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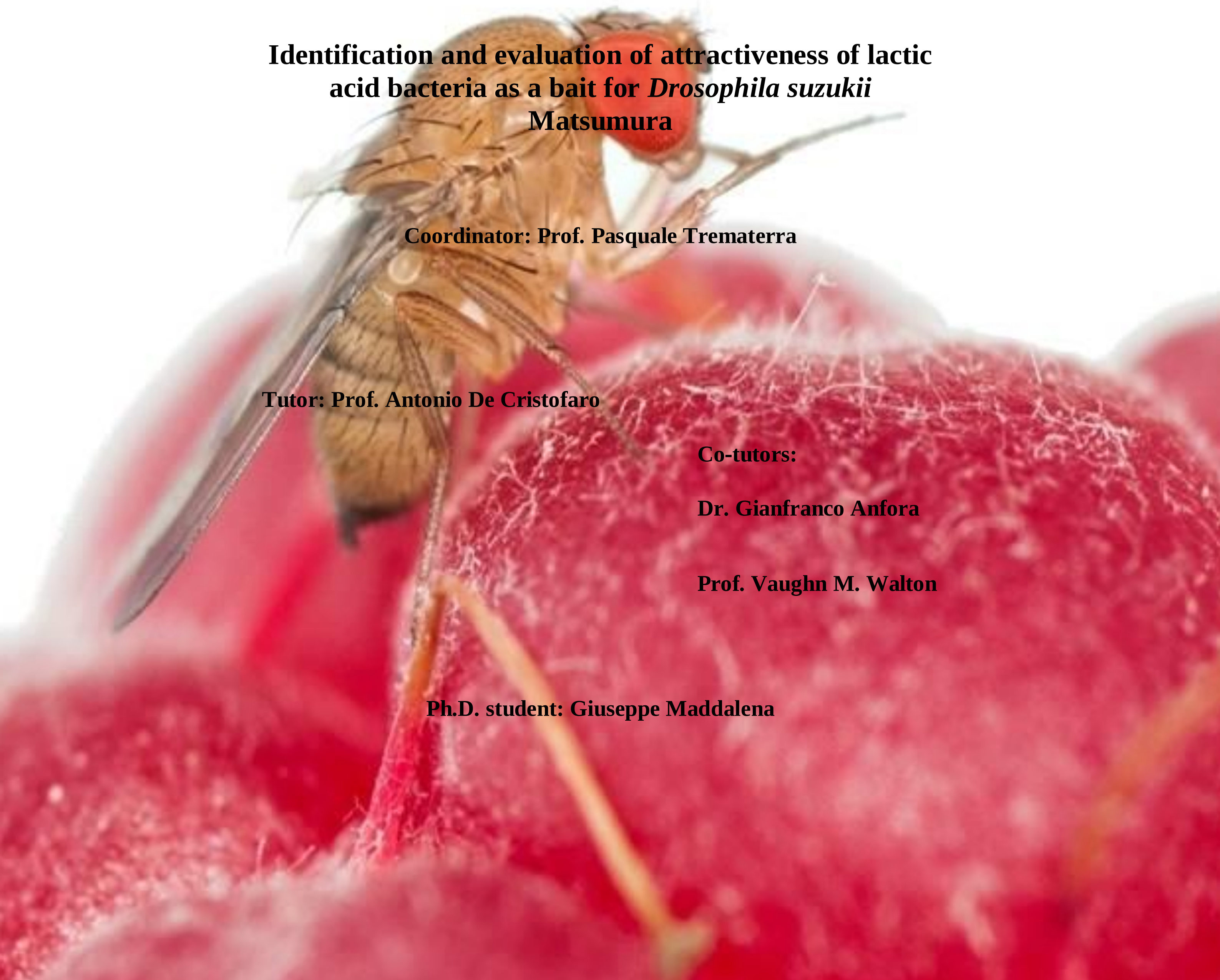

Identification and evaluation of attractiveness of lactic acid bacteria as a bait for *Drosophila suzukii* Matsumura

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Riassunto

Il rapido incremento delle attività commerciali, il perpetuarsi delle migrazioni umane, il turismo, i trasporti e i viaggi sempre più celeri, nel corso dell'ultimo secolo hanno notevolmente favorito la diffusione di numerose specie invasive, che hanno superato barriere geografiche, anche considerevoli, in un ristretto arco temporale. Questa tendenza è destinata ad aumentare ulteriormente, e le specie invasive provenienti da altri Paesi, prima sconosciute, sono da annoverare tre le principali minacce per la biodiversità e la produzione alimentare in tutto il mondo.

Gli insetti rappresentano la maggioranza degli organismi viventi e, quindi, costituiscono larga parte del problema delle specie invasive, tanto che in molte aree, come Nord America, Australia, Asia e Sud Africa, gli insetti dannosi alloctoni sono considerati importanti quanto, e forse più, di quelli autoctoni. Tradizionalmente l'Europa ha subito danni meno ingenti da parte di insetti di provenienza esterna, tendenza che è notevolmente cambiata in anni recenti e che ha portato ad un interesse sempre maggiore nei confronti degli insetti alieni che hanno colonizzato il territorio comunitario.

Drosophila suzukii Matsumura (Diptera: Drosophilidae), nota anche come moscerino dei piccoli frutti o spotted-wing drosophila (SWD), originaria del sud-est asiatico, è un fitofago di recente introduzione in Europa, Stati Uniti e Canada, ove i danni sono stati rinvenuti su numerose colture come ciliegio, vite, uva spina, lampone e frutti di bosco in genere, pesco, susino, cachi, pomodoro, olivo, gelso e nespolo, di cui attacca i frutti.

Gli adulti di *D. suzukii* sono moscerini lunghi 2-3 mm, con occhi rossi, torace marrone chiaro o giallastro e strisce nere sull'addome. Sono caratterizzati da uno spiccato dimorfismo sessuale; i maschi mostrano, infatti, una macchia scura al margine anteriore di ogni ala, le femmine un grande ovipositore seghettato. Le uova sono bianco latte, oblunghe (0,5-0,7 x 0,2 mm) e con due filamenti ad un'estremità; dell'uovo, deposto sotto l'epicarpo, sono visibili dall'esterno solo i due filamenti sporgenti (ca. 0,4

mm) dalla cicatrice di ovideposizione. Le larve si sviluppano attraverso tre età, sono di colore bianco traslucido e vivono all'interno del frutto ospite, nutrendosi della polpa e dei microrganismi naturalmente presenti, causandone il deterioramento; le cicatrici di ovideposizione, infatti, consentono l'ingresso di patogeni, come batteri e funghi, che comportano la cascola precoce dei frutti danneggiati.

I danni economici causati da *D. suzukii* sono stati stimati in circa 2 miliardi di dollari in America, oltre 4 miliardi in Europa e 500 milioni in Asia.

Appare scontato pensare che gli insetticidi di sintesi possano rappresentare il mezzo principale per il controllo e la gestione di *D. suzukii* ma, data la novità rappresentata da questo fitofago, deve essere individuata, intanto, una soglia economica di danno. Insetticidi di sintesi quali organofosfati, piretroidi e spinosad, hanno dimostrato una certa efficacia contro *D. suzukii*; l'altra faccia della medaglia mostra come questi principi attivi ad ampio spettro di azione siano tossici per gli insetti utili, oltre che essere responsabili dell'aumento dei residui presenti nei frutti, con tutti i rischi conseguenti per la salute umana.

Risultati incoraggianti sono attesi dall'uso di agenti di biocontrollo come funghi, batteri, virus e altri nemici naturali del carpofago, ovvero predatori e parassitoidi, che potrebbero essere usati per limitarne le popolazioni.

Il metodo attualmente più utilizzato per il monitoraggio di *D. suzukii* è basato sull'impiego di trappole costituite da un bicchiere di plastica forato contenente aceto di sidro di mele, un tensioattivo e un pannello adesivo di colore giallo. Tali trappole sono inefficaci per un monitoraggio precoce di *D. suzukii*, le cui popolazioni raggiungono rapidamente densità tali da arrecare danni, in quanto progettate per catturare altri insetti, in particolare Drosophilidae.

Al fine di mettere a punto strategie efficaci di cattura massale, sono necessari attrattivi altamente efficaci e selettivi. Per *D. suzukii*, osservazioni preliminari confermano che l'insetto adulto è richiamato dalla frutta lesionata e preferibilmente in stato di fermentazione, come suggerisce la naturale attrazione esercitata da una varietà di liquidi fermentati, tra cui vino, aceto, succhi di frutta e derivati, nonché dall'associazione, recentemente riportata, di questo insetto con una comunità di diverse specie di lieviti, come *Hanseniaspora uvarum* (Niehaus) Shehata *et al.* Allo stato attuale, le trappole più efficaci per catturare *D. suzukii* sono innescate con diversi tipi di aceto e vino, substrati entrambi derivanti dalla fermentazione mediata sia da lieviti che da batteri. Una delle esche liquide commerciali, il cosiddetto "Droskidrink", ha fornito risultati promettenti sia nel monitoraggio di *D. suzukii*, condotto negli anni 2011-2013 in provincia di Trento, che in prove preliminari di cattura massale.

L'obiettivo principale di questo lavoro, pertanto, è stato quello di studiare alcuni aspetti poco noti di *D. suzukii*, in particolare quelli inerenti la percezione di stimoli di varia natura e il riconoscimento di substrati di interesse per la messa a punto di esche innovative e altamente efficaci da utilizzare nel monitoraggio e in strategie di controllo del carpofago. In particolare, gli studi sono stati indirizzati alla caratterizzazione di parametri biologici, fisici e chimici utili per valutare l'efficacia in campo dell'attrattivo noto come "Droskidrink", l'esca commerciale raccomandata contro *D. suzukii* anche in pieno campo. Le prove di campo sono state condotte nella regione Trentino-Alto Adige, dove il fitofago ha recentemente causato danni ingenti. La ricerca ha sviluppato un nuovo tipo di esca, in cui l'attrattivo commerciale "Droskidrink" è risultato fortemente potenziato nella sua azione grazie all'associazione con microrganismi in grado di rilasciare sostanze volatili biologicamente attive su *D. suzukii*.

Gli studi sono proseguiti presso l'Oregon State University, dove è stata saggiata la funzionalità della trappola innescata con il nuovo attrattivo anche in ambienti ecologicamente diversi, con l'intento di accelerare la ricerca e il trasferimento tecnologico di nuovi metodi ecosostenibili di controllo di *D. suzukii* basati, ad esempio, su tecniche di cattura massale.

Nel presente lavoro è stato possibile dimostrare l'importanza dei microrganismi, in particolare batteri lattici, e più propriamente ceppi appartenenti alla specie *Oenococcus oeni* (Garvie) Dicks *et al.*, nel miglioramento della capacità di cattura del "Droskidrink", dovuto a sostanze prodotte durante la fermentazione malolattica. Gli studi compiuti hanno anche condotto alla messa a punto di un nuovo modello di trappola che sembra agire in modo sinergico con la miscela attrattiva utilizzata e che, tra l'altro, è in grado di ridurre i tempi necessari per la quantificazione e la determinazione degli individui catturati. Interessante è risultata la capacità di questo nuovo modello di trappola di catturare un consistente numero di individui, specialmente di sesso femminile, in periodi caratterizzati da temperature al limite della sopravvivenza di *D. suzukii*, ovvero in presenza di basse densità di popolazione del carpofago. Questi risultati permettono di ipotizzare una buona efficacia dell'applicazione precoce delle tecniche di cattura massale o di lotta attratticida, che potrebbero condurre, come auspicato, ad una significativa riduzione del numero di individui presenti in campo al momento della comparsa dei frutti adatti all'ovideposizione.

Abstract

The spotted-wing drosophila (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), native of Eastern Asia, is an invasive alien species in Europe and the Americas and is one of the main emerging pests of valuable crops, including soft fruits and wine grapes. The conventional approach to handle infestations of SWD involves the use of commercially available insecticides, but these do not seem able to ensure effective results; consequently, alternative strategies are strongly required. Mass trapping uses a high density of traps baited with a lure and is one of the most promising methods studied and tested so far. Consequently, an improvement in the bait composition is obligatory to guarantee reliable attractivity to SWD. This study was aimed precisely to investigate and improve upon one of the best attractant mixtures for SWD, the "Droskidrink" (DD). The goal of the work was centred on the exploitation of lactic acid bacteria as a biological catalyst in the production of organic volatile molecules attractive to SWD, thanks to the fermentation of sugars and organic acids present in the liquid bait. Strains of lactic acid bacteria were chosen because they are usually involved in the fermentation of wine and vinegar, historically the most attractive liquids for species of Drosophilidae. A series of preliminary field tests was coupled with laboratory tests to describe the behaviour and performance of various kinds of lactic acid bacteria, in this particular application. Afterwards, we focused our analysis on different biotypes of the bacterium *Oenococcus oeni* (Garvie) Dicks *et al*. that revealed a high resistance to stressful environmental conditions of the liquid bait and, at the same time, a promising attractive capacity to SWD. Attention was then moved to a new model of trap able to produce a synergistic effect between the trap and the liquid bait. Our results showed that the attractiveness of DD was greatly increased by the addition of *O. oeni* to the standard mixture. Malolactic fermentation due to *O. oeni* released volatile substances attractive to SWD. In particular, the new trap-bait combination provided excellent results, increasing the number of catches, especially with regard to female individuals and particularly during the cold seasons, when SWD has low population density. In addition, this new trap design is simpler and faster to

service, compared to the traps used previously. The long-term goal is to accelerate research and technology transfer toward the development of mass trapping and attract-and-kill strategies based on the use of traps baited with this new attractant. Moreover, through the development of a trap utilizing the best characteristics identified in this study, we were able to obtain excellent levels of catches during the whole year.

Dedication

To Antonella, with all my heart.

Contents

1. Introduction

1.1 Invasive alien insect species

Invasive alien species are recognized as one of the leading threats to biodiversity and food production worldwide. The ways in which non-native species affect native species and ecosystems are numerous and usually irreversible (Parker *et al.*, 1999). Non-indigenous species also impose enormous costs on agriculture, forestry and human health (Pimentel *et al.*, 2002). Rapidly accelerating human trade, tourism, transport and travel over the past century have dramatically enhanced the spread of invasive species, allowing them to surmount geographic barriers. Since this tendency is likely to increase (Levine & D'Antonio, 2003), national and international strategies are required to assess the full scope of the threat of invasive alien species and to deal with it effectively. Insects represent the majority of living organisms and, hence, form a large part of the alien species problem. In many regions, such as North America, Australasia and South Africa, exotic insect pests are considered as important as native pests, if not more so (Pimentel, 2002). Traditionally, problems have been less severe in Europe (Niemelä & Mattson, 1996). However, in recent years, several pests of economic importance have invaded Europe, inducing more interest in the issue of alien insects. For example, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), the Asian chestnut gall wasp, is a major pest of chestnuts, *Castanea* spp. (Fagaceae) (Zhang *et al.*, 2009), or even *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), that is causing extensive economic damage to small fruits (Cini *et al.*, 2012; Rota-Stabelli *et al.*, 2013; CABI, 2014; Asplen *et al.*, 2015). As for the control, in addition to the use of insecticides, that are relatively easy to use and have generally provided safe and effective pest control, biological control may represent one alternative to the use of insecticides. Biological control is the conscious use of living beneficial organisms, called natural enemies, for the control of pests. Virtually all pests have natural enemies and appropriate management of natural enemies can effectively control many pests. Although biological control will not control all pests all of the time, it should be the foundation of an approach called integrated pest management (IPM), which combines a variety of pest control methods. Biological control can be effective, economical, and safe, and it should be more widely used than it is today (Mahr *et al*., 2008).

1.2 *Drosophila suzukii* Matsumura

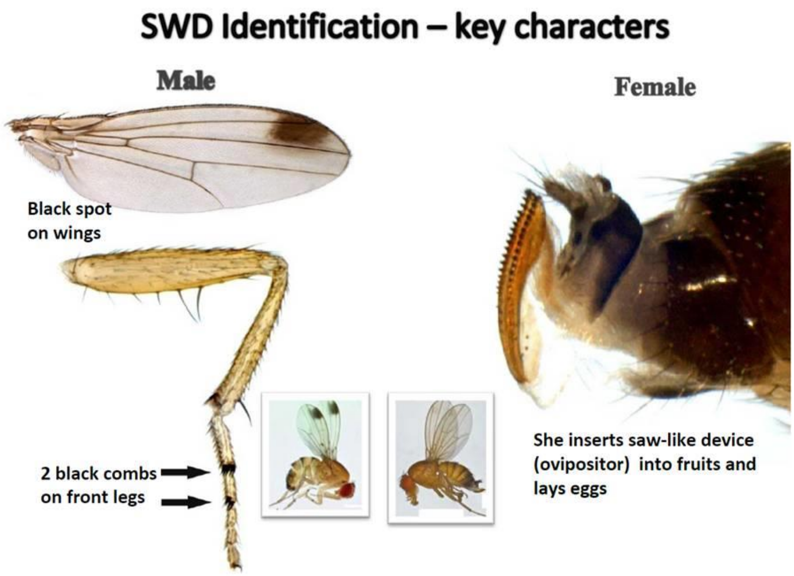

Fig. 1 - Key characters for the identification of *D. suzukii*. Photo: Oregon State University.

D. suzukii is commonly known as the spotted-wing drosophila (SWD). SWD originates from Southeast Asia, is a recently introduced pest in the continental United States, Canada and Europe that has caused significant damage to cherries and other soft-skin fruits (Hauser, 2011). It is thought to have originally had ancestors with short, weakly sclerotized ovipositors (typical of the *melanogaster* species), which later evolved to have the long, strongly sclerotized ovipositors, characteristic of *D. suzukii* (Kimura & Anfora, 2011). SWD adults are drosophilid flies (2-3 mm long) with red eyes, a pale brown or yellowish brown thorax and black stripes on the abdomen. Sexual dimorphism is evident: males display a dark spot on the leading top edge of each wing and females possess a large serrated ovipositor (Kanzawa, 1939; Walsh *et al*., 2011; CABI, 2014). Despite these evident features, the identification of SWD presents several challenges: adults can be easily misidentified, as it occurred for example in California, where it was initially erroneously identified as *Drosophila biarmipes* Malloch (Hauser, 2011; Cini *et al*., 2012). *D. suzukii* differs from *Drosophila melanogaster* Meigen in that the adult males have a black spot near the tip of their wings (which may be faded or completely absent if the insect is too young), and females have an ovipositor with dark sclerotized teeth (Hauser, 2011). The males also have two sets of black combs on their front legs, one on the first tarsal segment and one on the second, which appear as bands (Hauser, 2011; Cini *et al*., 2012). The distinguishing features of the two sexes (serrated ovipositor and black wing spots) are present in other *Drosophila* species, thus making species identification difficult in areas where they are sympatric. For example, *Drosophila subpulchrella* Takamori *et* Watabe males' black spots are very similar in shape and position to those of SWD (Takamori *et al*., 2006). Instead, other characteristics, such as the sex combs on the foretarsi may guide to identification (Cini *et al*., 2012). Other species within the *Drosophila* genus have smaller, less sclerotized ovipositors and tend to lay their eggs on rotting plant matter or overripe fruits and vegetables (Fellows & Heed, 1972; Jaenike, 1983; Markow & O'Grady, 2005). Within the family Drosophilidae, *Zapronius indianus* Gupta is another direct pest of a variety of fruits including figs and citrus, but are easily distinguishable from *D. suzukii* due to distinctive dorsal white stripes that extend from the head to the tip of the thorax (Steck, 2005). The eggs are milky white and oblong, 0.5-0.7 mm in length, about 0.2 mm in width with two filaments at one end. When the egg is laid under the skin of the fruit, all that is visible from the exterior is the two 0.4 mm filaments projecting from the oviposition scar (Kanzawa, 1935). The larvae are typical of the *Drosophila* genus; they grow from about 0.6 mm in length when first emerged from the egg and develop through three instars to 5.5 mm in length and 0.8 mm in width. The larval body is translucent white with distinguishable yellowish entrails. The black mouthparts are visible in the head. Two tan respiratory organs protrude from the posterior end and curve upwards. The larvae live inside the host fruit and feed on the fruit and the microorganisms present as the fruit deteriorates

(Kanzawa, 1935). When the larva is ready to pupate it may leave the host fruit, but pupation inside the fruit is more common (Walsh *et al*., 2011). The resulting pupa is brown and oblong, with respiratory organs on the anterior and posterior sides. The posterior respiratory organs are similar to the posterior respiratory organs of the larva. The anterior respiratory organs are visible as two protrusions, from either side of the head, with a whorl of 7-8 spikes around the termination of the spiracle (Kanzawa, 1935). This pest species has the potential to cause severe economic damage to fruit crops, because it has a wide host range and fast generation time (Kanzawa, 1939). The fruit is damaged as the three larval instars develop inside the fruit (Kanzawa, 1939), and the oviposition scar allows entrance by secondary pests, fungal and bacterial pathogens leading to decay of unripe fruits (Walsh *et al*., 2011; Hamby *et al*., 2012). The host range of SWD is widely varied and includes both crop and non-crop hosts. Kanzawa (1935) reported that SWD can infest cherries, grapes, gooseberries, raspberries, peaches, plums, persimmons, tomatoes, olives, mulberries, and loquats. Infestation levels in intact ripe fruit were compared to the levels in fruit left to shrivel or rot after harvest date, which indicated higher infestation levels in ripe fruit rather than overripe or rotting fruit (Kanzawa, 1935). In recent studies of susceptibility of some fruits at varying stages, oviposition began with fruit coloration and increased as the fruit ripened to maturity. Indeed, fruits susceptibility was shown to increase along the fruit ripening as a function of the balance between sugar and acidity. However, the severity of infestation depends mostly on the penetration resistance of the fruit skin (Lee *et al*., 2011a; Burrack *et al*., 2013; Ioriatti *et al*., 2015).

Fig 2 - Blueberry and cherries damaged by *D. suzukii*. Photos: Regional Pest Alert (Oregon State University).

The mobility of the fly and the wide range of hosts allow SWD to fully utilize a landscape of crop and wildland hosts that ripen throughout the year (Lee *et al*., 2011a). Important are the economic damage worldwide (Fig. 3) and they are estimated at more than 2 billion dollars in America, over 4 billion in Europe and 500 million in Asia (Liburd, 2015).

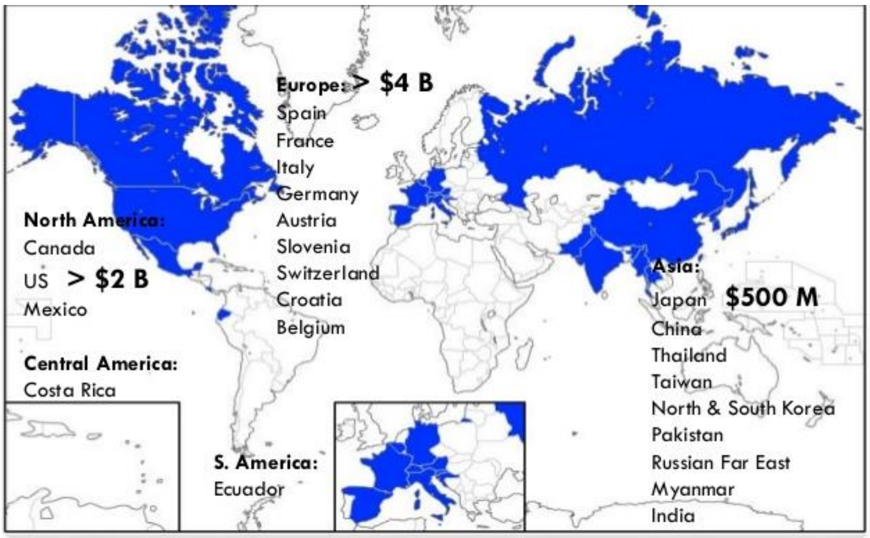

Fig. 3 - Economic damage by *D. suzukii* worldwide (Liburd, 2015).

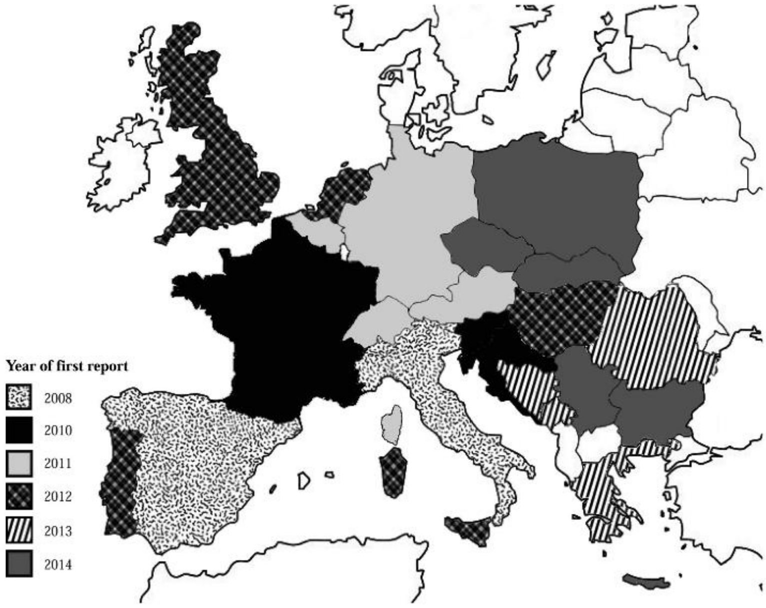

Fig. 4 - Current European SWD distibution map (as of May 2015). Countries are indicated accordingly to the years of the first SWD report (Asplen *et al*., 2015).

1.3 Management

Insecticides are the primary means of controlling and managing *D. suzukii*. Although an economic threshold for the insect has yet to be developed, it is recommended that upon first capture of the insect, treatments should begin for all susceptible crops in an area (Burgess, 2013). Organophosphate, pyrethroid and spinosyn insecticides have proven partly effective against *D. suzukii*, however these broad-spectrum compounds also kill beneficial insects, and application at the ripening stage may increase residues in the fruit (Burrack *et al*., 2012; Cini *et al*., 2012). Insecticide-alternative management strategies are also currently being investigated. Biological controls in the form of predators, parasitic wasps, symbiotic microorganisms in the form of *Wolbachia* bacteria are under investigation for the control of *D. suzukii* (Chabert *et al*., 2012; Siozios *et al*., 2013; Miller *et al*., 2015; Rossi Stacconi *et al*., 2015; Woltz *et al*., 2015). Changes in cultural practice, crushing or cold treatment of contaminated fruits, pesticide rotation to minimize pesticide resistance, installment of physical barriers such as fine netting, landscape management, and better sanitation practices such as removal of dropped fruit may also help control the insect (Dreves & Langellotto-Rhodaback, 2011; Lee *et al.*, 2011a; Cini *et al.*, 2012). However, due to the lack of specific insecticides against *D. suzukii* larvae within fruits, research has been focused on treatments based on chemicals targeting adults. Kanzawa (1939) found that camphor oil was the most effective of the treatments he employed, followed by nicotine sulphate, kerosene emulsion, and neoton but no treatment totally prevented oviposition and most of these materials are no longer used or acceptable. Recent laboratory and field studies both in the USA and in EU revealed that among the registered insecticides, organophosphates, timely applications of pyrethrins and spinosyns can provide good contact activity and residual impact for up to 12-14 days (Beers *et al*., 2011; Bruck *et al*., 2011; Profaizer *et al*., 2012). In contrast, the efficacy of the neonicotinoids as adulticides was not satisfactory (Bruck *et al*., 2011). Initial trials in the Province of Trento indicated that only lambda-cyhalothrin provided an adequate level of control. However, at high population densities repeated applications by alternating pyrethrins and spinosad in strawberry plantations only reduced the damage immediately after the treatment and had negligible impact at the end of the harvest time (Grassi *et al*., 2012). Therefore, future research needs to address not only identification of effective chemicals, but must also consider how protocols for delivery of chemicals can be optimized. In addition, growers should undertake a pesticide rotation, in order to avoid or at least delay the evolution of insecticide resistance, which can be easy in *Drosophila* species (even associated with a single resistance allele, see Ffrench-Constant & Roush, 1991) also thanks to the numerous generations per year. As mentioned before, there are multiple potential biocontrol agents (fungi, bacteria, viruses and other natural enemies of the pest, such as predators and parasitoids) that could be employed in IPM for *D. suzukii*. Biocontrol activity of microorganisms: recently, DNA viruses have been isolated also in *Drosophila* species (Unckless, 2011) and were found to be related to other viruses used for pest control. These findings open the way for the evaluation of SWD control based on viral pathogens and research is urgently needed on this subject. Research on arthropods as biocontrol agents is ongoing, although a control approach based on arthropod natural enemies would

probably be very difficult for a high-reproduction species like SWD. Nevertheless, several valuable studies on this topic have been performed in Japan, in the native range of the species. Potentially, results from these studies could help identify novel management strategies based on introduction and permanent establishment of natural enemies of SWD from its native range for a long-term control (Cini *et al.*, 2012). Nevertheless, another approach for the biological control of SWD would be to enhance the effect of beneficial organisms already present in the newly invaded areas, generalist and widespread species having *D. suzukii* in their host range or, otherwise, species able to adapt their selection strategies to the invader. The most promising candidates found in Europe so far are *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae), *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) and *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae) (Rossi Stacconi *et al.*, 2013, 2015; Gabarra *et al.*, 2015; Miller *et al.*, 2015).
However, in any IPM system, it is important to use multiple strategies to manage key pests. For example, in the Province of Trento, Northern Italy, it has been assessed that before the adoption of an IPM strategy, the potential revenue losses by *D. suzukii* were about 13% of the industry's output, while after the implementation of an IPM strategy including mass trapping, field sanitation and insecticide programs, the sum of losses and associated control costs decreased to about 7% of industry's output (De Ros *et al.*, 2015).

1.4 Volatile attraction

An insect's environment is made up of not only the tactile landscape that it encounters, but largely of chemical signals given off by other insects, plants, and animals. Volatile chemical cues indicate the presence of mates, food, oviposition sites, danger and other important factors in the surroundings. Carey and Carlson (2011) reviewed in detail the mechanisms by which odours are sensed, how insect behaviour is altered by odour, and how olfaction is utilized in insect management. Insects sense chemical cues with their antennae, which are covered by sensory hairs called sensilla (Shanbhag *et al.*, 1999). These sensilla respond to different stimuli by producing an electrical signal that is sent to the mushroom body and lateral horn regions of the insect's brain. The mushroom body is responsible for olfactory learning and memory, and the lateral horn is responsible for innate olfactory behaviours (Masse *et al.*, 2009). Depending on the species and chemical cues to be detected by the antennae, the number of sensilla can range from about 400 in *D. melanogaster* to more than 100,000 in *Manduca sexta* (L.) (Sanes & Hildebrand, 1976; Shanbhag *et al.*, 1999). There can also be sexual dimorphism in the number of sensilla arising due to the differences in necessity of host- or mate-seeking (Zwiebel & Takken, 2004). Along with the olfactory system that insects possess, they also have a gustatory system for sensing chemical cues that they come in contact with. In *Drosophila*, this system is comprised of the two labial palps covered with sensilla on the proboscis, taste pegs in the pharynx that make contact with food as it passes, and 16 taste bristles along the legs and the anterior margin of the wings (Amrein & Thorne, 2005). The sensilla of the gustatory system function similarly to those of the olfaction system, sending electrical responses to the fly's brain when detectable compounds are encountered (Stocker, 1994). The two chemosensory systems do not always work together to influence the behaviour of the insect. *D. melanogaster* females have been shown to have an egg-laying preference for substrates containing acetic acid that is mediated by the gustatory system in conjunction with a positional avoidance of substrates containing acetic acid that is driven by the olfaction of the fly (Joseph *et al.*, 2009). Insect behaviour is driven by taking in chemical cues present in the air or on the substrate it is in contact with and responding to those signals. The first step in determining the behavioural response of an insect to a chemical cue is determining if the compound is biologically active to the insect. Since the sensilla respond to chemicals with an electrical signal, that signal can be measured using an electroantennogram (EAG) (Mayer *et al.*, 1984). An insect is immobilized and electrodes are attached to an antenna, one at the severed tip of the antenna and another at the base of the antenna or into the base of the decapitated head. The antenna is exposed to various odours and an electrical response corresponds to the insect's detection of a compound (Arn *et al.*, 1975). Compounds can be presented singly or in series from a mixture of compounds that has been separated by gas chromatography (GC-EAD) (Struble & Arn, 1984). This techniques elucidate which compounds the insect can detect, but not the behavioural response they will elicit. Therefore, further testing of the EAG-active compounds is required to determine if it has an attractive, deterrent, or no effect. Plant-insect interactions are mediated by semiochemicals that can have many different effects on insect behaviour. Kairomones are chemical cues released from the plant that changes insect behaviour with no benefit to the plant. Three types of kairomones have been described: attractants (which draw the insect to the plant), arrestants (that slow down or stop the movement of the insect) and excitants (that cue the insect to feed or oviposit) (Metcalf & Metcalf, 1992). Leaf volatiles act as attractants, dispersing through air to attract insects from long-range. Once the insect is near or in contact with the attractive plant, close range volatiles elicit an arrestant or excitant effect to stop movement and induce feeding or oviposition. Fruit flies of the family Tephritidae use fruit odours to find suitable hosts for oviposition; females seek out ripe fruit and use their ovipositor to create a cavity where between four and ten eggs are laid (Ioannou *et al.*, 2012). Flies are lured to the plant by the fermentation volatiles, only to become unwilling pollinators. For communication between insects of the same species, pheromones are produced by individuals to elicit a response from another individual of the same species. Female-produced sex pheromones disperse very far, and the males of the species can detect tiny amounts of the pheromone and are directed toward the point source by following the concentration gradient (Cardé & Knols, 2000). Long distance male-produced pheromones are less common, usually attracting both females and males, and are referred to as aggregation pheromones (Landolt, 1997). These aggregation pheromones signal that the male is seeking a mate and also tend to identify sites that are appropriate for feeding or oviposition. *Drosophila* species have been shown to release aggregation pheromones to increase the density of oviposition at a site, which increases larval survival (Wertheim *et al.*, 2002). Along with volatile pheromones, species of the *D. melanogaster* subgroup utilize contact and close range pheromones, which are expressed on the insect’s cuticle (Cobb & Jallon, 1990). Female produced pheromones orient males to females and induce male courtship behaviours such as touching and wing vibrations (Shorey & Bartell, 1970). The *D. melanogaster* male produced pheromone 11-*cis*-vaccenyl acetate promotes aggressive behaviour between males and is transferred to females during mating to deter multiple mating with a mated female (Wang & Anderson, 2010). Interestingly, most of the species of the *Drosophila* subgroup share the same aggregation pheromone, 11-*cis*-vaccenyl acetate, while in SWD, which has the peculiar ecology to rely to fresh unwounded fruits for oviposition, it is lacking and the epicuticular hydrocarbon profile is isomorphic between sexes. However, SWD is still able to perceive it, likely providing a signal of a fermenting substrate already occupied by other *Drosophila* species, not attractive for mating and laying eggs but only for feeding purposes (Dekker *et al*., 2015). Another pheromone produced by males, 7-tricosene, increases receptiveness of females to male courtship (Grillet *et al*., 2006).

1.5 Monitoring

Currently, the most common method for monitoring and detecting SWD is through the use of traps constructed from a plastic cup with holes punched around the exterior, containing apple cider vinegar, a surfactant and a yellow sticky card (Burrack *et al*., 2012; Burgess, 2013). However, by the time this trap detects SWD, the insect has already established itself, populations are typically very high, and the insect has already done its damage (Bolda *et al*., 2010). Such traps are ineffective in early detection of SWD because they are designed to catch other pests and Drosophilidae in general (Cini *et al*., 2012). These traps utilize a vinegar-based bait which is appropriate for catching most Drosophilidae as the majority perform all aspects of their life cycle infermenting fruit (Cini *et al*., 2012). SWD however, uses fermenting fruit to feed, but also follows cues of ripening fruit when searching for oviposition sites (Cini *et al*., 2012; Dekker *et al*., 2015). If a trap more specific to SWD could be developed, which would include odorants found in ripening fruit, SWD could be detected earlier, and action could take place in attempts to control this insect dispersal and its population levels (Cini *et al*., 2012; Revadi *et al*., 2015). Mass trapping of pests has been used routinely on more than 10 million hectares of commercial crops around the world, predominantly against Lepidoptera, Coleoptera, Diptera and Hemiptera (Witzgall *et al*., 2010). A variety of lures are used to attract them, including food, colour, kairomones and pheromones, either alone or in combination (Howse, 1998). Insects respond to various stimuli that can be used alone or in combination to attract them: thermostimuli, photostimuli, mechanostimuli, and chemostimuli (Dethier, 1947). Research into trapping of *Drosophila* tends to be in manipulating color (Hottel, 2011) and odour (Hutner *et al*., 1937; Cha *et al*., 2012). Pheromones, plant-based kairomones and combinations of the two are used as attractants in traps for monitoring and mass-trapping purposes. Pheromone lures mimic female calling signals and are widely used in the trapping of moths, beetles, and some Hymenoptera species (Dethier, 1947). Kairomone attractants are used to draw insects to plant odours that signal food or oviposition site rather than the direct signal of a mate (Metcalf & Metcalf, 1992). In some cases, the combination of pheromone and kairomone is needed to achieve adequate attraction (Landolt & Phillips, 1997) or a combination of a food odour and oviposition host kairomone is most attractive (Landolt *et al*., 2012). Attractants are also used for the direct control of pests in similar programs of mass trapping and attract and kill by luring insects to either a trap where they are contained or a surface from which they can feed with an insecticide applied to it (El Sayed *et al*., 2006). The key objective of mass trapping is to capture the maximum number of insects before they reproduce or cause damage to crops in the specific control area (El Sayed *et al*., 2006; Suckling *et al*., 2015). Effective trapping requires the use of lures that are able to attract fruit flies more effectively than natural food sources, such as calling virgin females, mating aggregations or food sources, including efficient traps or stations or formulations for killing the attracted insects and using lures and non-saturating traps that are effective during the entire period of adult emergence and mating (Suckling *et al*., 2015). Consequently, traps need to be visually attractive and capable of capturing and retaining flies long enough to deliver a lethal dose of toxicant or otherwise prevent their escape, e.g., by drowning or starvation (Lasa *et al*., 2014). Evaluations of fruit fly trap designs have

focused on the influence of color, size, and shape on efficiency (Cytrynowicz *et al*., 1982; Economopoulos, 1989; Sivinski, 1990; Robacker, 1992; Lopez Guillen *et al*., 2009). However, other specific features related to the accessibility of the trap entrance and retention of captured flies have attracted less attention, despite the role played by these features in trap efficacy. The ability of a trap to retain flies is likely to be influenced by the bait and the retention system used. Wet traps use a liquid bait to retain flies that drown inside the trap, whereas dry traps retain flies using adherents or chemical insecticides (Lasa *et al*., 2014). As such, the overall efficacy of the trap involves a complex interaction between trap design, lure combination, and retention method (Robacker & Czokajlo, 2005; Diaz-Fleischer *et al*., 2009). The material and labour costs of trapping or killing may be lower than the costs of other treatments. Mass trapping has been successfully used to manage even such ubiquitous pests as blow flies, while the identification of new attractants can present opportunities for developing more environmentally benign pest management tactics (Suckling *et al*., 2015).
Mass trapping therefore can be a useful stepping stone towards a cost-effective control system, as it is possible to determine the number of flies that need to be removed in order to achieve a reduction in damage (Suckling *et al*., 2015).
However, in order to obtain effective mass trapping strategies, highly powerful and selective attractive baits are required. In *D. suzukii*, preliminary experimental observations confirm that this insect is attracted, in terms of nutritional sources, by wounded and presumably fermenting fruits, as suggested by the attraction exerted by a variety of fermented liquids, including wine, vinegar, and liquid derivatives of fruits (Kanzawa, 1935; Cha *et al.*, 2012, 2013, 2014; Landolt *et al.*, 2012) and by the association, recently reported, of this insect with a community of yeast species basically formed of *Hanseniaspora uvarum* (Niehaus) Shehata *et al*. (Hamby *et al*., 2012). Indeed, at present, the most effective traps for capturing *D. suzukii* are those baited with different types of vinegar and wine (Landolt *et al.*, 2012). Both these substrates result from fermentation, mediated both by yeast and by acetic bacteria.

According to this knowledge, traps are marketed containing wine vinegar, apple vinegar, wine, fruit juices (Asplen *et al.*, 2015). In order to warrant stability and marketability, such matrices are sterilized via pasteurization during the production step. One of these commercial liquid baits, the so-called Droskidrink, showed preliminary promising results in both monitoring SWD during the period 2011-2013 in the province of Trento and in first mass trapping trials (G. Anfora, personal communication). In summary, the traps known so far are not sufficiently active against SWD and feature a short duration in time.

Mass trapping or monitoring can also be useful for IPM program. Pest control can be taken only when the economic threshold is surpassed by pests, not by looking at calendar or plant physiology. Applying insecticides when needed reduces pesticide residues in the crop and saves time and money by limiting applications, decreases the chance of secondary pest infestations, reduces the effect of the pesticide on the environment and promotes human health and safety (Kogan, 1998).

1.6 The importance of trap design

Trap structure and design can also influence the ability of a trap to attract and retain insects. Trap color, contrast (such as trap striping), size of entry holes, as well as trap shape, may influence the attractiveness of a trap to SWD (Cini *et al.*, 2012; Basoalto *et al.*, 2013). Trap colour is important, as drosophilids have color vision and often use it to discriminate between different host and food options (Basoalto *et al.*, 2013). The colors red and black appear to be most effective in attracting SWD thus far, as these colors mimic ripe fruit (Basoalto *et al.*, 2013). Striping can also improve traps, as traps with a black stripe surrounded by red have been shown to significantly enhance trap captures over all-red or all black-traps (Basoalto *et al.*, 2013). It has also been suggested that SWD may prefer traps with mesh openings, which have increased entry areas (Basoalto *et al.*, 2013). Trap designs are frequently built upon plastic cups, however other traps which have been tested involve tents, or have taken the form of a dome (a.k.a. McPhail or

MultiLure® traps) (Leblanc *et al.*, 2010; Landolt *et al.*, 2012; Lee *et al.*, 2012; Basoalto *et al.*, 2013). The MultiLure® trap (designed by Better World Manufacturing, Fresno, California), is yellow on the bottom third and clear on the top two thirds with a large hole for insect entry (a.k.a. modified McPhail trap) and has been used and found to attract SWD (Leblanc *et al.*, 2010; Landolt *et al.*, 2012).

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2. Objectives

2.1 Main goal of the thesis

Based on the background described in the previous sections, the main goal of my thesis was to elucidate unknown processes of *D. suzukii* perception and recognition of attractive substrates in order to develop innovative and highly effective baits with the potential to implement in both monitoring and control strategies for this pest. In particular our studies were focused at characterizing either the physical, chemical and biological parameters or the efficacy in field conditions of Droskidrink, the commercial SWD food bait recommended in Trentino region, Italy. Such knowledge has led to develop a new concept of trap, in which the attractiveness of Droskidrink would be strongly increased by the combination of microorganisms releasing biologically active volatiles to SWD. The long term perspective is to accelerate research and technology transfer towards new environmentally friendly pest control methods based on the use of traps baited with this new lure, such as mass trapping and attract and kill.

2.2 Specific aims and list of main achievements and related publications

1) Currently there is little information on the chemical ecology of *D. suzukii*. However, SWD adults are attracted to and probably feed on damaged and presumably fermented fruits, as suggested by attraction to a variety of types of fermented sweet foods, such as wine and vinegar. This led to the development of different commercial food baits, like the liquid bait Droskidrink empirically set-up in Trentino.We have compared its attractiveness and control efficacy with that of other available food baits for SWD. The results obtained in this preliminary part of the work shall justify the necessity to investigate further chemical and microbiological characteristics of Droskidrink in relation to the mechanisms of the SWD adult attraction, in order to produce baits more effective.

- Grassi A., Anfora G., Maistri S., **Maddalena G.**, De Cristofaro A., Savini G., Ioriatti C., 2015. Development and efficacy of Droskidrink, a food bait for trapping *Drosophila suzukii*. IOBC Bulletin, 109: 197-204.

2) Some volatiles derived from the fermentation of carbonaceous compounds are present both in fermenting liquids attractive to *D. melanogaster* (McKenzie & Parsons, 1972; Becher *et al*., 2010, 2012; Barata *et al*., 2012) and SWD baits (Cha *et al*., 2012, 2013, 2014; Landolt *et al*., 2012). Among these volatiles, acetyl methyl carbinol, butyrate, and 2-phenyl ethanol, methionol, isoamyl lactate and dietyl succinate are the most frequently detected in the headspace of SWD baits, even though in low quantity. All these compounds are released in high amounts from the fermentation of sugars by yeasts and/or lactic bacteria (Nielsen & Richelieu, 1999; Cordente *et al*., 2012). Therefore, in this second step of the thesis we have focused our attention on the interactions between SWD and lactic bacteria, which are poorly studied with respect to yeasts. Furthermore, lactic acid bacteria do not appear in the microbiota usually isolated inside traps. Therefore, we aimed at developing a new trap for catching SWD characterized by the addition of viable culture of different species and strains of lactic acid bacteria as bio-catalyzers of the production of the mentioned biologically-active volatiles.

- Guzzon R., **Maddalena G.**, De Cristofaro A., Grassi A., Ioriatti C., Anfora G., 2016. Exploitation of lactic acid bacteria for the improvement of a bait for *Drosophila suzukii* trapping. Submitted to Journal of Applied Entomology.

3) Based on the outcomes collected in stages 1 and 2, I was included in the list of the inventors at Fondazione Edmund Mach submitting a patent application to the Italian Patent and Trademark Office on 06/11/2014. The application has been extended to the International Patent System under the Patent Cooperation Treaty (PCT) on 05/11/2015. The invention is regarding the use of lactic bacteria belonging to the *O. oeni* species for preparing a bait for use in traps aimed at monitoring and controlling *D. suzukii*. The main purpose of this patent proposal was to protect this innovative product from unwanted copying. Such a patent has been an incentive for further research and will have an important role in technology transfer and in future cooperations with industries.

- **Maddalena G.**, Guzzon R., Grassi A., Ioriatti C., Anfora G., 2014-2015. Use of an active culture of lactic bacteria for preparing a bait aimed at monitoring and controlling *Drosophila suzukii*. Classificazione internazionale dei brevetti (IPC): INV. A01N37/02 A01N65/00.

4) The last part of the research has been carried out at Oregon State University, US, where I have been host in the laboratory of Entomology coordinated by Prof. Vaughn M. Walton at the Department of Horticulture. During step 2 and 3 of the thesis, the lactic bacterium *O. oeni* has been selected in both laboratory and field trials as the most active to *D. suzukii*. Therefore, our main goal in the last year of the study was to evaluate the attractiveness of different *O. oeni* strains and to develop a specific trap-bait combination. This will lead to the adoption of a new type of bait trap resulting in better trapping before fruit appears in the field. This may lead to the early detection and reduction in populations of SWD.

- **Maddalena G.,** Dalton D., Guzzon R., Mazzoni V., Anfora G., Ioriatti C., De Cristofaro A., Walton V.M., 2016. Combined effect of trap design and Droskidrink mixture improved with different strains of *Oenococcus oeni* bacteria for early detection of *Drosophila suzukii*. To be submitted.

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Cha D.H., Adams T., Werle C.T., Sampson B.J., Adamczyk Jr J.J., Rogg H., Landolt P.J., 2014. A four-component synthetic attractant for *Drosophila suzukii* (Diptera: Drosophilidae) isolated from fermented bait headspace. Pest Manag. Sci., 70: 324-331.

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3. Development and efficacy of Droskidrink, a food bait for trapping *Drosophila suzukii* (Chapter 1)

3.1 Introduction

Drosophila suzukii (Diptera: Drosophilidae), the spotted-wing drosophila (SWD), native of South East Asia, is a pest of fresh fruits since it is one of the few Drosophilid with serrated ovipositor, which enables it to oviposit in unwounded fresh fruits thereby making them unmarketable. SWD is highly polyphagous and, at present, infests various soft skinned fruits including cherry, blueberry, blackberry, strawberry, raspberry, apricot and grapes (Cini *et al*., 2012). Recently, SWD invaded western countries and is now a threat to both European and American fruit industry (CABI, 2014). A highly attractive lure is an important part of IPM strategies. Currently there is little information on the chemical ecology of SWD. However, they are attracted to and probably feed on damaged and presumably fermented fruits, as suggested by attraction to a variety of types of fermented sweet foods, such as wine and vinegar and syntethic volatiles identified from such substrates (Kanzawa, 1935; Landolt *et al*., 2012; Cha *et al*., 2012, 2013, 2014). However, initial trap designs for monitoring SWD utilized apple cider vinegar, grape wine, yeasts and sugar water mixtures, or a vinegar/wine mixture as bait. We report here the results of several field experiments (2011-2014) that led to the development of Droskidrink, the food bait recommended in Trentino and that compared its attractiveness and control efficacy with that of other available food baits for SWD.

3.2 Material and methods

3.2.1 Perimeter mass trapping - 2011

The main goal of this trial was to evaluate the efficacy of a first version of Droskidrink bait used in a mass trapping technique against *D. suzukii*. Originally, the bait was a mixture of 75% apple cider vinegar (Azienda Agricola Prantil, Priò, Trento, Italy) and 25% red wine (Merlot, Conad supermarket). White plastic 1000 mL flasks (Kartell S.p.a., Noviglio, Italy) with 6 holes of 5 mm diameter, were used as trap, loaded with 200 mL of the bait mixture.

The experimental field was a small highbush blueberry plot (cv. Elliott) of about 550 square meters, located in Canezza (Trento, Italy). The traps (42 bottles) were positioned around the perimeter of the plot on July 12th (week 28), in correspondence of the fruit reddening phenological phase. Traps were hung directly on the bushes at about 60-80 cm from the soil level and about 2 meters apart.

Traps were serviced weekly. The bait was replaced and the adults captured were sexed and counted under dissecting microscope. The fruit infestation (fruits with eggs or larvae) was also weekly assessed during the whole harvest period on 100 commercially ripe fruits sampled either from the borders or from the inner part of the plantation. Fruits were inspected one by one under a dissecting microscope.

3.2.2 Attractiveness of attract-and-kill baits - 2011

In August 2011 we carried out a field survey in order to assess the efficacy of the Droskidrink used as an attract-andkill bait with comparison with other baits (Table 1). The trial was carried out in a highly SWD infested highbush blueberry plantation (cv. Elliott) of about 1100 square meters, rain protected with plastic tarpaulin, located in Samone (Trento, Italy). Either Droskidrink or other attractants were baited on both the sides of plastic yellow sticky panels (10 x 20 cm) using a toothbrush at about 09:00 am of the 8th of August 2011. The panels were hung vertically on the bushes at about 60 cm from the ground level. The adults caught on the panels were sexed and counted directly in the field using a hand magnifier (10x), then removed. Inspections were made at different intervals after the panel positioning. Each treatment was replicated 3 times in a randomized block design. A sticky panel without bait was used as a control. Baits composition is shown in Table 1.

For each time interval data on *D. suzukii* captures for each bait type were first $\sqrt{x+0.5}$-transformed in order to meet the assumption of homogeneous variances and then compared using ANOVA followed by Tukey’s *post hoc* test to separate the means.

Bait A	0.03 g USA bait (from Oregon State University)+0.03 g brewer’s yeast+25 mL water
Bait B	4 times concentrated bait A
Bait C	12.5 mL Spintor Fly (Dow Agroscience, 0.024% spinosad)+25 mL water+3 g unrefined brown sugarcane+25 mL apple cider vinegar
Bait D	0.5 mL Laser (Dow Agroscience, spinosad 480 g/lt)+3 g unrefined brown sugarcane +25 mL apple cider vinegar+25 mL water
Bait E	12.5 mL Spintor Fly (Dow Agroscience, 0.024% spinosad)+25 mL water+3 g unrefined brown sugarcane+18 mL apple cider vinegar+7 mL red wine

Table 1. Composition of the mixtures used in August 2011 for the evaluation of attractiveness as attract-and-kill baits.

During the period 2011-2012 results from several trials showed that red bottle traps generally performed better than clear bottles in catching SWD when baited with apple cider vinegar (results not shown). Therefore, during the following seasons (2012-2014), Droskidrink added with unrefined sugarcane and its combination with red bottles, was adopted as a tool for the monitoring and control efforts in Trentino.

3.2.3 Control efficacy of attract-and-kill baits - 2013

In 2013, an open field trial was carried out with the aim to assess the efficacy of daily sprays of an attract-and-kill bait formulated using 80% Droskidrink (produced by Azienda Agricola Prantil, Priò, Trento, Italy – 75% apple cider vinegar and 25% red wine), 20 g/liter of unrefined brown sugar and 20% Spintor Fly (Dow Agroscience – 0.024% spinosad). The treated plot was a small autumn-fruiting raspberry plantation (cv. Heritage) of about 350 square meters, rain protected, located in Canezza (Trento, Italy). Another raspberry plantation of cv. Erika, was selected in the same location, at about 350 m from the treated plot, and was used as a control plot.

No insecticides were applied for *D. suzukii* control in both the orchards. The bait was sprayed by means of a 1.5 liters hand sprayer, the nozzle was adjusted in order to release quite large droplets (around 3 mm diameter) on the grass at the base of the canes, on their basal leaves and on shrubs and trees around the field. The bait was daily distributed at 07:00-07:30 am, alternating 2m-long sprayed and unsprayed bands along the perimeter and the inner rows of the plantation. The first treatment was carried out at the beginning of the harvest time, and a total of 33 sprayings were made. The efficacy was assessed twice a week, determining the number of fruits infested with eggs or larvae on a sample of 50 commercially ripe raspberries randomly collected from both untreated and treated plots. Fruits were inspected one by one under dissecting microscope.

3.2.4 Mass trapping - 2013

The combination of Droskidrink and red traps was tested in 2013 in a mass trapping trial carried out in a highbush blueberry orchard (cv. Brigitta) of about 1600 square meters, located in Samone, (Trento, Italy). The experimental field was divided in two blocks, a treated and an untreated (control) plot: in this last one, the pest was managed only with insecticides, while in the treated plot insecticides were combined with mass trapping.
Red painted 1000 mL Kartell flasks with 6 holes of 5 mm diameter were used as traps, loaded with 200 mL of Droskidrink and 4 g of unrefined sugarcane. A step by step procedure was used to deploy the traps: at first (week 25), only 5 bottles were positioned on the surrounding wild vegetation. At the beginning of the fruit reddening phenological stage (week 27), 55 traps deployed 2 meters apart were hung at about 1 m from the ground level on the blueberry bushes along the perimeter of the plot and a sentinel trap was deployed in the middle. As the first adult was caught in the sentinel trap, 19 traps 4 meters apart were positioned along the internal rows of the mass trapping plot. The bait in all the traps was weekly renewed till the end of the trial (week 35) and the adults caught were sexed and counted. The efficacy of the technique was evaluated determining the percentage of berries infested with eggs or larvae in a sample of 100 commercially ripe

fruits randomly collected both from the inner and perimeter part of treated and untreated plot. Fruits were inspected one by one under dissecting microscope in laboratory.

3.2.5 Attractiveness of baits in monitoring traps - 2014

During August 2014 a trial was carried out in order to compare the attractiveness of some of the most common and commercially available lures and food baits for *D. suzukii*. The test was conducted in a 2000 square meters surface sweet cherry orchard located in Pergine Valsugana (Trento, Italy). Due to the severe SWD damage, the owner did not harvest most of the fruits that ripened in July. The following treatments were compared:

1) 200 mL of Droskidrink + 4 g unrefined sugarcane + a drop of Triton ™ X-100 (as surfactant agent);
2) 200 mL of Apple Cider Vinegar (Azienda Agricola Prantil, Priò, Trento, Italy) + a drop of Triton ™ X-100 (as surfactant agent);
3) 200 mL of Droskidrink + 4 g unrefined sugarcane + a drop of Triton ™ X-100 (as surfactant agent) + Pherocone® SWD dual lures (Trecè);
4) 200 mL of Apple Cider Vinegar (Azienda Agricola Prantil, Priò, Trento, Italy) + a drop of Triton ™ X-100 (as surfactant agent) + Pherocone® SWD dual lures (Trecè).

The different attractants were tested baiting the same trap, the Droso-trap®, 2013 version (Biobest, Westerlo, Belgium). To improve its selectivity and make easier the adults counting, a grid of mesh (with 3 x 2.5 mm openings) was fixed on the entrance holes. Each trap was filled with 200 mL of the bait. Traps were hung on the cherry trees at about 150-160 cm from the ground level. Trecè dispensers (Pherocone® SWD dual lures) were fixed under the lid of the trap using a scotch tape. Each treatment was replicated 5 times in a completed randomized experimental design. The experiment lasted 3 weeks and traps were serviced at weekly interval. Traps were positioned 2 meters apart: the distance between replications was at least 20

m. The position of the traps in each weekly service was changed with a randomized sequence.

Weekly captures of SWD in the tested baits were compared using ANOVA followed by Tukey's post hoc test to separate the means.

3.3 Results and discussion

3.3.1 Perimeter mass trapping - 2011

In July 2011, a first version of Droskidrink, prepared with only apple cider vinegar and wine, showed a good efficacy in limiting the damage in the inner part of experimental plots treated with the perimeter mass trapping technique as a control method (Table 2). However, a higher damage level was observed at the end of the harvest period, corresponding to an increase in the number of immigrating adults in the plantation.

	SWD adults		% infested fruits		
week	central trap	border traps	from central bushes	from border bushes	
21	*posit.*				
22-27	0				
28	0	*posit.*	0		**H**
29	0	11	0		**A**
30	0	84	0	7	**R**
31	1	156	0.8	5	**V**
32	1	111	0	2.2	**E**
33	0	1048	9	18	**S**
34	0	681	32	34	**T**
35	1	***end***	30		

Table 2. Captures of *D. suzukii* (SWD) adults and percentage of fruit infestation in mass trapping trial on highbush blueberry during 2011.

3.3.2 Attractiveness of attract-and-kill baits - 2011

A further improvement of Droskidrink, obtained by adding unrefined sugarcane, was observed in August 2011, when the food attractant was deployed as a bait in a preliminary experiment aimed at the development of an attract-and-kill technique (Figure 1). Despite all the compared baits showed a very fast drop of attractiveness, the bait prepared with 75% apple cider vinegar, 25% red wine and unrefined sugarcane (bait E) showed the highest attractive efficacy. Indeed, a significantly higher number of catches was recorded for bait E in comparison with control and bait B, while only a tendency to be higher with respect to bait A, C and D for the data corresponding to 20 min interval (ANOVA; $F=4.84$; d.f.=17; $P<0.05$). No significant differences were shown for the other time intervals (8 hours: $F=0.85$; d.f.=17; $P=0.54$ – 24 hours: $F=0.71$; d.f.=17; $P=0.63$ – 48 hours: $F=0.71$; d.f.=17; $P=0.63$).

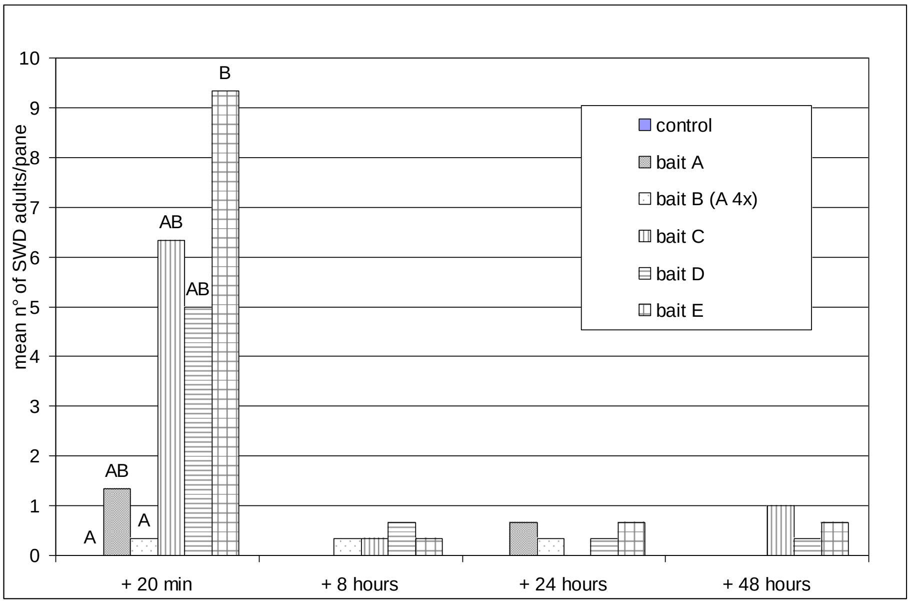

Figure 1. Evaluation of the attractiveness of attract-and-kill baits in a highbush blueberry orchard in 2011. Attractants were baited on both the sides of plastic yellow sticky panels and are described in Table 1. Inspections were made at different intervals after the panel positioning (n=3). Different letters above columns within the same time interval indicate significant differences (ANOVA, Tukey's test).

3.3.3 Control efficacy of attract-and-kill baits - 2013

Promising results were obtained with the complete formulation of Droskidrink as attract-and-kill (Table 3) and mass trapping devices (Figure 2).

date	week n°	% fruits with SWD eggs		
		A&K treated plot	control plot	% efficacy
9/8	32	6.8		
19/8	34	2.9	18.9	85
26/8	35	12.8	50	74
30/8	35	5.4	51	98
2/9	36	8.6	46.6	81
6/9	36	8.3	20.3	59
10/9	37	26.8	34.6	22
13/9	37	10	38.2	74

Table 3. Results of 2013 open field attract-and-kill trial as percentage of fruits with *D. suzukii* (SWD) eggs. The grey table cells indicate the period of the daily bait applications.

During the open field attract-and-kill trial in 2013, a considerable decrease in the control efficacy was recorded at the beginning of week 37, after the grower removed infested wild blackberry fruits from surrounding vegetation. Therefore, it is likely that many SWD adults moved from these external sources to the crop and damaged the cultivated fruits while no wild hosts were yet available.

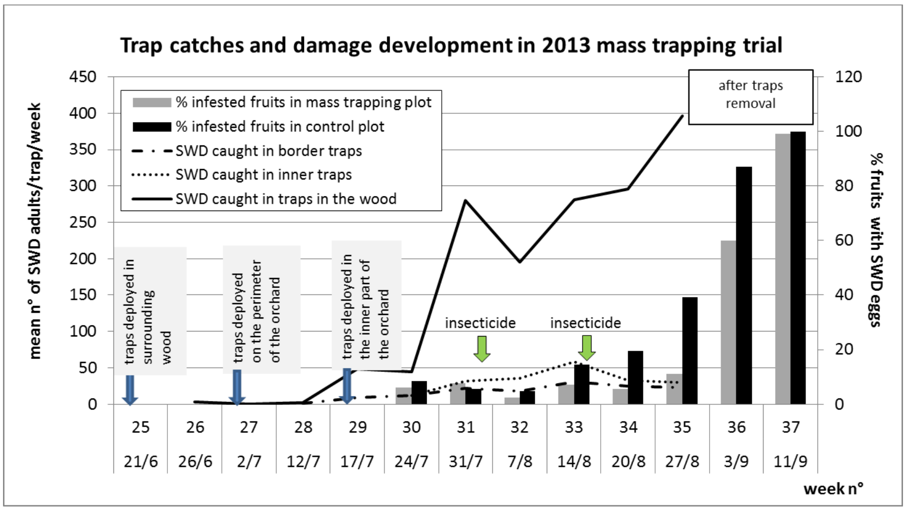

Figure 2. Results of the mass trapping trial carried out in 2013 in a highbush blueberry orchard.

The efficacy of the mass trapping treatment in comparison with the untreated plot (Figure 2) peaked up 72% during the weeks 34 and 35. After the traps removal, at the end of the harvest time, the damage rapidly increased and reached the same levels of the untreated block, confirming the previous buffer effect of the traps. It is also interesting to observe the high number of captures recorded during the trial in the traps deployed in the surrounding wood, indicating the crucial role of this wild environment, very common in adjacency of the soft fruit orchards in Trentino, for the behaviour and development of the pest.

3.3.4 Attractiveness of baits in monitoring traps - 2014

In 2014 comparative field trials, traps baited with only Droskidrink caught more *D. suzukii* adults than both commercially available *D. suzukii* lures and food baits recommended in other fruit growing regions (Figure 3). In particular, Droskidrink was significantly more attractive than ACV and ACV+Pherocone Dual Lure in correspondence of the first trap control (week 31: $F=5.79$; d.f.=19; $P<0.01$), ACV at the second control (week 32: $F=3.95$; d.f.=19; $P<0.05$), and ACV and ACV+Pherocone Dual Lure at the last control (week 33: $F=4.91$; d.f.=19; $P<0.05$), respectively.

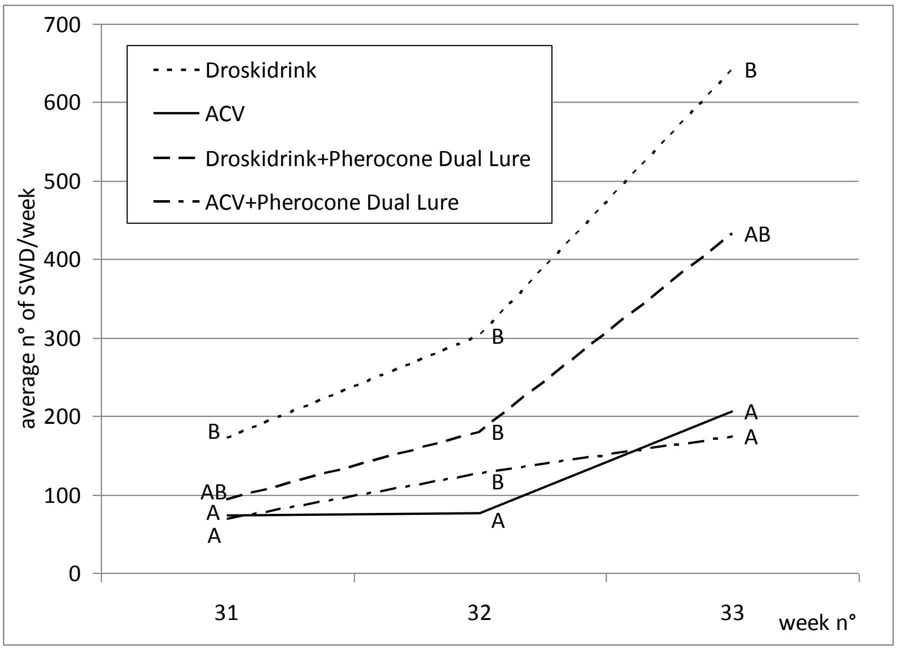

Figure 3. Results of the different baits and lures comparison trial carried out in August 2014 (n=5). For each weekly trap control different letters indicate significant differences (ANOVA, Tukey's test).

3.4 Conclusions

Trials carried out from 2011 to 2014 in Trentino region, demonstrated that Droskidrink is highly effective as food attractant for *D. suzukii* and showed the potentiality to be used both in insect direct control strategies (mass trapping, attract-and-kill) and in monitoring. Hence, results obtained in these trials justify the necessity to investigate further the chemical and microbiological characteristics of Droskidrink in relation to the mechanisms of the *D. suzukii* adult attraction, in order to produce bait even more effective and a delivery device easy to manage.

3.5 References

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4. Exploitation of lactic acid bacteria for the improvement of a bait for *Drosophila suzukii* trapping (Chapter 2)

4.1 Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), the spotted-wing drosophila (SWD), is an insect native of South-Est Asia. This insect has recently invaded both Europe and Americas, and it is causing serious losses in the production of the most relevant soft fruits (Lee *et al*., 2011b; Cini *et al*., 2012; Rota-Stabelli *et al*., 2013; Asplen *et al*., 2015). Eggs and larvae were detected in many valuable cultivated fruits, such as sweet cherry, apricot, blueberry, strawberry, raspberry, blackberry, and wine grape. High reproduction potential and adaptability to many environmental conditions make *D. suzukii* one of the major pests in agriculture of western countries, capable of causing production losses even higher than 90% (Cini *et al*., 2012). The management of SWD was implemented mainly with insecticides, such as pyrethroids, spinosyns, and organophosphates, sanitation and net exclusion (Beers *et al*., 2011, Cini *et al*., 2012; Asplen *et al*., 2015). At present, all these methods often show drawbacks and failures related to the pest population density and to the specific social and agronomical context. Other environmentally safe methods, such as mass trapping or biocontrol with natural enemies are still only matter of investigations (Grassi *et al*., 2015; Miller *et al*., 2015).

Understanding better biology and ecology of SWD is indeed a fundamental prerequisite to set up and/or optimize control techniques based on the interference with the insect behaviour. From this point of view, some useful hints may be obtained by the tremendous knowledge gained with the par excellence model organism, *Drosophila melanogaster* Meigen. With regard to the interactions with microorganisms, Becher *et al*. (2012) observed that the activity of the microbiota present in the oviposition and feeding sites plays a key role for the recognition and orientation of *D. melanogaster*. Moreover, it is proved that this insect improves its fitness when the food substrate is contaminated by yeasts. However, the information available for *D. melanogaster* can only partly help understanding the behaviour of *D.*

suzukii, due to its peculiar ecology. Indeed, SWD is attracted by rotting and fermenting fruits for adult feeding (Cha *et al*., 2013) but has the unique characteristic for the *Drosophila* genus of laying eggs in fresh soft fruits, breaking the skin of ripening and undamaged fruits using its serrated ovipositor (Cini *et al*., 2012; Atallah *et al*., 2014). Regarding the microorganisms involved in the trophic interactions of SWD, some evidence is present about the role of yeasts. As a fact, SWD is attracted for feeding purposes by fermenting substrates, as suggested by the action exerted by wine, vinegar, and fermented fruit juice (Kanzawa, 1935; Cha *et al*., 2012, 2013, 2014; Landolt *et al*., 2012). Also, a community of yeasts composed by *Hanseniaspora uvarum* (Niehaus) Shehata *et al*. and other secondary species was found in some vital organs of this insect (Hamby *et al*., 2012). Further confirmation of the interactions between SWD and yeasts was provided by field tests, where baits inoculated with *Saccharomyces cerevisiae* Meyen ex E.C. Hansen showed higher attraction with respect to the same, but sterile, bait (Knight *et al*., 2013). On the contrary, to date, there is no published evidence about the positive interactions, in the sense of increasing of attractiveness, of bacteria involved in the fermentation of wine or vinegar.

In many insects, a key role in the processes of choice of the oviposition and feeding sites is played by volatile compounds released by the microbiota interacting with the target substrates (Schoonhoven *et al*., 1998; Dicke & van Loon, 2000; Bruce *et al*., 2005; Hilker & McNeil, 2007; Bruce & Pickett, 2011). The behavioural activity driven by this group of volatile compounds can be therefore exploited for the development of control methods for *D. suzukii* based on baits, such as mass trapping and "attract and kill" (Grassi *et al*., 2015). At present, the most effective traps are those baited with vinegar and wine, or by synthetic compounds identified in their headspace (Asplen *et al*., 2015). These latter substances derived from the fermentation, mediated by various microorganisms, of carbonaceous compounds present in such liquids (McKenzie & Parsons, 1972; Becher *et al*., 2010, 2012; Barata *et al*., 2012). However, tests performed comparing the attractiveness of pure ethanol and acetic acid with a mixture of wine and vinegar suggested that other secondary compounds contribute in the attraction of SWD (Cha *et al*., 2013). Among these volatiles, acetyl methyl carbinol (also known as acetoin), butyrate, and 2-phenyl ethanol are frequently detected in the headspace of wine vinegar, while methionol, isoamyl lactate and dietyl succinate were already found in wine (Cha *et al.*, 2013). All these compounds derivate from the fermentation of sugars by yeasts and/or lactic bacteria (Nielsen and Richelieu, 1999; Cordente *et al*., 2012), confirming the importance of these microbes in the mechanisms of recognition of food and oviposition sites in *D. suzukii*. Hence, in this work we have focused our attention on the interactions between SWD and lactic bacteria, which are poorly studied with respect to yeasts (Guzzon *et al*., 2014). These molecules are present only in low concentration in the headspace of *D. suzukii* commercial baits, and lactic acid bacteria do not appear in the microbiota usually isolated inside traps.

Therefore, we aimed at developing a new trap for catching SWD characterized by the addition of viable culture of lactic acid bacteria as bio-catalyzers of the production of the mentioned biologically-active volatiles. Field tests and laboratory experiments have been carried out preliminarily in order to assess the performance of different genera of lactic acid bacteria. Afterwards, we selected three biotypes of *Oenococcus oeni* (Garvie) Dicks *et al*. that revealed a high resistance to the harsh environmental condition of the liquid baits and, at the same time, showed a significant attractive capacity *vs.* SWD. Results are discussed taking into account the practical implications of our findings for *D. suzukii* management.

4.2 Materials and methods

4.2.1 Insect rearing

A *D. suzukii* population collected in Trento Province was reared on a standard *Drosophila* semiartificial diet (*Drosophila* species stock center, https://stockcenter.ucsd.edu/info/ food_cornmeal.php,2013) at the temperature of 23-25°C, relative humidity (R.H.) of 65±5% and 16L:8D photoperiod.

4.2.2 Composition of the Droskidrink (DD) bait for *D. suzukii* trapping

The commercial *D. suzukii* bait chosen for the experiments described in the following sections is called Droskidrink (DD) and is composed by a mixture of 30% of red wine (Tavernello, Caviro, Italy), 70% of apple vinegar (Prantil, Italy) and 5 g/L of brown sugar cane. For details see Grassi *et al*. (2015).

4.2.3 Field assessment of different lactic acid bacteria species and strains

A preliminary trapping experiment was conducted during summer 2013 both in a commercial vineyard at the bottom of the Adige Valley (San Michele all'Adige, 46°19'01''N, 11°13'73''E, 230 m a.s.l.). The experiment was set up in order to compare the following baits: *A)* DD with a pH regulated at 4.00, and inoculated with the strain of *O. oeni* ATCC BAA-331. *B)* DD at pH 4.00 with a *Pediococcus* spp. strain. *C, D, E)* DD at pH 4.00 and added with 3 different strains of *Lactobacillus*. *F)* DD without bacteria. *G)* Commercial DD (Prantil). *H)* The same of *G* but pasteurized at 70°C for 30 min. *I)* The same of *G* added with 10 mL/L of cicloeximide (cicloeximide aqueous solution 0.01%, Oxoid, United Kingdom) for preventing the contamination of lure by environmental yeast. All the baits were tested by using 200 mL of the adjusted DD in red plastic traps (Droso-Trap, Biobest, Belgium). A drop of TritonTM X-100 (Sigma-Aldrich, USA) drowning solution was also added to each 200 mL of liquid bait in order to break the surface tension of the liquid. The nominal concentration of microorganisms of the DD traps inoculated with bacteria was adjusted at 10^6 cfu/mL. A randomized complete block design was used, with 3 replicate blocks. During the 7 weeks of trapping period, traps were serviced weekly by removing and counting the insects and by replacing the drowning solutions.

The mean weekly number of *D. suzukii* captured per trap over the 7-week study period in each treatment was compared among treatments using

Friedman (two-way ANOVAs with replicas), followed by Wilcoxon Pairwisw test for posthoc comparisons of means.

4.2.4 Laboratory evaluation of *Oenococcus oeni* performance to the DD conditions

We tested 14 strains of *O. oeni* for their resistance and performance to the physical and chemical conditions of DD considering its main limiting factors: low pH, high concentration of ethanol and acetic acid, and low temperatures likely encountered during the field exposure of traps. The *O. oeni* strains were belonging to the Edmund Mach Foundation collection; all bacteria were cultured in modified MRS broth (Oxoid) in a 96 micro volume (200 µL) plate (Starstedt, Germany) at 25°C (with the exception of temperature test). According to the feature of DD, the composition of MRS broth was modified taking into account the following parameters: pH (3.8), acetic acid (45 g/L), ethanol (5% v/v), and temperature (15°C). Each parameter was studied singularly. The bacterial growth was measured every 24 h, by increases of optical density (620 nm) of cell culture by a PowerWave HT Microplate Spectrophotometer (BioTek, USA). Each measurement was replicated 3 times. The time of incubation was adjusted according to the OIV methods for the analysis of this bacteria (OIV, 2014). Obtained data were analysed by a PCA.

4.2.5 Headspace characterization of DD inoculated with different *O. oeni* strains by Gas Chromatography - Mass Spectrometry (GC-MS)

The characterization of volatile compounds of DD was performed considering two different sampling approaches, the Headspace and the Closed Loop Stripping (CLS) analyses. In both cases samples of DD were inoculated with 1% v/v of bacterial strain culture and incubated at 25°C for 1 week. In the Headspace approach, 5 mL of DD was inserted prior the incubation into hermetic closed glass vials (20 mL) and the withdrawal of volatile compounds was performed by a multifunctional auto sampler (Multi-Purpose Sampler MPS, Germany). Each vial was automatically introduced in an incubator at 38°C for 20 min, then 1 mL of air was removed from the headspace by a gas syringe pre heated at 80°C, and further injected in the column of GC. In the CLS approach the sample of DD (50 mL) was transferred into a proper glass jar with a plastic top having two holes. One hole was hosting a miniature 12 V vacuum graphite pump (Fürgut GmbH, Germany) which made air circulating from the jar containing the liquid to the second tube loaded with a CLSA carbon filter (Brechbühler AG, Switzerland). After 60 minutes of air flux, volatiles concentrated in the CLSA filter were eluted with 100 µL of dichloromethane (J.T. Baker, Holland). The samples were stored in 1 mL vials for chromatography (CS-Chromatographie Service GmbH, Germany) prior GC analysis. The GC-MS analysis was performed by a 7890A Gas Chromatograph (Agilent Technologies, USA) equipped by a hp-5ms column (Agilent Technologies), and coupled with a 5975 inert XL mass selective detector (Agilent Technologies). Helium was used as carrier gas (flow rate of 1.2 mL/min); the thermal cycle provided 5 min at 30°C, a temperature ramp of 3.5°C/min until the 240°C, and 2 min at 240°C. The total run time was 44.87 minutes. Data acquisition and analysis were done by a ChemStation software (Agilent Technologies).

4.2.6 Electroantennography responses (EAG) of *D. suzukii* females to the headspace collected from DD inoculated with different *O. oeni* strains

EAG responses to volatile compounds were recorded on mated *D. suzukii* females (n = 5) by means of a standard EAG apparatus (Syntech), as previously described (Revadi *et al.*, 2015). Each stimulus, represented by the eluted samples obtained by CLS analysis, was prepared by absorbing 25 µL of a solution on a piece (1.5 cm^2) of filter paper (Albet® 400, Scharlab SL, Spain) inserted into a Pasteur pipette. The solution was loaded directly on the filter which has the function to absorb and release the volatiles. The Pasteur pipettes were closed on the thinner side with a 1 mL-blue-tip. Three pipettes were used as blank controls (one empty pipette, one filled with paraffin oil solvent, one filled with dichloromethane solvent) while other two were filled with 1-hexanol and 2-hexanal solutions. These compounds are known to be very effective in eliciting antennal responses in SWD, hence they were used as references. Each synthetic stimulus was prepared by absorbing 25 µl of a dichloromethane solution at the concentration of 1 µg/µl on the filter paper. The solvents were allowed to evaporate for 10 min before starting the experiments.

A stimulus controller (CS-01; Syntech, Hilversum, The Netherlands) was used to keep the flow over the antenna constant during injection. A glass capillary indifferent electrode filled with Kaissling solution containing 5g/l polyvinylpyrrolidone K90 was inserted in the severed fly's head. The different electrode was a similar capillary, brought into contact with the distal end of fly's antenna. After the antenna preparation, the chemical stimulus output was directly delivered on the antenna with the Pasteur pipette through the air flow, activated by operating an external pedal linked to the stimulus air control. Each odour pipette was replaced every 3 trials. EAG responses were analysed with EAG 2000 software programme (Syntech, Hilversum, The Netherlands), and evaluated by measuring the maximum amplitude of negative deflection (mV) elicited by a given stimulus.

EAG responses were compared across treatments by means of parametric one-way ANOVAs, followed by Tukey's test for posthoc comparisons of means. Homogeneity of variance had been determined previously with Levene's test.

4.2.7 Field test with DD activated by three strains of *O. oeni*

Field trapping tests were carried out in 2 different orchards in the area of Pergine Valsugana (Trento Province, Italy): *A)* a sour-cherry orchard at Zivignago (490 m a.s.l.; 46°04'15,89"N; 11°14'26,04"E); *B)* a repository soft-fruit orchard at Casalino including blueberries, blackberries, raspberries and red currant in different rows (669 m a.s.l.; 46°02'50,90"N; 11°14'26,04"E). The field tests were performed using strains of *O. oeni* belonging to three subpopulations that showed the best performances in laboratory tests carried out in the limiting growth conditions of DD (see section above). Bacteria were hence multiplied in a 100 mL flask containing MRS broth (Oxoid) for 7 days at 20°C, then the culture was centrifuged (4000 rpm, 15 min) to remove the growth medium, and cells were added to the DD used to fill traps, previously corrected by adjusting the pH at 4.00 value. The bacterial concentration in DD at the beginning of field tests was adjusted to 10^6 cfu/mL. Red plastic traps (Droso-Trap) were baited with 200 mL of DD containing bacteria. A drop of Triton TM X-100 (Sigma-Aldrich) drowning solution was added to each 200 mL of liquid bait, in order to reduce the surface tension and to facilitate capture and submersion of trapped insects (Landolt *et al.*, 2012). The traps were placed randomly along the rows of the orchards (each 2 m and 1,5 m from the ground), and the position of different traps was changed randomly each week. Three replications and 5 replications were deployed in test A and B, respectively. Test A was conducted from the cherry flowering period until the period of "veraison" of the fruits (7 weeks, from 10th April to 30th May 2014). Test B was conducted from "veraison" of the first fruits until the fruits were over-ripen on the plants (7 weeks, from 19th June to 6th August 2014). After 1 week of exposition, the liquid bait was filtered and sent to the microbiological analysis. The insects caught in the liquid bait were identified by optical observation using a stereoscope (Optika, Italy). Two commercial baits, the Pherocon SWD (Trécé, USA) and the conventional DD (Prantil) were employed as references.

The mean weekly number of *D. suzukii* captured per trap over the 7-week study period in each treatment was $\sqrt{2}(x + 0.5)$-transformed and compared among treatments using one-way ANOVAs, followed by Tukey's test for posthoc comparisons of means.

4.3 Results and discussion

4.3.1 Field assessment of different lactic acid bacteria species and strains

Figure 1 reports the total number of *D. suzukii* adults caught by the different baits during the preliminary tests, aimed at confirming the hypothesis about the attractiveness of volatiles produced by the fermentation metabolism of lactic bacteria. We explored the attractive performance of different genera of lactic acid bacteria with regard to biochemical changes of the bait, such as the increase of the standard pH of Droskidrink (typically about 2.50) up to 4.00, a value recognized as ideal for the heterofermentative activity of wine lactic acid bacteria (Lonvaud-Funel, 2001; Liu, 2002). Overall, the DD baits inoculated with the different species of lactic bacteria caught a significantly higher number of SWD adults, in comparison to the other baits (Friedman: $F=6$; $P<0.0001$; d.f.= 31.6). In particular, the bait inoculated with *O. oeni* showed the highest attractive activity (Figure 1). Indeed, *O. oeni*-baited traps were able to catch a significantly higher number of *D. suzukii* adults than that recorded in traps baited with both the combination of DD and other genera of lactic bacteria and the commercial DD alone. The differences, in terms of attractiveness of the baits inoculated bacteria increased over the time of trap exposure (Figure 2). Conversely, control theses represented by either the pasteurized DD bait or the DD bait added with antibiotic are far less attractive for SWD, likely due to the inhibition of microbiological processes in the liquid bait. Concerning the different genera of lactic acid bacteria, it has been already reported that *Oenococcus* spp. have a remarkable hetero-fermentative activity and resistance in a low-pH environment (Lonvaud-Funel, 2001; Liu, 2002). Therefore, their enhanced biological activity and effectiveness in trapping SWD is not unexpected. As a consequence, the regulation of the value of pH in the liquid bait is a key point for the improvement of the trap efficacy. The standard value of pH in DD, that is about 2.5, is unsuitable for the development of most of the lactic acid bacteria species and thus the pH level has to be increased in order to provide them an appropriate growing environment. On the other hand, too high pH values would favour a non-selective growth of contaminant microbes (yeasts, bacteria or mold), causing a rapid depletion of the nutrients contained in the bait. Furthermore, the pH value of 4.0, adopted in these experiments, has been shown in oenological trials to induce the shift of the metabolic activity of lactic bacteria from omo- to hetero-fermentation of sugars, increasing their energetic yield (Lonvaud-Funel, 2001; Liu, 2002). However, the pH 4.00 represents a threshold level for the optimal growth of both *Lactobacillus* spp. and *Pediocccus* spp., and this would partly explain their minor efficacy with respect to *O. oeni*. The poor performances of the baits containing a very limited if any microbial community after either pasteurization or antibiotic treatments further supported the hypothesis of the crucial role played by the microbiota in the regulation of the attractiveness of feeding substrates for SWD. The non-linear increase of traps catches over the time of field exposure could be explained considering that the traps were only refilled every week to compensate the bait evaporation and hence the liquid bait was not completely replaced during the entire duration of field test in order to maintain the original population of bacteria inside it (Figure 2). In these conditions the lactic acid bacteria added to DD were likely adapting to the harsh liquid bait conditions. We may speculate that during the first weeks of field exposure the effect of the initial high cellular concentration (about 10^6 cell/mL) inoculated in the traps was able to allow a satisfactory attractiveness. In the following weeks the composition of DD could have imposed a reduction of cell viability, and consequently a performance decrease of the traps baited with bacteria with respect to the commercial DD and other control baits, until the adaptation of bacteria at the DD environment over the 5^{th} week of exposure would have favored a new considerable bacterial growth and the consequent recovery of the trapping capacity. Therefore, this first experiment clearly indicates *O. oeni* as the most promising species among the tested lactic bacteria for the improvement of the efficacy of DD traps and it has been thus selected for further laboratory and field tests.

4.3.2 Laboratory evaluation of *O. oeni* performance to the DD conditions

O. oeni has been found in few fermented materials, in particular wines (Dicks *et al*., 1995; Li *et al*., 2006). Despite this, a large variability, in terms of resistance to environmental limiting factors and biosynthetic capacity, has been reported among strains of *O. oeni* (Zapparoli *et al*., 2012). However, the use of *O. oeni* as bio-catalyzer of the production of *D. suzukii* biologically-active volatiles inoculated in DD is completely new and, therefore, an assessment of the behaviour of different *O. oeni* strains in these peculiar conditions was needed. We took into account four main characteristics of DD that, reasonably, would act as limiting factors for the metabolism of *O. oeni* strains (Guzzon *et al*., 2009). In all cases the "wild" strains of *O. oeni*, isolated from wines, showed significantly higher growth rates in the conditions set up in the syntethic media, with respect to the standard strains employed in the first field test (Figure 4), supporting the hypothesis of a gradual adaptation of bacteria to harsh environmental conditions. The four considered variables allowed a satisfactory classification of *O. oeni* strains, according to the resistance to such limiting parameters; the cumulative percentage of the total variance explained by the first two factors was about 80%. Figure 3 shows the scatterplots of both the variables (2A) and the cases (2B) in the plane defined by the Factors 1 and 2. A first large group (Figure 3B, G1), containing 9 strains, was characterized by a substantial tolerance of low temperature and ethanol, a second group (Figure 3B, G2) composed by 4 strains showed, on the contrast, a poor tolerance at the liming factors. Finally, the strain 6 showed a peculiar behaviour, resulting largely resistant to the pH and acetic acid and, therefore, was grouped alone (Figure 3B, G3). This last result is particularly interesting considering that acetic acid has a double influence in our experimental conditions. It is able to lower the pH level (that in this test was about 2.50), but it is also an end-product of many metabolic reactions of *O. oeni* (Lonvaud-Funel, 2001) and, as such, can inhibit the bacterial activity unbalancing the ratio between substrates and catabolites. On the contrast, the low selective pressure induced by ethanol, at the values considered in these tests, is not surprising since this outcome is in line with what has been previously observed about the interactions of bacteria with wine. In conclusion, the laboratory tests identified 3 candidate sub-populations of *O. oeni* strains that showed different adaptation at the peculiar composition of DD. These 3 groups of strains have been selected and utilized in an extensive field assessment, after having checked their ability to produce volatile compounds with a biological activity to SWD by the fermentative metabolism in DD.

4.3.3 Headspace characterization of DD inoculated with different *O. oeni* strains by Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS analyses were done in order to characterize the volatile compounds released by the DD, inoculated by different *O. oeni* sub-populations. We selected the strain 5 (G1 group), 6 (G3 group), and 7 (G2 group), according to the results shown in the sections above, and the type strain, included in this test as reference. In Table 1 the relative quantification of volatiles emitted by the baits inoculated with the *O. oeni* strains is shown, referring to the amount of each compound emitted by a standard unfermented DD sample. Thirteen compounds were detected: ethanol, 2-butanone, acetic acid, acetidin, acetoin, 3-methyl-1-butanol, isoamyl alcohol, 2-butil acetate, isobutyl acetate, ethyl butyrate, isoamyl acetate, ethyl caproate, and ethyl octanoate. No qualitative differences were found among the baits, and the same compounds were detected in all samples. However, the relative quantity of compounds showed relevant differences. G1 sample is characterized by a high concentration of acetic acid (+ 560% than the standard DD) and ethanol (+ 55% than the standard DD). G2 and G3 strains showed a considerable reduction of acetic acid (- 45% of DD release) emission, while ethanol increases about the 30%. The emission rate of 3-methyl-1-butanol, reported as “non-target” attractive volatile for a wide range of moths (Landolt *et al.*, 2011), is higher than in the standard DD in the case of G2 and G3 samples, while being unvaried in the case of G1. It is likely that acetoin is one of the key compounds for the attraction of SWD to

DD (Cha *et al*., 2012; Guzzon *et al*., 2014). The amount of this molecule showed a decrease of 55% in the G1 group headspace when compared to standard DD, while its concentration was very high in the case of G2 (+85%) and G3 (+70%). The 2-butanone followed a similar trend, with a relevant reduction in the G1 bait and an increase in the G2 and G3 samples. In the case of compounds belonging to esters the impact of bacterial activity on their release rate appears more uniform, with a generalized decrease in comparison to DD, except the case of G3 sample that showed a slightly increase of 2-butyl acetate and ethyl octanoate production.

Overall, the GC results provide a very complex, and somehow unexpected, analytical framework of relationship between bacterial strains and baits features. Moreover, it is necessary to take into account that an excessive concentration of some compounds characterizing the components of DD, may induce a negative response into the olfactory system of the insect. Cha *et al*. (2012) reported indeed that several EAD active compounds released from wine and vinegar have deterrent effects at high concentration on *D. suzukii* attraction in the laboratory two-choice bioassay. One of these compounds is isoamyl acetate that is probably released by the epiphytic community on fruits surface as well as in fermenting substrates (Cha *et al*., 2012; Witzgall *et al*., 2012; Revadi *et al*., 2015). When it was tested singly, isoamyl acetate was attractive only within a concentration range similar to that emitted by fresh fruit (Revadi *et al*., 2015), whereas the 100-fold higher release rates from wine and vinegar were behaviourally repellent (Cha *et al*., 2012). In our experiments the released rate of isoamyl acetate has been remarkably reduced after bacterial inoculum and lactic fermentation, which could be one of the reasons of the augmented attractiveness to SWD. All this evidence further corroborates the commonly accepted theory that the absolute amount of ubiquitous volatiles is the critical factor mediating the recognition and the orientation of polyphagous insects to feeding and oviposition sites (Bruce *et al*., 2005).

Therefore, the quantitative variations induced in the DD by the added bacteria, both in the cases of increase and of decrease of initial amount of each volatile compound should be carefully considered as well as the interactions of the trap mixture with environmental variables (temperature, humidity, insect catches) along field ageing of traps. However, the relevant difference among the three *O. oeni* sub-populations warranted to carry out the further field trials in order to select the best attractive blend for SWD.

4.3.4 Electroantennography responses (EAG) of *D. suzukii* females to the headspace collected from DD inoculated with different *O. oeni* strains

EAG results are shown in Figure 4. As expected, the reference compounds, 1-hexanol and 2-hexanal elicited strong responses to *D. suzukii* antennae. There were not statistical differences between our samples (Droskidrink, G1, G2, G3 and reference strain), but all the baits were statistically different from both the solvents and the blank controls, except for G2 sample, which showed a significant lower response compared to the other baits and which resulted not statistically different from the blanks (ANOVA: d.f. = 47; F= 8.6; $p < 0.001$). All our samples, except G2, elicited EAG responses statistically equal to 1-hexanol, the compound that induced the highest absolute responses. Interestingly, 1-hexanol was already detected among the volatile bouquet of fresh mature host fruits of SWD and elicited its antennal responses in gas chromatographic analysis coupled with elettroantennographic detection experiments (GC-EAD) (Revadi *et al.*, 2015). On the other hand, the results obtained with the bacterial bait G2 was unexpected, since it did not show either to be less attractive in the field trials or to release low amounts of VOCs in the GC-MS analysis. However, EAG analysis confirmed a remarkable insect sensitivity to the both standard DD and DD inoculated with *O. oeni*. However, additional GC-EAD experiments are warranted in the future, in order to understand which single compounds are able to be perceived by the olfactory system of SWD.

4.3.5 Field test with DD activated by three strains of *O. oeni*

Two consecutive field tests were performed in order to evaluate the effect of the addition of bacterial *O. oeni* strains belonging to the G1 (strain 5), G2 (strain 6), and G3 (strain 7) groups in the traps in different periods of the *D.*

suzukii host-fruit ripening season, early spring in a sour-cherry orchard, and full summer in a mixed soft-fruit orchard in the same area. In Figure 5A the results of the spring test are reported, expressed as sum of the insects captured for each type of bait. This experiment was generally characterized by a low number of SWD catches, since it was carried out in the early season, when the SWD population density in the fruit growing areas of Trento Province is still relatively low (Wiman *et al*., 2014). However, the aim of this test was to evaluate the potential use of both DD and DD inoculated with *O. oeni* as an early warning tool able to provide a reliable information on the first infestation by SWD of cultivated fruits, i.e. sour-cherry. The number of insect caught in traps inoculated with both bacterial sub population, G1, G2, and G3 did not differ from the captures recorded in the standard DD or in the trap baited with the Trecè dispenser. The low activity of the baits inoculated with bacteria can be explained by the observation that, during traps exposure, the mean daily temperatures were generally below the threshold of 15°C, considered not suitable for an adequate growth of *O. oeni* (Liu, 2002; Guzzon *et al*., 2009) and, as a consequence, for the biotransformation of the liquid substrate and the production of volatile metabolites attractive to SWD. In any case, the low temperatures do not kill the microorganisms but only cause the slowing down of the metabolic activity that would recover as soon as the proper temperature would be re-established.

A similar observation was reported by Beers *et al*. (2011) regarding yeast baits, which have comparable environmental needs for optimal growing. In general, all microorganisms potential candidates for the production of attractive volatile metabolites for SWD (yeasts, acetic bacteria and lactic acid bacteria) are considered mesophilic, that is with an optimum of activity between 15 and 30°C. Interestingly, dissections of adult females captured during the early season field experiment showed that about 80% contained fully developed eggs in their ovaries (data not shown), supporting the hypothesis that SWD overwinter mainly as adult mated females. Therefore, during this portion of the season food baits, such as DD and *O. oeni*-inoculated DD, are supposed to be particularly efficient since either gravid females would be looking for feeding substrates before oviposition after the winter population bottleneck or food baits are not yet in strong competition with many natural sources of attraction, such as ripening fruits (Ometto *et al*., 2013; Wiman *et al*., 2014). A high number of catches in this part of the season thus will result in a delay of the population outbreak during the rest of the growing season, allowing reduced damage of the ripening crops. It is therefore crucial in future experiments to optimize trap architecture and bait components in order to keep the temperature within the optimal range and hence provide an effective tool for trapping control strategies even off season.

The second field experiment was performed during summer, for 7 consecutive weeks. In this case the temperatures were suitable for a massive bacterial growth, remaining stable over the daily average of 20°C. This different situation immediately reflected in the catches of insects which peaked up overall 3000 individuals a week (Figure 5B). Differences in attractiveness of inoculated baits with respect to commercial references are quite evident along all the duration of the experiment. In particular, the bait inoculated with the G3 strain showed basically the best trap performance, even though we did not find any statistical difference (ANOVA: d.f. = 55; $F = 0.18$; $p=0.99$). As mentioned before, the general greater summer-performance of bacterial baits, compared to the low activity earlier in the season can be explained by the effect of increased temperatures.

In conclusion, we believe that the knowledge provided in this work paved the way to develop new concept of trap, in which the attractiveness of DD would be strongly increased by the combination of microorganisms releasing biologically active volatiles to SWD. The long term perspective is to accelerate research and technology transfer towards the set up of new environmentally friendly pest control methods based on the use of traps baited with this new lure, such as mass trapping and attract and kill.

4.4 Table and figures

Volatile compound	Retention time	Extracted ions	*O. oeni* type strain	G1	G2	G3
	Min	m/z	%			
Acetic acid	2.20	60	-52.2	559.3	-44.1	44.3
Ethanol	1.55	45	26.1	54.7	38.5	29.3
3-methyl-1-butanol	4.67	70	8.3	0.9	70.0	21.1
Isoamyl alcohol	4.70	41	23.7	9.6	88.1	36.7
Acetoin	3.78	88	49.4	-55.4	84.8	69.2
Acetidin	2.27	88	-95.0	-96.1	-93.0	94.5
2-butyl acetate	5.55	87	14.6	-31.3	12.5	5.5
Isobutyl acetate	6.17	56	-29.3	-56.2	6.8	30.8
Ethyl butyrate	7.36	71	-35.1	-48.3	-9.2	37.8
Isoamyl acetate	10.84	43	-59.8	-70.5	-57.8	62.8
Ethyl caproate	16.73	88	-4.2	-49.4	47.4	7.5
Ethyl octanoate	25.35	88	43.4	-27.4	141.4	35.6
2-butanone	2.13	72	39.4	-15.0	64.7	49.3

Table 1. Relative quantification of volatiles emitted by the baits inoculated with 3 strains of *O. oeni* (G1-G2-G3). Data are expressed as difference (%) with respect to amount measured in the standard unfermented Droskidrink.

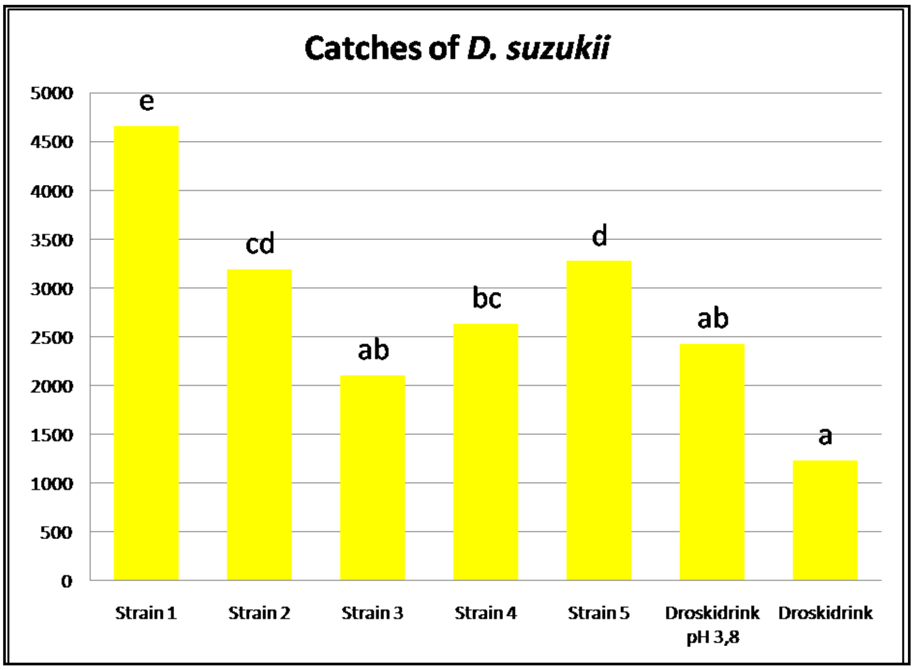


Figure 1. Overall number of *D. suzukii* catches in the preliminary field tests with traps baited with attractive mixtures (Droskidrink) inoculated with different species and strains of lactic acid bacteria. Different letters indicate significant differences (Wilcoxon pairwise test, P = 0.0001).

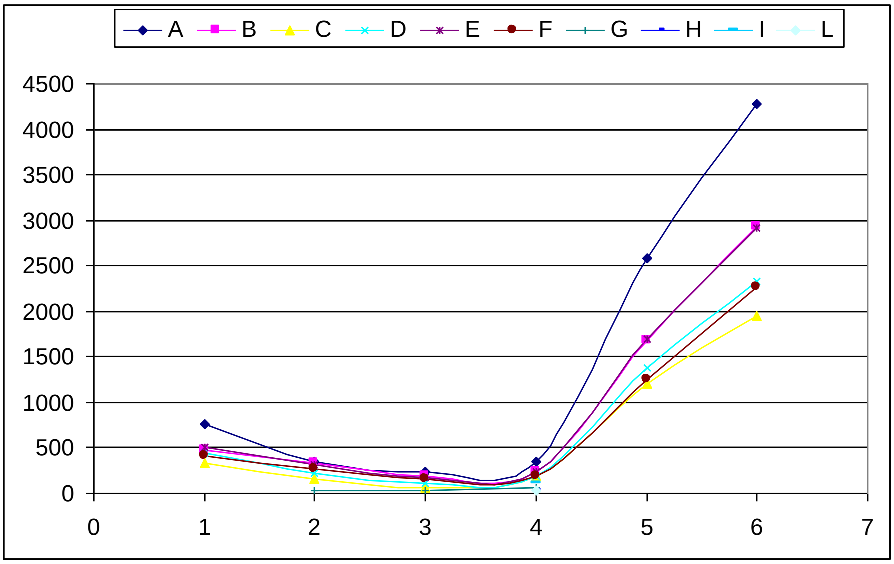


Figure 2. Catches trend week by week of different bacterial strains and different commercial baits.

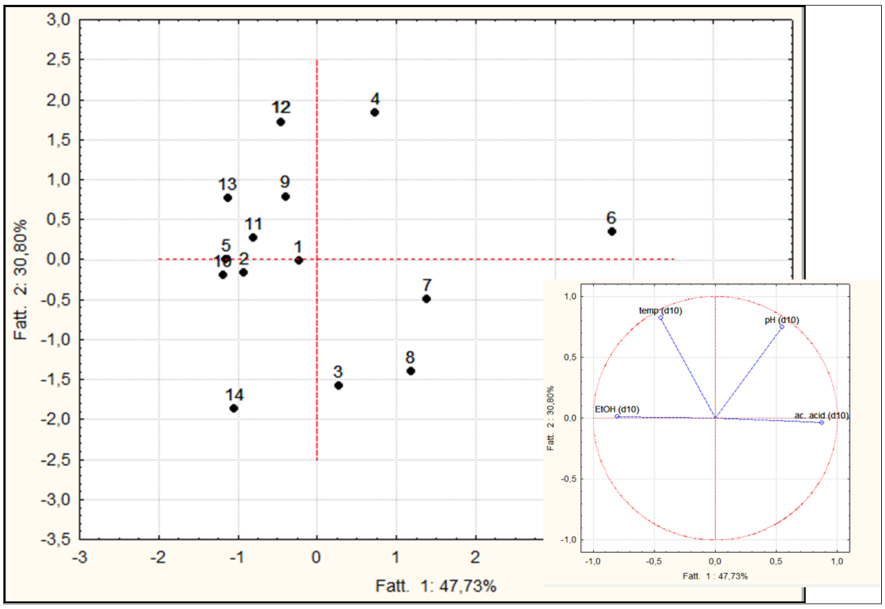


Figure 3. PCA of data of bacterial growth in synthetic media considering the 4 main limiting factors for *O. oeni* growth characteristic of DD: pH (4.00), ethanol (4%), acetic acid (45 g/L), and temperature (15°C). A) Scatterplots of the 4 variables in the plan defined by the factors 1 and 2. B) Scatterplots of the 4 variables in the plan defined by the factors 1 and 2.

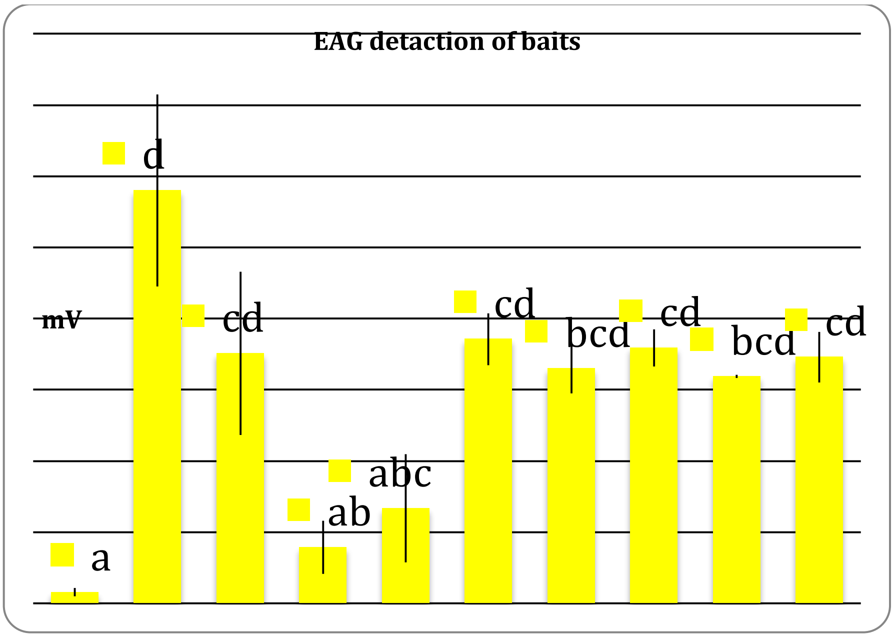


Figure 4. Mean EAG responses (mV) of *D. suzukii* mated female antennae elicited by commercial DD and DD inoculated with different *O. oeni* strains. Control stimuli: empty blank, paraffin oil, dichloromethane. Reference compounds: 1-hexanol, 2-hexanal. Baits: commercial Droskidrink, *O. oeni* reference strain (MRI 10000), G1, G2, G3 strains. The standard deviation of the means is reported. Istograms with the same letters are not significantly different (ANOVA: d.f. = 39; F= 9.5; p< 0.05).

5A

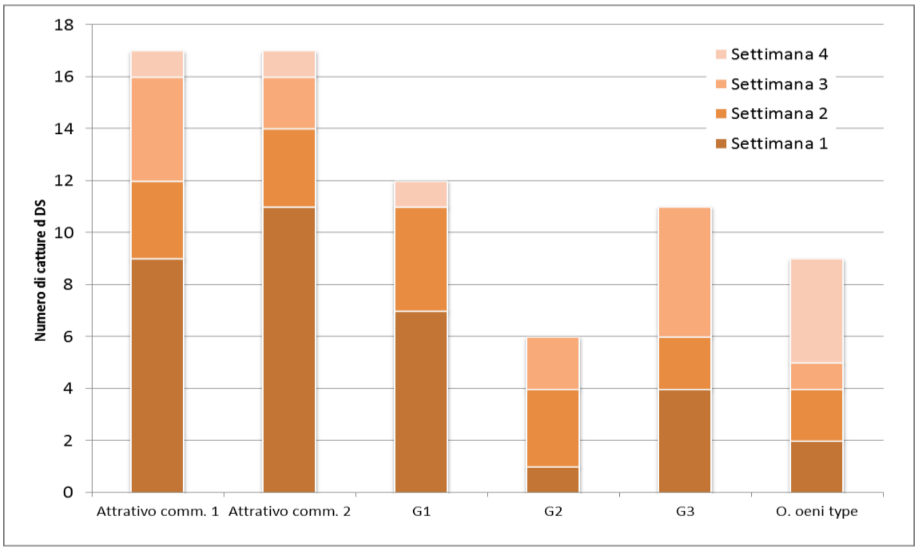
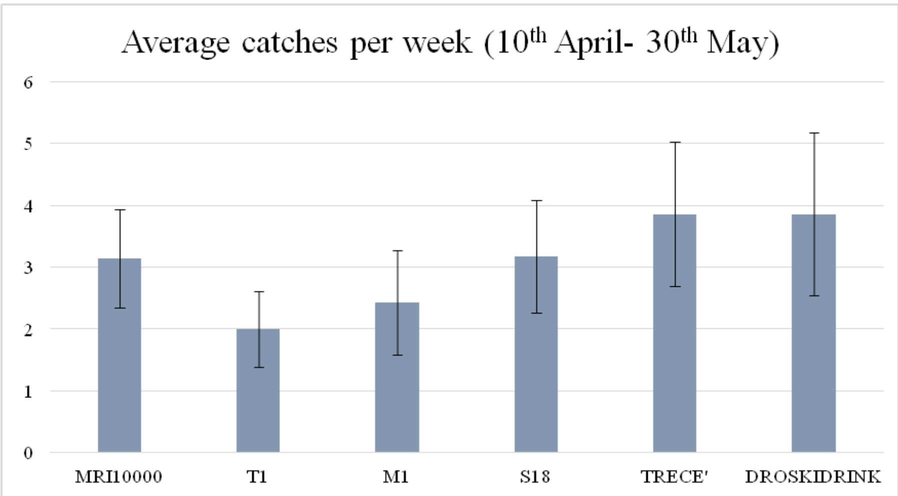
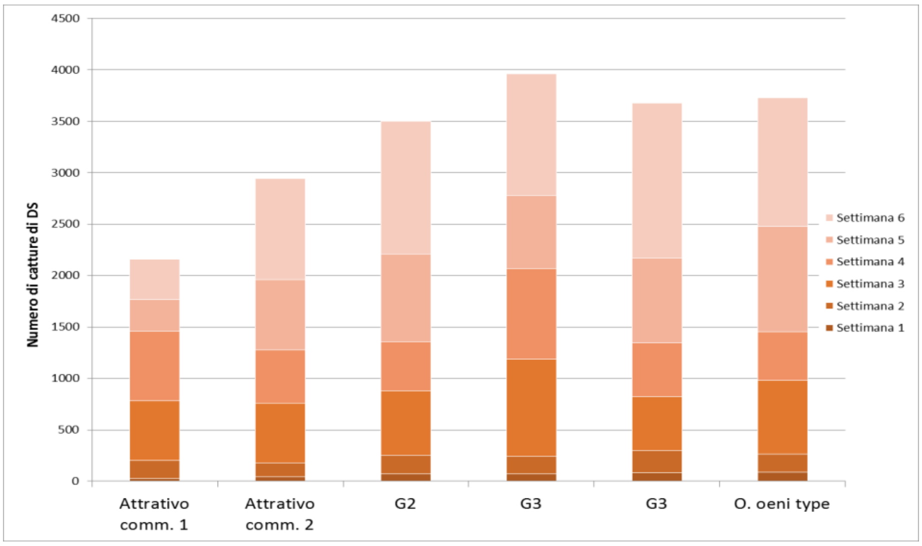


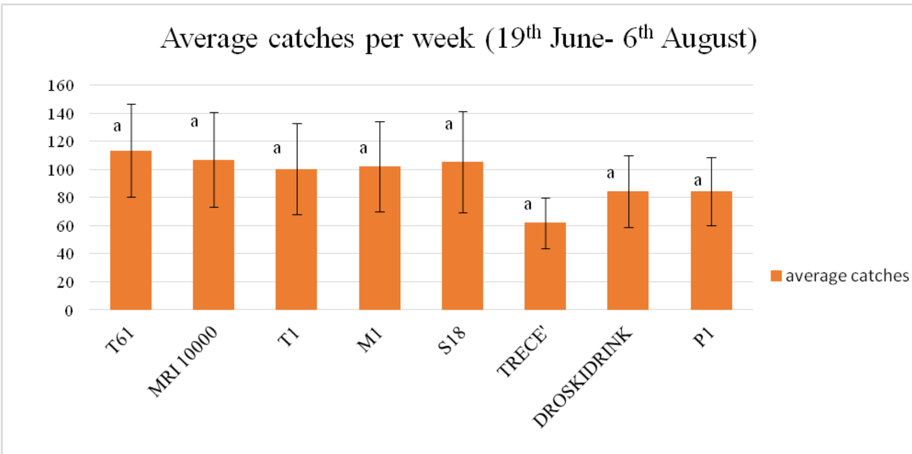
Figure 5. Comparison of *D. suzukii* catches by different baits; data are expressed as sum of fly catches during the entire period of tests. A) Early spring test. B) Summer test.



The graph shows the differences in catches between different baits in early season, in sour-cherry fields. The standard error of the means is reported.

5 B





The graph shows the mean of catches of the different baits tested in berries field, during seven weeks of trial (19th June- 6th August). Peak abundances with the same letters are not significantly different by ANOVA: d.f. = 55; F= 0.18; p=0.99. The standard error of the means is reported.

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5. Use of an active culture of lactic acid bacteria for preparing a bait aimed at monitoring and controlling *Drosophila suzukii* and its respective compound to be put in appropriate traps (Chapter 3)

5.1 Object of the invention

The use of lactic acid bacteria, belonging to the *Oenococcus oeni* (Garvie) Dicks *et al*. species, for preparing a bait for monitoring and controlling *Drosophila suzukii* Matsumura (Diptera, Drosophilidae), the spotted-wing drodophila (SWD), and its respective compound to be put in appropriate traps.

5.2 Scope of the invention

The scope of the invention is to propose a new, effective, economical, and long lasting preparation alternative to the treatments based on chemical pesticides for controlling *D. suzukii*. This scope is achieved by using a culture of lactic acid bacteria, belonging to the *O. oeni* species, and put in a condition to warranty the production of metabolites strongly attractive for SWD for a long period of time, thanks to the original composition of the attractive blend contained in a trap.

5.3 Background of the invention

D. suzukii is an insect capable of causing substantial damage in different high-value arboreal, frutescent and vegetable cultures, including soft fruits, drupaceous fruits, and grape. Aggressiveness and adaptability to numerous contexts make it one of the major adversities in the agricultural field, capable of causing step-downs of production even greater than 90%. At present, the struggle against this insect is performed through the use of insecticides, massive capture traps, and other agronomic practices. None of these solutions led to a resolution of the problem.

The researches published so far highlighted that the information concerning fundamental aspects for survival of insects, including the research for nutritional resources, escape from enemies, reproduction, and identification of the oviposition sites are mainly mediated by volatile chemical compounds perceived by olfaction (Schoonhoven *et al*., 1998; Dicke & Van Loon, 2000; Bruce *et al*., 2005; Hilker & McNeil, 2007).

Numerous studies demonstrated the importance of the volatile compounds, produced by the host plant and by other organic substrates, in helping insects in identifying the foods and the oviposition site (Bruce *et al*., 2005; Bruce & Pickett, 2011).

More recently for the *Drosophila* genus, some studies clarified the contribution given by the microbic component present in the substrates commonly frequented by the insects of this group, in producing chemical compounds capable of influencing their behaviour. For instance, Becher *et al*. (2012) observed that such microbic component strongly influences *Drosophila melanogaster* Meigen in searching oviposition and foodsites and that this insect features better performances, in terms of reproduction, whenever the substrate is contaminated by yeasts.

The experimental data clearly shows that *D. melanogaster* is more attracted by substrates being in fermentation, thanks to the presence of the *Saccharomyces cerevisiae* Meyen ex E.C. Hansen yeast, as compared to what observed in tests where the same, non-yeasting substrates were present.

Little information is present in the literature on the chemical ecology of SWD. With respect to *D. melanogaster* and to all remaining species of the same genus, SWD has a peculiarity in that it lays eggs on unwounded and healthy fruits, whereas it is attracted by marcescent and fermenting substrates for feeding purposes (Cini *et al*., 2012). Consequently, the knowledge acquired on the *D. melanogaster* model insect and on other closely-related species cannot provide an exhaustive information for understanding the behaviour of *D. suzukii*. Preliminary experimental observations confirm that this insect too is attracted, in terms of nutritional sources, by wounded and presumably fermenting fruits, as suggested by the attraction exerted onto SWD by a variety of fermented liquids, including wine, vinegar, and liquid derivatives of fruits (Kanzawa, 1935; Cha *et al*., 2012, 2013, 2014; Landolt *et al*., 2012) and by the association, recently

reported, of this insect with a community of yeast species basically formed of *Hanseniaspora uvarum* (Niehaus) Shehata *et al*. (Hamby *et al*., 2012).
A further confirmation of the interactions between SWD and microorganisms was found in capture tests, wherein baits inoculated with *S. cerevisiae* gave improved results as referred to sterile baits (Knight *et al*., 2013). Conversely, no evidences have been published about the positive interactions, in terms of a greater attractiveness, of the insect with bacteria originating from the ambient or involved in the fermentation processes that is the base of the attractive mix commonly used in traps. However, tests carried out by the applicant demonstrate that the attractive capacity exerted by matrices activated with lactic acid bacteria belonging to species not commonly isolated in traps or in the agricultural context where these are located, is significantly higher than that observed by the mentioned species of yeast, the conditions being equal.
At present, the most effective traps for capturing *D. suzukii* are those baited with different types of vinegar and wine (Landolt *et al*., 2012). Both these substrates result from fermentation, mediated both by yeast and by acetic bacteria, of carbonaceous compounds, mainly hexose sugars present in the raw material (McKenzie & Parsons, 1972; Becher *et al*., 2010, 2012; Barata *et al*., 2012).
Other secondary compounds are also apparently relevant in attracting these insects, which justifies the greater effectiveness observed in the case of using food matrices like vinegar and wine to bait traps, with respect to that of pure compounds, for instance acetic acid and ethanol.
Among these secondary compounds it is worth mentioning, according to the present references, acetyl methyl carbinol, butyrate, 2-phenyletanol, present both in wine and in vinegar, methionol, isoamyl lactate, and diethylsuccinate present in wine. All of these compounds are produced by the fermentation of yeasts and/or bacteria (Nielsen & Richelieu, 1999; Cordente *et al*., 2012), which confirms the importance of the microflora in forming a set of volatile molecules attractive to SWD.
According to this knowledge, traps are marketed containing wine vinegar, apple vinegar, wine, fruit juices (Grassi *et al*., 2014). In order to warrant

stability and marketability, such matrices are sterilized via pasteurization during the production step.
Tests carried out by the applicant demonstrated that the liquid matrices used so far in traps feature a limited activity, are not capable of warranting a sufficient effectiveness in the control strategies (mass trapping, attract and kill) and have a rather low duration in time, in the order of one week.
In summary, the traps known so far are not sufficiently active against SWD and feature a short duration in time. In order for the control strategies to provide acceptable results, it is therefore necessary to use a high number of traps per hectare, which can be estimated to amount to different thousands. Furthermore, the traps known so far require weekly service of the bait. The high number of traps and the complex operativity necessary for their maintenance make the current solution ineffective and unsustainable from the economical point of view.
In summary, the present invention refers to a bait aimed at monitoring and controlling SWD, which comprises the use of an active culture of lactic acid bacteria, preferably of the *O. oeni* species, preferably of the type used in oenology. In a preferred preparation the concentration of the lactic acid bacteria equals at least 10^8 cells/g and the mixture also comprises at least apple cider vinegar or wine vinegar or wine or fruit juices taken individually or added with at least one organic acid, sugar, preferably a fermentable one, and a fermentation activator, or with all of the three additives, namely organic acid, preferably fermentable sugar, and a fermentation activator.
The pH of the preparation ranges from 3.5 to 5.0 and is cold sterilized, preferably by filtration, before the first use.
According to an even more preferable preparation, the active culture of lactic acid bacteria is included in an organic matrix, preferably formed of an organic polymer of an alginate stabilized by bond to an element in a cationic form. Such containment form of microorganisms makes it possible to optimize their functionality, meant as a biosynthetic activity and a resistance to environmental stresses, and to warrant a use thereof extended in time as compared to the devices currently in use (Avnir *et al*., 2006; Guzzon *et al*., 2012).

The scientific works mentioned in the present document are listed below for the sake of completeness.

5.4 Disclosure of the invention

D. suzukii is an invasive species capable of causing major damages to cultivated fruits. For this reason it is presently managed by using chemical insecticides with big risks of damages to the environment, to the operator's health and to the population who stands on the cultivation areas of the cultures infected by SWD. Among the few existing alternatives there is the mass trapping of the population of insects by means of specific traps. However, so far traps did not demonstrate to be capable of solving the problem, as it appears from the major losses of production recorded even in the presence of these devices, because an adequately attractive and long-lasting bait has not been identified yet. Studies carried out by the applicant demonstrate that SWD is particularly sensitive to some molecules like acetoin and diacetyl. Such molecules are not naturally present in significant quantities in the attractive mixes presently used, however they can be produced in high quantities by *O. oeni*, a species of lactic acid bacteria of a typically oenological origin (Versari, 1999). Such species is the only one belonging to the *Oenocccus* genus and its presence has been exclusively described in oenological environments, in particular in wines during the malolactic fermentation (Liu, 2002). The addition of lactic acid bacteria belonging to the *O. oeni* species, which is the scope of the present invention, in traps baited with properly modified mixes of vinegar and wine, makes it possible very effective massive captures, capable of representing an alternative to chemical treatments.

The very restricted environmental allocation of the species under consideration, as well as its poor intraspecific variability, is due to some of its physiological characteristics. In particular, *O. oeni* exhibits a poor vigor and a very low growth rate, thereby being rapidly overtaken in the colonization of the common fermentable substrate, including sugary juices of vegetal origins, by other species of bacteria or yeasts. Contrary to other species *O. oeni* exhibits an excellent resistance to low pH values and to the presence of ethanol in the environment, thereby resulting the dominant species in wine at the end of alcoholic fermentation, wherein it finds the organic acids, among which in particular the malic acid, as a primary feeding source (Liu, 2002). Its characteristic metabolism consists of the decarboxylation of the malic acid into lactic acid, from which *O. oeni* is capable of taking energy thanks to the protonic gradient that is thus generated; it also produces secondary matabolites, including acetoin, diacetyl, and acetaldehyde (Liu, 2002). Such peculiar deacidification activity has been considered so far of an exclusively oenological interest, in that the action usually exerted by lactic acid bacteria in food matrices entails an acidification of the means through a consumption of sugars (e.g. dairy productions) and production of acid.

The disadvantages of the known traps are therefore solved by the present invention. It is based on the original use of *O. oeni* in capturing *D. suzukii* thanks to the surprising increase in attractiveness of the traps that this microorganism, added to bait, causes. Such application is totally surprising with respect to the current and one context of use of this microbial species, i.e. malolactic fermentation into wine (Liu, 2002).

O. oeni is also capable of warranting an increased attractive capacity of the conventional traps already in use, as demonstrated by experiments carried out by the applicant. The applicant experimentally ascertained that adding *O. oeni* to the common attractants present in the traps for SWD induces a higher number of captures than that observed with traps baited with other microorganisms or other attractive formulations; such difference can be attributed, according to the present references and to experiments carried out by the applicant, to the production by *O. oeni* of volatile molecules that are attractive to SWD, for instance acetoin or diacetyl, significantly higher with respect to other species of microorganisms. On the contrary, other experiments performed by the applicant demonstrated a non statistically significant intraspecific variability, i.e. internally to the *O. oeni* species, with respect to the attractive activity toward SWD. Such poor variability is reasonably to be attributed to the fact that the main differences highlighted between strains belonging to this species are in charge to genes encoding resistances to environmental stress factors and do not concern the energetic metabolisms essential to life of cells, which the production of the mentioned attractive molecules is related to.

An increased capability of traps in terms of attractiveness, thanks to the addition of *O. oeni*, is already evident by using traps already available on the market.

The applicant observed that the common attractants, especially that most commonly used in the Province of Trento, called Droskidrink, consisting of apple cider vinegar, wine vinegar, wine, fruit juice staken individually or mixed together, do not enable *O. oeni* to express its metabolic activity at the best. Conversely, a better result is achieved with completely new modifications in the composition of the attractant, consisting of adding to a 3:1 mix of apple cider vinegar and red wine of exogenous components like organic acids, for instance citric acid and L-malic acid, cane sugar, and nutritional factors including nitrogenous sources and vitamins. The acidity of the mix has also been corrected, by adjusting its pH to a value ranging from 3.5 to 5.0 in order to warrant a maximum efficiency, in energy terms, of the decarboxylation reaction of the malic acid. Such liquid bait is stabilized via a sterile filtration and is not thermally treated because it has been possible to experimentally demonstrate that the attractiveness of baits containing non thermally-treated substrates is greater, probably thanks to the preservation of the compounds with high biological activity, but thermolabile, secondary constituents, for instance vitamins. Also, the use of an immobilized bacterial culture makes it possible to recover the biomass in the periodical service and/or replacement of the attractive substrate, making it possible its use in subsequent batches with surprising increases in the activity period of the trap. Note that the current time of use of the commercial traps is as low as one week.

5.5 Experimental section

Figure 1 graphically represents a gaschromatography recording coupled with an electroantennography (GC-EAD) carried out by the applicant. By this technique, the individual compounds eluted by the gaschromatograph reach the antenna of an insect specifically prepared in an electroantennograph. The substances perceived by the antenna of an insect induce an electrical response which is displayed as a depolarization. In this recording a solution of volatile compounds collected from a sample of Droskidrink added with a culture of *O. oeni* (red line on the upper part) has been injected into a gaschromatograph and coupled with an antenna of a female *D. suzukii* (black line on the bottom side). As compared to the tests carried out in the absence of lactic acid bacteria, this preparation emits high quantities of diacetyl (the arrow indicates the peak of diacetyl at approximately 10min of retention time) and of acetoin (the arrow indicates the peak of acetoin at approximately 13min of retention time). Both compounds induce significant electroantennographic responses (their corresponding arrows on the bottom side).

The applicant performed a field test in an applicant's experimental vineyard of the Teroldego variety, simple pergola trailing system and located in the municipality of San Michele all'Adige (Trento) to evaluate the attractive effectiveness to SWD of a bacterial culture of *O. oeni* combined with the Droskidrink commercial product, i.e. the commercial attractant commonly used in the province of Trento and in other fruit growing regions. Droskidrink is normally formed of a mix of apple cider vinegar and red wine in a proportion 3:1 with the addition of 4.0 g of raw cane sugar. The pH of the commercial Droskidrink is approximately 3.0.

Bottles provided with side holes filled with 200 mL of liquid attractant have been used in the test.

The following theses have been evaluated:

1) a Droskidrink whose pH had been increased up to a value of approximately 4.0 through the addition of KOH (such pH value makes the growth of lactic acid bacteria easier) added with approximately 3.5 mL of a culture medium with *O. oeni*;
2) a Droskidrink featuring a pH of approximately 4.0;
3) an unmodified Droskidrink featuring a pH of approximately 3.0 (commercial Droskidrink);
4) a commercial Droskidrink added with 1.0 g of tetracycline (an antibiotic).

Every thesis has been repeated 3 times on different rows in a randomized block. Service of traps has been made weekly and the number of SWD captures are expressed as an average of the 3 traps, account being taken of the summation of males and females of the insect.

The traps baited with *O. oeni* at the moment of the weekly control have been filtered from the captured insects and refilled with a quantity of Droskidrink up to reaching the quantity of 200 mL again. Conversely, the traps of the theses containing Droskidrink only, according to the commercial protocol, have been completely emptied and baited again with a fresh attractant.

The results have been analyzed via a Levene test to check the standard distribution of the values. Analysis of variance (ANOVA) and LSD test have been performed and plotted in the following figures.

Figure 2 shows the average number of captures of SWD by means of traps (no. 3/theses; 4/weeks 5) by using an attractive mix inoculated with *O. oeni*, the result of the previously described test. The values are represented in a logarithmic scale. Letters that are different for every week of checks indicate significant differences (ANOVA, Levene test; LSD test, P=0.05).

The traps baited with a Droskidrink inoculated with a culture of *O. oeni* are always significantly more attractive than the remaining theses containing Droskidrink only in different conditions. Also, the presence of the bacteric culture in active growth made it possible to keep the trap effective for all the duration of the test without the need for completely replacing the liquid mix but just weekly refilling the contents lost by evaporation with new Droskidrink.

Figure 3 shows the results of a test carried out by the applicant similar to that previously described wherein different species of lactic acid bacteria, in particular of the *Pediococcus* and *Lactobacillus* genera, have been compared to each other. The diagram shows the total number of captures during the 5 weeks of test of such species as compared to *O. oeni*, which results to always be significantly more attractive to SWD.

5.6 Claims

1. A use of an active culture of lactic acid bacteria for preparing a bait aimed at monitoring and controlling *D. suzukii*.
2. A use according to claim 1, wherein the active culture of the lactic acid bacteria is of the type used in oenology.
3. A use according to claim 1, where in the lactic acid bacteria of the compound belong to the *O. oeni* species.
4. A use according to claim 1, wherein the concentration of the lactic acid bacteria in the compound is of at least 10^8 ufc/g.
5. A use according to claim 1, wherein the active culture of lactic acid bacteria is included in an organic matrix.
6. A use according to claim 5, wherein the organic matrix is formed of an organic polymer.
7. A use according to claim 1, wherein said compound comprises said lactic acid bacteria in association with at least one of the following liquids: vinegar, wine, fruit juices.
8. A use according to claim 1, wherein said compound comprises said lactic acid bacteria in association with at least one of the following liquids: fruit vinegar, wine vinegar, wine, fruit juices,and added with at least one of the following elements: an organic acid, a fermentable sugar, a fermentation activator.
9. A use according to claim 5, wherein said compound is cold stabilized.
10. A use according to claim 5, wherein said compound is cold stabilized by filtration.
11. An attracting compound aimed at monitoring and controlling *D. suzukii*, characterized in that it comprises an active culture of lactic acid bacteria belonging to the *O. oeni* species associated with at least one of the following liquids: fruit vinegar, wine vinegar, wine, fruit juices.

12 A compound according to claim 11, characterized in that the compound is added with at least one of the following elements: organic acid, sugar, fermentation activator.

13 A compound according to claim 11, characterized in that the compound is added with an organic acid and a sugar, and a fermentation activator.

14 A compound according to claim 10, characterized in that the compound features at a pH ranging from 3.5 to 5.0.

5.7 Figures

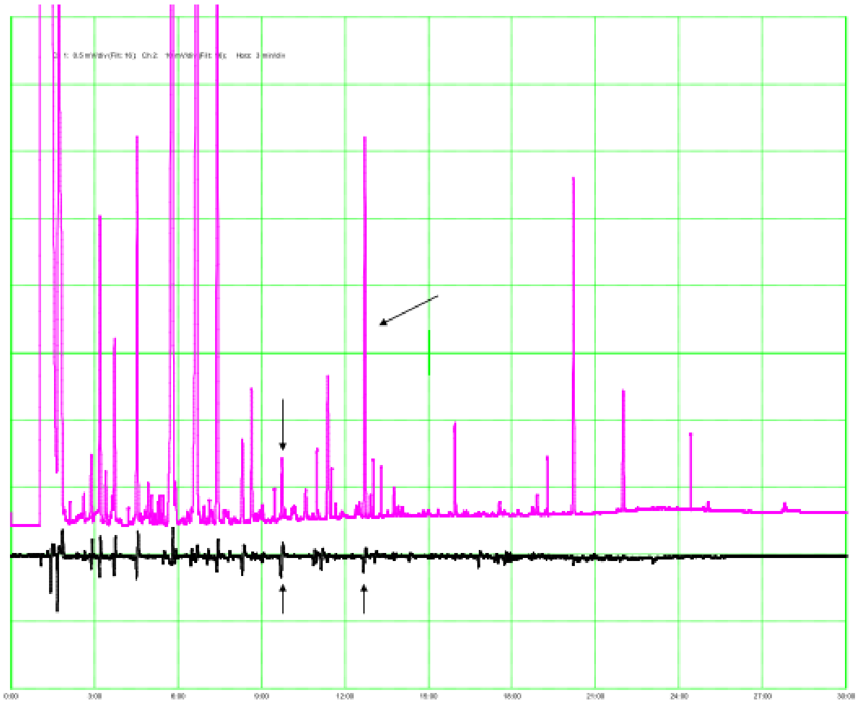

Fig. 1 - Gaschromatography recording coupled with an electroantennography (GC-EAD) carried out by the applicant.

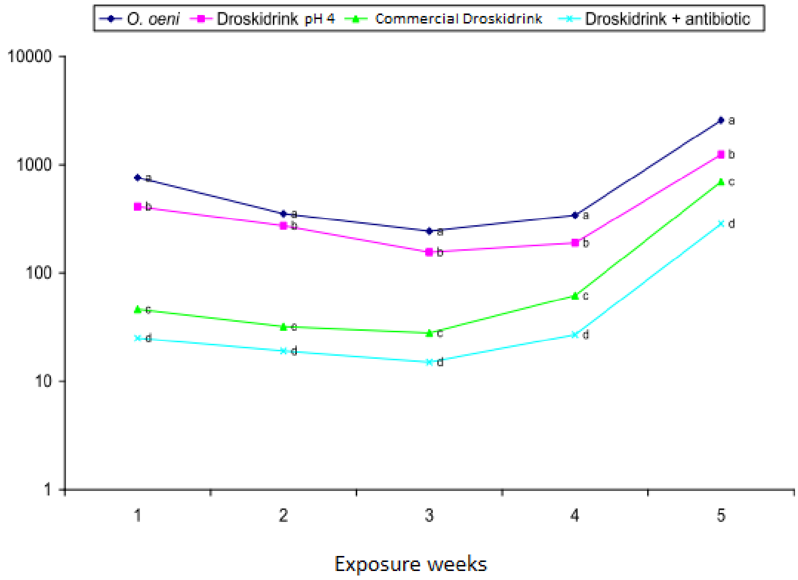

Fig. 2 - Average number of captures of *D. suzukii* by means of traps using an attractive mix inoculated with *O. oeni*.

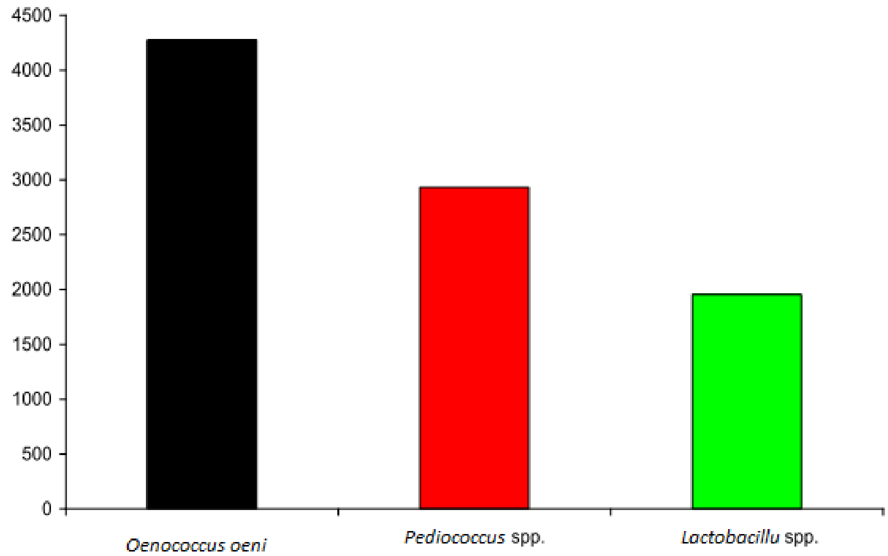

Fig. 3 - Total catches of *D. suzukii* with different species of lactic acid bacteria.

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6. Combined effect of trap design and Droskidrink mixture improved with different strains of *Oenococcus oeni* bacteria for early detection of *Drosophila suzukii* adults (Chapter 4)

6.1 Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), the spotted-wing drosophila (SWD), is an economic pest of small and stone fruit in major production areas including North America, Asia and Europe (Walsh *et al*., 2011; Cini *et al*., 2012). SWD females have a serrated ovipositor and exhibit a preference for ovipositing in ripe and ripening intact fruit as opposed to the overripe and blemished fruit that are infested by other *Drosophila* species (Lee *et al*., 2011a). Oviposition by SWD may reduce quality of fresh fruit and cause downgrading or rejection at processing facilities (Wiman *et al*., 2014). Adaptability to different environments makes SWD one of the major pests in agriculture. This species also causes production losses that may even exceed 90% (Cini *et al*., 2012). Economic damage is estimated at more than \$2 billion in North America, over \$4 billion in Europe and \$500 million in Asia (Liburd, 2015). *D. suzukii* originates from Southeast Asia. It is still unclear whether SWD is native to Japan, but in any case populations have likely been established there since 1916 (Kanzawa, 1939; Mitsui *et al*., 2010). Pesticide applications have been the primary control technique against SWD in both North America and Europe (Wiman *et al*., 2014). Mass trapping and other alternative agronomical practices may also affect populations (Beers *et al*., 2011, Cini *et al*., 2012). However, currently used methods and techniques do not sufficiently manage commercial infestations.

The alternative management strategies to insecticide applications are currently being investigated (Cini *et al*., 2012). These strategies are increasingly being used to reduce the risk of pesticide resistance development by insect populations and to decrease damages to beneficial organisms (Walsh *et al*., 2011). Monitoring SWD is the first step to an integrated pest management program to determine where and when populations are present in the field and when to enact control measures (Cini *et al*., 2012). Currently, an accepted commonly used monitoring method for

SWD involves the use of traps constructed from a plastic cup with holes at the top for fly access. Inside the cup is a yellow sticky card and a mixture of apple cider vinegar (ACV) and a surfactant (Burrack *et al*., 2012; Burgess, 2013). This trap has the problem of detecting SWD after the insect population has already established. By the point of the season when ACV traps detect SWD, populations are generally very high, and eradication or suppression of the pest population is a serious challenge (Bolda *et al*., 2010). Another drawback of these traps is that they are not specific to SWD and tend to capture non-target Drosophilids and other pests (Cini et al., 2012). For these reasons, there is a need to create a trap that functions better. A species-specific trap containing odorants emitted by host fruit during ripening would likely attract SWD earlier in the season, allowing action to control the pest on a local level to suppress population numbers (Cini *et al*., 2012). One purpose of trapping is to capture the maximum number of insects before they reproduce or cause damage to crops (El Sayed *et al*., 2006; Suckling *et al*., 2015). Another purpose of trapping is to indicate whether the pest is present or absent. Effective trapping requires the use of lures that are able to attract fruit flies more effectively than natural food sources, such as calling virgin females, mating aggregations or food sources, including efficient traps or stations or formulations for killing the attracted insects and using lures and non-saturating traps that are effective during the entire period of adult emergence and mating (Suckling *et al*., 2015). Accordingly, the traps must be visually attractive and capable of capturing and retaining flies sufficiently long enough to provide a lethal dose of toxicant or to otherwise prevent the escape by drowning or starvation (Lasa *et al*., 2014). The capacity of a trap for retaining flies is likely to be influenced by the bait and the retention system used. At present, the most effective traps are those baited with vinegar and wine, synthetic compounds identified in their headspace (Landolt *et al*., 2012) or Droskidrink (apple vinegar ¾, red wine ¼, cane sugar 20 g/L). Control methods based on the activity of volatile compounds, such as mass trapping and "attract and kill," are among the most promising methods to control SWD (Grassi *et al*., 2014). Traps baited with a combination of apple cider vinegar, red wine and sugar gave the best results when they were inoculated with lactic acid bacteria; SWD showed sensitivity to certain molecules produced by the degradation of the sugars present after malolactic fermentation (Guzzon *et al*., 2014).

6.2 Materials and methods

To compare the effect of novel formulations of Droskidrink (DD) on fly capture, standard DD mixture was compared to DD variants containing bacterial strains that are used in malolactic fermentation. In this experiment, the standard DD mixture contained 20 g of sugar dissolved in 750 mL apple cider vinegar (Sysco Corporation, Houston, Texas), 250 mL Merlot red wine (Peter Vella, Modesta, California), and a drop of soap as a surfactant to prevent captured flies from escaping. Separate DD treatments were inoculated with two strains of the bacteria *Oenococcus oeni* (Garvie) Dicks *et al.* (Enoferm Beta Lallemand, Enoferm Alpha Lallemand).

6.2.1 Fermentation in open field

In the first experiment, the fermentation process occurred in the field. Eight treatments (Tab. 1) were tested. Treatment A was composed of DD inoculated with 0.5 g/L of Enoferm Beta. For this treatment, pH was raised to 3.8 by using KOH (monohydrate granular AR [ARS]) in order to create an optimum environment for colonization and reproduction of the bacteria. Treatment B was standard DD inoculated with 0.5 g/L of Enoferm Alpha, with pH adjusted to 3.8 using KOH. For Treatment C, the same protocol was used as in Treatment A, with an additional 1.0 g /L of citric acid (pellets AR [ARS]) added to the mixture. Adding citric acid to the mixture results in malolactic fermentation that leads to increased production of acetoin, diacetyl and 2,3-butanediol (Lonvaud-Funel, 1999). These compounds might improve attraction of SWD. Treatment D represented the control standard DD bait. Treatment E was composed of normal bait DD with pH increased to 3.8 by using KOH. Treatment F was the commercially developed Cha-Landolt bait. Treatments G and H had the same composition as Treatment A, but the trap designs were different.

Treatment	Trap
A= Droskidrink, Enoferm Beta, KOH, soap.	Cup
B= Droskidrink, Enoferm Alpha, KOH, soap.	Cup
C= Droskidrink, Enoferm Beta, citric acid, KOH, soap.	Cup
D= Droskidrink, soap.	Cup
E= Droskidrink, KOH, soap.	Cup
F= Cha-Landolt solution.	Cup
G= Droskidrink, Enoferm Beta, KOH, soap.	Delta
H= Droskidrink, Enoferm Beta, KOH, soap.	Delta

Tab. 1 - Treatments used to test the efficiency of different baits, bait dipensers and traps.

For Treatments A, B, C, D, E, and F, identical traps were used. For these treatments, a red plastic cup (532.3 mL) with white lid was used. Polystyrene cups were placed inside each cup, to provide a barrier for temperature change. To provide access for SWD, six holes (0.5 cm diameter) were punched into each cup. Cups were filled with approximately 200 mL of the appropriate mixtures as described above.

Corrugated plastic Delta traps (Suterra, Bend, Oregon) containing a white sticky card were used for Treatments G and H. The Delta traps were modified with an open hole at the bottom centre of the traps. The head of a bottle (Camelbak, Petaluma, California) containing Treatment A was inserted inside the hole of each modified Delta trap. This bottle contained a pressure valve that allowed for the release of volatile compounds during fermentation. Treatments G and H were distinguished by the different type of bottle used in the Delta trap. For treatment G, insulated water bottles were used, whereas for Treatment H, the water bottles were not insulated. All traps were tested with the aim to search a new model trap, which could achieve even better results.

The experiments were conducted in a blueberry field site in Salem, Oregon (44°54'34"N; 123°06'51"W). The traps were placed randomly, at a distance of about 10 meters apart in the row, were replicated 4 times, and traps were placed every 3 rows. The experiment was conducted in the centre of the blueberry field, avoiding the perimeters, so as to ensure a homogenous environment.

The experiment was conducted over four weeks (from August 28 to September 25), and the traps were checked on a weekly basis. The mixtures and traps were filtered and contents returned to the lab in 70% EtOH. Sticky cards (Treatments G and H) were covered by plastic film and transported to the laboratory; male and female SWD were counted using a dissecting microscope.

As previously mentioned, treatments inoculated with bacteria were left in the field for the four-week duration of the experiment, while Treatments D and E were replaced with fresh bait every week , the temperatures of the bait of Treatments A, B, C, D, G and H, were monitored, so as to check if the conditions were ideal for fermentation.

6.2.2 Fermentation in the laboratory under controlled temperatures and different trap design and bait assessment

The second stage of the work was characterized by the replacement of the liquid bait on a weekly basis for all treatments. In addition, the amount of bacterial inoculum used for the preparation of the treatments was reduced considerably from 0.5 g to 0.2 g/L, because in a controlled environment, treatments had lower risk of contamination, and moreover, all were generated under optimal conditions to ensure colonization by the bacteria and the resulting malolactic fermentation. The room temperature was controlled at 22 ± 2°C. Eight treatments (Tab. 1) were tested. The same traps were used for Treatments A, B, C, D, E, and F, whereas the traps used for Treatments G and H were significantly changed. The plastic Delta traps were modified to allow insertion of small 30 mL cups (Dart Container Corporation, Mason, Michigan) in the centre, while the water bottles were eliminated. For Treatment G about 20 mL of DD (Treatment A) were used, then the cups were closed with specially prepared covers to exclude entry of insects, but which would allow the exit of volatile compounds. For Treatment H, approximately 15 mL of DD were used, 2 cotton balls were inserted inside the cups, and no lids were used.

The same blueberry field was used for the second experiment, traps were spaced apart and randomized as was done in the first experiment.

The experiment was conducted over 4 weeks (from 25 September to 23 October), traps were checked on a weekly basis, and the composition of the traps was identified in the laboratory.

6.2.3 New trap design

The last phase of this work involved the assessment and development of a different kind of trap, and the improvement of those traps already used in previous experiments. With this test, it was decided to assess the possible power of attraction, and whether this new model of the trap could further enhance the results that were achieved by the mixtures of DD inoculated with bacteria.

The experiment involved the comparison of 6 treatments (Tab. 2). Treatments A, B and C were obtained with the same preparations as in the previous experiment, but in this third experiment, the test included the use of the most promising trap from the previous studies: the plastic Delta trap with 30 mL cup inside. This cup was equipped with 2 cotton balls and 15 mL of the test mixture. While Treatment D was always composed of the same trap, but it was used as normal DD control. Treatments E and F used red plastic cups (532.3 mL) with white lid. Treatment E was baited with DD, while Treatment F was baited with the commercial Cha-Landolt solution. The trial lasted seven weeks and took place in the period between October 30 and December 18, during which the temperatures were usually low and, consequently, the population of SWD was also reduced relative to the periods of the previous experiments.

Treatment	Trap
A= Droskidrink, Enoferm Beta, KOH, soap.	Delta
B= Droskidrink, Enoferm Alpha, KOH, soap.	Delta
C= Droskidrink, Enoferm Beta, citric acid, KOH, soap.	Delta
D= Droskidrink, soap.	Delta
E= Droskidrink, soap.	Cup
F= Cha-Landolt solution.	Cup

Tab. 2 - Treatments used to test the efficiency of different baits and traps.

Also in this case, as in previous experiments, traps were checked weekly and the determination of the individuals took place under the dissecting microscope in the laboratory.

6.3 Results and discussion

6.3.1 Fermentation in open field

In the first experiment the mixture inoculated with strains of *O. oeni* was left in the field throughout the period of the test. This helped to assess the ability of bacteria to perform malolactic fermentation directly under field conditions. In an attempt to create optimal temperatures for malolactic fermentation (20°C), styrofoam cups were used to buffer against temperature changes. The temperatures of the liquid bait were monitored with portable data loggers (HOBO pendant loggers, Onset Computer Corporation, Bourne, MA). The results showed that the mixtures inoculated with bacteria (Treatments A, B, C) were less attractive than standard DD (Treatment D) and DD pH adjusted to 3.8 (Treatment E). Although the inoculated treatments did capture decent numbers of flies, the reason underlying their poor performance relative to uninoculated treatments may be linked to the temperatures reached by the liquid during the night and during the hottest hours of the day. The temperatures of the mixtures fluctuated as much as 50°C over the course of the 4-week trial, reaching a minimum temperature of 10°C and a maximum temperature of more than 60°C. These temperatures are far outside the range required by bacteria to carry out malolactic fermentation.

During testing, it was noted that some treatments experienced fluctuations in the number of catches over the weeks of testing. In particular, the increase showed very interesting result obtained from Treatment D. The catch obtained by using this treatment showed appreciable and significant increases (Fig. 4) throughout the experimental period. These increases in catches may be linked to the weekly replacement of the liquid bait. This confirms, once again, the importance of microbial activity for the attractiveness of the mixture. In fact, in the treatments inoculated with bacteria and left in the field for the entire period of the experiment, the temperature reached by the liquid bait certainly inhibited the development of bacterial flora, especially for Treatments A and C that maintained a fairly constant number of catches over the 4-week experimental period. Treatment B showed a significant increase in catches (Fig. 4) over the weeks of testing. Thus, in this test it seems clear that the bacteria inoculated in Treatment B are better adapted to high temperatures. Based on the number of flies caught in the traps, they are disappointing both in respect to the treatment A and C against Treatment D. Even Treatment F showed significant increases in the number of catches from a statistical point of view (Fig. 1). However, if catches remain insufficient, use of this type of bait cannot be justified. The excellent results obtained from Treatment D are probably due to the weekly replacement of liquid bait, which probably made it possible to maintain a higher concentration of autochthonous bacteria within the liquid bait.

As for treatments G and H, which included the use of DD inoculated with bacterial strains, but using a different model of the trap, numbers of captured flies were disappointing (Fig. 1).

6.3.2 Fermentation in the laboratory under controlled temperature and different trap design and bait assessment

Given the progress achieved in the first experiment, the methodology was adapted in second trial to replace all attractants weekly.

The results obtained with the second phase of the experiment shows that mixtures inoculated with bacteria, and in particular Treatment A, had better capture than in the first trial. In this second round of field trials, Treatment A had the highest number of the total catch during the 4-week trial. Moreover, this mixture was found achieve better results not only for the total of catches made over the 4 weeks of testing, but also for every individual week. The data obtained confirm once again, the important action of microbial activity and particularly of lactic acid bacteria to increase the attractiveness of the base DD mix. In fact, unlike the first cycle of tests, the mixtures were maintained in the laboratory at a temperature that allowed the microbial component to produce lactic acid capable of attracting SWD during fermentation. Treatment A turned out to be significantly more attractive than Treatment F (Fig. 2), while it appears to be more attractive than Treatment D (Fig. 2), though not in significant ways.

Compared to the previous test, Treatments G and H showed an important increase of catches. The effective capture that characterizes this model of trap bodes well for its use in future monitoring efforts. In addition, Treatment H showed a significant increase in catches from week to week (Fig. 5). Catches increased relative to the total number of flies captured as the temperature decreased and the population of SWD fell. Indeed, Treatment H had the highest number of catches in the final week (Fig. 5), when weather conditions were becoming prohibitive for fly activity as opposed to survival because the fly population normally does not die out in the Western Oregon winter climate.

For these reasons, it was decided to try the modified Delta trap with all inoculated mixtures of standard DD.

6.3.3 New trap design

For the third and final field test, the modified Delta trap (as it was used in Treatment H of the second field study) was evaluated using different modifications of DD mixture. The results bode well for further improvement of the trap. Mixtures inoculated with lactic acid bacteria, in fact, provided excellent results throughout the 7-week trial in this experiment. The best results were achieved with the use of Treatment A, but all treatments inoculated with bacteria outperformed the control treatments D and F (Fig. 3).

The new trap design was compared with the standard “cup” trap that was used during the two previous tests. Also in this case, the numbers of SWD trapped with the new design were significantly greater than the numbers trapped in the standard cup (as a control was used the mixture attractiveness DD, Treatment D on the two models of traps) over the course of 7 weeks (Fig. 3). Positive confirmations have occurred with regard to the number of catches, when the thermal conditions are at the limit of the tolerance for SWD. Another interesting fact revealed in this test, unlike the previous two, is linked to the catch of female individuals, in fact, these were found to be much more abundant compared to males.

Further confirmation of a more rapid method of identification and counting of insects was obtained during this test. In fact, the estimated time for the control of this new trap model was about ⅓ lower than the traps cup.

6.4 Conclusions

In conclusion, it was confirmed that, during the whole period of experimentation, the malolactic fermentation operated by lactic acid bacteria strains added to variants of DD, appears to be instrumental in boosting catches of SWD. It is also clear that, in addition to all other limiting factors such as pH, concentration of SO_2 etc., certain temperatures must be maintained to ensure that the fermentation takes place in an adequate manner. For this reason, it is appropriate that the mixtures, once prepared, are kept in a place with controlled temperature and that they are maintained so as not to suffer any contamination by other microbial species that could trigger undesirable fermentations, such as what may occur when leaving the liquid bait in the field for several weeks. Also from the results obtained it is clear that the bacterial strain Enoferm Beta appears to provide the best results, and that the addition of citric acid is a limiting factor and not an improvement for this kind of bait.

Another factor to be taken into account is the new trap design. This fact could be functional for monitoring, because of the speed with which it takes to place the monitoring of catches compared to installing the cup traps. But above all, this new trap design could be very important if used as a component of mass trapping. The characteristics of the trap demonstrated during the trial leave no doubt about its great potential during the fall and winter seasons when populations of SWD are low. Catches of relatively many individuals during this time period could lead to a substantial reduction in the number of individuals (especially females) of SWD in the spring and summer seasons.

These traps could be further improved by exploiting the intermediate characteristics of the two trap designs, so that only one trap would be even more attractive and able to guarantee excellent levels of catches during all seasons.

6.5 Figures

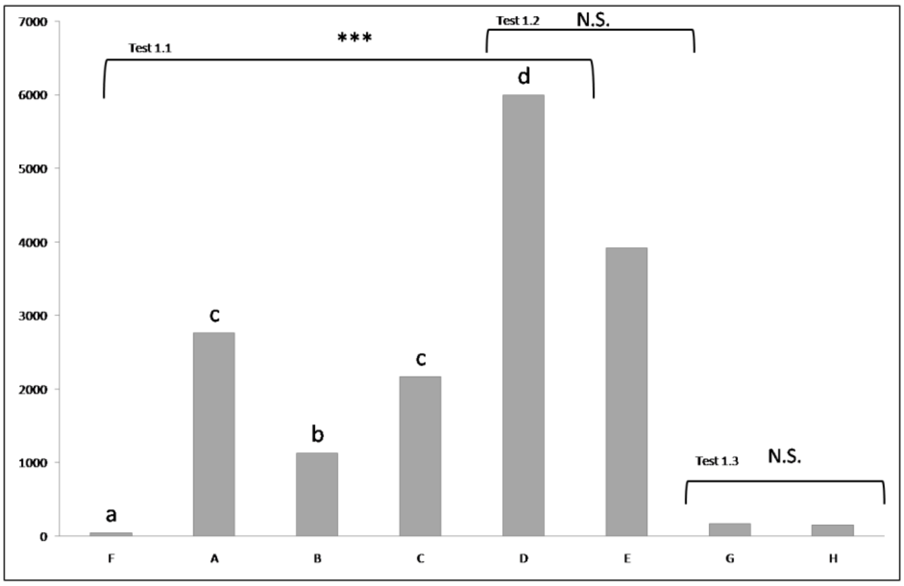

Fig. 1 - Total catches of *D. suzukii* referred to a 4-weeks period, from August 28 to September 25.

F= Cha-Landolt bait; A= DD + E. β; B= DD + E. α; C= DD + E. β + citric acid; D= DD; E= DD pH 3.8; for each treatment was used the cup trap; G= DD + E. β; Delta trap with bottle insulated. H= DD + E. β; Delta trap with bottle not insulated.

Different letters indicate significant differences (Siegel & Castellan, 1988) after post hoc Friedman test with replicas.

Test 1.1: differences between different treatments adding with bacteria and control ($F=7$; $P<0.001$; d.f.= 98.4). Test 1.2: difference between Droskidrink and Droskidrink pH adjusted ($F=1$; $P<0.05$ N.S.; d.f.= 0.75). Test 1.3: differences between different design trap ($F=1$; $P<0.05$ N.S.; d.f.= 1.05). ***($P<0.001$), **($P<0.01$), *($P<0.05$), N.S. (no statistical differences).

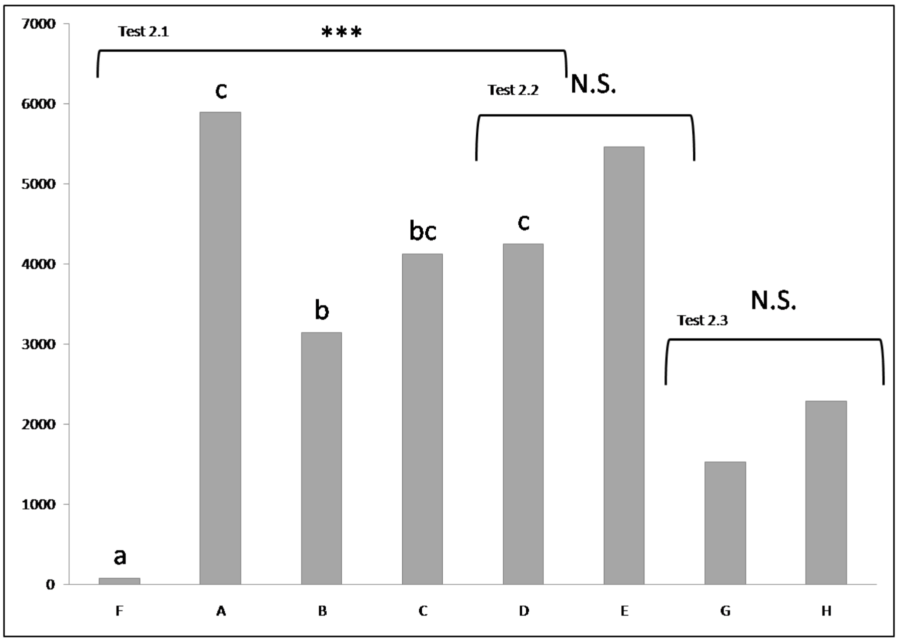

Fig. 2 - Total catches of *D. suzukii* referred to a 4-weeks period, from September 25 to October 23.

F= Cha-Landolt bait; A= DD + E. β; B= DD + E. α: C= DD + E. β + citric acid; D= DD; E= DD pH 3.8, cup trap; G= DD + E. β, Delta trap without cotton balls; H= DD + E. β, Delta trap with cotton balls.

Different letters indicate significant differences (Siegel & Castellan, 1988) after post hoc Friedman test with replicas.

Test 2.1: differences between different treatments adding with bacteria and control ($F=7$; $P<0.001$; d.f.= 57). Test 2.2: difference between droskidrink and droskidrink pH adjusted ($F=1$; $P<0.05$ N.S.; d.f.= 0.08). Test 2.3: differences between different design trap ($F=1$; $P<0.01$; d.f.= 6.78). ***($P<0.001$); **($P<0.01$); *($P<0.05$); N.S. (no statistical differences).

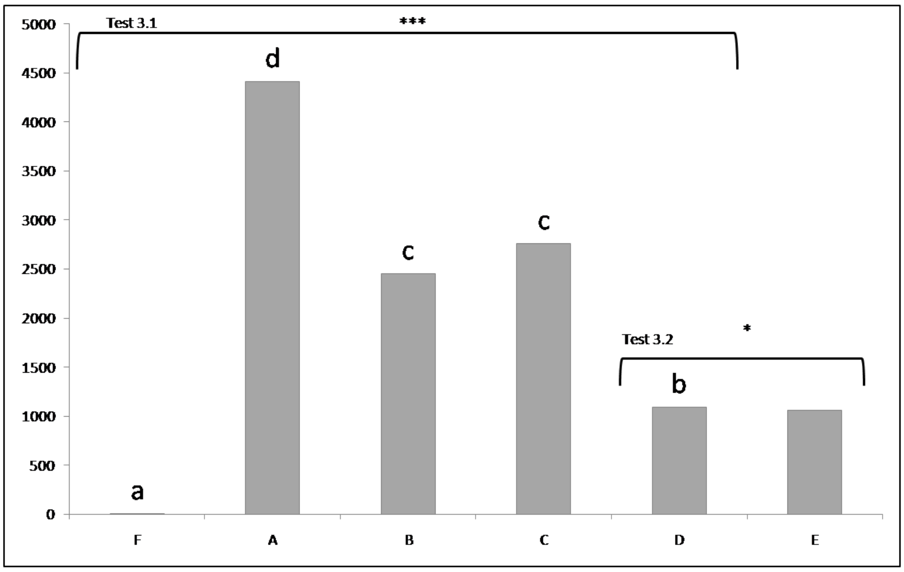

Fig. 3 - Total catches of *D. suzukii* referred to a 7-weeks period, from October 30 to December 18.

F= Cha-Landolt bait; A= DD + E. β; B= DD + E. α; C= DD + E. β + citric acid; D= DD, Delta trap with cotton balls; E= DD, cup trap.

Different letters indicate significant differences (Siegel & Castellan, 1988) after post hoc Friedman test with replicas.

Test 3.1: Differences between different treatments adding with bacteria and control (F=5; P<0.001; d.f.= 95.3). Test 3.2: differences between different trap designs (F=1; P<0.05; d.f.= 6.2). ***(P<0.001); **(P<0.01); *(P<0.05).

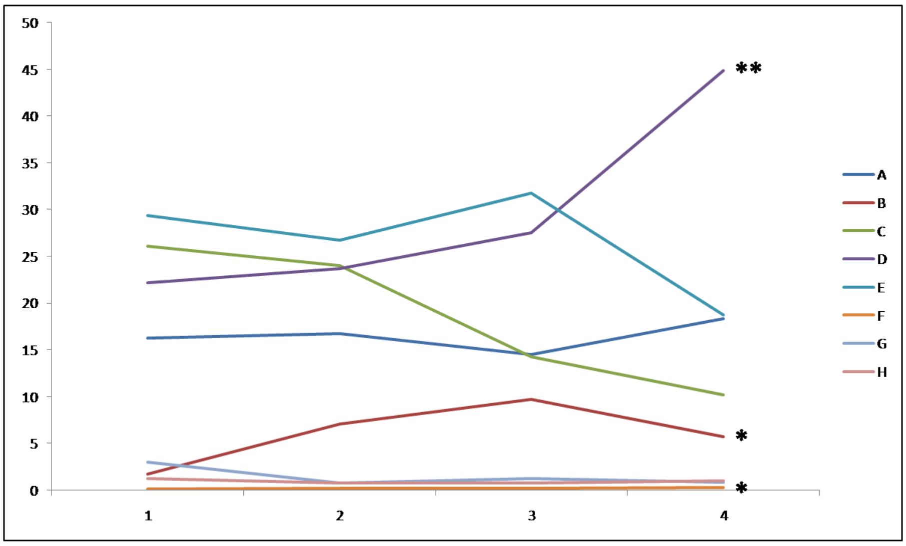

Fig. 4 - Trend of catches of *D. suzukii* (as percentage) during a 4-weeks period (from August 28 to September 25) observed in traps baited with different attractant mixtures.

F= Cha-Landolt bait; A= DD + E. β; B= DD + E. α; C= DD + E. β + citric acid; D= DD; E= DD pH 3.8, cup trap; G= DD + E. β, Delta trap with bottle insulated; H= DD + E. β, Delta trap with bottle not insulated.

Differences in the trend (either increasing or decreasing) catches of *D. suzukii* analysed by Jonkheere test; ***(P<0.001), **(P<0.01), *(P<0.05).

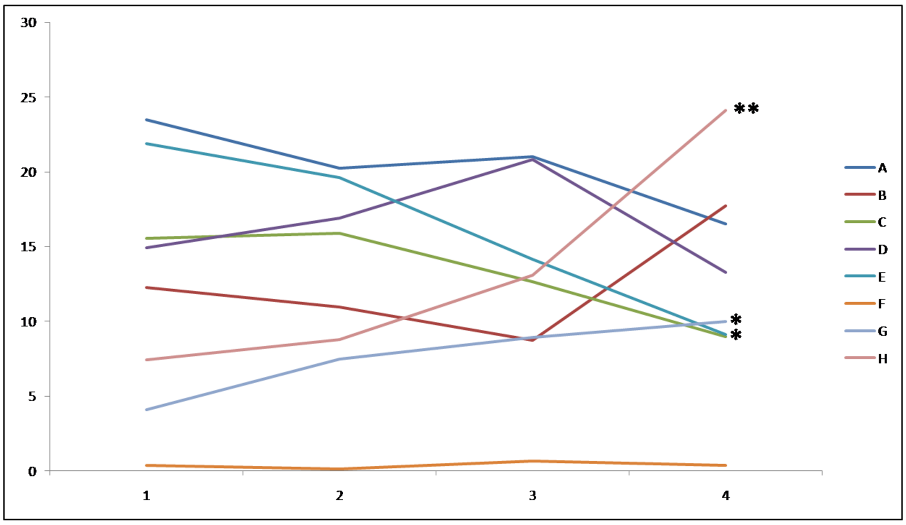

Fig. 5 - Trend of catches of *D. suzukii* (as percentage) during a 4-weeks period (from September 25 to October 23) observed in traps baited with different attractant mixtures.

F= Cha-Landolt bait; A= DD + E. β; B= DD + E. α; C= DD + E. β + citric acid; D= DD; E= DD pH 3.8, cup trap; G= DD + E. β, Delta trap without cotton balls; H= DD + E. β, Delta trap with cotton balls.

Differences in the trend (either increasing or decreasing) catches of *D. suzukii* analysed by Jonkheere test; ***(P<0.001), **(P<0.01), *(P<0.05).

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7. Final considerations

The results obtained from the present study appear to be very promising and make a further contribution to the research on *D. suzukii*. During the study, it was demonstrated that the technique for monitoring and mass trapping will be held in high esteem to reduce the damage caused by *D. suzukii*. These techniques have been already used for the control of *D. suzukii* showing interesting preliminary results and were based largely on the use of vinegar and wine mixtures and synthetic volatile compounds (Landolt *et al*., 2012). The Droskidrink (apple cider vinegar 75%, red wine 25% and brown sugar 20g /L) attractant mixture seemed to be the one that can guarantee the best results, at least in Italy (Grassi *et al*., 2015). By the present work, it was therefore possible to evaluate the importance of the biological component of the mixture Droskidrink. It is well known that the Drosophilidae have attraction privileged to the wine and, in particular to the vinegar substrates deriving from the fermentation made by yeasts and bacteria. Accordingly, the thesis had as objective the study of the bacterial component present in the above mentioned mixture, since its effect had not yet considered, while the importance of the metabolites released during the fermentation produced by yeast for *D. suzukii* attraction was already taken into account.

The preliminary phase was conducted in the Trentino Alto Adige Region, that have already faced considerable damage by *D. suzukii* (Cini *et al*., 2012). We worked on the characterization of bacterial species already present in the Droskidrink mixture. The trials have been held both in the field and in the laboratory conditions, and due to these tests it was possible to identify different species of lactic acid bacteria, in particular *O. oeni* which was the species eliciting the best catching performances. Such experiments have also shown that the treatment of these baits with antibiotics or microfiltration, which hence deprived the microbiological components, reduced dramatically the number of catches. This evidence emphasized in unequivocal manner, the importance of microorganisms producing volatile compounds by fermentation for the attractiveness of the mixture Droskidrink to *D. suzukii*.

After the characterization of the *O. oeni* strains, we focused our efforts in the control and manipulations of the physical and chemical factors which potentially may inhibit the metabolism of these bacteria, with the aim to further improve the field performance. During this second phase of work, we were able to obtain important information that allowed us to confirm and improve the results obtained previously.

Another essential step was to confirm the importance of *O. oeni*, and prove its biological activity towards *D. suzukii* in different environmental conditions. As a matter of fact, in the experiments carried out in a blueberry orchard in Salem (Oregon, the United States) at Oregon State University excellent results have been obtained. These results further supported the accumulating evidence of the huge potential that this bacterial species as *D. suzukii* bait shown in Italy. One of the crucial factors behind the improved outcomes observed in the trials performed in Oregon was that the bacterial fermentations within the liquid baits were mainly made in laboratory conditions and not in the field. This has allowed us to overcome the problems previously found that had prevented the total reliability of this strategy in the initial stage.

During the period at the Oregon State University, much attention has been made to the realization of a trap able to improve the efficacy of this type of control. The use of a trap that would ensure more easily release of volatile compounds, so as to reach a greater area has been a very important key that has allowed us to get a good combined effect between the trap and the mixture attractiveness. One of the most important results was obtained in this work was design of an innovative trap architecture that triggered a more powerful attraction and enabled us to capture a much greater number of *D. suzukii* with respect to other tools. In particular, there has been a consistent capture of female individuals during the colder periods of the *D. suzukii* flight season comparing to other traps. Moreover, the time needed for identification and count of the trapped individuals is practically halved by the use of this new trap.

For the reasons listed above, we believe that the present work will give a relevant contribution to further improve all the monitoring and control approaches based on attractive baits either for *D. suzukii* or could be a model for other insect pests.

Indeed, our results, especially off-season in relatively cold climatic conditions, suggest that the use of the *O. oeni*-baited trap may provide an important contribution for a significant reduction of *D. suzukii* population. We advocate that population control methods based on behavior manipulation and applicable at a wide territorial scale, such as mass-trapping, attract-and-kill and push-and-pull, should be maximized for example close to winter shelter areas (reservoir during diapause) as well as in wild environments flanking fruit growing areas susceptible to *D. suzukii* attacks. In addition, trapping control methods carried out before the start of the flowering and fruiting season and targeting mainly gravid females have the potential to be extremely effective because of the lack of competition between natural sources and bait traps.

Furthermore, a great advantage of the model of trap developed in this thesis is represented by the fast and easy service which would help to take timely actions for the use of insecticides and other control strategies against *D. suzukii*.

In conclusion, future studies are warranted in order to further ameliorate this new tool, considering the many factors contributing to the determination of the efficacy of the trap-blend. In perspective, the scale-up of the product for the biocontrol market in collaboration with companies that have already expressed their interest to the invention under patent registration is one of the main goal of our research team.

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