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PhD thesis

SELECTION OF MICROORGANISMS TO DEVELOP NEW TOOLS FOR THE MANAGEMENT OF *DROSOPHILA SUZUKII*

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Abstract

Drosophila suzukii Matsumura is an alien invasive pest of soft fruits. It has spread across the Americas and Europe since the late 2000s with adverse economic effects on berries. This pest received special attention because it is one of the most successful invasive species of the genus *Drosophila*, utilizing different food resources and showing ecological adaptation to variable climatic conditions. Monitoring, as a component of pest management, is the key to control this pest. Hence, it is important to use the best lure and trap available to obtain reliable monitoring. At present, current fermentation products and synthetic lures are not adequately selective and effective for monitoring. Moreover, no efficient monitoring tools have been developed yet. There is a need to improve the attractiveness of commercially available lures that are currently used to monitor this pest and developing a new trapping system for efficient pest management. The present work aims to develop the prototype of a highly attractive lure for developing a new trapping system intended for pest monitoring. Therefore, olfactory and trapping studies which included a series of behavioral bioassays were conducted under laboratory and semi-field conditions. Two- choice olfactometer was used to evaluate the behavioral response of female flies towards crop and non-crop host fruits and lactic acid bacteria strains associated with fruits surface and *D. suzukii* gut microbiota. Subsequently, cage assays were used to evaluate the behavioral response of flies towards synthetic volatile compounds, associated with host fruits and microbial fermentation, that elicit a behavioral response in adult flies. Lastly, a synthetic blend was evaluated for fly's attraction and used in the design of the prototype of a highly attractive lure for developing a new trapping system intended for pest monitoring. Our results showed that flies were significantly more attracted to crop and non-crop host fruits more than control in olfactometer bioassay. Blackberry fruits were the most attractive fruits. Moreover, flies exhibited a positive response to volatiles emitted by lactic acid bacteria strains inoculated into Droskidrink food bait. The most attractive strains, *Lactobacillus kunkeei* 84 and *Oenococcus oeni* LS, showed a significant attraction to females when combined and inoculated into food bait. In cage assays, the attractiveness of a commercial lure, Dros'Attract, was improved using a blend of plant-based volatiles (geraniol) and microbial fermentation volatiles (dimethyl sulfide). Therefore, a prototype of a more attractive lure was developed comprising the commercial lure and both compounds. Ultimately, a new trapping system was developed which is comprised of Dros'Attract lure combined with volatile compounds and specialized Droso-Trap. The obtained data provide knowledge on the importance of combining host fruit volatiles with microbes' volatiles to increase the attractiveness of existing attractive lures. Also, it increases our understanding of *D. suzukii* olfactory responses to synthetic volatile compounds as sources of attractants which may help in the development and adoption of behaviourally based tools for pest monitoring and eco-friendly management strategies.

Keywords: Drosophila, lactic acid bacteria, behavior, lure, trap, monitoring.

Riassunto

Drosophila suzukii Matsumura è un parassita alieno invasivo dei frutti di bosco. Si è diffuso nelle Americhe e in Europa dalla fine degli anni 2000 con effetti economici negativi sui frutti di bosco. Questo parassita ha ricevuto un'attenzione speciale perché è una delle specie invasive di maggior successo del genere Drosophila, che utilizza diverse risorse alimentari e mostra un adattamento ecologico alle condizioni climatiche variabili. Il monitoraggio, come parte della gestione dei parassiti, è la chiave per controllare questo parassita. Quindi, è importante utilizzare le migliori esche e trappole disponibili per ottenere un monitoraggio affidabile. Al momento, gli attuali prodotti di fermentazione e le esche sintetiche non sono adeguatamente selettivi ed efficaci per il monitoraggio. Inoltre, non sono stati ancora sviluppati strumenti di monitoraggio efficienti. È necessario migliorare l'attrattiva delle esche disponibili in commercio che sono attualmente utilizzate per monitorare questo parassita e sviluppare un nuovo sistema di cattura per una gestione efficiente dei parassiti. Il presente lavoro mira a sviluppare il prototipo di un'esca altamente attraente per lo sviluppo di un nuovo sistema di cattura destinato al monitoraggio dei parassiti. Pertanto, studi olfattivi e di intrappolamento che includevano una serie di saggi biologici comportamentali sono stati condotti in condizioni di laboratorio e semi-campo. L'olfattometro a due scelte è stato utilizzato per valutare la risposta comportamentale delle mosche femmine verso frutti ospiti coltivati e non raccolti e ceppi di batteri lattici associati alla superficie dei frutti e al microbiota intestinale di *D. suzukii*. Successivamente, sono stati utilizzati saggi in gabbia per valutare la risposta comportamentale delle mosche verso composti volatili sintetici, associati ai frutti ospiti e alla fermentazione microbica, che provocano una risposta comportamentale nelle mosche adulte. Infine, una miscela sintetica è stata valutata per l'attrazione delle mosche e utilizzata nella progettazione del prototipo di un'esca altamente attraente per lo sviluppo di un nuovo sistema di cattura destinato al monitoraggio dei parassiti. I nostri risultati hanno mostrato che le mosche erano significativamente più attratte dal raccolto e dai frutti ospiti non raccolti più del controllo nel saggio biologico olfattometrico. I frutti di mora erano i frutti più attraenti. Inoltre, le mosche hanno mostrato una risposta positiva ai volatili emessi dai ceppi di batteri lattici inoculati nell'esca alimentare Droskidrink. I ceppi più attraenti, *Lactobacillus kunkeei* 84 e *Oenococcus oeni* LS, hanno mostrato un'attrazione significativa per le femmine quando combinati e inoculati in esche alimentari. Nei saggi in gabbia, l'attrattiva di un'esca commerciale, Dros'Attract, è stata migliorata utilizzando una miscela di sostanze volatili di origine vegetale (geraniolo) e volatili della fermentazione microbica (dimetil solfuro). Pertanto, è stato sviluppato un prototipo di un'esca più attraente che comprende l'esca commerciale ed entrambi i composti. Alla fine, è stato sviluppato un nuovo sistema di cattura che comprende un'esca Dros'Attract combinata con composti volatili e Droso-Trap specializzato. I dati ottenuti forniscono conoscenze sull'importanza di combinare i volatili della frutta ospite con i volatili dei microbi per aumentare l'attrattiva delle esche attraenti esistenti. Inoltre, aumenta la nostra comprensione delle risposte olfattive di *D. suzukii* ai composti volatili sintetici come fonti di attrattivi che possono aiutare nello sviluppo e nell'adozione di strumenti basati sul comportamento per il monitoraggio dei parassiti e strategie di gestione ecocompatibili.

Parole chiave: Drosophila, batteri lattici, comportamento, esca, trappola, monitoraggio.

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List of Abbreviations

Abbreviation	Significance
AAB	Acetic Acid Bacteria
CFU	Colony- Forming Unit
cm	centimeter
cv.	cultivar
DD	Droskidrink
df	degree of freedom
F/M	Female to Male ratio
g/L	gram/Liter
h	hour
IPM	Integrated Pest Management
km	kilometer
L	Liter
LAB	Lactic Acid Bacteria
L:D	Light: Dark cycle
m	meter
M	Molarity
Max.	Maximum
µg	Microgram
min.	minute
Min.	Minimum
ml	milliliter
mm	millimeter
MRS	De Man, Rogosa and Sharpe agar
mVOCs	microbial Volatile Organic Compounds
PBS	Phosphate-Buffered Saline
ppb	Parts per billion
ppm	Parts per million
PRA	Pest Risk Analysis
oz.	ounces
RH	Relative Humidity
rpm	revolutions per minute
s	second
SE	Significant Error
sem	Standard Error Mean
spp.	species
SWD	Spotted Wing Drosophila
UNIMOL	University of Molise
var.	variety
VOCs	Volatile Organic Compounds

List of Symbols

Abbreviation	Significance
˚C	Celsius
&	and
®	Registered
™	Trademark
%	percent
~	approximate
≥	greater than or equal
≤	less than or equal
<	less than

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Dedication

To my ever loving and caring mother without whom I won't go that far in the journey of my life. Her spiritual presence and blessings made my life an inclined success.

More importantly to the departed soul of my father, Mahmoud Awwad Alawamleh, whose dream has been made true by holding the honorable title of Doctorate of Philosophy. I am always uplifted emotionally and professionally through his blessings throughout my life.

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Amani M. Alawamleh

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CHAPTER 1

1. Introduction

Alien insect pests are organisms that are native to different biogeographical area and whose introduction causes economic, environmental, or human health damages (Vaes-Petignat and Nentwig, 2014). Such kinds of organisms are known by their ability to invade and outbreak in vulnerable environments, threatening indigenous flora. Globalization and international trading have contributed to a considerable extent in the spread of alien pest species, making it a critical threat for food and economic security.

Soft fruit production is an important industry in the European agriculture sector which is mainly dominated by berries i.e. raspberries, blueberries, blackberries, cherries, and strawberries. Similarly, berries cultivation and production were reported officially by the International Food and Agriculture Organization of United Nations (FAO). In 2019, Poland has been observed to be the largest producer of berries in Europe. Additionally, the country has the largest cultivated area and yield for berries, which includes mainly strawberries, raspberries, and blueberries (FAOSTAT, 2019)

Basically, the increased production of berries can be related to the higher consumer demand. Raspberry has been reported to be the highest in demand compared to other berries such as blueberry and blackberry. Likewise, fresh berries production is a global and highly competitive market. It provides the farmers with a profitable margin in the markets, however, it is important to maintain the quality of these fruits throughout the entire value chain (Greblikaite *et al*., 2019).

For several decades, European berries producers are encountering many serious threats to production such as economic yield losses due to fruit flies' infestations (Cini *et al*., 2012). Since 2009, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), has been the most damaging fruit fly among the other known pests of soft fruits (Calabria *et al.*, 2012). *Drosophila suzukii*, an invasive fruit fly of Asian origin, invaded Europe rapidly causing great losses in soft fruits production (Rota-Stabelli *et al*., 2013; Asplen *et al.*, 2015).

The rapid invasive spread of *D. suzukii* across Europe has been expanded to temperate/cool climates causing severe damages in fruit crops and economic threat to small soft fruits industries (Cini *et al*., 2012). Fruit damage is caused by larva feeding and development within the infested fruits, making them non-saleable. *Drosophila suzukii* is a highly polyphagous pest that infests a broad range of soft-skinned fruits i.e. blackberry, raspberry, blueberry, strawberry, and grapes (Rota-Stabelli *et al*., 2013).

Monitoring, as a part of pest management, is the key to control this pest. It assists in detection the occurrence of *D. suzukii* in host crops and provides information about feeding patterns, behaviour throughout the year and population dynamics. Hence, it is important to use the most efficient lure and trap available to obtain the most adequate monitoring.

Primarily, monitoring *D. suzukii* populations is being implemented by using differentially shaped and colored traps baited with fermentation products such as apple cider vinegar, wine, or yeast/sugar as attractive lure (Baroffio *et al.*, 2014; Iglesias *et al*., 2014; Vaccari *et al.*, 2014; Mazzetto *et al.*, 2015; Cha *et al.*, 2018; Tonina *et al.*, 2018; Clymans *et al.*, 2019). At present, current fermentation products and synthetic lures are not adequately selective and effective for *D. suzukii* monitoring (Tonina *et al.*, 2018). Likewise, no efficient monitoring tools have been developed yet.

The present work contained olfactory and trapping studies which included a series of behavioral bioassays under laboratory and semi-field conditions. Two- choice olfactometer was used to evaluate behavioral response of female flies towards crop and non-crop host fruits and bacterial strains associated with fruits surface and *D. suzukii* gut microbiota. Subsequently, cage assays were used to evaluate behavioral response of flies towards synthetic volatile compounds, associated with host fruits and microbial fermentation, that elicit behavioral response in flies.

Lastly, a synthetic blend was evaluated for fly's attraction and used in design of the prototype of a highly attractive lure for developing a new trapping system intended for monitoring *D. suzukii*. The obtained data provide knowledge on the importance of combining host fruit volatiles with microbes' volatiles to increase attractiveness of current attractive lures. Also, it increases our understanding of *D. suzukii* olfactory responses to synthetic volatile compounds as sources of attractants which may help in the developing of behaviorally based tools for pest monitoring and management strategies.

CHAPTER 2

2. Literature Review

2.1. *Drosophila suzukii* (Matsumura)

Drosophila suzukii Matsumura, 1931, known as cherry drosophila in Japan and Spotted Wing Drosophila (SWD) in the United States of America, is an alien invasive pest of soft fruits (Walsh *et al.*, 2011). It is assumed that SWD is native to East and South East Asia, including China, Japan, and Korea (Walsh *et al*., 2011). The reported evidence on its geographical origin shows that it was introduced to Japan in the beginning of the 20^{th} century (Hauser, 2011). It has spread across Americas and Europe since the late 2000s with adverse economic effects on berries (Asplen *et al.*, 2015). This pest deserves special attention because it is one of the most successful invasive species of the genus *Drosophila*, utilizing different food resources and showing ecological adaptation to variable climatic conditions (Cini *et al.*, 2014).

Drosophila suzukii is an excellent model for research on biological invasions and pest management (Cini *et al.*, 2014). A great interest in this species persists due to its colonizing ability, and therefore offers a useful experimental model for investigating the changes in population genetics which are involved in adapting to a new environment (Cini *et al*., 2012). Genetic changes could be helpful to disclose *D. suzukii* colonization patterns and dynamics and to mark the invasion routes, which could allow prevention of recurrent pest introductions. Based on preliminary genetic findings Calabria *et al.*, (2012) proposed that the *D. suzukii* invasions in North America and in Europe could be correlated.

This fly is unusual among species of the family Drosophilidae in being a serious major pest of thin-skinned berries (e.g. blackberries, blueberries, strawberries, raspberries) and stone fruits (e.g. cherries, peaches, plums) (Cini *et al*., 2012). Additionally, a wide range of alternative host plants including wild and ornamental non-crop species have been recently listed in both American and European studies further showing the high polyphagy of *D. suzuki* (Lee *et al.*, 2015; Kenis *et al.*, 2016). *D. suzukii* is currently the target of intense research due to its huge impact on soft fruit industry in Europe and North America.

Since 2011, *D. suzukii* is listed by the European and Mediterranean Plant Protection Organization (EPPO) in A2 alert list of pests locally present in EPPO region and recommended for regulation as quarantine pest. Therefore, pest risk analysis (PRA) is recommended to identify the potential risk and propose phytosanitary measures to mitigate those risks. Subsequently, monitoring of *D. suzukii* populations was implemented intensively in several countries, and early warning systems has been announced in detection areas.

Several studies on biological, ecological, and genetic aspects of *D. suzukii* were carried out since its invasion in Europe. These studies suggested that an effective response to *D. suzukii* invasion requires an appropriate development of management tools and efficient transfer of information and technology to stakeholders (Lee *et al.*, 2011a).

2.2. Scientific classification

Drosophila suzukii Matsumura (Diptera: Drosophilidae), is classified in the subgenus *Sophophora*, species-group *melanogaster* and subgroup *suzukii*. The *Drosophila suzukii* species subgroup is considered polyphyletic (Kopp and True, 2002). Molecular phylogenetic analyses suggested *D. biarmipes* as a sister species of *D. suzukii* (Chiu *et al.*, 2013; Rota-Stabelli *et al*., 2013). *D. suzukii* adult flies were observed in Japan as early as 1916 by T. Kanzawa and they were described in 1931 for the first time by Matsumura (Walsh *et al.*, 2011).

2.3. Host range

Drosophila suzukii is a highly polyphagous pest. It has a broad range of host plants including soft-skinned fruits i.e. berry fruits such as blackberry (*Rubus* spp.), blueberry (*Vaccinium* spp.), raspberry (*Rubus idaeus*), strawberry (*Fragaria ananassa*) and grapes (*Vitis vinifera*), and stone fruits such as sweet cherries (*Prunus avium*), peaches (*Prunus persica*), plums (*Prunus domestica*) (Walsh *et al.*, 2011; Cini *et al*., 2012).

Additionally, wild berry fruits can serve as alternative non-crop hosts affecting crop risk for higher numbers of *D. suzukii*. High infestation rates on wild berry fruits were found in the genera *Prunus*, *Rubus*, *Sambucus*, *Vaccinium* and *Morus* (Lee *et al.*, 2015; Kenis *et al.*, 2016). *D. suzukii* can potentially utilize locally available non-crop hosts to increase population levels that later infest crop hosts (Elsensohn and Loeb, 2018).

Drosophila suzukii can infect fresh and ripen fruits of host plants in the native and in invaded areas. As fruits ripening start progressing, they become more susceptible to pest infestation. The physiological changes that occur during ripening, such as color change, decrease in skin hardness, increase in sugar content and decrease in acidity, play a main role in host susceptibility (Burrack *et al.*, 2013; Baser *et al.*, 2018). However, fruit susceptibility to pest infestation depends on both species and variety (Lee *et al.*, 2011b).

Furthermore, *D. suzukii* can attack damaged or deteriorating fruits as egg-laying site substitutes when healthy host fruits are limited or absent. Given the extreme dietary plasticity by *D. suzukii*, growers need to assess cultivation practices to limit risk of pest infestation. Application of effective methods for disposal of dropped and damaged fruits (e.g. composting, burial, cultivation, burning, etc.) could reduce access by *D. suzukii* to potential reproductive sites (Bal *et al*., 2017; Kienzle *et al.*, 2020).

To date, *D. suzukii* infestation has been confirmed in 198 plant species representing 75 genera in two botanical families. Plant species that produce fruits in spring or early summer are less vulnerable than plants produce fruits in late summer or autumn in temperate regions when *D. suzukii* populations are larger (Kenis *et al.*, 2016).

2.4. Geographical distribution

Drosophila suzukii is an invasive species endemic to South East Asia. It was described for the first time by Matsumura in 1931 on cherries in Japan. It has a high dispersal potential which was confirmed by its rapid spread and successful colonization in several continents mainly North America and Europe (Fig. 1). The First reports of this pest in Europe were in forestry localities in Rasquera, Spain in 2008 and Trentino, Italy in the following year 2009 (Calabria *et al.*, 2012). A bit later, the first reported damage to small soft fruits i.e. raspberry, highbush blueberry and strawberry in Europe was found in Trentino Province, Italy during 2009 (Grassi *et al*., 2011).

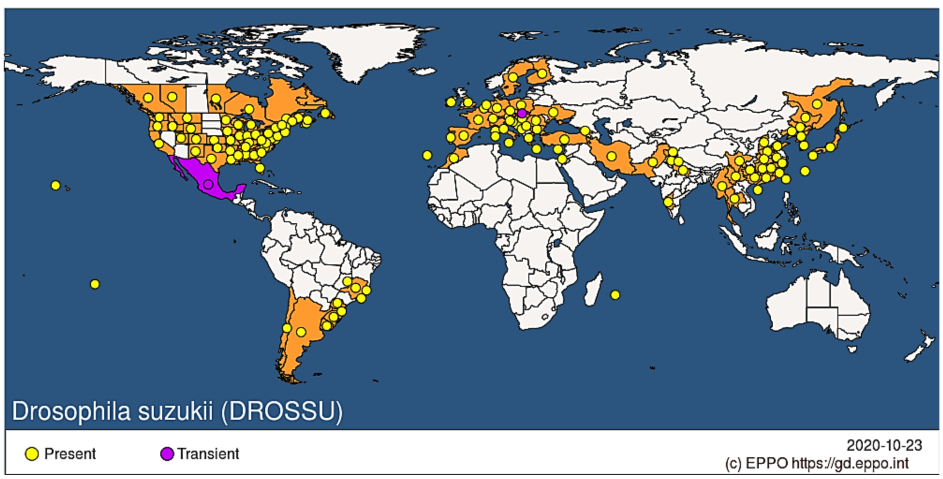

Figure 1. Geographical distribution map of *D. suzukii* in the world (EPPO, 2020).

Afterward, it started expanding its' geographic range across Europe to temperate/cool climates which can be related to ecological adaptation of this species (Asplen *et al*., 2015). This rapid invasive spread of *D. suzukii* with damages in fruit production in Europe makes economic threat to soft fruits industries (Walsh *et a*l., 2011; Calabria *et al*., 2012; Cini *et al*., 2012).

In Italy, several infestations were reported in the subsequent five years of the first detection starting in 2010 till 2015 from the northwestern regions of Piedmont, Liguria, Lombardy, and Aosta Valley, and northeastern regions of Bolzano, Veneto, and Emilia Romagna, also central regions of Marche, Umbria and Latium. In addition to southern regions of Campania, Calabria, Apulia and Islands of Sicily and Sardinia (Cini *et al.*, 2012; Tait *et al.*, 2017). These infestations caused economic yield losses in small soft fruits (De Ros *et al.*, 2013).

Based on the predictions of distribution models of *D. suzukii*, a shift in the ecological niche in pest populations, emphasizing the importance of using presence, and local environmental data. According to the models, precipitation and low temperatures are the key limiting factors for *D. suzukii* distribution, which implies that this species requires a humid environment and mild winters to establish a permanent population in its invasive range (Ørsted and Ørsted, 2019).

Conclusively, *D. suzukii* can colonize both temperate and subtropical regions, therefore, it can invade successfully, establish rapidly, and expand its distribution range in the continents of Asia, Europe and North and South America. The potential for further invasions in African and Australian continents is predicted due to the environmental suitability for this species (Dos Santos *et al.*, 2017).

2.5. Morphology and biology

The effective management strategy for the invasive pest *D. suzukii* relies on a complete understanding of its biology and ecology. Field surveys along with comparative morphology and genetic studies are useful approaches for better understanding of the complex winter biology in *D. suzukii*. The detailed knowledge on the biological aspects in *D. suzukii* may help in modeling early warning system and in defining a precise and reliable management practices (Rossi-Stacconi *et al.*, 2016).

A reliable morphological identification of *D. suzukii* can only be performed on adult specimens. Immature stages (eggs, larva and pupa) currently are not reliably identifiable using morphology, and they can only be identified by molecular techniques i.e. DNA barcoding (Hauser, 2011). Morphological variations in pest populations is an intrinsic property of *D. suzukii*, reflecting its capacity to adapt and utilize a broader range of resources and microhabitats in a single area (Little *et al.*, 2020).

2.5.1. Key morphological characteristics

Adults of *D. suzukii* are small drosophilid flies approximately 2-3 mm long (females slightly larger than males), with red eyes, a pale brown or yellowish-brown thorax and black stripes on the abdomen. Sexual dimorphism is evident: males display a characteristic dark spot on the leading top edge of the males' wings and females possess a large, serrated ovipositor enabling them to break the skin of healthy fruits (Fig. 2 and 3). The males' dark spot on each wing began developing 10 h after adult emergence but took up to two days to be fully formed and become obvious. Moreover, males are readily characterized by two short sex combs on the 1st and 2nd segment of fore tarsi of front legs (Hauser, 2011; Walsh *et al.*, 2011).

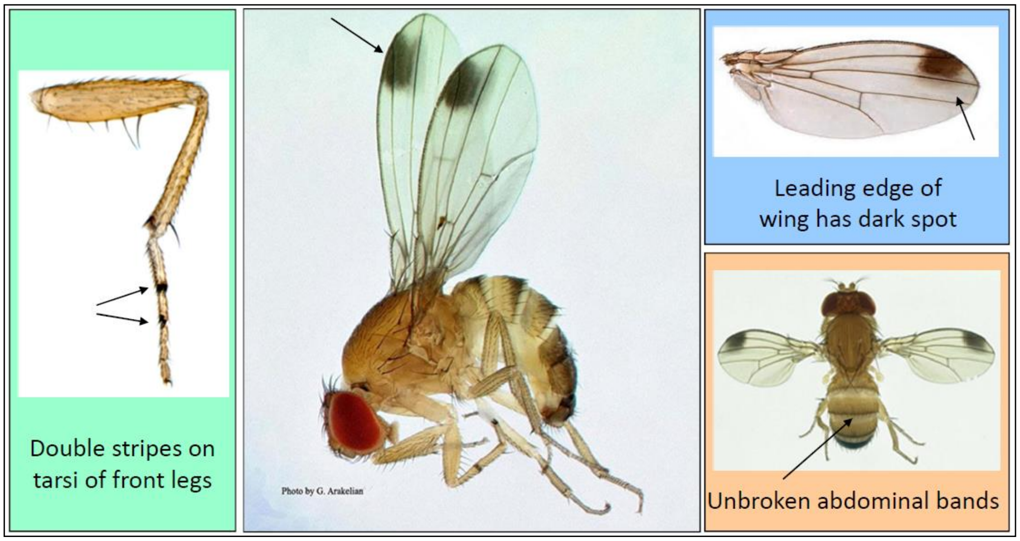

Figure 2. Morphological characteristics of *D. suzukii* male (Hauser, 2011).

Figure 3. Morphological characteristic of *D. suzukii* female ovipositor depicting a large and serrated structure with two even rows of teeth that are much darker than rest of parts of ovipositor (Hauser, 2011).

Eggs are milky white, glossy, and semi-transparent. The most important characteristic of the egg is the presence of two subapical respiratory tubes. The larvae are white to creamy in color with visible internal organs and black cephalopharyngeal skeleton; mandibles (mouth hooks) of the third instar larva densely serrated ventrally. There are three larval instars that vary in size. The pupae are creamy becoming tan-brown in color. The horn-shaped respiratory tubes are protrusions of the anterior spiracles on both sides of the head. Each respiratory tube bears seven to eight radially arranged branches at the ends (Walsh *et al.*, 2011; EPPO, 2013).

2.5.2. Life cycle and development

Drosophila suzukii has a high biotic potential and a short life cycle; the biological characteristics may vary according to geographical location (for the latitude, longitude, and altitude). Fruit identity and composition influence different aspects of the life cycle, including oviposition preference, emergence rate, development time, and number of emerging adult flies (Olazcuaga *et al.*, 2019). However, different laboratory and field studies were carried out on the development and life cycle of *D. suzukii* and the findings are summarized as follows:

Oviposition: Adults of *D. suzukii* reach maturity one or two days after emergence during the warm season. Mating occurs from the first days of life and females start oviposition between 1 day and 4 days after emergence. Female lays 1–3 eggs per oviposition site (healthy and ripening fruits), averaging 400 eggs over a lifetime. Eggs hatch within 2 to 72 h after being laid inside fruits (Lee *et al.*, 2011a).

Life cycle: *D. suzukii* develops through three larval instars, and development time from egg-laying to adult emergence ranges between 8 to10 days at 25 °C, and between 21 to 25 days at 15 °C (Fig. 4). This short generation time implies that *D. suzukii* can complete several generations in a single cropping cycle. The number of generations per year can vary from 3 to 16, depending upon the climatic conditions (Lee *et al.*, 2011).

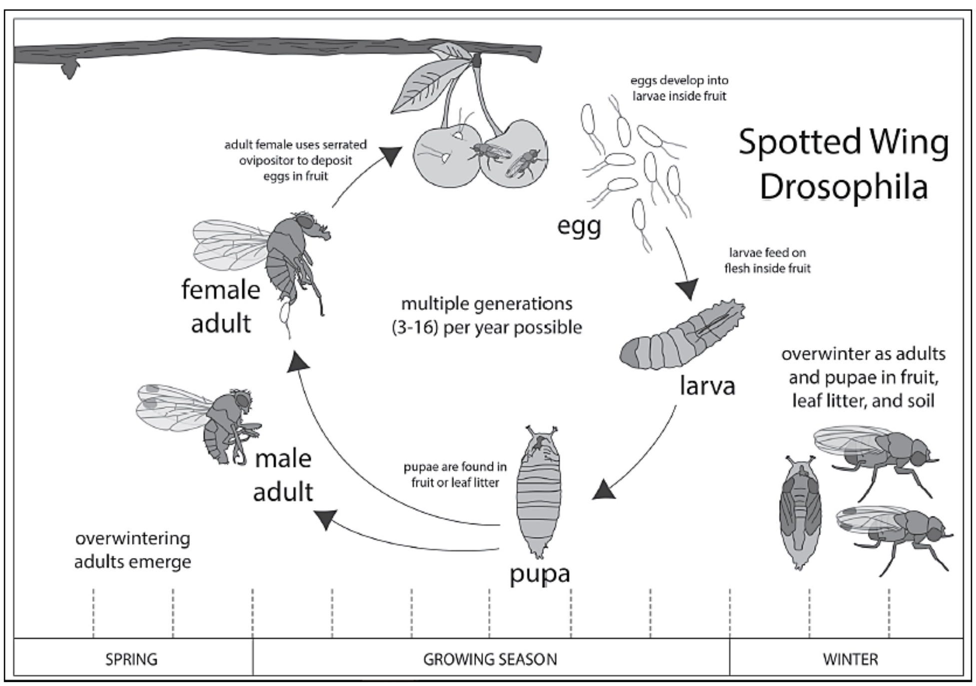

Figure 4. Distinctive lifecycle stages of *D. suzukii* (spotted wing drosophila), locations of each life stage, overwintering stages, and number of generations per year (Cannon, 2017).

Development conditions: It is known that the ambient temperature has a significant impact on development time, adult longevity, and productivity of *D. suzukii* populations. The response to temperature can affect the geographical distribution and seasonal abundance of this species. The optimal temperatures for *D. suzukii* development ranges between 22.6 and 28.2°C; however, a complete development is most reliable at constant temperatures of 20–26°C. *D. suzukii* can thus be considered a species with high thermal tolerance, being both heat and cold tolerant (Little *et al.*, 2020). Larval development and adult emergence can occur within a wide range of 8.1–30.9 °C. Larvae develop most quickly at 26–28°C.

Developmental temperatures also affect adult morphology, particularly wing size and shape, which deliberately affects flight ability. Additionally, low humidity levels limit survival of all development stages of *D. suzukii* (Asplen *et al.*, 2015). Obviously, *D. suzukii* can adapt physiologically and behaviorally to tolerate a wide range of temperature and humidity levels. As the effects of climate change become more proclaimed, fluctuations in both temperature and humidity will be more generic over an extended geographical range, providing further scope to *D. suzukii* for locating favorable habitats (Little *et al.*, 2020).

Reproductive diapause: *D. suzukii* adults and pupae are common overwintering life stages; however, adult females have been detected during winter period lacking mature fertile eggs. They showed a reproductive diapause and different ovarian maturation stages. Female flies can survive and reproduce in less favorable environments through behavioral plasticity, by making short-distance movements in and out of adjacent favorable microclimates. However, oogenesis usually resumes when temperatures and photoperiod increase, and overwintered females appeared early in the season (Wallingford *et al*., 2016; Grassi *et al.*, 2017).

Recent field studies revealed that overwintered females were bearing mature eggs as early in spring at 7 degree-days and were physiologically able to lay eggs at 87 degree-days which corresponded with the detection of the first infested hosts in early spring. These findings indicate that overwintered *D. suzukii* females are the main source for the infestations in the first available fruit crops of the season (Panel *et al*., 2018).

2.6. Chemical ecology

Chemical ecology is generally defined as the study of chemically mediated interactions between living organisms of the same species as well as other species along with their environment. It is driven by chemical signals that mediate interactions between individuals of the same species (i.e., intraspecific interactions) and/or between individuals of different species (interspecific interactions) (Karban and Baldwin, 1997).

The conventional chemical ecology workflow for studying chemical signals requires diverse methods, techniques, and procedures. Several experimental and empirical steps can be described in this workflow including laboratory/ field behavioral experiments, sampling of extracts, bioassays for evaluation of extract activity, analytical techniques for identification of extract components, discrimination of electrophysiologically active compounds, laboratory/ field behavioral bioassays of individual active compound or blend and chemical synthesis for scaling up the amount to be used in further assays (Barbosa-Cornelio *et al.*, 2019).

In the last two decades, the rapid breakthrough in understanding of the molecular basis of insect olfaction has raised the reverse chemical ecology approach. The concept of reverse chemical ecology is based on the identification of olfactory proteins i.e. odorant binding proteins (OBPs) and expression of their genes from target insect using molecular/ bioinformatics-based tools. This modern approach constitutes a reliable and efficient procedure for screening volatile organic compounds that serve as signals mediating biological processes (Barbosa-Cornelio *et al.*, 2019).

The chemical ecology of plant-insect interaction is a significant subfield of chemical ecology which integrates biology with chemistry. It provides a deep understanding of the molecular, physiological, and behavioral interactions regulated by naturally occurring specific organic compounds known as semiochemicals (Meiners, 2015). These organic compounds act as chemical signals used by insects to enable intra- and inter-specific chemical communications. They are biologically active at very low concentrations in the environment, therefore their chemical characterization is complicated (El-Shafie and Faleiro, 2017).

Nevertheless, the development of static and dynamic techniques for headspace collection of volatiles organic compounds in combination with gas chromatography–mass spectrometry (GC-MS) analysis has significantly provided a more reliable identification and characterization of semiochemical compounds (Tholl *et al*., 2006). Semiochemicals are classified in relation to their role in specific interactions. They are divided into pheromones (which mediate intraspecific interactions) and allelochemicals (which mediate interspecific interactions).

Allelochemicals are subdivided into kairomones, allomones, synomones and apneumones. The kairomones include plant odors which are used by herbivorous insects as attractants to locate host species for feeding and oviposition. These volatile attractive compounds, either alone or in combination, have been utilized for monitoring and trapping several insect species. However, the interest in research studies on volatile compounds for pest monitoring using traps has obviously evolved due to the fact that they are species-specific and safe to the environment making them promising tools for pest management (Bakthavatsalam, 2016).

2.6.1. Volatile cues

Volatile Organic Compounds (VOCs) play an important role in insect host location process. The recognition of a host plant by olfactory signals occurs by specific ratios of ubiquitous compounds due to central processing of olfactory signals by the insect, rather than their initial detection. Perception of these compounds is mediated by Olfactory Receptor Neurons (ORNs) in sensilla, located primarily on the insect antennae, which can recognize individual molecular structures. Numerous electrophysiological studies on several phytophagous insects have revealed that their peripheral receptors are modulated to the detection of ubiquitous plant volatile compounds. Chemoreception of these compounds, which comprise a range of fatty acid derivatives, phenylpropanoids and isoprenoids, is widespread (Bruce *et al*., 2005).

Furthermore, blends of volatiles play a crucial role in odor coding as there is a significant effect on insect behavioral responses. There are several studies where insect behavioral responses to host volatile blends have been shown to exceed the responses to individual volatile compound (Zhu *et al*., 2003; Alagarmalai *et al*., 2009; Anfora *et al*., 2009; Cunningham *et al*., 2016). In general, the number of volatile compounds used for host recognition by an insect ranges between 3–10 compounds which are key for host recognition (Bruce and Pickett, 2011).

In the past two decades, researchers have achieved a better understanding of *D. suzukii* attraction to host plant volatiles. Several studies have identified volatile compounds that attract adult *D. suzukii*, as well as compounds that elicit antennal responses in electrophysiological assays. Significant progress has been made in developing commercial lures based on volatile compounds for flies' attraction. Also, efforts have been devoted to improving trap designs for monitoring this pest under field conditions (Cloonan *et al.*, 2018).

Host plant volatiles can be produced and emitted from multiple sources. The primary sources of the emission of host fruit volatiles are the fruit metabolism and microbial fermentation associated with fruit surface. However, the physiological state of *D. suzuki* adult flies which includes feeding, mating and oviposition affects their response to these volatiles. *D. suzukii* flies employ these volatiles for distinct behaviors such as fruit volatiles for oviposition behaviors and yeast volatiles for feeding behaviors. Collectively, fruit and microbe volatiles contribute to attract female flies towards oviposition sites. However, leaf volatiles may be involved in host and mate finding and courtship behaviors (Cloonan *et al.*, 2018).

2.6.1.1. Host plant volatiles

Little is known about the role of host fruits volatiles and their attraction to *D. suzukii*. Mori *et al.* (2017) exhibited that mated female flies attracted to blueberry fruits for egg laying. Also, fruit volatiles attracted more mated than unmated females in the combined feeding–oviposition assay. Therefore, fruit volatiles are important in attraction of mated females which are the key life stage for pest population control.

The behavioral preference of *D. suzukii* towards host fruit volatiles i.e. raspberry, blackberry, cherry, blueberry, strawberry, bayberry and mulberry was assessed in behavioral bioassays using olfactometer. It was found that adult flies are attracted to volatiles emitted from ripe intact fruits (Revadi *et al.*, 2015; Liu *et al.*, 2018; Clymans *et al.*, 2019), fruits extract (Abraham *et al.*, 2015), or cut fruits (Yu *et al*., 2013). Hence, host fruit volatiles apparently play a crucial role in the flies' host-seeking behaviors for feeding and oviposition.

To characterize these attractive fruit volatiles, Abraham *et al.* (2015) further identified the antennally active compounds from raspberry extract, the most attractive fruit in olfactometer assays, using gas chromatography- mass spectrometry (GC-MS) and coupled gas chromatography-electroantennographic detection (GC-EAD) as well. *D. suzukii* antennae could detect 11 compounds from the headspace including butyl acetate, hexanol, 2-heptanone, 3-methyl- 1-butanone, trans-2- exanal, 3-methyl-2-butenyl acetate, 2-heptanol, hexanol, cis-3-hexanol, 6-methyl- 5-hepten-2-ol, and linalool.

Further choice assays were performed to test a synthetic blend comprising the EAD-active compounds identified from raspberry extract on flies' attraction. The blend attracted more *D. suzukii* than a blank control but was not as attractive as raspberry extract. Similarly, Revadi *et al.* (2015) performed GC-EAD assays with intact fruit of raspberry, strawberry, blueberry, and cherry.

GC-EAD recordings found 20 antennally active compounds in the headspace of raspberry fruit including acetic acid, hexanoic acid, ethanol, (Z)-3-hexen-1-ol, 1-octanol, 1-octen-3-ol, β-phenylethanol, nonanol, ethyl acetate, isoamyl acetate, ethyl butanoate, ethyl hexanoate, (Z)-3-hexenyl acetate, methyl salicylate, α-phellandrene, β-phellandrene, limonene, p-cymene, (±)-linalool, and (E)-caryophyllene.

Likewise, mated female flies were significantly attracted to isoamyl acetate, when tested as synthetic compound at 10 µg in a rubber septum, which has a release rate comparable to that of fresh fruits. Thus, the identification of the bioactive volatile compounds can contribute to the development of selective and efficient attractive lure (Abraham *et al.*, 2015; Revadi *et al.*, 2015).

On the other hand, less is known about *D. suzukii* flies' attraction to volatiles released from other parts of host plants such as leaves, stems, and roots. Keesey *et al*., (2015) found that *D. suzukii* behavioural preference in laboratory two-choice trap assays was towards the strawberry leaf compound β-cyclocitral, a volatile isoprenoid. Thus, it has been suggested that this leaf volatile is used as a possible long-range cue in attracting *D. suzukii* to their host plants.

Recently, *D. suzukii* preference between the commonly used fermentation product (apple cider vinegar) and host fruits (strawberry), taking into consideration the effect of flies' physiology, was investigated in both laboratory and field experiments. It was found that *D. suzukii* populations undergo a seasonal shift in olfactory preference between fermentation volatiles in search of food during autumn, winter and spring to fruit cues in search of oviposition sites during summer (Clymans *et al.*, 2019).

Consequently, developing highly efficient monitoring tools and control strategies should include both the fermentation volatiles and host fruits behaviorally bioactive compounds. This combined blend of volatile compounds can attract adult flies in the whole season, since the olfactory preference of *D. suzukii* flies is dependent on their physiological status i.e. feeding, mating and reproductive status and seasonal morphology which in population terms varies throughout the year (Wong *et al.*, 2018; Clymans *et al.*, 2019).

For a clear understanding of the importance of fruits' volatiles in flies' attraction, volatiles emitted by fruits should be examined apart from microbial volatiles released by microorganisms associated with fruits surface. In this regard, fruits surface sterilization can be applied for direct microbial inactivation using thermal or non-thermal processes. There are a variety of physical and chemical methods that can be used in non-thermal processes.

Chemical sterilization has been considered an efficient method aimed at reducing microbial populations on fruits and vegetables (Beuchat *et al.*, 1998; Sanz *et al.*, 2002; Oliveira *et al.*, 2012). Different protocols have been used for chemical sterilization of fruits surface, and they include several treatment times, kind of sanitizers and their concentrations. The efficacy of sanitizers can be affected by several factors mainly temperature, pH, microbial attachment, and biofilm formation (Sapers, 2001).

Chlorine and its various forms, particularly liquid chlorine and hypochlorite are the most used sanitizers in food processing due to its bactericidal properties. Currently, sodium hypochlorite (NaOCl) has widely been used as chemical sanitizer of fruits (Hashemi, 2017). Several studies have confirmed strong antibacterial activity of sodium hypochlorite against bacterial pathogens on artificially inoculated strawberries (Lukasik *et al.*, 2003), and minimally processed table grapes (Ergun and Dogan, 2018).

2.6.1.2. Microbial volatiles

Insects have evolved different strategies to feed on plants including associations with mutualistic symbionts, which can play an important role as mediators in insect-plant interactions. Microbial mutualistic symbioses may affect host plant range and enable insects to manipulate plant physiology for their own benefit.

However, the role of microbial symbionts as hidden players in insect-plant interactions may be greater than is currently recognized (Frago *et al*., 2012).

The invasion of new insect pests has often been enabled by their mutualists through allowing their hosts to exploit novel ecological niches such as non-native plants. Therefore, manipulating symbionts may be used to improve pest control strategies (Frago *et al*., 2012). Though, microbial mutualistic symbioses can release a plethora of volatiles and it appears that microbial volatiles play a substantial and often overlooked role in insect behavioral ecology (Davis *et al.*, 2013).

The microbial volatile organic compounds (mVOCs) are naturally occurring compounds, produced by microorganisms as part of their metabolism. They were often considered to be by-products of primary metabolism. Systematic exploration of mVOCs and characterization of their biological functions and ecological roles will likely provide opportunities for better control and utilization of microorganisms. Consequently, mVOCs can be exploited as pest control agents in ecofriendly and sustainable management strategies (Kanchiswamy *et al*., 2015).

The microbial volatiles belong to different chemical classes including alkenes, alcohols, ketones, benzenoids, pyrazines, sulfides, and terpenes. To quote a few examples of mVOCs, furfural, butanoicacid, propanoicacid, 5-hydroxy-methyl-furfural, β-caryophyllene, geosmin, 2-methylisobor-neol, 1-octen-3-ol, α-pinene, camphene, camphor, methanol, and acetaldehyde are among the most frequently emitted volatile compounds (Kanchiswamy *et al*., 2015).

There is increasing evidence indicating that mVOCs mediate attraction to oviposition sites, and food resources, as well as their roles in eliciting avoidance behaviors. It has been suggested that bacterial volatiles were particularly important for signaling the suitability of oviposition sites for successful larval development. Thus, the presence of volatiles from a specific microbial community may be a critical cue for eliciting oviposition (Davis *et al.*, 2013).

Significant progress has been made in understanding of *D. suzukii* response to volatiles from their associated microorganisms and host fruits. Several compounds have also been identified that elicit *D. suzukii* antennal responses in electrophysiological assays. Hence, commercial lures based on fermentation and microbial volatiles have been developed to attract *D. suzukii* flies. Moreover, efforts have been devoted to improving trap designs for pest monitoring under field conditions. Currently, the most attractive volatile compounds are being evaluated for monitoring, mass trapping, and attract & kill technique to manage *D. suzukii* populations (Fig. 5) (Cloonan *et al.*, 2018).

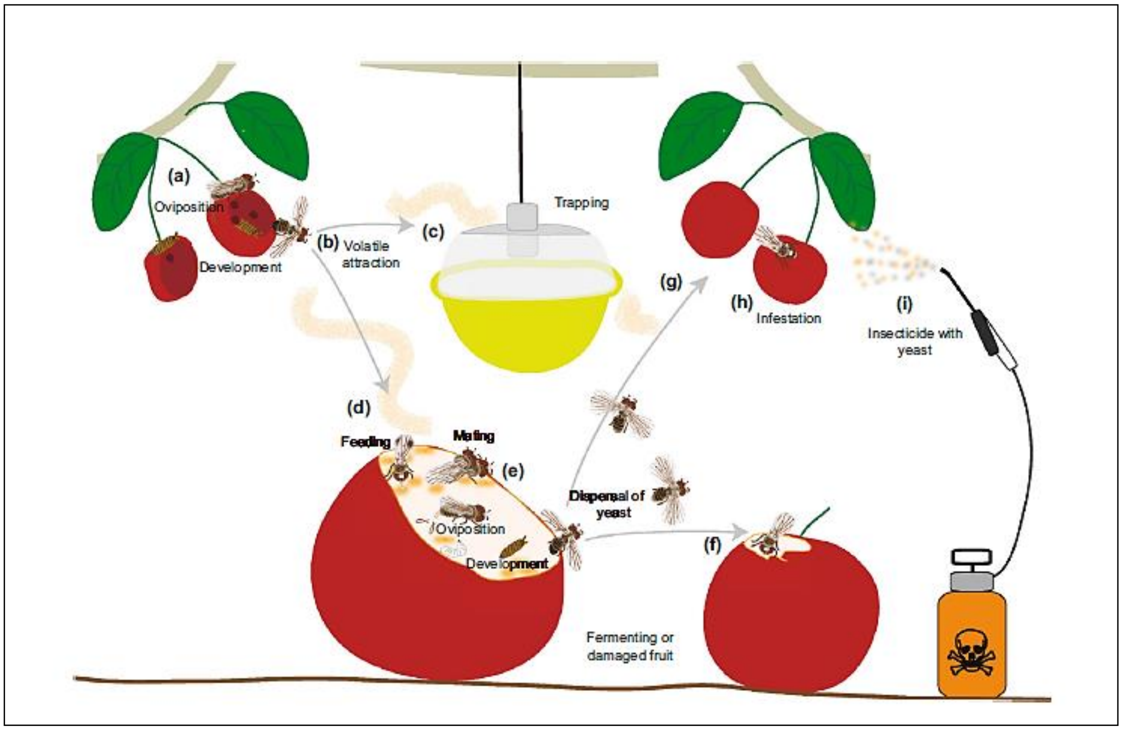

Figure 5. Illustration of interactions between *D. suzukii* and microbes and their use for pest management. (a) infestation of ripening fruit. Larvae develop inside the fruit, pupate, and emerge as adult flies. (b) female flies are attracted to food odors like yeast volatiles. (c) volatiles-based lure in traps are used for pest monitoring or management strategies, or (d) guide the flies to natural resources. (e) overripe fruit is a food resource and habitat for *D. suzukii* but also it visits fermenting fruit. Drosophilds vector yeasts and bacteria to (f) damaged fruit and (g) fresh fruit. (h) sexually mature females can infest fresh fruit. (i) as male and female *D. suzukii* are attracted to fermentation odors, yeast can be applied as attractant and biostimulant in combination with killing agents (Hamby and Becher, 2016).

2.6.1.2.1. Microorganisms associated with fruit surface

Fruits can harbor diverse populations of microorganisms including yeast and bacteria. As part of behavioral and ecological evolution, Drosophila flies are responsible for dispersal of these microorganisms apart from wind and other sources (Hamby and Becher, 2016). Indeed, microorganisms associated with fruit surface have a significant impact on Drosophila flies in terms of behavior, physiology, and ecology.

Some yeasts and bacteria are food sources to Drosophila flies and act as potential indicators of a habitat quality. It has already been proven that microbial volatiles induce strong attraction between *D. suzukii* larvae and flies (Venu *et al.*, 2014; Scheidler *et al.*, 2015). This interaction can be expressed as the female flies feed and mate on the fermented or rotten fruits and then track the volatiles of the freshly ripened fruits to oviposit (Cloonan *et al.*, 2018).

A recent study demonstrated the attractive effect of yeast VOCs on *D. suzukii* under field conditions. Yeast species were isolated from *D. suzukii*- infested fruits i.e. *Hanseniaspora* species and were used in field trapping trials. Traps were baited with live cultures of *H. uvarum*, and *H. opuntiae* and a commercial lure. It was found that these traps attract high numbers of *D. suzukii*. Further work on identification of volatiles profile of yeast-based baits showed that volatiles were dominated by ethyl acetate, ethanol, and acetoin. These findings provide the basis for improved design of lures for *D. suzukii* management (Bueno *et al.*, 2019).

On the other hand, Bueno *et al.* (2019) revealed that bacterial species isolated from *D. suzukii*- infested fruits influence flies' response. Acetic Acid Bacteria (AAB) species belong to the family Acetobacteraceae were isolated from infested fruits. Field traps placed in a raspberry orchard were baited with cultures of *Gluconobacter cerinus* and a commercial lure. It was found that these baits trapped relatively high numbers of flies with high selectivity for *D. suzukii*. Subsequently, it was found that VOCs associated with these baits were dominated by acetic acid and acetoin. These findings indicate that bacterial volatiles may function as a reliable cues of habitat suitability for fly feeding and oviposition.

2.6.1.2.2. Mutualistic yeasts

Since microbes associated to *D. suzukii* comprise a considerable part of the known microbial flora, and *D. suzukii* is closely associated with yeasts mainly as a food resource, studies focused on the role of mutualistic yeast species in modulating flies' preferences and attractive effects of their volatiles which may be utilized for improving effectiveness of baits and lures.

For instance, flies' preference to six yeast species, associated with *D. suzukii* in mutualistic association, was determined both in behavioral laboratory experiments and field preference experiment. It was found that *H. uvarum*, the most frequently cultured species from field-collected adult flies, induced the highest flies' captures among all isolated yeast species in laboratory and field traps. Furthermore, chemical analysis of volatile compounds from all yeast species revealed unique profiles demonstrating quantitative and qualitative differences (Scheidler *et al.*, 2015).

On the other hand, yeast species associated with host fruits were isolated from infested and uninfested samples of cherry and raspberry fruits. Interestingly, *H. uvarum* is the predominant yeast species across all sample types, suggesting it as a good candidate species for a selective attractive lure (Hamby *et al.*, 2012).

Mori *et al.* (2017) demonstrated that mated female flies attracted to *H. uvarum* for feeding and to blueberry fruits for egg laying. Also, both yeast and fruit volatiles attracted more mated than unmated females in the combined feeding-oviposition assay. Therefore, yeast and fruit volatiles are necessary for attracting mated females which are the targeted life stage in pest control approaches.

2.6.1.2.3. Symbiotic bacteria

Adult *D. suzukii* flies harbor an inconstant microbiota including acetic acid and lactic acid bacteria. Symbiotic bacteria impact flies' food quality, development time and reproductive output (Vacchini, 2014). They have been shown to play an important role in the production of volatile compounds which mediate flies' attraction particularly, gut-associated bacteria belong to family Acetobacteraceae. However, only few studies have been performed under laboratory and field conditions to evaluate the effects of the bacterial volatiles on *D. suzukii* response (Mazzetto *et al.*, 2016; Bueno *et al.*, 2019).

2.6.1.2.3.1. Acetic acid bacteria

Acetic Acid Bacteria (AAB) are a group of gram-negative bacteria. They are obligately aerobic bacteria within the family Acetobacteraceae, widespread in carbohydrate-rich food in an aerobic and acidic environment. AAB are known for their ability to partially oxidize a variety of carbohydrates and produce acetic acid during fermentation process (Mamlouk and Gullo, 2013). Recent research in microbe-insect symbiosis has shown that AAB ascertain symbiotic relationships with Drosophilid flies. AAB establish symbiotic associations with the flies' midgut, a niche characterized by the availability of diet-derived carbohydrates and oxygen and acidic pH which support AAB growth (Crotti *et al.*, 2010).

At a recent time, the attractiveness of bacterial volatiles has been investigated in behavioral bioassays, along with chemical characterization of volatiles profile. Strains and species of symbiotic AAB of commonly found genera in *D. suzukii* Italian populations (*Acetobacter*, *Gluconobacter* and *Komagataeibacter*) were tested in Y-tube olfactometer. Female flies showed a significant attraction for some strains of *Gluconobacter* and *Komagataeibacter* species which produced the most attractive volatiles that were proposed to be a useful tool for developing sustainable control strategies (Mazzetto *et al.*, 2016).

On the other hand, Bueno *et al.* (2019) proved that AAB species mediate *D. suzukii* attraction under field conditions. AAB species within the family Acetobacteraceae were isolated from wild *D. suzukii* flies. Traps in a raspberry orchard were baited with cultures of *Gluconobacter oxydans* and a commercial lure. It was found that these baits trapped relatively high numbers of flies with very high selectivity for *D. suzukii*. Moreover, it was found that VOCs associated with these baits were dominated by acetic acid, acetoin, and other carboxylic acids, and lacked detectable ethyl acetate. Hence, AAB volatiles can be a reliable tool for improving attractiveness and selectivity of available lures for effective pest management strategies.

2.6.1.2.3.2. Lactic acid bacteria

Lactic Acid Bacteria (LAB) are widespread microorganisms which play a significant role in a variety of food fermentation processes. They ferment carbohydrates and produce lactic acid as the main product of fermentation. They are gram-positive and acid-tolerant microorganisms that grow anaerobically. LAB include several species i.e. the genus *Lactobacillus*, as well as the genera *Pediococcus*, *Leuconostoc*, *Streptococcus* and *Oenococcus*. The fast-growing characteristics of LAB strains and their metabolic activities are the keys of LAB benefits and applications (Teuber, 1993).

Although AAB are considered the major component of *D. suzukii* bacterial community, symbiotic LAB species were identified in the gut of *D. suzukii* i.e. *L. plantarum* and *L. brevis* (Vacchini, 2014). The *D. suzukii* attraction towards volatiles emitted by LAB species has been rarely assessed. The potential use of LAB species as a source of *D. suzukii* attraction may contribute to developing a more attractive lure for effective pest monitoring and control.

In a previous study, Maddalena (2016) revealed that LAB species are capable to produce bioactive volatiles for *D. suzukii* flies' attraction. The species *O. oeni*, *Lactobacillus* spp., and *Pediococcus* spp. were inoculated individually into a commercial food bait and applied in field trapping trials. Among all tested LAB species, *O. oeni* showed a significant attraction towards *D. suzukii* flies. Additionally, *D. suzukii* flies exhibited antennal responses towards volatiles emitted by a commercial food bait inoculated with *O. oeni* strains using Electroantennography (EAG).

2.7. Damages to the soft fruits

It has been known that *D. suzukii* females oviposit in fresh intact fruit as opposed to fruit that is damaged or overripe. Upon insertion of the females' serrated ovipositor, a physical damage to the host fruit can be observed. Very often oviposition wounds, provide access to secondary infection by both insects and pathogens including fungi, yeasts, and bacteria causing additional losses. Likewise, eggs develop into larvae within the fruit, causing it to become soft and rot rapidly, resulting in reduced crop yields and significant economic losses (Walsh *et al.*, 2011).

2.8. Pest management strategies against *D. suzukii*

Effective management of this invasive pest is a challenge owing to the wide host range, high fertility, short generation time and high dispersal potential. Indeed, *D. suzukii* biology and ecology impose, to obtain an effective crop protection, the integration of several control tools and the transfer of knowledge and technology to growers. There is an increasing evidence of the importance of long-term and environmentally friendly management approaches which allow a sustainable control of this pest (Cini *et al*., 2012).

2.8.1. Monitoring of *D. suzukii*

A reliable pest monitoring is the first step for a successful Integrated Pest Management (IPM). An effective monitoring is fundamental to detect the presence of the fly and verify the characteristics of the pest population. Primarily, monitoring *D. suzukii* populations is being implemented by using differentially shaped and colored traps baited with fermentation products such as apple cider vinegar, wine, or yeast/sugar as attractive lure (Baroffio *et al.*, 2014; Iglesias *et al*., 2014; Vaccari *et al.*, 2014; Mazzetto *et al.*, 2015; Cha *et al.*, 2018; Tonina *et al.*, 2017; Clymans *et al.*, 2019).

Field surveys carried out in several European countries and the United States of America using fermented baits, revealed that *D. suzukii* captures were significantly greater in early spring and late summer more than in winter and reached higher peaks in autumn (Hamby *et al.*, 2014; Mazzetto *et al.*, 2015; Briem *et al.*, 2018; Cha *et al.*, 2018). Understanding the *D. suzukii* behaviour in the field is of high importance to estimate pest population density and to define pest management strategies.

2.8.2. Control methods

Current control efforts for *D. suzukii* rely heavily on the use of conventional chemical insecticides. Among the registered insecticides, organophosphates, spinosyns and pyrethrins, in timely applications, can provide adequate level of control (Pavlova *et al.*, 2017; Cahenzli *et al*., 2018; Shawer *et al.*, 2018). Among the environmentally safe strategies those based on the interferences with the insect communication are often provide efficient control i.e. mass trapping and attract & kill. These approaches act by reducing the survival of pest population (Rice *et al*., 2017; Spies and Liburd, 2019).

On the other hand, *D. suzukii* populations could be reduced by exploiting biocontrol agents (fungi, bacteria, viruses) and other natural enemies of the pest, such as parasitoids and predators (Rossi Stacconi *et al.*, 2013; Cuthbertson *et al*., 2014; Girod *et al.*, 2017; Ibouh *et al.*, 2019). Reducing pest population is also possible by inundative releases of sterile insects. The development of the Sterile Insect Technique (SIT) represents a breakthrough in pest management science (Schetelig *et al*., 2017). However, research is underway for the evaluation of these control methods in laboratory and field assays.

Moreover, current sustainable control strategies include field sanitation measures. These measures are based on removal of any possible food source or breeding site of *D. suzukii* either inside or outside fruit orchard such as dropped or leftover fruits, wild hosts and ornamental plants in backyard gardens. It is a simple but crucial measure in order to prevent re-infestations on local scale, and thus represents a key step in IPM (Walsh *et al.*, 2011; Cini *et al*., 2012).

However, several options for effective disposal of potentially infested fruits have been proposed, e.g. solarization, disposal in closed containers, cold treatment, bagging and burial (Walsh *et al.*, 2011). Furthermore, the use of physical barriers has proven to be efficient to exclude *D. suzukii* from its host plants. Exclusion netting has shown delaying and reduction in overall pest infestation in commercial orchards (Leach *et al*., 2016).

2.8.3. Trapping system of *D. suzukii*

Trapping system is based on conventional tools available for fruit flies in general, i.e. plastic traps and attractive lures. The efficacy of trapping is highly influenced by the lure composition and trap type in terms of shape and color. However, with the development of an effective lure, optimizing trap design, and improved trapping protocols, the pest monitoring can be more reliable (Renkema *et al*., 2014). The position of the trap is also considerable which is either on the ground or hung near the fruits in a shady portion with low temperature (Walsh *et al.*, 2011).

2.8.3.1. Attractive synthetic lures

Adult *D. suzukii* populations are monitored by traps loaded with fermentation products such as apple cider vinegar, wine, or yeast as baits. Apple cider vinegar has been used frequently because it is easily available, inexpensive, simple to apply in the field, and transparent enough for clear identification of caught flies in the bait solution (Walsh *et al*., 2011; Lee *et al*., 2012). A mixture of sugar water with baker's yeast has been reported to be highly attractive to *D. suzukii* in field applications (Walsh *et al*., 2011; Iglesias *et al*., 2014).

However, adding wine to apple cider vinegar increases the attractiveness of the bait towards *D. suzukii*, resulting in captures comparable to the yeast and sugar baited traps (Landolt *et al*., 2012; Iglesias *et al*., 2014; Mazzetto *et al*., 2015). Synthetic lures are available commercially for *D. suzukii* monitoring in fruit production, including Droskidrink® (Prantil, Trento, Italy), Scentry® lures (Scentry Biologicals Inc., Billings, Montana, USA), and Pherocon® SWD Dual-Lure (Trécé Inc., Adair, Oklahoma, USA). These products have been reported as an effective attractant in the early studies carried out in different countries (Cha *et al.*, 2018; Tonina *et al.*, 2018; Harmon *et al.*, 2019).

Previous studies on *D. suzukii* behavioural and olfactory responses to specific volatile compounds have identified several attractive blends, i.e. a four-component synthetic blend of ethanol, acetoin, acetic acid and methionol (Cha *et al.*, 2013), and a five-component blend derived from fermented apple juice (acetoin, ethyl octanoate, acetic acid, phenethyl alcohol and ethyl acetate) (Feng *et al.*, 2018). These findings provide an opportunity to develop a highly attractive and selective chemical lure for monitoring and management of *D. suzukii*.

2.8.3.2. Trap types

Till now, several types of traps have been developed and tested for monitoring *D. suzukii*. The most commonly used homemade traps are plastic cups or bottles with multiple small lateral holes (diameter ~5-10 mm) loaded with the liquid bait.

The addition of a small drop of surfactant or the placement of sticky cards inside the trap enhances trapping efficiency by preventing the escape of flies (Walsh *et al.*, 2011). Homemade traps can also comprise of transparent plastic cup (~20 oz.) with red mesh coverings on the two side openings, or a sticky yellow card to capture flies. Similarly, a red plastic cup (~12 oz.) with numerous entry holes around the lid can be used (Cloonan *et al.*, 2018).

During trap evaluation studies, red and black have been shown to be the most attractive colors, hence colored traps are recommended. In addition, lure and entry holes were more important in determining attraction than trap color (Lee *et al.*, 2012; Tonina *et al.*, 2018). Recently, different commercial traps have been made available to farmers i.e. Droso-Trap® (Biobest, Westerlo, Belgium), Dome traps® (Trappitt trap, Agrisense Ltd., Pontypridd, UK), Pherocon trap® (Trécé Inc., Adair, Oklahoma, USA), that have been proven with high capture ability.

However, economic and highly effective trap-lure combination is necessary to develop a successful monitoring. The best combinations could enable high attractiveness, selectivity, ease of use, low costs and environmental impacts. Hence, an efficient combination can be used to reduce pest population size using mass trapping and attract & kill technique as eco-friendly alternative control methods for pest management (Tonina *et al.*, 2018).

CHAPTER 3

3. Objectives

Although significant advances in the chemical ecology of *D. suzukii* have been made in the last decade, and some progress has been made in providing growers with monitoring tools for *D. suzukii*, there is still a need to develop a highly attractive lure specific for *D. suzukii*. Thus, filling knowledge gaps will help in improving the efficacy and reliability of existing synthetic lures and adoption of behaviour-based tools at wide scales for monitoring and pest management strategies.

The main objective of this thesis is to develop the protoype of an efficient synthetic lure for developing a new trapping system intended for monitoring *D. suzukii.* Specifically, the objectives of the present research work are as follows:

1. Conduct olfactory studies under laboratory conditions which include:
   a) Evaluation of behavioral responses of *D. suzukii* female flies towards odors released by fresh and ripen fruits of crop and non-crop hosts.
   b) Evaluation of behavioral responses of *D. suzukii* female flies towards odors released by selected LAB strains associated with host fruits and *D. suzukii* gut microbiota.

2. Conduct trapping studies under semi field conditions which include:
   a) Evaluation of selected volatile compounds for trapping *D. suzukii* flies.
   b) Evaluation of VOCs-blend for trapping *D. suzukii* flies.

3. Design and test a prototype of highly attractive chemical lure and develop a new trapping system for pest monitoring.

CHAPTER 4

4. Materials and Methods

4.1. Olfactory studies

Studies of olfaction in Drosophila have provided key insights into the sense of smell and associated olfactory-driven behaviors. Fly species of the genus *Drosophila* are dependent on olfactory cues emitted by suitable substrates such as decaying fruits or microorganisms living in and of the fruit, to survive and reproduce in an optimal way. Therefore, olfactory studies were carried out including laboratory assays on behavioral responses of *D. suzukii* to host plant and microbial volatiles. The attractiveness of female adult flies towards the volatiles was investigated using a choice bioassay approach in an olfactometer in the Entomology Laboratory, University of Molise (UNIMOL), Campobasso, Italy, during March 2018- February 2019.

4.1.1. Behavioral response to host plant volatiles

Drosophila suzukii behavioral response, in relation to olfactory sensation, towards crop host fruits has been evaluated enormously (Yu *et al.,* 2013; Abraham *et al.*, 2015; Revadi *et al.*, 2015; Liu *et al.*, 2018; Clymans *et al.*, 2019), but has not been assessed towards non-crop hosts. Therefore, the experiment was carried out to provide data on its behavioral response towards crop hosts, as well as non-crop hosts in the southern regions of Italy. The experiment was performed during May-September 2018.

4.1.1.1. Insect rearing

Insect rearing was carried out at Insect Rearing Lab of UNIMOL. Pupae of *D. suzukii* were provided by Fondazione Edmund Mach (FEM, Trento, Italy), from where source colonies were started in September 2017. The colony was reared on a cornmeal-yeast-agar diet (17 g/L yeast flakes, *Saccharomyces cerevisiae* (KI Group SpA); 15 g/L sugar; 71 g/L cornmeal; 10 g/L soyabean meal; 5.6 g/L agar powder, 2.5 g/L multivitamin mixture (MP Biomedicals, LLC); 4.7 ml propionic acid ≥99.5% and 1L tap water). The colony was maintained at (24±1°C), (65±5%) RH, under a (16:8 L:D) photoperiod. Newly-eclosed flies were collected daily between 9:00 and 12:00 h, males and females were kept in the same glass jars (16 cm diameter × 14 cm) with access to food and water and allowed to mate for 6 days.

4.1.1.2. Experimental flies

Since the response of *D. suzukii* to attractive odors is affected by the physiological status i.e. age, feeding, mating and ovipositional status (Wong *et al.*, 2018), only naive (had no prior exposure to odors) mated female flies were used once in the behavioral bioassays. Prior to behavioral bioassay, mature female flies at seven days old were kept in 200 ml plastic container and starved on water-soaked cotton swab for 1 h to enhance the flies' response towards odors. Hunger was supposed not to motivate fly choices between potential odor and control source during test period (Little *et al.*, 2017).

4.1.1.3. Fruits collection

The fruits collected were crop and non-crop hosts of *D. suzukii* with agricultural importance and commonly grown in South of Italy. Blackberry as a preferred host and table grapes as a susceptible host undergo complete development of flies were selected for choice tests. Field trips were conducted to different fruit tree orchards during July- September 2018 in six different locations in southern regions of Italy. Fruits of different varieties of the selected hosts were obtained from conventional agricultural fields while fruits of non-crop hosts were collected from different parks in the southern regions of Italy (Table 1).

Table 1. Crop and non-crop hosts that were sampled for bioassays during their summer season of 2018 in the southern regions of Italy.

Host Type	Host Species	Common Name	Variety	Location
Crop	*Vitis vinifera*	White grapes	Victoria	Bari, Apulia
Crop	*Vitis vinifera*	Black grapes	Black magic	Bari, Apulia
Crop	*Rubus fruticosus*	Blackberry	Thornfree	Bari, Apulia
Non-crop	*Morus alba*	White mulberry	-	Napoli, Campania
Non-crop	*Prunus avium*	Wild cherry	-	Napoli, Campania
Non-crop	*Rubus fruticosus*	Wild bramble	-	Campobasso, Molise

Intact (non-infested) fruits were freshly picked at ripe stage and stored in Polypropylene (PP) containers which have no influence on fruits' odors after harvesting (Giuggioli *et al*., 2015), and were kept at laboratory temperature for 1 h before conducting the tests.

4.1.1.4. Chemical treatment

The selected fruits were tested as non-sterilized and sterilized fruits. Chemical sterilization was carried out by using commonly used sanitizer, sodium hypochlorite solution (NaOCl), to eliminate the effect of microorganisms, associated with the fruits' surface, emitting fermentation by-products as volatile compounds responsible for *D. suzukii* attraction to host fruits. Sodium hypochlorite solution was prepared by diluting a 5% commercial bleach solution (5.0% sodium hypochlorite; ACE, Fater SpA, Pescara, Italy) with distilled water.

The pH of the solution was adjusted to 7.0 with 1.0 M solution of hydrochloric acid to ensure adequate antimicrobial activity of chlorine. Solutions' pH was measured using a digital pH meter (Mettler Toledo MP225). The solution was prepared immediately prior to application and was used within 30 min. Whole fruit was dipped into sodium hypochlorite solution 1% at room temperature for 60 s, then triple rinsing of fruit with tap water followed by distilled water. Afterwards, the berries were placed in sterile Petri dishes and air-dried in a laminar flow cabinet to avoid any microbial contamination.

4.1.1.5. Olfactometer set-up

The behavioral responses of *D. suzukii* female flies to host fruits odors were examined in a closed system in two-choice bioassays with odor measurement apparatus. Briefly, the apparatus consisted of Y-shaped glass tube olfactometer (stem length 29.0 cm; arm length 22.0 cm; arm angle 60°; internal diameter 4.5 cm) (Germinara *et al*., 2011). Each arm was connected to a glass cylinder (9.0 cm long, 3.0 cm and 4.5 cm internal diameter) as a container of odor source and equipped with porous Teflon barrier to exclude any possible visual cues. Also, stem was connected to a glass cylinder (9.0 cm long, 3.0 cm and 4.5 cm internal diameter) that serves as a release chamber.

The dual-choice olfactometer was placed into a wooden-frame observation chamber (115×75×60 cm) whose inner sides were covered with white paint to reflect illumination from top white, fluorescent neon providing uniform lighting inside the tube. A purified (activated charcoal-filtered) and humidified air was pumped uniformly through each arm at a constant flow (20 ml/min) using a vacuum pump (NEWA Tecno Industria srl, IT).

At the beginning of the tests, illuminance was measured with a luxmeter (HD 9221, Delta OHM, Padova, Italy) and the rate of airflow was measured with a digital flowmeter (DFC-HR™, Alltech Associates Inc., Korea). One arm of Y-tube held 30 g of fresh intact fruits which were randomly allocated to reduce effects of spatial influence on flies' choice, and the other arm served as a blank control with clean humidified air.

4.1.1.6. Two-choice bioassay using host plants

Fly preferences for host fruits were tested in a controlled, small-scale, and short duration bioassay. The bioassay was carried out as follows: *D. suzukii* female flies were introduced into the Y-tube at the entrance of the stem simultaneously and tested in groups of ten (Fig. 6). Since fruits odors may intensified over time as the test progressed leading to increase in fly's response (Little *et al.*, 2017), flies were observed for 1 h. after which flies were retrieved, irrespective of their choice. The observation time included the first 30 min to allow the odor to reach the arm at a constant release rate and the other 30 min for flies' response (Turlings *et al*., 2004).

A choice was recorded when the female fly moved 4.0 cm up an arm of the tube, crossing the red line marked on both arms as a choice decision line and stayed behind that line for more than 30 s. The number of flies that migrated from the release chamber to the stem (active flies), and the number of flies inside the fruit and control arms was documented. Tests were undertaken between 11:00 AM and 17:00 PM, the period of the day in which flies showed the maximum activity in our preliminary observation trials of searching fly's behavior.

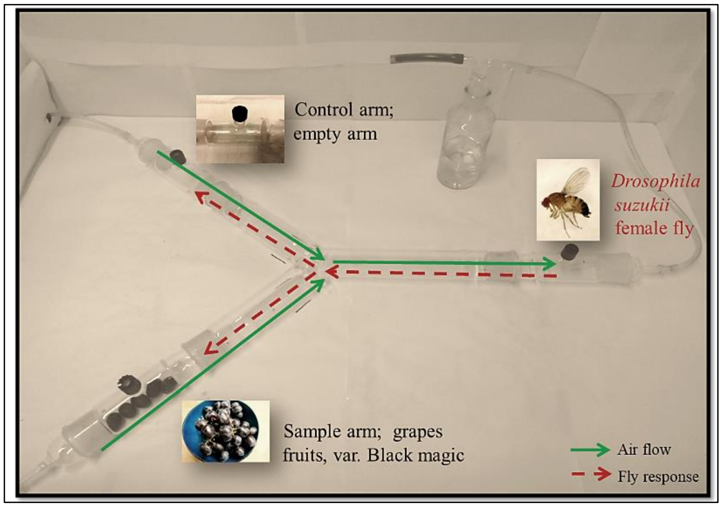

Figure 6. Functionality figure of Y-tube olfactometer used in two-choice bioassay to evaluate behavioral response of flies towards host volatiles.

After each test of 10 flies, the olfactometer was washed with distilled water and baked out in an oven for at least 1 h at 150 °C. Each host fruit type was tested separately in the olfactometer and each choice test was replicated 10 times on consecutive days at the same time. Thus, the olfactory responses of 100 flies per host fruit type in comparison to treatment were tested. The tests were conducted in a laboratory at (23±1°C) and relative humidity of (65±5%), with ~1000 lux light luminance. Prior conducting the bioassays, we tested 30 female flies with clean air in both arms to overcome the asymmetry of olfactometer and exclude any bias in the set-up.

4.1.1.7. Data analysis

Data were recorded based on the first choice made by a fly. For each treatment, mean percentages of active flies, flies inside fruit arm, and control arm were calculated, as well as corresponding standard errors. Flies that did not make a choice were excluded from the analysis. Chi-square test was used to compare the flies' response to treatments and control at $P < 0.05$, and to verify significant differences between treatments and control (Statistix, version 8.1; Analytical software, Tallahassee, Florida).

4.1.2. Behavioral response to microbial volatiles

Evaluation of the preference of *D. suzukii* for microbial volatiles, will improve fly attractiveness of food baits. Different strains of LAB species have been evaluated for their attractiveness for *D. suzukii* female flies. LAB species were used as bio-catalyzers of the production of biologically active compounds to *D. suzukii*. The experiment was performed during October 2018- February 2019. The use of active cultures of bacterial strains is considered as a very promising environment friendly tool for pest management.

4.1.2.1. Insect colony

Drosophila suzukii adult flies were obtained from a laboratory colony established in September 2017 for conducting the olfactory studies. Experimental flies were selected and used following the same protocol described in the first experiment.

4.1.2.2. Bacterial strains

LAB are widespread microorganisms which can be found in any environment rich mainly in carbohydrates, such as plants and fermented foods. Different species of LAB including *Lactobacillus plantarum*, *Lactobacillus kunkeei*, *Fructobacillus fructosus* and *Oenococcus oeni* were used. Isolation and characterization of LAB species were reported in different flowers and fruits i.e. grapes, cherry, and strawberry which are known as crop hosts of *D. suzukii* (Table 2).

Table 2. Research studies involving LAB species, used in this study, isolated from different species of fruits and flowers.

LAB species	Source of isolation	Scientific name	References
Lactobacillus plantarum	Guava	*Pisidium guajava*	Ruiz Rodríguez *et al.*, 2019
	Prunes	*Prunus* spp.	Di Cagno *et al.*, 2011a
	Kiwifruit	*Actinidia deliciosa*	Di Cagno *et al.*, 2011a
	Papaya	*Carica papaya*	Di Cagno *et al.*, 2011a
	Grapes	*Vitis vinifera*	Abubakr and Al-Adiwish, 2017
	Grapes	*Vitis vinifera*	Groenewald *et al*., 2006
	Barbados cherry	*Malpighia glabra*	Garcia *et al.*, 2016
	Sweet cherry	*Prunus avium*	Di Cagno, Surico, *et al.*, 2011b
	Strawberry	*Fragaria* spp.	Garcia *et al.*, 2016
	Strawberry	*Fragaria* spp.	Naeem *et al.*, 2012
	Plum	*Prunus domestica*	Naeem *et al.*, 2012
	Caper berries	*Capparis* spp.	Pulido *et al.*, 2012
	Mulberry	*Morus australis*	Chen et al., 2010
Lactobacillus kunkeei	Azalea	*Rhododendron* spp.	Endo *et al*., 2009
	Narcissus	*Narcissus* spp.	Endo *et al*., 2009
	Cosmos	*Cosmos* spp.	Endo et al., 2009
	Wild flowers	-	McFrederick *et al.*, 2017
	Wine grapes	*Vitis vinifera*	Bae et al., 2006
	Kumquat	*Fortunella margarita*	Neveling et al., 2012
Fructobacillus fructosus	Fig	*Ficus carica*	Ruiz Rodríguez *et al.*, 2019
	Azalea	*Rhododendron* spp.	Endo *et al*., 2009
	Wild flowers	-	McFrederick *et al.*, 2017
Oenococcus oeni	Grapes	*Vitis vinifera*	Franquès *et al.*, 2017
	Mango juice	-	Ethiraj and Suresh, 1985
	Stone fruit mashes	-	Bridier *et al.*, 2010

Generally, LAB are diverse bacterial groups and have various growth characteristics. The species, selected in this experiment, belong to fructophilic lactic acid bacteria (FLAB) group which prefers fructose as growth substrate. They share several unique biochemical characteristics when compared to other LAB species (e.g. high malolactic activity, production of volatile compounds, growth rate) (Endo e*t al*., 2018). *Oenococcus oeni* is the most well-adapted species to the harsh wine conditions due to its ability to tolerate low pH, high concentration of ethanol and sulphite (Capozzi *et al.*, 2010). Recently, *O. oeni* strain Enoferm Beta was patented by Fondazione Edmund Mach (FEM) for its efficiency in increasing attractiveness of a food bait for *D. suzukii* (Guzzon *et al*., 2016).

Moreover, the selected species has a specific association with Drosophila flies. The commensal species commonly found in metazoan gut affecting olfactory behaviour in Drosophila (Qiao *et al.*, 2019), *L. plantarum,* was identified in *D. suzukii* microbiome (Violetta, 2014; Bing *et al.*, 2018). These characteristics of LAB species distinguish them among other bacterial species, making them good candidates for a more attractive and selective lure.

The four different LAB species used in this experiment were selected from the bacterial culture collection of the DiAAA (Dept. of Agricultural, Environmental and Food Science, UNIMOL). The strains were selected based on previous phenotypic and genetic characterizations (Iorizzo *et al.*, 2016a; Iorizzo *et al.*, 2016b; Succi *et al.*, 2017). Additionally, a commercial starter strain of *O. oeni* was used in this experiment. However, a total of five strains of *L. plantarum*, three strains of *L. kunkeei*, one strain of *F. fructosus*, and one strain of *O. oeni* were used in this experiment, as indicated in Table 3.

Table 3. Lactic acid bacteria isolates selected for the experiment. LAB strains were identified based on blast comparison in GenBank. ∗Accession number of the sequence of the closest relative found by blast search.

LAB species	Isolates	Source*
Lactobacillus plantarum	B3	DKJ917253.1
Lactobacillus plantarum	B11	KJ917253.1
Lactobacillus plantarum	T5	JF728278.1
Lactobacillus plantarum	100	DQ860149.1
Lactobacillus plantarum	26	KJ921814.1
Lactobacillus kunkeei	84	NZJPUI01000014
Lactobacillus kunkeei	44	NZJPUI01000014
Lactobacillus kunkeei	Lk55	KU359947.1
Fructobacillus fructosus	109	KR704467.1
Oenococcus oeni	LS	Commercial strain (Viniflora® LS CiNe™)

Strains, stored at -80°C in MRS broth (Oxoid, Milan, Italy) with 15% glycerol, were propagated twice in MRS broth at 28°C prior their use. Then, 10 ml of each culture, grown in MRS broth at 28°C overnight, were centrifuged at 8000 rpm for 5 min at 4 °C. The pellet was washed 2 times with 1X phosphate buffer (1X PBS), adjusted to a concentration of 1 x 108 CFU/ml, and resuspended in 20 ml of Droskidrink (DD), incubated at 25°C for 24 and 48 h.

The commercially available food bait Droskidrink® (Prantil, Trento, Italy) was used as a substrate for bacterial strains. DD is a highly effective food attractant for *D. suzukii* with a potentiality to be used in monitoring and control strategies i.e. mass trapping and attract & kill. DD consists of mixture of apple cider vinegar (75%), red wine (25%) and 20 g/l of unrefined brown sugar (Grassi *et al.*, 2015).

4.1.2.3. Two-choice bioassay using bacterial strains

The behavioral response of *D. suzukii* females to the selected bacterial strains was evaluated by using the Y-tube olfactometer with similar set-up and conditions used in the first experiment. One arm of Y-tube hold 1 ml of DD inoculated with a strain and the other arm served as a blank control containing 1 ml of DD (Fig. 7). Briefly, the bioassay was conducted by placing a filter paper disk (1 cm diameter) loaded with 1 ml of bacterial filtrate in sample arm. Another filter paper disk (1 cm diameter) was loaded with 1 ml of DD and placed in control arm. The filter paper disks were raised at height of 2.25 cm at the center of each arm.

Female adult flies (n=10) were introduced at once to the stem of Y-tube and were observed for 1 h. Flies were retrieved from olfactometer after 1 h, irrespective of the choice. After each test of 10 flies, the olfactometer was washed with distilled water and baked out in an oven for at least 1 h at 150°C. A choice was recorded when the female fly moved 4.0 cm up an arm of the tube, crossing the red line marked on both arms as a choice decision line and stayed behind that line for more than 30 s.

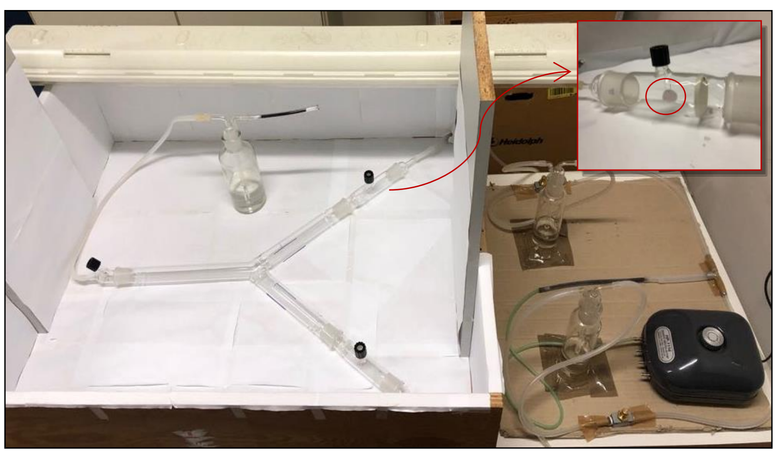

Figure 7. Lateral view of Y-tube olfactometer used in two-choice bioassay to evaluate behavioral response of flies towards bacterial strains volatiles released on filter paper desk, indicated by a red circle in the top right photograph.

The number of flies that migrated from the release chamber to the stem (active flies), and the number of flies inside the sample and control arms was documented. Tests were undertaken between 11:00 AM and 17:00 PM. Each strain was tested separately in the olfactometer, and each choice test was replicated 10 times at 24 and 48 h of bacterial growth in DD bait. Thus, the olfactory responses of 100 flies per species were tested. Based on the results of the experiment (Chapter 5. Results), two strains were the most attractive of all. Hence, they were used for further experimentation to evaluate the behavioral response of *D. suzukii* females using the Y-tube olfactometer.

4.1.2.4. Two-choice bioassay using a combination of bacterial strains

Following the bioassays using bacterial strains, the most attractive strains among all tested strains were tested in two-choice bioassay. The behavioral response of *D. suzukii* females to the most attractive bacterial strains was evaluated by using the Y-tube olfactometer with similar set-up and conditions used in the second experiment. Based on the results of the previous experiment, the highest attractiveness was recorded in *L. kunkeei* strain 84 and *O. oeni* strain LS at 48 h incubation period.

In Y-tube olfactometer, one arm held 1 ml of DD inoculated with a combination of the two bacterial strains and the other arm served as a blank control containing 1 ml of DD. Female adult flies (n=10) were introduced at once to the stem of Y-tube and were observed for 1 h. The number of flies inside the sample and control arms was recorded. Flies were retrieved from olfactometer after 1 h, irrespective of the choice. Each choice test was replicated 10 times at 24 and 48 h of bacterial growth in D. Thus, the olfactory responses of 100 flies were tested. After each test of 10 flies, the olfactometer was washed with distilled water and baked out in an oven for at least 1 h at 150°C.

4.1.2.5. Data analysis

Data were recorded based on the first choice made by a fly. The number of flies in each arm was expressed as a total number of flies tested. Flies that did not make a choice were recorded as “no choice”. Chi-Square test was used to compare the attractiveness of flies towards treatments and control at $P < 0.05$ (STATISTIX, version 10; Analytical software, Tallahassee, Florida).

4.2. Trapping studies

From laboratory experiments to field trials, this work investigated the efficiency of new formulation of a commercial attractive lure based on the use of VOCs for developing an effective trapping system for monitoring *D. suzukii*. Thus, the objectives of the trapping studies were: (1) to determine the *D. suzukii* attraction towards selected volatile compounds associated with fruit ripening and microbial fermentation; (2) to assess a new VOCs- blend attractiveness; (3) to design the prototype of an efficient attractive lure and develop a new trapping system. The studies were conducted in behavioral bioassay approach under semi field conditions in green lab compartment at Biobest Group N.V., Westerlo, Belgium during March- August 2020.

4.2.1. Insects

The *D. suzukii* culture used in the trapping experiments originated from multiple collections of adults in a private garden (Breendonk, Belgium, 51°03'01.5"N, 4°20'00.0"E) on cherries during May-June 2019. Adult flies were provided by Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium from where source colonies were started in May 2019. The laboratory colony was maintained on a cornmeal-yeast-agar diet at (23±2)°C, (65±5)% RH, and a (16:8) L:D photoperiod in a rearing chamber at Biobest Group N.V., Westerlo, Belgium. Newly eclosed flies were collected daily between 9:00 and 12:00 h, males and females were kept in the same plastic containers (18 cm × 12 cm) with access to food and water and allowed to mate for 6 days.

4.2.2. Volatile organic compounds

A total of six synthetic volatile compounds were tested at different concentrations for their attractiveness to *D. suzukii* (Table 4). The compounds are associated primarily with fruit ripening and microbial fermentation. For example, isopentyl acetate, also known as isoamyl acetate, is a ubiquitous compound present in ripening, ripe and early fermenting fruits. All compounds had ≥95% purity and where purchased from Sigma-Aldrich, Overijse, Belgium.

Table 4. Main chemical and physical properties and concentrations of the volatile compounds tested in trapping experiments.
*Odor descriptors and natural occurrence of compounds other than microbial fermentation are from PubChem https://pubchem.ncbi.nlm.nih.gov/, and the good scents database http://www.thegoodscentscompany.com.

Compound	Chemical class	CAS number	Molecular formula	Purity	Concentration	Odor*	Natural occurrence*
β-cyclocitral	Terpenoid	432-25-7	$C_{10}H_{16}O$	≥95%	100 ppm	tropical, saffron, herbal	food items such as safflower, saffron
Dimethyl sulfide (DMS)	Sulfur	75-18-3	C_2H_6S	≥99%	154 ppb	Unpleasant odor of wild radish, cabbage-like	food items such as garden onion, potato
Ethyl Acetate (EA)	Esters	141-78-6	$C_4H_8O_2$	≥99.5%	130 ppm	Ether-like, fruity odor	food, associated with tobacco as a natural component
Geraniol	Terpenoid/ alcohol	106-24-1	$C_{10}H_{18}O$	≥97%	1.5 ppm	rose odor, pleasant geranium-like odor, pleasant, floral odor	Fruits such as grapes, plums
Isopentyl acetate	Esters	123-92-2	$C_7H_{14}O_2$	≥97%	13.1 ppm	Fruity, banana-like odor	food items such as fruits
Linalool	Terpenoid	78-70-6	$C_{10}H_{18}O$	≥97%	6.6 ppm	floral, spicy, woody odor	Flowers and spices such as cinnamon, laurel

The tested volatile compounds were detected in the metabolic profiling of LAB species used earlier in our olfactory studies (Table 5). LAB species explore different metabolic activities that are associated with production of beneficial compounds i.e. volatile organic compounds that may improve the aroma profiles of the fermented food.

Table 5. List of volatile compounds used in this study emitted by the LAB species used in our olfactory studies.

Compound	Bacterial species	Fermentation matrix	Biological function	Reference
β-cyclocitral	*L.* plantarum	Fermented vegetables (Kimchi)	determining the taste and flavor of Kimchi product	Choi *et al.*, 2019
	L. plantarum	Carrot juice	improve sensory qualities of juice	Zhang *et al.*, 2019
Dimethyl sulfide (DMS)	*L. plantarum*	Fermented vegetables (Kimchi)	determining the flavor quality of Kimchi products	Kang *et al.*, 2003
Ethyl Acetate (EA)	*L. plantarum*	Black olives	microbiological stability of olives through controlled fermentation	Panagou *et al.*, 2008
	L. plantarum	Apple juice	Impart different aroma profiles to fermented juice	Chen *et al.*, 2019
	L. plantarum	Elderberry juice	enrich the typical aroma of juice	Ricci *et al.*, 2018
	O. oeni	Grape wines	bio-catalyzer in malolactic fermentation, produce VOCs that influence aroma	Lee *et al.*, 2009
Geraniol	*O. oeni*	Grape juice	release of terpene alcohols	Michlmayr *et al.*, 2012
	O. oeni	Wine	improve the typical aroma of Riesling wine	Michlmayr *et al.*, 2012
	L. plantarum	Malt based beverages	improve the fruity flavor of malt based beverages	Nsogning Dongmo *et al.*, 2017
Isopentyl acetate	*O. oeni*	Grape wines	bio-catalyzer in malolactic fermentation, produce VOCs that influence aroma	Lee *et al.*, 2009
Linalool	*L. plantarum*	Mixed berry juice	increase antioxidant activity after LAB fermentation	Park *et al.*, 2017
	O. oeni	wine	change the odor profile of wine	Hernandez-Orte *et al.*, 2009
	O. oeni	Grape juice	release of terpene alcohols	Michlmayr *et al.*, 2012
	L. plantarum	Malt based beverages	improve the fruity flavor of malt based beverages	Nsogning Dongmo *et al.*, 2017

Moreover, the tested volatile compounds were characterized previously in cultivated and wild blackberry fruits which showed the highest attractiveness towards *D. suzukii* flies among other host fruits in our olfactory studies (Table 6).

Table 6. Review of the recent studies on volatile compounds used in this study, detected in wild and cultivated variety of blackberry. +, positive detection.

Compound	Cultivated blackberry Thornless variety	Wild blackberry	Reference
β-cyclocitral	+	+	Wajs-Bonikowska *et al*., 2017
Dimethyl sulfide (DMS)	+	+	Klesk and Qian, 2003
Ethyl Acetate (EA)	+	+	Georgilopoulos and Gallois, 1987
	+	+	Revadi *et al.*, 2015
	+	+	Klesk and Qian, 2003
Geraniol	+	+	Georgilopoulos and Gallois, 1987
Isopentyl acetate	+	+	Du *et al*., 2010
	+	+	Revadi *et al.*, 2015
Linalool	+	+	Georgilopoulos and Gallois, 1987
	+	+	Revadi *et al.*, 2015
	+	+	Klesk and Qian, 2003

4.2.3. Trapping system

Droso-Trap® (Biobest, Westerlo, Belgium) was deployed for trapping experiment. In brief, the commercial trap that is designed for trapping Drosophila flies is consisting of transparent lid with wire hanger and red plastic base. The base contains three inlet tubes, each tube has seven holes of 5 mm diameter for flies' entry (Fig. 8).

Figure 8. Droso-Trap® used in trapping experiments.

Based on our observations which coincide with the findings of a previous study on the large headspace volume that may facilitate *D. suzukii* survival and eventual escape from traps (Renkema *et al*., 2014), we made a modification on trap design. A yellow card (12.5 x 10 cm), sticky on both sides, was placed upright inside the trap to immobilize captured flies and allow immediate vision of flies (Renkema *et al*., 2014; Cruz-Esteban, 2020).

The commercial attractive lure Dros'Attract® (Biobest, Westerlo, Belgium) was used as a drowning solution and base matrix for VOCs. This trapping system is rated as the most effective monitoring tool currently available both in in-house and independent studies.

4.2.4. Individual- compound behavioral assay

The attractiveness of *D. suzukii* adult flies for volatiles emitted by synthetic compounds was assessed with semi field trials. Bioassays were performed in screen cages (W:150 cm, L: 250 cm, H: 200 cm; non-metallic net, 0.5 mm mesh) which were placed in a greenhouse compartment at average temperature and humidity (22±2)°C, (60±5)% RH, respectively. Each cage contained four Droso-Traps, with two controls containing Dros'Attract and two treatments containing Dros'Attract spiked with a single compound at concentrations found in Table 4.

The volume of Dros'Attract used in both the treatment and control traps was 100 ml. All traps were placed diagonally opposite each other at one-meter height above the ground. 25 adult flies of each sex were released at ground level in the center of each cage at the age of 7-10 days (Fig. 9A and B). After 24 hours, the flies were collected, sexed, and counted for each trap. The experiment was repeated 15 times per compound over 30 experimental days, with differing trap arrangements to eliminate position bias.

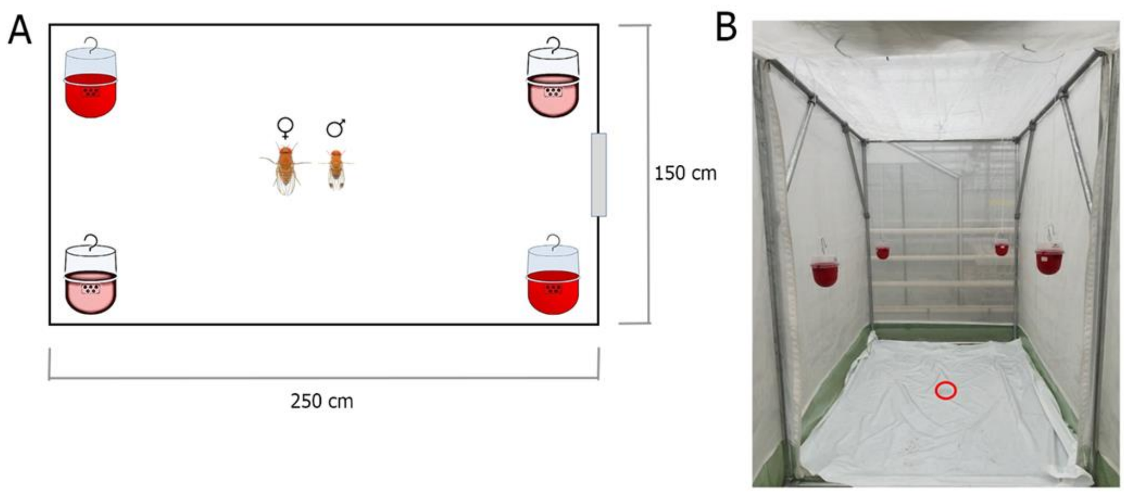

Figure 9. Experimental setup for individual-compound behavioral assay under greenhouse conditions. Diagram (A) and photograph (B) represent the 150 x 250 x 200 cm cages that were placed in a greenhouse compartment. Each cage contained four Droso-Traps, with two controls containing Dros'Attract (red) and two treatments containing Dros'Attract spiked with a single compound (pink). 25 male and 25 female *D. suzukii* adult flies were released at ground level from the middle of the cage, indicated by a red circle in the photograph.

4.2.4.1. Data analysis

Tests for significant differences between control and treatment groups were performed, per sex, per compound, using the Wilcoxon signed-rank test, with the Holm–Bonferroni method for multiple corrections. Responses of *D. suzukii* females and males were estimated by calculating a Preference index (PI), defined by Pham and Ray, 2015, as: PI = (number of flies in treatment traps - number of flies in control trap)/ total number of flies caught in treatment and control traps. PI indices were scaled to zero mean and unit variance. PI values equal to 0 indicate no effects of VOCs on base matrix. PI values less than 0 indicate repellent effects, and values greater than 0 indicate attractive effects. The statistical analyses were performed using STATISTIX, version 10 (Analytical software, Tallahassee, Florida).

4.2.5. VOCs- blend behavioral assay

The Dros'Attract lure combined with the most attractive compounds, DMS and geraniol, was tested preliminary in behavioral assay with similar set-up and conditions used in individual- compound behavioral assay. In each cage, the four traps included one control loaded with Dros'Attract and three treatments loaded with Dros'Attract spiked with geraniol, DMS, and a blend of both compounds at concentrations found in Table 2. A total of 50 flies (1:1 F:M) were released at ground level in the center of each cage at the age of 7-10 days. The experimental design was Randomized Complete Block Design (RCBD) (Fig. 10). After 24 hours, the flies were collected, sexed, and counted for each trap. The experiment was repeated 15 times with differing trap arrangements to eliminate position bias.

Replicate	RCBD layout			
1	B1	A1	C1	D1
2	A2	D2	B2	C2
3	A3	C3	D3	B3
4	B4	C4	A4	D4
5	A5	B5	C5	D5
6	D6	A6	C6	B6
7	C7	D7	B7	A7
8	A8	B8	D8	C8
9	D9	A9	B9	C9
10	B10	D10	C10	A10
11	C11	A11	B11	D11
12	D12	B12	A12	C12
13	B13	A13	D13	C13
14	C14	B14	D14	A14
15	C15	D15	A15	B15

Figure 10. Schematic presentation of traps locations in each cage used in VOCs- blend behavioral assay with 15 replications. (A) DMS; (B) Geraniol; (C) Control; (D) Blend of A and B.

4.2.5.1. Data analysis

Data were analyzed by a one-way analysis of variance (ANOVA) for RCBD. The male and female data were separated during the counts and analyzed separately to determine the effects of the treatments on each sex of *D. suzukii*. The treatment means were separated by Tukey's HSD (honestly significant difference) test at $\alpha=0.05$. The statistical significance was determined as $P \leq 0.05$. The statistical analyses were performed using STATISTIX, version 10 (Analytical software, Tallahassee, Florida).

4.2.6. Prototype design and developing a new trapping system

The development of the prototype of an efficient synthetic lure for developing a new trapping system intended for monitoring *D. suzukii* is the main objective of this research project. Therefore, the attractiveness of a commercial lure, Dros'Attract, was improved through a series of behavioral bioassays using individual compound and later a blend of volatile compounds. Among all tested compounds, geraniol and DMS significantly enhanced the behavioral response of *D. suzukii* to the lure. Moreover, both compounds showed a consistent performance from single treatment through blend, and trapping system continued to perform well in behavioral assays (Chapter 5. Results).

Therefore, a prototype of a more attractive lure was developed comprising the commercial lure and both compounds. Furthermore, a new trapping system was developed as a reliable and selective method for trapping *D. suzukii*. The system is comprised of a Dros'Attract lure combined with volatile compounds (DMS and geraniol) and Droso-Trap equipped with a sticky yellow card. This used lure combine microbial fermentation volatile compound (DMS) along with plant-based volatile compound (geraniol) which are needed to elicit high levels of response in female and male *D. suzukii*.

CHAPTER 5

5. Results

5.1. Olfactory studies

5.1.1. Behavioral response to host plant volatiles

Non-sterilized fruits were significantly more attractive to *D. suzukii* than control (*P*<0.05) (Fig. 11). Blackberry, white grapes, black grapes, and wild bramble fruits showed statistical difference in response of the flies among all tested fruits. However, flies showed higher response to wild cherry fruits more than wild mulberry fruits with no significant difference in flies' response in both host fruits (Table 7).

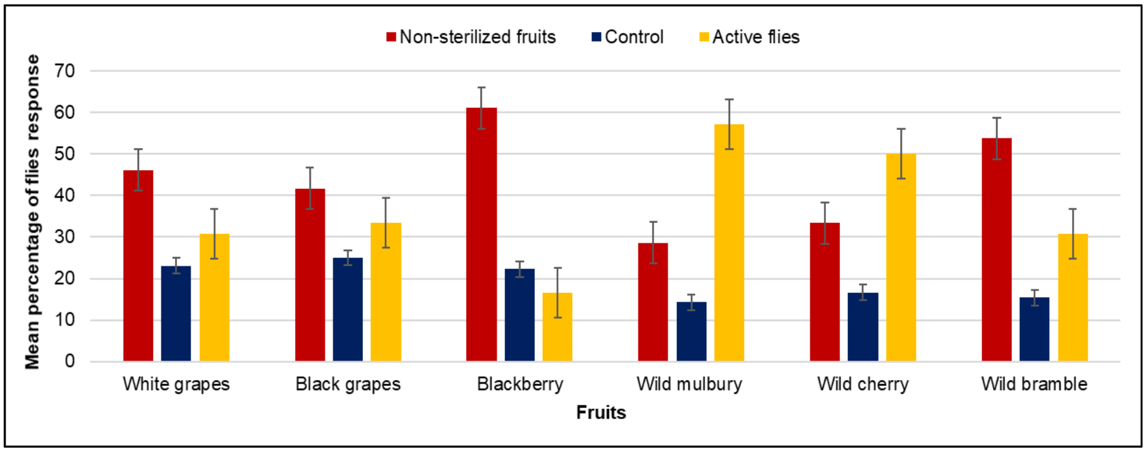

Figure 11. Mean percentages and SE of *D. suzukii* flies response towards crop and non-crop host fruits in non-sterilized treatment and control in a Y-tube olfactometer. Each treatment was tested in 10 replicates.

Table 7. Results of the statistical analysis of behavioral responses of *D. suzukii* females in a Y-tube olfactometer towards fruits and control (n=100, P < 0.05).

Treatment	**Fruit type**	**Flies response (%)**	**χ^2; Significance (Friedman-ANOVA)**
Non-sterilized fruits vs. control			
	White grapes	6	11.04; 0.0009
	Black grapes	5	4.77; 0.0290
	Blackberry	11	76.15; 0.0000
	Wild mulberry	2	1.04; 0.3083
	Wild cherry	2	1.04; 0.3083
	Wild bramble	7	28.07; 0.0000

Sterilized fruits vs. control	White grapes	1	8.18; 0.0042
	Black grapes	1	0.00; 1.0000
	Blackberry	2	4.27; 0.0387
	Wild mulberry	1	3.81; 0.0508
	Wild cherry	2	17.27; 0.0000
	Wild bramble	2	1.04; 0.3083

χ^2 values from the Friedman-ANOVA, performed to evaluate the differences between the number of flies that chose the fruits or the control, are reported with their significance (df = 1 in all tests).

When comparing sterilized fruits with control, fly's attraction to sterilized fruits varied according to fruits type (Fig. 7). Attraction was only significant more than the control in wild bramble with no statistical difference (P <0.05) (Table 7). On the other hand, black grapes fruits had similar attractiveness as control presenting no significant difference (P <0.05). Blackberry, white grapes, wild mulberry, and wild cherry fruits showed a statistical difference in response of the flies among all tested fruits in sterilized treatment (Table 7). However, the highest response of flies was towards wild bramble and the lowest response was towards white grapes.

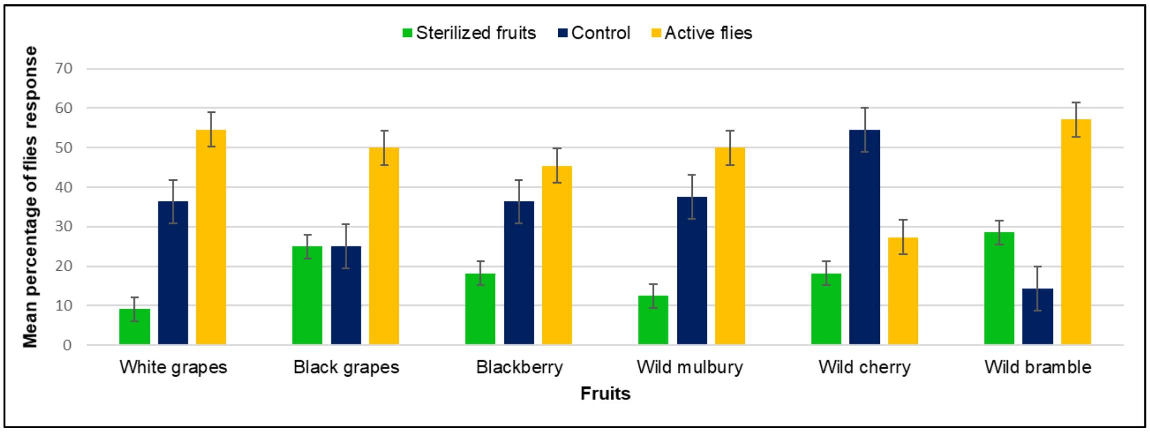

Figure 12. Mean percentages and SE of *D. suzukii* flies response towards crop and non-crop host fruits in sterilized treatment and control in a Y-tube olfactometer. Each treatment was tested in 10 replicates.

Thereafter, flies response towards non-sterilized fruits was compared with response towards sterilized fruits. Flies showed a significant preference for non-sterilized fruits of white grapes, black grapes, blackberry, wild mulberry, and wild bramble over the sterilized fruits (Table 8). Flies response towards these non-sterilized fruits had the highest percentage on blackberry fruits and the lowest percentage on wild mulberry (Fig. 13).

Table 8. Results of the statistical analysis of behavioral responses of *D. suzukii* females in a Y-tube olfactometer towards two treatments of crop and non-crop host fruits (n=100, P < 0.05).

Fruit type	Flies response (%)/ non-sterilized fruits	Flies response (%)/ sterilized fruits	χ^2; Significance (Friedman-ANOVA)
White grapes	6	1	22.73; 0.0000
Black grapes	5	1	14.34; 0.0002
Blackberry	11	2	98.62; 0.0000
Wild mulberry	2	1	10.4; 0.3083
Wild cherry	2	2	0.00; 1.0000
Wild bramble	7	2	28.07; 0.0000

χ^2 values from the Friedman-ANOVA, performed to evaluate the differences between the number of flies that chose the sterilized or non-sterilized fruits, are reported with their significance (df = 1 in all tests).

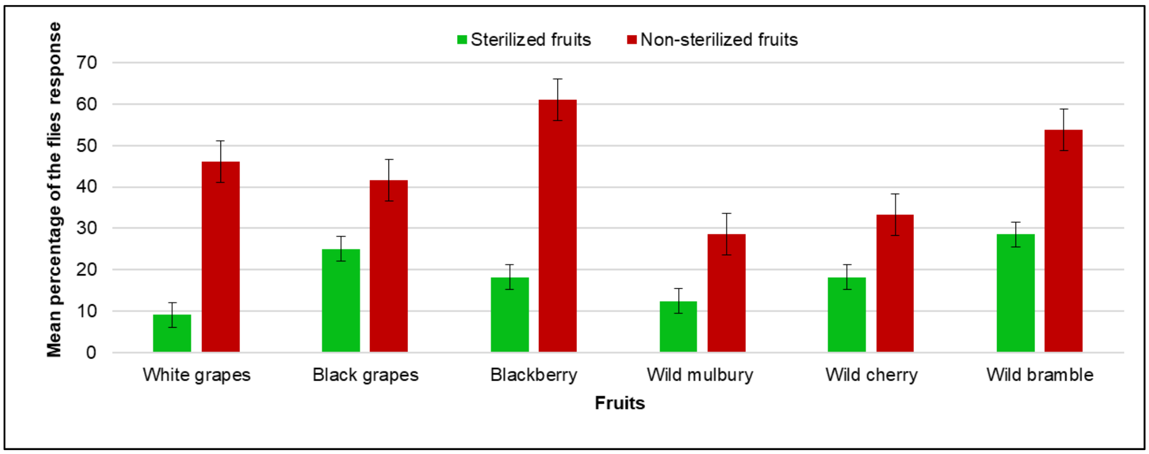

Figure 13. Mean percentages and SE of *D. suzukii* flies response towards crop and non-crop host fruits in sterilized and non-sterilized treatments in a Y-tube olfactometer. Each treatment was tested in 10 replicates.

Moreover, when non-sterilized fruits were tested in olfactometer, flies activity was high when non-crop host fruits were presented in the fruit arm. Active flies that left the release chamber and moved into the stem were at highest numbers in wild mulberry fruit tests as shown in Fig. 11. However, the overall flight activity was significantly increased when host fruits were tested in sterilized treatment but surprisingly it was reduced in wild cherry tests as presented in Fig. 12. The highest activity of flies was recorded in wild bramble fruits among all sterilized fruits.

5.1.2. Behavioral response to microbial volatiles

Behavioral bioassays with selected LAB strains revealed that all strains were significantly more attractive to *D. suzukii* females than the control at 24 and 48 h incubation period (P <0.05) (Table 9). Among all tested strains, flies showed a significant behavioral response to *L. kunkeei* 84, and *O. oeni* LS over the control at 24 incubation period (Fig. 14). However, the lowest response was towards *F. fructosus* 109 and *L. plantarum* 100 at 24 incubation period.

Table 9. Results of the statistical analysis of behavioral responses of *D. suzukii* females in a Y-tube olfactometer towards LAB strains at 24 and 48 h incubation period in Droskidrink (DD) food bait (n=100, P < 0.05).

LAB strain	χ^2; Significance (Friedman-ANOVA)	
	24 h	**48 h**
L. plantarum B3	29.00; 0.0000	28.00; 0.0000
L. plantarum B11	23.00; 0.0000	38.00; 0.0000
L. plantarum T5	24.00; 0.0000	22.00; 0.0000
L. plantarum 100	11.00; 0.0009	11.00; 0.0009
L. plantarum 26	30.00; 0.0000	26.00; 0.0000
L. kunkeei 84	42.00; 0.0000	46.00; 0.0000
L. kunkeei 44	33.00; 0.0000	15.00; 0.0001
L. kunkeei Lk55	34.00; 0.0000	19.00; 0.0000
F. fructosus 109	11.00; 0.0009	9.00; 0.0027
O. oeni LS	40.00; 0.0000	45.00; 0.0000

χ^2 values from the Friedman-ANOVA, performed to evaluate the differences between the number of flies that chose the LAB strains or the control, are reported with their significance (df = 1 in all tests).

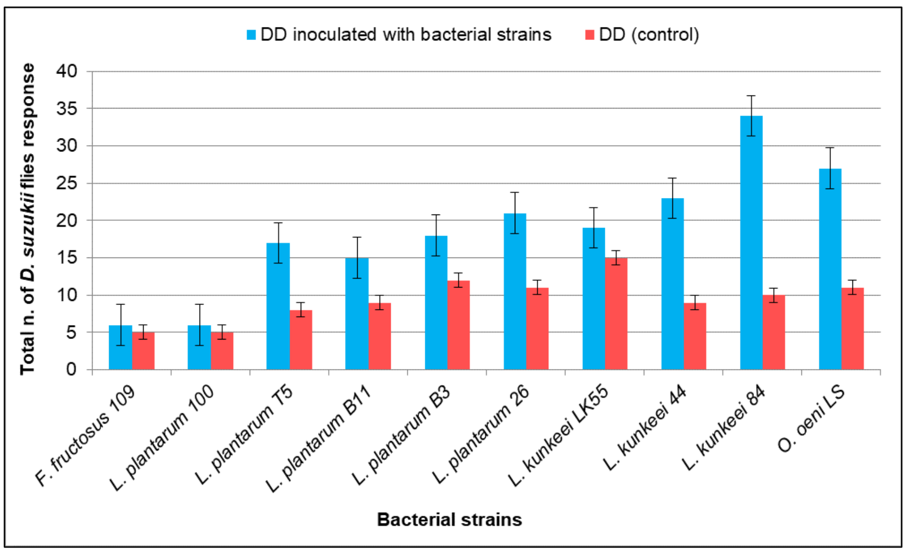

Figure 14. Total number of *D. suzukii* flies response towards LAB strains at 24 h incubation period in Droskidrink (DD).

Thereafter, the results of bioassays conducted at 48 h incubation period, showed that flies had a similar significant response towards the two most attractive LAB strains at 24 h incubation period i.e. *L. kunkeei* 84 and *O. oeni* LS. However, this response towards the two LAB strains was higher at 48 h incubation period than that at 24 h incubation period. Moreover, the lowest response was towards the same LAB strains as of the earlier bioassays conducted at 48 h incubation period i.e. *F. fructosus* 109 and *L. plantarum* 100. On the other hand, there was no major differences between flies' response towards *L. plantarum* strains in both bioassays, excepting *L. plantarum* 11 which had a higher attractiveness in bioassays conducted at 48 h than 24 h incubation period (Fig. 15).

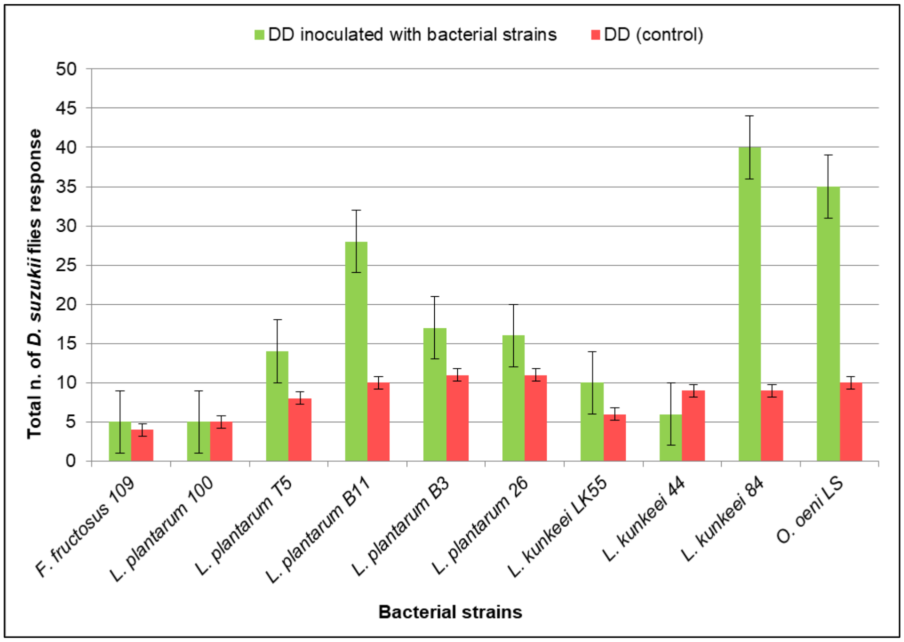

Figure 15. Total number of *D. suzukii* flies response towards LAB strains at 48 h incubation period in Droskidrink (DD).

Furthermore, flies attractiveness towards the combination of the most attractive strains in the earlier bioassays, *L. kunkeei* 84 and *O. oeni* LS, was found at 24 h incubation period (Fig. 16). Both strains showed significant differences (*L. kunkeei* 84: $\chi^2 = 57.00$, df = 1, P = 0.0000; and *O. oeni* LS: $\chi^2 = 38.00$, df = 1, P = 0.0000). However, there was no major differences between flies' response towards the combined strains and single strain i.e. *L. kunkeei* 84 and *O. oeni* LS, tested at 24 h incubation period.

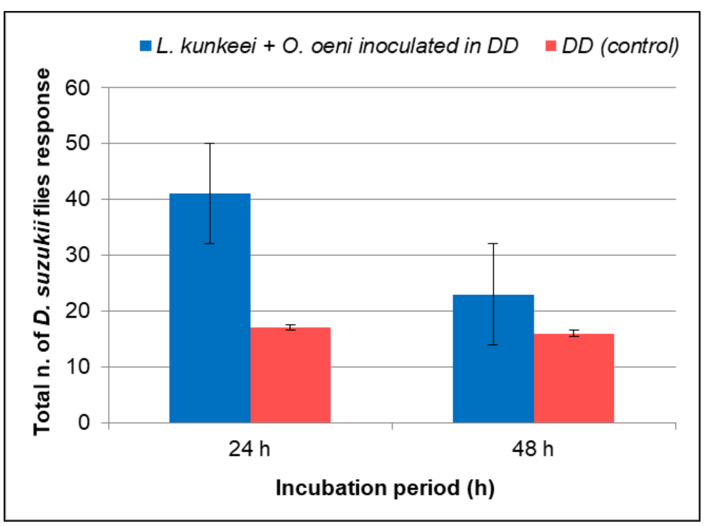

Figure 16. Total number of *D. suzukii* flies response towards LAB strains (*L. kunkeei* strain 84 and *O. oeni* strain LS) at 24 and 48 h incubation period in Droskidrink (DD).

5.2. Trapping studies

5.2.1. Individual- compound behavioral assay

The preferences of adult *D. suzukii* flies for a commercial lure (Dros'Attract) spiked with individual volatile compound in behavioral assays are shown in Fig. 17.

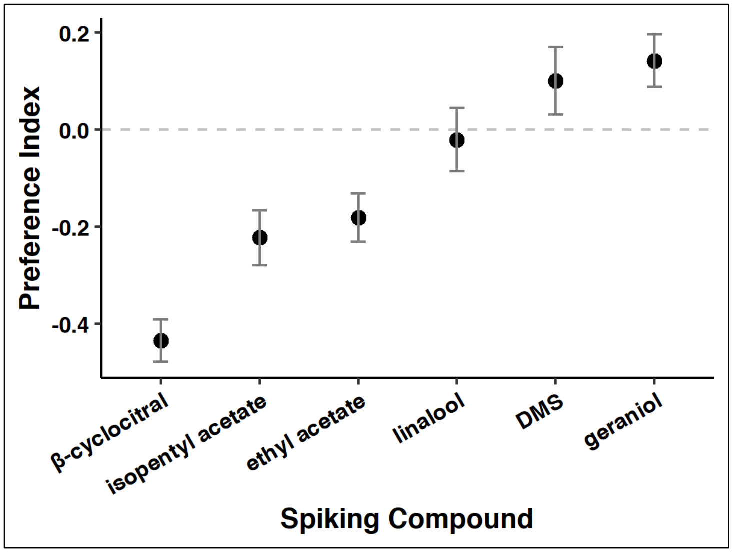

Figure 17. Adult *D. suzukii* preference for a commercial lure (Dros'Attract) spiked with a single volatile compound. Preference indices of flies (males and females) in response to Dros'Attract lure spiked with a single compound separately in six treatments. Values shown are mean±sem for each compound (n=25 pairs). Tests for significant differences between control and treatment groups were performed using the Wilcoxon signed-rank test, with the Holm–Bonferroni method for multiple corrections ($P<0.05$). Error bars represent standard errors.

Wilcoxon signed-rank test revealed significant differences among treatments in the order: geraniol > DMS > linalool > ethyl acetate > isopentyl acetate > β-cyclocitral. Significant differences were observed between treatment and control traps ($P=0.0000$). *D. suzukii* flies showed a significant preference for traps baited with lure and DMS or geraniol. Conversely, flies showed a repellent activity to traps baited with lure and four compounds i.e. linalool, ethyl acetate, isopentyl acetate and β- cyclocitral with different preference indices.

The differences in the response of both sexes of *D. suzukii* was observed throughout the whole behavioral assays. Significantly, more females, but not males, preferred traps baited with lure and DMS, geraniol and linalool compounds. Traps baited with lure and isopentyl acetate and ethyl acetate had no significant effects on females. The highest preference index was recorded from females for traps baited with lure and DMS. Moreover, females had the lowest preference index for ethyl acetate (Fig. 18).

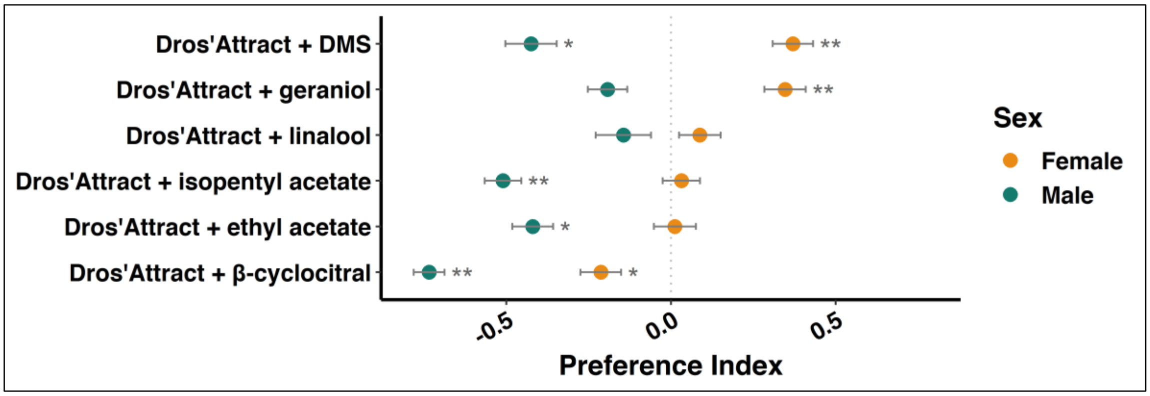

Figure 18. Volatile compounds increase female *D. suzukii* attraction to a commercial lure (Dros'Attract). Preference indices of male (green) and female (orange) *D. suzukii* in response to Dros'Attract lure spiked with a single compound. Values shown are mean±sem for each compound and sex (n=25 pairs). Tests for significant differences between control and treatment groups were performed using the Wilcoxon signed-rank test, with the Holm–Bonferroni method for multiple corrections. $^{*}P < 0.05$, $^{**}P < 0.01$.

In contrast, *D. suzukii* males reacted adversely to all traps baited with the lure and tested compounds. Likewise, both females and males were significantly repelled by traps baited with lure and β- cyclocitral. Obviously, the attractiveness of Dros'Attract lure to female flies was enhanced significantly by the addition of DMS and geraniol to the lure.

5.2.2. VOCs- blend behavioral assay

The mean values of *D. suzukii* captures in all treatments and control are presented in Fig.19. The trap baited with Dros'Attract lure and blend of two compounds (DMS and geraniol) captured significantly more flies than other traps and control. Traps baited with lure and DMS or geraniol showed similar performance with no significant difference ($P \leq 0.05$).

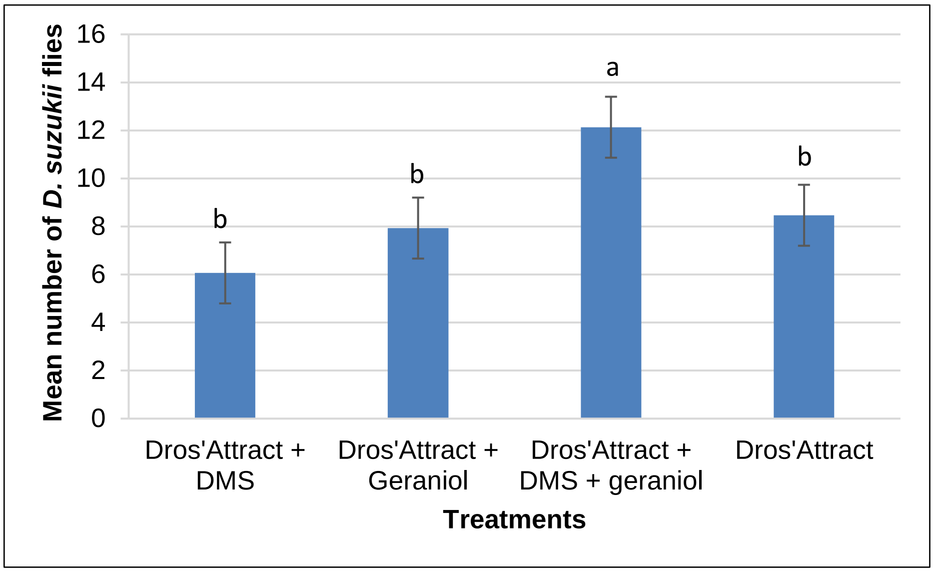

Figure 19. Mean numbers of *D. suzukii* captured per Droso-Trap® baited with different treatments in semi-field behavioral assays. Tests for significant differences between control and treatment groups were performed using Tukey's HSD test ($P \leq 0.05$). Different letters denote statistically significant differences among all treatments. Vertical bars indicate SE.

With regards to sex, the trap baited with Dros'Attract lure and blend of two compounds captured more females than males with no significant difference (Table 10). Similarly, the same trap captured significantly more females when compared with other traps in female captures. None of the other traps were significantly different from each other in females captures.

There were less males than females captured in all traps baited with lure and treated with compounds. There were significant differences in male captures among all traps. However, the highest mean number of females captured was presented by trap baited with lure and blend of two compounds at 6.93 ± 0.64 flies. Similar trend was found in the highest mean number of males captured in the same trap at 5.20 ± 0.50 flies (Table 10).

Table 10. Mean numbers of *D. suzukii* captured in treatments and control used in behavioral assay. Mean (±SE) number of adult flies within columns with different letters are significantly different from each other ($P \leq 0.05$; Tukey's HSD test).

Treatments	Number of flies captured (Mean ± SE)	
	Females	**Males**
Dros'Attract + DMS	3.26 ± 0.47b	2.80 ± 0.34b
Dros'Attract + geraniol	4.46 ± 0.55b	3.46 ± 0.37ab
Dros'Attract + DMS + geraniol	6.93 ± 0.64a	5.20 ± 0.50a
Dros'Attract	5.13 ± 0.54ab	3.33 ± 0.40b

In comparison to control, significantly more females and males were captured in the trap baited with Dros'Attract lure and blend of two compounds (Fig. 20). While trap baited with lure and DMS showed significant difference in female captures and no significant difference in male captures when compared with control. Trap baited with lure and geraniol showed significant differences in both female and male captures when compared with control trap captures.

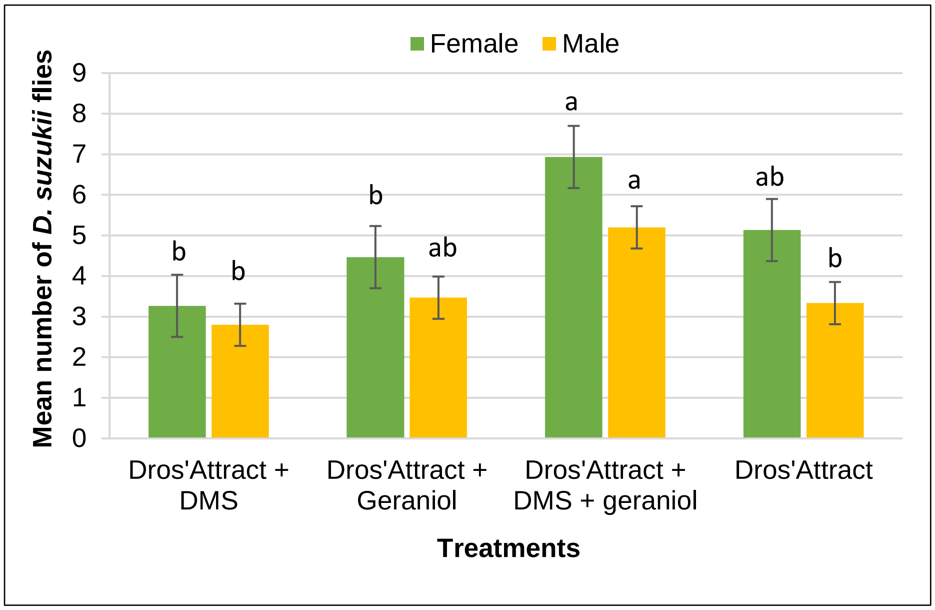

Figure 20. Mean numbers of males and females *D. suzukii* captured per Droso-Trap® baited with different treatments in semi-field behavioral assays. Tests for significant differences between control and treatment groups were performed using Tukey's HSD test ($P \leq 0.05$). Different letters denote statistically significant differences among all treatments. Vertical bars indicate SE.

CHAPTER 6

6. Discussion

6.1. Olfactory studies

6.1.1. Behavioral response to host plant volatiles

The present study reports for the first time the behavioral response of *D. suzukii* mated female to odors emitted by sterilized and non-sterilized fruits of crop and non-crop hosts. Overall, the attractiveness of female flies towards different types of host fruits, crop and non-crop, in our study reconfirms that *D. suzukii* can infest a wide range of hosts (Bellamy *et al*., 2013). Our bioassays data showed that non-sterilized fruits are more attractive than sterilized fruits or control and the highest attractiveness was recorded in blackberry fruits and the lowest attractiveness was in wild mulberry fruits. *D. suzukii* response to host fruits can be ranked as follows: blackberry > wild bramble > white grapes > black grapes > wild cherry mulberry > wild mulberry.

Although female flies showed higher attractiveness towards non-sterilized fruits more than sterilized fruits or control, it is noted that non-sterilized fruits of crop hosts i.e. blackberry and table grapes showed a statistical difference among all tested fruits. These findings coincide with outcomes of a dual-choice behavioral bioassays using Y-tube olfactometer to investigate mated female flies' response to host volatiles emitted by blackberry, raspberry, cherry, blueberry and strawberry (Revadi *et al.*, 2015).

Similar results were obtained by Bellamy *et al*. (2013) using fruit extracts of seven different reported hosts (blackberry, blueberry, sweet cherry, table grapes, peach, raspberry, strawberry) in multiple choice oviposition bioassays at population level, that showed no statistical difference in female flies choices. Moreover, in no-choice assays to assess oviposition preferences of *D. suzukii* in different varieties of common hosts (blackberry, blueberry, raspberry, strawberry), the fruits were infested at different rate where the highest oviposition was in raspberry fruits and blackberry varieties had no significant difference in infestation rate (Burrack *et al.*, 2013).

Furthermore, our results are in agreement with other studies which investigated the susceptibility of different varieties of table grapes to *D. suzukii*. The varieties i.e. Victoria var. were grown in Bari, Apulia Region in south-eastern Italy; the same location of our tested table grape varieties. A series of no-choice oviposition experiments showed that Victoria var. is susceptible to *D. suzukii* and it provided a suitable substrate for complete insect development (Baser *et al.*, 2018). Accordingly, our outcomes anticipate that table grapes Victoria var. is likely to be highly infested and severely damaged by *D. suzukii* in Apulia region where the first presence of this pest was reported in an organic table grape vineyard cultivated with Red Globe, Italia and Victoria varieties (Baser *et al.*, 2015).

However, this attraction towards non-sterilized fruits can be related to perceiving female flies different ubiquitous volatile compounds from fruits during host-location process for feeding and oviposition purposes. Similarly to phytophagous insects with different host preferences, *D. suzukii* can show different behavioral responses due to central processing of olfactory signals by using similar peripheral receptor systems for volatiles (Bruce *et al*., 2005). Therefore, effective attractive bait for management of this pest can be based on the selection of key volatile compounds including oviposition and feeding attractants. Such powerful baits can be used in the unripe stage of fruit development for reducing pest population.

In contrast, overall flies' attraction towards sterilized fruits was lower than control which indicates that the fruits lack the active microorganisms that may attract the flies. Treatment with 1% sodium hypochlorite for 60 s inactivated microorganisms' activity and growth. We used concentration that thought to have negligible deleterious effects on the fruit itself. The efficacy of the used sanitizer was optimized to obtain a reasonable inactivation of microorganisms associated with fruits surface. Differential temperatures of used water and fruits was kept at minimum rate to prevent infiltration and internalization of bacteria within fruits (Ibarra-Sánchez *et al.*, 2004), also solution' pH was kept at a range of (6.5-7.5) to maintain the bactericidal effect of chlorine (Sapers, 2003), with a short exposure time i.e. 1–2 min.

Furthermore, during the chemical treatment, chlorine was applied within the typical concentration range from 50 to 200 mg/L, so it dissolves in the water forming hypochlorous acid (HOCl), an efficient oxidizer for microbes' inactivation. However, sodium hypochlorite is capable of inactivating microbial populations on fresh fruits by more than 90% to 99% under optimum conditions (Sapers, 2003). Many researchers have frequently used household bleach containing 5.25% sodium hypochlorite for surface sterilization of fruits at different levels of concentration and contact time.

For table grapes, a concentration of 50 mg/L sodium hypochlorite was used for 1 min to sterilize the surface of berries and control microbial growth effectively without altering their intrinsic characteristics (Ergun and Dogan, 2018). Also, sodium hypochlorite was applied on artificially contaminated strawberries and significantly reduced bacterial counts (>98%) at concentration of 50 to 300 ppm of free chlorine and 2 min contact time (Lukasik *et al.*, 2003).

Additionally, preliminary trials (data not shown) were undertaken to observe the behavior of female flies in response to sodium hypochlorite solution in Y-tube olfactometer. It was clearly noticed that flies' mobility/activity in all arms of olfactometer was not affected by the tested solution even though flying of flies was rarely seen during the trials. These observations demonstrate that there is no repellent effect of sodium hypochlorite solution on female flies during the observation period. Moreover, the sterilization protocol was firm creating no chlorine scent on the treated fruits.

Hence, the attraction of flies towards sterilized fruits can provide useful information on suitability of sodium hypochlorite to efficiently inactivate the microbial growth potentially on soft fruits. Besides, it is likely that sodium hypochlorite solution can maintain fruits biological functionality and bioactive compounds responsible for flies' attraction. In reference to previous studies, the total inactivation of microorganisms is unachievable in chemical treatment using sodium hypochlorite which is attributed to microbial attachment to fruits' surface as a result of irregular surface structures. In addition, microorganisms may form biofilms, or become internalized within fruits tissues (Beuchat *et al.*, 1998; Sapers, 2001; Sanz *et al.*, 2002; Ijabadeniyi *et al*., 2011).

Thus, the morphological structure of fruits surface could explain the higher attractiveness in sterilized fruits of blackberry and mulberry (having irregular surface) among other sterilized fruits (having smooth surface). Apparently, flies' attraction towards sterilized fruits can be related to both fruits and microbial volatiles. Our findings concur with previous published data on the model organisms *Drosophila melanogaster*. Becher *et al.*, 2012 observed that flies have strong preference to baker's yeast *Saccharomyces cerevisiae* for oviposition and volatiles emitted by grape fruits were only of secondary importance since baker's yeast without fruit induced the same fly response as yeast on fruit.

A recent study revealed that *D. suzukii* mated female flies are attracted to blueberry volatiles for egg laying and to yeast *Hanseniaspora uvarum* volatiles for feeding in combined feeding-oviposition bioassays. This interference between yeast and fruit stimuli has a vital role on flies behavior and attraction (Mori *et al.*, 2017). Therefore, combining host fruit volatiles with microbial volatiles can be exploited for modulation *D. suzukii* olfactory behavior which is fundamental for the development of an efficient control method. However, further studies on representative microbial species including bacteria are needed for better understanding of the attraction, feeding and oviposition response of *D. suzukii* to fruit and microbes' volatiles.

Generally, flies' preferences between sterilized and non-sterilized fruits was determined primarily by their physiological status i.e. feeding and mating status which was confirmed in new studies. Clymans *et al.*, 2019 illustrated that olfactory preferences between fermentation and fruit volatiles of summer and winter morphs of *D. suzukii* are dependent on their physiological status. It is therefore likely that *D. suzukii* populations afford a seasonal shift in olfactory preference from fermentation cues during autumn, winter, and spring to fruit cues during summer.

Another newly published study reported that *D. suzukii* selects fermented fruit for feeding and ripe fruit for oviposition (Cai *et al.*, 2019). Hence, we believe that developing environmentally safe control strategies as mass trapping and attract & kill can be attained by blending host fruits volatiles and microbes' volatiles in one attractant lure which enables efficient capture of flies during the whole season of crop and non-crop host fruits.

On the other side, attraction towards non-crop host fruits coincides with previous works including field surveys and laboratory bioassays i.e. oviposition and olfactometry bioassays. Kenis *et al.*, 2016 reported high infestation in wild bramble in Northern Italy, and Lee *et al.*, 2015 identified wild cherry and white mulberry as hosts of *D. suzukii* based on infestation rate. A previous olfactometry study showed that *D. suzukii* female flies showed a response to mulberry fruits odors in a four-choice olfactometer and serve as a host (Yu *et al*., 2013).

In the current study, we found that wild bramble fruits manifested the highest attractiveness among all tested non-crop host fruits in both treatments. Bramble fruits were collected from Campobasso where no official records on the presence of *D. suzukii* were made in Molise region. Therefore, we endorse the informal reports on presence of *D. suzukii* in Molise region in the Central South of Italy based on behavioral responses of female flies towards non-crop host fruits (wild bramble).

Consequently, we highly recommend a preliminary survey to detect the presence of *D. suzukii* in Molise region using trapping tools available for a reliable monitoring of this pest. In addition to cultivated hosts, this survey should take in consideration wild habitats of both known and potential host plants for better understanding of pest population dynamics and identifying potential high-risk areas.

Researchers suggested that non-crop hosts may play an important role in sustaining *D. suzukii* populations since they serve as alternative hosts when suitable crop hosts are not available in the field (Wang *et al*., 2019). This has implications for management of this pest, particularly in backyard and mixed fruit orchard cases (Yu *et al*., 2013). Therefore, habitat management within-field or landscape level is essential approach for pest control in integrated pest management programs (IPM).

Eventually, although our experimental design exhibits a simple and basic approach, the results provide evidence that fruit volatiles can play an important role in flies' attraction which may contribute in developing highly attractive and specific attractant lure for monitoring and control of *D. suzukii* populations. Also, our findings highlight the importance of examining volatiles of different varieties of crop hosts and wild hosts and microbes' volatiles in bioassays under laboratory conditions taking into consideration fruits characteristics as size, color, and surface topography. Then, transporting the results to field situations. However, different techniques, including olfactometer, are being used to evaluate olfactory preferences i.e. electroantennography (EAG) and gas chromatography coupled with electroantennographic detection.

6.1.2. Behavioral response to microbial volatiles

In this study we explored, for the first time, the behavioral response of *D. suzukii* female flies towards lactic acid fermentation of DD bait in laboratory bioassays. As indicated in the previous experiment, microbial volatiles along with host fruits volatiles play an important role in fly attraction. The recent study showed that flies always exhibited positive response which is mediated by volatiles emitted by LAB strains in food bait. Thus, LAB may contribute significantly to changes of volatiles profile in blackberry fruits, the most attractive host crop in previous experiment, and subsequently increase flies' attraction.

Despite that the LAB species used in our study have not been reported in blackberry fruits (Di Cagno *et al.*, 2011), we anticipate that LAB can be transferred to fruits surface by insects and then contribute in fermentation process of infested fruits. However, the fermentation reaction is undertaken in the presence of malic acid, fructose and glucose which were reported in high quantities on blackberry fruits var. Thornfree (Milivojević *et al.*, 2011).

Therefore, identification of LAB species on infested (fermented) and non-infested blackberry fruits is necessary for better understanding flies' attraction towards intact fruits for oviposition and infested fruits for feeding. It is therefore adorable to screen LAB isolates from different berry host fruits associated with *D. suzukii* attraction to explore the bioactive compounds emission capacities. The use of LAB identified from different fruit origins could also expand the potential volatiles enrichment in food baits and the possibility to increase flies' attraction.

Our findings imply that the utilization of the selected LAB species in food bait was carried out successfully in behavioral bioassays. This is not surprising considering that they are an extremely important group of industrially related bacteria, and their behavior and robustness under stressful conditions. LAB strains were used in our study for fermentation liquid bait and production of bioactive volatile compounds that are associated with *D. suzukii* attraction. Therefore, the type and concentration of volatiles present in DD bait are greatly affected by the strain of LAB and incubation time.

So far, no such studies have been performed on laboratory behavioral bioassays for *D. suzukii* and LAB volatiles. For this reason, we compared our results with outcomes of previous field trials and electrophysiological experiments using different LAB species in DD bait for *D. suzukii* attraction. Furthermore, we compared our findings with the outcomes of previous laboratory studies focused on behavioral preferences of *D. suzukii* to volatiles produced by other bacterial groups i.e. AAB, and behavioral responses of *D. melanogaster*, a closely related species, towards LAB volatiles in laboratory bioassays.

As a first step towards understanding the attractive effect of LAB volatiles on *D. suzukii* response, we tested each LAB strain separately in DD bait at 24 and 48 h incubation period using Y-olfactometer. We found that the tested LAB strains were significantly more attractive to *D. suzukii* mated female flies than DD bait at both incubation periods. Also, we found that the highest attractiveness was towards *L. kunkeei* 84 and *O. oeni* LS inoculated into DD bait among all strains tested at both incubation periods.

Our results agree with the findings reported by Maddalena (2016) who recorded a significant attraction of DD baits inoculated with different LAB species i.e. *O. oeni*, *Lactobacillus* spp., and *Pediococcus* spp. in preliminary field trials, and DD inoculated with *O. oeni* was the most attractive bait among all tested baits. According to the female flies response, the use of *O. oeni* was efficient in both our laboratory bioassays and field trials with variations in capturing levels which could be related to environmental conditions that may affect the fermentation process of the liquid bait and consequently its attractiveness.

These outcomes indicate that LAB strains used were capable of producing behaviorally active volatile compounds that mediate attraction of *D. suzukii* and significantly contribute to enhancing the attractiveness of DD bait. However, the effect of LAB metabolism on the production of bioactive compounds in the liquid bait was apparent at different incubation periods.

Generally, it seems that volatiles enrichment at 24 h inoculation time was higher than that at 48 h. Similar trend was observed with the combination of the most attractive strains tested at 24 and 48 h incubation periods which can be related to LAB adaptation and good performance in the bait matrix. While most of the volatile compounds associated with LAB strains were retained or enriched after 24 h incubation period, it is possible that some volatile compounds were generated by LAB at 48 h incubation period. Considerably, *O. oeni* LS, *L. kunkeei* 84 and *L. plantarum* B11 affected the volatile profiles of the liquid bait more than the other strains.

LAB can produce a wide variety of volatile compounds during fermentation process. Bouquets of compounds have been reported and series of biologically active compounds towards fruit flies have been tested for their attractiveness (Schulz and Dickschat, 2007). It is expected that volatile compounds that are most prevalent in the main components of DD (wine and vinegar) i.e. methanol, ethanol, acetic acid, and ethyl acetate, were enriched with LAB fermentation at 48 h incubation period.

Moreover, the presence of unrefined brown sugar in DD bait is a suitable growth substrate for different LAB species used i.e. FLAB (*F. fructosus* and *L. kunkeei*). The unrefined brown sugar is a sugar product with a distinctive brown color containing at least 88% of sucrose, up to 10% molasses, and monosaccharides (glucose and fructose). On the other hand, volatile flavor compounds and organic acids in brown sugar include acetic acid, ethanol and DMS (Asikin *et al*., 2014). Such compounds may contribute to enrichment of DD bait with bioactive volatiles for *D. suzukii*.

Ricci *et al.* (2018) reported that the use of *L. plantarum* strains for fermentation of elderberry juice showed an optimal growth performance with an increase of total volatile compounds after 48 h of fermentation. These findings support our results which showed that the *L. plantarum* strains were more attractive at 48 h than 24 h incubation period. However, the volatile profile of LAB fermented juices was characterized by the presence of 82 volatile compounds related to different classes: alcohols, terpenes, organic acids, ketones, and esters. The most abundant compounds were acetoin, acetic acid, hexanol, and ethyl acetate which are commonly known to be attractive compounds to *D. suzukii*.

Due to the different fermentation capacities of LAB strains, it seems that LAB have various metabolic patterns in the process of fermentation, resulting in different types and possibly concentrations of volatile compounds in DD bait that influence flies' attraction. Obviously, our results provide evidence on the biological activity of volatiles in LAB metabolic profiling for *D. suzukii* attraction. However, the capability of LAB species to produce bioactive volatiles which are functional towards Drosophila flies' attraction was evaluated in previous studies.

The species *L. plantarum* was identified from larval gut in *D. melanogaster*, and their emitted volatile compounds were evaluated for flies' attraction. In behavioral bioassays, both larvae and adult flies showed a significant attraction to volatiles released from food substrates occupied by larvae, indicating that olfactory mechanism is involved in these preference behaviors. Furthermore, adults were more attracted to bacterial volatiles released from food substrates occupied by larvae than that of unoccupied substrates (Venu *et al.*, 2014).

Recent studies provided a better understanding of how gut bacteria affect olfactory behavior in *D. melanogaster* and offers an ecological basis for flies preferences for different microbes in their natural environment (Qiao *et al*., 2019). Additionally, *D. melanogaster* flies exhibited a strong behavioral preference towards a microbe co-culture consisting of yeast (*S. cerevisiae*), acetic acid bacteria AAB (*Acetobacter malorum*), and LAB (*L. plantarum*) due to metabolite exchange of microbes when grown together. The microbial community of yeast, AAB and LAB produced higher levels of acetoin and attracted more flies than co-culture of yeast and AAB, indicating the significant influence of *L. plantarum* on flies behavior (Fischer *et al.*, 2017).

In agreement with the aforementioned studies of gut microbes' impact on olfactory behavior of Drosophila flies, we found that *D. suzukii*-associated gut microbe, *L. plantarum*, has a significant impact on behavioral preferences of female flies. More specifically, the response of flies towards the five tested strains of *L. plantarum* was higher than the control except one strain i.e. *L. plantarum* 100.

These results suggest that *D. suzukii* flies exhibit behavioral preferences towards volatiles produced by the larval gut bacteria i.e. *L. plantarum.* Such bacterial volatiles are released from food substrates inhabited by larvae and serve as attractants for *D. suzukii* adult flies. Thus, LAB volatiles are involved in flies' recognition of suitable feeding and oviposition sites. Moreover, the use of heterogenous culture (LAB strains combination) in our study may support LAB tolerance towards certain stresses in DD bait.

Moreover, our obtained data on symbiotic LAB of *D. suzukii*, *L. plantarum*, coincide with outcomes of previous research to investigate female flies' response to volatiles emitted by symbiotic AAB species of *D. suzukii* populations in Y-olfactometer behavioral bioassays (Mazzetto *et al.*, 2016). Possibly, the interaction among volatile compounds emitted by symbiotic LAB and AAB can distinguish the most attractive volatiles for *D. suzukii*. So further investigations on characterization of bacterial volatiles and flies' responses to individual volatile compound and later to blend of volatiles are necessary for developing a selective and attractive bait for pest management.

Regarding incubation period, the highest attractiveness was observed in the baits inoculated with *O. oeni* LS and *L. kunkeei* 84 at 48 h incubation period. These results correspond to data recorded by Zhang and Lovitt (2005) and Endo *et al.* (2012) who found a higher fermentation of sucrose by *O. oeni* and *L. kunkeei* at 48 h more than 24 h incubation time. Thus, with increasing incubation time, these strains affected the volatiles profile of DD bait more than the other LAB strains. On contrary, baits inoculated with other LAB strains showed lower attractiveness at 48 h than those at 24 h incubation period which can be related to limited metabolic activity adopted by LAB in the liquid bait.

Our findings are in agreement with those reported by Guzzon *et al*. (2016), who obtained a higher attractiveness of DD bait by the addition of specific wine-LAB species (*O. oeni*). This high attractiveness is due to the production of volatiles from microbial fermentations. However, the performance of different *O. oeni* strains in DD bait was assessed in earlier laboratory tests, where these strains showed a suitable growth under peculiar conditions of DD (Maddalena, 2016). In our study, *O. oeni* showed a well-adapted behavior to the harsh bait conditions due to its ability to tolerate low pH, high concentrations of ethanol and sulfite compounds (Capozzi *et al*., 2010).

Moreover, our results reconfirm the olfactory antennal responses of *D. suzukii* females to volatiles emitted by DD inoculated with *O. oeni* strains by using Electroantennography (EAG) (Maddalena, 2016). Additionally, the most attractive LAB strains represent an important commercial starter culture for food fermentation (*O. oeni* LS), and a commensal species naturally found in honeybee gut (*L. kunkeei* 84). The *L. kunkeei* 84 has the ability to produce high levels of acetic acid, volatile compound associated with *D. suzukii* attraction, and can inhibit yeast fermentation and pathogen growth in fermented substrates (Lerm *et* *al*., 2011; Bisson *et al.*, 2017). Such characteristics and metabolic properties of LAB strains recognize them as suitable agents for a more effective food bait.

On the other hand, our laboratory behavioral bioassays showed *D. suzukii* to be attracted to volatiles released by a combination of the most attractive LAB strains i.e. *O. oeni* LS and *L. kunkeei* 84. Although the total number of flies respond to combined LAB strains was lower than those respond to each single strain separately, the trends in fly's behavioral response were similar, indicating a consistent response of flies towards LAB strains. Such differences could be explained by the fermentation process and volatile profiles; but more importantly, they could be inherent to the bacterial signaling, known as quorum sensing, which is mediated by small peptides used as auto-inducing molecules that allow each strain to communicate intra-specifically. However, quorum sensing plays a role in many complex processes such as biofilm formation, secretion of virulence factors, sporulation, production of bacteriocins and antimicrobial compounds (Albuquerque *et al.*, 2020).

However, these outcomes highlight the differences in volatiles production during fermentation process due to different metabolic behaviors of LAB strains used. Each strain exerts different metabolic pathways for volatiles production, being able to differently metabolize liquid bait. Likewise, LAB controlled fermentation affected volatile profile of bait matrix, enriching it with the main LAB metabolic compounds i.e. acetic acid, and ethanol. Thus, LAB can be foreseen as microbial factories to produce volatile compounds in food baits, characterized by new bioactive properties and potential innovative attractive value for *D. suzukii*.

As our best LAB strains were of higher attractiveness than that of the DD bait, we anticipate that their volatiles can be used to improve the attractiveness of commercially available baits for *D. suzukii*. However, the main volatile compounds produced by LAB, acetic acid and ethanol, were tested in laboratory bioassays and field trapping experiments for *D. suzukii* attraction (Cha *et al.*, 2013; Kleiber *et al.*, 2014). Although these compounds exhibited a successful trapping of *D. suzukii* in fields, it was suggested that additional volatile compounds other than acetic acid and ethanol such as ethyl acetate, isoamyl acetate and linalool will contribute in developing a highly attractive chemical lure with potential for use in detection and management of *D. suzukii* (Cha *et al.*, 2012; Abraham *et al.*, 2015). However, such volatiles known to be attractive to *D. suzukii* and associated with LAB metabolic profiling are useful for enhancement the attractiveness of DD bait, and therefore they have been selected and tested in field trials.

Our results provide an important basic knowledge for future attempts to the improvement of liquid baits attractiveness and developing new tools for efficient pest management focusing on the exploitation of bioactive volatiles. Lactic acid fermentation through selected LAB strains can therefore be considered as a promising source of novel volatiles with attractive potential to *D. suzukii*. Besides, additional work for a better explanation of *D. suzukii* preferences is needed to identify key volatile compounds produced during LAB fermentation process. All these works could assist in obtaining more selective and attractive food bait for monitoring and management strategies of *D. suzukii*.

Although we could not show overall attraction pattern of LAB strains with respect to the optimal LAB growth conditions and their metabolic profile in liquid bait, we showed that a combination of two different LAB species (*L. kunkeei* 84 and *O. oeni* LS) produce volatiles that could improve attractiveness of DD bait towards *D. suzukii* females. However, investigation on the fermentative behavior of these LAB strains, and variation in their volatile profiles in the liquid bait may bring insights to the significant differences observed in this study.

6.2. Trapping studies

6.2.1. Individual- compound behavioral assay

In the current study, six volatile compounds were tested individually for their attractive effect to *D. suzukii* in a commercial lure Dros'Attract. The synthetic compounds are associated primarily with fruit-ripening and microbial fermentation. They were selected based on reported positive physiological and behavioral responses of adult *D. suzukii* (Keesey *et al*., 2015; Revadi *et al*., 2015). In general, our results showed that the *D. suzukii* olfactory response ranged from no or negative responses to positive responses. DMS and geraniol, evoked behavioral preference in greenhouse assays. Terpenoid and ester compounds (ß-cyclocitral, ethyl acetate, isopentyl acetate and linalool) have shown inconsistent effect on females and males.

In previous studies, *D. suzukii* behavioral preference for strawberry leaves was investigated in comparison with its close relative species i.e. *D. biarmipes* and *D. melanogaster*. Keesey *et al*. (2015) found that *D. suzukii* was more attracted to strawberry leaf odors than other species. This behavioral preference seems to be linked to β-cyclocitral, a volatile isoprenoid associated with leaf tissue. Further trap assays showed that *D. suzukii* was more attracted to the single compound β-cyclocitral than other species and thus appears to be species specific compound.

Surprisingly, in our study β- cyclocitral repelled *D. suzukii* (females and males) when added to the commercial lure Dros'Attract. Similar repellant effect has also previously been observed in other behavioral assays under laboratory and field conditions. A recent study showed that the single component lure (β- cyclocitral) was not more attractive than control in single-lure behavioral assays. Moreover, adding β- cyclocitral did not increase *D. suzukii* attraction to a fermentation lure which is composed of four synthetic compounds (acetic acid, ethanol, acetoin, and methionol) in combined- lure behavioral assays (Cloonan *et al.*, 2019).

Later, this effect of ß- cyclocitral was confirmed in a 2-year field study conducted across multiple crops including blueberry, cherry, blackberries, and raspberries.

ß- cyclocitral did not improve the attractiveness of the fermentation lure or its selectivity in field traps during the growing season. In our study, the non-attractive effect of ß- cyclocitral can be due to the used concentration of the compound, its interference with lure components, and conditions in behavioral assays. Additionally, the physiological status of flies i.e. mated, unmated, satiated or starved plays an important role in their response to volatiles (Cloonan *et al.*, 2019).

Previous studies have also shown that individual attractive compounds do not always elicit an increased behavioral response in adult *D. suzukii*. In laboratory behavioral bioassays, Cha *et al.* (2012) found that adding isoamyl acetate (known as isopentyl acetate) to a mixture of acetic acid and ethanol significantly reduced *D. suzukii* fly attraction. Our obtained data agree with these findings where isopentyl acetate reduced flies' preference to the lure.

Findings of a new study indicated a slight positive responses of *D. suzukii* females (but not males) to isopentyl acetate when tested singly in behavioral assays. Moreover, isopentyl acetate was significantly more attractive to females than β-cyclocitral when both compounds were tested against each other (Piñero *et al.*, 2019). Furthermore, the addition of isopentyl acetate did not improve the attractiveness or selectivity of a fermentation lure in laboratory assays and field trials (Cloonan *et al.*, 2019). These findings support our results which showed that only females were attracted to isopentyl acetate and lure attractiveness did not improve significantly.

Hence, the attractive effect of isopentyl acetate noted for females suggests that this compound may be involved in host finding behaviors as it was identified from ripe strawberry fruits (Keesey *et al.*, 2015). Likewise, it was demonstrated that virgin females were more likely to seek out yeast volatiles i.e. isopentyl acetate for feeding purposes (Mori *et al.*, 2017). Such volatiles that trigger feeding behavior could be useful in developing a more effective and attractive lure.

Similarly, adding ethyl acetate to Dros'Attract lure showed no significant effects on females but strong repellent effects on the males. This agrees with previous work, showing this yeast compound to be unattractive to *D. suzukii* when added to a mixture of acetic acid and ethanol or apple cider vinegar in field traps (Cha *et al.*, 2012; Kleiber *et al.*, 2014). A recent work reported similar results on the fruit-based volatile compound, ethyl acetate, that did not prompt behavioral attraction by both male and female *D. suzukii*, despite the positive electrophysiological responses in EAG tests (Revadi *et al.*, 2015; Bolton *et al.*, 2019).

Subsequently, a monoterpene volatile compound, linalool, has shown inconsistent effect among females and males *D. suzukii*. While the behavioral activity of linalool was attractive to females, at the same time it was repellent to males indicating contradictory behavioral response. These findings coincide with previous scientific investigations on the bioactivity of plant volatiles i.e. linalool against *D. suzukii*. Linalool was identified from different ripe host fruits i.e.

raspberry, blueberry, cherry, and strawberry (Abraham *et al.*, 2015; Keesey *et al*., 2015), and consistently elicited strong antennal responses in female *D. suzukii.* Furthermore, linalool contributed in the high attractiveness of a synthetic blend in choice behavioral bioassays, but it did not improve attraction when used alone in cherry fruit extract (Abraham *et al.*, 2015).

On the other hand, previous reports demonstrated that there were some inconsistencies in repellent activity of linalool against *D. suzukii*. Erland *et al*. (2015) observed no significant oviposition deterrent activity for the three major monoterpene constituents of lavender essential oil (1,8-cineole, 3-carene and linalool), despite their good repellence towards *D. suzukii*. Our behavioural responses are largely in agreement with the aforementioned data which implies the unpredictable behavioural activity of some volatile compounds to female and male *D. suzukii*.

Interestingly, geraniol was behaviorally attractive to females *D. suzukii* and DMS elicited a stronger positive response in females. Geraniol is a terpenoid volatile compound with floral odor, widely emitted by flowers and fruits such as blueberry and grapes, which may explain the high female preference observed for this compound. However, few studies have been conducted to evaluate its attraction effect on *D. suzukii* adults. It was reported that geraniol is present in volatile profile of blueberry fruits and induced antennal responses in female *D. suzukii* (Abraham *et al.*, 2015) which endorse our results. This attraction effect was also reported for other insect species i.e. whitefly *Bemisia tabaci* with geraniol encapsulated in nanoparticle formulation using olfactometer bioassays indicating the potential use of it in trapping systems (De Oliveira *et al.*, 2018).

On contrary, geraniol, a common constituent of several essential oils, exhibited other biological activities against *D. suzukii* flies such as repellent. Recently, researchers have shown geraniol to be a plant-based repellent for *D. suzukii* females and males. In laboratory bioassays, geraniol was moderately repellent to female flies only up to 6 h and males for 24 h after individual application in choice and no-choice bioassays. Moreover, geraniol did not improve repellency of a synthetic blend of thymol, citronellol, and menthol (Renkema *et al*., 2017; Dam *et al*., 2019). Our obtained data agree with these findings where geraniol repelled males throughout the 24 h testing period.

A volatile sulfur compound, DMS, has been described in a wide range of food products as well as in beer and wine. Although DMS does not present fruity aroma, it has an olfactory impact on fruity aroma expression in fruits mixture. DMS produced an enhancing effect, increasing the perception of typical fruity aromatic character in berry-fruit syrups (Lytra *et al.*, 2014). Based on this evidence, we hypothesized that DMS played an important role for enhancing the attractiveness of the lure for the females.

However, DMS behavioral activity has not yet been tested for *D. suzukii* flies either in laboratory behavioral choice assays or field trials. Moreover, information on detection of Volatile Sulfur Compounds (VSCs) i.e. DMS in host fruits of *D. suzukii* is still limited due to their low detection thresholds. In general, these volatiles encounter difficulties in detection and identification due to their presence at trace levels and are difficult to quantify among the other volatiles present in higher concentrations in fruits (Du *et al*., 2012).

Therefore, the use of appropriate analytical instrumentation and sample preparation technique can provide a reliable detection and identification of VSCs i.e. DMS in host fruits. In this regard, employing the proper sampling conditions in headspace solid- phase microextraction technique (HS-SPME) coupled with gas chromatography and mass spectrometry (GC-MS) or gas chromatograph equipped with pulsed flame photometric detector (GC-PFPD) is recommended (Du *et al*., 2012; Dziekońska-Kubczak *et al.*, 2020). Different studies have proven the attraction effect of DMS on several fruit flies including *D. suzukii*.

Lately, a study was conducted to investigate the efficacy of fermentation treatment in disinfesting the fruit waste. Noble *et al.* (2017) measured the mean concentration of DMS in fermented strawberry fruit waste which harbor viable life stages of *D. suzukii* at (9.66 ± 8.7) ppm. This fermented fruit waste was found attractive for *D. suzukii* egg-laying and thereafter able to support a further life cycle. Additionally, DMS showed attraction activity for tephritid fruit flies in laboratory bioassays. Robacker (2007) reported the detection of DMS in volatiles collection of numerous species of symbiotic bacteria associated with tephritid fruit flies and recorded attraction effect of DMS on Mexican fruit fly. Here, it should be noted that DMS was identified previously in volatile profile of fruits that were fermented by LAB i.e. *L. plantarum*, a symbiotic LAB species associated with *D. suzukii* (Kang *et al.*, 2003; Vacchini, 2014).

Obviously, when DMS and geraniol was added individually to Dros'Attract lure, it significantly enhanced the response of females to the lure, suggesting that those compounds play a role in *D. suzukii* olfactory behavior and improve attraction to Dros'Attrac. These results suggest that both plant-based volatiles (geraniol) and microbial fermentation volatiles (DMS) are needed to elicit high levels of response in females seeking for food or oviposition resources.

Overall, females used in our behavioral assays were sexually mature and presumably mated therefore those females were considered to have a high oviposition drive. For this reason, females (not males) showed preference to five compounds out of six. Males, however, did not show preferences to all compounds a result that suggests that those compounds may play a role in male repellence from host plants. Although negative behavioral responses were observed for five tested compounds, positive responses may still be found within a blend of those compounds. Previous research has demonstrated that *D. suzukii* attracted to a specific blend and can be unaffected by or even repelled by the single compounds of that blend (Cha *et al.*, 2012; Bolton *et al.*, 2019). Therefore, we suggest that a combination of DMS and geraniol can improve attractiveness of Dros'Attract lure that is superior in attractiveness.

In addition, it has to be considered that the flies used in our behavioral assays had not been previously exposed to the tested compounds. It is possible that compounds that did not elicit an innate response in our assays, may elicit a conditioned response as a result of associative learning, when flies experience these volatile compounds in association with feeding or oviposition actions. Besides, a conflict between these compounds and lure components may be responsible for the decreased attraction that was observed in our behavioral assays.

Our findings increase our understanding of male and female *D. suzukii* olfactory responses to synthetic volatile compounds as sources of attractants. Further studies are needed to evaluate bioactivity of tested compounds at different concentrations to clarify their behavioral role for *D. suzukii*. A series of laboratory and field trails are necessary to determine compounds enhancing *D. suzukii* captures and to ascertain their role for developing a more species-specific lure.

6.2.2. VOCs- blend behavioral assay

Generally, our findings indicate that the Droso-Trap baited with Dros'Attract lure combined with DMS and geraniol was the best performing traps in behavioral assays conducted under greenhouse conditions. Traps baited with the lure and a single compound, either DMS or geraniol, also performed well initially even there was no significant difference. Moreover, the trend in flies' response was similar to that in earlier behavioral assays indicating a consistent performance of these compounds from single treatment through blend, and trapping system continued to perform well. However, variations in the attraction can be related to the several endogenous factors, such as nutritional and mating status, sexual development, age, and sex, which influence fly physiological status and directly impact the effectiveness of lure and trapping system.

Likewise, there was no significant differences in the numbers of females and males captured in our trapping system indicating the interference between DMS, geraniol and lure components making them perceived as an attractive host stimulus. These results demonstrate that volatile compounds naturally present in different sources increase *D. suzukii* attraction to attractive lure, providing opportunities to construct a synthetic blend to attract or preserve natural enemies of the pest at the greenhouse or field scale.

In our study we developed the prototype of a highly attractive lure which provided consistent captures of *D. suzukii* throughout the semi field assays and attracted both females and males. The effective performance of the prototype is related to the physical characteristics of the trap, including color, shape, selectivity, and the increased attractiveness of the lure. Consequently, a new trapping system was developed as a reliable and selective method of trapping *D. suzukii*. The system is comprised of a Dros'Attract lure combined with volatile compounds (DMS and geraniol) and Droso-Trap equipped with a sticky yellow card.

The attractiveness of our trapping system is related to both trap design and lure. Even though we did not evaluate trap selectivity, but we consider that the optimized trap design combined with Dros'Attract lure provide high selectivity. The trap is red on the bottom and clear on the top with several lateral openings. During trap evaluation studies, *D. suzukii* showed a strong visual preference for red traps which outperformed clear and yellow traps in laboratory and field tests (Renkema *et al*., 2014; Rice *et al.*, 2016). Moreover, integrating a yellow sticky card, placed upright inside the trap, enhanced flies' captures as a visual stimulus and allowed immediate vision of immobilized captured flies (Renkema *et al*., 2014; Cruz-Esteban, 2020).

Additionally, the increased number of lateral openings contribute to a better evaporation of lure and reduced number of non drosophilid large flies catches. Indeed, the efficiency of Droso-Trap was approved in different field studies. Overall, the trap is affordable, durable, and can hold high volumes of lure to maximize *D. suzukii* captures (Renkema *et al*., 2014; Tonina *et al.*, 2018). Another feature is the large size of trap, particularly the red hemispherical cup which was found the preferred size and shape for *D. suzukii* in semi field and field tests (Rice *et al.*, 2016). On the other hand, Dros'Attract is a special food-attractant developed by Biobest for use in Droso-Trap for monitoring *D. suzukii*. Droso-Trap loaded with Dros'Attract caught on average 3.3 times more *D. suzukii* than apple cider vinegar traps.

Generally, *D. suzukii* is known to use visual and chemical cues to search for host plants. For this reason, the combination of olfactory and visual stimuli has been used in our trapping system to stimulate the behavior responses of flies. Several researchers approved a synergistic interaction between chemical cues i.e. strong attractive stimulus (yeast species) and visual stimulus (red color) suggesting that combined stimuli can improve trapping tools and monitoring protocols (Lasa *et al.*, 2017).

Indeed, developing highly attractive lure was prioritized over improving selectivity in our trapping system. The selectivity of the most attractive commercial lures tested in a previous study was not constant over time and changed greatly among sites with the *D. suzukii* and non-target insect population density. This indicates that attractiveness and selectivity are not related to each other, thus it was suggested implementing different lure types in different periods for different purposes i.e. monitoring or mass trapping to reduce pest populations (Tonina *et al.*, 2018).

However, to develop an effective monitoring method, it is necessary to determine the combination of trap and lure enabling high attractiveness in conditions with low densities of pest population. Droso-Trap is an effective, suitable commercially available trap. Among other commercial traps, Droso-Trap with a commercial liquid bait i.e. Droskidrink showed the best performance in terms of early season captures and total number of catches (Vaccari *et al.*, 2015). Therefore, we recommend our trapping system for early season monitoring to detect *D. suzukii* at low population levels.

Our obtained data could provide essential information for developing a new trapping system comprised of a highly attractive lure and selective trap. This system will be useful as a reliable approach for early monitoring of *D. suzukii* population. However, chemical analysis of volatile profile of the lure is needed to identify key compounds responsible for fly's attraction. Further work is necessary to optimize trap design, lure, and field trapping protocols for *D. suzukii* adults for developing behavioral-based management options. Possibly, optimizing the ratios of lure components i.e. volatile compounds will contribute to maximizing the behavioral responses of flies and minimizing responses of nontarget insect species.

However, to unambiguously show the attractiveness of the new trapping system, studies should be conducted with infested host plants. Additional field studies will be needed to evaluate the efficacy of the trapping system for potential use in field application. In addition to the aspects of attractiveness, it is important to evaluate additional issues such as selectivity and environmental impacts. Selectivity should be accurately assessed and integrated into effective monitoring and pest management programs. High selectivity minimizes detrimental effects on biodiversity of nontarget insect species, simultaneously enables the ease sorting of specimens (Burrack *et al.*, 2015).

Furthermore, research is needed to compare the new system with different commercial trapping systems over a large range of climates, crops, and regions to determine factors affecting flies' capture. Understanding the factors driving behavioral responses in *D. suzukii* population may contribute to optimizing trapping systems for monitoring purposes. Ultimately, simpler is better: using a combination of more attractive lure and more selective trap design may have the practical benefit of an increased number of captured flies of *D. suzukii* and reduced number of nontarget insect species captured in traps. Use of this new trapping system will be a reliable approach for monitoring pest population.

CHAPTER 7

7. Conclusions and Recommendations

7.1. Olfactory studies

To our knowledge, this is the first study of a behaviorally response of *D. suzukii* mated females to odors of crop and non-crop host fruits and LAB strains associated with host fruits surface and *D. suzukii* gut microbiota. Flies were more attracted to non-sterilized fruits than chemically sterilized fruits which were sampled from southern regions of Italy. Blackberry fruits were the most attractive host crop fruits in olfactometer bioassay. Furthermore, flies always exhibited positive response to volatiles emitted by LAB strains in DD food bait. The most attractive strains, *L. kunkeei* 84 and *O. oeni* LS, showed a significant attraction to females when combined and inoculated into food bait.

Overall, this attractiveness can be related to volatile compounds emitted by fresh and ripen fruits, microorganisms associated with fruits' surface and LAB fermentation volatiles. Collectively, the obtained data demonstrate that olfaction plays a major role in *D. suzukii* host selection and increase our understanding of behavioral response of female and male flies. Moreover, the recent findings provide evidence on the biological activity of volatile compounds in host fruits and LAB metabolic profiling for *D. suzukii* attraction. Obviously, the unique characteristics and metabolic properties of tested LAB strains contribute to improving the attractiveness of a commercial lure and identify them as suitable agents for a more effective lure.

In conclusion, choice behavioral bioassays highlight the importance of combining host fruit volatiles with microbes' volatiles to increase attractiveness of current attractive lures. Therefore, further investigations on chemical analysis of volatile profiling of host fruits and LAB strains is required to identify key compounds which can elicit flies' behavioral activity. Data obtained may help in the developing of behaviorally based tools for pest monitoring. Subsequent work on flies' responses to individual volatile compound and later to blend of volatiles is necessary for expanding the potential volatiles enrichment in existing lures and the possibility to increase flies' attraction.

Besides, the identification of LAB species on infested and non-infested fruits is crucial for better understanding of flies' attraction towards intact fruits for oviposition and infested fruits for feeding. As a recommendation we suggest the exploitation of host fruit and LAB volatiles for developing a new concept of trapping based on a highly attractive lure combined with a selective trap. Such trapping has a potential application to monitoring programs and pest management techniques such as mass trapping and attract & kill.

7.2. Trapping studies

The development of the prototype of an efficient synthetic lure for developing a new trapping system intended for monitoring *D. suzukii* is the main objective of this research project. Therefore, the attractiveness of a commercial lure, Dros'Attract, was improved through a series of behavioral bioassays using individual compound and later a blend of volatile compounds. Among all tested compounds, geraniol and DMS significantly enhanced the behavioral response of *D. suzukii* to the lure. However, both compounds showed a consistent performance from single treatment through blend, and trapping system continued to perform well in behavioral assays. Therefore, a prototype of a more attractive lure was developed comprising the commercial lure and both compounds.

Ultimately, a new trapping system was developed as a reliable and selective method of trapping *D. suzukii*. The system is comprised of a Dros'Attract lure combined with volatile compounds (DMS and geraniol) and Droso-Trap equipped with a sticky yellow card. As indicated in the previous studies, microbial volatiles along with host fruits volatiles play important role in fly attraction. The recent study suggests that both plant-based volatiles (geraniol) and microbial fermentation volatiles (DMS) are needed to elicit high levels of response in females seeking for food or oviposition resources.

A conclusion of these trapping studies is that the use of a highly attractive lure with significant attraction of females over males may considerably contribute to reducing fruit damage and yield losses caused by female behaviors i.e. feeding and oviposition. Another interesting conclusion is that attractiveness and selectivity of trapping system can be obtained by combining a highly attractive and specific lure (Dros'Attract) combined with a commercial trap of optimized design (Droso-Trap). Therefore, we recommend our novel trapping system for early season monitoring to detect *D. suzukii* at low population levels. Also, we recommend further improvements on this system for a potential use in mass trapping and attract & kill techniques. Our findings increase our understanding of male and female *D. suzukii* olfactory responses to synthetic volatile compounds as sources of attractants.

Further studies are needed to evaluate bioactivity of tested compounds at different concentrations to clarify their behavioral role for *D. suzukii*. A series of laboratory and field trails, including a comparison with different commercial trapping systems, are necessary to determine endogenous and exogenous factors affecting *D. suzukii* captures. Further research is required to be conducted for chemical characterization and identification of key volatile compounds in lure responsible for fly's attraction. Further work is necessary to optimize trap design, lure, and field trapping protocols for *D. suzukii* adults for developing behavioral-based management options. Possibly, optimizing the ratios of lure components i.e. volatile compounds will contribute to high selectivity to maximize the behavioral responses of flies and minimize responses of nontarget insect species.

CHAPTER 8

8. References

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CHAPTER 9

9. Scientific Publications

This thesis is based on the work contained in the following papers.

Alawamleh A., Di Stefano M.G., Ganassi S., Hashmi M.M., Mancini M., Đurović G., Wäckers F., Anfora G., and De Cristofaro A. (2019). Comparative study of *Drosophila suzukii* females' behavioral responses to fruit odors of two varieties of *Vitis vinifera*. *IOBC-WPRS Bulletin*, 146: 138-142.

Alawamleh A., Đurović G., Maddalena M., Guzzon R., Ganassi S., Hashmi M.M., Wäckers F., Anfora G., and De Cristofaro A. (2021). Liquid baits with *Oenococcus oeni* increase captures of *Drosophila suzukii* (Part I: selection of lactic acid bacteria species and strain). *Insects*. (submitted and under review).

Đurović G., **Alawamleh A.**, Carlin S., Maddalena G., Guzzon R., Mazzoni V., Dalton D.T., Walton V.M., Suckling D.M., Butler R.C., Angeli S., De Cristofaro A. and Anfora G. (2021). Liquid baits with *Oenococcus oeni* increase captures of *Drosophila suzukii*. *Insects*, 12, 66. https://doi.org/10.3390/insects12010066.