Administrative unit: Università degli Studi di Padova

Department: Territorio e Sistemi Agro-Forestali (TESAF)

PhD Program: Land, Environment, Resources, Health (LERH)

Batch: XXIX

Thesis title

IMPACT OF TRADITIONAL PESTICIDES AND NEW CONTROLLED RELEASE FORMULATIONS ON *Drosophila suzukii*

PhD Program Coordinator: Prof. Davide Matteo Pettenella

Supervisor: Prof. Mario Aristide Lenzi

Co-supervisor: Prof. Nicola Mori

PhD candidate: Rady Shawer

Università degli Studi di Padova

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Territorio e Sistemi Agro-Forestali (TESAF)

CORSO DI DOTTORATO DI RICERCA IN: TERRITORIO, AMBIENTE, RISORSE E SALUTE

CICLO: XXIX

TITOLO TESI

IMPACT OF TRADITIONAL PESTICIDES AND NEW CONTROLLED RELEASE FORMULATIONS ON *Drosophila suzukii*

Tesi redatta con il contributo finanziario del Erasmus Mundus Fatima Al-Fihiri

Coordinatore: Prof. Davide Matteo Pettenella

Supervisore: Prof. Mario Aristide Lenzi

Co-Supervisore: Prof. Nicola Mori

Dottorando: Rady Shawer

TABLE OF CONTENTS

LIST OF THE FIGURES

LIST OF THE TABLES

SMMARY

The spotted wing drosophila, *Drosophila suzukii*, has become a great threat to European and American producton of soft and stone fruits. Laboratory and field experiments were performed to identify and optimize effective strategies to protect fruit crops from *D. suzukii*. The first experiment aimed at identification of the efficacy of most commonly insecticides used in Italy to control *D. suzukii* on cherries. As well as different chemical control strategies applied at commercial cherry orchards in Verona province, North-Eastern Italy, during the growing seasons of 2013, 2014 and 2015 were carried out to determine whether insecticide-based management programs and their timing can provide sufficient crop protection. Moreover, the adherence of those applied pesticides to their maximum residue levels' (MRLs) requirements set in legal EU regulation for the marketed products was measured. Pre-treating cherries bioassay results revealed that pyrethroids (lambda-cyhalothrin, deltamethrin), spinosyns (spinosad, spinetoram), organophosphates (phosmet, dimethoate) and diamide (cyantraniliprole) were highly efficious, resulting in excellent (>90%) adult *D. suzukii* mortalities. Moreover, they were able to significantly suppress female fecundity, eggs laying and hatching, immature stages development, and adult emerging as well. Conversely, neonicotinoids (acetamiprid, thiamethoxam, thiacloprid, imidacloprid), *Beauveria bassiana*, and emamectin-benzoate caused unsatisfactory results. However, dipping infested-cherries bioassays suggested that cyantraniliprole, dimethoate and phosmet (Spada® WDG) can providemore than 10 days residual control for cherries. They caused complete activity, suppressing eggs hatching into larvae. Spinetoram and phosmet (Spada® 200 EC) provided good residual control. While, neonicotinoids (acetamiprid, thiamethoxam, thiacloprid), emamectin-benzoate and pyrethroids (lambda-cyhalothrin, deltamethrin) caused moderate impacts. Moreover, field results proved that two or three insecticide applications were insignificantly able to protect major cherry crops during three consecutive seasons of 2013, 2014 and 2015. Thus, effective *D. suzukii* control program can be achieved by timely of four applications of insecticides belonging different mode-of-action chemical groups. Except for dimethoate, all residue levels detected in cherries were lower than and completely adherence to their MRLs in force the European Union. It can be concluded that, spinosyns, diamides, organophosphates and pyrethroids may have an important role to protect cherry crop. Neonicotinoids and *Beauveria bassiana* suggested insignificant activities.

The second experiment identified the efficacy of most commonly insecticides registered in Italy against *D. suzukii* on strawberries. An open field trial at a commercial strawberry orchard in Verona province, North-Eastern Italy in 2014, and two laboratory bioassay trials in March and September 2015 were performed to determine whether chemical control strategy can provide significant crop protection

from *D. suzukii.* All strategies applied in the field trial significantly succeeded to decrease the damage of strawberries compared to the untreated plants either at 14 or 21 DAFA. The field findings suggested that two treatments of spinosad may provide a suffiecient strawberry-crop protection. Results of pretreating strawberries bioassays confirmed that pyrethroids, spinosyns, avermectin (emamectin-benzoate) and diamide (cyantraniliprole) caused excellent activities, providing adult mortality higher than 90 and 97% at 1 and 2 DAT, respectively. They also provided significant residual activities against *D. suzukii* life stages emerging after treatment. Incontrast, neonicotinoids, and *Beauveria bassiana* showed insignificantly results. The same trend of pretreating strawberries bioassay was repeated within the dipping infested-strawberries bioassay, except that acetamiprid showed good residual control against the *D. suzukii* individuals emerging.

The third experiment investigated potential of the entomopathogenic bacterium, *Photorhabdus luminescens* as biological agent on *D. suzukii*. Efficacy of *P. luminescens* was assessed at different bacterial cell concentrations against third-instar larvae and pupae of *D. suzukii* under laboratory conditions. Larvae at 4 DAT were significantly affected by bacterial treatments when fed toxins; dipping bioassay was less effective. Following oral and dipping bioassays at concentration of 3.5×10^{8} cells mL^{-1}**,** total mortalities of 97 and 87% were recorded, respectively. For pupae, the concentration of 3.5×10^{8} cells mL^{-1} caused a pupae mortality of 64 and 47%, and a total mortality of 100 and 73.33%, respectively in the direct-spray and dipping bioassays. It could be concluded that *P. luminescens* may play a vital role for managing *D. suzukii*.

The last work principally focused on preparing and characterizing new controlled release formulations of lambda-cyhalothrin to improve its biological performance against *D. suzukii*. Chitosan (CS) loaded lambda-cyhalothrin (LC) nanoparticles were prepared using the ionotropic gelation. Tripolyphosphate (TPP) and alginate (ALG) were used as crosslinking agents with CS. The optimum encapsulation efficiency (73.6%) and loading capacity (51.4%) were obtained by a 0.4% CS high molecular weight, 0.3% ALG cross-liking agent, and LC concentration of 1% and at stirring rate of 500 rpm. The nanoparticle size of this formulation was about 416 nm (polydispersity index: 0.447) and a zeta potential of -19.8. Transmission electron microscope (TEM) imaging showed a spherical, smooth and almost homogenous structure for nanoparticles. Fourier transform infrared (FTIR) spectroscopy confirmed linking between tripolyphosphoric groups of TPP with ammonium groups of chitosan, and between ALG and CS in the nanoparticles. The release profile of LC loaded CS nanoparticles cross-linked with TPP exhibited an initial burst release of about 30-40% in the first hour followed by

controlled release of 50-60% for the subsequent 5 hours. However, the release profile of LC loaded CS nanoparticles cross-linked with ALG showed a constant sustained release of the pesticide among the time of the release study. All prepared formulations significantly caused adult mortality at 2 and 16 HAT, with a best activity in the formulation of lowest nanoparticle size (278 nm). Most prepared controlled release formulations based on LC suggested activity greater than the efficacy of the commercialize insecticide, Karate-zeon® (lambda cyhalothrin 10% CS).

Chapter I

Introduction

1 Concept

Fruit and vegetables represent 17% of the EU's agricultural production, with a total production value estimated to be more than €50 billion, and the fruit and vegetables supply chain has an estimated turnover of more than €120 billion with over 550,000 employees and around 1.4 million growers (**Freshfel, 2012**). Pests cause fruit losses determined to be over €10 billion in revenue and 3 million tonnes of produce to the EU fruit industry (**Kenis and Branco, 2010)**.

The spotted winged Drosophila, *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), a new emerging pest that has been recently introduced into Europe, has been listed in the European Plant Protection Organization (EPPO) as a quarantine pest and major risk (**EPPO, 2013a**), posing a major challenge to fruit production. *Drosophila suzukii*, native to Southeast Asia, is an invasive, polyphagous and destructive fruit pest (**Delfinado and Hardy, 1975; Hauser, 2011)**, infesting many small fruits, especially from the genera *Vaccinium, Rubus, Prunus, Fragaria, Vitis, Ficus, Actinidia, Rhamnus, Lonicera, Sambucus* and also many others. It has many different plant species from 15 families as host plants **(Kanzawa, 1939; Grassi *et al*., 2012; Seljak, 2011; Walsh *et al*., 2011).** *Drosophila suzukii* preferably infests small and thin-skinned fruits including cherry, blueberry, raspberry, grape and strawberry **(Sasaki *et al*., 1995; Lee *et al*., 2011a; Lee *et al*., 2011b; Walsh *et al*., 2011; Bellamy *et al*. 2013; Cuthbertson *et al*., 2014)**, causing extensive economic damages as it is one of only two species of vinegar flies that is known to oviposit in healthy fruits (**Goodhue *et al*., 2011**), as opposed to most species of drosophilids which only infest overripe, fallen and rotting fruits. *D. suzukii* females possess a serrated ovipositor (figure 1 & 2), which enables them to lay eggs in healthy, ripening fruits (**Rossi Stacconi *et al.*, 2013**). Furthermore, it is spreading rapidly and economic losses are severe, thus it is rapidly becoming a pest of great concern (**Cini *et al*., 2012**). *D. suzukii* was introduced into North America by 2008, is now widespread in the eastern and western USA, as well as southern British Columbia, Canada (**Hauser, 2011**). It was notified for the first time in Trentino-Alto-Adige region, north-eastern Italy in September 2009 (**EPPO, 2010**), causing an economic damage. In 2010 and 2011, it caused losses over €8 million in fruit crops in Northern Italy (**AGW, 2012**) and more than €1.5 million for French strawberries in 2011 **(Agustí *et al*., 2011)**. A loss of just 10% (1.1 million tonnes) of the strawberry crop in the EU represents €50 million in lost income. These pests and diseases are also a major concern in countries outside Europe. When first introduced into the Pacific fruit growing regions of the USA, the estimated damage due to *D. suzukii* was over €400 million/year (**Goodhue *et al*., 2011**).

2 State of knowledge

2.1 Description

D. suzukii adults are little flies about 2.25–4.0 mm long with a wing span of 6–8 mm and red eyes (figs. 3, 4). Males are usually slightly smaller (2.0–3.5 mm) and more active than females (2.5–4.0 mm) (**Kanzawa, 1939**). Males have a dark spot on the leading top edge of each wing (fig. 3), while females possess a large serrated ovipositor (**CABI, 2016**) (figs. 1, 2, 4).

Eggs are milky-white, prismatic, semi-transparent and oval (0.62 X 0.18 mm wide). Each egg has two subapical respiratory filaments (**Kanzawa, 1939; EPPO, 2013b; CABI, 2016**) (fig. 5). The larvae are white to cream in colour with black mouthparts. They develop throughout three larval instars, with a third instar ranges in size on average 3.94 X 0.88 mm (**Kanzawa, 1939; CABI, 2016**) (fig. 6). Pupae are about 3.5 mm long by 1.2 mm wide, creamy getting reddish-brown in colour; and have two pedicels with small projections **(EPPO, 2013b)** (fig. 7).

2.2 Identity

Drosophila suzukii was observed for the first time in June 1916 in Yamacho, Higashi Yamanashi County, Yamanashi Prefecture, Japan (Kanzawa, 1935) infesting cherries. The species was first described in 1931 by Dr. Shounen Matsumura as Drosophila suzukii Matsumura, giving it the common name of cherry drosophila (Kanzawa, 1935; CABI, 2016). The scientific and common names, synonym, and taxonomic of the species tree are described (Kanzawa, 1935; EPPO, 2013b; CABI, 2016; EPPO Global Database, 2016) as follow:

Scientific name: *Drosophila suzukii* (Matsumura, 1931).

Common names: Cherry vinegar fly, spotted wing drosophila, cherry fruit fly, cherry drosophila, drosophile du cerisier (French), Kirschessigfliege (German), yīng táo gǔo yíng (Chinese).

Synonym: *Leucophenga suzukii* **Matsumura, 1931**.

Taxonomic position:

Domain: Eukaryota
Kingdom: Metazoa
Phylum: Arthropoda
Subphylum: Uniramia
Class: Insecta
Order: Diptera
Family: Drosophilidae
Genus: Drosophila
Species: *Drosophila suzukii*

Figure 1. *D. suzukii* ovipositor with strong black teeth (EPPO, 2013).

Figure 2. *D. suzukii* female with strongly sclerotized saw-like ovipositor (EPPO, 2013).

Figure 3. Male of *D. suzukii* (EPPO, 2013).

Figure 4. Female of *D. suzukii* (EPPO, 2013).

Figure 5. *D. suzukii* eggs (EPPO, 2013).

Figure 6. *D. suzukii* third instar larva (EPPO, 2013).

Figure 7. *D. suzukii* pupae (EPPO, 2013).

2.3 Biology and Ecology

The biology of *D. suzukii* was minutely described in 1935 by **Kanzawa (1935).** Previous studies have shown that *D. suzukii* is highly fertile since females lay on average almost 400 eggs (**Kanzawa, 1936; Kanzawa, 1939; Hooven *et al*., 2012**), has up to 15 generations per year, one generation can be as short as 10 days under ideal conditions (**EPPO, 2010**). Its lifespan lasts between 9-10 days and 21-25 days, respectively at 25°C and 15°C (**Kanzawa, 1939; CABI, 2016**). Females actively lay eggs in the ripening fruits using its serrated ovipositor, eggs hatch in 1-3 days. Oviposition usually takes place from April to November. Larvae mature in 3-13 days, overrunning three larval instars. Larvae can pupate either in the fruit or in the soil **(EPPO, 2010; CABI, 2016**). Pupation stage ends between 4 and 43 days **(CABI, 2016**). Adults can be active all the year over 10ºC, while it overwinters as adults (**Dalton *et al*., 2011; CABI, 2016**) in the hidden places. **Walsh (2009)** mentioned that adults emerge from overwintering once the temperature gets approximately 10 °C.

Kanzawa (1939) reported that males of *D. suzukii* get sterile at 30 °C and population size becomes limited in regions of that temperature. Moreover, the generations of *D. suzukii* hatched after September have lifespans longer than generations that hatched early in the year.

D. suzukii clearly prefers high humidity and moderate temperatures. Its establishment in Northern China and the Southern part of Hokkaido (Japan) has proved that it can be survival in low temperatures (**EPPO, 2010**).

Mitsui *et al.* (2010) and **Tochen *et al*. (2014)** did not note any reproductive activity from *D. suzukii* individuals that were kept their whole life in the laboratory at temperatures below 10°C. Likewise, low or no levels of reproduction were found at temperatures above 30°C **(Tochen *et al*., 2014; CABI, 2016).**

Kinjo *et al.* (2013) conducted an experiment to elucidate whether oviposition behavior varies among blueberry cultivars having different firmness of fruit, and found that more eggs tended to be laid in berries of cultivars possessing softer fruits than in those having firmer fruits, moreover softer fruits were more vulnerable to *D. suzukii* females than firmer fruits.

Emiljanowicz *et al*., (2014) studied different biological beheviours of a North American ecotype of *D. suzukii* under optimal laboratory conditions and indicated that the mean total lifespan was 86.1 ± 4.25 d, with a maximum value of 153.7 days female fecundity was 5.7 ± 0.24 eggs per day, with a mean total lifetime production of 635.6 eggs. The gross reproductive rate was 317.8 daughter eggs per female and the net reproductive rate was 240.4 daughter eggs per female. The intrinsic rate of natural increase

was 0.179. The stable age distribution was comprised of 51% larvae, 25% eggs, 16% pupae, and 8% adults. The sex ratio over time was about 1:1.

2.4 Host plant species

Drosophila suzukii is able to infest both cultivated and wild hosts (**EPPO, 2010)**. It has a wide host range and can attack many fruit crops, including small fruit crops, fruit trees and grapevine. Plants on which significant economic damage has been reported are *Actinidia* spp.(kiwis), *Arbutus unedo, Cornus* sp*., Cotoneaster lacteus, Diospyros kaki* (persimmons), *Eriobotrya japonica, Eugenia involucrate, Elaeagnus umbellate, Lindera benzoin, Lonicera caerulea, Fragaria anannassa* (strawberries), *Ficus carica* (figs), *Malus domestica* (apples), *Mahonia aquifolium, Morus sp., Rubus laciniatus* (evergreen blackberries), *Rubus spectabilis, Rubus ursinus* (marionberries), *Rubus armeniacus* (Himalayan blackberries), *Rubus loganobaccus* (loganberries), *Rubus idaeus* (raspberries), *Rubus ursinus* (marionberries), *Rubus fruticosus,* and other blackberries (*Rubus* spp.), *Prunus armeniaca* (apricots), *Prunus avium* (sweet cherries), *Prunus persica* (peaches), *Prunus laurocerasus, Prunus lusitanica, Prunus domestica* (plums), *Pyrus pyrifolia* (Asian pears), *Sambucus nigra, Sarcococca confuse, Symphoricarpos albus, Solanum dulcamara, Solanum villosum, Vitis vinifera* (table and wine grapes), and *Vaccinium* spp. (blueberries) (**EPPO, 2010; EPPO Global Database, 2010; CABI, 2016).**

2.5 Global distribution

Drosophila suzukii, a pest native to Asia, has recently colonized fruit growing areas in America and Europe since its discovery (**Hauser, 2011; Walsh *et al*., 2011; Cowles *et al*., 2015**) attracting great concern. It was observed for the first time in June 1916 in Yamacho, Japan (**Kanzawa, 1935**) infesting cherries. Furthermore, it was also noted in Japan in 1931, causing damage to several fruit crops, including cherries and blueberries. In 1980 it was reported from Hawaii, USA, without causing economic damage (**CABI, 2016**). In September 2008, it was first detected in a raspberry field in Santa Cruz, California, USA. After that, it had spread to more than 20 counties and subsequent detections followed in Oregon, Washington, Florida and British Columbia (Canada) in 2009 (**CABI, 2016**); in Utah, North Carolina, South Carolina, Michigan and Louisiana in 2010; and in Virginia, Montana, Wisconsin, Pennsylvania and New Jersey and Mexico in 2011 **(Lee *et al*., 2011b).** In 2012, it has rapidly invaded other nine States and a further two in 2013 (**CABI, 2016**). By the end of 2013, only eight US states were not invaded by *D. suzukii*: Arizona, Nevada, New Mexico, Oklahoma, Kansas, Nebraska, South Dakota and Wyoming **(Burrack *et al*., 2012; CABI, 2016**).

In Europe, it is reported for the first time by **Cini *et al.* (2012).** In 2008, flies of *D. suzukii* were firstly collected at the same time in Rasquera Province, Spain (**Calabria *et al.*, 2012**; **CABI, 2016**) and in the Tuscany region, Italy (**Raspi *et al.*, 2011; CABI, 2016**). Adults were then trapped in 2009 from other regions of Spain (Bellaterra, near Barcelona), France (Montpellier and Maritimes Alpes) and Italy (Alto-Adige, Trentino Province) (**Grassi *et al.*, 2009; Mandrin *et al.*, 2010; Calabria *et al.*, 2012; CABI, 2016**). By 2010-2011, it intensively invaded several regions and countries into Europe (**CABI, 2016**) including; other locations in Italy and France (**Cini *et al.*, 2012**; **Weydert *et al.*, 2012**); Switzerland (**Baroffio and Fisher, 2011**), Slovenia (**Seljiak, 2011**), Croatia (**Milek *et al.*, 2011**), Austria (**Lethmayer, 2011**), Germany (**Vogt *et al.*, 2012**), Belgium (**Mortelmans *et al.*, 2012**), the Netherlands (**NPPO, 2012**), the UK (**EPPO, 2012**) and Hungary (**Kiss *et al.*, 2013**). Following its initial discovery in Italy by 2009, a considerable damage to blueberries, raspberries, strawberries and blackberries in its distribution areas has occurred **(Grassi *et al.*, 2009; EPPO Global Database, 2010)**.

According to **EPPO Global Database (2016)**, the presence of *D. suzukii* has now been reported in at least 40 countries over the world, and 40 States in the USA (figure 8), probably as a result of the movement of infested plant material and fruit, as well as by natural spread. Its occurrence in South America has been reported for the first time in southern Brazil in 2012 (**Deprá *et al.*, 2014**).

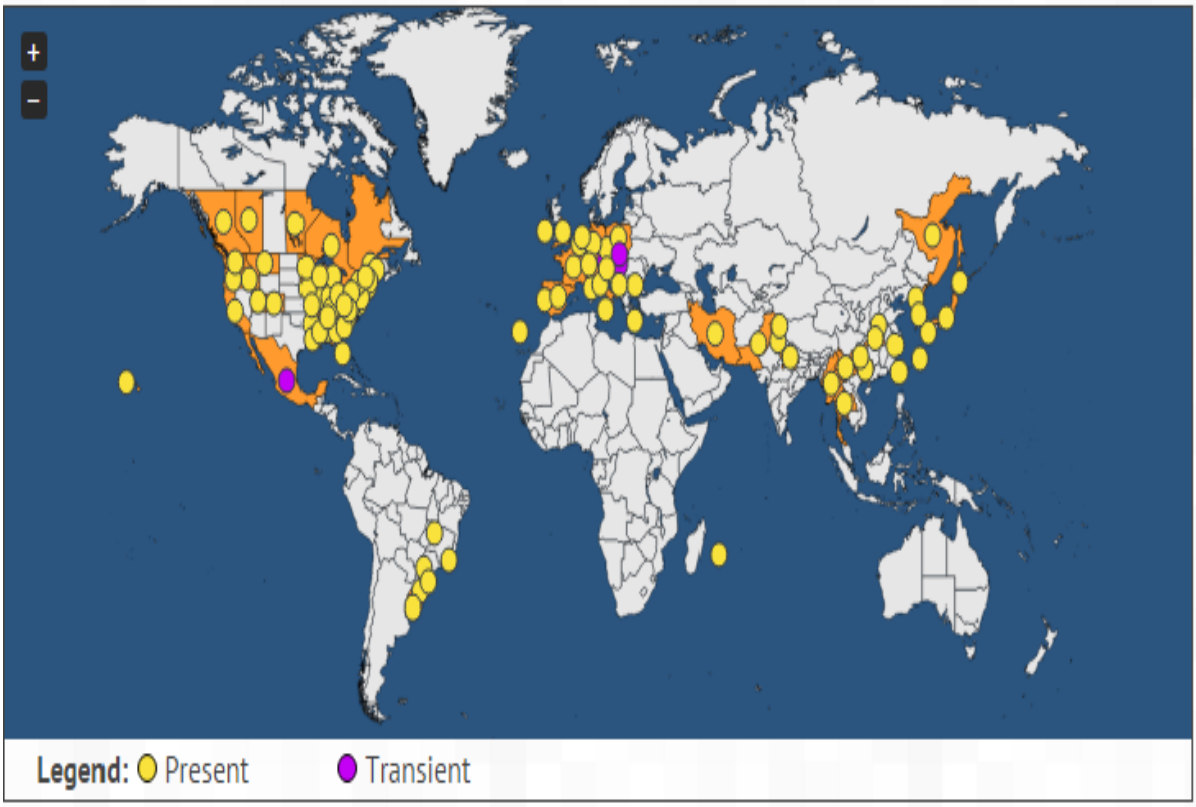


Figure 8. Map of the global distribution of *Drosophila suzukii* (EPPO Global Database, 2016).

2.6 Damage

Despite the majority of *Drosophila* species are not considered fruit pests as larvae only develop in damaged or rotting fruits, *D. suzukii* is one of the very few Drosophila species which can be able to feed and lay eggs on healthy ripening fruits making holes with its serrated ovipositor (figs. 9, 10). Infestation of fruit, therefore, reveals small scars or stings (figs. 11, 12) and indented soft spots or bruising on the fruit surface. Larvae rapidly feed on fruit pulp inside the fruit, collapsing and causing it to get soft, liquid, and rot quickly around the feeding site. Thereafter, secondary fungal or bacterial infections may contribute to further fruit deterioration (figs. 13, 14) (**EPPO global database, 2010**). Since SWD has established in the USA and Europe, considerable damage has been caused on a range of economic fruit crops. Yield losses have exceeded, sometimes, up to 90% on sweet cherries, 80% on strawberries and raspberries and 30-40% for blackberries and blueberries. In the USA, it caused losses for California, Oregon, and Washington at 40% for blueberries, 50% for caneberries, 33% for cherries and 20% for strawberries in 2008. A loss of just 20% account for $US 33.4 million in revenue losses for the strawberry crop, $US 56.7 million for blueberries, $US 156.6 million for caneberries and $US 174.8 million for cherries in California, Oregon, and Washington combined (**Bolda *et al*., 2010**). In Europe in 2010, losses of up to 80% were caused in strawberry crops of the Alpes Maritimes region of southern France (**private communication Reynaud, 2010**). In Northern Italy, it has caused up to 100% losses on many fruit crops and has also been reported to attack apricots, currants, figs and grapes (**Lee *et al.*, 2011a, Weydert *et al*., 2012**).

2.7 Control strategies

2.7.1 Monitoring

Trapping is highly considered the most efficient and quicker method for the first detection of *D. suzukii* flies. 250-750 ml plastic containers with four 5 mm-diameter holes on the side allowing flies to enter through these holes, can be designed and used as traps **(EPPO, 2010).** Traps should contain a preferable bait to attract the flies inside. It seems that the apple cider vinegar is considered to be highly effective, and the most practical bait to use.

Landolt *et al.* (2012) also indicated that chemicals in vinegar mixed with acetic acid, and chemicals in wine contained ethanol, are good attractants for *D. suzukii* since he found that the apple cider vinegar and the Merlot wine mixtures trapped flies higher than a mixture of acetic acid with ethanol.

Figure 9. *D. suzukii* oviposition holes in blueberry (T. Hueppelsheuser; EPPO, 2010).

Figure 10. *D. suzukii* - infested blueberry fruit with pupae (T. Hueppelsheuser; EPPO, 2010).

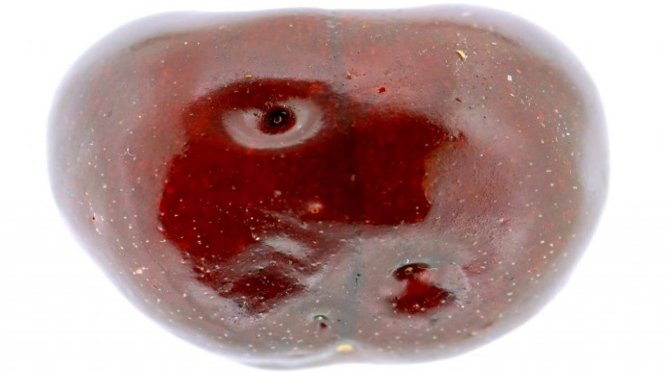


Figure 11. *D. suzukii*: oviposition scars on a cherry (Dr Martin Hauser; EPPO Global Database, 2016).

Figure 12. *D. suzukii*: oviposition scar on a cherry (Dr. Martin Hauser; EPPO Global Database, 2016).

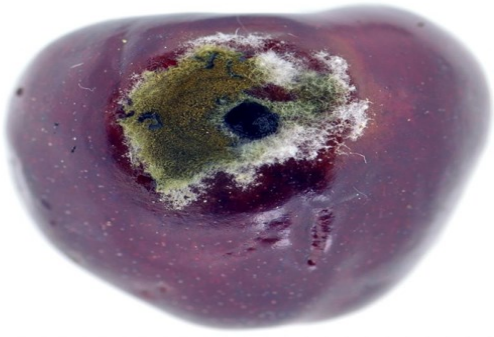


Figure 13. *D. suzukii* damage followed by secondary damage fungal rot. (Dr. Martin Hauser; EPPO Global Database, 2016).

Figure 14. *D. suzukii* on blueberry causing fruit drop (Matteo Maspero and Andrea Tantardini; EPPO Global Database, 2016).

Individuals of *D. suzukii* trapped with the SWD lure were greater compared with apple cider vinegar baits at Washington and New York sites, however were comparable with numbers of *D. suzukii* captured with a wine plus vinegar bait at a Germany site **(Cha *et al.*, 2013)**, suggesting that the four-component SWD chemical lure is an effective attractant for *D. suzukii* and could be used in place of

fermented food-type baits. Furthermore, **Cha *et al.* (2014)** proved that acetic acid, ethanol, acetone, and methanol are key olfactory cues for *D. suzukii* when attracted to wine and vinegar. They conclude that those four components which may be food-finding behavior leading flies to fermenting fruit in nature can be used as a highly attractive chemical lure for detecting and managing of *D. suzukii*.

Basoalto *et al.* (2013) concluded that *D. suzukii* flies were attracted to dark colours ranging from red to black. Likewise, flies captured in 237-ml plastic jars with ten 0.48-cm holes baiting with apple cider vinegar were significantly higher in jars with red or black than white caps.

Lee *et al.* (2013) studied the response of *D. suzukii* flies to trap physical features, assessing five colors, two bait surface areas, and a top and side position for the fly entry point at 16 sites spanning seven states in the USA. Apple cider vinegar was the standard bait in all trap types. They concluded that yellow-colored traps were more than clear, white, and black traps; and red traps captured more than clear traps. Moreover, the greater bait surface area (90 cm) was slightly more significant than the smaller area (40 cm). The traps with a side-mesh entry, with or without a protective rain tent, trapped more *D. suzukii* than the trap with a top-mesh entry and tent As well.

2.7.2 Biological control

Earlier studies evaluated the efficacy of *Phaenopria* spp. (Hymenoptera: Diapriidae) against *D. suzukii* under laboratory conditions, and insignificant results were obtained **(Kanzawa, 1939; CABI, 2016**). Recently several investigates regarding the potential performance of most available biological control agents for managing *D. suzukii* were performed both in North America and in Europe (**Brown *et al*., 2011; Chabert *et al*., 2012; Rossi-Stacconi *et al*., 2013; CABI, 2016**).

Several parasitoid species of *Ganaspis, Asobara*, and *Leptopilina* can attack *D. suzukii* larvae in fresh cherry fruits. While, Ganaspis was the only species able to parasitize *D. suzukii* in wild cherry fruits **(Kasuya *et al*. 2013; Nomano *et al*. 2015; Wiman *et al*., 2016).**

In France, under laboratory conditions, it is reported that two larval parasitoids, *Leptopilina heterotoma* and *Leptopilina boulardi*, and two pupal parasitoids, *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) and *Trichopria drosophilae* (Hymenoptera: Diapriidae) were successfully able to attack *D.suzukii*. Whereas, both *Leptopilina* parasitoids displayed high parasitism rates on *D. suzukii* (**Chabert *et al*., 2012; CABI, 2016**).

In field and laboratory studies performed in 2012 in Trento Province, Northern Italy, and Oregon in the Pacific Northwest of the USA, the generalist parasitoid, *P. vindemiae* (Rondani) attacked *D. suzukii* pupae **(Rossi-Stacconi *et al.*, 2013).** Furthermore, **Gabarra *et al*. (2015)** confirmed that the two parasitoid species; *P. vindemmiae* and *Trichopria* cf. *drosophilae* (Perkins) (Hymenoptera: Diapriidae) were able to parasitize *D. suzukii* pupae.

Several species of the bug genus *Orius*, a generalist predator, were observed feeding on *D. suzukii* larvae in backyard raspberries in the autumn of 2009 (**Walsh *et al*., 2011; CABI, 2016**); however, these results disagree with the recent observations of **Gabarra *et al*. (2015)** that the predator, *Orius laevigatus* (Fieber) (Hemiptera: Antochoridae) is able to prey only on *D. suzukii* eggs but not on larvae.

Whereas, the soil predator *Labidura riparia* Pallas (Dermaptera: Labiduridae) consumed *D. suzukii* larvae and pupae in laboratory tests. Likewise, the pupal ectoparassitoid *P. vindemiae* has also been noted in other studies related with *D. suzukii* in orchards and vineyards, both in the USA and in Europe **(Brown *et al*., 2011; Rossi-Stacconi *et al*. 2013; CABI, 2016).** Likewise, in both the USA and Italy, endosymbiotic bacterium strains, *Wolbachia* associated with *D. suzukii* have been found, anticipating the possibility of control of *D. suzukii* based on pathogens **(Siozios *et al*., 2013; Tochen *et al*., 2014; CABI, 2016**).

Within field surveys carried out in North Italy **(Mazzetto *et al*., 2016)**, six parasitoid species; *Leptopilina boulardi*, *L. heterotoma* (Hymenoptera: Figitidae), *Pachycrepoideus vindemiae* (Hymenoptera: Pteromalidae), *Trichopria* cf. *drosophilae* (Hymenoptera: Diapriidae), *Asobara tabida* (Hymenoptera: Braconidae), and *Spalangia erythromera* (Hymenoptera: Pteromalidae) were identified as parasitoids of drosophilids. A high mortality was recorded in SWD larvae exposed to parasitoid, *L. heterotoma* that failed to develop on *D. suzukii.* In contrast, *T.* cf. *drosophilae* developed successfully on *D. suzukii*, with no significant differences between the exotic and indigenous hosts

.2.7.3 Chemical control

Up to date, insecticides are considered the most adequate method for managing *D. suzukii*, with a wide range of available insecticides from different chemical groups, including spinosyns, organophosphates, pyrethroids and neonicotinoids. However, the larvae are developed within fruits, this assumes that the chemical control of *D. suzukii* should be mainly targeted adults, facing the challenge in

which of all insecticides have to be applied on the ripening fruits, which of course lead to increase residues in fruits (**Cini *et al*., 2012; CABI, 2016**). Moreover, a great deal of highly efficient broad-spectrum pesticides is being progressively banned. As a result, major commercial soft and stone fruits are seriously threatened since only a few natural products, with low or unknown activity against *D. suzukii,* are allowed (**Walsh *et al*., 2011**).

As it is a new emerging pest, *D. suzukii*, it is highly recommended to identify most available insecticides for their performance to manage this insect effectively in both conventional and organic production systems in different locations of its establishment. Moreover, several laboratory bioassays and field studies assessing a number of insecticides, belonging different modes of action chemical groups for managing *D. suzukii* were carried out. Overall, laboratory bioassays and field trials of certain products proved a same trend of results in which spinosyns, organophosphates and pyrethroids achieved high-level mortalities following direct spray bioassay, likewise provided range of 5–14 days of residual control of *D. suzukii* when they were exposed to fresh residue of those insecticides **(Bruck *et al.*, 2011).** Furthermore, similar results were then confirmed in semi-field trials in which those fresh residues of organophosphate, pyrethroid, and spinosyn insecticides caused a strong initial activity on flies, with varying levels of residual protection against fruit infestation **(Van Timmeren and Isaacs, 2013).** Exposing of *D. suzukii* adults to one-day field aged residue of malathion, bifenthrin and spinetoram caused high mortality levels as well (**Beers *et al*., 2011; Bruck *et al*., 2011**). Lambda-cyhalothrin performed well achieving an appropriate activity for controlling *D. suzukii* in Trentino Province, Italy (**Grassi *et al*., 2012; Cini *et al*., 2012).** Females of *D. suzukii* that were exposed to spinetoram, lambda-cyhalothrin, and carbaryl laid a number of eggs in cherries fewer than the untreated (**Beers *et al*., 2011; CABI, 2016**). Tolfenpyrad exhibited relatively acceptable activity following topical bioassay **(CABI, 2016**). An excellent potential of spinosad (100%) and chlorantraniliprole (93%) was performed upon *D. suzukii* flies following pre-dipping treatment for blueberries, however, all products were unsatisfactory when flies were directly sprayed **Cuthbertson *et al.* (2014)**. It seems that addition of sugar or sugar-yeast bait to insecticides significantly increases fly mortality **(Knight *et al*., 2013; Cowles *et al*. 2015).**

On the other hand, neonicotinoids, pyrethrin, azadiractin, and *Beauveria bassiana* were not satisfactory as adulticides (**Beers *et al*. 2011; Bruck *et al*., 2011; Gargani *et al.*, 2013; Van Timmeren and Isaacs, 2013; CABI, 2016**). However, neonicotinoids and the systemic organophosphate dimethoate seem to have control performance against eggs or larvae in fruit (**Beers *et al*., 2011)**. Low and moderate mortalities of adults were achieved by cyazypyr, respectively at 16 and 40 hours after

exposure **(CABI, 2016**). However, the low residual activity of imidacloprid, acetamiprid (up to five days) and cyazypyr on fruit, the number of emerging adult significantly decreased because of their systemic property (**Beers *et al*., 2011; Van Timmeren and Isaacs, 2013; CABI, 2016**). Studies performed by **Beers *et al*. (2011)** support that two or more preharvest pesticide applications should be done for managing of *D. suzukii* in California cherry orchards.

3 Research justifications and objectives

The principal objective of this work was to look for effective strategies to protect fruit crops from *D. suzukii*.

To achieve this overall objective, the following specific and technical objectives were met through the delivery of a serious of interrelated studies over three years.

a. To identify the potential of compounds appropriate for use on fruit in Europe against *D. suzukii* on cherries and strawberries either in the laboratory or in field conditions.
b. To evaluate the potential of an entomopathogenic bacterium, *Photorhabdus luminescens* against *D. suzukii* as a biological control agent.
c. To prepare and investigate bioactivity of new controlled pesticide formulations against *D. suzukii*.

To deliver those stated objectives, different serial studies were consequently carried out over last three years, addressing all raised questions and research aspects. The sitting work can be divided into four main parts, each consists of one or group of experimental trials as follow:

The first part (chapter 2) aimed at; (i) identification of the performance of most commonly pesticides used in Italy for managing *D. suzukii* through laboratory bioassays on cherries. (ii) compare chemical control strategies applied at commercial cherry orchards in Verona Province, North-Eastern Italy during the growing seasons of 2013, 2014 and 2015 to determine whether insecticide-based management programs and their timing provide most significant control of *D. suzukii*. (iii) measurement the adherence of chemicals used to the maximum residue levels' (MRLs) requirements set in legal EU regulation for the marketed products. To meet these objectives, two laboratory experiments were performed in 2014 and 2015 to identify most available insecticides against *D. suzukii* through laboratory bioassays on cherries. Moreover, three open-field experiments were conducted during June and July of cherry growing seasons of 2013, 2014 and 2015. As well as, residue levels of chemicals used in field investigations were determined in cherries.

The second part (chapter 3) deals with determining the efficacy of most commonly chemicals used in Italy for managing *D. suzukii* on strawberries under laboratory and field conditions. Therefore, two

laboratory experiments were conducted against *D. suzukii* on strawberries in March and September 2015. As well, two field trials were performed at commercial strawberry orchards in Verona Province, North-Eastern Italy during the growing seasons of 2014 and 2015.

Part three (chapter 4) investigated the virulence of the entomopathogenic bacterium, *Photorhabdus luminescens* against *D. suzukii* under laboratory conditions. The efficacy of *P. luminescens* as a biological agent was investigated for their insecticidal activity against *D. suzukii*. Oral and direct spray bioassays of *P. luminescens* bacterial cells against larvae and pupae of *D. suzukii* were tested.

The fourth part (chapter 5) principally focused on improving the biological performance of certain controlled release insecticide formulations on *D. suzukii*. Chitosan lambda-cyhalothrin nanoparticles were prepared by the ionotropic gelation technique to solve the problem of poor solubility and improve permeability, and bioactivity of the lambda-cyhalothrin pesticide. As well as, the bioactivity of prepared nanoparticles against *D. suzukii* were evaluated.

Chapter II

Chemical control of *Drosophila suzukii* and insecticide residues on cherries

---

Presented in the International Plant Protection Congress (IPPC), Berlin, Germany as:

R. Shawer, L. Tonina1, E. Gariberti, N. Mori (2015). Efficacy of insecticides against *Drosophila suzukii* on cherries, International Plant Protection Congress, 24-27 August, 2015, Berlin. Book of abstracts. http://www.ippc2015.de/.

Abstract

Since its emergence into Europe and USA, **s**potted wing drosophila (SWD), *Drosophila suzukii*, economic damages to commercial soft and stone fruits has been substantially caused. Cherry represents a very important rule in agriculture production and growers' revenue in Italy. However, it is highly susceptible to *D. suzukii* infestations. A wide range screening of available chemicals and field practical tactics for managing this serious pest are strongly recommended. Three open-field experiments in Marano di Valpolicella**,** Verona, North-Eastern Italy, during June and July of cherry-growing seasons of 2013, 2014 and 2015; in addition two laboratory experiments in 2014 and 2015 were performed to identify the efficacy of most commonly insecticides used in Italy and to determine whether chemical control strategies and their application timing can provide efficacious SWD control. As well as, residue levels of chemicals used in field investigations were determined in cherries to measure their adherence to the maximum residue level (MRLs) requirements set in legal EU regulation for the marketed products. Pyrethroids (lambda-cyhalothrin, deltamethrin), spinosyns (spinosad, spinetoram), organophosphates (phosmet, dimethoate) and diamide (cyantraniliprole) were highly efficious following the pretreating cherries bioassay, resulting in excellent (>90%) adult *D. suzukii* mortalities. Moreover, they were able to significantly suppress female fecundity, eggs laying and hatching, immature stages development, and adult emerging. Conversely, neonicotinoids, *Beauveria bassiana*, and emamectin-benzoate indicated unsatisfactory results. Furthermore, dipping infested cherries bioassay revealed that cyantraniliprole, dimethoate and phosmet WDG caused more than 12 days residual control, since they were able to cause complete activity, preventing eggs hatching into larvae, this was followed by spinetoram and phosmet EC. Neonicotinoids, emamectin-benzoate and pyrethroids caused moderate impacts. Field results proved that two or three insecticide applications were insignificantly able to protect major cherry fruit crops during three consecutive seasons of 2013, 2014 and 2015. Thus, effective *D. suzukii* control program can be achieved by timely of four applications of insecticides belonging different mode-of-action chemical groups. Except for dimethoate, all residue levels detected in cherries were lower than and completely adherence to their MRLs in force the European Union. It can be concluded that, spinosyns, diamides, organophosphates and pyrethroids may have an important role in reducing infestations of *D. suzukii* in cherry orchards. Neonicotinoids and *Beauveria bassiana* proved insignificant activities.

1. Introduction

The spotted winged Drosophila, *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), a direct pest of sweet cherry, was notified for the first time in Trentino-Alto-Adige region, north-eastern Italy in September 2009 **(EPPO, 2010)**, infesting thin-skinned fruits including cherry, blueberry, raspberry, grape and strawberry **(Sasaki *et al*., 1995; Lee *et al*., 2011a; Lee *et al*., 2011b; Walsh *et al*., 2011, Bellamy *et al*. 2013; Cuthbertson *et al*., 2014**). Its overrunning into Europe and North America as a soft fruit pest in 2008 has caused crop losses of up to 50% **(Goodhue *et al*., 2011; Walsh et al., 2011; Cini et al., 2012; Cuthbertson et al., 2014**). In Northern Italy, losses to fruit crops were estimated to be over €8 million in 2010 and 2011. Moreover, In Trento, Italy, annul losses in small fruit production were assumed €3.3 m per year **(De Ros *et al*., 2013; Haye *et al*., 2016).**

Cherry, *Prunus avium* (L.), is greatly considered one of the most important fruit crops all over the world. Europe represents 36.7% of the global cherries production with a total quantity of 841338 tons in 2013. Italy is globally ranked the fourth cherries producer after Turkey, USA, and Iran, producing 131.2 thousand tons in 2013 **(FAO, 2015)**. Cherry represent a very important source for growers' revenue in Italy; however, it is at a significant risk from this pest **(Cuthbertson *et al*., 2014).**

In the USA, the SWD has been reported to cause damage to sweet cherries for the first time in southern Santa Clara and northern San Benito counties of California in May 2009 (**Beers *et al*., 2011**), resulting in severe losses for cherry production (estimated at 25% state-wide in 2009) **(EPPO Global Database, 2016)**. Consequently, it was detected in the primary cherry production region of Washington in mid-summer of 2010.

SWD attacks cherry fruits at the ripening stage (close to harvest) taking advantage of the strong serrated ovipositor of its females, puncturing the fruit skin and lay eggs in healthy and ripening fruits (**Rossi Stacconi *et al.*, 2013**). The fruit puncturing provides a gateway for secondary infections with bacteria and fungi pathogens or additional pests **(Louise *et al*. 1996; Walsh *et al*. 2011; Haye *et al*., 2016).** Thereafter, damage is mainly caused by larval feeding, resulting in the degradation of fruits (**EPPO Global Database, 2016; Haye *et al*., 2016)**. Given that the fruit damage is amplified by the development of bacteria and fungi on the pulp and the damage on recently-infested fruits can't be observed at the harvest time but only after 2-3 day of shelf life (following eggs hatching), this could reduce growers' ability to export their cherry crop. Seeing that, only one or two females SWD are enough to deteriorate the whole production. Thereupon, the damage threshold becomes very low,

virtually zero. Conversely, the cherry fruit fly, *Rhagoletis cerasi* attacks cherries from the time the cherries turn pale yellow, so growers are easily able to take notice of infested cherries, and apply management programs enough time before the harvest.

In most of all regions of its distribution, several studies on the efficacy of registered insecticides for managing *D. suzukii* have been undertaken, including laboratory-based assays along with field evaluations where experimental plots are treated and sampled for control of the fly **(Beers *et al*., 2011; Bruck *et al*., 2011; Van Timmeren and Isaacs, 2013; Cuthbertson *et al*., 2014; Haye et al., 2016**). A wide range of insecticides including pyrethroids (bifenthrin, beta-cyfluthrin, permethrin, zeta-cypermethrin), organophosphates (malathion, diazinon) and spinosyns (spinosad, spinetoram) caused excellent control of adult *D. suzukii* following direct application **(Beers *et al*., 2011; Bruck *et al*., 2011; Haye *et al*., 2016**); however, neonicotinoids and the systemic organophosphate dimethoate were be able to kill eggs or larvae in fruit **(Beers *et al*., 2011).** A semi-field bioassay trial confirmed that fresh residues of organophosphate, pyrethroid, and spinosyn insecticides have a strong initial activity on flies, with varying levels of residual protection against fruit infestation. The neonicotinoid insecticide, acetamiprid provided activity for up to five days; pyrethrum was less effective **(Van Timmeren and Isaacs, 2013).** Spinosad, chlorantraniliprole and several coded products were highly effective against SWD, following pre- or post-dipping treatment of blueberries **(Cuthbertson et al., 2014; AGS Cuthbertson unpublished data; Haye *et al*., 2016).** As well, spinetoram and dimethoate have also been screened for efficacy in Italian cherry orchards **(Profaizer *et al*., 2015).**

Italian cherry orchards are infested with *R. cerasi,* and thus growers used to spray cherry with only one insecticide application at 20 to 30 days before the harvest. Since SWD has established into Italian cherry, at least two pre-harvest insecticide treatments (close to harvest) have been required (**Bolda *et al*., 2010; Beers *et al*., 2011; Haviland and Beers, 2012).** The times of insecticide applications required to cover the whole period from the first capture of SWD till harvest complete can be increased to be five till eight depending on different factors **(Van Timmeren and Isaacs, 2013).**

Making the situation extremely problematic is that the use of chemical insecticides is limited by the high risk of residues in fruits, insect resistance development and negative impact on beneficials **(Stark and Banks, 2003; Desneux *et al*., 2007; Haviland and Beers, 2012)**. However, violations of MRLs for specific pesticides can render the fruit unmarketable **(Goodhue *et al*., 2011),** growers have little choice but to protect their crop from infestation (**Haviland and Beers, 2012**). **Haviland and Beers (2012)**

studied the adherence of six insecticides (with PHI_s ranging from 3 to 14 d.) applied at 7 or 21 days before the harvest in sweet cherry orchards in the USA, to their MRLs required for export by measuring their residue degradation curves. They concluded that lambda-cyhalothrin, spinosad and malathion have favorable characteristics for SWD control, allowing producers to incorporate the principles of efficacy, fruit susceptibility, and resistance management and still allows for the export of fruit to all major export markets.

However, a great deal of insecticide evaluations against SWD has been initiated in most major regions of its distribution, majority under laboratory conditions (**Van Timmeren *et al*., 2013)**, more approaches on a broader scale towards potential efficacy of these insecticides against SWD on cherries under open-field conditions, especially in Europe, along with timing of insecticide applications, commitment of used chemicals to their MRLs, insect resistance development and their effect on pollinators and natural enemies are still highly required before strong conclusions or recommendations to the fruit growing industry can be made (**Haye *et al*., 2016**). Therefore, the present study identified efficacy of most insecticides approved for use on cherries in Europe for control SWD under laboratory conditions. In addition to determine whether insecticide-based management programs applied at commercial cherry orchards under open-field Italian conditions, can provide most sufficient crop protection. Here we also aimed at improving our understanding of the insecticide application timing appropriate for SWD control and the adherence of those insecticides to the MRL requirements set in legal EU regulation for the marketed products.

2. Material and methods

2.1 Laboratory colony

Drosophila suzukii used in the experiments originated from wild specimens from Northern Italy, collected in the autumn of 2013. Male and female *D. suzukii* adults (mixed ages) were placed into 50 mL plastic culture vials (diameter 30 mm, length 115 mm) with ~ 15 mL artificial diet of *Drosophila melanogaster* (Bloomington Drosophila Stock Center, Indiana University). Diet components (75 g raw cornmeal, 17 g dry-yeast, 15 g sucrose, 12 g soybean meal, 5.6 g agar and water adjusted to 1000 ml) were thoroughly mixed and cooked for 20 minutes at about 100°C, then 5 mL-propionic acid was added at a temperature of less than 50°C and 15 mL of prepared diet poured into each plastic vial. Cultures were maintained in climate chambers at 23±1 C°, 70±10% RH and 16:8 h L:D regime. Wild *D. suzukii* were introduced into the colony on multiple occasions in 2014 and 2015 to ensure that the genetic make-up of the individuals screened in the laboratory was representative of the field population.

2.2 Laboratory bioassays

To identify the efficacy of insecticides registered for use on cherry in Italy, laboratory bioassays were performed using formulated products (Table 1). Trials were arranged in a randomized complete block design with five replications of each. Cherries used were collected from insecticides free plants covered with insect-proof nets in orchards located in Verona province, North-Eastern Italy.

2.2.1 Pre-treating cherries

In 2014 and 2015, cherries were first dipped in the in field-rates of all the products (50 cherries per product). They were then placed on 9cm diameter Petri dishes (5 fruits per dish) and allowed to air dry for 2 hours. Cherries dipped in water acted as controls. The petri dishes were then placed into 10 cm diameter plastic deli-pots with ventilated lids. Ten adult *D. suzukii* (5 male and 5 female) were then introduced to the cherries contained within the deli-pots. All were maintained in a CE cabinet at 23°C. Mortality of the introduced adult flies was assessed at 1 and 2 days after application (DAA). The cherries were maintained at 23°C for a further 10 days at which time adult fly emergence was determined. Eggs laying, larvae developing and flies' emerging were investigated. Female fecundity per day was calculated according to **Blümel and Hausdorf (2002)** as follow:

$$\text{Fecundity} = \left[\frac{\text{Deposited eggs no. after 48 h}}{\left[\frac{\text{intial female no.} + \text{survival female no. after 48 h}}{2}\right]}\right] 2day$$

$$\text{Deposited eggs/fruit} = \frac{\text{Sum number of (emerged larvae + pupae + adults)}}{\text{Total number of deposited eggs after 72 h}}$$

$$\%\text{Eggs hatching} = \frac{\text{Sum number of emerged larvae, pupae and adults}}{\text{Total number of deposited eggs after 72 h}}$$

$$\%\text{Immature stages} = \frac{\text{Sum number of emerged larvae and pupae}}{\text{Total number of deposited eggs after 72 h}}$$

$$\%\text{Adults emerging} = \frac{\text{Sum number of emerged adults}}{\text{Total number of deposited eggs after 72 h}}$$

2.2.2 Dipping infested cherries

In June 2015, according to **Cuthbertson *et al.* (2014)** previously infested cherries (400 in total infested for 72 hours within four bug-dorm cages containing each approximately 100 mixed-sex adult *D. suzukii*) were randomly dipped (full emersion) in field-rate concentrations of all the products. A water treatment acted as control. 25 cherries were dipped in each control product. After dipping, cherries were placed into 10 cm diameter ventilated plastic deli-pots and placed into a Controlled Environment (CE)

cabinet and incubated for 10 days at 23°C. The pots were then assessed for presence of adult flies and the cherries dissected to inspect for presence of larvae and/or pupae development.

Table 1. Insecticides used for controlling *D. suzukii* in 2014 and 2015 laboratory bioassays.

Active ingredient	Trade name	Formulation	Rate (AI hL^{-1})	IRAC MoA group
dimethoate [a,b]	Danadim®	40%EC	37.5 mL	Organophosphates (1B)
phosmet [a,b]	Spada® 200 EC	20% EC	60 mL	Organophosphates (1B)
phosmet [a,b]	Spada® WDG	25% WDG	250 g	Organophosphates (1B)
deltamethrin [a]	Decis EVO®	2.8% EW	50 mL	Pyrethroids (3A)
deltamethrin [a,b]	Meteor®	15.70% EC	80 mL	Pyrethroids (3A)
lambda cyhalothrin [a,b]	Karate Zeon®	10% CS	30 mL	Pyrethroids (3A)
pyrethrin[a]	Pyganic®	12.91% EC	250 mL	Pyrethrins (3A)
thiamethoxam [a,b]	Actara®	25% WG	35 g	Neonicotinoids (4A)
thiacloprid [a,b]	Calypso®	40% SC	20 mL	Neonicotinoids (4A)
acetamiprid [a, b]	Epik®	5% SL	120 mL	Neonicotinoids (4A)
imidacloprid [a]	Kohinor®	17.1% SL	50 mL	Neonicotinoids (4A)
spinosad [a,b]	Laser®	48% SC	30 mL	Spinosyns (5)
spinetoram [a,b]	Delegate®	25 WG	35 mL	Spinosyns (5)
cyantraniliprole [b]	Exirel®	10%SE	75 mL	Diamides (28)
Beauveria bassiana [a,b]	Naturalis®	7.16% SC	150 mL	Fungal entomopathogens[c]
paraffinc oil[a]	Ultra Fine Oil	98.8 % EC	2000 mL	Mineral Oil[c]

[a] Insecticides used in 2014-laboratory bioassays.
[b] Insecticides used in 2015-laboratory bioassays.
[c] Excluded from IRAC MoA classification.

2.3 Open-field trials

Three open-field experiments were set up during 2013, 2014 and 2015 growing seasons in cherry orchards located in Marano di Valpolicella, Verona, Veneto Region, North-Eastern Italy, in a typical cherry growing area. Due to the presence of many wild host plants of SWD in the surrounding area, the infestation level was very high. The plants, CV Giorgia, Ferrovia, Kordia, were trained open center tree system (rows spacing: 6m, plant spacing within the row: 4m) and the plant age was 13/18 years old. Considering that the fruits start to be attractive when fruits changing color (BBCH 81), the applications were made during the ripening period. Six, six, and five strategies were assessed, respectively in 2013, 2014 and 2015, using formulated insecticides products registered in Italy on cherry (Table 2). Each strategy consisted of at least zero, two, three or four insecticide-application treatments. The first pesticide application of each strategy was performed at 21, 21, and 28 DBH, exactly on 18, 4 and 3 June, respectively in 2013, 2014 and 2015 trials. A strategy for organic farmers was considered each year. Where, during the ripening period, two or three applications with spinosad were considered, followed by pyrethrins or *B. bassiana* applied at 4 DBH. At the application time, the pre harvest interval (PHI) of used insecticides was considered.

Trials were strictly designed according to EPPO guideline "Efficacy evaluation of insecticides PP1/281(1) *Drosophila suzukii*) (**EPPO, 2013a**). Untreated plots were randomized together with the other treatments, arranged with one way complete randomized blocks design with four replications. Each plot consisted in five-plant, plot size 94 m^2, treatment size 376 m^2. The equipment used for the foliar applications was a motorized sprayer, with 12 bar of pressure, the water volume used was 1000 L ha^{-1}.

The pest's presence was checked weekly from fruit enlargement phenological growth stage (BBCH 75) till one week after the end of the harvest period (BBCH 89). R. *cerasi* and *D. suzukii* presence was evaluated using yellow sticky trap and red trap lured with Droskydrink® (mixture of Apple cider vinegar, red vine and brown sugar) respectively for cherry fruit fly and spotted-wing drosophila adults, while eggs and larvae were sampled directly by cherry observations. Pest's infestation was evaluated during the harvest period at the first and the last fruit collection. At each sampling 100 ripe cherries per plot were observed directly in the field and % of damage by *R. cerasi* and *D. suzukii* larvae was recorded. Moreover the observed fruits were collected, dissected and processed in brine solution in order to extract the larvae. At harvest time the residue levels in cherries were determined according to (UNI EN 15662:2009 Method 360, 361), and were then compared to the MRLs in force the European Union (EU Pesticide database).

Table 2. Chemical control strategies evaluated in 2013, 2014 and 2015 for *D. suzukii* management in cherry crops.

Strategy	2013			2014			2015		
	Treatment*	Rate ha^{-1}	DBH[3]	Treatment*	Rate ha^{-1}	DBH	Treatment*	Rate ha^{-1}	DBH
1st	Untreated	-	-	Untreated	-	-	Untreated	-	-
2nd	spinosad	120	21	-	-	-	spinosad	480	28
	spinosad	120	14	spinosad	120	14	spinosad	480	14
	spinosad	120	7	spinosad	120	7	spinosad	480	7
	pyrethrin	322.8	4	pyrethrin	322.8	4	*B. bassiana*	107.4	4
3rd	acetamiprid	60	21	acetamiprid	60.0	21	acetamiprid	100	28
	-		-	spinetoram	100	14	phosmet EC	120	14
	etofenprox [1]	150	7	spinosad	120	7	spinosad	480	7
	pyrethrin	322.8	4	deltamethrin EC	125.6	4	deltamethrin EC	25.2	4
4th	acetamiprid	60.0	21	acetamiprid	100	21	acetamiprid	100	28
	phosmet EC	120	14	phosmet EC	120	14	phosmet EC	120	14
	thiamethoxam	87.5	7	deltamethrin EC	125.6	7	spinetoram	75	7
	pyrethrin	322.8	4	-		-	deltametrin EC	25.2	4
5th	acetamiprid	60	21	acetamiprid	60	21	acetamiprid	100	28
	-		-	phosmet EC	120	14	cyantraniliprole	750	17
	etofenprox	150	7	spinetoram	100	7	cyantraniliprole	750	7
	-		-	deltamethrin EC	125.6	4	deltamethrin EC	25.2	4
6th	spirotetramat[2]	144	21	acetamiprid	60	21	-	-	-
	thiacloprid	80.0	14	dimethoate	150	14	-	-	-
	l-cyhalothrin	25	7	-	-	-	-	-	-
	-		-	-	-	-	-	-	-

[*] Refer to Table 1 for specific information on the formulated insecticides products
[1] Trebon Up® 30% SL
[2] Movento® 48 g/L SC
[3] DBH= days before harvest

2.4 Data analysis

Data were analysed using one-way analysis of variance (ANOVA) followed by means separation with Tukey's least significant difference (LSD) test using SAS (1978) V. 8 for laboratory and Costat for field data. Percentages of adult mortality were transformed to arcsin (radq (%)) before analysis to stabilize variance and reported means were returned back to percentages for presentation. Data were expressed as mean ± standard error (S.E.). Differences were considered significant at p 0.05 level.

3. Results

3.1 Laboratory trials

3.1.1 *Pre-treating cherries*

In 2014 trial (Table 3), the tested insecticide products significantly caused adult *D. suzukii* mortality at 1 DAA (F=36.61; df=15; P <0.0001), with excellent (≥ 85%) activity in deltamethrin EC formulation, lambda cyhalothrim, spinosad and spinetoram. At 2 DAA, a significant (F=23.67; df=15; P < 0.0001) adult mortality was observed; all products showed an excellent mortality except pyrethrin, imidacloprid and mineral oil. The application of insecticides significantly decreased the number of laid eggs (F=12.63; df=15; P <0.0001) and the female fecundity (F=10.87; df=15; P <0.0001) at 2 DAT. Pyrethroids (lambda cyhalothrin, deltamethrin) were highly effective; while neonicotinoids (thiacloprid, imidacloprid, acetamiprid) insignificantly caused low activity. Treatments used significantly suppressed adults *D. suzukii* from emergence after incubation of treated fruits for 10 days (F=5.65; df=15; P <0.0001), with full activity in organophosphates and lambda-cyhalothrin.

Table 3: Efficacy of insecticides on SWD mortality, eggs laying, fecundity and flies emerging following pre-treating-cherries bioassay in 2014 trial.

Treatment*	Percent adult mortality		Laid eggs 2 DAA	Fecundity 2 DAA	% flies emerging 10 DAA
	1 DAA	2 DAA			
Untreated	12.8±2.4 f	38.1 ± 11.6 c	35.7 ± 6.2 a	35.7± 2.9 a	9.7±2.1 b
dimethoate	64.2±2.5 cde	97.5 ± 2.5 ab	9.3 ± 1.5 abcde	9.3± 0.6 bcd	0.0±0.0 b
phosmet EC	71.9±7.3 abcde	100.0 ± 0.0 a	9.2 ± 2.6 abcde	9.2 ± 1.1 bcd	0.0±0.0 b
phosmet WDG	72.5±2.5 abcde	100.0 ± 0.0 a	5.5 ± 2.2 cdef	505 ± 0.9 d	0.0±0.0 b
deltamethrin EC	87.5±4.8 ab	87.5 ± 4.8 ab	0.8 ± 0.3 fg	0.8 ± 0.1 d	5.0±5.0 b
deltamethrin EW	72.5±4.8 abcde	95.0 ± 2.9 ab	1.7 ± 0.3 efg	1.7 ± 0.1 d	19.6±12.2 b
lambda cyhalothrin	91.9±4.9 a	100.0 ± 0.0 a	0.2 ± 0.2 g	0.2 ± 0.1 d	0.0±0.0 b
pyrethrum	57.5±2.5 e	77.5 ± 2.5 ab	3.6 ± 2.8 efg	3.6 ± 0.8 d	66.0 ± 21.0 a
thiamethoxam	79.7±3.9 abcd	92.5 ± 2.5 ab	13.6 ±1.2 abcd	13.6 ± 0.5 bcd	34.6±6. ab
thiacloprid	59.2±3.4 de	87.2 ± 2.4 ab	22.7 ± 4.6 abc	22.7 ±1.4 abc	10.3±2.2 b
acetamiprid	80.0±0.0 abcd	90.0 ± 0.0 ab	24.0 ± 4.6 ab	24.0 ± 1.9 ab	13.6±2.1 b
imidacloprid	62.5±4.8 de	75.0 ± 2.9 b	26.4 ± 5.4 ab	26.4 ± 2.4 ab	4.3±0.6 b
spinosad	84.4±5.2 abc	89.7 ± 4.1 ab	3.2 ± 0.7 defg	3.2 ± 0.3 d	7.3±4.8 b
spinetoram	92.5±2.5 a	92.5 ± 2.5 ab	6.9 ± 0.8 bcde	6.9 ± 0.3 cd	8.1±4.9 b
B. bassiana	70.0±5.8 bcde	92.5 ± 2.5 ab	5.9 ± 1.1 bcdef	5.9 ± 0.4 cd	8.0±0.8 b
mineral oil	5.0±2.9 f	22.5 ± 10.3 c	8.1 ± 4.4 def	8.1 ± 0.4 d	8.6 ± 5.1 b

Means within each column followed by the same letter (s) are not significantly different (Tukey's LSD test; p=0.05).
* Refer to Table 1 for specific information on the formulated insecticide products .
DAA= days after application.

In 2015 pre-treating cherries bioassay (Table 4), the insecticide applications significantly caused adult mortality at 1 and 2 DAA (1 D.: F=51.78; df=12; *P* <0.0001, 2 D.: *F*=39.89; df=12; *P* <0.0001). Organophosphates, pyrethroids, spinosad, spinetoram and cyantraniliprole showed a good activity, while neonicotinoids and *B. bassiana* had a low efficacy. The insecticides significantly reduced eggs laying (*F*=22.97; df=12; *P* <0.0001) and female fecundity (*F*=9.81; df=12; *P* <0.0001). Deltamethrin EW, lambda-cyhalothrin, spinosad, spinetoram and cyantraniliprole showed the best activities. Treatments significantly reduced the number of *D. suzukii* adults emerged at 10 DAA (*F*=11.56; df=12; *P* <0.0001). The dimethoate and phosmet EC were highly effective, resulting in full activity.

Table 4: Efficacy of insecticides on SWD mortality, eggs laying, fecundity and flies emerging following pretreating-cherries bioassay in 2015 trial.

Treatment*	Percent adult mortality		Laid eggs/fruit	Fecundity	% flies emerging
	1 DAA	2 DAA	2 DAA	2 DAA	10 DAA
Untreated	2.0 ± 2.0 d	6.0 ± 4.0 d	17.6 ± 2.4 a	7.9 ± 0.7 ab	57.8 ± 5.4 ab
dimethoate	100.0 ± 0.0 a	100.0 ± 0.0 a	4.4 ± 0.9 cd	4.4 ± 0.9 bcde	0.0 ± 0.0 d
phosmet EC	74.2 ± 14.1 ab	88.0 ± 7.3 a	9.9 ± 1.6 abc	8.9 ± 1.5 a	0.0 ± 0.0 d
phosmet WDG	96.0 ± 2.5 a	98.0 ± 2.0 a	6.2 ± 0.9 bcd	5.6 ± 0.9 abcd	2.4 ± 1.1 d
deltamethrin EW	56.0 ± 2.5 bc	70.0 ± 7.1 ab	2.3 ± 0.3 d	1.4 ± 0.2 de	41 ± 9.1 abc
lambda-cyhalothrin	95.6± 4.4 a	100.0 ± 0.0 a	0.5 ± 0.2 d	0.6 ± 0.3 e	66.7 ± 23.6 a
thiamethoxam	14.5 ± 7.2 d	16.5 ± 6.5 cd	15.0 ± 1.4 a	7.6 ± 0.8 ab	36.7 ± 3.2 abc
thiacloprid	15.6 ± 6.1 d	43.3 ± 15.6 bc	10.1 ± 1.3 abc	6.4 ± 0.6 abc	41.7 ± 4.9 abc
acetamiprid	28.7 ± 9.8 cd	28.7 ± 9.8 cd	14.6 ± 2.9 ab	8.2 ± 1.4 ab	39.3 ± 4.6 abc
spinosad	100.0 ± 0.0 a	100.0 ± 0.0 a	2.5 ± 0.7 d	2.5 ± 0.7 cde	41.6 ± 7.1 abc
spinetoram	100.0 ± 0.0 a	100.0 ± 0.0 a	2.7 ± 0.6 d	2.6 ± 0.6 cde	5.1 ± 1.4 cd
cyantraniliprole	100.0 ± 0.0 a	100.0 ± 0.0 a	5.2 ± 1.4 cd	5.0 ± 1.1 abcd	15.4 ± 2.3 bcd
B. bassiana	2.0 ± 2.0 d	2.0 ± 2.0 d	8.0 ± 1.0 abc	4.0 ± 0.5 bcde	74.5 ± 8.8 a

Means within each column followed by the same letter (s) are not significantly different (Tukey's LSD test; p=0.05).
* Refer to Table 1 for specific information on the formulated insecticide products .
DAA= days after application.

3.1.2 *Dipping infested cherries*

In 2015 dipping infested cherries bioassay (Table 5), treatments significantly affected eggs hatching (*F*=34.54; df=12; *P* <0.0001), immature stages development (F=11.78; df=12; P <0.0001) and *D. suzukii* emerging (*F*=33.98; df=12; *P* <0.0001). Dimethoate, cyantraniliprole, and phosmet WDG provided the greatest activity, resulting in 100% reduction. Spinetoram and phosmet WDG showed excellent controls as well. While, *B. bassiana* caused the lowest activity; however, it was able to significantly induce the hatching rate of eggs and number of adults emerging compared to the untreated plots.

Table 5. Efficacy of insecticides on eggs hatcing, immature stages development and adults emerging of SWD following dipping infested-cherries bioassay in 2015 trial.

Treatment*	hatching rate	% Immature stages	% adults emerging
Untreated	73.8±7.6 a	16.4±4.7 a	57.4±3.7 a
dimethoate	0.0±0.0 d	0.0±0.0 d	0.0±0.0 d
phosmet EC	4.2±1.7 cd	0.5±0.5 cd	3.7±1.4 cd
phosmet WDG	0.0±0.0 d	0.0±0.0 d	0.0±0.0 d
deltamethrin EW	22.8±6.7 b	7.5±2.7 ab	15.2±4.4 b
lambda-cyhalothrin	21.7±2.9 b	7.5±1.0 ab	14.2±2.2 b
thiametoxam	21.6±4.5 b	3.4±0.9 bcd	18.2±4.1 b
thiacloprid	9.9±1.8 bc	1.9±1.0 bcd	8.0±2.1 bc
acetamiprid	23.4±4.8 b	6.2±1.7 ab	17.2±4.6 b
spinosad	14.6±3.6 bc	5.8±1.8 ab	8.8±3.2 bc
spinetoram	0.5±0.3 d	0.0±0.0 d	0.5±0.3 d
cyantraniliprole	0.0±0.0 d	0.0±0.0 d	0.0±0.0 d
B. bassiana	19.0±4.1 b	5.0±1.9 bc	14.0±2.7 b

Means within each column followed by the same letter (s) are not significantly different (Tukey's LSD test; p=0.05).
* Refer to Table 1 for specific information on the formulated insecticide products .
DAA= days after application.

3.2 Open field trials

R. *cerasi* and *D. suzukii* presence in the experiment fields was high and representative of the cherry growing area (Figure 15). The cherry fruit fly adults were captured in May –June and the peak was observed at fruits changing color. *D. suzukii* abundance was relatively high before and during cherry flowering (data not reported in the figure) probably due to the attractiveness of the flowers that provide food after the overwintering period (**Tochen *et al.*, 2016**). After blooming the captures decreased probably due to climatic factors affecting the mortality of overwintering individuals (**Hamby *et al.*, 2016; Zerulla *et al.*, 2015**) and for the competition between the lures and the fruits (**Asplen *et al.*, 2015**). After fruit collection the captures sharply increased due to the new emerging adults and the end of the fruit-lure competition (**Wang *et al.*, 2016**)

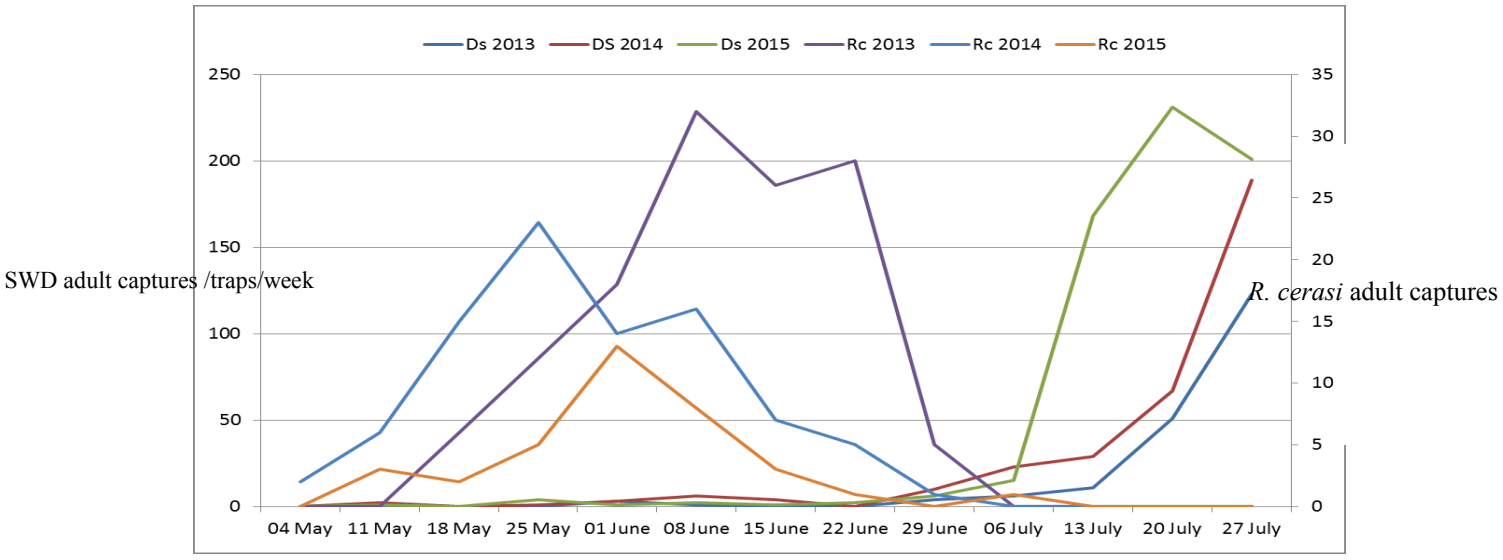


Figure 15. *R. cerasi* and *D. suzukii* captures in the experiment fields. First application was made on 18th, 04th and 03rd June and the harvest on 09th July, 25th June and 01st July respectively in 2013, 2014 and 2015.

3.2.1 Efficacy of chemical-control strategies

In 2013 (Table 6), at the begining of the harvest period (4 days after last application (DALA)), more than 16% of the cherries were infested by *D.suzukii*, one week later about half of the fruits were damaged with 0.5 SWD larva per cherry. The organic strategy and the strategy spirotetramat/thiacloprid/L-cyhalothrin insignificantly reduced the damage in comparison with the untreated plots. The 2013 results showed that the efficacy against SWD is related to the number of insecticide applications made during the period of maturation close to harvest. At 10 DALA, no significant differences were observed. Two and three pesticide applications were insufficient to control *D. suzukii* in cherry crops. Regarding *R. cerasi*, the neonicotinoids applied at fruit changing color showed a good control activity; the subsequently applications have reduced the damage until the end of the harvest.

In 2014 (Table 7), due to the favorable environmental conditions *D. suzukii* infestation was very high: more than 80% of the cherries were infested by 2-3 larvae. The organic strategy (2spinosad/pyrethrin) showed an efficacy reduction of 50% without significant differences compared with untreated plots. The 2014 result underline the efficacy of the applications made close of the harvest in the D. suzukii control. With strategies with the same number of applications, the best efficacy was obtained using persistent products such as organophosphates in EC formulation instead of pyrethroids or spinosin. One application with dimethoate was a good activity in the number of larvae reduction. Regarding *R. cerasi*, all strategies reduced the fruit damages and larval infestation. Against this pest, the applications made 2-3 week before harvest had the best activity, probably due to cherry fly phenology and adults' presence.

In 2015 (Table 8), the *D. suzukii* damages increased during the ripening period: 5% infested cherries at the first fruit picking (4DALA), almost 40% after 1 week at the end of harvest period (11 DALA). All strategies reduce the damages and the number of larvae with statistical differences between the conventional ones and untreated plot. The strategy with two cyantraniliprole applications, 17 and 7 before harvest, showed the best results in SWD control. No statistical differences were observed in the *R. cerasi* efficacy, due to the low population density of the cherry fly.

Table 6. Percent of cherries damage and number of *D.suzukii* larvae infested cherry fruits following chemical control strategies applied in 2013 field trial.

N°	Strategy*	Application time (DBH)	*D. suzukii*				*R. cerasi*				Residue (mg/kg)	MRLs
			% Damaged cherries		larvae N° /100 cherries		% Damaged cherries		Larvae N° /100 cherries			
			4 DALA	11 DALA	4 DALA	11 DALA	4 DALA	11 DALA	4 DALA	11 DALA		
1	Untreated	-	16.6 a	46,5 a	12.2 a	44.3 a	16.5 a	4.8 a	14.5 a	4.8 a	-	-
2	spinosad	21	9.7 ab	34.3 ab	8.7 ab	10.3 a	13.5 ab	4.0 a	12.0 a	3.5 ab	0.08	1.00
	spinosad	14									0.08	1.00
	spinosad	7									0.08	1.00
	pyrethrin	4									0.00	1.00
3	acetamiprid	21	1.8 cd	13.3 ab	0.9 c	20.8 a	2.5 b	2.0 ab	1.5 b	2.0 c	0.02	1.50
	etofenprox	7									0.20	1.00
	pyrethrin	4									0.00	1.00
4	acetamiprid	21	0.3 d	7.8 b	0.1 c	17.3 a	0.5 c	0.0 c	0.5 c	0.0 c	0.05	1.50
	phosmet EC	14									0.03	1.00
	thiamethoxam	7									0.18	1.00
	pyrethrin	4									0.01	1.00
5	acetamiprid	21	6.7 bc	28.3 ab	4.9 b	38.0 a	4.5 bc	3.8 a	3.5 bc	3.0 b	0.02	1.50
	etofenprox	7									0.10	1.00
6	spirotetramat	21	12.6 ab	16.3 ab	10.8 ab	24.5 a	9.8 ab	2.8 ab	7.5 ab	2.0 c	0.00	3.00
	thiacloprid	14									0.10	0.30
	l-cyhalothrin	7									0.03	0.30

Means within each column followed by the same latter are not significantly different (Tukey's LSD test; p=0.05).

* Refer to Tables 1 and 2 for specific information on the formulated insecticides and applied strategies.

DBH= Days Before Harvest. DALA= Days After Last Application

Table 7. Percent of cherries damage and number of *D.suzukii* larvae infested cherry fruits following chemical control strategies applied in 2014 field trial.

N°	Strategy*	Application time (DBH)	% Damaged cherries by D. suzukii		N° D. suzukii larvae/ 100 cherries		% Damaged cherries by R. cerasi		N° R. cerasi larvae/ 100 cherries		Residue (mg/kg)	MRLs
			4 DALA	11 DALA	4 DALA	11 DALA	4 DALA	11 DALA	4 DALA	11 DALA		
1	Untreated	-	23.5 a	82.5 a	8.5 a	234.0 a	11.0 a	12.5 a	9.5 a	9,5 a	0.000	-
2	spinosad	14	13.0 a	37.8 ab	4.0 ab	78.8 ab	5.5 ab	6.3 b	4.8 ab	2.5 a	0.034	1.000
	spinosad	7									0.034	1.000
	pyrethrum	4									0.010	1.000
3	acetamiprid	21	10.6 a	14.0 b	1.5 bc	12.8 bc	1.5 b	2.0 b	0.5 b	1.0 a	0.028	1.500
	spinetoram	14									0.010	0.050
	spinosad	7									0.038	1.000
	deltamethrin EC	4									0.023	0.200
4	acetamiprid	21	9.50 a	28.5 b	2.0 bc	25.8 bc	2.0 b	2.5 b	1.5 b	3.5 a	0.055	1.500
	phosmet EC	14									0.022	1.000
	deltamethrin EC	7									0.017	0.200
5	acetamiprid	21	3.50 a	11.8 b	0.3 c	24.8 c	3.0 b	3.8 b	4.0 ab	5.0 a	0.040	1.500
	phosmet EC	14									0.026	1.000
	spinetoram	7									0.021	0.050
	deltamethrin EC	4									0.018	0.200
6	acetamiprid	21	10.0 a	13.5 b	0.8 bc	20.8 bc	2.0 b	5.0 b	3.5 ab	3.8 a	0.050	1.500
	dimethoate	14									0.590	0.200

Means within each column followed by the same latter are not significantly different (Tukey's LSD test; p=0.05).

* Refer to Tables 1 and 2 for specific information on the formulated insecticides and applied strategies.

DBH= Days Before Harvest. DALA= Days After Last Application

Table 8: Percent of cherries damage and number of *D.suzukii* larvae infested cherry fruits following chemical control strategies applied in 2014 field trial.

N°	Strategy*	Application time (DBH)	% Damaged cherries by D. suzukii		N° D. suzukii larvae/ 100 cherries		% Damaged cherries by R. cerasi		N° R. cerasi larvae/ 100 cherries		Residue (mg/kg)	MRLs
			4 DALA	11 DALA	4 DALA	11 DALA	4 DALA	11 DALA	4 DALA	11 DALA		
1	Untreated	-	5.00 a	38.75 a	4.00 a	40.25 a	3.0 a	2.0 a	1.0 a	0.5 a	0.000	-
2	spinosad	28	2.75 ab	22.50 ab	2.75 ab	24.25 ab	1.8 a	1.8 a	0.5 a	0.5 a	0.039	1.000
	spinosad	14									0.072	1.000
	spinosad	7									0.072	1.000
	B. bassiana	4									n.a.	-
3	acetamiprid	28	1.50 b	18.75 ab	0.75 b	17.25 ab	0.3 a	0.3 a	0.0 a	0.0 a	0.048	1.500
	phosmet EC	14									0.025	1.000
	spinosad	7									0.035	1.000
	deltamethrin EC	4									0.018	0.200
4	acetamiprid	28	0.50 b	13.75 b	0.50 b	12.50 b	0.0 a	0.0 a	0.0 a	0.0 a	0.041	1.500
	phosmet EC	14									0.026	1.000
	spinetoram	7									n.a.	0.050
	deltametrin EC	4									0.015	0.200
5	acetamiprid	28	0.25 b	10.25 b	0.25 b	9.75 b	0.0 a	0.0 a	0.0 a	0.0 a	0.043	1.500
	cyantraniliprole	17									n. a.	-
	cyantraniliprole	7									n. a.	-
	deltamethrin EC	4									0.020	0.200

Means within each column followed by the same latter are not significantly different (Tukey's LSD test; p=0.05).
* Refer to Tables 1 and 2 for specific information on the formulated insecticides and applied strategies.
DBH= Days Before Harvest. DALA= Days After Last Application.

3.2.2 Insecticide residue levels

Open-field results revealed that (Tables 6, 7, 8), residue levels of insecticides detected into cherries were lower than their MRLs and were completely compliant with their MRLs in force the European Union (**EU Pesticide database, 2013, 2014, 2015**), except for dimethoate product of the 6th strategy of 2014-open field trial which exceeded its MRLs.

4. Discussion

In the current study, we highlighted the efficacy of insecticides against SWD approved for use on cherries under Italian conditions. This included laboratory assays along with open-field chemical-control programs applied in cherry fruit crop. Laboratory assays were a good indicator for selecting products to be used in the field control strategies (**Bruck *et. al*., 2011**). Four classes of insecticides, namely spinosyns (spinosad, spinotram), organophosphates (dimethoate, phosmet), pyrethroids (lambda-cyhalothrin, deltamethrin)

(Beers *et al.*, 2011; Van Timmeren and Isaacs, 2013; Cuthbertson *et al*., 2014) and diamide (cyantraniliprole) (**Cuthbertson *et al*., 2014, Shawer *et al*., 2015)** were highly effective with varying levels of control against various life stages of SWD when used in either pre- or post-treating cherries bioassays. Each of those chemical classes had its own way to achieve this excellent activity. The acute impact of pyrethroids as adulticides and their short residual effects were highly noted. In contrast, a systemic action (long-term effect) of organophosphates was powerfully appeared, in addition to significant effect as adulticides (acute effect) especially at 2 DAA. In laboratory bioassays, pyrethroid products significantly affected fecundity of SWD female which deposited low number of eggs; however, high numbers of laid eggs were hatched, immature stages were developed and therefore high numbers of adults were then emerged (Table 3). In contrast, despite organophosphates (phosmet EC, dimethoate) were less effective on female SWD fecundity and high levels of eggs were laid, they significantly disrupted ability of eggs to get larvae. Spinosyns caused excellent acute and good long-term (residual) activities (tables 4, 5) (**Van Timmeren and Isaacs, 2013; Cuthbertson *et al*., 2014)**. Neonicotinoids evaluated (acetamprid, imidacloprid, thiacloprid and thiamethoxam) suggested moderate SWD adult mortality following dipping infested cherries bioassays and were less effective in the pretreating cherries bioassays **(Bruck *et. al*., 2011)**. However; the low effect of acetamiprid and imidacloprid as adulticides in cherry assays allowed higher levels of oviposition to occur, their systemic effect resulted in reducing development of immature stages to get adults **(Beers *et al.,* 2011)**. Field strategies that started with acetamiprid as a first application at 28 - 21 DBH indicated SWD control greater than these were started with an organic product (spinosad or spinotram). As well as, spraying acetamiprid as a first application at cherries turn pale yellow (28-21 DBH) showed a good activity against *R. cerasi* **(Profaizer *et al*., 2015)**, the subsequently applications have reduced the damage until the end of the harvest. This systemic mode of effect of acetamiprid was also appeared in laboratory strawberries bioassays where high efficacy against SWD life stages was performed **(Shawer *et al*., unpublished data).** Acetamiprid and thiamethoxam caused intermediate adult *D. suzukii* mortality within insecticide bioassays on strawberries; however, they reduced the larval infestation upto 86% (**Andreazza *et al*., 2016).** This mode of effect of neonicotinoids can be attributed to their brief lethal-contact impact as it is readily absorbed by plants,

systemic properties and its potentially beneficial long-term sublethal such as antifeeding, repellency and ovipositional deterrence **(Barry and Polavarapu, 2005; Wise *et al*., 2006; Beers *et al.*, 2011; Bruck *et al*., 2011)**.

Before *D. suzukii* invaded our Veneto region, Italy, cherry growers were only applying one pesticide application 20 - 30 DBH for managing *R. cerasi*. The present study confirms that the effective SWD control is highly subjected to the number of insecticide applications made within the maturation period of cherries close to harvest, two or three insecticide applications were not efficacious (**Bolda *et al*., 2010; Beers *et al*., 2011; Haviland and Beers, 2012; Van Timmeren and Isaacs, 2013)**. To maintain a protective residue throughout the harvest period, four applications with effective insecticides belonging different mode-of-action groups are strongly recommended, provided that the first application should be applied when monitoring traps indicate the presence of *D. suzukii* populations. The organic products were not able alone to protect cherries from SWD damage, as well. We suggest starting with a first application at 28 or 21 DBH (depending on the first capture in traps) with the systemic neonicotinoid, acetamiprid taking advantage of its long-term residual effects to reduce the population and infestation of both *D. suzukii* and *R. cerasi* **(Profaizer *et al*., 2015).** The second application should be applied at 21-14 DBH with a member of diamide (cyantraniliprole) or organophosphates (phosmet). This can be followed with a third application sprayed at 14-7 DBH with a product from spinosyns or diamide (depending on the used product in the 3rd application). The fourth application is highly recommended to be applied with a pyrethroid product (lambda-cyhalothrin, deltamethrin) at 7 - 4 DBH, benefiting its prompt activity as a adulticide and meeting MRLs market requirements. This increase in times of insecticide application must led up growers to face the extremely challenge of protecting pollinators, complying with restricted entry interval, preharvest interval and maximum residue limit regulations (**Bruck *et. al*., 2011**). There were no detectable residues exceeded MRLs required by the European Union (**EU Pesticide database, 2015**) except a case of dimethoate. Rotation and diversity in applying of insecticides with different and unique modes of action are greatly necessary to combat or at least delay widespread insecticide resistance. Cyantraniliprole, phosmet, spinotram, deltamethrin, lambda-cyhalothrin **(Haviland and Beers, 2012)** and spinosad **(Haviland and Beers, 2012)** had favorable characteristics for SWD control, allowing producers to

incorporate the principles of efficacy, fruit susceptibility, and resistance management and still allows for the export of fruit to all major export markets. Laboratory and field trials confirmed the excellent acute activity of the new registered diamide, cyantraniliprole as adulticide along with a high long-term activity, even if very few eggs were able to get larvae and then adults (tables 4, 5, 8). Based upon its positive insecticidal effect, highly selectivity and its novel mode of action, integration the diamide insecticide, cyantraniliprole into SWD IPM programs is highly recommended.

5. conclusion

It could be concluded that pyrethroids (lambda-cyhalothrin, deltamethrin), spinosyns (spinosad, spinotram), organophosphates (phosmet EC, phosmet WDG, dimethoate) and diamide (cyantraniliprole) are recommended to use against *D. suzukii* since they caused excellent activities, concerning adult mortality (>90%), female fecundity, eggs laying and hatching, immature stages development and adults emerging within laboratory bioassays. Conversely, neonicotinoids, *Beauveria bassiana*, and emamectin-benzoate indicated unsatisfactory results. Moreover, field results proved that effective *D. suzukii* control program can be achieved by timely of four applications of insecticides belonging different mode-of-action chemical groups. Commitment to regulations of restricted entry interval, preharvest interval and maximum residue limit must be considered. Further studies regarding the side-effects of these chemicals used to control this invasive pest, SWD on non-target organisms are highly needed. As well as increasing the secreening of alternative biological agents of SWD should be considered.

Chapter III

Drosophila suzukii management: insecticides laboratory and field studies in strawberry crops

Abstract

Two laboratory trials in March and September 2015 were performed to identify the most commonly pesticides used in Italy against *D. suzukii* on strawberries. As well as, the efficacy of different chemical control strategies to protect strawberry fruits from the damage caused by *D. suzukii* were evaluated through an open-field trial in Veneto region, North-Eastern Italy during summer of 2014. Based upon pre-treating strawberries bioassay results, pyrethroids, spinosyns, avermictin (emamectin benzoate) and diamide (cyantraniliprole) caused excellent activities, concerning adult mortality higher than 90 and 97% at 1 and 2 days after treatment (DAT), respectively. They also provided a significant residual activity against *D. suzukii* life stages emerging after treatment. Incontrast, neonicotinoids, and *Beauveria bassiana* showed insignificantly activities. The same trend of pre-treating strawberries bioassay was repeated within the dipping infested-strawberries bioassay, except that acetamiprid showed good residual control against the *D. suzukii* individuals emerging. All strategies significantly succeeded to decrease the damage of strawberries compared to the untreated plants either at 14 or 21 days after first application (DAFA). The field findings confirmed that the two treatments of spinosad provided the best strawberry crop protection.

1. Introduction

Fruit and vegetables account for 17% of the value of the EU's agricultural production, with a total production value estimated to be more than €50 billion, and the fruit and vegetables supply chain has an estimated turnover of more than €120 billion with over 550,000 employees and around 1.4 million growers (**Freshfel, 2012**). Strawberry, *Fragaria anannassa*, one of the most adaptable and important fruit crops all over the world, is grown from the tropics to near the Arctic Circle **(Barney, 1999)**. It is the most important berry crop in the EU, being produced in all member states. Forasmuch, world produces about 7.73 million tonnes of strawberry per year, Europe represents 26.6% of its global production, exceeding 1.48 million tonnes per year (**FAO stat, 2016)**. Strawberry production requires specialised knowledge, high energy costs and high external inputs, and it was estimated that pesticide applications exceed 1Kg/ha/year. Fruit losses from pests and pathogens estimated to be over €10 billion in revenue and 3 million tonnes of produce to the EU fruit industry (**Kenis and Branco, 2010)**.

The spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), an Asian pest of strawberries and other thin-skinned fruit, has recently introduced into USA (**Bolda *et al*., 2010; Walsh *et al*., 2011**) and Europe (**Cini *et al.*, 2012**). It was notified for the first time in Trentino-Alto-Adige region, north-eastern Italy in September 2009 (**EPPO, 2010**). It has become a pest of great concern, causing considerable damage to thin-skinned fruits including cherry, blueberry, raspberry, grape and strawberry **(Sasaki *et al*., 1995; Lee *et al*., 2011a; Lee *et al*., 2011b; Walsh *et al*., 2011, Bellamy *et al*. 2013; Cuthbertson *et al*., 2014)**. Yeald losses of up to 80% occurred in the strawberry crops of southern France in 2010 (**Lee *et al*., 2011a; Orhan *et al*., 2016).** Thus, fruit growers are obligated to adopt intensive spray programs to protect fruit from its infestation **(Cowles *et al*., 2015)**.

Since *D. suzukii* has established into USA (**Hauser *et al*., 2009; Steck *et al*., 2009**) and Europe (**Cini *et al*., 2012**) in 2008, a great concern with respect to screening of several products and identifying their potential for controlling this pest have been received over the last years and a number of insecticide-efficacy trials have been performed on stone fruits and berry crops (**Bolda *et al*., 2010; Beers *et al*., 2011; Bruck *et al*., 2011; Haviland and**

Beers, 2012) in most major regions of its distribution (**Van Timmeren *et al*., 2013**). Despite these trials have addressed the lack of information on the efficacy of certain products in some regions of its distribution on some main host plants, a great deal of research and further investigations concerning a thorough screening of several products to assess the potential effect, the environmental fate, side effects on microorganisms, and residual effects in crop fruits and soil of most commonly used insecticides on the main infested crops under different environmental conditions are largely required. The present study went for investigation the efficacy of most commonly pesticides used in Italy for managing *D. suzukii* on strawberries under laboratory and Italian field conditions.

2. Material and methods

2.1 Laboratory colony

Drosophila suzukii individuals used in these experiments were reared in the previous work (chapter 2, page: 30) **(Shawer *et al*., 2015).**

2.2 Laboratory bioassays

To investigate the potential efficacy of most products registered for use on strawberries in Italy against *D. suzukii*, two laboratory bioassay experiments were performed in March and September of 2015 season (Table 9). Fruit bioassays were carried out with strawberries collected from untreated-pesticides orchards, Verona, North-Eastern Italy. Strawberries selected for bioassays were immediately checked out under stereomicroscopes and the infested or/and injured ones were displaced. To make sure that the fruits free of pesticide residues, a subsample of collected fruit was tested 1 day prior to performing the experiments. Any sample with evidence of active pesticide residue (>5% mortality after 24 h exposure) was discarded (**Bruck *et al.*, 2011**). Strawberries were then dipped into 2% proprionic acid (methylacetic acid) for 5 seconds as a mold inhibitor and air dried. All tested insecticides were prepared immediately in deionized water at the equivalent of 1000 L water ha^{-1}. Deionized water acted as the untreated control. The experiments were arranged in a randomized complete block design with ten replications of each treatment and two fruits per each replicate. Experiments were repeated five times. Six and eight insecticides at their recommended label rate were evaluated in March and September trials, respectively. Two-fruit bioassays were considered as follow:

2.2.1 Dipping infested strawberries bioassay

Following dipping infested strawberries bioassay previously described in chapter 2 (**Cuthbertson *et al*., 2014; Shawer *et al*., 2015**), strawberries were infested, cleaned of flies and inspected for presence of *D. suzukii* individuals. Fruits were then dipped into pesticide solutions for 30 seconds, dried, incubated under controlled-environmental conditions. Pots were then checked out for emergence of flies and fruits were dissected to investigate for presence of immature stages development. Percent of adults emerging and immature stages presence were considered according abovementioned equations **(chapter 2, page 30)**.

2.2.2 Pretreating strawberries bioassay

To determine a pesticide's residal toxicity, pretreating strawberries bioassay previously described in chapter 2 (**Bruck *et al.*, 2011; Shawer *et al.*, 2015)** was performed. Briefly, strawberries were immersed into insecticide solutions, air dried, placed into plastic pots. 10 *D. suzukii* flies (5 males + 5 females) were then added into the plastic pots, maintained for 10 days under controlled-environmental conditions. Mortality of *D. suzukii* adults were recorded at 1 and 2 DAT. The pots were also checked out for emergence of adult flies and the strawberries were dissected to investigate for immature stages presence. Percentages of immature stages presence, and adult flies emerging were calculated according the above mentioned equations in chapter 2, page 30 (**Blumel and Hausdorf, 2002)**

Table 9. Names, formulations, manufacturers and application rates of insecticides used in laboratory bioassays for controlling *Drosophila suzukii* on strawberries.

Trade name	Formulation	Chemical name	Chemical group	Manufacturer	Rate (AI g ha^{-1})
Epik®	5% SL	acetamiprid[a]	Neonicotinoids	Sipcam Italia	60
Decis EVO®	2.8% EW	deltamethrin[a,b]	Pyrethroids	Bayer CropScience	14
Karate Zeon®	10% CS	lambda cyhalothrin[a,b]	Pyrethroids	Syngenta Crop Protection	25
Laser®	48% SC	spinosad[a,b]	Spinosyns	DOW-Agrosciences	120
Success®	11.6% SC	spinosad[b]	Spinosyns	DOW-Agrosciences	116
Delegate®	25 WG	spinetoram[b]	Spinosyns	DOW-Agrosciences	87.5
Affirm®	0.95% SG	emamectin benzoat[a,b]	Avermectins	Syngenta Crop Protection	23.75
Naturalis®	7.16% SC	*Beauveria bassiana*[a,b]	Biopesticides	CBC Biogard	107.4
Exirel®	10% SE	cyantraniliprole[b]	Diamides	DuPont	750

[a] chemical used in March 2015- laboratory bioassays for controlling *D. suzukii*.

[b] chemical used in September 2015- laboratory bioassays for controlling *D. suzukii*.

2.3 Open-field trials

An open field trial in Zevio**,** Verona province, Veneto region, North-Eastern Italy using formulated insecticides was performed during August-September of the growing season of 2014 to investigate different chemical control strategies of registered pesticides (table 10) to control *D. suzukii* in strawberry fruit crops. Trail was accurately designed following the EPPO standard PP 1/281 efficacy evaluation of insecticides (**EPPO, 2013a**). Five strategies of four plots (replicates) were designed. The first and second pesticide applications were at 28 August and 4 September 2014, respectively. Individual plots were 4 m wide and 6 m length, arranged in a randomized complete block design. Treatments were applied in the equivalent of 800 L water ha^{-1} using a knapsack Fox 320 with water pressure of 8 bar and with three hollow cone-nozzles. Untreated control was sprayed with water only. At the time of application, the population level of the pest and its stage of development were trapped using yellow-sticky and red color-bottle, containing vinegar, traps. 100-ripening strawberries were randomly collected at 14 and 21 days after first application (DAFA), inspected for damage caused by *D. suzukii*, dissected and the percentage of both fruit damage and numbers of immature stages were recorded. Strawberries were after that incubated for 48 hours in climate cells at 25°C to allow eggs hatching and larvae to grow to a larger size. Larvae were then sieved out from fruits by immersing in a 10% NaCl solution. Direct effects (phytotoxic and/or any positive effects) on strawberry crops, effects on other pests and on non-target organisms were accurately observed and noted.

Table 10. Chemical control strategies evaluated in 2014 for the management of *D. suzukii* infesting strawberry crops.

Strategy	Trade name	Common name	Pesticide application		
			Rate (mL. hL^{-1})	Times	Dates
1st	Untreated		-	0	0
2nd	Karate-Zeon® 10% CS	Lambda cyhalothrin	250 mL/hL	1	28-Aug
3rd	Delegate® 25 WG	Spinetoram	300 mL/hL	1	28-Aug
4th	Laser® 48% SC	Spinosad	250 mL/hL	2	28-Aug, 4-Sep
5th	Decis-EVO® 2.8% EW	Deltamethrin	60 ml/hl	2	28-Aug, 4-Sep

2.4 Data analysis

Laboratory and field data were analysed by one-way analysis of variance (ANOVA) followed by means separation with Fisher's least significant difference (LSD) for laboratory bioassays and Tukey's LSD for field data, using the appropriate models for a completely randomized design (SAS Institute, 2010). Percentages of adult mortality were transformed

to an arcsine (radq (%)) before analysis to stabilize variance and reported means were back-transformed to percentages for presentation. Data were expressed as mean ± standard error (S.E.). Differences were considered significant at *p* 0.05 level.

3. Results

3.1 Laboratory screening

3.1.1 Pretreating strawberries bioassay

Results of the pretreating strawberries bioassay of March 2015 revealed that, after exposing insects to pesticide-residual on strawberries, the tested insecticides resulted in significant (*F*=146.48; *DF*=6; *P* <.0001) percent mortality against adult *D. suzukii* at 1 DAT (Table 11). All insecticides except *B. bassiana* significantly caused total adult mortality greater than the untreated, with excellent (≥89) activity in spinosyns, pyrithroids, and emamectin products at 1 DAT. The same trend occurred with females (*F*=115.5, *DF*=6, *P* <.0001) as males (*F*=115.25; *DF*=6; *P*<.0001) at 1 DAT (fig. 16).

Table 11. Percent adult mortality (male, female, total) at 1 and 2 DAT, fecundity, and individuals emerging of *D. suzukii* following pre-treating cherries bioassay of Spring 2015 trial.

	Percent adult mortality		**Fecundity**	**Individuals emerging at 10 DAT**		
Treatment	**1 DAT**	**2 DAT**		**Immatuare**	**adult**	**Total**
Untreated	20.1 ±4.3 c	34.5 ± 5.4 c	1.5±0.2 a	1.0 ± 0.3 a	14.2 ± 2.5 a	15.2 ± 2.5 a
Acetamiprid	61.1 ±4.1 b	79.0 ± 3.8 b	0.8±0.2 b	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 c
B. bassiana	21.6±4.1 c	36.0 ± 5.2 c	1.0±0.2 b	1.4 ± 0.7 a	9.8 ± 1.8 b	11.2 ± 2.0 b
Deltamethrin	89.0 ±1.8 a	98.0 ± 0.7 a	0.1±0.0 c	0.0 ± 0.0 b	0.3 ± 0.1 c	0.3 ± 0.1 c
Emamectin benzoat	90.8±2.1 a	97.0 ± 1.2 a	0.3±0.1 c	0.0 ± 0.0 b	0.1 ± 0.0 c	0.1 ± 0.0 c
Lambda-cyhalothrin	99.5±0.3 a	100.0 ±0.0 a	0.2 ± 0.2 c	0.0 ± 0.0 b	0.1 ± 0.1 c	0.2 ± 0.1 c
Spinosad[b]	99.0±0.7 a	100.0±0.0 a	0.0±0.0 c	0.0 ± 0.0 b	0.1 ± 0.1 c	0.2 ± 0.1 c

a Means within each column followed by the same letter (s) are not significantly different (Fisher's LSD test; $p<0.05$).
b Laser®.

Percent mortality of adults at 2 DAT performrd by all evaluated chemicals were also highly significant (*F*=86.70; *DF*=6; *P*<.0001) greater than the untreated, except for the *B. bassiana,* with full activity (100%) in spinosad and lambda-cyhalothrin. Moreover, deltamethrin and emamectin benzoat accomplished excellent mortalities of 98 and 97%, respectively. Whereas, a moderate mortality recoded by acetamiprid. Mortality caused in females (*F*=74.35; *DF*=6; *P* <0.0001) were significant similar as males (*F*=76.41; *DF*=6; *P*<.0001). Only acetamiprid killed 20% of male *D. suzukii* higher than female either at 1 or 2 DAT (figs. 16, 17).

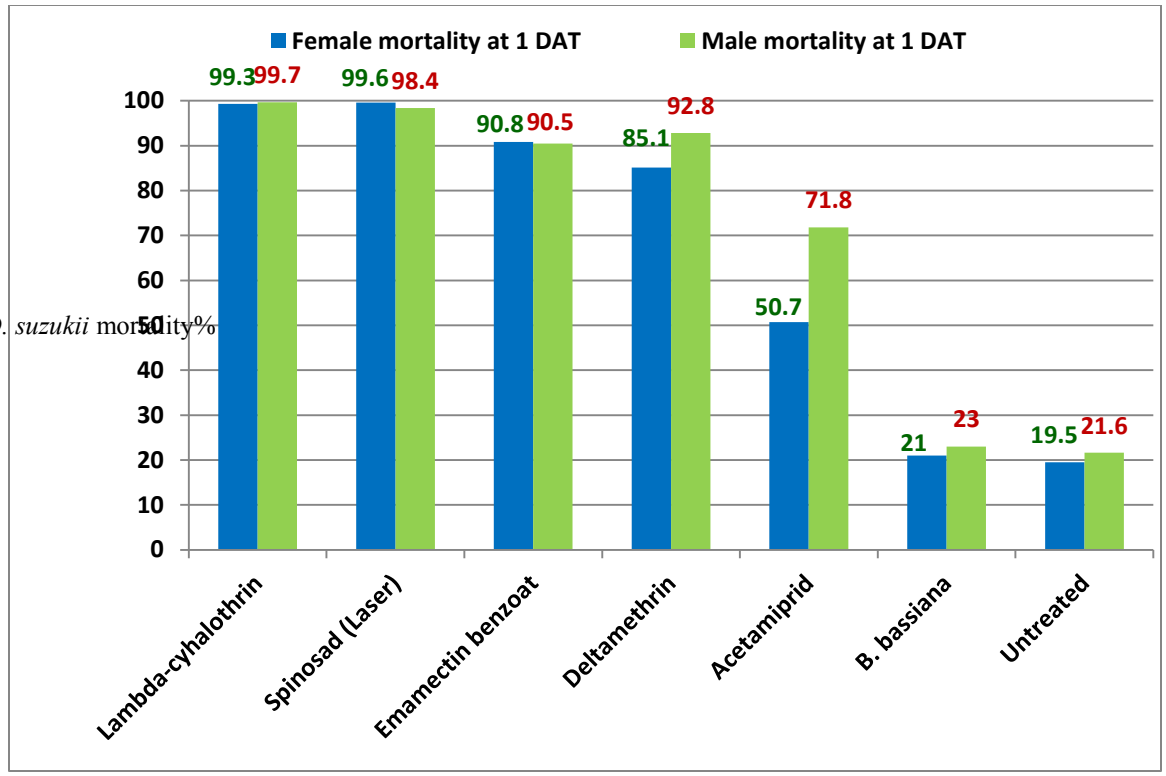


Figure 16. Percent mortality of male and female *D. suzukii* following exposure to various pesticide residuals in March laboratory bioassays at 1 DAT.

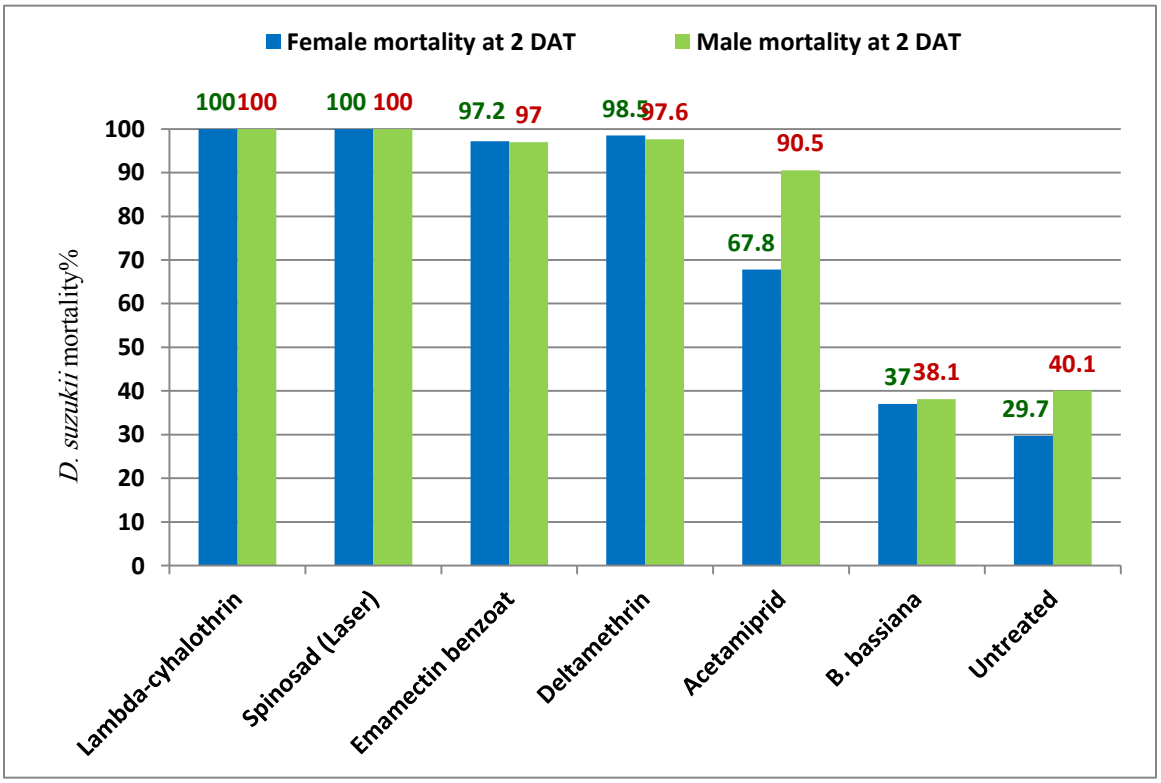


Figure 17. Percent mortality of male and female *D. suzukii* following exposure to various pesticide residuals in March laboratory bioassays at 2 DAT.

Fecundity of *D. suzukii* female was significantly (F=12.54; DF=6; P<0.0001) affected by the exposure to insecticides. Spinosyns, pyrethroids, and emamectin benzoate showed significantly fecundity lower than the untreated, with full activity (0.0) in spinosad.

For total *D. suzukii* individuals emerging at 10 DAT, all insecticides significantly (F=27.70; DF=6; P <0.0001) reduced the numbers of adults and total individuals fewer than the untreated. The neonicotinoid insecticide; acetamiprid suggested the best results giving full (0.0) efficacy on the total emerged individuals, followed by emamectin benzoate, Lambda-cyhalothrin, and deltamethrin. *B. bassiana* was less effective.

Based on the pretreating strawberries bioassay of September 2015 trial, highly significant (F=43.21; DF=8; P <.0001) mortalities were achieved by insecticides greater than the untreated except *B. bassiana* at 1 DAT (Table 12 & figs. 18, 19). Diamide, spinosyns, pyrethroids, Avermectin chemical groups provided excellent (≥98%) mortality. Cyantraniliprole, spinetoram, and spinosad (Success®) provided complete (100%) mortality; however, *B. bassiana* suggested droopy (28%) activity against adults. At 2 DAT, all treatments significantly (F=14.22; DF=8; P <0.0001) reduced the number of adults lower than the untreated. Cyantraniliprole, spinetoram, lambda-cyhalothrin, spinosad (Success®), spinosad (Laser®), and emamectin benzoate caused a full activity (100%). As well as a mortality of 98% in deltamethrin was recorded; while, a moderate effect (54%) in *B. bassiana*.

Table 12. Percent adult mortality (male, female, total) at 1 and 2 DAT, and fecundity following pre-treating cherries bioassay of September 2015 trial.

Treatment	Percent adult mortality		Fecundity	Individuals emerging after 10 DAT		
	1 DAT	2 DAT		Immature	Adult	Total
Untreated	9.8±7.8 b	17.8±15.7 c	0.7±0.0 ab	13.2±1.1 a	18.8±2.3 a	32±2.7 a
B. bassiana	27.7±13.7 b	54.3±17.4 b	0.5±0.1 abc	7.4±0.7 b	15.4±1.50 a	22.8±1.0 b
Cyantraniliprole	100.0±0.0 a	100.0±0.0 a	0.4±0.0 bcd	0±0.0 c	0.8±0.12 b	0.8±0.12 c
Deltamethrin	98.0±2.0 a	98.2±1.82 a	0.1±0.0 de	1±0.24 c	0±0.0 b	1±0.24c
Emamectin benzoate	98.0±2.0 a	100.0±0.0 a	0.8±0.1 a	0.4±0.10 c	0±0.0	0.4±0.10 c
Lambda-cyhalothrin	97.8±2.2 a	100.0±0.0 a	0.0±0.0 d	0±0.0 c	0.6±0.13 b	0.6±0.13 c
Spinosad[b]	98.0±2.0 a	100.0±0.0 a	0.7±0.1 ab	2.2±0.3 c	0±0.0 b	2.2±0.3 c
Spinetoram	100.0±0.0 a	100.0±0.0 a	0.2±0.0 cde	1.2±0.23 c	0±0.0 b	1.2±0.23 c
Spinosad[c]	100.0±0.0 a	100.0±0.0 a	0.1±0.0 cd	1.2±0.23 c	0±0.0 b	1.2±0.23 c

a Means within each column followed by the same letter (s) are not significantly different (Fisher's LSD test; p<0.05).
b Laser®
c Success®

Female fecundity significantly (F=5.78; DF=8; P <0.0001) affected by exposure to insecticides; the best effect (0.0) resulted in lambda-cyhalothrin, followed by deltamethrin, success®, and spinetoram. Overall, *D. suzukii* individuals emerging after 10 DAT were highly significantly (F=15.01; DF=8; P <0.0001) suppressed by exposure to insecticides lower than the untreated. Emamectin benzoate showed the fewer (0.4) individuals, followed by lambda-cyhalothrin, cyantraniliprole deltamethrin, and spinosyns. In contrast, *B. bassiana* appeared to be the lowest (22.8) activity.

Treatments provided significant mortality against female and male *D. suzukii* at 1 DAT (Female: F=27.03, DF=8, P <0.0001; male: F=40.27, DF=8, P <0.0001). The same pattern remained at 2 DAT as well (Female: F=11.62, DF=8, P <0.0001; male: F=13.04, DF=8, P <0.0001). However, there were no considerable differences between the percent mortality of females and males *D. suzukii* either at 1 or 2 DAT except that *B. bassiana* caused significantly mortality higher than the untreated only in females, recording 24% mortality greater than in males at 2 DAT (figs. 18, 19).

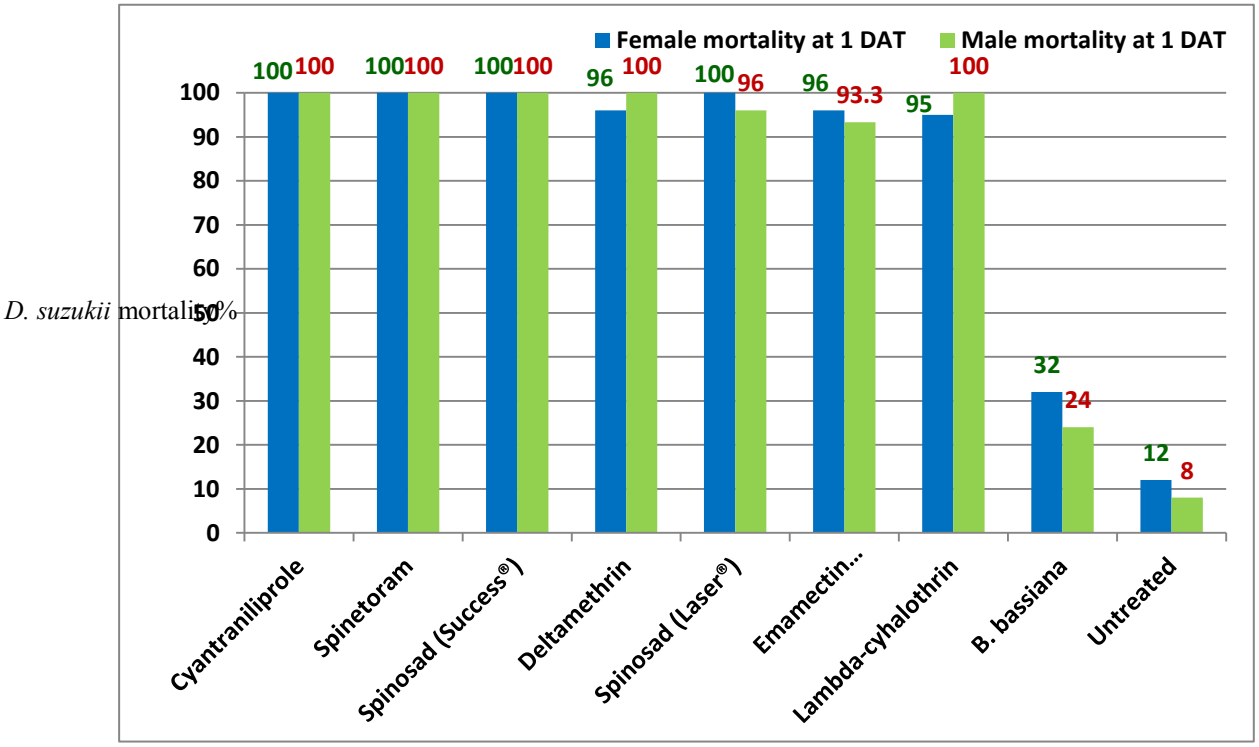


Figure 18. Percent mortality of male and female *D. suzukii* following exposure to various pesticide residuals in September laboratory bioassays at 1 DAT.

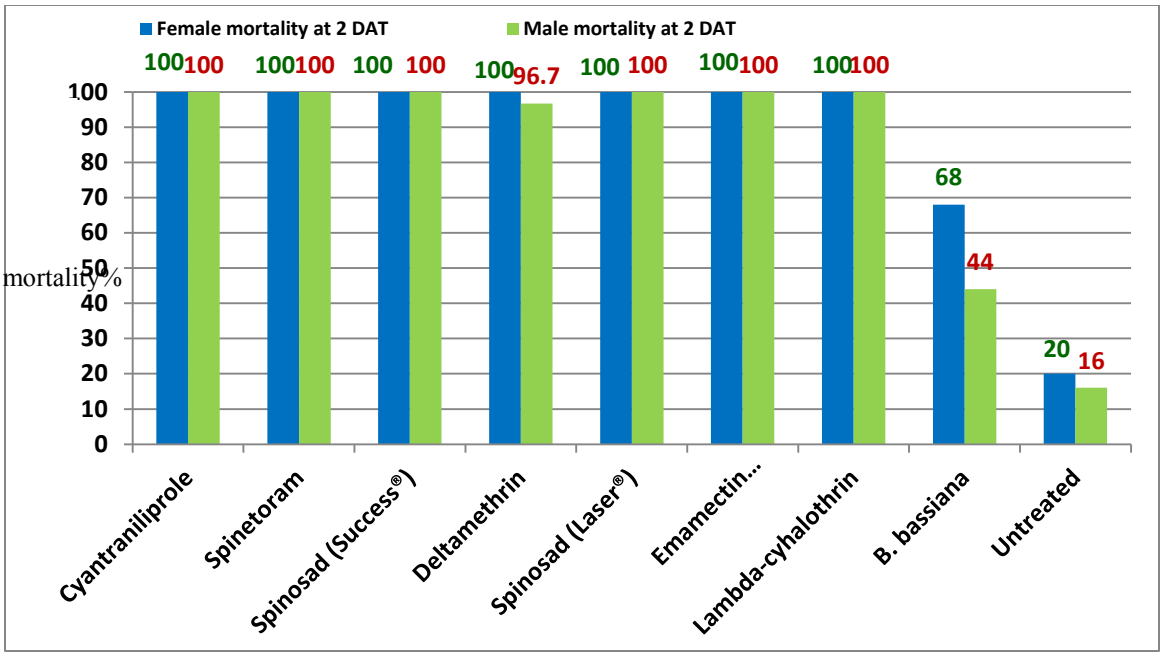


Figure 19. Percent mortality of male and female *D. suzukii* following exposure to various pesticide residuals in September laboratory bioassays at 2 DAT.

3.1.2 Dipping infested strawberries

Development of *D. suzukii* life stages after dipping infested strawberries into the insecticide solutions for 30 seconds is illustrated in Table 13. The tested pesticides significantaly reduced the number of adult *D. suzukii* emerging compared to the untreated (F=96.6; DF=6; *P*<0.0001). Full activity was achieved by emamectin benzoate against adults emerging, followed by spinosad (Laser®), acetamiprid, lambda-cyhalothrin, and deltamethrin.Significant response occurred in females (F=91.4; DF=6; *P*<0.0001) as males (F=87.5; DF=6; *P*<0.0001). In general, all evaluated insecticides significantly (F=101.3; Df=6; *P*<0.0001) supressed the life stages of *D. suzukii,* with full activity in emamectin-benzoate, followed by positive efficacy in spinosad, acetamiprid, lambda-cyhalothrin, and deltamethrin. Efficacy of *B. bassiana* was insufficient.

Table 13. *D. suzukii* life stages emerging after dipping 72-hour infested strawberries into different insecticide solutions within March 2015 dipping bioassay.

Treatment	Adults emerging			Immatures	Total individuals
	Female	Male	Total		
Untreated	32.9±2.2 a	27.2±1.9 a	60.1±3.9 a	1.8±0.6 a	61.9±4.0 a
Acetamiprid	1.8± 0.5d	1.3±0.3 cd	3.2±0.7 d	0.1±0.0 c	3.2±0.7 d
B. bassiana	22.0±2.4 b	18.4±2.2 b	40.3±4.5 b	1.0±0.3 b	41.3±4.4 b
Deltamethrin	6.8±1.2 c	4.4±0.7 c	11.1±1.8 c	0.2±0.1 c	11.4±1.9 c
Emamectin benzoate	0.0±0.0 d	0.0±0.0 d	0.0±0.0 d	0.0±0.0 c	0.0±0.0 d
Lambda-cyhalothrin	2.7± 0.6d	2.4±0.5 cd	5.0±1.1 cd	0.2±0.1 c	5.2±1.1 cd
Spinosad[b]	0.2± 0.1d	0.1±0.1 d	0.3±0.2 d	0.3±0.2 bc	0.6±0.3 d

Means within each column followed by the same letter (s) are not significantly different (Fisher's LSD test; $p<0.05$).
b Laser®.

Table 14 shows the mean of *D. suzukii* life stages emerging after dipping the infested strawberries into different pesticide solutions in September 2015 trial. All tested insecticides, except *B. bassiana,* significantly suppressed total adult emerging (F=23.95; DF=8; *P*<0.0001), with complete activity achieved in cyantraniliprole, emamectin benzoate, lambda-cyhalothrin, spinetoram, spinosad (Laser®) and spinosad (Success®). *D. suzukii* emerging significantly affected in females (F=25.4; DF=8; *P*<0.0001) as males (F=18.0; DF=8; *P*<0.0001) compared to the untreated. Except *B. bassiana,* all insecticides were significantly able to prevent eggs to get larvae (F=7.7; DF=8; *P*<0.0001). Overall, diamide (cyantraniliprole) spinosyns (spinosad and spinetoram), pyrethroid (lambda-cyhalothrin), and avermectin (emamectin-benzoate) significantly suggested full activity, suppressing development of *D. suzukii* individuals (F=51.8; DF=8; *P*<0.0001). Deltamethrin caused an excellent efficacy as well. In contrast, *B. bassiana* presented insignificant effect along with the exeperiment.

Table 14. *D. suzukii* life stages emerging after dipping 72-hour infested strawberries into different pesticide solutions within September 2015 bioassays.

Treatment	Adults emerging			Immatures	Total individuals
	Female	Male	Total		
Untreated	18.8± 4.1 a	11.8± 2.2 a	30.6±6.3 a	7.2 ±2.0 b	37.8±6.3a
B. bassiana	15.8± 2 a	9.0± 2.4 a	24.8±4.2 a	12.4±4.4 a	37.2±2.7 a
Cyantraniliprole	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
Deltamethrin	0.2 ± 0.2 b	0.0 ± 0.0 b	0.2±0.2 b	0.0 ± 0.0 b	0.2±0.2 b
Emamectin benzoate	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
Lambda-cyhalothrin	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
Spinosad[b]	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
Spinetoram	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
Spinosad[c]	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b

a Means within each column followed by the same letter (s) are not significantly different (Fisher's LSD test; $p<0.05$).
b Laser®
c Success®

3.1.3 2014 open-field trial

Figure 20 showes the number of *D. suzukii* individuals that weekly trapped during period of conducting the open-field experement from 4 August till 15 september 2014. The first pesticide application was performed in 28 August and the second was in 4 September. The number of captured flies started to increase by the second and third weeks of August. In the fourth week, after first pesticide application (28 August), number of cought adults gradually decreased.

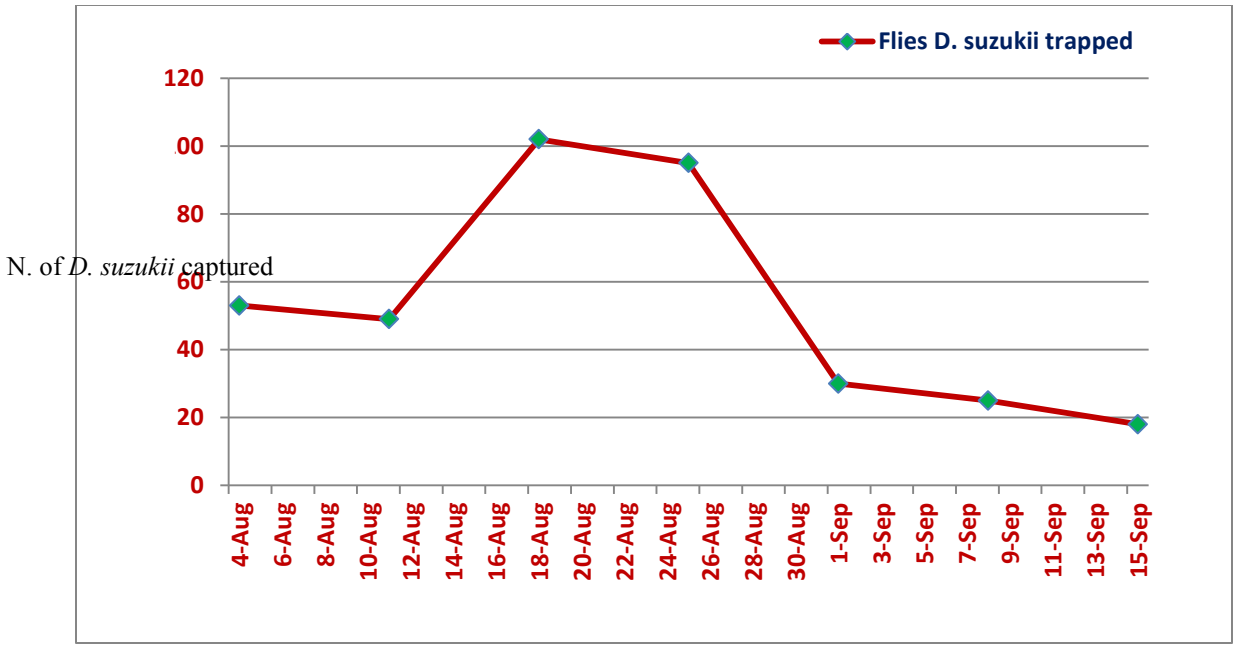


Figure 20. Adult *D. suzukii* captured during the 2014 open-feld trial of strawberry crop.

The efficacy of different chemical control strategies to protect strawberry fruits from the damage caused by *D. suzukii* were evaluated (Table 15). All strategies significantly succeeded to decrease the damage of strawberries compared to the untreated plants either at 14 or 21 DAFA. At 14 DAFA, the fifth strategy caused the highest fruit protection, followed by third, second, and fourth strategies. The fifth one remained to provide the best activity at 21 DAFA, followed by fourth, second, and third strategies. Regarding the percent number of larvae, all strategies significantly decreased the number of larvae compared to the untreated at 14 DAFA, with a highest activity in the second and the fourth strategies. At 21 DAFA, the second strategy only recorded significant percent number of larvae fewer than the untreated, giving the best activity; however, the fifth, fourth, and third strategies reduced the number of larvae lower than the untreated but without significance.

Table 15. Percent of both strawberries damaging and number of larvae following different chemical control strategies of *D. Suzukii* in 2014 open-field trial.

Strategy	Percent strawberries damaging		Percent number of larvae	
	14 DAFA	21 DAFA	14 DAFA	21 DAFA
1st	18.8 a	36.5 a	2.5 a	3.7 a
2nd	7.1 b	20.0 b	0.3 b	1.8 b
3rd	6.7 b	23.0 b	0.4 b	2.9 ab
4th	8.2 b	16.0 b	0.3 b	2.7 ab
5th	5.4 b	15.8 b	0.6 b	2.5 ab

a Means within each column followed by the same letter (s) are not significantly different (Fisher's LSD test; $p<0.05$).

4. Discussion

The present studies focuced on identifying the best chemical control strategy to combact *D. suzukii* on strawberries. Hence, most commonly pesticides registered in Italy and chemical control strategies under laboratory and field conditions were studied. Laboratory bioassays on strawberries greatly supported our earlier studies on cherries presented in chapter 2 which were prior confirmed that pyrethroid (lambda-cyhalothrin), spinosyns (spinosad and spinetoram) **(Bruck *et al*., 2011; Van Timmeren and Isaacs, 2013; Shawer *et al*., 2015)** and diamide (cyantraniliprole) **(Shawer *et al*., 2015**) suggested excellent activity and provided residual controls from 5 to 14 days against adults *D. suzukii.* Those insecticides caused mortality higher than 80% to adults and larvae of *D. suzukii* following topical and dip-strawberry bioassays. And they exhibited a positive effect as toxic baits only for adults *D. suzukii* (**Andreazza *et al*., 2016).** Fecundity of *D. suzukii* female was also significantly affected by the exposure to spinosyns, pyrethroids, and emamectin benzoate. As opposed to prevoius studies, the avermectin (emamectin-benzoate) product showed an excellent adult mortality greater than 91%, reducing adults emerging although female fecundity was high compered to other treatments. However, neonicotinoid (acetamiprid) provided a moderate adult mortality, it suggested significant activity preventing development of *D. suzukii* lifestages. This phenomenon was also confirmed by **Andreazza *et al*. (2016)** who stated that neonicotinoids (acetamiprid and thiamethoxam), and pyrolle (chlorfenapyr) caused intermediate adult *D. suzukii* mortality; however, they reduced the larval infestation upto 86%. This can be attributed to its brief lethal-contact impact as it is readily absorbed by plants, systemic properties and its potentially beneficial long-term sublethal such as antifeeding, repellency and ovipositional deterrence **(Barry and Polavarapu, 2005; Wise *et al*., 2006; Bruck *et al*., 2011)**. Activity of insecticides almost occurred with females as males, except that acetamiprid killed 20% of male *D. suzukii* higher than female; however *B. bassiana* caused about 24% mortality in females higher than males. However, results published by **Smirle *et al*. (2016)** suggested that malathion was more significantly toxicant to older male SWD were than females. *B. bassiana* showed somewhat adult mortality activity **(Cuthbertson *et al*., 2014).** The field chemical control strategies of strawberry suggested that the two treatments of spinosad provided a significant strawberry crop protection. Inclusion of such spinosyn and diamide insecticides in *D. suzukii* management

programs may alleviate the strong selection pressure currently being imposed on a few mode-of-action insecticide classes used by growers to maintain fly suppression over long continuous harvest periods of mixed cultivars **(Knight *et al*., 2016)**.

5. Conclusion

Laboratory and field trials were performed to identify the most effective chemical control strategy to suppress *D. suzukii* on strawberries. Pretreating strawberries bioassay results proved that pyrethroids (Lambda-cyhalothrin, deltamethrin), spinosyns (spinosad, spinetoram), avermictin (emamectin benzoate) and diamide (cyantraniliprole) caused excellent adult mortality higher than 89 and 97% at 1 and 2 DAT, respectively. They also provided a significant residual activity against *D. suzukii* life stages emerging after treatment. Incontrast, neonicotinoids, and *Beauveria bassiana* showed insignificantly activities. The same trend of pretreating strawberries bioassay was repeated within the dipping infested-strawberries bioassay, except that acetamiprid showed good residual control against the *D. suzukii* individuals emerging. All strategies significantly succeeded to decrease the damage of strawberries compared to the untreated plants either at 14 or 21 DAFA. The field findings confirmed that the two treatments of spinosad provided the best strawberry crop protection. It could be concluded that pyrethroids, spinosyns, diamide (cyantraniliprole) and avermictin (emamectin benzoate) should be considerd into the integrated control strategies of *D. suzukii*. However, commitment to regulations of restricted entry interval, preharvest interval and maximum residue limit of insecticides must be considered.

Chapter IV

Efficacy of the entomopathogenic bacteria, *Photorhabdus luminescens* on *Drosophila suzukii*

Abstract

Since its establishment in America and Europe, *Drosophila suzukii* (Matsumura) has caused considerable economic damage to small and thin-skinned fruits including cherry, blueberry, raspberry, grape, and strawberry. In Northern Italy, losses to fruit crops were estimated to be over €8 million in 2010 and 2011. *D. suzukii* attacks fruits at the ripening stage and therefore, the use of chemical pesticides is limited by the high risk of residues in fruits. Moreover, the intensive use of pesticides leaded to the development of pest resistance. Thus biological control agents are expected to play an essential role in managing this pest. A number of bacterial strains and toxins have been widely used for pest control. More recently, the gram positive bacterium, *Photorhabdus luminescens* and its symbiotic *Heterorhabditis* spp.-nematode have been shown to be highly pathogenic to insects, having the potential to replace pesticides for suppressing several pests. Pathogenicity of *P. luminescens* was assessed at different bacterial cell concentrations against third-instar larvae and pupae of *D. suzukii* under laboratory conditions. Larvae at 4 DAT were significantly affected by bacterial treatments when fed toxins; dipping bioassay was less effective. Following oral and dipping bioassays at concentration of 3.5×108 cells mL^{-1} total mortalities of 97 %and 87%, respectively were recorded. For pupae, the concentration of 3.5×108 cells mL^{-1} caused a pupae mortality of 64 and 47%, and a total mortality of 100% and 73.33%, respectively in the direct-spray and dipping bioassays. It could be concluded that *P. luminescens* may play a vital role for managing *D. suzukii*.

1. Introduction

The spotted winged Drosophila, *Drosophila suzukii* **(Matsumura, 1931)** (Diptera: Drosophilidae), has recently colonized fruit growing areas in America and Europe attracting a great concern since its discovery (**Hauser, 2011; Walsh *et al*., 2011; Cowles *et al*., 2015)**. The European Plant Protection Organization (EPPO) has listed the species as a quarantine pest and major risk (**EPPO, 2013)**, posing a major challenge to fruit production. *Drosophila suzukii* is a devastating pest to fruit crops **(Delfinado and Hardy, 1975; Hauser, 2011**) attacking small and thin-skinned fruits including cherry, blueberry, raspberry, grape and strawberry (**Sasaki and Sato, 1995; Lee *et al*., 2011a; Lee *et al*., 2011b, Walsh *et al*., 2011; Bellamy *et al*., 2013; Cuthbertson *et al*., 2014)**, causing extensive economic losses (**Goodhue *et al*., 2011**). It was notified for the first time in Trentino-Alto-Adige region, north-eastern Italy in September 2009 (**EPPO, 2010**). In 2010 and 2011, it caused losses of over €8 million in fruit crops in Northern Italy (AGW, 2012) and more than €1.5 million in strawberries in 2011 in France **(Agusti *et al*., 2011**).

Pesticides are the main pest control method. Due to the intensive use of these chemicals health and environmental hazards are dramatically increasing, as is the development of pest resistance. Alternative control strategies are being sought as a consequence **(Rahoo *et al.*, 2011).**

Photorhabdus luminescens, a Gammaproteobacteria, is a gram-negative and mutualistic bacterium that lives in the gut of entomopathogenic nematodes (Heterorhabditidae) (**Waterfield *et al*., 2001**). Both *P. luminescens* alone and its symbiotic *Photorhabdus*-nematode complex are known to be highly pathogenic to insects **(Guo *et al*., 1999).** Once the nematode infects an insect, *P. luminescens* is rapidly released into the insect haemocoel, where it secretes enzymes and high-molecular-weight toxin complexes **(Blackburn *et al*., 1998**) that disintegrate and bioconvert the body of the infected insect into nutrients which can be consumed by both nematode and bacteria. The infected insect host is killed within 48 hours by producing lethal toxins, such as TcA **(Gotz *et al*.; 1981; Balcerzak, 1991; Guo *et al*., 1999; Blackburn *et al*., 2005)**, and a proteic toxin through the expression of a single gene called makes caterpillars floppy (mcf) (**Daborn *et al*., 2002)**. *P. luminescens* alone are able to kill if they are injected into the insect hemocoel; however,

the nematodes may play a role in insect mortality **(Gotz *et al*., 1981; Balcerzak, 1991; Guo *et al*., 1999**). *P. luminescens* excretes a range of toxins with both injectable and oral insecticidal activity against insect host as well **(Ffrench-Constant *et al*., 2003; Dowling and Waterfield, 2007)**. In laboratory studies *P. luminescens* caused 99% mortality of *Galleria mellonella* larvae at a concentration of 4 x 10^7 cells mL^{-1} (at 30°C and a moisture level of 20 %) (**Rahoo *et al*., 2011**). Moreover, two *P. luminescens* strains showed pathogenic potential against *Caenorhabditis elegans* (Nematoda: Rhabditidae), reducing development rate, survival and reproduction (**Sicard *et al*., 2007)**.

Entomopathogenic symbiotic bacteria may have the potential to replacee pesticides for suppressing of several pests **(Rahoo *et al*., 2011)**. Here we assess the pathogenic potential of *P. luminescens* at different bacterial cell concentrations against larvae and pupae, and their coming life stages of *D. suzukii,* under laboratory conditions.

2. Material and methods

2.1 Insect colony

Drosophila suzukii individuals used in these experiments were reared in the previous work (chapter 2, page: 30) **(Shawer *et al*., 2015).**

2.2 Bacteria colony

P. luminescens Subsp. *akhurstii* W14, (DSM15138) was isolated from soil samples collected from Kiwi-fruit orchards. The bacterial colonies were sub-cultured until pure colonies of uniform morphology. Bacteria were then identified using 16S gene sequencing. The bacteria were inoculated with sterile needles onto sterilized (15psi at 121°C for 30 mins) tryptone soya broth (TSB) agar (17.5 g pancreatic digest of casein, 5.0g NaCl, 3.0g Pancreatic digest of soybean meal, 2.5g K_2HPO4, 2.5g Glucose, 15 g agar; volume brought to 1.0L with distilled water, adjusted to pH 7.3 ± 0.2 at 25°C**) (Atlas, 2010)**, the agar plates were incubated at 28℃ in the dark for 24 h **(Rahoo *et al*., 2011).**

2.3 Bacterial suspensions for bioassays

Methods described by **Mahar *et al.* (2008)** and **Rahoo *et al.* (2011)** were used. Briefly, a single pure colony of *P. luminescens* was inoculated into a 200 mL solution of

sterilized TSB in a laminar flow chamber and placed in a shaking incubator at 28^{o}C and 150 rpm for 3 days in dark conditions. The optical density of broth containing bacteria was then measured using a spectrophotometer at 600 nm wave-length. The concentration of bacterial cells in broth was determined by making 10-fold serial dilutions of the broth, plating and counting colony forming units (cfus) after three days. The concentration of bacteria cells in broth suspension for use in experiments was adjusted to 3.5 x10^{8} cells mL^{-1}, a concentration higher than the concentration of 4 x 10^{7} cells mL^{-1} shown to be effective against larvae and pupae of several insects **(Elawad, 1998; Mahar *et al*., 2008; Rahoo *et al*., 2011)**. Three laboratory bioassays assessed the efficacy of *P. luminescens* for their insecticidal activity at different bacterial-cell concentrations against larvae and pupae of *D. suzukii*.

2.4 larval oral bioassay

For oral bioassay **(Blackburn *et al*., 1998; Waterfield *et al*., 2001),** six bacterial-cell concentrations (3.5 x 10^{8}, 3.5 x 10^{7}, 3.5 x 10^{6}, 3.5 x 10^{5}, 3.5 x 10^{4}, 3.5 x 10^{3} cells mL^{-1}) of *P. luminescens* were evaluated against the third-instar larvae of *D. suzukii*. Ten mL sterilized (15psi at 121^{o}C for 30 mins) *D. suzukii* diet was placed into 9 cm diameter Petri-dishes, allowed to dry, 1 mL of bacterial-cell concentrations was evenly spread on the surface and allowed dry for approximately 30 min. under the laminar flow. Ten 3rd-instar larvae of similar size, age and colour were put in each Petri-dish. All Petri-dishes were covered, sealed with Para film before incubation at 25ºC. A TSB treatment acted as control. Each treatment was replicated thrice. Mortality of larvae at 2, 3 and 4 days after treatment (DAT) was recorded. Development of surviving larvae into pupae and emerged adults were followed. Mortality was also recorded in the emerged pupae and adults previously treated with *P. luminescens* as larvae. Percent mortality was then calculated for immature stages [sum. mortality of larvae (4 DAT) and pupae (10 DAT)], and total life stages [sum. mortality of larvae (4 DAT), pupae (10 DAT), and adults (10 DAT)] of *D. suzukii*. Larvae were considered dead if they were not able to move when lightly touched with a needle (**WHO, 1981; da Silva *et al*., 2013**). While, emerged pupae were considered dead if they were not able to get alive adults. Deformed pupa was also considered dead as well.

2.5 Larval and pupal dipping bioassay

Dipping bioassays were used for insecticidal activity of *P. luminescens* at concentrations of 3.5 x 10^8, 3.5 x 10^7 and 3.5 x 10^6 cells mL^{-1} against larvae and pupae of *D. suzukii*. The same procedures for larvae-oral bioassay were followed except larvae and pupae were fully immersed in bacterial cells for 30s before being placed into Petri dishes. Controls were dipped in TSB. Survival was determined as development to succeeding stages. As mentioned earlier, larvae mortality at 2, 3 and 4 DAT was recorded. Mortalities of emerged pupae (10 DAT) and adults (10 DAT) that were treated as larvae were taken as well. For pupal-dipping bioassay, mortalities of both pupae at 9 DAT, and emerged adults at 7, 8, and 9DAT were considered.

2.6 Pupal direct-spray bioassay

Six bacterial-cell concentrations (3.5 x 10^8, 3.5 x 10^7, 3.5 x 10^6, 3.5 x 10^5, 3.5 x 10^4, and 3.5 x 10^3 cells mL^{-1}) of *P. luminescens* were tested against mature pupae of *D. suzukii* (7 days old) (**Abdel-Razek, 2003)**. Pupae were directly sprayed with 1 ml of bacterial-cells using an atomizer after being placed into Petri dishes. Then, Petri-dishes were covered, sealed, and incubated at 25ºC. Percent mortalities of pupae at 9 DAT and emerged adults at 7, 8, and 9 DAT were noted.

2.7 Data analysis

Bioassay data were analysed by one-way analysis of variance (ANOVA) followed by means separation with Fisher's least significant difference (LSD), using the appropriate models for a completely randomized design (laboratory bioassays) (SAS Institute, 2010). Percentages of adult mortality were transformed to an arcsine (radq (%)) before analysis to stabilize variance and reported means were back-transformed to percentages for presentation. Data were expressed as mean ± standard error (S.E.). Differences were considered significant at p 0.05 level.

Figure 21. Dead *D. suzukii* larva that had fed on diet contains *P. luminescens*.

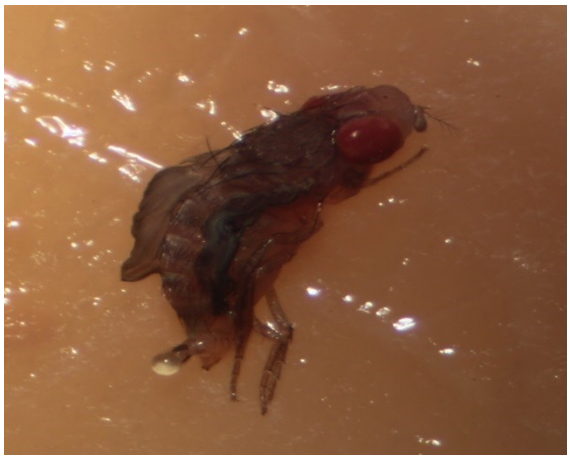


Figure 22. Dead *D. suzukii* adult that had fed on diet contains *P. luminescens*.

Figure 23. Deformed *D. suzukii* adult previously dipped into *P. luminescens* solution as a pupa.

Figure 24. Alive *D. suzukii* female pupa after dipping the larva into TSB medium as a control.

3. Results

3.1 Potential of *P. luminescens* on *D. suzukii* larvae

The influence of feeding *D. suzukii* larvae on diet containing different cell concentrations of *P. luminescens* on larval mortality and on subsequent life stages of *D. suzukii* (pupae and adults) were investigated following the oral toxicity (table 16 and fig. 21). There were no significantly (F=0.87; df=6; P=0.5425) differences between all treatments on larval mortality at 2 DAT; however, the highest three concentrations (3.5×10^8, 3.5×10^7, 3.5×10^5 cells mL^{-1}) suggested mortality higher than control. At 3 DAT, all treatments showed mortality higher than the control, with significantly activity (30%) in the concentration 3.5×10^8 cells mL^{-1}. The number of larvae was significantly (F=3.54; df=6; P=0.0240) affected by treatments at 4 DAT. Whereas, the best effect (36.7%) was caused by

the concentration of 3.5×10^8 cells mL^{-1}, followed by the concentration 3.5×10^7 cells mL^{-1} (33.3%).

Table 16. Percent mortalities of larvae, emerged pupae, and adults *D. suzukii* following the larval-oral bioassay of *P. luminescens*.

Con. (cells mL^{-1})	Percent mortality						
	Larvae			Emerged pupae	Emerged adults		Immatures
	2 DAT	3 DAT	4 DAT	10 DAT	9 DAT	10 DAT	10 DAT
3.5×10^8	10.0± 0.0* a	30± 0.0 a	36.7±1.63 a	10.0±2.5 a	50.0± 1.4 a	50.0± 1.4 a	46.7±2.2 a
3.5×10^7	3.3± 0.8 a	16.7± 2.2 ab	33.3±2.16 ab	3.3±0.8 a	46.7± 3. 6a	53.3 ±4.3 a	36.7±2.9 a
3.5×10^6	6.7± 1.6 a	10± 1.4 b	10±1.4 c	0.0±0.0 a	46.7± 3.6 a	53.3± 4.3 a	10.0 ±1.4 b
3.5×10^5	6.7± 1.6 a	6.7±1.6 b	13.3± 0.8 bc	0.0±0.0 a	46.7± 3.3 a	53.3± 2.9 a	13.3± 0.8 b
3.5×10^4	0.0± 0.0 a	6.7±1.6 b	6.7± 1.6 c	6.7±1.6 a	33.3± 1.6 ab	46.7± 1.6 a	13.3±1.6 b
3.5×10^3	0.0± 0.0 a	6.7±1.6 b	10 ± 2.5 c	3.3±0.8 a	20.0± 2.8 ab	46.7± 2.2 a	13.3± 2.2 b
Control	3.3± 0.8 a	3.3±0.8 b	6.7± 0.8 c	0.0±0.0 a	6.67± 0.8 b	6.7± 0.8 b	6.7± 0.8 b

* Means followed by the same letter (s) within columns are not significantly different ($P<0.05$).

The number of emerged pupae at 10 DAT was insignificantly supressed by feeding their larvae on *P. luminescens* at tested concentrations. However, immature individuals were significantly (F=4.08; df=6; P=0.0141) affected by treatments. The concentrations of 3.5×10^8 and 3.5×10^7 cells mL^{-1} significantly provided 46.7 and 36.7 % mortality in immature stages, respectively. Moderate mortalities (≥ 46.7%) in the emerged adults at 9 DAT were significantly caused by treating their larvae by the bacteria at the concentrations of 3.5×10^8, 3.5×10^7, 3.5×10^6, and 3.5×10^5 cells mL^{-1} compared to the control. While all treatments significantly reduced their numbers at 10 DAT over the control, with a best reduction (50%) in 3.5×10^8 cells mL^{-1} treatment. Overall, all treatments were highly significantly (F=12.53; df=6; P <0.0001) caused total mortality of *D. suzukii* life stages (larvae at 4 DAT, pupae at 10 DAT, and adults at 10 DAT) greater than the control (fig. 25), with excellent activities in treatments of 3.5×10^8 (96.7%) and 3.5×10^7 cells mL^{-1} (90%). While, other treatments showed moderate percent total mortalitiy ranging 60 – 73.3%.

For larval-dipping bioassay, all the tested concentrations of *P. luminescen* were not able to significantly decrease the number of treated larvae at 2, 3, and 4 DAT; however, somewhat mortality higher than the untreated were suggested by the treatments of 3.5×10^8 cells mL^{-1} at 3 and 4 DAT, and 3.5×10^7 cells mL^{-1} at 4 DAT (Table 17 and fig. 25). A significantly decrease in the number of the emerged pupae that were prevoiusly treated as larvae was caused in the treatment 3.5×10^8 cells mL^{-1} at 10 DAT compared to the control, giving pupal mortality of 60%. There were no significant differences appeared in the mortality of the emerged adults at 9 or 10 DAT. Moreover, the highest mortality (30%) in

those emerged adults was performed in 3.5×10^6 cells mL^{-1}, maybe becouse it had the lowest mortality (20%) individuals in the previous imature stages. By the tenth day, tested treatments significantly (F=4.25; df=3; P =0.0451) reduced the number of immature stages, with a significantly greatest activity (70%) caused in the concentration of 3.5×10^8 cells mL^{-1} compared to control.

Table 17. Percent mortality of larvae, emerged pupae and adults *D. suzukii* following dipping the larvae into *P. luminescens* at different concentrations.

Con. (cells mL^{-1})	Percent mortality (DAT)						
	Larvae			Emerged pupae	Emerged adults		Immatures
	2 DAT	3 DAT	4 DAT	10 DAT	9 DAT	10 DAT	10 DAT
3.5×10^8	6.7±0.8* a	6.7±0.8*a	10±0 a	60.0±3.7 a	3.3±0.8 a	16.7±2.9 a	70± 3.7a
3.5×10^7	3.3±0.8 a	6.7±0.8 a	10±1.4 a	36.7±0.8 ab	3.3±0.8 a	16.67±0.8 a	46.7±2.2ab
3.5×10^6	3.3±0.8 a	3.3±0.8 a	3.3±0.8 a	16.7±4.1 b	6.7±1.63 a	30.0±2.5 a	20±3.7b
Control	3.3±0.8 a	3.3±0.8 a	3.3±0.8 a	13.3±2.2 b	3.3±0.8 a	10.0±0.0 a	16.7±1.6b

* Means followed by the same letter (s) within columns are not significantly different (P<0.05).

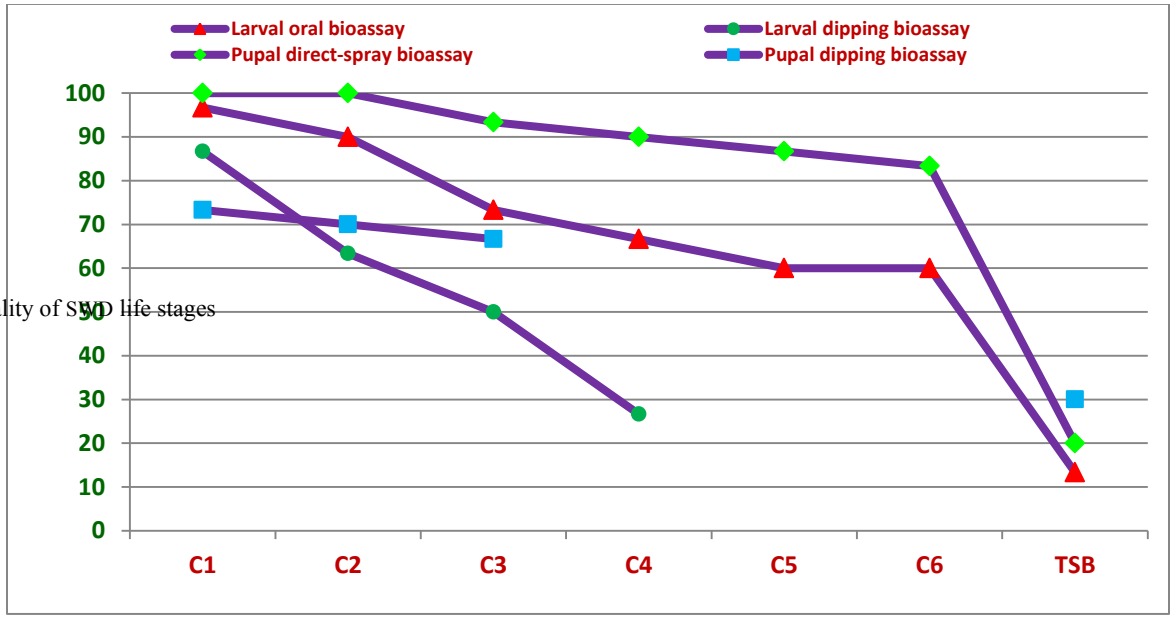


Figure 25. Total mortalities of *D. suzukii* life stages following the larval and pupal bioassays of *P. luminescen*. While, C1=3.5 x 10^8; C2=3.5 x 10^7; C3= 3.5 x 10^6; C4= 3.5 x 10^5; C5=3.5 x 10^4; C6=3.5 x 10^3 cells mL^{-1}, and TSB=controls.

3.2 Potential of *P. luminescens* on *D. suzukii* pupae

In the pupal direct-spray bioassay (Table 18 and fig. 25), significantly (F=4.38; df=6; P =0.0107) reductions in the pupae numbers at 9 DAT ranges 50-63.3% were caused by all concentrations compared to control. Except for 3.5×10^8 cells mL^{-1}, the number of emerged adults was significantly (F=2.86; df=6; P =0.0491) suppressed at 7 DAT in the evaluated treatments higher than the control. At 8 DAT, the number of emerged adults was significantly affected by treating pupae at concentrations of 3.5×10^8, 3.5×10^7, and 3.5×10^3

cells mL^{-1} compared to the control; however, the treatments of 3.5×10^{8}, and 3.5×10^{7} cells mL^{-1} were only significantly different at 9 DAT than the control. The best reduction (50%) in the emerged adults was caused in 3.5×10^{7} cells mL^{-1}. Overall, excellent significant (F=32.96; df=6; P =0.0001) differences in the total mortality of *D. suzukii* individuals over the pupation and adulthood stages were caused in all treatments as a result of spraying pupae with different bacterial concentrations, with full activity (100%) in 3.5×10^{8}, 3.5×10^{7} cells mL^{-1} (fig. 25).

Table 18. Mortalities of pupae, and emerged adults *D. suzukii* following spraying the pupae with *P. luminescens* at different concentrations.

Con. (cells mL^{-1})	Percent pupae mortality	Percent emerged adults mortality		
	9 DAT	7 DAT	8 DAT	9 DAT
3.5×10^{8}	63.3 ± 3.3* a	20 ± 1.4 ab	33.3 ± 2.9 ab	36.7 ± 3.3 a
3.5×10^{7}	50± 3.7 a	30±2.5 a	50± 3.7 a	50± 3.7 a
3.5×10^{6}	63.3± 1.6 a	23.3± 0.8 a	30.00± 1.4 abc	30± 1.4 ab
3.5×10^{5}	63.3± 0.8 a	23.3± 1.6 a	23.3± 1.6 c	26.7± 0.8 ab
3.5×10^{4}	56.7±0.8 a	30± 1.4 a	30± 1.4 abc	30± 1.4 ab
3.5×10^{3}	50± 2.5 a	33.3± 0.8 a	33.3± 0.8 ab	33.3±0.8 ab
Control	10± 0 b	3.3±0.8 b	6.7± 0.8 c	10± 0b

* Means followed by the same letter (s) within columns are not significantly different (P<0.05).

For the pupal dipping bioassay, a significantly decreasing in the number of pupae at 9 DAT was provided in both 3.5×10^{8} and 3.5×10^{7} cells mL^{-1} treatments greater than the control, recording a same activity (46.67%) (Table 19 and figs. 23, 24, 25). The emerged adults were not significantly decreased by the tested bacterial concentrations at 7, 8, and 9 DAT; however, they all constantly caused percent mortality higher than control. Generally, all treatments caused total mortality of *D. suzukii* individuals over the pupation and adulthood stages than the control due to dipping their pupae in different bacterial concentrations, with significantly total mortality (≥70%) were suggested in 3.5×10^{8}, 3.5×10^{7} cells mL^{-1} (fig. 25).

Table 19. Percent mortalities of pupae, and emerged adults *D. suzukii* following dipping the pupae into *P. luminescens* at different concentrations.

Con. (cells mL^{-1})	Percent pupae mortality	Percent emerged adults mortality		
	9 DAT	7 DAT	8 DAT	9 DAT
3.5×10^{8}	46.7 ± 0.8* a	10 ±1.4 a	26.7 ±4.1 a	26.7 ±4.1 a
3.5×10^{7}	46.7 ±2.2 a	16.7 ±2.2 a	20.0 ±2.8 a	23.3 ±3.6 a
3.5×10^{6}	43.3 ±2.2 ab	13.3 ±0.8 a	20± 0.0 a	23.3 ±0.8 a
Control	23.3 ±0.8 b	6.7± 0.8 a	6.7 ±0.8 a	6.7 ±0.8 a

* Means followed by the same letter (s) within columns are not significantly different (P<0.05).

4. Discussion

The entomopathogenic bacteria, *P. luminescens* has gained a considerable attention being lethal to several insect pests (**Dowds and Peters, 2002; Rahoo *et al*., 2011)**. As well as, the discovery of Tc's, lethal toxins produced by *P. luminescens* into the infected insect host **(Gotz *et al*.; 1981; Balcerzak, 1991; Guo *et al*., 1999; Blackburn *et al*., 2005),** has led to great interest in their development as replacements for Bt. **(Dowling and Waterfield, 2007**).Nevertheless, there are no previous studies available concerning the potential of *P. luminescens* against *D. suzukii.* While, this is the first study on this target, it has added to the knowledge base of what species can act as biological agents on *D. suzukii* **(Cuthbertson *et al*., 2014; Naranjo-Lázaro *et al*., 2014; Cuthbertson and Audsley, 2016)**. However, there are many publications which confirm the excellent efficacy of *P. luminescens* bacteria against larvae and pupae of several pests including; *Galleria mellonella* larvae **(Rajagopal and Bhatnagar, 2002; Rahoo *et al*., 2011)**, *Plutella xylostella* pupae (**Abdel-Razek, 2003)**, *Leptinotarsa decemlineata* and *Bemisia tabaci* (**Blackburn *et al*., 2005**), *Spodoptera litura* (**Rajagopal and Bhatnagar, 2002)**, *Manduca sexta* (**Blackburn *et al*., 1998)**. Therefore, the current study establishes new knowledge towards the use of bacteria *P. luminescens* to control larvae and pupae *D. suzukii.* The larval bioassays provided significantly activities of *P. luminescens* against *D. suzukii* life stages, suggesting excellent total mortality (86.67 – 100%) of *D. suzukii* life stages at 3.5×10^{8} cells mL^{-1} treatment. Parallel findings were obtained by **(Rahoo *et al.*** , 2011) who reported that *P. luminescens* caused 99% mortality of *Galleria mellonella* larvae at a concentration of against 4 x 10^{7} cells mL^{-1}. A culture of *P. luminescens* (Strain GPS 12) caused 76 % female mortality of the mushroom mite within 2 DAT; however, another strain of *P. luminescens* showed 83 % mortalities of *L. perniciousus* within 3 DAT (**Bussaman *et al*., 2006)**. Two sets (group was fed on pet food and the other was unfed) of larvae *Aedes aegypti* were exposed to a suspension containing *P. luminescens* which caused 73 % and 83% mortality, respectively of fed and non-fed larvae (d**a Silva *et al*., 2013**). Within 24 h, the treated larvae of *D. suzukii* were briefly feeding on the diet, stopped feeding and produced feces less than usual. After cessation of feeding, larvae body color was changed from green to yellow or black (fig. 23). However, larvae can stay alive for several days after stop feeding. This explains why the significant effect of *P. luminescens* bacteria began to appear at days 3 and 4 after treating larvae. Those results are

in the same trend with those previously obtained by (**Blackburn *et al*., 1998)** who observed similar symptoms and responses form first-instar larvae of *Manduca sexta* after they were exposed to diet treated with 1 µg of Tca, a toxin complex secretes by *P. luminescens.* Moreover, the injection of 100 cells of *P. luminescens* (K-1) isolate was found to be lethal for *G. mellonella* larvae in 48 hours. Likewise, the bacteria toxin that was secreted into the growth medium as the culture supernatant also killed the insect in 48 hours **(Rajagopal and Bhatnagar, 2002)**. Moreover, two *P. luminescens* strains showed pathogenic potential against *Caenorhabditis elegans* (Nematoda: Rhabditidae), reducing the worm's developmental, survival and reproductive rate **(Sicard *et al*., 2007).**

For pupal bioassay, all concentrations were able to reduce pupae individuals compared to the control, achieving mortality ranges from 43.33 - 63.33%. A 100 and 73.33% total mortality was caused by 3.5×10^8 cells mL^{-1} following pupal direct-spray and dipping bioassays, respectively. These findings are in agree with those found by **Abdel-Razek (2003)** towards the effect of both *X. nematophilus* and *P. luminescens* against pupae of *P. xylostella* causing mortalities of 40 and 60 %, respectively. According our visual observations, there were a lot of deformed adults (fig. 25) in pupae were treated with *P. luminescens* cells (data not shown). Most of those deformed adults were not able to fully emerge and normally pursuit its life stages. No deformed adults were noted in the control. Those notes confirmed the same results previously obtained by **Abdel-Razek (2003)** who recorded 24–8% deformed *P. xylostella* adults within different concentrations of *P. luminescens*. Based on those results, it could be concluded that *P. luminescens* bacteria may have a vital role for managing *D. suzukii* as an IPM element.

5. conclusion

The current study establishes new knowledge towards the use of bacteria *P. luminescens* to control larvae and pupae *D. suzukii.* The larval bioassays provided significantly activities of *P. luminescens* against *D. suzukii* life stages, suggesting excellent total mortality (86.67 – 100%) of *D. suzukii* life stages at 3.5×10^8 cells mL^{-1} concentration. Moreover, pupal bioassay concluded that all concentrations were able to reduce pupae individuals compared to the control, achieving mortality ranges from 43.33 - 63.33%. A 100 and 73.33% total mortality was caused by 3.5×10^8 cells mL^{-1} following pupal direct-spray

and dipping bioassays, respectively. We can conclude that *P. luminescens* bacteria may represent a vital role in the biological control of *D. suzukii;* however, further investigations are recommended.

Chapter V

Preparation and characterization of lambda cyhalothrin-chitosan nanoparticles and their bioactivity against *Drosophila suzukii*

Abstract

The newly advanced technology of pesticides encapsulation within nanoparticles is one of the prime objectives of the sustainable agriculture. Lowering the applied pesiticides concentrations and the fewer replicates of applications decrease the toxicity to non-targeted organisms and in turn decrease the risk of wider environmental contamination. Chitosan is a natural polysaccharide polymer that has been widely used as a carrier for controlled drug delivery due to its chemical properties such as biodegradability, biocompatibility, bioactivity, and polycationicity. Chitosan/tripolyphosphate and chitosan /alginate lambda-cyhalothrin nanoparticles were prepared and subjected to physicochemical charcaterization. The optimum encapsulation efficiency (73.6%) and loading capacity (51.4%) were obtained with the formula composed of 1% w/v lambda-cyhalothrin concentration, 0.4%w/v high molecular weight chitosan, 0.3%w/v alginate (anioinic cross-liking agent) and at stirring rate of 500 rpm. The particle size, polydispersity index and zeta potential of prepared nanoparticles were 416 nm, 0.447 and -19.8, respectively. Transmission electron microscope showed a spherical, smooth and almost homogenous structure for nanoparticles. The Fourier transform infrared spectroscopy confirmed tripolyphosphoric groups of TPP linked with ammonium groups of chitosan in the nanoparticles. Release profile of LC loaded CS nanoparticles cross-linked with TPP exhibited an initial burst release of about 30-40% in the first hour followed by controlled release of 50-60% for the subsequent 5 hours. However, the release profile of LC loaded CS nanoparticles cross-linked with ALG showed a constant sustained release of the drug among the time of the release study. All prepared formulations significantly caused adult mortality at 2 and 16 hours after treatment (HAT), with a best activity in the formulation of lowest nanoparticle size (278 nm).

Introduction

Alarms sounded following the 2007-8 food price increases regarding our ability to feed the world in 2050. Some said we need to double food production *(***Wise, 2013).** In order to meet the demand of growing population explosion and diet changes that expected to reach 9.15 billion in 2050, global food production must increase by 70–100% (**Schmidhuber, 2010**). In the 21st century, agriculture productions faced multiple challenges: production of more food with a smaller rural labour force, presence of more feedstocks for a potentially huge bioenergy market, contribution to the overall development in many agriculture-dependent developing countries, adaptation with climate change to get more efficient and sustainable agricultural production methods (**Schmidhuber, 2010**).

On the other hand, agricultural production is facing the biggest challenge by large number of variable insect, pests, diseases, and weeds accounting for 40% losses to the tune (US $2000 billion per year) (**Pimentel *et al*., 2009; Rai and Ingle, 2012; Kashyapa *et al*., 2015).**

The increased use of chemical pesticides on horticultural crops has raised a number of economic, ecological and health concerns. In most developing countries, economic concern arises from farmers that apply conventional pesticides at excessive and indiscriminative quantity. Application of pesticides at concentration greater than recommended dosage, unfortunately lead to ecological problems of common pests developing resistance, elimination of natural enemies and other beneficial arthropods (degradation of agro-ecosystems), and environmental pollution. Human health concerns focus on risks from large deviations from recommended doses and situations that allow for more than 80% loss of pesticide to air, excessive run-off to the soil and water sources, deterioration of soil health, residual toxicity, pest outbreaks and adverse impact on beneficial non-target organism **(Enkerli *et al*., 2004; Sahayaraj and Namasivayam, 2008; Karungi *et al*., 2011).**

However, to ensure and maintain export compliance, grower and consumer safety, and environmental integrity; farmers, government and development partners are developing programmes designed to improve pesticides usage, regulation and management on horticultural crops. The current WTO regime revealed the absolute necessity to limit the use of chemical pesticides to remain in the world market and sustain the competition (**Bharani**

***et al*., 2014)**. Recently, nanotechnology delivery systems were emerged as promising option for pesticide delivery. It involves the manufacture, processing, and application of structures, devices and systems that control the shape and size at the nanometer scale **(Donaldson and Seaton, 2007; Scott, 2007; Bouwmeester, 2009**; **Bharani *et al*., 2014)**.

Application of nanotechnology in agriculture field is relatively recent compared to their use in drug and pharmaceuticals delivery. Thus, nanotechnology had captured the imagination of researchers, manufacturers, and even the general population for producing phytopharmaceutical formulations able to compete crop pathogens, minimize nutrient losses in fertilization, improve crop productivity, decrease hazardous to the end-user and at the same time efficient in pest control (**Bharani *et al*., 2014).** Smart delivery of nutrients, pesticides, herbicides, biocontrol agents and sensors for the diagnosis of plant diseases are some of the emerging topics of nanotechnology for agriculture (**Garcia *et al.*, 2010; Bharani *et al*., 2014)**.

Polymeric nanopesticides formulations have received a greatest attention over the last two years. Encapsulating active ingredients such as fertilizers, herbicides, fungicides, insecticides, and micronutrients in controlled release matrices is one of the most challenging and viable options in the area of agricultural sustainability and food security **(Kah and Hofmann, 2014).**

Lambda-cyhalothrin (LC) is a pyrethroid non-systemic insecticide, belonging to the 3A-IRAC mode of action group that acts by contact and ingestion at nervous system level as a sodium channel modulator (**IRAC, 2016)**. Because of its lipophilic nature, it penetrates the biological tissues; more particularly penetrates the insect cuticle, disrupting nerve conduction in a very short time **(He *et al*., 2008; Tukhtaev *et al*., 2012).** It is reported as very poor water soluble insecticide (5 × 10g/L at 25 °C) that is highly effective against a wide range of pests (**He *et al*., 2008**). It acts as contact and stomach action and repellent properties, giving rapid knockdown and long residual activity **(NPIC, 2001).**

Chitosan (CS) is synthetic polymer obtained by removing the acetate moiety from chitin through amide hydrolysis under alkaline conditions or through enzymatic hydrolysis in the presence of chitin deacetylase (fig. 26) **(Bansal *et al.,* 2011; Suh and Matthew,**

2000; Kashyapa *et al*., 2015). It is available at wide range of molecular weights (500–1400 kDa) and degrees of acetylation. It is a weak base (pK 6.2–7) that has low solubility at physiological pH of 7.4 and free solubility in aqueous acidic media that allow it to acquire a cationic character due to amine groups formation (**Kashyapa *et al*., 2015).** Chitosan was emerged as one of the most promising polymers for efficient delivery of agrochemicals and micronutrients because of its proven biocompatibility, biodegradability, non-toxicity, and adsorption or adhesive properties (**Kashyapa *et al*., 2015)**.

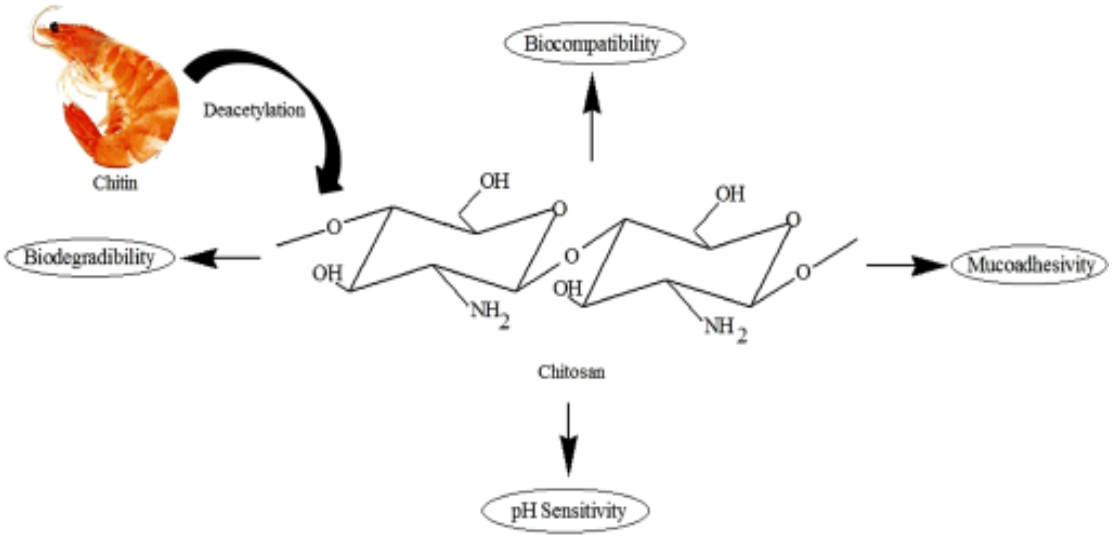


Figure 26. Chemical structure of chitosan along with its characteristic properties (**Saikia *et al*., 2015**).

Agro-chemicals encapsulated into CS matrices have the ability to function as a protective reservoir for the active ingredients (**Kashyapa *et al*., 2015)**. They showed major advantages over traditional forms including; facilitate active molecule uptake through the cell membrane, protect ingredients from the surrounding environment, serve as efficient gene delivery systems for plant transformation or controlled release of pesticides (**Kashyapa *et al*., 2015)**. Absorption enhancing effect of chitosan nanoparticles improves the molecular bioavailability of the active ingredients contained within the nanoparticles **(Tiyaboonchai, 2003; Varshosaz *et al*., 2013**). Most of the characteristic properties of chitosan are attributed to the high content of primary amino groups with a pKa of 6.3. At low pH, the positive charge on the-NH_3^+ groups converts chitosan to a water-soluble cationic polyelectrolyte; when pH increases to above 6.0 the positive charge on the amino groups is lost and chitosan becomes insoluble **(Pillai *et al*., 2009; Kumirska *et al*., 2010).** Chitosan's amine groups allow it to readily complex with a variety of oppositely charged

polymers such as poly (acrylic acid), sodium salt of poly (acrylic acid), carboxymethyl cellulose **(Sonia and Sharma, 2011; Kashyapa *et al*., 2015)** xanthan, carrageenan, alginate and pectin, etc **(Varshosaz *et al*., 2013; Kashyapa *et al*., 2015).**

The ionotropic gelation technique, as the most important technique for ionic cross-linking of CS with low molecular weight counterions, hydrophobic counterions and high molecular weight ions, involve sodium tripolyphosphate, too (**George and Abraham, 2006; Stoica *et al*., 2013**). CS microspheres as a potential carrier for drugs **(Sinha *et al*., 2004; Ion *et al*., 2007; Stoica *et al*., 2013),** like anticancer agents and vaccines **(Susan *et al*., 2011; Stoica *et al*., 2013)** are the most widely studied systems. Chitosan–alginate (CS–ALG) polyionic complexes are formed through the ionic gelation via interactions between the carboxyl groups of alginate and the amine groups of CS **(Yan *et al.*, 2001; Motwani *et al*., 2008).**

Despite the great progress of using chitosan in area of medical and pharmaceutical sciences, there is still a wide gap regarding the potential application of chitosan for encapsulation of active ingredients in agriculture. Therefore, the present study is aimed at prepare of CS nanoparticles loaded with lambda-cyhalothrin by ionotropic gelation technique. The effect of formulation variables such as pesticide concentration, crosslinking type and concentrations, CS molecular weights and concentrations, stirring rate and stirring time on the nanoparticles characteristics will be studied. Factorial design program was applied for minimize the number of prepared formals. The bioactivity of prepared lambda-cyhalothrin-chitosan nanoparticles against *Drosophila suzukii* was investigated.

2. Material and methods

2.1. Materials

Lambda-cyhalothrin 97% technical grade was purchased from Orient Resources International Co., Ltd. (ORICO), China. Chitosan of high and low molecular weights, sodium tripolyphosphate and sodium alginate were purchased from Sigma-Aldrich, Italy. Karate-zeon® 10% CS was provided by Syngenta, Italy. Tween 80 and sucrose were obtained from El-Gomhoria Co., Egypt. All other used reagents were of analytical grade.

2.2. Preparation of lambda cyhalothrin loaded CS-nanoparticles

The LC loaded CS nanoparticles were prepared using ionotropic gelation technique previously described **(Werle *et al*., 2009; Mohammadpour *et al*., 2012)**. Firstly, CS solutions (high and low molecular weight) at concentration 0.4% w/v were prepared by dissolving with 1% v/v acetic acid and kept overnight at room temperature ***(*Gazori *et al.,* 2009*;* Leelapornpisid *et al*., 2010; Masalova *et al*., 2013)**. Tween 80 (1% v/v) was added into the solution as a surfactant to decrease surface tension, facilitate the solution break down and reduce nanoparticles hydrodynamic diameter (**Masalova *et al*., 2013**). Specified weights of the core material (LC powder) at concentration levels of 1 and 1.5% w/v were added to CS solutions under magnetic stirring until homogenous CS-LC dispersions were obtained. Crosslinking agent solutions of sodium tripolyphosphate and alginate were separately dissolved in distilled water at concentration 0.3%w/v and 0.2 % w/v, respectively **(Leelapornpisid, *et al*., 2010)**. Nanoparticles were formed instantaneously upon the dropwise addition of the crosslinking solution to a certain volume of chitosan-LC dispersion. The LC nanoparticle suspensions were gently stirred for 60 minutes at room temperature at 500 or 1500 rpm. Sucrose (5% w/v) was added to prepared nanosuspension to act as a cryoprotectant. The nanoparticles were separated by cooling centrifugation at 20000 rpm at 4 ºC for 30 minutes and followed by freeze drying at 20 Pa and −50 ◦C, respectively for 24h (Freeze drier Alpha 1-2 LD Martin- Christ- Germany).

The CS solutions (0.4% w/v) were prepared by dissolving each high and low molecular weight CS in 1%v/v acetic acid solution (pH 3) and kept overnight at room temperature. The concentration of CS was chosen based on the work of **Masalova *et al*. (2013)** who deduced that a higher than 0.75 % concentrations of CS is not practical because viscosity of the solution becomes too high. As a consequence, a homogeneous distribution of the added cross-linking agent and pesticide is impossible; it would have led to the formation of agglomerates. Whereas, CS concentration less than 0.3% showed low ability for pesticide encapsulation.

2.3. Factorial design and statistical analysis

An experimental 2^{4-1} fractional factorial design was tailored to evaluate and optimize the effect of the formulation variables on the physicochemical properties of the CS-LC nanoparticles. Fractional factorial design reduced considerably the number of preparations (from 16 preparations to eight, in the present case of four factors at two levels each) which is economic and time saving **(Montgomery, 1991).** The four factors were CS type, crosslinking agent, drug concentration, and stirring rate at two levels as shown in Table 20. The design expressed the effect of variables in eight different formulations illustrated in Table 21. Statistical analysis of the experimental data was performed by an ANOVA test using JMP software version 4.0.4 (SAS Institute, Cary, NC, USA). Adult mortality in laboratory bioassay was analysed by one-way analysis of variance (ANOVA) followed by means separation with Fisher's least significant difference (LSD) using the appropriate models for a completely randomized design (SAS Institute, 2010). Percentages of adult mortality were transformed to an arcsine (radq (%)) before analysis to stabilize variance and reported means were back-transformed to percentages for presentation. Data were expressed as mean ± standard error (S.E.). Differences were considered significant at p 0.05 level.

Table 20. Factors and levels of factorial design.

Independent variables		Levels	
		Low	High
CS type (0.4%w/v)	Q	Low Mwt	High Mwt
Crosslinking agent	Q	TPP (0.3%w/v)	ALG (0.2%w/v)
Drug concentration (%w/v)	C	1	1.5
Stirring rate (rpm)	C	500	1500

*The letters *Q* and *C* denote a qualitative factor (cannot be varied continuously) and a continuous factor (can be varied continuously) respectively.

Table 21. Factors and forming materials of lambda-cyhalothrin nanoparticles formulas.

Code	CS MW (0.4% w/v)	Cross linking agent (%w/v)	Drug concentration (% w/v)	Stirring rate (rpm)
F1	High	TPP (0.3)	1.5	1500
F2	High	TPP (0.3)	1	500
F3	Low	TPP (0.3)	1.5	1500
F4	Low	TPP (0.3)	1	500
F5	High	Alginate (0.2)	1.5	1500
F6	High	Alginate (0.2)	1	500
F7	Low	Alginate (0.2)	1.5	1500
F8	Low	Alginate (0.2)	1	500

2.4. Characterization of developed nanoparticles

2.4.1. Determination of percentage yield of LC formulations

The isolated LC loaded CS nanoparticles were weighed and referred to the weight of initial components according to the following equation:

$$\text{Yield percentage (YP)} = \frac{\text{Weight of nanoparticles}}{\text{Total initial solids weight}} \times 100$$

2.4.2. Determination of percentage pesticide loading and entrapment efficiency

Percent pesticide loading (%PL) refers to percentage of pesticide relative to amount of solid polymer as follow:

$$\%PL = \frac{\text{Amount of pesticide entrapped}}{\text{Total weight of polymer incorporated}} \times 100$$

The %EE was determined by direct method prevouisly described by **Acikgoz *et al.* (1996)** with little modification**.** Five milligrams of freeze dried LC loaded CS nanoparticles were dissolved in 20ml of 1% acetic acid overnight. The precipitated LC residue was separated by disposing the supernatant and then dissolved in 10 ml of methanol. The concentration of LC in the solution was measured by UV- spectrophotometer (JASCO, Japan) at wave length 278 nm. The %EE was then calculated using the following equation.

$$\%\ EE = \frac{\text{Amount of drug actually present}}{\text{Total amount of drug added}} \times 100$$

2.4.3 Determination of particle size, PDI and zeta potential

Based on the dynamic light scattering (DLS) technique, particle size, PDI and zeta potential of the developed formulas were measured by photon correlation spectroscopy (Malvern zetasizer, UK). One mg sample of the developed nanoparticles was diluted in 10 mL double distilled water. The samples were sonicated for 5 min prior to size determination. All measurements were taken as average of triplicate.

2.4.4 Morphological examination

Transmission electron microscope (TEM) analysis was performed to examine the morphological characteristics of LC nanoparticles (JEOL, JEM-1230, Japan). The sample was diluted appropriately with 0.1M phosphate buffer to capture the particles image

2.4.5 Fourier transform infrared

The structural features of nanoparticles were measured by Fourier transform infrared (FTIR) spectrometer (FTIR- 4100®, Jasco), using KBr pellets **(Mohammadpour *et al*., 2012).**

2.4.6 *In-Vitro* Release Study

Release of LC from developed formulas was investigated in distilled water, using a modified USP apparatus-I dissolution tester (Hanson Research Dissolution tester, Chatsworth, LA, USA). A specified weight of each sample equivalent to 5 mg loaded LC was placed in 100 ml dissolution medium kept at room temperature 25º C at the stirring speed of 50rpm. At predetermined time intervals, 1ml of dissolution medium was withdrawn and replaced by the same volume of fresh media. The concentration of LC in the collected samples was measured spectrophotometricaly at measurement at λ278 nm. The release study was performed in triplicate for each sample for a period of 6 hr.

3.4.7 Bioactivity of nanoformulations

The potential efficacy of prepared controlled release formulations of LC loaded CS was examined against *D. suzukii* througon strawberries-dipping bioassay as previously described (chapter 2; page: 29) **(Shawer *et al*., 2015)**. *Drosophila suzukii* individuals used in the present experiment were reared in the previous work (chapter 2; page: 29) **(Shawer *et al*., 2015)**. Application rate of prepared formulations used in bioassays was performed based on concentration of the active ingredient in karate-zeon® at its recommended label rate, attempting to unify the percentage of active ingredient in all tested transactions (Table 22). Initial active-ingredient concentration of nanoparticles (1.5 or 1%) was considered for the prepared formulations. The experiment was arranged in a randomized complete block design with ten replications of each treatment and a fruit per each replicate. Water treatment acted as control. *D. suzukii* mortality was recorded at 2 and 16 HAT.

Table 22. Application rate of tested formulation treatments

Formulation code	Active ingredient (% w/v)	Application rate hL^{-1}
F1	1.5	166.7 g
F2	1	250 g
F3	1.5	166.7 g
F4	1	250 g
F5	1.5	166.7 g
F6	1	250 g
F7	1.5	166.7 g
F8	1	250 g
Karate-zeon® CS	10	25 mL

3. Results

3.1 Yield percentage of LC–CS formulations

Ionic gelation is a very simple method that employs the use of oppositely charged complexes (polyanions) to bond with the oppositely charged amino groups of CS (NH3+). The CS nanoparticles produced through ionotropic gelation method are known to be stable, non-toxic and organic solvent free **(Saharan *et al.*, 2013)**. The amounts of yielded nanoparticles compared to amount of excipient used proved the reproducibility of the preparation process as shown in Table 27. The values of %yield ranged from 50 to 80% approximately for all preparations. From Table 27 it could also be concluded that all the formulations prepared with high stirring rate (1500 rpm) showed higher %yield than those prepared with low stirring rate (500 rpm). The formulations coded F1 (high m.wt CS, TPP, 1.5%pesticide, 1500 rpm) and F5 (high m.wt CS, ALG, 1.5%pesticide, 1500 rpm) showed significantly higher %yield 79.6 and 68.5 respectively.

3.2 Percentage of pesticide loading and entrapment efficiency

In most nanoparticle delivery systems, the pesticide carrying capacity is defined as encapsulation efficiency. The results determined for %PL and %EE were illustrated in Table 4 and Figure 5. It was clearly found that the formulations prepared with high molecular weight CS cross-linked with either TPP or ALG showed higher %PL and %EE compared to low molecular weight CS. The results also illustrated an inverse relationship between pesticide concentration and %EE for example F1 showed 22.86 and 53.14% for %PL and %EE respectively compared to 33.45 and 68.93 %PL and %EE respectively in the formula coded F2 .The other factor that imparted significant effect on %PL and %EE was stirring speed, where F2, and F6 showed %PL and %EE 33.45, 68.93 and 51.2, 73.56 respectively.

3.3 Particle size, PDI and zeta potential

Manipulation of formulation parameters can be used to give some control over size range that is obtained to favor the maximum yield of nanoparticles of desired size. The respective average diameters, measured by Zetasizer, of LC-loaded CS nanoparticles ranged from approximately 278 nm and 416 nm (Table 23). The formula coded F7 (low molecular weight CS cross-linked with ALG under low stirring 500 rpm) showed the least particle size

(278 nm) among the other formulations. The results illustrated the significant effect of stirring speed on the particle size and PDI of LC-CS nanoparticles. From Table 23 we can also conclude that high molecular weight CS showed increase in particle size of nanoparticles. All formulations showed PDI <0.5 indicating homogeneity and narrow range of distribution between particles loaded. The PDI value of chitosan nanoparticles was 0.219 while that of insecticide- chitosan nanoparticles was 0.429, thus indicating a narrow and favorable particle size distribution (PDI < 0.5) (Table 23). Zeta potential of CS nanoparticles can greatly influence their stability in suspension by means of electrostatic repulsion between the particles. Our results demonstrated respective zeta potentials ranged between -2.3 and -23.1 mV. These results showed that chitosan molecules are likely to adopt a diffuse conformation in the solution because of electrostatic repulsion force existing between amine groups along the molecular chain. The carboxyl groups on the surface of a large protein molecule may form hydrogen bonds with amine groups at certain sites along the chitosan chain, but still maintain a compact 3D structure without diffusing in the relatively acidic solution so as to keep an inner hydrophobic core.

Table 23. Physical characterization of different LC loaded CS nanoparticles.

Formula	%Yield	%DL	%EE	Particle size(nm)	PDI	Z-potential (mv)
F1	79.64±2.1	22.86±1.4	53.12±3.5	346±21	0.528±0.01	-23.1±0.5
F2	58.44±3.1	33.45±2.7	68.93±2.9	414±13	0.448±0.02	-19.3±0.4
F3	61.62±1.7	24.8±1.7	36.53±1.9	308±5	0.341±0.01	-5.69±0.5
F4	57.31±2.0	18.9±1.8	24.95±1.3	363±12	0.457±0.003	-2.84±0.3
F5	68.55±3.2	48.41±2.2	59.56±2.1	353±8	0.39±0.007	-11.3±0.2
F6	57.48±1.9	51.42±1.6	73.56±4.3	416±16	0.447±0.005	-19.8±0.6
F7	65.60±2.5	20.15±0.9	29.32±1.2	278±11	0.472±0.01	-21.1±1.2
F8	59.29±4.0	24.11±0.8	36.12±1.1	289±9	0.453±0.01	-11.2±0.4

3.4 Morphological examination

In the present study, optical photographs and TEM images have shown the morphological properties and surface appearance of nanoparticles. The nanoparticles have nearly spherical shape, smooth surface as shown in figs. 27 and 28.

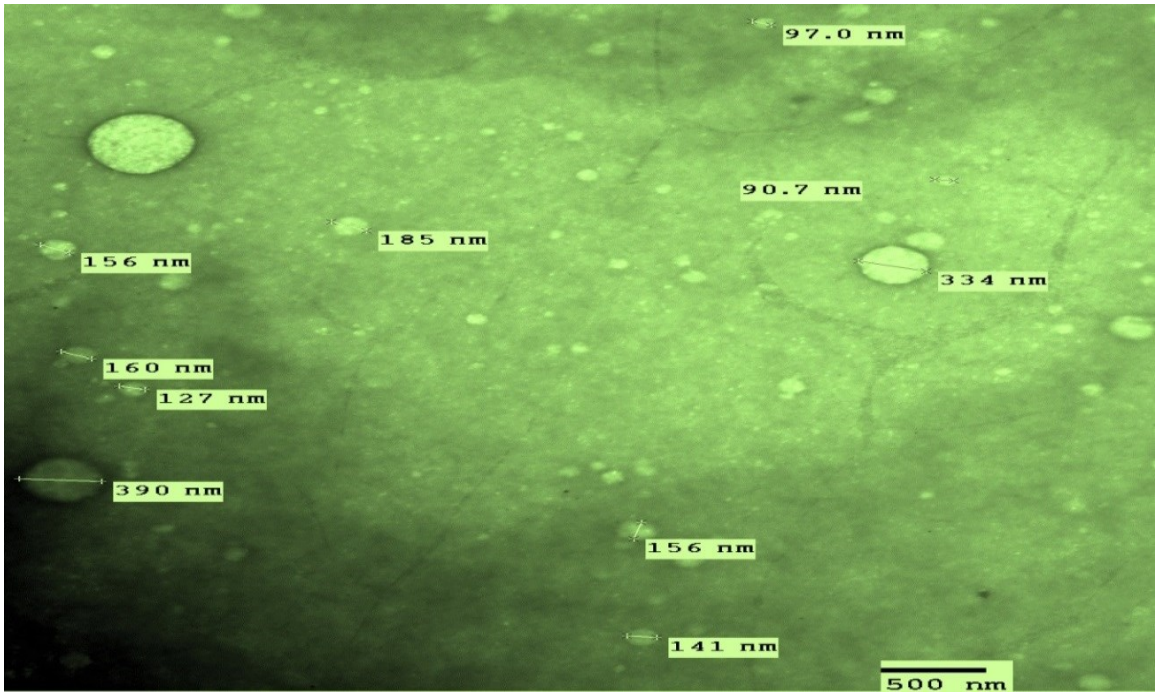


Figure 27. TEM micrograph conforming the spherical shape of LC loaded CS nanoparticles.

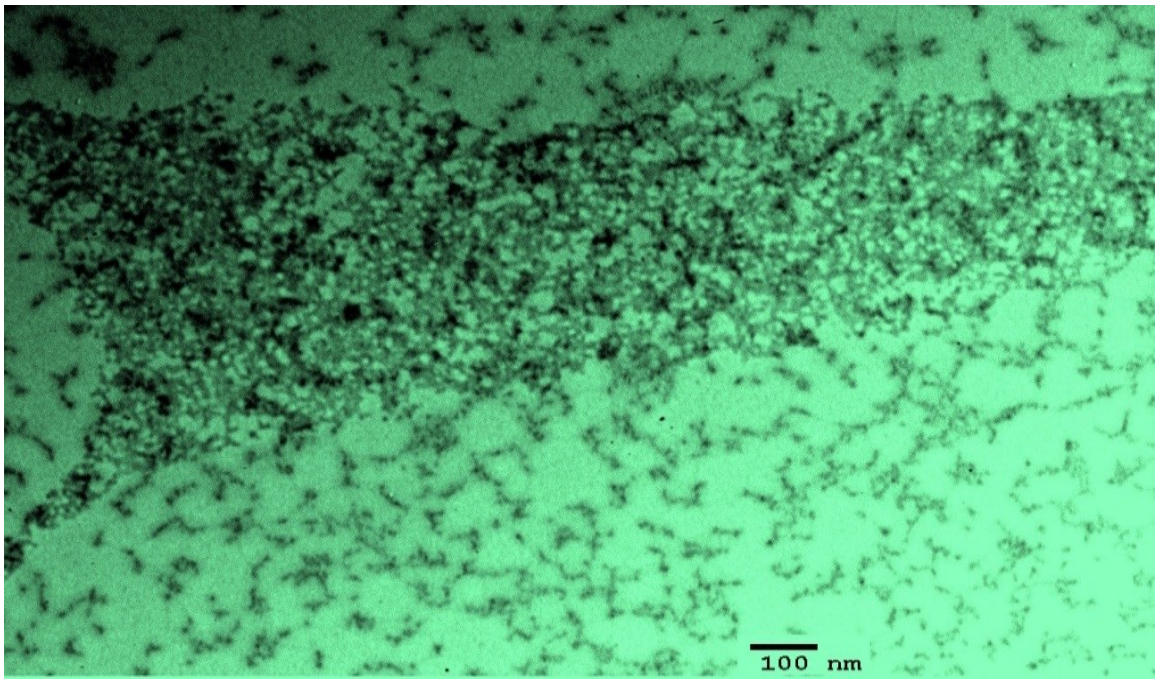


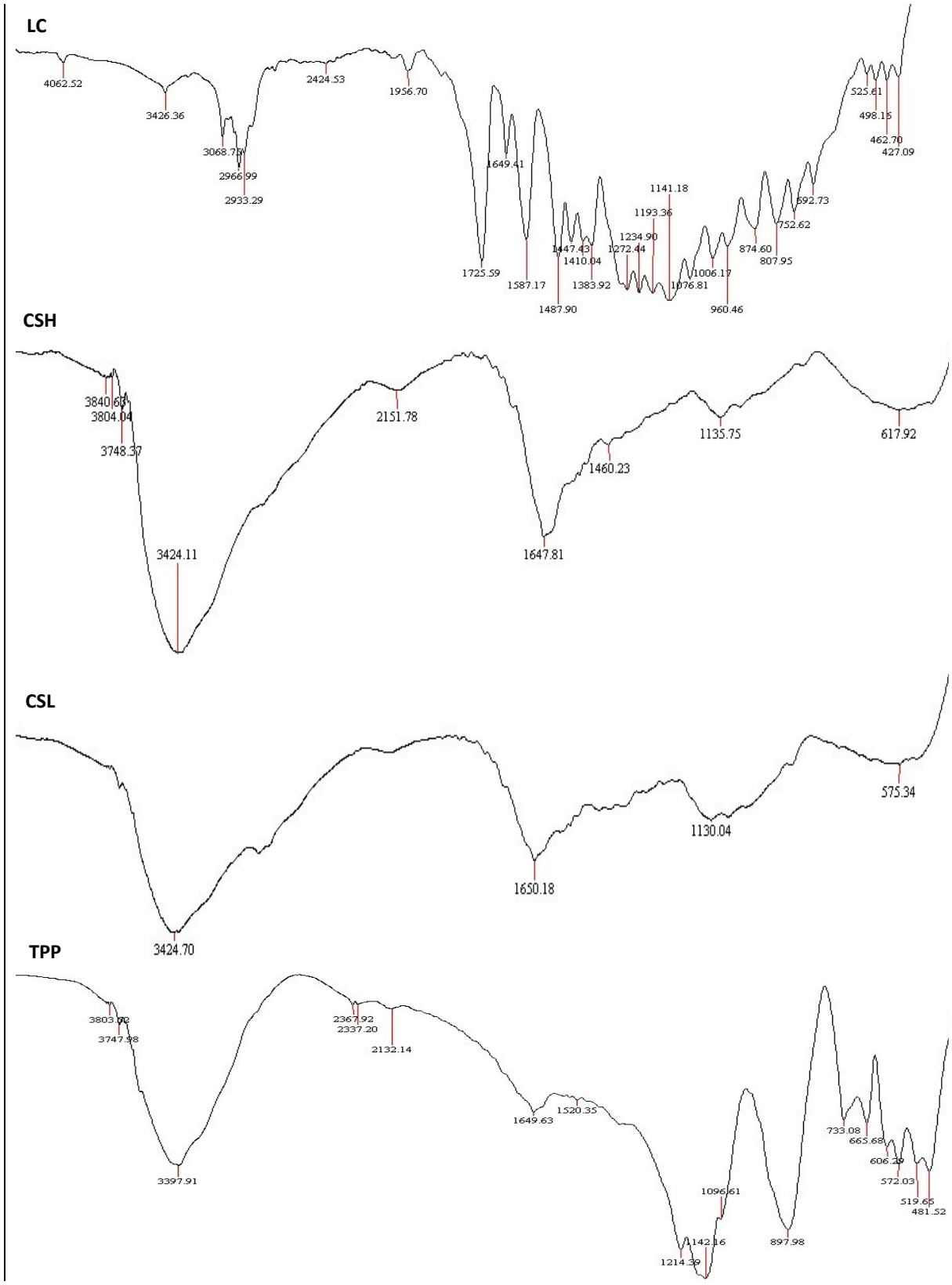
Figure 28. TEM micrograph of LC loaded CS nanoparticles.

3.5 FTIR

The ability of ionic gelation process to form LC loaded CS nanoparticles was assessed by employing FTIR to determine LC-CS interactions and to ensure cross-linking. On the other hand the compatibility between pesticide and other excipients was evaluated too. The FTIR spectra of CS high and low molecular weight, TPP, ALG, and LC loaded chitosan nanoparticles were shown in Fig. 29. In the chitosan spectra (high and low molecular weight), the strong and wide peak in the 3500-3300 area is attributed to hydrogen-bonded O-H stretching vibration. The peaks of N-H stretching from primary amine and type II amide are overlapped in the same region (**Yu *et al*., 1999**). The peak for asymmetric stretch of C-O-C is found at around 1135 and 1130 cm^{-1} in CS high and low molecular weights, respectively.

The spectrum of LC, the peak appeared at 3068 cm^{-1} is attributed C-H bond in the aromatic ring; however, C-H bond in aliphatic chain presented at 2900-3000 cm^{-1} area. The C≡N bond appeared at the area of 2100-2300 cm^{-1}, and C-O bond at 1100-1300 cm^{-1} area. The strong peak at 1587 cm^{-1} is attributed to C=O bond stretching vibration. The spectrum of ALG showed a broad peak for O-H at (3200-3500 cm^{-1}) where the sharp peaks at (1650-1550 cm^{-1}) and (1100-1300 cm^{-1}) presented the C=O and C-O bonds respectively. The characteristic peaks of LC were not affected by encapsulation in CS nanoparticles as shown in figure 31, indicating high compatibility between pesticide and other excipients.

In CS-TPP nanoparticles (F3) the tip of the peak of 3450 cm^{-1} has broadened and shifted to 3300 cm^{-1} with increased relative intensity indicating development of hydrogen bonding. In nanoparticles the peaks for N-H bending vibration of amine I at 1600 cm^{-1} and the amide II carbonyl stretch at 1650cm^{-1} shifted to 1540 cm^{-1} and 1630 cm^{-1}, respectively. The cross-linked CS-TPP also showed a P=O peak at 1170 cm^{-1}. These results have been attributed to the linkage between phosphoric and ammonium ion. So we conclude that the tripolyphosphoric groups of TPP are linked with ammonium groups of chitosan. The inter- and intra-molecular actions are enhanced in chitosan nanoparticles.


LC
4062.52
3426.36
3068.73
2966.99
2933.29
2424.53
1956.70
1725.59
1649.41
1587.17
1487.90
1447.43
1410.04
1383.92
1272.44
1234.90
1193.36
1141.18
1076.81
1006.17
960.46
874.60
807.95
752.62
692.73
525.61
498.15
462.70
427.09
CSH
3840.60
3804.04
3748.37
3424.11
2151.78
1647.81
1460.23
1135.75
617.92
CSL
3424.70
1650.18
1130.04
575.34
TPP
3803.02
3747.98
3397.91
2367.92
2337.20
2132.14
1649.63
1520.35
1214.39
1142.16
1096.61
897.98
733.08
665.68
606.29
572.03
519.65
481.52

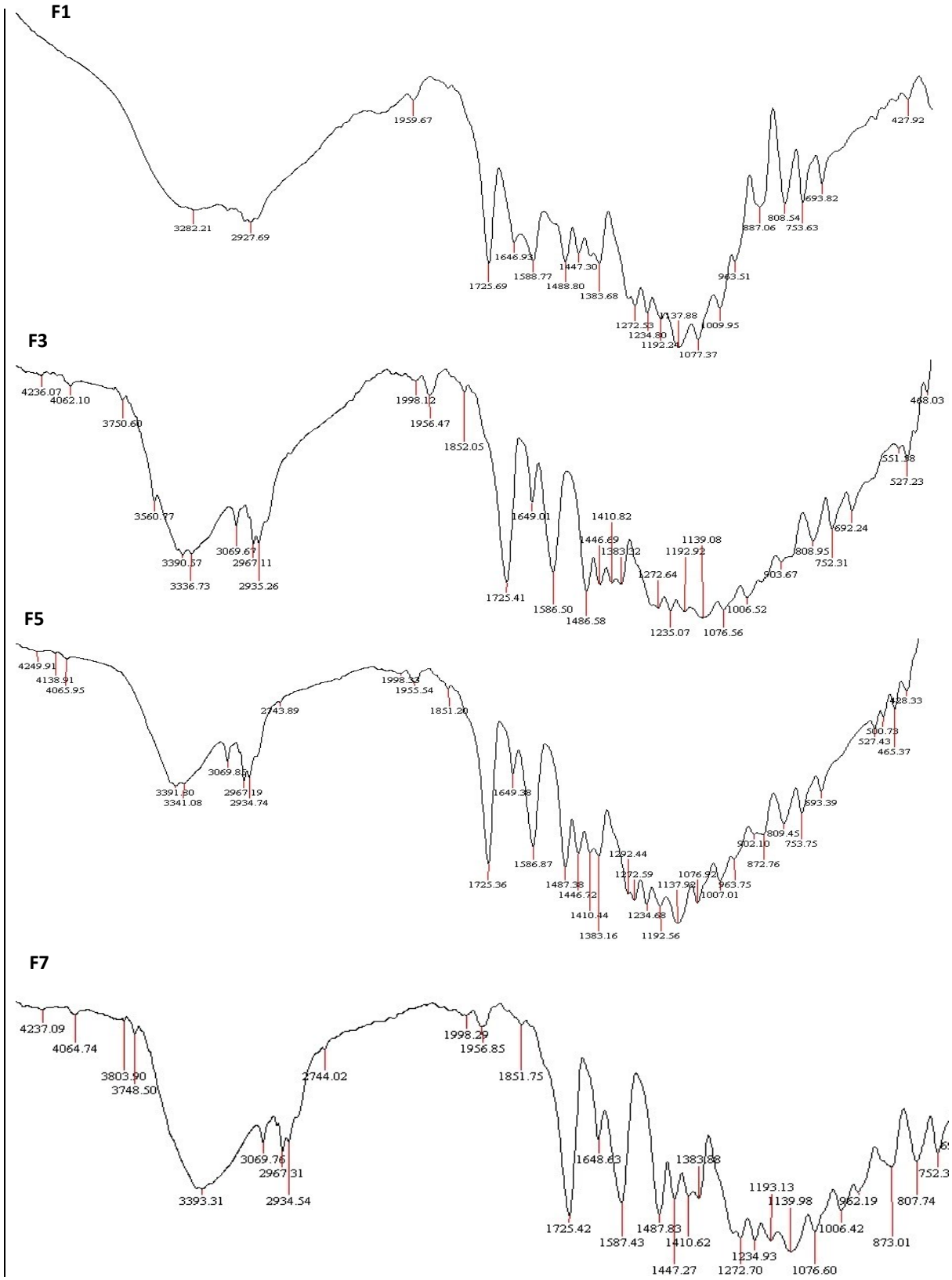


Figure 29. FTIR spectra of LC, CS, TPP, ALG, LC-loaded CS-TPP nanoparticles and LC-loaded CS-ALG nanoparticles. LC=lambda-cyhalothrin; CSH=chitosan high molecular weight; CSL= chitosan low molecular weight; TPP= Tri poly phosphate; ALG=alginate; F1= formula 1; F2=formula 3; F5=formula 5; F7=formula 7.

In ALG/ CS nanoparticles spectra, the peaks for N-H bending vibration of amine I at 1600 cm^{-1} and the amide II carbonyl stretch at 1650cm^{-1} shifted to 1587 cm^{-1} and 1648 cm^{-1}, respectively, this can be attributed to CS and ALG cross-linking.

3.6 *In-Vitro* Release Study

The ability of the prepared nanoparticles to retard pesticide release in the agricultural environment was assessed by conducting pesticide release studies. Our observations showed that about 90% of the loaded LC was released within 6 hours of incubation in distilled water. The release profile of LC loaded CS nanoparticles Cross-linked with TPP exhibited an initial burst release of about 30-40% in the first hour followed by controlled release of 50-60% for the subsequent 5 hours (Figure 30). The observed burst effect can be attributed to dissociation of pesticidemolecules that were loosely bound to the surface of CS nanoparticles. In addition, the effect of diffusion of pesticide molecules dispersing close to the surface of nanoparticles in the first rapid release is undeniable **(Zhou *et al*., 2001**).

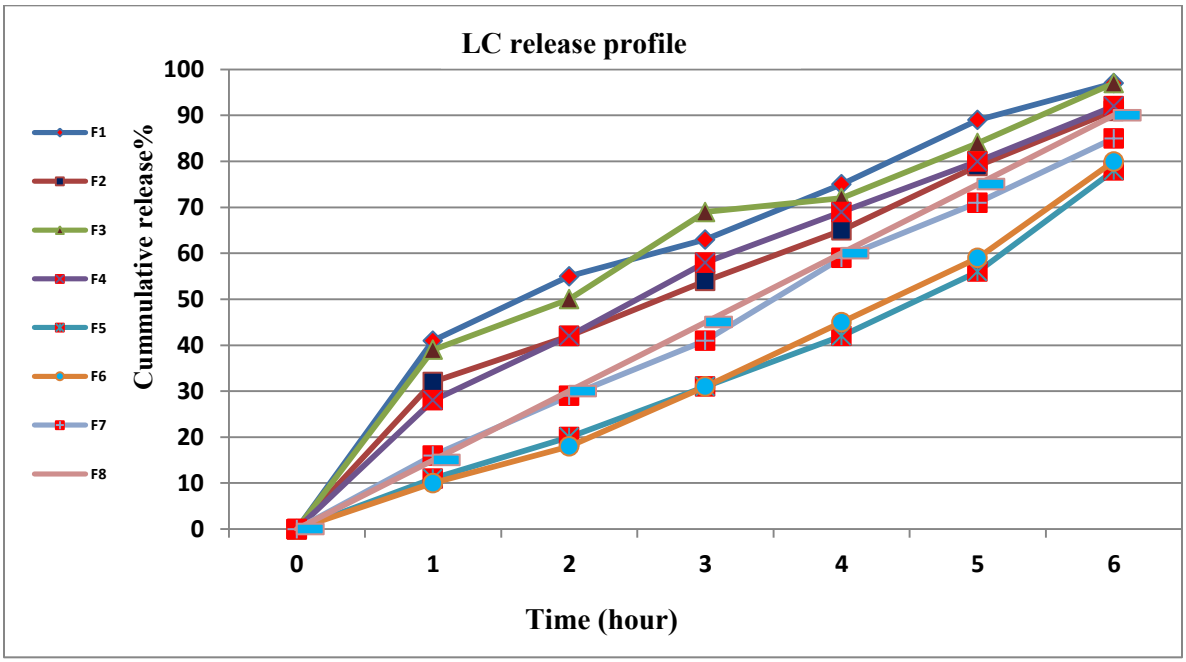


Figure 30. LC release profile from prepared LC-loaded CS nanoparticles.

3.7 Biological performance of prepared CS-LC nano-particles

The eight-prepared lambda-cyhalothrin formulations based on CS-nanoparticles were evaluated and compared to the commercialized insecticide, lambda cyhalothrin (Karatae-zeon®) as a standard towards their efficacy against *D. suzukii* (Table 24). Pre-treating

strawberries bioassay results revealed that all prepared formulations and Karate zeon® significantly (F=12.91; DF=9; P <0.0001) caused *D. suzukii* male mortality higher than control at 2 HAT. As well as the prepared nanoparticle formulations provided percent mortality greater than the comercial product (Karate-zeon®), with a best activity (86%) in F7. The same pattern occurred with females (F=14.82; DF=9; P <0.0001) as males at 2 HAT. At 16 HAT, excellent significantly (males: F=327.26, DF=9, P <0.0001; females: F=180.90; DF=9; P <0.0001) differences were performed by treatments in *D. suzukii* male and female. All prepared formulas recorded activity higher than Karate-zeon®.

Table 24. Percent mortality of male and female *D. suzukii* exposed to 1 h residual strawberries treated with prepared formulations.

Formula	Rate g hL^{-1}	Percent mortality			
		2 h		16 h	
		Male	Female	Male	Female
F1	166.67	84± 5.8 a	78±8.7 ab	100±0.0 a	98±2.0 a
F2	250	74± 7.9 ab	86± 5.2 a	100±0.0 a	100±0.0 a
F3	166.67	74± 6.0 ab	78± 7 ab	100±0.0 a	100±0.0 a
F4	250	82± 6.3 ab	70.9±6.3 ab	100±0.0 a	100±0.0 a
F5	166.67	70± 9.1 ab	60±10.3 b	100±0.0 a	100 ±0.0 a
F6	250	68 ±9.0 ab	80±4.2 a	100±0.0 a	100±0.0 a
F7	166.67	86 ±4.3 a	84±5 a	100±0.0 a	100±0.0 a
F8	250	78± 6.3 ab	68±7.4 ab	100±0.0 a	100±0.0 a
Karate-zeon®	25	64± 7.8 b	74±6.0 ab	98 ±2.0a	90±5.4 b
Control	0	2± 2.0 c	0.0±0.0 c	12±4.4 b	10±3.3 c

4. Discussion

We prepared lambda-cyhalothrin chitosan nanoparticles based on the ionic gelation technique. TEM photographs confirmed that loaded nanoparticles have nearly a uniform spherical shape and smooth surface. Results of CS/TPP nanoparticles spectra concluded that the tripolyphosphoric groups of TPP are linked with ammonium groups of chitosan. As well, the spectra of ALG/ CS nanoparticles proved cross-linking between CS and ALG. All the formulations prepared with high stirring rate (1500 rpm) showed higher yield percentage than those prepared with low stirring rate (500 rpm). These results agreed with **Fabregasa *et al.* (2013)** who found that the stirring speed during ionic gelation significantly affect reaction yield. The results also showed a positive significant effect for pesticide concentration on the amount of yielded nanoparticles. Formulations prepared with high molecular weight CS cross-linked with either TPP or ALG showed higher %PL and %EE

compared to low molecular weight CS which contradicted the results previously published by **Kouchak *et al.* (2012)** who explained the low EE of high MW CS nanoparticles. They reported that the higher viscosity of high MW chitosan solution containing the longer fragments made its free amino groups harder to protonate and restricted the ionic interaction. The results also illustrated an inverse relationship between pesticide concentration and %EE. The results of nanoparticle size (278 - 416 nm) illustrated a significant effect of both stirring speed and CS molecular weight on the particle size and PDI of LC-CS nanoparticles. The formula of low molecular weight CS cross-linked with ALG under low stirring 500 rpm showed the least particle size (278 nm). **Mohammadur Dounighi *et al*. (2016)** prepared optimum venom-loaded nanoparticles with size range of 300-400 nm using low molecular weight at 2 mg/mL, and chitosan/TPP ratio 2:1.

The release profile of LC loaded CS nanoparticles Cross-linked with TPP exhibited an initial burst release of about 30-40% in the first hour followed by controlled release of 50-60% for the subsequent 5 hours. The observed burst effect can be attributed to dissociation of pesticide molecules that were loosely bound to the surface of CS nanoparticles **(Amidi *et al*., 2006; Mohammadpour Dounighi *et al*., 2012)**. The second part of the release profile is related to the slow release of entrapped LC molecules at an approximately constant rate that arises from the slow degradation of nanoparticles **(Dailey *et al*., 2005; Mohammadpour Dounighi *et al*., 2012).** However, the release profile of LC loaded CS nanoparticles Cross-linked with ALG showed a constant sustained release of the pesticide among the time of the release study. This release behavior may be referred to high density of the nanoparticle core and also increase in the diffusional path length, which the pesticide molecules have to traverse.

Pre-treating strawberries bioassay results revealed that the prepared nanoparticle formulations provided percent mortality at greater than the comercial product (karate-zeon®), with a best activity in the optimum formula which prepared by low molecular weight CS cross-linked with ALG under low stirring 500 rpm. This optimum formula showed the lowest particle size (278 nm), therefore it could be concluded that there is a relationship between the particle size and the biological performance of the prepared nano formulations. Those results are in agreement with those found by **Cini *et al*. (2012) and**

Grassi *et al*. (2012) who mentioned that lambda-cyhalothrin performed well achieving an appropriate activity for controlling *D. suzukii* in Trentino Province, Italy**.** Moreover, females of *D. suzukii* that were exposed to spinetoram, lambda-cyhalothrin, and carbaryl laid a number of eggs in cherries fewer than the untreated (**Beers *et al*., 2011; CABI, 2016**).

5. Conclusion

We prepared new controlled release formulations based on lambda-cyhalothrin loaded chitosan nanoparticles using the ionotropic gelation of tripolyphosphate or alginate with chitosan to improve their bioactivities. Optimum encapsulation efficiency (73.6%) and loading capacity (51.4%) were obtained by a 0.4% chitosan high molecular weight, 0.3% ALG cross-liking agent, and LC concentration of 1% and at stirring rate of 500 rpm. The nanoparticle size of this formulation was about 416 nm (polydispersity index: 0.447) and a zeta potential of -19.8. Transmission electron microscope imaging confirmed the spherical, smooth and almost homogenous structure for nanoparticles. FTIR study also confirmed the cross linking between the polymer and cross linking agents. The prepared controlled release formulations significantly caused adult mortality, with a best activity in the formulation of the lowest nanoparticle size (278 nm). Most prepared formulations performed activity greater than the commersialized pesticide (karate-zeon®).

Chapter VI

CONCLUSION

The impact of current registered insecticides in Europe for use on soft fruits, with either traditional or modern mode of action has been studied against *D. suzukii* on cherry and strawberry fruit crops. Those subsequental studies aimed at the identification and optimization of effective strategies to protect fruit crops from *D. suzukii*. Based upon laboratory bioassays and field experements performed on cherries and strawberries, pyrethroids (lambda-cyhalothrin, deltamethrin), spinosyns (spinosad, spinetoram), organophosphates (phosmet, dimethoate) and diamide (cyantraniliprole) caused excellent activities, causing more than 12 days residual control. On the other hand, neonicotinoids, *Beauveria bassiana*, and emamectin-benzoate indicated unsatisfactory results. Moreover, field results proved that two or three insecticide applications were insignificantly able to protect major cherry or strawberry fruit crops. Thus, we support an effective *D. suzukii* control program which can be timely achieved by four applications of insecticides belonging different mode-of-action chemical groups. Except for dimethoate, all residue levels detected in cherries were lower than and completely adherence to their MRLs in force the European Union. Therefore, we can conclude that, spinosyns, diamides, organophosphates and pyrethroids can be used within an IPM strategy to combact *D. suzukii*, considering the development of resistance against those chemical groups.

The potential of the entomopathogenic bacterium, *P.luminescen*s as biological agent against *D. suzukii* was also assessed against the third-instar larvae and pupae of *D. suzukii* under laboratory conditions. Larvae and pupae were significantly affected by bacterial treatments. This means that *P. luminescens* may play an importantrole in managing *D. suzukii*, even if further investigations are recommended.

New controlled release formulations of lambda-cyhalothrin loaded chitosan nanoparticles were prepared and characterized using the ionotropic gelation of tripolyphosphate or alginate with chitosan to improve their bioactivities against *D. suzukii*. The optimum encapsulation efficiency (73.6%) and loading capacity (51.4%) were obtained by a 0.4% chitosan high molecular weight, 0.3% ALG cross-liking agent, and LC concentration of 1% and at stirring rate of 500 rpm. The nanoparticle size of this formulation was about 416 nm (polydispersity index: 0.447) and a zeta potential of -19.8. Transmission electron microscope imaging showed a spherical, smooth and almost

homogenous structure for nanoparticles. Fourier transform infrared (FTIR) spectroscopy confirmed tripolyphosphoric groups of TPP linked with ammonium groups of chitosan in the nanoparticles. All prepared formulations significantly caused adult mortality at 2 and 16 HAT, with a best activity in the formulation of lowest nanoparticle size (278 nm). Most prepared formulations performed activity greater than the commersialized pesticide (karate-zeon®).

Chapter VII

BIBLIOGRAPHY

Abdel-Razek, A. (2003). Pathogenic effects of *Xenorhabdus nematophilus* and *Photorhabdus luminescens* (Enterobacteriaceae) against pupae of the Diamondback Moth, *Plutella xylostella* (L.). Anzeiger für Schädlingskunde, 76:108-111.

Açikgöz, M.; H. Kaş; M. Orman and A. Hincal (1996). Chitosan microspheres of diclofenac sodium: I. application of factorial design and evaluation of release kinetics. J. microencap., 13: 141-159.

Agusti, L; A. Bonaterra; C. Moragrega; J. Camps and E. Montesinos (2011). Biocontrol of root rot of strawberry caused by *Phytophthora cactorum* with a combination of two *Pseudomonas fluorescens* strains. J. Plant Patho., 363-372.

AGW (2012). A report of the commission expert working group on the annexes of Council 2000/29/EC (annexes working group- AWG) http://ec.europa.eu/environment/ppps/home.htmhttp://ec.europa.eu/environment/ppps/home.htm.

Amidi, M.; S. G. Romeijn; G. Borchard; H. E. Junginger; W. E. Hennink; W. Jiskoot (2006). Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. J. Con. Rel., 111(1-2):107-16.

Andreazza, F.; D. Bernardi; C. A. Baronio; J. Pasinato; D. E. Nava and M. Botton (2016). Toxicities and effects of insecticidal toxic baits to control Drosophila suzukii and Zaprionus indianus (Diptera: Drosophilidae). Pest Manag. Sci., doi:10.1002/ps.4348.

Asplen MK., Anfora G., Biondi A., *et al*. (2015) Invasion biology of spotted wing Drosophila (*Drosophila suzukii*): a global perspective and future priorities. Journal of Pest Science 88:469-494

Atlas, R. M. (2010). Handbook of microbiological media CRC press.

Balcerzak, M. (1991). Comparative studies on parasitism caused by entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. I. The roles of

the nematode-bacterial complex, and of the associated bacteria alone, in pathogenesis. Acta Parasitol. Polonica 4.

Bansal, V.; P. K. Sharma; N. Sharma; O. P. Pal and R. Malviya (2011). Applications of chitosan and chitosan derivatives in drug delivery. Advances in Biol. Res., 5 (1): 28-37.

Barney, D. L. (1999). Growing strawberries in the Inland Northwest & Intermountain West. Book: Page: 5. Available at:

Baroffio, C. and S. Fischer (2011). New threat to orchards and berry plants: the cherry fruit fly (Neue Bedrohung fur Obstplantagen und Beerenpflanzen: die Kirschessigfliege), UFA-Revue, 11:46-47.

Barry, J. D. and S. Polavarapu (2005). Feeding and survivorship of blueberry maggot flies (Diptera: Tephritidae) on protein baits incorporated with insecticides. Fla. Entomol., 88:268–277.

Basoalto, E.; R. Hilton and A. Knight (2013). Factors affecting the efficacy of a vinegar trap for *Drosophila suzikii* (Diptera; Drosophilidae). J. Appl. Entomol., 137(8): 561-570.

Beers, E. H.; R. A. Van Steenwyk; P. W. Shearer; W. W. Coates and J. A. Grant (2011). Developing *Drosophila suzukii* management programs for sweet cherry in the western United States. Pest Manag. Sci., 67: 1386-1395.

Bellamy, D. E.; M. S. Sisterson and S. S. Walse (2013). Quantifying host potentials: indexing postharvest fresh fruits for spotted wing drosophila, *Drosophila suzukii*. Plos One, 8:e61227.

Bharani, A. R. S.; S. K. R. Namasivayam and S. S. Shankar (2014). Biocompatible chitosan nanoparticles incorporated pesticidal protein beauvericin (Csnp-Bv) preparation for the improved pesticidal activity against major groundnut defoliator *Spodoptera Litura* (Fab.) (Lepidoptera; Noctuidae). Intern. J. ChemTech Res., 6(12): 5007-5012.

Blackburn, M. B.; J. M. Domek; D. B. Gelman and J. S. Hu (2005). The broadly insecticidal *Photorhabdus luminescens* toxin complex a (Tca): Activity against the Colorado potato beetle, *Leptinotarsa decemlineata*, and sweet potato whitefly, *Bemisia tabaci*. J. Ins. Sci., 5:32.

Blackburn, M.; E. Golubeva; D. Bowen and R. H. Ffrench-Constant (1998). A novel insecticidal toxin from *Photorhabdus luminescens*, toxin complex a (Tca), and its histopathological effects on the midgut of *Manduca sexta*. Appl. and Environ. Microbiol., 64:3036-3041.

Blumel, S. and Hausdorf, H. (2002). Results of 8th and 9th IOBC joint pesticides testing programme: persistence test with *Phytoseiulus persimilis* Athias Heriot (Acari: Phytoseiidae). Pesti. benefi. organisms IOBC/wprs. Bull., 25 (11): 43-51.

Bolda, M. P.; R. E. Goodhue and F. G. Zalom (2010). Spotted wing drosophila: potential economic impact of a newly established pest. Agric. Resour. Econ. Update, 13: 5–8.

Bouwmeester, H.; S. Dekkers; M. Noordam; W. Hagens; A. Bulder; C. Deheer; S. Tenvoorde; S. Wijnhoven; H. Marvin and A. Sips (2009). Review of health safety aspects of nanotechnologies in food production. Regulatory Toxicol. Pharmacol., 53 (1): 52-62.

Brown, P. H.; P. W. Shearer; J. C. Miller and H. M. A. Thistlewood (2011). The discovery and rearing of a parasitoid (Hymenoptera: Pteromalidae) associated with spotted wing drosophila, *Drosophila suzukii*, in Oregon and British Columbia. In: ESA 59th Annual Meeting, 2011 November 13-16, Reno, Nevada, USA.

Bruck, D. J.; M. Bolda; L. Tanugoshi; J. Klick; J. Kleiber; J. DeFrancesco; B. Gerdeman and H. Spitler (2011). Laboratory and field compaqrison of insecticides to reduce infestation of *Drosophila suzukii* in berry crops. Pest Manag. Sci., 67:1375-1385.

Burrack, H. J.; J. P. Smith; D. G. Pfeiffer; G. Koeher and J. Laforest (2012). Using volunteer-based networks to track Drosophila suzukii (Diptera: Drosophilidae) an invasive pest of fruit crops. J. Integ. Pest Manag., 3(4): 1-5.

Bussaman, P.; R. W. Sermswan and P. S. Grewal (2006). Toxicity of the entomopathogenic bacteria Photorhabdus and Xenorhabdus to the mushroom mite (*Luciaphorus* sp.; Acari: Pygmephoridae). Biocon. Sci. Tech., 16: 245-256.

CABI (2016). *Drosophila suzukii* (spotted wing drosophila), Datasheet (online). http://www.cabi.org/isc/datasheet/109283.

Calabria, G.; J. Máca; G. Bächli; L. Serra and M. Pascual (2012). First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. J. Appl. Entomol., 136(1/2):139-147.

Cha, D. H.; S. P. Hesler; R. S. Cowles; H. Vogt; G. M. Loeb and P. J. Landolt (2013). Comparison of a Synthetic Chemical Lure and Standard Fermented Baits for Trapping Drosophila suzukii (Diptera: Drosophilidae). Environ. Entomol., 42(5): 1052-1060.

Cha, D. H.; T. Adams; C. T. Werle; B. J. Sampson; J. J. Adamczyk; H. Rogg and P. J. Landolt (2014). A four-component synthetic attractant for Drosophila suzukii (Diptera: Drosophilidae) isolated from fermented bait headspace. Pest Manag. Sci., 70(2): 324-331.

Chabert, S.; R. Allemand; M. Poyet; P. Eslin and P. Gibert (2012). Ability of European parasitoids (Hymenoptera) to control a new invasive Asiatic pest, *Drosophila suzukii*. Biol. Con., 63(1):40-47.

Cini, A.; C. Ioriatti and G. Anfora (2012). A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. Bull. Insectol., 65, 149-160.

Cordova, D.; E. A. Benner; M. D. Sacher; J. J. Rauh; J. S. Sopa; G. P. Lahm; T. P. Selby; T. M. Stevenson; L. Flexner; S. Gutteridge; D.F. Rhoades; L. Wu; R. M. Smith and Y. Tao (2006). Anthranilic diamides: A new class of insecticides with a novel mode of action, ryanodine receptor activation. Pesti. Biochem. Physiol., 84, 196–214.

Cowles, R. S.; C. Rodriguez-Saona; R.Holdcraft; G. M. Loeb; J. E. Elsensohn and S. P. Hesler (2015). Sucrose improves insecticide activity against *Drosophila suzukii* (Diptera: Drosophilidae). J. Econ. Entomol., 108:640-653.

Cuthbertson, A. G. and N. Audsley (2016). Further screening of entomopathogenic fungi and nematodes as control agents for *Drosophila suzukii*. Insects, 7:24.

Cuthbertson, A. G.; D. A. Collins; L. F. Blackburn; N. Audsley and H. A. Bell (2014). Preliminary screening of potential control products against *Drosophila suzukii*. Insects, 5:488-498.

da Silva, O.S.; G. R. Prado; J. L. R. da Silva; C. E. Silva; M. da Costa and R. Heermann (2013). Oral toxicity of *Photorhabdus luminescens* and *Xenorhabdus nematophila* (Enterobacteriaceae) against *Aedes aegypti* (Diptera: Culicidae). Parasitol. Res., 112: 2891-2896.

Daborn, P.; N. Waterfield; C. Silva; C. Au and S. Sharma (2002). A single Photorhabdus gene, makes caterpillars floppy (mcf), allows *Escherichia coli* to persist within and kill insects. Proceedings of the National Academy of Sci., 99:10742-10747.

Dailey, L. A.; M. Wittmar and T. Kissel (2005). The role of branched polyesters and their modifications in the development of modern drug delivery vehicles. J. Con. Rel., 101(1-3):137-49.

De Ros, G.; G. Anfora; A. Grassi and C. Ioriatti (2013). The potential economic impact of Drosophila suzukii on small fruits production in Trentino (Italy). IOBC-WPRS Bull., 91: 317-321.

Delfinado, M. and D. Hardy (1975). A Catalog of the Diptera of the Oriental region. Vol. II. Suborder brachycera through division aschiza, Suborder cyclorrhapha, Uni. press Hawaii, Honolulu.

Deprá, M.; J. L. Poppe; H. J. Schmitz; D. C. De Toni and V. L. Valente (2014). The first records of the invasive pest *Drosophila suzukii* in the South American continent. J. Pest Sci., 87(3): 379-383.

Desneux, N.; A. Decourtye and J. M. Delpuech (2007). The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol., 52: 81-106.

Donaldson, K. and A. Seaton (2007). Nanosci Technol., 7(12), 432-438.

Dowds, B. C. and A. Peters (2002). 4 Virulence Mechanisms. Entomopath. nematol.:79.

Dowling, A. and N. R. Waterfield (2007). Insecticidal toxins from Photorhabdus bacteria and their potential use in agriculture. Toxicon, 49: 436-451.

Elawad, S. (1998). Studies on the taxonomy and biology of a newly isolated species of Steinernema (Steinernematidae: Nematoda) from the tropics and its associated bacteria. Ph. D. Thesis, Department of Agriculture, University of Reading, UK.

Emiljanowicz, L. M.; G. D. Ryan; A. Langille and J. Newman (2014). Development, reproductive output and population growth of the fruit fly pest *Drosophila suzukii* (Diptera: Drosophilidae) on artificial diet. J. econ. Entomol., 107(4): 1392-1398.

Enkerli, J.; F. Widmer and S. Keller (200). Long term field persistence of *Beauveria brongniartii* strains applied as biocontrol agents' European cockchafer larvae in Switzerland, Biol. Con., 29,115-123.

EPPO (2010). Report of a pest risk analysis for *Drosophila suzukii*. Available at: file:///D:/DROPSA%20PROJECT/Drosophila%20suzukii%20Articals/EPPO%20Methods/epp12059.pdf.

EPPO (2013a). EPPO Datasheet for efficacy evaluation of insecticides on *Drosophila suzukii*. OEPP/EPPO Bull., 43: 386–388.

EPPO (2013b). EPPO Standard PM 7/115 (1) *Drosophila suzukii*. OEPP/EPPO Bull., 43 (3): 417–424. file:///D:/DROPSA%20PROJECT/Drosophila%20suzukii%20Articals/EPPO%20Methods/epp12059.pdf.

EPPO Global Database (2010). First record of *Drosophila suzukii* in Italy: addition to the EPPO Alert List, EPPO Reporting Service no. 01- 2010 Num. article: 2010/007. Available at: https://gd.eppo.int/reporting/article-305.

EPPO Global Database (2016). *Drosophila suzukii*, Available at: https://gd.eppo.int/taxon/DROSSU/distribution.

EPPO, 2012. EPPO Reporting Service. EPPO Reporting Service. Paris, France: EPPO.http://archives.eppo.org/EPPOReporting/Reporting_Archives.htm.

EU pesticide database (2015). Available at: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN.

Fabregasa, A.; M. M˜ınarroa; E. Garcia-Montoyaa; P. Perez-Lozanoa; C. Carrillo; R. Sarratea; N. Sancheza; J. R. Ticoa; J. M. S˜une-Negrea (2013). Impact of physical parameters on particle size and reaction yield when using the ionic gelation method to obtain cationic polymeric chitosan–tripolyphosphate nanoparticles. Int. J. Pharm., 446: 199–204.

FAO (2015). Food and Agriculture Organization of the United Nations, FAO Statistic Division. Available at: http://faostat3.fao.org/browse/Q/QC/E.

FAO stat (2016). Food and Agriculture Organization of the United Nations, statistic division. http://faostat3.fao.org/home/E.

Ffrench-Constant, R.; N. Waterfield; P. Daborn; S. Joyce; H. Bennett; C. Au; A. Dowling; S. Boundy; S. Reynolds and D. Clarke (2003). Photorhabdus: towards a functional genomic analysis of a symbiont and pathogen. FEMS microbiol. reviews, 26:433-456.

Freshfel Europe Activity Report (2012). http://www.freshfel.org/asp/newsroom/.

Gabarra, R.; J. Riudavets; G. A. Rodríguez; J. Pujade-Villar and J. Arnó (2015). Prospects for the biological control of Drosophila suzukii. Biocon., 60 (3): 331–339.

Garcia, M.; T. Forbei and E. Gonzalez (2010). Potential applications of nanotechnology in the agro-food sector. Ciênc. Tecnol. Aliment. Campinas, 30(3): 573-581.

Gargani, E.; F. Tarchi; R. Frosinini; G. Mazza and S. Simoni (2013). Notes on Drosophilasuzukii Matsumura (Diptera: Drosophilidae): field survey in Tuscany and laboratory evaluation of organic products. Redia-Giornale Di Zoologia, 96: 85-90.

Gazori, T.; M. R. Khoshayand; E. Azizi; P. Yazdizade; A. Nomani and I. Haririan (2009). Evaluation of alginate/chitosan nanoparticles as antisense delivery vector: formulation, optimization and in vitro characterization. Carbohydrate Polymers, 77: 599-606.

George, M. and T. E. Abraham (2006). Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan-a review. J. Controlled Release, 114 (1) 1-14.

Goodhue, R. E.; M. Bolda; D. Farnsworth; J. C. Williams and F. G. Zalom (2011). Spotted wing drosophila infestation of California strawberries and raspberries: economic analysis of potential revenue losses and control costs. Pest manag. Sci., 67:1396-1402.

Gotz, P.; A. Boman and H. Boman (1981). Interactions between insect immunity and an insect-pathogenic nematode with symbiotic bacteria. Proceedings of the Royal Soci. of London B: Biol. Sci., 212:333-350.

Grassi, A.; L. Palmie and L. Giongo (2009). New pests of the small fruits in Trentino. (Nuovo fitofago per i piccoli frutti in Trentino.) Terra Trentina, 55(10):19-23.

Grassi, A.; L. Palmieri and L. Giongo (2012). *Drosophila* (Sophophora) *suzukii* (Matsumura), new pest of soft fruits in Trentino (North-Italy) and in Europe. IOBC/wprs Bulletin, 70: 121–128.

Guo, L.; R. O. Fatig; G. L. Orr; B. W. Schafer; J. A. Strickland; K. Sukhapinda; A. T. Woodsworth and J. K. Petell (1999). *Photorhabdus luminescens* W-14 insecticidal activity consists of at least two similar but distinct proteins purification and characterization of toxin A and toxin B. J. Biol. Chem., 274: 9836-9842.

Hamby K. A.; D. E. Bellamy; J. C. Chiu; J. C. Lee; V. M. Walton; N. G. Wiman and A. Biondi (2016). Biotic and abiotic factors impacting development, behavior, phenology, and reproductive biology of *Drosophila suzukii*. J. Pest Sci., 89:605-619.

Hauser M. (2011). A historic account of the invasion of *Drosophila suzukii* (Matsumura)(Diptera: Drosophilidae) in the continental United States, with remarks on their identification. Pest manag. Sci., 67: 1352-1357.

Hauser, M.; S. Gaimari and M. Damus (2009). *Drosophila suzukii* new to North America. Fly Times, 43: 12–15.

Haviland, D. R. and Beers, E. H. (2012). Chemical control programs for *Drosophila suzukii* that comply with international limitations on pesticide residues for exported sweet cherries. J. Integ. Pest Manag., 3(2): 1-6.

Haye, T.; P. Girod; A. G. S. Cuthbertson; X. G. Wang; K. M. Daane; K. A. Hoelmer and N. Desneux (2016). Current SWD IPM tactics and their practical implementation in fruit crops across different regions around the world. J. Pest Sci., 89(3): 643-651.

He, L.; J. Troiano; A. Wang and K. Goh (2008). Environmental chemistry, ecotoxicity, and fate of lambda-cyhalothrin. Rev. Environ. Contam. Toxicol.,195: 71-91.

Ion, R. M.; S. Dreve; D. V. Brezoi (2007). New technologies and products in machines manufacturing and technologies. Presented at the 14th Intern. Conf. - Tehnomus XIV, 4-5 May 2007, Suceava, Romania.

IRAC (2016). Insecticide resistance action committee, IRAC mode of action classification scheme. Ver. 8.1. Available at: http://www.irac-online.org/documents/moa-classification/?ext=pdf.

Kah, M. and T. Hofmann (2014). Nanopesticide research: current trends and future priorities. Environ. Intern., 63: 224-235.

Kanzawa, T. (1935). Research into the fruit-fly *Drosophila suzukii* Matsumura(preliminary report). Yamanashi Prefecture Agri. Exp. Station, Kofu, Japan.

Kanzawa, T. (1936). Studies on *Drosophila suzukii* mats. J. Plant Prot., 23, 66–70, 127–132, 183–191. Abstract in Rev. Appl. Entomol. 24, 315.

Kanzawa, T. (1939). Studies on *Drosophila suzukii* Mats. Kofu, Yamanashi agricultural experiment station 49 pp. Abstract Rev. Appl. Entomol., 29: 622.

Karungi, J.; S. Kyamanywa; E. Adipala and M. Erbaugh (2011). Pesticide utilisation, regulation and future prospects in small scale horticultural crop production systems in a developing country, pesticides in the modern world - pesticides use and management, Dr. Margarita Stoytcheva (Ed.), ISBN: 978-953-307-459-7, InTech, Available from: http://www.intechopen.com/books/pesticides-in-the-modern-world-pesticides-use-andmanagement/pesticide-utilisation-regulation-and-future-prospects-in-small-scale-horticultural-crop-productions.

Kashyapa, P. L.; X. Xiang and P. Heiden (2015). Chitosan nanoparticle based delivery systems for sustainable agriculture. Intern. J. Biol. Macromol., 77: 36–51.

Kasuya, N.; H. Mitsui, S. Ideo, M. Watada and M. T. Kimura (2013). Ecological, morphological and molecular studies on Ganaspis individuals (Hymenoptera: Figitidae) attacking *Drosophila suzukii* (Diptera: Drosophilidae). Appl. Entomol. and Zool., 48(1): 87-92.

Kenis, M. And M. Branco (2010). Impact of Alien Terrestrial Arthropods in Europe. In *BIORISK– Biodiversity & Ecosystem Risk Assessment* 4, 51–71.

Kinjo, H.; Y. Kunimi; T. Ban and M. Nakai (2013). Oviposition efficacy of *Drosophila suzukii* (Diptera: Drosophilidae) on different cultivars of Blueberry. J. Econ. Entomol., 106(4): 1767-1771.

Kiss, B.; G. Lengyel; Z. Nagy and Z. Kárpáti (2013). First record of spotted wing drosophila [*Drosophila suzukii* (Matsumura, 1931)] in Hungary. (A pettyesszárnyú muslica (*Drosophila suzukii*) elsodouble acute~ magyarországi elodouble acute~fordulása.) Növényvédelem, 49(3): 97-99.

Knight, A. L.; E. Basoalto; W. Yee; R. Hilton and C. P. Kurtzman (2016). Adding yeasts with sugar to increase the number of effective insecticide classes to manage *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in cherry. Pest. Manag. Sci., 72: 1482–1490. doi:10.1002/ps.4171.

Kouchak, M.; M. Avadi; M. Abbaspour and; A. Jahangiri and S. K Boldaji (2012). Effect of different molecular weights of chitosan on preparation and characterization of insulin loaded nanoparticles by ion gelation method. Intern. J. Drug Develop. Res., 4(2): 271-277.

Kumari, K.; K. K. Raina and P. P. Kundu (2008). Studies on the cure kinetics of chitosan-glutamic acid using glutaraldehyde as crosslinker through differential scanning calorimeter. J. appl. Polym. Sci., 108(1): 681-688.

Kumirska, J.; M. Czerwicka; Z. Kaczyński; A. Bychowska, K. Brzozowski, J. Thöming and P. Stepnowski (2010). Application of spectroscopic methods for structural analysis of chitin and chitosan. Marine drugs, 8(5): 1567-1636.

Landolt, P. J.; T. Adams; T. S. Davisi and H. Rogg (2012). Spotted wind drosophila, Drosophila suzukii (Diptera: drosophilidae), trapped with Combinations of Wines and Vinegars. Florida Entomologist, 95(2): 326-332.

Lee, J. C.; D. J. Bruck; A. J. Dreves; C. Ioriatti; H. Vogt and P. Baufeld (2011b). In focus: spotted wing drosophila, *Drosophila suzukii*, across perspectives. Pest manag. Sci., 67:1349-1351.

Lee, J. C.; D. J. Bruck; H. Curry; D. Edwards; D R. Haviland; R. A. van Steenwyk and B. M. Yorgey (2011a). The susceptibility of small fruits and cherries to the spotted-wing drosophila, *Drosophila suzukii*. Pest Manag. Sci., 67, 1358–1367.

Lee, J. C.; P. W. Shearer; L. D. Barrantes; E. H. Beers; H. J. Burrack; D. T. Dalton; A. J. Dreves; L. J. Gut; K. A. Hamby; D. R. Haviland; R. Isaacs; A. L. Nielsen; T. Richardson; C. R. Rodriguez-Saona; C. A. Stanley; D. B. Walsh; V. M. Walton; W.

L. Yee; F. G. Zalom and D. J. Bruck (2013). Trap Designs for Monitoring Drosophila suzukii (Diptera: Drosophilidae). Environ. Entomol., 42(6): 1348-1355.

Leelapornpisid, P.; P. Leesawat; S. Natakarnkitkul and P. Rattanapanadda (2010). Application of chitosan for preparation of arbutin nanoparticles as skin whitening. J. Metals, Mat. and Min., 20: 101-105.

Lethmayer, C. (2011). Gefhrliche fly on apple (Gefhrliche Fliegen fur Apfel & Co.) Bessers Obst, 12:4-5.

Mahar, A.; N. Jan; G. M. Mahar and A. Q. Mahar (2008). Control of insects with entomopathogenic bacterium *Xenorhabdus nematophila* and its toxic secretions. Int. J. Agric. Biol., 10:52-56.

Mandrin, J. F.; C. Weydert and Y. Trottin-Caudal (2010). Fruit falls victim to a newly-arrived pest: *Drosophila suzukii*. First reports of damage to cherry. (Un nouveau ravageur des fruits: *Drosophila suzukii*. Premiers dégâts observés sur cerises.) Infos-Ctifl, 266:29-33.

Masalova, O.; V. Kulikouskaya; T. Shutava and V. Agabekov (2013). Alginate and chitosan gel nanoparticles for efficient protein entrapment, Physics Procedia, (40): 69-75.

Matsumura, S. (1931). A revision of the Palaearctic and Oriental Typhlocybid-genera with descriptions of new species and new genera. Insecta Matsumurana, 6: 55-91.

Mazzetto, F.; E. Marchetti; N. Amiresmaeili; D. Sacco; S. Francati; C. Jucker; M. L. Dindo; D. Lupi and L. Tavella (2016). Drosophila parasitoids in northern Italy and their potential to attack the exotic pest *Drosophila suzukii*. J. Pest Sci., 89(3): 837-850.

Milek, T. M.; G. Seljak; M. Simala and M. Bjelis (2011). First record of *Drosophila suzukii* (Matsumara, 1931) (Diptera: Drosophilidae) in Croatia. (Prvi nalaz *Drosophila suzukii* (Matsumara, 1931) (Diptera: Drosophilidae) u Hrvatskoj.) Glasilo Biljne Zastite, 11(5):377-382.

Mitsui, H. and M. T. Kimura (2010). Distribution, abundance and host association of two parasitoid species attacking frugivorous drosophilid larvae in central Japan. European J. Entomol., 107(4):535-540.

Mohammadpour Dounighi, N.; R. Eskandari; M. R. Avadi; H. Zolfagharian; A. Mir Mohammad Sadeghi and M. Rezayat (2012). Preparation and in vitro characterization of chitosan nanoparticles containing *Mesobuthus eupeus* scorpion venom as an antigen delivery system. J. Venomous Ani.Toxins Including Trop. Dis., 18(1): 44-52.

Mohammadur Dounighi, N.; M. Mehrabi; M. R. Avadi; H. Zolfagharian and M. Rezayat (2016). Preparation, characterization and stability investigation of chitosan nanoparticles loaded with the *Echis carinatus* snake venom as a novel delivery system. Archives Razi Inst., 70(4): 269-277.

Montgomery, D. (1991). Design and Analysis of Experiments, Wiley, Chichester.

Mortelmans, J. and H. Casteels and T. Beliën (2012). *Drosophila suzukii* (Diptera: Drosophilidae): a pest species new to Belgium. Belgian J. Zool., 142(2):143-146.

Motwani, S. K.; S. Chopra; S. Talegaonkar; K. Kohli; F. J. Ahmad and R. K. Khar (2008). Chitosan–sodium alginate nanoparticles as submicroscopic reservoirs for ocular delivery: Formulation, optimisation and in vitro characterization, European J. Pharm. and Biopharm., 68: 513–525.

Naranjo-Lázaro, J. M.; M. A. Mellín-Rosas; V. D. González-Padilla; J. A. Sánchez-González; G. Moreno-Carrillo and H. C Arredondo-Bernal. (2014). Susceptibility of *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) to entomophatogenic fungi. Southwestern Entomol., 39:201-203.

Nomano, F. Y.; H. Mitsui and M. T. Kimura (2015). Capacity of Japanese Asobara species (Hymenoptera; Braconidae) to parasitize a fruit pest *Drosophila suzukii* (Diptera; Drosophilidae). J. Appl. Entomol., 139 (1-2) : 105-113.

NPIC (2001). National pesticide infirmation center, lambda-cyhalothrin (General Fact Sheet) technical fact sheet for more technical information. Available at: http://npic.orst.edu/factsheets/archive/l_cyhalotech.pdf.

NPPO (2012). First findings of *Drosophila suzukii*. NPPO., The Netherlands: National Plant Protection Organization.

Orhan, A.; R. Aslantas; B. Ş. Önder and G. Tozlu (2016). First record of the invasive vinegar fly Drosophila suzukii (Matsumura) (Diptera: Drosophilidae) from eastern Turkey. Turk. J. Zool., 40: 290-293.

Pillai, C. K. S.; W. Paul and C. P. Sharma (2009). Chitin and chitosan polymers: Chemistry, solubility and fiber formation. Progr. Polym. Sci., 34:641–678.

Pimentel, D.; R. Peshin and A. K. Dhawan (Eds.) (2009). Integrated pest management: Innovation-development, Springer, Dordrecht, Netherland, pp. 83–88.

Rahoo, A. M.; T. Mukhtar; S. R. Gowen and B. Pembroke (2011). Virulence of entomopathogenic bacteria *Xenorhabdus bovienii* and *Photorhabdus luminescens* against *Galleria mellonella* larvae. Pak. J. Zool., 43: 543-548.

Rai, M. and A. Ingle. (2012). Role of nanotechnology in agriculture with special reference to management of insect pests. Appl. Microbiol. Biotechnol., 94:287–293.

Rajagopal, R. And R. K. Bhatnagar (2002). Insecticidal toxic proteins produced by *Photorhabdus luminescens* akhurstii, a symbiont of *Heterorhabditis indica*. J. Nematol., 34:23.

Raspi, A; A. Canale; R. Canovai; B. Conti; A. Loni and F. Strumia (2011). Insects of the protected areas of the town of San Giuliano Terme (Insetti delle aree protette del comune di San Giuliano Terme). San Giuliano Terme, Pisa, Italy: Felici Editore.

Rossi Stacconi, M. V.; A. Grassi; D. T. Dalton; B. Miller; M. Ouantar; A. Loni, C. Ioriatti; V. M. Walton and G. Anfora (2013). First field records of *Pachycrepoideus*

vindemiae as a parasitoid of *Drosophila suzukii* in European and Oregon small fruit production areas. Entomolo., 1:11-16.

Saharan, V.; A. Mehrotra; R. Khatik; P. Rawal; S. S. Sharma and A. Pal (2013). Synthesis of chitosan based nanoparticles and their in vitro evaluation against phytopathogenic fungi. Int. J. Biol. Macromol., 62 (2013) 677–683.

Sahayaraj, K. and R. N. S. Karthick (2008). Mass production of entomopathogenic fungi using agricultural products and byproducts, Afr. J. Biotechnol., 17: 213-218.

Sasaki, M. and R. Sato (1995). Bionomics of the cherry drosophila, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) in *Fukushima prefecture* (Japan) , 3: Life cycle. Annu. Rep. Soci. Plant Prot. North Japan, 46:170-172.

Schmidhuber, J. (2010). FAO's Long-term Outlook for Global Agriculture—Challenges, Trends and Drivers. Intern. Food Agr. Trade Pol. Council. http://www.agritrade.org/events/documents/schmidhuber.pdf.

Scott, N. R. (2007). Nanotechnology opportunities in agriculture and food systems, Biol. Environ. Eng., Cornell Uni., NSF Nanoscale Sci. Eng. Grantees Conf., December 5, Arlington, VA.

Seljak, G. (2011). Spotted wing drosophila - *Drosophila suzukii* (Matsumura). (Plodova vinska musica - *Drosophila suzukii* (Matsumura). SAD, Revija za Sadjarstvo, Vinogradnistvo in Vinarstvo, 22(3).

Shawer, R.; L.Tonina; E. Gariberti and N. Mori (2015). Efficacy of insecticides against *Drosophila suzukii* on cherries, Intern. Plant Prot. Cong. (IPPC), Berlin, Germany.

Sicard, M.; S. Hering; R. Schulte; S. Gaudriault and H. Schulenburg (2007). The effect of *Photorhabdus luminescens* (Enterobacteriaceae) on the survival, development, reproduction and behaviour of *Caenorhabditis elegans* (Nematoda: Rhabditidae). Environ. Microbiol., 9:12-25.

Sinha, V. R.; A. K. Singla; S. Wadhawan; R. Kaushik; R. Kumria; K. Bansal and S. Dhawan (2004). Int. J. Pharmaceut. 274: 1-33.

Siozios, S.; A. Cestaro; R. Kaur; I. Pertot; O. Rota-Stabelli and G. Anfora (2013). Draft genome of the Wolbachia endosymbiont of *Drosophila suzukii*. Genome Announc., 1(1):e00032-13.

Smirle, M. J.; C. L. Zurowski; M. M. Ayyanath; I. M. Scott and K. E. MacKenzie (2016). Laboratory studies of insecticide efficacy and resistance in *Drosophila suzukii* (Matsumura)(Diptera: Drosophilidae) populations from British Columbia, Canada. Pest. Manag. Sci.. doi:10.1002/ps.4310

Sonia, T. A. and C. P. Sharma (2011). Chitosan and its derivatives for drug delivery perspective. Chitosan for biomaterials I. Springer Berlin Heidelberg, 23-53.

Stark, J. D. and J. E. Banks (2003). Population-level effects of pesticides and other toxicants on arthropods. Ann. Rev. Entomol., 48(1): 505-519.

Steck, G. J.; W. Dixon and D. Dean (2009). Spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), a fruit pest new to North America. Pest Alerts http://www.fl.dpi.com/enpp/ento/*drosophila_suzukii*.html. (Accessed on 01/08/2014).

Stoica, R.; R. Şomoghi and R. M. Ion (2013). Presentation of chitosan-tripolyphosphate nanoparticles for the encapsulation of polyphenoles extracted from rose hips. Digest. J. Nanomat. Biostructures, 8 (3): 955 – 963.

Suh, J. K. F. and H. W. Matthew (2000). Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomater., 21(24): 2589-2598.

Susan, M.; I. Baldea; S. Senila; V. Macovei; S. Dreve; R. M. Ion and R. Cosgarea (2011). Photodamaging effects of porphyrins and chitosan on primary human keratinocytes and carcinoma cell cultures. Int. J. Dermatol., 50 (3): 280-286.

Tiyaboonchai, W. (2003). Chitosan nanoparticles: a promising system for drug delivery. Naresuan Uni. J., 11 (3): 51-66.

Tochen, S.; D. T. Dalton; N. G. Wiman; C. Hamm; P. W. Shearer and V. M. Walton (2014). Temperature-related development and population parameters for *Drosophila suzukii* (Diptera: Drosophilidae) on cherry and blueberry. Environ. Entomol., 417-424.

Tochen, S.; V. M. Walton and J. C. Lee (2016a). Impact of floral feeding on adult *Drosophila suzukii* survival and nutrient status. J. Pest Sci., 89(3):793–802.

Tukhtaev, K.; S. T. N. Zokirova and N. Tukhtaev (2012). Effect of long term exposure of low doses of lambda-cyhalothrin on the level of lipid peroxidation and antioxidant enzymes of the pregnant rats and their offspring. Med. Health Sci. J. (MHSJ), 13: 93-98.

Van Timmeren, S. and R. Isaacs (2013). Control of spotted wing drosophila, *Drosophila suzukii*, by specific insecticides and by conventional and organic crop protection programs. Crop Prot.; 54: 126-133.

Varshosaz, J.; N. Tavkoli; F. Moghaddam and E. Ghassami (2013). Polyelectrolyte complexes of chitosan for production of sustained release tables of bupropion HCL. Farmacia, Vol. 63 (1): 65-71.

Vogt, H.; P. Baufeld; J. Gross; K. Kopler and C. Hoffmann (2012). *Drosophila suzukii*: a new threat feature for the European fruit and viticulture - report for the intern. Conf. Trient, 2, December 2011. J. fur Kulturpflanzen, 64:68-72.

Walsh, D. (2009). Spotted wing drosophila could pose threat of Washington fruit growers. Washington State Uni. Extension. http://sanjuan.wsu.edu/Documents/SWD11.09.pdf.

Walsh, D. B.; M. P. Bolda; R. E. Goodhue; A. J. Dreves; J. Lee; D. J. Bruck; V. M. Walton; S. D. O‟Neaand and F. G. Zalom (2011). *Drosophila suzukii* (Diptera: Drosophilidae): Invasive pest of ripening soft fruit expanding its geographic range and damage potential. Intern. J. Pest Manag., 2: 1–7.

Wang, X. G.; T. J. Stewart; A. Biondi; B. A. Chavez; C. Ingels; J. Caprile and K. M. Daane (2016). Population dynamics and ecology of *Drosophila suzukii* in Central California. J. Pest Sci., 89(3):701-712

Waterfield, N.; A. Dowling; S. Sharma; P. J. Daborn; U. Potter and R. H. Ffrench-Constant (2001). Oral toxicity of P*hotorhabdus luminescens* W14 toxin complexes in *Escherichia coli*. Appl. Environ. Microbiol., 67:5017-5024.

Werle, M.; H. Takeuchi and A. Bernkop-Schnürch (2009). Modified chitosans for oral drug delivery. J. Pharmaceut. Sci., 98: 1643-1656.

Weydert, C.; J. F. Mandrin and B. Bourgouin (2012). *Drosophila suzukii*: a report on the pest status in tree-fruit growing and small fruits. (Le ravageur *Drosophila suzukii*: point sur la situation en arboriculture fruitière et petits fruits.) Infos-Ctifl, 279:45-52.

WHO (1981). Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides.

Wiman, N. G.; D. T. Dalton; G. Anfora; A. Biondi; J. C. Chiu; K. M. Daane and A. Grassi (2016). *Drosophila suzukii* population response to environment and management strategies. J. Pest Sci., 1-13.

Wise, J. C.; A. B. Coombs; C. Vandervoort; L. J. Gut; E. J. Hoffmann and M. E. Whalon (2006). Use of residue profile analysis to identify modes of insecticide activity contributing to control of plum curculio in apples. J. Econ. Entomol., 99: 2055–2064.

Wise, T. A. (2013). Can we feed the world in 2050. A scoping paper to assess the evidence (Working Paper no. 13-04). Tufts Uni., Global Develop. Environ. Inst. http://ase.tufts.edu/gdae/Pubs/wp/13-04WiseFeedWorld2050.pdf.

Yan, X. L.; E. Khor and L.Y. Lim (2001). Chitosan-alginate films prepared with chitosans of different molecular weights. J. Biomed. Mater., 58:358–365.

Yu, J. H.; Du, Y. M. and H. Zheng (1999). Blend films of chitosan-gelation. Wuhan Univ. J. Nat. Sci., 45:440-4.

Zerulla F. N.; S. Schmidt; M. Streitberger; C. P. W. Zebitz and R. Zelger (2015). On the overwintering ability of *Drosophila suzukii* in South Tyrol. Journal of Berry Research 5:41-48.

Zhou, S. B.; Deng, X. M. and Li, X. (2001). Investigation on a novel core-coated microspheres protein delivery system. J. Con. Rel., 75(1-2): 27-36.