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MOLECULAR, GENETIC AND FUNCTIONAL CHARACTERIZATION OF PESTICIDE RESISTANCE MECHANISMS OF *Tetranychus urticae*

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Σας ευχαριστώ πολύ όλους! Τίποτα δεν είναι τυχαίο!

Curriculum Vitae

Maria Riga was born on 20 September 1985 in Alexandroupoli, Greece. After taking nationwide exams, Maria registered in 2003 to the Department of Biology, University of Crete for her bachelor which she completed in 2008. On 2009, Maria started her Master Studies in Environmental studies, Department of Biology at the University of Crete. In March 2012 Maria started her PhD project entitled "MOLECULAR, GENETIC AND FUNCTIONAL CHARACTERIZATION OF PESTICIDE RESISTANCE MECHANISMS OF Tetranychus urticae" at the laboratory of Molecular Entomology (Vontas lab), in the Department of Biology, the University of Crete.

List of publications

1. **Maria Riga,** Sabina Bajda, Christos Themistokleous, Stavrini Papadaki, Maria Palzewicz, Wannes Dermauw, John Vontas and Van Leeuwen, 2017**.** The relative contribution of target-site mutations in complex acaricide resistant phenotypes as assessed by marker assisted backcrossing in *Tetranychus urticae*. *Scientific Reports.*

2. **Maria Riga**, Antonis Myridakis, Dimitra Tsakireli, Evangelia Morou, Eyripidis G. Stefanou, Ralf Nauen, Thomas Van Leeuwen, Vasilis Douris, John Vontas, 2015. Functional characterization of the Tetranychus urticae CYP392A11, a cytochrome P450 that hydroxylates the METI acaricides cyenopyrafen and fenpyroximate. *Insect Biochemistry and Molecular Biology* 65: 91-99.

3. Nena Pavlidi, Vasilis Tseliou, **Maria Riga**, Ralf Nauen, Thomas Van Leeuwen, Nikolaos E. Labrou, John Vontas, 2015. Functional characterization of glutathione S – transferases associated with insecticide resistance in *Tetranychus urticae. Pesticide Biochemistry and Physiology* **121**: 53 – 60.

4. **Maria Riga**, Dimitra Tsakireli, Aris Ilias, Evangelia Morou, Antonis Myridakis, Evripidis G. Stefanou, Ralf Nauen, Wannes Dermauw, Thomas Van Leeuwen, Mark Paine, John Vontas, 2014. Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in *Tetranychus urticae. Insect Biochemistry and Molecular Biology* 46: 43-53.

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List of Abbreviations

BSA	Bovine Serum Albumin
BSA	Bulk Segregant Analysis
CCE	Carboxylesterase
CPR	Cytochrome P450 reductase
CrispR	Clustered Regularly Interspaced
CTAB	Hexadecyltrimethylammonium bromide
DCJW	Decarbomethoxyllated JW062
DEF	S,S,S-tributyl phosphorotrithioate
DEM	Diethyl maleate
Dm	*Drosophila melanogaster*
dsRNA	double-stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FAD	Flavine adenine dinucleotide
FAO	Food and Agriculture Organisation
FMN	Flavin mononucleotide
GluCl	Glutamate gated chloride channel
GSTs	Glutathione - S – transferases
HPLC	High Performance Liquid Chromatography
IC50	Inhibition Concentration 50
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRAC	Insecticide Resistance Action Committee
K*cat*	Catalytic Constant
Km	Michaelis - Menten Constant

LC_{50}	Lethal Concentration 50
METI	Mitochondrial Electron Transport Inhibitor
MS	Mass Spectrum
NADPH	Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate
P450	Cytochrome P450
PBO	Piperonl Butoxide
P-gp	P- glycoprotein
PMSF	phenylmethylsulfonyl fluoride
qPCR	quantitative Polymerase Chain Reaction
QTL	Quatitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction fragment length polymorphism
RH	Relative Humidity
RNAi	RNA interference
RR	Resistance Ratio
SDS	Sodium dodecyl sulfate
SR	Synergism Ratio
TBS	Tris-Buffered Saline
TSE	Tris-Sucrose-EDTA
UAS	Upstream Activator Sequence
VGSC	Voltage Gated Sodium Channel

Contents

Abstract 1
Περίληψη 3
Chapter 1: Introduction 5
1.1 Biology and ecology of the spider mite *Tetranychus urticae* 5
1.2 Control of the spider mite *Tetranychus urticae* 6
1.3 Insecticide Resistance 9
1.3.1 Behavioral resistance 10
1.3.2 Cuticle resistance 10
1.3.3 Target site (or toxicodynamic) resistance 11
1.3.4 Metabolic (or toxicokinetic) resistance 12
1.3.4.1Cytochrome P450 monooxygenases 12
1.3.4.2 Carboxylesterases (CCEs) 14
1.3.4.3 Glutathione – S – transferases (GSTs) 15
1.3.5 Insecticides/Acaricides resistance reports and mechanisms in *T. urticae* 15
1.4 Approaches and techniques for analysing insecticide resistance 18
1.4.1 Establishing the resistance phenotype 18
1.4.2 Association studies to specific molecular markers 19
1.4.3 Validation of the association between markers and resistance phenotype 20
1.5 Insecticide Resistance Management 22
1.5.1 Practices to prevent resistance 22
1.5.2 Evidence based resistance management 22
Aim of the PhD study 23

Chapter 2: Functional characterization and expression *in vitro* of *Tetranychus urticae* cytochrome P450s associated with high levels of acaricide resistance
2.1 Introduction 24
2.2 Materials and Methods 26
2.2.1 Strains 26
2.2.2 Extraction of RNA and cDNA synthesis 26

2.2.3 Cloning and co-expression of CYPs with *Tetranychus urticae* CPR, and preparation of membranes 26
2.2.4 P450 activity and ligand IC50 determination 28
2.2.5 HPLC – MS analysis of insecticide metabolism and preparative purification of metabolites 29
2.2.5.1 Metabolism assays with abamectin, identification and isolation of the metabolite (CYP392A16) 29
2.2.5.2 Metabolism assays with Cyenopyrafen and Fenpyroximate (CYP392A11) 30
2.2.6 Peptide Antibody Development against CYP392A16 31
2.2.6.1 Western Blot Analysis 31
2.3 Results 32
2.3.1 Functional expression of P450s 32
2.3.2 Enzyme activity and ligand IC50 determination 33
2.3.3 Detection of Metabolism and Enzyme characterization 35
2.3.4 Anti-CYP392A16 43
Conclusions – Discussion 45

Chapter 3: Heterologous expression of *T. urticae* P450s and homologous CPR inR *Drosophila* in order t investigate the functional role of genes in resistance *in vivo*

3.1 Introduction 47
3.2 Materials and Methods 49
3.2.1 Construction of the transgenic fly strains 49
3.2.2 Over-expression of CYP392A16, CYP392A11 and/or TuCPR in *Drosophila melanogaster* 50
3.2.3 Toxicity Assays 50
3.3 Results 51
3.3.1 Transgenic expression of CYP392A16, CYP392A11 and CPR of *T. urticae* in *Drosophila* 51
3.3.2 Toxicity Assays 52
Conclusions – Discussion 54

Chapter 4: The relative contribution of target-site mutations in complex acaricide resistant phenotypes as assessed by marker assisted backcrossing in *Tetranychus urticae*
4.1 Introduction 56
4.2 Materials and Methods 59
4.2.1 Acaricides 59
4.2.2 Spider mite strains 59
4.2.3 Backcrossing experiments 59
4.2.4 Single mite DNA extraction 60
4.2.5 Genotyping 61
4.2.6 Toxicity bioassays 61
4.3 Results 63
4.3.1 Establishment of congenic lines 63
4.3.2 Toxicity assays of Parental and Backcrossed strains 64
4.3.2.1 Abamectin and Milbemectin 64
4.3.2.2 Pyrethroids 65
Conclusions – Discussion 67
General Discussion – Future plans 70
References 74
Ammendum 84

Abstract

Tetranychus urticae (Koch) is one of the most destructive agricultural pests. Its control is based mainly on the use of insecticides / acaricides. Due to the extensive use of chemical compounds and the life history traits (short cycle, high proliferation high fecundity and arrhenotokous parthenogenesis) they develop resistance quickly, showing striking phenotypes in some cases. Among them, several studies report resistance to abamectin, METIs and pyrethroids.

Both target site resistance and metabolic resistance have been associated with abamectin, METIs and pyrethroids resistance. Gene expression data on abamectin and METI resistant populations compared to susceptible populations indicated that many detoxification genes and particularly cytochrome P450s are over - expressed in the resistant strains and they are associated with the phenotype.

In this study (Chapter 2) a functional link between cytochrome P450 metabolism and multi-insecticide resistance was investigated by expressing several P450s (CYP392A16, CYP392A11, CYP392A12, CYP392D8 and CYP392D10) in an *E. coli* model system. I showed that the cytochrome P450 CYP392A16, which is strongly associated with abamectin resistance at the gene expression level, is capable to metabolise abamectin to a less toxic compound (24- or 26-hydroxymethyl metabolite) as confirmed by bioassays with the purified metabolite. An antibody was developed against CYP392A16, and successfully tested on resistant and susceptible spider mite homogenates showing high specificity and sensitivity in detecting the elevated levels of the 55 kDa CYP392A16 protein. Also, CYP392A11, a cytochrome P450 strongly associated with METI resistance at the gene expression level, is capable to metabolise two METI acaricides, fenpyroximate and cyenopyrafen, a novel METI recently introduced in the market, but never used against the mite strains analysed in this study. It was shown that fenpyroximate metabolism produces a non toxic compound ("metabolite M5"), while cyenopyrafen was metabolized to a hydroxylated compound.

In Chapter 3 I employed the GAL4/UAS system for ectopic co-expression of *T. urticae* cytochrome P450s (CYP392A16 and CYP392A11) and CPR in *Drosophila*, in order to validate their role in resistance to abamectin and METIs, *in vivo*. The transgenic lines co-expressing CYP392A16; TuCPR under GAL4 driver were successfully generated, and toxicity bioassays showed that they were resistant to abamectin in comparison to the control line (CYP32A16; TuCPR x w^{1118}). Also, TuCPR; CYP392A11 x GAL4 line was tested against fenpyroximate

showing 2.6 folds resistance to the specific insecticide / acaricide compared to the control line (TuCPR; CYP392A11 x w^{1118}).

In the last chapter (Chapter 4), I examined the relative contribution of known target-site mutations that have been associated with the resistance phenotype to abamectin and pyrethroids by undertaking a genetic approach. I introduced G314D, G326E on glutamate gated chloride channels and L1024V and F1538I on sodium channel in a susceptible *T. urticae* genetic background through multiple genetic crosses, in order to obtain homozygous lines that carry these mutations, alone or in combinations. Their contribution of the specific target site mutations to resistance was examined with toxicity assays. It was indicated that 314D and 326E alone have minor effect to abamectin and milbemectin resistance. On the other hand, their combination (G314D; G326E) provides higher resistance levels to abamectin and milbemectin, approximately 10-20 folds. Hpwever additional mechanisms are possibly involved in resistance to abamectin. The same methodology was followed for the investigation of the relative contribution of sodium channel mutations (L1024V and F1538I) in pyrethroid resistance. The results indicate that both L1024V and F1538I mutations provide high resistance levels to all pyrethroids tested (bifenthrin, fluvalinate and fenpropathrin) and their presence in populations alone is enough to cause field failure after acaricide treatment.

The findings, their impact on insecticide resistance research and Insecticide Resistance Management (IRM) strategies, and some future research directions are discussed in the last session (general discussion).

ΠΕΡΙΛΗΨΗ

Ο τετράνυχος, *Tetranychus urticae* (Koch) αποτελεί σημαντικό εχθρό πολλών θερμοκηπιακών και υπαίθριων καλλιεργειών. Η κύρια μέθοδος καταπολέμησής του είναι με τη χρήση εντομοκτόνων / ακαρεοκτόνων ουσιών. Όμως, η εντατική χρήση των εντομοκτόνων σε συνδυασμό με τα ιδιαίτερα χαρακτηριστικά του οργανισμού αυτού, όπως ο σύντομος κύκλος ζωής, το υψηλό αναπαραγωγικό δυναμικό, οι πολλές γεννεές μέσα στο χρόνο και η αρρενοτόκος παρθενογέννεση, έχουν ως συνέπεια την εμφάνιση υψηλών επιπέδων ανθεκτικότητας σε ένα μεγάλο αριθμό εντομοκτόνων / ακαρεοκτόνων, όπως αβερμεκτίνες, METIs και πυρεθροειδή. Η ανθεκτικότητα του τετρανύχου στα εντομοκτόνα είναι πιθανότατα αποτέλεσμα της μεταβολικής ανθεκτικότητας (υπερέκφραση ενζύμων αποτοξικοποίησης) ή/ και αλλαγών στο στόχο του εντομοκτόνου (ανθεκτικότητα στόχου).

Ανάλυση δεδομένων γονιδιακής έκφρασης από πληθυσμούς που εμφανίζουν ανθεκτικότητα στις αβερμεκτίνες και στα METIs ακαρεοκτόνα δείχνουν υψηλά επίπεδα έκφρασης γονιδίων αποτοξικοποίησης και κυρίως P450s. Στη συγκεκριμένη μελέτη (Κεφάλαιο 2) εκφράστηκαν οι P450s CYP392A16, CYP392A11, CYP392D2, CYP392D8 και CYP392D10, σε βακτηριακό (*E. coli*) σύστημα. Δύο από αυτές, η CYP392A16 και η CYP392A11 εκφράστηκαν λειτουργικά με επιτυχία. Φάνηκε πως η CYP392A16 είναι ικανή να μεταβολίζει το εντομοκτόνο abamectin παράγοντας έναν μη τοξικό υδροξυλιωμένο μεταβολίτη (24- or 26-hydroxyl metabolite), όπως επιβεβαιώθηκε και από πειράματα βιοδοκιμών με το συγκεκριμένο μεταβολίτη που απομονώθηκε από το HPLC. Επίσης, αναπτύχθηκε αντίσωμα για το συγκεκριμένο ένζυμο με υψηλή εκξειδίκευση για την CYP392A16 σύμφωνα με πειράματα ανοσοαποτύπωσης (western), το οποίο μπορεί να αξιοποιηθεί για ανάπτυξη διαγνωστικού και μελέτη της ιστοειδικής έκφρασης του ενζύμου στον τετράνυχο

Επίσης, η CYP392A11 εκφράστηκε λειτουργικά και φάνηκε πως το συγκεκριμένο ένζυμο μεταβολίζει δύο δραστικές ουσίες που ανήκουν στα METI ακαρεοκτόνα, το fenpyroximate και το cyenopyrafen. Ο μεταβολισμός του fenpyroximate οδηγεί στη δημιουργία ενός μη τοξικού μεταβολίτη, τον «M-5». Ο μεταβολίτης του cyenopyrafen είναι ένα υδροξυλιωμένο προϊόν, αλλά δεν ήταν δυνατό να ταυτοποιηθεί η ακριβής θέση στην οποία γίνεται η υδροξυλίωση.

Στο Κεφάλαιο 3, με τη χρήση του GAL4/UAS συστήματος δημιουργήθηκαν διαγονιδιακά στελέχη δροσόφιλας στα οποία συν-εκφράζονται οι CYP392A16 ή CYP392A11 με τη CPR από τον τετράνυχο και αξιολογήθηκε η δράση αυτών των ενζύμων σε σχέση με τον φαινότυπο της ανθεκτικότητας. Συγκεκριμένα, το στέλεχος που εκφράζει την CYP392A16 και CPR (CYP32A16; TuCPR x GAL4) εμφανίζει ανθεκτικότητα στο εντομοκτόνο abamectin σε σύγκριση με το

στέλεχος αναφοράς (CYP32A16; TuCPR x w^{1118}). Επίσης, δημιουργήθηκε το στέλεχος TuCPR; CYP392A11 x GAL4 το οποίο εμφανίζει ανθεκτικότητα στο fenpyroximate σε σχέση με το στέλεχος αναφοράς (TuCPR; CYP392A11 x w^{1118}). Η ανθεκτικότητα του στελέχους TuCPR; CYP392A11 δεν μελετήθηκε με το ακαρεοκτόνο cyenopyrafen, επειδή το συγκεκριμένο ακαρεοκτόνο δεν είναι τοξικό για τη δροσόφιλα.

Στο Κεφάλαιο 4 αξιολογήθηκε η σχετική επίδραση γνωστών μεταλλαγών που έχουν βρεθεί σε κανάλια στόχους στον τετράνυχο στον φαινότυπο της ανθεκτικότητας. Για να ελεγχθεί ο ρόλος τους στον φαινότυπο της ανθεκτικότητας έγινε εισαγωγή της κάθε μεταλλαγής σε ευαίσθητο γενετικό υπόβαθρο μέσω πολλαπλών διασταυρώσεων. Συγκεκριμένα μελετήθηκαν οι μεταλλαγές G314D, G326E που εντοπίζονται στα κανάλια χλωρίου και σχετίζονται με την ανθεκτικότητα στις μακροκυκλικές λακτόνες και οι μεταλλαγές L1024Vκαι F1538I που έχουν βρεθεί στο κανάλι νατρίου και έχουν συσχετιστεί με την ανθεκτικότητα στα πυρεθροειδή. Τα αποτελέσματα έδειξαν πως η εισαγωγή των μεταλλαγών G314D και G326E ξεχωριστά προσδίδουν χαμηλά επίπεδα ανθεκτικότητας στα εντομοκτόνα abamectin και milbemectin. Ωστόσο, ο συνδυασμός τους προσδίδει περίπου 10-20 φορές υψηλότερα επίπεδα ανθεκτικότητα στα εντομοκτόνα abamectin και milbemectin. Τα επίπεδα ανθεκτικότητας αυτών των στελεχών είναι χαμηλά σε σχέση με αυτά του ανθεκτικού πατρικού πληθυσμού (>1000 φορές) γεγονός που υποδηλώνει πως οι μεταλλαγές αυτές δεν αποτελούν τον μοναδικό μηχανισμό που συνεισφέρει ανθεκτικότητα στο abamectin. Η ανθεκτικότητα στα πυρεθροειδή μελετήθηκε σε ομόζυγα στελέχη που φέρουν τις μεταλλαγές L1024V και F1538I. Τα αποτελέσματα έδειξαν πως η εκάστοτε μεταλλαγή (L1024V και F1538I) προσδίδει υψηλά επίπεδα ανθεκτικότητας στα εντομοκτόνα bifenthrin (τύπου I), fenpropathrin και fluvalinate (τύπου II) υποδεικνύοντας πως η παρουσία τους μπορεί να προκαλέσει μη επιτυχημένο έλεγχο στο πεδίο.

Τέλος, στην τελευταία ενότητα (γενική συζήτηση) συνοψίζονται τα ευρήματα της μελέτης, με έμφαση τη συμβολή τους στην έρευνα για την ανθεκτικότητα αλλά και στη διαχείριση του φαινομένου στον αγρό καθώς και συζητούνται πιθανές μελλοντικές ερευνητικές κατευθύνσεις.

Chapter 1: Introduction

1.1 Biology and ecology Tetranychus urticae

Two spotted spider mite, *Tetranychus urticae* (Koch), belongs to Acari subclass and the family of Tetranychidae. It is a phytophagous species and infests more than 1000 plant species. Most of them are of economically important crops such as cotton, maize, tomatoes, sweet pepper and ornamentals (Van Leeuwen et al., 2013).

The damage of the leaf is caused by feeding. Mites are mostly appeared on the underside of the leaves by puncturing and sucking out their contents. These leaves are turning yellow inhibiting the photosynthesis process and in great damages the plant is getting destroyed (http://www.biologicalservices.com.au/pests/two-spotted-mite-90.html). Spider mites produce web which serves as protection from abiotic and biotic conditions, egg shelter, communication via pheromone production and dispersal (Van Leeuwen et al., 2013).

The life cycle of *T. urticae* is comprised of 5 stages: egg, larva, protonymph, deutonymph and adult (Figure 1.1). Adult female mites show high fecundity. One female can lay up to 200 eggs. The eggs are translucent and spherical with a diameter of 100-150 µm. As the development takes place the eggs become opaque and yellowish. There are three immature mobile stages (larva, protonymph and deutonymph) each one followed by a quiescent stage (protochrysalis, deutochrysalis and teliochrysalis) before reaching adulthood. The larva has 3 pairs of legs whereas the other stages have four pairs of legs. During the resting stage, spider mites anchor to the leaf and a new cuticle is prepared in order to discard the old one (ecdysis process). Usually males mature before females, thus locating themselves near or on the female teliochrysalis until the latter one emerges. Copulation takes place immediately after emergence of the female. The duration of the development of *T. urticae* is mainly temperature dependent, but is also correlated to other factors such as humidity, host plant and photoperiod. The eggs hatch 3 - 4 days after deposition under ideal conditions (25 – 30 C, 50-55% RH, 16:8 L:D). As the temperature increases the duration of the life stages decreases (Bounfour and Tanigoshi, 2001; Tehri, 2014). Spider mites reproduce by arrhenotokous parthenogenesis, a process that unfertilized females produce male offspring whereas fertilized females produce female offspring. The males have one set of chromosomes (haploid) whereas the diploid females have two sets of chromosomes. If a mutation occurs in the population it will be immediately expressed in the male irrespective of the dominant or recessive status of the mutation (Horowitz et al., 2003). Through natural selection the mutation will be established quickly in the population and the characteristics it confers will be expressed. This

will lead to the development of insecticide / acaricide resistance on genetic basis and due to their high proliferation, the short life cycle and the continuous selection with pesticides in order to control them, resistance will develop in short time.

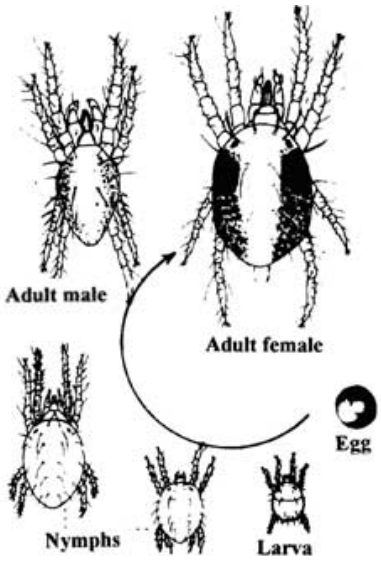


Figure 1.1: Life cycle of two-spotted spider mite (*T. urticae*)

1.2 Control of the spider mite, *Tetranychus urticae*

Spider mites are major enemies of greenhouse plants and ornamentals and their control is of great importance. There are several methods in order to control spider mites such as i) cultivation measures, ii) biological control and iii) chemical control.

i) Cultivation methods and resistant plant varieties:

Cultivation methods (addition) might be adapted in order to control spider mites. Resistant plant varieties could be/is an approach in order to control the spider mite population that infests crops. For example, Miyazaki et al. (2012) tested several cotton genotypes for their susceptibility against spider-mites. They demonstrated that there are 3 cotton genotypes that are mite-resistant in terms of low mite density and low damage of the plant. Another method is the control of atmosphere in the greenhouse. Increased humidity levels affect the spider mite population (Attia et al., 2013).

ii) Biological Control:

Biological control is based on the use of natural enemies. Phytoseeid mites such as *Phytoseiulus permisilis* and *Neioseiulus californicus*, the gall midge *Feltiella acarisuga* and the ladybeetle *Stethorus punctillum* are major predators of spider mites and are successfully used in greenhouses (Attia et al., 2013; McMurty and Croft, 1997; Zhang, 2003). Also,

entomopathogenic fungi could lead to efficient management of spider mites. Fungi applications such as *Beauveria bassiana* and *Paecilomyces fumosoroseus* on spider mites cause high mortality rates (Attia et al., 2013). The application of mineral oils is a physical and a biological method for controlling spider mite populations. Some oils act as an oviposition repellent and their effect has been documented on many arthropod pests including spider mites (Vincent et al., 2003). Garlic essential oil is known for its acaricidal activity. It causes high mortality rates and low fecundity levels when it is applied in spider mites (Attia et al., 2012). The use of oils will be a great addition in controlling spider mites as resistance to these compounds has not reported up to date (Attia et al., 2013, Vincent et al., 2003)

iii) Chemical Control:

Acaricides are the major weapon against spider mites. Several of those compounds, such as the organophosphates and the pyrethroids, act against both insects and acari, while other are specific to acari. Some insecticides / acaricides act on specific life stages, e.g. all life stages (fenpyroximate), just eggs or immature stages (hexythiazox). Also, most of the insecticides target on muscle /nervous system (pyrethroids, macrocyclic lactones) and other affect the mitochondrial respiratory chain (METIs) or regulate the growth (Figure 1.2).

Among several insecticides which have been used against mites there are three chemical groups, the avermectins, the METIs and the pyrethroids which have been used more often in several geographical regions such as Belgium, Turkey, Greece, Korea, Brazil and other countries.

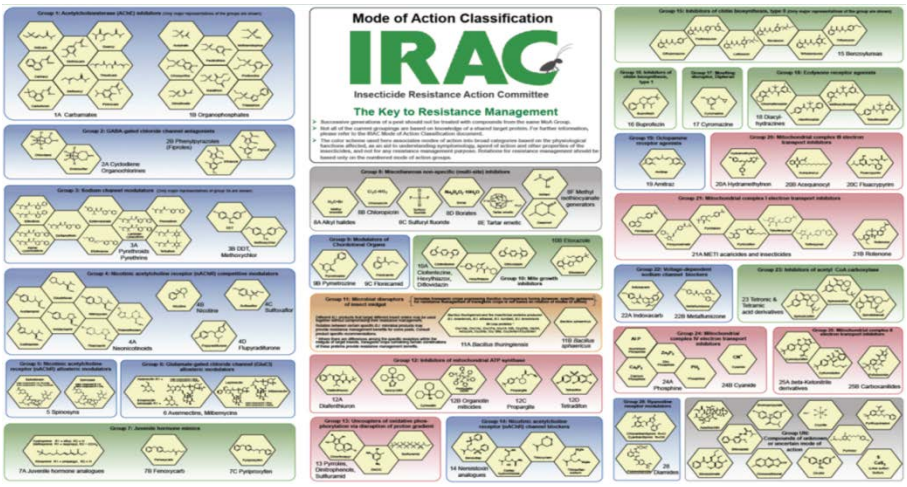


Figure 1.2: Insecticide Resistance Action Committee (IRAC) insecticide classification according to the mode of action of insecticides. Colors represent the targeted physiology. Blue: nerve and muscle, green: growth and development, red: respiratory, brown: midgut, grey: unknown (source: http://www.irac-online.org/content/uploads/econnection36.pdf).

Avermectins: Avermectins belong to the class of macrocyclic lactones. They are produced from the fermentation of the soil microorganism *Streptomyces avermitilis* (Clark J. M. et al., 1994; Van Leeuwen et al., 2009b). The potential activity of avermectins as insecticides and acaricides, except their excellent activity against nematodes, was found by Merck at 1975 (Shoop et al., 1995). They act on glutamate-gated chloride channels, leading to the activation of the chloride ion channel, causing paralysis in the target pest. Avermectins are used as antiparasitic drugs for animal health as well as controlling insect pests belonging in different orders such as Coleoptera, Isoptera, Hymenoptera, Diptera, Lepidoptera (McKellar and Benchaoui, 1996; Putter et al., 1981). Abamectin is frequently used to control spider mites in many crops.

METIs (Mitochondrial Electron Transport Inhibitors): METI insecticides were launched in the early 1990s. Four compounds (pyridaben, fenpyroximate, tebufenpyrad and fenazaquin) were developed preferably or specifically for spider mite control. Although these compounds belong to different chemical families (quinazolines, pyrimidinamines, pyrazoles and pyridazinones), they show the same mode of action by inhibiting the complex I of the mitochondrial respiratory chain (Van Leeuwen et al., 2009). Particularly, these compounds lead to the translocation of the proton from NADH to ubiquinone oxidoreductase (Hollingworth and Ahammadsahib, 1995; Lummen, 2007). Recently, two beta-ketonitrile derivatives were developed which target the complex II of mitochondrial respiratory system inhibiting the succinic dehydrogenase (Nakahira, 2011). Fenpyroximate, which targets complex I, is very effective against all life stages of *T. urticae* (Koch) and *Panonychus citri* (Motoba et al., 1992), showing very low toxicity against beneficial insects, animal-parasitic mites and soil-living mites (Motoba et al., 1992; Van Leeuwen et al., 2010). Cyenopyrafen (acts on complex II) is a relatively new developed and commercialized acaricide which shows strong acaricide activity and very low toxicity against beneficial insects and bees (Yu et al., 2012).

Pyrethroids: The introduction of synthetic pyrethroids was in 1970's (Khambay and Jewess, 2010). They are synthetic analogues of the natural insecticidal esters of chrysanthemic acid (pyrethrins I) and pyrethric acid (pyrethrins II), originally found in the flowers of *Chrysanthemum cinerariaefolium* (Davies et al., 2007). Pyrethroids traget on voltage gated sodium channel (VGSC). Either they enhance activation or inhibit inactivation and deactivation of them, resulting in prolonged opening, thus leading to paralysis and death of the targeted pest (Dong et al., 2014). There are two types of pyrethroids, Type I and Type II. Type I pyrethroids, such as bifenthrin, lack the α-cyano group, which is present in

Type II pyrethroids. Pyrethroids are used for controlling mosquitoes but they are also used against Lepidoptera, mites and aphids (Khambay and Jewess, 2010).

1.3 Insecticide Resistance and mechanisms

Insecticide resistance is termed as a heritable phenomenon where population's susceptibility to a toxin decreases, as the toxin is applied for successive generations (Heckel, 2012). Alternatively, resistance is defined by IRAC as "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/).

Insecticide resistance is an evolutionary process. Random mutations occur in the population. These mutations are rare in the population. However, their frequency increases under selection pressure, imposed by the insecticide applications. Through preferential survival, the mutations increase in frequency throughout the population. As the mutation becomes common among the individuals of the population, the effectiveness of the insecticide is reduced thus after several generations resistant alleles spread in the population, the susceptible phenotypes decrease and resistance to insecticides develops (Metcalf, 1989).

Populations exhibit variable levels of resistance to insecticides, which scales up to 1000 fold. In some cases they might exhibit resistance to more than one insecticides, without being previously exposed to the latter one. In this case, a single gene or mechanism is responsible for this phenotype. This situation is called crossed resistance (Metcalf, 1989). Also, the phenomenon of multi-resistance may occur. In this situation the population shows resistance to many unrelated insecticides due to the effect of multiple mechanisms (http://www.entsoc.org/PDF/2013/EPAResistanceTerms-2013.pdf).

The first case of resistance documented in 1914 was involved the development of resistance i*n Quadraspidiotus perniciosus* to lime sulphur. The cases of resistance were sporadic until the mid-40s (Sparks and Nauen, 2015). During 1940 DDT was introduced and in 1947, resistance was confirmed in houseflies. From mid-40s until today many insecticides have been produced and they have been commercially available. However, numerous mosquitoes (Vontas et al., 2012) and agricultural pests, such as Lepidoptera (Qian et al., 2008, Pu et al., 2009), Hemiptera (Wang and Wu, 2007) and Acarina have developed resistance to a broad spectrum of compounds in the same pace with insecticide discovery (Table 1.1 – Sparks and Nauen, 2015).

Table 1.1: Resistant pest species

Species	Common name	Order	No. of compounds
Tetranychus urticae	Two-spotted spider mite	Acari	93
Plutella xylostella	Diamondback moth	Lepidoptera	91
Myzus persicae	Green peach aphid	Hemiptera	75
Musca domestica	House fly	Diptera	58
Bemisia tabaci	Sweet potato whitefly	Hemiptera	54
Leptinotarsa decemlineata	Colorado potato beetle	Coleoptera	54
Aphis gossypii	Cotton aphid	Hemiptera	48
Panonychus ulmi	European red mite	Acari	48
Helicoverpa armigera	Cotton bollworm	Lepidoptera	47
Boophilus[c] *microplus*	Southern cattle tick	Ixodida	44
Blattella germanica	German cockroach	Blattodea	43
Spodoptera litura	Mediterranean climbing cutworm	Lepidoptera	38

The mechanisms of insecticide resistance are classified into four main categories: behavioral resistance, penetration resistance, target site resistance and metabolic resistance. Metabolic and target site resistances are the most well studied ones.

1.3.1 Behavioral resistance is defined as the development/evolution of behaviors that reduce an insect's exposure to toxic compounds or that allow an insect to survive in a harmful environment (Sparks et al., 1989). Behavioral resistance mechanisms can be subdivided in two categories:

i) Stimulus - dependent mechanisms include both irritancy and repellency which require contact with the insecticide and

ii) Stimulus - independent mechanisms is the situation when the population prefers habitats other than those normally treated in order to avoid contact with the insecticide (Chareonviriyaphap et al., 2013; Sparks et al., 1989).

Behavioral resistance has been documented in several classes of insecticides, including pyrethroids (Lockwood et al., 1985; Russell et al., 2011).

1.3.2 Cuticle resistance (reduced penetration or increased excretion) is a heritable characteristic that reduces the effective dosage of the insecticide that reaches the hemolymph, via reduced penetration of the toxin through the cuticle or increased excretion. This gives more time to the detoxification enzymes to metabolize the toxin before it reaches the target (Pittendrich et al., 2007; Strycharz et al., 2013). Decreased penetration has been associated with increased thickness of the cuticle (Wood et al., 2010) because of increased

secretion of lipids, chitin and hydrocarbons (Plapp and Hoyer, 1968; Juarez and Fernadez, 2007).

1.3.3 Target site (or toxicodynamic) resistance is termed as the situation where modification of the target site occurs, either by mutation(s) that alter the protein structure or by mechanisms that lead to changes of the expression of the target site (Van Leeuwen and Dermauw, 2016). Thus, binding of the insecticide on the active site of the target is prevented resulting in reduced effect of the toxic compound (http://www.irac-online.org/about/resistance/mechanisms/).

Modifications of the target that the insecticide directly interacts have been documented in many cases. These modifications regard aminoacid substitution(s) on the target protein. Alteration of the glutamate gated chloride channel has been reported in several species*. T. urticae,* unlike insects, express 5 GluCl genes that might be putative targets of abamectin. Two non-synonymous mutations have been detected (G314D and G326E) in two of the five channels and associated with abamectin resistance (Dermauw et al., 2012). Additionally in insects, such as *Plutella xylostella* a deletion of 36 bp has been associated with the specific phenotype (Liu et al., 2014). A single mutation in *Drosophila melanogaster* (P299S) has been associated with resistance to ivermectin (14 folds) (Kane et al., 2000).

Numerous cases have linked amino acid substitutions on sodium channel with resistance to pyrethroids. Although the vast number of mutations identified in VGSC, not all of them contribute conclusively to pyrethroid resistance. Two mutations (L1014 and M918) alter the structure of VGSC in insects with 81 reported cases carryin one or both mutations (Feyereisen et al., 2015). For instance, a leucine to phenylalanine substitution at position 1014 (segment 6 of Domain II) has been associated with resistance to pyrethroids in many insect species, such as *Anopheles gambiae* (Jones et al., 2012), *Myzus persicae* (Martinez-Torres et al., 1999), *Plutella xylostella* (Sonoda et al., 2008), *Tuta absoluta* (Haddi et al., 2012), *Musca domestica* (Williamson et al., 1996) and other insects. In some cases a combination of L1014F and other mutation(s) has been identified providing higher resistance levels. For example, a combination of mutations has been found in *Musca domestica* that is L1014F and M918T. Further work showed that when each mutation is expressed alone in Dm sodium channel causes 5-10 folds reduction of sensitivity to deltamethrin, whereas when both are co-expressed sensitivity to deltamethrin is abolished (Dong et al., 2014). Substitution of phenylalanine to isoleuine (F1538I) that is positioned in segment 6 of Domain III was identified in resistant to pyrethroids *Rhipicephalus microplus* (He et al., 1999) and its expression in cockroach sodium channel shows high insensitivity to different types of pyrethroids (Tan et al., 2005).

1.3.4 Metabolic (or toxicokinetic) resistance is the situation when the organism over-expresses enzymes that are capable of sequestering (binding) and/or detoxifying the insecticide into a non- or less-toxic compound. There are three key detoxification enzyme families which are usually divided in two categories, Phase I and Phase II enzymes. The detoxification enzymes that play important role in metabolic resistance are the cytochrome P450 mono-oxygenases (P450s / Phase I enzymes), the carboxylesterases (CCEs / Phase I enzymes) and the glutathione – S – transferases (GSTs / Phase II enzymes – (Perry et al., 2011)). The mechanism underlying the over-expression of these enzymes is unknown in many cases, but some causes are gene amplification (Grigoraki et al., 2015; Puinean et al., 2010) and mutation on cis- or trans- regulators (Feyereisen et al., 2015). Also, aminoacid substitutions have been reported to affect the activity of the detoxification enzymes (Campbell et al., 1998; Cui et al., 2011).

1.3.4.1 Cytochrome P450 monooxygenases are involved in a number of metabolism reactions of compounds (Scott and Wen, 2001). Their feature is a characteristic absorbance near 450 nm (called Soret peak) of their Fe-CO complex from which they were named (Feyereisen, 2012). They catalyze a number of reactions but these enzymes are mostly known for their mono-oxygenase reaction, transferring one atom of molecular oxygen to a substrate (RH), reducing the other to water (Feyereisen et al., 2012 – eq. 1)

$$RH+O_2+NADPH+H^+ \rightarrow ROH+H_2O+NADP^+ \text{ (eq. 1 from Feyereisen, 2012)}$$

P450s are classified in two main classes depending on the manner that electrons are delivered from NADPH to their catalytic site (Werck-Reichhart and Feyereisen, 2000). Class I P450s require both FAD-containing reductase and an iron sulfur redoxin. In this class belong some bacterial and the mitochondrial P450s (Figure 1.3 upper row – Reichart Feye, Paine 2005). Class II P450s are the most well-studied and common ones in eukaryotes. They are responsible for a series of catalytic reactions such as the metabolism of xenobiotic substances as well the biosynthesis of hormones. They are ER-bound proteins and their function depends on their redox partner, cytochrome P450 reductase (CPR), which contains FAD- and FMN- cofactors. CPR is also anchored in the ER membrane (Figure 1.3 – down left). Also, the cytochrome b_5 is an important co-factor in the P450 monooxygenase system which may enhance the activity and catalytic efficiency of P450 enzymes. Moreover, there are P450 enzymes that are self sufficient and have evolved from the fusion of the P450 and CPR (Bernhardt, 2006; Paine et al., 2005) – Figure 1.3 – down right). These fused enzymes are found in bacteria and fungi, with the most known example of P450-BM3 isolated from *Bacillus megaterium*.

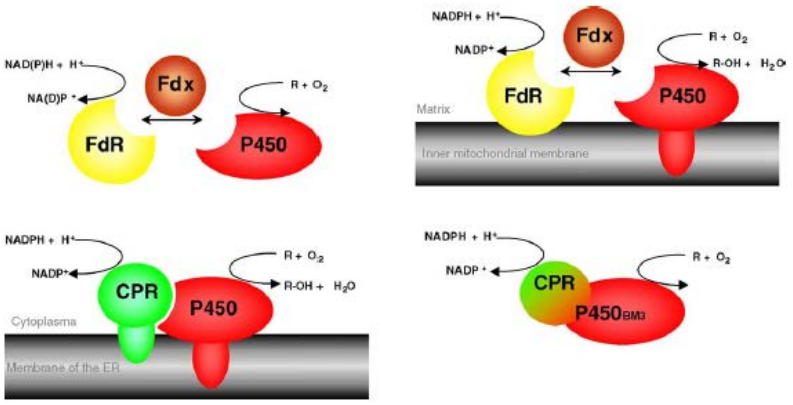


Figure 1.3: Classes of P450s. Upper part depicts bacterial and mitochondrial P450s (Class I). Lower part left shows the ER anchored P450s (Class II). Lower part right shows the self sufficient system (P450-BM3/adapted from Bernhard 2006).

The CYPome size of insects and mites is variable (Table 1.2). The sequencing of *T. urticae* genome revealed that this organism has eighty-six cytochrome P450 (CYP) genes, where most of them (48 P450 genes) belong to CYP2 clan in comparison to other insect and crustacean species and are lineage specific (Grbic et al., 2011a). The *T. urticae* CYP2 clan consists of intron-less genes which belong to a new family, CYP392.

Metabolism assays indicate that P450s by either microsomal preparation (Yoon et al., 2002) or by functional expression of recombinant P450s (Ding et al., 2013; Karunker et al., 2009; Muller et al., 2008; Stevenson et al., 2011; Zhu et al., 2010) are capable of metabolizing several classes of insecticides.

Table 1.2: CYPome size of various species (retrieved from Feyereisen, 2012).

	CYPome size	Reference
Insecta		
Drosophila melanogaster	88	Tijet et al., (2001)
Anopheles gambiae	105	Ranson et al., (2002a)
Aedes aegypti	160	Strode et al., (2008)
Culex quinquefasciatus	170	Arensburger et al., (2010)
Pediculus humanus	36	Lee et al., (2010)
Bombyx mori	85	
Apis mellifera	46	Claudianos et al., (2006)
Nasonia vitripennis	92	Oakeshott et al., (2010)
Camponotus floridanus	132	Bonasio et al., (2010)
Harpegnathos saltator	93	Bonasio et al., (2010)
Tribolium castaneum	134	Tribolium Genome Sequencing Consortium (2008)
Acyrthosiphon pisum	64	
Pediculuc humanus	36	Lee et al., (2010)
Crustacea		
Daphnia pulex	75	Baldwin et al., (2009)
Acari		
Tetranychus urticae	86	Gbric et al., (2011)

1.3.4.2 Carboxylesterases (CCEs) are enzymes that can hydrolyse ester bonds from various substrates with a carboxylic ester and generate an alcohol and carboxylate products (Figure 1.4). CCEs are involved in detoxification of insecticides as they are considered as Phase I enzymes but they can also sequester insecticides and delays or prevents the interaction of the compound with the target site (Wheelock et al., 2005). Resistance to insecticides has been associated with over-expression or aminoacid substitution of these enzymes. Over-expression of esterases in resistant strains usually occurs by gene amplification and has been associated with insecticide resistance in *Myzus persicae* (Devonshire et al., 1998) and mosquitoes (Grigoraki et al., 2015). Single point mutations of the esterases are thought to play role in increased metabolism. Campbell et al. (1998) reports that E3 confers resistance to malathion in *Lucilia cuprina* through Trp251Leu substitution, whereas the Gly139Asp substitution of E3 confers resistance to abroad spectrum of organophosphates.

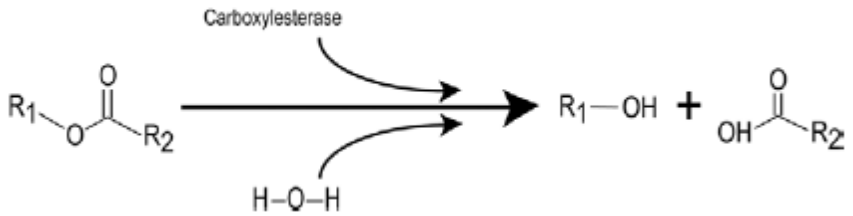


Figure 1.4: Hydrolysis reaction catalysed by CCEs (from ref. (Montella et al., 2012))

1.3.4.3 Glutathione – S – Transferases (GSTs) are a large family of multifunctional enzymes that are involved in xenobiotic metabolism by catalyzing the conjugation of electrophilic compounds with the thiol group of reduced glutathione (GSH). The resultant products are more water-soluble and excretable than the non-GSH conjugated substrates (Enayati et al., 2005). These enzymes have been associated with resistance to many insecticides, such as pyrethroids, organochlorines and organophosphates (Huang et al., 1998; Pavlidi et al., 2015; Stumpf and Nauen, 2002; Vontas et al., 2001). It has been indicated that GSTs are capable of detoxifying insecticides, such as DDT and organophosphates. The most well-known case is GSTe2 from *Anopheles gambiae* that metabolises DDT into the non-insecticidal compound DDE (Ranson et al., 2001).

1.3.5 Insecticide / Acaricide resistance reports and mechanisms in *Tetranychus urticae*

As presented in Table 1.1, *T. urticae* is the most resistant species in terms that it has developed resistance to many compounds of different mode of action, showing over 200 cases of resistance (Sparks and Nauen, 2015). Resistance of *T. urticae* to avermectins, METIs and pyrethroids has been reported in several cases.

Spider mites show elevated resistance levels to abamectin (Sato et al., 2005; Vassiliou and Kitsis, 2013). Cross resistance between abamectin and milbemectin has been observed in Brazilian populations of spider mites (Nicastro et al., 2010), although it is a matter of debate. Genetic studies of abamectin resistance on spider mites imply its recessive or intermediate and polyfactorial nature ((Argentine et al., 1992; Dermauw et al., 2012; He et al., 2009; Pu et al., 2009; Yorulmaz and Ay, 2009). One mechanism that has been associated with abamectin resistance is the target site modification. Kwon et al. (2010c) investigated resistance to abamectin in *T. urticae* showing that a mutation (G314D) on glutamate gated chloride channel is responsible for this phenotype. Dermauw et al. (2012) identified six orthologous genes encoding for glutamate gated chloride channels and reported the existence of an additional mutation on glutamate gated chloride channels, G326E. This mutation has been associated with high resistance levels to abamectin.

Biochemical and bioassay data supported that detoxification enzymes play also a role in abamectin resistance. Kwon et al. (2010c) showed that P450s and esterases are highly expressed in abamectin resistant strain of *T. urticae*. Also, Stumpf and Nauen (2002) examined several spider mite populations collected from the field. Three of these populations (populations from Netherlands, from Brazil and from Colombia) showed moderate resistant

levels to this compound. The populations from Netherlands were subjected to synergism studies indicating that P450s and GSTs are involved in resistance to abamectin. All three populations were used for biochemical studies indicating positive correlation between the phenotype and elevated activity of P450s and GSTs. Recently, (Piraneo et al., 2015)) tested by qPCR the relative expression levels of 3 cytochrome P450s from field populations showing resistance to abamectin. Among these P450s, CYP392D8 was constitutively over-expressed in all field populations tested. Genome wide microarray analysis and subsequent validation by qPCR, of the multi- resistant strains Mar-ab, which is highly resistant to abamectin and to other compounds also, and MRVP revealed that several P450s are associated with resistance to abamectin (Demaeght et al., 2013). Finally, Pavdili et al. (2015) demonstrated that a glutathione-S-transferase from the Mar-ab strain is inhibited by abamectin, indicating a putative role of this enzyme in abamectin resistance.

METI insecticides/acaricides have been used successfully for several years against *T. urticae*. However, there have been reports for development of resistant phenotypes (Van Leeuwen et al., 2010). Cross resistance among METIs has been also observed (Stumpf and Nauen, 2001; Sugimoto and Osakabe, 2014; Van Pottelberge et al., 2009b). For example, field selection with tebufenpyrad led to high cross resistance to pyridaben, fenazaquin and fenpyroximate (Devine et al., 2001; Stumpf and Nauen, 2001; Van Pottelberge et al., 2009b). Also, laboratory selections with fenpyroximate conferred cross resistance between METIs (Kim et al., 2004). Genetic experiments indicate that resistance to pyridaben and fenpyroximate is completely dominant and monogenic, whereas resistance to tebufenpyrad is dominant but under the control of more than one genes (Van Pottelberge et al., 2009, Devine et al., 2001). Sugimoto and Osakabe (2014) studied the cross – resistance between the recently developed cyenopyrafen and pyridaben, investigating the mode of inheritance for both compounds. Their results showed that mode of inheritance for both cyenopyrafen and pyridaben resistance is (in)completely dominant. Experiments with synergists showed that resistance to cyenopyrafen and pyridaben is, at least partially, due to P450 and esterase activity. Khalighi et al. (2014) examined resistance levels of cyenopyrafen and cyflumetofen (another recently developed beta keto-nitrile) in a number of laboratory strains and field-collected populations. The multi-resistant strain (MR-VP) showed the highest resistance levels against cyenopyrafen and cyfluometofen (>30-folds, and relevant for field efficacy). Cyenopyrafen resistance in both MR-VP and TU008R strains was synergised by PBO, indicating the possible involvement of P450 oxidases in the phenotype.

Biochemical and synergism studies indicated that METI-resistance is associated with elevated P450 activity in many different strains (Stumpf and Nauen, 2001; Tirello et al., 2012; Van Pottelberge et al., 2009b). MR-VP strain, which is resistant to pyridaben, fenpyroximate and tebufenpyrad, showed 20-fold higher P450 activity measurements with the model substrate 7-EFC (Van Pottelberge et al., 2009). Also, toxicity assays with PBO showed suppression of the phenotype almost 100-fold for the field collected MR-VP strain (Van Pottelberge et al., 2009) and over 300-folds for the selected strain FR-20 (Kim et al., 2004).

High resistance levels to pyrethroids have been reported in several studies (Ay and Gurkan, 2005, Van Leeuwen et al., 2005, Tsagkarakou et al., 2009, Kwon et al., 2010). The mode of inheritance of resistance to bifenthrin is completely recessive as it is indicated by Tsagkarakou et al. (2009).Aminoacid substitutions on the sodium channel were investigated in two greek resistant populations to pyrethroids (Tsagkarakou et al., 2009). This study indicated that a mutation takes place on segment 6 of domain III on voltage gated sodium channel, where a phenylalanine (F) is substituted by isoleucine (I) at position 1538 (Musca domestica numbering). Similarly, Kwon et al., (2010) showed that another mutation occurs at segment 6 of Domain II of voltage gated sodium channel in a fenpropathrin resistant population. In this study a leucine (L) is substituted by valine (V) at position 1024 of amino acid sequence. In both of these reports it is mentioned that these mutations are accompanied by a mutation on II/III linker of sodium channel (A1215D). Also, M918T mutation was identified for the first time in spider mite populations (*T.evansi)* collected from Malawi. These populations show moderate resistance levels to bifenthrin (Nyoni et al., 2011).

Biochemical evidence pointed to elevated activity of esterases and/or P450s (Ay and Gurkan, 2005, Van Leeuwen et al., 2005, Tsagkarakou et al., 2009) implicating these detoxifications genes in resistance to pyrethroids. Feng et al. (2011a) examined the expression levels of two esterases (TCE1 and TCE2) in pyrethroid, abamectin and omethoate resistant strains indicating that only TCE2 is highly over-expressed in the resistant strains compared to the susceptible one. Also, groups of individuals from the susceptible population were induced with those insecticides showing that TCE2 is expressed after induction.

1.4 Approaches and techniques for analysing insecticide/acaricide resistance

Analysis of the insecticide/acaricide resistant phenotypes and link to metabolic and/or target-site resistance, as well as specific genes and mutations, can be performed by several approaches and techniques, which are briefly described below:

1.4.1 Establishing the resistance phenotype

Bioassays: This is the initial test in order to detect and measure the intensity of the phenotype, as well as associate it with a putative resistance mechanism. More specifically:

(a) Conventional bioassays: It is the first step in order to determine the levels of resistance to one or more insecticide compounds, in comparison to the susceptible strain. The compounds tested could be of different mode of action and/ or chemical group in order to evaluate the cross – or multi-resistance spectrum. This is informative for the mode of action of the insecticides and by extension an indication about the mechanisms that play role in resistance. For example, DDT and pyrethroids act at the same domain on voltage gated sodium channel although they belong to different chemical classes; cross resistance between those active ingredients, indicate the presence of target site resistance.

(b) Combined bioassays - Synergism studies: Synergists are generally non-toxic compounds that enhance the toxicity of an insecticide. These kind of studies are used as first indicator of metabolic resistance mechanisms. The most common enzyme inhibitors that synergise toxicity are Piperonyl Butoxide (PBO) for inhibition of Cytochrome P450, diethyl maleate (DEM) that inhibits GSTs and S,S,S-tributyl phosphorotrithioate (DEF) which acts against CCEs. Generally, if detoxification enzymes (e.g. P450s) play role in resistance, the application of synergist (PBO) should reduce the resistant levels.

Biochemical assays: The involvement of metabolic resistance can be analysed by using diagnostic/model substrates in order to detect enzyme activity on crude insect homogenates. There are several general model substrates in order to identify if metabolic resistance is due to the elevated activity of Cytochrome P450s, GSTs and /or esterases. These substrates do not provide any further information on which particular P450, for example, enzyme(s) is / are highly active but only an indication about the mechanism that is responsible for the resistant phenotype. In case of P450s, it has been reported that the model substrates 7-ethoxy-4-

trifluoromethylcoumarine, 7-methozyresorufin and 7-ethoxyresorufin are good candidates for measuring P450 activity in *T. urticae* (Van Pottelberge et al., 2008).

1.4.2 Association studies to specific molecular markers

Identification of gene mutations associated with resistance: In cases where the mode of action of the insecticide and its target site is known, mutations on the gene encoding the target protein that the insecticide binds can be identified by sequencing of this gene and comparing resistant versus susceptible sequences. Examples of target site mutations have been listed in sections 1.3.3 and 1.3.5.

Gene expression: With this approach candidate detoxification genes are identified for metabolic based resistance by microarrays and/or next generation sequencing (e.g. RNA seq). The expression levels of these genes are compared between resistant and susceptible phenotype. Genes with high expression levels are validated by qPCR technique. Microarray analysis data between a resistant and susceptible strain of *T. urticae* revealed the upregulation of several detoxification genes/transcripts, such as P450s, GSTs, CCEs and dioxygenases, indicating their putative role in acaricide resistance. Further validation of them by qPCR technique designates their probable involvement in acaricide phenotype (Dermauw et al., 2013, Khalighi et al., 2016). In *Trialeurodes vaporariorum,* a resistant to pyriproxifen strain showed several detoxification genes to be over-expressed. qPCR validation experiments indicated that a P450 gene is over-expressed in the resistant strain (Karatolos et al., 2012). More recently, next generation sequencing of *Aedes albopictus* revealed the up-regulation of esterases in the temephos resistant strain. Subsequent qPCR experiments indicated the association of esterase gene amplification with resistance to temephos (Arouri et al., 2015; Grigoraki et al., 2015; Karatolos et al., 2012).

Genetic Mapping and genetic association studies: An important tool, once the genome is available or it is about to be, is the mapping of the loci on the genome. This approach gives insights to the genetic structure of a phenotypic trait, locate and identify candidate genes responsible for the specific trait as well as provides information how the phenotype may be evolving (QTL mapping) (Hawthorne, 2003; Heckel, 2003). Several studies have exploited the existence of known markers in order to identify genomic regions associated with insecticide resistance (QTL – Quantitative Trait Loci). Usually a dense linkage map is used in combination with cross experiments between two strains that differ in one or more traits. For example, (Ranson et al., 2000)) identified two loci (*rtd1* and *rtd2*) responsible for DDT resistance in *A. gambiae* that are not linked to any known GST, which is believed that plays role in pyrethroid

resistance. (Wondji et al., 2007)) associated pyrethroid resistance in *An. funestus* to a locus (*rp1*) existing on chromosome 2R. This locus is linked to a cluster of CYP6 P450 genes hypothesizing that one or more of these P450s provide resistance to pyrethroid, as earlier studies has shown that phenotype of resistance is not due to target site mutations. Another technique for QTL mapping is Bulk Segregant Analysis (BSA). BSA was developed by plant geneticists in order to identify rapidly markers in a specific genomic region. The underlying principle is that groups of individuals, ideally inbred lines, are studied against the same genomic region that is responsible for the phenotypic trait, which is dissimilar between the groups. The samples under study are screened for differences with markers (RFLP, RAPD) and those that are polymorphic between the pools are linked to the loci (Michelmore et al., 1991). Based on that, Van Leeuwen et al. (2012) and Demaeght et al. (2014) adapted BSA mapping in order to identify the locus responsible for etoxazole, clofentezine and hexythiazox resistance phenotype, which is located on scaffold 3 and chitin synthase gene is on the same scaffold, indicating that a non synonymous point mutation in the etoxazole resistant strain is responsible for the phenotype. Also, BSA analysis coupled with RNA – seq used by (Park et al., 2014) in order to identify mechanisms responsible for resistance, as genomic resources for *S. exigua* are scarce. Through BSA, by using as reference the genome of *Bombyx mori*, and expression studies they identified a region that contains 3 ABC type C transporters (ABCC1, ABCC2, ABCC3) and a deletion in one of them (ABCC2). These characteristics together with experiment of partial silencing of these genes render them the major mechanisms for resistance to Cry1Ca (Bt toxin). Possibly, BSA will be a valuable tool for locating resistance and nonresistance traits.

The contribution of known target site mutations to the phenotype of resistance could be examined by genetic association studies, e.g. by using isogenic lines (same genetic background between the strains) or introduction of the mutation in a susceptible genetic background. Kwon et al. (2010c) examined abamectin resistance phenotype between a field resistant spider mite strain and the susceptible one that originated from the field strain, keeping it under no selection pressure (isogenic lines) and they identified the amino acid substitution on GluCl. Also, (Brito et al., 2013) conducted genetic crosses for several generations in mosquitoes so as to introduce two kdr mutations into susceptible genetic background and then examined the role of these mutations in pyrethroid resistance as well the fitness cost might confer.

1.4.3 Validation of the association between markers and resistance phenotype

In silico studies of the binding of the insecticide to the altered target and/or detoxification enzymes by using closely related crystal structures (O'Reilly et al., 2006) is an important step, as many new non-silent mutations are discovered and are usually associated with insecticide resistance. Molecular docking prediction could show possible interactions between the insecticide and the P450 enzyme. For instance Karunker et al. (2009) showed that CYP6M1vQ should be capable of binding imidacloprid and hydroxylate it in at least one position.

***In vitro* assays:** Involve the isolation and expression of detoxification enzymes in heterologous systems, such as bacteria, baculovirus and yeast in order to identify their ability to metabolize insecticides. Subsequently, these studies are coupled with mass spectrometry analysis of the identified metabolite in order to detect the position of hydroxylation, in the case of P450s. There are many examples in literature that P450s, for example, are capable of metabolizing insecticides and by MS analysis the position of the addition of molecular oxygen was identified on the metabolite (Demaeght et al., 2013; Ding et al., 2013; Joussen et al., 2012; Karunker et al., 2009). Also, molecular docking of the insecticide is used in these studies (Karunker et al., 2009)

Electrophysiological studies are used to examine the properties of altered target site to the phenotype of resistance by *in vitro* expression in Xenopus oocytes. Mutations of sodium channel have drawn great attention as this is the target of pyrethroids, a compound used majorly for controlling mosquitoes and other pests. Tan et al. (2005) showed that L993F and F1519W in cockroach reduced pyrethroid binding on sodium channel. Also, (Jiang et al., 2015) showed that two novel mutations from indoxacarb resistant Lepidoptera (*P. xylostella*) are positioned on segment 6 of Domain IV. Xenopus oocytes expressing the mutated channels show reduced sensitivity in the sodium channel blockers used for this study (indoxacarb, DCJW and metaflumizone).

In vivo functional assays: These kind of assays are of utmost importance. In this way the role of the candidate for resistance enzyme could be directly observed either on the same organism or by ectopic expression.

I) RNAi: Silencing of RNA has been used successfully in *T. castaneum* (Zhu et al., 2010) indicating that over-expression of a P450 enzyme is the major factor in deltamethrin resistance. Also, through RNAi in mosquito, it was validated that GSTe7 and GSTe2 play role in pyrethroid resistance. However, RNAi technique has many difficulties as dsRNA is not always

systemically distributed, injury of the organism by injections for inserting the molecule and silencing in alternative tissues. The disadvantage of injury has been outreached in some cases as administration of the molecule has been done via feeding and the use of transgenic plants (Mao et al., 2011; Shi et al., 2016). However, RNAi approach is not easily done and replicated in some species like *T. urticae*.

II) Transgenesis: The method of the ectopic expression of detoxification genes from major pests and mosquitoes in *Drosophila* is the most commonly used one according to literature (Daborn et al., 2012; Riveron et al., 2013; Zhu et al., 2010). This insect has many advantages including the easiness of mapping of insertion of the gene on the chromosome. Moreover, amino acid alteration of the target gene via CrispR in Drosophila will shed light on the contribution of target site mutation on the phenotype of resistance *in vivo*.

1.5 Insecticide Resistance Management

1.5.1 Practices to prevent resistance

FAO and IRAC suggest several practices in order to prevent the development of resistance to insecticides. These practices include sanitation measures, use of resistant crop varieties, avoidance of year-round cultivation, use and protection of beneficial insect and acari populations as well as preserve refugees of susceptible insect and acari populations. Also, the application rates of the approved for use insecticides should be according to the guidelines indicated on the insecticide label. A good approach is the rotation of chemical compounds that are unrelated, in terms they have different mode of action. This approach assumes that if resistance exists in one insecticide it will decline when the second is applied (Figure 1.5). Once the mechanism and the target that the insecticide acts are known, the rotating insecticides could be chosen wisely (FAO, 2012, IRAC, 2006).

1.5.2 Evidence based resistance management

The knowledge of the mode of action, the underlying mechanisms and genetics behind insecticide resistance are very important steps in order to fight with this global phenomenon.

It is very important to monitor the field populations for appearance of resistance cases. This could be done by insecticide dose-response curves (bioassays) as well as the development and introduction of molecular tools/ diagnostics to assess resistance levels in the field. The use of the diagnostics will be helpful for early detection of field resistance, a case for easier and early decision management. The knowledge of all the parameters regarding the action of the

insecticide (target-site, mechanism, genetics) with the contribution of diagnostics tools (molecular, immunological) are major components for management of resistance in the field, based on scientific evidence and is helpful for preventing development of resistance cases in the future(FAO, 2012; IRAC, 2006; Ramasubramanian et al., 2005).

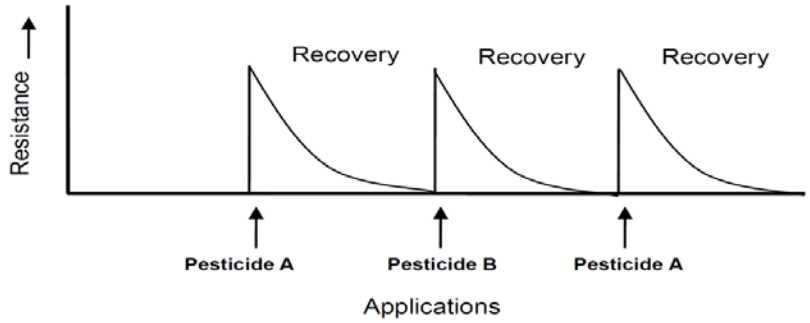


Figure 1.5: Effect of insecticides of different mode of action in a rotation system for insecticide resistance management (from FAO, 2012).

Aim of the PhD study

The aim of the study is to determine/ examine the underlying resistance mechanisms of spider mites to abamectin, METIs and pyrethroids. Specifically the three research chapters of this study are dealing with:

1. Functional characterization and expression *in vitro* of *Tetranychus urticae* cytochrome P450s associated with high levels of acaricide resistance with emphasis on their ability to metabolise – detoxify insecticides.
2. Heterologous expression of *T. urticae* P450s and homologous CPR in *Drosophila* in order to investigate the functional role of genes in resistance *in vivo*
3. Genetic approaches to determine the relative contribution of individual target site mutations in the resistance phenotype of *T. urticae.* I introduced known mutations in susceptible genetic background, and determined the effect of individual mutations, alone or in combination, to the phenotype of resistance

Chapter 2: Functional characterization and expression *in vitro* of *Tetranychus urticae* cytochrome P450s associated with high levels of acaricide resistance

(This chapter has been redrafted from:

Riga, M., Tsakireli, D., Ilias, A., Morou, E., Myridakis, A., Stephanou, E. G., Nauen, R., Dermauw, W., Van Leeuwen, T., Paine, M., Vontas, J., 2014. Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in *T. urticae.* Insect Biochem Mol Biol 46: 43-53

Riga, M., Myridakis, A., Tsakireli, D., Morou, E., Stephanou, E. G., Nauen, R., Van Leeuwen, T., Douris, V., Vontas, J., 2015. Functional characterization of the *Tetranychus urticae* CYP392A11, a cytochrome P450 that hydroxylates the METI acaricides cyenopyrafen and fenpyroximate. Insect Biochem Mol Biol 65, 91-99.

2.1 Introduction

A vast number of P450 enzymes have been implicated in insecticide resistance of many pests (Feyereisen, 2012) including spider mites. Dermauw et al. (2013) revealed that CYP392A16, CYP392D8 and CYP392D10 are over-expressed in the adult MAR-AB resistant to abamectin *T. urticae* strain and possibly they are associated with the specific phenotype. Additionally, genome-wide gene expression experiments by using strains resistant to METI acaricides revealed that CYP392A11 and CYP392A12 are upregulated, rendering them as candidate genes for METI resistant phenotype, including the new METI acaricide, cyenopyrafen (Khalighi et al., 2016).

Several P450s have been functionally characterized *in vitro* by over-expression in bacteria, baculovirus or yeast system (Table 2.1 – adapted from Feyereisen, 2012).

The expression by using bacteria has the advantage of low cost production of high amounts of P450s and purification of them and their redox partners. However, purification and reconstitution of them are difficult and possibly not suitable if the aim under examination is their catalytic effect. This disadvantage has been outreached by leading both the P450 and the redox partner to the bacterial membranes, allowing the production of high amounts of membrane bound P450, avoiding that way the formation of inclusion bodies.

Baculovirus expression system allows the study of an insect P450 in an insect environment without the need of purification. The total amount of the P450 produced by this system does not play important role as the highest activity is achieved by the optimal P450/P450 reductase

ratio. However, the interactions with the redox partner are not easily manipulated and the amount of P450 produced is not that high as in *E. coli*, although addition of hemin or δ-aminolevulenic acid can increase the amount of P450.

Yeast expression system carries all the advantages of production in *E. coli* with the additional advantage of P450 production and study in eukaryotic system. However, expression of insect P450s in yeast cells is not a common technique as the ones originating from plants (Feyereisen, 2012).

Table 2.1: In vitro expression systems of P450s. (Adapted from Feyereisen 2012).

Expression system	P450	Organism	Substrate	Redox partner	Reference
E.coli	CYP6A1	*Musca domestica*	aldrin, heptachlor, sesquiterpenoids	MdCPR (Mdb5)	Andersen et al., (1994)
	CYP6A1	*Musca domestica*	diazinon, testosterone, progesterone	MdCPR (Mdb5)	Andersen et al., (1997)
	CYP6A5	*Musca domestica*	benzphetamine, p-chloro-N-methylaniline, methoxyresorufin	MdCPR	Feyereisen (2005)
	CYP12A1	*Musca domestica*	aldrin, amitraz, azinphosmethyl, diazinon, heptachlor, progesterone, testosterone and 7-pentoxycoumarin	Adrenodoxin CPR/Adrenodoxin	Guzov et al., (1998)
***E. coli* membranes**	CYP6CM1	*Bemisia tabaci*	imidacloprid, alkoxycoumarins and resorufins	AgCPR Agb5	Karunker et al., (2009)
	CYP6A1	*Nilaparvata lugens*	imidacloprid	MdCPR	Ding et al., (2013)
	CYP69b	*Anopheles funestus*	pyrethroids	AgCPR Agb5	Riveron et al., (2013)
	CYP329E10	*Tetranychus urticae*	spirodiclofen	AgCPR Agb5	Demaeght et al., (2013)
	CYP6Z2	*Anopheles gambiae*	alkoxyresorufins	AgCPR	McLaughlin et al., (2008)
	CYPM2	*Anopheles gambiae*	pyrethroids	AgCPR Agb5	Stevenson et al., (2011)
Baculovirus	CYP329E10	*Tetranychus urticae*	spirodiclofen		Demaeght et al., (2013)
	CYP6B1	*Papillo polyxenes*	furanocoumarins	MdCPR	Wen et al., (2003)
	CYP6BQ9	*Tribolium castaneum*	deltamethrin	DmCPR	Zhu et al., (2010)
	CYP4G2	*Musca domestica*	alkanes	MdCPR	Qiu et al., (2012)
	CYP6A2	*Drosophila melanogaster*	aldrin, heptachlor, diazinon	MdCPR	Dunkov et al., (1997)
Yeast	CYP9A12	*Helicoverpa armigera*	p-nitroanisole, methoxyresorufin, esfenvalerate	ScCPR	Yang et al., (2008)
	CYP9A14	*Helicoverpa armigera*	p-nitroanisole, methoxyresorufin, esfenvalerate	ScCPR	
	CYP6Z8	*Aedes aegypti*	α-naphthoflavone, resveratrol, 3-phenoxybenzoic alcohol	AeCPR	Chandor-Proust et al., (2013)

Recently, Demaeght et al. (2013) expressed functionally in *E. coli* (bacteria) and characterized CYP392E10, a P450 from *Tetranychus urticae* which is capable of metabolizing spirodiclofen and spiromesifen.

In this study, the genes encoding the following P450 enzymes, CYP392A16, CYP392A11, CYP392A12, CYP392D8 and CYP392D10 were isolated and cloned from a greek multi-resistant strain of *T. urticae* (Mar-AB) and expressed functionally in order to examine their catalytic properties and metabolism potential to a number insecticides/acaricides.

2.2 Materials and Methods

2.2.1 Strains

The Mar-AB strain was isolated from a heavily sprayed rose greenhouse near Athens in 2009, and it has been maintained under abamectin selection (10 mg/L abamectin) every two generations since then. The London strain, which was used as a reference susceptible strain, was previously described (Khajehali et al., 2011). *T. urticae* strains were mass reared on potted kidney bean plants at 25°C, 60% relative humidity (RH) and 16:8 h light:dark photoperiod. Oligonucleotides and chemicals were obtained from Sigma Aldrich unless otherwise indicated. Enzymes for RNA/DNA work were supplied by New England Biolabs, and HPLC solvents from Fisher Scientific. Analytical grade insecticides were purchased from Sigma-Aldrich. Commercial formulation of abamectin (Vertimec 1.8EC) used in this study.

2.2.2 Extraction of RNA and cDNA synthesis

Total RNA was extracted from about 100 adult female or pools of 300 deutonymphs of each *T. urticae* strain using RNeasy Mini kit (Qiagen). Extracted RNAs were treated with Turbo DNase (Ambion) to remove any genomic DNA contamination, and were consequently used to make first strand cDNA using oligo-dT primers with Superscript III reverse transcriptase (Invitrogen).

2.2.3 Cloning and co-expression of CYPs with *Tetranychus urticae* CPR, and preparation of membranes

The cDNA sequences encoding CYP392D8 (Tu ID: tetur03g05070), CYP392D10 (Tu ID: tetur03g05110), CYP392A16 (Tu ID: tetur06g04520), CYP392A11 (Tu ID: tetur03g00970), CYP392A12 (Tu ID: tetur03g00830) and Cytochrome P450 Reductace (CPR) (Tu ID: tetur18g03390) were isolated by RT-PCR using RNA purified from adult *T. urticae* from the MAR-AB strain, and the primers listed in Table 2.1.

Primers used for the amplification of the full length of CYP392D8, CYP392D10, CYP392A11, CYP392A12 and CYP392A16 introduced a NgoMIV restriction site before the ATG codon, and downstream the stop codon a EcoRI site, SacI and HindIII according to gene sequence and the multi-cloning site of the expression vector (Table 2.1). The P450 genes were isolated from mite cDNA (MAR-AB strain) and were ligated to pCW-OmpA2 (McLaughlin et al., 2008) to create pCW_CYP392D8, pCW_CYP392D10, pCW_CYP392A12, pCW_CYP392A12 and pCW_CYP392A16. Primers used for the amplification of the full CPR ORF (Table 2.2) introduced

an NcoI restriction site before the ATG codon and a XhoI site downstream the stop codon. CPR was amplified from mite cDNA (London strain) and was initially ligated into pET22b vector (Novagen). Using the restriction enzymes NdeI/XhoI we managed to isolate the full CPR ORF with pelB leader sequence. The pelB-TuCPR sequence was then ligated into pACYCDuet-1 expression vector (Novagen) to create pACYC-TuCPR. PCR-products and expression vectors were sequenced to confirm identity.

For functional expression of CYP392D8, CYP392D10 and CYP392A16 competent *E.coli* JM109 cells were co-transformed with pCW_CYP392D8, pCW_CYP392D10, pCW_CYP392A12, pCW_CYP392A12 and pCW_CYP392A16 and pACYC-TuCPR. Transformed cells were grown in terrific broth with ampicillin and chloramphenicol selection until the optical density at 595nm reached ~1cm^{-1} whereupon the heme precursor δ-aminolevulinic acid was added to a final concentration of 1mM. Induction was initiated with the addition of isopropyl-1-thio- β-D-galactopyranoside (IPTG) to a final concentration of 1mM. Spheroplasts were prepared by adding TSE buffer (0.1 M Tris acetate, pH 7.6, 0.5 M sucrose, 0.5 mM EDTA) containing 0.25 mg/ml lysozyme to the cell pellet and gentle mixing for 60 min at 4°C. The solution was centrifuged at 2800 xg for 25 min at 4°C and the spheroplast pellet was resuspended in spheroplast resuspension buffer (0.1 M potassium phosphate buffer, pH 7.6, 6 mM magnesium acetate, 20% glycerol) containing 0.1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonylfluoride (PMSF), 1 mg/ml aprotinin and 1 mg/ml leupeptin. The suspension was sonicated and the membrane fraction was pelleted by ultracentrifugation at 180,000 xg for 1 h, at 4°C. Membrane preparations were diluted in TSE buffer and stored in aliquots at -80 °C and assayed for, total protein concentration (Bradford assay with BSA standards), P450 concentration (Omura and Sato, 1964), and CPR activity by monitoring cytochrome C reduction (Strobel and Dignam, 1978).

Table 2.2: Primers used for the amplification of the cytochrome P450s and TuCPR

Gene	TeturID	Primer (c, e)[a]	Sequence (5'-3')[b]	Product size (bp)
CYP392A16	Tetur06g04520	F(c/e)	GCCGGCATGTTTTTAATTAGTAATTTGCTGTCAT	1512
		R(c/e)	AAGCTTTTAGTTGGAATTGGAAATTTTCTC	
CYP392A11	Tetur03g00970	F(c/e)	GCCGGCATGCAAAAAGTTATGTCTTTATTGG	1524
		R(c/e)	AAGCTTTCAGTCAGAATTGGAAATTTTCTC	
CYP392A12	tetur03g00830	F(c/e)	GCCGGCATGTTTTCAATTAATAATTTGTTTGAA	1512
		R(c/e)	GAGCTCTCAGTCAGAATTGGATATTTTCAC	
CYP392D10	tetur03g05110	F(c/e)	GCCGGCATGCTTCTCGATCATTTCAAATC	1473
		R(c/e)	GAGCTCTTAAAGACTCAAAATGTGAAAATG	
CYP392D8	tetur03g05070	F(c/e)	GCCGGCATGTTTCTCGATCATTTCAACG	1503
		R(c/e)	GAATTCTTAATGCTTCAAAGTGTGAAAATT	
CPR	tetur18g03390	F(c/e)	CCATGGAAGAATCGCCTAATCAA	2004
		R(c/e)	CTCGAGTAACTCCACACATCAGCA	

c: primers used for cloning; e:primers used for making expression constructs

[b] Underlying sequence denotes the introduction of restriction sites to facilitate cloning

2.2.4 P450 activity and ligand IC_{50} determination

P450 activity measurements with six fluorogenic substrates (ethoxycoumarin, 7-ethoxy-4-trifluoromethylcoumarin, and the resorufin ethers, methyl, ethyl, pentyl, and benzyl) and seven luciferin-based substrates (P450 Glo™ proluciferin substrates, Luciferin-H, Luciferin-ME, Luciferin-CEE, Luciferin-H EGE, Lucifern-PFBE, Luciferin-PPXE, Luciferin-ME EGE, Promega) were also tested. Enzyme activity measurements were performed in 50 mM potassium phosphate pH 7.4, 5 µM substrate, bacterial membranes containing 1 pmol of P450 (or mite homogenates) and 0.1 mM NADPH. Plates were pre-warmed for 5 min at 30 °C before reactions were initiated by addition of NADPH and determinations were carried out in 96-well plates (Nunc MaxiSorp) using a SpectraMax M2e multimode microplate reader (Molecular Devices, Berkshire, UK). Luciferin reactions were run for 30 min before quenching as described by the P450-Glo kit (Promega). The endpoint signal was then measured by a single tube luminometer (Berthold Detection Systems FB12 Luminometer) and the turnover was calculated. Three replicates of positive and negative control reactions were run for each P450/substrate combination. For ligand IC50 determinations, assays were performed in a final volume of 100 µl consisting of 100 mM potassium phosphate pH 7.4; 1 pmol/ml recombinant cytochrome P450, substrate concentration equal to the Km of the particular P450, and variable concentrations of test ligand. Ligand stocks were dissolved in methanol or acetonitrile and a solvent control was included to correct for any solvent effects across the dilution range. IC50 values were calculated using GRAFIT 3.0.3 (Erithacus Software Ltd., Surrey, U.K.).

2.2.5 HPLC – MS analysis of insecticide metabolism and preparative purification of metabolites

2.2.5.1 Metabolism assays with abamectin, identification and isolation of the metabolite (CYP392A16)

HPLC Analysis

Abamectin 98.7% (Sigma–Aldrich, Technical) was incubated with bacterial membranes containing 20 pmole recombinant P450 in 100ul Tris-HCl buffer (0.2M, pH 7.4) containing 0.25mM $MgCl_2$. The incubation was performed in the presence or absence of NADPH generating system: 1 mM glucose-6-phosphate (Sigma-Aldrich), 0.1 mM $NADP^+$ (Sigma-Aldrich), 1 unit ml^{-1} glucose-6-phosphate dehydrogenase (G6PDH, Sigma-Aldrich). Reactions were carried out at 30^0C with 1250rpm shaking. Reactions were stopped at different elapsed time intervals varying from 5min to 4 hours with 100ul of acetonitrile and incubated for further 30 min to ensure that all insecticide was dissolved. The quenched reactions were centrifuged at 10.000rpm for 10min before transferring the supernatant to glass HPLC vials. 100ul of the supernatant was injected at a flow rate of 1.4 ml /min at 40^0C. Abamectin and its metabolite were separated on a C18 column (Acclaim^R 120, Dionex, 4.6 X 250mm, 5µm 120Å). Time–trial reactions were run with an isocratic program 80% A: 20% B (A: 0.1 % acetic acid in acetonitrile, B: 0.1 % acetic acid in water) for 22min. Abamectin elution was monitored by absorption at 245nm and quantified by peak integration (Chromeleon, Dionex). For enzyme reaction kinetics varying concentrations of abamectin (1–150 µM) were used. Rates of substrate turnover from three independent reactions were plotted versus substrate concentration. Km, Vmax and Kcat were determined using SigmaPlot 12.0 (Systat Software Inc., London, UK).

HPLC-MS analysis

Prior to the analysis all the samples were desalted with solid phase extraction (C18 Waters SEP PAK cartridges) as follows: initially the cartridges were preconditioned with 3 mL 100% acetonitrile, followed by 3mL 2.5% acetonitrile in water and then samples were diluted to 4mL with water and loaded to the cartridges; cartridges were subsequently washed with 1mL water and samples were eluted with 2mL acetonitrile. All analyses were performed on an LC-MS/MS system consisting of an RP-HPLC chromatograph coupled to a mass spectrometer. Sample injection was performed via a Surveyor Autosampler (Thermo Finnigan, USA). The

chromatographic separation was achieved using a Surveyor LC system (Thermo Finnigan, USA), equipped with a Perfectsil ODS (5 µm, 250 mm × 4.6 mm) analytical column by Thermo Scientific, (USA). The mass detection was achieved with a TSQ Quantum triple quadrupole with ESI source (Thermo Finnigan, USA) operated in positive mode. The system was controlled by the Xcalibur software, which also used for the data acquisition and analysis. The optimum mass spectrometer parameters were set as follows: spray voltage at 4500V, for sheath gas pressure at 49 arbitrary units, for capillary temperature 270°C and for source collision induced dissociation at 8 eV. Sheath/auxiliary gas was high purity nitrogen and collision gas was high purity argon. For MS/MS analysis, collision energy was set at 40 eV.

2.2.5.2 Metabolism assays with of Cyenopyrafen and Fenpyroximate (CYP392A11)

We performed metabolism assays for the METIs acaricides cyenopyrafen and fenpyroximate. Cyenopyrafen 97,3% (Fluka, PESTANAL,analytical standard) and fenpyroximate 99, 4% (Fluka, PESTANAL,analytical standard) were incubated with bacterial membranes containing 25 pmol recombinant P450 in 100 µl Tris-HCl buffer (0.2M, pH 7.4) containing 0.25mM $MgCl_2$. The incubation was performed as described previously for abamectin metabolism assay.

Prior to the HPLC-MS analysis, the samples were desalted with solid phase extraction (Bond Elute LRC-C18, 200 mg cartridges, Agilent, USA) as follows: initially the cartridges were pre-conditioned with 3 mL 100% acetonitrile, followed by 3 mL 2.5% acetonitrile in water and then samples were diluted to 15 mL with water and loaded to the cartridges; cartridges were subsequently washed with 1 mL water and samples were eluted with 1 mL acetonitrile. Eluents were transferred to HPLC autosampler vials, 250 µL water was added to enhance chromatographic separation and were analysed with an HPLC-MS/MS system. Sample injections (20 µL loop) were performed via a Surveyor Autosampler (Thermo Finnigan, USA). The chromatographic separation was achieved using a Surveyor LC system (Thermo Finnigan, USA), equipped with a Gemini C18 (3 µm, 100 mm × 2 mm) analytical column (Phenomenex, USA). An isocratic elution was applied with 80% acetonitrile-20% water, both containing 0.1% acetic acid and flow rate was set at 200 µL/min. The mass detection was achieved with a TSQ Quantum triple quadrupole (Thermo Finnigan, USA) with positive electrospray ionisation (ESI) as ionisation source. Mass spectrometer was operated in full scan, single ion monitoring and product ion scan modes. The system was controlled by the Xcalibur software, which also used for the data acquisition and analysis. The optimum mass spectrometer parameters were set as follows: spray voltage at 4500 V, sheath gas pressure

at 20 arbitrary units, auxiliary gas pressure at 10 arbitrary units, capillary temperature at 300 °C and source collision induced dissociation at 26 eV. Sheath/auxiliary gas was high purity nitrogen and collision gas was high purity argon. For MS/MS analysis, collision energy was set at 5 eV.

For enzyme reaction kinetics varying concentrations (0.5-100μM) of fenpyroximate or cyenopyrafen were used. Rates of the substrate turnover from two independent reactions were plotted versus substrate concentration. *Km*, V_{max} and K_{cat} were determined using SigmaPlot 12.0 (Systat Sofware Inc, London, UK).

2.2.6 Peptide Antibody Development against CYP392A16

The amino acid sequence of CYP392A16 was blasted against other P450s of *Tetranychus urticae* that share high sequence similarity. According to blast results there is a region in CYP392A16 (aa 161-178) that is the most differentiated among the homologs tested. A hydrophobicity test was also carried out in order to check that the selected peptide does not correspond to a transmembrane domain. The peptide (SALENNGKPADFEKCISH) was chemically synthesized and used to raise specific antibodies in rabbits (Davids Biotechnologie, GmbH, Germany). Furthermore, antibodies were affinity purified (Davids biotechnologie) and used in western blot analysis.

2.2.6.1 Western Blot Analysis

Pools of 500 female mites from each strain (resistant Mar-ab, susceptible London) were homogenized in 100 μl of 0.1M Tris-HCl, pH:7.4. The samples were centrifuged at 1000 xg, at 4C for 10 min. The supernatants were transferred in new tubes and they were used for measuring the total protein concentration according to Bradford assay.

Upon addition of Laemli Buffer (1x) and boiling at 95^0C for 5 min, 100 μg total proteins from each homogenate were separated on a 12% SDS – acrylamide gel and electrotransferred on a PVDF membrane, pre-activated with methanol. After transfer, the membrane was blocked with blocking solution (5% milk in 1xTBS-Tween) for 1h at room temperature and then, incubated with anti-CYP392A16 (1:500 dilution in 3% milk), by shaking at 4^0C overnight. Antibody binding was detected with 1:1000 dilution of goat anti-rabbit IgG coupled to horse-radish peroxidase (Invitrogen) and ECL (Amersham ECL Western Blotting Detection Reagents).

2.3 Results

2.3.1 Functional expression of P450s

Bacteria were co-transformed with the expression vectors containing the P450 gene and the TuCPR.

In order to check if the enzymes have the correct folding, the CO-difference spectrum method was used (Omura and Sato, 1964). CYP392A16 and CYP392A11 showed a characteristic peak at 450nm, indication of a good quality P450. Although, there were series of optimization efforts for the rest of P450s it was not possible to get spectra that indicate appropriate folding and instead they were expressed as P420 (Figure 2.1). The membranes over-expressing CYP392A16 and CYP392A11 contained 5 µM and 2 µM P450, respectively and the activity of P450 reductase (TuCPR) was 3000-4500 µmol cytochrome c reduced/min/g protein and 1500-2000 µmol cytochrome c reduced/min/g protein, respectively.

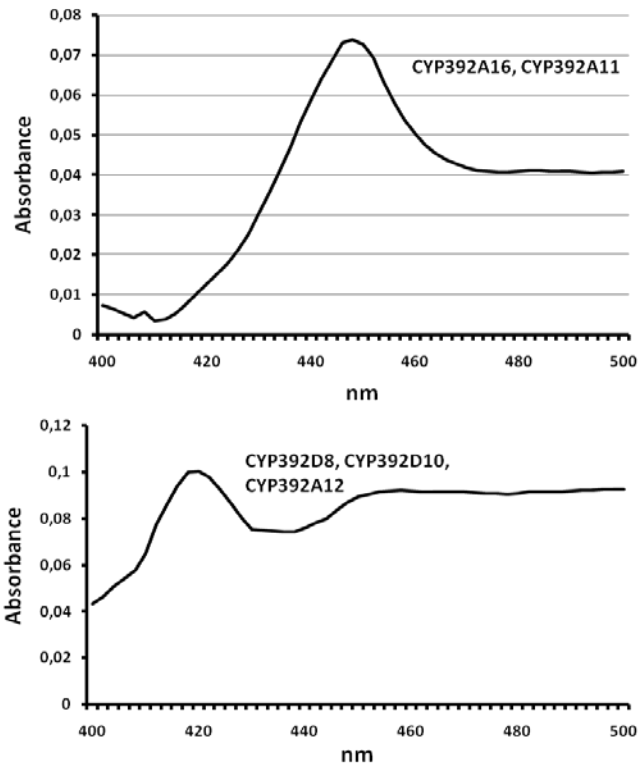


Figure 2.1: CO-difference spectrum of bacterial membranes expressing CYP392A16, CYP392A11 (UP), CYP392D8, CYP392D10 and CYP392A12 (DOWN).

2.3.2 Enzyme activity and ligand IC50 determination

Numerous fluorogenic (7-ethoxycoumarin, 7-ethoxy-4-trifluotomethylcoumatin, methoxy-, ethoxy-, pentoxy- and benzoxy-resorufin) and chemi-luminescent (Lucifenin - ME EGE, Luciferin - H EGE, Luciferin – H, Luciferin – PFBE, Luciferin – PPXE, Luciferin – ME and Luciferin - CEE) model substrates were used so as to determine the catalytic activity of CYP392A16 and CYP392A11. The results are listed on Table 2.3. Ethoxycoumarin shows the highest activity among the fluorogenic substrates that were used. Metabolism of Luciferin - ME EGE by both P450s shows the highest activity rates among the seven chemi-luminescent substrates we used. We proceeded to the determination of inhibition constants by using the chemi-luminescent substrate Luciferin - ME EGE. The Km of the model substrate is 3 μM for CYP392A16 and 2.5 μM for CYP392A11.

Table 2.3: Catalytic activity of model substrates by CYP392A16 and CYP392A11.

Compound/Substrate	**Specific activity** [a]	
Fluorescent substrates	CYP392A16	CYP392A11
Ethoxycoumarin	0.036±0.018	0.11±0.01
Ethoxy-4-trifluoromethylcoumarin	0.026±0.014	0.02±0.01
Methoxyresorufin	Nd	Nd
Ethoxyresorufin	Nd	Nd
Pentoxyresorufin	Nd	Nd
Benzoxyresorufin	Nd	Nd
Chemiluminescent substrates		
L-ME EGE	12.88 ± 0.6	3.2 ± 0.15
L-H	0.03 ± 0.001	0.02 ± 0.001
L-ME	0.17 ± 0.008	0.5 ± 0.02
L-CEE	0.015 ± 0.0007	Nd
L-PFBE	0.15± 0.007	0.2± 0.01
L-PPXE	0.023 ± 0.001	0.02 ± 0.001
L-H EGE	0.63 ± 0.031	0.17 ± 0.008

[a]Fluorescent substrates as pmol product/min/pmol P450 (±SEM), Chemiluminescent substrates as pmol D-Luciferin/min/ pmol P450 (±SEM), nd: not detected activity under assay's conditions.

Insecticides of different mode of action were used in order to determine their ability to inhibit P450-mediated Luciferin ME-EGE activity through IC50 measurement. That is the value at which the compound inhibits the P450 activity at 50%. The results are in a low micromolar range and are shown on Table 2.4. Three of the insecticides (abamectin, pyridaben and hexythiazox) inhibit CYP392A16 significantly, whereas bifenthrin and clofentezine do not show any inhibition. CYP392A11 is inhibited by all the insecticides we used under the assay conditions.

Table 2.4: Inhibition of CYP392A16 and CYP392A11 by diverse insecticides of different mode of action.

Compound	**IC50 (µM)***		**Mode of Action (MoA)**
	CYP392A16	**CYP392A11**	
Abamectin	3.82±0.91	1.14 ± 0.17	Avermectin
Pyridaben	6.25±0.43	7.6 ±0.2	METI
Cyenopyrafen	-	4.9±0.19	METI
Fenpyroximate	-	3.7±0.2	METI
Bifenthrin	Ndi	-	Pyrethroid
Hexythiazox	7.37±0.74	7.8±0.4	Thiazolidin/ Growth regulator
Clofentezin	Ndi	3.9±1.9	Growth regulator

*Assays were performed in a final volume of 100 µl consisting of 100 mM potassium phosphate pH 7.4, 1 pmol/ml recombinant CYP392A16, 3 µM Luciferin-ME EGE substrate, and variable concentrations of test ligand. ndi: not detected inhibition, under assay conditions; MoA: Mode of Action; IGR: Insect Growth Regulator, METI: Mitochondrial Electron Transport Inhibitor, Ndi: Not detected inhibition

2.3.3 Detection of Metabolism and Enzyme characterization

Both P450 proteins (CYP392A16 and CYP392A11) were tested against several insecticides, especially the ones that showed strongest inhibition as well as reduced toxicity levels in the Mar-ab strain.

In this study, it was shown that CYP392A16 is capable of metabolizing abamectin, revealing a metabolite peak in earlier elution time when NADPH was added in the reaction. When NADPH was absent, there was not any formation of the metabolite peak and only the parental compound eluted (Figure 2.2).

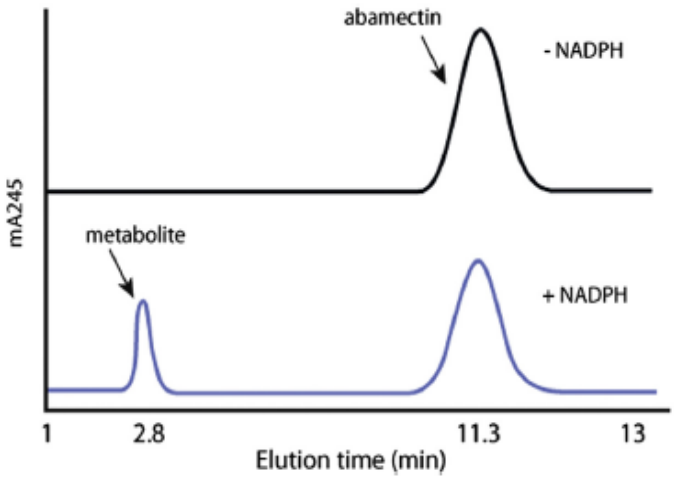


Figure 2.2: Metabolism of abamectin by CYP392A16. Abamectin depletion (eluting at 11.3 min) and metabolite formation (eluting at 2.8 min) observed at the sample when supplemented with NADPH. In the absence of NADPH, no change was detected on the chromatogram

It was also found that the formation of the metabolite and the depletion of the parental compound is time dependent reaction and 40% of abamectin was metabolized in 4h. Incubation of the enzyme with different concentrations of substrate took place in order to examine the substrate dependent reaction rates. The depletion of abamectin in response to abamectin concentration revealed Michaelis-Menten kinetics: V_{max}= 10.7 pmol depleted abamectin/min, Km=45.9 μM and k_{cat}=0.54 pmol depleted abamectin/min/pmol P450 (Figure 2.3).

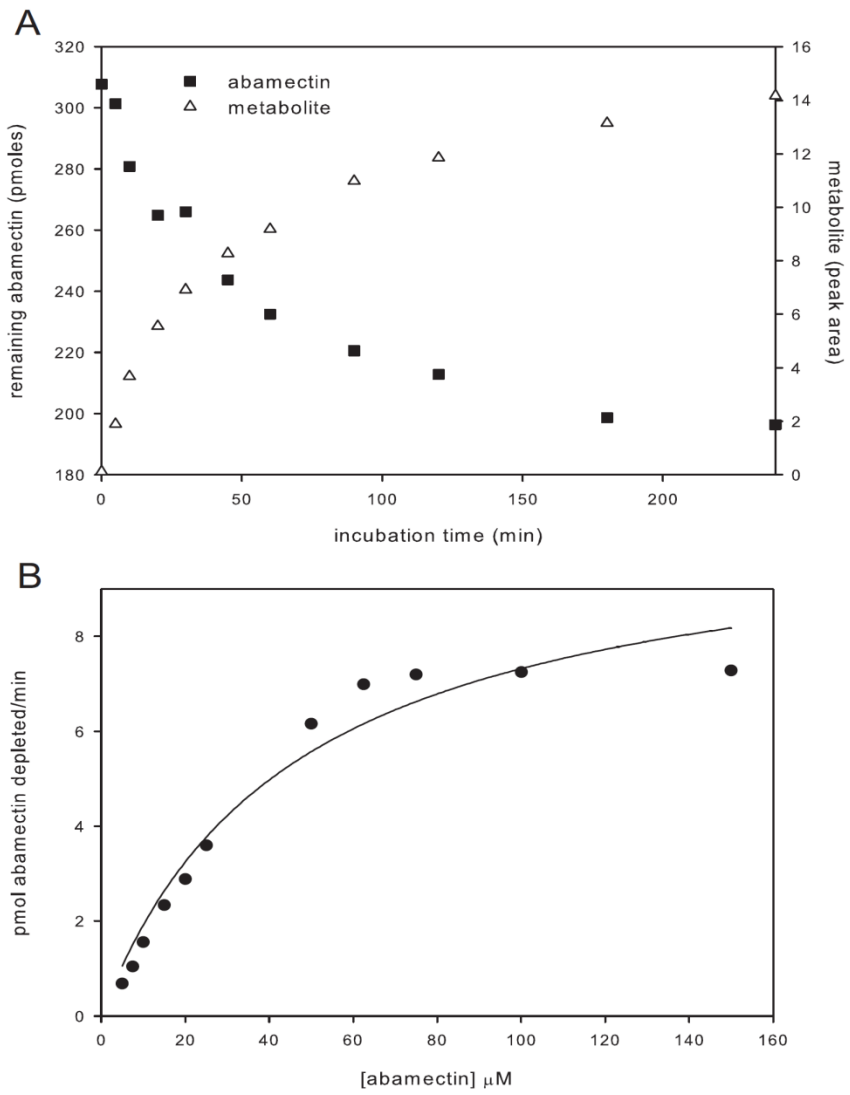


Figure 2.3: Kinetics of abamectin metabolism. A: Time course of abamectin depletion (squares) and the formation of metabolite (triangles). Approximately 40% of abamectin was metabolized in 4h. Reactions carried out at 30C supplemented with 62.5 μM of insecticide. B. Michaelis – Menten kinetics of abamectin metabolism by CYP392A16. Values represent the mean of duplicates. Curves were calculated by non-linear regression.

Moreover, the metabolite was isolated and HPLC-MS analysis confirmed the generation of a hydroxylated metabolite, hydroxyl-abamectin. Electrospray ionization mass spectrum of the metabolite revealed the molecular ion peaks at m/z [M+H]+:889.6, [M+NH4]+: 906.6 and [M+Na]+: 911.5 that are 16m/z units higher than the corresponding ones of the parental substrate (m/z [M+H]+:873.7, [M+NH4]+: 890.6 and [M+Na]+: 895.6) (Figure 2.4). The MS/MS spectrum of the metabolite pseudomolecular ion [M+Na]+: 911.5 shows fragmentation that corresponds to either the 24—OH or 26-OH isomer of hydroxyl abamectin (Figure 2.5). This result indicates the addition of molecular oxygen to abamectin, thus forming a hydroxyl group.

Toxicity assays were carried out with the isolated metabolite and the parental compound, by using the susceptible strain London. The concentration of both metabolite and the parental compound was 0.5 µg/ml. The percentage mortality was 98.3% for the spider mites sprayed with the parental compound, whereas low levels of mortality (approximately 8.5%) were observed when the hydroxyl-abamectin was used.

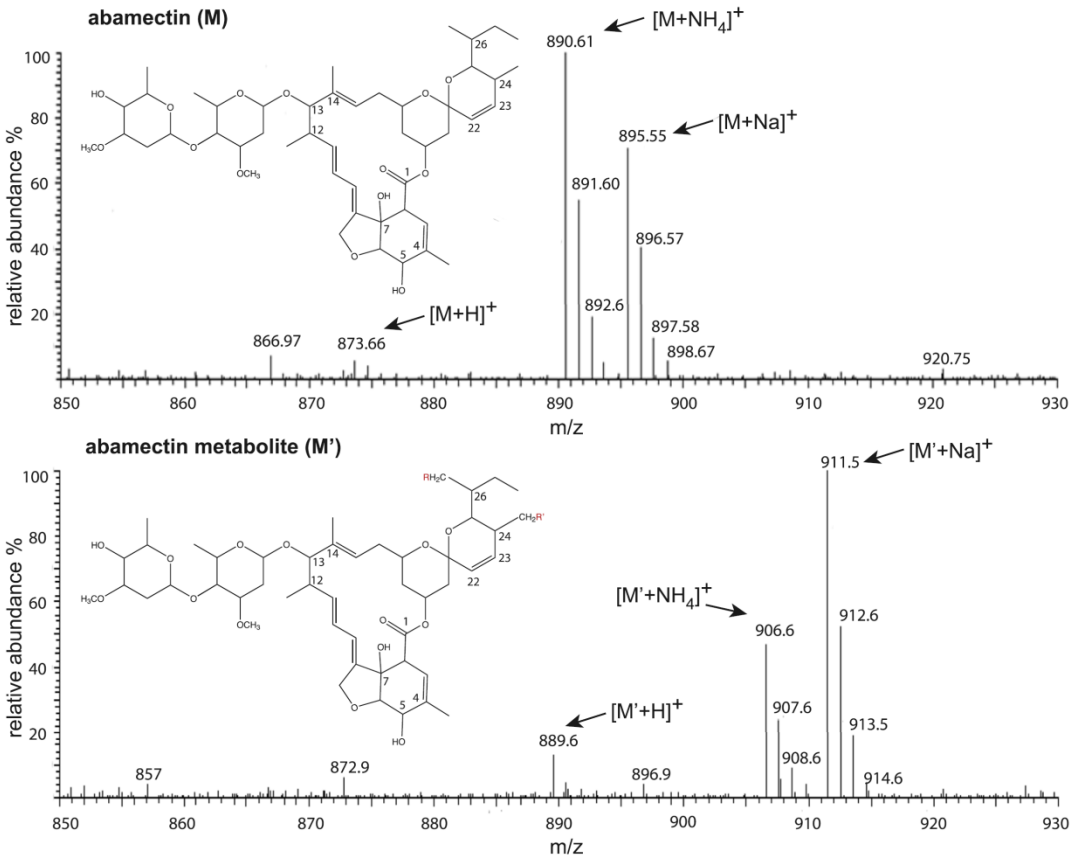


Figure 2.4: Electrospray ionization mass spectrum of abamectin and the metabolite, hydroxyl-abamectin. UP: Mass spectra of abamectin. Incubation of 20 pmol CYP392A16 and 62.5 µM abamectin for 1 h in the absence of NADPH. DOWN: Mass spectra of the metabolite (red letters on the structure indicate the OH-group). Incubation of 20 pmol CYP392A16 and 62.5 µM abamectin for 1 h in the presence of NADPH.

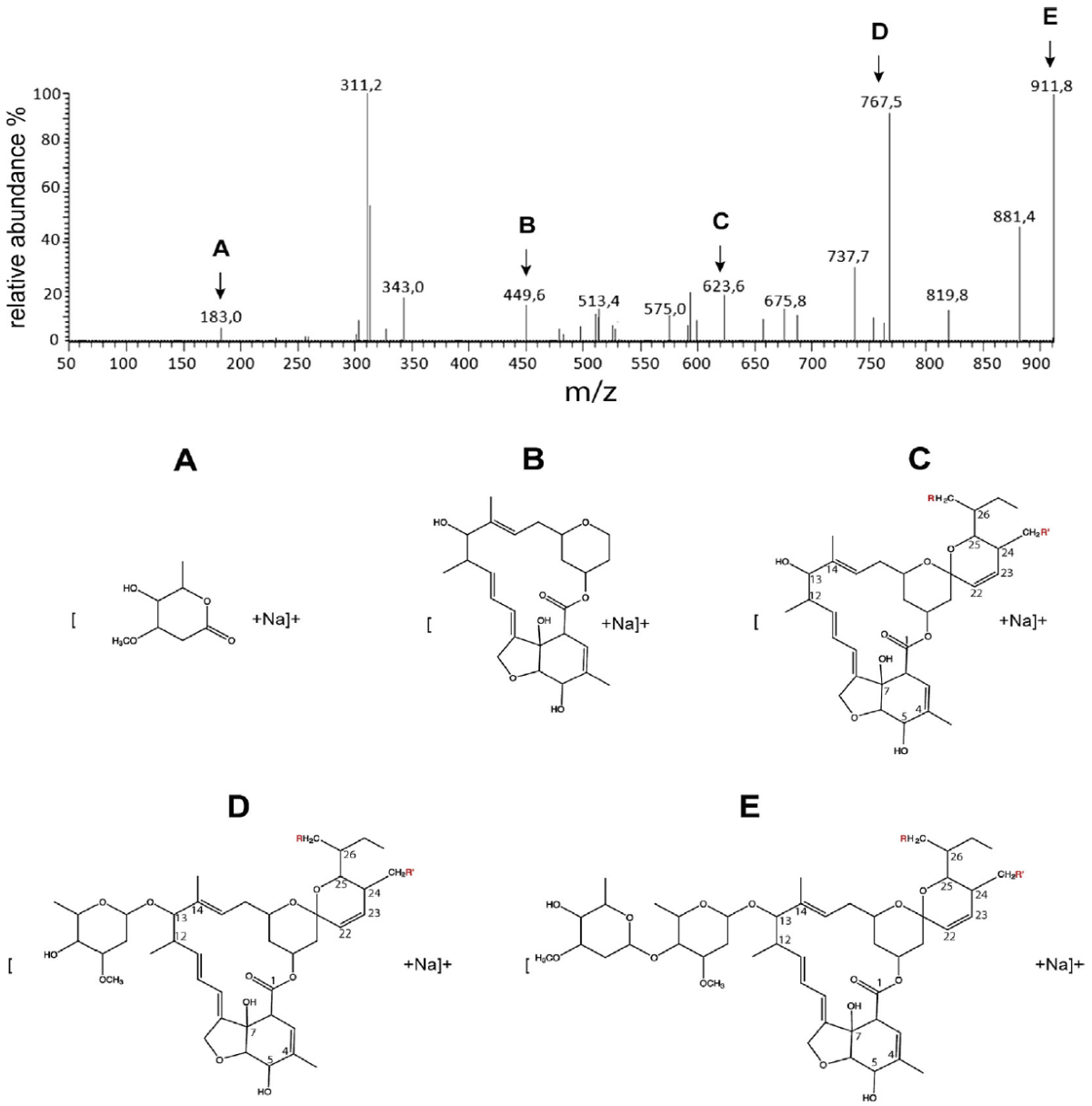
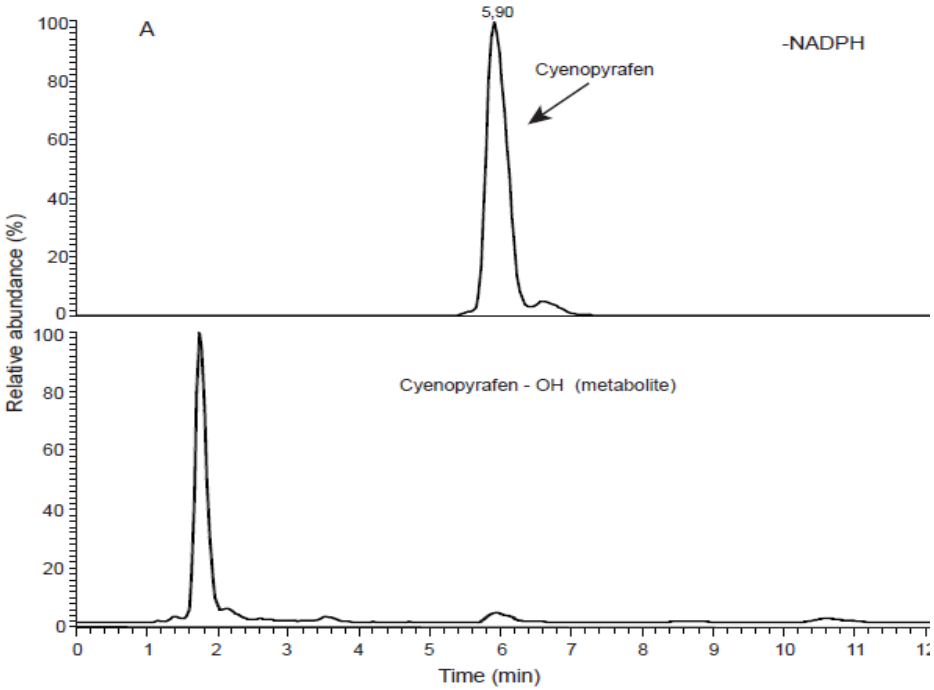


Figure 2.5: Fragment ion spectrum of the metabolite hydroxyl-abamectin. Chemical structures of fragment ions of hydroxyl-abamectin are shown below the mass-spectra graph. OH- groups correspond either to R or R' on the chemical structures (depicted by red color). The metabolite pseudomolecular ion [M'+Na]+: 911.5 shows a fragmentation pattern that probably cprresponds to either 24-OH or 26-OH isomer of hydroxyl-abamectin.

CYP392A11 was tested against several acaricides that belong to different groups. The results indicate that the specific enzyme is catalyzing the metabolism of two METI acaricides, cyenopyrafen and fenpyroximate.

More specific, mass spectrometric analysis of cyenopyrafen showed a molecular peak at [MH]+: 394 m/z revealing two more isotopic peaks at 395 m/z and 396 m/z. Single ion analysis was applied to the metabolism assay samples in order to monitor only the peaks mentioned above and the possible ions of hydroxylated cyenopyrafen. The results showed the formation of hydroxylated metabolite (plus 16 m/z that equals to oxygen) giving ion peaks at 410, 411 and 412 m/z only in the reactions supplied with NADPH, revealing a peak at 3.5 min (Figure 2.6). We were not able to do structural identification of the hydroxylated metabolite as the signal intensity was not intense for MS/MS analysis.

Fenpyroximate metabolism reactions by CYP392A11 were also applied to full scan mode revealing a molecular peak [MH]+ at +422 m/z/. In order to indentify the possible molecular ions that Motoba et al. (2000) described (m/z: +382, +408, +217, +438, +366, +452) single ion monitoring was applied. Metabolite formation was not detected in the minus NADPH samples, whereas the plus NADPH reactions revealed an ion peak at +382 m/z which probably corresponds to the metabolite M-5 of Motoba et al. (2000) and this peak appears at 3.2min. This metabolite was further characterized and it was revealed a fragment at +364 m/z, which corresponds to loss of H2O molecule (minus 18 m/z) of the structure proposed by Motoba et al. (2000) (Figure 2.7).



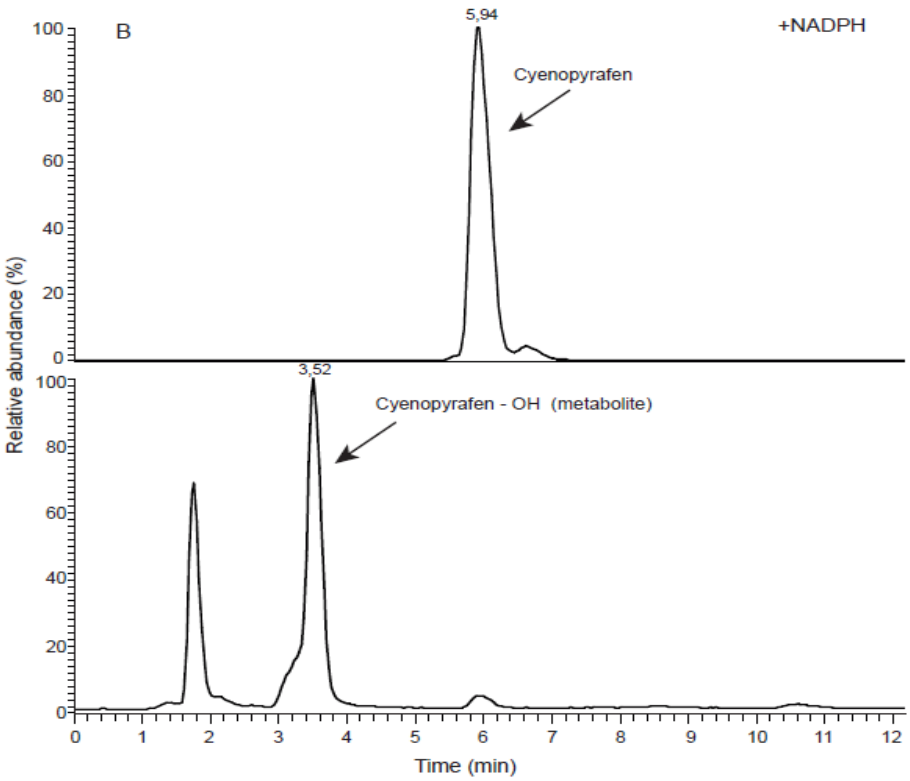


Figure 2.6: Hydroxylation of cyenopyrafen by CYP392A11 – Single ion chromatograms. A: Incubations carried out in the absence of NADPH and they showed no change in the control chromatogram of cyenopyrafen (eluting at 5.94 min, m/z:+396). B. Incubations carried out in the presence of NADPH and they showed cyenopyrafen depletion and formation of metabolite (eluting at 3.52 min, m/z:+412).

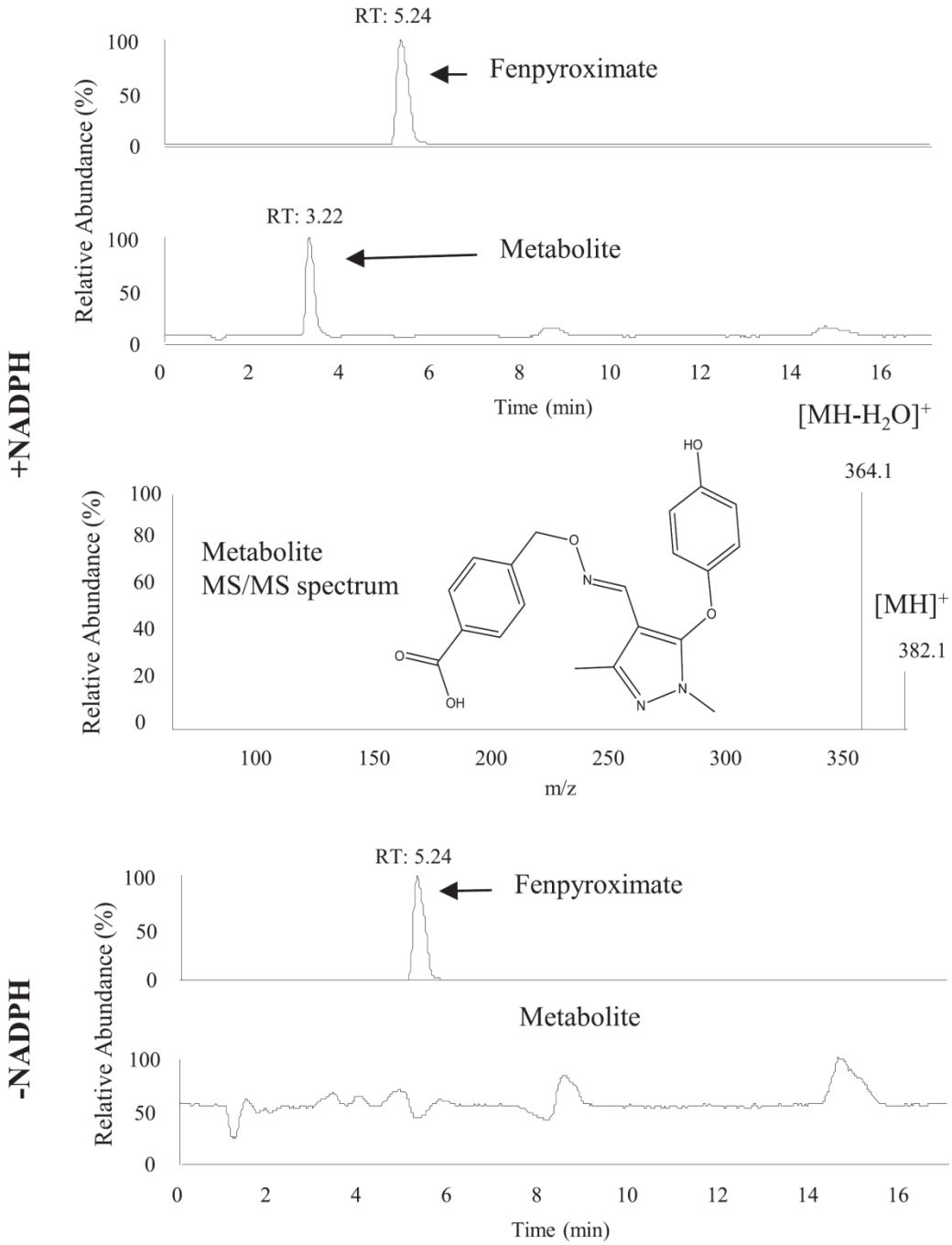


Figure 2.7: Hydroxylation of fenpyroximate by CYP392A11. UP: Incubations carried out in the presence of NADPH and they showed fenpyroximate depletion (eluting at 5.2 min) and metabolite formation (eluting at 3.2 min). Product ion scan (+ 382 m/z) at + NADPH samples. Peak at +364.1 m/z corresponds to water loss and matches with the proposed structure. DOWN: Incubations carried out in the absence of NADPH and they showed no change in the control chromatogram of fenpyroximate (eluting at 5.2 min).

Finally, the enzyme kinetic parameters were obtained by measuring the rate of cyenopyrafen and fenpyroximate depletion in response to cyenopyrafen and fenpyroximate substrates, respectively, which revealed Michaelis-Menten kinetics. The values Km, V_{max} and k_{cat} for cyenopyrafen are the following: Km=65.6 µM, Vmax = 59.3 pmol depleted cyenopyrafen/min and kcat=2.37 pmol depleted cyenopyrafen/min/pmol P450. The values Km, Vmax and Kcat for fenpyroximate are the following: Km=65.0 µM, Vmax = 46.2 pmol depleted cyenopyrafen/min and kcat=1.85 pmol depleted cyenopyrafen/min/pmol P450. The Michaelis-Menten kinetics are shown in Figure 2.8 for cyenopyrafen and in Figure 2.9 for fenpyroximate.

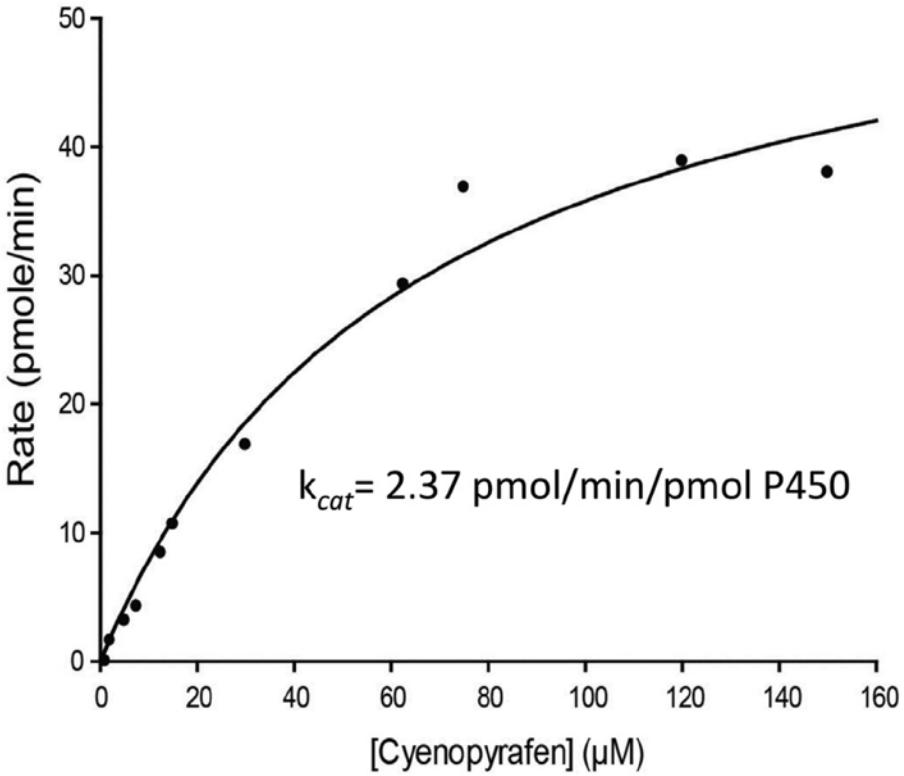


Figure 2.8: Kinetics of cyenopyrafen metabolism. Michaelis – Menten kinetics of cyenopyrafen metabolism by CYP392A11. Values represent the mean of duplicates. Curves were calculated by non-linear regression.

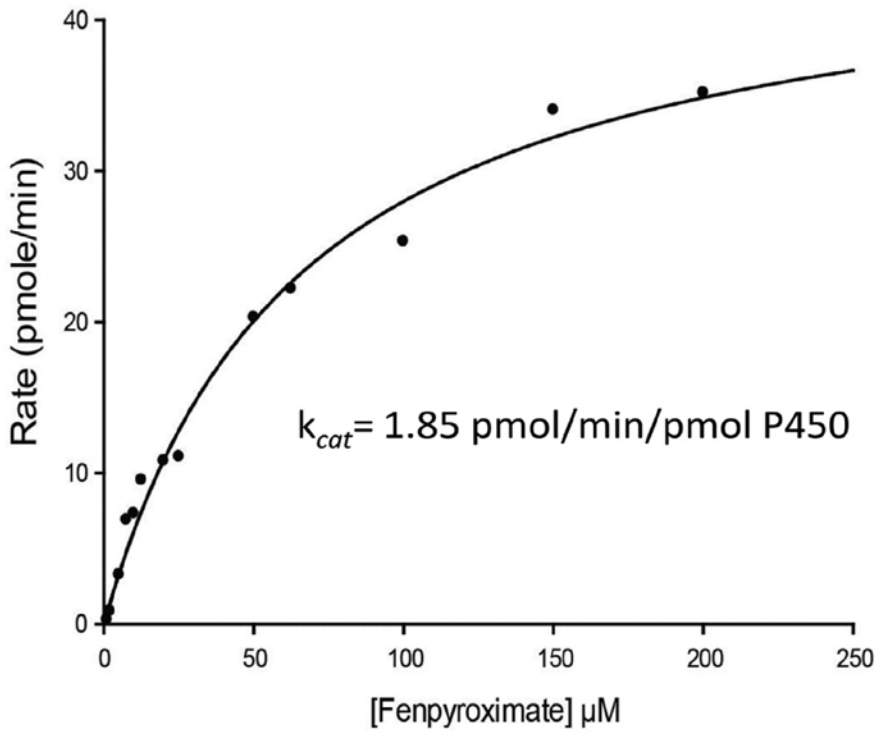


Figure 2.9: Kinetics of fenpyroximate metabolism. Michaelis – Menten kinetics of fenpyroximate metabolism by CYP392A11. Values represent the mean of duplicates. Curves were calculated by non-linear regression.

2.3.4 Anti-CYP392A16

In order to detect if CYP392A16 is over-expressed in the resistant strain Mar-ab in relation to the susceptible one (London strain), clear homogenates from both strains and equal amounts of protein were loaded on SDS-PAGE. The existence of CYP392A16 in the samples was checked by using the antibody against CYP392A16 (Figure 2.10). A polypeptide at 55 kDa (calculated molecular weight of CYP392A16 is at 57.4 kDa) was detected only in the homogenate from the resistant population whereas nothing was detected on the one from susceptible population (Lanes 2 and 3 respectively). It was also identified a band of similar size (55 kDa) from bacterial membranes that over-express CYP392A16 (Lane 1). Probably this band corresponds to CYP392A16 which is highly expressed in the resistant population compared to the susceptible one, as any other signal was not detected under the experimental conditions. The result indicates that the successful development and production of a specific antibody against CYP392A16 which could be used as a diagnostic tool/marker for screening resistance to abamectin in the field.

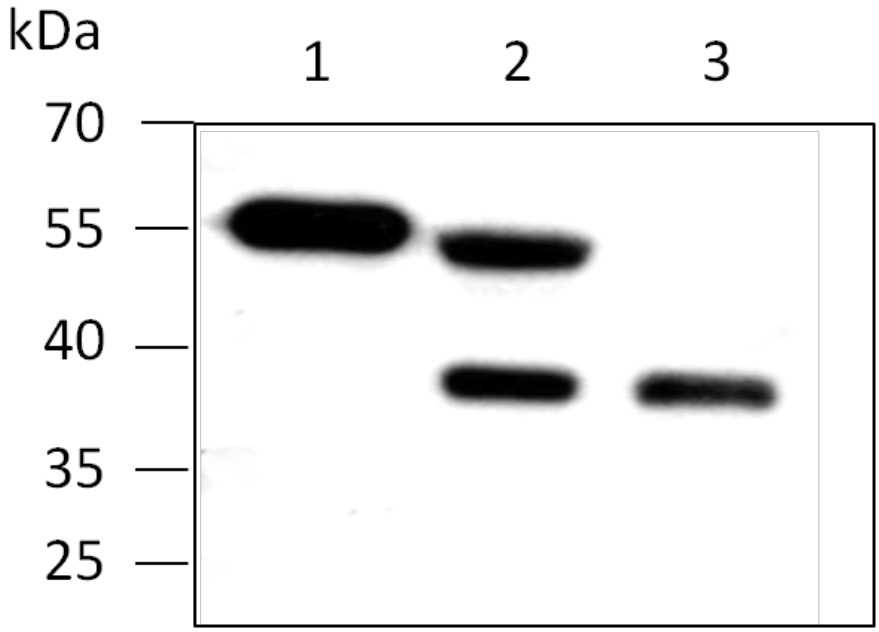


Figure 2.10: Western blot analysis of CYP392A16 expression on mass homogenates from *Tetranychus urticae*. Homogenates from Mar-ab strain (Lane 2) and London strain (Lane 3) were checked with anti-CYP392A16. Bacterial membranes over-expressing CYP392A16 (Lane 1, 10 µg loaded on the gel) served as control. Calculated Molecular Weight of CYP392A16: 57.4 kDa.

Conclusions – Discussion

Recent microarray data from multi resistant strains (Mar-ab and MR-VP) with striking resistance phenotypes to several acaricides / insecticides, such as abamectin and METIs, revealed that the majority of the upregulated detoxification genes belong to P450s. Among these, CYP392A16, CYP392A11, CYP392A12, CYP392D8 and CYP392D10 were found to be highly expressed by microarray data and subsequent qPCR analysis (Dermauw et al., 2013, Khalighi et al., 2016). Based on these results, functional expression and characterization of the aforementioned P450 genes was performed. The P450 genes were amplified from the resistant strain Mar-ab. CYP392A16 and CYP392A11 were successfully co-expressed with TuCPR in bacteria, while I could not express functionally CYP392A12, CYP392D8 and CYP392D10, despite several optimization efforts (change of temperature, different time of incubation, CPR-InFusion system).

I showed that CYP392A16 is active against several model substrates, showing the highest activity with Luciferin ME – EGE. CYP392A16 was subsequently incubated with insecticides that belong to different groups, according to ligand assays and reduced toxicity data on the resistant strain. I showed that CYP392A16 is capable of metabolizing abamectin only. That is in contrast with other P450 enzymes that have been studied up to date, as several of them are capable of metabolizing toxic compounds that belong to the same or even different group. For example, CYP6CM1 from *Bemisia tabaci* is capable of metabolizing neonicotinoids (imidacloprid, thiacloprid and clothianidin) and the insecticide pymetrozine which belongs to pyridine azomethine group (Karunker et al., 2009; Nauen et al., 2013). Nevertheless, this result may indicate that CYP392A16 might be active against abamectin only.

Subsequent experiments took place in order to detect the structure of the produced metabolite. According to *in vitro* and *in vivo* metabolism assays in vertebrates, there are three possible metabolites: 24-hydroxymethyl (24-OH), 26-hydroxymethyl (26-OH) and 3’’-O-Desmethyl (3’’ DM) (Zeng et al., 1996). Based on the *in vitro* data presented in this study, it is concluded that the formation of metabolite is a derivative of mono-oxygenation reaction, thus giving hydroxylated compound (24-OH or 26-OH) and not the 3’’DM metabolite. One step further, the isolated metabolite was used for toxicity assays. According to these data, the hydroxylated metabolite is less toxic in comparison to the parental compound as the metabolite did not have any toxic effect on spider mites.

Moreover, a specific antibody was developed against CYP392A16. According to the results, signal was detected in the homogenates from the resistant population but not from the

susceptible one, under the assay conditions. Presumably, the detected signal from the homogenates from the resistant population is CYP392A16, compared to the bacterial membranes that over-express the specific cytochrome P450. It is obvious that the spider mites from the resistant population over-express CYP392A16. This antibody is specific for the CYP392A16 of *Tetranychus urticae* and it could be used, possibly, as a diagnostic tool for the detection of metabolic resistance in the field.

Also, CYP392A11 shows the higher metabolism rates against the model substrate Luciferin ME – EGE, than several others tested. According to microarray data from a multi-resistant strain (Khagelhi et al., 2016) CYP392A11 is a candidate gene for the detoxification of METI acaricides. CYP392A11-TuCPR complex was incubated with insecticides that belong to different groups, such as tebufenpyrad, cyfluometofen, pyridaben, abamectin, bifenthrin and hexythiazox. The results revealed that CYP392A11 is catalyzing the metabolism of fenpyroximate, an acaricide that belongs to METIs and target complex I, and cyenopyrafen a new METI acaricide that targets complex II of the mitochondrial respiratory chain. Metabolism of fenpyroximate by CYP392A11 leads to the formation of a metabolite, which according to HPLC-MS results and published data from Motoba et al. (2000), corresponds to a non toxic metabolite, specifically metabolite M-5 (Motoba et al., 2000). Furthermore, CYP392A11 is capable of metabolizing the new METI acaricide, cyenopyrafen. The HPLC-MS analysis of this study indicates that CYP392A11 hydroxylates cyenopyrafen at high rates (kcat: 2.37 pmol depleted cyenopyrafen/min/pmol P450) but it was not possible to determine the exact hydroxylation position of the parental compound as further identification of these ions with MS/MS was not possible. Interestingly, CYP392A11 has been found to be over expressed in the multi-resistant strains Mar-ab and MR-VP and is correlated with resistance to cyenopyrafen,(Khalighi et al., 2016). Bioassays with the multi- resistant strains indicate cross resistance to cyenopyrafen. These strains show resistance to fenpyroximate also (Table I Ammendum). Both of these strains have never been exposed to cyenopyrafen, as it has not been used in Europe. Probably the over expression of CYP392A11 and its cross resistance to cyenopyrafen is due to previous selection to fenpyroximate, as this acaricide was used extensively in Europe. Luciferin ME-EGE was used in mite homogenates showing that P450s are highly active in resistant mites compared to the susceptible (Table I Ammendum). The substrate could be a potential diagnostic tool for monitoring P450-based resistance in the field.

Chapter 3: Heterologous expression of *T. urticae* P450s and homologous CPR in *Drosophila* in order to investigate the functional role of genes in resistance *in vivo*

3.1 Introduction

Drosophila provides plenty advantages as a laboratory rearing insect as it has low dietary requirements, easy manipulation, quick and high proliferation and short life cycle. Most important is that Drosophila is a well studied organism conferring additional benefits for research such as genomic, genetic and molecular tools. Although Drosophila is not considered as a pest, it is a powerful tool for studying insecticide/pesticide resistance (target-site and metabolic) and potentially addressing this global phenomenon, (Perry et al., 2011).

The ease of producing transgenic drosophila makes this insect a valuable tool. Many researchers have employed *Drosophila* system in order to study metabolic resistance, as well target site alteration, from several pests, as these kind of experiments are difficult to take place in the very same organisms. Generally, the GAL4/UAS system has been used extensively for gene expression in Drosophila, providing a temporal and spatial gene expression (Perry et al., 2011). It is based on the yeast GAL4 transcriptional regulator (driver) which binds to the Upstream Activator Sequence (UAS) of the gene of interest causing the initiation of transcription (Duffy, 2002). The system is separated in two transgenic Drosophila lines. The one line carries the GAL4 driver which is downstream of Drosophila's tissue specific enhancer (Perry et al., 2011). The other transgenic line carries the UAS-gene which is the GAL4 – responder (Rorth, 1998). When the flies are crossed, thus GAL4/UAS-gene of interest are combined in the progeny, expression of the target gene takes place in a tissue specific manner (Rorth, 1998) – (Figure 3.1).

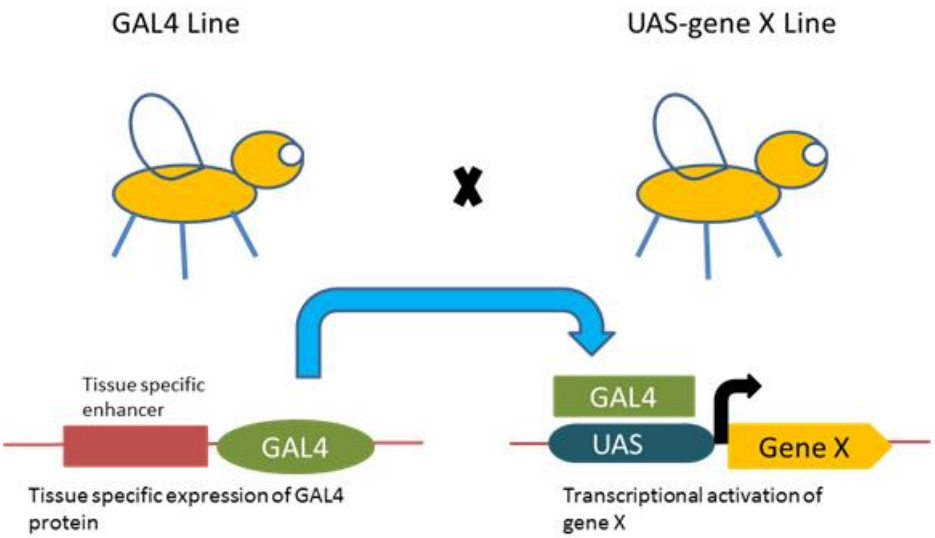


Figure 3.1: GAL4/UAS gene expression in *Drosophila melanogaster.* The GAL4 line contains the yeast GAL4 gene downstream of Drosophila's tissue specific enhancer. The UAS-gene X line contains the gene of interest downstream of UAS site. In the progeny, the GAL4 protein binds to UAS site activating the transcription of the gene of interest in specific tissues.

Specifically, the GAL4/UAS system has been used for driving the over-expression of Drosophila's CYP6G1 in specific tissues (Chung et al., 2007) showing the role of this gene in insecticide resistance. Daborn et al. (2007) over-expressed in Drosophila eight P450s from the very same organism, in order to evaluate their role in insecticide resistance, by using this system. Moreover, genes originating from resistant pests have been ectopically expressed in Drosophila by using the GAL4/UAS system (Daborn et al., 2012, Pavlidi et al., 2012, Zhu et al., 2010).

In this chapter I validated the role of CYP392A16 and CYP392A11 in the resistance *in vivo,* by using *Drosophila* as a model

3.2 Materials and Methods

3.2.1 Construction of the transgenic fly strains

Kapa Taq DNA Polymerase (Kapa Biosystems) was used for the amplification of the CYP392A11 ORF, using as template a plasmid containing this gene. CYP392A11 was sub-cloned using the primer pair CYP392A11_Dm F (5'GGAATTCATGCAAAAAGTTATGTCTTTATTGGA 3') and CYP392A11_Dm R (5' AAGGAAAAAAGCGGCCGCTCAGTCAGAATTGGAAAT 3'). The conditions used were 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min followed by a final extension step for 2 min. The sub-cloning of the target gene into pUAST vector was performed as previously described (Pavlidi et al., 2012) and clones were sequence-verified. A clone of the correct sequence was chosen in order to transform the germ-line of *Drosophila melanogaster* yellow-white strain using standard techniques. Independent transformed lines were made homozygous and crossed with balancers for the 2nd (Cyo/Gla; yw) and the 3rd chromosome (Sb/DgL3; yw) and different lines with insertion of the gene were mapped in the relevant chromosome.

Moreover, transgenic lines with CYP392A16 and the CPR of *T. urticae* were created. As template, plasmids containing CYP392A16 ORF and TuCPR ORF were digested with BglII and EcoRI, respectively, and inserted to pUAST by digesting it with the same restriction enzymes. A sequence-verified clone was used for the generation of transgenic flies in germ-line of D. melanogaster yellow-white strain. Independent transformed lines were made homozygous and crossed with balancers for the 2nd (Cyo/Gla; yw) and the 3rd chromosome (Sb/DgL3; yw) and different insertions of the genes were mapped in the relevant chromosome.

In order to generate homozygous transgene flies that would conditionally express both CYP392A11 and TuCPR, and CYP392A16 and TuCPR I selected a line containing UAS-CYP392A11 in the 3rd chromosome and another line containing UAS-CPR in the 2nd chromosome, and CYP392A16 in the 2nd chromosome and TuCPR in the 3rd chromosome. The homozygous male flies were crossed with a strain carrying multiple balancer chromosomes [w; if/CyOwglacZ; MKRS Sbe/TM6 Tbe] so as to generate independent lines for CYP392A11-TuCPR and CYP392A16-TuCPR and discriminate by varying progeny phenotype. By using standard genetic crossing techniques, we obtained heterozygote progeny (virgin females and males) carrying both transgenes against balancer chromosomes; these were inter-crossed in order to obtain the line carrying both transgenes in homozygous state.

3.2.2 Over-expression of CYP392A16, CYP392A11 and/or TuCPR in *Drosophila melanogaster*

The GAL4/UAS system was used to express CYP392A11, CYP392A16 and TuCPR in the transgenic flies, as previously described (Daborn et al., 2007). HR-GAL4 driver was used in order to drive the expression of CYP392A11, CYP392A16 and/or TuCPR in specific tissues (malpighian tubules, midgut and fat body) (Chung et al., 2007). The driver was constructed in the w^{1118} Drosophila strain and is homozygous for the 6g1-HR-GAL4 construct inserted on the 3rd chromosome. Transgenic UAS-TuCPR; UAS-CYP392A11 and UAS-CYP392A16; UAS-TuCPR virgin females were crossed with HR-GAL4 males and the progeny were treated with insecticides. Progeny from the crosses of UAS-TuCPR;UAS-CYP392A11 and UAS-CYP392A16; UAS-TuCPR virgin females with males of w^{1118} strain (i.e. not driving expression by GAL4) served as control for the (over)-expression of P450 and were also supplied with insecticide.

3.2.3 Toxicity assays

Several bioassays were used to investigate response to acaricides in Drosophila, including “adult feeding bioassays”, “adult contact bioassay” and “larvae bioassays”.

In the “adult feeding” bioassay used in order to investigate response to pesticides in Drosophila, 20 adult flies (10 males and 10 females) aged 2 to 4 days per replicate were used for the toxicity assay. Flies were collected in plastic vials and the insecticide was provided to them through wettex (or cloth). The insecticide was diluted in 5% sucrose. Each dose was tested in 3 replicates and 5% sucrose only served as control. Mortality was scored after 24 h. Five to 6 concentrations that cause 5 to 95% mortality were used. Contact adult bioassays and larvae bioassays for assaying the toxicity of acaricides in Drosophila were conducted as previously described (Daborn et al., 2007; Pavlidi et al., 2012).

A Chi-squared test was used to assess how well the individual LC_{50} values observed in the bioassays agreed with the calculated linear regression lines, and the results were analyzed with PoloPlus (LeOra Software, Berkeley, CA). The LC_{50} values and RR (resistance ratio) were considered significant if the 95% confidence limits (CL) did not include 1.

3.3 Results

3.3.1 Transgenic expression of CYP392A16, CYP392A11 and CPR of *T. urticae* in *Drosophila*

Transgenic UAS-TuCPR flies showing the strongest white phenotype (using as reference the intensity of eye color – Figure 3.2) were chosen for the creation of transgenes that express ectopically both CYP392A16 - TuCPR and CYP392A11 - TuCPR. Subsequently, the homozygous for both genes lines were crossed with HR-GAL4 strain in order to drive the expression in the specific tissues (malpigian tubules, fad body and midgut – Daborn et al., 2007).

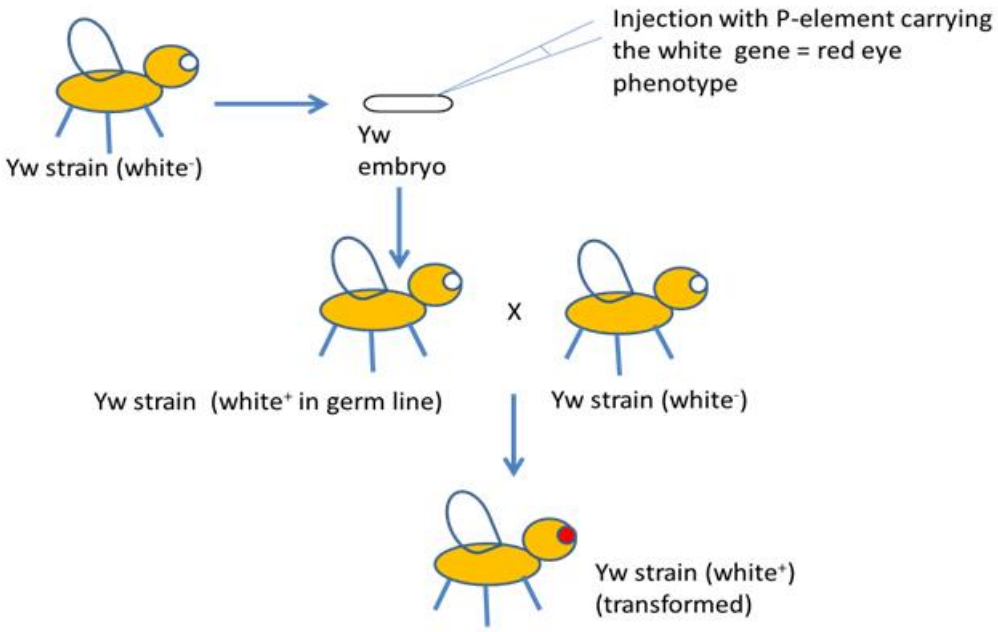


Figure 3.2: Eye phenotype in transgenic lines carrying both P450 and TuCPR. The P-element which carries the white+ gene (gives red eye color) is inserted in the germ line. After crossing the progeny and mapping the position on the chromosome of each P450 and TuCPR, the transgenic flies with intense eye color were chosen for further experiments.

3.3.2 Toxicity Assays

A feeding bioassay on adults was conducted by using 20 individuals per concentration of the supplied insecticides. The transgenic virgin females that carry both CYP392A16 and TuCPR (UAS-CYP392A16; UAS-TuCPR) were crossed with HR-GAL4 males to drive the expression in specific tissues in the progeny line. The offspring originated from the cross between virgin females of UAS-CYP392A16; UAS-TuCPR with w^{1118} males served as control for the toxicity assay with abamectin, as the offsprings from both crosses have the same genetic background. The same approach was followed for the examination of resistance levels to fenpyroximate and cyenopyrafen with the UAS-TuCPR; UAS-CYP392A11 transgenic flies.

The results (Table 3.1) indicate that the co-expression of each P450 and the TuCPR under GAL4 driver shifts toxicity levels for abamectin and fenpyroximate in comparison to the control line. Specifically, LC_{50} value of UAS-CYP392A16; UAS-TuCPR x w^{1118} for abamectin is at 31.2 mg/L (95% CL: 14.7 – 37.6), whereas UAS-CYP392A16; UAS-TuCPR x HR-GAL4 LC_{50} is at 53 mg/L (95% CL: 47.4 – 57.6), giving a resistance ratio of 1.69 folds (95% CL: 1.4 – 2.06) when these two lines are compared. As far as the toxicity assays with fenpyroximate are concerned, the results are in the same line as the ones for abamectin. Specifically, 2473 mg/L fenpyroximate (95% CL: 1672 -3135) need to dispatch the 50% of UAS-TuCPR; UAS-CYP392A11 x w^{1118} progeny, whereas when CYP392A11 and TuCPR are induced by HR-GAL4 (UAS-TuCPR; UAS-CYP392A11 x HR-GAL4) there is a shift in toxicity levels showing LC_{50} levels at 6597 mg/L (95% CL: 6012 - 7437) fenpyroximate. The resistance ratio between these two lines, that share the same genetic background, is 2.6 folds (95% CL: 2.17 – 3.26).

Feeding bioassays with cyenopyrafen were also conducted (up to 10000ppm) with CYP392A11-TuCPR transgenes, but this compound is not toxic for Drosophila.

Table 3.1: Toxicity Bioassay of transgenic *D. melanogaster* with abamectin and fenpyroximate.

Compound	Transgenic Lines	Regression parameters			
		LC50 (mg/L)(95% CI)	Slope±SE	χ^{2a} (df)	RR (95% CI)
Abamectin	UAS-CYP392A16; UAS-TuCPR x HR-GAL4	53 (47.4-57.6)	6.8 ± 0.87	14 (15)	1.69 (1.4 – 2.06)
	UAS-CYP392A16; UAS-TuCPR x w1118	31.2 (14.7-37.6)	6.3 ± 1.4	30.9 (14)	-
Fenpyroximate	UAS-TuCPR.32; UAS-CYP392A11 x HR-GAL4	6,597 (6,012-7,437)	5.6 ± 0.98	8.3 (10)	2.6 (2.17-3.26)
	UAS-TuCPR.32; UAS-CYP392A11 x w1118	2,473(1,672-3,135)	3.3 ± 0,4	17.9 (10)	-

[a] A χ^2 (Chi-squared) test was used to assess how well the individual LC_{50} values observed in the bioassays agreed with the calculated linear regression lines (LeOra Software, 1987)

Conclusions - Discussion

In this study, the successful ectopic expression of two P450s from *T. urticae* in Drosophila is reported, in presence of Cytochrome P450 Reductase (CPR) from the same species. The results indicated that the expression of CYP392A16 together with CPR alters levels of resistance to abamectin, as well the co-expression of CYP392A11 and TuCPR indicated resistance to fenpyroximate.

Many studies employed ectopic expression of detoxification genes and examination of their role in insecticide resistance in Drosophila as this insect has been proven a valuable tool.

The majority of the research has been focused on the ectopic expression of detoxification genes in Drosophila either originating from the same organism or from other pest species. More specifically, Pavlidi et al. (2012) employed the GAL4/UAS system in order to express and validate the role of CYP9J28 from *A. aegypti.* The resistance ratio was 7 folds between the induced line by GAL4 and the control (not induced by GAL4). Although, mosquitoes show a range of resistance to pyrethroids from 30 to >1000 mg/L (Vontas et al., 2012), when CYP9J28 was expressed in Drosophila the resistance ratio was low. Also, CYP6CM1 from *Bemisia tabaci* was expressed under GAL4/UAS system in Drosophila (Daborn et al., 2012). Even though the resistance ratio of *Bemisia tabaci* to imidacloprid was high, the ectopic expression of CYP6CM1 in Drosphila provides low resistance levels (2 to 3 folds resistance). The results of this study are in accordance to the ones presented here, as over-expression of CYP392A16; TuCPR resulted 1.69 folds resistance to abamectin and TuCPR;CYP392A11 show 2.6 folds resistance to fenpyroximate. Moreover, Riveron et al. (2013) expressed ectopically 2 P450s from *A. funestus* (CYP69a and CYP69b) showing that these genes play important role to pyrethroid resistance. Furthermore, Daborn et al. (2007) over-expressed eight P450s from *Drosophila melanogaster* under the GAL4/UAS system in order to examine their contribution to insecticide resistance. They indicated that over-expression of CYP6G1 gene gives medium to high resistance levels to DDT, dicyclanil and nitenpyram. Specifically, resistance to DDT is 4.06 folds, 1.96 folds resulted for nitenpyram and 2.23 folds for dicyclanyl. These results are in agreement to the ones presented in this study, as the transgenic flies tested for resistance to pesticides showed low resistance levels.

Also, other detoxification genes apart from P450s have been expressed ectopically in Drosophila. Daborn et al. (2012) expressed two detoxification enzymes originating from different pests, a carboxyl-esterase from *Lucilia cuprina* and GstE2 from *Anopheles gambiae.* The carboxyl-esterase encoded by Rma-1 allele originated from *Lucilia cuprina* gives resistance ratio 3 to 5 folds for diazinon and 600 to malathion, whereas Rop-1 allele confers 10 to 16

folds resistance to diazinon and not detectable resistance to malathion. Ectopic expression of these two alleles in Drosophila, toxicity assay results indicated that Rop-1 confers 6 to 7 folds resistance to diazinon and no resistance to malathion, whereas Rma-1 allele confers 2 folds resistance to diazinon and 29 to 30 folds resistance to malathion. It is clear that resistance ratios for both alleles are lower in Drosophila compared to the target's pest resistance. The results obtained by this study are in agreement to the previously reported ones. The resistance levels of transgenic flies to both abamectin and fenpyroximate are low, whereas *Tetranychus urticae* shows >1000 and >9 folds resistance to abamectin and fenpyroximate, respectively.

Drosophila serves for heterologous and ectopic expression of detoxification genes in order to examine their potential role in insecticide resistance. However, there are many limitations on assessing resistance levels *in vivo* by heterologous expression in Drosophila, e.g. phylogenetic distance between Drosophila and the pest under examination, different bioassay methods between Drosophila and the pest of interest, genetic distance between the resistant and susceptible pest strain might affect the resistance levels, detection of the specific tissue that the detoxification enzyme is expressed in the pest might be different from that in Drosophila, additional detoxification genes may contribute to insecticide resistance in the pest under examination, cis- or trans-regulators might induce the expression of detoxification genes (Feyereisen, 2012) and/ or even the co-operation of major counterparts, such as CPR. Taking into account the successful conditions of in vitro metabolism assays (co-expression of TuP450s with TuCPR / Chapter 2), it was decided to co-insert the P450s and CPR from *T. urticae*. The reason that resistance ratios to both insecticides tested in this study, by co-expressing the P450s with the homologous CPR, are low might lie on the phylogenetic distance between these two organisms or other reasons referred above (e.g. cis-, trans- regulators). In this study, CYP392A16 and CYP392A11 from *T. urticae* were successfully expressed in *D. melanogaster.* Their role in the phenotype of resistance to abamectin and fenpyroximate, respectively, was validated *in vivo*. It is the first time that genes from a distant phylogenetic organism are expressed in Drosophila. This system could be used as a standard method for validating the role of other detoxification enzymes *in vivo*, without replacing the *in vitro* heterologous expression which helps for the enzyme characterization.

Chapter 4: The relative contribution of target-site mutations in complex acaricide resistant phenotypes as assessed by marker assisted backcrossing in *Tetranychus urticae*.

4.1 Introduction

Insecticide resistance is a major threat for the chemical control of insects and mites in public health and agriculture. At present, the Insecticide Resistance Action Committee (IRAC) distinguishes between at least fifty-five different chemical classes and more than twenty-five distinct mode of action (MoA) groups (Sparks, 2015). MoA diversity is of key importance for effective Insecticide Resistance Management (IRM). However, the costs involved in the discovery, development and marketing of chemicals with new properties, increased immensely and slow down the development of compounds with new MoA. In addition, concerns about the environment and human health, integrated in new regulations, demand molecules with better selectivity. To preserve the utility and diversity of available and newly developed insecticides/acaricides, it is of utmost importance to understand the resistance mechanisms against these compounds (Sparks and Nauen, 2015) and develop diagnostic tools that support monitoring activities and resistance management.

A number of mechanisms have been shown to underlie insecticide resistance, most often quantitative or qualitative changes in major detoxification enzymes and transporters (pharmacokinetic mechanisms) and/or target-site mutations (pharmacodynamic mechanisms) (Feyereisen et al., 2015; Li et al., 2007; Van Leeuwen and Dermauw, 2016). When resistance is caused by a combination of factors (polygenic resistance), the overall resistance levels may be the sum of contribution of each individual factor (Bohannan et al., 1999; Raymond et al., 1989) but synergistic or antagonistic interactions between resistance loci also occur (Moore and Williams, 2005; Williams et al., 2005; Zhang et al., 2016). The relative contribution of each individual resistance locus to complex insecticide/acaricide resistance phenotypes has only been sporadically investigated (Hardstone and Scott, 2010). In particular, the relative importance and strength of target-site mutations is often hard to assess by merely associating a phenotype with mutation frequency in field populations, where prolonged selection may have led to the accumulation of additional resistance mechanisms. Furthermore, the majority of studies that look into epistatic interactions

and/or resistance levels confirmed by a single genetic factor, are sometimes difficult to interpret if resistance alleles are not investigated in a common genetic background (Liu and Pridgeon, 2002; McEnroe and Naegele, 1968; Peyronnet et al., 1994; Shi et al., 2004; Zhang et al., 2016). Therefore, analysis of a resistance trait requires the studied strains to be identical, except for its causal gene (Georghiou, 1969; McKenzie et al., 1982). Functional validation of resistance mutations has been reported after recombinant expression. Inhibitor-protein interactions are then quantified via enzymatic reactions or ligand binding assays such as voltage-clamp electrophysiology. Although they provide strong evidence of the effect of a mutation on the affinity for the compound to the target-site, they are less suitable to assess the relative phenotypic consequences *in vivo* (Cully et al., 1994; Ludmerer et al., 2002). A more precise way to determine the effect of a mutation *in vivo* is to introduce it in a defined susceptible genetic background, by utilizing genome editing techniques, such as CRISPR-Cas9 (Douris et al., 2016; Zimmer et al., 2016), in species where this approach is applicable. In species where genome editing tools are not yet available, a more feasible alternative is to repeatedly backcross resistant individuals with susceptible ones (Georghiou, 1969; McCart et al.,2005; Roush and Mckenzie, 1987). Marker-assisted backcrossing provides a straight-forward and relatively precise method to untangle a mutation of interest from other mechanisms that might have been co-selected. The impact of a modifier or interactions between modifiers can be then analyzed by comparing the genetically identical strains that differ only in a small region on the chromosome, which harbors the resistant locus of interest (Bajda et al., 2017; Brito et al., 2013).

The two-spotted spider mite, *Tetranychus urticae* (Chelicerata: Acari: Acariformes) is an important agricultural pest, that thrives on more than a 1,000 plant species (Jeppson et al., 1975; Migeon and Dorkeld, 2006-2015). Its short life cycle, high fecundity and haplo-diploid system facilitates a rapid evolution of acaricide resistance. Today, *T. urticae* has developed resistance to more than 90 different chemical compounds, including major groups of currently used acaricides (Sparks and Nauen, 2015; Van Leeuwen et al., 2013; Van Leeuwen et al., 2009a). In *T. urticae* and other related spider mites, very high resistance ratios (RRs) have been reported for a number of compounds (RR>10,000) (Kramer and Nauen, 2011; Van Leeuwen et al., 2009a) with numerous cases of cross-resistance to newly introduced acaricides, for example, (Khalighi et al. (2014). Several target-site mutations have been uncovered and were associated with acaricide resistance in populations of *T. urticae*, recently summarized in (Van Leeuwen and Dermauw (2016). These include mutations leading to amino acid substitutions in acetylcholinesterase (*AChE*) (G119S, A201S, T280A,

G328A and F331W) that are associated with resistance to organophosphates and carbamate (Khajehali et al., 2010). The L1024V and A1215D + F1538I substitutions in the voltage-gated sodium channel (VGSC) have been linked to resistance to Type I (absence of α-cyano group) and Type II (presence of α-cyano group) pyrethroids (Kwon et al., 2010a; Tsagkarakou et al., 2009). Six orthologous glutamate–gated chloride channel (GluCl) genes have been reported in spider mites and substitutions in G314D and G326E in GluCl1 and GluCl3, respectively, were associated with resistance to abamectin (Dermauw et al., 2012; Kwon et al., 2010b). The G126S, I136T, S141F, D161G, P262T substitutions (in different combinations) identified in the cytochrome b (cytb) cause strong bifenazate resistance (Mitochondrial Qo inhibitors: QoI) (Van Leeuwen et al., 2008). A substitution I1017F in the chitin synthase 1 gene (*CHS1*) has been linked with high levels of resistance to mite growth inhibitors, etoxazole, clofentezine and hexythiazox (Demaeght et al., 2014; Van Leeuwen et al., 2012). Most recently, an H92R substitution in the PSST subunit of the Mitochondrial Respiratory Complex I, has been associated with resistance to pyridaben, tebufenpyrad and fenpyroximate (Mitochondrial Electron Transport Inhibitors, site I, METI-I) (Bajda et al., 2017). As resistance in spider mites often has a polygenic basis, the relative contribution of target-site resistance to the overall resistance levels is currently unknown. One notable exception for *T. urticae* is the H92R mutation in the PSST subunit, which was introduced into a susceptible background by repeated backcrossing and shown to confer moderate levels of METI resistance (Bajda et al., 2017).

In this study, the relative contribution of four known target-site mutations conferring resistance to abamectin and pyrethroids was investigated. The method of (Bajda et al. (2017) was adopted and succeeded in generating 12 congenic resistant and susceptible lines of *T. urticae.* When a combination of mutations in homologous genes was reported, the phenotypic levels of resistance were examined for both the single mutations, as well as their combination.

4.2 Materials and Methods

4.2.1 Acaricides

Acaricides used in this study were commercial formulations of abamectin (Vertimec 18 g l^{-1} EC), milbemectin (Milbeknock 10 g l^{-1} EC), bifenthrin (Talstar 100 g l^{-1} EC), fluvalinate (Mavrik 240 g l^{-1} EW) and analytical grade fenpropathrin (Sigma Aldrich).

4.2.2 Spider mite strains

The susceptible Wasatch strain is an inbred line, originally collected from tomato in a greenhouse near Salt Lake City, Utah, USA. The pyrethroid susceptible strain KOP8 is an inbred line derived from the Houten strain (Chatzivasileiadis and Sabelis, 1997). Wasatch does not contain any of the so far described mutations. KOP8 harbors the A1215D substitution, potentially associated with pyrethroid resistance. The GH strain carries the L1024V genotype (*Musca domestica* numbering) of the VGSC gene and was collected from greenhouse grown maize in Utah USA. The TuSB9 strain carrying the A1215D and F1538I mutations (*Musca domestica* numbering) in VGSC was previously described (Tsagkarakou et al., 2009). The MAR-AB strain carrying G314D and G326E substitutions (*Tetranychus urticae* numbering) in GluCl1 and GluCl3, respectively, was previously described in (Dermauw et al. (2012). An overview of strains is presented in Table 1. All *T. urticae* strains were maintained on 3-week old potted kidney bean plants (*Phaseolus vulgaris L.*) in a climatically controlled room or incubator at 25 ± 1°C, 60% relative humidity, and 16:8 light : dark photoperiod.

4.2.3 Backcrossing experiments

To assess the relative resistance levels associated with mutations, we used a marker assisted backcrossing approach to produce near-isogenic sister lines (Fig. 4.1 and Table 4.1). The crossing procedure was previously outlined in (Bajda et al. (2017). In short, a haploid male of the resistant strain was crossed with a virgin female of the susceptible strain. The resulting heterozygous virgin females were backcrossed to susceptible males and heterozygote genotypes were identified by a TaqMan molecular assay or PCR and sequencing as it is described in section 4.2.5. This process was repeated for nine generations. In the last generation, a cross was carried out between the backcrossed heterozygous virgin females and their first born sons representing either a susceptible (absence of mutation) or the resistant (presence of mutation) genotype. This finally resulted in congenic homozygous lines for the mutation and the wild type allele. The final crosses were performed as follows (see Table 4.1): For the mutations in GluCls, G314D in GluCl1 and G326E in GluCl3, MAR-AB

males were crossed with Wasatch virgin females in order to separate the mutations in different lines, as they are inherited independently(Dermauw et al., 2012), after which they were introgressed separately: ♀ 314D/314G x ♂ 314D or ♂ 314G to generate GluCl1_R1-R3 and GluCl1_C, ♀326E /326G x ♂ 326E or ♂326G to produce homozygous congenic GluCl3_R1-R3 and GluCl3_C respectively. Mutations were later joined in a single line by dedicated crosses as follows: ♀GluCl1_R1 x ♂GluCl3_R1, ♀GluCl1_R2 x ♂GluCl3_R2, ♀GluCl1_R3 x ♂GluCl3_R3 and ♀GluCl1_C x ♂GluCl3_C to produce GluCl1+3_R1,R2,R3 and C respectively. For the mutations in VGSC; the ♀1024V/1024L x ♂1024V or ♂1024L were crossed to obtain homozygous congenic lines VGSC_R1-R3 and VGSC_C1 respectively, ♀1215D+1538I/1215D+1538F x ♂1215D+1538I or ♂1215D+1538F to obtain homozygous congenic VGSC_R4-R6 and VGSC_C2 respectively.

Table 4.1: Summary of crosses performed to create congenic *T. urticae* lines. VGSC mutations were numbered according to *Musca domestica* numbering, whereas GluCl1 and GluCl3 substitutions according to *Tetranychus urticae* numbering. *IRAC mode of action group number is shown between brackets.

Strain	***Resistant to****	***Target-site mutation***	***Crossed to***	***Backcrossed lines***	
MAR-AB	abamectin (6)	GluCl1 (G314D)	Wasatch	GluCl1_C, GluCl1_R1, R2, R3	GluCl1+3_C, GluCl1+3_R1, R2, R3
MAR-AB	abamectin (6)	GluCl3 (G326E)	Wasatch	GluCl3_C, GluCl3_R1, R2, R3	
GH	pyrethroids (3A)	VGSC (L1024V)	Wasatch	VGSC _C1, VGSC _R1, R2, R3	
TuSB9	pyrethroids (3A)	VGSC (F1538I + A1215D)	KOP8	VGSC_C2, VGSC_R4, R5	

4.2.4 Single mite DNA extraction

In order to perform single mite genotyping for G314D, G326E (MAR-AB) and F1538I, A1215D (TuSB9), L1024V (GH) single mite DNA was extracted following the CTAB method (Navajas et al., 1999). In short, individual mites were homogenized in 200 µl of extraction buffer (2% CTAB, 1.4M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris – HCl, pH:8.0) and incubated at 65 °C for 15 min. Equal volume of chloroform: isoamylalcohol (24:1) was used in order to remove proteins. The DNA was precipitated by isopropanol and

washed with 75% ethanol. The pellet was air-dried and resuspended in 20 µl DEPC treated water.

4.2.5 Genotyping

Single mite genotyping was performed with standard PCR and sequencing (L1024V) and/or TaqMan method (Ilias et al., 2017) (mutations F1538I, G314D and G326E). PCRs were conducted in 30 µl final volume with 3 µl 10x Kapa Taq Buffer A, 0.2 mM of each dNTP, 0.5 µM each primer, 2µl template, 0.3 µl Kapa Taq polymerase and 1.8 µl DMSO with cycling conditions; 5 min at 95 °C followed by 40 cycles 30 sec at 95 °C, 30 sec at 55 °C, 40 sec at 72 °C and 2 min of final extension. Reactions were performed in BIOER GENEPRO Thermal Cycler. PCR products were purified with Nucleospin Gel and PCR Clean-Up purification kit (Macherey – Nagel) and sequenced at Macrogen sequencing facility (Amsterdam). Sequencing data were analyzed using BioEdit 7.0.1 software (Hall, 1999). Primers used for the PCR reactions and sequencing are listed in Table 4.2.

TaqMan assay was performed as previously described (Ilias et al., 2017). In short, all assays were carried out in 15 µl total volume containing 2 µl of genomic DNA, 7.5 µl TaqMan Universal PCR Master Mix, 0.8 µM of each primer and 0.2 µM of each probe. Samples were run on CFX Connect, Real-Time PCR Detection System (Biorad) using the temperature cycling conditions of: 10 min at 95˚C followed by 40 cycles of 92˚C for 15 sec and 60˚C for 1 min. The increase in VIC and FAM reporter dyes, representing individuals with the resistant and susceptible alleles respectively, was monitored in real time using the CFX Manager software. Positive and negative template controls were included in each run to aid genotype scoring. Primers and probes used for the TaqMan assay are listed in Table 4.2.

4.2.6 Toxicity bioassays

To assess the toxic effects of abamectin, milbemectin and pyrethroids 20–30 young adult female mites were transferred on the upper side of 9 cm^2 square-cut kidney bean leaf discs on wet cotton wool. Plates were sprayed with 1 ml of spray fluid at 1 bar pressure with a Potter Spray Tower (Burkard Scientific, UK) to obtain a homogenous spray film (2 mg deposit / cm^3). Experiments were then placed in a climatically controlled room at 25±0.5°C, 60% RH and 16/ 8 h (light/dark) photoperiod. Three to four replicates of at least five serial dilutions of each acaricide and a control (deionized water or 1:100 dilution of the mixture of N, N-dimethylformamide and emulsifier W, depending on the acaricide used) were tested. Fenpropathrin was of technical grade and formulated in 3:1 v/v mixture of N, N-

dimethylformamide and emusulfier W and subsequently diluted in deionized water as previously described (Van Leeuwen et al., 2007). Mortality was assessed after 48h for all acaricides used. Mites were scored as being alive if they could walk twice the distance of their body size after being prodded with a camel's hair brush (Sato et al., 2005). All mortalities obtained for control treatment were lower than 10%. LC_{50} values, slopes, RRs and 95% confidence limits were calculated by probit analysis (POLO, LeOra Software, Berkeley, USA) (Robertson, 1992). In case 5,000 mg l^{-1} did not cause 50% mortality, no further attempts were made to determine LC_{50}s and RR was calculated by dividing 5,000 mg l^{-1} by the LC_{50} of susceptible strain. The effect of the treatment on the susceptible parent and the experimental line was considered significantly different if the hypothesis of equality of slopes and intercepts was rejected (p value=0.05) (Robertson et al., 2007). If a regression line - illustrating dose response - could not be derived (LC_{50} of the experimental line was found to be higher than 5,000 mg l^{-1}), the effect of treatment was considered different when the LC_{90} of the susceptible control was lower than 5,000 mg l^{-1}.

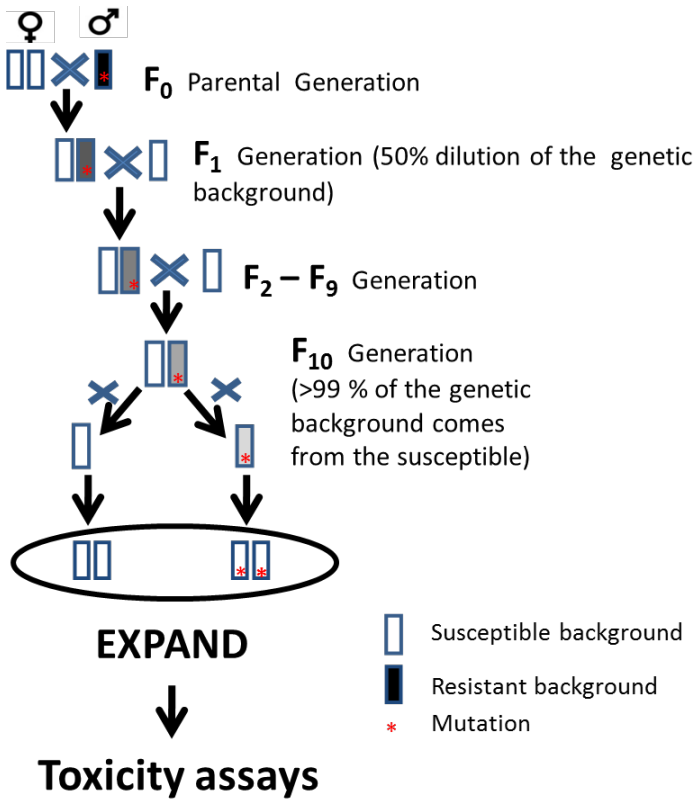


Figure 4.1: Procedure of genetic crosses.

Table 4.2: Probes and primers used for the study (from Ilias et al., 2016; Dermauw et al., 2012; Khajehali et al., 2010)

Name	Sequence (5'-3')	Description
G314D_F (Primer)	CACGTCAAATATCAGGAATCAATGCAT	GluCl1 mutation (G314D)
G314D_R (Primer)	GGCAAATTCAATGAGAGCACCAAAA	
G314D_VIC (Probe)	TTGACATTTGGACAGATTG	
G314D_FAM (Probe)	TGACATTTGGACAGGTTG	
Tu_GluCl1_diag_F	TTGGATTGACCCTAACTCAGCA	
Tu_GluCl1_diag_R	TTGCACCAACAATTCCTTGA	
G326E_F (Primer)	TCCACCGGTCAGTTACATTAAAGC	GluCl3 mutation (G326E)
G326E_R (Primer)	CAAACTCTAGGAGGGCACCAAAA	
G326E_VIC (Probe)	TTGGACCGAAGTCTG	
G326E_FAM (Probe)	TTTGGACCGGAGTCTG	
Tu_GluCl3_diag_F	CCGGGTCAGTCTTGGTGTTA	
Tu_GluCl3_diag_R	CACCACCAAGAACCTGTTGA	
F1538I_F (Primer)	AACAACCAGTTTATGAAAATAGTATTCTGATGTACTTA	VGSC mutation (F1538I)
F1538I_R (Primer)	CACCTCCTTTCTTTTTTTGTTCATTAAAATTATCAATAATG	
F1538I_VIC (Probe)	TTTTTGGCTCTTTTATCACAC	
F1538I_ FAM (Probe)	TTTTGGCTCTTTTTTCACAC	
kdrF4	CAACATTCAAAGGTTGGACAAT	VGSC mutation (L1024V)
kdr R1	TCTTCCGTCATCAACATCTCC	
kdrF5	TGATTGTTTTCCGTGTCCTG	
kdrR5	CTGCGAAGCTGCTTAAGTCC	
kdrF2	TGCATCTCAATTGTCCAAGG	VGSC mutation (A1215D)
kdrR2	GTTTCTTCCAGGCAACATGG	

4.3 Results

4.3.1 Establishment of congenic lines

The initial crosses between parental resistant and susceptible strains are outlined in Table 4.1. Briefly, the susceptible strain Wasatch, which does not carry any of the mutations studied here, was used for the most of the backcrossing experiments (Table 4.1). To study the mutations in GluCl1 (G314D) and GluCl3 (G326E) associated with abamectin resistance, virgin females of Wasatch were crossed with males of the abamectin resistant strain MAR-AB carrying both GluCl mutations. Similarly, for the L1024V mutation associated with pyrethroid resistance, Wasatch virgin females were crossed with males of the pyrethroid resistant strain GH that carries L1024V. The effect of A1215D + F1538I mutations in pyrethroid resistance was examined through crossing males of TuSB9 with females of the parental susceptible strain KOP8 (carrying the A1215D only).

For the nuclear encoded mutations, the final cross between heterozygous backcrossed females and their sons resulted in congenic homozygous lines with either the mutation fixed or absent (Figure 4.1, Table 4. 1, see paragraph 4.2.3 for outline experimental setup). Since mutations in GluCl1 and GluCl3 are not genetically linked(Dermauw et al., 2012), the impact of each mutation could be assessed separately. Once homozygous backcrossed lines carrying a mutation either in GluCl1 (GluCl1_R1-R3) or in GluCl3 (GluCl3_R1-R3) and their respective congenic control lines (GluCl1_C and GluCl3_C) were generated, the mutations were joined again by dedicated crosses, giving rise to GluCl1+3_R1-R3. The susceptible control GluCl1+3_C was obtained with the cross GluCl1_C x GluCl3_C. One replicate with genotype A1215D + F1538I (pyrethroid resistance mutations) was lost during backcrossing and only two biological replicates VGSC_R4, R5 could be analyzed for each genotype.

4.3.2 Toxicity assays of Parental and Backcrossed strains

4.3.2.1 *Abamectin and Milbemectin*

Abamectin and milbemectin were tested against the parental susceptible strain, Wasatch and the resistant strain, MAR-AB (G314D + G326E), with the latter one exhibiting high resistance levels to abamectin (1354.9 fold) and moderate resistance to milbemectin (71.7 fold) in comparison to Wasatch (Table III Ammendum).

The introgressed strains carrying resistance mutation in only one of the GluCls (either GluCl1 or GluCl3) showed minor resistance to abamectin and milbemectin with RR values up to 3.3 and up to 1.6, respectively (Figure 4.3, Table III Ammendum). However, when mutations were joined by dedicated crosses, individuals carrying both mutations (GluCl1+3_R1-3 congenic lines) showed higher resistance levels to both compounds. The RR values obtained for abamectin and milbemectin were up to 19.8 and 13.7 fold, respectively (Figure 4.3, Table III Ammendum).

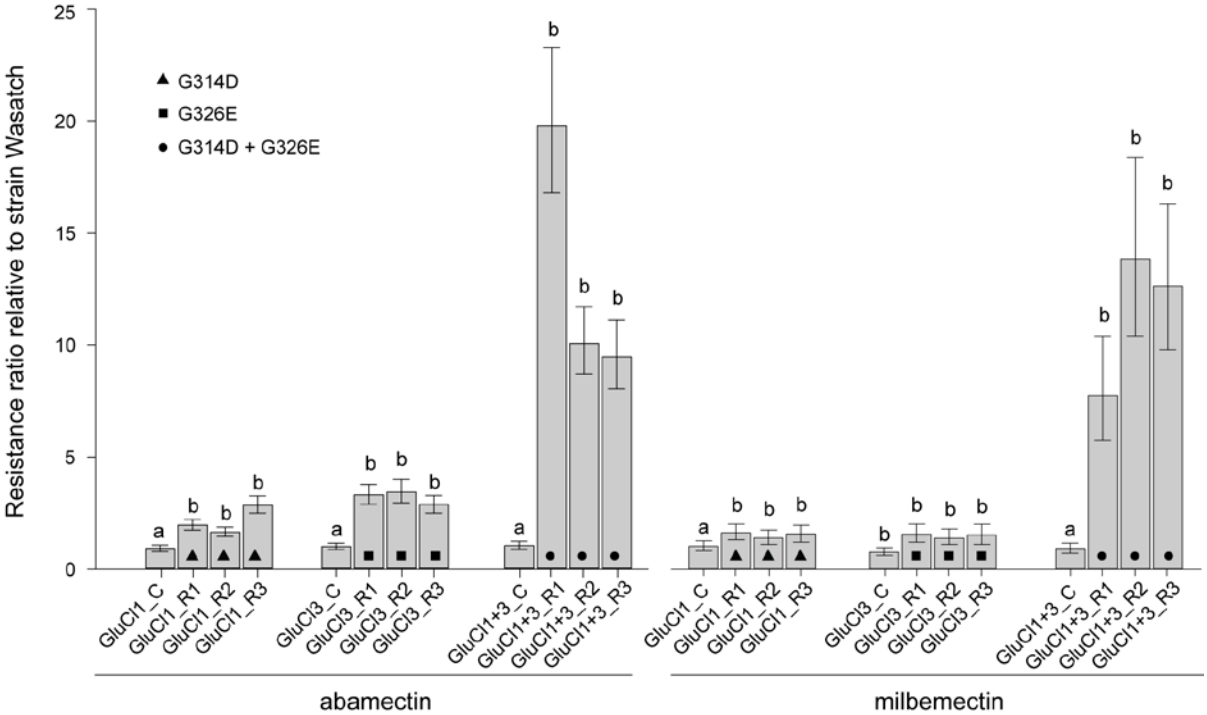


Figure 4.3: Susceptibility levels of backcrossed *T. urticae* lines GluCl1_R1-R3 (G314D), GluCl1_C, GluCl3_R1-R3 (G326E), GluCl3_C, GluCl1+3_R1-R3 (G314D+G326E), GluCl1+3_C to abamectin and milbemectin. The RRs were calculated as the LC_{50} values of the backcrossed lines divided by the LC_{50} of the parental susceptible strain Wasatch. Error bars represent the 95% confidence limit calculated by probit analysis. Letters above bars indicate lines where acaricide treatment had statistically the same (a) or different (b) effect comparing to Wasatch (PoloPlus LeOra Software).

4.3.2.2 *Pyrethroids*

The parental susceptible strains, KOP8, which carries only the A1215D VGSC substitution, and Wasatch showed high susceptibility to bifenthrin, fluvalinate and fenpropathrin whereas the GH (L1024V) and TuSB9 (A1215D + F1538I) resistant strains were highly resistant to the aforementioned pyrethroids (Table 4.3, Fig.I and II Ammendum).

The backcrossed strains VGSC_R1-3 and VGSC_R4,5 exhibited high levels of resistance to all pyrethroids used in this study (bifenthrin, fluvalinate and fenpropathrin), with RR values being greater than 200 fold in some cases. In contrast, the backcrossed susceptible lines VGSC_C1 and VGSC_C2 were susceptible to all three compounds (Table 4.3, Fig. I and II Ammendum).

Table 4.3: Toxicity of pyrethroids (bifenthrin, fluvalinate and fenpropathrin) to adult females of backcrossed lines VGSC_C1, VGSC_R1-R3 (L1024V genotype), VGSC_C2, VGSC_R4,5 (F1538I+ A1215D genotype) and their parental strains (Wasatch, GH, KOP8, TuSB9). [a] Number of mites used in toxicity tests. [b] RR compared to Wasatch in case of GH, VGSC_C1 and VGSC_R1-3 or KOP8 in case of TuSB9, VGSC_C2 and VGSC_R4,5 lines. a: Treatment effect was significantly different when compared to Wasatch or KOP8.

Compound	*Strain*	*Genotype*	*N*[a]	*LC_{50} mg l^{-1} (95% CI)*	*Slope (±SE)*	*χ^2 (df)*	*RR (95% CI)*[b]
Bifenthrin	Wasatch	L1024	404	3.8 (2.1; 4.7)	3.9 (± 0.8)	17 (13)	-
	GH	L1024V	443	1031.0 (721.7; 1406.8)a	1.5 (± 0.1)	14 (13)	271.8 (185.3; 398.8)
	KOP8	A1215D+F1538	354	4.1 (3.0; 4.8)	3.2 (± 0.6)	8 (16)	-
	TuSB9	A1215D+F1538I	517	1,715.8 (696.5; 2474.8)a	2.3 (± 0.4)	24 (16)	423.5 (272.4; 658.4)
	VGSC_C1	L1024	382	5.09 (3.4; 6.2)a	4.9 (± 0.8)	26 (13)	1.3 (1.0; 1.8)
	VGSC_C2	A1215D+F1538	436	4.6 (3.3; 5.5)	4.8 (± 0.8)	29 (16)	1.1 (0.9; 1.5)
	VGSC_R1	L1024V	670	353.3 (277.1; 410.3)a	3.7 (± 0.6)	20 (19)	93.2 (69.1; 125.7)
	VGSC_R2	L1024V	560	328.2 (260.7; 390.5)a	3.0 (± 0.5)	13 (18)	86.5 (63.1; 118.8)
	VGSC_R3	L1024V	427	405.4 (329.8; 466.5)a	3.8 (± 0.7)	13 (13)	106.9 (79.4; 143.9)
	VGSC_R4	A1215D+F1538I	554	508.9 (261.6; 670.8)a	2.6 (± 0.6)	16 (12)	125.6 (87.5; 180.3)
	VGSC_R5	A1215D+F1538I	435	538.8 (380.6; 670.2)a	3.6 (± 0.5)	21 (12)	134.0 (100.4; 176.1)
Fluvalinate	Wasatch	L1024	479	102.2 (82.7; 118.5)	3.9 (± 0.6)	18 (17)	-
	GH	L1024V	118	>5,000a	-		>45
	KOP8	A1215D+F1538	294	92.4 (67.3; 117.5)	4.7 (± 1.1)	15 (11)	-
	TuSB9	A1215D+F1538I	186	>5,000a	-	-	>50
	VGSC_C1	L1024	436	83.0 (63.2; 98.5)	3.7 (± 0.6)	16(15)	0.8 (0.6; 1.0)
	VGSC_C2	A1215D+F1538	508	87.0 (69.3; 102.4)	3.7 (± 0.5)	19 (15)	0.9 (0.8; 1.2)
	VGSC_R1	L1024V	188	>5,000a	-	-	>45
	VGSC_R2	L1024V	180	>5,000a	-	-	>45
	VGSC_R3	L1024V	213	>5,000a	-	-	>45
	VGSC_R4	A1215D+F1538I	194	>5,000a	-	-	>50
	VGSC_R5	A1215D+F1538I	161	>5,000a	-	-	>50
	Wasatch	L1024	360	21.3 (15.8; 26.9)	3.1 (± 0.5)	23 (19)	-
Fenpropathrin	GH	L1024V	97	>5,000a	-	-	>230
	KOP8	A1215D+F1538	297	13.7 (11.0; 16.9)	2.8 (± 0.5)	8 (15)	-
	TuSB9	A1215D+F1538I	182	>5,000a	-	-	>360
	VGSC_C1	L1024	476	35.2 (26.2; 44.2)a	2.1 (± 0.3)	5(16)	1.7 (1.2; 2.3)
	VGSC_C2	A1215D+F1538	396	21.5 (15.9; 26.8)a	3.5 (± 0.5)	15 (19)	1.6 (1.1; 2.2)
	VGSC_R1	L1024V	153	>5,000a	-	-	>230
	VGSC_R2	L1024V	155	>5,000a	-	-	>230
	VGSC_R3	L1024V	180	>5,000a	-	-	>230
	VGSC_R4	A1215D+F1538I	171	>5,000a	-	-	>360
	VGSC_R5	A1215D+F1538I	156	>5,000a	-	-	>360

Conclusions - Discussion

Field collected *T. urticae* strains often exhibit very high levels of resistance to multiple acaricides used for their control. Due to the identification of acaricide target-site sequences (Grbic et al., 2011b; Van Leeuwen et al., 2013) and implementation of recently developed genetic mapping tools (Bajda et al., 2017; Van Leeuwen et al., 2012; Van Leeuwen and Dermauw, 2016), a number of mutations has been uncovered in the target-site of frequently used acaricides. However, to what extent these mutations determine the resistant phenotype is mostly unknown. Resistant field strains investigated so far, typically display a broad altered transcriptional response with the putative involvement of many detoxifying enzymes and transporters that might affect acaricide toxicity (Demaeght et al., 2013; Dermauw et al., 2013; Khalighi et al., 2016). Crossing experiments have revealed that a complex genetic make-up typically underlies resistance, implying the additive effect of multiple mechanisms (Dermauw et al., 2012; Van Pottelberge et al., 2009a; Van Pottelberge et al., 2009b). Moreover, the extent by which resistant alleles confer resistance can also vary according to the genetic background in which they are expressed (McKenzie et al., 1982; Schrag et al., 1997).

Several studies have used congenic backcrossed lines to assess insecticide related fitness cost/advantage and pleiotropic effects (Arnaud et al., 2002; ffrench-Constant and Bass, 2017; Helle, 1962; Wang and Wu, 2014; Xiao et al., 2017; Yuan et al., 2017). By substituting phenotypic selection with molecular marker-assisted backcrossing, the potential accumulation of alleles with additive effect can be uncoupled (Roush and Mckenzie, 1987). Such a setup has been previously used to assess the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost (Brito et al., 2013) and recently, to investigate resistance levels to METI-I acaricides caused by a mutation in the PSST subunit of complex I in *T. urticae* (Bajda et al., 2017).

Here, the relative phenotypic contribution of target-site resistance mutations, previously uncovered in highly resistant *T. urticae* field populations, was analyzed. A marker-assisted backcrossing procedure was adopted, as has been described in Bajda et al. (2017), in order to untangle the target-site resistance loci from potential complex additive genetic mechanisms. Although a possible effect of closely linked loci connot be excluded (Hospital, 2001), previous research involving resistance gene mapping by means of bulk segregant analysis, revealed a high recombination rate in *T. urticae* (Demaeght et al., 2014; Van

Leeuwen et al., 2012) which suggests that the procedure performed here, resulted in near-isogenic lines.

Abamectin resistance mutations

Both abamectin and milbemectin resistance has been reported frequently in spider mite populations worldwide (Nicastro et al., 2010; Sato et al., 2005; Yorulmaz and Ay, 2009) exhibiting >1000 fold resistance in some cases (Dermauw et al., 2012). These molecules target both GluCls and GABA gated chloride channels (GABACl), although GluCls are considered the main target (Clark et al., 1994; Wolstenholme, 2010). In contrast to insects with a single copy, the genome of *T. urticae* harbors six orthologous GluCl genes (Dermauw et al., 2012). Two non-synonymous mutations have been associated with resistance to abamectin, the G314D in GluCl1 and G326E in GluCl3 (Dermauw et al., 2012; Kwon et al., 2010b). When G314D and G326E were introgressed separately, only low levels of resistance remained. However, when both mutations were joined by dedicated crosses, resistance levels increased to 10-20 fold. These resistance levels are comparable with a previous study, where an abamectin resistant strain homozygous for both GluCl mutations was investigated. Resistance levels in that strain reached only 20-fold (Kwon et al., 2015; Kwon et al., 2010b), suggesting that target-site mutations were the only factor contributing to resistance. A possible explanation for the relatively low resistance levels conferred by the combination of two GluCl mutations may lie in the number of genes involved in channel assembly. Glutamate-gated chloride channels typically consist of five subunits, which in *T. urticae* can be encoded by 5 different GluCl genes. Hence, if the channel consists of a combination of subunits carrying the resistance associated substitution (GluCl1 and/or GluCl3) and a GluCl2 subunit (GluCl2 does not carry a resistance associated substitution, while GluCl4 and GluCl5 naturally carry substitutions that interfere with abamectin binding see Dermauw et al. (2012)), abamectin binding might still be possible. In addition, we cannot exclude the possibility of heteromeric channel assembly, consisting of GluCls and GABACl (Cully et al., 1994; Ludmerer et al., 2002). In such case, the existence of mutations in GluCl1 and GluCl3 alone would also not be capable to fully prevent channel blocking. Consequently, our results also reconfirm the importance of additional mechanisms in abamectin resistance (Clark et al., 1994; Pavlidi et al., 2015; Riga et al., 2014). Studies with synergists and biochemical tests have previously implied the involvement of detoxification enzymes in resistance in many field collected strains worldwide (Campos et al., 1996; Pavlidi et al., 2015; Stumpf and Nauen, 2002). For instance, a P450 (CYP392A16) was reported to be overexpressed in abamectin resistant strains and detoxifies abamectin rapidly (Riga et al., 2014). Therefore

very high abamectin resistance levels in the MAR-AB strain (Table III Ammendum) may be attributed to a joint action of P450 detoxification and decreased sensitivity of the target-site, potentially even acting synergistically.

Milbemectin belongs to the same insecticidal class as abamectin and acts on the same target-site. Whether cross-resistance might occur between both compounds is therefore of crucial importance, and still a matter of debate. Here, we show that the combination of both GluCl mutations confers resistance levels of about 10-fold, indicating potential cross-resistance risks between milbemectin and abamectin, as has been previously suggested (Nicastro et al., 2010; Sato et al., 2005).

Pyrethroid resistance mutations

Pyrethroid resistance has been documented globally in *T. urticae* with resistance levels exceeding 10,000 folds in some cases (Herron et al., 2001; Van Leeuwen et al., 2005). Unlike most other arthropods, spider mites have mutations in unique positions on VGSC (Ding et al., 2015; Kwon et al., 2010a; Tsagkarakou et al., 2009), instead of the known kdr (L1014F) and super-kdr (M918T) mutations (*Musca domestica* numbering). The super-kdr mutation has been identified only once in a *Tetranychus evansi* strain (Nyoni et al., 2011). Three point mutations have been located in the sodium channel of spider mites, L1024V and F1538I in combination with A1215D (Kwon et al., 2010a; Tsagkarakou et al., 2009). Backcrossing experiments indicated the major effect of both L1024V and A1215D + F1538I mutations in pyrethroid resistance. Interestingly, the KOP8 strain has the A1215D mutation uncoupled from F1538I and is susceptible to all pyrethroids, indicating that the mutation alone has no effect on pyrethroid toxicity. So far, the mutation F1538I has been studied most thoroughly and its effect in resistance to pyrethroids has been confirmed by electrophysiological studies (Tan et al., 2005). Here, we showed that both L1024V and A1215D + F1538I mutations confer high resistance levels to all pyrethroid compounds, irrespectively of their type, i.e. presence of α-cyano group and/ or extended halogenated acidic moiety, suggesting that the sodium channel mutations can cause field failure of the pyrethroids.

General Discussion – Future plans

Previous studies had indicated that both target site resistance mutations and detoxification enzymes are involved in resistance to abamectin and METIs (Ilias et al., 2014, Van Pottelberge et al., 2009). Microarray analysis of resistant populations, compared to the susceptible, revealed high expression levels of P450 genes, such as CYP392A11, CYP392A12, CYP392A16, CYP392D8 and CYP392D10, indicating potential role in resistance to these insecticides / acaricides (Dermauw et al., 2013; Kahlighi et al., 2016), while target site mutations had been associated with abamectin and pyrethroid resistance (Dermauw 2012, Tsagkarakou 2009).

I) Summarizing Results

CYP392A16 and CYP392A11 were expressed successfully in *E. coli* in vitro. They were tested against several model substrates showing the highest activity with L ME-EGE. Metabolism assays showed that CYP392A16 catalyzes the metabolism of abamectin, leading to the production of a non-toxic hydroxylated metabolite as it was confirmed by toxicity assays. A peptide antibody was developed against CYP392A16 and specific signal was detected in crude homogenates from resistant population as it was revealed by Western blot analysis. Similar experiments were conducted for CYP392A11 characterization, showing that it metabolizes two METI acaricides that act on different complexes of the mitochondria respiratory chain, fenpyroximate and cyenopyrafen. Both of these compounds are hydroxylated to less/non toxic metabolites. Toxicity assay data in multi-resistant strains with cyenopyrafen indicated cross resistance to this active ingredient.

CYP392A16, CYP392A11 and TuCPR were subsequently co-expressed ectopically in *Drosophila melanogaster* in order to validate their role in the phenotype of resistance to both abamectin and METI acaricides (Chapter 3). I employed the GAL4/UAS system which drives the expression of these genes in specific tissues (midgut, malpigian tubules and fat body). The results indicated that transgenic lines expressing CYP392A16; TuCPR are resistance to abamectin while lines expressing TuCPR; CYP392A11 are resistant to fenpyroximate. Application of cyenopyrafen did not confer any toxicity to the parental strain used for the generation of the transgenic lines. Although resistance levels in both cases are low, the results are not that different from those in other studies (Daborn et al., 2007, 2012). It was demonstrated that *D. melanogaster* could be used for the validation of the role of detoxification enzymes, originating from a phylogenetically distant organism, *in vivo*.

In the last Chapter, a marker-assisted backcrossing approach was employed in order to look at the phenotypic effect of the main and currently relevant target-site mutations reported to confer resistance to abamectin and pyrethroids. Mutations in VGSC confer high levels of resistance and their presence in populations alone is enough to cause field failure after acaricide treatment. In contrast, although the functional importance of GluCl mutations and the cumulative effect of mutations in multiple channels was confirmed, mutations in only two channels genes does not lead to the high resistance levels that have been reported for abamectin resistance. Overall, our results functionally validate the importance of mutations that have been inferred from correlation analysis and genetic mapping.

II) Impact of this study on resistance research and insecticide resistance management

The main points of this study, regarding its possible impact on insecticide resistance research and management are summarized below:

- CYP392A11 found to be over-expressed in multi-resistant strains was shown to be capable to metabolise both fenpyroximate and cyenopyrafen. These data indicate cross – resistance to cyenopyrafen a compound they have never been exposed (Table I – Ammendum)
- Novel insights and functional validation concerning the contribution of specific target site resistance mutations, alone or in combination, in the resistance phenotype were produced
- The utility of *Drosophila* as a model to in vivo validate mite genes in resistance was demonstrated
- The basis for novel biochemical diagnostics was provided: The L ME-EGE P450 substrate which was utilised by the CYP392A11 and CYP392A16 might be used as a diagnostic substrate for the detection of both abamectin and METI P450-mediated resistance in the field. Indeed, mite homogenates from the resistant and susceptible strains were tested enzymatically with the model substrate L ME-EGE and the P450 activity was remarkably high in the resistant strain in comparison to the susceptible (Table II Ammendum). An additional diagnostic tool for detecting abamectin resistance could be based on the the specific antibody against CYP392A16. Elisa based-diagnostic and / or lateral flow test (Nauen et al., 2015) could be developed for the specific detection of elevated

CYP392A16 protein levels associated with resistance, thus facilitating the management of resistance.

III) Future Plans

Towards this direction, several P450s associated with resistance, were characterized. However CYP392D8, CYP392D2 and CYP392A12 were not expressed functionally in bacteria. An alternative expression system, such as baculovirus and yeast could be used for their expression and further characterization.

The homozygous lines generated in this study could be further used in fitness experiments in order to study the influence of the mutations on the life cycle of the spider mites and other parameters, such as fecundity and longevity. This kind of knowledge would be a great addition for the management of these phenotypes in the field.

Further studies of the exact role of detoxification genes in resistance to abamectin, such as CYP392A16 and GSTd14 (Pavlidi et al., 2015), will provide better understanding of the detoxification mechanisms and pathways involved in resistance to abamectin. Localization studies, using the CYP392A16 antibody will reveal the specific tissues that detoxification occurs, to provide novel insights into the physiology of acaricide detoxification (tissue localizaton, barriers to target site). Also, CrispR method will facilitate the study of target site mutations together with heterologous expression of detoxification genes in Drosophila. In this way, the role of each mechanism that contributes to the phenotype of resistance will be evaluated. Currently, CrispR is employed in order to generate mutations on target sites of certain insecticides, e.g. mutation on GluCl, in Drosophila and in combination with the line that expresses CYP392A16 the relative contribution of each factor to the abamectin resistance phenotype will be determined *in vivo* (Douris, personal communication). Transgenesis of spider mites will be a breakthrough as no model organism exists among Chelicerata. *Tetranychus urticae* is the best candidate organism for transgenesis because of its high proliferation, high fecundity, its short life cycle and the easiness to rear it under laboratory conditions. Such a development will be a milestone for both applied and basic research and especially on the section of acaricide resistance as the functional role of detoxification genes could be validated on the same organism. The use and development of CrispR method in *T. urticae* will elucidate the contribution of metabolic and target site mechanisms in resistance to insecticides / acaricides *in vivo,* as both of these mechanisms co-exist in multi-resistant strains. For instance, disruption of detoxification genes and

conversion of known mutations to the wild type in the resistant population will provide insights for the role of these genes in the phenotype of resistance in the very same organism *in vivo*.

Finally, the development of a multiplex molecular diagnostic platform, such as the Labdisk currently developed for mosquitoes (www.dmc-malvec.gr) that will combine the detection of mutations of insecticides targets as well as over-expression of genes linked to resistance phenotypes will be an important tool for the early detection of specific resistance traits in the field, to guide the implementation of evidence based insecticide resistant management strategies.

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Ammendum

Table I: Toxicity of cyenopyrafen (and fenpyroximate), against multi-resistant strains Tetranychus urticae strains which overexpress CYP302A11, but have never been exposed to cyenopyrafen

Insecticide/Strain	Regression parameters			
	LC50 (mg/L)	Slope ± SE	χ2	RR
Cyenopyrafen				
London (susc. Ref)	7.5	1.32 ± 0.15	16.7	1
MARATHONAS	134.8	1.86 ± 0.14	36.6	18
MRVP(a)	255.34	2.88 ± 0.21	25.7	34
Fenpyroximate				
Loondon	340	1.35 ± 0.15	21.6	1
MARATHONAS	3098	1.15 ± 0.2	17.8	9.1
MRVP(b)	10581	1.50 ± 0.1		32

a: Data from Khalighi et al., (2016)

b: Data from Van Pottelberge et al., (2009)

Table II: The activities of esterases, glutathione – S – transferase (GST) and cytochrome P450 monooxygenases (P450s) in the laboratory susceptible (London) and the abamectin-resistant (Mar-ab) *Tetranychus urticae* strains. The results are presented as the means ± SE (n=3). All enzymatic assays were repeated at least 3 times and compared by one way analysis of variance (ANOVA). An asterisk indicates significant difference. The activities of the enzymes are given in: nmol/min/mg protein for esterases; FU/mg protein for GST and pmol D-Luciferin/min/mg protein for P450s.

Strains	Esterases		GST	P450s
	a-naphthyl acetate	β-naphthyl acetate	Monochlorobimane	L ME-EGE
Londnon	235.4 ± 10.3	267.5 ± 11.6	29 ± 1.4	0.0475 ± 0.0024
Mar-ab	229.9 ± 9.8	269.6 ± 11.1	153.4 ± 7.7*	1.4 ± 0.069*

Table III: : Toxicity of abamectin and milbemectin to adult females of backcrossed lines GluCl1_C (G314/G314), GluCl1_R1-3 (G314D/G314D), GluCl3_C (G326/G326), GluCl3_R1-3 (G326E/G326E), GluCl1+3_C (G314/G314; G326/G326), GluCl1+3_R1-R3 (G314D/G314D; G326E/G326E) and their parental strain (Wasatch, MAR-AB). [a] Number of the mites used in toxicity tests. [b] Resistance ratio compared to Wasatch. a: Treatment effect was significantly different when compared to Wasatch

Compound	*Strain*	*Genotype*	*N*[a]	*LC_{50} mg l^{-1} (95% CL)*	*Slope (±SE)*	*χ^2 (df)*	*RR (95% CL)*[b]
Abamectin	Wasatch	G314;G326	545	0.4 (0.3 ; 0.4)	5.0 (± 0.6)	9 (16)	-
	MAR-AB	G314D;G326E	425	512.2 (430.8 ; 578.7)a	4.3 (± 0.8)	14 (16)	1,354.9 (1,147.9 ; 1,599.3)
	GluCl1_C	G314	370	0.3 (0.3 ; 0.4)	4.3 (± 1.0)	11 (13)	0.9 (0.8 ;1.1)
	GluCl3_C	G326	474	0.4 (0.3 ; 0.4)	6.5 (± 1.3)	18 (16)	1.0 (0.9 ; 1.2)
	GluCl1+3_C	G314;G326	659	0.4 (0.4 ; 0.5)	4.1 (± 0.6)	22 (16)	1.1 (0.9 ; 1.3)
	GluCl1_R1	G314D	555	0.7 (0.7 ; 0.8)a	5.5 (±1.0)	21 (19)	1.9 (1.7 ; 2.2)
	GluCl1_R2	G314D	394	0.6 (0.6 ; 0.7)a	6.9 (± 0.9)	15 (13)	1.7 (1.5 ; 1.9)
	GluCl1_R3	G314D	447	1.1 (1.0 ; 1.2)a	6.5 (± 1.1)	10 (13)	2.9 (2.5 ; 3.3)
	GluCl3_R1	G326E	519	1.3 (1.1 ; 1.4)a	6.8 (± 0.8)	9 (16)	3.3 (2.9 ; 3.8)
	GluCl3_R2	G326E	466	1.3 (1.1 ; 1.5)a	4.7 (±0.6)	15 (16)	3.4 (3.0 ; 4.0)
	GluCl3_R3	G326E	502	1.1 (1.0 ; 1.2)a	5.9 (± 0.7)	8 (16)	2.9 (2.5 ; 3.3)
	GluCl1+3_R1	G314D;G326E	513	7.5 (6.4 ; 8.5)a	3.7 (± 0.4)	8 (16)	19.8 (16.8 ; 23.3)
	GluCl1+3_R2	G314D;G326E	399	3.8 (3.3 ; 4.3)a	5.7 (± 0.8)	11 (13)	10.1 (8.7 ; 11.7)
	GluCl1+3_R3	G314D;G326E	396	3.6 (3.1 ; 4.0)a	5.1 (± 0.7)	8 (13)	9.5 (8.1 ; 11.1)
Milbemectin	Wasatch	G314;G326	416	0.9 (0.6 ; 1.1)	5.5 (± 1.2)	15 (13)	-
	MAR-AB	G314D;G326E	409	65.4 (52.1 ; 76.4)a	3.9 (± 0.6)	14 (12)	71.7 (55.9 ; 92.0)
	GluCl1_C	G314	448	0.9 (0.8 ; 1.0)	7.5 (± 1.0)	7 (13)	1.0 (0.8 ; 1.3)
	GluCl3_C	G326	436	0.7 (0.6 ; 0.8)a	7.4 (± 1.3)	17 (16)	0.8 (0.6 ; 0.9)
	GluCl1+3_C	G314;G326	417	0.8 (0.7 ; 1.0)	6.4 (± 1.0)	18 (13)	0.9 (0.7 ; 1.2)
	GluCl1_R1	G314D	479	1.5 (1.3 ; 1.6)a	6.8 (± 1.5)	13 (16)	1.6 (1.3 ; 2.0)
	GluCl1_R2	G314D	444	1.3 (1.1 ; 1.4)a	6.2 (± 1.1)	18 (16)	1.4 (1.1 ; 1.7)
	GluCl1_R3	G314D	452	1.4 (1.3 ; 1.6)a	4.1 (± 0.7)	7 (16)	1.6 (1.2 ; 2.0)
	GluCl3_R1	G326E	532	1.4 (1.2 ; 1.7)a	2.6 (± 0.4)	10 (16)	1.6 (1.2 ; 2.0)
	GluCl3_R2	G326E	388	1.3 (1.0 ; 1.5)a	3.2 (± 0.6)	21 (16)	1.4 (1.1 ; 1.8)
	GluCl3_R3	G326E	431	1.4 (1.1 ; 1.7)a	2.8 (± 0.5)	9 (16)	1.5 (1.1 ;2.0)
	GluCl1+3_R1	G314D;G326E	360	7.0 (5.3 ; 9.1)a	1.9 (± 0.2)	17 (13)	7.7 (5.7 ; 10.3)
	GluCl1+3_R2	G314D;G326E	472	12.6 (10.1 ; 15.1)a	2.6 (± 0.3)	12 (16)	13.7 (10.3 ; 18.2)
	GluCl1+3_R3	G314D;G326E	517	11.4 (9.7 ; 13.4)a	3.5 (± 0.4)	15 (16)	12.5 (9.7 ; 16.1)

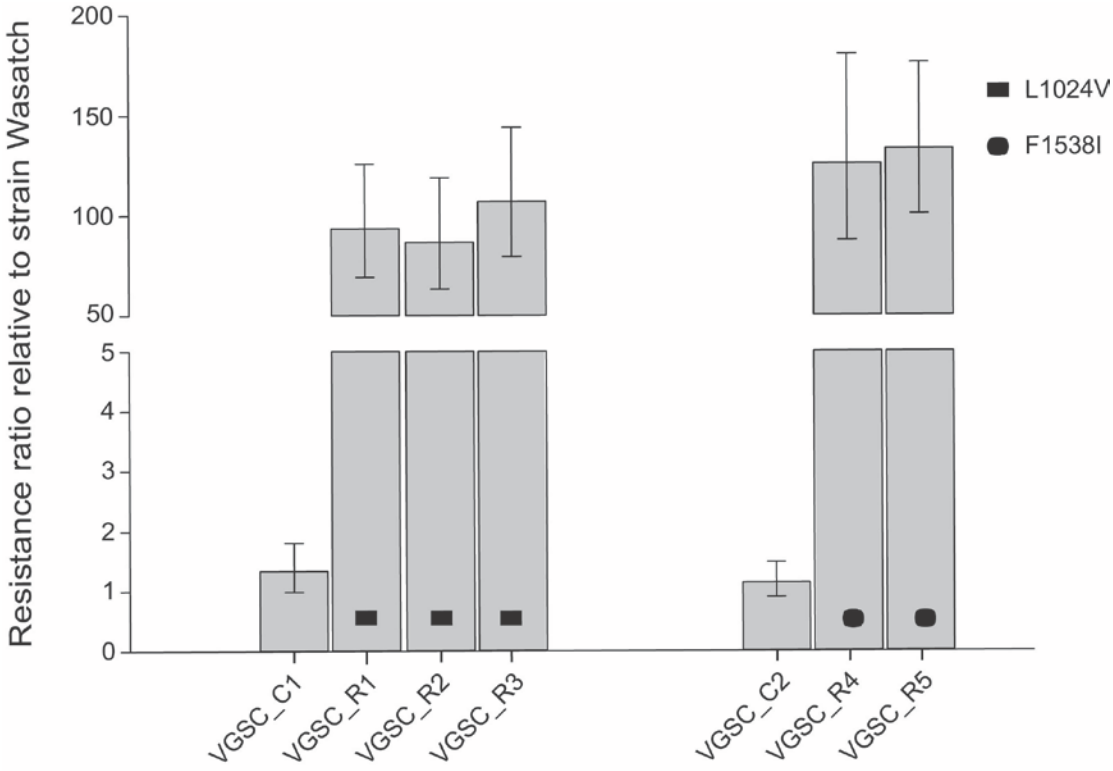

Figure I: Susceptibility of backcrossed *T. urticae* lines VGSC_R1-R3 (L1024V) and VGSC_R4, R5 (A1215D+F1538I) to bifenthrin. The RRs were calculated as the LC_{50} values of the backcrossed lines divided by the LC_{50} of the parental susceptible strain Wasatch.. Error bars represent 95% confidence limits calculated by probit analysis (PoloPlus LeOra Software). This graph and dataset was produced in collaboration with Christos Themistokleous and part of the data has been also included in his Diploma Thesis, which I supervised.

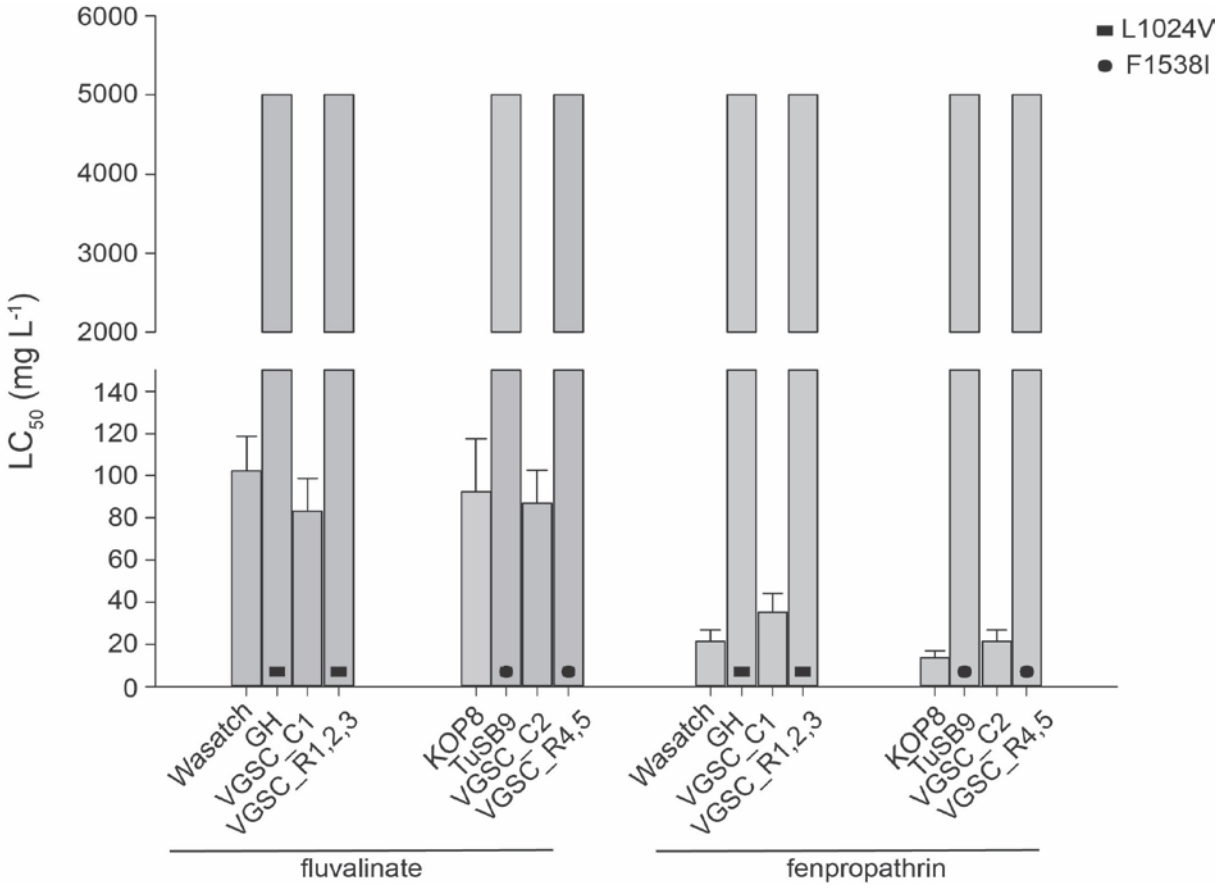

Figure II: Susceptibility of backcrossed *T. urticae* lines VGSC_R1-R3 (L1024V), VGSC_C1, VGSC_R4,5 (A1215D+F1538I), VGSC_C2 and their susceptible and resistant parental strains, to pyrethroids fluvalinate and fenpropathrin. Bars represent the acaricide concentration at which 50% of the individuals are affected. Error bars represent the 95% confidence limit calculated by probit analysis. As LC_{50} values exceeded 5,000 mg l^{-1} for all VGSC lines for each pyrethroid tested, only one bar depicts LC_{50}s. This graph and dataset was produced in collaboration with Christos Themistokleous and part of the data has been also included in his Diploma Thesis, which I supervised.