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CHARACTERISATION OF INSECTICIDE RESISTANCE MECHANISMS IN ITALIAN POPULATIONS OF THE GREEN PEACH APHID *MYZUS PERSICAE* (SULZER)

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SUMMARY

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ABBREVIATIONS

1-NA: 1-naphthyl acetate

1-NB: 1-naphthyl butyrate

1-NP: 1-naphtol

7-MFC: 7-methoxy-4-trifluoromethylcoumarin

Ach: acetylcholine

AChE: acetylcholinesterase

ATChI: acetylthiocholine iodide

AZA: azamethiphos

DBLS

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid)

E4: esterase isoenzyme

EcoSyn: ecofriendly synergists for insecticide formulations

FBRR: Fast Blue RR salt

FE4: variant of E4

FU: fluorometric units

GABAR: GABA gated-chloride channels receptors

GSTs: glutathione-S-transferases

IC_{50}: inhibitor concentration required to give 50% inhibition of enzyme activity

IRAC MoA: IRAC mode of action

IRAC: insecticide resistance action committee

IRM: Integrated resistance management

kdr: knock-down resistance

LC_{50}: lethal concentrations required to kill 50% of the population

MACE: modified acetylcholinesterase

MDP: methylenedioxyphenyl

MFOs: mixed function oxidases

mOD: milli optical density

nAChR: nicotinic acetylcholine receptors

nAChRβ1: Arg to Thr substitution in position 81 in the loop D region of the nicotinic acetylcholine receptor β1 subunit

NADHP: nicotinamide adenine dinucleotide phosphate tetrasodium salt

OPs: organophosphate

P450s: cytochrome P450s monooxygenases

PASA-PCR: allele specific polymerase chain reaction amplification

PBO: piperonyl butoxide

Pgp: p-glycoprotein transporters

pNA: 4-nitrophenylacetate

RF: resistance factor

RyR: ryanodine receptors

SAR: structure activity relationship

SF: synergism factor

s-kdr: super-kdr

SNPs: single nucleotide polymorphisms

VGSC: voltage-gated sodium channels

CHAPTER 1

GENERAL INTRODUCTION

1. INSECTICIDE RESISTANCE

1.1 WHAT IS INSECTICIDE RESISTANCE?

Insecticide resistance is an evolutionary process resulting from genetic adaptation of pest populations due to selection pressure exerted by pesticides (Mota-Sanchez *et al*. 2002). After the first report at the beginning of the last century (Melander 1914), the frequency of resistant cases grew during the years, with an exponential increase during the late 1970s and early 1980s (Georghiou *et al*. 1991). This trend has continued into the 21st century and pesticide resistance has become a subject of interest for the scientific community, as demonstrated by the high number of scientific contributors, including international health organizations and agrochemical companies. For these reasons, the definition of resistance has been adapted and refined over the years. In 1957, a panel of the WHO (World Health Organization) experts defined resistance as "*the development of an ability in a strain of pests to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species*" (http://whqlibdoc.who.int/Hq/1998/WHO_CDS_CPC_MAL_98.12.pdf).

However, the definition was focused on a population view rather than individuals. A few years later, J.F. Crow proposed a more flexible version that considered the survival of single individuals within a population: "*Resistance marks a genetic change in response to selection*" (Crow 1960). In 1987, R.M. Sawicki expanded this definition that became "*a genetic change in response to selections by toxicants that may impair control in the field*" (Sawicki 1987). With their interpretations, both authors introduced the important concept of resistant management: focusing on

single specimens, it is possible to encourage an early detection of resistance instead of initiate countermeasures after field failures of a product.

The most recent revision of resistance definition is the one proposed by IRAC (Insecticide Resistance Action Commitee) that gives a more practical interpretation of resistance: "*Resistance is a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species*" (http://www.irac-online.org/about/resistance). The interpretation differs slightly from the others, giving a restricted criteria by which the establishment of resistance could be reported only after confirmation of field failures and in a strict linkage with product dosages. Even if this is the more recent definition, the approach adopted can be problematic and leaves open questions (an early detection of low frequencies of resistant alleles in a population may not represent a threat and the acceptance of resistance is strictly linked to the registered product dosages), showing how difficult it is to contain the nuances of pesticide resistance in a single definition.

1.2 IMPACT OF RESISTANCE IN ARTHROPODS

Resistance is a widespread phenomenon within the phylum Arthropoda. Over the years, the increased use, overuse and even misuse of pesticides has led to the selection of resistance in more than 500 from the estimated 10000 arthropod pest species, most of which have been recorded over the last 60 years of intensive pesticide use. Michigan State University developed an online database (APRD) (http://www.pesticideresistance.com) to enumerate the resistance cases reported from 1914 to the present, in order to provide an up to date list based on the published literature and help the management resistance practices.

Within the phylum Arthropoda, insects represent the major class. The order with the highest number of resistant species is Diptera (27%) followed by Lepidoptera (25%), Homoptera (15%), Coleoptera (10%) and Hemiptera (2.0%) (Whalon *et al.*

2012). Most of the species in these orders represent a serious threat for agricultural production, although a consistent number of them, mainly among the Dipteran, have also medical and veterinarian importance. The use of insecticides plays an important role in controlling populations of insect pests, but as a result of the continued applications over time many resistance mechanisms allowing survival have evolved and have been selected. The possibility and success in resistance development depends upon a variety of genetic, biochemical and ecological factors such as generation time, fecundity rate, dispersal ability or fitness costs, together with the frequency, the dosage or the persistence of insecticide applications (Brattsen *et al.* 1986; Hemingway *et al.* 2002).

The presence of different genotypes in a population can explain how some individuals have a selective advantage and survive after insecticide exposure. As a result of continued insecticide application, the proportion of resistant insects increases compared to the susceptible and the population becomes increasingly difficult to control (Nauen 2007). Resistant specimens could be killed by using new products with different modes of action or by increasing the application rates and frequencies. Both these hypothetical solutions are not really feasible: the development of new compounds is slow and expensive, whilst the use of higher amounts of insecticides is not allowed, because it results in environmental contamination and higher risk of exposure to insecticides. Resistant management strategies must follow indications provided by the product labels reporting recommended doses to be used in the field.

1.3 INSECTICIDES MODES OF ACTION

The development and occurrence of insect resistance have been widely studied by academics as well as by several organizations and action committees. In order to describe insecticide resistance data and encourage homogeneus reporting of resistance cases, IRAC developed a “Mode of Action (MoA) classification” of insecticides (http://www.irac-online.org/documents/moa-classification). The more

recent update (release 7.3, February 2014) reports 27 different MoA groups. Insecticide compounds included in the active list must have a minimum of one registered use in at least one country. Active ingredients are allocated to specific groups according to their targets:

- **Nerve and muscle targets**.

  Most of the insecticides that are now available act on nervous and muscular systems. They are generally fast acting, causing hyperexcitation, convulsion, nerve block or paralysis.

- **Growth and development targets**.

  Growth regulator insecticides act directly against cuticle formation, lipid biosynthesis or by mimicking one of the principal hormone that control insect development.

- **Respiration targets**.

  Several insecticides are known to interfere with mitochondrial respiration by the inhibition of electron transport or oxidative phosphorylation.

- **Midgut targets**.

  Some insecticides act as microbial disruptors of Lepidoptera/Coleoptera midgut membranes.

- **Unknown or non-specific targets**.

  Several insecticides are known to affect less well-described target-sites or functions, or to act non-specifically on multiple targets.

The assignment usually involves the identification of the target protein responsible for the biological effect; nevertheless until now for several modes of action the target protein can only be supposed or remains unknown. The aim of this classification is to provide a complete guide that can help farmers, growers and other professional staff in the product selection for resistance management strategies (Nauen 2007).

1.4 INSECTICIDE RESISTANCE MECHANISMS

Despite the large diversity in insect species showing insecticide resistance, only a few resistance mechanisms have been identified. Resistance could be achieved by biochemical and molecular mechanisms, causing the sequestration or the disruption of the toxic agent or involving changes in its site of action (see sections 1.4.1 and 1.4.2) or by behavioural and physiological adaptations (see section 1.4.3). Generally, these resistance factors do not occur alone but interact with each other to enhance the level of resistance.

The presence of combinations of different resistance mechanisms has been demonstrated in many insect populations and also for single individuals within a population. “Cross-resistance” occurs when a single defence mechanism against one insecticide is capable of conferring resistance to other insecticides, even if the insect has not been previously exposed to the latter product. This phenomenon can result from physical factors, that can affect chemically unrelated compounds, or non specific enzymes, that attack functional groups of insecticides rather than specific molecules; indeed it is not only restricted to a specific chemical class but can involve insecticides with different mode of actions. “Multiple-resistance” occurs when different resistance mechanisms coexist and confer resistance to different insecticides to which the organism has been exposed (Oppenoorth & Welling 1976; Yu 2008). The occurrence of both cross-resistance and multiple-resistance is of particular importance, because they result in great difficulty in pest control. Clearly, because pest insect populations are usually large in size and breed quickly, there is always a risk that insecticide resistance may evolve, especially when insecticides are misused or over-used (Soderlund & Bloomquist 1990).

1.4.1 Metabolic resistance

Metabolic resistance is a common defence mechanism, based on enzymatic systems that protect the insect by detoxifying insecticide molecules. The involved enzymes are the same defence mechanisms that insects have developed as protection

against naturally occurring plant toxins (allelochemicals) such as alkaloids, terpenes and phenols, in order to overcome the potential toxicity of the plants they feed on. This could explain the rapid development of metabolic resistance against a very broad spectrum of insecticides.

Enzymes can detoxify xenobiotics by changing their molecular structure into a non-toxic compound and/or into a form more suitable for a rapid elimination from the body. Resistant insects metabolise the insecticide faster because they possess more effective forms of the enzyme with a higher catalytic rate, or higher quantities of the involved enzymes resulting from increased trascription or gene amplification. Detoxification can be divided into phase I (primary) processes, consisting of hydrolysis or oxidation, and phase II (secondary) processes, consisting of conjugation of phase I products with endogenous compounds, like glutathione, and their subsequent excretion from the body (Li *et al.* 2007; Hollingworth & Dong 2008; Yu 2008).

1.4.1.1 Esterases

Esterases are a large group of phase 1 metabolic enzymes that are able to metabolise a variety of exogenous and endogenous substrates. Their involvement in detoxifying insecticide molecules is well documented and it has already been demonstrated that they can act against a broad range of products, including pyrethroids, organophosphates and carbamates (Hollingworth & Dong 2008).

Detoxification can occur through degradation or sequestration of the insecticide molecules. Esterases catalyse the hydrolysis of ester insecticides into their corresponding acid and alcohol compounds (Fig. 1.1); this increases the polarity of the insecticidal metabolites that can then be excreted more easily from the insect body. They can also sequester insecticides through the formation of stable compounds such that the toxic molecules are no longer available for chemical reactions (Devonshire & Moores 1982; Oakeshott *et al.* 2005; Wheelock *et al.* 2005).

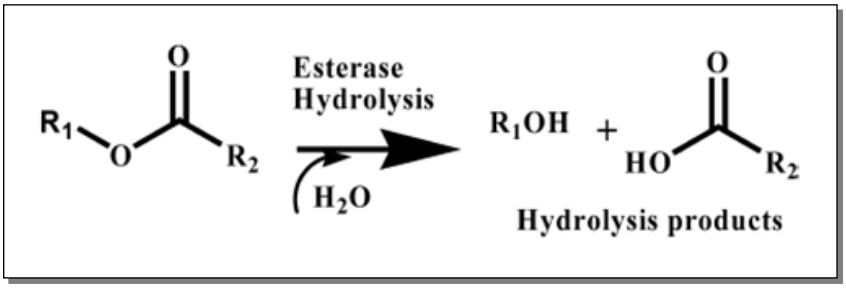

Figure 1.1: Esterase hydrolysis reaction. Esterases hydrolyse an ester by the addition of water to form the corresponding alcohol and acid.

Esterases have been associated with insecticide resistance in many insect species. It can occur from both quantitative and qualitative changes, consisting in the overproduction of the enzymes or in modifications of their structures (Li *et al.* 2007).

Esterase overexpression can be due to either gene amplification or upregulation, or a combination of both. The most extensively studied example of insecticide detoxification by gene amplification is the overproduction of a specific carboxylesterase in the green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Field *et al*. 1988) but amplified esterases associated with insecticide resistance have been also found in mosquitoes of the *Culex* genus (Diptera: Culicidae) (Hemingway *et al*. 2004) and other species, for example in the brown planthoppers *Nilaparvata lugens* Stal (Hemiptera: Delphacidae) (Small & Hemingway 2000). In other species, like *Aphis gossypii* Glover (Hemiptera: Aphididae) or B-biotype *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), the increased expression of esterases results from increased transcription levels, due to upregulation of the corresponding gene (Cao *et al*. 2008; Alon *et al.* 2008).

Esterase-based resistance can occur also through qualitative changes of the enzyme, which confers an enhanced ability to metabolise the insecticide. This mechanism was first described in the housefly *Musca domestica* L. (Diptera: Muscidae) and it became known as the “mutant ali-esterase theory” (Oppenoorth & van Asperen 1960). The resistant insects showed a decreased esterase activity

compared to the susceptibles, showing that structural modifications of the enzyme can facilitate the hydrolysis of the insecticide but prevent or reduce the hydrolysis of the model substrates conventionally used to determine the esterase activity. It was subsequently demonstrated the presence of two amino-acid substitutions (Gly137Asp and Trp251Leu) in those resistant houseflies as well as in other insect species belonging to the order of Diptera (Campbell *et al*. 1998; Claudianos *et al*. 1999; Carvalho *et al*. 2006).

1.4.1.2 Monooxygenases

Mixed function oxidases (MFOs), or microsomal oxidases, are a large family of phase 1 enzymes involved in the detoxification of xenobiotics, but also in the metabolism of endogenous substances such as hormones, pheromones or fatty acids. They are able to convert lipophilic compounds into polar metabolites that can be easily eliminated from the body; for that reason, they are mainly located in the digestive apparatus (Feyereisen 2005).

Cytochrome P450s monooxygenases (P450s) are microsomal oxidases that belong to the group of the hemethiolate proteins and are so named because they show a characteristic absorbance peak at 450 nm (Soret peak) in their reduced form when complexed with carbon monoxide. They catalyse the transfer of one atom of molecular oxygen to a substrate and the reduction of the second atom of oxygen in water; the process requires the transfer of two electrons provided by NADPH cytochrome P450 reductase (Feyereisen 2005; Guengerich 2008). The reaction is commonly described as:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Due to the large number of enzymes and their substrate specificity, P450s are able to catalyse different reactions like epoxidation, hydroxylation, N-dealkylation, O-dealkylation or desulfurization; for that reason they play an important role in the

metabolism of many insecticide classes, including carbamates, organophosphates, pyrethroids and DDT (Yu 2008).

A single P450 is named as CYP followed by an arabic number to designate the family, a capital letter to designate the subfamily and an arabic number to designated the individual protein; each form is coded by its own gene. To date, more than 600 insect P450 genes have been characterised and genes belonging to the families *CYP4*, *CYP6*, *CYP9* and *CYP12* have been associated with insecticide resistance (Feyereisen 2005; Li *et al.* 2007).

Because of the complexity of the P450 system and the difficulties in purifying these enzymes (due to their instability or to the difficulty to obtain high yields), it is not easy to determine the mechanisms underlying resistance. However, it has already been demonstrated that resistant insects can show increased levels of P450s and an enhanced monooxygenase activity. Many cases of resistance correlated to overexpression of P450 activity have been reported in the literature and it is generally caused by gene upregulation, probably through changes in the regulatory elements (Feyereisen 2005). Although this is the main mechanism described, cases of gene amplification or qualitative changes have also been reported in other species (Amichot *et al.* 2004; Wondji *et al.* 2009; Puinean *et al*. 2010).

1.4.1.3 Glutathione-S-transferases

Glutathione-S-transferases (GSTs) are a group of multifunctional phase II enzymes involved in the detoxification of several hydrophobic endogenous or exogeneous compounds. They catalyse the conjugation of the reduced glutathione (GSH) with electrophilic substrates, converting those reactive molecules into more water-soluble and non-toxic conjugates that can be more readily excreted from the body (Hayes *et al*. 2005).

Insect GSTs are divided in two different groups according to their location within the cell, microsomal and cytosolic, but only the latter is implicated in the metabolism of insecticides. Due to the broad range of substrates of the individual

enzymes, they play an important role in resistance to different classes of insecticides, including organophosphates and pyrethroids; a DDT-dehydrochlorinase GST is also responsable for DDT resistance in houseflies and mosquitoes (Enayati *et al*. 2005). GST-based resistance is generally due to an increased amount of enzyme, resulting either from gene amplification or overexpression (Vontas *et al*. 2002; Ranson & Hemingway 2005). GSTs may also protect against pyrethroid toxicity in insects by sequestering the insecticide (Kostaropoulos *et al*. 2001).

1.4.2 Target-site resistance

Target-site resistance is one of the most important mechanisms that determine resistance to different classes of insecticides. It is based on alterations in the sequences of genes encoding for the insecticide target proteins, reducing the binding affinity of the toxic compound. Conserved target-site mutations conferring different levels of insensitivity to the insecticides have already been detected in genes encoding for voltage-gated sodium channel, acetylcholinesterase, nicotic receptor, GABA receptor and ryanodine receptor (Hollingworth & Dong 2008; Yu 2008).

1.4.2.1 Voltage gated sodium channels

Voltage-gated sodium channels (VGSC) are large trans-membrane spanning proteins that are essential for electrical signalling in nerve cell membranes. They are composed of one pore-forming α-subunit of about 260 kDa and up to four smaller β-subunits of about 30-40 kDa. The α-subunit is the principal structural element and consists of a single transmembrane polypeptide chain with four internally repeating homologous domains (I to IV), each with six hydrophobic transmembrane segments (S1 to S6) connected by intracellular or extracellular loops. S5 and S6 helices form the central pore, whilst S1-S4 helices form the voltage sensing domains. This structure mediates the sodium ion permeability that is essential for the normal

transmission of nerve impulses (Catterall 2000). Because of their critical role in electrical signalling, sodium channels have been used as important target of a variety of natural or synthetic neurotoxins, including pyrethroid insecticides. DDT, pyrethrins and pyrethroids act on the VGSC and modify the gating kinetic, mainly by slowing channel deactivation; it results in a prolonged opening of the individual channel that stimulates the production of repetitive discharges, causing the paralysis and the consequent death of the insects (Davies *et al*. 2007; Soderlund 2012).

A large number of target-site mutations in the sodium channel protein have been identified in several insect species. More than 30 of these substitutions (or combinations of them) have been detected in more than one species, whilst others are unique. A certain number of these mutations have been functionally expressed in *Xenopus* oocytes, confirming their role in reducing sodium channel sensitivity to pyrethroids; however, most of them remain uncharacterised (Rinkevich *et al*. 2013).

Non synonymous mutations were first described in *M. domestica* and indicated as "knock-down resistance" traits, as they confer resistance to the paralytic effect (knock-down) caused by DDT and pyrethroids and reduce their efficacy (Busvine 1951; Williamson *et al*. 1996).

The most common amino acid substitutions is from Leu to Phe, identified in the housefly (L1014F) and in the German cockroach (L993F); it is located in domain IIS6 and termed as "knock-down resistance" (*kdr*). In addition to this mutation, a second amino acid substitution from Met to Thr has been detected in highly pyrethroid resistant housefly (M918T); it is located in the IIS4-S5 linker and termed as "super-kdr" (*s-kdr*) (Williamson *et al*. 1996; Davies 2007). Those two mutations have been documented in most of the major arthropod pests frequently treated with pyrethroids. Variability in amino acid substitutions of both these residues (L1014H/S/C/W and M918I/L/V) is documented in different species and some of these replacements have been functionally associated with resistance to pyrethroid insecticides. In addition, in a few cases, it is possible to find more than one of these possible alternatives in the same species (Rinkevich *et al*. 2013).

1.4.2.2 Acetylcholinesterases

Acetylcholinesterase (AChE) is the enzyme that catalyses the hydrolysis of the excitatory neurotransmitter acetylcholine (ACh) which is responsible for the nerve impulse transmission across the cholinergic synapses. Organophosphate (OP) and carbamate insecticides interfere in this process because they are able to phosphorylate or carbamylate the critical serine residue in the active site, that is subsequently incapable of hydrolysing its normal substrate. The inhibition of AChE activity impairs the removal of ACh, which accumulates in the synapse causing a continuous stimulation, resulting in the death of the insect (Eldefrawi 1985; Casida & Quistad 2003).

Modified AChE (MACE), with alterations in the primary structure of the enzyme, results in a reduced sensitivity of AChE to OPs and carbamates and provides to the insect some levels of resistance (Fournier & Mutero 1994). Sequencing of AChE genes (*ace*) of resistant insect has revealed the presence of several point mutations that result in amino acid substitution of residues located in the active site of the enzyme, close to the catalytic triad. These mutations confer different levels of resistance and the effects among different insecticide products can vary considerably (Fournier 2005). Higher Diptera have only one gene (*ace*) for AChE, whereas it has been shown that in the majority of the insect species there are two genes, *ace-1* (paralogous to *ace*) and *ace-2* (orthologous to *ace*) encoding for two different acetylcholinesterases, AChE1 and AChE2. Different point mutations have been discovered in *ace-1* genes of several insect species, providing some degrees of resistance (Fournier 2005).

1.4.2.3 Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChR) are proteins commonly found in the insect nervous system and are so called because they are particular responsive to nicotine. They belong to the “Cys-loop” superfamily of ligand-gated ion channels and are composed of 5 subunits (usually 2 identical α subunits and 3 β subunits)

arranged around a central ion pore. At least two ACh-binding sites are present and they must be occupied to start the channel opening (Karlin 2002).

Nicotine and the neonicotinoid insecticides mimic the neurotransmitter ACh and act as agonists activating the receptor and causing an influx of sodium ions with the generation of action potentials. Normally the synaptic action is terminated by the enzyme AChE, that hydrolyses the neurotrasmitter; because the insecticides are not destroyed, the persistant activation leads to hyperexcitation, convulsion, paralysis and death of the insect (Jeske and Nauen 2005). Modified nAChR has recently been documented in different insect species and a correlation between the presence of target-site mutations and alteration in the sensitivity to the effect of the insecticides has been reported (Nauen & Denholm 2005; Crossthwait *et al*. 2014).

1.4.2.4 GABA receptors

GABA gated-chloride channels receptors (GABAR) are membrane-bound proteins located in the central nervous system and also at the peripheral neuromuscolar junctions. GABAR belong to the superfamily of ligand-gated channels known as "Cys-loop" receptors and consist of 5 subunits that form a central ion pore; each subunit has a long N-terminal domain that contributes to the GABA binding site. γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter and it is responsible for the nerve impulse inhibition when it links to GABAR (Buckingham & Sattelle 2005). Those ligand-gated ion channels are blocked by the linkage of cyclodiene insecticides, which act as receptor antagonists leaving GABA neurotransmitter unable to bind the same receptors and to stop the impulse transmission. The absence of synaptic inhibition leads to hyperexcitation, convulsions and consequent dead of the insect (Bloomquist 2001).

Modified GABAR, caused by a target-site mutation in the gene encoding for that protein, results in a reduced affinity of the receptor for the insecticide. The gene involved in resistance, termed *Rdl* (Resistance to Dieldrin), was isolated from a field collected population of *Drosophila melanogaster*; homologous mutations have

already been described in many other insects and correlated with a certain level of resistance (fFrench-Constant *et al.* 1993; Buckingham & Sattelle 2005).

1.4.2.5 Ryanodine receptors

Ryanodine-sensitive calcium release channels, commonly known as ryanodine receptors (RyR), are large tetrameric proteins found in sarcoplasmic/endoplasmic reticulum membrane in muscles and nervous tissue. They show the same basic structure of the sodium channels, with 4 homologous domains arranged around a central ion pore, and play a key role in calcium homeostasis. Calcium is a universal intracellular messanger and its release from intracellular stores is modulated by channels such as RyR and is important for many physiological activities including muscle contraction (Hamilton 2005).

RyR have been taken into consideration as a potential insecticide target for decades. Plant extracts from *Ryania speciosa* (Flacourtiaceae) proved to be quite efficacious against many targets (Rogers *et al*. 1948; Molinari *et al*. 2005). However it is only recently that economically relevant compounds have been developed: flubendiamide and anthranilic diamides, both particularly active against lepidopteran pest species. They are selective RyR activators that stimulate calcium release causing impaired regulation in the contraction of insect muscles, which culminates in paralysis and subsequent mortality (Nauen 2006). Resistance to these insecticides has recently been reported in the diamondback moth *Plutella xylostella*, a global lepidopteran pest of cruciferous crop, and the association with a target-site mutation in the membrane-spanning domain of the RyR has been demonstrated (Troczka *et al*. 2012).

1.4.3 Other resistance mechanisms

Target site and metabolic resistance are the main mechanisms by which resistance is achieved. In addition, there are several other mechanisms that may contribute at

a more modest level but relatively little attention has been paid to them. Although individually they may be only moderate in their impact, they can act as important intensifiers of resistance when combined with the major mechanisms in the same insect.

1.4.3.1 Pgp pumps

P-glycoprotein (Pgp) transporters are integral membrane proteins that belong to the ATP binding cassette (ABC) superfamily, which utilise the energy derived from ATP hydrolysis to translocate a variety of different metabolites and xenobiotics across cellular membranes (Hollenstein *et al.* 2007). The action of Pgp pumps in removing a broad range of toxic compounds from cells is well established as a mechanism of antibiotic resistance in bacteria and of fungicide resistance in fungi (Lage 2003); in contrast very little is known about their physiological functions in insects. Only recently ABC transporters in insects have emerged as a putative mechanism which can contribute to resistance by facilitating efflux transport of insecticides and their metabolites derived from phase I and II reactions (O'Donnell 2008). The involvement of Pgp pumps in insecticide resistance has been documented in several insect species and it has been correlated to increased expression of genes encoding ABC transporters (Porretta *et al*. 2008; Aurade *et al*. 2010; Bariami *et al.* 2012). A survey of cases where the involvement of ABC transporters in insecticide resistance is suggested has been recently reviewed by Dermauw & Van Leeuwen (2014). ABC transporters have been associated with resistance to insecticides with different modes of action, with evidences based on the quantification of transcript or protein levels and by synergism studies using ABC inhibitors (Buss & Callaghan 2008; Dermauw & Van Leeuwen 2014). In addition, a mutant allele in different lepidopteran species has recently been discovered and confers resistance to the pore-forming Cry1Ac toxin from *Bacillus thuringiensis* (*Bt*) by a mechanism that is not related to toxin extrusion, but because it causes the loss of Cry1Ac binding to membrane vesicles (Gahan *et al*. 2010; Heckel 2012).

1.4.3.2 Penetration resistance

To reach its target, an insecticide must first penetrate the cuticle of the insects. Penetration resistance occurs when the insects have physico-chemical alterations to the structure of their cuticle that results in a slower absorption of the chemicals or in a reduced amount of the insecticide passing through these physical barriers. This mechanism protects insects from a wide range of insecticides, but on its own it confers low levels of resistance. Indeed, it is usually found in combination with other forms of resistance, enhancing their effects. For example a delayed and slower penetration can provide more time for the detoxification of the insecticide (Oppenoorth & Welling 1976; Scott 1990).

1.4.3.3 Behavioural resistance

Behavioural resistance consists of the adaptation of insect behaviour in order to avoid the insecticide. This phenomenon is stimulus dependent and resistant insects can detect or recognise the danger and simply stop feeding or leave the treated area, walking or flying away. They can respond to lower concentrations of insecticide than normal insects, indicating the presence of receptors that allow the development of the ability to better detect the presence of insecticides (Sparks *et al*. 1989; Yu 2008).

1.5 SYNERGISTS

Synergists are compounds that can be used in combination with insecticides to increase their efficacy against resistant insect pests. These molecules are themselves non-toxic at the doses applied and can inhibit enzymes usually involved in xenobiotic detoxification, temporarily restoring the susceptibility of resistant insects or making the susceptible more sensitive (Metcalf 1967). For that reason, synergists have been used for a long time in laboratory bioassays to determine the presence of metabolic resistance in insect pests. The efficacy of a synergist is

commonly expressed as the synergism factor (SF), which is the ratio between the LC_{50} value of an insecticide applied alone and the LC_{50} value obtained when the insecticide is mixed with a synergist (Metcalf 1967; Ishaaya 1993).

Studies on insecticide synergists started in 1940s, with the observation of an enhanced insecticidal activity of pyrethrum when combined with sesame oil; the active components were identified as sesamin and sesamolin (Fig. 1.2), two methylenedioxyphenyl (MDP) compounds (Haller *et al*. 1942). Since then, many MDP compounds have been investigated for their synergistic effects, including piperonyl butoxide (PBO) (Fig. 1.3).

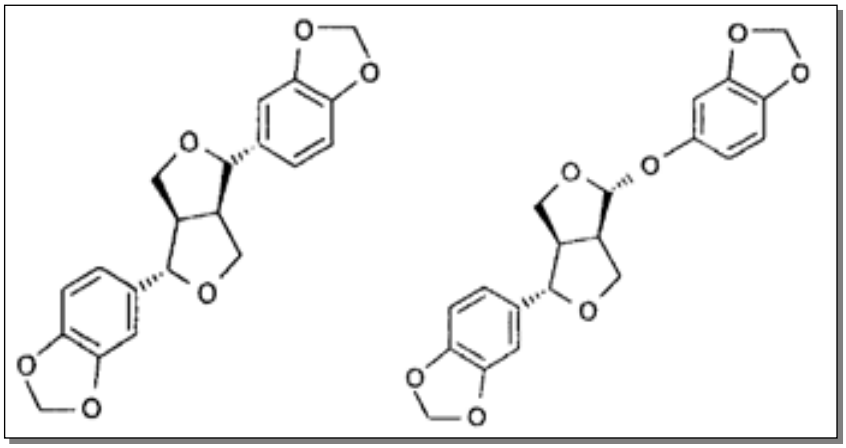

Figure 1.2: Sesamin and sesamolin structures.

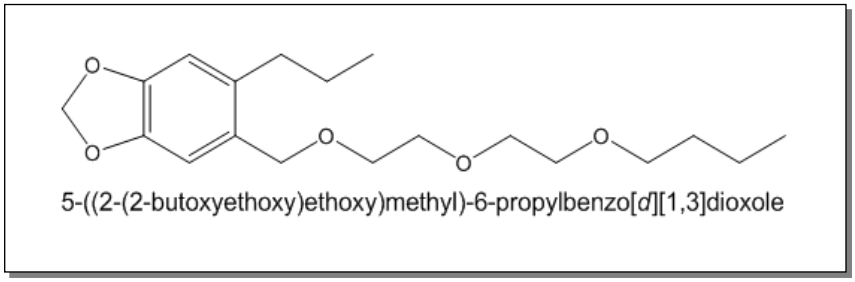

Figure 1.3: PBO structure.

Today PBO is probably the best known synergist, widely used in the household insecticide market but with a limited application in agriculture. At present registered PBO formulations for agriculture are available only in Australia and USA.

In Europe the status of PBO and synergists in general is still not completely defined by the legislation. Currently, mixtures between PBO and natural pyrethrins are, at least in Italy, registered and commonly used in organic farming; another registered application of PBO is to synergise synthetic pyrethroids to protect stored cereals (http://www.fitogest.it).

PBO was developed in the 1950s, first synthesised using the raw material safrole and later produced synthetically. Initially it was reported to be a specific inhibitor of cytochrome P450 enzymes (Casida 1970; Wilkinson 1984; Hodgson *et al*. 1998), but more recently it has been demonstrated to be involved also in esterase-based resistance in some agriculturally important pests (Gunning *et al*. 1998; Young *et al*. 2005).

The synergistic effect of PBO has been demonstrated with different classes of insecticides, including pyrethroids, OPs, carbamates and recently also with neonicotinoids (Casida 1970; Bingham *et al.* 2008). Furthermore, it has been shown how these effects can be enhanced with an appropriate pre-treatment time, depending on the insect pest. This phenomenon is known as “temporal synergism” and refers to a delay between the application of the synergist and the insecticide, allowing full inhibition of the specific metabolic enzymes involved in resistance prior to the addition of the insecticidal component (Moores *et al*. 2005). This concept has led to the development of microencapsulated insecticides with PBO, that initially release PBO and several hours later the insecticide. For example, microencapsulated formulations of PBO with α-cypermethrin and bifenthrin were found to be effective against a few important agricultural insect pest species (Bingham *et al*. 2007; Mazzoni *et al*. 2010).

2. *MYZUS PERSICAE* (SULZER) (HEMIPTERA: APHIDIDAE)

2.1 THE GREEN PEACH APHID

The green peach or peach potato aphid *Myzus persicae* (Sulzer) (1776) (Hemiptera: Aphididae) is one of the key pests of many agricultural and horticultural field and glasshouse crops. It causes significant damage by direct feeding, transmission of many plant viruses and honeydew production. This aphid is distributed worldwide (http://www.cabi.org/isc/datasheet/35642) and highly polyphagous, with a host range of more than 400 species in 40 different plant families including economically important crop plants (Blackman & Eastop 2000). In Europe, the main crops that can be infested are peach, potato, pepper and oilseed rape. In Italy, the most damaged cultivations are peach orchards and open-field cultures such as sugar beet, potato and tobacco (Barbagallo *et al*. 2007).

The life cycle of *M. persicae* depends on the climate and on the availability of its primary winter host *Prunus* spp., expecially peach (*P. persica*) and nectarine (*P. persica* var *laevis*) (Blackman 1974). In temperate latitudes, both cold winters and the presence of overwintering hosts allow the green peach aphid to be holocyclic, with a sexual phase in autumn and a parthenogenetic (asexual) reproduction in spring and summer. Sexual morphs mate after an autumn migration on primary host, where they lay cold-resistant eggs that represent the overwintering stage; the fundatrices that hatch from the eggs start a succession of parthenogenetic all-female generations, with wingless or winged forms that can migrate on a wide number of secondary hosts. In warm climate and in the absence of the primary host, the life cycle is usually anholocyclic, with the loss of sexual reproduction and a continual parthenogenesis all year. Considering the short generation time, parthenogenesis allows a rapid increase of the population under favourable conditions, causing high density infestations and quick damage to plants.

2.2 INSECTICIDE RESISTANCE IN THE GREEN PEACH APHID

M. persicae is now considered one of the most widely and strongly resistant species worldwide (http://www.pesticideresistance.com). The control of this pest is achieved mainly by the application of chemical insecticides, often with multiple applications each year. In order to control this pest, several insecticides have been used: carbamates (IRAC MOA 1A), organophosphates (IRAC MOA 1B), pyrethroids (IRAC MOA 3A), neonicotinoids (IRAC MOA 4A) and feeding inhibitors (IRAC MOA 9). These continuous treatments have selected resistant populations showing a variety of resistance mechanisms, well documented in the literature. After the first report of resistance to organophosphates in this species (Anthon 1955), the frequency of resistant cases grew during the years and today is reported to most classes of insecticide including organophosphates, cyclodienes, carbamates, pyrethroids and in the last few years also neonicotinoids (Bass *et al*. 2014) (Fig. 1.4).

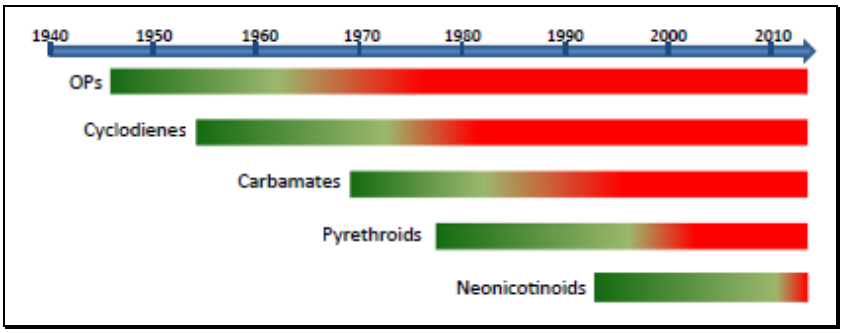

Figure 1.4: Timeline of resistant development in* M. persicae*. Green bars: years when insecticides provide good control; red bars: years when cases of compromised resistance control provided by the same insecticides (Bass* et al*. 2014).

2.2.1 Resistance to organophosphates and carbamates

Metabolic resistance against organophosphates and carbamates (and to a less extent against pyrethroids) based on enhanced levels of esterases has been well studied and was the first resistance mechanism described in *M. persicae*. It was demostrated biochemically over 40 years ago, by the observation that resistant strains had an enhanced ability to hydrolyse the model substrate 1-naphthyl acetate

(1-NA) compared to the susceptible ones (Needham & Sawicki 1971). It was subsequently explained that this was due to the increased esterase production (Devonshire & Moores 1982), which occurs by gene amplification (Field *et al*. 1988).

In *M. persicae* two different esterase variants, E4 and FE4, have been described. Their genes show 99% sequence identity of the coding region and both have seven introns of the same size and in identical positions. The two proteins differ by only 9 amino acid substitutions and have a molecular mass of 65 kDa for E4 and 66 kDa for FE4, respectively, as a consequence of a non-sense mutation bringing a premature stop codon in the E4 gene (Field *et al*. 1993 and 1996). Despite their very similar molecular weights, the FE4 isoform appears slightly faster when run on electrophoresis gels (Devonshire & Moores 1982; Devonshire *et al.* 1983).

The E4 variant is widespread in North Europe populations and is correlated with a particular translocation between autosome 1 and 3; FE4 is mainly diffused in the Mediterranean regions and is not associated with chromosomal rearrangements. Usually just one of the two paralogues genes is amplified in individual aphids; amplification of both forms is very rare (Blackman *et al*. 1996 and 1999). However, in recent years, such cases of gene flow have been suggested from field collected aphids around Europe. In England, two clones of *M. persicae* with both E4 and FE4 amplified genes were found in 2002 (Field & Foster, 2002); in Greece, analysis on populations collected during the years 2002-2007 showed marked changes in the frequencies of E4 and FE4 genes compared to old data obtained in the middle of 1990s (Kati *et al*. 2014). In addition, FE4 genes associated with the A1,3 translocation have been reported in samples collected in central-southern Italy (Rivi *et al*. 2013). These are indications of gene flow between sexually reproducing FE4 genotypes and parthenogenetic populations carrying the translocation and/or the E4 genes.

Furthermore, “revertant” clones have been described in populations with amplified E4 genes: it has been demonstrated that demethylation can cause gene silencing and consequent loss of esterase-based resistance (Field 2000).

2.2.2 Resistance to dimethylcarbamates

Dimethylcarbamates are members of the carbamate class of insecticide and represent excellent aphicides, acting specifically on their AChE. Pirimicarb is the most important insecticide in this group, widely used because it is very efficacious and is less effected by high levels of esterase. Nevertheless, highly pirimicarb resistant *M. persicae* clones were detected in Greece in the early 1990s and their AChE insensitivity to pirimicarb was demonstrated biochemically (Moores *et al*. 1994).

Sequencing of *ace1* and *ace2* (respectively paralogue and orthologue of *ace* gene in dipteran *D. melanogaster*/*M. domestica*) of resistant and susceptible samples of *M. persicae* revealed the presence of a point mutation in *ace1* causing a single amino acid substitution (S431F) in the acyl pocket of AChE (Nabeshima *et al*. 2003). Further experiments with both wild-type or mutated recombinant enzyme confirmed its correlation with resistance to pirimicarb because it strongly affects insecticide binding to the enzyme (Benting & Nauen 2004). Sequences derived from non-aphid species and vertebrates showed a high conserved residue of Phe in the same position, whilst in other wild-type aphid species the presence of a Ser residue was confirmed. This demonstrates the strong selectivity of pirimicarb and its excellent specificity on aphid pests and also that the mutation associated with pirimicarb-insensitivity restores the residue to that normally found in other organisms (Andrews *et al*. 2004).

2.2.3 Resistance to cyclodienes

Although now cyclodiene insecticides like endosulfan have been phased out, they have been used for many years as aphicides on a range of crops, in rotation with other products. Resistance to this insecticide class was detected for the first time in clones collected from peach and nectarine orchards in US (Unruh *et al*. 1996). It results from a mutation in the gene encoding for the GABA receptor, which causes a single amino acid substitution in position 302. In a wide range of insects the Ala

residues are replaced with a Ser whilst in some *M. persicae* clones it has been found to be either a Ser or a Gly. Southern blot analysis revealed the presence of two independent loci, one carrying alleles A or G and the other carrying allele S. Only allele G has been correlated with resistance to cyclodienes, whilst the functional significance of the other allele is still unclear, although it is present in all studied clones regardless of their resistance status (Anthony *et al*. 1998).

2.2.4 Resistance to pyrethroids

For a long time pyrethroids have been one of the main insecticide classes used against *M. persicae* and it has led to the development of populations showing high levels of resistance, both metabolic and target site. Metabolic resistance is conferred by esterases and monooxygenases. These enzymes are responsible for the sequestration (Devonshire *et al*. 1998) or detoxification of pyrethroid molecules by ester group cleavage or through oxidative mechanisms, respectively (Berge *et al*. 1998; Wheelock *et al*. 2005). Target-site resistance causes pyrethroid insensitivity through mutations in the sodium channel protein and it is considered the most important resistance mechanism against pyrethroids (Devonshire *et al*. 1998).

L1014F (*kdr*) and M918T (*s-kdr*) are the two most common target-site mutations described in this pest. M918T has always been found in combination with L1014F, leading to high levels of pyrethroid resistance (Martinez-Torres *et al*. 1999; Eleftherianos *et al*. 2008). Also a few other mutations have been reported (F979S and L932F), but their possible involvement in resistance is only partially or not at all characterised (Criniti *et al*. 2008; Fontaine *et al*. 2011). Additionally, another amino acid substitution (M918L) in the *s-kdr* locus has recently been identified in French populations of *M. persicae* and linked to a resistant phenotype even in the absence of the classic *kdr* (Fontaine *et al*. 2011).

Although in most insect species *kdr* and *s-kdr* mutations have been shown to be inherited as recessive traits, *M. persicae* heterozygous clones display a resistant

phenotype to a range of pyrethroids (Eleftherianos *et al*. 2008; Fontaine *et al*. 2011).

2.2.5 Resistance to neonicotinoids

After their introduction on the market in the early 1990s, neonicotinoid insecticides became one of the key components of pest management strategies used by growers against many crop pests and in recent years they have been considered the main effective control measure adopted against *M. persicae* on many crops (Nauen & Denholm 2005). Nevertheless, recent field-collected samples of this aphid showed a significant level of resistance to neonicotinoids.

The involvement of detoxifying enzymes was confirmed with bioassays in a clone collected in 2007 from Greece that exhibited 30-60-fold resistance to different neonicotinoids when compared to a reference susceptible strain (Philippou *et al*. 2010). Microarray analysis revealed the constitutive overexpression of a single P450 gene (CYP6CYP3), data confirmed by quantitative PCR (Puinean *et al*. 2010).

Some further findings suggested the contribution of additional mechanisms in conferring resistance to neonicotinoid insecticides. Enzyme inhibitors did not restore complete susceptibility; LC_{50} values were lower in feeding bioassays compared to the topical applications; up-regulation of ESTs encoding for cuticular proteins in microarrays compared between resistant and susceptible clones: these observations are evidence of a reduced cuticular penetration of the insecticide in resistant aphids (Puinean *et al*. 2010).

Later, in a population collected from Southern France expressing extremely high resistance to neonicotinoids, a new single point mutation causing an Arg to Thr substitution in position 81 (R81T) in the loop D region of the nicotinic acetylcholine receptor β1 subunit (nAChRβ1) was discovered. This substitution confers a vertebrate-like character to the insect and results in a reduced sensitivity to neonicotinoids because it changes their binding affinity to the nicotinic acetylcholine receptor (Bass *et al*. 2011).

2.2.6 Further considerations

Despite the primary importance of *M. persicae* in agriculture, limited genomic informations were available for this species so far. However, recent advances in DNA sequencing allowed to rapidly acquire data about cDNA libraries and to create expressed sequence tags (ESTs) databases to make in silico predictions of differentially expressed genes, SNPs identification or microarrays creation for gene expression studies (Ramsey *et al*. 2007). The produced *M. persicae* EST collections together with the pea aphid *Acyrthosiphon pisum* (Harris) genome (International Aphid Genomics Consortium, 2010) allowed, for example, a direct comparison of xenobiotic detoxification enzymes in those two related insect species with different feeding habits (Ramsey *et al*. 2010).

The International Aphid Genomics Consortium (IAGC) has recently released a working draft of the genome assembly version of *M. persicae* clone O (GPA_Ov1, the green peach aphid clone that is prevalent in UK) and scaffolds are now available for BLAST searches (http://www.aphidbase.com/download). The availability of genome sequence could be very powerful to find new potential targets involved in insecticide resistance. For example, it could allow phylogenetic analysis among different insect species, in order to infer orthologous relationships that might suggest conserved function and thus to better investigate mechanisms that have already been described in other insect species, as it has been recently done in studies on ABC transporter gene family (Dermauw & Van Leeuwen 2014). Furthermore, genome assembly will represent an important source for increasing understanding of insecticide resistance mechanisms that have already been characterised. For example, if it is well know that metabolic resistance occurs by enhanced production of specific enzymes, to date the mechanism(s) by which detoxification genes are amplified has not been identified, as well as how genes are copied and moved around the genome. The interrogation of genome sequence will help to better exploring those and other issues.

CHAPTER 2

AIM OF THE PROJECT

Insect pests represent a serious threat for agricultural production, human health and animal protection, because of crop damage and transmission of several diseases. Chemical insecticides have been used to control these pests for many decades and today they remain essential to ensure a supply of affordable food and as part of disease vector control strategies. The green peach aphid *M. persicae* is a globally significant crop pest that has evolved high levels of resistance to almost all classes of insecticide. Due to its widespread distribution, the host range of over 400 species, the short life cycle and the high capacity of dispersion, it is now considered one of the major arthropod pests worldwide (Whalon *et al*. 2008). Work spanning over 40 years has shown its ability to evolve mechanisms that overcome the toxic effect of insecticides, leading this insect to be regarded as one of the most important "case studies" for the evolution of insecticide resistance (Bass *et al*. 2014).

Despite the numerous papers present in the literature regarding *M. persicae*, only a small number relate to investigations on the Italian presence and impact of insecticide resistance in this aphid. The last data available were published by Criniti *et al.* in 2008 considering populations collected around Italy in the previous years. Since then changes in resistance management strategies have been established, with different guidelines based on the fact that some products have been phased out whilst others were introduced into the market. Among them, neonicotinoid insecticides represented a key element for many agricultural systems, allowing a diversification of the selection pressure due to a different chemistry and mode of action. In recent years, cases of reduced efficacy of this class have been reported and new mechanisms of resistance have been described. The aim of this project is to investigate the main biochemical and molecular mechanisms underlying

resistance in Italian populations of *M. persicae*, in order to provide an update of the current situation, taking into account these recent topics.

The first part of this thesis (Chapters 4 and 5) is related to target-site resistance and aims to analyse the distribution of the main mutations responsible for resistance. In particular, the present work is focused on target-site mutations that have already been associated to neonicotinoid resistance (involving nicotinic acetylcholine receptors) and pyrethroid resistance (involving voltage gated sodium channels), compromising those insecticides that in recent years have been the main components of pest management strategies used by growers against the green peach aphid. The present survey doesn't include data about the presence of another well-known mutation located in the acetylcholinesterase enzyme, conferring target-site insensitivity to pirimicarb (a dimethylcarbamate). Despite its importance in many part of Europe, where this mutation is predominant in analysed populations, preliminary data collected at the beginning of this work indicated that its frequency has not increased since the last Italian survey in 2008. In addition, the use of pirimicarb in peach orchards is now greatly reduced in Italy although it is still permitted in the legislation and included in official Integrated Pest Management guidelines. This choise depends primarly on the fact that pirimicarb easily produce residues that can influence the good quality of the productions although they remain lower that the maximum values allowed. Also, the small portion of resistant insects can survive to the treatment and then generate rapid reinfestations in the field. Results obtained by this investigation will be important to develop proper insecticide resistance management strategies, in order to avoid ineffective applications and improve the long-term sustainability of chemical control against *M. persicae*.

The second part of this thesis (Chapter 6 and 7) is focused on metabolic resistance in order to verify the involvement of the main detoxyfing systems responsible of resistance. Again, literature data have already shown that esterases and monoxygenases are the main mechanisms responsible for the detoxification or

sequestration of pyrethroid and neonicotinoid insecticides; the present work aims to investigate the involvement of both these two enzymatic classes in resistant populations recently collected in Italy.

Furthermore, the possibility of overcome this kind of resistance has been taken in account with analysis conducted within the framework of the European project called “EcoSyn” (Ecofriendly synergists for insecticide formulations - Grant Agreement no: 605740). This is a FP7 project that aims to characterise the interactions between the main detoxifying metabolic systems, esterases and monoxygenases, and synergistic compounds that are able to inhibit these enzymes and thus metabolic resistance mediated by an enhanced production. The present work analyses the “in vitro” interaction between purified esterase or recombinant P450 enzymes of the green peach aphid with a wide range of compounds; structure activity relationship (SAR) analysis will then aid the design of bespoke structures, to be tested with “*in vivo*” bioassays and field trials, in order to select novel compounds with high inhibition potency. The last aim is the production of new synergists for use with insecticides, and thus allowing a reduction of the active ingredients in agricultural applications.

CHAPTER 3

GENERAL MATERIALS AND METHODS

1. INSECTS

1.1 APHID REARING

M. persicae populations were maintained on pea-seedlings (cv Meraviglia d'Italia) in controlled environmental conditions (21 ± 0.5 °C with a 16:8 h light:dark photoperiod) to be reared as colonies of parthenogenetic females according to a previously reported procedure (Mazzoni & Cravedi 2002) (Fig. 3.1).

Figure 3.1:* M. persicae *rearing collection. Different aphid populations are maintained isolated in small plastic boxes containing 3-5 pea-seedlings 5 days old.

1.2 FIELD COLLECTION

M. persicae populations were collected in different areas of Italy, mainly from peach orchards but also from herbaceous hosts. In most cases populations were collected after control failures but a detailed list of insecticide treatments was not available. Usually, according to local aphid management practices, aphids collected from peach survived at least to a neonicotinoid and in many cases also to a pyrethroid application; collection from secondary host generally occurred after pyrethroid failures. During field sampling, specimens were randomly collected from infested leaves. Some were directly stored in acetone at -20 °C and then used for DNA

extraction and molecular analysis, while others were put on pea-seedlings and reared as colonies of parthenogenetic females in controlled environmental conditions as described above. A full list of *M. persicae* populations collected in 2012 and 2013 is reported in table 3.1.

Region	Area	Host	n° populations	
			2012	2013
Emilia-Romagna	Bologna	peach	8	3
Emilia-Romagna	Forlì-Cesena	peach	16	10
Emilia-Romagna	Forlì-Cesena	eggplant	2	
Emilia-Romagna	Forlì-Cesena	pepper	1	1
Emilia-Romagna	Ravenna	peach	17	3
Emilia-Romagna	Rimini	peach	1	
Abruzzo	Chieti	peach	1	1
Calabria	Cosenza	peach	1	
Campania	Salerno	tobacco	1	
Lazio	Latina	peach		1
Lazio	Latina	pepper		1
Lazio	Viterbo	peach		1
Lazio	Roma	peach	1	1
Puglia	Foggia	peach	1	3
			Tot. 50	Tot. 25

Table 3.1: Geographical and host distribution of* M. persicae *populations collected in 2012/2013.

1.3 REFERENCE CLONES

Aphid parthenogenetic lineages available in the rearing were used as references in full dose-response bioassay and molecular analysis. The fully susceptible strain 1X was collected in Tuscany in 1995 on peach and was lab-reared without any insecticide selection pressure. Three clones (92H6, 99H1 and 175H2) were selected

from resistant populations collected after serious neonicotinoid control failures respectively in 2010 in Cesena (92H6), in 2011 in Ravenna (99H1) and in 2013 in Chieti (175H2). Clone 384C heterozygous for the M918L mutation was kindly provided by Séverine Fontaine (Unité Résistance aux produits phytosanitaires, Lyon, France).

A full list of the clones used as references is reported in table 3.2. Their genotypes were assessed by direct sequencing of the amplicons encompassing the target-site mutations under investigation, according to previously described protocols (Cassanelli *et al*. 2005; Bass *et al*. 2011).

clone	*nAChR* (R81T) aga-->aca	*Kdr* (L1014F) ctc-->ttc	*s-kdr* (M918T) atg-->acg	*s-kdr* (M918L) atg-->ttg	*s-kdr* (M918L) atg-->ctg
1X	SS	SS	SS	SS	SS
62H		SR	SR		
92H6	SS	RR	RR	-	-
97H1	SR				
99H1	RR				
384C		SS	SS	SR	SS
175H2		SR	SR	SS	SR

Table 3.2: Reference clones of* M. persicae *used in bioassays and molecular analysis. Genotypes of interest are reported for each clone.

2. BIOASSAYS

Susceptibility towards different insecticide products with or without synergists was investigated by dip-test bioassays. Pea seedlings were dipped for 5 s in insecticide (or insecticide plus synergist) solutions and after 15 min batches of 10-15 individuals of *M. persicae* were transferred onto the plants. Each experiment was replicated at least 4 times and with 5-6 different concentrations of insecticide (or insecticide plus

synergist); test subjects were selected randomly from all available and different groups were assigned randomly to each concentration. In each replication a control group was included, with the same number of subjects selected from the population with the same criteria and treated with water.

Tests with insecticide products used in combination with a synergist were performed starting from a tank mix 1:5 ratio of active ingredient: synergist. The choise of a constant ratio instead of a variable one that is a fixed amount of the synergist added to variable doses of the insecticidal component is to better simulate field conditions and thus getting results more suitable from an applicative point of view. Also, ratio 1:5 has been decided because it is a commercially cost-effective ratio often used in household products (Farnham 1998).

Samples were maintained at 21 ± 0.5 °C with a 16:8 h photoperiod. Mortality assessment was done 24 h after pyrethroid application or 72 h after neonicotinoid application. Data recorded for each combination of clone/insecticide/concentration were pooled together and concentration-mortality relationships were estimated by probit analysis (Finney 1971). Data were processed using POLO-Plus software (LE-ORA Software). Concentrations required to kill 50% of the population (LC_{50}), 95% confidence limits (CL95%), slopes with standard errors (SE), chi-square (χ^2) and degrees of freedom (df) were estimated. The LC_{50} values were used to calculate a "resistance factor" (RF) and a "synergistic factor" (SF) as follow:

- $RF = LC_{50}$ resistant population / LC_{50} susceptible population

  It estimates the effect of a certain treatment if tested against both resistant and susceptible populations. Generally it indicates how much more insecticide is required to provide equal control against a resistant population compared to a susceptible one.

- $SF = LC_{50}$ without synergist / LC_{50} with synergist

  It estimates the effect of a synergist when used in conjunction with an insecticide on a particular insect population (either resistant or susceptible). The toxicity of the insecticide normally increases with the relative amount of synergist in the synergist/insecticide treatment.

2.1 INSECTICIDES

- imidacloprid (Confidor, 200 SL; Bayer CropScience S.r.l., Milan, Italy) (neonicotinoid)
- bifenthrin (Brigata Flo; SIPCAM, Pero, Milan, Italy) (type I pyrethroid)
- *λ*-cyhalothrin (Karate Zeon 1.5; Syngenta Crop Protection, Gallarate, Milan, Italy) (type II pyrethroid)
- DDT (Sigma Aldrich)

2.2 SYNERGISTS

Technical PBO and analogues of PBO with modifications in the methylenedioxyphenyl (MDP) mojety, alkyl and polyether side chains were supplied by Endura SpA, Italy.

- structures of EN 1-14, 1-16, 1-40, 1-42, 1-101, 1-162, 1-93, 1-129, 1-180, 1-186, 1-175, 1-179, 1-164, 1-125, 1-163, 1-183, 1-181, 1-182, 16-05, 16-06, 14-05, 16-17, 16-18, 25-10, 25-35, 25-37, 25-36: see Philippou *et al*. 2013;
- structure of EN 1-126: see Philippou & Moores 2014;
- structures of EN 18-05, 1-48, 1-44, 25-08, 25-09: Endura SpA, personal communication.

3. BIOCHEMICAL AND MOLECULAR ANALYSIS

3.1 SUBSTRATES AND CHEMICALS

- 1-NA (1-naphthyl acetate), MW: 186.21 (Sigma-Aldrich)
- 1-NB (1-naphthyl butyrate), MW: 214.26 (Sigma-Aldrich)
- 1-NP (1-naphtol), MW: 144.17 (Sigma-Aldrich)
- FBB (Fast blue B), MW: 475.46 (Carlo Erba)
- FBRR (Fast Blue RR salt), MW: 387.89 (Sigma-Aldrich)
- pNA (4-nitrophenylacetate), MW: 181.15 (Sigma-Aldrich)

- ATChI (acetylthiocholine iodide), MW: 289.2 (Sigma-Aldrich)
- DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), MW: 396.3 (Sigma-Aldrich)
- 7-MFC (7-methoxy-4-trifluoromethylcoumarin), MW: 244.17 (Sigma-Aldrich)

3.2 BUFFERS

- 0.02 M sodium phosphate buffer pH 7.0
- 0.02 M Tris/HCl buffer pH 8.5
- TNES (Tris 50 mM, NaCl 400 mM, EDTA 20 mM, SDS 0.5%) buffer pH 7.5

3.3 DNA EXTRACTION PROTOCOL

Genomic DNA was extracted from a single specimen by a "salting-out" protocol, as already described (Sunnucks & Hales 1996; Guillemaud *et al*. 2003). Individual adults were homogenised using a QIAGEN TissueLyser LT for 30 s at 50 Hz in a 2 mL tube containing one stainless steel bead and TNES buffer pH 7.5 (300 µL) with proteinase K (100 µg mL^{-1}). The homogenate was heated at 55 °C for 1 h and then proteins were precipitated with 5 M NaCl (85 µL) and pelleted at 16000 x g for 5 min. DNA was isolated from the supernatant by ethanol precipitation and resuspended in sterile water (50 µL).

The DNA concentration was assessed using a Qubit Fluorimeter 2.0 instrument (Quant-iT ds DNA HS Assay kit; Invitrogen, Carlsbad, CA, USA). The amount of genomic DNA obtained was in the range 2-50 ng μL^{-1}. For each population, DNA extraction was carried out from 5-10 specimens randomly selected from previously collected and acetone-preserved samples.

3.4 PRIMERS

A full list of the primers used is reported in table 3.3:

Table 3.3: Primers sequences.

Primer name	Sequence (5'-3')	References	Notes
kdr-F1	TCGTGGCCCACACTGAATCT	Cassanelli *et al*. 2005	Primers used for *kdr* (L1014F) characterisation by pasa-PCR. *(See Chapter 4)*
kdr-R4	GTTCATGTAAGATACATGAATTC	Cassanelli *et al*. 2005	
Sd2	CTACTGTTGTCATTGGTAACC	Guillemaud *et al.* 2003	
Rv2	ATAGTACTTATACATACCACGAA	Guillemaud *et al.* 2003	
MpSK-F25	TGAAACTGATGGCGATGAGCCCTA	Cassanelli S, p. comm.	Primers used for *s-kdr* (M918T) characterisation by pasa-PCR. *(See Chapter 4)*
MpSK-R3292	GTAGGTTCTGGATAGCAATTGTTGC		
MpSKs-RE	GCACCGATGGTTCGACCCA		
MpSKr-FW	GCCCACACTGAATCTTTTAATATCCATAAC		
MpNACR-F52	ATTGTTCGTATAGTTACAGAATC		Primers used for R81T characterisation by pasa-PCR. *(See Chapter 4)*
MpNACR-R514	GAGATAAATCGCTGAGTAGATTTC		
MpNACRs-FW	GATAATGAAATCAAACGTTTGGTTGAG		
MpNACRr-RE	TATATTAAGTAGGTTACTCACAAGTG		
MpSK-R21	TCCCGTCACCAATGTCATCTCCA	Cassanelli S, p. comm.	Primer used togheter with MpSK-F25 for amplification and sequencing of locus encompassing *kdr* and *s-kdr* mutations. *(See Chapter 5)*

Tab 3.3: cont.

Primer name	Sequence (5'-3')	References	Notes
65-F	GCCCACACTGAATCTTTTAA	Nauen R, p. comm.	Primers used for PCR reaction before pyrosequencing assay. *(See Chapter 5)*
98-R-btn	CATACCCATGACGGCAAATA	Nauen R, p. comm.	
116-seq	TGAATCTTTTAATATCCAT	Nauen R, p. comm.	Primer used for pyrosequencing assay. *(See Chapter 4)*
T7	TAATACGACTCACTATAGGG	pGEM-T Easy Vector (Promega)	Primers used for the cloning experiment. *(See Chapter 5)*
Sp6	TATTTAGGTGACACTATAG	pGEM-T Easy Vector (Promega)	

CHAPTER 4:

TARGET-SITE RESISTANCE TO PYRETHROIDS AND NEONICOTINOIDS: AN ITALIAN SURVEY

DETECTING THE PRESENCE OF TARGET-SITE RESISTANCE TO NEONICOTINOIDS AND PYRETHROIDS IN ITALIAN POPULATIONS OF *MYZUS PERSICAE*.

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Abstract

BACKGROUND: *Myzus persicae* is a key pest of peach, which in commercial orchards is mainly controlled by chemical treatments. Neonicotinoids represent the main control strategy, but resistance monitoring programmes in Southern Europe have shown the widespread presence of populations highly resistant to this insecticide class in peach orchards. Moreover, in Italy reports of neonicotinoid application failures are increasing. This work describes the status of the main target-site mutations associated with neonicotinoid and pyrethroid resistance in Italian populations collected in 2012.

RESULTS: R81T mutation linked with neonicotinoid resistance was found in 65% of analysed aphids (35.5% with a homozygous resistant genotype). For the first time, R81T was found in samples collected from herbaceous hosts. Bioassays on a few genotyped populations also revealed the involvement of P450-based metabolic resistance. Only a few individuals without *kdr* (L1014F) and *s-kdr* (M918T) target-site mutations were collected. A new single nucleotide polymorphism in the *s-kdr* locus producing M918L substitution was found.

CONCLUSION: Target-site resistance to neonicotinoids is common in specialised peach-growing areas, and it is spreading in other Italian regions and on herbaceous hosts. The high frequency of target-site mutations and data obtained from bioassays confirm the presence of multiple resistance mechanisms and suggest the importance of coordinated control strategies.

1. INTRODUCTION

The control of the green peach aphid has relied almost exclusively on the use of chemical insecticides and during the last years pyrethroids and neonicotinoids represented the main products used by growers. In particular, since their recent introduction, neonicotinoids have been considered the key elements for the control strategies, because of the different chemistry and mode of action.

However, recently field-collected samples of *M. persicae* showed a significant level of resistance to neonicotinoids and the involvement of detoxifying enzymes, with the overexpression of a single cytochrome P450 gene (CYP6CY3), was demonstrated (Philippou *et al*. 2010; Puinean *et al*. 2010). The situation changed significantly when in 2009 a clone of *M. persicae* exhibiting extremely high resistance to neonicotinoids was collected in France (IRAC newsletter, issue 25). In this clone a new single point mutation causing an Arg to Thr substitution at position 81 (R81T) in the loop D region of the nicotinic acetylcholine receptor β1 subunit (nAChR β1) was discovered (Bass *et al*. 2011). This substitution reduces the binding affinity of neonicotinoids to the nicotinic acetylcholine receptor, giving a target-site resistance that compromises the efficacy of these insecticides against *M. persicae* populations in peach orchards (Slater *et al*. 2012).

Following these discoveries, monitoring programmes were established in France and Spain in order to investigate the geographical distribution and widespread presence of resistant populations of *M. persicae*, sampling peach orchards as well as some other herbaceous crops. In Italy, some failure cases were reported in 2010, and resistance was confirmed in 2011 (IRAC newsletter, issue 29; Mazzoni *et al*. 2012). In order to provide additional data for this survey, and in view of the increasing number of reports concerning the loss of efficacy of insecticide treatments, in spring 2012 we started a screening of *M. persicae* populations collected in different areas of Italy, focusing mainly on the most important peach-growing area (Emilia-Romagna). This work aims to investigate the diffusion of the main target-site mutations underlying the insensitivity to neonicotinoids and pyrethroids, both used in peach orchards against aphids and other pests.

2. MATERIALS AND METHODS

2.1 APHID SAMPLES

M. persicae populations were collected from spring to autumn 2012 in different areas of Italy, mainly from peach orchards (n=46), but also from a few herbaceous hosts (n=4) (Table 4.1).

Region	Area	Host	n° populations
Emilia-Romagna	Bologna	peach	8
Emilia-Romagna	Forlì-Cesena	peach	16
Emilia-Romagna	Forlì-Cesena	eggplant	2
Emilia-Romagna	Forlì-Cesena	pepper	1
Emilia-Romagna	Ravenna	peach	17
Emilia-Romagna	Rimini	peach	1
Abruzzo	Chieti	peach	1
Calabria	Cosenza	peach	1
Campania	Salerno	tobacco	1
Lazio	Roma	peach	1
Puglia	Foggia	peach	1
			Tot. 50

Table 4.1: Total number of* M. persicae *populations collected in Italy in 2012, divided by site and host of collection.

2.2 INSECTICIDE BIOASSAYS

Susceptibility towards the neonicotinoid imidacloprid (Confidor, 200 SL; Bayer CropScience S.r.l., Milan, Italy) and towards imidacloprid plus the synergist piperonyl butoxide (PBO) (PBO 80 EC; Endura, Bologna, Italy) was investigated by a

dip-test bioassay on reference clones 1X, 92H6 and 99H1, as previously described (see chapter 3, section 2).

2.3 DNA EXTRACTION

Genomic DNA was extracted from a single specimen by a “salting-out” protocol as already described (see chapter 3, section 3.3).

2.4 BI-PASA PCR

The presence of single point mutations was assessed with allele specific polymerase chain reaction amplification (PASA-PCR). This method allows the genotyping of a particular site in a target gene with a single reaction, through the combination of four different primers: two common primers are used to amplify the region of interest, while the other two internal specific primers are used for the specific amplification of the susceptible or resistant allele. The 3’ of the internal primers are designed specifically on the wild-type or mutate nucleotide and have different directions. Thus, in combination with the external primers, asymmetrically located in respect to the mutation of interest, they produce fragments of different size corresponding to homozygous or heterozygous genotypes (Liu *et al*. 1997).

Results of bi-PASA-PCR were validated by sequencing the control fragments directly amplified with the external primers, encompassing the mutations under investigation. This check was performed previously on the reference clones (see chapter 3, section 1.3) and on at least five different aphid samples for each target, randomly selected.

2.4.1 Characterisation of R81T mutation

In order to detect the presence of the substitution associated with neonicotinoid resistance (Bass *et al*. 2011), four new primers were designed (see chapter 3, table

3). Primer MpNACRs-FW is specific for the wild-type allele and in combination with MpNACR-R514 amplifies a177 bp fragment; primer MpNACRr-RE is specific for the resistant allele and in combination with MpNACR-F52 amplifies a 332 bp fragment (Fig. 4.1). The PCR reaction (25 µL) contained 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific, Milan, Italy), 0.4 µM of each primer and 1 µL of genomic DNA. Amplification started with 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 45 s, with a final elongation at 72 °C for 5 min.

2.4.2 Characterisation of *kdr* and *s-kdr* mutations

In order to detect the presence of the *kdr* mutation (L1014F), primers *kdr*-F1 and *kdr*-R4 (Cassanelli *et al*. 2005) were used in combination with primers Sd2 and Rv2 (Guillemaud *et al*. 2003) (see chapter 3, table 3). Primer Sd2 is specific for the wild-type allele and in combination with *kdr*-R4 amplifies a 195 bp fragment; primer Rv2 is specific for the *kdr* allele and in combination with *kdr*-F1 amplifies a 380 bp fragment (Fig. 4.1). The PCR reaction (25 µL) contained 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific), 0.4 µM of each primer and 1 µL of genomic DNA. Amplification started with 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 45 s, with a final elongation at 72 °C for 5 min.

In order to detect the presence of the *s-kdr* mutation (M918T), four new primers were designed (see chapter 3, table 3.3). Primer MpSKs-RE is specific for the wild-type allele and in combination with MpSK-F25 amplifies a 310 bp fragment; primer MpSKr-FW is specific for the *s-kdr* allele and in combination with MpSK-R3292 amplifies a 495 bp fragment (Fig. 4.1). The PCR reaction (25 µL) contained 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific), 0.4 µM of each primer and 1 µL of genomic DNA. Amplification started with 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 45 s, with a final elongation at 72 °C for 5 min.

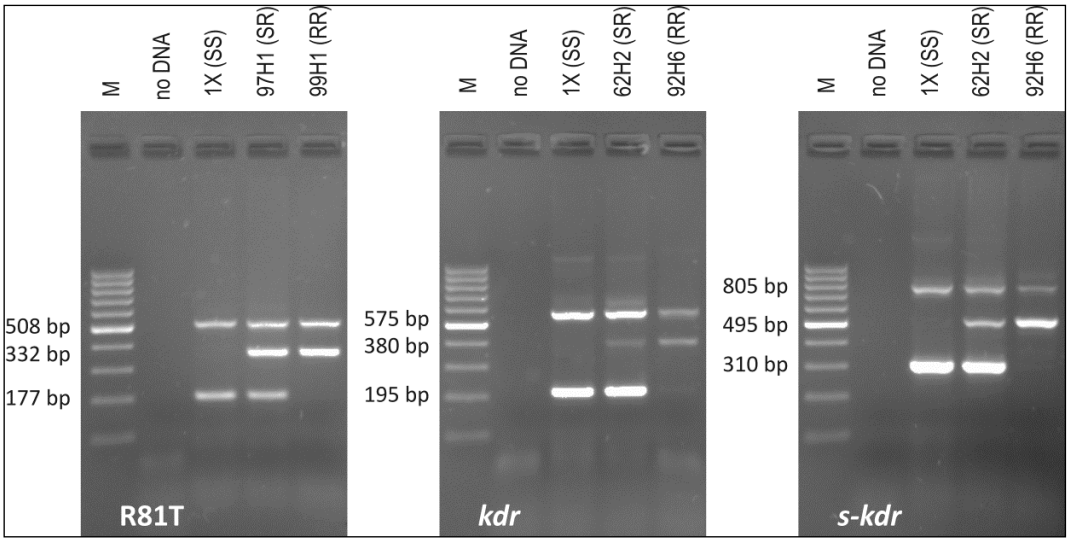

Figure 4.1: Bi-PASA-PCR products on R81T,* kdr *(L1014F) and* s-kdr *(M918T) mutations. Amplification profile of the possible genotypes: R81T: SS (508 bp and 177 bp); SR (508 bp, 332 bp and 177 bp), RR (508 bp and 332 bp);* kdr*: SS (575 bp and 195 bp); SR (575 bp, 380 bp and 195 bp), RR (575 bp and 380 bp);* s-kdr*: SS (805 bp and 310 bp); SR (805 bop, 495 bp and 310 bp), RR (805 bp and 495 bp).

2.4.3 M918L detection

In order to detect polymorphisms in *s-kdr* locus reported by other authors (Fontaine *et al*. 2011), direct PCR was assembled using primers *kdr*-F1 and *kdr*-R4 (see chapter 3, table 3.3) that encompass the codon of interest, as already described (Cassanelli *et al*. 2005). Amplicons were sequenced in both directions. This check was performed on wild-type aphid samples for both the common *kdr* (L1014F) and *s-kdr* (M918T) mutations, because, as reported in the literature, the new M918L (atg→ttg) was not found to be linked with them and because primers used in the bi-PASA (MpSKs-RE and MpSKr-FW) could not detect this nucleic substitution (a/t) (EMBL accession number FR774834).

3. RESULTS

3.1 NEONICOTINOID RESISTANCE

Probit analysis was used to estimate an imidacloprid baseline for the reference clone and indicated differences of the two neonicotinoid-resistant populations (92H6 and 99H1) compared with the susceptible population (1X). Results obtained from probit analysis are indicated in table 4.2.

Strain	Treatment	LC_{50} (µg mL $^{-1}$)	CI 95%		slope	d.f.	χ2	R.F.	R.S.
1X	I	0.49	0.35	0.64	2.46 ± 0.21	39	93.6		
92H6	I	5.72	3.55	7.73	3.20 ± 0.47	45	91.7	11.6	
	I + P	2.58	1.51	3.63	2.23 ± 0.35	45	94.1	5.3	2.2
99H1	I	297	n.c.	n.c.	1.21 ± 0.32	21	66.2	605	
	I + P	54.1	7.70	243	0.97 ± 0.21	13	28.7	110	5.5

Table 4.2: Log-dose probit-mortality data for imidacloprid (± PBO) against adults of different clones of* M. persicae *in pea seedling dip bioassays. LC_{50}: lethal concentration that is expected to cause 50% of mortality; CI 95%: confidence interval limits at 95%; d.f.: degree of freedom; R.F.: resistance factor (calculated as the ratio between LC_{50} of resistant strain and LC_{50} of susceptible strain 1X); R.S.: synergism factor (calculated as the ratio between LC_{50} without PBO and LC_{50} with PBO).

In both "resistant" clones the LC_{50} was significantly higher than the LC_{50} of the susceptible clone, as indicated by the non-overlapping of the confidence limits. The higher value was in clone 99H1, carrying the target-site mutation R81T in homozygous form, as confirmed by cDNA sequencing. The LC_{50} of clone 92H6, with susceptible genotype (R81) and presumed to be quite resistant to neonicotinoids owing to metabolic resistance, was lower; nevertheless it was significantly higher than the LC_{50} of the susceptible strain. Resistance factors (RFs) were calculated and were equal to 12 for strain 92H6 and more than 600 for strain 99H1. These values were significantly reduced by the use of the synergist PBO. The synergism factors,

2.2 for 92H6 and 5.5 for 99H1, suggest a possible involvement of an oxidative metabolic resistance mechanism, at least for clone 99H1.

Furthermore, a dose-response curve comparison of clones 1X and 92H6 does not reject the hypothesis of parallelism (χ^2=2.84; df=1; *P*=0.092), while for curves of clones 1X and 99H1 this hypothesis is rejected (χ^2=5.63; df=1; *P*=0.018), confirming the involvement only of metabolic resistance in the former and a combination of metabolic and target-site resistance in the latter (Robertson *et al*. 2007).

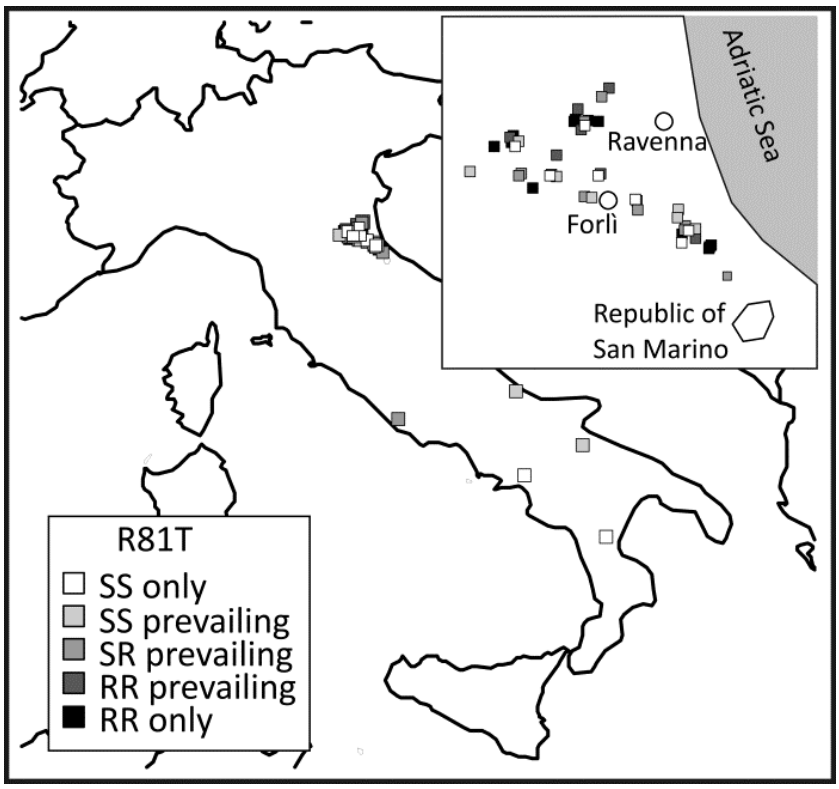

Figure 4.2: Distribution map of collection sites showing the presence of neonicotinoid target-site resistance genotypes.

Bi-PASA analysis of field samples collected in 2012 showed the presence of the R81T mutation in more than half of the populations: 35.5% of the individuals were homozygous, 29.5% were heterozygous and 35.0% showed a homozygous wild-type form. Homozygous resistant aphids were found only in Emilia-Romagna (Fig. 4.2)

and it was the only genotype present in 13 out of 45 samples collected in this region. At the same time, in the same areas, 7 wild-type populations were found. A fairly high percentage of heterozygous resistant specimens (70%) was found in sample from Lazio, near Rome, while in Abruzzo and Puglia they were present but in a lower percentage, 10 and 30% respectively. No aphids with this mutation were found in samples from Campania and Calabria. Specimens carrying this mutation were also found in 2 samples collected in autumn from herbaceous hosts: eggplant (100% homozygous resistant) and pepper (80% homozygous resistant, 20% heterozygous). (Table 4.3).

3.2 PYRETHROID RESISTANCE

Molecular analysis showed the presence of the common *kdr* and *s-kdr* mutations in almost all the populations collected, with higher percentages in Emilia-Romagna compared with the central and southern areas (Table 4.3).

Kdr (L1014F) was found in 97.4% of analysed aphids (*n*=467) (62.9% showed a homozygous genotype and 34.5% a heterozygous one). A few wild-type individuals were observed only in 3 of the 50 populations, coming from Emilia-Romagna (eggplant), Abruzzo (peach) and Campania (tobacco). *s-kdr* (M918T) was found in 94.4% of analysed aphids (50.1% showed a homozygous genotype and 44.3% were heterozygous). Wild-type individuals were observed only in 9 of the 50 populations. These aphids were collected from peach as well as from herbaceous hosts (tobacco and eggplant); they were present in all the populations coming from central and southern Italy and in a few populations collected in Emilia-Romagna.

s-kdr mutation was detected only in insects with the *kdr* mutation. When *kdr* was found in the homozygous form, *s-kdr* was homozygous or heterozygous; when *kdr* was in the heterozygous form, the *s-kdr* homozygous form was never detected (Table 4.4). Considering target-site resistance to neonicotinoids, R81T genotypes are quite uniformly distributed among *kdr* resistant genotypes (Table 4.5).

		s-kdr			
		RR	**SR**	**SS**	***Total***
kdr					
	RR	50.1%	12.4%	0.4%	63.0%
	SR		31.9%	2.6%	34.5%
	SS			2.6%	2.6%
	Total	50.1%	44.3%	5.6%	

Table 4.3: Percentage of specimens with different* kdr *and* s-kdr *genotypes combinations.

		R81T			
		RR	**SR**	**SS**	***Total***
kdr					
	RR	21.8%	20.3%	20.8%	62.9%
	SR	13.7%	9.0%	11.8%	34.5%
	SS		0.2%	2.4%	2.6%
	Total	35.5%	29.5%	35.0%	

Table 4.4: Percentage of specimens with different* kdr *and R81T genotypes combinations.

Only 12 specimens were fully susceptible for both *kdr* and *s-kdr* mutations. They were investigated for the presence of the other *s-kdr* mutation (M918L). This was indicated only in one specimen, collected from peach in Abruzzo, but differing from literature reports (Fontaine *et al*. 2011). Two differences were found: (1) it was caused by an a/c nucleic substitution instead of the a/t already documented; (2) the mutation was in homozygous form. This specimen was the only *kdr* wild-type aphid bearing R81T.

Table 4.5: **M. persicae** ***populations collected in Italy in 2012.***

Sample (code)	Site (province)	Host	Pop. (n.)	R81T genotype (%)			*kdr* genotype (%)			*s-kdr* genotype (%)		
				RR	SR	SS	RR	SR	SS	RR	SR	SS
132	RA	peach	10	50.0	30.0	20.0	80.0	20.0	0.0	80.0	20.0	0.0
133	RA	peach	10	0.0	100.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
150	RA	peach	10	80.0	10.0	10.0	100.0	0.0	0.0	100.0	0.0	0.0
107	RA	peach	10	100.0	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0
108	RA	peach	9	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
121	RA	peach	10	0.0	80.0	20.0	90.0	10.0	0.0	90.0	10.0	0.0
149	RA	peach	9	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
129	RA	peach	9	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
106	RA	peach	9	0.0	0.0	100.0	66.7	33.3	0.0	66.7	33.3	0.0
115	RA	peach	9	66.7	33.3	0.0	33.3	66.7	0.0	22.2	77.8	0.0
122	BO	peach	10	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0
141	BO	peach	10	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0
143	BO	peach	10	50.0	50.0	0.0	60.0	40.0	0.0	60.0	40.0	0.0
140	BO	peach	10	100.0	0.0	0.0	30.0	70.0	0.0	0.0	100.0	0.0
142	BO	peach	10	0.0	20.0	80.0	100.0	0.0	0.0	100.0	0.0	0.0
127	BO	peach	10	100.0	0.0	0.0	70.0	30.0	0.0	50.0	50.0	0.0
109	BO	peach	10	0.0	0.0	100.0	60.0	40.0	0.0	50.0	10.0	40.0
128	RA	peach	10	50.0	50.0	0.0	70.0	30.0	0.0	70.0	30.0	0.0
111	BO	peach	10	10.0	40.0	50.0	70.0	30.0	0.0	70.0	30.0	0.0
131	RA	peach	10	0.0	80.0	20.0	100.0	0.0	0.0	20.0	80.0	0.0
151	RA	peach	10	0.0	60.0	40.0	100.0	0.0	0.0	100.0	0.0	0.0
136	RA	peach	10	0.0	100.0	0.0	10.0	90.0	0.0	10.0	90.0	0.0

Table 4.5: cont.

Sample (code)	Site (province)	Host	Pop. (n.)	R81T genotype (%)			*kdr* genotype (%)			*s-kdr* genotype (%)		
				RR	SR	SS	RR	SR	SS	RR	SR	SS
146	RA	peach	9	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0	0.0
147	RA	peach	9	0.0	11.1	88.9	77.8	22.2	0.0	77.8	22.2	0.0
117	FC	peach	10	90.0	10.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
144H	FC	peach	10	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0	0.0
139	RA	peach	8	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0
134	FC	peach	8	0.0	100.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
126	FC	peach	10	0.0	20.0	80.0	100.0	0.0	0.0	90.0	10.0	0.0
116	FC	peach	10	0.0	100.0	0.0	10.0	90.0	0.0	10.0	90.0	0.0
137	FC	peach	10	0.0	0.0	100.0	0.0	100.0	0.0	0.0	100.0	0.0
138	FC	peach	9	0.0	100.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
110	FC	peach	10	0.0	10.0	90.0	80.0	20.0	0.0	20.0	80.0	0.0
113	FC	peach	9	0.0	22.2	77.8	44.4	55.6	0.0	22.2	66.7	11.1
112	FC	peach	10	0.0	90.0	10.0	80.0	20.0	0.0	50.0	50.0	0.0
119	FC	peach	10	30.0	70.0	0.0	80.0	20.0	0.0	50.0	50.0	0.0
135	FC	peach	10	0.0	20.0	80.0	80.0	20.0	0.0	0.0	90.0	10.0
125	FC	peach	8	100.0	0.0	0.0	50.0	50.0	0.0	50.0	50.0	0.0
148	FC	peach	10	0.0	0.0	100.0	0.0	100.0	0.0	0.0	80.0	20.0
118	FC	peach	10	100.0	0.0	0.0	50.0	50.0	0.0	40.0	60.0	0.0
130	FC	peach	10	100.0	0.0	0.0	70.0	30.0	0.0	70.0	30.0	0.0
120	RI	peach	10	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0

Table 4.5: cont.

Sample (code)	Site (province)	Host	Pop. (n.)	R81T genotype (%)			*kdr* genotype (%)			*s-kdr* genotype (%)		
				RR	SR	SS	RR	SR	SS	RR	SR	SS
154	FC	eggplant	6	0.0	0.0	100.0	0.0	16.7	83.3	0.0	16.7	83.3
152	FC	eggplant	6	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
153	FC	pepper	5	80.0	20.0	0.0	60.0	40.0	0.0	60.0	40.0	0.0
114	CH	peach	10	0.0	10.0	90.0	50.0	40.0	10.0	20.0	70.0	10.0
155	RO	peach	10	0.0	70.0	30.0	80.0	20.0	0.0	50.0	20.0	30.0
145	FG	peach	10	0.0	30.0	70.0	60.0	40.0	0.0	10.0	70.0	20.0
124	SA	tobacco	6	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
123	CS	peach	9	0.0	0.0	100.0	0.0	100.0	0.0	0.0	88.9	11.1

4. DISCUSSION

In recent years, in Italy, neonicotinoids and to a lesser extent pyrethroids have been the key components of the pest management strategies used by growers against the peach potato aphid *M. persicae*. Neonicotinoids were considered to be a key instrument in resistance management strategies (Elbert *et al*. 2008) but, in time, cases of reduced efficacy were reported. Recent monitoring programmes in Southern Europe have revealed the widespread distribution of resistant populations, posing a serious threat to the long-term efficacy of these insecticides. The present work aimed to investigate the current Italian status of genomic mutations linked to neonicotinoid and pyrethroid insecticide target-site resistance. Emilia-Romagna was the main area monitored because it is the most important cultivation area for peach in Italy, but the survey was extended also to other regions. Great attention was paid to those instances where neonicotinoid treatments were not efficacious and peach growers observed consistent re-infestations of the pest.

The present data confirm a quite worrying situation in the more specialised peach-growing area of Italy. Aphids carrying the R81T substitution in the loop D region of the nAChR β1 were very common and uniformly distributed in Emilia-Romagna. A few samples from different parts of Italy indicated the presence of a potentially critical situation in other regions also: the mutation was found in many specimens of populations collected in Lazio, Abruzzo and Puglia. Only aphids from Calabria and Campania showed a complete wild-type genotype. Until now this mutation has been described only in populations collected from peach (Slater *et al*. 2012; IRAC newsletter, issue 29). Here, for the first time, its presence also in *M. persicae* samples collected from herbaceous hosts is described. It is worth noting that, in one case, aphids collected on the same farm but at different times from peach (in spring) and from eggplant (in autumn) showed the same genotype (100% homozygous resistant). This underlines the fact that resistant aphids, spreading on secondary hosts, can also affect any neonicotinoid-based management strategy on these crops.

The present data also confirm the compromised effectiveness of pyrethroids, the employment of which in *M. persicae* control strategies must be avoided owing to the extremely high frequencies of *kdr* and *s-kdr* mutations. Only in one population, collected in Campania from tobacco plants, all the analysed specimens were wild type.

In comparison with previously published data concerning the presence of different resistance mechanisms in Italian populations of *M. persicae* (Criniti *et al*. 2008), here emerges a huge increase in the percentage of pyrethroid-resistant populations. In particular, *s-kdr* specimens are much more abundant and aphids with homozygous resistant genotype have been found. This could be explained by the fact that in Italy pyrethroids remain one of the few solutions against thrips on nectarines, and they are usually applied pre- or post-flowering in a period that is also critical for insecticide application against *M. persicae*, so giving, at least indirectly, a significant selection pressure with this mode of action.

Furthermore, the new *s-kdr* mutation (M918L) has been found, although for the moment it seems to be quite rare in Italy. In fact, our data described only one specimen carrying that mutation in an homozygous form. Actually, this survey considered the presence of that mutation only in wild-type aphids for both the original *kdr* and *s-kdr* mutations; nevertheless it is important to consider the possibility that it could be present also in samples with different genotypes, i.e. heterozygous for the same mutations, in order to verify different hypothetical combinations of them, because of a possible location on different chromosomes. In addition, literature data showed that M918L can affect pyrethroid resistance (Fontaine *et al*. 2011). For that reason, its spread should be considered and monitored more in details in further sampled field populations.

CHAPTER 5

TARGET-SITE RESISTANCE: ALTERNATIVE *S-KDR* MUTATIONS IN THE VOLTAGE-GATED SODIUM CHANNEL

PRESENCE AND IMPACT OF ALLELIC VARIATIONS OF TWO ALTERNATIVE *S-KDR* MUTATIONS, M918T AND M918L, IN THE VOLTAGE-GATED SODIUM CHANNEL OF THE GREEN PEACH APHID *MYZUS PERSICAE*.

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Abstract

BACKGROUND: Pyrethroids have been widely employed in order to control several agricultural pests, including *M. persicae*. Target-site resistance is the main mechanism that confers insensitivity to this class of compounds. The most common amino acid substitutions are *kdr* (L1014F) and *s-kdr* (M918T), but recently another mutation in the *s-kdr* locus (M918L) has been described in French and Korean populations of *M. persicae*.

RESULTS: Molecular analysis of several Italian populations of *M. persicae* by pyrosequencing revealed the presence of the new *s-kdr* mutation (M918L) in different forms. It was found in two possible nucleotide polymorphisms (a/t or a/c substitution), in heterozygous or homozygous status, and also in combination with the classic *kdr* and *s-kdr*. Bioassays on populations carrying M918L mutation show that it strongly affects pyrethroid efficacy, particularly of type II pyrethroids such as *λ*-cyhalothrin, whilst it has no effect against DDT.

CONCLUSION: This work contributes to add more information about the new *s-kdr* M918L mutation in *M. persicae*, describing a more complicated situation due to the possible combination with the classic L1014F and M918T. Our data open new questions on the origin of these new genotypes with different combinations of target-site mutations and also on their possible influence on control strategies.

1. INTRODUCTION

Pyrethroids is one of the main insecticide classes widely used for both agricultural and public health purposes, in order to control several pests and vectors of human diseases. This class of synthetic insecticides affects the insect nervous system, acting on the voltage-gated sodium channels (VGSC), large trans-membrane spanning proteins that are essential for electrical signalling in nerve cell membranes (Davies *et al*. 2007).

For a long time pyrethroids have been used against *M. persicae* and the continuous treatments have led to the development of populations showing high levels of resistance (Devonshire *et al*. 1998). L1014F (*kdr*) and M918T (*s-kdr*) are the two most common target-site mutations described in this pest; until now, M918T has always been found in combination with L1014F, leading to high levels of pyrethroid resistance (Martinez-Torres *et al*. 1999; Eleftherianos *et al*. 2008).

Another amino acid substitution (M918L) in the *s-kdr* locus has recently been identified in French and Korean populations of *M. persicae* and linked to a resistant phenotype even in the absence of the classic *kdr* (Fontaine *et al*. 2011; Roy *et al*. 2013; Kim *et al.* 2014). Interestingly, literature data describe two different polymorphisms responsible for this mutation: in samples collected in France an a/t nucleotide substitution (atg → ttg) was found only in a heterozygous status (Fontaine *et al*. 2011), whilst in Italian populations an a/c nucleotide substitution (atg → ctg) was described in a homozygous status (Panini *et al*. 2014, see chapter 4).

In this work we document the contemporary presence of both allelic variants of the M918L mutation together with the *kdr* L1014F, resulting in novel *M. persicae* genotypes not yet described by other authors. The discovery underlines the importance of these critical sites in the voltage gated sodium channels, where the main target-site mutations are localised, and also provide additional information regarding the evolution of target-site resistance in the green peach aphid.

2. MATERIALS AND METHODS

2.1 APHID SAMPLES

M. persicae populations were collected from spring to autumn 2012 (n=50) and 2013 (n=25) in different areas of Italy, mainly from peach orchards (n=69), but also from secondary hosts (n=6) (Table 5.1).

Region	Area	Host	Populations 2012		Populations 2013	
			n° tot	M918L	n° tot	M918L
Emilia-Romagna	Bologna	peach	8		3	
Emilia-Romagna	Forlì-Cesena	peach	16	2	10	1
Emilia-Romagna	Forlì-Cesena	eggplant	2	1		
Emilia-Romagna	Forlì-Cesena	pepper	1		1	
Emilia-Romagna	Ravenna	peach	17	3	3	1
Emilia-Romagna	Rimini	peach	1			
Abruzzo	Chieti	peach	1	1	1	1
Calabria	Cosenza	peach	1	1		
Campania	Salerno	tobacco	1			
Lazio	Latina	peach			1	1
Lazio	Latina	pepper			1	1
Lazio	Viterbo	peach			1	
Lazio	Roma	peach	1		1	
Puglia	Foggia	peach	1	1	3	3
			Tot. 50	Tot. 9	Tot. 25	Tot. 8

Table 5.1: Geographical and host distribution of* M. persicae *populations collected in 2012/2013. For each year the total number of sampled populations and the number of them in which M918L mutation was detected are reported.

2.2 DNA EXTRACTION

Genomic DNA was extracted from a single specimen by a "salting-out" protocol as already described (see chapter 3, section 3.3).

2.3 PYROSEQUENCING ASSAY FOR *S-KDR* GENOTYPING

Pyrosequencing is a DNA sequencing-by-synthesis technology that allows the detection of single nucleotide polymorphisms (SNPs). This method is based on the luminometric detection of pyrophosphate (PPi) released upon nucleotide incorporation (Ahmadian *et al*. 2000). Target-site mutations located in the *s-kdr* locus (M918T/L) were investigated with this technique.

A short gene fragment of 109 bp was amplified by PCR from genomic DNA. Primers 65-F and 98-R-btn (see chapter 3, table 3.3) were designed using the "Assay Design Software" (PSQ-Biotage AB, Uppsala, Sweden); the reverse primer was biotinylated at the 5' end. The PCR reaction (50 µL) contained 1X Taq enzyme reaction mix (RedTaq JumpStart Master Mix, Sigma Aldrich, Milan, Italy), 0.4 µM of each primer and 5 µL of genomic DNA. Amplification started with 1 min at 94°C, followed by 45 cycles of 94 °C for 30 s, 52 °C for 45 s and 72 °C for 30 s, with a final elongation at 72 °C for 10 min. PCR products were controlled in 2% agarose gel.

The biotinylated single strands of the PCR products were obtained by using the Vacuum Prep Tool (Biotage AB) in combination with streptavidin coated beads (Streptavidin Sepharose High Performance beads, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The pyrosequencing reactions were performed with the PSQ 96MA System, using dedicated PSQ 96 SNP Reagent Kit and HS Plate (Qiagen) and according to the manufacturer's instructions. The genotyping was assessed with the sequence-primer 116-seq (see chapter 3, table 3.3), starting one nucleotide upstream of the site of interest. Pyrograms were analysed using the PSQ96 MA SNP Software.

2.4 CLONING

DNA fragments 600 bp long were PCR-amplified with primers *kdr*-F1 and *kdr*-R4 (see chapter 3, table 3.3) and then cloned in pGEM-T Easy Vector (Promega, Madison, WI, USA) using T4 DNA ligase, according to the manufacturer's instructions. Ligated plasmids were transformed into *E. coli* High Efficiency Competent Cells (Promega). Selection of recombinant clones was made with white:blue colonies screening on Luria-Bertani agar containing X-gal (40 μg mL^{-1}), IPTG (0.4 mM) and ampicillin (0.05 mg mL^{-1}) (Sambrook *et al*. 1989).

White colonies were re-suspended in 15 μL of sterile distilled water and lysed at 95 °C for 10 min. The presence of cloned insert was evaluated by Colony PCR using T7/Sp6 primers (see chapter 3, table 3.3). PCR reactions (25 μL) contained 12.5 μL of DreamTaq Green PCR Master Mix (Thermo Scientific, Milan, Italy) and 0.4 μM of each primer. Amplification started with 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 50 °C for 40 s, 72 °C for 1.5 min and a final extension at 72 °C for 10 min, using GeneAmpR PCR System 2700 (Applied Biosystems, Life Technologies, USA, Foster City, CA, USA). Plasmids were extracted from positive clones using an alkaline lysis method (Sambrook *et al*. 1989). Inserts were sequenced using a BigDye v3.1 Sequencing kit according to the manufacturer's instructions (Applied Biosystems). After unincorporated dye terminators removal, sequences were loaded and run on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

2.5 cDNA SEQUENCING

Total RNA was isolated from 15 mg of fresh aphids using TRI Reagent (Sigma-Aldrich), according to the manufacturer's instructions. RNA pellets were dissolved in DEPC water and their quality was assessed by denaturing electrophoresis in TBE/formamide 1.2% agarose gel. The concentrations were determined using a Qubit Fluorimeter 2.0 instrument (Qubit RNA Assay Kit; Invitrogen). All the samples were diluted in DEPC water to obtain stocks 1 μg $μL^{-1}$. First-strand cDNA was

synthesized from 1 µg of the total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol.

In order to detect the presence of the mutations under investigation, primers MpSK-F25 and MpSK-R21 were used (see chapter 3, table 3.3). PCR reactions (25 µL) contained 2 µL of cDNA, 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific), 0.4 µM of each primer. Amplification started with 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, with a final elongation at 72°C for 10 min. PCR products obtained were purified with GenElute PCR Clean-Up Kit (Sigma-Aldrich) and direct sequenced in both strands using the same primers.

2.6 INSECTICIDE BIOASSAYS

Susceptibility toward the type I pyrethroid bifenthrin (Brigata Flo; SIPCAM, Pero, Milan, Italy), the type II pyrethroid *λ*-cyhalothrin (Karate Zeon 1.5; Syngenta Crop Protection, Gallarate, Milan, Italy) and DDT (Sigma Aldrich) was assessed by a dip-test bioassay (see chapter 3, section 2). DDT was emulsified in water using rapeseed oil and ethylene glycol monostearate. Piperonyl butoxide (PBO) (PBO 80EC; Endura, Bologna, Italy) was used to synergize pyrethroid efficacy.

3. RESULTS

3.1 CHARACTERISATION OF M918L MUTATION

The *s-kdr* mutation M918L was detected in all regions where populations were collected, except Campania (Table 5.1). There are a few cases of samples carrying this mutation that were collected in the same orchards in 2012 and 2013, confirming its persistence over the years. Furthermore, the mutation was found not

only in populations collected from peach, but also from secondary hosts (eggplant and pepper).

Two different polymorphisms, giving the same amino acid substitution, were found. The most common is the a/c substitution, already documented in Italian populations of *M. persicae* (Panini *et al*. 2014, see chapter 4); the other is the a/t substitution, already detected in populations collected in other areas of Southern Europe and Korea and reported here for the first time also in Italy. The presence of the mutation was checked in total in 293 aphids and the percentage of specimens carrying it was about 20%. In particular both polymorphisms, a/c and a/t, account for 15% and 5% of the total analysed cases, respectively. The a/t substitution was detected only in the heterozygous form, whilst the a/c substitution was present also in the homozygous form. Indeed the latter seems to be very rare: only two specimens were found and both samples were collected from the same orchard in Abruzzo.

3.2 M918L AND M918T COMBINATION

Analysing the *s-kdr* locus, in some specimens, contemporary nucleotidic substitutions in both the first and second position of the "atg" methionine codon have been detected: a/c or a/t in position 1 and c/t in position 2 (Fig. 5.1).

Their combination can theoretically encode for other aminoacid substitutions ("ccg" for a proline and "tcg" for a serine) and not necessary for M918L or T. In order to characterise the true amino acid substitutions encoded by such mutations and to confirm or exclude the presence of new mutations, never reported in literature in this locus, a fragment encompassing the *kdr* and *s-kdr* locus was cloned and sequenced. According to the cloning experiment results, the only codons detected were, "acg" (threonine) and "ctg" or "ttg" (leucine) whilst other possible codons were not found.

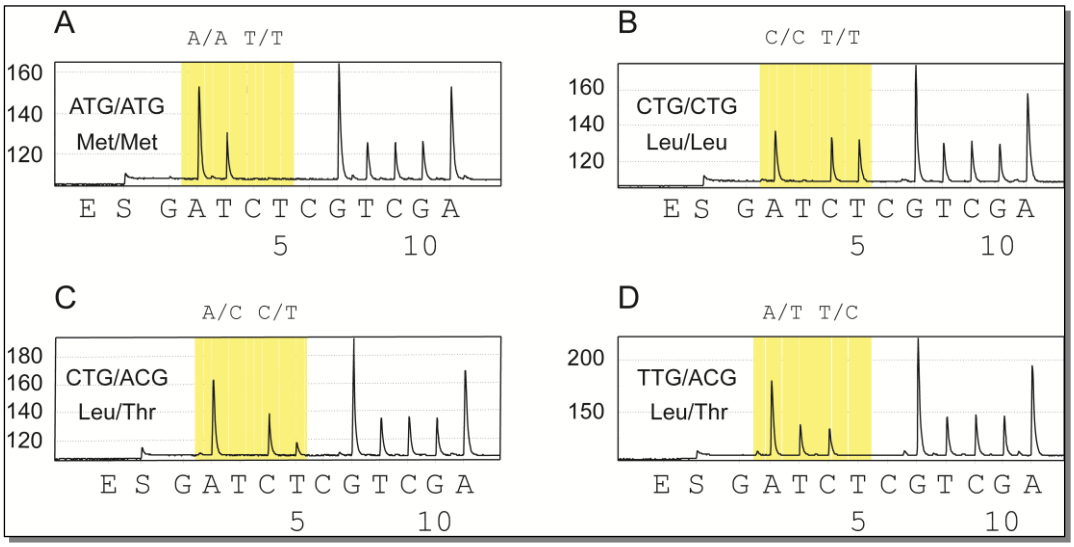


Figure 5.1: Pyrosequencing of the short fragment of genomic DNA encompassing the* s-kdr *locus in* M. persicae*. Highlighted areas evidence the nucleotides of interest, showing alternative polymorphisms in different clones. A: wild-type; B: homozygous for the nucleic substitution responsible for the M918L mutation; C: heterozygous for both* s-kdr *mutations (M918L and M918T) with the a/c substitution; D: heterozygous for both* s-kdr *mutations (M918L and M918T) with the a/t substitution.

As confirmed by the sequencing of the cloned fragment, the two nucleotidic substitutions are located on different alleles. Moreover "ctg" or "ttg" (M918L) was never found linked with the classic *kdr* mutation, whilst "acg" (M918T) was always linked with L1014F, confirming literature data already available (Lee *et al.* 1999; Soderlund 2008; Rinkevich *et al.* 2013). So, for the first time, the simultaneous presence of M918T and M918L mutations, located on different alleles, in the same individual (Fig. 5.2) is described.

This evidence is also important to justify the homozygous status of M918L mutation only in samples that are wild-type (L1014 and M918). cDNA sequencing demonstrates that both alleles are transcribed in clones with both *s-kdr* mutations (M918T and M918L) as well as in heterozygous clones (wild-type and M918L) (Fig. 5.3).

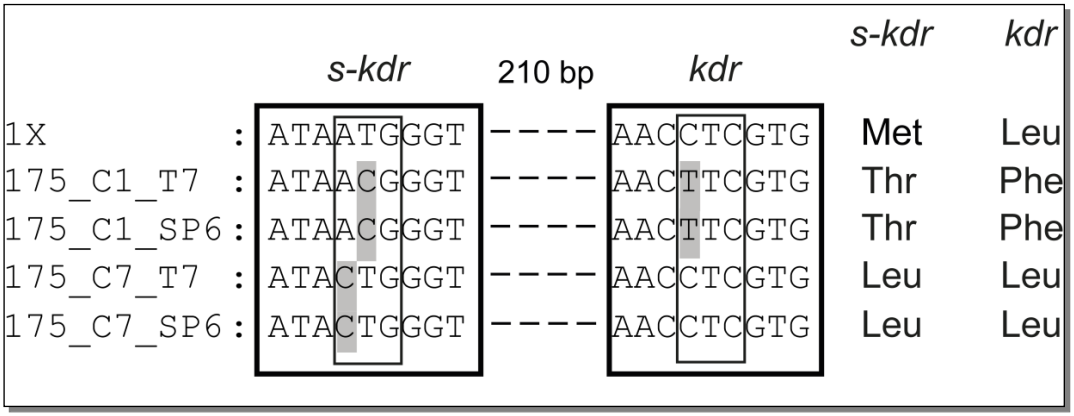


Figure 5.2: Alignment of sequences derived from cloning experiments. Only the regions encompassing* kdr *and* s-kdr *mutations are reported and sites of amino acid substitutions are boxed. Nucleotide substitutions are highlighted.

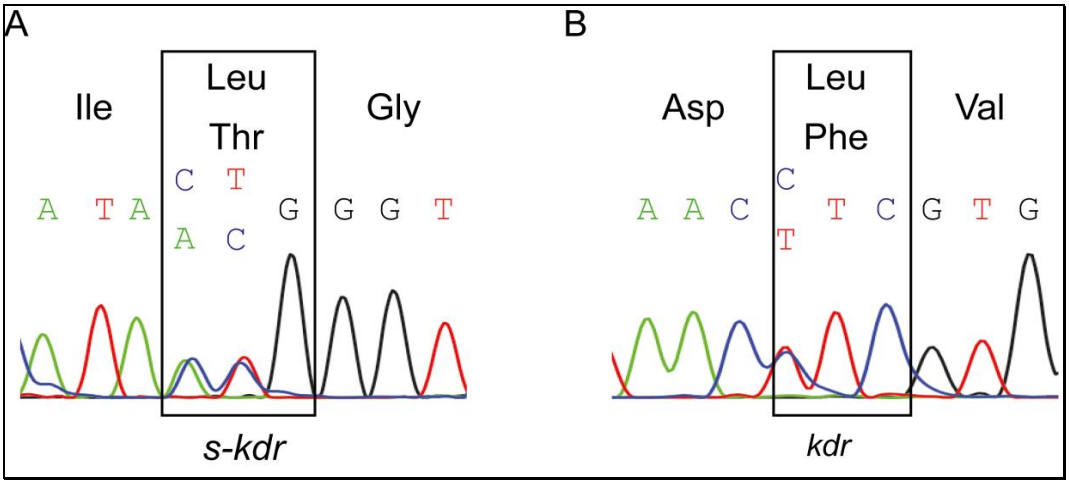


Figure 5.3: cDNA sequencing of* s-kdr *(A) and* kdr *(B) loci. Sites of amino acid substitutions are boxed. Alternative SNPs are in correspondence with double peaks.

A summary of all the possible *s-kdr* genotypes detected in analysed aphids are listed in table 5.2, together with the percentages of their frequencies.

mutation(s)	genotype	codon(s)	2012	2013
wild type	homozygous	atg	17.1%	17.0%
M918T only	heterozygous	acg + atg	59.0%	45.5%
	homozygous	atg	2.6%	17.6%
M918T + M918L	heterozygous	acg + ctg	14.5%	8.0%
	heterozygous	acg + ttg	2.6%	2.8%
M918L only	heterozygous	atg + ctg	3.4%	4.5%
	heterozygous	atg + ttg		4.0%
	homozygous	ctg	0.9%	0.6%
		specimens (n)	117	176

Table 5.2:* s-kdr *genotypes and their frequencies detected in* M. persicae *populations collected in 2012/2013.

3.3 BIOASSAYS

The influence of different *s-kdr* genotypes on pyrethroid and DDT efficacies was investigated with full dose-response bioassays in four clones: a fully susceptible (1X), a L1014F+M918T clone (92H6), a M918L clone (384C) and a M918T+M918L+L1014F clone (175H2) (see chapter 3, table 3.2). Parameters of baselines for bifenthrin, *λ*-cyhalothrin and DDT, estimated by probit analysis, are summarised in table 5.3.

Estimated LC_{50} values for bifenthrin in clones 384C and 175H2 were significantly different from LC_{50} values of the susceptible clone (1X), as indicated by non-overlapping confidence limits. The LC_{50} value of bifenthrin in clone 92H6 was extremely high and it was not possible to calculate its confidence limits. Among clones with a M918L mutation the highest resistance factor (more than 1400-fold)

was observed in clone 175H2 (M918T and M918L simultaneously) that showed also the highest synergistic ratio when combined with PBO (about 20). Clone 384C (M918L only) showed a much lower resistance factor (RF = 86) and synergistic ratio (SF = 3.2).

Resistance to λ-cyhalothrin was higher and it was not possible to achieve significant regression estimates for clones 384C and 175H2 using the insecticide alone. As a consequence, resistance and synergistic ratios could not be calculated. On the contrary, in bioassays with λ-cyhalothrin/PBO it was possible to estimate significant regression coefficients and to calculate resistance factors, however again clone 175H2 showed the highest resistance factor (RF = 1466).

In summary, for both M918L clones resistance factors calculated for λ-cyhalothrin+PBO were higher than those calculated for bifenthrin+PBO but resistance factors of clone 384C was always lower for both insecticides than the corresponding resistance factors of clone 175H2.

In DDT bioassays no differences were observed between the susceptible clone and clone 384C. The hypothesis of equality (equal slopes, equal intercepts) evaluated with probit analysis was not rejected ($P>0.05$; χ^2: 4.93, degrees of freedom: 2). In clone 175H2 a resistant factor of 3.5 was observed and baseline comparison rejects the equality as well as parallelism hypothesis and confidence limits for LC_{50} values are completely separated from those of the susceptible clone.

Treatment	Clone	LC_{50} (µg mL^{-1})	CI 95%		slope	d.f.	χ2	R.F.	S.F.
bifentrin	1X	1.07	0.56	1.67	1.65(± 0.31)	8	6.2		
bifentrin + PBO	1X	0.46	0.22	0.72	1.69 (± 0.34)	8	6.8		2.3
bifentrin	384C	92.3	15.5	256	0.77 (± 0.16)	13	19.7	86.4	
bifentrin + PBO	384C	28.8	14.6	45.8	1.85 (± 0.36)	13	14.7	63.3	3.2
bifentrin	175H2	1534	383	10800	0.67 (± 0.11)	18	65.8	1436	
bifentrin + PBO	175H2	77.8	40.8	123	1.57 (± 0.20)	18	20.2	171	19.7
bifentrin	92H6	10900	-	-	0.71 (± 0.24)	28	36.4	>10000	
bifentrin + PBO	92H6	124	70.2	200	1.34 (± 0.18)	28	41.1	273	87.6
λ-cyhalothrin	1X	1.93	0.85	3.00	1.70 (± 0.32)	23	40.4		
λ-cyhalothrin + PBO	1X	0.63	0.10	1.35	1.34 (± 0.18)	18	77.2		3.1
λ-cyhalothrin	384C	-	-	-	0.25 (± 0.14)*	15	14.9	n.e.	
λ-cyhalothrin + PBO	384C	99.6	57.0	154	1.46 (± 0.17)	13	18.1	158	n.e.
λ-cyhalothrin	175H2	-	-	-	-0.40 (± 0.62)*	18	27.6	n.e.	
λ-cyhalothrin + PBO	175H2	923	243	1000	2.43 (± 0.66)	14	16.2	1466	n.e.
DDT	1X	112	67.2	139	3.08 (± 0.89)	11	11.1		
DDT	384C	131	85.0	186	1.83 (± 0.27)	18	20.4	1.2	
DDT	175H2	390	169	829	0.95 (± 0.10)	21	58.8	3.5	

Table 5.3: Log-dose probit-mortality data for bifenthrin (± PBO), λ-cyhalothrin (± PBO) and DDT against adults of different clones of* M. persicae *in pea seedling dip bioassays (24h) (Legend: * = not significant; n.e. = not estimable).

4. DISCUSSION

In this work we describe the presence and distribution of the *s-kdr* mutation M918L in Italian populations of the green peach aphid. It has been found to be not a rare mutation, as suggested from previous preliminary data available (Panini *et al*. 2014, see chapter 4), but on the contrary, it is present throughout the country and, even if our samples were collected mainly from peach, it has been detected also in populations from secondary hosts. The discrepancy with the previous survey is explained by the different methodology adopted, that allows to evaluate at the same time all the SNPs present in the *s-kdr* locus giving a more clear view of the presence of this mutation.

Fontaine *et al*. (2011) pointed out a significant increase of resistance to λ-cyhalothrin (type II pyrethroid) due to the presence of the M918L mutation in French populations of *M. persicae*. Our data confirm the resistance and show that also the efficacy of type I pyrethroids such as bifenthrin is strongly affected by this mutation. A comparison of label field rates for both pyrethroids and the corresponding estimated LCs values confirm the possibility for that populations with M918L mutation (even if in heterozygous status) to survive to field treatments. Furthermore, the contemporary presence of both mutations (M918T and M918L) in individual aphids increases the resistance levels to both types of pyrethroids (even if metabolic resistance could play a role as suggested by synergism observed in bioassays with PBO). Resistance factors are different between type I and type II pyrethroids, i.e. resistance ratios to λ-cyhalothrin are higher. This is in agreement with earlier findings suggesting an important role of mutations especially in the *s-kdr* locus, and reporting higher resistance levels to type II pyrethroids like *λ*-cyhalothrin (Eleftherianos *et al*. 2008).

Interestingly, the presence of M918L alone, in heterozygous form, does not confer resistance to DDT in our bioassays since baseline data for the susceptible clone are not statistically different from that of clone 384C. This finding is in line with recent data showing that the presence of *s-kdr* (M918T) does not affect DDT sensitivity (Usherwood *et al*. 2005).

No data of the influence on resistance levels of the homozygous genotype for the M918L mutation are available. This genotype seems to be quite rare and has not yet been described in lab established populations used to isolate clones for bioassays. It is likely produced by the mating of heterozygous adults but it may have high fitness costs and as a consequence it could be less competitive in co-existence with the other genotypes. This can explain its extinction in lab reared populations and its low frequency in the field: indeed homozygous M918L samples have been detected early in the season but they were never collected later in secondary colonies from peach or from secondary hosts.

Our data show for the first time the combination of M918L and M918T in the same specimen. The presence of the double mutation could be explained by the sexual holocycle of *M. persicae* on peach, i.e. it is possible that, in autumn, sexual morphs with classical *kdr* and *s-kdr* mutations (L1014F & M918T) mate with M918L specimens and produce offspring with both mutations in the *s-kdr* locus. This genotype apparently does not suffer severe disadvantages compared to other genotypes as it represents more than 50% of the samples collected with the M918L mutation in Italy.

Artificial crosses of *M. persicae* clonal populations with different combinations of resistant alleles could be important to demonstrate what mentioned above and better investigate the heredity of resistance. Unfortunately, crosses between aphids require a lot of efforts and time, due to difficulties to induce sexual stages under laboratory conditions and, above all, to get eggs hatching as they usually require cold accumulation and suffer high mortality (Blackman *et al*. 1996). Nevertheless, these experiments could allow to confirm the origins of the M918L homozygous genotype as well as the combination of M918L and M918T in the same aphid.

The potential effect of M918L mutations on control strategies are yet to be fully understood, especially for secondary hosts that could rely, more than peach, on pyrethroid application against *M. persicae*. Our data revealed that, at least for type I pyrethroids, resistance conferred by the M918L mutation is lower than that conferred by homozygous L1014F+M918T.

The presence of M918L genotypes with multiple resistance mechanisms towards other insecticide modes of actions also needs to be considered. Some preliminary data about the presence in Italy of neonicotinoid and dimethyl-carbamate target-site resistance do not show special association with M918L, but further surveys are needed and are currently in progress. Data presented here and in the previous chapter clearly demonstrate the coexistence of different resistance mechanisms within the same aphid, resulting in populations that are resistant to multiple insecticides. Literature works have already shown a reduction in aphid fitness in populations with one or two resistance mechanisms (Foster *et al*. 1996, 1997). It has been also demonstrated that *kdr* mutation is responsible of altered sodium channel gating properties. This results in a general reduction of the excitability of the nervous system and thereby potential disruption in the perception and behavioural responses to various stimuli that could be important for aphid survival, like an altered response to the alarm pheromone B-farnesene (Foster *et al*. 1999, 2007). Clonal lineages carrying multiple resistant mechanisms, including different *kdr* and *s-kdr* (M918L and M918T) allelic combinations, as well as others like neonicotinoid target-site resistance or metabolic resistance, could be established for *ad hoc* designed experiments to test possible interactions of resistance mechanisms and their impact on fitness costs.

CHAPTER 6:

METABOLIC RESISTANCE IN ITALIAN POPULATIONS

1. INTRODUCTION

In *M. persicae*, esterases and cythocrome P450s are the main classes of enzymes involved in the detoxification of insecticides used against this pest. Both these two phase I enzymatic systems are capable of acting directly on the intact insecticide molecules and the enhanced production of these enzymes results in the metabolism or sequestration of the insecticide before it reaches the target protein (Devonshire *et al*. 1998; Bass *et al*. 2014).

Enhanced levels of the resistance-associated esterase (E4 or FE4) confers a broad-spectrum of resistance against organophosphates, carbamates and pyrethroids and results from gene amplification (Needham & Sawicki 1971; Devonshire & Moores 1982; Field *et al*. 1988). Different approaches exist to demonstrate the increased production of esterases. Total esterase activity of aphid homogenate can be easily measured by biochemical tests using model substrates such as 1-naphthyl acetate (1-NA) or 1-naphthylbutyrate (1-NB) (Devonshire *et al.* 1992). The same substrates can also be used for staining a native polyacrylamide gel following electrophoresis in order to detect the esterase banding pattern (Devonshire & Moores 1982). Also, "*in vivo*" dose-response bioassays with insecticide products, used alone or in combination with esterase-inhibitors, represent an indirect method to determine the involvement of esterases in resistant populations. If insecticide toxicity increases in the presence of the inhibitor, esterases are being blocked by the action of the inhibitor and are no longer available to sequester or detoxify insecticide molecules.

Increased production of one single P450 enzyme (CYP6CY3) has been correlated to high levels of resistance to neonicotinoid (Puinean *et al*. 2010). Measurement of P450 activity is difficult due to the large number of enzymes with a very high substrate specificity. Although biochemical assays have been successfully used in

many insect species to measure monooxygenase activity, e.g. O-deethylation activity with the model substrate 7-ethoxycoumarin, they cannot be used directly with *M. persicae*: no appreciable activity has been found in aphid microsomal preparations, possibly due to high levels of oxidase inhibitors present in the homogenates (Philippou *et al*. 2009). Since monooxygenase-inhibitors are available, monooxygenases studies can alternatively be performed with "*in vivo*" insecticide bioassays, that can indirectly suggest the involvement of P450s enzymes in resistance.

Piperonyl butoxide (PBO) is a well-known monooxygenase-inhibitor (Casida 1970; Wilkinson 1984), often used in bioassays for monooxygenases studies. It has been reported that it can also inhibit esterase activity (Gunning *et al*. 1998; Young *et al*. 2005). The ability of PBO to inhibit both major metabolic resistance enzymes makes it an ideal inhibitor to provide evidences of metabolic mechanisms in resistant populations, but cannot characterise between esterase or P450 involvement.

In addition, some analogues of PBO derived from modifications in the molecule structure are now available. In *M. persicae*, the analogue EN 16/5-1 used in combination with technical α-cypermethrin showed a specific ability to inhibit esterase activity, with a greatly reduced activity against microsomal oxidases (Moores *et al*. 2009). Another analogue EN 1-126 has been investigated recently against a clone of *M. persicae* exhibiting 90-fold resistance to imidacloprid. The compound showed the capacity to greatly reduce the resistant factors of the resistant population in comparison to a susceptible one and thus it can be very effective against monooxygenases (Moores & Philippou 2014).

This thesis aims to investigate the involvement of esterase and monooxygenase enzymes in Italian populations of *M. persicae*. A combination of "*in vitro*" biochemical assays and "*in vivo*" dose-response bioassays with neonicotinoid and pyrethroid products in the presence of PBO and PBO analogues will enable the characterisation of these metabolic resistance mechanisms in some recently collected populations.

2. MATERIALS AND METHODS

2.1 APHID SAMPLES

M. persicae populations collected between 1999 to 2013 from different areas of Italy were maintained as parthenogenetic lineages in controlled environmental conditions as described previously (see chapter 3, section 1.1).

2.2 INSECTICIDE BIOASSAYS

Susceptibility towards the pyrethroid bifenthrin (Brigata Flo; SIPCAM, Pero, Milan, Italy) and the neonicotinoid imidacloprid (Confidor, 200 SL; Bayer CropScience S.r.l., Milan, Italy) was investigated by a dip-test bioassay as described previously (see chapter 3, section 2). Susceptibility toward bifenthrin or imidacloprid in combination with the synergist piperonyl butoxide (PBO) (PBO 80 EC; Endura, Bologna, Italy) and PBO analogues EN 16/5-1 and EN 1-126 was evaluated with the same procedures.

2.3 DETERMINATION OF TOTAL ESTERASE ACTIVITY

M. persicae clonal populations available in our rearing facility were screened for total esterase activity using a colorimetric assay as described by Devonshire (1977) and adapted for microplates by Devonshire *et al*. (1992). Single aphids were homogenized on ice in 100 µL of 0.02 M phosphate buffer pH 7.0 containing 0.1% Triton X-100 and centrifuged at 4 ˚C at 10000 x g for 10 min. The supernatant was used as an enzyme source and 20 µL were transferred to single wells of a 96-well microplate containing 30 µL of buffer/Triton. The reaction was started with the addition of 150 µL/well of 0.3 mM 1-naphthyl acetate (1-NA) previously diluted in buffer/ Triton from an acetone stock (30 mM). Diazo blue lauryl sulphate reagent (DBLS) was prepared by dissolving 0.015 g of Fast Blue B salt (tetra-azotized o-

dianisidine/$ZnCl_2$) in 5 mL of 3.5% SDS (sodium dodecyl sulphate). After 5 min of incubation at 25 ˚C, 25 µL of DBLS were added to each well and the microplate left in the dark for 20 min. Esterase activity was assayed by measuring the rate of hydrolysis of 1-NA to 1-naphthol (1-NP) and acetic acid. DBLS and 1-naphthol create a complex that can be detected at 620 nm. Three different measurments were done for each aphid and at least three aphids were assayed for each populations; incubations without enzyme were used as negative controls. Measurements were performed in a Genios pro microplate reader (Tecan, Mannedorf, Switzerland) taking endpoint readings at 620 nm.

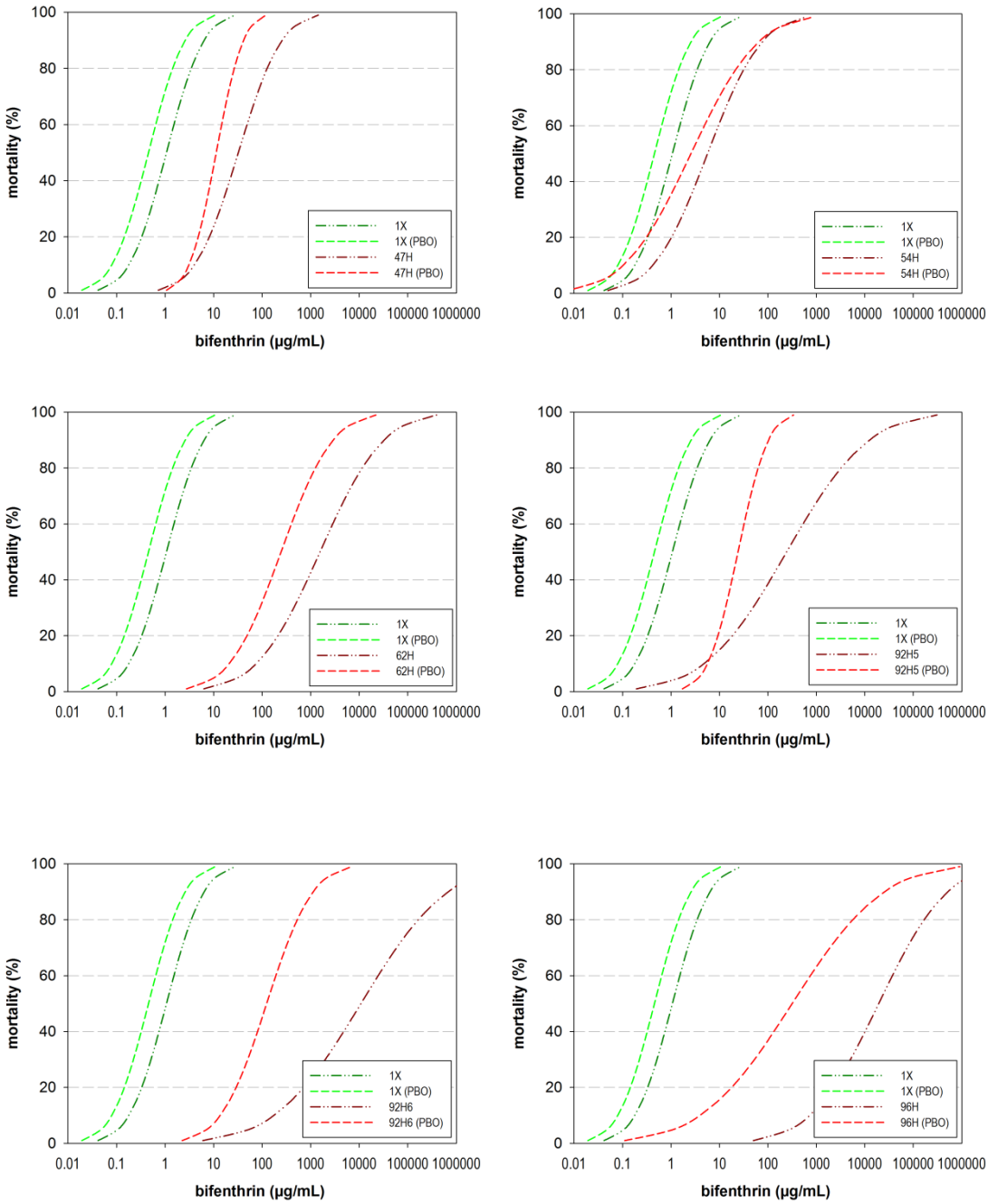
Absorbance values (OD_{620}) were converted to 1-naphthol per protein unit (nmoles mg^{-1}) using a 1-naphthol standard curve. Dilutions of 1-NP were prepared in acetone, from 25 mM to 0.5 mM stock solutions and then diluted 1:100 in buffer to obtain final concentrations ranging from 0.25 mM to $5X10^{-3}$ mM with 1% acetone. Measurements were performed in a Tecan Genios pro reader taking endpoint readings at 620 nm. Total protein content was measured with the Bradford assay (Biorad Protein Assay Kit), according to the manufacturer's instructions.

3. RESULTS

3.1 INDIRECT DETERMINATION OF METABOLIC RESISTANCE IN BIOASSAYS

Inhibition studies with PBO were performed on selected populations available in our rearing facility. Some had been collected recently (164H and 175H2 in 2013; 96H in 2011; 92H5 and 92H6 in 2010) whilst others had been reared long-term (47H, 54H and 62H from 1999). In addition, a fully susceptible strain (1X) was included. These populations were reared in large numbers to obtain sufficient aphids to set up dose-response bioassays. Probit analysis was used to estimate bifenthrin and imidacloprid baselines for the reference clone 1X and populations mentioned above; LC_{50}s, resistance factors (RFs) (LC_{50} for resistant clone / LC_{50} for

susceptible clone) and synergistic factors (SFs) (LC_{50} insecticide / LC_{50} insecticide + synergist) were calculated (Fig. 6.1 and 6.2). The use of PBO combined with insecticide revealed a synergistic action in all analysed clones, suggesting the involvement of metabolic resistance mechanisms. The highest synergistic factors were found in bioassays with bifenthrin for two clones: 92H6 (SF: 87.6) and 96H (SF: 61.1) (Table 6.1 and 6.2).



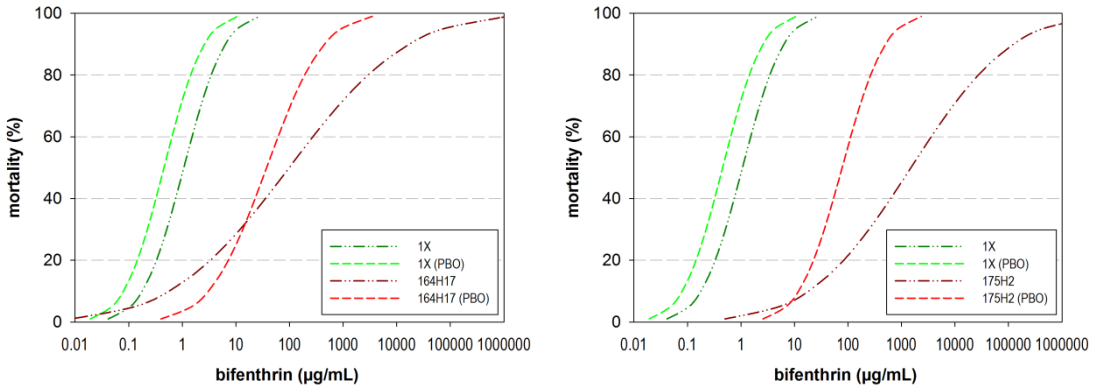


Figure 6.1: Bifenthrin and bifenthrin + PBO baselines for clones 47H, 54H, 62H, 92H5, 92H6, 96H, 164H17 and 175H2. The susceptible clone 1X has been included as reference.

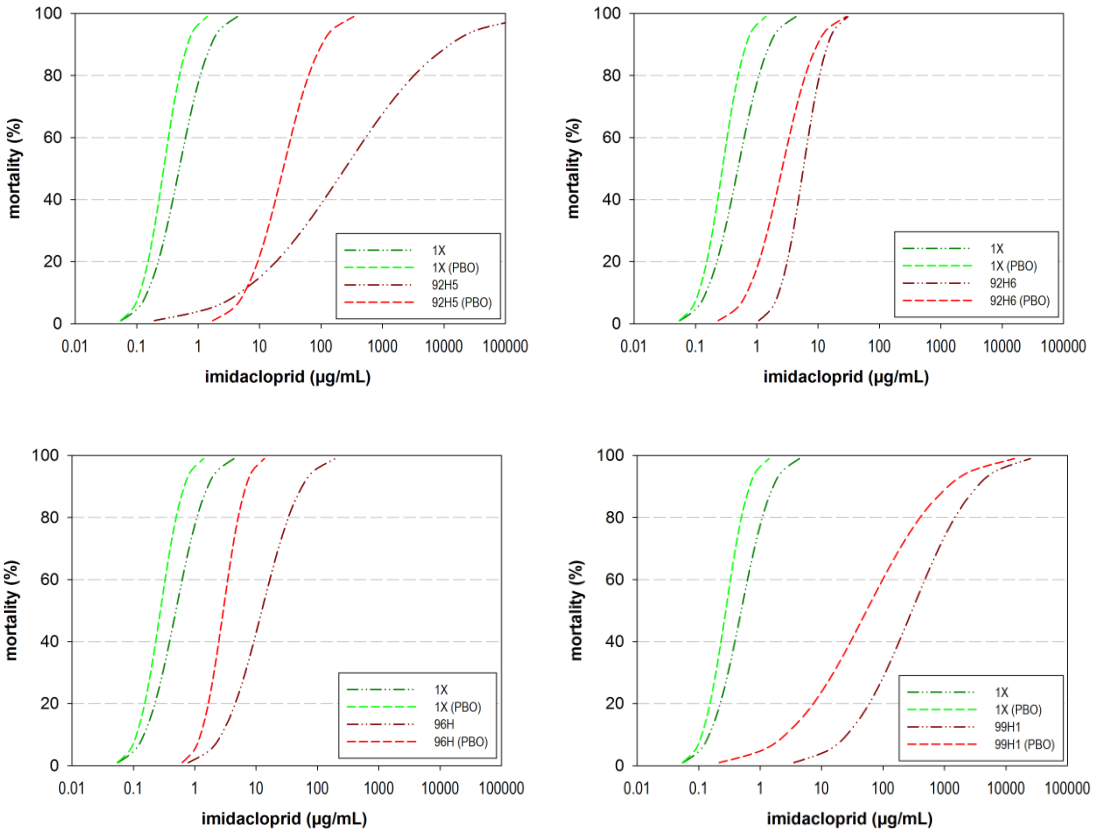


Figure 6.2: Imidacloprid and imidacloprid + PBO baselines for clones 92H5, 92H6, 96H, 99H1. The susceptible clone 1X has been included as reference.

Clone 92H6 was selected for further bioassays with an analogue of PBO, EN 1-126, in combination with both bifenthrin and imidacloprid (Fig. 6.3) and the calculated synergistic factors were compared to those obtained for PBO. They were very similar in bioassays with imidacloprid (2.2 for PBO and 2.9 for EN 1-126) whilst a difference was observed in bioassays with bifenthrin (87.6 for PBO and 29.4 for 1-126) (Table 6.3).

A further set of bioassays were performed using clone 62H and bifenthrin only, but incorporating two PBO analogues (EN 1-126 and EN 16/5-1) (Fig. 6.3). In this case, the synergistic factor for EN 1-126 was slightly higher than PBO (6.6 and 6.3), whilst the third analogue, EN 16/5-1, more than 3X lower (1.9) (Table 6.3).

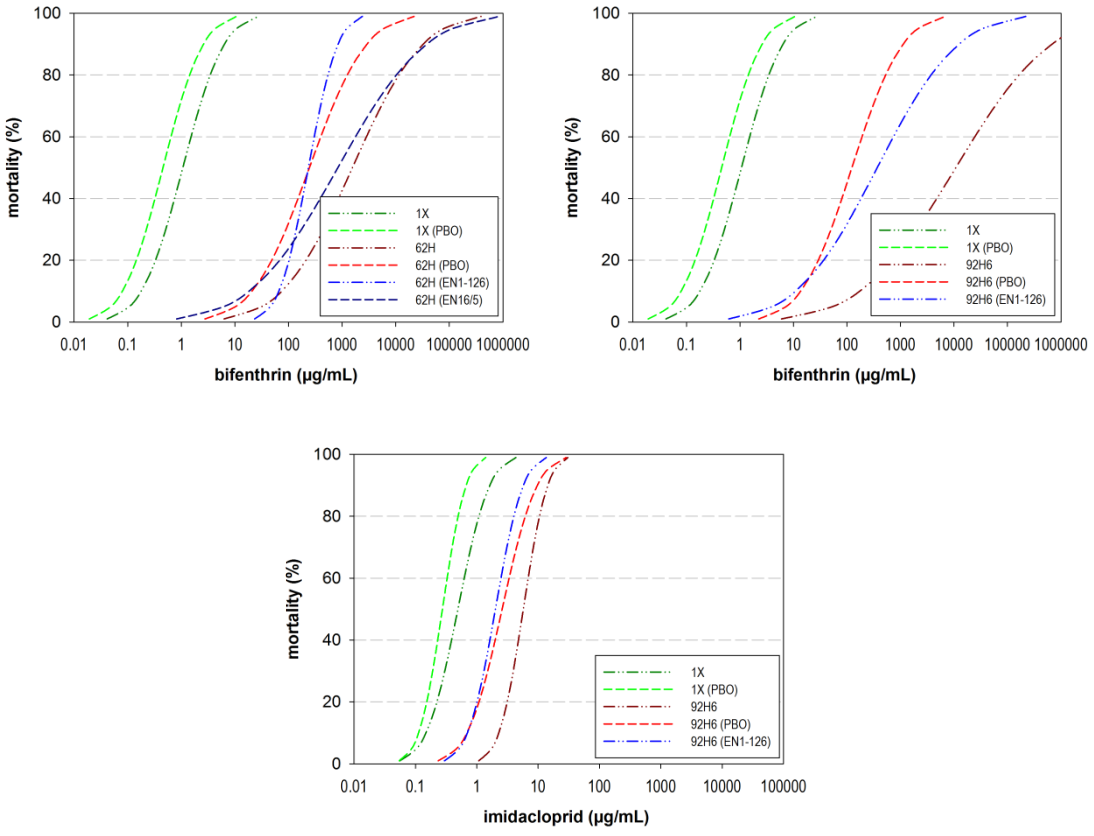


Figure 6.3: Bifenthrin and bifenthrin + PBO, EN 1-126 or EN 16/5-1 baselines for clone 62H. Bifenthrin and bifenthrin + PBO or EN 1-126 baselines for clone 92H6. Imidacloprid and imidacloprid + PBO and EN 1-126 baselines for clone 92H6. The susceptible clone 1X has been included as reference.

Treatment	Clone	LC_{50} (mg mL^{-1})	CI 95		slope	d.f.	χ2	R.F.	S.F.
bifentrin	1X *	1.07	0.56	1.67	1.65 ± 0.31	8	6.2		
bifentrin + PBO	1X *	0.46	0.22	0.72	1.69 ± 0.34	8	6.8		2.3
bifentrin	47H	32.3	18.5	52.3	1.41 ± 0.21	18	21.6	30.2	
bifentrin + PBO	47H	11.3	8.25	14.6	2.27 ± 0.29	30	45.1	24.9	2.8
bifentrin	54H	5.69	0.98	16.6	1.13 ± 0.16	18	65.7	5.3	
bifentrin + PBO	54H	2.59	0.49	8.12	0.91 ± 0.09	24	130	5.7	2.2
bifentrin	62H	1563	898	3300	0.97 ± 0.13	32	57.2	1463	
bifentrin + PBO	62H	246	75.7	517	1.19 ± 0.14	25	105	542	6.3
bifentrin	92H5	245	77.8	1734	0.75 ± 0.10	20	50.7	229	
bifentrin + PBO	92H5	24.2	13.8	39.5	2.03 ± 0.44	13	8.3	53.1	10.1
bifentrin	92H6 *	10900	-	-	0.71 ± 0.24	28	36.4	10181	
bifentrin + PBO	92H6 *	124.1	70.2	200	1.34 ± 0.18	28	41.1	273	87.6
bifentrin	96H	19463	-	-	0.90 ± 0.53	18	11.3	18223	
bifentrin + PBO	96H	319	57.9	1224	0.67 ± 0.11	18	57.6	700	61.1
bifentrin	164H17	98.8	2.23	367.2	0.57 ± 0.10	13	40.7	92.5	
bifentrin + PBO	164H17	37.4	13.9	78.3	1.18 ± 0.12	13	30.3	82.2	2.6
bifentrin	175H2 *	1534	383	10763	0.67 ± 0.11	18	65.8	1436	
bifentrin + PBO	175H2 *	77.8	40.8	122	1.57 ± 0.20	18	20.2	171	19.7

Table 6.1: Results obtained from probit analysis: bifenthrin ± PBO. LC_{50}: lethal concentration that is expected to cause 50% mortality; CI 95%: confidence interval limits at 95%; d.f.: degree of freedom; R.F.: resistance factor (calculated as the ratio between LC_{50} of the resistant clone and LC_{50} of the susceptible clone 1X; S.F.: synergism factor (calculated as the ratio between LC_{50} without PBO and LC_{50} with PBO).*data reported in chapter 5 section 3.3).

Treatment	Clone	LC_{50} (mg mL^{-1})	CI 95%		slope	d.f.	χ2	R.F.	S.F.
imidacloprid	1X *	0.49	0.35	0.64	2.46 ± 0.21	39	93.6		
imidacloprid + PBO	1X *	0.27	0.19	0.35	3.30 ± 0.46	13	18.1		1.8
imidacloprid	92H5	3.24	2.46	4.16	1.87 ± 0.20	31	47.6	6.6	
imidacloprid + PBO	92H5	2.22	1.02	3.77	2.59 ± 0.41	9	31.5	8.2	1.5
imidacloprid	92H6 *	5.72	3.55	7.73	3.20 ± 0.47	45	91.7	11.7	
imidacloprid + PBO	92H6 *	2.58	1.52	3.63	2.23 ± 0.35	45	94.1	9.6	2.2
imidacloprid	96H	12.4	-	-	1.94 ± 0.87	17	51.9	25.2	
imidacloprid + PBO	96H	2.90	-	-	3.47 ± 0.81	8	18.6	10.8	4.3
imidacloprid	99H1 *	297	-	-	1.21 ± 0.32	21	66.2	606	
imidacloprid + PBO	99H1 *	54	7.70	243.20	0.97 ± 0.21	13	28.7	200	5.5

Table 6.2: Results obtained from probit analysis: imidacloprid ± PBO. LC_{50}: lethal concentration that is expected to cause 50% mortality; CI 95%: confidence interval limits at 95%; d.f.: degree of freedom; R.F.: resistance factor (calculated as the ratio between LC_{50} of the resistant clone and LC_{50} of the susceptible clone 1X; S.F.: synergism factor (calculated as the ratio between LC_{50} without PBO and LC_{50} with PBO). *data reported in chapter 4 section 3.1).

Treatment	Clone	LC_{50} (mg mL^{-1})	CI 95%		slope	d.f.	χ2	R.F.	S.F.
bifenthrin	92H6	10874	70.2		0.71 ± 0.24	28	36.4	10181	
bifenthrin + PBO	92H6	124	174	200	1.34 ± 0.18	28	41.1	273	87.6
bifenthrin + 1-126	92H6	370		836	0.84 ± 0.1	15	45.7		29.4
imidacloprid	92H6	5.72	3.55	7.73	3.20 ± 0.47	45	91.7	11.7	
imidacloprid + PBO	92H6	2.58	1.52	3.63	2.23 ± 0.35	45	94.1	9.6	2.2
imidacloprid + 1-126	92H6	2.01	0.77	2.99	2.81 ± 0.39	13	49.6		2.9
bifenthrin	62H	1563	898	3300	0.97 ± 0.13	32	57.2	1464	
bifenthrin + PBO	62H	246	75.73	517	1.19 ±0.14	25	105.4	542	6.3
bifenthrin + 1-126	62H	236			2.31 ± 0.6	9	18.8		6.6
bifenthrin + 16/5-1	62H	837	474	1736	0.77 ± 0.1	16	23.6		1.9

Table 6.3: Results obtained from probit analysis: bifenthrin or imidacloprid (± PBO or EN 1-126 or EN 16/5-1). LC_{50}: lethal concentration that is expected to cause 50% mortality; CI 95%: confidence interval limits at 95%; d.f.: degree of freedom; R.F.: resistance factor (calculated as the ratio between LC_{50} of the resistant clone and LC_{50} of the susceptible clone 1X; S.F.: synergism factor (calculated as the ratio between LC_{50} without PBO (or EN 1-126 or EN 16/5-1) and LC_{50} with PBO (or EN 1-126 or EN 16/5-1)).

3.2 BIOCHEMICAL EVALUATION OF ESTERASE-BASED RESISTANCE

Total esterase activity was measured in several populations available in the rearing facility, some of which were reared long-term and others recently collected (Table 6.4). The susceptible clone 1X was used as reference. Esterase activity calculated for this clone was compared with another susceptible clone (33H) provided by Rothamsted Research and used as susceptible reference in the literature. The two clones showed similar values (data not shown).

Sample (code)	Site (province)	Collection (year)	Host	Esterase activity (nmoles μg^{-1} min^{-1})
1X *	PI	1995	peach	0.130 ± 0.004
4 *	RA	1998	peach	0.551 ± 0.032
6 *	PC	1998	peach	0.527 ± 0.035
10 *	PC	1997	peach	0.385 ± 0.027
11 *	FE	1998	peach	0.451 ± 0.041
12 *	FE	1997	peach	0.753 ± 0.083
14 *	CN	1997	peach	0.603 ± 0.016
15 *	RA	1997	peach	0.585 ± 0.045
16 *	PC	1997	peach	0.466 ± 0.032
17 *	PC	1996	tomato	0.360 ± 0.020
18H	RA	1998	peach	0.809 ± 0.016
19 *	PC	1996	tomato	0.113 ± 0.004
22 *	CS	1997	peach	0.580 ± 0.030
23 *	CZ	1998	potato	0.114 ± 0.006
24 *	BO	1998	peach	0.397 ± 0.029
26 *	PI	1996	peach	0.110 ± 0.002
27H	RA	1998	peach	0.484 ± 0.038
39 *	FC	1999	peach	0.344 ± 0.006
43 *	RA	1999	peach	0.411 ± 0.007
44 *	RA	1999	peach	0.535 ± 0.066
46 *	TO	1999	peach	0.605 ± 0.035
48 *	BN	1999	tobacco	0.121 ± 0.005
49 *	SA	1999	tobacco	0.127 ± 0.006

Table 6.4: Total esterase activities (nmoles μg^{-1} min^{-1}) of* M. persicae *populations (*: data published in Rivi* et al*. 2013).

Sample (code)	Site (province)	Collection (year)	Host	Esterase activity (nmoles μg^{-1} min^{-1})
50 *	SA	1999	tobacco	0.121 ± 0.003
51 *	SA	1999	tobacco	0.113 ± 0.006
52 *	CH	1999	tobacco	0.123 ± 0.007
53 *	TO	1999	peach	0.504 ± 0.013
54 *	PD	1999	peach	0.462 ± 0.062
57 *	PE	1999	tobacco	0.130 ± 0.002
58	AP	1999	peach	0.434 ± 0.009
62H *	RA	1999	peach	0.453 ± 0.032
63 *	LO	1999	peach	0.519 ± 0.009
64 *	CH	1999	tobacco	0.128 ± 0.002
65 *	CH	1999	tobacco	0.130 ± 0.002
66 *	CH	1999	tobacco	0.128 ± 0.003
67H	BO	2000	peach	0.484 ± 0.033
69 *	PC	2000	aubergine	0.673 ± 0.038
70 *	CS	2000	peach	0.510 ± 0.015
92H5 *	FC	2010	peach	0.478 ± 0.014
92H6	FC	2010	peach	0.376 ± 0.032
96H	BO	2011	peach	0.378 ± 0.001
97	BO	2011	peach	0.445 ± 0.030
99H1	RA	2011	peach	0.339 ± 0.021
114	CH	2012	peach	0.387 ± 0.006
125	FC	2012	peach	0.215 ± 0.002
149	RA	2012	peach	0.397 ± 0.004
152	FC	2012	eggplant	0.376 ± 0.028
164H17	FG	2013	peach	0.419 ± 0.020
175H2	CH	2013	peach	0.319 ± 0.010
188	RA	2014	peach	0.416 ± 0.007

Table 6.4: cont.

The mean value of the esterase activities obtained for clones collected from 1996 to 2000 (0.381 nmoles μg^{-1} min^{-1}) is very close to the mean value of esterase activities calculated for the recent clones sampled from 2010 to 2014 (0.379 nmoles μg^{-1} min^{-1}). In general, esterase activities of the older clones present a higher variability, with a few strains reaching very high values (0.67 nmoles μg^{-1} min^{-1} for clone 69H and

0.81 nmoles μg^{-1} min^{-1} for clone 18H) and others very close to the susceptible strain (e.g. 0.113 μg^{-1} min^{-1} for clone 19 and 0.121 μg^{-1} min^{-1} for 48). Also, populations collected from secondary hosts have, in general, lower esterase activity (0.183 μg^{-1} min^{-1}) to those derived from peach (0.484 μg^{-1} min^{-1}) (Fig. 6.4).

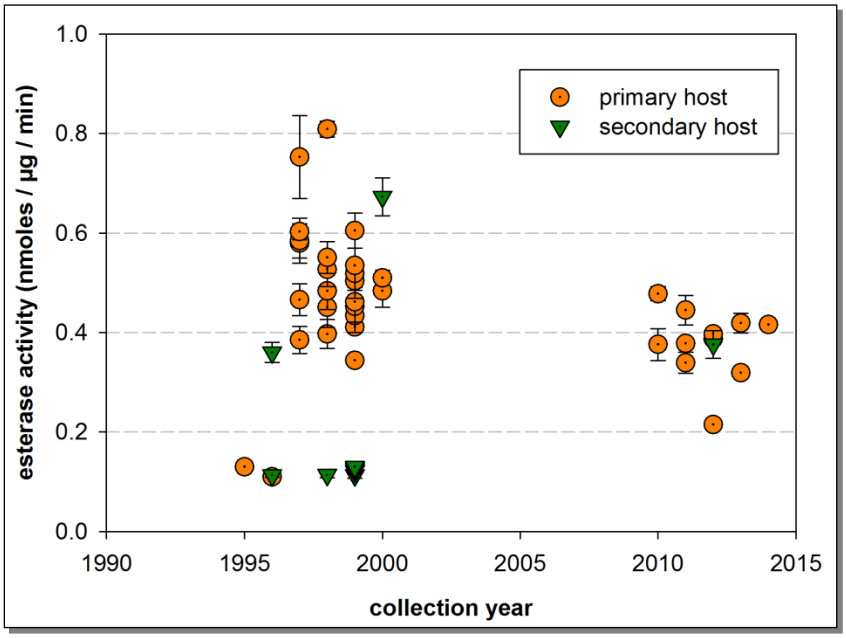


Figure 6.4: Esterase activities: comparison between "old" and "recent" populations, collected from peach (in orange) or other herbaceous hosts (in green).

Some of the long-termed reared populations used in this study were screened previously by Mazzoni & Cravedi (2002) and Criniti *et al*. (2008) and classified as susceptible (S) or resistant (R1, R2 or R3) according to absorbance ranges described by Devonshire *et al*. (1992). Comparing those results with the esterase activities here reported, it is possible to observe that populations showing activity values lower than 0.2 nmoles μg^{-1} min^{-1} (Fig. 6.4) were classified by the other authors as susceptible (S) or intermediate between susceptible and low resistant (S/R1). It could be then suggested 0.2 nmoles μg^{-1} min^{-1} as possible discriminant esterase activity value to identify resistant clones.

4. DISCUSSION

In this thesis the contribution of metabolic resistance in Italian populations of the green peach aphid has been considered. In order to detect the involvement of both/either major enzymatic classes, esterases and monooxygenases, inhibition studies were performed with "*in vivo*" bioassays using the well-known inhibitor PBO, in combination with pyrethroid or neonicotinoid insecticides. Recent (collected from 2010 to 2013) and old (1996 - 2000) populations, maintained under controlled conditions as parthenogenetic clones, were included in the investigation.

Piperonyl butoxide acted as a synergist in all the analysed clones and for both the tested products, bifenthrin and imidacloprid. The calculated synergistic factors were different among the considered clones which suggests a different involvement of enzymatic systems for the detoxification of the insecticide molecules. The contribution of each enzymatic class cannot be discriminated by these bioassays, since it has been reported that PBO has effects not only on microsome oxidases but also on esterases.

Additional bioassays were then performed with different PBO analogues, EN 1-126 (reportedly very active against P450s conferring resistance to imidacloprid) and EN 16/5-1 (reportedly more specific against esterases). Clone 62H was the only clone tested with all three inhibitors. The lowest synergistic factor was obtained for EN 16/5-1 (SF 1.9), suggesting that esterases are responsible for less metabolic resistance than monooxygenases in the instance of bifenthrin detoxification.

Clone 92H6 was tested with PBO and EN 1-126 only, but using both bifenthrin and imidacloprid. In this case, PBO showed a considerably higher synergism with bifenthrin than imidacloprid; in contrast, EN 1-126 performed slightly better than PBO in imidacloprid bioassays, agreeing with published data relating to bioassays with imidacloprid, in which EN 1-126 was the more effective analogue tested against a strain highly resistant to this insecticide (Philippou & Moores 2014). This suggests that the two different clones respond in different ways when treated with the same products. Looking at the high resistant factors showed by clone 92H6, it could be hypothesized that different monooxygenases are involved, for which PBO

has a higher affinity than 1-126. Another possible explanation is the involvement of further mechanisms, like cuticular changes, that can alter the permeability of the inhibitor and thus influencing its efficacy. Such alternative mechanism may act differentially on PBO and EN 1-126.

In order to specifically detect esterase activities, a colorimetric assay was performed. The biochemical assay adopted allowed the rapid detection of enzymatic activities using aphid homogenate as a source of enzymes. Since there have been changes in the control strategies adopted against this pest during the years, a comparison between "old" and "recent" populations was performed. Esterases are enzymes able to act against carbamate, organophosphate and pyrethroid insecticides (Hollingworth & Dong 2008). The first two classes of products, widely used in the past, have been almost completely phased out recently in order to reduce exposure risks and environmental contamination problems. For this reason, the hypothesis of a reduction in the esterase-based resistant mechanisms is suggested. However, populations more recently collected (without exposure to OP or carbamate products) showed esterase activities similar to those collected in the past. Literature data report that FE4 variant esterases can act against pyrethroids and also, in a lesser extent, against neonicotinoid imidacloprid (Philippou & Moores 2010). The high esterase activities observed in recent populations suggest that the maintainance of this detoxifying mechanism is important and could be involved in resistance against pyrethroids and possibly also neonicotinoids.

Unfortunately, the parallel biochemical assay for P450 activity cannot be used with *M. persicae*, and it was not possible to quantify their specific contribution as detoxification system. Although the specific P450 enzyme responsible of neonicotinoid resistant in *M. persicae* has been characterised (CYP6CY3), the quantification of the gene copy numbers of the considered populations was not performed during this research. Furthermore, it is not possible to exclude the involvement of other P450s in different populations.

Although a combination of *"in vivo"* synergist bioassays can provide important information, they are very time consuming and require high numbers of live aphids. This is the main reason for which, in the present study, only a few populations were analysed with this method and not all the combination of bioassays have been performed. Further bioassays on different aphid strains could provide important data for a better description of this phenomenon. However, the development of a biochemical assay able to detect monooxygenase activity will be very important for a better and more rapid quantification of metabolic resistance in field collected samples.

CHAPTER 7

METABOLIC RESISTANCE: IDENTIFICATION OF NOVEL SYNERGISTS

NOVEL SYNERGISTS: CHARACTERISATION OF INTERACTIONS WITH DETOXIFYING ENZYMES FE4 AND CYP6CY3.

EU project "EcoSyn: ecofriendly synergists for insecticide formulations".

7th Framework Programme for research, technological development and demonstration.

Research for the Benefit of SMEs; grant agreement no 605740 (October 2013 - October 2015)

1. INTRODUCTION

Synergists are non-toxic compounds that act to increase the effectiveness of the insecticides they are used with. Their combination can enable a reduction in the amount of the insecticidal active applied in control strategies. They inhibit the metabolic system(s) that insects use to metabolise or sequester insecticide molecules; as a result, insect sensitivity increases and it is possible to overcome or delay the metabolic resistance conferred by the enhanced level of detoxifying enzymes (Ishaaya 1993).

The proposed project "EcoSyn" aims to develop novel "ecofriendly" synergists for insecticide formulations and deployment strategies, in order to enable insect pest control with lower insecticide rates, without adverse effect on non-target insects, such as bees, or the environment (http://www.ecosyn.eu). Piperonyl butoxide (PBO) is a well-known synergist widely used in the household market and also in a niche market in agriculture. Many studies in literature document its interaction with phase 1 metabolic enzymes, initially only cytochrome P450s (Casida 1970; Wilkinson

1984; Hodgson *et al*. 1998) and subsequently also esterases (Gunning *et al*. 1998; Young *et al*. 2005). Starting from the current knowledge concerning the biochemical mechanism of PBO, a range of existing analogues will be explored and evaluated for their inhibition efficacy against detoxifying enzymes of a variety of insect targets. Based on experimental laboratory and field results, novel analogues will be developed and tested for their potential synergistic activity, with particular evaluation of possible effects on beneficial insects and the cost-effectiveness for a wider use in agriculture and public health.

M. persicae is one of the target insects that have been included in the project. It has been selected because it is one of the most economically important crop pests worldwide, with a rapid population growth due to its high fecundity and short generation time. Literature data are currently available on the interaction of PBO with the resistance-associated esterase E4 (Philippou *et al*. 2013) but no studies have been made on the Mediterranean variant FE4. Furthermore, it has recently been demonstrated that a single cytochrome P450 enzyme (CYP6CY3) is overexpressed in a resistant strain of this aphid (Puinean *et al*. 2010), but no research with the recombinant enzyme have been reported. This work aims to investigate the interactions between these detoxifying enzymes and PBO or other existing PBO analogues; experimental results derived from inhibition efficacies and structure activity relationships will help to design novel structures with hypothetical higher synergisms and thus possible agricultural applications.

2. MATERIALS AND METHODS

2.1 ESTERASE FE4 PURIFICATION

Esterase FE4 was purified from aphid samples using size exclusion and ion exchange chromatography, as described by Philippou *et al*. (2013). Aphids (1 g), stored at -20 ˚C, were homogenised on ice in 20 mL of 0.02 M phosphate buffer pH 7.0 and

centrifuged at 10000 x g for 5 min. The supernatant was collected, filtered through cheesecloth and passed through a Sephadex G-25 column (Fine; Amersham Biosciences), diameter 3 cm x height 12 cm, to separate low-molecular-weight material. Proteins were eluted using 0.02 M Tris/HCl buffer pH 8.5 and then loaded onto an ion exchange column, DEAE Sepharose TM (Fast Flow; Amersham Biosciences), diameter 2.5 cm x height 8 cm. Fractions (5 mL) were eluted and collected using a linear 0-0.35 NaCl gradient in 500 mL of Tris/HCl buffer.

Fractions were tested for total esterase activity using the colorimetric assay as described by Grant *et al*. (1989) and modified by Philippou *et al*. (2013). Briefly, 20 µL of each fraction were transferred into a single well of a 96-well microplate (Nunc, maxisorb) and mixed with 200 µL of substrate solution (1-NA and FBRR). The substrate solution was prepared by adding 250 µL of the model substrate 1-naphthyl acetate (1-NA) (stock 30 mM in acetone) to 25 mL of Fast Blue RR solution (FBRR) (30 mg dissolved in 50 mL of 0.02 M phosphate buffer pH 7.0), to give final concentrations of 0.3 mM and 1.5 mM respectively.

Esterase activity was assayed by measuring the rate of hydrolysis of 1-NA to 1-naphthol (1-NP) and acetic acid. FBRR and 1-NA create a complex that can be detected at 450 nm. The assay was performed in a Tmax kinetic microplate reader (Molecular Devices, Corporation; Menlo Park, CA) taking readings every 20 s at 450 nm for 5 min. The integrated software programme Softmax Pro version 5.4 was used to fit linear regressions. The slopes were recorded as a rate of milli optical density change per minute at 450 nm (mOD_{450} min^{-1}).

Fractions with the highest activities were pooled together and passed again through a Sephadex G-25 column (Fine; Amersham Biosciences) to desalt and exchange to 0.02 M phosphate buffer pH 7.0 for storage. Aliquots were frozen at -20 °C.

2.2 ESTERASE INTERFERENCE ASSAY: INDIRECT DETECTION OF FE4 INHIBITION

The interference assay is an indirect method to measure the binding affinity of an inhibitor to purified esterases. Aliquots of the enzyme (500 µL) were pre-incubated

at 4 ˚C in Eppendorf tubes with the inhibitor (5 µL) prepared in acetone (10 mM stock solution); enzyme with acetone only was used as control. After 16 h of incubation, the esterase interference assay was carried out as described by Khot *et al*. (2008), using a 96-well microtitre plate (Nunc, maxisorb). Briefly, 25 µL of enzyme (or enzyme + inhibitor) were incubated for 1 h with 25 µL of two-fold serial dilutions of Azamethiphos (Aza) (stock 10^{-6} M in acetone) in 0.02 M phosphate buffer pH 7.0 (to give final concentrations ranging from 20 nM to 0.01 nM). Buffer only was used as control.

At the same time, *M. domestica* head homogenate was prepared. 10 frozen housefly heads were homogenised in 1 mL of 0.02 M phosphate buffer pH 7.0, centrifuged at 10000 x g for 1 min and the supernatant used as a source of acetylcholinesterase (AChE): 25 µL were added to every well and incubated for 10 min at room temperature. Acetylcholinesterase activity was measured using a colorimetric assay (Ellman *et al*., 1961) according to Moores *et al*. (1996): 100 µL of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 100 µL of the model substrate acetylthiocholine iodide (ATChI) were added to every well (final concentrations 0.5 mM). The release of thiocholine was detected at 405 nm through its reaction with DTNB.

A kinetic assay was performed using a Tmax kinetic microplate spectrophotometer, taking readings every 10 s for 10 min. Acetylcholinesterase only and buffer only were used as positive and negative controls respectively and each assay was repeated in triplicate. The integrated software programme Softmax Pro version 5.4 was used to fit the linear regressions. The slopes were recorded as a rate of milli optical density change per minute at 405 nm (mOD_{405} min^{-1}).

In the absence of FE4, Aza inhibits AChE activity; in the presence of the esterases, the insecticide is sequestered by them and Aza is unable to acts against AChE. If an inhibitor is present, it binds the esterases and they became unable to sequester the insecticide, so it remains available to inhibit AChE activity. Acetylcholinesterase activity remaining was calculated by dividing the activity measured in the presence of the inhibitor by the activity in the absence of inhibitor (uninhibited control) and

multiplying by 100. Remaining activities (%) was then plotted against inhibitor concentrations. Indirect determination of FE4 inhibition was analysed by calculating the Aza concentrations required to inhibit 50% of AChE activity (IC_{50}), using Grafit software (Leatherbarrow, R.J., Version 5.0.10, Erithacus Software Limited).

2.3 DETECTION OF FE4 INHIBITION: 1-NA AND 1-NB ASSAY

Inhibition of FE4 esterase activity was tested using the conventional esterase assay previously described (see section 2.1) using the model substrate 1-naphthyl acetate (1-NA) and a similar substrate 1-naphthyl butyrate (1-NB). Both the substrates were prepared adding 250 µL of the stock 30 mM in acetone to 25 mL of FBRR solution. Two-fold serial dilutions of the inhibitor were prepared in acetone, starting from a 10 mM stock solution and then added to 0.02 M phosphate buffer pH 7.0 and dispensed into a 96-well microplate (Nunc, maxisorb). Aliquots (10 µL) of the purified enzyme were added to each well and incubated with 2.5 µL of the inhibitors at room temperature for 10 min, followed by the addition of 200 µL of the substrate solution (1-NA or 1-NB and FBRR). Final concentrations of inhibitor ranged from 0.5 mM to $5x10^{-4}$ mM, with 1% acetone per well (total volume per well: 250 µL); enzyme in the presence of 1% acetone was used as control.

The integrated software programme Softmax Pro version 5.4 was used to fit linear regressions. The slopes were recorded as a rate of milli optical density change per minute at 450 nm (mOD_{450} min^{-1}). Activity remaining was calculated by dividing the activity measured in the presence of inhibitor by the activity in the absence of inhibitor (uninhibited control) and multiplying by 100. Remaining activities (%) was then plotted against increasing concentrations of the inhibitor.

2.4 DETECTION OF FE4 INHIBITION: PNA ASSAY

Inhibition of FE4 esterase activity was tested using 4-nitrophenyl acetate (pNA) as an alternative substrate, according to the method described by Pocker & Stone

(1967). A 100 mM pNA stock was prepared in acetone and added to 0.02 M phosphate buffer pH 7.0 (final concentration 2 mM). Different concentrations of the inhibitor were prepared in acetone, diluted in buffer and dispensed into a 96-well microplate (Nunc, maxisorb), as described previously (see section 2.3). Aliquots (10 µL) of the purified enzyme were added to each well and incubated at room temperature for 10 min. 100 µL of 2 mM pNA were then added (final concentration 0.8 mM; total volume per well: 250 µL) and esterase activity was determined by measuring the rate of hydrolysis of pNA to 4-nitrophenol and acetate. Enzyme in the presence of acetone was used as the uninhibited control. The assay was performed in a Tmax kinetic microplate reader, taking readings every 10 s at 405 nm for 5 min.

The integrated software programme Softmax Pro version 5.4 was used to fit the linear regressions. The slopes were recorded as the rate of milli optical density change per minute at 405 nm (mOD_{405} min^{-1}). Activity remaining was calculated by dividing the activity measured in the presence of inhibitor by the activity in the absence of inhibitor (uninhibited control) and multiplying by 100. Remaining activities (%) were plotted against inhibitor concentrations. Incubation of the enzyme with the inhibitor was repeated at different times (0, 5, 10, 30 and 60 min) to confirm the reversibility of the reaction.

2.5 DETECTION OF CYP6CYP3 OXIDASE ACTIVITY: 7-MFC ASSAY

Recombinant P450 CYP6CY3 was provided by Rothamsted Research, Biological Chemistry and Crop Protection (BCCP) department, partner of the "Ecosyn" consortium. Gene constructs encoding for CYP6CY3 (Genbank accession no. AHB52756.1) and its redox partner NADPH cytochrome P450 reductase (CPR) from *M. domestica* (Genbank accession no. Q07994.1) were obtained by gene synthesis (Geneart, CA, USA) optimised for lepidopteran insect cell lines to ensure highest translation efficiency. Constructs were inserted in into the pDEST8 expression vector (Invitrogen); vector with no inserted DNA and vector with CPR only were used as negative controls. The recombinant baculovirus DNA was produced and

transfected into insect cells as already described (Bass *et al*. 2013). Microsomes of the membrane fraction were prepared according to standard procedures and P450 expression and functionality was estimated by measuring CO-difference spectra in reduced samples (Philips & Shephard 2006).

CYPCY3 enzymatic activity was tested for oxidase activity using a fluorometric assay according to Ullrich and Weber (1972) and adapted to the microplate format as described by De Sousa *et al.* (1995), but with a different model substrate: 7-methoxy-4-trifluoromethylcoumarin (7-MFC). Briefly, 7-MFC was dissolved in DMSO to make a 20 mM stock solution and then diluted by the addition of 0.1 M sodium phosphate buffer pH 7.8 to give a concentration of 0.5 mM. Recombinant enzyme (10 µL) was added to a 96-well microplate (OptiPlate^{TM} Perkin Elmer, white) and diluted to 50 µL with 0.1 M phosphate buffer pH 7.6, followed by the addition of 80 µL of 0.5 mM 7-MFC; incubations without enzyme were used as negative controls. The microplate was incubated for 5 min at 30 °C and the reaction was initiated by the addition of 10 µL of 9.6 mM NADPH in 0.1 M sodium phosphate, pH 7.8.
Enzyme activity was read in a Spectramax Gemini EM for 60 min, with readings taken every 2 min, using an excitation wavelength of 410 nm and an emission wavelength of 510 nm, with a 495 nm cut-off filter. O-demethylation activity was measured by the rate of production of 7-hydroxy-4-trifluoromethylcoumarin and expressed as fluorimetric units per minute (FU min^{-1}). The integrated software programme Softmax Pro version 5.4 was used to fit the linear regressions. The enzymatic activity was compared to the control containing CPR only, following the same procedure.

2.6 DETECTION OF CYP6CYP3 INHIBITION

Inhibition of recombinant P450 enzymes was measured following the method already described by Moores *et al*. (2009) but using the alternative substrate 7-MFC. Dilution of the recombinant enzymes (50 µL) were dispensed into a 96-well microplate (OptiPlate^{TM} Perkin Elmer, white), mixed with 3 µL of inhibitor (stock 0.1

M in acetone, final concentration 30 µM) and incubated 10 min at room temperature. Acetone only was used as control. After the incubation, 80 µL of 0.5 mM 7-MFC was added, followed by 10 µL of 9.6 mM NADPH and the reaction was monitored as described above (see section 2.5).

3. RESULTS

3.1 ESTERASE FE4 PRODUCTION

Different *M. persicae* clones were selected from the collection available in our rearing facility and screened for total FE4 esterase activity (see Chapter 6). Among clones that showed high activities compared to the susceptible clone 1X, two of them were selected and multiplied in large number to obtain a sufficient amount of aphids to purify the resistance-associated FE4 esterase (Mp18H and Mp27H). Both these clones have already been verified by PCR-RFLP to have the FE4 esterase variant (Bizzaro *et al*. 2005; Rivi *et al*. 2013).

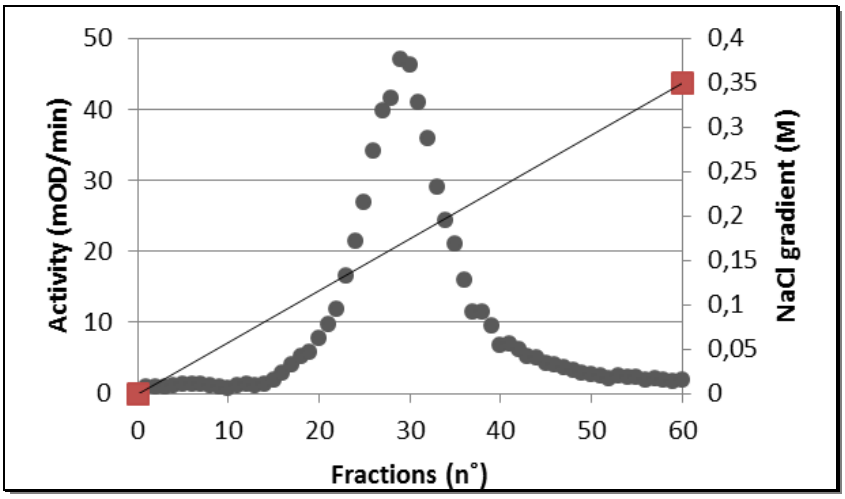


Figure 7.1: Esterase purification profile following size exclusion and ion exchange chromatography. Fractions were eluted using a linear 0-0.35 NaCl gradient and tested for the total esterase activity. Tubes 27-31, showing the highest activities, were pooled together.

From each clone, 1 g of aphids were collected and maintained at -20 °C before homogenisation on ice. After size exclusion and ion exchange chromatography, the eluted fractions were screened for total esterase activity and those with the highest values were pooled together (Fig. 7.1), desalted and stored at -20 °C after a final check on the esterase activity (Fig. 7.2).

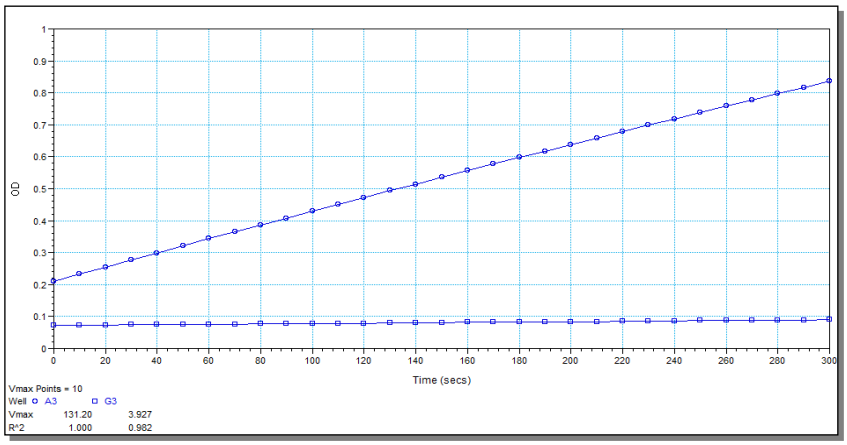


Figure 7.2: Monitoring the hydrolysis of 1-naphtyl acetate (1-NA) by the purified esterase FE4. The slope was recorded as a rate of milli optical density change per minutes at 450 nm (mOD_{450} sec^{-1}).

3.2 INDIRECT DETERMINATION OF FE4 INHIBITION BY PBO OR ITS ANALOGUES

The ability of PBO or its analogues to bind esterase FE4 was tested using the esterase interference assay, an indirect method that measures the sequestration of azamethiphos (Aza), a potent AChE inhibitor. This can be evaluated by calculating the concentration of Aza required to obtain 50% inhibition of AChE activity (IC_{50}). In the absence of FE4, housefly AChE is inhibited by Aza and the IC_{50} is low. In the presence of FE4, Aza is sequestered before the addition of AChE and the IC_{50} increases: it means that FE4 "protects" the AChE and higher concentrations of Aza are needed to inhibit the housefly enzyme. When FE4 is pre-incubated with PBO or its analogues, it is blocked by them and becomes unable to protect AChE from the action of the insecticide and the IC_{50} decreases again.

Philippou *et al*. (2013) demonstrated that there is no direct inhibition of housefly AChE with the synergists. Thus, the IC_{50} values reflect the affinity for the purified

FE4 and allow the comparison between different compounds. Piperonyl butoxide and four other analogues of variable efficacy were compared and their inhibition curves are shown in Fig. 7.3.

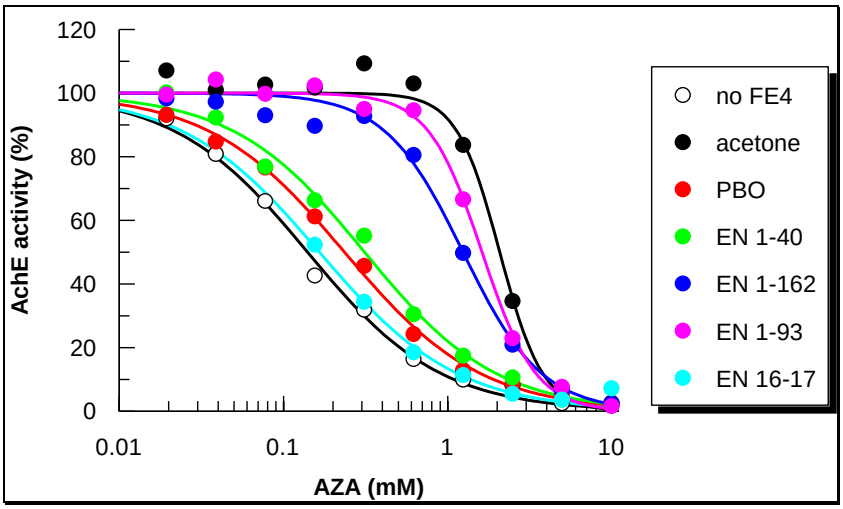


Figure 7.3: AChE activity remaining (%) calculated for different concentrations of Azamethiphos (Aza), in the presence of PBO and other analogues. Controls without FE4 (no FE4) and FE4 without analogues (acetone) are reported.

Analogue	IC_{50} (nM)	s.e.
No FE4	0.140	0.007
acetone	2.072	0.109
EN 16-17	0.166	0.014
PBO	0.235	0.008
EN 1-40	0.312	0.020
EN 162	1.239	0.076
EN 1-93	1.617	0.052

Table 7.1: Results of esterase interference assays with PBO and its analogues showing the concentration of azamethiphos required to inhibit 50% of the AChE activity (IC_{50}) following incubation of the synergists with FE4.

EN 16-17 showed the highest affinity for FE4, with the lowest IC_{50} value; it is followed by PBO, EN 1-40 and EN 1-162; analogue EN 1-93 gave the lowest inhibition, with an IC_{50} value very close to the acetone control (Table 7.1).

3.3 DIRECT DETERMINATION OF FE4 INHIBITION BY PBO: SUBSTRATE COMPARISON

PBO was tested for its ability to inhibit FE4 activity using a conventional, spectrophotometric assay. Three different substrates were tested: 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB) and 4-nitrophenyl acetate (pNA). For the model substrate 1-NA and the alternative substrate 1-NB no evidence of inhibition was detected with increasing concentrations of the inhibitor: FE4 activity increased using 1-NA and remained stable with 1-NB. On the contrary it was possible to observe a decrease of esterase activity with pNA as an alternative substrate. The resultant curves derived from the FE4 activity remaining after inhibition by increasing concentrations of PBO are shown in Fig. 7.4.

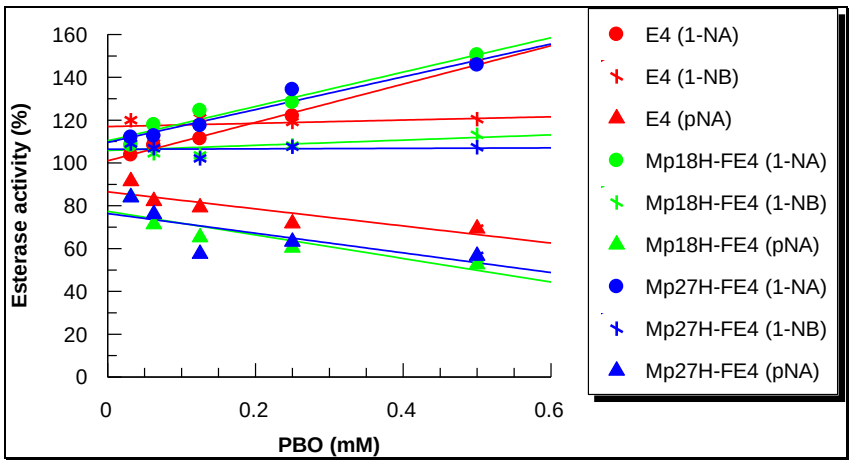


Figure 7.4: FE4 activity remaining (%) after inhibition by increasing concentrations (mM) of PBO. E4 is the esterase variant used as control. FE4-Mp18H and FE4-Mp27H are the esterase FE4 variants purified from two different* M. persicae *populations. 100% of activity corresponds to the uninhibited enzyme.

3.4 DIRECT DETERMINATION OF FE4 INHIBITION BY PBO AND ITS ANALOGUES: PNA ASSAY

Before doing inhibition tests using the alternative substrate pNA, the reversibility of the interaction between FE4 and PBO has been verified. As already described for the E4 variant, kinetic data collected after different incubation periods confirmed the same for the FE4 variant, as shown in table 7.2.

		mOD/min				
		0 min	**5 min**	**10 min**	**30 min**	**1h**
no PBO	**1 repl.**	/	154.11	161.78	144.84	151.86
	2 repl.	/	154.25	165.20	151.27	149.86
	3 repl.	/	166.80	165.10	146.11	156.55
	mean		158.39	164.02	147.41	152.76
PBO (100 µM)	**1 repl.**	/	63.90	68.47	65.71	62.07
	2 repl.	/	62.87	71.64	64.73	63.71
	3 repl.	/	66.18	68.40	67.49	63.67
	mean		64.32	69.50	65.98	63.15

Table 7.2: Esterase activities (mOD/min) measured after different period of incubations with the enzyme alone or with PBO.

PBO and other 4 analogues were tested with a direct spectrophotometer assay, using pNA as substrate. Inhibition by increasing concentrations of the inhibitors and the resultant curves are shown in Fig 7.5.

The concentration of synergist at which 50% of esterase activity is inhibited (IC_{50}) was calculated for each compound. EN 16-17 showed the highest affinity for FE4, with the lowest IC_{50} value; it is followed by PBO and EN 1-40; EN 1-162 and EN 1-93 gave the lowest inhibition, as already shown by their inhibition curves, both overlapped the acetone control (Table 7.3).

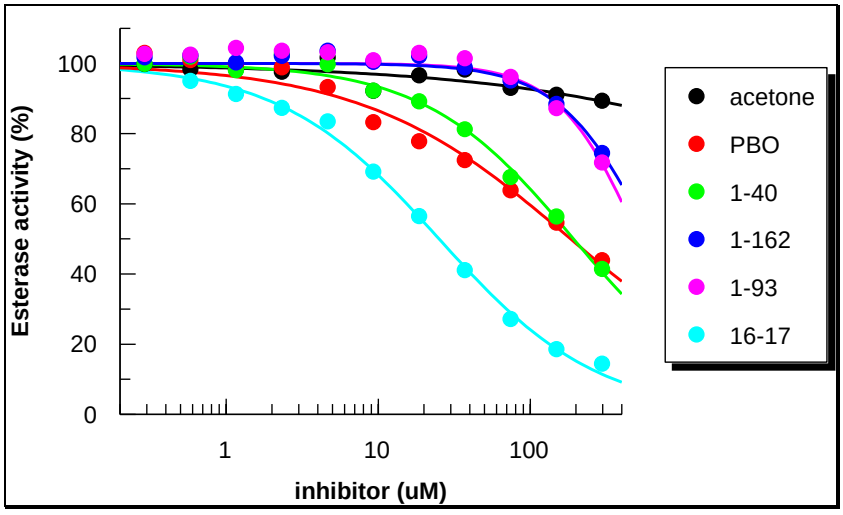


Figure 7.5: Esterase activity remaining (%) calculated for different concentrations of PBO or other analogues. Control without analogues (acetone) is shown.

Analogue	IC_{50} (μM)	s.e.
acetone	n.c.	-
EN 16-17	25.11	1.04
PBO	184.30	22.40
EN 1-40	192.70	12.48
EN 1-93	518.00	77.32
EN 1-162	620.20	77.47

Table 7.3: Results of the direct esterase assay with PBO and its analogues, using pNA as substrate. The concentration of synergist required to inhibit 50% of the FE4 activity (IC_{50}) and standard error (s.e.) are shown.

3.5 CORRELATION BETWEEN DIRECT AND INDIRECT ESTERASE INHIBITION ASSAYS

Esterase assay using pNA as substrate revealed that it is possible to observe directly the esterase-inhibiting capabilities of selected synergists. Results obtained with this method (see section 3.4, table 7.3) ranked the 5 analogues identically to the

interference assay (see section 3.2, table 7.1). Therefore, a diagnostic concentration of inhibitor (stock 10 mM in acetone, final concentration 100 µM) was chosen and the assay was used for further analysis with a large number of other analogues. Results are shown in Fig. 7.6.

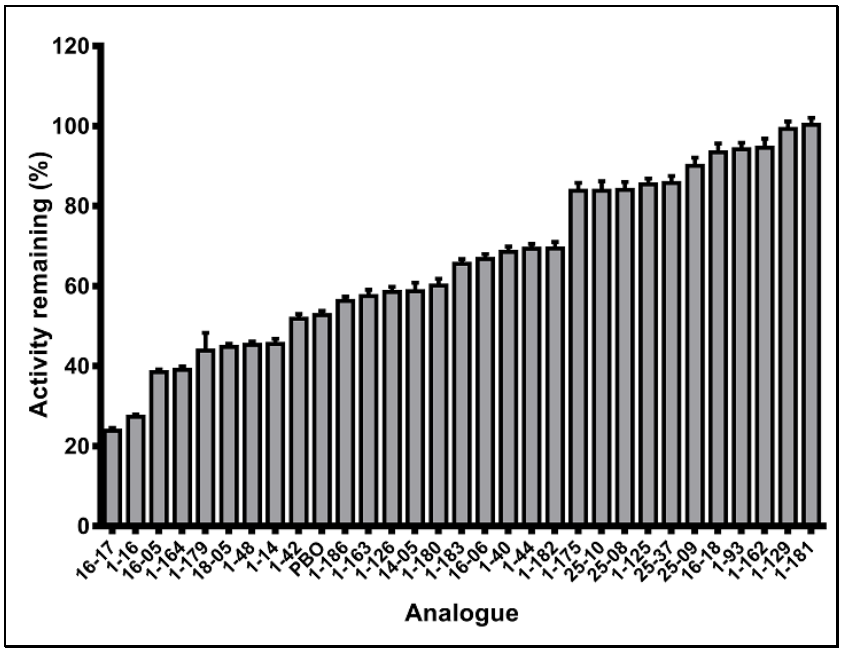
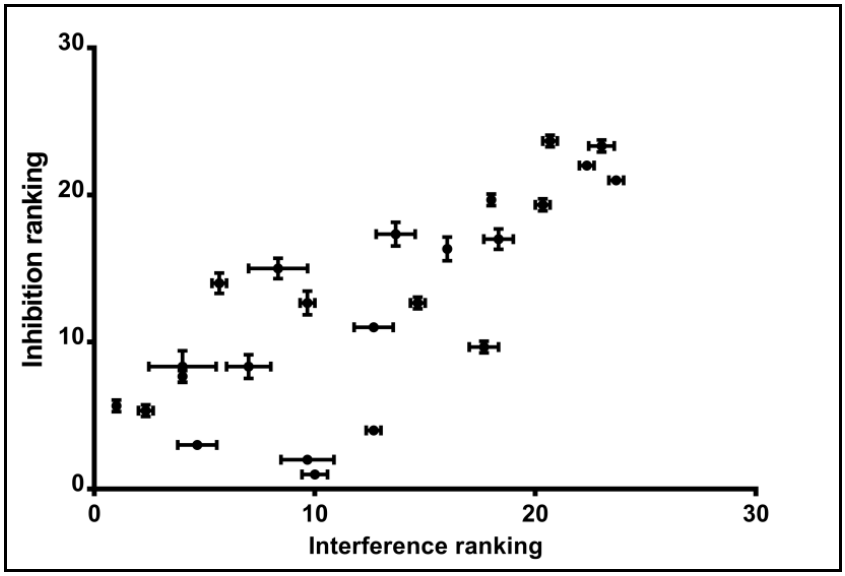


Figure 7.6: FE4 activity remaining (%) calculated for different analogues. Activity remaining was calculated by dividing the activity measured in the presence of inhibitor by the activity in the absence of inhibitor (uninhibited control, not shown) and multiplying by 100.

Inhibition data on esterase FE4 derived from this assay were compared to data already published on the variant esterase E4 obtained with the indirect interference assay (Philippou *et al*., 2012). A significant positive correlation was found between the two experiments, as shown in Fig. 7.7.



	Interference rank VS Inhibition rank
Spearman r	0.7700
95% confidence interval	0.5225 to 0.8978
P value	
P (two-tailed)	<0.0001
P value summary	****
Exact or approximate P value?	Approximate
Significant? (alpha = 0.5)	Yes
Number of XY pairs	24

Figure 7.7: Correlation between the esterase interference assay (X-axis) and the direct esterase inhibition assay with pNA (Y-axis). Analogue's ranking position derived from each assay are plotted, single points represent each analogue. The list of the interference assay results was derived from literature data (Philippou* et al*. 2012) and replicate values were considered thanks to Philippou D., personal communication.

3.6 CYTOCHROME P450 CYP6CY3 PRODUCTION

Microsomal preparations containing the recombinant cytochrome P450 CYP6CY3 were prepared by Rothamsted Research (BCCP Department) in 1.5 mL aliquots and stored at -80 °C. The quality of the enzyme was previously estimated by measuring CO-difference spectra in reduce samples (Philips & Shephard 2006): the classic peak at 450 nm (Soret peak) was indicative of a stable enzyme (data not reported). CYP6CY3 enzymatic activity was checked by O-dealkylation activity on the fluorescent substrate 7-MFC (Fig. 7.8).

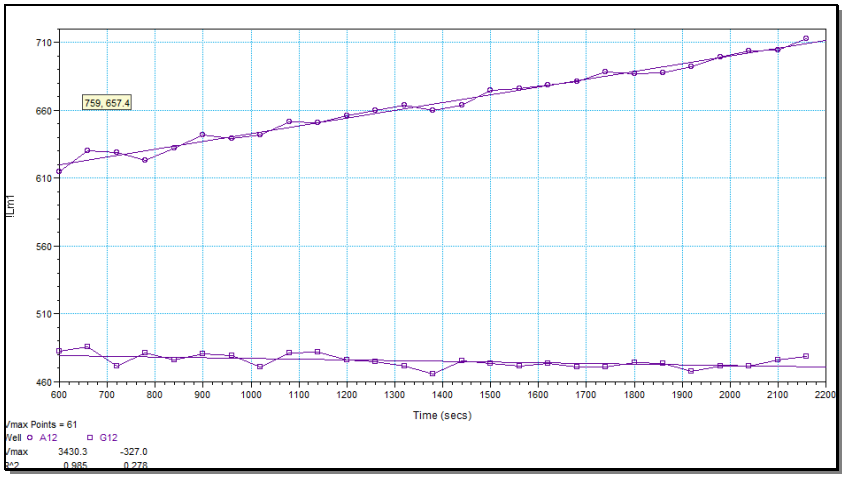


Figure 7.8: Monitoring of 7-hydroxy-4-(trifluoromethyl) coumarin (7-HFC) produced by CYP6CY3 incubated with NADPH and 7-MFC.

3.7 DIRECT DETERMINATION OF CYP6CY3 INHIBITION BY PBO AND ANALOGUES

The purified enzyme was then used to characterise the interactions with piperonyl butoxide (PBO) or its analogues and to determine their ability to inhibit CYP6CY3 activity. Inhibition of oxidase activity can be detected directly using 7-MFC substrate.

A diagnostic concentration of inhibitor (stock 0.1 mM in acetone, final concentration 2 µM) was chosen and the assay was used for analysis with PBO and a large number of other putative inhibitors. Results are shown in Fig. 7.9.

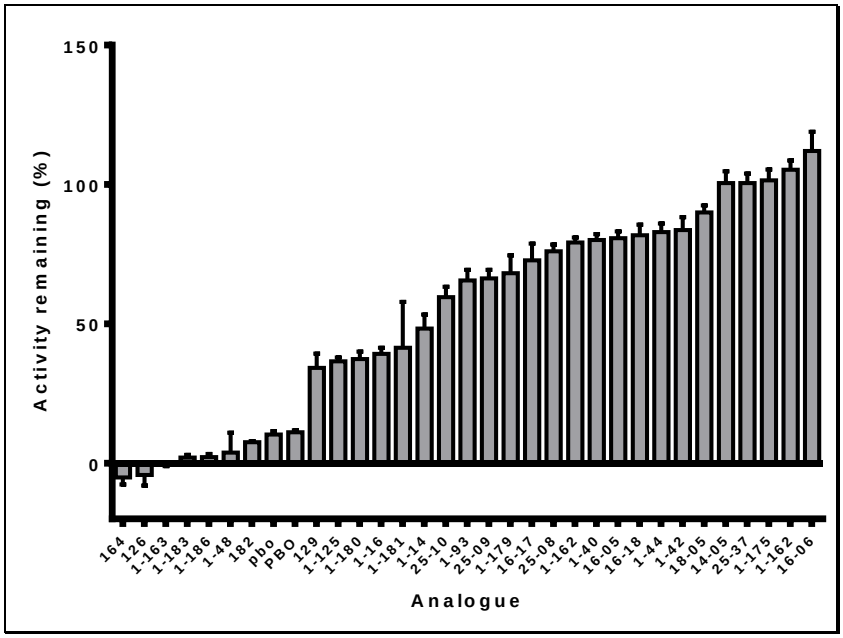


Figure 7.9: CYP6CY3 activity remaining (%) calculated for different analogues. Activity remaining was calculated by dividing the activity measured in the presence of inhibitor by the activity in the absence of inhibitor (uninhibited control, not shown) and multiplying by 100.

4. DISCUSSION

Synergists are non-toxic compounds that are able to act against detoxifying enzymes and thus increase the effectiveness of the insecticidal actives they are combined with. The first part of the EU project "Ecosyn" aims to fully characterise the interactions between the standard synergist PBO (or novel synergists) and esterase or P450 enzymes derived from chosen agriculturally-important insect pests, including *M. persicae*. Only enzymes that have been confirmed in the literature to confer resistance to insecticide have been considered. The present work focuses on the green peach aphid and aims to investigate the interaction of PBO with the cytochrome P450 CYP6CY3 and the resistant-associated esterase FE4. Currently, some works have been done regarding the interaction between PBO and the major human detoxifying P450 CYP3A4 (Moores *et al*. 2011) but no assays have

been performed using CYP6CY3 from *M. persicae*, that has been demonstrated to be the enhanced P450 enzyme involved in insecticide detoxification (Puinean *et al*. 2010). However, results from in vitro test are already available regarding its capacity to metabolize nicotine and two neonicotinoids (imidacloprid and clothianidin) into less-toxic metabolites (Bass *et al*. 2013). The efficacy of PBO (or its analogues) against this enzyme have been evaluated through inhibition of enzyme activity directly detected using the fluorimetric substrate 7-MFC. Data obtained from this assay show a very high inhibition efficacy of some analogues, with very low oxidase activity remaining.

Much progress had already been made regarding the interaction of PBO (and its analogues) with the resistant-associated esterase E4, commonly found in Northern Europe, but no data were available on the other Mediterranean variant FE4. Because of the glycosylation sites within the enzymes, recombinant production of E4 or FE4 was not considered and the evaluations were made on enzymes purified from insects. As already described for E4, direct inhibition of FE4 esterase activity could not be measured by simple colorimetric assays using classic model substrates. In fact, PBO does not interact with the active site of the enzyme, but binds to an adjacent site in such a way that enhances substrate entry instead of occluding it, so it can still be hydrolysed. Therefore, initially the "esterase interference assay" was carried out, as being the only method described in the literature (Khot *et al.* 2008, Philippou *et al.* 2013). However, other esterase substrates were assessed and pNA was found to be suitable for directly monitoring inhibition. The assay was then used to screen PBO and other analogues for their inhibition efficacy against FE4. Results were found to correlate with the E4 data obtained with the interference assay (Philippou *et al.* 2013), confirming the same mechanism of action against both esterase variants; in addition, different periods of incubation of FE4 with the inhibitors showed the reversibility of the reaction, as already described for E4.

The possibility of measuring inhibition of FE4 directly using a model substrate presents several advantages over the indirect interference assay. Firstly, the latter is relatively time-consuming, requiring several incubation steps, one of which is

overnight; secondly, the interference assay is more dependent upon an initial purification of the esterase. For both assays, the resistance-associated esterase (FE4) would ideally be purified, but with the direct assay a simple homogenisation step will suffice to give approximate indications, whilst with the interference assay the FE4 must be at least partially purified for the assay to be completed. If insect availability is a factor, this could be important.

Based on the results of the inhibition assays, a structure activity relationship (SAR) study will constructed to predict novel inhibitor structures for future testing. The structures showing the most potent inhibition efficacy will be selected and tested in "*in vivo*" bioassays and then in field experiments, with the expectation to derive bespoke structures that are capable of high synergism to be used in agricultural applications.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Insect pests represent a serious threat to agricultural production and vector disease control. A high number of insect species are responsible for important agricultural yield losses which poses problems for maintaining future food security. An ongoing challenge for agricultural production is the presence of insecticide resistance, with more than 500 species for which at least one reported case of insecticide failure has been described (http://www.pesticideresistance.com). This is an important issue considering the increasing human population which will require more food from limited resources suitable for agricultural production. In order to maintain good yields from the existing production areas, the use of chemical insecticides for controlling insect pests represented the key tool of the last 60 years and remain essential for guaranteeing a supply of nutritious and affordable food for the near future. However, the worldwide use of high levels of insecticides over many years has led to increased selection for resistance populations. In addition, wasteful and ineffective insecticide treatments have contributed to environmental contamination and represent a health risk for operators and consumers.

The understanding of insecticide resistance mechanisms has made remarkable advances in the last years. It has been shown that resistance can evolve by two main mechanisms: the enhanced production of metabolic enzymes, which sequester or detoxify the insecticide, and mutations of target proteins, which make them less sensitive to the insecticide. A number of subsidiary physiological mechanisms giving a contribute to enhance the insecticidal effects have also been described, e.g. a reduced penetration of the chemicals. A variety of different chemical classes has been developed (http://www.irac-online.org/documents/moa-classification), which act on different biological targets. However during the years insects have evolved mechanisms to overcome the toxicity of most classes of

insecticide. Thus, the possibility that they could evolve resistance also against potential new products with different mode of actions has to be taken in account.

Aphids are an ideal model to study the complexity of insecticide resistance and increase understanding of how resistance evolve. They demonstrate coexistence of different reproductive modes, either as continuous and obligate parthenogenesis or alternating parthenogenetic reproduction in spring and summer with amphigonic reproduction in autumn, depending on the areas and the primary host availability. Parthenogenesis allows the rapid enstablishment of specific mutations, due to both the high fecundity and short life cycle of the aphids. On the contrary mating is responsible for the production of different genotype combinations, possibly explaining the coexistence of cross and multiple resistance and the presence of different resistance mechanisms within individuals, each contributing to enhance resistance factors.

The green peach aphid is a particularly interesting example because of its widespread distribution, high efficacy as a virus vector, wide variability of properties such as color, life-cycle, host-plant relationships and ability to overcome insecticide treatments, with several resistance mechanisms already reported and described in this species (Blackman & Eastop 2000). It is considered one of the major agricultural pests, causing damages on many agricultural and horticultural crops resulting in significant yield losses if populations are not kept under economic damage thresholds. The first reports of resistance were to organophosphates (OPs) and carbamates by amplification of esterase genes, followed by resistance to dimethylcarbamates due to a target-site mutation in the acetylcholinesterase enzyme. Later, reduced sensitivity to pyrethroids was associated to target-site mutations in the voltage gated sodium channel and in lesser extent to esterase detoxification or sequestration (Devonshire *et al*. 1998). Finally, duplications of CYP6CY3 P450 gene and target-site mutation in a subunit of the acetylcholine receptor have recently been identified as responsible for neonicotinoid resistance (Bass *et al*. 2014).

For almost 50 years, the control of the green peach aphid has relied mainly on three insecticide classes: OPs and carbamates, acting of AchE, and pyrethoids, acting on the voltage-gated sodium channel. Their intensive use, together with only two biological targets being involved, has led to the development of multiple and widespread forms of resistance against them. To date, OPs and carbamates have been phased out, at least in Europe, because of their unfavourable toxicological and environmental profile. Dimethylcarbamates, like the established pirimicarb, are still permitted but today their use is severely restricted, at least in Italy. Treatments with pyrethroids have been reduced in the last few years because of the extent of target-site mutations causing insensitivity to this class of insecticides; nevertheless, they are still applied in peach orchards against other pests (they remain one valid alternative in pre-flowering control strategies against trips on nectarines) and thus they continue indirectly to exert selection pressure on *M. persicae* populations.

These aphicides have been mainly replaced by neonicotinoids which represent the fastest-growing class of insecticides since their introduction on the market, due to the novelty and specificity of their target. Acting on the insect central nervous system as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs), there is little or no cross-resistance to older insecticide classes, providing the growers new tool for managing a wide range of destructive crop pests, including *M. persicae* (Jeschke & Nauen 2005 and 2008). Also, neonicotinoids are specifically toxic to insects compared to mammals, due to fundamental structural differences in their receptor subunits (Tomizawa & Casida 2002).

Although neonicotinoids have been considered remarkably resilient to resistance and were thus adopted as the key element of the control measures also against *M. persicae* (Nauen 2005), resistant aphids against this class of insecticide have recently been detected in Southern Europe, which poses a serious threat to their long-term efficacy (IRAC newsletters, issues 25 and 29; Philippou & Moores 2009). Furthermore, a European ban on certain neonicotinoid insecticides (imidacloprid, thiamethoxam and clothianidin) was introduced in December 2013 because of the possibility that they can affect bees and other insect pollinators (Regulation EU No

485/2013). In view of these restrictions together with neonicotinoid treatment failures, control strategies have to be reviewed taking into account both the crop and local guidelines.

Monitoring and detection of insecticide resistance is currently one of the important factors of insect pest management. Previously, the discovery of resistance was linked to insecticide treatment failures when the proportion of resistant individuals was quite high. However, the significant amount of research carried out in recent years has provided several advances in understanding the evolution of resistance mechanisms and a variety of molecular and biochemical techniques have been developed allowing a rapid detection of resistance in specimens collected directly from the field.

Aphid monitoring surveys in peach orchards have revealed that after more than 20 years of neonicotinoid use on many crops, neonicotinoid resistant populations of the green peach aphid *M. persicae* exist in Southern France and Northern Spain. Data carried out in this thesis work have confirmed not only the presence but also the widespread distribution of resistant aphids in Italy. This is true not just in areas important for peaches but also in a small number of samples collected from secondary hosts. This poses a serious threat for countries where *M. persicae* reproduces only by parthenogenesis on herbaceous crop. Although the recent restrictions related to a few neonicotinoid products, they remain one of the main effective control measures adopted by growers. This emphasizes the importance of continuously monitoring the distribution and impact of resistant aphids, and the necessity to collaborate with local experts to create and implement effective resistance management strategies.

Resistance management guidelines have been proposed by IRAC for stone fruits in Sothern Europe following the results of surveys from 2010 to 2012 (http://www.irac-online.org/documents/myzus-persicae-irm-english), which took in consideration that the management of neonicotinoid resistant green peach aphid on peach and other fruit crops is complicated by the presence of pyrethroids and carbamate resistance aphids in the same areas. Where no loss of performance to

neonicotinoids has been reported, a maximum of one neonicotinoid application per crop cycle is recommended. This will minimize the further spread and intensification of resistance and maintain effectiveness of neonicotinoids. Where a decline of neonicotinoid efficacy has occurred, use of different insecticides with alternative modes of action is recommended as a preventive measure, depending on local registrations. Alternative products, i.e. pymetrozine, flonicamid, spirotetramat and diamide insecticides, with different modes of action (IRAC MOAs 9B, 9C, 23 and 28) to reduce the selection pressure exerted by neonicotinoids are available. They have been proved to be efficacious when applied after neonicotinoid failures and can therefore be considered and included in control strategy programmes, by rotating different insecticide modes of action rather than applying the same chemicals on consecutive generations of *M. persicae*.

Historically, the problem of insecticide resistance has been overcome by continuously introducing new active ingredients to replace those which resulted in a loss of efficacy. Although there is an appreciable number of products available on the market, their modes of action are limited and recent phasing out of several actives has further reduced the MOA portfolio. Since the development of new compounds is slow and expensive, it is important to prolong the useful life of the existing and still effective insecticides by a judicious use of them. A possible solution to take in account is to enhance their actions by using synergic molecules in combination with them, allowing a reduction in the amount of insecticide active applied or overcoming metabolic resistance and maintaining the same level of control at the registered doses.

This concept represents the central issue of the European project “EcoSyn: ecofriendly synergists for insecticide formulations” (http://www.ecosyn.eu), which is to investigate possible interactions between PBO/analogues and metabolic enzymes conferring insecticide resistance in different insect species with different insecticide resistance profiles. *M. persicae* has been included in the project and the current thesis presents preliminary data obtained by “*in vitro*” tests identifying

potential PBO analogue candidates to consider because of their high inhibition efficacies against detoxifying enzymes.

Piperonyl butoxide is probably the best known synergist and it is widely used in the household market. Although literature data demonstrate its efficacy as an insecticide synergist on several crop pests, in Europe it is still not registered for use in the field. However, since it has been recognized as specific esterase and MFO inhibitor, it has been used for research purposes to characterise metabolic resistance using "*in vivo*" laboratory bioassays. This study confirms that in the green peach aphid PBO and other analogues, which have modifications in the MDP moiety and side chain, can inhibit both these phase 1 metabolic enzymes. Structure activity relationship studies (SAR) are currently underway to find bespoke parameters that can contribute to the design of more potent inhibitor structures to give higher binding affinities to the enzymes. Such results will then be confirmed in "*in vivo*" bioassays on resistant populations and finally tested in field experiment.

At this stage of the study it can be concluded that some analogues, among the wide range of compound that were investigated, showed the potential to enhance insecticide efficacy. Such compounds could be possibly used as insecticide synergists in insect resistance management (IRM) programmes in order to maintain effective utility of current insecticides by preventing or slowing development of resistance. Also, considering differences in the enzyme structures among different insect species, specific chemicals could be designed for particular insects and thus minimize undesired effects on non-target species, in particular beneficial insects such as honey bees.

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