ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΤΜΗΜΑ ΒΙΟΛΟΓΙΑΣ

Molecular, genetic and immunolocalization approaches for analyzing insecticide resistance in major agricultural pests

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Abstract

Tetranychus urticae is one of the most economically important and damaging pest in agriculture worldwide. Its high fecundity, arrhenotokous reproduction and short life cycle in combination with intensive chemical spraying lead to limited control of this pest. In the present survey, the resistant levels of a Greek *Tetranychus urticae* population, derived from Trizinia, against a range of chemicals with different mode of action were investigated. Trizinia population exhibited high resistance levels against pesticides of different chemical classes. Moreover, the molecular mechanisms underlying abamectin resistance were also examined. By using genetic crosses, toxicity bioassays genotyping we investigate involvement of a novel mutation (I310T) in the GluCl3 in abamectin resistance.

In parallel, lack of knowledge for tissues implicated in detoxification physiology led us to investigate the localization pattern of detoxification enzymes within the organism. Delta GSTs and CYP392A16 are detoxification enzymes directly associated with cyflumetofen and abamectin resistance in *T. urticae*, respectively. Immunofluorescence experiments indicated that both of these enzymes were located mainly in midgut. In addition, delta GSTs were also expressed underneath the cuticle.

In the last chapter of this thesis, the frequency of A2001V mutation in ACCase of *Bemisia tabaci*, strongly associated with ketoenol resistance, was monitored in whitefly populations collected from different Mediterranean countries. Additionally, a simple PCR-RFLP molecular diagnostic was developed and may be applied as a valuable tool for the early and accurate detection of the mutation in the field.

Περίληψη

Ο τετράνυχος *Tetranychus urticae* αποτελεί έναν από τους πιο σημαντικούς εχθρούς των καλλιεργούμενων φυτών παγκοσμίως. Το υψηλό αναπαραγωγικό δυναμικό, η αρρενοτόκος παρθενογένεση και ο μικρός κύκλος ζωής, σε συνδυασμό με την εκτεταμένη χρήση χημικών σκευασμάτων έχουν οδηγήσει στην ανάπτυξη ανθεκτικότητας και κατ' επέκταση στο περιορισμένο έλεγχο του εχθρού. Σε αυτή την έρευνα, μελετήθηκαν τα επίπεδα ανθεκτικότητας ενός Ελληνικού πληθυσμού τετράνυχου από την Τροιζηνία, έναντι ενός εύρους χημικών σκευασμάτων με διαφορετικό τρόπο δράσης. Ο πληθυσμός εμφάνισε υψηλά επίπεδα ανθεκτικότητας σε σκευάσματα διαφορετικού τρόπου δράσης. Επιπλέον, ελέγχθηκαν σε μοριακό επίπεδο οι υπομοριακοί στόχοι του abamectin για ύπαρξη μεταλλαγών ανθεκτικότητας στόχου. Στον ανθεκτικό πληθυσμό εντοπίστηκε μια νέα μεταλλαγή στο τρίτο κανάλι χλωρίου (I310T). Ο ρόλος της μεταλλαγής στον φαινότυπο της ανθεκτικότητας αξιολογήθηκε με κλασικές μεθόδους γενετικής και γονοτύπηση.

Η έλλειψη γνώσης των ιστών που εμπλέκονται στην φυσιολογία αποτοξικοποίησης οδήγησε στην ανίχνευση του εντοπισμού των ενζύμων αποτοξικοποίησης μέσα στον οργανισμό. Οι GSTs της δέλτα κλάσης και η CYP392A16 είναι ένζυμα που σχετίζονται με την ανθεκτικότητα στο cyflumetofen και στο abamectin του *T. urticae*, αντίστοιχα. Μέσω πειραμάτων ανοσοφθορισμού, και τα δύο ένζυμα εντοπίστηκαν κυρίως στο μεσέντερο του τετρανύχου. Επιπλέον, οι GSTs της δέλτα κλάσης εντοπίστηκαν και κάτω από την επιδερμίδα.

Στο τελευταίο κεφάλαιο αυτής της εργασίας, μελετήθηκε η συχνότητα μιας νέας μεταλλαγής (A2001V) στο γονίδιο της ACCase του *Bemisia tabaci* σε πληθυσμούς του εντόμου από διάφορες χώρες της Μεσογείου. Η ανάπτυξη μοριακών διαγνωστικών (PCR/RFLP) μπορεί να χρησιμοποιηθεί για την έγκαιρη παρακολούθηση της ανθεκτικότητας στις κετοενόλες στο πεδίο συμβάλλοντας στην διαχείριση και στην πρόληψη της ανθεκτικότητας.

Chapter 1 Introduction

1.1 Biology of *Tetranychus urticae* & *Bemisia tabaci*

1.1.1 *Tetranychus urticae* Koch

The two-spotted spider mite, *Tetranychus urticae* Koch, is one of the most important cosmopolitan agricultural pests. It belongs to Chelicerata which is the second largest group of animals, in Acari subclass and Tetranychidae family. It is also known as two-spotted spider mite because of its two distinct dark spots localized in the internal gut content, visible through the semitransparent cuticle (Bensoussan et al., 2018). It was originally described from European specimens, as temperate zone species, but there are some findings in subtropical regions as well. The name 'spider' reveals their ability to produce silk-like webbing used to establish a colonial micro-habitat as shelter against abiotic agents, protect from predators, transport and communication via pheromones production (Grbić et al., 2011). Their webbings are very large and complex. *Tetranychus urticae* represents one of the most polyphagous arthropod herbivores with more than 1,100 host plant species. It is a major pest in greenhouse production and field crops, infesting economically important crops such as cotton, maize, tomatoes, sweet peppers, strawberries, apples, citrus and ornamentals (Van Leeuwen, Dermauw, Grbic, Tirry, & Feyereisen, 2013). The mites feed on leaves causing the appearance of chlorotic spots (loss of pigmentation) on them and finally resulting in their total destruction (Park & Lee, 2002).

The typical short life cycle of *T. urticae* is composed of the egg, the larva, two nymphal stages, (protonymph and deutonymph) and the adult (Figure 1.1). Among the immature mobile stages, three inactive phases, protochrysalis, deytochrysalis and teliochrysalis are interposed. Mites shed their skins as they grow, ecdysis process and a new cuticle is prepared. Under ideal conditions, 25^{o}C temperature, 60% relative humidity (RH) and 16:8 h light/dark photoperiod, mites' developmental stages last from 5 to 20 days, while their life span range from two to four weeks. Adult females can lay 50-300 eggs during their lifespan and they are about 0.5mm long with dark spots in either side of the body, while males are smaller (Fasulo & Denmark, 2016).

Besides the short life cycle of *T. urticae,* other important characteristics are the arrhenotokous parthenogenesis and high fecundity. Unfertilized females produce male offspring, which are haploid, whereas diploid females are developed from fertilized eggs. Short life cycle, arrhenotokous reproduction and high fecundity are the three most important factors resulting in the rapid resistance development.

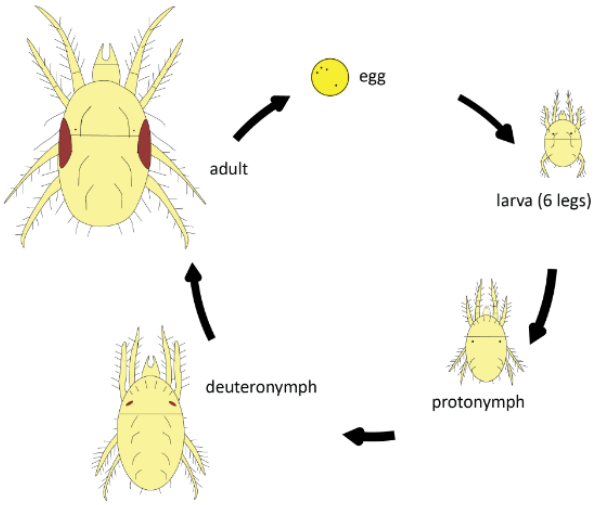

Figure 1.1 Life cycle of Tetranychus urticae (Image taken Sonya Broughton)

1.1.2 *Bemisia tabaci*

The phloem-sucking whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is one of the most major cosmopolitan insect pest of numerous protected, field crops and ornamentals (Anastasia Tsagkarakou et al., 2009). It is also known as sweet potato or tobacco whitefly. About 1.500 species from the family of Aleyrodidae have been described (Martin, Mifsud, & Rapisarda, 2000). They penetrate and suck cell contents of the plants. Big amounts of honeydew are excreted causing increasing of sooty mould fungal development (Ia & F-h, 2015). Whiteflies are also important vectors of a wide range of plant viruses belonging to genus Begomovirus and Ipovirus (Jones, 2003). *Bemisia tabaci* has a wide geographic distribution and among them, the Middle East-Asia Minor 1 (MEAM1) and Mediterranean (MED) species are the most invasive species. They are referred to as *B. tabaci* B and Q biotypes respectively, and particularly destroying by transmitting viruses, which cause serious crop disease (Peng et al., 2017) (Ilias et al., 2015). The variation in the species is a result of the different host range, feeding behavior, types of symbionts and viruses they vector (A. Tsagkarakou, Tsigenopoulos, Gorman, Lagnel, & Bedford, 2007).

The typical life cycle of *B. tabaci* is composed of the egg, four instar stages, pupa and adult (Figure 1.1). Under optimum conditions, whiteflies developmental stages last approximately 30 days. These conditions are about 25°C temperature, 60% relative humidity (RH) and 16:8 h light/dark photoperiod. Eggs are laid often in circular groups on the undersides of leaves (Ia & F-h, 2015).

The result of the continuous exposure to insecticides in combination with the occurrence of several biotypes and its high reproductive rate, is the rapid development of

resistance to a wide range of xenobiotics, such as neonicotinoids and ketoenols (Bielza et al., 2019).

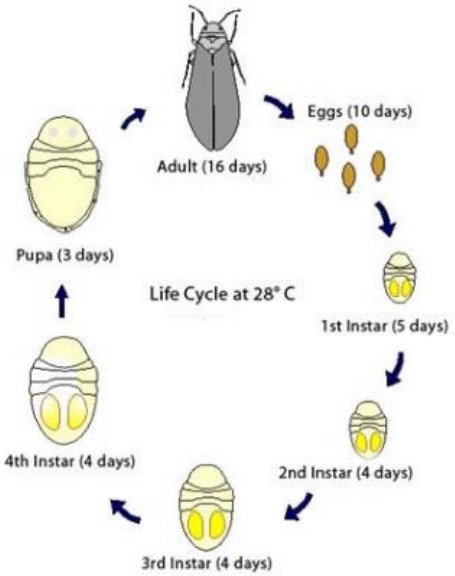

Figure 1.2 Life cycle of Bemisia tabaci

1.2 Pest control of insects and mites

Insects and mites are responsible for serious yield losses in field and greenhouse cultivations. The more efficient pest management strategies against both species are via biological and chemical control. Biological control is an environmentally friendly manner of controlling pest populations mainly by the use of natural enemies - antagonists of the pests. Chemical management via both of insecticides and acaricides is the most commonly used control method for controlling pest populations. In any case, it is very essential to know the enemy in order to select the appropriate control method (Haines, 1973).

1.2.1 Biological control

Biological control implicates the use of natural enemies such as parasites, pathogens and predators, which are antagonists of insects and mites. The success in biological control is found in the knowledge of the relationship between pests, their natural enemies and the environment. This biological control has expanded the late years because it is environmentally friendly and highly effective in greenhouses. The development of insecticide resistance in greenhouse crops may appear rapidly, compared to open field crops. Insecticide resistance and food and water contamination due to the extend use of chemicals will always boost the development of new biological methods (Perdikis, Kapaxidi, & Papadoulis, 2008).

1.2.2 Chemical control

Insecticides and acaricides are most frequently used for insects and mite control. They are used to kill, repel or differentiate the developmental stages of animals and classified in various categories such as systemic, contact, microbial and insect growth regulators (IGRs). Pesticides are divided in different chemical groups, based on their mode of actions (IRAC). The factors that affect their efficacy are timing of application, weather conditions and resistance development by the pests (Horton, Fuest, & Cravedi, 2008). Regardless of the cost effectiveness, they are often harmful for humans and the environment, cause water and crops contamination and the pests can rapidly develop resistance to them. Avermectins, pyrethroids, tetronic acids, carbamates and mitochondrial electron transport inhibitors (METIs) are some chemical groups used against insects worldwide.

1.3 Insecticide resistance and resistance mechanisms

Insecticide resistance is a heritable phenomenon. Prior to organism's exposure to a stressor factor such as insecticides, rare individuals with one or more resistance alleles already exist in a population. These are often polymorphisms in the resistance allele or increased expression of the resistance allele, which allow them to survive in pesticide exposure. Under the stress of intensive chemical spraying, the frequency of the individuals carrying the resistance polymorphisms or alleles is increased. The offspring with the resistance allele sequence will have an increased survival rate and finally they will be the predominant group in the field population (Liu, 2015). Arthropods present different levels of resistance to various insecticides and this poses significant environmental and health obstacles resulting in disruption of ecosystem. (Pérez et al., 2009).

Resistance mechanisms implicate either the increased metabolism or the modification of the insecticide target. These two mechanisms are called toxicokinetically and toxicodynamically derived resistance, respectively. There are four major categories of resistance mechanisms, behavioral resistance, reduced penetration through the cuticle, target site resistance and metabolic resistance. Among arthropods *T. urticae* has the highest incidence of pesticide resistance (Grbić et al., 2011).

1.3.1 Target site resistance

Target site resistance is the consequence of the structural modifications of target genes to insecticides (Liu, 2015). It occurs either by the presence of mutations which modify the protein structure or by mechanisms which result to change the expression of the target site (Van Leeuwen & Dermauw, 2016). Due to the modification of the target toxic compound cannot interact and bind on the active site. Numerous resistance cases have been associated with amino acid substitutions on target proteins, such as the mutations found in voltage gated sodium channel - resistance to pyrethroids, glutamate chloride channels - resistance to avermectins or acetyl-coenzyme A carboxylase - resistance to tetronic acids (Dermauw et al., 2012; Feyereisen, Dermauw, & Van Leeuwen, 2015; Karatolos et al., 2012).

1.3.1.1 Glutamate gated chloride channels (GluCls)

The cys-loop ligand-gated ion channel (cysLGIC) super family includes the permeable nicotinic acetylcholine receptors (nAChRs), the γ-aminobutyric acid (GABA)-gated channels, the glutamate-gated chloride channels (GluCls), the histamine-gated chloride channels (HisCls) and the pH-sensitive chloride channels (pHCl). Members of these neurotransmitter receptors are often target of pesticides. Most insects like *Drosophila melanogaster* have only one glutamate-gated chloride channel subunit, whereas *T. urticae* has six orthologous genes, *Tu_GluCl*1-6. *Tu_GluCl*5 and 6 may be result of a duplication event. GluCls are targets of macrocyclic lactone compounds, such as avermectin and milbemycin in both insects, mites and nematodes. Electrophysiological experiments showed that the binding of the drug to GluCls causes irreversible opening of the channels, resulting to an influx of chloride ions, hyperpolarization and paralysis of the pest (Dermauw et al., 2012; Wolstenholme & Rogers, 2005). A variety of point mutations on these channels have been linked to resistance in pests, whereas point mutations G314D and G326E are strongly linked with abamectin resistance in *T. urticae* (Dermauw et al., 2012; Kwon, Yoon, Clark, & Lee, 2010; Mermans, Dermauw, Geibel, & Van Leeuwen, 2017).

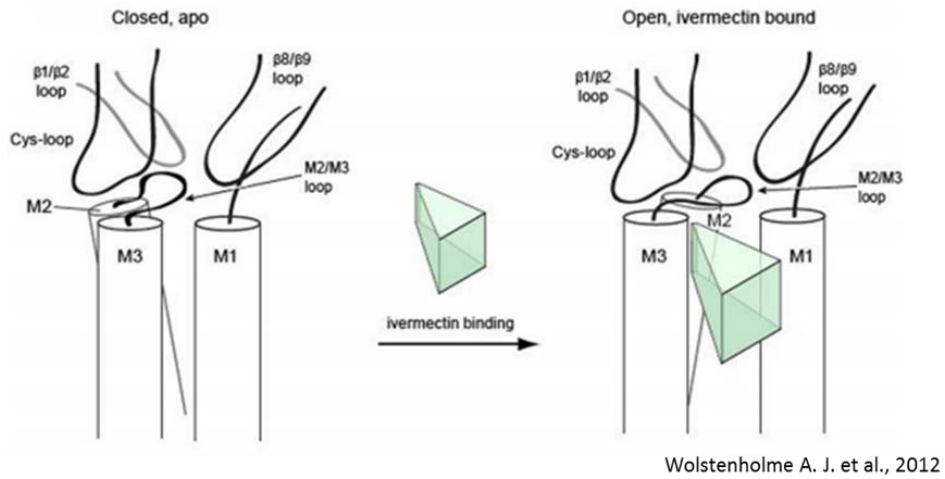

Figure 1.3The irreversible opening of GluCl channel by macrocyclic lactone compounds (like ivermectin) (Adrian J. Wolstenholme, 2012)

1.3.1.2 Acetyl-coenzyme A carboxylase (*ACCase*)

Acetyl-CoA carboxylases are crucial enzymes for the metabolism of fatty acids, playing an important role in lipid biosynthesis. They constitute the target of commercial herbicides and insecticides, such as spiromesifen, spirodiclofen and spirotetramat. Ketoenols act as inhibitors of ACCase, resulting in reduction in lipid biosynthesis (Nauen, Bretschneider, Elbert, Fischer, & Tiemann, 2003). More specifically, the carboxyltransferase (CT) domain of ACC is their site of action (Zhang, Tweel, & Tong, 2004). The first report of target site resistance to this class of pesticide in insects is reported in the whitefly *Trialeurodes vaporariorum*. The point mutation E645K in the *ACCase* of *T. vaporariorum* is the only resistance mutation linked with spiromesifen resistance until now (Karatolos et al., 2012). Recently, extremely high resistance levels against spiromesifen has been reported in B. tabaci population form Spain. Authors concluded that target site resistance as the most likely mechanism responsible for the observed resistance phaneotype (Bielza et al., 2019).

1.3.2 Metabolic resistance

Metabolic resistance is the ability of an organism to make toxic products to less or non-toxic and more water soluble form in order to be more readily excreted from the cell (Enayati, Ranson, & Hemingway, 2005). There are many reports of resistance associated with overexpression of genes, such as cytochrome P450 mono-oxygenases (P450s), glutathione S-transferase (GSTs), and carboxylesterases (CCEs), causing increased metabolism. Numerous cases of insecticide resistance have been associated with increased levels of detoxification genes in insecticide resistance populations. In most cases, the mechanisms which are

implicated in metabolism are gene duplication, amplification or up-regulation (mutation on either on cis or trans regulators) (Feyereisen et al., 2015).

1.3.2.1 Glutathione S-Transferases (GSTs)

The glutathione S-transferases (GSTs) are a large and diverse family of enzymes implicated in the detoxification of endogenous and xenobiotics compounds. These enzymes can also conduct intracellular transport, biosynthesis of hormones and defense to oxidative stress. Their basic catalyzing reaction is the conjugation of electrophilic compounds with the thiol group of the tripeptide glutathione. There are two major groups of GSTs in insects, microsomal and cytosolic, but only cytosolic GSTs are implicated in insecticide resistance. In insects, they are classified in six classes, delta, epsilon, zeta, omega, theta and sigma (Enayati et al., 2005). For example, a delta GST of *Nilaparvata lugens* has reported to confer resistance to pyrethroid, detoxifying lipid peroxidation products (Vontas, Small, & Hemingway, 2001). In acari, GST genes belonging to mu-class are also present. There are thirty one GSTs in *Tetranychus urticae* genome, while no epsilon class is identified (Grbić et al., 2011). Another example of delta GSTs correlated with insecticide resistance is the TuGSTd05. TuGSTd05 is able to metabolize cyflumetofen catalyzing the conjugation of ionized glutathione to this insecticide (Pavlidi et al., 2017). Previous reports have showed that GSTs have been reported in the fat body and midguts of insects, suggesting to play an essential role in insecticide detoxification (Enayati et al., 2005).

1.3.2.2 Cytochrome P450 mono-oxygenases (P450s)

Cytochrome P450 genes have their characteristic name from the absorbance peak at 450nm of their Fe-CO complex. Their basic reaction is the mono-oxygenase, where one atom of molecular oxygen is transferred to a substrate, reducing the other to water. (Feyereisen, 2012). They are classified into four groups, whereas members of the second class are most commonly implicated with metabolic resistance. P450s are usually localized in ER membrane and act with the cytochrome P450 reductase (CPR), their redox partner (Bernhardt, 2006). P450s are the best characterized detoxification enzymes family and specifically the CYP392 family within the CYP2 clan is highly associated with acaricide resistance (Van Leeuwen & Dermauw, 2016). *T. urticae* genome has eighty-six P450s genes and most of them belong to CYP2 clan (Grbić et al., 2011). For instance, CYP392E10 metabolizes spirodiclofen, CYP392A16 metabolizes abamectin and CYP392A11 hydroxylates cyenopyrafen and fenpyroximate in *T. urticae* (Demaeght et al., 2013; Riga et al., 2015, 2014).

1.4 Anatomy of *Tetranychus urticae*

A typical female body of *Tetranychus urticae* is about 0.5mm and is divided in gnathostome, which contains the mouthparts and idiosoma, which is the rest of the body (Fasulo & Denmark, 2016). It has eight legs and plenty of setae, which are playing a mechano- and chemo sensory role (AUGER, MIGEON, UECKERMANN, Edward A. TIEDT, & NAVAJAS, 2013; McEnroe, 1969). The central nervous system is called syngaglion and is surrounded by silk, dorsal podocephalic gland, midgut and ovary (Blauvelt, 1944). The alimentary system of *T. urticae* is well characterized and offers useful information about xenobiotic physiology. It is divided in foregut, midgut and hindgut (Bensoussan et al., 2018). Furthermore, in the anterior and dorsal podocephalic glands, there are the salivary proteins, indicating a molecular interface between phytophagous mites and their host plants. This report in *T. urticae* is the first complete among phytophagous chelicerates (Jonckheere et al., 2016).

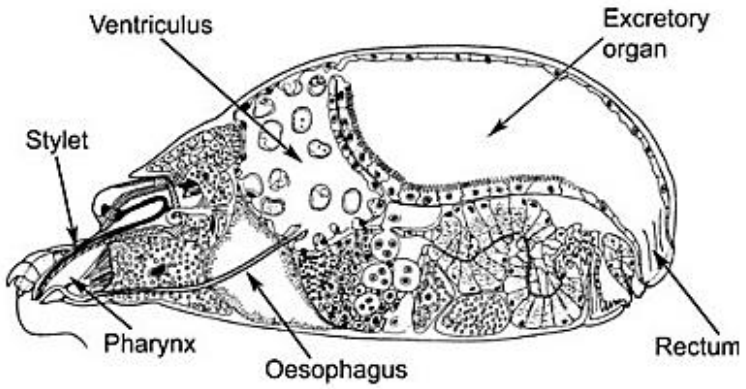

Figure 1.4 Schematic representation of a longitudinal section through a female *Tetranychus urticae* (Occhipinti & Maffei, 2013)

1.4.1 Digestive system of *Tetranychus urticae*

Digestive system of *Tetranychus urticae* is divided into foregut, midgut and hindgut. The foregut includes the stylet, buccal cavity, pharynx, esophagus and esophageal valve. The ventriculus, caeca and posterior midgut are in the midgut and the hindgut consists of rectum and anal slit. The midgut is comprised of a single-layered epithelium, which encircles the midgut lumen. Caeca includes three types of epithelial cells, which are discriminated based on their shape: generative cells (GCs), ventricular epithelial cells (VEs) and large epithelial cells (LCs). Generative cells are the most abundant cell type in the midgut epithelium. They are cuboidal, forming the outer midgut wall and then detach from the epithelial wall, forming free-floating vesicles, called residual digestive cells (RDC) in the lumen. Ventriculus cells are located at the cranial caeca and ventriculus, forming a thin squamous epithelium, while large epithelial cells

are rounded and line the caudal caeca in contact with the posterior midgut (Bensoussan et al., 2018). The chlorophyll is catabolized and the byproducts are excreted in the form of black pellets and shows autofluorescence. Moreover, two purines that are the main nitrogenous waste products in arthropods, are named guanine in spiders and this guanine is localized in the excretory organ pellets and in the white pellets excreted by the mites (Occhipinti & Maffei, 2013). The rapid excretion ability of *T. urticae* assists in the reduction of the accumulation of xenobiotic compounds and thus in insecticide resistance (Bensoussan et al., 2018).

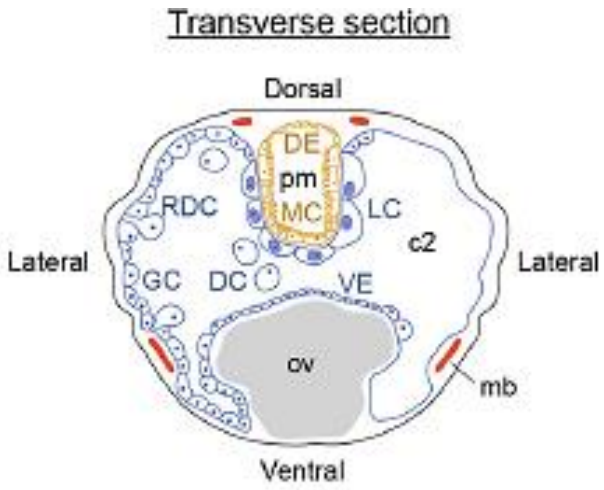

Figure 1.5 Schematics of spider mite internal anatomy DE-dorsal epithelium, MC-microvilli cell, pm-posterior midgut, VE-ventricular epithelium, GC-generative, cell RDC-residual digestive cell, DC-digestive cell, LC-lateral cell, c2-caudal caeca, ov-ovary, mb-muscle (Bensoussan et al., 2018)

Aim of this study

The aim of the study is the molecular, genetic and immunolocalization approaches for analyzing insecticide resistance in major agricultural pest. More specifically, we focused on the following:

1) Characterization of Trizinia resistance levels to acaricides of different mode of action and association of a novel mutation (I310T) in abamectin resistance

2) Detection of detoxification enzymes in *Tetranychus urticae* cryosections in order to better understand the physiology of detoxification.

3) Monitoring of ketoenol associated resistance mutation (A2001V) in Acetyl- coenzyme A carboxylase (ACCase) in *Bemisia tabaci* populations from Mediterranean countries

Chapter 2 Characterization of Trizinia resistance levels to acaricides of different mode of action and the potential role of a novel mutation in abamectin resistance

2.1 Introduction

Tetranychus urticae is famous for the rapidly resistance development to xenobiotics, such as pesticides. As it mentioned before, its high fecundity, in parallel with its short lifecycle and arrhenotokous reproduction lead to high resistant levels to insecticides. It is considered as the most resistant species, in terms of the total number of insecticides to which populations have become resistant (Leeuwen, Vontas, Tsagkarakou, Dermauw, & Tirry, 2010). In this part of the study, the resistance levels of one *T. urticae* field population against insecticide from different chemical classes were examined. The pesticides used in the toxicity assays were mite growth inhibitors, inhibitors of acetyl CoA carboxylase (inhibition of lipid biosynthesis), glutamate-gated chloride channel allosteric modulators (irreversibly activation of an inhibitory neurotransmitter) and inhibitors of the electron transport chain in mitochondria. Moreover, molecular mechanisms underlying abamectin resistance were further analyzed in order to investigate the presence of already known (G314D, G326E) or and novel mutations in target proteins potentially associated with increased levels of abamectin resistance. Our sequencing results revealed the presence of a novel mutation in GluCl3 gene of Trizinia population, which involvement in abamectin resistance is analyzed by reciprocal crosses, toxicity bioassays and genotyping.

2.2 Materials and methods

2.2.1 Spider mite strains

Three strains of *T. urticae* were used in this study. The Trizinia strain, a red form of *T. urticae* collected from a gypsum greenhouse in Trizinia on summer of 2018, with heavy pesticide application history (abamectin, clofentezine and bifenazate). The London inbred strain, a green form of *T. urticae,* which was used as a reference susceptible strain, originating from Vineland region (Ontario, Canada) (Khajehali, Van Nieuwenhuyse, Demaeght, Tirry, & Van Leeuwen, 2011). The Spain strain is a reference susceptible red strain which was used in crossing experiments (kindly provided by Prof. Thomas Van Leeuwen). *T. urticae* strains were reared on potted kidney bean plants at 25^{o}C temperature, 60% relative humidity (RH) and 16:8 h light/dark photoperiod.

2.2.2 Toxicity bioassays

Full dose response toxicity assays (adulticide, larvicide and ovicide bioassays) were performed to estimate the resistance status of Trizinia population against a range of pesticide with different mode of action. Adult bioassays (Abamectin, Bifenazate, Cyflumetofen, Pyridaben and Cyenopyrafen) were performed on bean leaves as follows: bean leaves were placed on wet cotton in plastic Petri dishes and the edges of the leaf discs were covered with wet filter paper to prevent mites from escaping. Twenty adult females were transferred onto a leaf disc and sprayed with 1 ml aqueous solution of insecticide at 1 bar pressure in order to obtain a homogenous spray film. The leaf discs with mites were subsequently placed in a climatically controlled room at 25^{o}C temperature, 60% relative humidity (RH) and 16:8 h light/dark photoperiod. Serial dilutions of each acaricide were tested in 3 replicates, including a water sprayed control. Mortality was assessed after 24 hours for Cyflumetofen, Pyridaben and Cyenopyrafen or 48 hours for Abamectin and Bifenazate.

Egg (Clofentezine) and larval (Spirodiclofen, Etoxazole and Spirotetramat) bioassays were performed on bean leaves as follows: 20 adult female mites were placed on the leaf discs and permitted to lay eggs for 24 h, after which they were removed. This resulted in approximately 80 eggs per leaf disk (the eggs were counted). After removal of the adults (egg bioassay) or just before hatching of the eggs (larval bioassay), the leaf discs were sprayed as described previously. The plates were then placed in the climatically controlled room. Serial dilutions of each acaricide were tested in 3 replicates, including a water sprayed control. Mortality was assessed after the emergence of adults on the control leaves in egg and larval bioassays. 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).

Commercial formulations of Abamectin (Vertimec 1.8EC), Clofentezine (Apollo 50SC), Spirodiclofen (Envidor 240SC), Bifenazate (Floramite 240SC), Etoxazole (Baroque 11SC), Cyflumetofen (Nealta 20SC), Pyridaben (Nexter 20EC), Spirotetramat (Movento 150OD) and Cyenopyrafen (Starmite 30SC) were used in this study.

2.2.3 Backcrossing experiments

To estimate the dominance of resistance, individuals of the Spain and Trizinia strains were reciprocally crossed to produce hybrid F1 generation. More specifically, 15 female teleiochrysalis of one strain and 35 adult males of the other strain were placed on a bean leaf

on wet cotton in a Petri dish. Three days after colonization, fertilized females were transferred on fresh bean leaves and were allowed to lay eggs for 10 days. Every day, the egg laying females were collected and placed on a fresh leaf. The F1 females were collected (approximately 10 days after hatching) and were used in toxicity bioassay with the appropriate concentrations of abamectin. The experiment was repeated twice. The degree of dominance was determined from the LC50 of the parents and the respective F1 progeny by using the formula of Stone:

$$D = \frac{2X_2 - X_1 - X_3}{X_1 - X_3}$$

in which X_1 is the log of the LC_{50} of the resistant strain, X_2 is the log of the LC_{50} of the F1 females and X_3 is the log of the LC_{50} of the susceptible strain. This formula will result in a value of -1 if resistance is fully recessive, a value of 0 if resistance is intermediate and a value of +1 if resistance is fully dominant.

The number of genes implicated with abamectin resistance was assessed by the dose response of F2 female progeny. Therefore, reciprocal crosses were made by mattings susceptible (Spain) and resistant parents (Trizinia) to produce F2 female progeny. Because males are haploid and inherit their genes only from the mother, the F2 progeny obtained from the crosses were genetically equivalent to backcross progeny. F2 females were collected and their mortality was assessed with dose response bioassays with the appropriate concentrations of abamectin. To test for monogenic resistance, the observed response in bioassays was compared to the expected response with the formula of Georghiou (1969): C= 0.5 w (parent 1) + 0.5 w (parent 2), where C is the expected mortality and w the observed mortality of the parental types at a given concentration. A χ^2 goodness-of-fit analysis was used to examine any deviation between the observed response and the expected response under monogenic resistance.

2.2.4 Extraction of genomic DNA and single mite genotyping by sequencing

In order to perform single mite genotyping for I310T, single mite DNA was extracted following the Cethyl Trimethyl Ammonium Bromide (CTAB)-based extraction method (Navajas et al., 1999) as follows: 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 20 min. Equal volume of chloroform: isoamylalcohol (24:1) was used in order to remove proteins. Upon centrifugation at 8.000rpm for 10min at RT, the upper face was collected and the DNA was precipitated by isopropanol for 60 min -20°C. Upon

centrifugation at 15.000rpm for 15min at 4°C DNA pellet was washed with 75% cold ethanol. The pellet was air-dried and resuspended in 20 µl DEPC treated water.

Single mite genotyping of F2 survival vs F2 control individuals of both crosses (RS x S) and (RS x R) was performed with standard PCR and sequencing by using gene-specific primers. PCRs were conducted in 20 µl final volume with 2 µl 10x Minotech Taq Buffer, 0.5 ul of 10 mM dNTPs, 0.3 ul of 10 µM each primer, 2µl template and 0.2 µl Minotech Taq polymerase with cycling conditions: 2 min at 94 °C followed by 35 cycles 45 sec at 94 °C, 30 sec at 56 °C, 30 sec at 72 °C and 5 min of final extension. Reactions were performed in BIO RAD T100™ Thermal Cycler. PCR reactions were purified with Nucleospin Gel and PCR Clean-Up purification kit (Macherey – Nagel) and sequenced at CeMIA SAM. Sequencing data were analyzed using BioEdit 7.0.5.3 software (Hall, 1999). Primers used for the PCR reactions and sequencing were the forward GluCl3_F (5'-GATCCAAATGCTATTCCTGCC-3') and GluCl3_R (5'-GTGGTGGTATGGGTTTCCCTC-3').

2.3 Results

2.3.1 Characterization of Trizinia strain resistance levels to acaricides of different mode of action

(Maria-Kyriaki Papapostolou has contributed equally to this work)

The estimation of resistance levels of Trizinia population against pesticides abamectin, clofentezine, spirodiclofen, bifenazate, etoxazole, cyflumetofen, pyridaben, spirotetramat and cyenopyrafen, was performed via full dose toxicity bioassays. Trizinia population was previously treated with abamectin, clofentezine and bifenazate in the grower's greenhouse. Toxicity bioassays were conducted using the London inbred as reference laboratory susceptible strain. The outcome of these bioassays revealed high resistance ratio to etoxazole (RR~5000), clofentezine (RR>1666), cyflumetofen (RR~500), cyenopyrafen (RR=117) and abamectin (RR=90) (Table 1). Resistance to bifenazate (RR=13), spirodiclofen (RR=20), pyridaben (RR=28) and spirotetramat (RR=37) was considerably, but rather low compared to previous pesticides. The London inbred strain was fully susceptible in almost every acaricide used.

Table 2.1 Toxicity bioassays of insecticides with different mode of action in Trizinia versus London

Compound	*Strain*	*Slope ± se*	*LC50 (95% CI) µg/ml*	*X² (df)*	*RR (95% CI)*
Abamectin (Vertimec 18EC)	London	5.748±0.788	0.190	11.597 (13)	1
	Trizina	4.769±0.602	17.126	6.866 (10)	90
Clofentezine (Apollo 50SC)	London	2.357±0.157	6.341	34.748 (15)	1
	Trizina		>10000		>1666
Spirodiclofen (Envidor 240 SC)	London	13.485 ±1.565	6.449	45.674 (12)	1
	Trizina	2.745±0.203	131.306	58.141(18)	20
Bifenazate (Floramite SC)	London	5.982±0.631	2.323	44.381 (16)	1
	Trizina	3.633±0.368	30.622	9.933 (16)	13
Etoxazole (Baroque 11SC)	London		~2		1
	Trizina		>10.000		~5.000
Cyflumetofen (Nealta 20SC)	London	8.265±0.994	19.986	11.369 (13)	1
	Trizina		>10.000		>500
Pyridaben (Nexter 20EC)	London	2.316±0.558	29.066	18.320 (15)	1
	Trizina	1.465±0.183	813.114	8.159 (10)	28
Spirotetramat (Movento 150OD)	London	5.316±0.681	1.167	27.473 (10)	1
	Trizina	1.088±0.060	42.246	71.110 (12)	37
Cyenopyrafen (Starmite 30SC)	London	2.402±0.384	3.889	6.480 (10)	1
	Trizina	3.217±0.430	453.745	10.177 (12)	117

LC, lethal concentration expressed in mg/L; χ^2, Chi-square testing linearity; RR, resistance ratio

2.3.2 Association of a novel mutation (I310T) in Tu_GluCl3 in abamectin resistance of *Tetranychus urticae*

Due to the detected relatively high levels of abamectin resistance in Trizinia, all the GluCls genes were sequenced in order to investigate the existence of previously known or/and novel mutations on GluCl channels (Dermauw et al., 2012; Kwon et al., 2010). Sequencing revealed the presence of a novel mutation in Trizinia population, the substitution of isoleucine to threonine at a position 310 of Tu_GluCl3. In all other positions known to be associated with abamectin resistance, identical residues were found in both strains. The investigation of association of this novel mutation with abamectin resistance was conducted by the previously described reciprocal crosses between Trizinia and Spain. The F_1 female progeny were collected and bioassays with the appropriate concentrations of abamectin were performed. The chosen doses were 0, 1, 2, 4, 5, 10 ppm for females of both F_{1a} Trizinia♀ x Spain♂ and F_{1b} Spain♀ x Trizinia♂. The concentration mortality outcome for F_{1a} females from Trizinia♀ x Spain♂ and F_{1b}

Spain♀ x Trizinia♂ reciprocal crosses, showed that resistance in abamectin is incompletely recessive (not maternally inherited) and the estimated degree of dominance (*D)* was -0.17 and -0.23, respectively (Figure 2.1 and Table 2.2). This result was in accordance with previous surveys, which indicated that the inheritance of abamectin resistance is incompletely recessive (Dermauw et al., 2012; Kwon et al., 2010).

Backcrossing of F_{1a} females (Trizinia♀ x Spain♂) with Trizinia♂ and Spain♂ and dose-response relationship of abamectin toxicity in both F2 females revealed that resistance is polygenic and not under monogenic control (Figure 2.2). X^2 for F_2 (F_{1a}♀ x Trizinia♂) was 22.86 ($p < 0.05$, df=4) and for $F_{2'}$ (F_{1a}♀ x Spain♂) was 35.78 ($p < 0.05$, df=4) (Table 2.2).

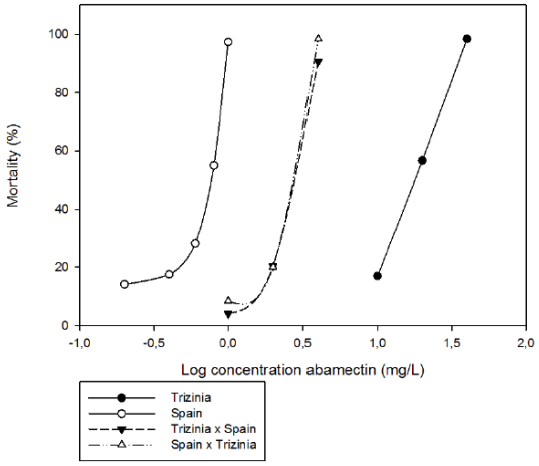

Figure 2.2 Abamectin concentration-mortality curves for Spain, Trizinia and reciprocal crosses F1a♀ (Trizinia♀ x Spain♂) and F1b♀ (Spain♀ x Trizinia♂)

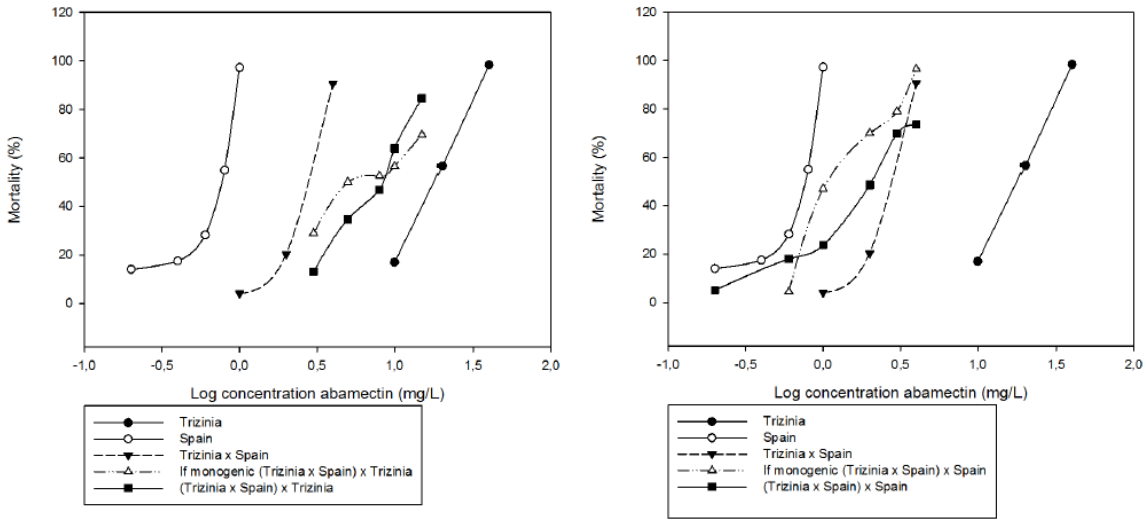

Figure 2.3 Abamectin concentration-mortality curves for Spain, Trizinia, reciprocal crosses F1a♀ (Trizinia♀ x Spain♂) and backcrosses (F1a♀ (Trizinia♀ x Spain♂)) x Trizinia ♂ on the left and (F1a♀ (Trizinia♀ x Spain♂)) x Spain ♂ on the right. The triangles represent the response expected if abamectin resistance was determined by a single major gene in both cases.

Table 2.2 Probit statistics for the reciprocal crosses (F1, F2) tested against abamectin

strain	n	LC50 (95%CI)	slope ± SE	dF	χ^2	RR (95%CI)	D
Spain	250	0,763	13,380 ± 3,231	11	14		
Trz	238	17,126	4,769±0,602	10	7	22,46	
F1a♀(Trz♀Spain♂)	365	2,755	5,854 ± 0,791	12	15		-0,17
F1b♀(Spain♀Trz♂)	283	2,501	10,423 ± 1,515	10	4,97		-0,23
F2(F1a♀Trz♂)	627	8,222	2,773 ± 0,358	25	32		
F2'(F1a♀Spain♂)	326	1,992	2,231± 0.377	12	4,182		

N, number of mites tested; dF, degree of freedom; *D*, degree of dominance

To evaluate the role of I310T in abamectin resistance individual F2 female progeny survived at high abamectin concentrations vs F2 control individuals for both F2 crosses were genotyped. Mites survived from toxicity bioassays at 10 mg L^{-1} and 3 mg L^{-1} were collected from F2 crosses F1a♀ x Trizinia♂ and F1a♀ x Spain♂ respectively. Preliminary genotyping results of some of the survivals at 10 mg L^{-1} abamectin indicated an enrichment of the homozygous Thr/Thr Tu_GluCl3 genotype compared to the respective control. Although, our results are preliminary and additional genotyping is needed, it seems that I310T mutation is potentially linked with abamectin resistance.

2.4 Discussion

Tetranychus urticae, as one of the most resistant pests worldwide, causes significant yield losses in many economically important crops. Because of its rapidly resistance development, *T. urticae* has unique potential to become a good model to study resistance evolution and plant interactions (Van Leeuwen et al., 2013). A new population of *T. urticae,* which is called Trizinia, was collected from gypsum greenhouse in Trizinia and aim of this survey was the characterization of toxicological profile against acaricides of different mode of action. The toxicity assays revealed that Trizinia strain exhibited multi-resistance against several pesticides with different mode of action, showing high resistance ratios to etoxazole, clofentezine, cyflumetofen, cyenopyrafen and abamectin relative to the susceptible London inbred strain. Furthermore, the macrocyclic lactone abamectin has been frequently linked with non-synonymous substitutions, G314D and G326E, in the glutamate-gated chloride channel (GluCl) subunits TuGluCl1 and TuGluCl3 respectively. Sequencing of GluCl gene subunits revealed the absence of these two mutations, but the existence of a novel non-synonymous

substitutions (I310T) in GluCl3 gene. This mutation has not been reported in mites of insects previously.

In order to investigate the role of this mutation in abamectin resistance reciprocal crosses performed between individuals of Trizinia and Spain (a susceptible red form of *T. urticae*). The concentration mortality outcome for F_{1a} females revealed that resistance to abamectin was inherited by incomplete recessive trait. Incomplete recessive inheritance of abamectin resistance was also reported in *T. urticae* previously (Dermauw et al., 2012; Kwon et al., 2010). F1 female mites were backcrossed and the dose-response relationship of abamectin toxicity in F2 female mites showed that resistance was under polygenic control, indicating an additional resistance mechanism, such as metabolic via P450s or GSTs. Preliminary genotype analysis of back-crossed F2 survivors in high abamectin dose indicated that the majority of resistant mites possessed homozygous Thr/Thr alleles. The substitution of a neutral Ile residue for a polar Thr residue in TuGluCl3 most likely resulted in functional changes that probably reduced abamectin sensitivity. This outcome imply that I310T mutation might confer resistance to abamectin. It is very possible that GluCl genes play important physiological roles and the alteration of their structure by amino acid substitutions may lead to important functional changes.

Chapter 3 Immunolocalization of detoxification enzymes in *Tetranychus urticae*

3.1 Introduction

Tetranychus urticae is a polyphagous species indicating the ability of high digestive physiology adaptation. This acute response proves its wide range of defenses and the ability of the xenobiotic detoxification as weapon against pesticides (Bensoussan et al., 2018). Genome characterization showed that these adaptations are implicated with the expansion of detoxification genes (Grbić et al., 2011). The role of detoxification enzymes such as P450s and GSTs is well established by previous reports. Specifically, GSTd05 metabolizes the complex II inhibitor cyflumetofen, while CYP392A16 metabolizes abamectin (Pavlidi et al., 2017; Riga et al., 2014). Characterization of the tissues and cells, which are related to detoxification physiology are necessary to better understand detoxification mechanisms. The description of the alimentary track was a useful support for detoxification physiology (Bensoussan et al., 2018). The aim of this part of the study was the delineation of the localization pattern of these enzymes in *T. urticae*.

3.2 Materials and Methods

3.2.1 Spider mite strains

Two strains of *T. urticae* were used in this study. Marathonas strain (Mar-ab), a green form of *T. urticae* collected from a highly sprayed rose greenhouse in Marathonas, Athens in 2009. This population has been maintained in the lab under abamectin selection (70mg/L) every one generation. The second was the London inbred strain, a green form of *T. urticae,* which was used as a reference susceptible strain, originated from a wild-collected *T. urticae* population from the Vineland region (Ontario, Canada). *T. urticae* strains were reared on potted kidney bean plants at 25^{o}C, 60% relative humidity (RH) and 16:8 h light/dark photoperiod.

3.2.2 Western Blot analysis

In this series of experiments 150-250 adult females were collected from the Mar-ab and London strain respectively and homogenized in 100-150 ul of protein extraction buffer, containing 0.05M Tris-HCl pH=7.5, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 0.2M PMSF, 0.25M EDTA and 1mM protease inhibitor cocktail. The mites were homogenized mechanically using a plastic-pestle homogenizer and a grinder. Upon centrifugation at 10.000rpm for 5min at 4^{o}C, supernatant was collected and the total protein concentration was determined by Bradford assay (Sigma-Aldrich B6916) with bovine serum albumin (BSA) used as standard. Finally, 1x

Laemlie buffer was added to the samples. The purified His_6-TuGSTd05 protein was used as positive control. Samples (homogenate/positive controls) were boiled at 95°C for 5min and they were loaded on a gel 12.5% SDS-PAGE. Upon separation, polypeptides were transferred to a polyvinylidene difluoride (PVDF) membrane. Upon blocking of the membrane with 5% milk in 1XTBST for 1 hour at room temperature with shaking, two antibodies produced in rabbits against delta GSTs were used (1:5000 dilution in 3% milk/1X TBST), for 12-16 hours at 4°C. The first antibody recognized the whole protein (anti-GSTd05) and the second one was raised against a single peptide of the protein (anti-GSTd05pep) (peptide sequence: YFPIVRQGVKPDPAVATLFMDKVKLLDEALAK). Upon incubation with the antibody dilutions the membranes were washed extensively with 1X TBST, followed by one-hour incubation with a goat anti-rabbit antibody (conjugated with horse peroxidase) in 1:10.000 dilution in 1% milk/1XTBST (Cat.No 7074S, Cell Signalling). Mouse anti-β-tubulin (Santa Cruz Biotechnology (SC-365791)) was also used as a loading control in 1:250 dilution in 3% milk/1X TBST.

3.2.3 Immunofluorescence and Confocal Microscopy

Adult females (100-150) were collected from both Mar-ab and London strains and were fixed with 1X phosphate-buffered saline (PBS) supplemented with 4% paraformaldehyde (16% Formaldehyde (w/v) methanol-free, 10 X 1 ml, Cat.No 28906, Thermo Scientific LSG (Pierce)) for 1h at RT on a rotating wheel. Then, the fixative was removed and the samples were incubated 12-16 hours with a cryo-protective solution of 30% sucrose in 1X PBS at 4°C on a rotating wheel again. The fixed mites were immobilized in Optimal Cutting Temperature compound (OCT, Tissue-Tek; Sakura) and stored at -80° C until use. 6μm sections were obtained using the Leica Cryotome CM 1850, mounted on microscope slides (SuperFrost Plus microscope slides, O. Kindler GmbH D-79110) and finally stored at -20°C until immunostaining.

Immunostaining of the obtained sections was performed with an initial step of antigen retrieval. Briefly, sections of the freshly frozen mite specimens were incubated with a solution of Na_2HPO_4-citric acid pH 4 (3 x 5min). In the case of the detection the localization of CYP392A16, antigen retrieval was not performed. Then, immunostaining was based on Ingham et al. 2014 as follows: Sections were washed with 0.2% Tween20 in 1X PBS (3 x 5min) followed by a single wash step with 0.2% Triton X-100 in 1X PBS for 10min and the again with 0.2% Tween20 in 1X PBS (3 x 5min). The samples were blocked with blocking buffer in 0.2% Triton X-100/1X PBS and 5% BSA for 1h at RT. Tissues were incubated with both delta GST antibodies or CYP392A16 antibody in 1:500 dilution, in blocking buffer at 4°C 12-16 hours. As negative control, pre-immune serum (in 1:1000 dilution) was used to ensure specificity of our signal.

Three washes were performed with 0.2%Triton X-100/1X PBS for 5min (3 x 5min) and Alexa Fluor 488-conjugated anti-rabbit antibody IgG (Invitrogen) was used as the secondary antibody in 1:1000 dilution in blocking serum for 1h at RT in the dark. Three 5min washes were performed with 0.2%Triton X-100/1X PBS, two 5min washes with 0.2% Tween20/1X PBS and nuclei were stained with DAPI (PanReac AppliChem), 1:100 in 0.2%Triton X-100/1X PBS for 20min at RT in the dark. Three 5min washes were performed with 0.2% Tween20 in 1X PBS and finally samples were mounted in Vectashield (Vector Laboratories, H-1000-10) on SuperFrost+ slides[120-123] (Ingham et al., 2014). Imaging was conducted using Leica SP8 laser scanning confocal microscope (Institute of Molecular Biology and Biotechnology, IMBB Microscope Facility of Foundation for Research & Technology-Hellas).

3.3 Results

3.3.1 Expression levels of delta GSTs across strains with different insecticide resistance profiles

The protein expression levels of delta GSTs in mite strains were investigated through Western blot analysis, using the two different antibodies against this class of GSTs. Both antibodies (anti-GSTd05 and anti-GSTd05pep, 1:500 dilution) recognized one protein band with approximate molecular mass of 28-30 kDa in the protein extracts of adult mites from both of Marathonas and London strains (The calculated molecular weight of the GST is 25KDa). Purified His_6-TuGSTd05 protein, used as positive control, was recognized again by both antibodies, migrating slight slower compared to the band on the crude extracts, most probably because of the extra His6 linker that the recombinant protein beared. As loading control in this series of experiments, a mouse anti-β-tubulin (1:250) was used (Figure 3.1).

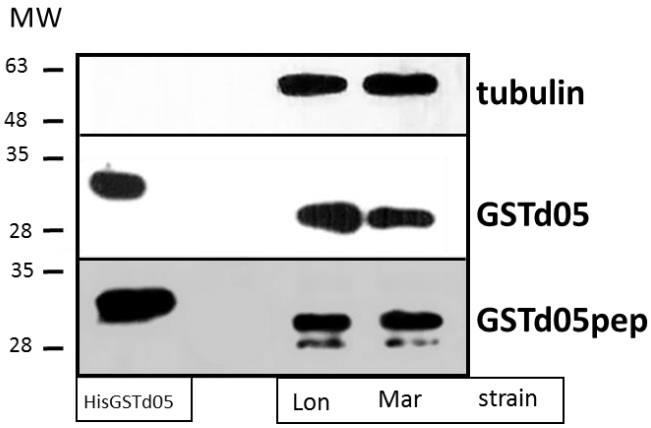

Figure 3.1 Western blot analysis of anti-GSTd05 (1:5000) and anti-GSTd05pep (1:5000) against positive controls and protein extracts from Marathonas (Mar) and London (Lon) strains. His_6-TuGSTd05 purified protein (tetur01g02510): 36ng, Mar and Lon protein extracts: 25μg. Anti-tubulin (1:250) was used as loading control.

3.3.2 Immunolocalization of delta GSTs in *T. urticae*

For a more detailed analysis of delta GSTs tissue localization, immunostaining experiments were conducted in Marathonas strain sections. First of all, in order to detect and better understand the presence of autofluorescence of chlorophyll degradation products in our experiments, cryosections of mites were immunostained only with Dapi, which stained the nuclei of the cells. A red false color was detected (Figure 3.2, up), derived most probably from the chlorophyll digested from the spider mites, as previous findings indicated (Bensoussan et al., 2018; Occhipinti & Maffei, 2013). Moreover, rabbit anti-actin, (Cat.No A2066, Sigma Aldrich) was used as positive control in our survey. The intensity of anti-actin signal was strong and actin was detected specifically in muscles (Figure 3.2, down).

Cryosections from adults *T. urticae* were immunostained with both anti-GSTd05 and anti-GSTd05pep in order to detect the localization of delta GSTs in mites. The intensity of anti-GSTd05pep signal was stronger than the one coming from anti-GSTd05, but in both cases delta GSTs were detected underneath the cuticle and in all three cell types of caeca, generative, ventricular epithelial and large epithelial cells in mite midgut (Figure 3.3). The specificity of our antibodies was checked by using pre-immune serums against TuGSTd05 and TuGSTd05pep in our mite sections. Pre-immune serums of both antibodies tested did not stain *T. urticae* cryosections, as expected (Figure 3.4).

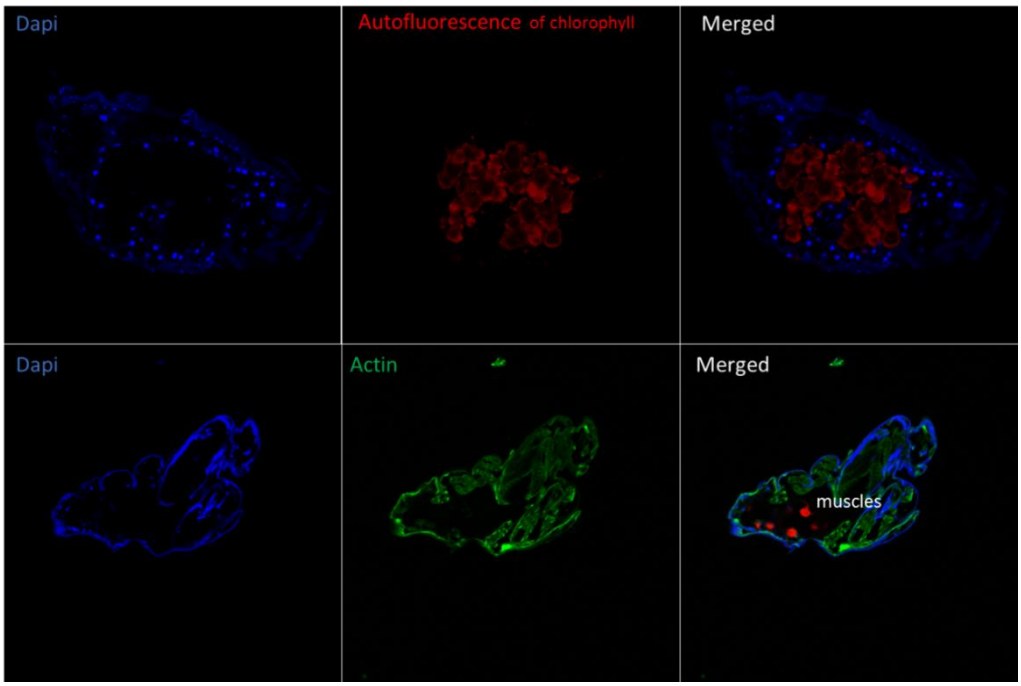

Figure 3.2 Confocal images of autofluorescence of chlorophyll in *T. urticae* sections, red false color (upper). Immunohistochemical localization of *T. urticae* actin in muscles, as positive control. Dapi stained the nuclei (blue). Cryosections were immunostained with anti-Actin antibody (green) (lower). Pictures were obtained using confocal microscopy (40x).

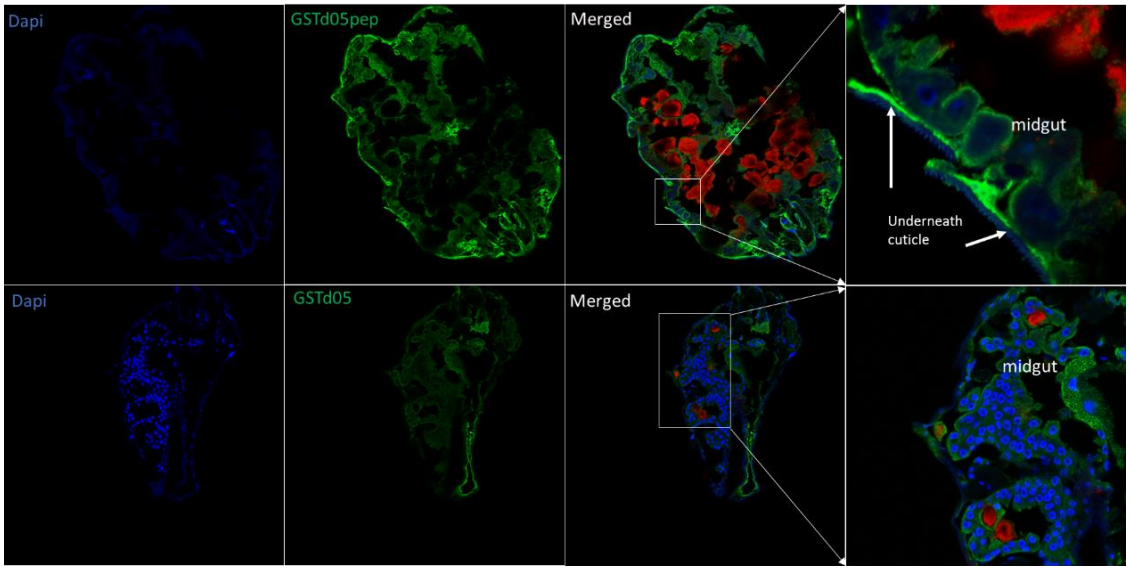

Figure 3.3 Immunohistochemical localization of *T. urticae* delta GSTs both in the cytoplasm of gut cells and underneath the cuticle, using anti-GSTd05pep antibody (1:500) (upper) and anti-GSTd05 (1:500) (green) (lower). Dapi stained the nuclei (blue) and the autofluorescence was represented by red false color. Pictures were obtained using confocal microscopy (40x).

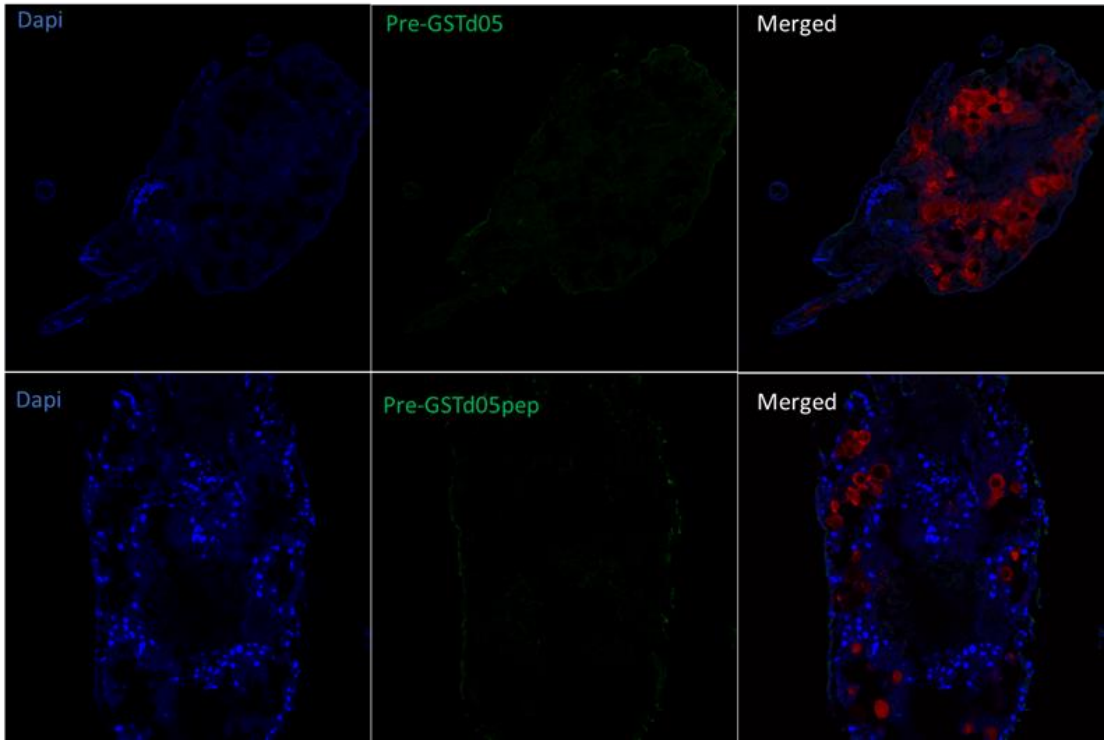

Figure 3.4 Pre-immune serums against GSTd05 and GSTd05pep (1:1000) were used as negative control (green). Dapi stained the nuclei (blue) and the autofluorescence was represented by red false color. Pictures were obtained using confocal microscopy (40x).

3.3.3 Immunolocalization of CYP392A16 in *T. urticae*

Next, we wanted to further analyze the tissue localization of CYP392A16 within mite body in order to reveal which tissues participate in detoxification in this organism. Cryosections from fixed adult mites were immunostained with α-CYP392A16 and CYP392A16 was detected only in generative cells (GC) in midgut. The specificity of our antibody was verified by using pre-immune serum against CYP392A16 in our mite sections. Pre-immune serum did not stain *T. urticae* cryosections, as expected (Figure 3.5).

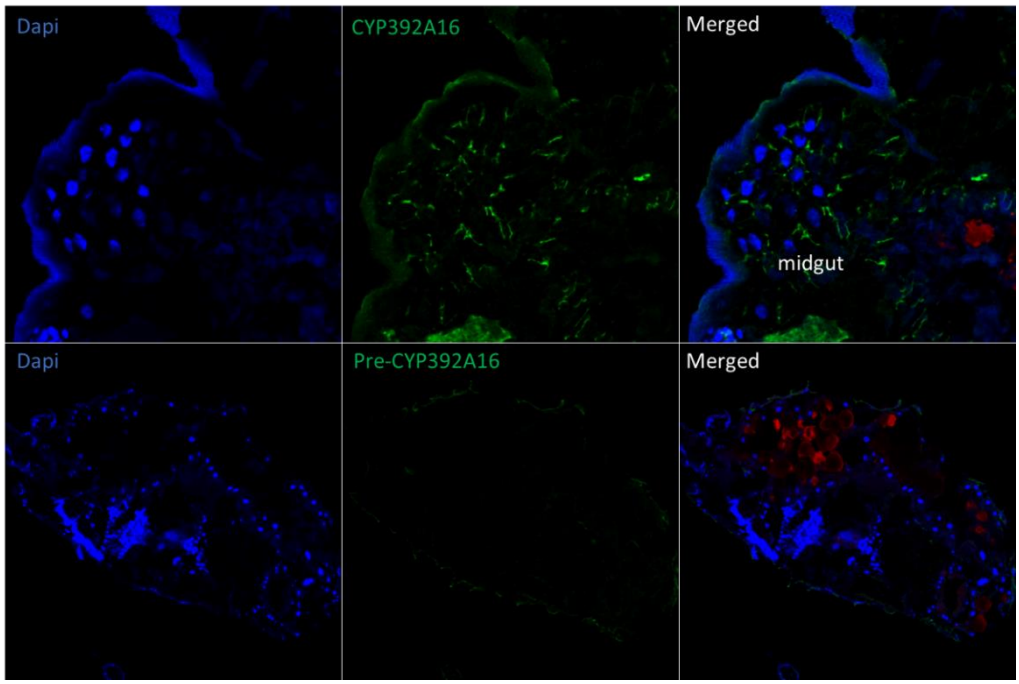

Figure 3.5 Immunohistochemical localization of CYP392A16 in gut cells of *T. urticae*, using anti-CYP392A16 (1:500) (upper) (green). Pre-immune serum against CYP392A16 (1:1000) was used as negative control (green) (lower). Dapi stained the nuclei (blue) and the autofluorescence was represented by red false color. Pictures were obtained using confocal microscopy (40x).

3.4 Discussion

The acute response of *T. urticae* against xenobiotic compounds indicates that *T. urticae* has developed wide range of defenses and capabilities for xenobiotic detoxification. Mites tissues that specialize in detoxification are still unknown (Bensoussan et al., 2018; Blauvelt, 1944). The present study focused on a better description of *T. urticae* digestive physiology, analyzing the expression of delta GSTs and CYP392A16 in midgut epithelial cells. Previous studies revealed the localization of detoxification enzymes in midgut of other arthropods, such as *D. melanogaster* and *Ae. albopictus* (Chung et al., 2009; Grigoraki et al., 2016). A tissue-

specific staining, using immunohistochemical procedure, targeting the midgut epithelium could validate the hypothesis that the best detoxification candidate is the midgut. Delta GSTs were also detected underneath the cuticle, indicating the direct protection from insecticides-induced reactive oxygen species. This finding is in accordance with a previous study which revealed the expression of another GST, gst-4, in hypodermis of *Caenorhabditis elegans*, as an antioxidant defense agent (Hu, Macneil, Walhout, & Kubiseski, 2017). The potentially different localization of these two enzymes may indicate their distinct role in xenobiotic metabolism.

Chapter 4 Monitoring of ketoenol associated resistance mutation (A2001V) in Acetyl-coenzyme A carboxylase (ACCase) in Bemisia tabaci populations from Mediterranean countries

4.1 Introduction

The whitefly *Bemisia tabaci* is developing rapidly insecticide resistance through the years in very novel and effective compounds, such as spiromesifen and spirotetramat. Apart from this continuous exposure to insecticides, *B. tabaci* has some characteristics, like several biotypes and high reproductive rate, which lead to rapid resistance development. The mechanisms which confer this high resistance levels to ketoenols were unknown (Bielza et al., 2019; Peng et al., 2017). Recently, a novel mutation (A2001V) in *B. tabaci* from Spain has been reported to be highly correlated with spiromesifen resistance. The aforementioned mutation has been validated via CRISPR/Cas9 in *D. melanogaster* to confer resistance to both spiromesifen and spirotetramat (Douris et al, unpublished data). In the present chapter, the frequency of this novel mutation was estimated in *B. tabaci* populations originating from different Mediterranean countries.

4.2 Materials and Methods

4.2.1 *Bemisia tabaci* samples from Mediterranean countries

Insects from seventeen *B. tabaci* populations were collected the last eleven years (2007-2018) mainly from greenhouses of different regions of Mediterranean countries, such as Greece, Italy, Spain and France. These samples were used to monitor the frequency of spiromesifen associated resistance mutation (A2001V) in ACCase gene by the development and application of novel RFLP diagnostic assay. More specifically the used populations were derived from Crete (Ierapetra and Tympaki), Nafplio, Lazio, Sardegna, Barcelona, Perpignan and Saint Hippolyte (more details concerning the collection date, the host plant and the number of individuals were shown in Table 1. *B. tabaci* samples were either preserved in 90% ethanol or stored in -20°C, until use.

4.2.2 Extraction of genomic DNA and individual genotyping by sequencing

Genomic DNA (gDNA) was extracted using the DNAzol® reagent as follows: individual whiteflies were homogenized by using a plastic-pestle homogenizer and a grinder in total volume of 200μl reagent. Upon centrifugation at 10.000rpm for 10min at RT, the supernatant (SN) was transferred into a new 1.5ml Eppendorf and the DNA was precipitated with equal volume of 100% ethanol. Upon centrifugation at 13.000rpm for 20min at RT, the supernatant

was discarded and the DNA pellet was washed with 300ml of 75% ethanol. Upon centrifugation at 13.000rpm for 5min at RT, the supernatant was discarded and the DNA pellet was re-suspended in 30µl H_2O.

Primer pair was designed for the amplification of a 141bp part of ACCase gene fragment, encompassing A2001V resistance mutations and amplified by polymerase chain reaction (PCR). Primers used for the PCR reactions and sequencing were the forward ACC_F (5'-AAATTCGGTGCCTACATCGT-3') and the reverse ACC_R (5'-GTCGTTGcCCCAACCATCAAC-3'). PCRs were conducted in 20 µl final volume with 2 µl 10x Minotech Taq Buffer, 0.5µl 10 mM dNTPs, 0.5µl 10 µM of each primer, 2µl template and 0.2 µl Minotech Taq polymerase with cycling conditions: 2 min at 94 °C followed by 35 cycles 45 sec at 94 °C, 30 sec at 57 °C, 45 sec at 72 °C and 10 min of final extension. Reactions were performed in BIO RAD T100™ Thermal Cycler. PCR products were purified with Nucleospin Gel and PCR Clean-Up purification kit (Macherey – Nagel) and sequenced at CeMIA SAM. Sequencing data were analyzed using BioEdit 7.0.5.3 software.

Table 3.1 Origin, host plant, and collection date of the 17 field *B. tabaci* populations from Mediterranean countries.

Code	N	Country/location	Host plant	Collection date
Ie1	23	Greece-Ierapetra	Eggplant	2018
Ie2	29	Greece-Ierapetra	Pepper	2018
Ty3	30	Greece-Tympaki	Eggplant	2018
Ty4	28	Greece-Tympaki	Eggplant	2018
Na5	31	Greece-Nafplio	Eggplant	2018
Bt455	15	Greece-Ierapetra	Eggplant	2012
Bt457	15	Greece-Ierapetra	Eggplant	2012
Bt541	12	Italy-Lazio		2016
Bt537	13	Italy-Lazio		2016
Bt535	5	Italy-Lazio		2016
Bt539	25	Italy-Sardegna		2016
Bt543	8	Spain		2016
Bt545	9	Spain		2016
Bt549	19	Spain		2016
ES199	20	Spain-Barcelona		2007
F21	15	France-Perpignan	Tomato	2007
F22	10	France-Saint Hippolyte	Tomato	2007

4.2.3 Development of a PCR-PFLP assay for genotyping the A2001V mutation in ACCase

The diagnostic PCR-restricted fragment length polymorphism (RFLP) assay designed to detect ketoenol resistance mutation, enabling the reliable distinguishing between susceptible (A2001) and resistant (V2001) ACCase alleles. It has been noticed that wild type *B. tabaci*

alignments could also have a silent (synonymous) single nucleotide polymorphism (SNP). The assay is based on the PCR amplification of a 141 bp fragment using the forward primer ACC_F (5'-AAATTCGGTGCCTACATCGT-3') and the reverse ACC_R (5'-GTCGTTG**C**CCCAACCATCAAC-3') with twenty one base pairs tail (5'-CAGGGCGGCGGGGGCGGCGCC-3') and two restriction site enzymes, HaeIII and BstAPI (New England Biolabs, NEB) (Figure 4.1). The bold/underlying C in the reverse primer represents a mismatch with the genomic DNA template. This allows the creation of a BstAPI site in the PCR product, for the detection of this silent SNP in susceptible alleles (2001A'). PCRs were performed in 20 µl final volume with 2 µl 10x Minotech Taq Buffer, 0.5µl 10 mM dNTPs, 0.5µl 10 µM of each primer, 2µl template and 0.2 µl Minotech Taq polymerase with cycling conditions: 2 min at 94 °C followed by 35 cycles 45 sec at 94°C, 30 sec at 57 °C, 45 sec at 72 °C and 10 min of final extension. Reactions were performed in BIO RAD T100^{TM} Thermal Cycler. Fifteen microlitre aliquots of the PCR product were independently digested with 3 units of HaeIII and 5 units of BstAPI in 20µl total volume, for 2h at 37^{o}C and at 60^{o}C respectively. Digestion products were visualized in 3.5% (w/v) agarose gel. Digestion with HaeIII yields a restriction pattern of two fragments (97 and 44 bp) for the susceptible allele (2001A), one fragment (141 bp) for the resistant allele (2001V) or silent susceptible allele (2001A') and three fragments (141, 97 and 44 bp) for the heterozygous allele. Digestion with BstAPI yields a restriction pattern of two fragments (103 and 38 bp) for the silence susceptible allele (2001A'), one fragment (141 bp) for the resistant allele (2001V) or the susceptible allele (2001A) and three fragments (141, 103 and 38 bp) for the heterozygous allele (Figure 4.2). Combining the restriction profiles of both RLFP assays we were able to genotype all samples used in our survey. The 44 and 38 bp fragments could not be practically detected in an agarose gel. For the validation of this diagnostic assay and before applying them in the screening of field *B. tabaci* collections, genomic DNA templates of known genotypes were used.

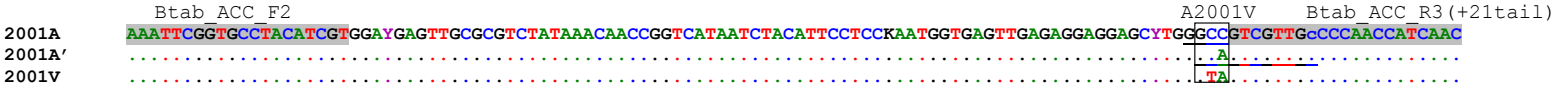

Figure 4.1 Diagnostic PCR-RFLP assay for genotyping the A2001V mutation in ACCase gene. Primer ACC_R creates restriction site for BstAPI enzyme for the detection of this silent SNP in susceptible (A'2001) allele

HaeIII Bs tAPI

GGCC *GCANNNNNTGC*

s TGGGCCGTCGTTGcCCCA

s' TGG*GCAGTCGTTGc*CCCA

r TGGGTAGTCGTTGcCCCA

Expected Bands (bp)		
Genotype	**HaeIII**	**BstAPI**
s/s	97-44	141
s'/s'	141	103-38
s/s'	141-97-44	141-103-38
s/r	141-97-44	141
r/r	141	141
s'/r	141	141-102-38

Figure 4.2 The size of the expected fragments for all possible alleles. The HaeIII and BstAPI recognition sites are shown above the DNA sequences (underlined for HaeIII and both underlined and Italian for BstAPI). The three possible alleles present in our samples at 2001 position are shaded in grey.

4.3 Results

4.3.1 Detection of A2001V mutation by sequencing and application of a new PCR-PFLP diagnostic assay

Bemisia tabaci populations from different Mediterranean countries were extracted and part of the carboxyltransferase (CT) domain of ACCase gene was amplified with the previous described primers. Ten samples from each population were sent for sequencing in order to detect the A2001V mutation. Sequencing results indicated the presence of this nucleotide polymorphism among the samples and the existence of an alternative silent SNP susceptible allele. Sequencing revealed the presence of resistant allele (r/r) GTA/GTA, the wild type allele (s/s) GCC/GCC and the alternative wild type allele (s'/s') GCA/GCA.

Moreover, a novel diagnostic PCR-RFLP assay was developed for genotyping all the possible alleles among these populations. HaeIII restriction enzyme recognizes one restriction site in wild type allele (s), while the recognition site is absent in resistant allele (r) and alternative wild type allele (s'). The second restriction enzyme, BstAPI recognizes one site in the alternative wild type allele (s') and similarly the recognition site is absent in the resistant allele (r) and the wild type allele. Based on both PCR-RFLP profiles, all possible alleles were able to be

distinguished (Figure 4.5). It was not detected neither heterozygous (r/s') nor homozygous (s'/s') allele among the examined samples (Table 4.2).

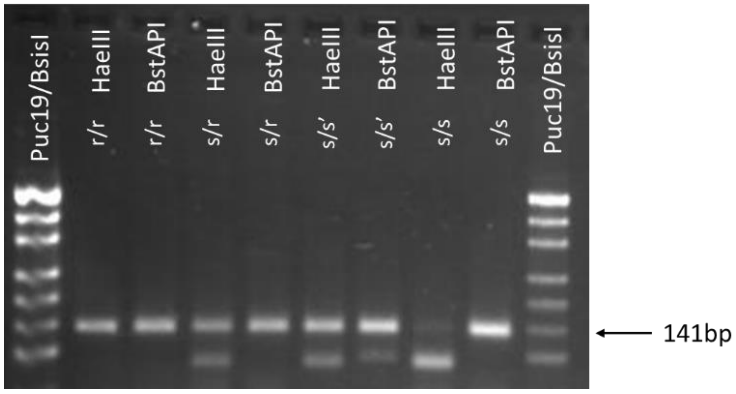

Figure 4.5 A representative agarose gel of the PCR-RFLP assay for genotyping A2001V mutation of ACCase gene. Digestion with HaeIII yields a restriction pattern of two fragments (97 and 44 bp) for the susceptible allele, one fragment (141 bp) for the resistant and three (141, 97 and 44 bp) for the susceptible with the silent SNP allele. Digestion with BstAPI yields a restriction pattern of two fragments (103 and 38 bp) for the susceptible allele, one fragment (141 bp) for the resistant and three (141, 103 and 38 bp) for the susceptible with the silent SNP allele. The 44 and 38 bp fragments could not be detected as they were small enough to be visualized by electrophoresis.

4.3.2 Frequency of the ACCase A2001V mutation in Mediterranean countries

The frequency of the A2001V mutation in the ACCase gene, strongly associated with ketoenols resistance, was investigated in field collected populations from Greece, Italy, Spain and France, via PCR-RFLP diagnostic assay. The susceptible with the silent SNP allele was found only heterozygous in populations from Greece (Ie1, Ie2, Ty3, Ty4, Na5, Bt455 and Bt457), Italy (Bt537 and Bt539) and Spain (Bt549). The mutant allele was found only in two populations from Greece, Ie1 (67.4%) and Bt457 (16.7%), two from Italy, Bt537 (3.8%) and Bt539 (8%) and four from Spain Bt543 (25%), Bt545 (83.3%), Bt549 (76.3%) and ES199 (5%). A2001V mutation was absent from populations originating from France. Furthermore, in the populations Ie2, Ty3, Ty4, Na5 and Bt455 from Greece and Bt541 and Bt535 from Italy the resistance mutation was not detected. High frequency of the A2001V mutation was detected in Spain and Greece (>60%) (Table 4.2).

Table 4.2 Frequency of A2001V mutation in *B.tabaci* populations from Mediterrenean basin

Code	Country/location	N	genotypes				% allele frequency	
			s/s	r/r	s/s'	s/r	A2001	V2001
Ie1	Greece-Ierapetra	23	3	12	1	7	32.6	67.4
Ie2	Greece-Ierapetra	29	27	0	2	0	100	0
Ty3	Greece-Tympaki	30	21	0	9	0	100	0
Ty4	Greece-Tympaki	28	25	0	3	0	100	0
Na5	Greece-Nafplio	31	26	0	5	0	100	0
Bt455	Greece-Ierapetra	15	11	0	4	0	100	0
Bt457	Greece-Ierapetra	15	10	1	1	3	83.3	16.7
Bt541	Italy-Lazio	12	12	0	0	0	100	0
Bt537	Italy-Lazio	13	9	0	3	1	96.2	3.8
Bt535	Italy-Lazio	5	5	0	0	0	100	0
Bt539	Italy-Sardegna	25	18	1	4	2	92	8
Bt543	Spain	8	4	1	1	2	75	25
Bt545	Spain	9	0	6	0	3	16.7	83.3
Bt549	Spain	19	1	11	0	7	23.7	76.3
ES199	Spain-Barcelona	20	18	0	0	2	95	5
F21	France-Perpignan	15	15	0	0	0	100	0
F22	France-Saint Hippolyte	10	10	0	0	0	100	0

4.4 Discussion

B. tabaci is an increasingly important pest of many vegetables and ornamentals, such as potato and tobacco worldwide. This pest is the target of numerous insecticides, including ketoenols, and resistance has been recently developed. According to previous study, metabolic resistance was rejected as a possible resistance mechanism against ketoenols (Bielza et al., 2019). The presence of a novel mutation (A2001V) in ACCase gene, the target of ketoenols, was observed in *B. tabaci* population from Spain and it was proposed as the major resistance mechanism. A simple PCR-RFLP diagnostic assay was developed for easy and accurate detection of the mutation, and further applied in field collected *B tabaci* samples from Mediterranean basin. The ketoenol associated resistance mutation A2001V in the ACCase gene was detected in high frequency in Spain and Greece and lower in Italy, while it was not observed in the samples from France. It was also detected in Spain samples from 2007, before the massive exposure of ketoenols in the field, indicating the possible geographic origin of this mutation. Furthermore, the development of the diagnostic assay for this mutation will enable the routine resistance monitoring of the mutation. The ability of this assay to detect heterozygous alleles in very low frequency, such as the case of 3.8% frequency of resistant allele in Bt537 population, will enable the early detection on the field and the appropriate pest control strategy. This is an important tool to prevent ineffective insecticide applications and the resistance spread of *B. tabaci* population in greenhouse and open field worldwide.

Discussion-Future plans

The multi resistant Trizinia strain was currently characterized based on its different response against acaricides of different mode of action. This study gave a wide range of information about the resistance levels of a Greek *T. urticae* population. To better understand Trizinia resistant profile, toxicity bioassays with enzyme inhibitors, such as PBO, DEF and DEM, will give insights whether or not detoxification enzymes are involved with the resistance phenotype. Additionally, RNAseq analysis (in progress) revealed several detoxification gene (over-expressed in Trizinia population) as potential candidates for pesticide detoxification. Moreover, the high resistance ratio of Trizinia to cyflumetofen creates the hypothesis of presence of mutations in the target gene or/and other resistance mechanism. Further analysis of mitochondrial electron transport chain domains may reveal the "secret" of this resistant strain. In addition to previous described validation using reciprocal crosses of the role of I310T mutation to abamectin resistance, functional expression of GluCl3 wild type and GluCl3 310T followed by two-electrode voltage-clamp electrophysiology in Xenopus oocytes could validate its role in resistance. Furthermore, as CRISPR/Cas9 genome modification in *D. melanogaster*, using a marker-assisted backcrossing approach and reverse genetics approach in a defined genetic background, could provide valuable insight into the involvement of the mutation in abamectin resistance.

Lack of knowledge about tissues and cells that are responsible for chemical response creates plenty of queries about where xenobiotic metabolism takes place. In the present study and for the first time to our knowledge detoxification enzymes, delta GSTs and CYP392A16, were found to be expressed specifically in midgut epithelial cells, "protecting" insecticide target. A tissue-specific staining, using immunohistochemical procedure, targeting the midgut epithelium could be used as additional support to this finding. These results are in accordance with previous studies showing localization of detoxification enzymes in midgut of other arthropods, such as *D. melanogaster* and *Ae. albopictus* (Chung et al., 2009; Grigoraki et al., 2016). Moreover, delta GSTs are also expressed underneath the mite cuticle, having possibly an anti-oxidative stress role. For example, in *C. elegans* has been largely demonstrated that GST, a well-known stress indicator, is expressed in the hypodermis underneath the worms cuticle (Hu et al., 2017). Additional experiments are needed to reveal the detoxification sites in mites, such as feeding or/ contact bioassays with abamectin, metabolized by CYP392A16 and cyflumetofen, metabolized by GSTd05 (Pavlidi et al., 2017; Riga et al., 2014). Feeding bioassays will improve our understanding of the detoxification phenomenon in midgut, while contact bioassays will indicate the direct metabolism of the internalized insecticide underneath the

cuticle. This acute protection from oxidative damage depicting delta GSTs acting in the first line upon insecticide attack. To conclude, it seems that detoxification occurs in insecticide entry points, but toxicity bioassays will reveal the physiological barriers which influence insecticide toxicity.

Development of PCR-RFLP diagnostic assay for the detection of A2001V mutation in ACCase target gene in *B. tabaci* populations, is a useful tool for the rapid and early detection of resistance in the field. The periodical screening of this mutation within Mediterranean contributes to insecticide resistance management of *B. tabaci*. Furthermore, the high frequency of this mutation in Spain populations from different collection dates indicate the possible origin and geographic distribution of phenotype.

References

AUGER, P., MIGEON, A., UECKERMANN, Edward A. TIEDT, L., & NAVAJAS, M. (2013). Acarologia is proudly non-profit, with no page charges and free open access. *Acarologia*, *53*(4), 383–415. https://doi.org/10.1051/acarologia/20142144

Bensoussan, N., Zhurov, V., Yamakawa, S., O'Neil, C. H., Suzuki, T., Grbić, M., & Grbić, V. (2018). The digestive system of the two-spotted spider mite, tetranychus urticae koch, in the context of the mite-plant interaction. *Frontiers in Plant Science*, *9*(September), 1–18. https://doi.org/10.3389/fpls.2018.01206

Bernhardt, R. (2006). Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology*, *124*(1), 128–145. https://doi.org/10.1016/j.jbiotec.2006.01.026

Bielza, P., Moreno, I., Belando, A., Grávalos, C., Izquierdo, J., & Nauen, R. (2019). Spiromesifen and spirotetramat resistance in field populations of Bemisia tabaci Gennadius in Spain. *Pest Management Science*, *75*(1), 45–52. https://doi.org/10.1002/ps.5144

Blauvelt, W. E. (1944). The internal morphology of the common Red Spider Mite (Tetranychus telarius Linn.). *Cornell University Agricultural Experiment Station*, *270*, 1–44.

Chung, H., Sztal, T., Pasricha, S., Sridhar, M., Batterham, P., & Daborn, P. J. (2009). Characterization of Drosophila melanogaster cytochrome P450 genes. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(14), 5731–5736. https://doi.org/10.1073/pnas.0812141106

Demaeght, P., Dermauw, W., Tsakireli, D., Khajehali, J., Nauen, R., Tirry, L., … Van Leeuwen, T. (2013). Molecular analysis of resistance to acaricidal spirocyclic tetronic acids in Tetranychus urticae: CYP392E10 metabolizes spirodiclofen, but not its corresponding enol. In *Insect Biochemistry and Molecular Biology* (Vol. 43). https://doi.org/10.1016/j.ibmb.2013.03.007

Dermauw, W., Ilias, A., Riga, M., Tsagkarakou, A., Grbić, M., Tirry, L., … Vontas, J. (2012). The cys-loop ligand-gated ion channel gene family of Tetranychus urticae: Implications for acaricide toxicology and a novel mutation associated with abamectin resistance. *Insect Biochemistry and Molecular Biology*, *42*(7), 455–465. https://doi.org/10.1016/j.ibmb.2012.03.002

Enayati, A. A., Ranson, H., & Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. *Insect Molecular Biology*, *14*(1), 3–8. https://doi.org/10.1111/j.1365-2583.2004.00529.x

Fasulo, T. R., & Denmark, H. A. (2016). *Twospotted Spider Mite , Tetranychus urticae Koch (Arachnida : Acari : Tetranychidae) 1*. 1–5.

Feyereisen, R. (2012). Insect CYP Genes and P450 Enzymes. In *Insect Molecular Biology and Biochemistry* (Vol. 450). https://doi.org/10.1016/B978-0-12-384747-8.10008-X

Feyereisen, R., Dermauw, W., & Van Leeuwen, T. (2015). Genotype to phenotype, the molecular and physiological dimensions of resistance in arthropods. *Pesticide Biochemistry and Physiology*, *121*, 61–77. https://doi.org/10.1016/j.pestbp.2015.01.004

Grbić, M., Van Leeuwen, T., Clark, R. M., Rombauts, S., Rouzé, P., Grbić, V., … Van De Peer, Y. (2011). The genome of Tetranychus urticae reveals herbivorous pest adaptations. *Nature*, *479*(7374), 487–492. https://doi.org/10.1038/nature10640

Grigoraki, L., Balabanidou, V., Meristoudis, C., Miridakis, A., Ranson, H., Swevers, L., & Vontas, J. (2016). Functional and immunohistochemical characterization of CCEae3a, a carboxylesterase associated with temephos resistance in the major arbovirus vectors Aedes aegypti and Ae. albopictus. *Insect Biochemistry and Molecular Biology*, *74*, 61–67. https://doi.org/10.1016/j.ibmb.2016.05.007

Haines, E. C. (1973). Insect control [3]. *Science*, *182*(4111), 433–434. https://doi.org/10.1126/science.182.4111.433-b

Hall, T. A. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. https://doi.org/10.1039/c7qi00394c

Horton, D. L., Fuest, J., & Cravedi, P. (2008). Insects and mites. *The Peach: Botany, Production and Uses*, 467–504.

Hu, Q., Macneil, L. T., Walhout, A. J. M., & Kubiseski, T. J. (2017). *The Oxidative Stress Response in Caenorhabditis*. *206*(August), 1909–1922. https://doi.org/10.1534/genetics.116.198788/-/DC1.1

Ia, K., & F-h, W. (2015). Life history of Bemisia tabaci (Gennadius) (Homoptera: Aleyrodidae) biotype B on tomato and cotton host plants. *Journal of Entomology and Zoology Studies*, *3*(3), 117–121.

Ilias, A., Lagnel, J., Kapantaidaki, D. E., Roditakis, E., Tsigenopoulos, C. S., Vontas, J., & Tsagkarakou, A.

(2015). Transcription analysis of neonicotinoid resistance in Mediterranean (MED) populations of B. tabaci reveal novel cytochrome P450s, but no nAChR mutations associated with the phenotype. *BMC Genomics*, *16*(1), 1–23. https://doi.org/10.1186/s12864-015-2161-5

Ingham, V. A., Jones, C. M., Pignatelli, P., Balabanidou, V., Vontas, J., Wagstaff, S. C., … Ranson, H. (2014). Dissecting the organ specificity of insecticide resistance candidate genes in Anopheles gambiae: Known and novel candidate genes. *BMC Genomics*, *15*(1), 1–9. https://doi.org/10.1186/1471-2164-15-1018

Jonckheere, W., Dermauw, W., Zhurov, V., Wybouw, N., Van Den Bulcke, J., Villarroel, C. A., … Van Leeuwen, T. (2016). The salivary protein repertoire of the polyphagous spider mite tetranychus urticae: A quest for effectors. *Molecular and Cellular Proteomics*, *15*(12), 3594–3613. https://doi.org/10.1074/mcp.M116.058081

Jones, D. R. (2003). *Plant viruses transmitted by whiteflies.pdf*. 195–219.

Karatolos, N., Williamson, M. S., Denholm, I., Gorman, K., Ffrench-Constant, R., & Nauen, R. (2012). Resistance to spiromesifen in Trialeurodes vaporariorum is associated with a single amino acid replacement in its target enzyme acetyl-coenzyme A carboxylase. *Insect Molecular Biology*, *21*(3), 327–334. https://doi.org/10.1111/j.1365-2583.2012.01136.x

Khajehali, J., Van Nieuwenhuyse, P., Demaeght, P., Tirry, L., & Van Leeuwen, T. (2011). Acaricide resistance and resistance mechanisms in Tetranychus urticae populations from rose greenhouses in the Netherlands. *Pest Management Science*, *67*(11), 1424–1433. https://doi.org/10.1002/ps.2191

Kwon, D. H., Yoon, K. S., Clark, J. M., & Lee, S. H. (2010). A point mutation in a glutamate-gated chloride channel confers abamectin resistance in the two-spotted spider mite, Tetranychus urticae Koch. *Insect Molecular Biology*, *19*(4), 583–591. https://doi.org/10.1111/j.1365-2583.2010.01017.x

Leeuwen, T. Van, Vontas, J., Tsagkarakou, A., Dermauw, W., & Tirry, L. (2010). Acaricide resistance mechanisms in the two-spotted spider mite Tetranychus urticae and other important Acari : A review. *Insect Biochemistry and Molecular Biology*, *40*(8), 563–572. https://doi.org/10.1016/j.ibmb.2010.05.008

Liu, N. (2015). Insecticide Resistance in Mosquitoes: Impact, Mechanisms, and Research Directions. *Annual Review of Entomology*, *60*(1), 537–559. https://doi.org/10.1146/annurev-ento-010814-020828

Martin, J. H., Mifsud, D., & Rapisarda, C. (2000). The whiteflies (Hemiptera: Aleyrodidae) of Europe and the Mediterranean Basin. *Bulletin of Entomological Research*, *90*(5), 407–448. https://doi.org/10.1017/S0007485300000547

McEnroe, W. D. (1969). A tactile seta of the two-spotted spider mite Tetranychus urticae K. (Acarina: Tetranychidae). *Acarologia*, *11*(1), 29–31. Retrieved from http://www1.montpellier.inra.fr/CBGP/acarologia/article.php?id=3520

Mermans, C., Dermauw, W., Geibel, S., & Van Leeuwen, T. (2017). A G326E substitution in the glutamate-gated chloride channel 3 (GluCl3) of the two-spotted spider mite Tetranychus urticae abolishes the agonistic activity of macrocyclic lactones. *Pest Management Science*, *73*(12), 2413–2418. https://doi.org/10.1002/ps.4677

Nauen, R., Bretschneider, T., Elbert, A., Fischer, R., & Tiemann, R. (2003). Spirodiclofen and spiromesifen. *Pesticide Outlook*, *14*(6), 243–245. https://doi.org/10.1039/b314855f

Occhipinti, A., & Maffei, M. E. (2013). Chlorophyll and its degradation products in the two-spotted spider mite, Tetranychus urticae: Observations using epifluorescence and confocal laser scanning microscopy. *Experimental and Applied Acarology*, *61*(2), 213–219. https://doi.org/10.1007/s10493-013-9686-6

Park, Y., & Lee, J. (2002). *Leaf Cell and Tissue Damage of Cucumber Caused by Twospotted Spider Mite (Acari : Tetranychidae)*. 952–957.

Pavlidi, N., Khalighi, M., Myridakis, A., Dermauw, W., Wybouw, N., Tsakireli, D., … Van Leeuwen, T. (2017). A glutathione-S-transferase (TuGSTd05) associated with acaricide resistance in Tetranychus urticae directly metabolizes the complex II inhibitor cyflumetofen. *Insect Biochemistry and Molecular Biology*, *80*(2017), 101–115. https://doi.org/10.1016/j.ibmb.2016.12.003

Peng, Z., Zheng, H., Xie, W., Wang, S., Wu, Q., & Zhang, Y. (2017). Field resistance monitoring of the immature stages of the whitefly Bemisia tabaci to spirotetramat in China. *Crop Protection*, *98*, 243–247. https://doi.org/10.1016/j.cropro.2017.04.009

Perdikis, D., Kapaxidi, E., & Papadoulis, G. (2008). Biological control of insect and mite pests in

greenhouse solanaceous crops. *Eur J Plant Sci Biotechnol*, *2*, 125–144.
Pérez, C. J., Alvarado, P., Narváez, C., Miranda, F., Hernández, L., Vanegas, H., … Shelton, A. M. (2009). Assessment of Insecticide Resistance in Five Insect Pests Attacking Field and Vegetable Crops in Nicaragua. *Journal of Economic Entomology*, *93*(6), 1779–1787. https://doi.org/10.1603/0022-0493-93.6.1779
Riga, M., Myridakis, A., Tsakireli, D., Morou, E., Stephanou, E. G., Nauen, R., … Vontas, J. (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. https://doi.org/10.1016/j.ibmb.2015.09.004
Riga, M., Tsakireli, D., Ilias, A., Morou, E., Myridakis, A., Stephanou, E. G., … Vontas, J. (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*(1), 43–53. https://doi.org/10.1016/j.ibmb.2014.01.006
Tsagkarakou, A., Tsigenopoulos, C. S., Gorman, K., Lagnel, J., & Bedford, I. D. (2007). Biotype status and genetic polymorphism of the whitefly Bemisia tabaci (Hemiptera: Aleyrodidae) in Greece: Mitochondrial DNA and microsatellites. *Bulletin of Entomological Research*, *97*(1), 29–40. https://doi.org/10.1017/S000748530700466X
Tsagkarakou, Anastasia, Nikou, D., Roditakis, E., Sharvit, M., Morin, S., & Vontas, J. (2009). Molecular diagnostics for detecting pyrethroid and organophosphate resistance mutations in the Q biotype of the whitefly Bemisia tabaci (Hemiptera: Aleyrodidae). *Pesticide Biochemistry and Physiology*, *94*(2–3), 49–54. https://doi.org/10.1016/j.pestbp.2009.03.002
Van Leeuwen, T., & Dermauw, W. (2016). The Molecular Evolution of Xenobiotic Metabolism and Resistance in Chelicerate Mites. *Annual Review of Entomology*, *61*(1), 475–498. https://doi.org/10.1146/annurev-ento-010715-023907
Van Leeuwen, T., Dermauw, W., Grbic, M., Tirry, L., & Feyereisen, R. (2013). Spider mite control and resistance management: Does a genome help? *Pest Management Science*, *69*(2), 156–159. https://doi.org/10.1002/ps.3335
Vontas, J. G., Small, G. J., & Hemingway, J. (2001). Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. *Biochemical Journal*, *357*(1), 65–72. https://doi.org/10.1042/0264-6021:3570065
Wolstenholme, A. J., & Rogers, A. T. (2005). Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. *Parasitology*, *131*(SUPPL. 1). https://doi.org/10.1017/S0031182005008218
Zhang, H., Tweel, B., & Tong, L. (2004). Molecular basis for the inhibition of the carboxyltransferase domain of acetyl-coenzyme-A carboxylase by haloxyfop and diclofop. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(16), 5910–5915. https://doi.org/10.1073/pnas.0400891101