pheromones for the stink bugs *Chlorochroa uhleri* and *Chlorochroa sayi* (Hemiptera: Pentatomidae). *Journal of Economic Entomology*, 103(5), 1603-1612.

- Millar, J. G., Rice, R. E. (1998). Sex pheromone of the plant bug *Phytocoris californicus* (Heteroptera: Miridae). *Journal of Economic Entomology*, 91(1), 132-137.

- Mizutani, J. (1999). Selected allelochemicals. *Critical Reviews in Plant Sciences*, 18(5), 653-671.

- Moayeri, H., Ashouri, A., Poll, L., Enkegaard, A. (2007). Olfactory response of a predatory mirid to herbivore induced plant volatiles: multiple herbivory vs. single herbivory. *Journal of Applied Entomology*, 131(5), 326-332.

- Moraes, M. C., Millar, J. G., Laumann, R. A., Sujii, E. R., Pires, C. S., Borges, M. (2005). Sex attractant pheromone from the neotropical red-shouldered stink bug, *Thyanta perditor* (F.). *Journal of Chemical Ecology*, 31(6), 1415-1427.

- Moritya, S., Shiga, M. (1984). Attraction of the male brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae) for males and females of the same species. *Applied Entomology and Zoology*, 19(3), 317-322.

- Njihia T. N., Torto B., Murungi L. K., Irungu J., Mwenda D. M. and Babin R. (2017*).* Identification of kairomones of second instar nymphs of the variegated coffee bug *Antestiopsis thunbergii* (Heteroptera: Pentatomidae). *Chemoecology*, 27(6), 239-248.

- Olson, D. M., Ruberson, J. R., Zeilinger, A. R., Andow, D. A. (2011). Colonization preference of *Euschistus servus* and *Nezara viridula* in transgenic cotton varieties, peanut, and soybean. *Entomologia Experimentalis et Applicata*, 139(2), 161-169.

- Ondiaka, S., Migiro, L., Rur, M., Birgersson, G., Porcel, M., Rämert, B., Tasin, M. (2016). Sunflower as a trap crop for the European tarnished plant bug (*Lygus rugulipennis*). *Journal of Applied Entomology*, 140(6), 453-461.

- Palumbo, J. C., Perring, T. M., Millar, J. G., Reed, D. A. (2016). Biology, ecology, and management of an invasive stink bug, *Bagrada hilaris*, in North America. *Annual Review of Entomology*, 61, 453-473.

- Pan, H., Lu, Y., Xiu, C., Geng, H., Cai, X., Sun, X., Zhang, Y., Williams III, L., Wyckhuys, K. A., Wu, K. (2015). Volatile fragrances associated with flowers mediate host plant alternation of a polyphagous mirid bug. *Scientific Reports*, 5, 14805.

- Pavis, C., Malosse, C., Ducrot, P. H., Descoins, C. (1994). Dorsal abdominal glands in nymphs of southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae):

Chemistry of secretions of five instars and role of (*E*)-4-oxo-2-decenal, compound specific to first instars. *Journal of Chemical Ecology*, *20*(9), 2213-2227.

- Rather, A., Azim, M., Maqsood, S. (2010). Host plant selection in a pentatomid bug *Eurydema pulchrum* Westwood. *Journal of Plant Protection Research*, 50(2), 229-232.

- Reed, D. A., Newman, J. P., Perring, T. M., Bethke, J. A., Kabashima, J. (2013). Management of the Bagrada bug in nurseries. *Univ. California Coop. Ext., Agric. Nat. Resources, Ventura County*. (http://ceventura. ucdavis. edu/files/163391. pdf) (accessed 9 May 2014).

- Sánchez-Peña, S. R. (2014). First record in Mexico of the invasive stink bug *Bagrada hilaris*, on cultivated crucifers in Saltillo. *Southwestern Entomologist*, 39(2), 375-378.

- Santadino, M., Lucia, A., Duhour, A., Riquelme, M., Naspi, C., Masuh, H., Liljesthröm, G., Coviella C. (2017). Feeding preference of *Thaumastocoris peregrinus* on several *Eucalyptus* species and the relationship with the profile of terpenes in their essential oils. Phytoparasitica, 45(3), 395-406.

- Sant'Ana, J., Da Silva, R. F., Dickens, J. C. (1999). Olfactory reception of conspecific aggregation pheromone and plant odors by nymphs of the predator, *Podisus maculiventris*. *Journal of chemical ecology*, 25(8), 1813-1826.

- Schaefer, C. W., Panizzi, A. R. (2000). Economic importance of Heteroptera: a general view. *Heteroptera of Economic Importance*, 3-8.

- Schoonhoven, L. M., Van Loon, B., van Loon, J. J., Dicke, M. (2005). *Insect-Plant Biology*. Oxford University Press on Demand.

- Schwarz, J., Gries, R., Hillier, K., Vickers, N., Gries, G. (2009). Phenology of semiochemical-mediated host foraging by the western boxelder bug, *Boisea rubrolineata*, an aposematic seed predator. *Journal of Chemical Ecology*, 35(1), 58.

- Smith, R. F., Pierce, H. D., Borden, J. H. (1991). Sex pheromone of the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *Journal of Chemical Ecology*, 17(7), 1437-1447.

- Soldi, R. A., Rodrigues, M. A., Aldrich, J. R., Zarbin, P. H. (2012). The male-produced sex pheromone of the true bug, *Phthia picta*, is an unusual hydrocarbon. *Journal of Chemical Ecology*, 38(7), 814-824.

- Staddon, B. W., Abdollahi, A., Parry, J., Rossiter, M., Knight, D. W. (1994). Major component in male sex pheromone of cereal pest *Eurygaster integriceps* puton (Heteroptera: Scutelleridae) identified as a homosesquiterpenoid. *Journal of Chemical Ecology*, 20(10), 2721-2731.

- Suckling, D. M., Karg, G., Rechcigl, J., Rechcigl, N. (2000). Pheromones and other semiochemicals. *Biological and biotechnological control of insect pests*, 63-99.

- Sugie, H., Yoshida, M., Kawasaki, K., Noguchi, H., Moriya, S., Takagi, K., Fukuda, H., Fujiie, A., Yamanaka, M., Ohira, Y. (1996). Identification of the aggregation pheromone of the brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). *Applied Entomology and Zoology*, 31(3), 427-431.

- Takita, M., Sugie, H., Tabata, J., Ishii, S., Hiradate, S. (2008). Isolation and estimation of the aggregation pheromone from *Eysarcoris lewisi* (Distant) (Heteroptera: Pentatomidae). *Applied Entomology and Zoology*, 43(1): 11-17.

- Wang, Q., Millar, J. G. (2000). Mating behavior and evidence for male-produced sex pheromones in *Leptoglossus clypealis* (Heteroptera: Coreidae). *Annals of the Entomological Society of America*, 93(4), 972-976.

- Weber, D. C., Walsh, G. C., DiMeglio, A. S., Athanas, M. M., Leskey, T. C., Khrimian, A. (2014). Attractiveness of harlequin bug, *Murgantia histrionica*, aggregation pheromone:

Field response to isomers, ratios, and dose. *Journal of Chemical Ecology*, 40(11-12), 1251-1259.

- Weber, D., Khrimian, A., Blassioli-Moraes, M. C., Millar, J. (2018). Semiochemistry of Pentatomoidea. Biology of invasive stink bugs and related species. *CRC Press*, Boca Raton, FL, 677-725.

- Wennström, A., Hjulström, L. N., Hjältén, J., Julkunen-Tiitto, R. (2010). Mother really knows best: host choice of adult phytophagous insect females reflects a within-host variation in suitability as larval food. *Chemoecology*, 20(1), 35-42.

- Wertheim, B., van, Baalen E.-J. A., Dicke, M., Vet, L. E. (2005). Pheromone-mediated aggregation in nonsocial arthropods: an evolutionary ecological perspective. *Annual Review of Entomology*. 50, 321-346.

- White, W. H., Brannon, L. W. (1933). The harlequin bug and its control. *US Dep. Agr. Farmers Bull*, 1712.

- Williams, L., Blackmer, J. L., Rodriguez-Saona C., Zhu, S. (2010). Plant Volatiles Influence Electrophysiological and Behavioral Responses of *Lygus hesperus*. *Journal of Chemical Ecology*, 36(5), 467-478. http://dx.doi.org/10.1007/s10886-010-9778-2 http://dx.doi.org/10.1007/s10886-010-9778-2

- Zarbin, P. H., Fávaro, C. F., Vidal, D. M., Rodrigues, M. A. (2012). Male-produced sex pheromone of the stink bug *Edessa meditabunda*. *Journal of Chemical Ecology*, 38(7), 825-835.

- Zarbin, P. H., Reckziegel, A., Plass, E., Borges, M., Francke, W. (2000). Synthesis and biological activity of methyl 2, 6, 10-trimethyldodecanoate and methyl 2, 6, 10-trimethyltridecanoate: male-produced sexual pheromones of stink bugs *Euschistus heros* and *Piezodorus guildinii*. *Journal of Chemical Ecology*, 26(12), 2737-2746.

Chapter 2

The role of (*E*)-2-octenyl acetate as an intraspecific semiochemical of *Bagrada hilaris* (Burmeister): laboratory and field evaluation

This chapter is based on the work contained in the following article:

- Arif, M. A., Guarino, S., Colazza, S., Peri, E. (2020). The role of (*E*)-2-octenyl acetate as pheromone of *Bagrada hilaris* (Burmeister): laboratory and field evaluation. *Insects*, Submited.

Chapter 2

The role of (*E*)-2-octenyl acetate as an intraspecific semiochemical of *Bagrada hilaris* (Burmeister): laboratory and field evaluation

Abstract

Bagrada hilaris is a pest of cruciferous crop native to Asia and Africa and invasive in Europe and the Americas. Previous studies suggest that mate location and aggregation behavior of this species is mediated by volatile chemicals among which the main compound is (*E*)-2-octenyl acetate. Males emit larger amounts of this compound but its possible role as an aggregation or sex pheromone is still to be defined. In this study the response of *B. hilaris* females, males and nymphs to (*E*)-2-octenyl acetate using electroantennogram (EAG), olfactometer and field trap bioassays was evaluated. EAG recordings showed that this compound evokes greater antennal responses in *B. hilaris* females. Olfactometer behavioral responses showed that females and nymphs were attracted to (*E*)-2-octenyl acetate, while males showed no attraction. In field trap bioassays experiments captures were obtained in traps baited with the doses of 5 and 10 mg of (*E*)-2-octenyl acetate, while in traps loaded with 2 mg and control traps were not recorded catches. These results suggest the involvement of (*E*)-2-octenyl acetate in intraspecific interactions of this species.

Key words: Painted bug, olfactometer, EAG, Traps.

2.1 Introduction

The Painted bug, *Bagrada hilaris* (Burmeister), is an invasive stink bug which feeds mainly on brassicaceous hosts, and it is particularly detrimental to crops in recently invaded areas (Huang et al. 2014). This species is widely distributed across Africa, southern and central Europe, Pakistan, India, China, and parts of southeast Asia (Reed et al. 2013; Bundy et al. 2018). In 2008, the Painted bug was reported in California, probably introduced by commercial trade, and it rapidly expanded its range to the brassicaceous crops of coastal California and central Arizona (Palumbo and Natwick 2010), and then to Nevada, New Mexico, and Utah (Bundy et al. 2012). More recently, *B. hilaris* has been reported in Mexico (Sánchez-Peña 2014), Hawaii (Palumbo et al. 2016), and in Chile (Torres-Acosta et al. 2017). *Bagrada hilaris* has had a remarkable impact on agriculture in the Americas; it has been estimated that about 90 % of the broccoli acreage planted in the USA has been infested by the Painted bug, with yield losses often exceeding 10 % of production (Huang et al. 2014). A recent study by Guarino et al. (2017) showed that Painted bug infestation of cauliflower plant reduced leaf photosynthesis by about 40%. To date, there are no effective detection strategies or monitoring tools for the pest (Bundy et al. 2018; Guarino et al. 2018).

Several studies have focused on chemical communication in stink bugs, including the identification of sex pheromones (Baker et al. 1987; Aldrich et al. 1987, 1993; Borges and Aldrich 1994; Borges 1995; Borges et al. 2006). The great majority of pheromones described for these species are produced by adult males (Weber et al. 2018; Millar 2005), although nymphs are known to produce aggregation compounds (Fucarino et al. 2004).

In the case of *B. hilaris*, (*E*)-2-octenyl acetate, the main compound produced by adults, with significantly higher amounts emitted by males, was reported as a possible pheromone, as it might be involved the attraction process of females to males (Guarino et al. 2008). Although Bundy et al. (2018) report that attempts to use this compound as a monitoring were not successful, the attraction response of *B. hilaris* individuals to (*E*)-2-octenyl acetate has yet to be tested.

In this study the electroantennographic and behavioral response of *B. hilaris* to (*E*)-2-octenyl acetate was assessed in order to establish its suitability for further trials.

The study objectives study were to

1. Assess antennal response of *B. hilaris* females, males and nymphs to (*E*)-2-octenyl acetate using electrophysiological techniques,
2. Evaluate the attraction response elicited from (*E*)-2-octenyl acetate alone or in presence of a food source (host plant), using a two choice olfactometer,
3. Evaluate in field trials in caper orchards (*Capparis spinosa* L.) located in Pantelleria (Italy), the attraction response of *B. hilaris* to (*E*)-2-octenyl acetate.

2.2 Material and methods

2.2.1 Insects and plants

A colony of *B. hilaris* was established and restocked regularly with individuals collected from caper (*Capparis spinosa* L.) fields on the island of Pantelleria (Trapani, Italy). Insects were reared in an environmentally controlled room (30 ± 2 °C, 70 ± 10% RH, photoperiod 16L:8D), in wooden cages (25 × 25 × 40 cm) with two 5-cm diameter mesh-covered holes for ventilation. The colony was fed with cauliflower and cabbage plants, depending on seasonal availability. As *B. hilaris* females lay their eggs in the soil, dishes (6-cm Ø) with a mixture of sand, silt, and clay (33% for each soil component) were placed in the cages as oviposition sites. Dishes were changed weekly, and those with eggs were kept in separate cages until the emergence of nymphs. Adults and nymphs were used separately in experiments.

Seeds of *B. oleracea* var. botrytis (cauliflower) obtained from a local market (Palermo – Italy), were placed on cotton wool (5 g) soaked with distilled water and held in glass containers with a distance of circa 0.5 cm between seeds. The containers were placed in an environmentally controlled growth chamber (25 ± 1 °C, 70 ± 10% RH, photoperiod 16L: 8D). After 7 days, newly emerged seedling clusters were used in behavioral experiments.

2.2.2 Electroantennography

Dose-response experiments using (*E*)-2-octenyl acetate were carried out. Electroantennograms (EAG) were conducted using aliquots of 2µl of acetone control or test solution of (*E*)-2-octenyl acetate (Bedoukian Research, Danbury, CT) dissolved in acetone, pipetted onto a piece of filter paper (Whatman, grade 1). Tested doses of (*E*)-2-octenyl acetate were 0.02, 0.2, 2, 20 and 200 µg. The loaded paper was exposed to the air for 40s to allow the solvent to fade away, and then placed inside a glass Pasteur pipette. Puff stimuli were blown into an airstream that passed over the antennal preparation using a flow controller (model CS-05; Syntech, Hilversum, the Netherlands) to generate a 1.5-s stimulus at 1-min intervals, with a flow rate of 1.5 l min^{-1}. The signals generated by the antennae were passed through a high-impedance amplifier (model

IDAC-4, Syntech) and recorded with custom software (Syntech). For the EAG preparations, adult and nymphs *B. hilaris* were anesthetized by refrigeration at −4°C for 40s, the head was excised and mounted on a glass capillary tube reference electrode (1.5 mm diameter) filled with 0.1 M KCl solution and connected with silver wire to the amplifier. The recording electrode was a similar glass capillary in contact with the tip of an antenna. The capillary tubes were drawn to a fine point using a microelectrode puller (Narishige PC-10, Tokio, Japan) to achieve a diameter enabling insertion into the antennal tip (Fig. 1).

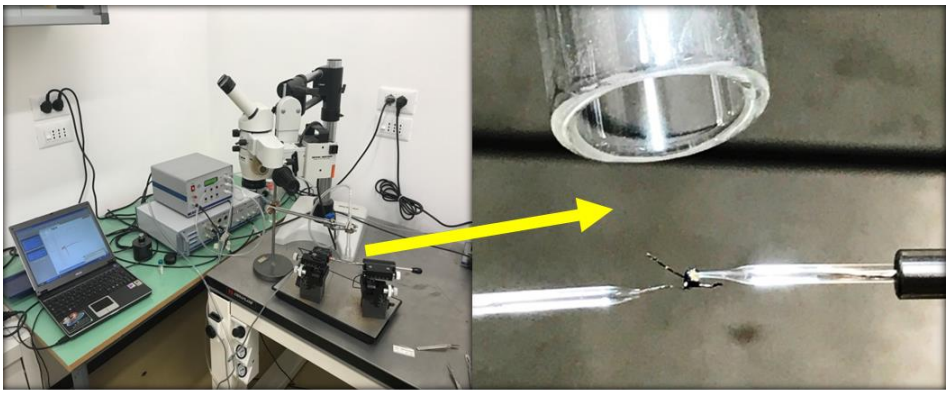

Figure 1. Electroantennography (EAG) device and preparation with bug's head excised and mounted on reference electrode and recording electrode touching the tip of the antenna

2.2.3 Open Vertical Y-shaped olfactometer

Bioassays were conducted with an open vertical Y-shaped olfactometer consisting of a brass rod (left and right arms 20 cm long, central arm 25 cm long, 1.00 cm diameter) (Fig. 2).

The behavioral bioassays were conducted testing the (*E*)-2-octenyl acetate alone or in presence of the host plant in two separate experiments. In the first experiment (*E*)-2-octenyl acetate was tested alone versus air used as control. In the second experiment (*E*)-2-octenyl acetate was tested in presence of a 7-days old seedling clusters (N=50) of *B. oleracea* var botrytis versus a seedling cluster alone used as control. The choice of this host plant species at seedling stage was

suggested by a recent study that evidenced its attractiveness *to B. hilaris* (Guarino et al. 2018). (*E*)-2-octenyl acetate was applied using 2 µl of a 10% acetone solution (200µg) on a filter paper disk (Whatman N. 1) and left 5 minutes for evaporation of the solvent before being placed in the test jar. The test stimuli were changed after 15 minutes. The left and right arms were covered with two glass tubes (18 cm long, 5 cm diameter) terminating in hose nipples connected by Tygon tubes to a high–purity air source, and air flow was controlled with a flow-meter at a rate of 0.2 l/min. The air flowed through two glass chambers (125 ml each), which held the test stimuli. Light was provided with a halogen lamp (Osram, 12V–35W, Münich, Germany) hanging 30 cm above the olfactometer. Experiments were carried out under ambient laboratory temperature and humidity conditions (25 ± 3 °C, and 50± 15% RH). For each replicate, a single adult or nymph was gently placed at the bottom of the central arm of the olfactometer with a paint brush and allowed 10 min to respond. The bugs moved from the bottom upward toward the light source and upon arriving at the Y junction, chose between the two different volatile stimuli. The criterion for a response was that the test bug walked in the test arm or the control arm for at least 5 cm past the Y junction (first choice). Bugs that did not move into one of the two arms during the 10 min trial were scored as non-responders and were not included in the analysis. After 8 replicates, the glass parts of the apparatus were washed with water and detergent, and then wiped with acetone and the brass rod was cleaned with distilled water and acetone and baked at 200°C for 60 min. Experiments were carried out from 2.00pm to 7.00pm.

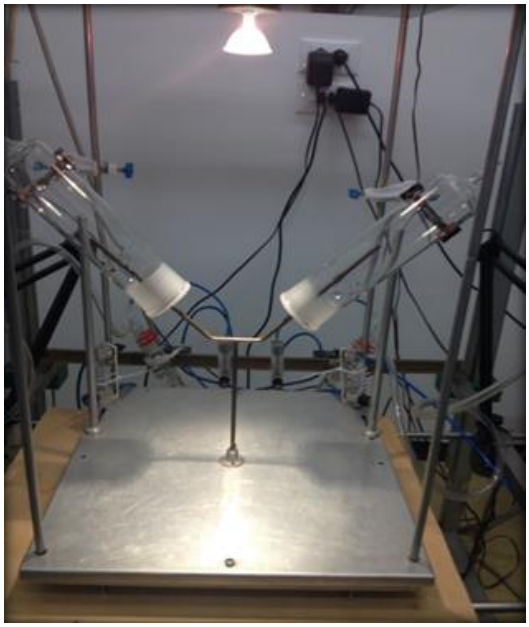

Figure 2. Open Vertical Y-shaped olfactometer.

2.2.4 (*E*)-2-octenyl acetate dispenser emission rate

The emission rate estimation of (*E*)-2-octenyl acetate was carried out in order to support the successive field trap experiment. In this chemical analysis was assessed the amount of this compound emitted with time from a commercial dispenser. As releaser was used a 0.5 ml polyethylene tube, chosen as one of the most commonly used for highly volatile semiochemicals as (*E*)-2-octenyl acetate. The polyethylene tubes were loaded with the same dose successively tested in the field, in the specific 2, 5 or 10 mg of (*E*)-2-octenyl acetate (N = 3 per each dose). The collection of (*E*)-2-octenyl acetate from polyethylene tubes was conducted in airstream by placing the dispensers in a cylindrical glass chamber (25 ml volume) and pumping through the air at 300 ml/min. A glass tube containing a plug of 100 mg of Porapak Q (80–100 mesh; Sigma-Aldrich) was used to collect the (*E*)-2-octenyl acetate emitted at different days from the releaser loading (Fig. 3). After collecting for 15 min, the traps were eluted with 1 ml of acetone. All replicates were carried out in a temperature-controlled room (25 ± 1°C). Extracts were stored at 4 °C in glass vials with Teflon cap liners until used for (GC/MS) analyses. GC-MS analyses were performed on an Agilent 6890 GC system interfaced with an MS5973 quadruple mass spectrometer. One µl of extract was injected onto a DB5-MS column 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 was set at 40°C for 5 min, and 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.

In order to estimate the quantitative amounts of (*E*)-2-octenyl acetate emitted from polyethylene tube, the integrated GC peaks were compared with a calibration curve carried out with standard solutions. Linearity was determined for this compound injecting in the GC concentrations of 6, 12, 25, 50 and 100 ng µl $^{-1}$, determining a calibration curve that had regression coefficients (R^2) of 0.9976 (Fig 4).

Figure 3. (*E*)-2-octenyl acetate collection system in air stream.

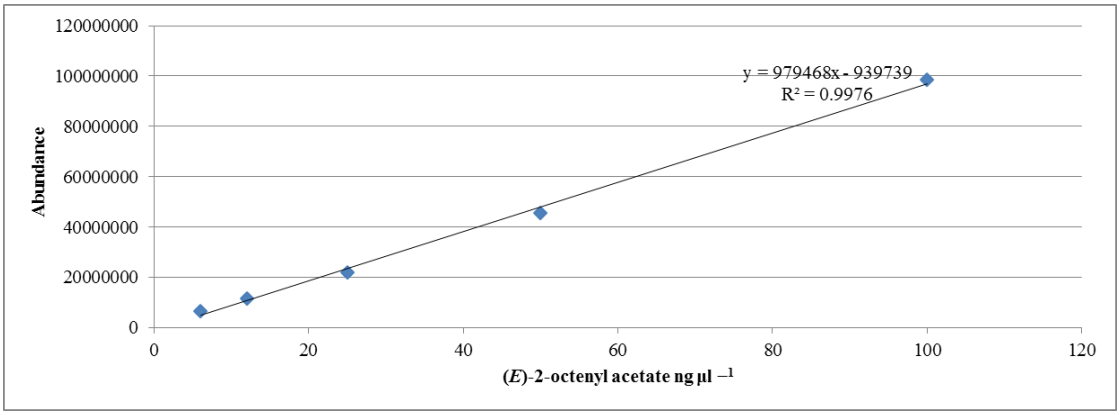

Figure 4. Calibration curve of (*E*)-2-octenyl acetate carried out by injecting doses of 6, 12, 25, 50 and 100 ng µl $-^{1}$.

2.2.5 Field bioassays

Field bioassays using (*E*)-2-octenyl acetate were conducted in a caper orchard in Pantelleria island (Italy) (36°46'15.2"N 11°57'44.1"E), infested with *B. hilaris*. Horizontal, dual funnel-sided plastic traps (25 x 15 x 15 cm) furnished by GEA S.r.L., (Settimo Milanese Milan – Italy) were used (Fig. 5). Paraffin oil was applied on the board of the inner part of the funnels to prevent insect escape. Traps were placed in the proximity of the caper plant and partially buried in order to facilitate insect entrance and to prevent wind damage (Fig. 6). Each trap was baited with a polyethylene tube containing either 2 mg, 5 mg or 10 mg of (*E*)-2-octenyl acetate dissolved in 100 µl of acetone. Distance among traps was approximately 8m. Three traps per dose and three acetone-only bait control traps were placed in the field using a Latin square design. Traps were inspected every 3 days and the number of trapped individuals (males, females or nymphs) recorded.

Figure 5. Horizontal trap furnished by GEA, (Settimo Milanese Milan – Italy).

Figure 6. Placement of the trap in the caper field.

2.2.6 Statistical analysis

EAG data, given as means of antennal responses in mV after subtraction of the responses to the solvent were root square transformed and analyzed by using one-way measure ANOVA followed by LSD pair-wise comparisons to evidence different response for each dose tested on for males, females and nymphs. Vertical Y-shaped olfactometer choice experiments were analyzed using χ^2 tests. Data of the emission curve of (*E*)-octenyl acetate from releasers loaded with different doses were compared for each day of sampling by one-way ANOVA, followed by Tukey test. For field experiments, the numbers of caught adults and nymphs were compared by one-way ANOVA, followed by Fisher's LSD test. All statistical analyses were performed using Statistica 10.0 for Window (Statsoft 2001, Vigonza, PD, Italy).

2.3 Results

2.3.1 Electroantennography

The results of EAG experiments are shown in Fig. 7. Overall antennae of females, males and nymphs showed dose-dependent responses to (*E*)-2-octenyl acetate but with a different sensitivity. In particular, females' antennae showed significant electroantennographic response already at 0.2 µg ($P < 0.05$; ANOVA followed by LSD test) and similar responses were observed at doses of 2 and 20 µg. In males the only significative response was observed at the dose of 2 µg ($P < 0.05$; ANOVA followed by LSD test). Nymphs showed significant response at the doses of 2 µg and 20 µg ($P < 0.05$; ANOVA followed by LSD test). All males, females and nymphs antennae reached saturation with the increasing doses and showed a decreasing response at the highest dose used (200µg).

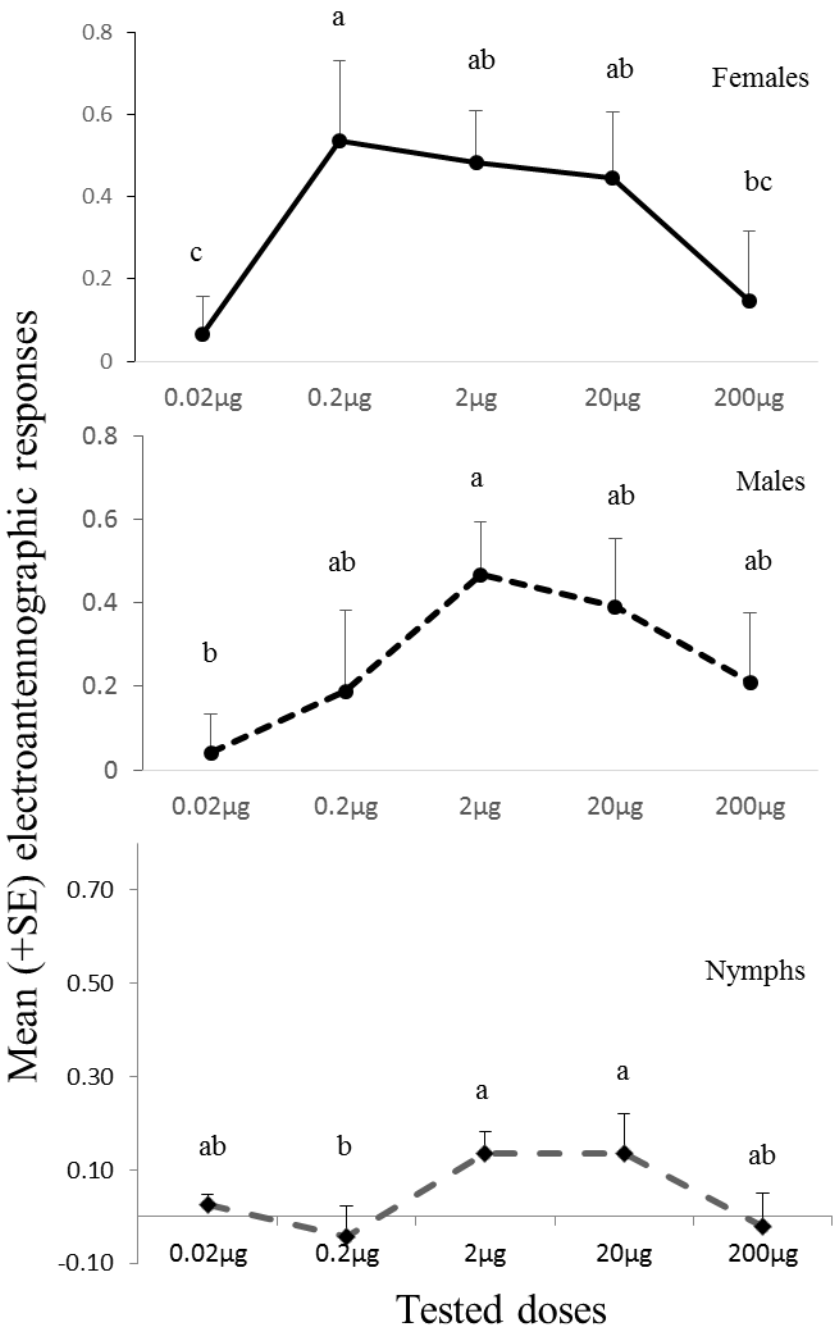

Figure 7. EAG dose–response curves of *B. hilaris* individuals (Female N=7; Male N= 7; Nymphs N= 6) to (*E*)-2-octenyl acetate at different doses. EAG amplitudes were adjusted to a control stimulus (acetone). Different letters indicate that values differ statistically at $P < 0.05$ (ANOVA, followed by LSD test).

2.3.2 Open Vertical Y-shaped olfactometer

Responses of *B. hilaris* adults and nymphs in open vertical Y-shaped olfactometer bioassays are shown in Fig. 8 and Fig 9. In the first experiment adult *B. hilaris* females were strongly attracted by (*E*)-2-octenyl acetate rather than from air (χ^2 = 10.52, df = 1, *P* < 0.001, N = 46) while males and nymphs were not attracted (χ^2 = 0.71, df = 1, *P* = NS, N = 36), nor nymphs (χ^2 = 0.64, df = 1, *P* = NS, N = 56) (Fig. 8). In the second experiment, conducted in presence of *B. oleracea* seedlings in both test and control arms, was evidenced a significant attraction response to the (*E*)-2-octenyl acetate rather to seedlings alone from both female (χ^2 = 5.22, df = 1, *P* = 0.02, N = 62) and nymphs (χ^2 = 7.22, df = 1, *P* = 0.07, N = 61). Differently, males were not attracted to (*E*)-2-octenyl acetate (χ^2 = 0.96, df = 1, *P* = 0.3, N = 66) (Fig. 9).

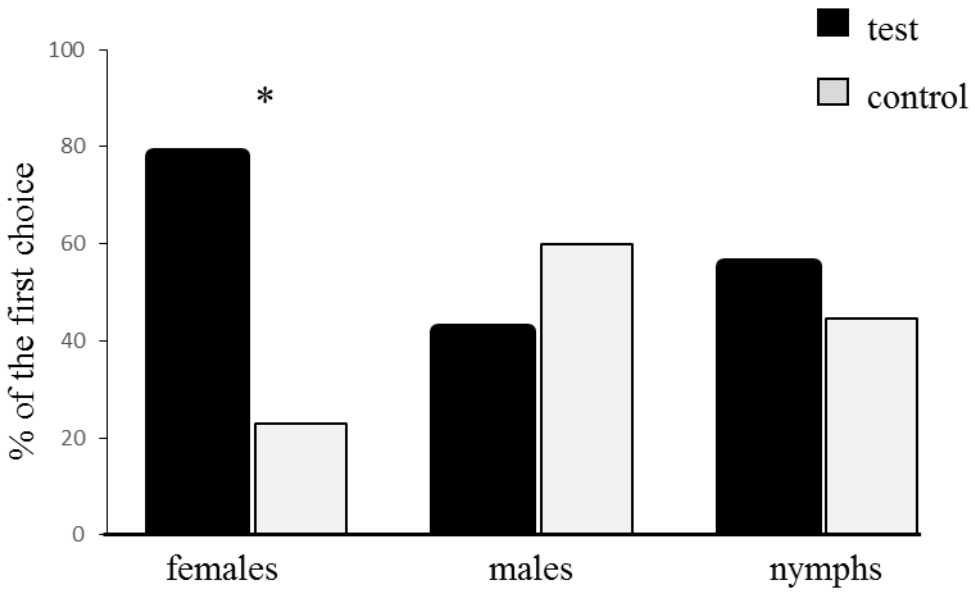

Figure 8. Response of *B. hilaris* individuals to (E)-2-octenyl acetate, (females N= 46; males N= 36; nymphs N= 56); * = P < 0.05.

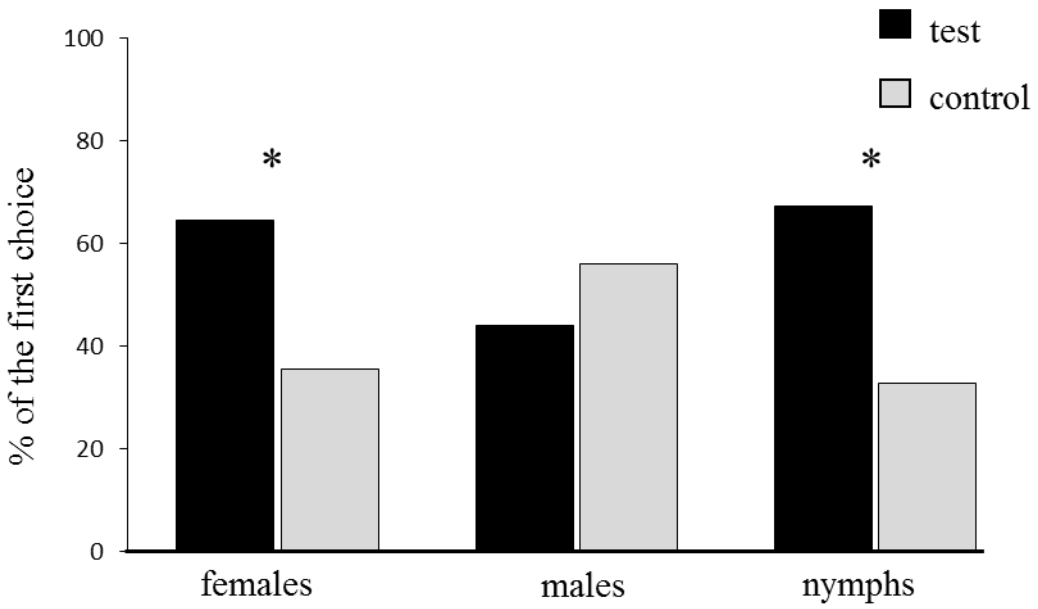

Figure 9. Response of *B. hilaris* individuals to (*E*)-2-octenyl acetate in presence of *B oleracea* seedlings (test) versus *B. oleracea* seedlings alone (control) (females N= 62; males N= 66; nymphs N= 61); * = $P < 0.05$.

2.3.3 (*E*)-2-octenyl acetate dispenser emission rate

The emission rate (ng/hr) of the polyethylene dispensers loaded with different doses of (*E*)-2-octenyl acetate is reported in Fig. 10. After one day from releaser preparation, the emission of (*E*)-2-octenyl acetate was similar in the different treatments. However, the dispensers loaded with 10 mg emitted a higher amount of (*E*)-2-octenyl acetate rather than 2 and 5 mg at the fourth day after from releaser loading ($P < 0.01$; Tukey test). Successively, at the days seven and ten, the dispensers loaded with 10 mg emitted a higher amount of (*E*)-2-octenyl acetate rather than dispensers loaded with 2 mg ($P < 0.01$; Tukey test) and similar to the ones loaded with 5 mg (P = NS). At the day thirteen the amount emitted was higher in 10 mg dispenser than to the others ($P < 0.01$; Tukey test), however the amount emitted from 5 mg dispenser was higher than the one emitted from 2 mg dispensers ($P < 0.01$; Tukey test).

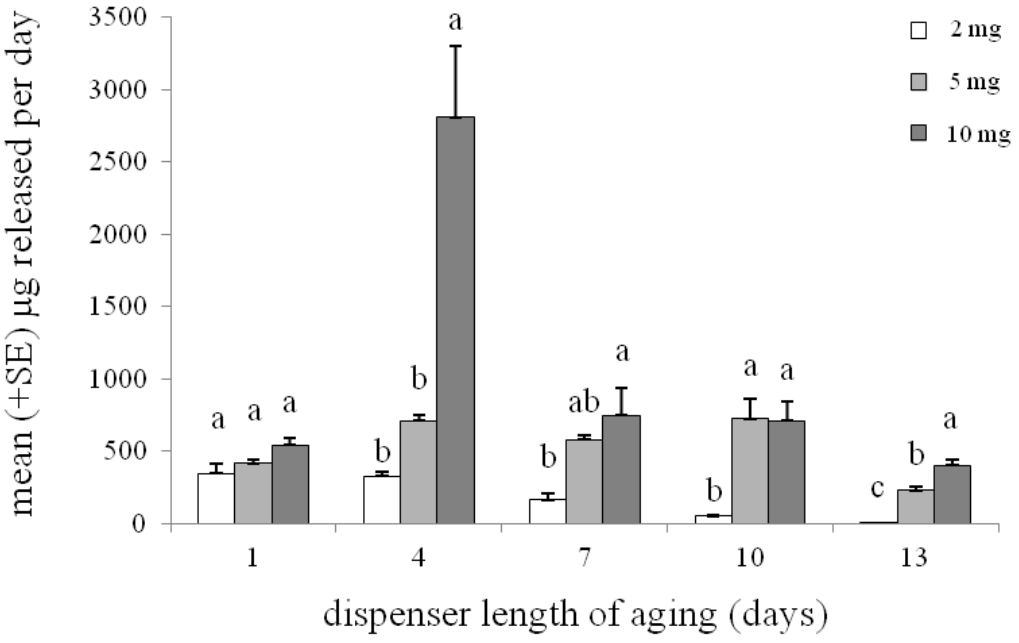

Figure 10. Emission rate of (*E*)-2-octenyl acetate from polyethylene tube dispensers loaded with doses of 2, 5, 10 mg. Different letters within the same day of sampling indicate statistical differences for $P < 0.01$ (ANOVA, followed by Tukey test).

2.3.4 Field bioassays

Results of field bioassays to (*E*)-2-octenyl acetate are reported in Fig. 11. The number of *B. hilaris* individuals captured during the field experiment was significantly different among treatments ($F = 2.90$; df = 3; $P < 0.05$, ANOVA). In the traps baited with 5 mg of (*E*)-2-octenyl acetate captured a mean (+SE) of 0.2+ 0.1 nymphs and with trap baited with 10 mg captured female and nymphs mean (+SE) 0.13 + 0.15 and 0.2 + 0.15 respectively per trap per 3 days. No captures were observed in the traps baited with 2 mg of (*E*)-2-octenyl acetate and control traps.

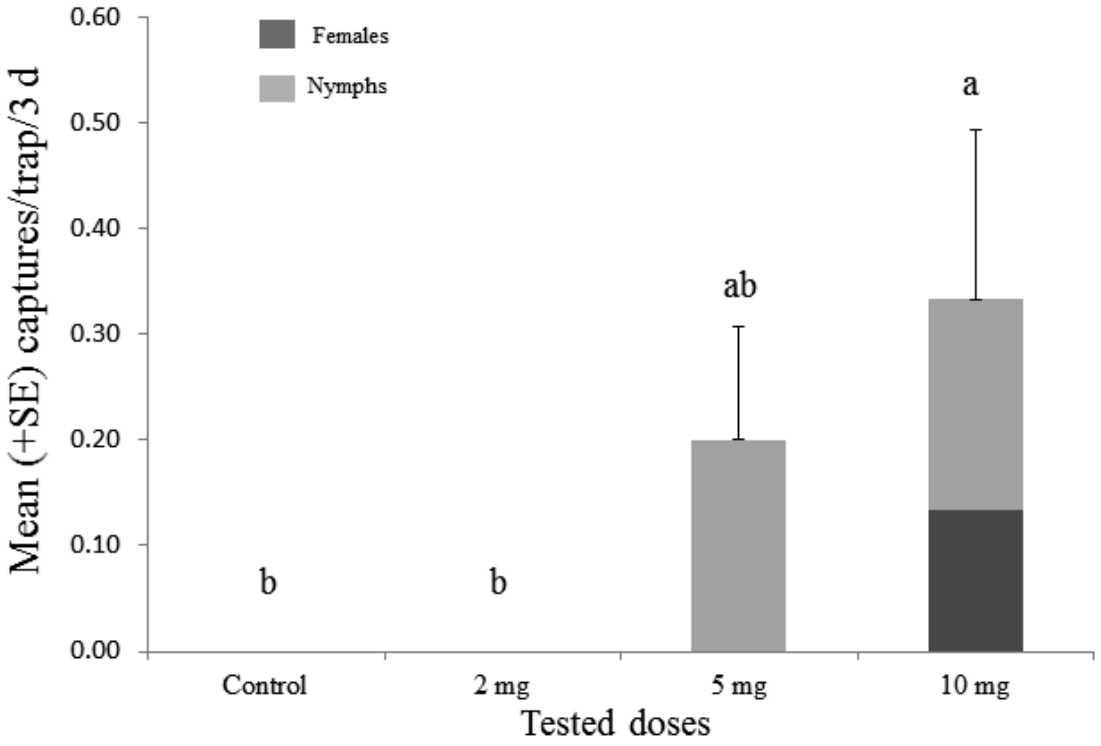

Figure 11. Mean (+SE) captured of B. hilaris individual by traps baited with (*E*)-2-octenyl acetate at different doses and control test (acetone). Different letters indicate that values differ statistically at P <0.05 (ANOVA, followed by LSD test).

2.4 Discussion

The results suggest that (*E*)-2-octenyl acetate is a semiochemical involved in the intraspecific communication of *B. hilaris*.

The data obtained from EAG experiments indicated a higher sensitivity of *B. hilaris* female's antenna to (*E*)-2-octenyl acetate in comparison with males and nymphs. Similar strong sensitivity of female's antenna has been reported in other stink bug species EAG experiments testing the sex pheromone as in the case of *Thyanta pallidovirens* (Stål) (Millar 1997), *Chinavia ubica* (Rolston) and *C. impicticornis* (Stål) (Blassioli-Moraes et al. 2012). Insects generally perceive odors by means of specially adapted sensillae on the antennae (Cork et al. 1990; Brézot et al. 1996; Innocenzi et al, 2004; Ventura and Panizzi, 2005; Silva et al. 2010; Ruschioni et al. 2015), so it is likely that, the females' antennae possess specific receptors for this compound in higher amount than males and nymphs. In other pentatomid bugs species, EAG recording evidenced females' responses to the sex pheromone such as in *Nezara viridula* (L.) (Brézot et al. 1994) and in *Chinavia spp*. (Blassioli-Moraes et al. 2012).

The results EAG bioassays were confirmed by the bioassays carried out in vertical Y-shaped olfactometer that indicated attraction response of females toward (*E*)-2-octenyl acetate and not response from males of *B. hilaris* testing the compound in presence or not of the host plant odor. In the same experiment, nymphs were attracted to (*E*)-2-octenyl acetate only in presence of the host plant, suggesting that host plant volatile can augment the response to the intraspecific semiochemical, as observed in other stink bugs as *Euschistus conspersus* Uhler (Krupke et al. 2001) and *Halyomorpha halys* Stål (Morrison III et al. 2018).

The release rate from the polyethylene dispensers loaded with different doses of (*E*)-2-octenyl acetate, evidenced in the specific a rapid exhaustion from the 2 mg dispenser in comparison with the 5 and 10 mg dispensers. This factor probably determined the different number of captures recorded in the field, suggesting that the attraction of *B. hilaris* females and nymphs to (*E*)-2-octenyl acetate is strictly linked with the amount perceived. Furthermore, the dispenser loaded

with 10 mg evidenced a high emission of the chemical in particular at day four from their preparation.

The overall low number of individuals captured in the traps in our experiments, limited to the two highest doses of (*E*)-2-octenyl acetate tested, could also be influenced by the complex behavior of stink bugs, that often lead to disappoint results in field test. In fact, similar difficulties have been observed during trials to bioassay possible pheromone components for other phytophagous bug species (Ho and Millar, 2001). For example, although the *N. viridula* pheromone determines strong attraction of females in laboratory bioassays (Aldrich et al. 1987; Baker et al. 1987), the field tests results have often not been confirmatory (Aldrich et al. 1993; Panizzi 1997). In several cases stink bug pheromone attracts the insect in the proximity of the traps but the number of the individual entering inside it is poor (Millar et al. 2002; Morrison et al. 2018). Moreover, several papers suggest that the commonly used insect trap designs are not effective for phytophagous stink bugs (Aldrich et al. 1991; Sugie et al. 1996). The tendency of *B. hilaris* habits to aggregate on the host plant and oviposit in the soil, provoked our use of a novel two-entrance trap, designed to be placed on the ground and in the proximity of the host plant.

Overall, our laboratory and field bioassays support the previous studies of Guarino et al. (2008) that first suggested a possible pheromonal role for (*E*)-2-octenyl acetate in this species. It is then likely that, similarly to all the other pentatomid bugs, *B. hilaris* female adults respond to a semiochemical that can bring in close proximity the two sexes where then other close-range courtship behavior can take place, including contact cues, as observed by Guarino et al. (2008). However, unlike other pentatomid bugs, the attraction is not determined by a specific compound emitted by mature males, but by a compound produced by both sexes (Guarino et al. 2008). Moreover, in other pentatomid bugs, male-produced compounds act as sex pheromones attracting only females, as in *Piezodorus guildinii* (Westwood) (Borges et al. 2007), *T. pallidovirens* (McBrien et al. 2002), *Oebalus poecilus* (Dallas) (de Oliveira et al. 2013), *Eysarcoris parvus* (Uhler) (Alizadeh et al. 2002), *Pallantia macunaima* (Grazia) (Fávaro et al. 2013). In other cases, the male produced compounds act as aggregation pheromones, attracting females, males,

and even nymphs as in *Murgantia histrionica* (Hahn) (Weber et al. 2014; Khrimian et al. 2014a), *N. viridula* (Aldrich et al. 1987), *Eysarcoris lewisi* (Distant) (Takita et al. 2008), *Euschistus tristigmus* (Say) (Aldrich et al. 1991; Millar 2005), *H. halys* (Stål) (Khrimian et al. 2014b).

However, as described in Guarino et al. (2008) in the case of *B. hilaris* the putative attractant semiochemical is produced by both sexes, albeit in different amounts. The lack of distinct differences in compounds produced by the two sexes is similar to results obtained with other Heteroptera species such as *Lygus lineolaris* (Palisot de Beauvois) (Aldrich 1988). (*E*)-2-alkenals are ubiquitous in Heteroptera, and are known to function as intra-specific signals such as aggregation and sex pheromones (Aldrich 1988; Millar 2005). For example, in the lygaeid *Geocoris punctipes* (Say), (*E*)-2-octenyl acetate is produced mainly by females and it plays a role as sex pheromone (Marques et al. 2000). Furthermore, in the alydid *Leptocorisa chinensis* (Dallas), a blend of (*E*)-2-octenyl acetate and octenol is produced by both sexes, but it is only attractive to males (Leal et al. 1996). Moreover, (*E*)-2-octenyl acetate together with other compounds, was found in the metathoracic gland contents and in aeration extracts of other stink bugs as *T. pallidovirens* and *E. heros* (F.) (McBrien et al. 2002; Pareja et al. 2007). In fact, in several cases, (*E*)-2-octenyl acetate is considered part of the defensive blend of Heteroptera species, having repellent effect toward predators (Noge et al. 2012). Defensive compounds have been found to function as alarm pheromones (Lockwood and Story, 1987) or even more specifically as sex pheromones (Ruther et al. 2001; Geiselhardt et al. 2008; Weiss et al. 2013). The use of a chemical substance for two or more functions is well-known and has been referred to as "semiochemical parsimony" (Blum 1996).

To conclude, the results obtained suggest that (*E*)-2-octenyl acetate is involved in intraspecific communication of *B. hilaris*. The weak attraction observed in the field tests can suggest that this semiochemical is probably not the only cues involved, as most phytophagous stink bugs are not strongly attracted into close proximity to pheromone sources where other sensory cues, such as visual and/or vibrational signals occur similarly to what observed in other stink bugs (Ota and Cokl, 1991; Ryan and Walter, 1992).

Our future efforts to develop an effective lure for this species, will focus on testing (*E*)-2-octenyl acetate together with plant derived attractant compounds recently identified in host plant such as the novel identified diterpene hydrocarbon found in seedling stages of *Brassica* spp. (Guarino at al. 2018).

2.5 References

- Aldrich, J. R. (1988). Chemical ecology of the Heteroptera. *Annual Review of Entomology*, 33(1), 211-238.

- Aldrich, J. R., Hoffmann, M. P., Kochansky, J. P., Lusby, W. R., Eger, J. E., Payne, J. A. (1991). Identification and attractiveness of a major pheromone component for Nearctic *Euschistus spp*. stink bugs (Heteroptera: Pentatomidae). *Environmental Entomology*, 20(2), 477-483.

- Aldrich, J. R., Numata, H., Borges, M., Bin, F., Waite, G. K., Lusby, W. R. (1993). Artifacts and pheromone blends from *Nezara spp*. and other stink bugs (Heteroptera: Pentatomidae). *Zeitschrift für Naturforschung C*, 48(1-2), 73-79.

- Aldrich, J. R., Oliver, J. E., Lusby, W. R., Kochansky, J. P., Lockwood, J. A. (1987). Pheromone strains of the cosmopolitan pest, *Nezara viridula* (Heteroptera: Pentatomidae). *Journal of Experimental Zoology*, 244(1), 171-175.

- Alizadeh, B. H., Kuwahara, S., Leal, W. S., MEN, H. C. (2002). Synthesis of the racemate of (Z)-exo-α-bergamotenal, a pheromone component of the white-spotted spined bug, *Eysarcoris parvus* uhler. *Bioscience, Biotechnology, and Biochemistry*, 66(6), 1415-1418.

- Baker, R., Borges, M., Cooke, N. G., Herbert, R. H. (1987). Identification and synthesis of (Z)-(1′ S, 3′ R, 4′ S)(–)-2-(3′, 4′-epoxy-4′-methylcyclohexyl)-6-methylhepta-2, 5-diene, the sex pheromone of the southern green stinkbug, *Nezara viridula* (L.). *Journal of the Chemical Society, Chemical Communications*, (6), 414-416.

- Blassioli-Moraes, M. C., Laumann, R. A., Oliveira, M. W. M., Woodcock, C. M., Mayon, P., Hooper, A., Pickett, J. A., Birkett, M. A., Borges, M. (2012). Sex pheromone communication in two sympatric neotropical stink bug species *Chinavia ubica* and *Chinavia impicticornis*. *Journal of Chemical Ecology*, 38(7), 836-845.

- Blum, M. S. (1996). Semiochemical parsimony in the Arthropoda. *Annual Review of Entomology*, 41(1), 353-374.

- Borges, M. (1995). Attractant compounds of the southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae). *Anais da Sociedade Entomologica do Brasil*, 24(2), 215-225.

- Borges, M., Aldrich, J. R. (1994). Attractant pheromone for Nearctic stink bug, *Euschistus obscurus* (Heteroptera: Pentatomidae): insight into a Neotropical relative. *Journal of Chemical Ecology*, 20(5), 1095-1102.

- Borges, M., Birkett, M., Aldrich, J. R., Oliver, J. E., Chiba, M., Murata, Y., Laumann, R, A., Barrigossi, J.A., Pickett, J, A., Moraes, M. C. (2006). Sex attractant pheromone from the rice stalk stink bug, *Tibraca limbativentris* Stal. *Journal of Chemical Ecology*, 32(12), 2749.

- Borges, M., Millar, J. G., Laumann, R. A., Moraes, M. C. (2007). A male-produced sex pheromone from the neotropical redbanded stink bug, *Piezodorus guildinii* (W.). *Journal of chemical ecology*, 33(6), 1235.

- Brézot, P., Malosse, C., Mori, K., Renou, M. (1994). Bisabolene epoxides in sex pheromone in *Nezara viridula* (L.) (Heteroptera: Pentatomidae): Role of cis isomer and relation to specificity of pheromone. *Journal of chemical ecology*, 20(12), 3133.

- Brézot, P., Tauban, D., Renou, M. (1996). Sense organs on the antennal flagellum of the green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae): sensillum types and numerical growth during the post-embryonic development. *International Journal of Insect Morphology and Embryology*, 25(4), 427-441.

- Bundy, C. S., Grasswitz, T. R., Sutherland, C. (2012). First report of the invasive stink bug *Bagrada hilaris* (Burmeister) (Heteroptera: Pentatomidae) from New Mexico, with notes on its biology. *Southwestern Entomologist*, 37(3), 411-415.

- Bundy, C.S., Perring, T.M., Reed, D.A., Palumbo, J.C., Grasswitz, T.R., Jones, W.A. (2018) *Bagrada hilaris*. In: McPherson JE (ed) Invasive Stink Bugs and Related Species (Pentatomoidea): Biology, Higher Systematics, Semiochemistry, and Managemen, *CRC Press*, Boca Raton, FL; 205–242.

- Cork, A., Beevor, P. S., Gough, A. J. E., Hall, D. R. (1990). Gas chromatography linked to electroantennography: a versatile technique for identifying insect semiochemicals. In *Chromatography and isolation of insect hormones and pheromones* (pp. 271-279). Springer, New York, NY.

- de Oliveira, M. W. M., Borges, M., Andrade, C. K. Z., Laumann, R. A., Barrigossi, J. A. F., Blassioli-Moraes, M. C. (2013). Zingiberenol,(1 S, 4 R, 1′ S)-4-(1′, 5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol, identified as the sex pheromone produced by males of the rice stink bug *Oebalus poecilus* (Heteroptera: Pentatomidae). *Journal of agricultural and food chemistry*, 61(32), 7777-7785.

- Fávaro, C. F., Soldi, R. A., Ando, T., Aldrich, J. R., Zarbin, P. H. (2013). (6 R, 10 S)-Pallantione: the first ketone identified as sex pheromone in stink bugs. *Organic letters*, 15(8), 1822-1825.

- Fucarino, A., Millar, J. G., McElfresh, J. S., Colazza, S. (2004). Chemical and physical signals mediating conspecific and heterospecific aggregation behavior of first instar stink bugs. *Journal of chemical ecology*, 30(6), 1257-1269.

- Geiselhardt, S., Ockenfels, P., Peschke, K. (2008). 1-Tridecene—male-produced sex pheromone of the tenebrionid beetle *Parastizopus transgariepinus*. *Naturwissenschaften*, 95(3), 247-251.

- Guarino, S., Arif, M. A., Millar, J. G., Colazza, S., Peri, E. (2018). Volatile unsaturated hydrocarbons emitted by seedlings of Brassica species provide host location cues to *Bagrada hilaris*. *PloS one*, 13(12), e0209870.

- Guarino, S., De Pasquale, C., Peri, E., Alonzo, G., Colazza, S. (2008). Role of volatile and contact pheromones in the mating behaviour of *Bagrada hilaris* (Heteroptera: Pentatomidae). *European Journal of Entomology*, 105(4), 613.

- Guarino, S., Peri, E., Colazza, S., Luchi, N., Michelozzi, M., Loreto, F. (2017). Impact of the invasive painted bug Bagrada hilaris on physiological traits of its host *Brassica oleracea* var botrytis. *Arthropod-Plant Interactions*, 11(5), 649-658.

- Ho, H. Y., Millar, J. G. (2001). Identification and synthesis of a male-produced sex pheromone from the stink bug *Chlorochroa sayi*. *Journal of Chemical Ecology*, 27(6), 1177-1201.

- Huang, T. I., Reed, D. A., Perring, T. M., Palumbo, J. C. (2014). Feeding damage by *Bagrada hilaris* (Hemiptera: Pentatomidae) and impact on growth and chlorophyll content of Brassicaceous plant species. *Arthropod-Plant Interactions,* 8*(*2), 89-100.

- Innocenzi, P. J., Hall, D. R., Cross, J. V., Masuh, H., Phythian, S. J., Chittamaru, S., Guarino, S. (2004). Investigation of long-range female sex pheromone of the European tarnished plant bug, *Lygus rugulipennis*: *chemical, electrophysiological, and field studies. Journal of Chemical Ecology*, 30(8), 1509-1529.

- Khrimian, A., Shirali, S., Vermillion, K. E., Siegler, M. A., Guzman, F., Chauhan, K., Aldrich, J. A., Weber, D. C. (2014a). Determination of the stereochemistry of the aggregation pheromone of harlequin bug, *Murgantia histrionica*. *Journal of Chemical Ecology*, 40(11-12), 1260-1268.

- Khrimian, A., Zhang, A., Weber, D. C., Ho, H. Y., Aldrich, J. R., Vermillion, K. E., Siegler, M.A., Shirali, S., Guzman, F., Leskey, T. C. (2014b). Discovery of the aggregation pheromone of the brown marmorated stink bug (*Halyomorpha halys*) through the creation of stereoisomeric libraries of 1-bisabolen-3-ols. *Journal of Natural Products*, 77(7), 1708-1717.

- Krupke, C. H., Brunner, J. F., Doerr, M. D., Kahn, A. D. (2001). Field attraction of the stink bug Euschistus conspersus (Hemiptera: Pentatomidae) to synthetic pheromone-baited host plants. *Journal of economic entomology*, *94*(6), 1500-1505.

- Leal, W. S., Ueda, Y., Ono, M. (1996). Attractant pheromone for male rice bug, *Leptocorisa chinensis*: semiochemicals produced by both male and female. *Journal of Chemical Ecology*, 22(8), 1429.

- Lockwood, J. A., Story, R. N. (1987). Defensive secretion of the southern green stink bug (Hemiptera: Pentatomidae) as an alarm pheromone. *Annals of the Entomological Society of America*, 80(5), 686-691.

- Marques, F. D. A., McElfresh, J. S., Millar, J. G. (2000). Female-produced sex pheromone of the predatory bug *Geocoris punctipes*. *Journal of Chemical Ecology*, 26(12), 2843-2855.

- McBrien, H. L., Millar, J. G., Rice, R. E., McElfresh, J. S., Cullen, E., Zalom, F. G. (2002). Sex attractant pheromone of the red-shouldered stink bug *Thyanta pallidovirens*: a pheromone blend with multiple redundant components. *Journal of Chemical Ecology*, 28(9), 1797-1818.

- Millar, J. G. (1997). Methyl (2*E*, 4*Z*, 6*Z*)-deca-2, 4, 6-trienoate, a thermally unstable, sex-specific compound from the stink bug *Thyanta pallidovirens*. *Tetrahedron Letters*, 38(46), 7971-7972.

- Millar, J. G. (2005). Pheromones of true bugs. In *The chemistry of pheromones and other semiochemicals II* (pp. 37-84). Springer, Berlin, Heidelberg.

- Millar, J. G., McBrien, H. L., Ho, H. Y., Rice, R. E., Cullen, E., Zalom, F. G., Uokl, A. (2002). Pentatomid bug pheromones in IPM: possible applications and limitations. *IOBC wprs Bulletin*, *25*(9), 241-250.

- Morrison III, W. R., Allen, M., Leskey, T. C. (2018). Behavioural response of the invasive Halyomorpha halys (Hemiptera: Pentatomidae) to host plant stimuli augmented with semiochemicals in the field. *Agricultural and forest entomology*, *20*(1), 62-72.

- Noge, K., Prudic, K. L., Becerra, J. X. (2012). Defensive roles of (E)-2-alkenals and related compounds in Heteroptera. *Journal of Chemical Ecology*, 38(8), 1050-1056.

- Ota, D., Čokl, A. (1991). Mate location in the southern green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae), mediated through substrate-borne signals on ivy. *Journal of Insect Behavior*, 4(4), 441-447.

- Palumbo, J. C., Natwick, E. T. (2010). The bagrada bug (Hemiptera: Pentatomidae): A new invasive pest of cole crops in Arizona and California. *Plant Health Progress*, 11(1), 50.

- Palumbo, J. C., Perring, T. M., Millar, J. G., Reed, D. A. (2016). Biology, ecology, and management of an invasive stink bug, *Bagrada hilaris*, in North America. *Annual Review of Entomology,* 61, 453-473.

- Panizzi, A. R. (1997). Wild hosts of pentatomids: ecological significance and role in their pest status on crops. *Annual Review of Entomology*, 42(1), 99-122.

- Pareja, M., Borges, M., Laumann, R. A., Moraes, M. C. (2007). Inter-and intraspecific variation in defensive compounds produced by five neotropical stink bug species (Hemiptera: Pentatomidae). *Journal of Insect Physiology*, 53(7), 639-648.

- Reed, D. A., Palumbo, J. C., Perring, T. M., May, C. (2013). *Bagrada hilaris* (Hemiptera: Pentatomidae), an invasive stink bug attacking cole crops in the southwestern United States. *Journal of Integrated Pest Management*, 4(3), C1-C7.

- Ruschioni, S., Riolo, P., Verdolini, E., Peri, E., Guarino, S., Colazza, S., Romani, R ., Isidoro, N. (2015). Fine structure of antennal sensilla of *Paysandisia archon* and electrophysiological responses to volatile compounds associated with host palms. *PloS One*, 10(4), e0124607.

- Ruther, J., Reinecke, A., Tolasch, T., Hilker, M. (2001). Make love not war: a common arthropod defence compound as sex pheromone in the forest cockchafer *Melolontha ippocastani*. *Oecologia*, 128(1), 44-47.

- Ryan, M. A., and Walter, G. H. 1992. Sound transmission in *Nezara viridula* (L.) (Heteroptera: Pentatomidae): Further evidence that signal transmission is substrate-borne. *Experientia* 48:1112– 1115.

- Sánchez-Peña, S. R. (2014). First record in Mexico of the invasive stink bug *Bagrada hilaris*, on cultivated crucifers in Saltillo. *Southwestern Entomologist*, 39(2), 375-378.

- Silva, C. C., de Capdeville, G., Moraes, M. C. B., Falcão, R., Solino, L. F., Laumann, R. A., Silva, J. P., Borges, M. (2010). Morphology, distribution and abundance of antennal sensilla in three stink bug species (Hemiptera: Pentatomidae). *Micron*, 41(4), 289-300.

- Sugie, H., Yoshida, M., Kawasaki, K., Noguchi, H., Moriya, S., Takagi, K., Fukuda, H., Fujiie, A., Yamanaka, M., Ohira, Y., Tsutsumi, T., Tsuda, K., Fukumoto, K., Yamashita, M., Suzuki, H. 1996. Identification of the aggregation pheromone of the brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). *Applied Entomology and Zoology,* 31(3), 427-431.

- Takita, M., Sugie, H., Tabata, J., Ishii, S., Hiradate, S. (2008). Isolation and estimation of the aggregation pheromone from *Eysarcoris lewisi* (Distant) (Heteroptera: Pentatomidae). *Applied Entomology and Zoology*, 43(1), 11-17.

- Torres-Acosta, R. I., Sánchez-Peña, S. R.,Torres-Castillo, J. A. (2017). Feeding by Bagrada Bug, *Bagrada hilaris*1, on Moringa oleifera (Brassicales: Moringaceae) in Mexico. *Southwestern Entomologist*, 42(3), 919-923.

- Ventura, M. U., Panizzi, A. R. (2005). Morphology of olfactory sensilla and its role in host plant recognition by *Neomegalotomus parvus* (Westwood) (Heteroptera: Alydidae). *Brazilian Archives of Biology and Technology*, 48(4), 589-597.

- Weber, D. C., Khrimian, A., Blassioli-Moraes, M. C., Millar, J. G. (2018). Semiochemistry of Pentatomoidea. *Invasive Stink Bugs and Related Species (Pentatomoidea): Biology, Higher*

Systematics, Semiochemistry, and Management, ed McPherson JE (CRC, Boca Raton, FL), 677-725.

- Weber, D. C., Walsh, G. C., DiMeglio, A. S., Athanas, M. M., Leskey, T. C., Khrimian, A. (2014). Attractiveness of harlequin bug, *Murgantia histrionica*, aggregation pheromone: Field response to isomers, ratios, and dose. *Journal of Chemical Ecology*, 40(11-12), 1251-1259.

- Weiss, I., Rössler, T., Hofferberth, J., Brummer, M., Ruther, J., Stökl, J. (2013). A nonspecific defensive compound evolves into a competition avoidance cue and a female sex pheromone. *Nature Communications*, 4, 2767.

Chapter 3

Novel diterpene hydrocarbons emitted by seedlings of *Brassica* species provide host location cues to *Bagrada hilaris*.

This chapter is based on the work contained in the following articles:

- Guarino, S., Arif, M. A., Millar, J. G., Colazza, S., Peri, E. (2018). Volatile unsaturated hydrocarbons emitted by seedlings of Brassica species provide host location cues to *Bagrada hilaris*. *PloS one*, 13(12), e0209870.
- Arriola, K., Guarino, S., Arif, M. A., Colazza, S., Peri, E., Millar, J. G. (2019) Identification of Brassicadiene, a Novel Diterpene Hydrocarbon Attractive to the Invasive Stink Bug *Bagrada hilaris,* from Volatiles of Cauliflower Seedlings, *Brassica oleracea* var botrytis. *Organic Letters,*(submited).

Chapter 3

Novel diterpene hydrocarbons emitted by seedlings of Brassica species provide host location cues to *Bagrada hilaris*

Abstract

Bagrada hilaris Burmeister, is a stink bug native to Asia and Africa and invasive in the United States, Mexico, and more recently, South America. This species can cause serious damage to various vegetable crops in the genus *Brassica*, with seedlings being particularly susceptible to *B. hilaris* feeding activity. In this study, the role of volatile organic compounds (VOCs) emitted by seedlings of three *Brassica* species on the host preference of *B. hilaris* was evaluated. In dual choice arena and olfactometer bioassays, adult Painted bugs preferred *B. oleracea* var. botrytis and *B. napus* over *B. carinata*. Volatiles from *B. oleracea* seedlings were collected and bioassayed with *B. hilaris* adults and late stage nymphs, using electroantennographic (EAG) and behavioral (olfactometer) techniques. When crude extracts of the VOCs from *B. oleracea* var. botrytis seedlings and liquid chromatography fractions thereof were bioassayed, *B. hilaris* adults and nymphs were attracted to the crude extract, and to a non-polar fraction containing hydrocarbons, whereas there were no responses to the more polar fractions. GC-MS analysis indicated that the main constituents of the non-polar fraction was an as yet unidentified diterpene hydrocarbon, with trace amounts of few other diterpene hydrocarbons. The major diterpene occurred in VOCs from both of the preferred host plants *B. oleracea* and *B. napus*, but not in VOCs of *B. carinata*. Using a combination of mass spectrometry, microchemical tests, and analysis of NMR spectra, the compound was identified as a novel tricyclic diterpene hydrocarbon and named brassicadiene. The results reported suggest that brassicadiene, alone or in combination with one or more of the minor compounds, is a key mediator in this insect-plant interaction, and could be a candidate for its use in lures for trapping *B. hilaris* in the field.

Key words: Painted bug; olfactometer; dual choice arena; EAG; diterpene hydrocarbon

3.1 Introduction

The Painted bug, *Bagrada hilaris* Burmeister, is an invasive stink bug which feeds mainly on brassicaceous hosts, and it seems particularly damaging to crops in recently invaded areas (Huang et al. 2014a). This species is widely distributed across Africa and southern Europe, and east through Pakistan, India, China, and parts of southeast Asia (Reed et al. 2013; Bundy et al. 2018). In 2008, the Painted bug was first reported in California, probably introduced by commercial trade, and it rapidly expanded its range to the brassicaceous crops of coastal California and central Arizona (Palumbo and Natwick, 2010), and then to Nevada, New Mexico, and Utah (Bundy et al. 2018). More recently, *B. hilaris* has been reported from Mexico (Sánchez-Peña, 2014), from the island of Maui in Hawaii (Palumbo et al. 2016), and in Chile (Torres-Acosta et al. 2017). *Bagrada hilaris* has had a major impact on agriculture in the Americas; it has been estimated that about 90% of the broccoli acreage planted in the USA has been infested by the Painted bug, with yield losses often exceeding 10% of production (Huang et al. 2014a). A recent study by Guarino et al. (2017a) showed that infestation of a cauliflower plant, *Brassica oleracea* var. botrytis L., by 40 adult Painted bugs reduces leaf photosynthesis by about 40%. To date, effective sampling strategies and good monitoring tools for this pest, for example based on semiochemicals, have not yet been developed (Bundy et al. 2018). Other stink bug species are currently monitored with traps baited with sex or aggregation pheromones (Cullen and Zalom, 2006; Leskey et al. 2015). In the case of *B. hilaris*, (*E*)-2-octenyl acetate was reported as a possible pheromone for this species (Guarino et al. 2008), but attempts to develop this compound into a reliable monitoring tool have not yet been successful (Bundy et al. 2018). Consequently, alternative lures based on host plant volatile organic compounds (VOCs) may be good candidates for use in monitoring traps. Host plant VOCs are known to be exploited by phytophagous pentatomid species as host location cues (Rather et al. 2010; Guarino et al. 2017b). However, information about the VOCs that might serve as host attractants for *B. hilaris* is limited (Guarino et al. 2017b). Recent electroantennographic studies showed that adults of this species perceive several plant VOCs, including octanal, nonanal, acetic acid, benzaldehyde, 3-butenyl isothiocyanate, and 4-pentenyl isothiocyanate (Palumbo et al. 2016; Guarino et al.

2017a). Among these chemicals, only isothiocyanates are characteristic to *Brassica* spp., and they are produced mainly upon damage to tissues rather than by undamaged plants (Guarino et al. 2017a; Mccormick et al. 2014; Chabaane et al. 2015). In field bioassays, *B. hilaris* were not consistently attracted to traps baited with various isothiocyanates, either singly or in blends (Palumbo et al. 2016), and to date, no consistently effective plant-derived attractants have been identified for this species (Bundy et al. 2018). Any search for candidate attractants for herbivorous pests should consider not only the preferred host plant species and their main VOCs, but also other plant traits, which may be indicators of host quality as food and/or shelter. These characteristics may change during plant development, and as a consequence, the attraction of *B. hilaris* may change with the development of its hosts (Boege, 2005).

In contrast to other stink bug specialists of Brassicaceae, such as *Murgantia histrionica* (Hahn), which prefers to feed on plants with at least 4-5 pairs of true leaves (White et al. 1933; Ludwig and Kok, 2001), the Painted bug appears to strongly prefer host plants at the stage of newly emerged seedlings (Huang et al. 2014b; Joseph et al. 2017). The feeding damage of *B. hilaris* on cotyledons and young tissues results in significant reductions in leaf area, chlorophyll content, and dry weight, if it does not kill the seedling outright (Fig. 1) (Huang et al. 2014a; Sánchez-Peña, 2014). This suggests that VOCs produced by seedlings of preferred *B. hilaris* host plants are likely to be particularly attractive to the bugs. Further clues as to the identities of host-produced attractants can be obtained by comparing the profiles of VOCs produced by more or less susceptible brassicaceous species and varieties. The identification of these VOCs could have several major benefits. First, it will lead to a better understanding of the ecology of *B. hilaris*, and the factors mediating its host selection behaviors. Second, given the demonstrated strong attraction of *B. hilaris* to *Brassica* seedlings, the attractant chemicals would be excellent candidates for development into tools for monitoring and attract-and-kill strategies. Third, identification of the attractants could inform traditional or molecular biological plant breeding efforts to eliminate the attractants from the host plant VOCs. Thus, the goal of this study was to characterize the volatile cue(s) exploited during host location by *B. hilaris*. Our specific objectives were:

1. Among possible host plants in the *Brassica* genus, to identify species that differed in attractiveness at the seedling stage to *B. hilaris* nymphs and adults;

2. To prepare active extracts of one or more attractive species;

3. To use bioassay-driven fractionation to individuate the attractant(s) present in extracts from the attractive species;

4. To purify and characterize the molecules of the active fraction(s).

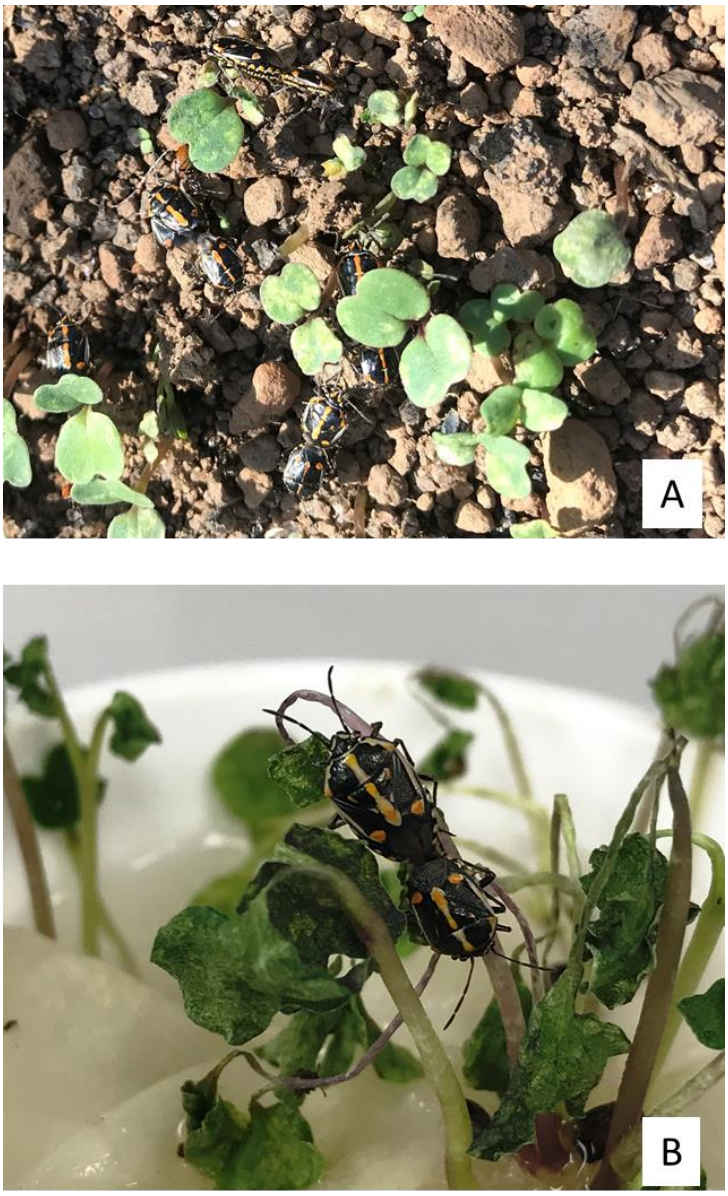

Figure 1. Feeding damage of *B. hilaris* on young seedlings (A = in the field, B= in the laboratory).

3.2 Material and methods

3.2.1 Insects

The colony of *B. hilaris* was established and restocked regularly with individuals collected from caper (*Capparis spinosa* L.) fields on the island of Pantelleria (Italy), with permission from the owner of the land. Insects were reared in an environmentally controlled room (30 ± 2 °C, 70 ± 10% RH, photoperiod 16L:8D), in wooden cages (40 x 25 × 25 × 40 cm) with two 5-cm diameter

mesh-covered holes for ventilation. The colony was fed with cauliflower and cabbage plants, depending on seasonal availability. Because *B. hilaris* lays eggs in the soil, dishes (6-cm Ø) with a mixture of sand, silt, and clay (33% for each soil component) were placed in the cages as oviposition sites. Dishes were changed weekly, and those with eggs were kept in separate cages until the emergence of nymphs. The nymphs were then kept in separate cages until the final molt to adults. Adults and 4^{th}-5^{th} instar nymphs were used separately in experiments.

3.2.2 Seedlings

Seeds of *B. oleracea* var. botrytis (cauliflower), *B. napus* (rapeseed), and *B. carinata* (Abyssinian cabbage), all obtained from a local market (Palermo – Italy), were placed on cotton wool (10 g) soaked with distilled water and held in glass containers with a distance of circa 0.5 cm between seeds. The containers were placed in an environmentally controlled growth chamber (25 ± 1 °C, 70 ± 10% RH, photoperiod 16L:8D) equipped with lights with a photosynthetic flux density (PPFD) of 600 mol photons m^{-2} s^{-1} placed above the foliage. To avoid desiccation, soaked seeds were covered with a Petri dish for 3 d until they germinated (Fig. 2). After 7 d, newly emerged seedling clusters at the cotyledon stage were wrapped around the base with aluminum foil for use in host preference bioassays, VOC collection, or behavioral experiments.

Figure 2. Seedlings 7 days old.

3.2.3 Dual choice arena

The dual choice arena consisted of a Plexiglas cage 50 cm wide, 30 cm high, and 30 cm deep with mesh for ventilation on opposing sides. Inside the cage, two clusters of 7-d old seedlings of the different plant varieties (N = 20) were placed 20 cm apart (Fig. 3). All three possible combinations of pairs of the three plant species were tested. Bioassays were carried out in static air, using in parallel 6 cages for each session of experiments. Adult bugs were released individually into each cage at 2:00 PM for each replicate and as a response the presence of the bug in one of the seedlings cluster was recorded after 20h, at 10:00 AM the next day (final choice). The position of the treatments was alternated between replicates. After each replicate, the Plexiglas cage was cleaned with water and dried. Bioassays were conducted under ambient laboratory temperature and humidity conditions (25 ± 3 °C, and 50 ± 15% RH).

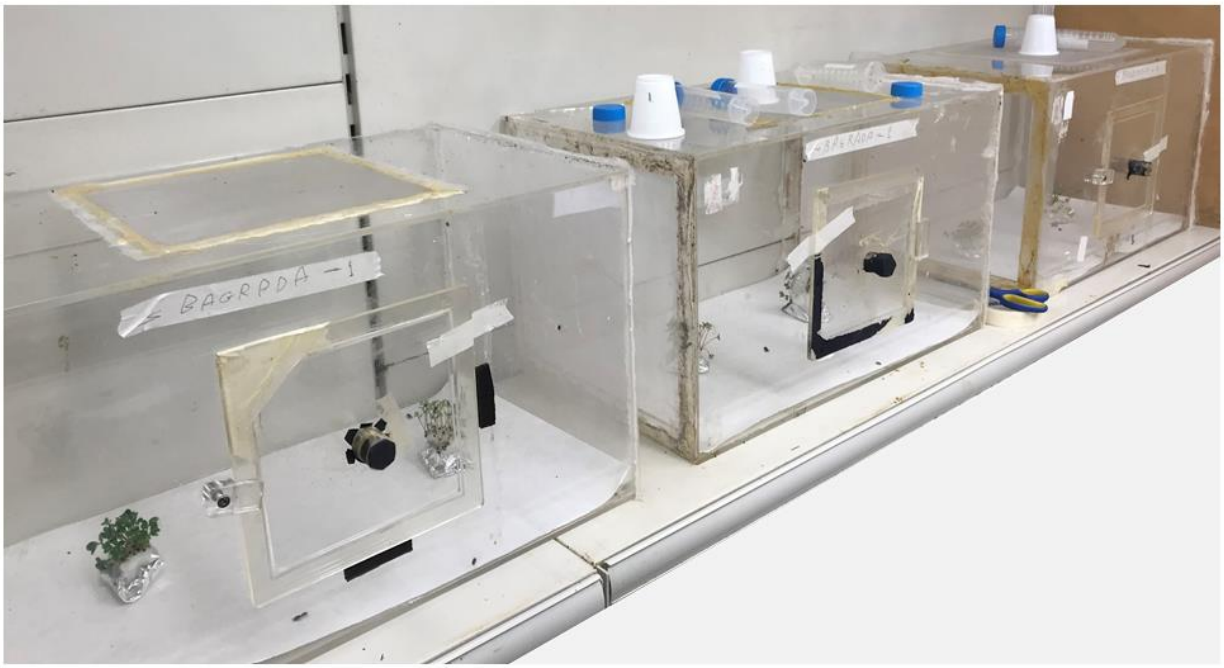

Figure 3. Plexigas cages used in dual choice arena bioassay.

3.2.4 Open Vertical Y-shaped olfactometer

Further bioassays were carried out with an open vertical Y-shaped olfactometer consisting of three connected brass rods (left and right arms 20 cm long, central arm 25 cm long, 1 cm diameter). The left and right arms were covered with two glass tubes (18 cm long, 5 cm diameter) terminating in hose nipples. Charcoal-purified air (0.2 l/min per chamber) was directed through two glass chambers (125 ml each), which held the test stimuli, consisting of seedling clusters (N=50), or extracts of headspace volatiles released by seedlings (see below). The outlets of the stimulus chambers were connected to the hose nipples on the respective arms of the olfactometer with Tygon tubing. Light was provided with a halogen lamp (Osram, 12V–35W, Münich, Germany) hanging 30 cm above the olfactometer (Fig. 4). Experiments were carried out under ambient laboratory temperature and humidity conditions (25 ± 3 °C, and 50± 15% RH). For each replicate, a single adult or nymph was gently placed at the bottom of the central arm of the

olfactometer with a paintbrush and allowed 10 min to respond. The bugs moved from the bottom upward toward the light source and upon arriving at the Y junction, choose between the two different volatile stimuli. The criterion for a response was that the test bug walked onto the test arm or the control arm at least 5 cm past the Y junction (first choice). Bugs that did not move onto one of the two arms during the 10 min trial were scored as non-responders and were not included in the analysis. After 8 replicates, the glass parts of the apparatus were washed with water and detergent, and then wiped with acetone and the brass rods were cleaned with distilled water and acetone and baked at 200°C for 60 min.

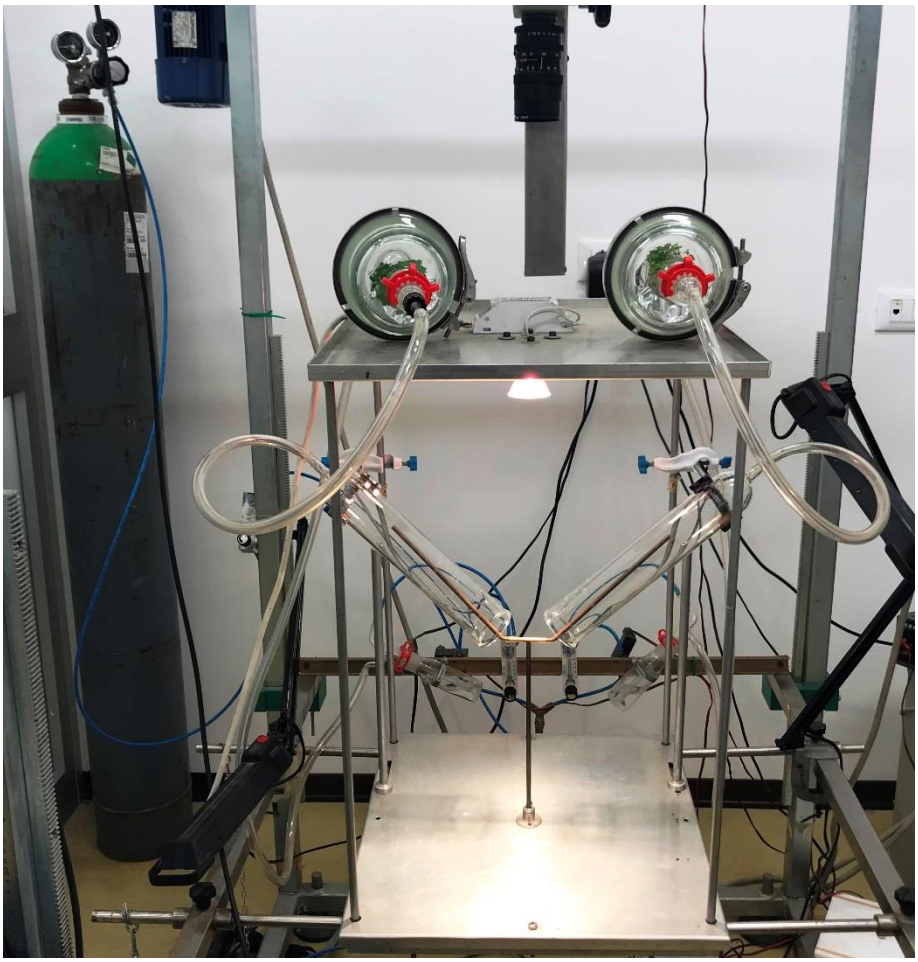

Figure 4. Open Vertical Y-shaped olfactometer.

3.2.5 Electroantennography

Electroantennograms (EAG) were conducted using an aliquot of 2 µl of each test or control stimulus solution pipetted onto a piece of filter paper (Whatman, grade 1). The loaded paper was exposed to the air for 30s to allow the solvent to evaporate, and then inserted into a glass Pasteur pipette. Puff stimuli were blown into an airstream that passed over the antennal preparation using a flow controller (model CS-05; Syntech, Hilversum, the Netherlands) to generate a 1.5-s stimulus at 1-min intervals, with a flow rate of 1.5 l min^{-1}. The signals generated by the antennae were passed through a high-impedance amplifier (model IDAC-4, Syntech) and recorded with custom software (Syntech). For the EAG preparations, adult and nymphs *B. hilaris* were anesthetized by refrigerating them at about −4°C for 40s, the head was excised and mounted on a glass capillary reference electrode (1.5 mm diameter) filled with 0.1 M KCl solution and connected with silver wire to the amplifier. The recording electrode was a similar glass capillary in contact with the tip of an antenna. The capillary tubes were drawn to a fine point using a microelectrode puller (Narishige PC-10, Tokyo, Japan) to achieve a diameter enabling insertion into the antennal tip.

3.2.6 VOC collection and fractionation

VOC collections were carried out using clusters of 7-d-old seedlings (N = 500) of *B. oleracea* var. botritys, *B. carinata,* and *B. napus*. Seedling clusters were placed in a cylindrical glass chamber (3 l volume) and a stream of air purified by passing through a filter of activated charcoal (0.5-1.0 mm, 18-35 mesh, Merck, Darmstadt, Germany), was pumped through the chamber at 400 ml/min. A glass tube containing a plug of 100 mg of Porapak Q (80–100 mesh; Sigma-Aldrich) was used to collect the VOCs (Fig. 5). After collecting for 20 h, the traps were eluted with 1 ml 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 (GC/MS) analyses and bioassay experiments. All replicates were carried out in a temperature-controlled room (25 ± 3°C, 60 ± 5% RH and photoperiod 16L: 8D). After each collection, the chamber was washed

with water and fragrance-free detergent, rinsed with acetone, and baked overnight at 150 °C. Blank aerations were carried out as controls to identify possible system contaminants.

Aliquots of 1 ml of VOC extracts from seedlings (ten aerations pooled together) were fractionated by liquid chromatography into non-polar and polar fractions by using a solid phase extraction cartridge (SPE, silica column DSC-SI 52652-U, 1 ml tube, Supelco) (Fig. 6). The silica gel cartridge was conditioned by rinsing with 1 ml of hexane. The extract was loaded onto the cartridge as a hexane solution, followed by elution with 1 ml of hexane to obtain a non-polar hydrocarbons fraction. The cartridge then was eluted with 1 ml of dichloromethane and 1 ml of ethyl alcohol to extract the medium and high polarity compounds respectively. The fractions were kept at -20 C° until used for GC/MS analyses or electrophysiological and behavioral bioassays.

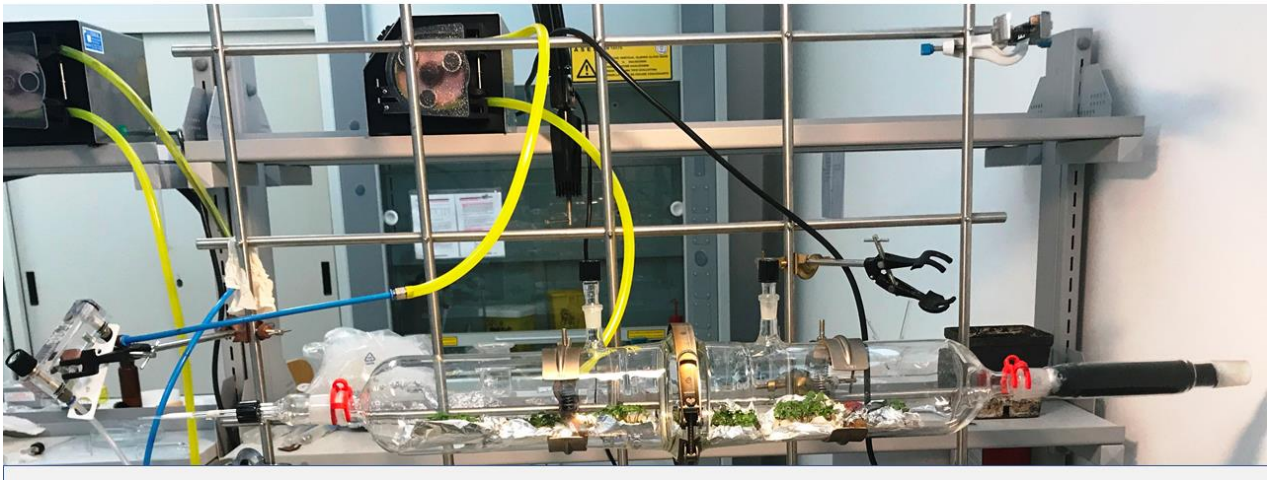

Figure 5. VOCs collection system.

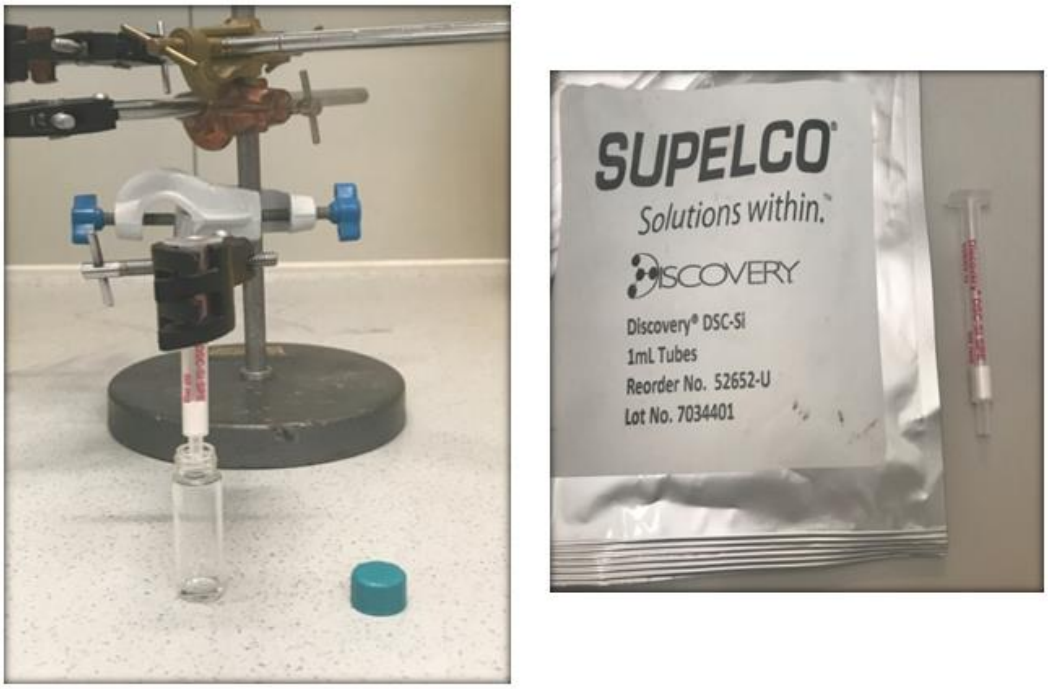

Figure 6. Solid phase extraction cartridge (SPE, silica column DSC-SI 52652-U, 1 ml tube, Supelco).

3.2.7 Behavioral bioassays

Host preference bioassays. This experiment was carried out to assess the host preference of *B. hilaris* adults (sex ratio 1:1) to different potential host plants in two-choice tests using both the dual choice arena and the open vertical Y-shaped olfactometer. The experimental design was: *B. oleracea* var. botritys vs *B. carinata*, *B. napus* vs *B. carinata*, *B. oleracea* var. botritys vs *B. napus*. The number of replicates for each experiment was respectively 88, 85 and 106 in the dual choice arena and 120 in the open vertical Y-shaped olfactometer.

Bioassays with B. oleracea var. botrytis VOCs. In this experiment, volatiles from *B. oleracea* var. botrytis were bioassayed with adults and nymphs using EAG recording and open vertical Y-shaped olfactometer tests. EAG experiments tested 2 µl of the VOC crude extract, corresponding to approximately 200 seedling-hours, as a stimulus, versus solvent controls (hexane) (N = 20

nymphs; N = 15 adults). In a second experiment, responses elicited by the non-polar, medium polarity, and high polarity fractions were compared, with values normalized by subtracting the response elicited from the respective solvent control (hexane, dichloromethane, or ethyl alcohol) (N = 20 nymphs; N = 18 adults).

In the Y-shaped olfactometer, VOCs emitted from *B. oleracea* var. botrytis were tested according to the following design: 1) seedlings of *B. oleracea* var. botrytis (test) vs air (control) (N = 116 nymphs; N = 133 adults); 2) VOC extracts, corresponding to 200 seedling-hours, (test) vs hexane (control) (N = 188 nymphs; N = 200 adults); 3) Non-polar fraction (test) vs hexane (control) (N = 159 nymphs; N = 164 adults; 4) Medium polarity fraction (test) vs dichloromethane (control) (N = 161 nymphs; N = 169 adults); 5) Highly polar fraction (test) vs ethyl alcohol (control) (N = 160 nymphs; N = 160 adults).

3.2.8 Chemical analysis

Coupled gas chromatography-mass spectrometry (GC-MS) analyses of the headspace extracts from *B. oleracea* var. botrytis, *B. napus,* and *B. carinata* were performed on an Agilent 6890 GC system interfaced with an MS5973 quadruple mass spectrometer (Fig. 7). One µl of extract was injected onto a DB5-MS column 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 was set at 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. Four replicates were carried out for each plant species.

An aliquot of a VOC extract in hexane was reduced by stirring with 5% Pd on charcoal catalyst under a hydrogen atmosphere for 1 h. After filtration through a plug of celite to remove the catalyst, the solution was analyzed by GC-MS as described above.

Figure 7. Gas chromatography-mass spectrometry (GC-MS).

3.2.9 Chemical identification

As the main compound found in the extract was a diterpene not matching with NIST11 spectra. A set of analysis was developed to identify this putative new compound. In order to have a larger sample of the compound, indispensable for identification operations, the diterpene compound was isolated from a composite crude extract, obtained by combining multiple VOC collections from *B. oleracea* var botrytis seedlings (Fig. 8).

In the specific, to isolate the diterpene, a composite sample from 200 VOC collections was concentrated to ~0.2 ml under a stream of nitrogen. A 12 mm diameter sintered glass funnel was loaded with 3 g of 230-400 mesh silica gel, oven dried at 120°C overnight and cooled in a sealed tube. The cooled silica gel was wetted with pentane, and the concentrated composite sample of VOC collections was loaded onto the column, rinsing it 3 times with 0.2 ml pentane. The column was then eluted with 18-1 ml aliquots of pentane, pulling the solvent through the bed with mild suction. The diterpene began eluting in the 8th fraction, with fractions 9-14 being >90% pure, and residual amounts eluting in fractions 15 and 16. Fractions 9-14 were combined and concentrated just to dryness under a gentle stream of nitrogen, and 0.2 ml of deuterated methylene chloride

(99.96% D, Aldrich Chem. Co., Minneapolis WI, USA) was added and blown down just to dryness. The process was repeated, and the residue was then dissolved in 20 µl CD_2Cl_2 and transferred to a 1 mm diameter microbore NMR tube (Bruker Biospin Corp., Fallenden, Germany). NMR spectra (^1H, ^{13}C, ^1H-^1H gCOSY, ^1H-^1H DQFCOSY, ^1H-^1H gHMBC, ^1H-^{13}C gHSQC, gNOESY, TOCSY) were taken on a Bruker Avance III 700 MHz spectrometer. Mass spectra were taken with a Hewlett-Packard 5890 GC interfaced to a 5973 mass selective detector, with electron impact ionization (70 eV). For further details about all the steps that were used during the identification process see Arriola et al. (2019).

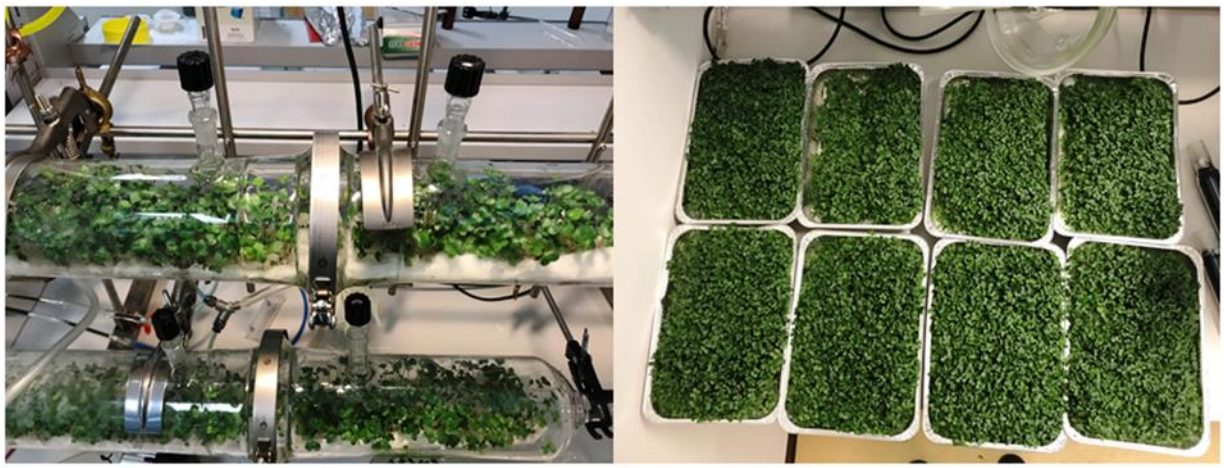

Figure 8. Multiple VOC collections from *B. oleracea* var botrytis seedlings.

3.2.10 Statistical analysis

Mean values of the antennal depolarization responses elicited by the various test stimuli in EAG trials with *B. hilaris* antennae were prior examined for normality distribution by Shapiro-Wilk test and then analyzed by one-way ANOVA followed by Tukey's test. Data from dual choice arena and open vertical Y-shaped olfactometer experiments, respectively, comparing the final and the first choice between the test and control, were analyzed with χ^2 tests. All the statistical analyses were performed using Statistica 7.0 for Window (Statsoft 2001, Vigonza, PD, Italy).

3.3 Results

3.3.1 Host preference bioassays

In dual choice arena bioassays (Fig. 9), *B. hilaris* adults preferred *B. oleracea* var. botrytis over *B. carinata* (χ^2 = 11.63, df = 1, *P* < 0.01, N = 88), and *B. napus* over *B. carinata* (χ^2 = 6.22, df = 1, *P* < 0.01, N = 85). Adults exhibited no significant preference between *B. oleracea* var. botrytis and *B. napus* (χ^2 = 1.84, df = 1, *P* = NS, N = 106).

In open vertical Y-shaped olfactometer bioassays (Fig. 10), *B. hilaris* adults also were more strongly attracted to *B. oleracea* var. botrytis than *B. carinata* VOC (χ^2 = 8.53, df = 1, *P* < 0.01 N = 120), and to *B. napus* than to *B. carinata* (χ^2 = 10.80, df = 1, *P* < 0.01, N = 120). However, adult bugs were equally attracted to volatiles from *B. oleracea* var. botrytis and *B. napus* (χ^2 = 2.27, df = 1, *P* = NS, N = 120).

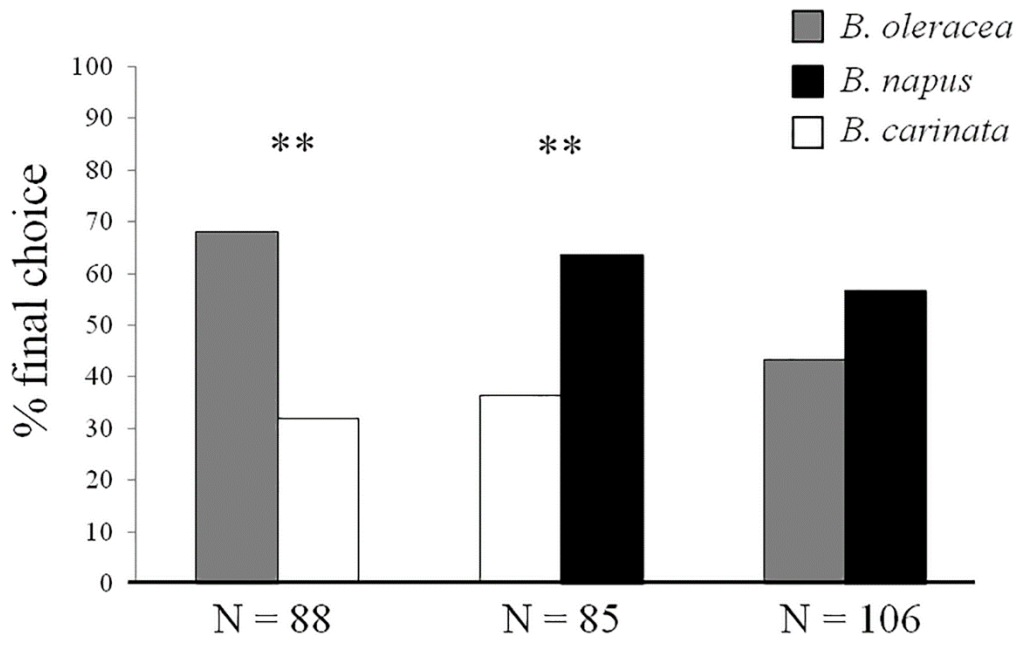

Figure 9. Dual choice arena bioassays: Host preference responses (% final choice) in of *B. hilaris* adults to seedlings of *B. oleracea* var. botrytis, *B. carinata*, and *B. napus*. N = number of replicates; ** = P < 0.01.

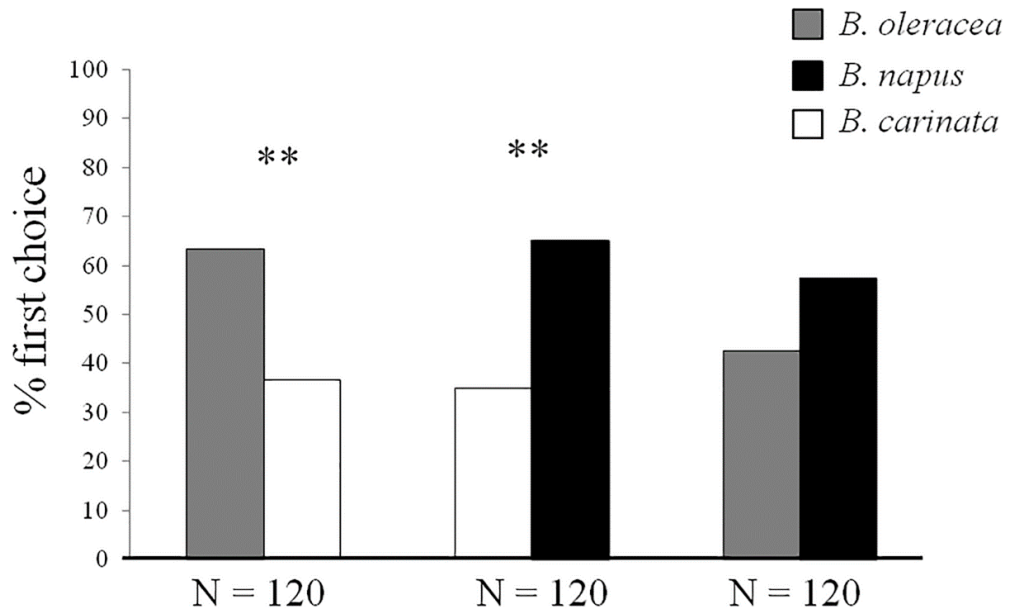

Figure 10. Y-shaped olfactometer bioassays: Host preference responses (% first choice) in of *B. hilaris* adults to seedlings of *B. oleracea* var. botrytis, *B. carinata*, and *B. napus*. N = number of replicates; ** = P < 0.01.

3.3.2 Bioassays with *B. oleracea* var. botrytis VOCs

The crude VOC extract of *B. oleracea* var. botrytis elicited significantly higher EAG responses from the antennae of *B. hilaris* nymphs (F =12.61, df = 1, $P < 0.001$, N = 20, ANOVA) (Fig. 11A) and adults (F =35.91, df= 1, $P < 0.0001$, N = 15, ANOVA) (Fig. 11B) than the controls. Significantly different responses were also recorded from the different fractions of the VOC extract: the non-polar fraction elicited stronger responses than the intermediate and highly polar fractions from the antennae of 4th and 5th instar nymphs (F = 21.05, df = 2, $P < 0.05$, N = 20, ANOVA) (Fig. 12A) and adults (F = 14.39, df = 2, $P < 0.0001$, N = 18, ANOVA) (Fig. 12B).

Responses of *B. hilaris* adults and nymphs in open vertical Y-shaped olfactometer bioassays are shown in Figs. 13-14. Seedlings of *B. oleracea* var. botrytis were significantly more attractive than controls (clean air) for 4th and 5th instar nymphs (χ^2 = 8.82, df = 1, $P < 0.05$, N = 116) and for adults (χ^2 = 9.21, df = 1, $P < 0.01$, N = 133). Similarly, the crude extract of VOCs from *B. oleracea* var. botrytis seedlings attracted both nymphs (χ^2 = 11.25, df = 1, P = 0.01, N= 188) and adults (χ^2 = 4.50, df = 1, $P < 0.05$, N = 200) in comparison to controls. When fractions of the crude extract

were tested, bugs were significantly attracted only to the non-polar fraction eluted with hexane (hexane fraction versus solvent control, nymphs, χ^2 = 3.93, df = 1, P < 0.05, N= 159, adults, χ^2 = 6.24, df = 1, P < 0.01, N = 164). Neither of the more polar fractions was significantly more attractive than the solvent controls to nymphs (dichloromethane fraction, χ^2 = 0.05, df = 1, P = NS, N = 161; ethyl alcohol fraction, χ^2 = 0.90, df = 1, P = NS, N = 160) or adults (dichloromethane fraction, χ^2 = 1, df = 1, P = NS, N = 169; ethyl alcohol fraction, χ^2 = 0.62, df = 1, P = NS, N = 160).

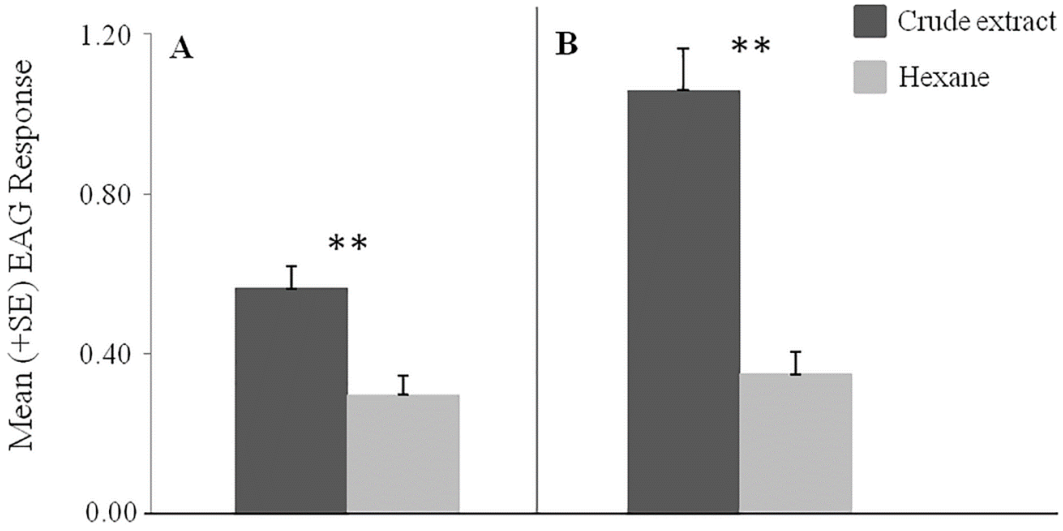

Figure 11. Electroantennogram responses (mV) (mean + SE) of *B. hilaris* 4th and 5th instar nymphs (N = 20) (A) and adults (N = 15) (B), to crude extracts of VOCs from *B. oleracea* var. botrytis seedlings; ** = P < 0.01; one-way ANOVA followed by Tukey's test.

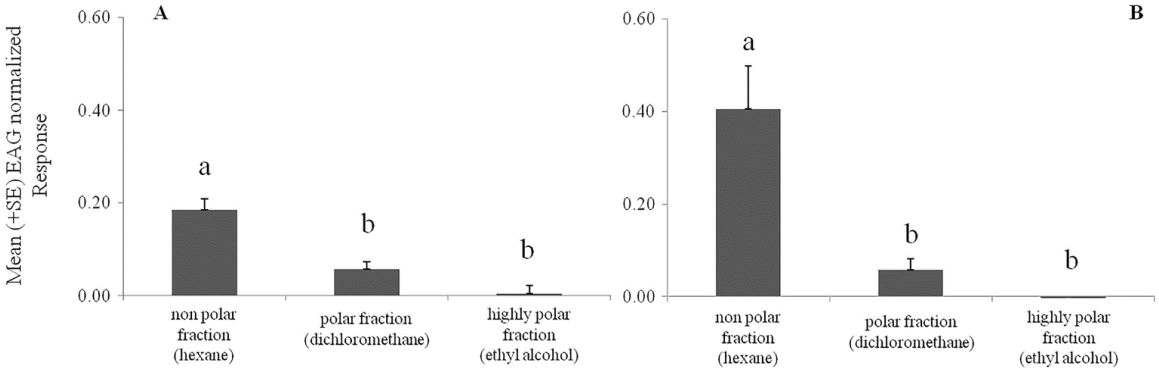

Figure 12. Electroantennogram responses (mV) (mean + SE) of *B. hilaris* 4th and 5th instar nymphs (N = 20) (A) and adults (N = 18) (B), to fractions of crude extracts of VOCs from *B. oleracea* var. botrytis seedlings; Different letters indicate that values differ statistically at P < 0.05; one-way ANOVA followed by Tukey's test.

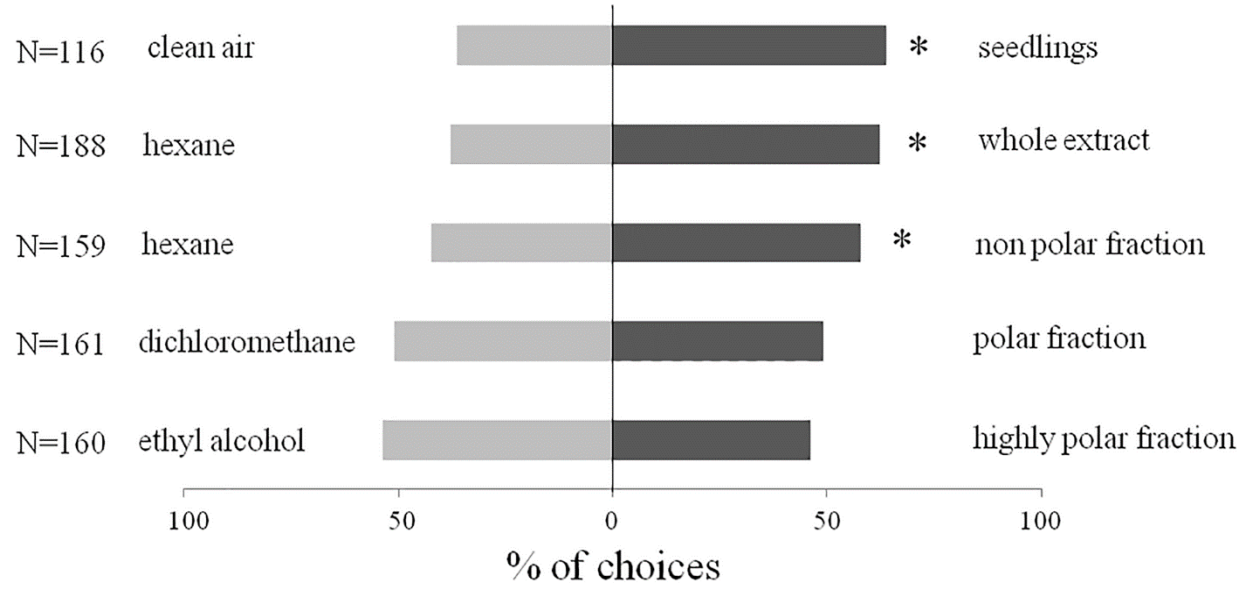

Figure 13. Responses (% first choice) *B. hilaris* 4th and 5th instar nymphs to *B. oleracea* var. botrytis seedlings, to crude extracts of seedling VOCs, and to fractions therein, versus controls, in open vertical Y-shaped olfactometer bioassays. N = number of replicates; * = $P < 0.05$.

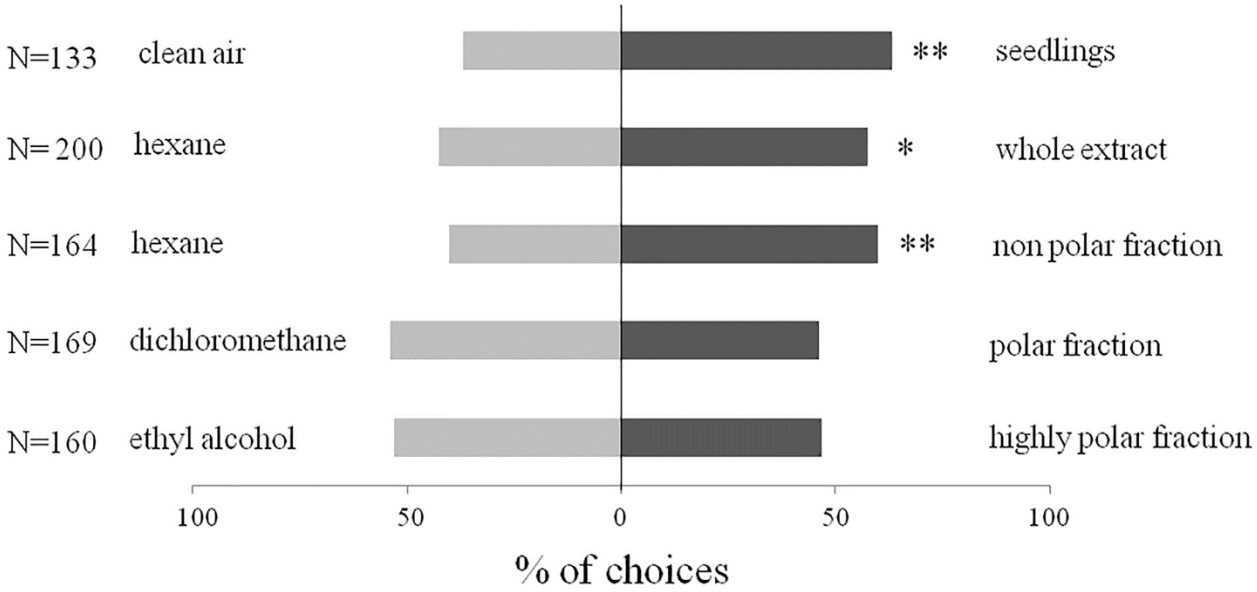

Figure 14. Responses (% first choice) *B. hilaris* adults to *B. oleracea* var. botrytis seedlings, to crude extracts of seedling VOCs, and to fractions of the VOC extract, versus controls, in vertical Y-shaped olfactometer bioassays. N = number of replicates; * = $P < 0.05$, ** = $P < 0.01$.

3.3.3 Chemical analysis

Representative chromatograms of VOCs collected from *B. oleracea* var. botrytis, *B. carinata*, and *B. napus* seedlings are shown in Fig. 15. One major and four minor compounds were observed in the VOCs of *B. oleracea* var. botrytis and *B. napus* that were not detected in VOCs of *B. carinata*. A crude VOC extract of *B. oleracea* var. botrytis was fractionated by liquid chromatography on silica gel, eluting sequentially with hexane, dichloromethane, and ethanol, providing fractions of low, medium, and high polarity. The five compounds all eluted in the hexane fraction, indicating that they were hydrocarbons. The compounds were tentatively identified as diterpene hydrocarbons based on their molecular weights of 272 daltons, corresponding to molecular formulae of $C_{20}H_{32}$. The most abundant compound had a Kovats retention index of 1989 (DB5). Catalytic reduction with Pd on carbon produced a single compound with molecular weight 276, indicating the parent compound likely had two C=C double bonds, and hence, three rings to account for the three remaining sites of unsaturation. The mass spectra of the parent compound (Fig. 16) and the product from catalytic reduction (Fig. 17) were both dominated by a base peak from loss of 43 mass units, indicating the presence of an isopropyl group that was readily lost during fragmentation. The mass spectrum of the parent compound produced no reasonable matches with any structures in the NIST 11 Mass Spectral Database. Similarly, a search of all 996 structures in Chemical Abstracts with a molecular formula of $C_{20}H_{32}$, two double bonds, three rings, and an isopropyl group, failed to turn up any candidate structures whose mass spectra were a reasonable match to that of the major compound. The major component has now been isolated in microgram quantities by a combination of liquid and preparative gas chromatography and subjected to microbore NMR spectrometry.

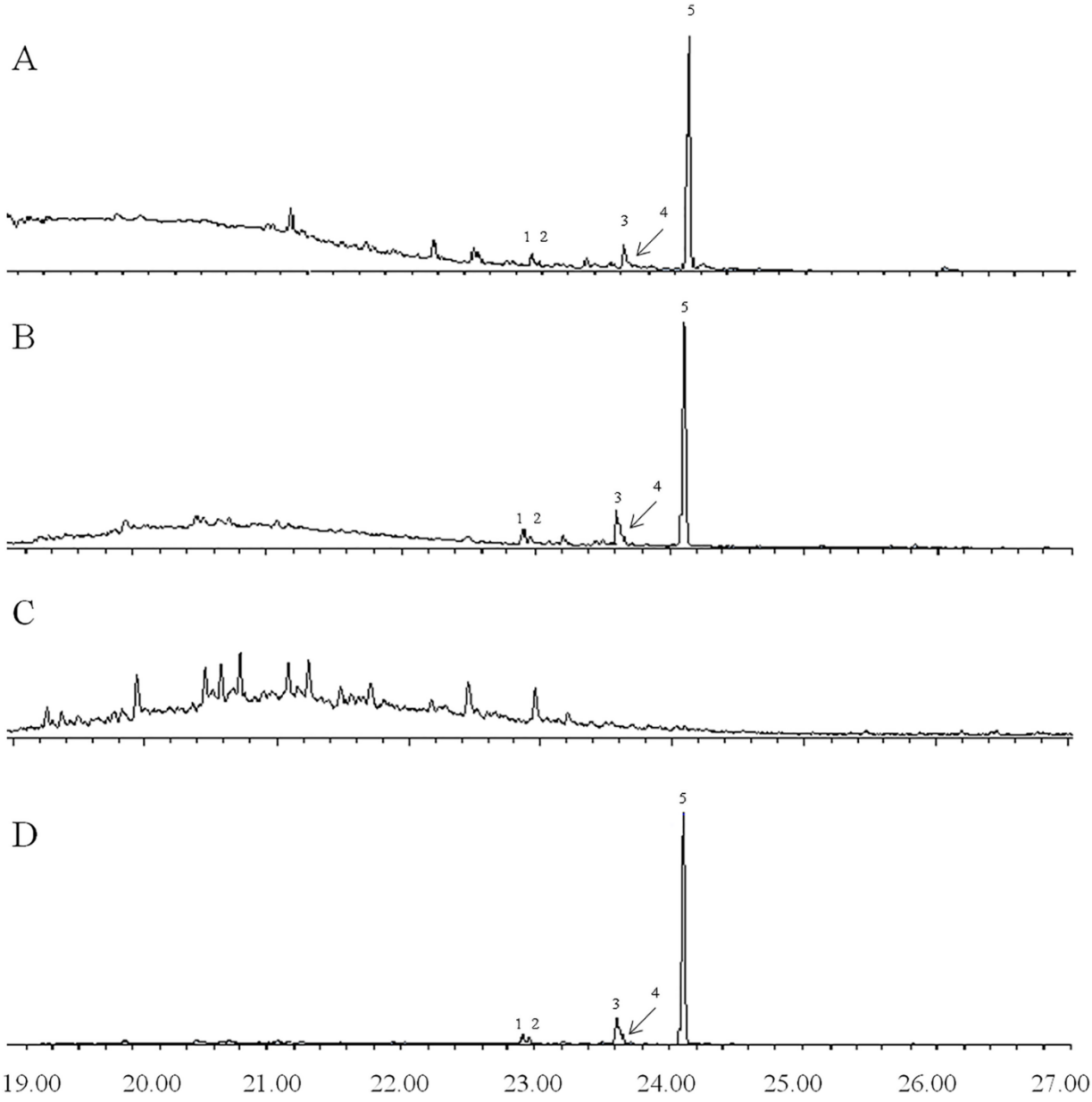

Figure 15. Representative gas chromatograms of VOCs from *B. oleracea* var. botrytis. (A), *B. napus* (B), *B. carinata* (C), and *B. oleracea* var. botrytis non-polar fraction (D). The peak numbers indicate the diterpene hydrocarbons detected in the analysis.

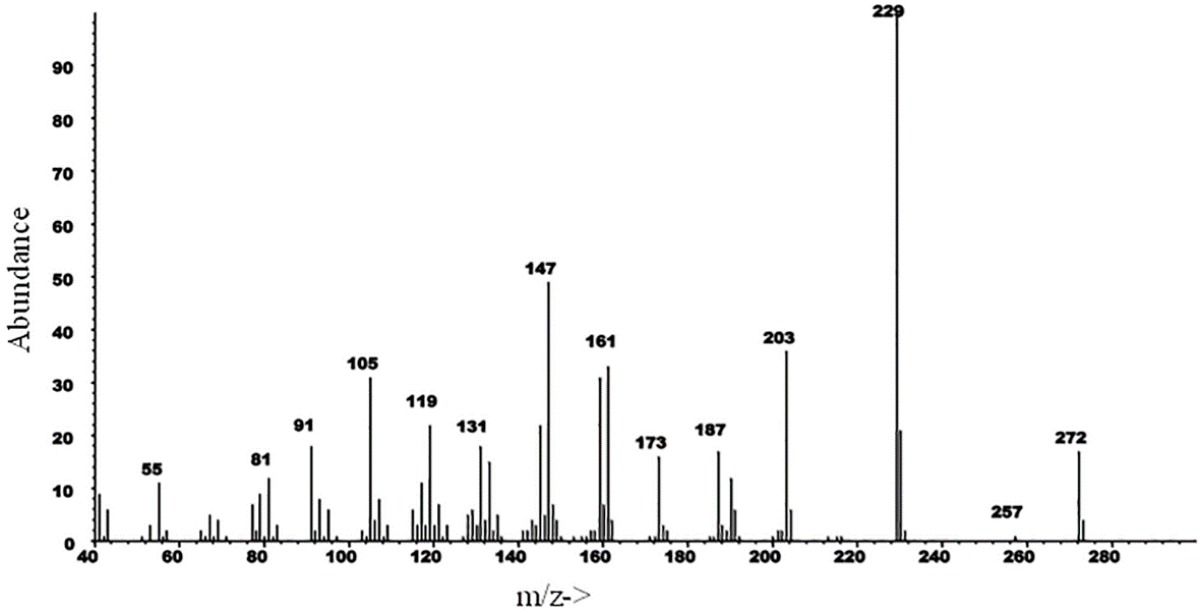

Figure 16. Electron ionization mass spectrum of the major compound in VOC extracts from *B. oleracea* var. botrytis.

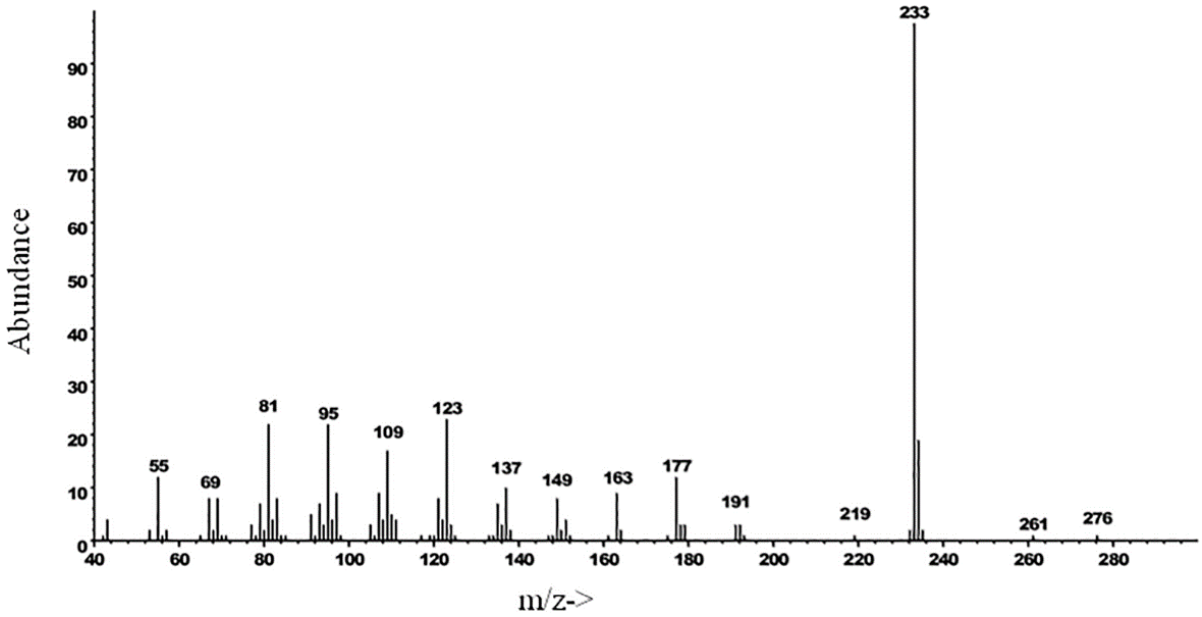

Figure 17. Electron ionization mass spectrum of the product from catalytic reduction of the major compound in VOC extracts from *B. oleracea* var. botrytis.

3.3.4 Chemical identification

The major component, isolated in microgram quantities by a combination of liquid and preparative gas chromatography and subjected a combination of mass spectrometry, microchemical tests, and analysis of NMR spectra the compound, was identified as a novel tricyclic diterpene hydrocarbon. This novel compound was named brassicadiene as was isolated from seedings of *B. oleracea* (Fig. 18).

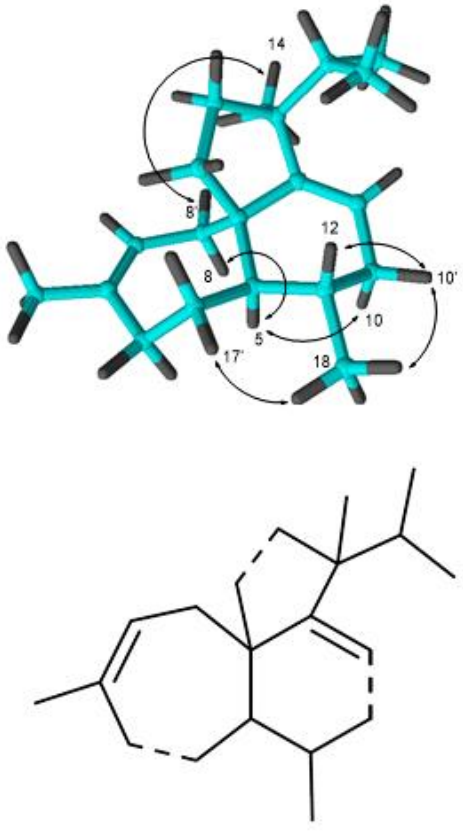

Figure 18. Model of Brassicadiene.

3.4 Discussion

The results described above provide strong evidence that host plant VOCs are exploited by both nymphs and adults of *B. hilaris* in location and possibly acceptance of their host plants. The dual choice arena bioassay results indicated that some *Brassica* species are preferred over others, with *B. hilaris* adults preferentially orientating toward seedlings of *B. oleracea* var. botrytis and *B. napus* rather than *B. carinata*. These results were confirmed by the olfactometer bioassays, which suggested that attraction to preferred hosts is primarily mediated by olfactory rather than visual cues. Other recent studies with *B. hilaris* had demonstrated that this species was attracted to seedlings of several brassicaceous species, including arugula (*Eruca sativa* L.), turnip (*B. rapa* L. var. rapa), mizuna (*B. rapa* L. nipposinica), kale (*B. oleracea* L. acephala), choi (*Brassica rapa* L. var. chinensis), broccoli (*B. oleracea* L. var. italica), cauliflower (*B. oleracea* L. var. botrytis), and to a lesser extent sweet alyssum (*Lobularia maritima* L.), as well as non-brassicaceous seedlings of species such as lettuce (*Lactuca sativa* L.) (Joseph et al. 2017; Huang et al. 2014b).

However, the present study is the first to show a direct relationship between host plant VOCs and Painted bug host preference via the demonstrated attraction of Painted bugs to extracts of VOCs. Analogous results have been observed with other pests of *Brassica* spp. plants (Himanen et al. 2017). Bioassays with fractions of the crude extract of *B. oleracea* var. botrytis VOCs then showed that the attraction of *B. hilaris* was mediated by one or more compounds contained in the non-polar fraction of the extract, consisting only of hydrocarbons.

In parallel with the behavioral trials, EAG recordings showed that the crude extract of volatiles from *B. oleracea* var. botrytis seedlings elicited significant responses from the antennae of *B. hilaris* adults and 4^{th}-5^{th} instar nymphs. Further EAG recordings then localized the activity to the non-polar fraction of the VOC extract, with no significant responses being elicited by either of the more polar fractions.

GC-MS analyses of the crude VOC extracts showed that the VOCs emitted by *B. oleracea* and *B. napus* seedlings were quite similar, whereas the VOCs of *B. carinata* seedlings were markedly different. Surprisingly, the common green leaf volatiles and monoterpenes observed in other

studies of *B. oleracea* var. botrytis volatiles using plants 4-5 weeks old or older (Guarino et al. 2017a; Conti et al. 2008; Bruinsma et al. 2009) were not observed in the VOCs from seedlings. This result suggests that plants at the seedling stage, with only cotyledon leaves, emit VOCs quite different from older plants with true leaves. Rather, the VOCs from seedlings of *B. oleracea* var. botrytis and *B. napus* were dominated by a single diterpene hydrocarbon, with lesser amounts of several analogs. The mass spectra of these diterpenes did not match any spectra in the NIST 11 mass spectral database or any published spectra of diterpenes with three rings, two double bonds, and an isopropyl group, all these information suggested that the diterpenes represented novel compounds. The main efforts were than focused on the characterization of the main diterpene through a combination of techniques that lead to describe a new compound, named brassicadiene to recall the plants where it was observed for the first time.

Several diterpenes recently have been reported from the roots of the brassicaceous plant *Arabidopsis thaliana* L., but because of the difficulty in identifying the complex, often multicyclic structures of these types of compounds, only a few of these compounds were fully characterized (Vaughan et al. 2013; Wang et al. 2016). Because the brassicadiene detected in this study constitute > 95% of the non-polar fraction of the VOCs of *B. oleracea* var. botrytis seedlings that attracted and elicited EAG responses from *B. hilaris* in our study, it seems likely that this may be the key compound exploited by *B. hilaris* in its strong attraction to seedlings of its host plants. However, at present, it is not possible to exclude the possibility that other hydrocarbons present in minor amounts in the non-polar fraction may be partly or wholly responsible for the activity seen.

The possibility that *B. hilaris* may exploit brassicadiene for host location is interesting, given that these diterpenes are generally thought to contribute to plant chemical defenses (Vaughan et al. 2013; Gershenzon and Dudareva, 2007). For example, the protective role of plant diterpenes in defense against biotic factors has been demonstrated by their antifeedant activity against *Pieris brassicae* L. (Chen et al. 2017) and *Heliothis armigera* Hübner, (Hua J et al. 2017), and for antifungal properties toward several phytopathogenic fungi (Koga et al. 1997; Salah et al. 2003; Bi and Yu, 2016). The presence of these defensive compounds in cotyledons, together with non-

volatile compounds such as glucosinolates, may be explainable based on the importance of such tissues for the future development of the plant; according to optimal defense theory, the most valuable parts of a plant should also be the most heavily defended (Rhoades, 1979; Badenes-Perez et al. 2014).

However, insect specialists on brassicaceous plant species, such as *B. hilaris*, may have evolved adaptations to excrete or detoxify such defensive plant compounds (Blight et al. 1995; Gols and Harvey, 2009). Taken one step further, this pest may have evolved to actually exploit this chemical defense as a cue to locate *Brassica* seedlings, analogous to other specialist herbivores which exploit their hosts' defensive chemistry rather than being deterred by it, such as lygaeid bugs which sequester toxic cardenolides from their milkweed hosts (Isman et al. 1977), or flea beetles which sequester glucosinolates from their brassicaceous hosts (Beran et al. 2014). In fact, *B. hilaris* may have further refined this strategy by evolving to find and exploit the particularly susceptible and nutrient-rich seedling stage of its hosts.

Several other studies on herbivore-plant interactions have elucidated the crucial role of such volatile secondary plant compounds that act as host location kairomones for herbivores (Metcalf and Kogan, 1987; Schoonhoven et al. 2005; Carrasco et al. 2015). In fact, the importance of these secondary plant substances as cues exploited for host plant selection was emphasized several decades ago by Fraenkel (Fraenkel, 1969) as "*the very heart of agricultural entomology*". However, in most cases, host plant location by phytophagous insects is likely determined by the perception of ubiquitous phytochemicals such as green leaf volatiles and monoterpenes which may be emitted from particular plant species in specific ratios (Bruce et al. 2005; Bruce and Pickett, 2011). In contrast, in interactions between herbivores and brassicaceous host plants, including the *B. hilaris – B. oleracea* system, attraction to hosts is often mediated by the perception of relatively uncommon compounds which are specific to one or a small group of closely related host species (Blight et al., 1995; Bartlet et al. 1993; Barker et al. 2006; Csonka et al. 2007).

From an ecological viewpoint, the work described here provides fundamental knowledge about the chemical cues exploited by *B. hilaris* for host location. For practical purposes, this work may also represent an important step in the discovery of useful attractants for monitoring this pest, particularly because no effective attractants are currently available for this species.

Moreover, as alternative IPM tool, seedlings of highly attractive varieties could possibly be used as a trap crop, attracting *B. hilaris* away from the cash crop, and/or to specific areas for treatment with pesticides. Furthermore, the fact that some *Brassica* species, such as *B. carinata*, apparently lack brassicadiene may provide opportunities for developing hybrids that do not emit these compounds, either by traditional plant breeding methods, or by genetic manipulation of the plants.

3.5 References

- Arriola, K., Guarino, S., Arif, M. A., Colazza, S., Peri, E., Millar, J. G. (2019). Identification of Brassicadiene, a Novel Diterpene Hydrocarbon Attractive to the Invasive Stink Bug *Bagrada hilaris,* from Volatiles of Cauliflower Seedlings, *Brassica oleracea* var botrytis. *Organic Letters,*(submited).

- Badenes-Perez, F. R., Gershenzon, J., Heckel, D. G. (2014). Insect attraction versus plant defense: young leaves high in glucosinolates stimulate oviposition by a specialist herbivore despite poor larval survival due to high saponin content. *PLoS One,* 9(4), e95766.

- Barker, A. M., Molotsane, R., Müller, C., Schaffner, U., Städler, E. (2006). Chemosensory and behavioural responses of the turnip sawfly, *Athalia rosae*, to glucosinolates and isothiocyanates. *Chemoecology*, 16(4), 209-218.

- Bartlet, E., Blight, M. M., Hick, A. J., Williams, I. H. (1993). The responses of the cabbage seed weevil (*Ceutorhynchus assimilis*) to the odour of oilseed rape (*Brassica napus*) and to some volatile isothiocyanates. *Entomologia Experimentalis et Applicata*, 68(3), 295-302.

- Beran, F., Pauchet, Y., Kunert, G., Reichelt, M., Wielsch, N., Vogel, H., Ramasamy, S. (2014). *Phyllotreta striolata* flea beetles use host plant defense compounds to create their own glucosinolate-myrosinase system. *Proceedings of the National Academy of Sciences,* 111(20), 7349-7354.

- Bi, Y., Yu, Z. (2016). Diterpenoids from Streptomyces sp. SN194 and their antifungal activity against *Botrytis cinerea*. *Journal of Agricultural and Food Chemistry*, 64(45), 8525-8529.

- Blight, M. M., Pickett, J. A., Wadhams, L. J., Woodcock, C. M. (1995). Antennal perception of oilseed rape, *Brassica napus* (Brassicaceae), volatiles by the cabbage seed weevil *Ceutorhynchus assimilis* (Coleoptera, Curculionidae). *Journal of Chemical Ecology*, 21(11), 1649-1664.

- Boege, K. (2005). Herbivore attack in *Casearia nitida* influenced by plant ontogenetic variation in foliage quality and plant architecture. *Oecologia*, 143(1), 117-125.

- Borges, M., Moraes, M. C. B., Peixoto, M. F., Pires, C. S. S., Sujii, E. R., Laumann, R. A. (2011). Monitoring the Neotropical brown stink bug *Euschistus heros* (F.)(Hemiptera: Pentatomidae) with pheromone-baited traps in soybean fields. *Journal of Applied Entomology*, 135(1-2), 68-80.

- Bruce, T. J., Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects–finding the right mix. *Phytochemistry*, 72(13), 1605-1611.

- Bruce, T. J., Wadhams, L. J., Woodcock, C. M. (2005). Insect host location: a volatile situation. *Trends in Plant Science*, 10(6), 269-274.

- Bruinsma, M., Posthumus, M. A., Mumm, R., Mueller, M. J., van Loon, J. J., Dicke, M. (2009). Jasmonic acid-induced volatiles of *Brassica oleracea* attract parasitoids: effects of time and dose, and comparison with induction by herbivores. *Journal of Experimental Botany*, 60(9), 2575-2587.

- Bundy, C. S., Perring, T. M., Reed, D. A., Palumbo, J. C., Grasswitz, T. R., Jones, W. A. (2018) *Bagrada hilaris*. In: McPherson JE (ed) Invasive Stink Bugs and Related Species

(Pentatomoidea): Biology, Higher Systematics,Semiochemistry, and Managemen, *CRC Press, Boca Raton*, *FL*, 205–242.

- Bundy, C. S., Grasswitz, T. R., Sutherland, C. (2012). First report of the invasive stink bug *Bagrada hilaris* (Burmeister)(Heteroptera: Pentatomidae) from New Mexico, with notes on its biology. *Southwestern Entomologist*, 37(3), 411-415.

- Carrasco, D., Larsson, M. C., Anderson, P. (2015). Insect host plant selection in complex environments. *Current Opinion in Insect Science*, 8, 1-7.

- Chabaane, Y., Laplanche, D., Turlings, T. C., Desurmont, G. A. (2015). Impact of exotic insect herbivores on native tritrophic interactions: a case study of the African cotton leaf worm, *Spodoptera littoralis* and insects associated with the field mustard *Brassica rapa*. *Journal of Ecology*, 103(1), 109-117.

- Chen, X. Q., Gao, L. H., Li, Y. P., Li, H. M., Liu, D., Liao, X. L., Li, R. T. (2017). Highly Oxygenated Grayanane Diterpenoids from Flowers of *Pieris japonica* and Structure–Activity Relationships of Antifeedant Activity against *Pieris brassicae*. *Journal of Agricultural and Food Chemistry*, 65(22), 4456-4463.

- Mccormick, A. C., Gershenzon, J., Unsicker, S. B. (2014). Little peaks with big effects: establishing the role of minor plant volatiles in plant–insect interactions. *Plant, Cell & Environment*, 37(8), 1836-1844.

- Conti, E., Zadra, C., Salerno, G., Leombruni, B., Volpe, D., Frati, F., Bin, F. (2008). Changes in the volatile profile of *Brassica oleracea* due to feeding and oviposition by *Murgantia histrionica* (Heteroptera: Pentatomidae). *European Journal of Entomology*, 105(5).

- Csonka, É., Tóth, M., Ujváry, I. (2007). Differences in host-plant related chemical communication of the flea beetles *Phyllotreta cruciferae* Goeze and *Ph. vittula* Redtenbacher (Coleoptera, Chrysomelidae). *Acta Phytopathologica et Entomologica Hungarica*, 42(2), 343-352.

- Cullen, E. M., Zalom, F. G. (2006). *Euschistus conspersus* female morphology and attraction to methyl (2E, 4Z)-decadienoate pheromone–baited traps in processing tomatoes. *Entomologia Experimentalis et Applicata*, 119(2), 163-173.

- Fraenkel, G. (1969). Evaluation of our thoughts on secondary plant substances. *Entomologia Experimentalis et Applicata*, 12(5), 473-486.

- Gershenzon, J., Dudareva, N. (2007). The function of terpene natural products in the natural world. *Nature Chemical Biology*, 3(7), 408.

- Gols, R., Harvey, J. A. (2009). Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. *Phytochemistry Reviews*, 8(1), 187-206.

- Guarino, S., De Pasquale, C., Peri, E., Alonzo, G., Colazza, S. (2008). Role of volatile and contact pheromones in the mating behaviour of *Bagrada hilaris* (Heteroptera: Pentatomidae). *European Journal of Entomology*, 105(4), 613.

- Guarino, S., Peri, E., Colazza, S (2017b). Plant and stink bug interactions at different trophic levels. In *Stinkbugs* (pp. 180-199). CRC Press.

- Guarino, S., Peri, E., Colazza, S., Luchi, N., Michelozzi, M., Loreto, F. (2017a). Impact of the invasive Painted bug *Bagrada hilaris* on physiological traits of its host *Brassica oleracea* var botrytis. *Arthropod-Plant Interactions*, 11(5), 649-658.

- Himanen, S. J., Li, T., Blande, J. D., Holopainen, J. K. (2017). Volatile organic compounds in integrated pest management of Brassica oilseed crops. *Wallingford, UK: CAB International* (pp. 3281-294).

- Hua, J., Luo, SH., Liu, Y., Liu, Y. C., Tan, Y. Y., Feng, L., et al. (2017) New Bioactive Macrocyclic Diterpenoids from *Euphorbia helioscopia*. *Chemistry & biodiversity*. 14(10), e1700327.

- Huang, T. I., Reed, D. A., Perring, T. M., Palumbo, J. C. (2014a). Feeding damage by *Bagrada hilaris* (Hemiptera: Pentatomidae) and impact on growth and chlorophyll content of Brassicaceous plant species. *Arthropod-Plant Interactions*, 8(2), 89-100.

- Huang, T. I., Reed, D. A., Perring, T. M., Palumbo, J. C. (2014b). Host selection behavior of *Bagrada hilaris* (Hemiptera: Pentatomidae) on commercial cruciferous host plants. *Crop protection*, 59, 7-13.

- Isman, M., Duffey, S., Scudder, G. E. (1977) Variation in cardenolide content of the lygaeid bugs, *Oncopeltus fasciatus* and *Lygaeus kalmii* kalmii and of their milkweed hosts (*Asclepias* spp.) in central California. *Journal of Chemical Ecology*. 3(6), 613–24.

- Joseph, S. V., Grettenberger, I. M., Godfrey, L. D., Zavala, N. (2017). Susceptibility of germinating cruciferous seeds to *Bagrada hilaris* (Hemiptera: Pentatomidae) feeding injury. *Arthropod-Plant Interactions*, 11(4), 577-590.

- Koga, J., Ogawa, N., Yamauchi, T., Kikuchi, M., Ogasawara, N., Shimura, M. (1997). Functional moiety for the antifungal activity of phytocassane E, a diterpene phytoalexin from rice. *Phytochemistry*, 44(2), 249-253.

- Leskey, T. C., Agnello, A., Bergh, J. C., Dively, G. P., Hamilton, G. C., Jentsch, P., Morrison III, W. R. (2015). Attraction of the invasive *Halyomorpha halys* (Hemiptera: Pentatomidae) to traps baited with semiochemical stimuli across the United States. *Environmental Entomology*, 44(3), 746-756.

- Ludwig, S. W., Kok, L. T. (2001). Harlequin bug, *Murgantia histrionica* (Hahn) (Heteroptera: Pentatomidae) development on three crucifers and feeding damage on broccoli. *Crop Protection*, 20(3), 247-251.

- Metcalf, R. L., Kogan, M. (1987). Plant volatiles as insect attractants. *Critical Reviews in Plant Sciences*, 5(3), 251-301.

- Palumbo, J. C., Natwick, E. T. (2010). The bagrada bug (Hemiptera: Pentatomidae): A new invasive pest of cole crops in Arizona and California. *Plant Health Progress*, 11(1), 50.

- Palumbo, J. C., Perring, T. M., Millar, J. G., Reed, D. A. (2016). Biology, ecology, and management of an invasive stink bug, *Bagrada hilaris*, in North America. *Annual Review of Entomology*, 61, 453-473.

- Rather, A., Azim, M., Maqsood, S. (2010). Host plant selection in a pentatomid bug *Eurydema pulchrum* Westwood. *Journal of plant protection research*, 50(2), 229-232.

- Reed, D. A., Palumbo, J. C., Perring, T. M., May, C. (2013). *Bagrada hilaris* (Hemiptera: Pentatomidae), an invasive stink bug attacking cole crops in the southwestern United States. *Journal of Integrated Pest Management*, 4(3), C1-C7.

- Rhoades, D. F. (1979). Evolution of plant chemical defense against herbivores. *Herbivores: their interaction with secondary plant metabolites*, 3-54.

- Salah, M. A., Bedir, E., Toyang, N. J., Khan, I. A., Harries, M. D., Wedge, D. E. (2003). Antifungal clerodane diterpenes from *Macaranga monandra* (L) Muell. et Arg.(Euphorbiaceae). *Journal of agricultural and food chemistry*, 51(26), 7607-7610.

- Sánchez-Peña, S. R. (2014). First record in Mexico of the invasive stink bug *Bagrada hilaris*, on cultivated crucifers in Saltillo. *Southwestern Entomologist*, 39(2), 375-378.

- Schoonhoven, L. M., Van Loon, B., van Loon, J. J., Dicke, M. (2005). *Insect-Plant Biology*. Oxford University Press on Demand.

- Torres-Acosta, R. I., Sánchez-Peña, S. R.,Torres-Castillo, J. A. (2017). Feeding by Bagrada Bug, *Bagrada hilaris*1, on Moringa oleifera (Brassicales: Moringaceae) in Mexico. *Southwestern Entomologist*, 42(3), 919-923.

- Vaughan, M. M., Wang, Q., Webster, F. X., Kiemle, D., Hong, Y. J., Tantillo, D. J., Tokuhisa, J. G. (2013). Formation of the unusual semivolatile diterpene rhizathalene by the Arabidopsis class I terpene synthase TPS08 in the root stele is involved in defense against belowground herbivory. *The Plant Cell*, 25(3), 1108-1125.

- Wang, Q., Jia, M., Huh, J. H., Muchlinski, A., Peters, R. J., Tholl, D. (2016). Identification of a dolabellane type diterpene synthase and other root-expressed diterpene synthases in Arabidopsis. *Frontiers in Plant Science*, 7, 1761.

- White, W. H., Brannon L. W. (1933). The harlequin bug and its control. *US Dep Agric Farmers Bull*, 1712, 1– 10.

Chapter 4

Attractiveness of volatile *Brassica* spp. seedling components to *Bagrada hilaris* and potential implications for developing future control strategies

This chapter is based on the work contained in the following article:

- Arif, M. A., Guarino, S., Colazza, S., Peri, E. (2020). Attractiveness of volatile Brassica spp. seedling components to *Bagrada hilaris* and potential implications for developing future control strategies. *Insects , special issue*, Submited.

Chapter 4

Attractiveness of volatile *Brassica* spp. seedling components to *Bagrada hilaris* and potential implications for developing future control strategies

Abstract

Caper bush (*Capparis spinosa*) is an important cultivation of marginal lands and arid environments as such those of Mediterranean islands. In particular in Pantelleria Island (Italy), caper is appointed with the protected geographical indication by EU. In this island, the caper crop is damaged by *Bagrada hilaris* (Burmeister), a stink bug native from Asia. Recent studies evidenced strong attraction of this species toward plants at the seedlings stage. Objective of this study was to evaluate different seedlings of brassicaceous plants as candidate trap crops for *B. hilaris*, to protect caper bush. The tested seedlings were *Brassica oleracea* var. botrytis, *Eruca sativa* and *Brassica carinata*. In laboratory bioassays, carried out using dual choice arena and olfactometer, adults of *B. hilaris* preferred to orient toward seedlings of *B. oleracea* var. botrytis and *E. sativa* L. over *B. carinata*. Seedlings of *B. oleracea* and *E. sativa* were then tested in the caper fields as attractant lures in traps, determining a substantial number of captures of individuals, respectively 6.29 and 9.54 per trap per inspection (3d). Finally, seedlings were also tested in trap cropping trials, by sawing them in artificial pots and placed in caper field infested by *B. hilaris*. The observations evidenced that hundreds of *B. hilaris* individuals were diverted from capers and attracted to pots of *E. sativa* and *B. oleracea* with a lesser extent to *B. carinata*. Overall, these results indicated that *B. oleracea* and *E. sativa* seedlings used as lure or as trap crop may be a useful tool in the management of *B. hilaris* populations.

Key words: Painted bug, olfactometer, dual choice arena, *Capparis spinosa*, trap crop

4.1 Introduction

Caper bush (*Capparis spinosa* L.) is a perennial shrub belonging to the family of Capparaceae, cultivated mainly for the flower buds known as capers, widely acknowledged for their pungent and strong aromatic properties (Sozzi 2001; Rivera et al. 2003). Caper plants have strong rusticity that allows them to survive in environments with dry summer climate typical of the islands of the Mediterranean Basin (Simoglou and Dioli 2017). For these reasons in Italy the caper cultivation is positively exploited in Sicily and its minor islands for several factors as the exploitation of marginal lands, its low initial cost and higher profit margins compared to other local crops (Infantino et al. 2007). In particular the caper grown in the island of Pantelleria (Italy) have been appointed with the protected geographical indication (PGI) for Commission Regulation No. 1107/96 (Infantino et al. 2007) and reach productions of 300/400 tons per year (Colazza et al. 2004). Caper plants are attacked by a large group of phytophagous insects as the dipterean *Capparimya savastanoi* (Martelli), *Asphondylia gennadii* (Marchal) and several pentatomid bugs as *Bagrada hilaris* (Burmeister), *Antheminia lunulata* (Goeze), *Eurydema ornata* L., *Eurydema ventralis* Kol., *Holcostethus punctatus* (Lindberg) and *Nezara viridula* L. (Infantino et al. 2007) etc as reported in table 1.

Table 1. Heteroptera: Pentatomidae species reported feeding of wild and cultivated capers (*Capparis* spp., Capparidaceae).

Species	Reference
Acrosternum millierei	(Gozuacik 2018)
Antheminia lunulata	(Infantino et al.2007)
Bagrada abeillei	(Gozuacik 2018)
Bagrada amoenula	(Gozuacik 2018)
Bagrada hilaris	(Colazza et al. 2004)
Carpocoris fuscispinus	(Gozuacik 2018)
Codophila varia	(Gozuacik 2018)
Dolycoris baccarum	(Gozuacik 2018)
Eurydema eckerleini	(Simoglou and Dioli, 2017)
Eurydema ornata	(Fernández et al. 1986)
Eurydema ventralis	(Infantino et al. 2007)
Holcostethus punctatus	(Infantino et al. 2007)
Nezara viridula	(Infantino et al. 2007)
Stenozygum coloratum	(Sarmra et al. 2015)

Among these pentatomids, the Painted bug *B. hilaris*, can be considered the key pest of caper plants in the island of Pantelleria. This species, after its unintentional introduction in 1978 (Carapezza 1981), extended progressively its area of diffusion in the island and caused increasing damages to the growers. The plant damage is determined by the feeding activity of adults and nymphs that with their pierce-sucking mouthparts, cause necrotic spots, that compromise the plant photosynthetic activity and deformations that negatively affect the marketability of flower buds (Colazza et al. 2004; Infantino et al. 2007). Furthermore, *B. hilaris* can form large aggregation of individuals on host plants, determining in some case the death of the plant itself (Colazza et al. 2004). To keep populations of *B. hilaris* below the level of economic threshold damage caper growers of Pantelleria have in the past relying on the use of chemical insecticides from four to five applications per year, sprayed after each harvest (Colazza et al. 2004). Moreover, the limited number of insecticide registered on caper plant and the increasing insects resistance suggest the use of alternative control methods for this pest. Finally, the management of this species is also compromised by the lack of effective sampling strategies and appropriate monitoring tools, for example based on semiochemicals that still haven't been developed (Bundy et al. 2018).

In this context, new tools for control *B. hilaris* population are urgent to reduce treatments, have pesticide-free products and give the opportunity to caper growers to switch to organic agriculture.

Among the possible alternative strategies for managing invasive insect pests, in recent years trap cropping has been receiving attention as having low environmental impact and useful in organic farming contexts (Shelton and Badenes-Perez, 2006). Trap cropping is technique based on the use of plant stands that are, by themselves deployed to attract, divert, intercept, and/or retain targeted insects in order to reduce damage to the main crop (Hokkanen 1991). This technique showed interesting results in the control of several stink bugs in both conventional and organic crop production systems (Mathews et al. 2017; Mizell III et al. 2008; Rea et al. 2002). For example, to manage *N. viridula* in organic sweet corn *Zea mays* (L.), were successfully used *Sinapis alba* (L.), *Pisium sativum* (L.) and *Brassica nigra* (L.) as trap crops (Rea et al. 2002).

Moreover, *N. viridula* and *Euschistus servus* (Say) in cotton orchards, were controlled using *Sorghum bicolor* (L.) exploited as a trap crop (Tillman et al. 2015).

A successful trap cropping relies on the fact that pests often show marked host plant preferences and strongly depend on how can the trap crop can divert the insect pest from the crop to protect. Consequently, the high attractiveness of the source used *i.e.* a preferred host in a specific stage, is of crucial importance (Hokkanen 1991; Cook et al. 2006).

In the case of *B. hilaris*, in the territories where this bug is widespread, as Africa and Pakistan, India, China and more recently in the Americas, this pest is feeding mainly on brassicaceous plants (Palumbo and Natwick 2010; Bundy et al. 2012; Reed et al. 2013; Torres-Acosta et al. 2017; Bundy et al. 2018). Among the Brassicaceae *B. hilaris* shows distinct preferences for different species and varieties such as *B. oleracea* var botrytis (Guarino et al. 2017), *Raphanus sativus* L. (Huang et al. 2014), and *E. sativa* L. (Joseph et al. 2017). Moreover, differently to other phytophagous stink bug of Brassicaceae, *B. hilaris* showed preference for the plants at seedling stage (Huang et al. 2014) as evidenced by Guarino et al. (2018) whom suggested that the *B. hilaris* attraction toward *Brassica* seedlings is determined by the emission of a few diterpene hydrocarbons, the main one recently identified and named brassicadiene (Arriola et al. in preparation). This attraction behavior suggests that young seedlings of brassicaceous species can be exploited as candidates for trap cropping of *B. hilaris*, also in consideration that they are readily available and cheap, as can be self-produced by the farmers (Ronoh et al. 2018).

In this study we investigated, in laboratory and field bioassays, the possibility to use brassicaceous seedlings as candidates for trap cropping *B. hilaris* individuals.

Our specific objectives were:

1. Evaluate different brassicaceae seedlings in laboratory behavioral bioassays, to identify the ones more attractive to *B. hilaris*,

2. Use attractant seedlings as lure in traps to verify if their volatile emitted can drive *B. hilaris* inside traps,

3. To bioassays the candidate seedlings in *B. hilaris*-infested caper fields to evaluate the ability of such seedlings to divert the bugs from the plant to protect to these attractive sources.

4.2 Material and methods

4.2.1 Insects

The colony of *B. hilaris* was established and restocked regularly with individuals collected from caper (*C. spinosa* L.) fields on the island of Pantelleria (Italy). Insects were reared in an environmentally controlled room (30 ± 2 °C, 70 ± 10% RH, photoperiod 16L:8D), in wooden cages (25 × 25 × 40 cm) with two 5-cm diameter mesh-covered holes for ventilation. The colony was fed with cauliflower and cabbage plants, depending on seasonal availability. Because *B. hilaris* lays eggs in the soil, paper dishes (6-cm Ø) with a mixture of sand, silt, and clay (33% for each soil component) were placed in the cages as oviposition sites. Dishes were changed weekly, and those with eggs were kept in separate cages until the emergence of nymphs. The nymphs were then kept in separate cages until the final molt to adults. Newly emerging adults were used separately in experiments.

4.2.2 Seedlings

Seeds of *Brassica oleracea* var. botrytis (cauliflower) L., *Eruca sativa* Mill. (rocket), and *Brassica carinata* Braun (Abyssinian cabbage), all obtained from a local market (Palermo – Italy), were placed on cotton wool (10 g) soaked with distilled water and held in glass containers with a density of circa 0.5 cm seeds/cm^2. The choice of *B. oleracea* var botrytis and *B. carinata* was determined by the previous experiments (Chapter 3), *E. sativa* was chosen because of the attractiveness toward *B. hilaris* reported in previous studies (Joseph et al. 2017). The containers were placed in an environmentally controlled growth chamber (25 ± 1 °C, 70 ± 10% RH, photoperiod 16L:8D) equipped with lights with a photosynthetic flux density (PPFD) of 600 mol photons m^{-2} s^{-1} placed above the foliage. Cotyledon stage seedling clusters were collected; the substrate was wrapped in aluminum foil to avoid substrate desiccation and then used for laboratory bioassays.

4.2.3 Laboratory bioassays

In order to evaluate a host preference response, seedlings clusters of *B. oleracea* var botrytis, *B. carinata* and *E. sativa*, were used as stimuli in two-choice test, carried out in both dual choice arena and open vertical Y-shaped olfactometer. In order to assess the response of the insect to seedlings at different age, the experiments were carried out separately using seedlings of 3 and 9 days old.

4.2.3.1 Dual choice arena

The arena consisted in a Plexiglas cage 50 cm wide, 30 cm high and 30 cm deep. The two opposite sides of the cage were made by with net for ventilation. Inside the arena, two clusters of 3 or 9 days old seedlings (N = 50) were placed 20 cm apart. Adult bugs were starved for 24h before the experiments. Individuals (N = 6 per replicate) were released into the cage at 2:00 PM and the total number of insects on each seedlings cluster was recorded after 20h, at 10:00 AM the next day (final choice). The position of the treatments inside the arena was alternated after each replicate. After each bioassay, the Plexiglas cage was cleaned with water and dried. Observations were conducted in static air, under ambient laboratory temperature and humidity conditions (25 ± 3 °C, and 50 ± 15% RH).

4.2.3.2 Open Vertical Y-shaped olfactometer

The open vertical Y-shaped olfactometer consisted of a brass rod (left and right arms 20 cm long, central arm 25 cm long, 1.00 cm diameter). The left and right arms were partially covered with two glass tubes (18 cm long, 5 cm diameter) terminating in hose nipples connected by Tygon tubes to a high–purity air source, and air flow was controlled with a flow-meter at a rate of 0.2 l/min. The air flowed through two glass chambers (500 ml each), which held the test stimuli (seedling clusters N = 50). Light was provided with a halogen lamp (Osram, 12V–35W, Münich, Germany) hanging 30 cm above the olfactometer. Experiments were carried out under ambient laboratory temperature and humidity conditions (25 ± 3 °C, and 50± 15% RH). For each

replicate, a single adult was gently placed at the bottom of the central arm of the olfactometer with a paintbrush and allowed 10 min to respond. The bugs moved from the bottom upward toward the light source and upon arriving at the Y junction, having the possibility to choose between the two different volatile stimuli. The criterion for a response was that the test bug walked in the test arm or the control arm for at least 5 cm past the Y junction (first choice). Bugs that did not move into one of the two arms during the 10 min trial were scored as non-responders and were not included in the analysis. After 8 replicates, the glass parts of the apparatus were washed with water and detergent, and then wiped with acetone and the brass rod was cleaned with distilled water and acetone and baked at 200°C for 60 min.

4.2.4 Field tests

4.2.4.1 Trap bioassays

Seedlings of *B. oleracea* var botrytis and *E. sativa* were tested in a caper field infested by *B. hilaris* as attractant lures in traps. A cluster of 50 seedlings (7 days old) with soaked cotton used as growing substrate, were placed inside the traps. For this experiment were used a new design of horizontal plastic trap (25 x 15 x 15 cm) furnished by GEA S.r.L., (Settimo Milanese Milan – Italy), with shaped-like funnel from both sides to allow insect entrance (Fig. 1). The trap used was specifically thought and designed for this experiment in consideration of *B. hilaris* behavior. Traps were placed in the proximity of the caper plant and partially buried in order to facilitate insect entrance and to prevent wind damages (Fig. 2). On the board of the inner part of the funnels was applied paraffin oil to avoid insects escape. Four traps per each seedling species and control (unloaded traps) were used by putting them in a Pantelleria caper field (36°46'15.5"N 11°57'43.9"E) infested by *B. hilaris*, using Latin square design. Distance between traps was approximately 8m. Traps were inspected every 3 days from emergence and the number of trapped individuals was counted.

Figure 1. Trap lured by *B. oleracea* or *E. sativa* seedlings before being closed.

Figure 2. Placement of the trap in the caper field lured by *B. oleracea* or *E. sativa* seedlings after closure.

4.2.4.2 Host preference bioassays

Host preference bioassays using *B. oleracea* var botrytis, *E. sativa* and *B. carinata* were carried out in a caper field in Pantelleria Island (Italy) (36°46'21.7" N 11°57'38.1" E). The caper field chosen for the experiment was 40 m long and 80 m wide and presented a heavy infestation from *B. hilaris*. The experiment carried out using pots made from aluminum (40 x 30 x 10) containing cotton soaked with water used as growing substrate each treatment was replicated three times for a total amount of twelve experimental pots. For each species were sown using 5 g of seeds per pot (Fig. 3).

The seeds were sown in July 2019, and irrigated every two days (Fig. 4). To assess the attractiveness of each seedling species, the number of total *B. hilaris* individuals present on each seedling pot, was counted and removed using paint brush at the 3rd, 6th, 9^{th,} 11th, 13th, and 15th days after seedlings emergence.

Figure 3. Preparation of *B. oleracea*, *E. sativa* and *B. carinata* seedling pots.

Figure 4. Placement of the seedling pots of *B. oleracea*, *E. sativa* and *B. carinata* in the caper field.

4.2.5 Statistical analysis

Data obtained from dual choice arena and open vertical Y-shaped olfactometer experiments were analyzed using χ^2 tests. Data obtained from field trap bioassays were analyzed by using a one-way ANOVA followed by Tukey test. Finally, a descriptive analysis of host preference *B. hilaris* toward the different seedlings tested in the field was conducted. All the statistical analyses were performed using Statistica 7.0 for Window (Statsoft 2001, Vigonza, PD, Italy).

4.3 Results

4.3.1 Laboratory bioassays

4.3.1.1 Dual choice arena

The response of *B. hilaris* adults to the different seedling clusters at 3 and 9 days old tested in dual choice arena is reported in Fig. 5.

Overall the seedling species that attracted the higher number of *B. hilaris* adults were *B. oleracea* var. botrytis and *E. sativa*, with *B. carinata* resulting the less preferred.

In the specific, using 3 days old seedlings, *B. hilaris* adults showed similar attraction response toward *B. oleracea* var. botrytis and *E. sativa* (χ^2 = 0.36, df = 1, P < 0.54, N = 44), while *B. hilaris* adults preferred *B. oleracea* over *B. carinata* (χ^2 = 6.75, df = 1, P < 0.009, N = 48), and *E. sativa* over *B. carinata* (χ^2 = 8.69 df = 1 P < 0.003, N = 46).

Furthermore, using 9 days old seedlings, *B. hilaris* adults preferred *B. oleracea* var. botrytis over *E. sativa* (χ^2 = 9.25, df = 1, P < 0.002, N = 39), and over *B. carinata* (χ^2 = 11.63, df = 1, P < 0.006, N = 50), as well as *B. hilaris* preferred *E. sativa* over *B. carinata* (χ^2 = 6.08, df = 1 P < 0.01, N = 37).

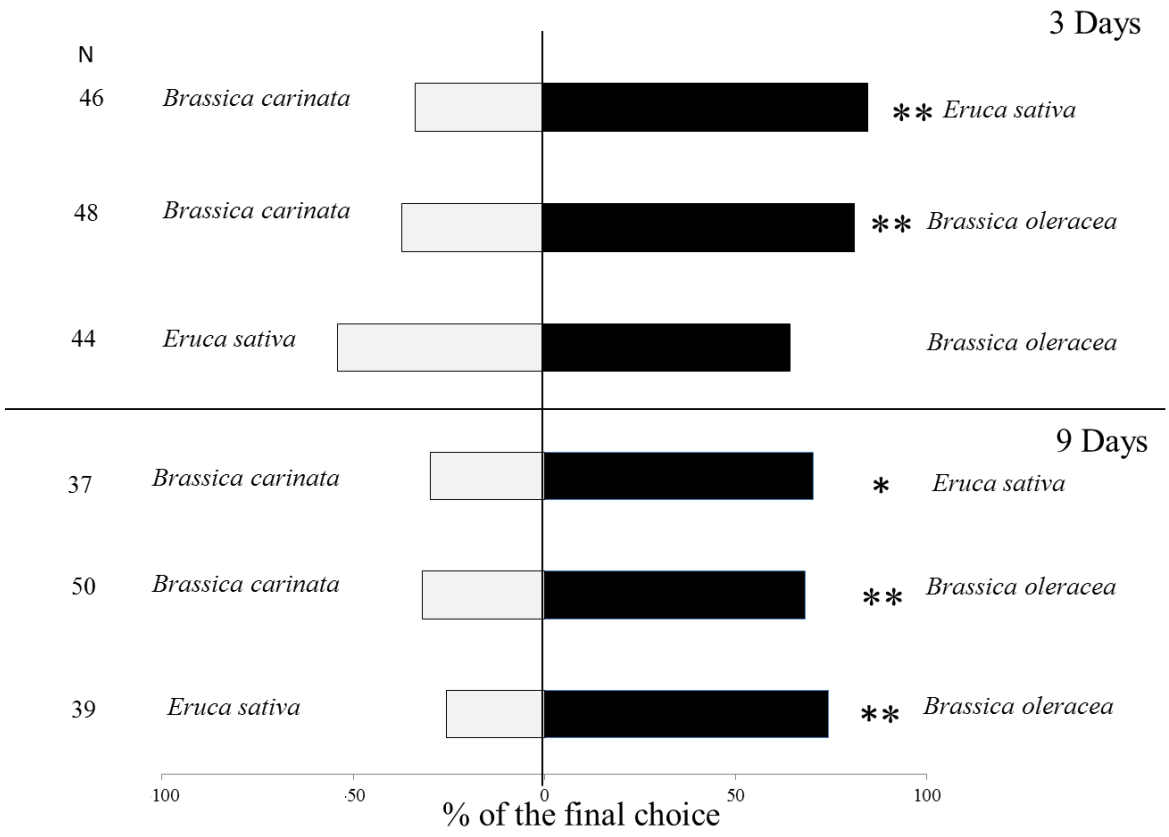

Figure 5. Dual choice arena bioassays: Host preference responses (% final choice) of *B. hilaris* adults to seedlings 3 and 9 days old of *B. oleracea* var. botrytis, *B. carinata* and *E. sativa*. N = number of replicates; * = P < 0.05; ** = P < 0.01.

4.3.1.2 Open Vertical Y-shaped olfactometer

The response of *B. hilaris* adults to the volatiles of the different seedling clusters 3 or 9 days old tested in open vertical Y-shaped olfactometer is reported in Fig. 6.

In experiments using 3 days old seedlings, *B. hilaris* adults oriented toward *E. sativa* over *B. carinata* volatiles (χ^2 = 6.52, df = 1 P < 0.01, N = 64), while *B. hilaris* adults did not show a significant responses between volatiles of *B. oleracea* var. botrytis and *E. sativa* (χ^2 = 0.40, df = 1, P < 0.52, N = 61), as well as *B. hilaris* did not show different among *B. oleracea* var. botrytis and *B. carinata* (χ^2 = 2.25, df = 1, P < 0.13, N = 64).

In experiments with seedlings of 9 days old, *B. hilaris* adults also were more strongly attracted to volatiles from *B. oleracea* var. botrytis than to volatiles of *B. carinata* VOC (χ^2 = 8.53, df = 1, *P* < 0.03, N = 120), and to *E. sativa* volatiles than to *B. carinata* volatiles (χ^2 = 7.71 df = 1 p < 0.005, N = 109). However, adult bugs were equally attracted to volatiles from *B. oleracea* var. botrytis and *E. sativa* (χ^2 = 2, 61 df = 1 *P* < 0.1, N = 98).

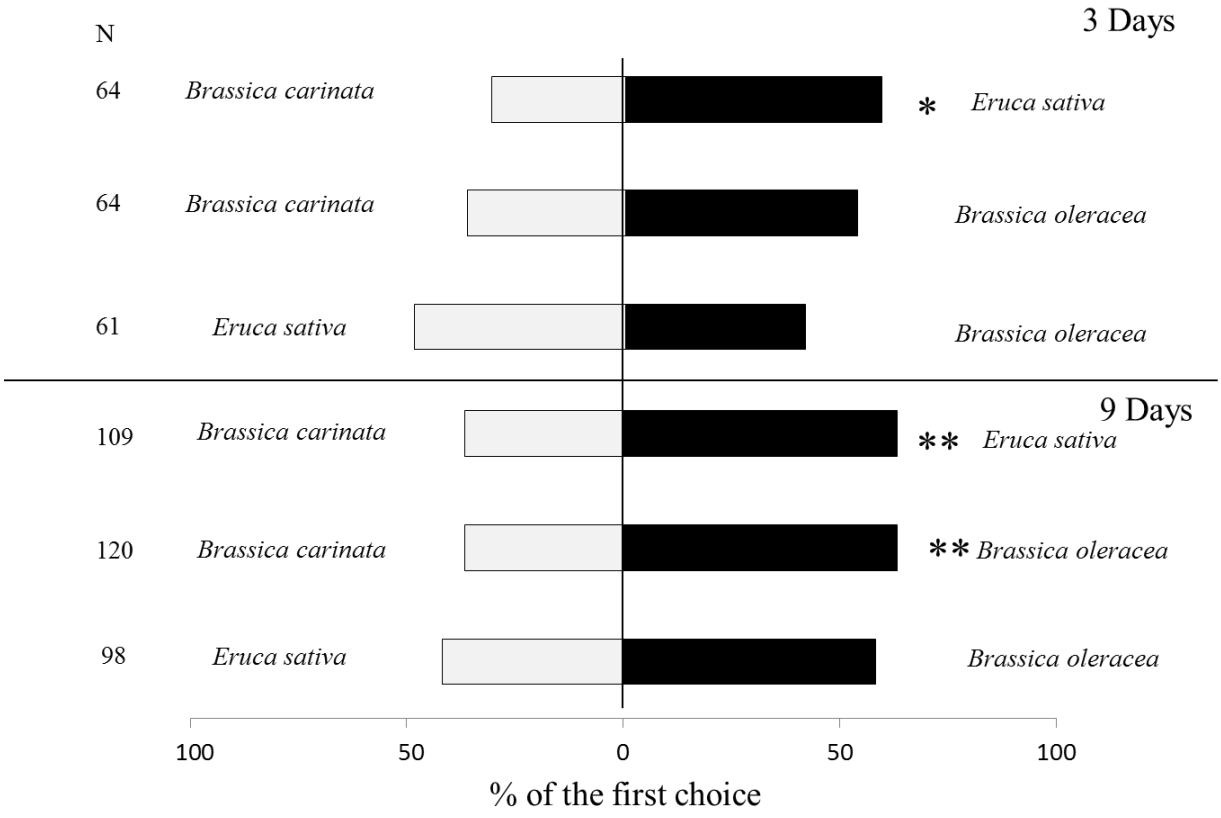

Figure 6. Y-shaped olfactometer bioassays: Responses (% first choice) of *B. hilaris* adults in to seedlings 3 and 9 days old of *B. oleracea* var. botrytis, *B. carinata* and *E. sativa* in. N = number of replicates; * = P < 0.05; ** = P < 0.01.

4.3.2 Field test

4.3.2.1 Trap bioassays

Results of trap bioassays, using living seedlings as lures are reported in Fig. 7. Overall, the traps lured with *B. oleracea* var botrytis and *E. sativa* seedlings captured more individuals than control (F = 16.03, df = 2, P < 0.0001). In the specific, traps loaded with *B. oleracea* and *E. sativa* seedlings captured respectively a mean (± SE) of 6.29 ± 1.2 and 9.54 ± 1.65 individuals per trap per 3 days, while no captures were recorded in control traps. However, the number of captures determined by *B. oleracea* and *E. sativa* seedlings did not differ statistically (P = 0.14; Tukey test).

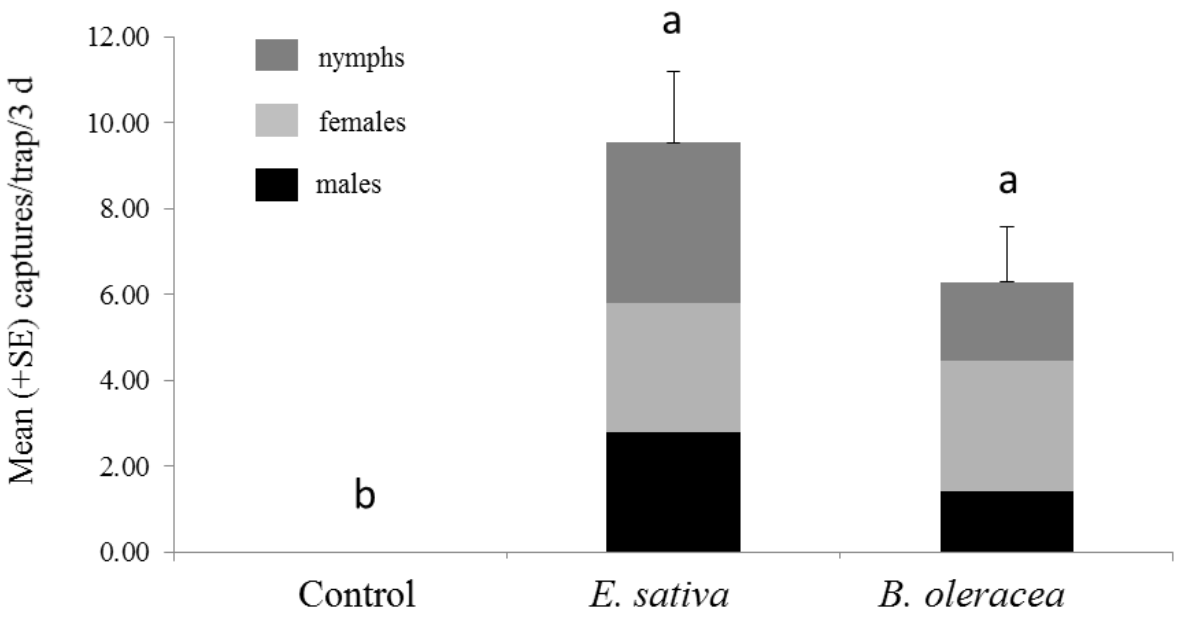

Figure 7. Mean (+ SE) *B. hilaris* individuals captured in horizontal trap lured by *B. oleracea* and *E. sativa* seedling or control traps. Different letters indicate that values differ statistically at P < 0.05 (One-way ANOVA, followed by Tukey test).

4.3.2.2 Host preference bioassays

Results of host preference bioassays with different brassicaceous seedlings are reported in Fig. 8. The pots with seedlings of *E. sativa* attracted a higher number of individuals of *B. hilaris* rather than *B. oleracea* var botrytis and *B. carinata* at the 5th and 7th day from emergence (Fig. 9). Differently, *B. oleracea* var botrytis young seedlings pots attracted a higher number of individuals at day 9th, 11th and at 13thfrom emergence. These latter results were also determined by the progressive decline and death of the majority of the *E. sativa* seedlings observed from day 9th. The attraction of *B. oleracea* and *E. sativa* seedlings decreased progressively after the 11th days from emergence. This was in part determined by strong damage by *B. hilaris* individuals that lead to the death of the majority of the seedlings. Differently, *B. carinata*, although showed to be less attractive in the first samplings, evidenced a stronger resistance to *B. hilaris* individuals.

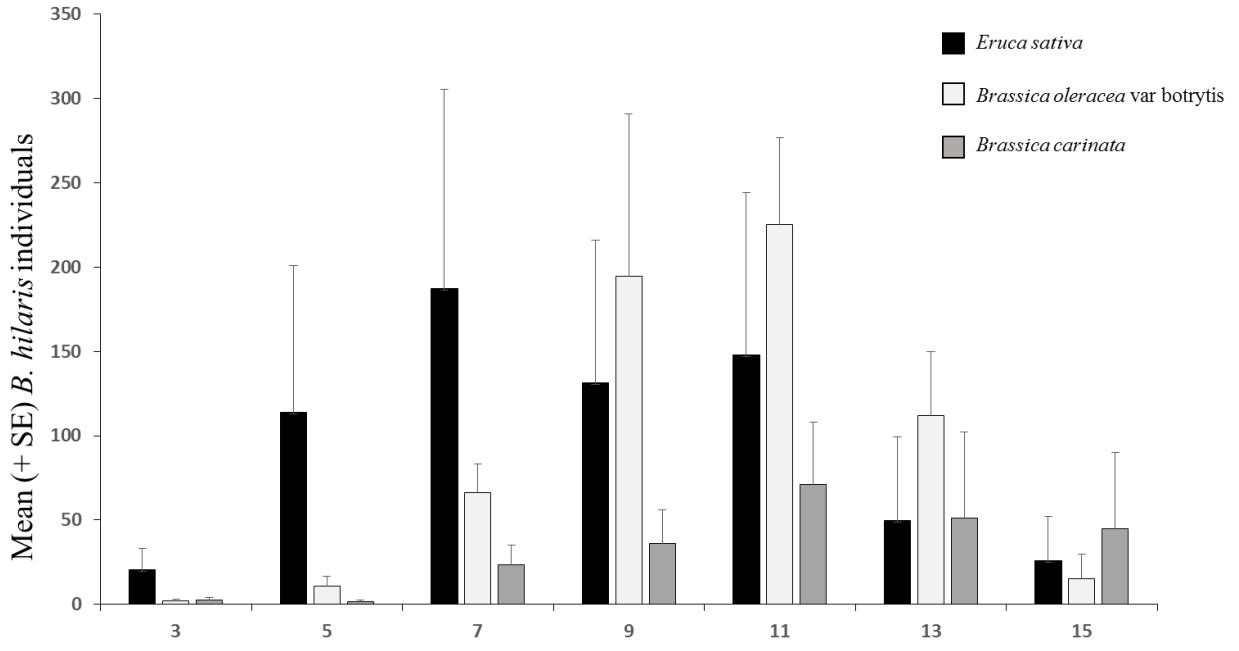

Figure 8. Mean (+ SE) *B. hilaris* individuals' attracted to the seedlings pots of *E. sativa, B. oleracea* and *B. carinata* in Pantelleria caper field.

Figure 9. *Bagrada hilaris* individuals attracted to the seedlings of *E. sativa* in Pantelleria caper field.

4.4 Discussion

The results described above provide evidences that seedlings of *B. oleracea* and *E. sativa* are good candidates as attractant plants for *B. hilaris* and may find application as lure for traps or in trap cropping techniques.

Attraction of *B. hilaris* toward these seedling is strictly related to the specific volatile organic compounds (VOCs) emitted from the seedlings species tested. In fact, the olfactometer bioassays firstly evidenced that the attraction to preferred hosts is mediated by olfactory rather than visual cues. In particular testing the seedlings at 3 and 9 days from emergence, *B. hilaris* adults oriented toward VOCs from *E. sativa* rather than from the ones of *B. carinata*.

As described by Guarino et al. (2018) *B. hilaris* is attracted to the seedlings VOCs of *B. oleracea* var. botrytis and *B. napus* which contained uncommon volatile diterpene hydrocarbons of novel identification (> 95%) and named brassicadiene, while were less attracted from the VOCs of *B. carinata,* which have a different chemical profile (see Chapter 3). Remarkably, the attraction in this case seem to be determined by few key odorant molecules rather than from blend of ubiquitary compounds as observed in the majorities of the phytophagous-plant interactions cases (Bruce and Pickett, 2011). Similarly, it is than possible that the attraction of *B. hilaris* adults toward *E. sativa* seedlings, already observed also from Joseph et al (2017), can be determined by few key odorant molecules, however yet not investigated.

Field bioassays carried out using living seedlings inside the traps as attractant lure, not only confirmed the high attraction of *B. hilaris* toward the seedlings of *B. oleracea* and *E. sativa*, but also pose the basis for the use a new type of attractant lure for monitoring this species. From these results was evidenced also the possibility to use living germinating seeds as lure. Even if the use of vegetal material as attractant lure for trapping insects has been already exploited for other pests as the read palm weevil (Giblin-Davis et al. 1994; Peri et al. 2017), the use of such seedlings as attractant is novel to our knowledge. One step forward, the use of such lures tested in our experiment combined with the particular type of trap used in the trials is particularly interesting. In fact, differently to other stink bug traps that are placed in vertical way, the trap

used in our trials is placed horizontally and partially buried and is more useful to catch a bug such *B. hilaris* whom oviposit in the soil, differently to other stink bugs (Taylor at al. 2014), and often mate on the ground at the host plant basis. Moreover, the placement of this trap can also protect it from wind, one of the climatic factors that mainly influence the efficiency of the trap (Rummel and Bottrell, 1976; Carroll and Rummel, 1985; Jones et al. 1992) and that in Pantelleria is particularly intense (Bivona et al. 2003).

Finally, the data obtained from host preference field experiments showed that both *E. sativa* and *B. oleracea* var botrytis seedlings can attract a large number of *B. hilaris* individuals with a lesser extent to *B. carinata*, diverting them from the surrounding caper plants. These results are encouraging in order to more in depth explore the possibility use such seedlings for trap cropping purposes to protect caper bush plants. Furthermore, in accordance with the laboratory bioassays results, *B. oleracea* and *E. sativa* elicited a different attraction on *B. hilaris* individuals with time, probably related to the seedling grown rate and the relative volatiles emitted. In the specific, while *E. sativa* seedlings attracted the highest number of individuals at the 5^{th} and 7^{th} day after emergence, *B. oleracea* var botrytis resulted more attractive in the following observations at the 9^{th} ,11^{th} and at 13^{th}day respectively. It is also to be pointed out that the decrease in seedling cluster attraction in the latest observations could be partially determined by the rapid mortality caused by the large aggregations of *B. hilaris* individuals on seedlings after the 7^{th} day from emergence and particularly evident in *E. sativa* seedling clusters. This may entail that, once *E. sativa* trap plants are rapidly dying, they need to be quickly destroyed or treated to avoid that *B. hilaris* individuals re-infest the caper bush plants. Differently, the *B. oleracea* var botrytis seedlings showed the higher attraction to the pest at day 9^{th} after emergence, evidencing increasing efficacy and higher endurance to the *B. hilaris* feeding damage.

Overall, these results suggest that the seedlings used might have potential use as trap crops to manage this pest also encouraged by the strong needs from Pantelleria caper growers to have alternative and reliable methods to control Painted bug populations. In our experiment we decided to carry out the experiment in the summer, when the *B. hilaris* population is at the highest level in order to attract the highest number of individuals, however due to the high

temperature it was necessary to irrigate periodically the young seedlings. In order to exploit the trap cropping technique successfully, and in consideration of the well-known water shortage in the island of Pantelleria (Manenti et al. 2013), it could be alternatively recommendable that brassiceous seeds are sown in occurrence of rainfall periods. This tactic was also suggested by Ludwig and Kok (1998) as to apply the trap crops during fall could limit the size of the population entering in winter diapause. However, alternatively or simultaneously, trap crop could be used in spring to catch the newly emerging colonizers before they infest the caper bush. In consideration of the fact that *B. hilaris* generally overwinter in the stone walls surrounding the caper fields (Colazza et al. 2004), this last technique, could be useful if the trap crop is planted around the border of the main crop, in order to apply the so-called "perimeter trap cropping method" (Boucher at al. 2003). In fact, as suggested from Shelton and Badenes-Perez (2006) "the potential success of a trap cropping system depends on the interaction of the characteristics of the trap crop and its deployment with the ecology and behavior of the targeted insect pest". To our knowledge, very few cases have been reported of commercially successful trap cropping application (Shelton and Badenes-Perez, 2006 and references therein). On the other hand, it is encouraging that the use of trap crops using *Brassica* plants to reduce infestation was reported successfully for other pest as *Psylliodes chrysocephala* (L.) (Coleoptera: Chrysomelidae) (Cabbage stem flea beetle) and *Ceutorhynchus pallidactylus* (Marsh.) (Coleoptera: Curculionidae) (Cabbage stem weevil) (Barari et al. 2005) and for *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Charleston and Kfir, 2000).

In conclusion, the results present in this study evidence the high attraction of *B. oleracea* var botrytis and *E. sativa* seedlings in both laboratory and field. These results have not only ecological value but also can be exploited to both use these as attractant lure in the traps for monitoring purposes that can be used in integrated management programs (IPM) to reduce the insecticide at the strong necessity. Finally, the demonstrated ability of these seedling to divert the *B. hilaris* from caper to the such attractant sources give the opportunity to explore them as tools for trap cropping, particularly needful for those caper growers that would approach organic farming methods.

4.5 References

- Bivona, S., Burlon, R., Leone, C. (2003). Hourly wind speed analysis in Sicily. *Renewable Energy*, 28(9), 1371-1385.

- Boucher, T. J., Ashley, R., Durgy, R., Sciabarrasi, M., Calderwood, W. (2003). Managing the pepper maggot (Diptera: Tephritidae) using perimeter trap cropping. *Journal of Economic Entomology*, 96(2), 420-432.

- Bruce, T. J., Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects–finding the right mix. *Phytochemistry*, 72(13), 1605-1611.

- Bundy, C. S., Grasswitz, T., Sutherland, C. (2012). First report of the invasive stink bug *Bagrada hilaris* (Burmeister) (Heteroptera: Pentatomidae) from New Mexico, with notes on its biology. *Southwestern Entomologist*, 37(3), 411-414.

- Bundy, C. S., Perring, T. M., Reed, D. A., Palumbo, J. C., Grasswitz, T. R., Jones, W. A. (2018), *Bagrada hilaris*. In: McPherson JE (ed) Invasive Stink Bugs and Related Species (Pentatomoidea): Biology, Higher Systematics, Semiochemistry, and Management, *CRC Press, Boca Raton*, FL, 205-242.

- Carapezza, A. (1981). Gli eterotteri dell'isola di Pantelleria (Insecta, Heteroptera). *Il Naturalista siciliano*, 5, 73-91.

- Carroll, S., Rummel, D. (1985). Relationship between time of boll weevil (Coleoptera: Curculionidae) emergence from winter habitat and response to Grandlure-baited pheromone traps. *Environmental Entomology*, 14(4), 447-451.

- Charleston, D. S., Kfir, R. (2000). The possibility of using Indian mustard, *Brassica juncea*, as a trap crop for the diamondback moth, *Plutella xylostella*, in South Africa. *Crop protection*, 19(7), 455-460.

- Colazza, S., Guarino, S., Peri, E. (2004). *Bagrada hilaris* (Burmeister)(Heteroptera: Pentatomidae) a pest of caper in the island of Pantelleria [*Capparis spinosa* L.; Sicily]. *Informatore Fitopatologico* (Italy).

- Cook, S. M., Smart, L. E., Martin, J. L., Murray, D. A., Watts, N. P., Williams, I. H. (2006). Exploitation of host plant preferences in pest management strategies for oilseed rape (*Brassica napus*). *Entomologia Experimentalis et Applicata*, 119(3), 221-229.

- Fernández, J., Jordano, D., Rodriguez, J. (1986). *Capparis spinosa*: a resource for insects during summer food shortage in Southern Spain. In Proc. 3*rd European Congress of Entomology, Nederlandse Entomologische Vereniging*, Amsterdam (pp. 259-262).

- Giblin-Davis, R. M., Weissling, T. J., Oehlschlager, A. C., Gonzalez, L. M. (1994). Field response of *Rhynchophorus cruentatus* (Coleoptera: Curculionidae) to its aggregation pheromone and fermenting plant volatiles. *Florida Entomologist*, 164-177.

- Gözüaçık, C. (2018). Pentatomid (Heteroptera) species damaging buds and fruits of wild and cultivated capers (capparaceae) and pentatomid egg parasitoids in southeastern anatolian region of turkey. *Munis Entomology and Zoology Journal*. Vol. 13, No. 2.

- Guarino, S., Arif, M. A., Millar, J. G., Colazza, S., Peri, E. (2018) Volatile unsaturated hydrocarbons emitted by seedlings of Brassica species provide host location cues to *Bagrada hilaris*. *PLos One* 13 (12), e0209870.

- Guarino, S., Peri, E., Colazza, S., Luchi, N., Michelozzi, M., Loreto, F. (2017). Impact of the invasive Painted bug *Bagrada hilaris* on physiological traits of its host *Brassica oleracea* var botrytis. *Arthropod-Plant Interactions*, 11(5), 649-658.

- Hokkanen, H. M. (1991). Trap cropping in pest management. *Annual Review of Entomology*, 36(1), 119-138.

- Huang, T. I., Reed, D. A., Perring, T. M., Palumbo, J. C. (2014). Host selection behavior of *Bagrada hilaris* (Hemiptera: Pentatomidae) on commercial cruciferous host plants. *Crop protection*, 59, 7-13.

- Infantino, A., Tomassoli, L., Peri, E., Colazza, S. (2007). Viruses, fungi and insect pests affecting caper. *The European Journal of Plant Science and Biotechnology*, 1(2), 170-179.

- Jones, R. W., Cate, J. R., Martinez, Mernandez, E., Treviño Navarro, R. (1992). Hosts and seasonal activity of the boll weevil (Coleoptera: Curculionidae) in tropical and subtropical habitats of northeastern Mexico. *Journal of economic entomology*, 85(1), 74-82.

- Joseph, S. V., Grettenberger, I. M., Godfrey, L. D., Zavala, N. (2017). Susceptibility of germinating cruciferous seeds to *Bagrada hilaris* (Hemiptera: Pentatomidae) feeding injury. *Arthropod-Plant Interactions*, 11(4), 577-590.

- Ludwig, S., Kok, L. (1998). Evaluation of trap crops to manage harlequin bugs, *Murgantia histrionica* (Hahn)(Hemiptera: Pentatomidae) on broccoli. *Crop Protection*, 17(2), 123-128.

- Manenti, F., Masi, M., Santucci, G., Manenti, G. (2013). Parametric simulation and economic assessment of a heat integrated geothermal desalination plant. *Desalination*, 317, 193-205.

- Mathews, C. R., Blaauw, B., Dively, G., Kotcon, J., Moore, J., Ogburn, E., Pfeiffer, D. G., Trope, T., Walgenbach, J. F., Welty, C. (2017). Evaluating a polyculture trap crop for organic management of *Halyomorpha halys* and native stink bugs in peppers. *Journal of Pest Science*, 90(4), 1245-1255.

- Mizell III, R. F., Riddle, T. C., Blount, A. S. (2008, December). Trap cropping system to suppress stink bugs in the southern coastal plain. In *Proceedings of the Florida State Horticultural Society* (Vol. 121, pp. 377-382).

- Palumbo, J. C., Natwick, E. T. (2010). The bagrada bug (Hemiptera: Pentatomidae): A new invasive pest of cole crops in Arizona and California. *Plant health progress*, 11(1), 50.

- Peri, E., Rochat, D., Belušic, G., Ilic, M., Soroker, V., Barkan, S., Guarino, S., Bue, P. L. Colazza, S. (2017). *Rhynchophorus ferrugineus*: Behavior, Ecology, and Communication. *Handbook of Major Palm Pests: Biology and Management*, 105-130.

- Rea, J. H., Wratten, S. D., Sedcole, R., Cameron, P. J., Davis, S. I., Chapman, R. B. (2002). Trap cropping to manage green vegetable bug *Nezara viridula* (L.)(Heteroptera: Pentatomidae) in sweet corn in New Zealand. *Agricultural and Forest Entomology*, 4(2), 101-107.

- Reed, D. A., Palumbo, J. C., Perring, T. M., May, C. (2013). *Bagrada hilaris* (Hemiptera: Pentatomidae), an invasive stink bug attacking cole crops in the southwestern United States. *Journal of Integrated Pest Management*, 4(3), C1-C7.

- Rivera, D., Inocencio, C., Obón, C., Alcaraz, F. (2003). Review of food and medicinal uses of *Capparis* L. Subgenus *Capparis* (capparidaceae) Revisión de los Usos Alimentarios y Medicinales de *Capparis* Subgénero *Capparis* (Capparidaceae). *Economic Botany*, 57(4), 515.

- Ronoh, R., Ekhuya, N. A., Linde, M., Winkelmann, T., Abukutsa-Onyango, M., Dinssa, F. F., Debener, T. (2018). African nightshades: Genetic, biochemical and metabolite diversity of an underutilised indigenous leafy vegetable and its potential for plant breeding. *The Journal of Horticultural Science and Biotechnology*, 93(2), 113-121.

- Rummel, D., Bottrell, D. (1976). Seasonally related decline in response of boll weevils to pheromone traps during mid-season. *Environmental Entomology*, 5(4), 783-787.

- Samra, S., Ghanim, M., Protasov, A., Mendel, Z. (2015). Development, reproduction, host range and geographical distribution of the variegated caper bug *Stenozygum coloratum* (Hemiptera: Heteroptera: Pentatomidae). *European Journal of Entomology*, 112(2).

- Shelton, A., Badenes-Perez F. (2006). Concepts and applications of trap cropping in pest management. *Annual Review of Entomology*. 51, 285-308.

- Simoglou, K. B., Dioli, P. (2017). First report of caper (*Capparis spinosa*) serious infestation by *Eurydema eckerleini* in Cyclades Islands, Greece (Hemiptera: Heteroptera, Pentatomidae). *Fragmenta eEntomologica*, 49(1), 65-69.

- Sozzi, G. O. (2001). Caper bush: botany and horticulture. *Horticultural reviews*, 27, 125-188.

- Taylor, M. E., Bundy, C. S., McPherson, J. E. (2014). Unusual ovipositional behavior of the stink bug *Bagrada hilaris* (Hemiptera: Heteroptera: Pentatomidae). *Annals of the Entomological Society of America*, 107(4), 872-877.

- Tillman, P., Khrimian, A., Cottrell, T., Lou, X., Mizell, III. R., Johnson, C. (2015). Trap cropping systems and a physical barrier for suppression of stink bugs (Hemiptera: Pentatomidae) in cotton. *Journal of Economic Entomology*, 108(5), 2324-2334.

- Torres-Acosta, R. I., Sánchez-Peña, S. R., Torres-Castillo J. A. (2017). Feeding by Bagrada Bug, *Bagrada hilaris*, on *Moringa oleifera* (Brassicales: Moringaceae) in Mexico. *Southwestern Entomologist*, 42(3), 919-922.

Chapter 5

Concluding remarks.

Chapter 5

Concluding remarks

Many Heteroptera Pentatomidae species (Stink bugs) are economically important pests to several crops around the world. The chemical ecology of these species is driven by intraspecific and interspecific semiochemicals that act like cues and lead them to find conspecifics or bring them in proximity of a suitable host plant. The use of such semiochemicals can be exploited for manipulate the behavior of these phytophagous insects. In particular pheromones and host plant volatiles can be used for in monitoring and control the Heteropteran herbivores in the programs of the integrated pest management aimed to reduce the use of insecticides.

In this dissertation, we focused on the chemical ecology of the pentatomid species *Bagrada hilaris* also know as Painted bug, to evidence the chemical cues exploited at intra / inter-specific level in order to develop new tools for monitoring and control this dangerous species.

In detail, on the first chapter is presented a general introduction on the strategy used by Heteroptera herbivore for mating and host searching behavior with emphasis on the role of the chemical compounds involved in the stink bugs chemical ecology. The chapter introduce also the chemical ecology of *B. hilaris*, the subject of this thesis.

The second chapter focuses on the role of (*E*)-2-octenyl acetate, the main volatile produced by adult Painted bugs, on the behavior of this species. In our experiments, in olfactometer was shown that females and nymphs are attracted to (*E*)-2-octenyl acetate, while males showed no attraction. Moreover, in field bioassays experiments was evidenced an overall small but significant number of captures of individuals (females and nymphs) in the traps baited with the higher doses of (*E*)-2-octenyl acetate. These results prove that (*E*)-2-octenyl acetate is involved in chemical ecology of insect species eliciting the attraction of females and nymphs. Further trials are advisable to investigate the optimal dose to use in the field trap test to assess the reliability of these chemical as attractant for monitoring purposes.

In the third chapter, we examined the volatile organic compounds determining the host preference behavior of *B. hilaris* individuals to three *Brassica* species at seedling stage. The observations showed that adult of Painted bugs preferred *B. oleracea* var. botrytis and *B. napus* over *B. carinata* and that this preference is strongly influenced by the difference in the bouquet of volatile organic compounds (VOCs) emitted. In the specific, chemical analysis of the VOCs of the three species collected in airstream, indicated a high amount of a diterpene (chemical formula $C_{20}H_{32}$) of novel identification present in VOCs from both of the preferred host plants *B. oleracea* and *B. napus* and not in VOCs of *B. carinata*. Furthermore, bioassays carried out after chemical fractionation of the VOC indicated that the non-polar fraction largely made by this novel diterpene was the responsible of this attraction behavior. The results reported suggest that this diterpene, alone or in combination with one or more of the minor compounds, is a key mediator in this interaction. Finally, the structure of this novel diterpene was characterized by using nuclear magnetic resonance techniques and the compound was named brassicadiene to recall the plants where it was observed for the first time.

The fourth chapter was focused to evaluate the possible use of different seedlings of brassicaceous plants as candidate for trap cropping *B. hilaris* in order to protect the caper bush (*Capparis spinosa* L.) in Pantelleria plantations. In a first step to evaluate in laboratory the brassicaeous seedling most attractive to *B. hilaris* laboratory, the results indicated Painted bugs prefer to orient toward to seedlings of *E. sativa* L. and to *B. oleracea* var. botrytis then in less degree to *B. carinata*. Field experiment were then carried out to test these seedlings as lure inside traps and also in pots placed in infested field to test their ability and to divert Painted bugs from caper plants. The results indicated that traps lured with living seedlings of *E. sativa* and *B. oleracea* can attract a consistent number of individuals inside the trap, suggesting the possibility to use this combination of lure and trap for monitoring *B. hilaris*. Finally, the pots with seedlings placed in the field determined the attraction of hundreds of individuals and suggest the possibility to explore this tool as trap crop, in particular for those caper growers who want to switch to organic farming in Pantelleria.

5.1 Future perspective

The introduction of semiochemical-based products as control methods the IPM of *B. hilaris* is highly recommendable. One important first step could be to optimize the use of (*E*)-2-octenyl acetate by testing different doses and different releaser type. Furthermore the synthesis of brassicadiene could give an opportunity to have a new attractant that could synergize the use of (*E*)-2-octenyl acetate to increase the trap efficacy.

In addition, in consideration that plant diterpenes have often defensive role against microorganism, the possibility to test the bioactivity properties of brassicadiene versus fungi and nematodes could be of particular interest.

In parallel, in order to have at short-term new tools for *B. hilaris* management in Pantelleria caper orchards, studies could focus in optimizing the use of seedlings as lure in traps, enlarging their life by using an optimal grown substrate and including the use of a systemic insecticide to avoid that *B. hilaris* could kill them once entered in the traps.

Finally, the seedlings have to be assessed as trap crop in Pantelleria, evaluating their sustainability in term of costs and benefits.