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STUDIES ON INSECTICIDE RESISTANCE IN *TUTA ABSOLUTA* (MEYRICK), WITH SPECIAL EMPHASIS ON CHARACTERISATION OF TWO TARGET SITE MECHANISMS

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Dedication

To My parents, with all my love

To my family members for their encouragement and support

To my late sister Sana, you will always live in our hearts.

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Abstract

Tuta absoluta Meyrick (Lepidoptera: Gelechiidae) is a primary pest of tomato plants and is native to South America. Since the first documented European case in 2006, it has spread throughout the Mediterranean basin and North Africa. Larval stages cause direct feeding damage and reductions to both yield and fruit quality. Chemical insecticides have been the main control tools used against *T. absoluta*, but decreasing efficacy has been attributed to the development of insecticide resistance.

During this study, leaf-dip bioassays were used to quantify responses of five field strains of *T. absoluta* to insecticides belonging to different chemical classes. The results showed significant variation in susceptibilities to organophosphates and pyrethroids, which are a major class of neurotoxic insecticides and acaricides used extensively over the last decades.

One important mechanism of resistance to pyrethroids, termed knockdown resistance (*kdr*), has been shown to arise through alterations (point mutations) in the *para*-type sodium channel protein leading to reduced sensitivity of the insect nervous system to the pyrethroids. Mutations in the *Ace* gene have also been reported to cause insensitivity to organophosphates.

A combination of PCR-based molecular methods and biochemical assays was used to investigate possible existence of any mutations in these two insecticides targets in several laboratory strains.

Cloning and sequencing of domains II, III, and IV of the *T. absoluta* sodium channel gene revealed the presence of several kdr mutations previously reported to confer reduced sensitivity in other arthropod species. These included L1014F, M918T, T929I and L925M mutations. Characterisation and sequencing of *Ace* gene revealed the existence of a single mutation A201S previously related to organophosphate insensitivity in several insect species.

Diagnostic tools that allow detection in individual larvae and adults were developed and used to screen field samples of diverse geographical origin and assess their distribution in global *T. absoluta* populations.

Key words: *Tuta absoluta,* insecticide resistance, mutation, sodium channel, acethylcholinesterase, Taqman.

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Abbreviations

ACh	acetylcholine	μM	micromolar
AChE	acetylcholine esterase	mg	milligram
ATChI	acetylthiocholine iodide	min	minute
bp	base pair(s)	ml	millilitre
cDNA	complementary DNA	mM	millimolar
CM	carbamates	mRNA	messenger RNA
DDT	dichlorodiphenyltrichloroethane	nAChR	nicotinic acetylcholine receptor
DNA	deoxyribonucleic acid	nM	nanomolar
dNTP	deoxynucleotide triphosphate	OP	organophosphate
DTNB	5,5'-dithiobis-2-nitrobenzoic acid	PCR	polymerase chain reaction
DTT	dithiothreitol	pmo	picomole
EDTA	ethylene diamine tetra-acetic acid	RACE	rapid amplification of cDNA ends
g	gram	*Rdl*	*Resistance to dieldrin*
g	acceleration of gravity	RNA	ribonucleic acid
GABA	γ-aminobutyric acid	rpm	revolutions per minute
gDNA	genomic DNA	RT	reverse transcription
GST	glutathione-S-transferase	SDS	sodium dodecyl sulphate
Ic_{50}	inhibitory concentration 50%	SDW	sterile de-ionised water
kb	kilobase pair	sec	second
kDa	kilodalton	skdr	super knockdown resistance
kdr	knock-down resistance	TBE	tris borate EDTA
l	litre	TE	tris-EDTA
LB	Luria-Bertani broth	TM	transmembrane domain
Lcch3	*Ligand-gated chloride channel homologue 3*	Tris	tris (hydroxymethyl)-aminoethane
Lc	lethal concentration	Triton X-100	octylphenyl-nonaoxyethylene
μg	microgram	UV	ultraviolet
X-gal	5–bromo–4–chloro–3–indolyl–β-D-alactopyranoside		

1 - Insecticide resistance

1.1. History and dilemma

One of the most challenging aspects of pest control strategies is the occurrence of insecticide resistance. Intensive application of pesticides results in the eventual selection of resistant individuals in a population. Further insecticide applications result in an increase in the number of resistant individuals in the population as they survive treatment but susceptible individuals are eliminated. The first report of a resistance case dates back to 1914, when Melander reported his 1908 observations, made in the Clarkston Valley of Washington, of an unusual survival capacity of the San Jose scale *Quadraspidiotus perniciosus* (Comstock) up to then controlled by lime-sulfur treatments.

The rate of resistance cases increased dramatically with the introduction of the use of DDT during the 1940s. One year after its introduction, populations of house fly (*Musca domestica* L.) showed resistance to DDT in Sweden and Denmark and one year later, populations of the mosquitoes *Aedes solicitans* (Walker), *Culex pipens* L. and the bed bug *Cimex lectularius* (Latreille) were reported to be resistant to DDT respectively in Florida, Italy and Hawaii (Forgash, 1984).

Forgash (1984) reported that prior to 1946 a new resistance case emerged only every 2 to 5 years. This rate increased steadily from an average of 1 to 2 species annually between 1946 and 1954 to the average of 17 per year between 1954 and 1960 and reached 428 resistant species by 1980. The same Author, adapting data by Georghiou (1980), reported that from 428 resistant species 260 species were crop pests and 168 species were medical and veterinary pests. In 1989, Georghiou and Lagunes-Tejeda (1991) counted over 500 arthropod species with strains showing resistance to different pesticides used in the field. Some years after Forgash's report, Roush (1993) reported that 56% of the pests showing a resistance were crop pests and 40% were medical and veterinary pests while only 4% were beneficial arthropods.

The importance of the resistance is not only realised by the number of resistant species but also by the diversity of insect orders and families and the pesticides classes involved. Works by Brown (1971), Georghiou and Taylor (1977) and Georghiou (1980), cited by Forgash (1984), indicated that the resistant insect and mite populations reported until 1967 belong to 14 orders of Arthropoda, with 91% belonging to six orders. In 1980, these same six orders represented 93% of the reported resistant species: Diptera (153 species), Lepidoptera (64 species), Coleoptera (64 species), Acarina (53 species), Hemiptera Homoptera and Heteroptera (respectively 42 and 20 species).

The Arthropods Resistant to Pesticides Database (ARPD; http://www.pesticideresistance.org/) is a comprehensive resource detailing pesticides resistance cases. By 2006, the database contained over 7,400 resistant cases from 550 species and by 2011, there were 8,123 cases within 308 genera and 568 species. The different orders of insects were resistant to different classes of pesticides. The chemicals involved included both the inorganic used before 1946, such as arsenicals and sulfurs, and the more recently introduced classes of organic chemicals, such as DDT, cyclodiene, organophosphates, carbamates and pyrethroids.

Georghiou (1980) indicated that, out of 428 resistant species detected in 1980, 229 species were resistant to DDT, 269 species against cyclodiene, 200 against organophosphates, 51 against carbamates, 22 against pyrethroids, 17 against fumigants and 41 against different other pesticides. It is clear that many insect species were already resistant to more than one class of pesticides. The comparative analysis made by Forgash (1984) between the data of Brown (1967) and Georghiou (1980) indicated that the number of resistant species totally increased for all classes of pesticides but the rate of increase was higher for more recently introduced classes, like pyrethroids, than for the older classes, like DDT, cyclodiene and organophosphates. On their analysis to the content of the ARPD database, Mota-Sanchez *et al*. (2002) reported that 44% of the cases involved organophosphates and 32% involved organochlorines.

The pesticides resistance is a serious problem faced by producers, pesticides companies and environmentalists. Four classes of pesticides accounted for 70% of pesticides used in 2000. These four classes, organochlorines, organophosphates, carbamates and

pyrethroids, have all been seriously compromised by resistance (Devine and Denholm, 1998). The increasing rate of insecticide resistance makes the need for new pesticides with novel modes of action greater; however, the production of a new pesticide is time and money consuming.

Tipically a new pesticide is intensively used for a relative short period until it can no longer control the pest populations. Farmers and growers often respond by increasing the doses and frequencies of application, usually leading to the faster build up of resistance and hence loss of efficacy of the product against that target pest. Once this scenario is reached, the only way to maintain control is to switch to a pesticide with a different mode of action, if available, and the cycle starts again. This complicated situation has been described by many authors as the "Pesticide treadmill".

1.2. Types of resistance

1.2.1. MODES OF ACTION OF INSECTICIDES

To understand the different types and mechanisms of resistance, it is worthy reviewing the major modes of action of the principal classes of insecticides (organophosphates, carbamates, pyrethroids and neonicotinoids). The majority of insecticides used are nerve poisons targeting key functions in the nervous system of the insect. The principal insecticide targets include the voltage-gated sodium channel, acetylcholine (Ach) esterase, the gamma-aminobutyric acid (GABA) receptor and the nicotinic acetylcholine receptor (nAChR).

The sodium channel is a voltage dependent sodium ion pore. The inactivation of the pore allows the selective entry of sodium into the neuron following a stimulus; then, a local depolarization between the outside and inside of the neuron makes the channel recover to the original state. This change provides the signal for neuron excitation. The acetylcholine (Ach) is a neurotransmitter that can either excite or inhibit its target at neuron levels. Its level in the synapse is regulated by the enzyme acetylcholine esterase (AChE). The GABA

is an inhibitory neurotransmitter converting the received signals to chloride channel opening of the GABA receptor. When activated at the synapse, GABA causes the nerve impulse to terminate. Other physiological functions are also targets of insecticides, including the respiratory function of mitochondria, cuticle formation and growth and development hormones action.

Generally, for a pesticide to be able to achieve the required result, it must firstly come into contact with the insect, secondly reach the biochemical target site within the insect and thirdly exert its action on the target. A failure to meet any of these three steps will result in failure to control the insect pest. Organophosphates and carbamates target AChE and are considered as cholinesterase inhibitors that stop the elimination of ACh at the synapse level, resulting in the over-stimulations of the nervous system and leading to insect death. Pyrethroids act on the voltage gated sodium channel level preventing its closing and resulting in a continual impulse transmission through the nerve. Organochlorines can act on the GABA receptor and result in the over-stimulation of the nervous system acting at GABA target by preventing the chloride channel from closing.

Insects can survive insecticides expositure by preventing the contact with these compounds trough modifiying their behavior (called behavioral resistance); through changes in the cuticle; that result in reduced penetration of insecticide; through mutation of the target site protein resulting in insensitive to the pesticide; through enhanced metabolism of insecticide before it reaches the target site.

1.2.2. TYPES OF RESISTANCE

1.2.2.1. Behavioral resistance

Behavioral resistance occurs when the insect shows behavioral changes that allow avoiding the contact with the pesticide to be avoided. Onstad (undated) defined the behavioral changes as movement of immature stages, adult dispersal, oviposition, feeding, or any social or non-social interaction in a population that permit avoidance.

1.2.2.2. Penetration resistance

Penetration resistance derives from changes to the composition of the insect's exoskeleton in ways that inhibit the penetration of insecticides. Price (1991) reported that reduced penetration is rarely cited as a sole mechanism of resistance and that it is more often associated with enhanced detoxication and provides additional time for insecticide metabolism to occur. When it is a significant factor of resistance, it usually confers cross-resistance to several insecticides since it is related to the physicochemical properties of molecules rather than insecticide mode-of-action.

1.2.2.3. Metabolic resistance

Metabolic resistance results from either enhanced detoxification of the insecticide or prevention of the metabolism of the applied compound into its toxic forms This is usually mediated by modification of the metabolic pathways render the insecticide to a less toxic form. Hemingway *et al.* (2004) described the three major groups of enzymes involved in metabolic resistance: esterases, P450 monoxygenases and Glutathione S-transferases (GSTs).

The over-production of esterases (such as carboxylesterases), can result in increased sequestration of insecticide while an an increase in the activity of the enzyme (through amino acid substitutions in the enzyme) can enhance hydrolysis of the insecticide. Both these qualitative and quantitative changes can play an important role in resistance of some classes of pesticides, such organophosphates and pyrethroids. The elevated activities of P450 and GST can enhance the metabolism and resistance of insecticides belonging to most chemical classes , P450s are usually involved in phase I metabolism whereas GSTs are involved in phase II metabolism.

1.2.2.4. Target-site resistance

Target-site resistance (or site-insensitivity) results from modification of the chemical sites of action (such as acetylcholinesterase, the GABA receptor or the voltage gated sodium

channel) resulting in a reduced sensitivity to the active form of insecticide. Acetylcholinesterase insensitivity or modified acetylcholinesterase (MACE) has been reported as a major mechanism of organophosphate and carbamate resistance. Mutation of the GABA receptor most commonly described as resistance to dieldrin (rdl) is a mechanism of resistance to chlorinated hydrocarbons other than DDT, while mutations in the voltage gated sodium channel (known as knockdown resistance - kdr) confer pyrethroid and DDT resistance (see section 2.4).

1.2.3. TARGET-SITE MUTATIONS

1.2.3.1. Voltage-gated sodium channel and knockdown resistance

Sodium channels are large transmembrane complexes containing α subunit of 240 – 280 kDa and one or more of three smaller β1, β2 and β3 polypeptide subunits of approximately 30 kDa (Zlotkin 1999; Morgan *et al,* 2000).

The α subunit has four homologous, repeated domains (I – IV) with each domain containing six α-helical transmembrane segments (S1–6). The four domains surround a central ion pore in a circular radial arrangement. The segments S1 to S4 of each domain are implicated in the voltage-sensing property of the channel responding to the level of membrane potential and controlling the opening of the pore (Jiang *et al.,* 2003; Yu and Catterall, 2003). A hydrophobic region in the intracellular loop between domains III and IV is required for rapid inactivation of the channel, while the extracellular loop between segments S5 and S6 contributes to the inner lining of the sodium channel pore (Delpech *et al.,* 2005).

Multiple sodium channel isoforms and variants are presently known. At least four candidate sodium channel genes have been found in the *Drosophila* genome (Littleton and Ganetzky, 2000) and an additional *D. melanogaster* (Meigen) subunit (tip-E) (Zlotkin, 1999); in the housefly (*Musca domestica* L.), the sodium channel gene Vssc1 (Williamson *et al.,* 1993; Knipple *et al.,* 1994) is an orthologue of the *para*-gene of *D. melanogaster.*

The sodium channels found in insect neurons are either background sodium channels that contribute to the resting membrane potential or voltage-sensitive sodium channels involved in action potential generation (Sattelle and Yamamoto, 1988) and the later mediates permeability of the cell membrane to the sodium ions.

The mediation starts at the resting state by receiving a depolarization signal that will cause a movement of gating charges on voltage sensors at S3 and S4 level, followed by a change on the formation of the channel protein leading the channel pore to open. At this stage the channel is active. The period of time it remains open is controlled by an inactivation state that occurs milliseconds after its opening and makes the channel unresponsive to further depolarization signals (Yu and Catterall, 2003; Davies *et al.,*2007 a and b; Dong, 2007).

Sodium channels are the primary target of DDT and pyrethroids. By delaying the normal voltage-dependent mechanisms of inactivation, these insecticides cause the persistent activation of the sodium channels (Soderlund and Bloomquist, 1989). The sodium channel is also targeted by oxadiazines which is a newer class of pyrazoline-like insecticides (Zlotkin, 1999; Cestele and Catterall, 2000; Silver and Soderlund, 2005; Wing *et al.,* 2005).

Because of their quick knockdown effects, high insecticidal potency, relatively low mammalian toxicity at recommended doses, pyrethroids have been used extensively to control insects of agricultural and public health importance for decades (Elliot *et al*., 1973; Leahey, 1985; Shono, 1985).

However due to the intensive use of DDT and pyrethroids, many pest populations have developed resistance. One major mechanism of resistance linked to reduced target-site sensitivity in the sodium channel is known as knockdown resistance (kdr). The first case of kdr linked resistance was detected by Busvine (1951) in DDT-selected houseflies, *M. domestica*. Kdr is caused by a change in the affinity of the sodium channel for pyrethroids/DDT and results from single or multiple amino acid substitutions in the binding site (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000a; reviewed in Hemingway *et al.,* 2004).

The most common kdr mutation in a range of resistant insect species is a single base change in the S6 segment of domain II of the sodium channel gene resulting in a leucine to phenylalanine (L1014F) substitution (Miyazaki, 1996; Williamson *et al.,* 1996). The related mutations L1014H and L1014S at the same position have also been reported (Park and Taylor, 1997; Martinez-Torres *et al.,* 1999).

Secondary mutations associated with higher levels of pyrethroid resistance called super-kdr mutations have also been described The first example of a skdr mutation was a methionine to threonine substitution (M918T) within domain II of the sodium channel of resistant house flies (Williamson *et al.,* 1996).

Soderlund (2008), quoting previous works in the literature (Gurrero *et al.,* 1997; Park and Taylor, 1997; Dong *et al.,* 1998; Martinez-Torres *et al.,* 1998 and 1999; Lee *et al.,* 1999; Zhiqi *et al.,* 2000; Bass *et al.,* 2004; Brun-Bale *et al.,* 2005), reported about 28 sodium channel mutations associated with pyrethroids resistance in various pest species, including *Myzus persicae* (Sulzer), the diamondback moth *Plutella xylostella* (Linnaeus), *Blattella germanica* (Linnaeus), *Anopheles gambiae* (Giles), *Haematobia irritans* (Linnaeus), *Heliothis virescens* (Fabricius), *Culex pipiens* (Linnaeus), *Cydia pomonella* (Linnaeus), *Leptinotarsa decemlineata* (Say), and *Ctenocephalides felis* (Bouché). He reviewed also a number of additional mutations inside and outside domain II of sodium channel, such as 929I, L925I, L932F, D1549V, E435K and C785R. They can be found combined with the Kdr mutation or as single mutation as in the case of L925I in *Bemisia tabaci* (Gennadius) (Morin *et al.,* 2002).

1.2.3.2. Insensitive acetylcholinesterase (AChE)

Acetycholinesterase (AChE) is a key synaptic enzyme of the nervous system. It plays an important role in the regulation of acetycholine (Ach) levels and terminating nerve impulses by catalyzing the hydrolysis of this neurotransmitter (Hemingway *et al.,* 2004; Cousin *et al.,* 2005; Fournier, 2005; Alout *et al.,* 2008). The inhibition of AChE by insecticides such as organophosphates and carbamates causes insect death. The inhibition is due to irreversible competition of insecticides for the enzyme acetylcholinesterease and

occurs by phosphorylation or carbamylation of the serine residues within the active site gorge leading to the accumulation of Ach in the synapses (Eldefrawi, 1985).

Resistance to organophosphates and carbamates has been mediated through target site resistance in many insects and has been termed insensitive acetycholinesterase. Smissaert (1964), cited by Price (1991), was the first one to suggest the existence of a form of AChE which is insensitive to inhibition by organophosphates and carbamates. Devonshire and Moores (1984) detected the existence of several forms of the insensitive enzyme. Since then this resistance mechanism has been shown to be very common in numerous pest species (Chen *et al.,* 2001; Fournier and Mutero, 1994; Li and Han, 2004; Menozzi *et al.,* 2004).

Fournier *et al.* (1992) showed that the insensitivity of the enzyme arose from point mutations in the gene encoding AChE. Point mutations in the AChE gene have been described for resistant strains of different species, including *Musca domestica* L., *Lucilia cuprina* (Wiedemann), *Bactrocera oleae* (Gmel.), *Leptinotarsa decemlineata* (Say), *Anopheles gambiae* (Giles), *Culex pipiens* (Linnaeus), *Aphis gossypii* (Glover), *Myzus persicae* (Sulzer), *Tetranychus urticae* (Koch) (Zhu *et al.,* 1996; Chen *et al.,* 2001; Walsh *et al.,* 2001; Vontas *et al.,* 2002; Anzawa *et al.,* 2003; Nabeshima *et al.,* 2003; Weill *et al.,*2003; Li and Han, 2004; Menozzi *et al.,* 2004; Fournier, 2005; Temeyer *et al.,* 2008).

Fournier (2005) reviewed all the mutations of acetylcholinesterase which have been reported as linked to insecticide resistance.

Feyereisen (1995) concluded, from his works on the characterization of the mutations in the coding gene encoding AChE (Ace) in *Drosophila,* that multiple mutations caused higher levels of insensitivity than single mutations and that multiple mutations may result from the accumulation of mutations or from the recombination between genes of individuals carrying single mutations. Hemingway *et al*. (2004) reported that individual mutations confer a low level of insensitivity, while a combination of mutations generate enhanced resistance, and that the mutations confer resistance as they constrict the active site gorge limiting insecticide access to the catalytic residues.

Most insects have two Ace genes: Ace1, paralogous to the *D. melanogaster* gene, and Ace2, the orthologus gene to the *Drosophila* Ace (Fournier *et al*., 1989; Weill *et al*., 2002). The resistance can be associated with single mutation in the Ace1 gene like in mosquito (Hemingway *et al*., 2004; Alout *et al*., 2008) or combinations of mutations on the unique Ace2 gene like in true flies (Mutero *et al*., 1994).

1.2.3.3. GABA

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter widely distributed in the synapses through the nervous system of insects. It comprises five subunits arranged around the central ion channel (Hemingway *et al*., 2004). Each subunit contains a large extracellular agonist-binding N-terminal domain, and four transmembrane domains (M1–M4) (Nakao *et al*., 2010). In insects, GABA receptors inhibit pre- and postsynaptic neuronal activity by increasing mainly chloride dependent events (Le Corronc *et al*., 2002).

In vertebrates, GABA receptors were classified into three structurally and pharmacologically distinct subtypes: GABAa, GABAb and GABAc. Even though many studies showed that the vast majority of GABA receptors in insects exhibit properties similar to GABAa in vertebrates, they do not fit into these classification as they are subject to allosteric modulation but are insensitive to bicuculline (Le Corronc *et al.,* 2002).

Hosie *et al*. (1997) reported that three cysloop receptor subunit classes were cloned from *Drosophila* and that they are encoded by three genes with high sequence identity to vertebrates GABA receptor subunits. The genes are called *Rdl* (resistance to dieldrin), *Gdr* (GABA and glycine-like receptor of *Drosophila*) and *Lcch3* (ligand-gated chloride channel homologue).

The GABA gated chloride channel / GABA receptors is the primary target site of the cyclodienes and fiprole classes of insecticides. Resistance to cyclodienes is relatively common in many insect pest species. Reduced sensitivity of the nervous system to cyclodienes was first demonstrated by Kadous *et al*. (1983). They found that the neuroexcitatory action of both dieldrin and picrotoxinin developed rapidly in nerve cords from susceptible German cockroaches but required a longer time to develop in nerve cords

from a resistant strain. The resistance to cyclodienes was explained by a change in the structure of the GABA receptors of resistant insects due to a single point mutation (ffrench-Constant, 1999).

Mutations at a single codon in the *Rdl* gene (encoding one receptor subunit), from an alanine to a serine or to a glycine, have been documented in several different insect species that are resistant to the cyclodiene aldrin (ffrench-Constant *et al*., 1998). This mutation occurs in the second transmembrane region of the RDL subunit and appears to confer both insensitivity to the insecticide and a decreased rate of desensitization (Hemingway *et al*., 2004).

1.2.3.4. The nicotinic acetylcholine receptor

The nicotinic acetylcholine receptor (nAChR) is a member of the ligand-gated ion channel class of receptors. It is responsible for fast transient transmission of impulses in the synaptic junctions (Hille, 1992; Galzi and Changeux, 1994). Unlike vertebrates, the insect nAChRs are present only in the central nervous (Liu and Casida, 1993). The nAChR is a pentameric transmembrane protein of 300kDa composed of five structurally similar subunits: α (2 subunits), β, γ and δ with two acetylcholine binding sites located between the α and γ, and the α and δ subunits (Corringer *et al.,* 2000).Each subunit is composed of three main regions including a large hydrophilic N-terminal carrying the ligand binding site, four hydrophobic transmembrane regions M1to M4 of and a hydrophilic segment between M3 and M4 carrying phosphorylation sites involved in regulation and desensitization of the receptor (Miles *et al.,* 1994; Hucho *et al.,*1996). The subunits are associated with the rapsyn, a protein thought to play a role in attaching the receptor to the cytoskeleton (Phillips, 1995).

The ion channel is formed from the five M2 helices from each subunit. The nAChR can exist in four distinct states: resting, active, intermediate desensitised and slowly desensitised. The channel is open in the active state and close in the desensitised state. The affinity for Ach is high when the channel is open and low when it is closed (Changeux and Edelstein, 1998).Miyazawa *et al.,* (2003) described the gating mechanism of the channel.

The gate is destabilised by rotations in the subunits triggered by the ACh entering the ligand binding domain resulting in the movement of helices to a confirmation that makes the receptor permeable to ions.

The nAChRs are the primary target for, relatively new class of insecticides, neonicotinoids such as imidacloprid. Cases of resistance in B. tabaci and other species have been reported (Nauen *et al.,* 2008).

1.2.4. METABOLIC MECHANISMS OF INSECTICIDES RESISTANCE

1.2.4.1. P450 monooxygenases

The cytochrome P450-dependent monooxygenases (P450s) are a very important and diverse family of hydrophobic heme-containing enzymes that are involved in the metabolism of numerous endogenous compounds such as hormones, fatty acids and steroids, and the catabolism and anabolism of exogenous compounds (Scott, 1999; Hemingway *et al.,* 2004). The term P450 derives from the discovery of a liver microsomal pigment (P) with an absorption peak at 450 nm (Feyereisen, 1999).

Insect genomes have been found to contain between 36 to over 150 genes encoding different P450 enzymes (Nelson et al, Feyerisen). The high number of cytochrome P450s in most insects allow a significant diversity of substrate reactions (Berge *et al.,* 1998). P450s are involved in endogenous metabolism and have several functional roles since they can oxidize a wide range of substrates. They are involved in growth, development and reproduction processes through the synthesis and degradation of steroid moulting hormones and juvenile hormones and also influence pheromones metabolism (Rendic and Di Carlo, 1997; Mansuy, 1998; Feyereisen, 1999). They are involved in the synthesis and degradation of more than 20 hydroxyecdysone and juvenile hormones (Scott, 1999 and the references cited).

P450s also metabolise exogenous compounds metabolism, and provide protection from many natural and synthetic xenobiotics. They are involved in plant toxin and pesticides tolerance and insecticide resistance. P450-mediated resistance is one of the most frequently

described insecticide resistance mechanisms (Openoorth, 1985; Scott, 1999). This mechanism was first implicated in the resistance when sesamex, a P450 inhibitor, was found to abolish the carbaryl resistance (Eldefrawi *et al.,* 1960 cited by Scott, 1999). Now, it is well established that in many cases the P450-mediated resistance results from elevated P450 expression and/or changes on the catalytic activity of the P450, both leading to pesticide detoxification (Oppenoorth, 1984; Field *et al.,*1988; Rauch and Nauen, 2003; Karunker *et al.,* 2008 and 2009; Philippou *et al.,* 2009; Barale *et al.,* 2010; Puinean *et al.,*2010).

The mechanism of P450 action usually involves the addition of oxygen molecule to a substrate and yields a hydrophilic molecule that is more easily metabolized by the organism (Danielson *et al.,* 1997; Feyereisen, 1999; Hemingway *et al.,* 2004). When the P450 enzyme binds molecular oxygen it receives electrons from NADPH to introduce an oxygen atom into the substrate and to form water with the other oxygen atom as shown in the following reaction:

Substrate (S) + NADH + H + O2→ SO + NADP+ H2O.

Scott *et al.* (1998) indicated that the involvement of a P450 in a resistance can be demonstrated if the insecticidal compound is proven to be detoxified by P450 in the resistant insect and that the P450 levels are higher in the resistant insect than in the susceptible one. Different methods can help to prove the involvement of P450 enzymes in a pesticide resistance. One of the most commonly used is the use of piperonylbutoxide, which is a P450 monooxygenase inhibitor that can lead to a partial or complete loss of resistance caused by enhanced P450-mediated detoxification when applied to resistant insects prior to insecticide application (Raffa and Priester, 1985; Devine and Denholm., 1998; Brooke *et al.,* 2001; Bues *et al.,* 2005; Moores *et al.,* 2008; Barale *et al.,* 2010).

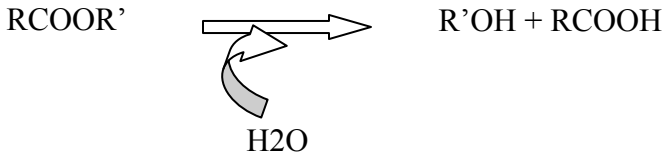
The direct comparison of the NADPH-dependent metabolism of the resistant and susceptible insect is another direct method to prove the involvement of P450-mediated detoxification but it requires radio-labbelled molecules (Berge *et al.,* 1998).

1.4.4.2. Carboxylesterases

Carboxylesterases (CaE) or esterases are very important enzymes involved in the detoxification of xenobiotics and one of the major mechanisms of insect resistance to older classes of insecticides such as organophosphates and carbamates (Hemingway and Karunaratne, 1998; Wheelock *et al.*, 2005; Zang *et al.*, 2010). Generally, the most used classification of CaE is the one presented by Aldrige (cited by Hemingway and Karunaratne, 1998 and Wheelock *et al.,* 2005) grouping them in three clades (A, B and C) according to their interaction with organophosphates. A-esterases are the enzymes that hydrolyze organophosphates compounds. B-esterases are the one inhibited by organophosphates such as paraoxon and C-esterases provide resistance to organophosphates but do not degrade them.

Esterases can confer resistance through either quantitative or qualitative changes in enzyme production or activity (Field *et al.,* 1999; Hemingway *et al.,* 2004; Cui *et al.,* 2007). Quantitative changes result from an over-expression of the esterase protein and are often achieved by structural amplification of CaE genes. In this case the carboxylesterases provide protection from insecticide exposure by delaying or preventing the interaction between the toxin and target site. Qualitative changes occur when the capability of enzymes to metabolize insecticides is enhanced or changed by amino acid substitutions in the esterase enzyme (Hemingway and Karunaratne, 1998; Wheelock *et al.,* 2005).

As their name implies, the carboxylesterases hydrolyze carboxyl esters (RCOOR') into the corresponding alcohol (R'OH) and carboxylic acid (RCOOH) via the addition of water, as described in the following reaction shows:



This reaction has an absolute requirement for three conserved amino acids within the active site of the enzyme: Serine (Ser), Histidine (His) and Glutamic acid (Glu). Wheelock *et al.* (2005) and Potter and Wadkins (2006) give a detailed description of the hydrolysis

reaction. Hydrolysis is initiated by nucleophilic attack transferring a proton from Ser to His and generating a serine nucleophile. His is stabilized by hydrogen bond formation to the Glu. The Ser nucleophile attacks the electron deficient carbonyl in the ester substrate, forming a tetrahedral intermediate that collapses to form the acyl-enzyme complex releasing the Ser and the alcohol portion of the substrate. Repeating the former process, a His activated water molecule attacks the acyl-enzyme complex and releases the acid portion of the substrate. The second serine provides structural support for the spatial orientation of the Glu and the catalytic triad.

1.2.4.3. Glutathione S-transferase (GSTs)

Metabolic resistance of insects can also be provided by the detoxifying activity of Glutathione S-transferases (GSTs) enzymes. GST-based resistance has been described for organophosphates, cyclodienes and pyrethroids but they are also able to dehydrochlorinate insecticides like DDT. In many cases, GSTs can act as a secondary resistance mechanism with monooxygenase or esterases based resistances.

GSTs belong to a super-family that includes 25 groups. A very simplified classification divides insect GSTs into two groups called class I and class II GSTs, where class I is very related to the mammalian theta class and class II to the pi class, without extending this relationship to the substrates specificity of each enzyme (Hemingway, 2000). The vast majority of GSTs are cytosolic dimeric proteins comprising two subunits and each subunit has two binding sites: G and H sites. The G site binds to tripeptide glutathione and the H site is a substrate binding site with higher variability of structures (Hemingway *et al*., 2004).

The GST-based resistance is due to either higher enzyme levels or increased activity of GSTs (Scott, 1995). Hemingway *et al.* (2004) reported that the higher enzyme activity is due to an increase in the amount of one or more GST enzymes as a result of either a gene amplification or increases in the rate of gene transcription.

GSTs can cause resistance through the conjugation of reduced glutathione (GSH) to insecticides or its toxic metabolic products. In the case of DDT dehydrochlorination, GSH acts as a co-factor and not as a conjugate (Hemingway, 2000). In organophosphate resistance, the detoxifications occurs by an O-dealkylation, where the glutathione is conjugated with the alkyl portion of the insecticide or by an O-dearylation as a reaction of the glutathione with the leaving group (Hemingway *et al*., 2004 and the references cited in).

Elevated GST activity has been first described for organophosphate (OP) resistance (Hayes and Wolf, 1988). DDT dehydrochlorinase is achieved by an abstraction of hydrogen catalysed by the thiolate anion generated in the active site of the GST, resulting in chlorine elimination from DDT and the generation of DDE (Hemingway *et al*., 2004). In pyrethroid resistance, GSTs seem to have no direct role in their metabolism but can confer the resistance to this class of insecticides by detoxifying lipid peroxidation subproducts or by protecting against pyrethroid toxicity via insecticide sequestration (Vontas *et al*., 2001; Kostaropoulos, 2001 cited by Hemingway *et al*., 2004).

2 - Tuta absoluta, new pest in Europe

2.1. Taxonomy

2.1.1. NAME

Tuta absoluta (Meyrick, 1917) Povolny, 1994

2.1.2. SYNONYMS

- *Gnorimoschema absoluta* (Meyrick, 1917) Clark, 1962.
- *Phthorimaea absoluta* Meyrick, 1917.
- *Scrobipalpula absoluta* (Meyrick, 1917) Povolny, 1964.
- *Scrobipalpuloides absoluta* (Meyrick, 1917) Povolny, 1987.

2.1.3. HISTORY OF NOMENCLATURE

Tuta absoluta is a micro-lepidopteran insect from the family Gelechiidae. It was originally described as *Phthorimaea absoluta* by Meyrick (1917). Later on, it has been assigned to the genera *Gnorimoschema*, by Clark (1962), and *Scrobipalpula*, by Povolny (1964); then, it was placed in the new genus *Scrobipalpuloides*, described by Povolny in 1987. The presently accepted assignment of the species to the genus *Tuta* has been proposed by the same Povolny in 1994.

Common names are used to describe *T. absoluta* and depend on the specific language. In English, it can be called tomato borer, South American tomato moth, tomato leaf miner, South American tomato pinworm. In Spanish, the common names used are: polilla del tomate, polilla perforadora, cogollero del tomate, gusano minador del tomate, minador de hojas y tallos de la papa. In Portuguese, traça-do-tomateiro.

2.2. Geographical distribution

T. absoluta was described for the first time in Peru by Meyrick in 1917 (Povolny 1975) and has since spread to most of South America, where significant damages are reported. According to García and Espol (1982), the insect was introduced from Chile to Argentina in 1964. Since 1960, it has spread out to Bolivia, Brazil, Colombia, Ecuador, Paraguay, Uruguay and Venezuela (Souza *et al*, 1986; Larrain, 1986). *T. absoluta* is not present in Andean regions at high altitudes (above 1000m), as low temperature is a limiting factor for its survival (Notz, 1992).

In Asia, and specifically in Japan, its presence is unconfirmed since no recent records have revealed its presence. Only an old record of *T. absoluta* attacking *Solanum lyratum* in Japan was reported by Clarke (1962). In Europe, *T. absoluta* was added in 2004 to the EPPO A1 action list of pests recommended for regulation as a quarantine pest and since this plants to be imported from countries where *T. absoluta* occurs must be free from the pest. Despite these precautions, *Tuta absoluta* has been recently reported in several European countries including Spain (2006) (Urbaneja *et al*., 2007), Italy (first found in 2008, when it has been reported from Calabria, Campania, Sardinia and Sicily) (Tropea Garzia *et al*., 2009; Viggiani *et al*., 2009), Netherlands (incidental finding in January 2009) (Potting *et al.,* 2009), France (first detected in 2008 in tomato crops) (Germain *et al.,* 2009 cited by Desneux *et al.,* 2010). Thus, from 2009 it has been moved to the EPPO A2 action list.

In April 2009, it was reported for the first time in Malta in a greenhouse at Dingli (South West region of Malta) (Malia, 2009). In North Africa, *T. absoluta* has been reported in Algeria (March 2008) (Guenaoui, 2008), Morocco (May 2008) (EPPO,2008) and in Tunisia (2008) (EPPO, 2009). By 2011, more than 20 countries in southern Europe, North Africa and Middle East have reported the presence of *T. absoluta* (EPPO, FERA2009; www.tutabsoluta.com.).

2.3. Morphology

T. absoluta is a grey-brown in color with silverfish-grey scales and black spots on anterior wings. It is approximately 6 mm long with a wingspan of about 10 mm,. The filiform antennae are about 10 mm long.

Figures 1-7, *Tuta absoluta* (Meyrick): 1 – adult; 2 - adults mating; 3 - eggs; 4 - mature egg; 5 - larva; 6 - mature larvae; 7 - pupae (Source: Monserrat, D.A., undated).

The egg is small (0.36 mm long and 0.22 mm large), cylindrical, creamy white to yellow.

Newly-hatched caterpillars are approximately 0.5 - 0.9 mm long and have a creamy yellowish color with dark head. When maturing, caterpillars turn yellow-green to light pink in the second to fourth instars and a black band develops behind the head. Fully-grown caterpillars are approximately 7.5 to 9 mm in size, with a pinkish color on the back. The pupa is light brown and approximately 6 mm long.

2.4. Biology

The biology of *T. absoluta* has been largely studied in different ecological situations in many countries of Latin America (Bahamondes and Malea, 1969; Coelho and França, 1987; Souza *et al*, 1992; Miranda *et al* 1998) as well as in European and Mediterranean environments (Guenaoui, 2008; Tropea Garzia *et al*., 2009; Monserrat, 2009; Viggiani *et al*., 2009; Abolmaaty *et al.,* 2010; Desneux *et al.,* 2010; Lacourdiere and Feuvrier, 2010; Roditakis *et al.,* 2010; Sannino and Espinosa, 2010). The life cycle consists of 6 stages: egg, 4 larval instars, pupa and adult stage.

T. absoluta has a high reproductive potential; there may be 10–12 generations per year. The biological cycle is completed in 29–38 days depending on environmental conditions, especially temperature. Studies in Chile have shown that development takes 76.3 days at 14°C, 39.8 at 19.7°C and 23.8 at 27.1°C (Barrientos *et al*., 1998).

Guenaoui *et al* (2008) reported that the life cycle can be completed in 3 weeks in the Mediterranean region at 27°C and Monserrat (2009) suggested that in Murcia (Spain) the life cycle can be completed in about 20 days under optimal conditions. The same authors also established the duration of all the life cycle stages as follows:

Table 1: Duration (in days) of different stages of the life cycle of *T. absoluta*.

	Egg	**Larvae**	**Pupa**	**Adult**	**Cycle (days)**
30 °C	4	11	5	9	20
25 °C	4	15	7	13	27
20 °C	7	23	12	17	42
15 °C	10	36	20	23	66

Adults are nocturnal and usually hide during the day among leaves. Females lay eggs on aerial parts of the host plants and a single female can lay a total of about 180 to 260 eggs during its lifetime. Eggs can be deposited on both sides of leaves. Hatching takes place after 4–5 days. Four larval instars develop feeding on the mesophyll as a miner in the leaf, stem or fruit but usually exit the mine to pupate after about 13–15 days. Larger caterpillars can sometimes be found outside the mine or fruit. Larvae do not enter diapause as long as food is available. Pupation may take place in the soil, on the leaf surface or within mines, depending on environmental conditions. The pupation duration is about 9–11 days. When *T. absoluta* does not pupate in the soil, a cocoon is usually built. The pest may overwinter as eggs, pupae or adults.

2.5. Host plants

T. absoluta is reported as oligophagous (Siquiera *et al.*, 2000), exclusively feeding on plants of the family Solanaceae (Vargas, 1970; Lietti *et al.*, 2005). The main host of *T. absoluta* is tomato, with spectacular damages. Potato is also reported to be a host (Galarza, 1984; Notz, 1992; CIP, 1996), together with eggplant, *Solanum melongena* (Viggiani *et al.,* 2009); sporadic attacks were reported on common bean, *Phaseolus vulgaris* (EPPO, 2009;

Sarra *et al.,* 2009). Other plants attacked include *Lycopersicon hirsutum, Solanum lyratum* and various wild solanaceous species, such as *Solanum nigrum, S. elaeagnifolium, S. puberulum, Datura stramonium, D. ferox* and *Nicotiana glauca*. In laboratory studies (Galarza, 1984), egg-plant was reported as a potential host (with other solanaceous species), but there are no references to its importance in the field. There is an old record of tobacco being attacked in Argentina (Mallea *et al.,* 1972).

2.6. Damage pattern and detection

All parts of the plant in different developmental stages can be infested by *T. absoluta*. In greenhouse conditions, all stages can be found throughout the entire growing season (Mallia, 2009).The caterpillars have a strong preference for leaves and stems but they may also be found in or under the crown of the fruit and in the fruit itself; small heaps of excrement are often found near the entrance hole (Mallia, 2009).

After hatching, neonate larvae penetrate into the fruits, leaves or stems where their feeding and development activity creates mines and galleries. Fruits can be attacked as soon as they are formed, and the galleries can be invaded by secondary pathogens leading to fruit rot. On leaves, young larvae feed only on mesophyll tissues, leaving the epidermis intact. Leaf mines are irregular and may later become necrotic. Galleries in stems alter the general development of the plants. Tomato plants can be attacked at any developmental stage, from seedlings to mature plants. The pest is generally easily found because it prefers apical buds, flowers or new fruits, on which the black frass is visible. On potato, only aerial parts are attacked and *T. absoluta* does not develop on tubers (Notz, 1992; Caffarini *et al*., 1999).

2.7. Economic impact

In Latin America, *T. absoluta* is considered a key pest of tomato both in the field and under protected conditions (Lopez, 1991; Apablaza, 1992; Barrientos *et al.,* 1998; Estay, 2000). Both yield and fruit quality can be significantly reduced by the direct feeding of the pest and the secondary pathogens which may enter through the wounds caused by the pest

(Moore, 1983; Silva *et al.,* 1998; Oliveira *et al.,* 2009). Severely attacked tomato fruits lose their commercial value. Losses as high as 50–100% have been reported on tomato (mainly under low rainfall). On potato, CIP (1996) considers that *T. absoluta* is one of the major pests of foliage, occurring in warm zones of low altitudes (below 1000m a.s.l.).

In European and Mediterranean regions, where tomato has a huge economic role, since its first detection in Spain, *Tuta absoluta* became a key pest of tomato and has caused serious damages in protected and open field crops in this regions (Germain *et al*., 2009; Desneux *et al.*, 2010).

Severe damages have been reported from different localities. In Italy, Sannino and Espinosa (2010) reported losses of up to 100% in protected tomato during the year 2009. The same authors reported 30 to 40 % of damages on protected eggplant crops and the findings of larvae in cans of processed tomato coming from the Province of Salerno. Serious damages were reported on potato, caused by simultaneous attacks of *T. absoluta* and *Phthorimaea operculella* (Zeller) (Sannino and Espinosa, 2010). In 2008, Sarra *et al,*(2009) reported that 3% of the 305 investigated farms in Sardinia (Italy) had to replant their tomato crop after a complete destruction by *T. absoluta*.

In Spain, and particularly in Murcia region (with a mild climate and counting more than 3000 ha of protected tomato fields), Monserrat (2007) reported a permanent establishment of *T .absoluta* with a continuous activity around the year. Damages varied between 50 and 100% in this area. In 2008, crop losses up to 100% have been reported in winter tomato crops in the Province of Valencia in Spain (EPPO, 2008).

Different other authors reported serious damages, in some cases up to 100%, from different major producing and exporting countries in the Mediterranean area, including Morocco (Ouardi, 2011), Algeria (Benddine, 2011), Tunisia (Nasraoui, 2011), Egypt (Temerak, 2011), Turkey (Ugurlu, 2011), France (Trottin, 2011) and Iran (Baniameri, 2011).

In Netherlands, Potting *et al.,* (2010) reported that estimated economic consequences according to the worst scenario of the establishment of *T. absoluta* for the local tomato sector can reach 25 million/year due to crop losses and € 4 million/year due to pest management

One more indicator of the economic importance of *T. absoluta* in the European and Mediterranean regions is its high ability to use various plants as secondary hosts (Desneux *et al.*, 2010). It has been reported in pepper and bean plant as well as in Cape gooseberry (*Physalis peruviana*) in Sicily (Italy) (EPPO, 2009; Tropea Garzia, 2009; Serra *et al.,* 2009).

Figure*:* 8-12 - *Tuta absoluta* (Meyrick): different damage patterns on leaves (1 and 2), on fruits (3 and 4) and under greenhouse (5) (Source: Monserrat*,* 2007).

2.8. Control strategies

Chemical control has been the main control strategy for this insect since the early 1980s (Souza and Reis, 1986). Some farmers have to perform up to 36 insecticide applications during a single crop cycle (Picanço *et al.,* 1995) but effective control is difficult to achieve because the larvae are internal feeders. As a consequence, reduced efficacy and insecticide failures in controlling this pest have been observed by producers.

Lietti *et al.* (2005) reported that firstly organophosphates and then pyrethroids were used during the 1970's and 1980's to control *Tuta absoluta*; then new products with different mode of action were introduced since the 1990's.

In Malta, Mallia (2009) reported that the use of indoxacarb, lufenuron, spinosad, thiacloprid, imidacloprid, abamectin and *Bacillus thuringiensis* is claimed to be effective against *T.absoluta*. In Italy, Tropea Garzia *et al.* (2009) mentioned the use of chlorpyrifos and pyrethrins for control. Indoxacarb, spinosad, imidacloprid, deltamethrin, and *Bacillus thuringiensis* var. *kurstaki,* were applied for the control of larval infestations in Spain (FERA, 2009). In France, only indoxacarb and *Bacillus thuringiensis* were recommended for use. In Agrentina, Riquelme (2006) reported the use of *Bacillus thuringiensis* and triflumuron in IPM programs while a larger number of insecticides were recommended for use in Brazil, including methamidophos, spinosad, and indoxacarb, chlorfenapyr, phenthoate, abamectin, and cartap (IRAC, 2007).

Recently, it has been reported that new classes of insecticides are providing good control of this pest and at least 12 classes of insecticides can provide good control of *Tuta absoluta.* They are summarised in the Table2.

Table2 - Mode of action groups, chemical groups and common names of the insecticides used to control *T. absoluta* (from: New Pest Response, GuidelinesTomato Leafminer (*Tuta absoluta),* USDA, 2011)

MOA Group	Chemical subgroup	Common Name
Acetylcholinesterase (AChE) inhibitors	1B: Organophosphates	Chlorpyrifos Methamidophos
Sodium channel modulators	3A: Pyrethroids	Deltamethrin
Nicotinic acetylcholine receptor (nAChR) agonists	4A: Neonicotinoids	Imidacloprid Thiacloprid
Nicotinic acetylcholine receptor (nAChR) allosteric activators	5:Spinosyns	Spinosad
Chloride channel activators	6:Avermectins	Abamectin, emamectin
Microbial disruptors of insect midgut membranes	11: *Bacillus thuringiensis*	*Bacillus thuringiensis*
Uncouplers of oxidative phosphorylation via disruption of the proton gradient	13:Chlorfenpyr	Chlorfenapyr
Inhibitors of chitin biosynthesis, type 0	15:Benzoylureas	lufenuron
Voltage-dependent sodium channel blockers	22A : indoxacarb 22B: Metaflumizone	Indoxacarb Metaflumizone
Ryanodine receptor modulators	28: Diamides	Chlorantraniliprole Flubendiamide
Unknown or act on multiple targets	Azadirachtin	Azadirachtin

Several Integrated Pest Management strategies have been developed in different countries. They include the use of pheromone and light traps for early detection, mass traping and pest populations monitoring (Filho *et al.,* 2000; Al Zaidi, 2009; Monserrat, 2009 and 2010; Arno and Gabarra, 2010; Desneux *et al.,* 2010; Jacobson, 2011). They include also crop rotations with non-host plants, biological control with *Trichogramma sp., T. achaeae* (Nagaraja and Nagarkatti)*, Macrolophus pygmaeus* (Rambur), *Nesidiocoris tenuis* (Reuter), *Nabis pseudoferus ibericus* (Remane) (Salvo and Valladares, 2007; Sanchez *et al.,* 2008 and 2009; Cabello *et al.,*2009a and b; Urbaneja *et al.,* 2009; Arnó and Gabarra, 2010; Sardegna, 2010) and, when needed, the use of compatible pesticides such as imidacloprid in irrigation, spinosad, indoxacarb and *Bacillus thuringiensis* (Robredo-Junco and Cardeñoso-Herrero, 2008; Arnó and Gabarra, 2010; Botto, 2011; http://www.tutaabsoluta.com/tuta-absoluta).

2.9. Tuta absoluta and insecticides resistance

Because of the short generation time and the frequent applications of insecticides to manage *T. absoluta,* resistance to several insecticides has developed. In many studies, researchers have shown reduced insecticide activity in the field (Souza *et al.,* 1992; Guedes *et al.,* 1994), suggesting the development of resistant populations to many of compounds used against the pest (Gonçalves *et al*., 1994).

In Chile, resistance to deltamethrin, metamidophos, esfenvalerate, lambdacyhalothrin and mevinphos have been reported by Salazar and Araya (1997). Lietti *et al*. (2005) reported a slight resistance to abamectin in one population and deltamethrin resistance in two populations from Argentina.

In Brazil, since 1999 significant resistance of *T. absoluta* to acephate and deltamethrin was reported by Castelo Branco *et al.* (2001). Control failures of abamectin, cartap, methamidophos and permethrin used against *T. absoluta* have been also reported by Guedes *et al*. (1994). In 2001, resistance to abamectin was additionally reported by Siqueira *et al*. (2000a).

Siqueira *et al*. (2000b) reported differences in susceptibility among Brazilian populations of *T. absoluta* to abamectin, cartap, methamidophos, and permethrin, which could indicate a development of resistance of this insect to these insecticides

In their recent study, Gerson *et al.,* 2011 surveyed resistance levels of populations of *T. absoluta* from the main tomato-producing regions in Brazil to the main insecticides currently used and recommended. They reported evidence of control failures for bifenthrin, permethrin, diflubenzuron, teflubenzuron, triflumuron and *B.thuringiensis*, moderate levels of resistance to indoxacarb and no resistance or only low resistance levels for bifenthrin and permethrin, abamectin, spinosad, *B. thuringiensis* and the mixture of deltamethrin and triazophos.

Up to date, in the European and Mediterranean region, works that dealt with resistance to insecticides of *T. absoluta* are very rare despite the importance of the pest for tomato crops in this region. Many works were focusing mainly on chemicals that can be used by the farmers to control the pest. One of the works that aimed to investigate the susceptibility of *T. absoluta* to insecticides was carried out by Roditakis *et al.* (2011), who used and validated the leaf-dip methodology. The test, conducted on field populations from various Greek regions, included eight insecticides (Rynaxapyr, Indoxacarb, emamectin benzoate, spinosad, metaflumizone, flubendiamide, chlorpyriphos and cypermethrin). The results showed low variability in the LC_{50} values among the tested populations but hight LC_{95} values in comparisons with the recommended field rates for chlorpyriphos, metaflumizone and cypermethrin, indicating possible existence of resistance to these compounds.

3. Materials and methods

3.1. Materials

3.1.1. TUTA ABSOLUTA STRAINS

Tuta absoluta strains used for the first set of bioassays, carried out at the University of Catania (Italy), were field collected from two regions in Italy (namely Campania and Sicily) and called using the name of localities they come from: Vittoria, Fiumefreddo, Caivano, Salerno, Marsala and Pachino.

For the second set of bioassays, carried at Rothamsted Research, Harpenden (UK), the strains were provided by Dr Pablo Bielza (Departamento de Produccion Vegetal, Universidad Politecnica de Cartagena, Spain) and called TA1 (Spain), TA2 (Spain), TA3 (Italy) and TA4 (Portugal). They were all field collected during the year 2010. The strain GA was provided by Bayer CropScience laboratories. It is a mix population field collected in 2008 from different areas of Brazil (São Paulo state and Minas Gerais state) and reared since then in the absence of any contact with insecticides.

The live strains were reared on tomato plants inside insect proof cages and under controlled conditions of temperature (26 ± 2 ˚C) and light (16h L/8h D).

A large collection of samples of different populations of *T. absoluta* from different origins, stored in ethanol 70%, was also provided by Dr Bielza for molecular studies. The list of the samples in this collection is given in Table 3.

Table 3: Samples of *Tuta absoluta* collected from different areas around the world and stored in ethanol 70% for molecular analysis.

Sample ID Nº	**Origin**	**Sampled from**
1	Tejina, Tenerife,**Is. Canarias**	Tomato
2	Guía de Isora, Tenerife,**Is. Canarias**	Tomato
3	Granadilla, Tenerife,**Is. Canarias**	Tomato
4	Arico,Tenerife,**Is. Canarias**	Tomato
5	Teulera, Mallorca, **Is Baleares**	Tomato
6	San Fangos, Mallorca, **Is.Baleares**	Tomato
7	Mostaganem, **Algeria**	Tomato under-protection
8	Mostaganem, **Algeria**	Tomato under-protection
9	Turín, **Italy**	Unknown
10	La Tola, Pichincha, **Ecuador**	Tomato
11	Tudela, Navarra, **Spain**	Tomato
12	Barrancas,Santa Fé, **Argentina**	Tomato under-protection
13	LaPrimavera, Mendoza, **Argentina**	Tomato
14	La Plata, Bs.As,**Argentina**	Tomato under-protection
15	Cagliari.S.Margherita di Pula,**Italy**	Tomato under-protection
16	La Palma, **Is. Canarias**	Unknown
17	Sicilia, **Italy**	Aubergine
18	Ramonete/Lorca, MU, **Spain**	Tomato
19	Curicó costa, **Chile**	Tomato
20	Antioquía/Rionegro, **Colombia**	Tomato
21	Heraklion/Creta, **Grecia**	Wild plants
22	Mazarrón, Murcia, **Spain**	Tomato
23	Canelones, **Uruguay**	Tomato
24	Chulacanas, Piura**, Perú**	Tomato
25	Valencia, **Spain**	Unknown
26	Maresme, Cataluña, **Spain**	Tomato
27	Silveira. **Portugal**	Tomato
28	Sele valley.Salerno.Campania, **Italy**	Tomato
29a	Beit hashita, israel valley (1), **Israel**	Unknown
29b	Ein hmifraz, Western Galilee (2), **Israel**	Unknown

3.1.2. INSECTICIDES

Pyrethroids are synthetic chemical analogues of pyrethrins which are natural compounds contained in the flowers of chrysanthemums (*Chrysanthemum cinerariaefolium*). They have been widely used to control insect pests in agriculture and public health.

By binding to the voltage-gated sodium channel, pyrethroids prevent the gate from closing normally which results in continuous nerve stimulation and tremors in poisoned insects. There are two groups of pyrethroids with distinctive poisoning symptoms, denoted as Type I and Type II (Li-Ming *et al.,* 2008 and the references cited).

Two insecticides from this class were used for the bioassays: Lambda-cyhalothrin is a mixture of highly active isomers of cyhalothrin (trade name: Lambda Cyhalothrin CS 10% from Syngenta). It penetrates the insect cuticle, disrupting nerve conduction within minutes and leading to loss of muscular control, paralysis, feeding cessation, and eventual death.

Tau fluvalinate (trade name: Tau Fluvinate TEC 91.7 % from Makhteshim) is a broad-spectrum insecticide and acaricide with contact and stomach action causing convulsion, intense hyperactivity and death.

Organophosphates (OPs) are a group of synthetic chemical compounds, containing different mixtures of phosphorus, carbon, and hydrogen. Organophosphate pesticides disturb the nerve function in insects by binding to the active site of the enzyme acetylcholinesterase in the synaptic cleft leading to its irreversible inactivation. They are widely used in pests' management programs in agriculture and public health.

From the OP class, Chlorpyrifos (trade name: Cyren) was used. It is a broad-spectrum, chlorinated organophosphate insecticide, acaricide and nematicide. It is a non-systemic insecticide effective by direct contact, ingestion, and inhalation and causing the inhibition of the breakdown of acetylcholine.

Neonicotinoids are synthetic derivatives of nicotine.They are a relatively new class of insecticides with a common mode of action that affects the central nervous system of insects, causing paralysis and death. This class contains seven compounds: acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam.

Their low toxicity to mammals is linked to their high affinity for insect nicotinic acetylcholine receptors (nAChRs). They are widely used in Agriculture and are effective against sucking and chewing insects (Yu, 2008).

Imidacloprid (trade name: Confidor 200, Bayer CropScience) is the most widely used insecticide of the group. Imidacloprid is effective by contact or ingestion. It is a systemic insecticide that translocates rapidly through plant tissues following application. It has long residual activity.

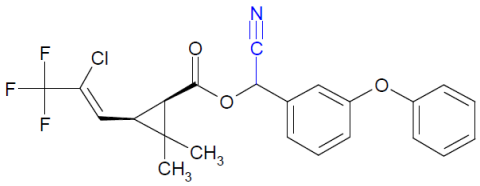
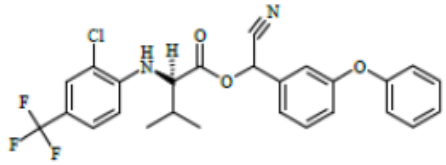
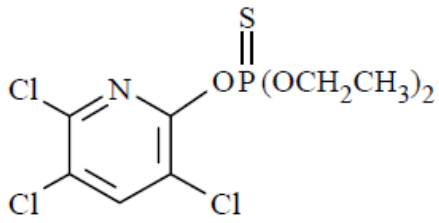
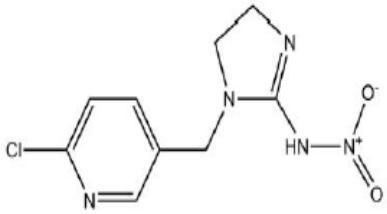
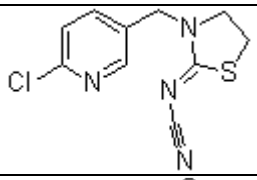
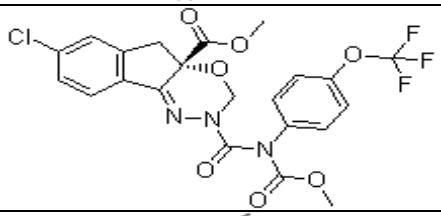
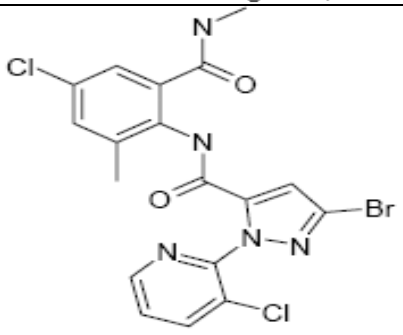
Thiacloprid (trade name: Biscaya 240 g a.i/l, Bayer CropScience) is another neonicotinoid with acute contact and stomach poisoning action, it also has systemic properties and broad-spectrum activity.

Oxadiazines are part of the latest generations of insecticides. They act as voltage-dependent sodium channels blockers. They include indoxacarb (trade name: Steward EC, Dupont) which is selectively active against lepidopteran pests (Wing *et al.,* 1998; FERA, 2009; Picanço, 2006; Sixsmith, 2009). It has ovicidal and larvicidal activity by entering the insect through the cuticle or digestive system. Following application, larvae cease feeding within 3-4 hours and either goes into mild convulsions or a passive paralysis and subsequently dies. The product is not systemic but shows translaminar movement into the mesophyll.

Anthranilic diamides represent a newly discovered class of insecticides with exceptional control potential through action on a novel target, the ryanodine receptor. Anthranilic diamides bind to insect ryanodine receptors in muscle cells causing the channel to open and a depletion of calcium ions from internal stores into the cytoplasm leading to feeding cessation, lethargy, muscle paralysis, and ultimately insect death (Lahm *et al.,* 2005*,* 2007; Cordova *et al .,*2006; Lahm, 2009)

From this new class of pesticides, Chlorantraniliprole (trade name: Rynaxypyr, Dupont) is characterized by high insecticidal potency, long-lasting activity and low toxicity to mammals. It is used as insecticide for the control of lepidoptera and selected other species (Dupont, 2008).

Table 4: The different insecticides used for the bioassays with their classes and chemical structures.

Class	Compound	Chemical structure
---	---	
Pyrethroids	Lambda Cyhalothrin	
Pyrethroids	Tau fluvalinate	
Organophosphates	Chlorpyrifos	
Neonicotinoid	Imidacloprid	
Neonicotinoid	Thiacloprid	
Oxadiazine	Indoxacarb	
Anthranilic diamides	Rynaxypyr	

3.1.3. PRIMERS

Degenerate primers were designed from the conserved regions of the target DNA regions (Sodium channel, Ache and P450) based on the alignment of known sequences of other species obtained from the NCBI database. Degenerate primers were used to obtain novel sequence of t these target genes from *T. absoluta,* then subsequently specific primers were designed based on the initial sequences obtained. All primers used were custom synthesised by Sigma – Aldrich life science. Upon reception, a stock solution of 100µM/µl was made by dissolving the primers in sterilised distilled water then 1/10th subsequent dilutions (10µM/µl) were made and used for PCR and sequencing. All primers solutions were stored at -20°C.

3.1.4. Molecular Kits and reagents

A range of molecular kits and reagents have been used for RNA/DNA extractions cleaning, cDNA synthesis, PCRs and RACE, gel slices clean up, transformation and cloning (see methods). These are detailed in the method protocols below. All were purchased from different suppliers including Ambion Applied Biosystems, Invitrogen (Life Technologies), Fermentas Life sciences, Promega, Bioline, Agilent Technologies (Stratagene Products).

3.2. Methods

3.2.1 BIOASSAYS

For each insecticide tested, a set of leaf dip bioassays were carried out to evaluate the susceptibility of the strains of *Tuta absoluta* in laboratory culture. Tomato leaves were immersed in six different concentrations of insecticide and allowed to air dry for at least one hour. One to two leaves were placed on slightly moistened filter paper inside Petri dishes (90mm diameter × 20 mm height).

Fifteen to twenty L2 instars were placed on the leaves in each Petri dish then placed in controlled conditions of temperature (26 ± 2 ˚C) and light (16hL/8hD). Three replicates

were used for each concentration of insecticide. Each bioassay was scored after 48 hours. Larvae were counted as dead if they showed signs of uncoordinated movements could not flip back to the crawling position or could not crawl for a distance at least equal to the double of their length.

3.2.2 RNA EXTRACTION

RNA was extracted from a pool of individuals of *Tuta absoluta* (10 adults or larvae) by grinding for 30 seconds in liquid nitrogen in a 1.5ml eppendorf tube using a micro-pestle. 0.5 ml of Trizol was added followed by 5 minutes incubation at room temperature. 0.1 ml of chloroform was then added to the mixtureshacken by hand for 15 seconds and then left for 3 minutes at room temperature. After 15 min centrifugation at 12000xg, the clear aqueous phase was transferred to a fresh tube for RNA precipitation using 0.25 ml of isopropanol. Samples were then incubated at room temperature and centrifuged for 15 min at 12000xg. The supernatant was removed and the pellet washed with 0.5ml ice-cold ethanol (70%) and centrifuged at 12000xg for 5 min. The ethanol was removed and the pellet left to air dry before being re-suspended in 20-25 µl of nuclease free water and stored at -80˚C.

3.2.3 DNA EXTRACTION

Single adult/larvae were grinded in 200 µl of DNAzol in a 1.5 ml eppendorf tube and left to stand for 1 min at room temperature. The mixture was centrifuged for 15-20 min at 12000xg and 4˚C. The supernatant was transferred to a fresh tube and 100 µl of ethanol 100% added. The tube was inverted several times and left for 5 min at room temperature. After the incubation, the mixture was centrifuged for 15 min, then alcohol was removed and 200 µl of ethanol 70% was added prior to centrifugation for 5 min at 12000xg.The ethanol was then removed and the pellet left to air dry for approximately 10 min before being re-suspended in an appropriate volume of sterilized distilled water. DNA was stored at -20˚C

3.2.4. c-DNA synthesis

Before cDNA synthesis, contaminating DNA was removedfrom RNA using the Ambion DNA free kit according to the manufacturer's recommendations. First strand cDNA was synthesized using superscript III reverse transcriptase (Invitrogen). 10 pg-5 µg of RNA template was mixed with 1 µl of oligo (dT) 20, 1 µl of 10mM dNTP mix and 13 µl of sterile distilled water. The mixture was heated to 65˚C for 5 minutes and incubated on ice for at least 1 minute.

To this mixture 4 µl 5X First Strand buffer, 1 µl of 0.1 MDTT, 1 µl RNase OUT and 1 µl of SuperScript III RT was added and mixed by gentle pipetting. The reaction was incubated at 50˚C for 30-60 min and inactivated by heating at 70˚C for 15 minutes. The cDNA was stored at -20˚C.

3.2.5. PCRs

First and nested PCRs were carried out in sterile 0.2 ml plastic tubes. For each 20 µl reaction mixture, 1 µl of template DNA was added to 1 µl of each primer (10 µM), 10 µl of GreenTaq (Fermentas) and 7 µl of sterile distilled water. PCRs were carried out using a thermal cycler with a heated lid. The mixture was held for 5 min at 95ºC for denaturation and run for 35 cycles of denaturation at 94 °C for 30 sec, annealing at 48- 58 °C for 60 sec and elongation at 72 °C for 90-120 sec.

For most PCR reactions a secondary (nested) round of PCR was carried out. Aliquots of 1 µl of each primary PCR were taken directly into secondary reaction using the same conditions except that one or both of the original primers was replaced with the a different inner primer.

3.2.6. RACE (Rapid Amplification of c-DNA Ends)

In an attempt to obtain the full length sequence of the AChE gene rapid amplification of cDNA ends (RACE) was performed.

The SMARTer RACE (Clontech) kit was used. The technique consisted on designing gene specific primers from the already known region that are oriented in the direction of the missing ends and PCR amplifying the region between the specific primers and the 3' and 5' ends. A reverse primer that can anneal to the existing poly (A) tail is used for 3' extensions while a modified lock-docking oligo(dT) is used for the 5' ends.

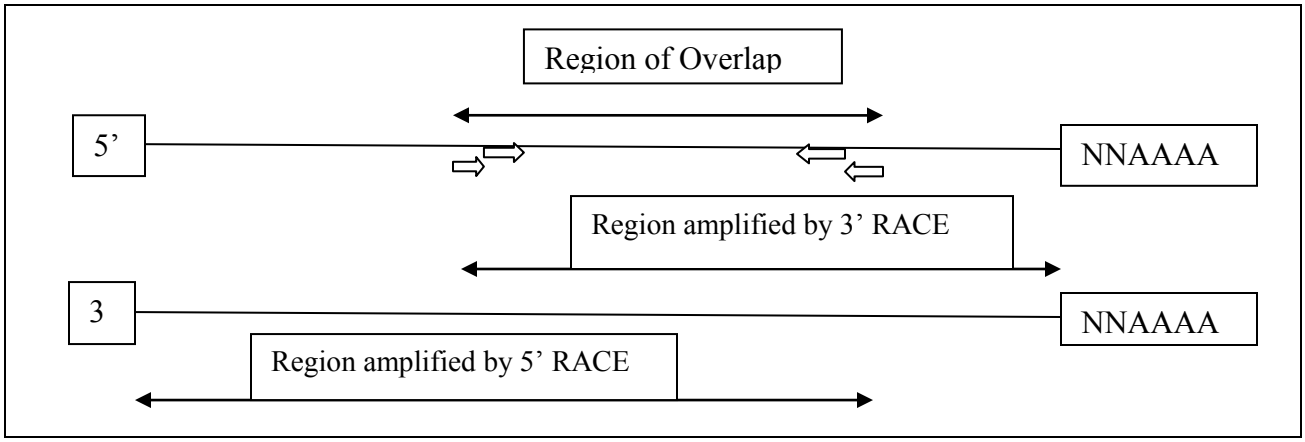


Figure 13: Simplified mechanism of Rapid Amplification of cDNA Ends (RACE) technique.

3.2.7. GEL ELECTROPHORESIS

After carrying out PCR reactions, products were loaded onto agarose gels for electrophoresis. Gels were made by dissolving 1.2% of agarose in appropriate volume of 1X TBE (Tris/Borate/EDTA) in a microwave. The dissolved agarose was left to cool for few minute before adding 1-3 µl of ethidium bromide (10mg/ml) per 100ml of gel. A marker (GeneRuler 100 bp or 1 kb ladder from Fermentas) was also loaded to estimate the size of fragments from PCR. The electrophoresis was carried out in 1X TBE for 45-60 min. Gels were visualized under UV light.

3.2.8. PCR PRODUCTS PURIFICATION

PCR products of the correct size were excised from agarose gels and purified using the membrane-based Wizard® SV Gel and PCR Clean-Up System from Promega. The excised slices of gel containing the DNA were dissolved in guanidine isothiocyanate (Membrane Binding Solution) before being spun through a silica membrane where the DNA binds. Following an alcohol based wash the DNA was eluted in 30ul of sterile distilled water.

3.2.9. PCR PRODUCTS LIDATION AND TRANSAFORMATION

Ligation reaction mixtures were prepared by combining (in order) 3 µl StrataClone Cloning Buffer, 2 µl of PCR product (5–50 ng) and 1 µl of StrataClone Vector Mix amp/kan. The sample was mixed gently by repeated pipetting and incubated at room temperature for 5 minutes. When the incubation was complete, the reaction was placed on ice.

To thawed tubes of StrataClone SoloPack competent cells, 1 µl of the cloning reaction mixture was added and the transformation mixture incubated on ice for 20 minutes, heat-shocked at 42°C for 45 seconds and incubated again on ice for 2 minutes.

250 µl of pre-warmed LB medium was added to the transformation reaction mixture before incubation for at least 1 hour at 37°C with agitation (200rpm).

After recovery, 100-150 µl of the transformation mixture and 40 µl of 2% X-gal was spread on LB plates containing 100µg/ml ampicillin and incubated overnight at 37°C.

3.2.10. SINGLE COLONY PCR AND PLASMID DNA RECOVERY

After overnight incubation, ampicillin-plates showed separate colonies with white and blue colorations. White colonies were checked that they contained an insert of the

correct size by PCR. A single colony was streaked onto a fresh numbered ampicillin-plate and put for incubation at 37C, while the remainder was vigorously shacked in 20ul of distilled sterilised water and used as template for PCR.

PCR was set up for each colony using 10 µl of Greentaq Mix, 7 µl of distilled sterilised water, 1 µl of template and 1 µl of each M13 primer (forward and reverse).

Gel electrophoresis of PCR products revealed thosecolonies with an insert of the correct size. From these an LB mini prep culture was set up for overnight incubation at 37C.

The mini prep cultures were purified using a GeneJET™ Plasmid Miniprep Kit from FRMENTAS. The kit uses a silica based membrane in spin columns. Harvested bacterial cells were re-suspended and plasmid DNA liberated using alkaline lysis with SDS (detergent sodium dodecyl sulphate). After neutralisation and centrifugation, the plasmid DNA was loaded onto the spin column for binding on the silica membrane. The adsorbed DNA was washed to remove contaminants, and is then eluted with 50ul of the Elution Buffer (10 mM Tris-HCl, pH 8.5).

3.2.11. SEQUENCING

Sample sequencing was done either at Rothamsted using an ABI 310 sequencer or sent to Eurofins MWG/Operon for sequencing

3.2.12. DATA ANALYSIS

Statistical analysis bioassay data was carried out to calculate mortality and the lethal concentration required to kill 50% of the strain (LC50).The GenStat 13th edition software was used to apply Abott's formula and carry out Probit transformation and regression.

The regions of similarities between sequences were searched using the BLAST algorithm at the NCBI web site (put url here). Sequences were analysed and aligned using Vector NTI (Invitrogen).

3.2.13. TAQMAN

High-throughput methods were developed in order to genotype Single nucleotide polymorphisms (SNPs) that confer resistance in *T. absoluta*. The TaqMan assay is a PCR method employing oligonucleotide probes that are dual-labelled with a fluorescent reporter dye and a quencher molecule. During PCR, the TaqDNA polymerase cleaves probe and liberates the reporter fluor from the quencher increasing net fluorescence. The TaqMan assay uses two probes: one specific for the wildtype allele labelled with VIC and the second, specific for the mutant allele labelled with FAM. The presence of two probes with different fluorophores allows the detection of both alleles in a single PCR reaction. PCR conditions for TaqMan assays were: 10 minutes at 95°C followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute.

The increase in fluorescence of either VIC or FAM will indicate a homozygous wild type or mutant while an increase in both signals indicates a heterozygote (Livak.1990; Rohan *et al.,* 2005; Twyman, 2005; Bass *et al.,* 2007; Hui *et al.,* 2008).

4- Phenotypic expression of insecticide resistance

4.1. Introduction

The biological responses of insect populations to chemical compounds are generally assessed in laboratory using dose-response bioassays. Dose-response bioassays are experiments where a population of insect is exposed to increasing doses of a single chemical and the responses ranging from no observable effect to extreme toxicity are recorded.

The responses are dose dependent since a higher effect is observed with higher doses. The responses are not linear but usually in form of a sigmoid curve deriving from the normal distribution of natural variability within the insect populations.

Mortality is one of the effects investigated in susceptibility to insecticide bioassays. There is often significant variability in measured mortality at different doses. Plotting concentrations of dose on a log scale and mortality responses on a probit scale mathematically transforms the data from a sigmoidal curve to appear linear and facilitates generating statistically the dose that kills 50% of the individuals, called LC50s, allowing to make comparisons between studied compounds or populations.

Many factors are of crucial importance for the validity of a bioassay such as simplicity, rapidity, repeatability, reproducibility and robustness. The conditions of bioassays should be as similar as possible to the natural conditions.

In this study and in order to investigate the susceptibility of *Tuta absoluta* to different insecticides, a leaf dip bioassay was adopted. The method is recommended by the Insecticide Resistance Action committee (IRAC) and consists in the immersion of tomato leaflets in serial dilutions of an insecticide before being air dried and allowing the larvae to feed on them.

Two sets of experiments were carried out not only to validate the assay method but also to investigate the susceptibility. In the first one, which served mainly as a validating test, six field strains collected from different locations in Italy were tested against two

known efficient insecticides: rynaxypyr and indoxacarb. The test was carried out at the University of Catania and was repeated twice for each strain and insecticide. In the second, realized in UK, five strains collected from different European countries and reared in Rothamsted Research were tested against five different insecticides belonging to both conventional (pyrethroids and organophosphates) and novel (neonicotinoids) classes of chemicals.

A major limitation faced during the two sets of experiments consisted on the unavailability of a known standard susceptible strain.

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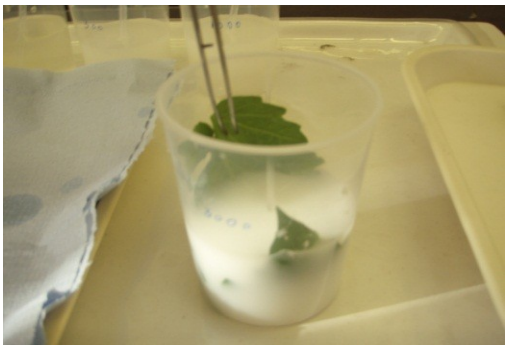
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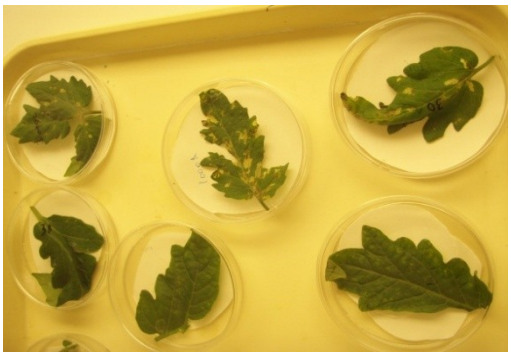
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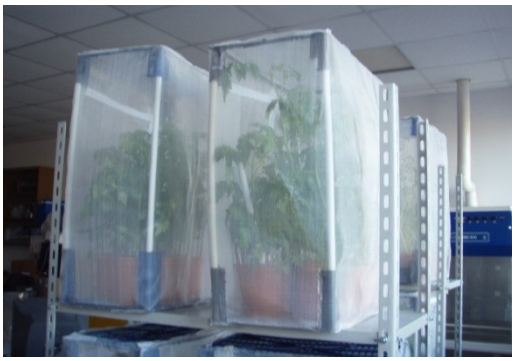
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Figures 14 to 21: Different steps of leaf dip bioassay (14 to 19) and rearing cages in Rothamsted Research (UK) - 20 and University of Catania (Italy) -21.

4.2. Results

The results of the first set of bioassays carried out in Italy (at the University of Catania) to test the susceptibility to indoxacarb and rynaxypyr are shown in tables 5 and 6 and in figures 22 and 23.

4.2.1. INDOXACARB

Comparing the data of the first and second repetitions for each strain, only Caivano strain shows some differences between the two repetitions. These differences are not statistically significant.

Significant differences are seen between the LC50 of the six strains tested ranging from 0.94 to 7.37 ppm (a.i). Fiumefreddo and Cavaino strains are showing higher LC50s than the other strains, with wider intervals of confidence with respectively 7.35 (5.37-9.64) and 7.37 (5.30-10.07) while Marsala strain scored the lowest LC50 with 0.94 (0.21-2.00). The differences were of 7 fold between the highest and lowest LC50.

Table 5: Relative toxicity of Indoxacarb to six Italian strains of *Tuta absoluta*.

Strains	Repetition	N	% mort. Untreated	Slope ± SE		LC_{50} (ppm) ($CI_{95\%}$)			χ^2	h
Vittoria	1	223	12.5	1.525	0.419	**2.18**	0.24	7.2	11.84	0.582
	2	223	9.4	1.533	0.388	**2.24**	0.42	6.7	11.03	0.493
	1+ 2	446	10.9	1.527	0.257	**2.21**	1.20	3.65	23.23	0.140
F.freddo	1	221	6.3	1.522	0.224	**8.12**	5.28	12.03	3.55	0.083
	2	192	6.3	1.578	0.254	**6.65**	3.77	9.89	2.92	0.099
	1+ 2	413	6.3	1.569	0.165	**7.35**	5.37	9.64	7.24	0.043
Caivano	1	222	12.9	1.552	0.372	**12.22**	7.46	21.51	3.21	0.221
	2	188	6.5	1.306	0.208	**4.42**	2.58	6.85	2.16	0.097
	1+ 2	410	9.7	1.420	0.174	**7.37**	5.30	10.07	9.97	0.057
Pachino	1	192	0.0	1.545	0.426	**1.23**	0.16	2.51	0.75	0.292
	2	192	0.0	1.081	0.292	**0.83**	0.04	2.26	2.71	0.281
	1+ 2	384	0.0	1.249	0.236	**0.98**	0.25	1.92	5.84	0.137
Salerno	1	192	0.0	1.186	0.267	**1.6**	0.3	3.4	1.08	0.195
	2	192	3.0	1.433	0.284	**3.1**	1.2	5.3	0.94	0.151
	1+ 2	384	4.7	1.305	0.194	**2.29**	1.09	3.65	2.66	0.085
Marsala	1	192	0.0	0.928	0.268	**0.62**	0.01	2.06	4.55	0.320
	2	192	3.1	1.164	0.284	**1.31**	0.17	2.97	2.74	0.228
	1+ 2	384	1.6	1.045	0.194	**0.94**	0.21	2.00	7.75	0.133

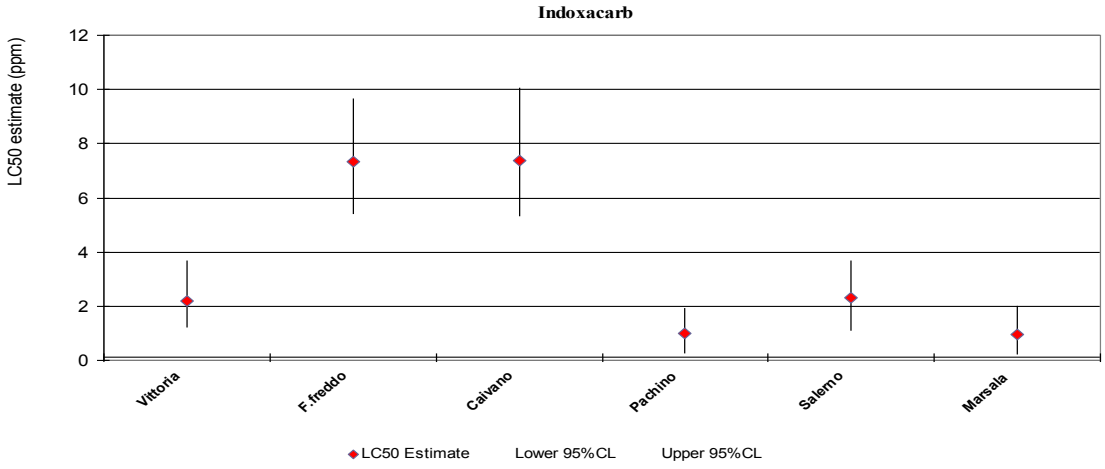


Figure 22: Indoxacarb Log concentrations (ppm) of LC_{50} estimates for six different Italian *T. absoluta* strains.

4.2.2. RYNAXYPYR

For rynaxapyr, no significant differences were found between the repetitions for all the strains and only Fiumefreddo strain showed differences of almost 8 folds with the most susceptible Salerno strain.

The LC50 ranged from 0.23 ppm (a.i) (0.08-0.48) and 1.80 (1.16-2.45) respectively scored by Salerno and Fiumefreddo.

Table 6: Relative toxicity of Rynaxypyr to six Italian strains of *Tuta absoluta*.

Strains	Repetition	N	% mort. untreated	Slope ± SE		LC_{50} (ppm) ($CI_{95\%}$)			χ^2	h
Vittoria	1	192	3.1	0.94	0.177	**0.74**	0.30	1.32	1.72	0.136
	2	224	3.1	1.07	0.148	**0.79**	0.46	1.25	1.06	0.073
	1+ 2	416	3.1	1.02	0.112	**0.78**	0.51	1.12	3.18	0.046
F.freddo	1	192	6.3	1.839	0.299	**0.280**	0.087	0.564	2.955	0.099
	2	192	15.6	1.581	0.265	**1.390**	0.102	4.051	4.73	0.115
	1+ 2	384	10.9	1.72	0.218	**1.80**	1.16	2.54	13.54	0.062
Caivano	1	223	12.5	1.05	0.167	**1.34**	0.72	2.25	2.83	0.097
	2	191	3.1	1.10	0.188	**0.51**	0.19	0.99	1.99	0.113
	1+ 2	414	7.8	1.08	0.116	**0.88**	0.55	1.29	2.83	0.097
Pachino	1	192	0	1.219	0.212	**0.657**	0.250	1.233	1.075	0.125
	2	192	21.8	1.310	0.310	**1.135**	0.200	2.568	1.984	0.257
	1+ 2	384	10.9	1.01	0.135	**0.45**	0.20	0.79	5.35	0.069
Salerno	1	192	6.3	0.885	0.192	**0.209**	0.028	0.557	2.206	0.203
	2	192	9.4	0.896	0.210	**0.239**	0.029	0.631	0.331	0.157
	1+ 2	384	7.8	0.92	0.135	**0.23**	0.08	0.46	2.99	0.083
Marsala	1	192	6.3	1.219	0.212	**0.657**	0.250	1.233	1.075	0.098
	2	192	9.4	1.538	0.253	**1.019**	0.492	1.715	1.146	0.164
	1+ 2	384	6.3	1.37	0.157	**0.83**	0.51	1.22	3.63	0.050

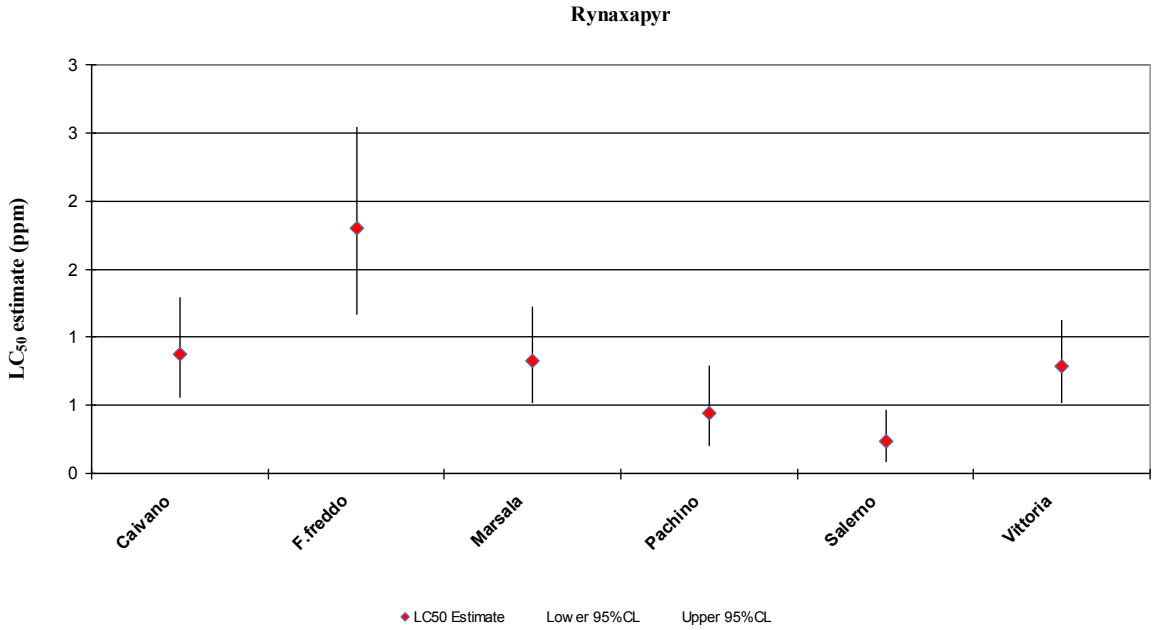


Figure 23: Rynaxapyr Log concentrations (ppm) of LC_{50} estimates for six different Italian *T. absoluta* strains.

The results from the leaf-dip bioassays carried out in UK (at Rothamsted Research, Harpenden) to evaluate the susceptibility of five field strains of *T. absoluta* (variously collected in Europe) to five compounds belonging to different class of pesticides are summarized in the table 7 and in figures 24 to 33.

The data show significant variation in susceptibility to the different compounds among the five strains based on the criterion of failure of 95% LC at LC50s to overlap. A general standard susceptible strain could not be identified. The comparisons and resistance ratios were based on the most susceptible strain and recommended field rates.

Table 7: Relative toxicity of λ-cyhalothrin, Tau-fluvalinate, Chlorpyriphos, Imidacloprid, and Thiacloprid to five strains of *Tuta absoluta*.

	Strain	LC50 (mg/l)	Lower 95%LC	Upper 95%LC	Slope	SE
λcyhalothrin	**TA1**	85	36.7	159	0.904	0.132
	TA2	350.5	176.3	507	2.256	0.577
	TA3	631	456	837	2.452	0.458
	TA4	700	472	957	1.859	0.34
	GA	1514	1137	2106	2.106	0.371
Tau fluvalinate	**TA1**	2047	1235	4483	1.8	0.5
	TA2	1952	1365	2841	2.4	0.6
	TA3	821	544	1177	1.765	0.358
	TA4	3716	1443	29334	0.7	0.2
	GA	9259	1403	61099	2	0.3
Chlorpyriphos	**TA1**	2141	1025	7467	0.706	0.15
	TA2	846	545	1242	1.531	0.296
	TA3	451	301.7	605	2.2	0.4
	TA4	635	358	1076	1.091	0.211
	GA	716	297	1448	0.9	0.2
Imidacloprid	**TA1**	1545	771	3592	1.444	0.432
	TA2	1582	905	3497	0.928	0.187
	TA3	221	44	1026	0.384	0.102
	TA4	6692	2437	113619	0.668	0.191
	GA	4595	1384	9.93 E+04	0.526	0.158
Thiacloprid	**TA1**	770	565	1018	2.302	0.381
	TA2	814	616.7	1053	3.129	0.584
	TA3	1100	844	1402	3.145	0.546
	TA4	970	748	1237	2.817	0.405
	GA	952	693.1	1224	3.339	0.689

4.2.3. LAMBDA CYHALOTHRIN

The five strains showed differences of susceptibility to lambda cyhalothrin with GA and TA1 having respectively the highest and lowest LC50s of 1514 and 85 ppm (a.i) respectively. The variability between GA and TA1 was moderately high (17 folds). There were no overlaps between the confidence intervals (lower and upper LC95) of the five strains, except between TA3 and TA4 showing similar LC50s.

4.2.4. TAU FLUVALINATE

TA3 has the lowest LC50 of 821 ppm (a.i). The other four strains have overlapping confidence intervals, with the highest LC50 of 9259 ppm (a.i) scored by GA. The resistance ratio between the most and less susceptible strains was 11 folds.

Both lambda cyhalothrin and tau fluvalinate belong to pyrethroids class and are broad-spectrum insecticide and acaricide, with high contact activity at low doses. The recommended field rates are around 25 and 50 ppm respectively for Lambda cyhalothrin and tau fluvaliante. The LC50 of all strains are far beyond the field rates, suggesting the existence of resistance.

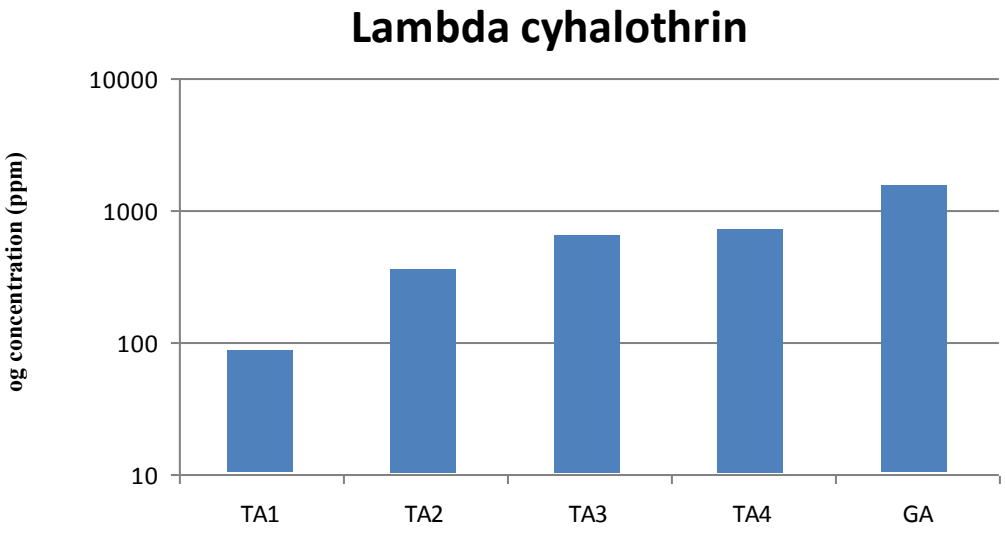


Figure 24: Lambda cyhalothrin Log concentrations (ppm) of LC_{50} estimates for five different *T. absoluta* strains.

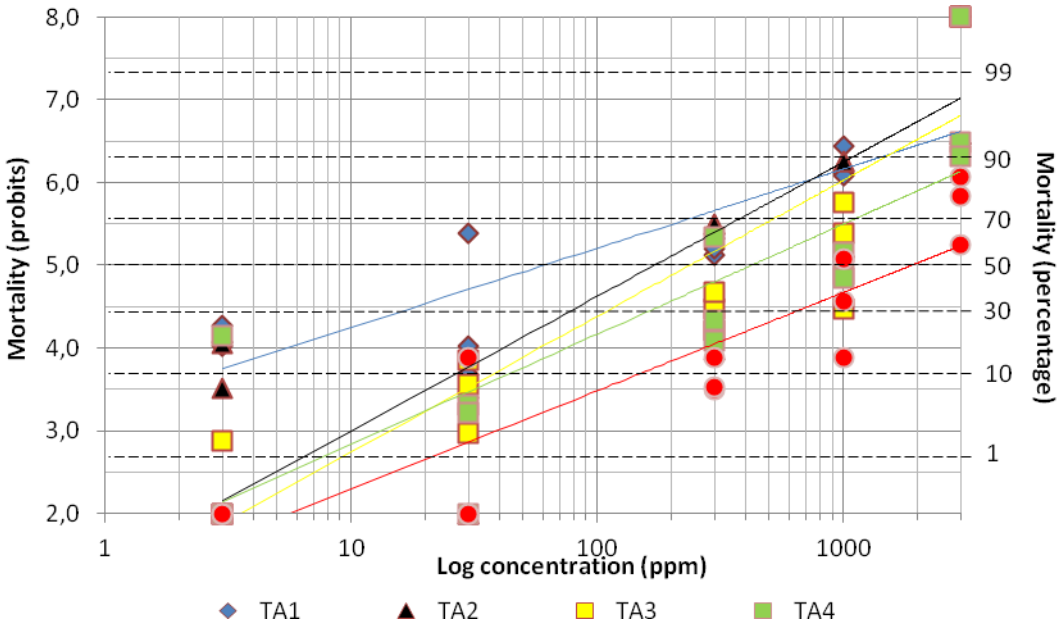


Figure 25: Dose–response curve of Lambda cyhalothrin expressed by mortality probit and log concentration (ppm) for five different *T. absoluta* strains.

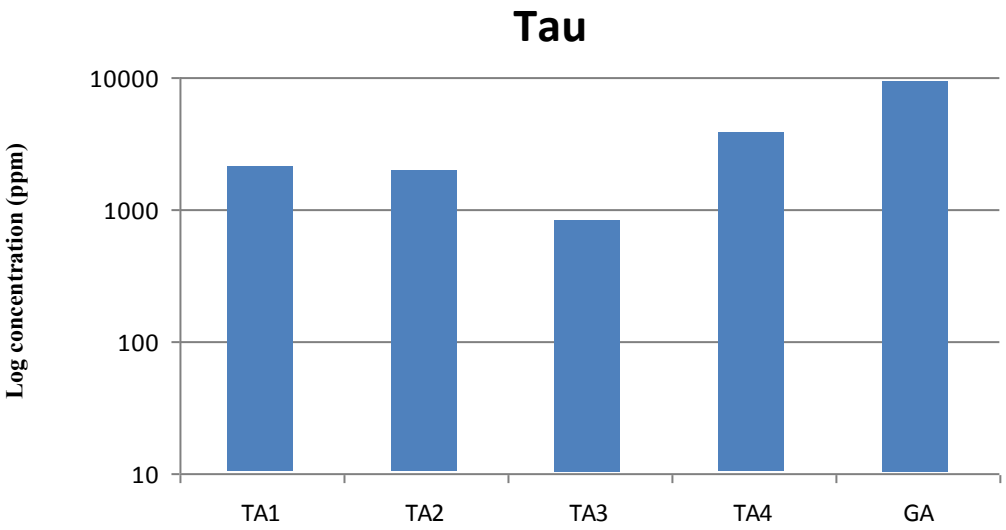


Figure 26: Tau fluvalinate Log concentrations (ppm) of LC_{50} estimates for five different *T. absoluta* strains.

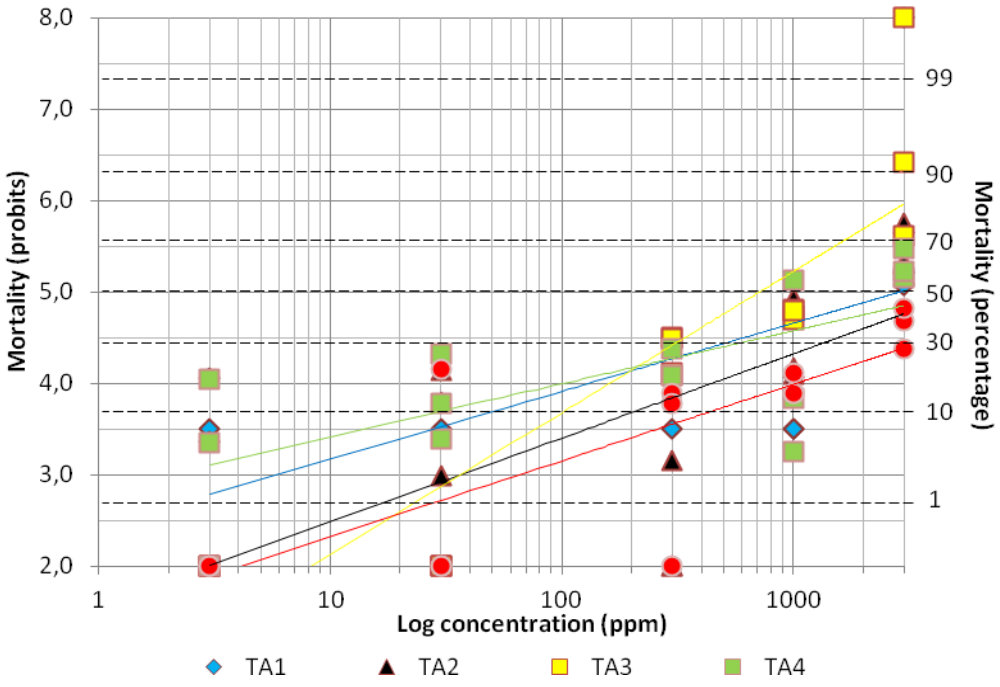


Figure 27: Dose–response curve of Tau fluvalinate expressed by mortality probit and log concentration (ppm) for five different *T. absoluta* strains.

4.2.5. CHLORPYRIPHOS

For chlorpyriphos, the variability observed in the LC50 values among the tested strains was low (under 5 folds). The highest LC50 was 2141 ppm (a.i) and was scored by TA1 while TA3 has the lowest LC50 of 451 ppm (a.i). The confidence intervals of all strains overlapped. The TA2, TA4 and GA strains scored 846, 635, and 716 ppm (a.i) respectively.

The field rates for chlorpyriphos are 560 ppm (a.i.) so all the strains have their LC50 higher except the TA3.

4.2.6. IMIDACLOPRID

The five strains showed significant differences of susceptibility to imidacloprid. The differences ranged from 7 to 30 folds. TA3 has the lowest LC50 with 221 ppm (a.i.), while

TA1 and TA2 have a higher and similar LC50, respectively of 1545 and 1582 ppm (a.i.). The highest LC50 was scored by TA4, reaching a value of 6692 ppm (a.i.).

The intervals of confidence were overlapping and very wide, suggesting a high heterogeneity of response from all the strains. The field rates for imidacloprid are 320 ppm (a.i.), thus only TA3 has a lower LC50.

4.2.7. THIACLOPRID

All the strains showed similar levels of LC50 from the lowest of 770 ppm (a.i.) for TA1 until the highest of 1100 ppm (a.i.) for TA3. The LC50s of all the strains were very high in comparison with the recommended field rate for the thiacloprid (96 ppm a.i.).

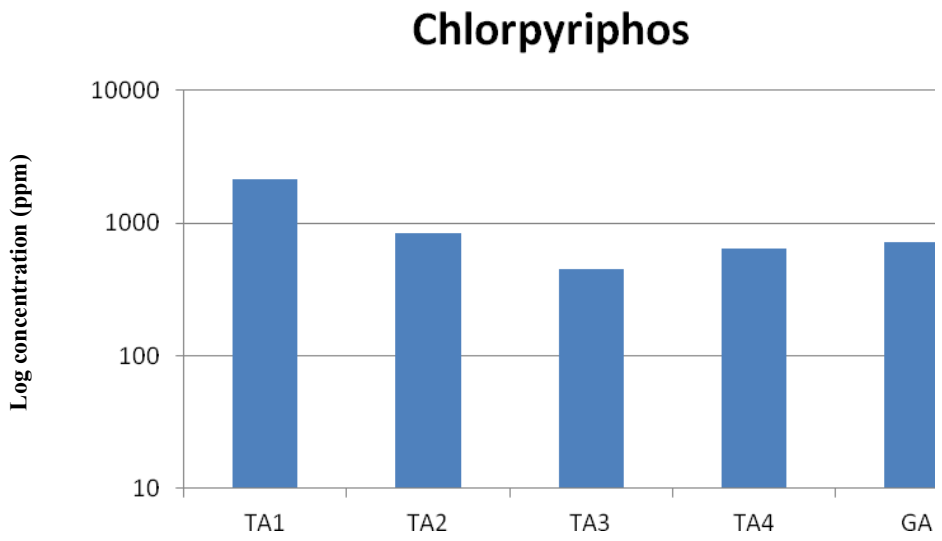


Figure 28: Chlorpyriphos Log concentrations (ppm) of LC_{50} estimates for five different *T. absoluta* strains.

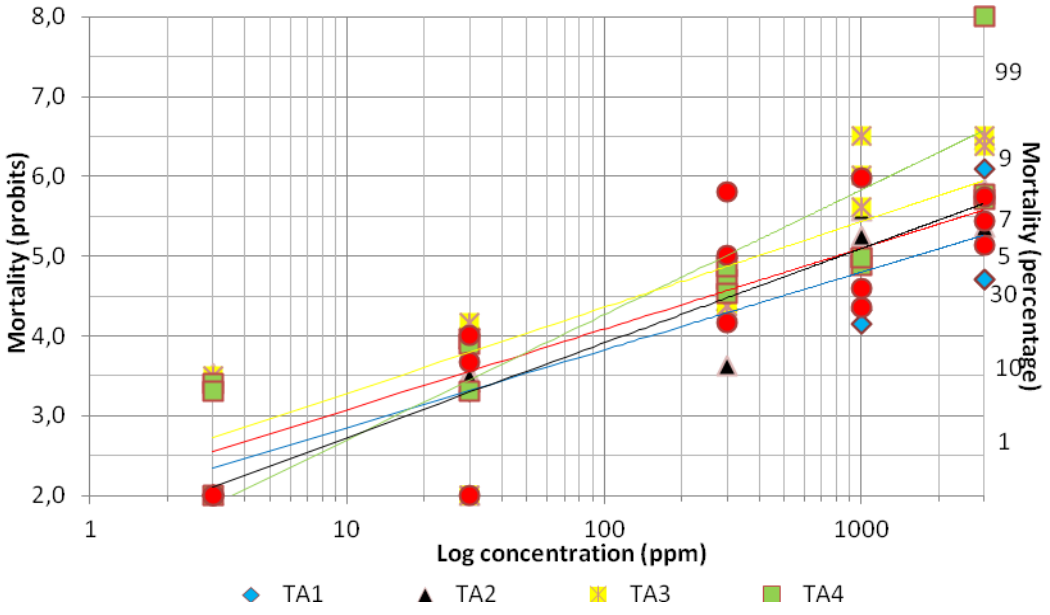


Figure 29: Dose–response curve of Chlorpyryphos expressed by mortality probit and log concentration (ppm) for five different *T. absoluta* strains.

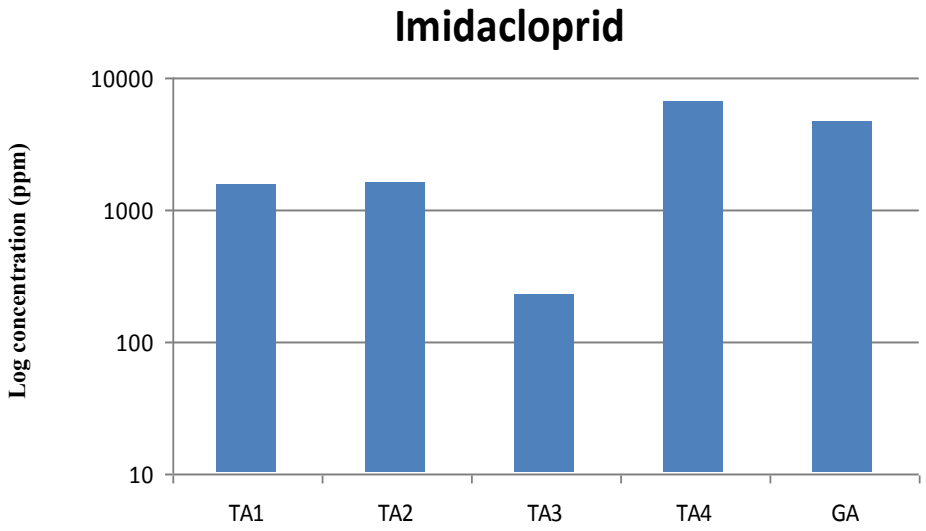


Figure 30: Imidacloprid Log concentrations (ppm) of LC_{50} estimates for five different *T. absoluta* strains.

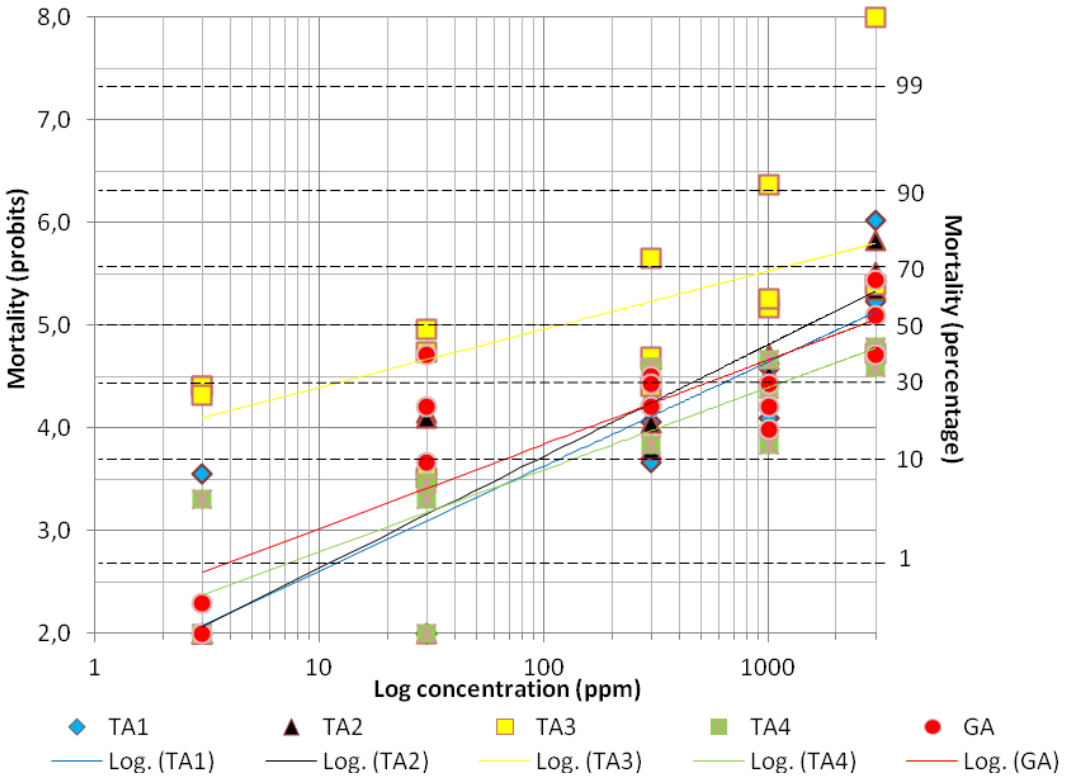


Figure 31: Dose–response curve of Imidacloprid expressed by mortality probit and log concentration (ppm) for five different *T. absoluta* strains.

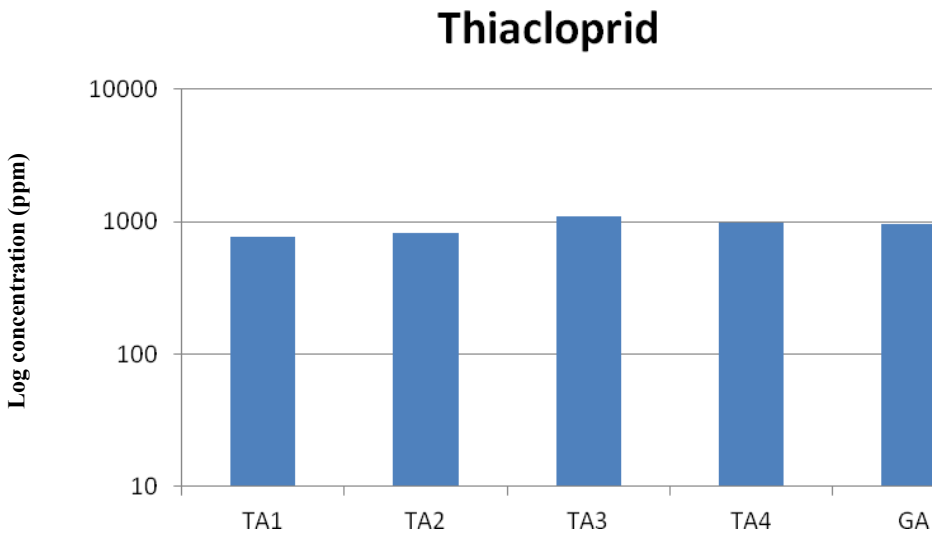


Figure 32: Thiacloprid Log concentrations (ppm) of LC_{50} estimates for five different *T. absoluta* strains.

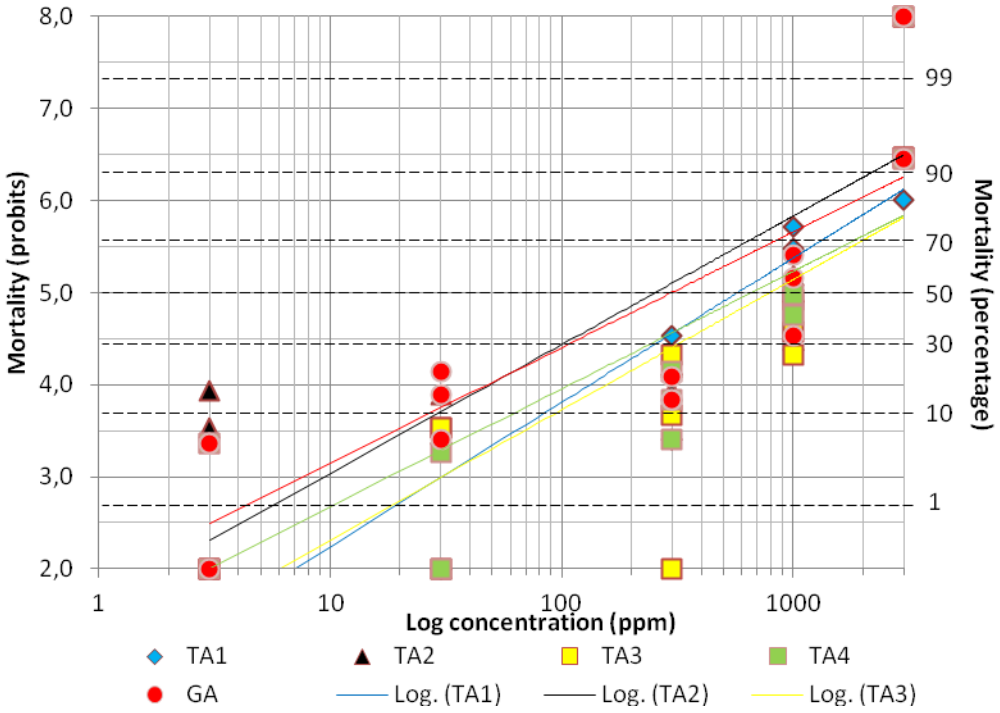


Figure 33: Dose–response curve of Thiacloprid expressed by mortality probit and log concentration (ppm) for five different *T. absoluta* strains.

4.3. Discussion

In order to investigate the susceptibility to insecticides of *Tuta absoluta* and other lepidopteran insects, different methods were used in the past by different authors. Salazar and Araya (1997) used a direct spray based method to compare the susceptibility of collected larvae of *T. absoluta* from Chile to several commonly used insecticide doses applied on two development stages of larvae groups. Siqueira *et al*. (2000) used an insecticide impregnated filter paper bioassay to study the resistance and synergism to cartap in populations of *T. absoluta* while Alvaro *et al.* (2001) used the same method to study in Brazil the susceptibility of *T. absoluta* to four insecticides.

More recently, IRAC adopted and recommended the leaf dip method for insecticides resistance studies for *T. absoluta*. Gerson *et al.* (2011), used the same method to survey resistance levels to the main insecticides currently used and recommended in populations of *T. absoluta* from the main tomato-producing regions in Brazil. Castelo Branco *et al.* (2001)

tried to evaluate the efficacy of the recommended field rates of some insecticides in laboratory bioassays for two Brazilian Tomato Pinworm populations and one Diamondback Moth population using also the leaf dip method. Roditakis *et al.* (2011) used the leaf dip method to study the susceptibility of two populations from Greece to seven insecticides.

In this study a leaf dip methodology was adopted. The first set of bioassays carried out in Italy aimed to test the validity of the method for investigating the susceptibility to insecticides of *Tuta absoluta*. Results showed good robustness and repeatability of the method. Similar conclusions were reported by Roditakis *et al.* (2011), confirming that the method is easy to perform, robust and repeatable.

In this first set of bioassays, the LC50s found for the six strains were generally similar for indoxacarb and rynaxypyr, both belonging to relatively novel class of insecticides. The levels of the LC50s found in this study were similar to those ones found by Roditakis, in Greece, and Bielza, in Spain (pers. comm.) conferring a strong reproducibility to the method. Even if Fiumefreddo and Caivano strains have higher LC50s than the other strains, none of them is considered as resistant since all the LC50s were far below the field rates.

The results from the second set of bioassays carried out in Rothamsted Research showed significant differences in the responses to five different compounds belonging to pyrethroids, organophosphates and neonicotinoids classes among five field strains of *Tuta absoluta*. The comparison between the most susceptible and the other strains showed that differences were ranging between 4 to 17 folds for lambda cyhalothrin, 2 to 11 folds for tau fluvalinate, 7 to 30 folds for imidacloprid and less than 5 for chlorpyriphos and thiacloprid. Similar LC50s were reported only for chlorpyriphos by Roditakis *et al.* (2011), while Salzar and Araya (2001) reported LC50 of 35.8 ppm (a.i.) for lambda cyhalothrin using a direct spray method.

A susceptible strain was not available and the results did not allow identifying a general standard susceptible strain from the ones tested.

The comparison with the recommended field rates for each insecticide suggested the existence of a possible resistance to Lambda cyhalothrin and tau fluvalinate (pyrethroids),

tolerance or even a possible resistance to chlorpyriphos (organophosphate) and more interestingly a possible resistance to imidacloprid and thiacloprid (neonicotinoids). Results did not suggest the existence of any cross resistance between the different insecticides and classes of insecticides.

Historically, *Tuta absoluta* has been controlled mainly with chemicals belonging to organophosphates and pyrethroids classes (Lietti *et al.,* 2005) but the intensive use of these insecticides led to the development of resistance. During the 1980's, resistance to deltamethrin, metamidophos, esfenvalerate, lambda cyhalothrin and mevinphos has been reported in Chile by Salazar and Araya (1997). In Brazil, since 1999, significant resistance of *T. absoluta* to acephate and deltamethrin was reported by Castelo Branco *et al.* (2001). Control failures of abamectin, cartap, methamidophos and permethrin used against *T. absoluta* have been also reported by Guedes *et al.* (1994). In 2001, resistance to abamectin was additionally reported by Siqueira *et al.* (2000a). In their recent study, Gerson *et al.* (2011) surveyed resistance levels in populations of *T. absoluta* from the main tomato-producing regions in Brazil to the main insecticides currently used and recommended. They reported evidence of control failures for bifenthrin, permethrin, diflubenzuron, teflubenzuron, triflumuron and *B.thuringiensis*, moderate levels of resistance to indoxacarb and no resistance or only low resistance levels for bifenthrin and permethrin, abamectin, spinosad, *B. thuringiensis* and the mixture of deltamethrin and triazophos.

The many previous control failures of *T. absoluta* from South America, the high reproductive and expansion potentials of the insect and the results about susceptibility to different classes of insecticides found in this study, yet to be confirmed, should be a warning signals for the urgent establishment of strict IPM programs for this pest.

5 – Target-site mechanisms of pyrethroid and indoxacarb resistance

5.1. Introduction

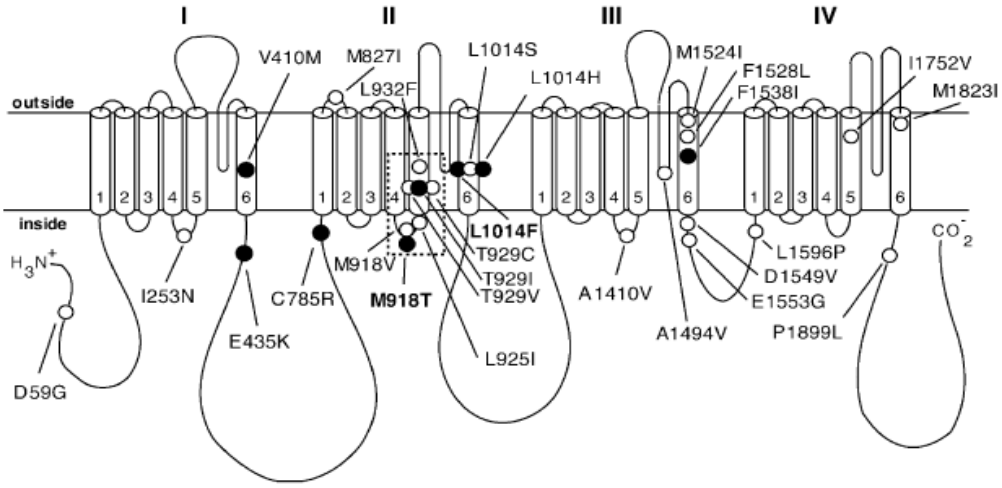
Chemical use has been the main control strategy against *T. absoluta* in South America (Souza and Reis, 1986; Picanço *et al.*, 1995), without significant efficacy and leading to a build-up of insecticide resistance. Main causes of this resistance to several insecticides are the short generation time and the frequent applications of insecticides to manage *T. absoluta*. In many studies, researchers have shown reduced insecticide activity in the field (Souza *et al.*, 1992; Guedes *et al.*, 1994), suggesting the development of resistant populations to compounds used against this insect from at least three different insecticides classes widely used: organophosphates, carbamates and pyrethroids (Gonçalves *et al.*, 1994).

Pyrethroids are a major class of neurotoxic insecticides that have been used extensively over the last decades to control a wide range of agricultural pests. Pyrethroids act on voltage-gated sodium channels at the insect nervous system level. They modify the normal operation of gate opening and closing, which results in continuous nerve stimulation in poisoned insects leading to paralysis and death. Sodium channel is also a primary target of newer classes of insecticides, like the oxidiazines (such as indoxacarb).

To date, the molecular mechanisms underlying resistance in *T. absoluta* have not been studied and characterized; however, research on other major crop pests has shown that insecticide resistance is most commonly caused by modification of the target-site protein or enhanced enzymatic detoxification.

One major mechanism of resistance linked to a reduced target-site and known as knockdown resistance (kdr) has been shown to arise through a single point mutation in the S6 segment of domain II of the sodium channel gene, resulting in a leucine to phenylalanine (L1014F) substitution (Miyazaki, 1996; Williamson *et al.*, 1996).

Different secondary mutations giving enhanced (super-kdr) resistance have also been identified singly or in combination with kdr within the domain II region of the channel, including M918T, T929I, L925I, L932F, D1549V, E435K and C785 (Figure34).



Anopheles gambiae (Meigen), *Aphis gossypii* (Glover), *Bemisia tabaci* (Gennadius), *Blattella germanica* (Linnaeus), *Boophilus microplus* (Canestrini), *Ctenocephalides felis* (Bouché), *Culex pipiens* (Linnaeus), *Drosophila melanogaster* (Meigen), *Aedes aegypti* (Linnaeus), *Helicoverpa armigera* (Hübner), *Heliothis virescens* (Fabricius), *Haematobia irritans* (Linnaeus), *Leptinotarsa decemlineata* (Say), *Musca domestica* (Linnaeus), *Myzus persicae* (Sulzer), *Pediculus capitis,* (De Geer), *Plutella xylostell* (Linnaeus), *Frankliniella occidentalis* (Pergande).

Figure 34: kdr mutations in sodium channels and insects reported to have one or more mutations (Ke Dong, 2007).

5.2. Specific methods

The leaf-dip bioassays carried out to evaluate the susceptibility of many strains of *T. absoluta* to different compounds belonging to different classes of pesticides, including pyrethroids and oxidiazines, showed significant differences in susceptibility to different compounds among the strains (see chapter 4), rising the need to give insight to determining mechanisms.

In the aim of cloning and sequencing the domain II region of sodium channel gene for *T. absoluta*, different PCR reactions were carried out, firstly on cDNA prepared from

pools of 15-20 individuals from each strain and using degenerate primers designed against conserved motifs within the IIS4 and IIS6 linker regions of the channel protein (as described by Martinez-Torres *et al.,*1997). A nested PCR approach was used based on primers DgN2 and DgN3 in a primary round of PCR and primers DgN1 and DgN3 in a secondary reaction (primer sequences are given in Table 8). Once the sodium channel sequence had been determined for the pools, specific primers were designed to perform direct PCR analysis from genomic DNA (see Table 8). These primers were also used to determine the positions and sizes of any introns within this region of the gene.

Total RNA was extracted from pools of 15-20 individuals using Trizol and following the manufacturer's instructions. Genomic DNA was removed by DNase I digestion using a DNA-free DNase treatment and removal reagent (Ambion). The quality and quantity of RNA pools were assessed by spectrophotometry (Nanodrop Technologies) and by running an aliquot on a 1.2% agarose gel. A quantity of 4 µg of RNA sample was then used for cDNA synthesis using Superscript III and random hexamers (Invitrogen) according to the manufacturer's instructions.

First and nested PCRs were carried out in sterile 0.2 ml plastic tubes. For each 20 µl reaction mixture, 1 µl of template DNA was added to 1 µl of each primer (10 µM), 10 µl of GreenTaq (Fermentas) and 7 µl of sterile distilled water. PCRs were carried out using a thermal cycler with heated lid and run for 35 cycles of 95 °C for 30 sec 48- 50 °C for 60 sec and 72 °C for 90-120 sec.

1.2% Agarose gel electrophoresis of PCR products was carried out in 1x TBE buffer and slices of DNA fragments of the correct size were excised. The Wizard SV gel and PCR clean up System from Promega was used to Recovery the DNA from gel slices according to manufacturer's recommendations.

The Strataclone PCR Cloning kit from Stratagene was used for cloning allowing a blue-white screening with plasmid pSC-A-amp/kan. After single colony PCR, the GeneJET Plasmid Miniprep Kit was used to purify the DNA. DNA samples were sent to Eurofins mwg/operon for sequencing.

Table 8: Oligonucleotide primer sequences used to PCR amplify DNA from the IIS4-6 domain of the *Tuta absoluta para*-type sodium channel gene. All primers are shown 5′ to 3′.

Name	Sequence
DgN1	GCNAARTCNTGGCCNACNYT
DgN2	GCNAARTCNTGGCCNAC
DgN3	YTTRTTNGTNTCRTTRTCRGC
DgN4	TTNGTNTCRTTRTCRGCNGTNGG
TutNaF1	TGGAYTGYTGYTGGCTNTGG
TutNaF2	GAYTTYTTYGYGTNATGGAYT
TutNaR1	AGACCATGACAATGACGAAG
TutNaR2	GACCATGACAATGACGAAGT
TAF1	GAAATCGTGGCCGAC
TAF2	GGCCGACGTTTAATTTACTC
TAF3	AGAATGGATTGAGAGTATGTGG
TAF4	GTATGTGGGACTGTATGTTGG
TAR1	CTACTGCCGACAACGACACC
TAR2	TCGACACCTACTGCCGATAAC
TAR3	GACCTACCACGATGGAACTT
TAR4	CCATTCTTCTTAGCCACCG
TAR5	GGTATGCAACTATTTGGGAA

5.3. Results

5.3.1. DOMAIN II OF SODIUM CHANNEL AND PYRETHROIDS RESISTANCE

The PCR amplification, cloning and sequencing a 420 bp fragment of the *Tuta absoluta para* gene using degenerate primers designed against conserved sequences within the domain II region of the insect *para* sodium channel, revealed high similarities between *Tuta absoluta* and other insects within this region.

The fragment sequenced included part of IIS4, all of IIS5/IIS6 and the first 16 residues of the II-III intracellular linker of the channel protein and included also each of the three main kdr/super-kdr mutation residues.

The alignment of encoded amino acid sequences of the IIS4–S6 region of the *Tuta absoluta para*-type sodium channel gene compared with other insects are shown in Figure 35, which also shows the Transmembrane segments (S4, S5, S6) and the positions of the L1014F, M918T and T929V mutations.

The Top hits from blast against NCBI sequences database, shown in Table 9, reveals that the similarities were high not only with insects from the Lepidoptera order, such as diamondback moth *Plutella xylostella,* silkworm *Bombyx mori,* corn earworm *Helicoverpa zea,* cotton leafworm *Spodoptera litura* and tobacco budworm *Heliothis virescens,* but also with insects from other orders including Diptera, such as fruit fly *Drosophila melanogaster,* house fly *Musca domestica,* the mosquitoes *Anopheles,* Hemiptera, such as peach-potato aphid *Myzus persicae,* Blattodea, such as German cockroach *Blattella germanica,* and Coleptera, such as Colorado potato beetle, *Leptinotarsa decemlineata.*

Overall sequence identities ranged between 77-88% for the different insect channels.

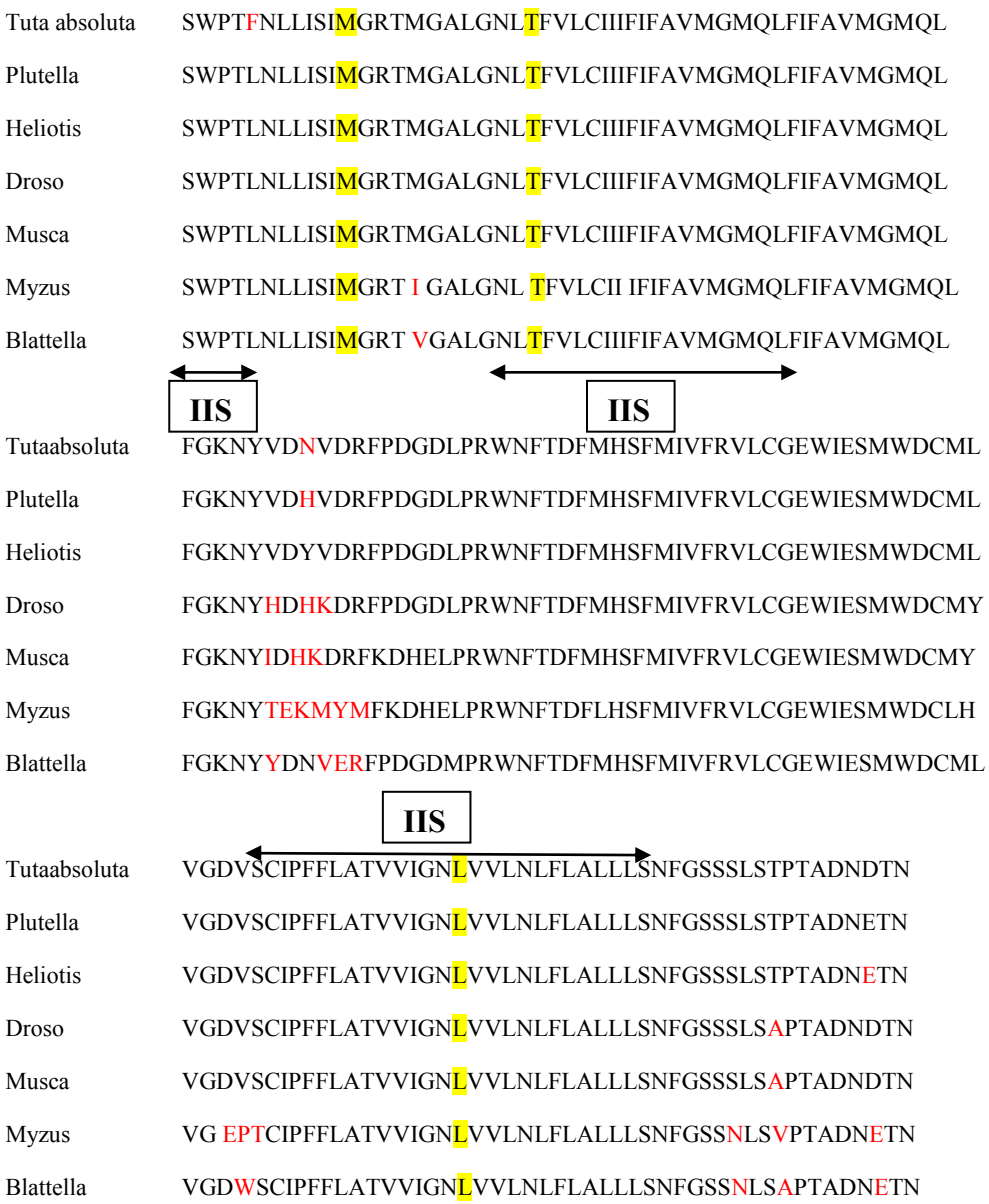


Figure 35: Alignment of the *Tuta absoluta* and other insect domain IIS4-IIS6 sodium channel amino acid sequences. Other sequences shown are *Plutella xylostell* (Linnaeus, 1758), *Heliothis virescens* (Fabricius, 1777), *Drosophila melanogaster* (Meigen, 1830), *Musca domestica* (Linnaeus, 1758), *Myzus persicae* (Sulzer, 1776) and *Blattella germanica* (Linnaeus, 1767).

Table 9: Top BLAST hits of similarities with different insects for the sequenced fragment of the *Tuta absoluta para*-type sodium channel gene.

Accession	***Description***	***Max score***	***Total score***	***Query coverage***	***Max ident***
GU574730.1	***Helicoverpa zea*** *voltage-gated sodium channel mRNA, partial cds, alternatively spliced*	*531*	*531*	*99%*	*88%*
DQ157447.1	***Spodoptera litura*** *voltage-gated sodium channel mRNA, partial cds*	*517*	*517*	*99%*	*87%*
GQ202023.1	***Bombyx mori*** *paralytic (PARA) mRNA, complete cds*	*444*	*444*	*99%*	*83%*
AF072493.1	***Heliothis virescens*** *voltage-gated sodium channel alpha subunit (hscp) mRNA, hscp-UCR-S allele, partial cds*	*426*	*426*	*99%*	*82%*
NM_001165908.1	***Tribolium castaneum*** *paralytic A (Para), mRNA >gb|GQ202024.1| Tribolium castaneum paralytic A (PARA) mRNA, complete cds*	*396*	*396*	*99%*	*80%*
AJ223279.1	***Plutella xylostella*** *mRNA for para-type voltage-sensitive sodium channel, FEN strain*	*383*	*383*	*81%*	*84%*
NM_001201722.1	***Drosophila melanogaster*** *paralytic (para), transcript variant BD, mRNA*	*354*	*354*	*99%*	*78%*
AF114489.1	***Leptinotarsa decemlineata*** *voltage-sensitive sodium channel mRNA, partial cds*	*354*	*354*	*99%*	*78%*
AF411452.1	***Musca domestica*** *para-like sodium channel mRNA, partial cds*	*342*	*342*	*99%*	*78%*
XM_001689051.1	***Anopheles gambiae*** *str. PEST AGAP004707-PB (AgaP_AGAP004707) mRNA, complete cds*	*334*	*334*	*100%*	*77%*

Specific primers were used in direct PCR analysis from genomic DNA to determine the positions and sizes of any introns within this region of the gene. Sequence characterisation of these introns is important because it facilitates the development of gDNA-based PCR diagnostic approaches to detect resistance-associated mutations. Previous work, characterizing the domain II S4-S6 region of the *para* gene from different insect species, has shown that this region contains two introns of variable size. Generally the

positions of these two introns are highly conserved but their sizes vary widely from one species to another. The sequencing showed the existence of two introns. The position of the introns and the primers used to characterize their sequences are shown in Figure 36.

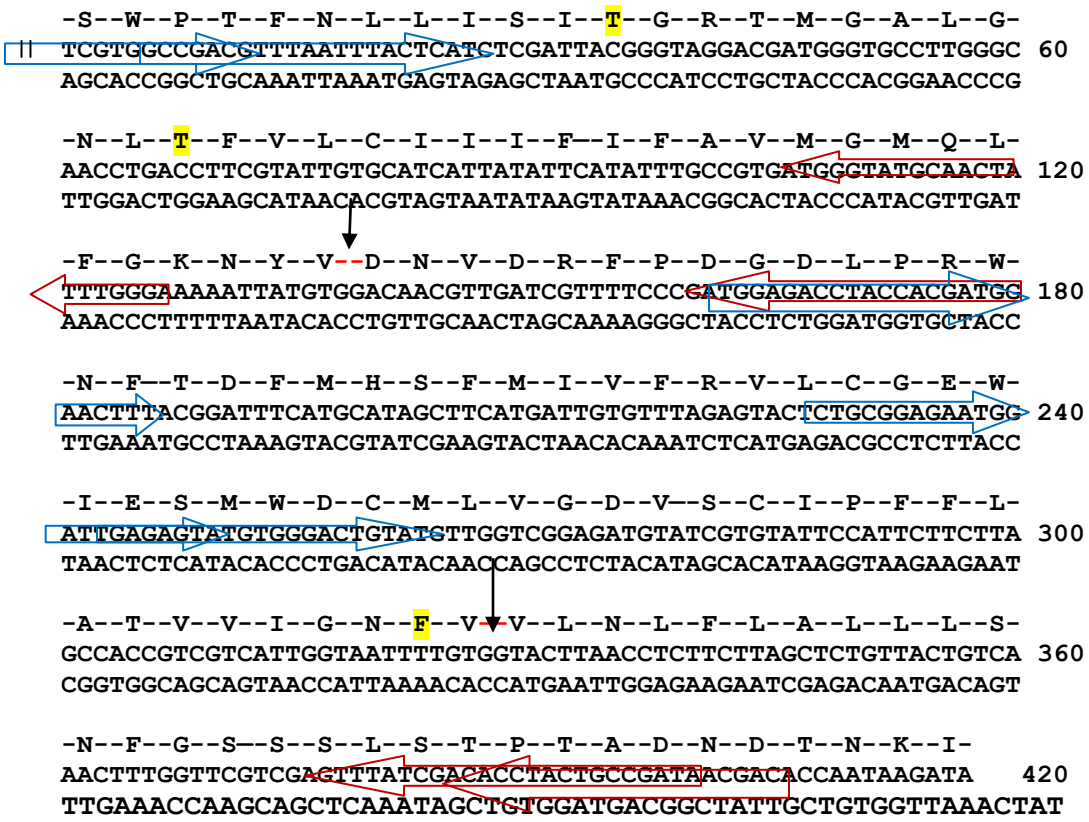


Figure 36: Domain IIS4-S6 *para*-type sodium channel cDNA sequence. Positions of known kdr/super-kdr mutations are in yellow. Vertical arrows indicate *para* gene intron positions. Red arrows highlight *Tuta absoluta* specific primers used for PCR/sequencing, blue arrows the position of degenerate primers.

The sequencing showed that the size of the large intron was 862 nucleotides and short intron was 106 nucleotides. Their complete sequences and position are shown in the Figure37. The sequences of the introns appear highly conserved across the different strains and no base differences were noted.

Figure 37: IIS4-S6 gDNA sequence of the sodium channel gene for *Tuta absoluta*. Transmembrane regions (S4, S5, and S6) sequences are in black. Position of known resistance mutations are in red. Introns sequences are shown in blue.

After the specific region of sodium channel were cloned and sequenced for *T.absoluta* and in order to investigate the existence of any mutations conferring possible resistance to insecticides in the five *T. absoluta* strains TA1, TA2, TA3, TA4 and GA reared in Rothamsted labs and already tested for their susceptibility to different chemical compounds, the specific primers designed were used to PCR amplify the domain II of sodium channel from cDNA of 15 to 20 individual pools from each strain.

The sequencing of the 420kb fragment (domains IIS5/IIS6) of the *para* sodium channel gene revealed the presence of three kdr-type mutations including an amino acid

substitution from leucine (CTT) to phenylalanine (TTT) at position 1014, a substitution of a methionine (ATG) to a threonine (ACG) at position 918 and a substitution of a threonine (ACG) to isoleucine (ATC) at position 929. The analysis of the chromatograms for all the strains showed that all the mutant alleles are found in the homozygous form for the mutation at position 1014 and in heterozygous forms for both the mutations at positions 918 and 929.

Figure 38 shows the positions and the nucleotides substitutions of the three mutations found for the five strains.

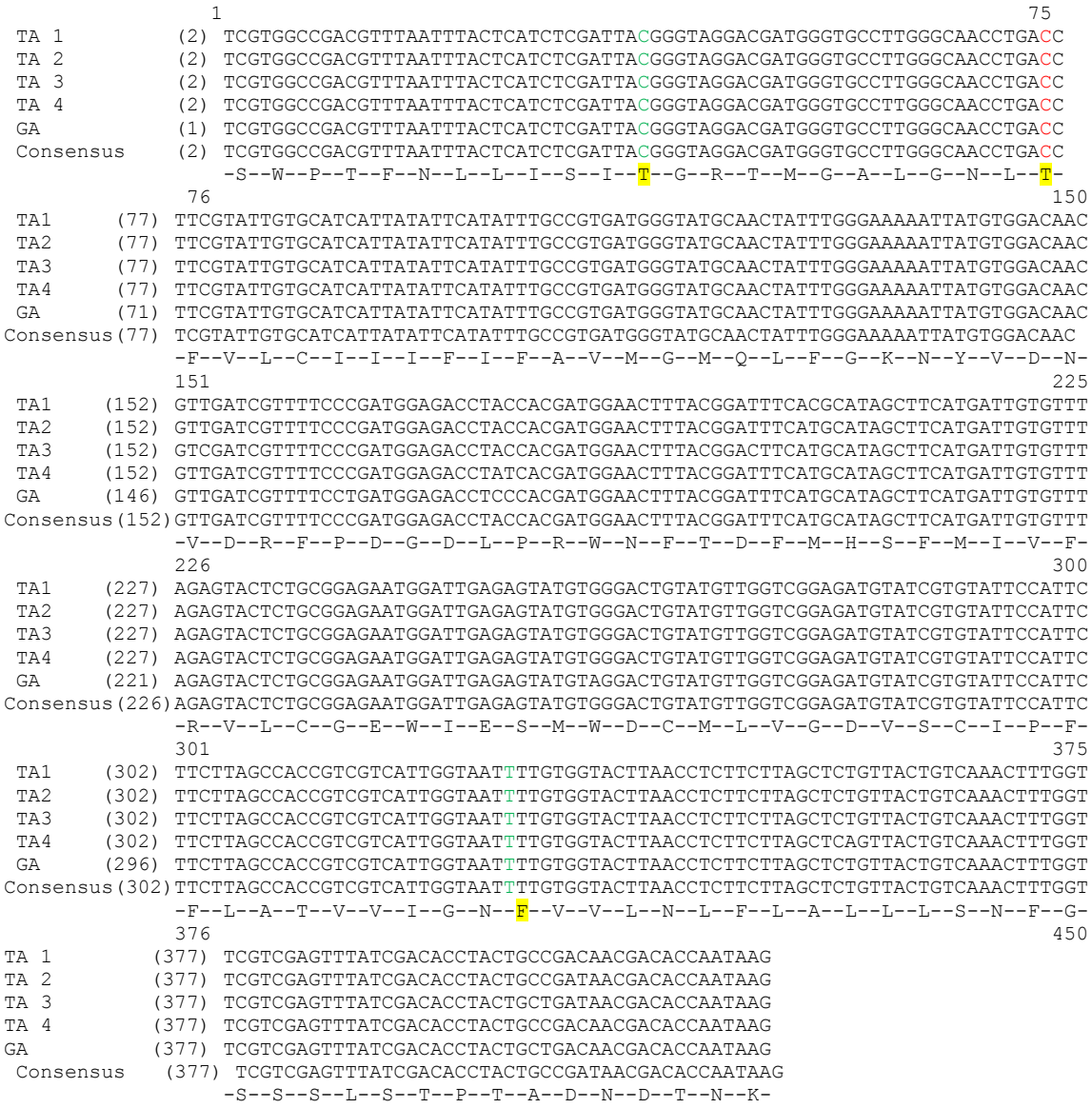


Figure 38: Positions and nucleotides substitutions of the three mutations found for the five laboratory strains.

To assess the frequencies of the three mutations within the different strains of *T. absoluta*, the genomic DNA of 10 single adults from each strain was extracted and used to amplify the fragment of interest using specific primers designed from the cDNA sequence.

This analysis allowed us to accurately genotype multiple individuals from each strain for each of the three mutations. The approximate frequency of the mutant alleles compared to the wild-type alleles for the three mutations based on the analysis of 10 individuals are summarized in Table10. The analysis confirmed that the L1014F mutation was widespread and present in all strains and all the individuals were homozygous for this mutation. Whereas for the two other mutations, the frequency of the T929I mutation is either higher or equal to the M918T mutation except for the TA4 strain.

Table 10: Frequencies of the kdr type mutations in the five reared strains of *Tuta absoluta* strains (genotypes expressed as a percentage of the population analyzed).

Strains	M918T			T929I			L1014F
	RR	RS	SS	RR	RS	SS	RR
TA1	10	50	40	40	50	10	100
TA2	30	40	30	30	40	30	100
TA3	0	40	60	50	40	10	100
TA4	0	90	10	10	70	20	100
GA	0	40	60	60	40	0	100

Table 11 shows the individual phenotypes for the two mutations at positions 918 and 929. The mutation at position 1014 was always homozygous for all the strains.

For both M918T and T929I mutations, 5 to 6 out of 10 adults were homozygous for all the strains, except for the TA4, where only one for the M918T and two for the T929I mutation were homozygous. 0 to a maximum of 3 wild-type alleles were detected for all the strains at position 929, while up to 6 individuals from both TA3 and GA strains showed a homozygote wild-type for the M918T mutation. Only one individual from TA1 and 3 from TA2 scored homozygous for the mutant allele at position 918, while 3 to 6 scored

homozygous for the mutant allele at position 929 and only TA4 did not score any mutant allele at that position.

Interestingly, the presence of the two mutations together in the same individual required it to be heterozygote at both positions.

Two individuals from the strain TA4 showing a wild type allele at position 929 and a mutant heterozygous allele at position 918 were carrying a 4th substitution at position 925 from a leucine (TTG) to methionine (ATG), and it is the first report of such mutation. Previous work reported mutations at that position but the substitution was from the amino acid leucine to isoleucine. The mutation found in this study can be considered a novel one and needs to be investigated further.

Table11: Phenotypes of 10 individuals from each of the five laboratory strains at mutations positions 918 and 929.

	TA1		TA2		TA3		TA4		GA	
Sample N°	**M918T**	**T929I**	**M918T**	**T929I**	**M918T**	**T929I**	**M918T**	**T929I**	**M918T**	**T929I**
1	S/S	R/R	S/S	R/R	S/S	R/R	R/S	R/S	S/S	R/R
2	S/S	R/R	S/R	S/R	S/S	R/R	R/S	R/S	S/S	R/R
3	R/S	R/S	R/R	S/S	S/S	R/R	R/S	S/S	R/S	R/S
4	R/S	R/S	R/S	R/S	S/S	R/R	S/S	R/R	S/S	R/R
5	R/S	R/S	R/R	S/S	S/S	R/R	R/S	R/S	S/S	R/R
6	R/S	R/S	S/S	R/R	R/S	R/S	R/S	R/S	R/S	R/S
7	S/S	R/R	S/S	R/R	R/S	R/S	R/S	R/S	S/S	R/R
8	S/S	R/R	R/S	R/S	R/S	R/S	R/S	R/S	R/S	R/S
9	R/S	R/S	R/S	R/S	S/S	R/R	R/S	R/S	S/S	R/R
10	R/R	S/S	R/R	S/S	R/S	R/S	R/S	S/S	R/S	R/S

5.3.2. DOMAIN III AND IV OF SODIUM CHANNEL AND OXIDIAZINES RESISTANCE

Suspecting a possible building up of a resistance to indoxacarb from the bioassays carried out in Italy and in the aim of investigating the existence of any other mutations, the domain III and IV of *Tuta absoluta para* sodium channel were cloned and sequenced using the same PCR based approach as for the domain II. The sequencing targeted a fragment starting from IIIS4 and ending at IVS6 and all the linkers in between. The degenerate primers used are shown in the Table12.

Table 12: Oligonucleotide primer sequences used to PCR amplify DNA from the IIIS1-IVS6 domain of the *Tuta absoluta para*-type sodium channel gene. All primers are shown 5′ to 3′.

Name	Sequence
DgNF1	CCITTYTGGCARGGITGG
DgNF2	GAAAATAAGTACTTCGAAACC
DgNF3	GGITGGATHCARATHATGAA
DgNF4	GCIACITTMAARGGITGGA
DgNR1	TACATRTCRTARTCRTCRTC
TutNaR2	TGCCADATYTCRTARTACAT

The PCR amplification of domain II and IV of *para* sodium channel of *T. absoluta* resulted in a fragment of 1751kb with high similarities with the same region from different other insects. The comparison of the fragment of amino acids sequenced with the reported mutations in that region did not show any amino acids substitutions linked with insecticides resistance.

The comparison of the fragment within different strain of *T. absoluta* did not show any differences between the more and less susceptible stains detected with the bioassays.

No introns have been sequenced. The alignment of encoded amino acid sequences of the IIIS1–IVS6 region of the *Tuta absoluta para*-type sodium channel gene is shown in Figure39.

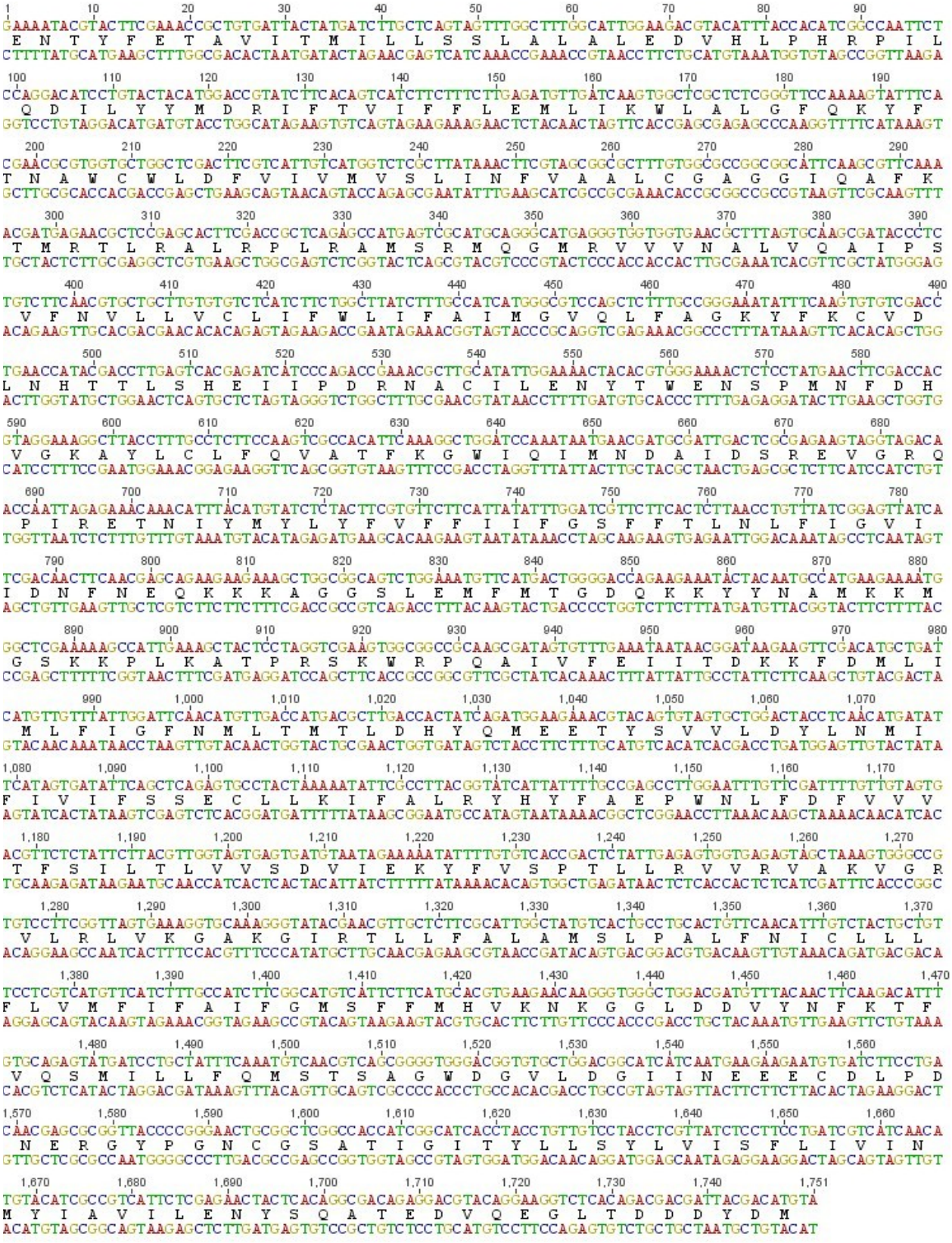


Figure 39: The alignment of amino acid, predicted protein and complementary sequences of the IIIS1–IVS6 region of the *Tuta absoluta para*-type sodium channel gene.

5.4. Discussion and conclusions

The results of this study have shown that the leucine to phenylalanine substitution found within the IIS6 segment of sodium channels in pyrethroid-resistant insects is also present in all studied stains of *Tuta absoluta*. All strains examined contained the L1014F kdr mutation with 100% of the mutant allele. Functional expression studies of L1014F kdr mutation using the frog *Xenopus laevis* oocytes showed the role of this substitution in conferring resistance and up to 17 folds to certain pyrethroids (Vais *et al.*, 2000; Smith *et al.*, 2001; Tan *et al.*, 2002; Soderlund and Knipple, 2003).

The L1014F mutation was reported in all strains combined with two other super-kdr mutations, namely M918T and T929I. The two mutations were found in heterozygous forms.

Using functional expression of channels of *D. melanogaster* in oocytes, Vais *et al,* (2000) found that the T929I mutation reduce the sensitivity of the sodium channel to deltamethrin by 10 folds and can confer more than 10,000 folds resistance to deltamethrin when inserted in the wild type.

So far M918T was never reported in the absence of L1014F mutation (Soderlund, 2008) but using *Xenopus oocyte* system Lee *et al.* (1999) showed that when inserted in sodium channel, the M918T mutation produced channels that were not detectably modified by high concentration of cismethrin and the insertion of the two mutations together gave channels that were completely insensitive to both cismethrin and cypermethrin at the highest concentrations that could be tested. In *Drosophila melanogaster* the combination of the two mutations reduced the sensitivity of sodium channels by approximately 100 fold (Soderlund *et al.,* 2001). Vais *et al.* (2000) reported a decrease of sodium channel sensitivity to deltamethrin approximately by 100 fold.

The combined effect of the three mutations together has never been reported and studied so far. Interestingly, in this study the existence of the three mutations together seems to be linked to the compulsory heterozygous form of the mutant alleles of the super-kdr mutations.

Morin *et al.* (2002) reported a novel amino acid substitution from leucine to isoleucine at position 925 in *Bemisia tabaci* tightly linked to pyrethroids resistance. In this study another novel mutation is reported at the same position conferred by the substitution of the leucine to methionine. This mutation was found only in two individuals from 10 analysed. It is found combined with both L1014F and the heterozygous form of M918T. However, the occurrence of this mutation should be treated with elements of caution and further investigations are needed to comprehend the occurrence and role of this mutation in insecticides resistance and any possible fitness costs linked to this amino substitution.

Finally, the widespread occurrence of the kdr and super-kdr mutations within strains of *Tuta absoluta* can explain the high LC50s resulting from the bioassays and confer resistance to pyrethroids. They suggest also that these mutations arose many years ago. Unfortunately no susceptible strain was available to be used as a reference for comparisons.

No mutations were found in the fragment sequenced from the domains III and IV where the site of action of indoxacarb (oxidiazine) is found. The differences in susceptibility reported in the bioassays could not be in anyway linked to any changes at the target site of this compound.

6- Target-site mechanisms of organophosphate resistance

6.1. Introduction

Acetylcholinesterase (AChE) plays an important role in nerve function as a key enzyme that terminates synaptic transmission at cholinergic synapses by breaking down the neurotransmitter acetycholine following it's release from the presynaptic membranes.

Acetylcholinesterase is the target of two widely used classes of insecticides, the organophosphates (OP) and carbamates (CMs). These compounds inhibit the AChE activity by phosphorylating or carbamylating the active site serine residue leading to impaired nerve function and death of the insect (Aldrige *et al.,* 1950 cited by Fournier 2005; Eldefrawi, 1985).

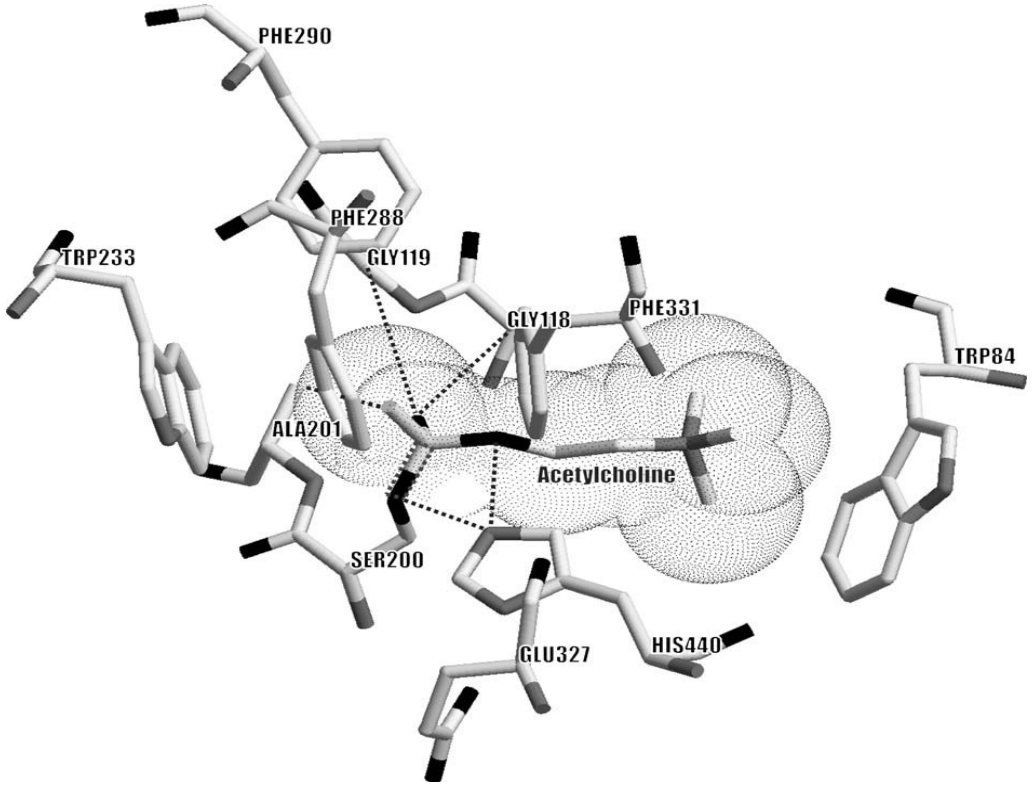


Figure 40: Schematic drawing of the active site of Torpedo AChE with the substrate, acetylcholine(see spacefill) and the positions of the catalytic triad (S200, G327, and H440), oxyanion hole (G118, G119, and A201), acyl pocket (Trp233, F288, and F290), and anionic binding site (Trp84) highlighted (From: Kono and Tomita, 2006).

Over the past six decades, the intensive use of OP and CM insecticides has led to the development of resistance in important agricultural andanimal health pests (Georghiou, 1990).

Resistance often results from modifications of the binding site of the AChE enzyme and many examples of this have been reported for insect and acarus species (Mutero *et al.,* 1994; Baxter and Barker, 1998; Chen *et al.,* 2001; Anazawa *et al.,* 2003; Weill *et al.,* 2003; Li and Han, 2004). Specific examples includes *Tetranychus urticae* (Koch) (Smissaert, 1964; Anazawa *et al.,* 2003), *Drosophila melanogaster* (Meigen) (Fournier *et al.,* 1992; Fournier & Mutero, 1994; Menozzi *et al.,* 2004), *Musca domestica* (Linnaeus), (Devonshire & Moores, 1984; Kozaki *et al.,* 2001; Walsh *et al.,* 2001), the mosquitoes of the genera *Anopheles* and *Culex* (Hall & Malcolm, 1991; Weil *et al.,* 2003, 2004; Alout *et al.,* 2008), *Aphis gossypii* (Glover) (Andrews *et al.,* 2004; Li and Han, 2004; Toda *et al.,* 2004), *Chilo suppressalis* (Walker) (Jiang *et al.,* 2009), *Plutella xylostella* (Linnaeus) (Lee *et al.,* 2007), *Cydia pomonella* (Linnaeus) (Cassanelli *et al.,* 2006) and *Bemisia tabaci* (Gennadius) (Alon *et al.,* 2008).

The cloning and sequencing of ace genes has enabledthe individual mutations that are responsible for resistance to be identified (see table 13). They are generally found within the active site gorge of the enzyme where they affect the binding of the larger insecticide compounds, but not the natural substrate acetylcholine (Fournier, 2005). These studies have also shown that combinations of mutations are often more effective in conferring resistance, and the existence of a particular mutation does not always confer resistance to all OPs and CMs (Walsh *et al.,* 2001; Menozzi *et al.,* 2004; Fournier, 2005).

Table13: Reported mutations in the Ace gene from different insects (From Fournier, 2005 with amendments from Hsu *et al.,*2006; Lee *et al.,*2007; Jiang *et al.,*2009¸Khajehali *et al.,* 2010). (*) Positions are according to *Topredo californica* numeration system.

Mutations	**Position***	**Insect**
E to G	73	Only in one *Drosophila* population.
• F to L • F to S	78	• *Aphis gossypii* • expressed in *Drosophila melanogaster, Lucilia cuprina* and *Aedes aegypti* recombinant enzymes.
E to K	82	Found only once, in a *Drosophila* population.
• G to S • G to S	119	• *Culex pipiens, Anopheles* gambiae and *Anopheles albimanus* • *Tetranychus urticae*
D to E	128	*Tetranychus urticae*
I to V	129	*Drosophila, Bactrocera oleae*, expressed in *Lucilia cuprina* and *Drosophila* recombinant enzymes
V to L	151	*Musca domestica*
A to S	201	*Aphis gossypii, Chilo suppressalis, Plutella xylostella, Tetranychus urticae, Bactrocera dorsalis*
G to A G to V	227	• *Drosophila,* • *Musca domestica*, has been expressed *in Drosophila melanogaster, Lucilia cuprina,Aedes aegypti* and *Musca domestica* recombinant enzymes
S to G	238	*Leptinotarsa decemlineata*,
F to Y	290	*Drosophila*, *Musca domestica*, has been expressed in *Drosophila, Musca domestica, Lucilia cuprina and Aedes aegypti* recombinant enzymes
G to A	328	*Drosophila, Musca domestica*

For *Tuta absoluta*, many studies showed reduced sensitivity to organophosphates and carbamates compounds in different countries meanly from South America and including metamidophos, mevinphos, acephate and cartap (Gudes *et al.,* 1994; Salazar and Araya, 1997; Seiqeura *et al.,* 2000; Branco *et al.,* 2001).

For *Tuta absoluta*, many studies have shown a reduced sensitivity to a range of organophosphate and carbamate compounds such as metamidophos, mevinphos, acephate and cartap (Gudes *et al.,* 1994; Salazar and araya, 1997; Seiqeura *et al.,* 2000; Branco *et al.,* 2001).Although the chlorpyriphos bioassays carried out in this study did not show significant differences between the strains that were investigated, the LC50 levels were nevertheless higher than the recommended field doses for this compound, suggesting that all the strains carry significant levels of resistance.

The objectives of the this study were firstly to investigate the insensitivity of the *T.absoluta* AChE to OPs and CMs using biochemical methods, and secondly to look for any mutations in the *T.absoluta* ace gene that might be responsible for this resistance.

6.2. Methods

The insensitivity of AChE to OP and carbamate insecticides has been characterised using biochemical assays in many different insect species (Oppenoorth, 1982; Raymond *et al.*, 1982; Hemingway *et al.,* 1986; Moores *et al.,* 1988; Byrne and Devonshire, 1993). Most of these studies were based on the Ellman method (Ellman *et al.,* 1961).

The protocol uses endogenous AChE to catalyse the hydrolysis of acetylthiocholine iodide (ATChI) to thiocholine, which reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to form a coloured product (2-nitro-5-thiobenzoate) that can then be measured by spectrophotometry.

6.2.1. ACHE INHIBITION

The AChE assay described by Ellman *et al.* (1961) was adapted as follow: 10 adults of *T. absoluta* from four laboratory strains (TA1, TA3, TA4, GA) were ground in 1.3 ml of phosphate/Triton (0.1 M phosphate buffer pH 7.5 containing 1% Triton X-100). TA2 was not tested, due to a short availability of samples at the time of this test.
The homogenate was briefly microfuged to pellet debris and the supernatant used as the enzyme source. A stock acetone solution of chlorpyrifos-oxon (0.01 M) was prepared and serial dilutions added to aliquots of insect homogenate using a 96 well NUNC microplate and incubated for 10 mins. Enzyme in buffer only acted as a control.
Remaining AChE activity was measured by addition of acetylthiocholine iodide (ATChI) substrate and detection of the released thiocholine colorimetrically (405 nm) by its reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The final concentration of both reagents was 0.5 mM. Kinetic assays to measure AChE activity were performed at 405nm using a Tmax kinetic microplate spectrophotometer, with readings taken automatically for 10 mins at 20 sec intervals. The rate (mODmin-1) was calculated by Sotmax Pro ver 5.4. IC_{50} values were calculated using Grafit 3.0 (erithacus Software).

The insecticide tested for the ability to inhibit AChE activity was chlorpyrifos-oxon, belonging to the OP class.

6.2.2. CLONING AND SEQUENCING ACHE OF T. ABSOLUTA

To clone and sequence the AChE of *T. absoluta* the same approach as for the sodium channel was used. Degenerate primers were designed against conserved regions of a consensus alignment of seven insects belonging to the Lipetopderan order (see Appendix A):*Bombyx mori* (Linnaeus)*, Helicoverpa armigera* (Hübner)*, Helicoverpa assulta (*Guenée)*, Cydia pomonella* (Linnaeus), *Chilo suppressalis* (Walker), *Spodoptera exigua* (Hübner), and *Spodoptera litura* (Fabricius). A single PCR approach was used based on primers DegAceF1and DegAceR3 (primer sequences are given in Table 14). PCRs were carried out using a thermal cycler with heated lid and run for 35 cycles of 95 °C for 30 sec 48- 50 °C for 60 sec and 72 °C for 90-120 sec. 1.2% Agarose gel electrophoresis of PCR products was carried out in 1x TBE buffer and slices of DNA fragments of the correct size were excised from the gel. The Wizard SV gel and PCR clean up System from Promega was used to Recovery the the PCR fragments from gel slices according to manufacturer's recommendations.The Strataclone PCR Cloning kit from Stratagene was tor clone the gel-purified fragments; this provides a blue-white screening with plasmid pSC-A-amp/kan to identify colonies with cloned inserts. After single colony PCR, the GeneJET Plasmid Miniprep Kit was used to purify the DNA. Plasmid DNA samples were sent to Eurofins MWG/Operon for sequencing.

Table 14: Degenarate oligonucleotide primer sequences used to PCR amplify cDNA from the Ace gene of the *Tuta absoluta*. All primers are shown 5′ to 3′.

Name	**Sequence**
DegAceF1	CCCAACACAGATATGCAGGA
DegAceF2	TCACACCTAGACCACGACCA
DegAceF3	TCTTTGGGGGTGGGTTTTAT
DegAceF4	GGGGCTGTGTCTGTTTCATT
DegAceR1	AATTTTTCCCGGGATTCAGA
DegAceR2	ATTTTTCCCGGGATTCAGAG
DegAceR3	GGGTTATTCTTACTACGATGCTTG
DegAceR4	ATGATGGGAACAAACGGAAA

Since the use of the degenerate primers resulted only in a partial sequence representing the middle part of the gene, which is relatively more conserved among the different insect species, a second approach was needed to complete the sequence of the rest of the gene. This was carried out using RACE (rapid amplification of cDNA ends) methodology (see chapter3). The technique involved designing gene specific primers, from the known sequence that are oriented in the direction of the missing ends, and PCR amplifying the region between the specific primers and the 3' or 5' ends. A reverse primer that can anneal to the existing poly (A) tail has been used for 3' extensions while a modified lock-docking oligo(dT) was used for the 5' ends. The sequences of the primers used for the RACE approach are given in Table 15.

Table 15: Oligonucleotide primer sequences used to PCR amplify cDNA from the Ace gene of the *Tuta absoluta* using a RACE method. All primers are shown 5′ to 3′.

Name	**Sequence**
RACE F1	TGATGCCTAAGGAAGAGAACG
RACE F2	TGCCTAAGGAAGAGAACGTG
RACE F3	TGGCTGAATCCAAATGATCC
RACE R1	CGC TCA TTC TCT TGT GTG ATG
RACE R2	CGGCAATATCATGCTGGATT
RACE R3	GAGTACAATAGACAGATACGGCAAT
RACE R4	GCTTCGGCACAACTATGTTT
RACE R5	CATTACTGCCGCATTCTTGG

Once the complete sequence of the gene was determined,, specific primers to *T. absoluta* were designed and used to PCR amplify the cDNA of the five other *T.absoluta* strains. The sequences of the specific primers are given in Table 16.

Table 16: Specific oligonucleotide primer sequences used to PCR amplify cDNA from the Ace gene of the *Tuta absoluta*. All primers are shown 5′ to 3′.

Name	Sequence
TAcheF1	TAATACGACTCACTATAGGGCAAGC
TAcheF2	AATACGACTCACTATAGGGCAAGC
TAcheF3	ATACGACTCACTATAGGGCAAGC
TAcheF4	CAGATATGCAGGAAGACTGTCTCTA
TAcheF5	CAAGAATGCGGCAGTAATGT
TAcheR1	CTGCTCGTGCAGTTCTTTGG
TAcheR2	TGCTGCCACAAGTAAACCAG

6.3. Results

6.3.1. INHIBITION SENSITIVITY

The strains of *T. absoluta* tested showed no significant variation in AChE sensitivity to chlorpyrifos-oxon. The plot showing percentage inhibition of AChE activity against increasing insecticide concentration of the insecticide is shown in Figure 41. The corresponding IC50 values are shown in Table 17.

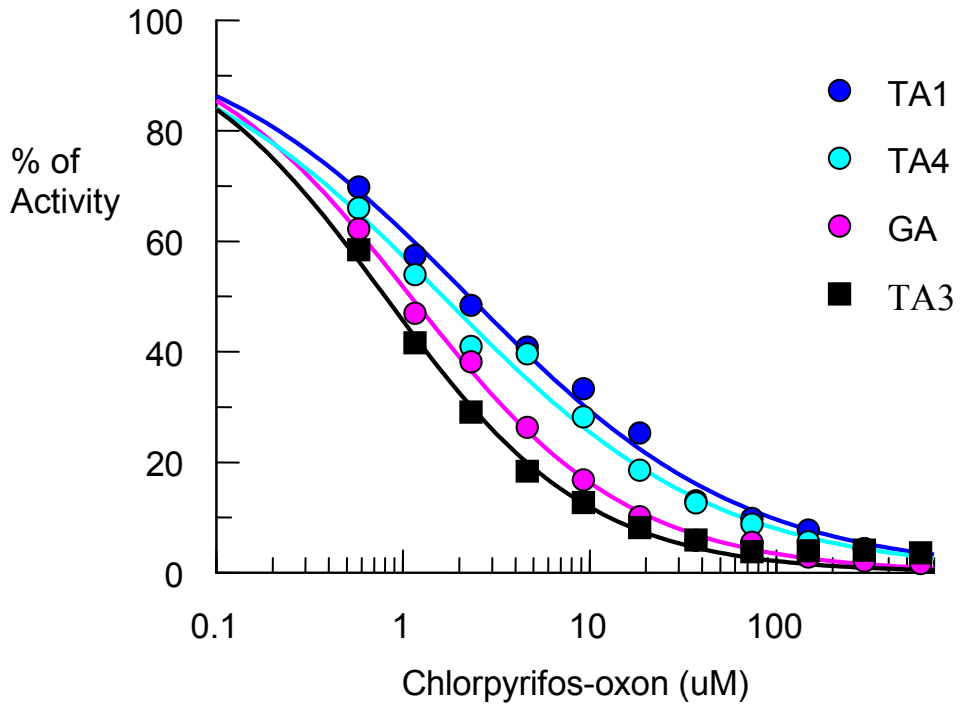


Figure 41: plot of the percentage inhibition of AChE enzyme against increasing chlorpyrifos-oxon concentration in different *Tuta absoluta* strains.

Table 17: IC50 values of AChE inhibitionby chlorpyrifos-oxon for different *Tuta absoluta* strains.

Strain	**IC50 (uM)**	**Std Error**
TA1	**2.26**	**0.15**
TA3	**0.80**	**0.05**
TA4	**1.64**	**0.13**
GA	**1.10**	**0.04**

No known susceptible strain was available to be used as a reference. Interestingly, although no large differences in sensitivity (about 3-fold) were found, the trend in AChE inhibition reflects the susceptibility found in bioassays. TA1 resulted as the most insensitive strain, while TA3 was the most sensitive. TA2 was not tested, due to a short availability of samples at the time of the test.

6.3.2. CLONING AND SEQUENCING THE ACE GENE

The PCR amplification, cloning and sequencing of the Ace gene was achieved in two steps approach. The first step used the degenerate primers against conserved AChE sequences and enabled the more highly conserved middle part of the gene to be cloned, while the second step used specific primers for RACE PCR amplification of the missing parts at the 3' and 5' ends. This approachwas very successful and yielded in a fragment of 1874 bp with high similarity to other insect AChEs, confirming that the *Tuta absoluta* AChE had indeed been cloned

The top BLAST hits for the similarities between *T. absoluta* and other insects for the Ace gene are reported in the Table 18. The alignment of the sequenced fragment of Ace gene amino acids from *T.absoluta* against Ace gene sequence of different insect is shown in Figure.42.

Table 18: Top BLAST hits of the sequenced Ace gene fragment of *Tuta absoluta* against different insects using NCBI (http://www.ncbi.nlm.nih.gov/).

Accession	Description	Max score	Total score	Query coverage	Max ident
DQ064790.1	***Helicoverpa armigera*** strain BK-77 (Ace1) mRNA, partial cds	1348	1348	91%	77%
EF453724.1	***Chilo suppressalis*** (Ace1) mRNA, complete cds	1315	1315	97%	76%
DQ001323.1	***Helicoverpa assulta*** (Ace1) mRNA, complete cds	1306	1306	95%	76%
FP340420.1	70A06_SfBAC_fin, ***Spodoptera frugiperda*** BAC, egg DNA	1256	1299	87%	86%
EU328261.1	***Bombyx mori*** (ace1) mRNA, complete cds	1211	1211	96%	74%
DQ267977.1	***Cydia pomonella*** strain S (Ace1) mRNA, complete cds	1180	1180	97%	74%
AY773014.2	***Plutella xylostella*** (ace1) mRNA, partial cds	1090	1090	84%	75%
FJ959384.1	***Spodoptera litura*** (Ace1) mRNA, partial cds	960	960	63%	77%
DQ280488.1	***Spodoptera exigua*** (Ace1) mRNA, partial cds	773	773	51%	77%
HQ260968.1	***Tribolium castaneum*** (Ace 1) mRNA, complete cds	609	609	74%	70%
DQ288249.1	***Blattella germanica*** ace1 mRNA, complete cds	609	609	75%	69%
XM_002430504.1	***Pediculus humanus corporis*** acetylcholinesterase, putative, mRNA	571	571	75%	69%

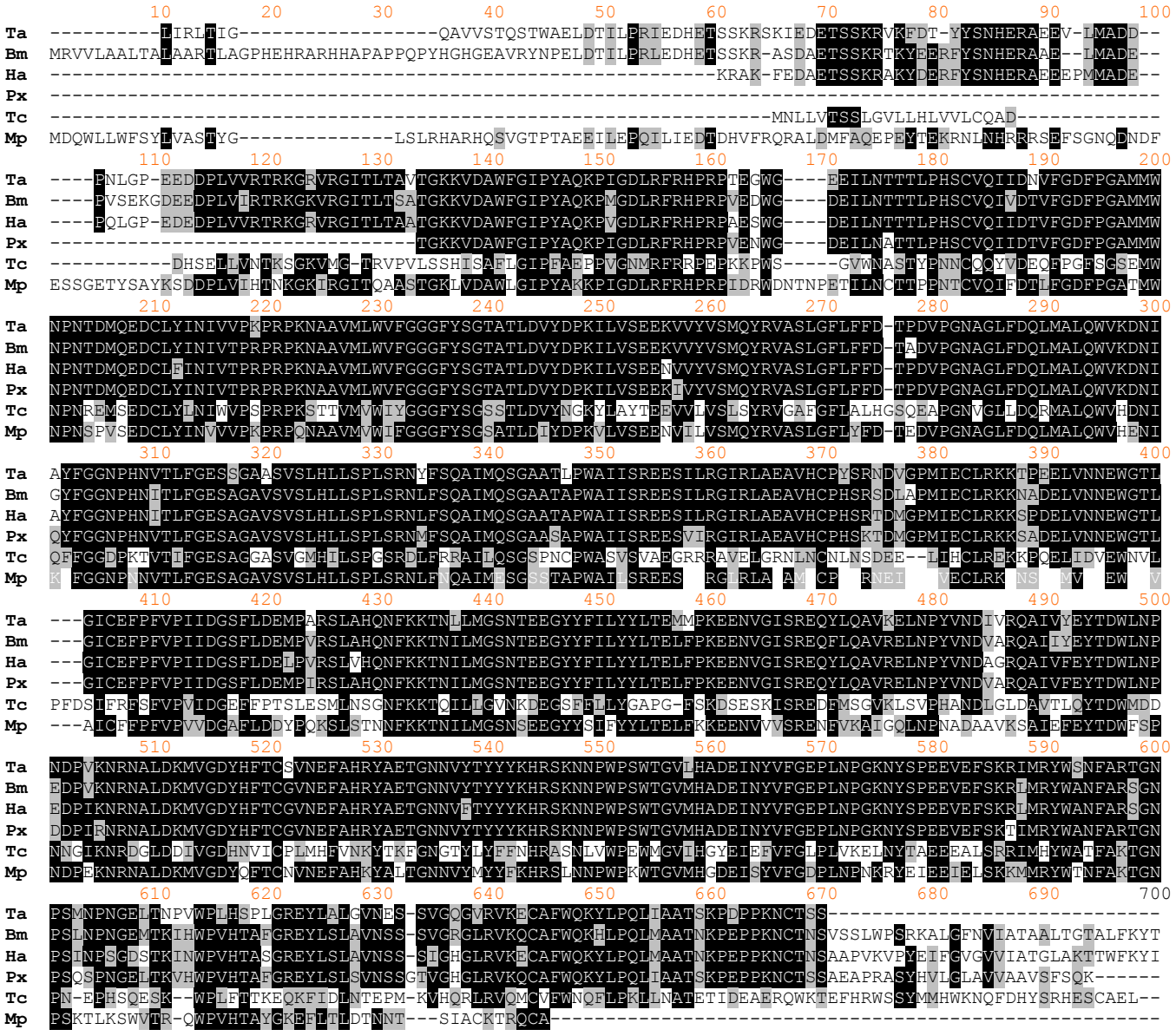


Figure42: Alignment of sequence fragment of Ace gene of *T.absoluta* against different insects: BM- *Bombyx mori* (Linnaeus); Ha- *Helicoverpa armigera* (Hübner); Px- *Pluttela xylophella* (Linnaeus); Tc-*Torpedo californica* (Houttuyn) and Mp- *Myzus persicae* (Sulzer).

The nucleotides sequence of the 1874 kb fragment, its amino acids translation and reverse complement sequence are given in figure 43.

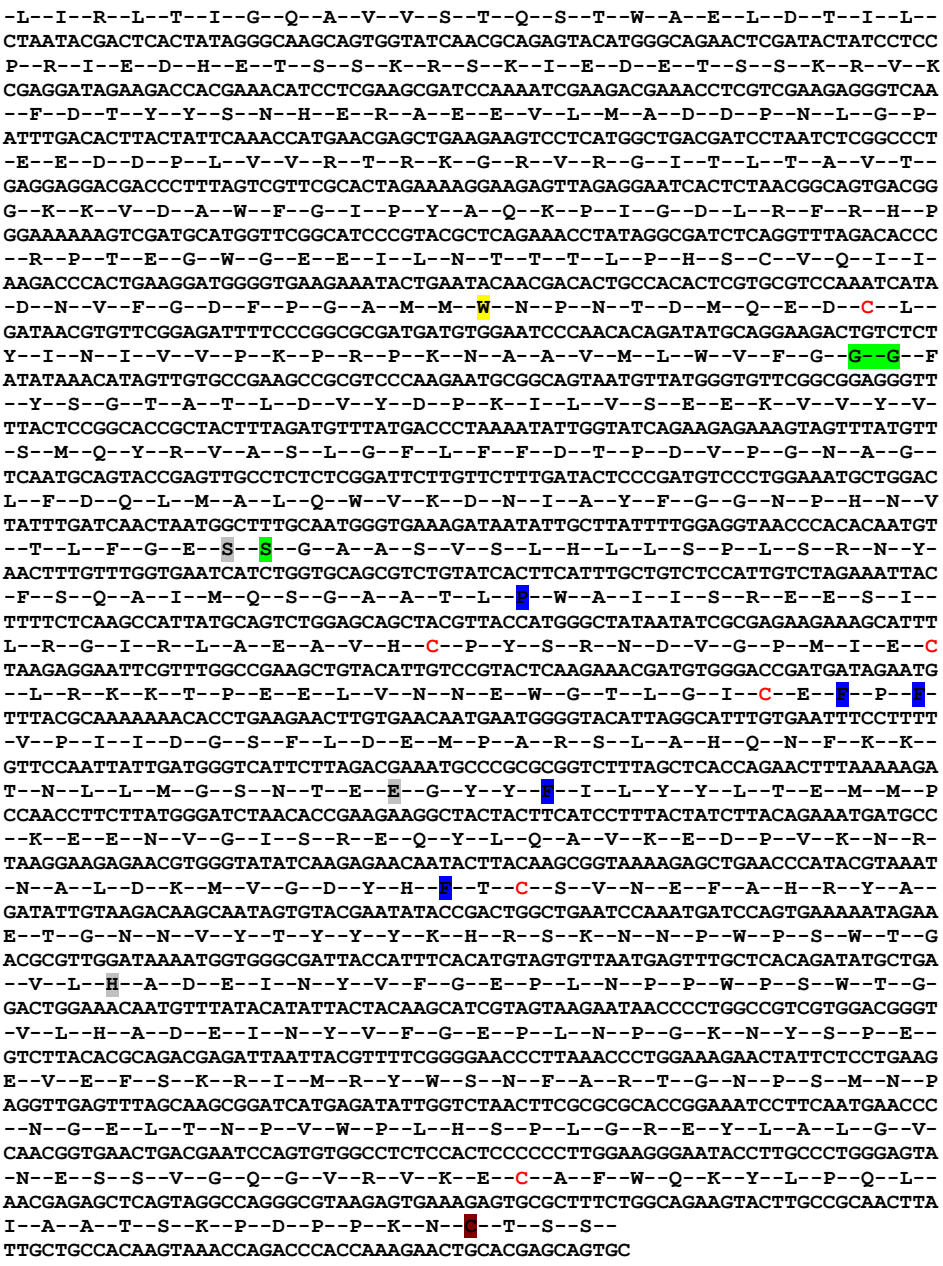


Figure43: The nucleotides sequence of the 1874 bp fragment and its amino acids translation of partial Ace gene of *Tuta absoluta*. C: cysteine pair forming intra-subunit disulfide bond; W: tryptophan of anionic binding site; C: cysteine making inter-subunit bond; X: amino acid of oxyanion hole; F: amino acid of acyl pocket; X: amino acid of catalytic triad.

In order to look for the existence of mutations that might be responsible for conferring resistance in the AChE sequences of the other *T. absoluta* strains, the specific primers designed against the *Tuta absoluta* cDNA were used to PCR amplify and sequence the ace gene from each of the five strains. The sequences were then aligned against *Torpedo californica* AChE which is used as the reference for all AChE resisdue numbering. The alignment is shown in figure 44.

The comparison shows the existence of one known resistance mutation at position 201. This mutation involves substitution of the amino acid alanine (GCC) to serine (TCT). This mutation is located next to the active site serine that forms the catalytic triad of the enzyme. All wild-type insect AChEs have alanine at this position, whereas the serine substitution seen in all the *T. absoluta* strains has also been reported for OP/CM resistant strains of *Aphis gossypii* (Glover), *Bactrocera dorsalis* (Hendel),*Plutella xylostella* (Linnaeus), *Chilo suppressalis* (Walker), *Tetranychus urticae* (Koch) (Li and Han, 2004; Toda *et al,*. 2004; Andrews *et al.*, 2004; Lee *et al.*, 2007; Jiang *et al.*, 2009; Khajehali *et al.*,2010) All the strains were homozygous for the mutation except for the GA strain. The sequencing of 10 individuals from that strain showed that they all carried the mutation in heterozygous form.

Figure 44: Alignment of the middle part of the ace gene for the five strains tested against the *Torpedo californica* sequence with the found mutation at position 201 in Grey.

6.4. Discussion and conclusions

A biochemical assay for characterising the sensitivity of AChE from *Tuta absoluta* to the OP insecticide chlorpyriphos-oxon was carried out. Four of the five laboratory strains were tested but no significant variations in Ic_{50} values were found (less than 3 fold difference between strains). The trend in AChE inhibition, however, did reflect perfectly the susceptibility of these strains as found in bioassays, confirming a good correlation between resistance and AChE insensitivity.

The past intensive use of the OP and carbamates insecticides as a control strategy against *T. absoluta* across South America (where this insect is thought to have originated) may well have led to selection of insensitive AChE variants that have subsequently been transported and established themselves in the Mediterranean region. Unfortunately, the absence of standardised, laboratory susceptible strain does make it more difficult to draw firm conclusions about the exact level of resistance in these populations.

It is reported here, for the first time, the PCR amplification, cloning and sequencing of 1874kb fragment of *Tuta absoluta* AChE cDNA sequence. Although the start and stop codons are missing from this sequence, the combined approach of degenerate primer PCRs and RACE methodology has enabled the successful cloning of over 80% of the gene coding sequence, including all regions in which mutations have been found in other pests.

The T absoluta sequence shows high similarities with the ace1 gene of many other insects, particularly those of other closely-related Lepidopterans.

The sequence shows all the characteristic features of AChEs, including the catalytic triad (S200, E327 and H440), the six cysteines (C67-C94, C254-C265 and C402-C521) forming the intrachain disulphide bonds and the residues (G118, G119 and A201) that form the oxy-anion hole which is important for stabilizing the tetrahedral transition stat molecule during catalysis (Soreq *et al.,* 1992 cited by Javed *et al.,* 2003; Zhang *et al.,* 2002). All the positions are named according to the *Torpedo californica* mature AChE protein, as is the convention for AChEs.

Alignment of the five AChE sequences from the T absoluta strains against other wild-type insect AChEs and *Torpedo californica* (Figure 44) reveals the presence of a single

mutation at residue 201 (A201S), that has previously been associated with insecticide-insensitive forms of AChE. It is unfortunate that we dod not have a laboratory susceptible strain for comparison, however given the extremely high conservation of the alanine (A201) in all other species, it does seem likely that this mutation is also conferring a level of resistance in the T absoluta strains.

The first report of the A201S mutation was by Toda *et al.* (2004) in a strain of *Aphis gossypii* (Glover) having a reduced susceptibility to two OPs: fenitrothion and malathion. For the same insect, Li and Han (2004) reported this same mutation combination with another mutation F139L in strains presenting ratios of resistance to methamidophos, monocrotophos, pirimicarb and thiodicarb of 301, 379, 35 and 32, respectively.Andrews *et al.* (2004) found the mutation A201S occurring in tandem with the mutation S331F in a strain of *Aphis gossypii* (Glover) with resistance to pirimicarb and organophosphates.Hsu *et al.* (2006) found tree mutations including A201S, D229G and G324A associated with resistance to organophosphate insecticides in *Bactrocera dorsalis* (Hendel), while Lee *et al.* (2007) reported the same three mutations D229G, A298S and G324A in a prothiofos-resistant strain of *Plutella xylostella* (Linnaeus).

In a strain of *Chilo suppressalis* (Walker), Jiang *et al.* (2009) found that the A201S mutation was consistently associated with the occurrence of triazophos (OP) resistance. Their results showed a strong correlation between frequencies of the mutation and phenotypic levels of resistance to triazophos. More recently, Khajehali *et al.* (2010) reported the A201S mutation in combination with another mutation (F331W) in OP resistant strain of the spider mite,*Tetranychus urticae* (Koch).

Using a three dimensional homology model of the catalytic site residues from *Myzus persicae* (Sulzer) Ace1 gene, Andrews *et al.* (2004) tried to investigate the role of the A201S mutation in changing the sensitivity of the enzyme to inhibition by carbamates and organophosphates. They explained that the substitution of serine to alanine to serine affects the formation of a tight hydrogen bond between the Oxygen of S200 and the amide nitrogens from G118 and G119. These three residues interact with the substrate carbonyl oxygen of ACh to form the oxyanion hole, stabilizing the enzyme-substrate transition state (Steitz and Schulman (1982).

In summary, although the *T. absoluta* strains tested in this study did not show significant differences in the insensitivity of AChE their field histories do nevertheless suggest that they carry a high inherent insensitivity to OPs and CMs, and this has been confirmed by the discovery of a known reistance mutation (A201S) in these strains. Further investigations are needed to evaluate the exact level of resistance conferred by this mutation, and whether it also carries any adverse affects (ie fitness penalties) on the T absoluta individuals that carry it.

7– Diagnostic assays for mutations associated with insecticide resistance

7.1. Introduction

Direct sequencing of both sodium channel and AchE genes showed a widespread occurrence of these mutations in the five laboratory strains tested, suggesting a similar widespread occurrence in populations of *Tuta absoluta* not only from around the Mediterranean area but also from its native area of origin (South America). To confirm this hypothesis, it was necessary to analyse and screen a larger number of *Tuta absoluta* individuals from various geographic origins, using the collection of samples kindly provided by Dr Pablo Bielza Lino (Departamento de Produccion Vegetal, Universidad Politecnica de Cartagena, Spain).

The screening of such a large collection needed the development of a diagnostic tool with high reliability in detection of sensitivity and specificity of mutations of interest.

Currently several methods exist for detecting changes in insects DNA related to insecticide resistance. They include Allele Specific PCR (ASPCR) (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000), Heated Oligonucleotide Ligation Assay (HOLA) (Lynd *et al.,* 2005), Sequence Specific Oligonucleotide Probe Enzyme-Linked ImmunoSorbent Assay (SSOP-ELISA) (Kulkarni *et al.,* 2006), PCR-Dot Blot (Kolaczinski *et al.,* 2000), Fluorescence Resonance Energy Transfer (FRET)/Melt Curve analysis (Verhaeghen *et al.,* 2006), PCR elongation with fluorescence (Tripet *et al.,* 2006) and the newly developed fluorescence-based assays based on TaqMan probes and high resolution melt (HRM) (Bass *et al.,* 2007).

SNP (Single-Nucleotide Polymorphisms) genotyping based on TaqMan assays has become recently a useful methodology for detecting insecticide resistance in insects such as malaria vector mosquitoes (Bass *et al.,* 2007, 2008, 2010). It is a PCR based method employing oligonucleotide probes that are dual-labelled with a fluorescent reporter dye and a quencher molecule. During PCR, the TaqDNA polymerase cleaves probe and liberates the

reporter fluor from the quencher, increasing net fluorescence. Two probes are used: one is specific for the wildtype allele, labeled with VIC; the second is specific for the mutant allele and is labeled with FAM (see figure45).

In this study, four TaqMan assays were designed and validated to be used for detection of the previously found mutations in the sodium channel (L1014F, M918T and I929T) and AchE (A201S) genes of *Tuta absoluta*. The same TaqMan was then used to screen the samples collection of *T. absoluta* provided by Dr Bielza. Partial and preliminary results will be showed and discussed only for the sodium channel mutations, since the TaqMan assay for the AchE was not completed by the time this thesis was written.

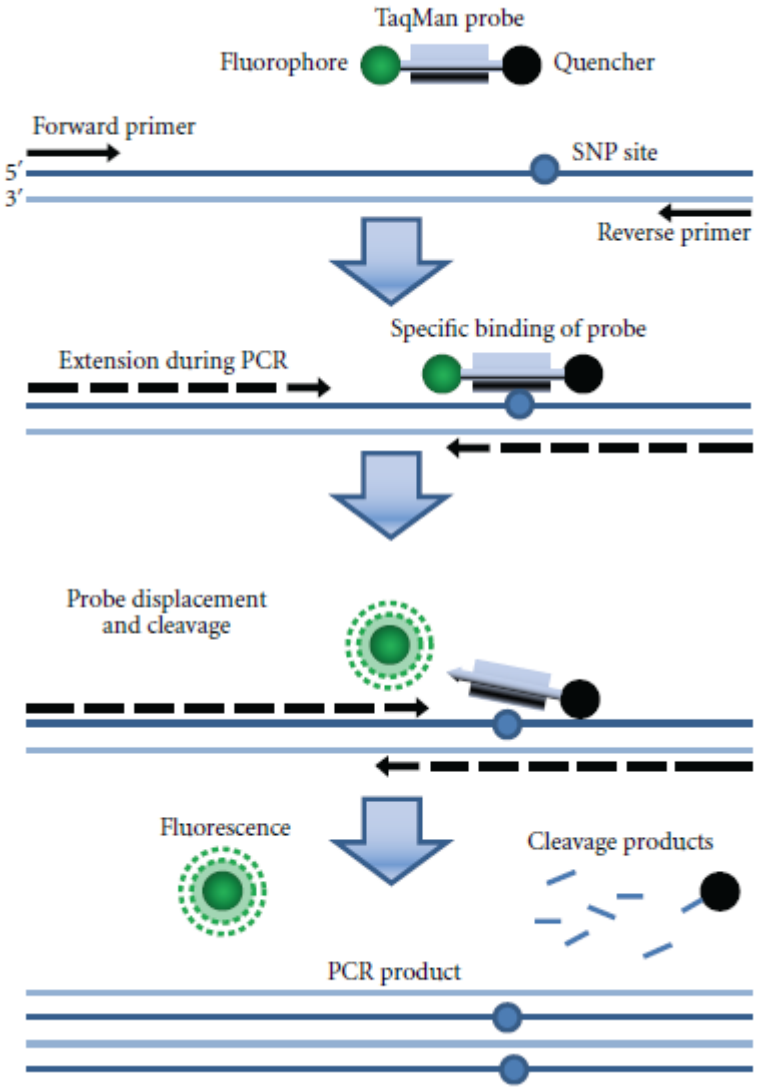


Figure45: Schematic description of TaqMan SNP genotyping method.

7.2. Specific methods

Forward and reverse primers and two probes were designed using the Custom Taqman Assay Design Tool (Applied Biosystems) . The first probe, labeled with VIC at the 5', was for detection of the wildtype allele, while the second probe, labeled with FAM, was for detection of mutant allele. Each probe also carried a 3' non-fluorescent quencher. The sequences of the primers and probes are given in the table19.

Table 19: Oligonucleotide primer sequences used in TaqMan assays to genotypes the different SNPs of the Tuta absoluta para-type sodium channel gene. All primers are shown 5′ to 3′.

Name	Sequence
TAkdr_F	CTTCTTAGCCACCGTCGTCATT
TAkdr_R	CGCTTTTACTGGTATATTGCAATAAAAAGCT
TAkdr VIC	AACCACAAGATTACC
TAkdr FAM	ACCACAAAATTACC
TAT929I_F	ACGATGGGTGCCTTGGG
TAT929I_R	TGCATACCCATCACGGCAAATAT
TAT929IVIC	CACAATACGAAGGTCAGGTT
TAT929IFAM	CACAATACGAAGATCAGGTT
TAM918T_F	TGGCCGACGTTTAATTTACTCATCT
TAM918T_R	TGCCCAAGGCACCCATC
TAM918TVIC	TCCTACCCATAATCG
TAM918TFAM	TCCTACCCGTAATCG
TA_ace_F	GGAGGTAACCCACACAATGTAACTT
TA_ace_R	ACAATGGAGACAGCAAATGAAGTGA
TA_aceVIC	TTGGTGAATCAGCTGGTG
TA_aceFAM	TTGGTGAATCATCTGGTG

PCR reactions (15 µl) contained 2 µl of genomic DNA extracted from individual insects using DNAzol reagent (see Chapter3), 7.5 µl of SensiMix DNA kit (Quantace), 0.375 of primer probe and 5.125 of sterile distilled water. Samples were run on a Rotor-Gene 6000™ (Corbett Research) using the temperature cycling conditions of: 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds.

The increase in fluorescence of the two probes VIC and FAM was monitored in real time by acquiring each cycle on the yellow (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 emission) of the Rotor-Gene respectively. Template controls (a wildtype homozygous, a mutant homozygous and a mutant heterozygous samples) were included in each run to determine the background level of fluorescence and the fluorescence values of these reactions were subtracted from values all samples. Template controls facilitate the interpretation of results and the scoring of unknown samples.

In order to optimize and validate the TaqMan method, and before applying it for screening the samples collection, genomic DNA templates of known genotypes from the five laboratory strains were used for the first runs of PCRs. Optimization parameters included the annealing temperature, the concentration of probes in the reaction and the number of temperature cycles.

7.3. Results

After the first optimization runs with the known template genotypes (TA1 and TA2 strains) the TaqMan assay proved to be a good tool for discrimination between the wildtype and the mutant alleles for the three mutations in the sodium channel (Figure 46).

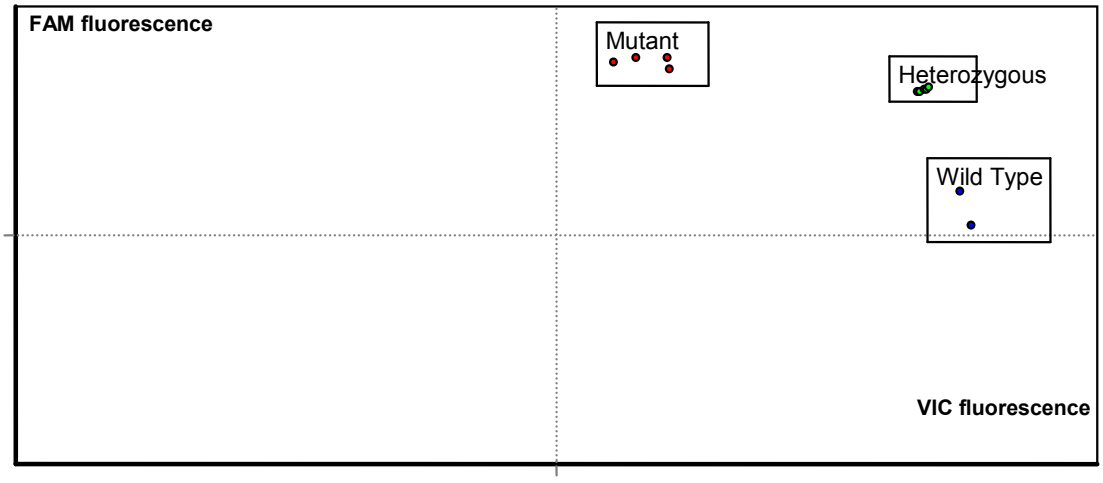
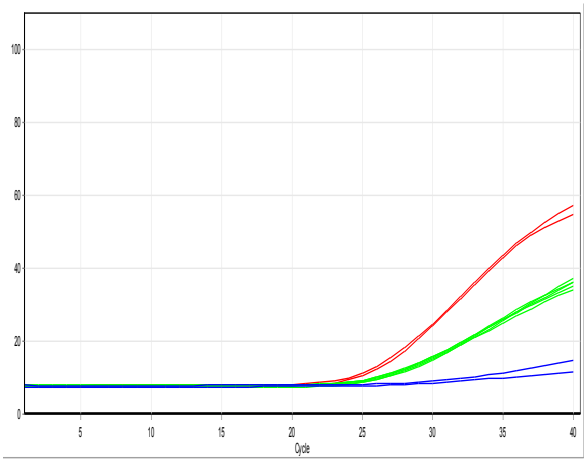


Figure:46: Example of Scatter plot analysis of TaqMan fluorescence data

Clear increase in VIC or FAM fluorescence indicates a homozygous form of wildtype and mutant alleles respectively, while the intermediate increase in both signals indicates a heterozygous form of alleles (Figures 47 to 52). X axis indicates the number of PCR amplification cycles and Y axis indicates the probe fluorescence levels.

The zygosity results obtained using the TaqMan assays for the used template genotypes (TA1 and TA2 strains) always matched the zygosity results previously obtained by PCR amplification and direct sequencing of the same samples.



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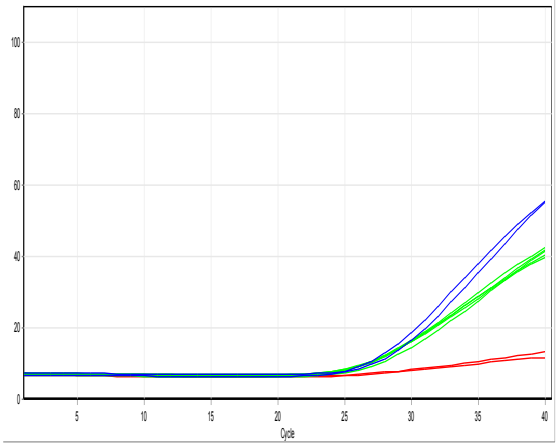

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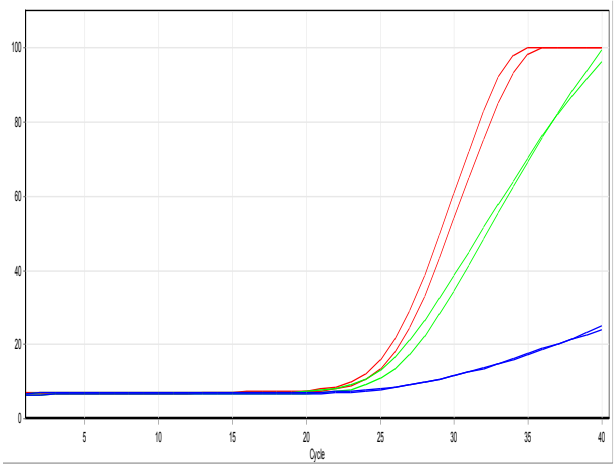

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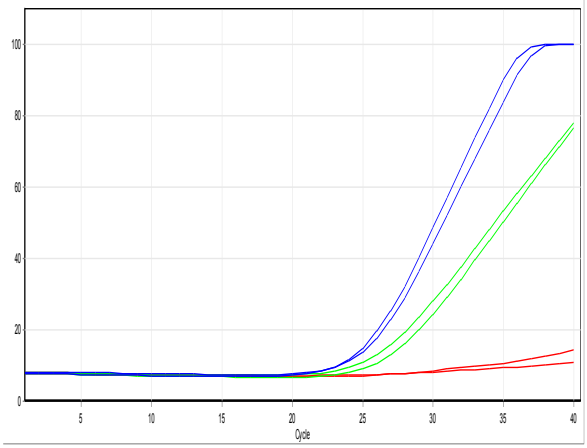

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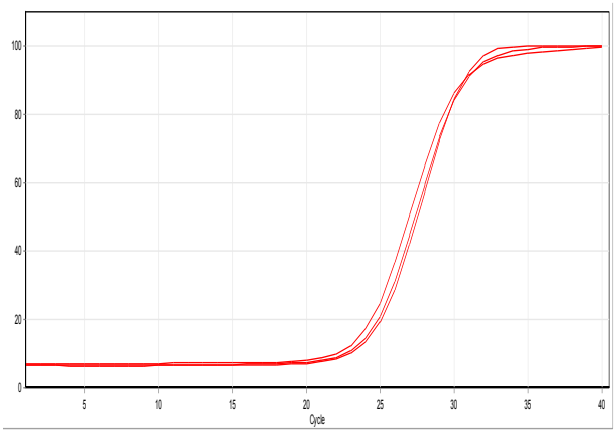

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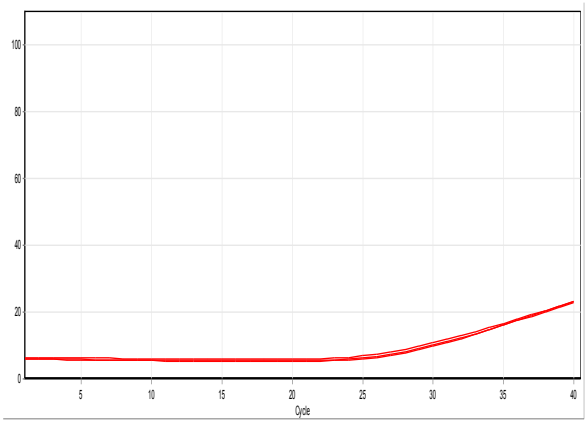


52

Figures 47 to 52: Examples of TaqMan detection for mutations T929I (A, B); M918T (C, D) and L1014F (E, F) for known genotypes from TA1 and TA2 strains using VIC (A, C, E) and FAM (B, D, F) probes. Wild type, mutant and heterozygous forms are indicated respectively with green, red and blue colours.

Once completed the optimization and validation of the assay as a reliable tool of allele's discrimination for the three studied mutations, the screening of the collection samples of *Tuta absoluta* was carried out. The results of the screening are presented, as frequencies of homozygous and heterozygous forms for each mutation, in table20.

During the gDNA extraction, the two samples 19 and 23 originating respectively from Chile and Uruguay have been mistakenly mixed up and were treated as a single sample.

Table20: Frequencies of homozygous and heterozygous forms for three mutations investigated for the sample collection of *T.absoluta* using TaqMan assays.

			L1014F			M918T			T929I		
Sample ID Nº	**Origin**	**Number of individulas tested**	**SS**	**RS**	**RR**	**SS**	**RS**	**RR**	**SS**	**RS**	**RR**
1	**Canary Islands**	**12**	0	0	100	17	50	33	33	50	17
2	**Canary Islands**	**10**	0	0	100	10	90	0	0	80	20
3	**Canary Islands**	**10**	0	0	100	30	70	0	10	60	30
4	**Canary Islands**	**8**	0	0	100	0	88	0	0	88	13
5	**Baleares Islands**	**3**	0	0	100	0	67	0	0	33	33
6	**Baleares Islands**	**3**	0	0	100	0	100	0	0	100	0
7	**Algeria**	**5**	0	0	100	40	60	0	0	40	20
8	**Algeria**	**5**	0	0	100	40	60	0	0	60	20
9	**Italy**	**3**	0	0	100	0	100	0	33	67	0
10	**Ecuador**	**11**	0	0	100	9	73	18	27	73	0
11	**Spain**	**8**	0	0	100	0	100	0	0	75	0
12	**Argentina**	**7**	0	0	100	100	0	0	0	0	100
13	**Argentina**	**8**	0	0	100	63	25	0	13	38	25
14	**Argentina**	**3**	0	0	100	0	100	0	0	100	0
15	**Italy**	**5**	0	0	100	60	40	0	0	40	60
16	**Canary Islands**	**7**	0	0	100	14	86	0	0	43	14
17	**Italy**	**14**	0	0	100	21	64	14	14	64	14
18	**Spain**	**6**	0	0	100	50	50	0	0	50	50
19	**Chile**	**8**	0	0	100	0	0	0	0	0	0
20	**Colombia**	**11**	0	82	18	0	91	9	9	91	0
21	**Greece**	**3**	0	0	100	33	33	0	33	33	0
22	**Spain**	**16**	0	0	100	50	50	0	0	38	63
23	**Uruguay**	**6**	0	0	100	0	0	0	0	0	0
24	**Peru**	**7**	0	0	100	14	71	14	14	71	14
25	**Spain**	**9**	0	0	100	22	67	11	33	44	22
26	**Spain**	**12**	0	0	100	25	50	25	25	50	17
27	**Portugal**	**5**	0	0	100	40	40	0	0	40	40
28	**Italy**	**17**	0	0	100	0	100	0	12	59	0
29a	**Israel**	**6**	0	0	100	17	83	0	17	67	17
29b	**Israel**	**6**	0	0	100	33	33	33	33	33	33

Interesting findings can be highlighted when analysing the results of TaqMan assays for the frequencies of different forms of zygosity for the three mutations in the sodium channel within the different samples. The widespread of kdr mutation (L1014F) among the

populations investigated is noteworthy. All samples showed a homozygous form of mutant allele for kdr except the sample Nº 20 originating from Colombia, where the heterozygous form is dominant. None of the populations showed the wildtype allele.

The three mutations are present simultaneously in all samples except one population from Argentina (sample 12), where only the kdr and T929I mutations are present. The two other mutations (M918T and T929I) exist dominantly in the heterozygous form for all the populations, except for the samples 12 and 15. The homozygous form of mutant allele of the mutation M918T seems to be the least frequent of all forms.

The fact that the mutations M918T and T929I never exist simultaneously as homozygous form in the same individual is extremely interesting. Similar findings have been already reported in the laboratory strains for the individuals investigated using direct sequencing.

No clear patterns neither in the frequencies of the mutations or in the zygosity forms could be detected between the two major areas of origin of the different samples, namely South America and European and Mediterranean region. Only two populations from South America showed particularities of carrying only two mutations (L1014F and T929I) for the Argentinean sample 12 and high dominance of the heterozygous form of resistance (L1014F) for the Colombian sample 20.

Regarding the two populations from Chile and Uruguay mistakenly mixed up and treated as single sample, the results were similar to other populations, with 100% of homozygous form for the kdr mutation and dominance of the heterozygous form for both the other mutations.

Finally, few tested samples showed ambiguous results and could not be assigned a clear form of zygosity. The percentage of these ambiguous samples was very low, scoring respectively 0, 3 and 9 % for L1014F, M918T and T929I mutations.

7.4. Discussion

In this study, TaqMan assays were developed, optimized and used for the first time to screen a large collection of samples of *T. absoluta* originated from different geographical areas for residue changes related to three mutations in the sodium channel gene.

The TaqMan assay is a PCR method employing probes with a fluorescent reporter dye and a quencher molecule. The parameters optimization of the PCR using template of known genotypes resulted in high sensitivity of TaqMan assays to discriminate between the wildtype and the mutant alleles for the three mutations investigated in the sodium channel.

The screening of a large collection of samples of *T. absoluta* showed a wide spread of the kdr (L1014F) mutation, generally coupled with two other mutations (M918T and T929I). This indicates the high selection pressure on the insect by the intensive use of pyrethroids in the past. It indicates also that the mutation occurred either very early after the first introduction in use of this class of insecticides and/or different mutations occurred in different localities in different dates. It surely shows the high propensity of the insect to mutate under insecticide use pressure.

It is also interesting how the two mutations M918T and T929I can coexist only in heterozygous forms, suggesting the existence of high fitness cost for the presence of the two mutations simultaneously in homozygous forms.

Early detection of insecticide resistance is of crucial importance for resistance management strategies. Different methodologies are available for detecting the DNA changes related to insecticides resistance but their cost, simplicity of use, rapidity and even reliability can be limiting factors for their use. The TaqMan assay is a real time PCR based method recently developed and optimized for use in insecticide resistance related mutations. Bass *et al.* (2007, 2008, 2010) used the TaqMan method for sensitive detection of mutations related to knock down resistance (kdr), insensitive acetylcholinesterase and resistance to dieldrin (Rdl) in mosquitoes vector of malaria. He reported that the real time TaqMan assays were the most sensitive (with the lowest number of failed reactions), the most specific (with

the lowest number of incorrect scores) than other methods and that they were quick to optimize and show the highest throughput of the assays being simple and quick to setup.

8 - Oxidative detoxification of insecticides

8.1. Introduction

P450s are an important and diverse class of hydrophobic and heme containing enzymes, with many functions ranging from biosynthesis to the metabolism of xenobiotics (Hemingway *et al.,* 2004; Feyereisen, 2006; Nelson, 2009). They are found in virtually all insect tissues (reviewed in Feyereisen, 1999).

The analysis of the available insect P450s sequences resulted in dividing them into four clades: CYP2 clade, CYP3 clade, CYP4 clade and mitochondrial CYP clade, with each one having different CYP families (Feyereisen, 2006). Berge *et al.* (2010) indicated that up to date insect P450s have been assigned to six CYP families, five are insect specific (CYP6, 9,12,18 and 28) and one (CYP4) is shared with other organisms.

The P450s have many functional similarities resulting from their common catalytic chemistry but their metabolic capabilities can be very divers (Schuler, 1996). Although their highly diverse array of protein sequences, the P450s share some characteristic structural features described by Werck-Reichhart and Feyereisen (2000) as follow: “The core of the protein is formed by helices, two sets of beta sheets and a meander. The conserved FXXGXXXCXG sequence of the heme-binding decapeptide contains a conserved cysteine that serves as a fifth ligant to the heme iron”. This domain is found on the C-terminus of a P450 protein. On the N-terminus, the DGXXT domain, which is associated with the formation of the oxygen-binding pocket of helix I, is needed to stabilize the core structure. Located between these two domains is the conserved EXXR pair, which corresponds to the proton transfer groove on the distal side of the heme (Figure 54).

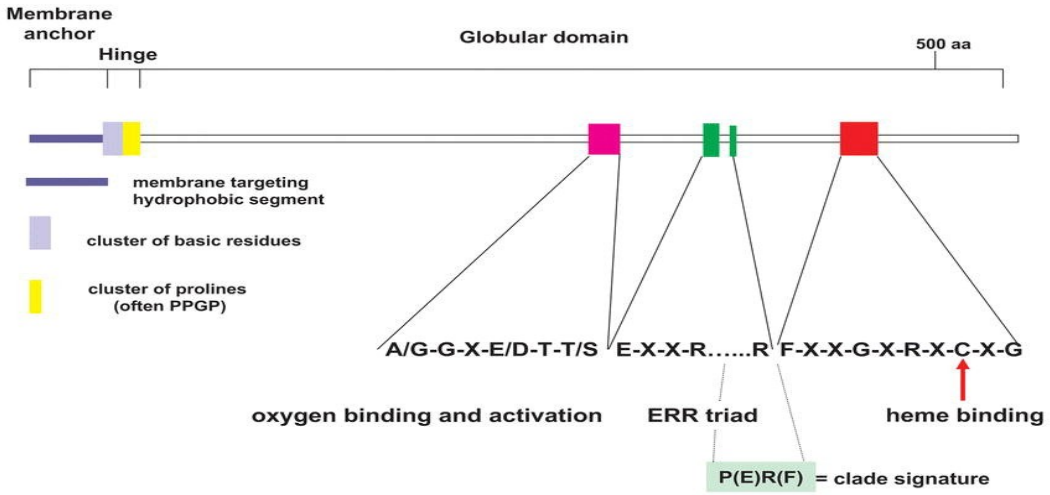


Figure 54: Characteristic structural features of the P450 proteins (from Werck-Reichhart *et al.,* 2002).

In insects, metabolic insecticide resistance is associated with increased levels of cytochrome P450s (monooxygenases), GSTs, esterases or a combination of more than one of them (Hemingway and Karunaratne, 1998; Huang *et al.,* 1998; Daborn *et al.,* 2002; Nikou *et al.,* 2003; Festucci-Buselli *et al.,* 2005). Synergism studies using piperonyl butoxide (PBO) that inhibits the cytochrome P450 suggest that P450-mediated detoxification is involved in insecticide resistance (Feyereisen *et al.,* 1999; Brooke *et al.*, 2001).

The role of P450 enzymes in insecticides resistance by enhanced detoxification was reported in many resistant species including: *Heliothis virescens* (Fabricius) (Rose *et al.,*1997), *Musca domestica* (Linnaeus) (Liu and Scott, 1998), *Anopheles mosquitoes* (Nikou *et al.,* 2003; Rodpradit *et al.,* 2005; Hemingway *et al.,* 2011 and references cited in; Stevenson *et al.,* 2011), *Blattella germanica* (Linnaeus) (Pridgeon *et al.*, 2003), *Drosophila melanogaster* (Meigen) (Daborn *et al.,* 2007), *Bemisia tabaci* (Gennadius) (Karunker *et al.,* 2008), *Helicoverpa armigera* (Hübner) (Song *et al.,* 2005; Zhang *et al.,* 2009; Brun-Barale *et al.,* 2010), *Myzus persicae* (Sulzer) (Puinean *et al.,* 2010) and *Nilaparvata lugens* (Stål) (Bass *et al.,* 2011). So far no works have been carried out for *T. absoluta*.

The present study aimed to firstly isolate some of P450 genes for *Tuta absoluta*, their characterisation and annotation. The aim of the current study did not include any expression investigation, due of the absence of a known susceptible strain.

8.2. Specific methods

The study of P450s can be based on different strategies, including the sequencing of the entire genome of the studied insect and the use of identified P450 from other insects as a probe to isolate new P450.

In this study, the PCR-based method was adopted for its speed and efficiency. The methodology is similar to the one used by Karunker *et al.* (2008) to investigate the over-expression of cytochrome P450 associated with the resistance to imidacloprid in *Bemisia tabaci*. He used degenerate primers designed according to the conserved amino acid sequences of CYP4 and CYP6 families of P450 genes. The degenerate reverse primer was based on the heme-binding motif (PFXXGXXXCXGXXF) region for both CYP families. The forward primer was designed within the family-specific 13-residues sequence of helix I (EVDTFMFEGHDTT) for the CYP4 family and conserved amino acids (FT/AL/IYEL/IA) downstream from the oxygen-binding motif AGXXT (helix I), provided the forward degenerate primer region for the CYP6 family. The sequences of the degenerate primers is shown in the table 21.

Table 21: Degenarate oligonucleotide primer sequences used to PCR amplify cDNA of CYP4 and CYP6 families of *Tuta absoluta*. All primers are shown 5′ to 3′.

Name	**Sequence**
CYP4F	TTY ATG TTY GAR GGI YAY GAY
CYP4R	AA YTT YTG ICC DAT RCA RTT
CYP6F	TTY RCI YTI TAY GAR YTI GC
CYP6R	RCA RTT YCK BGG ICC ITC ICC RAA

For each PCR reaction, 3 µl of template cDNA of *T. absoluta* was mixed with 1 µl of each degenerate primer (10 mM of concentration), 15 µl of TaqDNA polymerase and 15 µl of sterile distillate water.

The PCR conditions consisted of a first cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 46 °C for 1 min, 72 °C for 2 min and a final cycle for the extension at 72 °C for 10 min. PCR products of the expected size were purified from 1.2% Agarose gel using QIAquick gel extraction kit (Qiagen). To remove the excess of primers and nucleotides, a selective ethanol precipitation has been needed. For each 20 µl of purified PCR product, 40 µl of 4M NH4OAc and 200 µl of ethanol (100%) have been added, mixed well and microfuged for 20 minutes at room temperature; then the pellet was washed with200 µl of ethanol (75%) and left to dry, before being dissolved in sterile distilled water at 20ng/ µl.

After the ethanol precipitation, a 15 µl sequencing reaction was set up using 3 µl of BigDye mix (Applied Biosystems), 1 µl of primer 5 µl of PCR fragment, 1.5 µl of 5x sequencing buffer and 3.5 µl of sterile distilled water. The sequencing reaction was placed in thermal cycler under the following conditions: 96°C for 2 minutes, 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 60°C for 3.5 minutes.

The product was purified by adding to each sequencing reaction 35 µl of sterile distilled water, 5 µl of 3MNaOAc (pH 5.5) and 100 µl of ethanol (100%), followed by 20 minutes incubation at room temperature, 20 minutes of centrifugation. Then the pellet was washed with ethanol (75%) and dried thoroughly before adding 20 µl of deionised formamide (HiDi). The product was heated at 90°C for 2 minutes, chilled on ice and loaded onto 319/3100 sequencer.

The sequences obtained have been analysed using VectorNTI and compared to known sequences using the Blastx from NCBI (http://www.ncbi.nlm.nih.gov/).

8.3. Results

The PCR amplification, cloning and sequencing of the P450s of *Tuta absoluta* based on the method used by Karunker *et al*. (2004) resulted in more than 70 sequences. The alignment of these sequences using vector NTI resulted in 7 contigs (named from 1 to 7) and

2 singletons (named from 1 to 2). The size of these contigs and singletons varied between 324 and 699 kb.

The blastx of the contigs and singletons using NCBI website showed high similarities of the sequences with different P450s families belonging to different insect species. The similarities exceeded generally 60%. An example of the top hits similarities for the contig1 is shown in Table22.

Table 22: Top hits for the contig1 fragment of P450 of *Tuta absoluta* against different insects using BLASTX of NCBI (http://www.ncbi.nlm.nih.gov/).

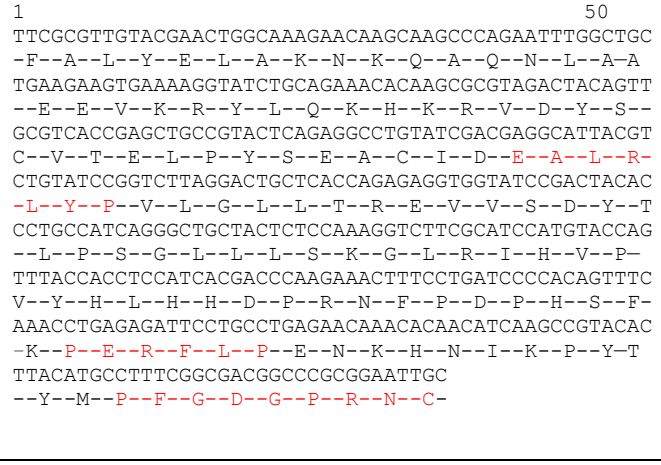
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
ACF17813.2	Induced Cytochrome P450 [***Ostrinia furnacalis***]	199	199	100%	7e-60	68%
ABB69054.1	Cytochrome P450 [***Helicoverpa armigera***]	193	193	100%	8e-58	68%
CBW30576.1	CYP6-1 protein [***Cnaphalocrocis medinalis***]	173	173	100%	3e-54	59%
NP_001104006.1	Cytochrome P450 [***Bombyx mori***]	182	182	100%	9e-54	64%
ADE05581.1	Cytochrome P450 6AE32 [***Manduca sexta***]	182	182	98%	2e-53	64%
AAP83689.1	Cytochrome P450 [***Depressaria pastinacella***]	182	182	100%	2e-53	63%
ABX64440.1	Cytochrome P450 CYP6AE9 [***Bombyx mandarina]***	171	171	100%	3e-49	58%

The predicted encoding proteins for all the contigs and singletons contained conserved domains characteristic of P450s such as the oxygen binding motif (helix I) ([A/G]GX[E/D]T[T/S]), the helix K motif (EXXRXXP), the heme-binding motif (PFXXGXXXCXG) and the motif (PXXFXP). The sequences of the contigs and singletons with the conserved characteristic motifs are given in figures 54 to 62. Contigs 1, 5, 6 and 7 do not contain the oxygen binding motif ([A/G]GX[E/D]T[T/S]) while contigs 2, 3, 4 and singletons 1 and 2 contained a variation at the start of the same motif where an L or E replaced the [A/G] respectively for the singleton 1 and the others.

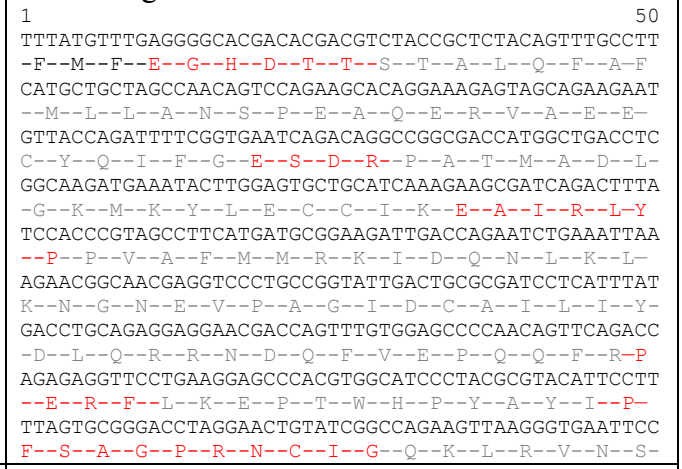
The results and analysis carried out using the NCBI website (http://www.ncbi.nlm.nih.gov/) and the P450 analysis website (http://p450.antibes.inra.fr/) revealed that the contigs and singletons belong to two CYP clades, namely CYP4 and CYP3 clades. Contigs 2, 3, 4 and singletons 1 and 2 belong to CYP4 clade and Contigs 1, 5, 6 and

7 belong to CYP3 clade and precisely to the family CYP6 (Contigs 1, 5 and 7) and the family CYP9 (contig6).

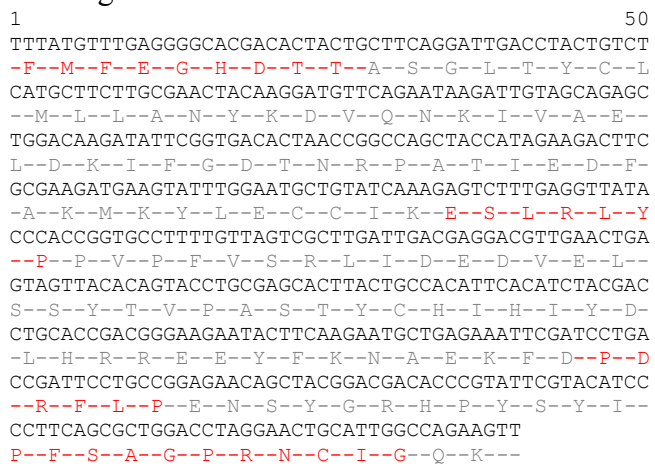
54: Contig1



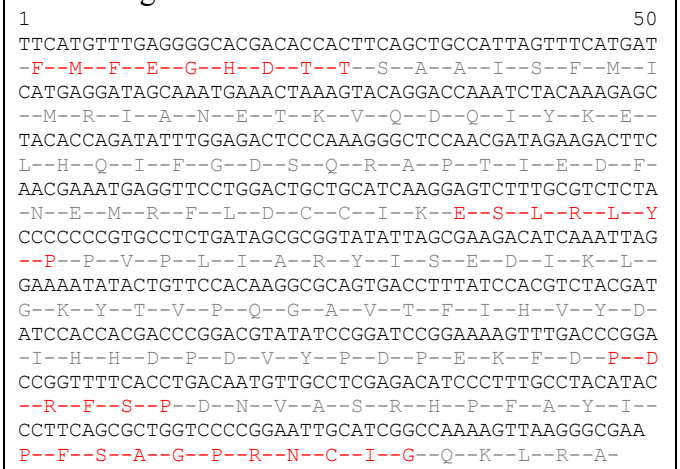
55: Contig2



56: Contig3



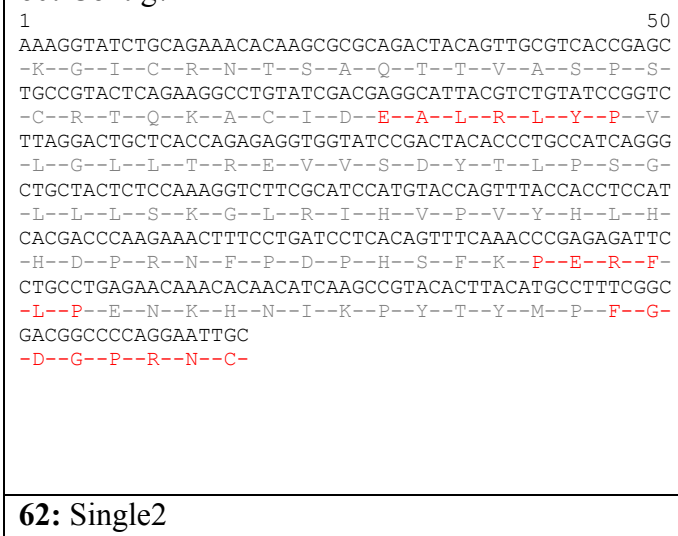
57: Contig4



58: Contig5

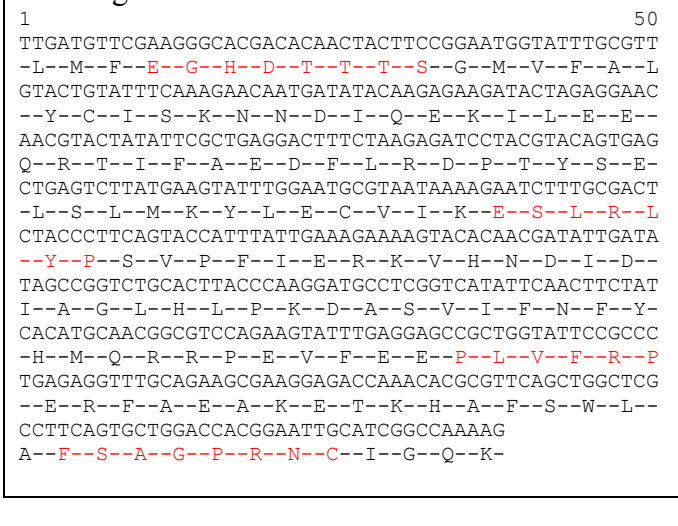
59: Contig6

60: Contig7



61: Single1

62: Single2



Figures 54-62: Sequences of contigs and singletons of P450s of *T. absoulta* with their predicted encoding proteins and the conserved motifs (in red).

A phylogenetic tree using the method of Neighbour-Joining (NJ) was constructed using Geneious Pro™ 5.5 software (Drummond *et al.,* 2011; http://www.geneious.com/**)** and the P450s library of *Bombyx mori* from the GeneBank database (NCBI; http://www.ncbi.nlm.nih.gov/) (Figure:63).

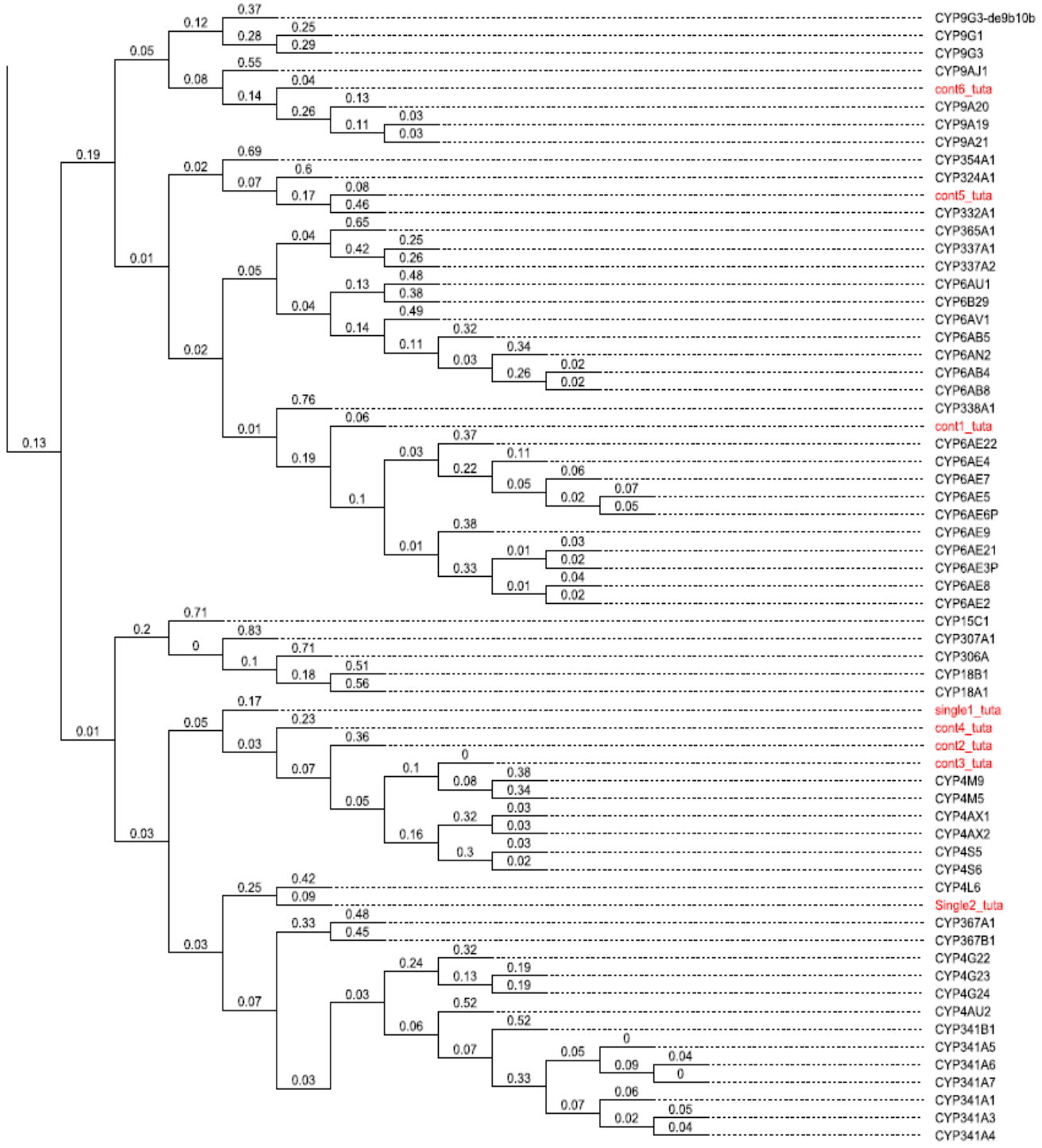


Figure 63: Phylogenetic relationship based on the amino acid sequence alignment of cytochrome P450s from various P450 families of *Bombix mori* and the sequences of contigs and singletons of *T. absoluta*.

8.4. Discussion

In this study, the PCR-based method was adopted to partially characterise for the first time some of P450s of *Tuta absoluta*. Seven contigs and 2 singletons were sequenced and were placed in two CYP clades (CYP3 and CYP4) using Neighbor-Joining method.

Cytochrome P450 is a wide and diverse class of enzymes with many biological functions. The up-regulated transcription of many P450 and mainly from the CYP6 family has been related to insecticides resistance. For example, the over-production of CYP6D1 and CYP6A2 were related respectively to resistance to pyrethroids and organophosphorus insecticides in *Musca domestica* (Dunkov et al., 1996; Kasai and Scott, 2000). In *Anopheles* mosquitoes, CYP6M2, CYP6P4 and CYP6P9 are associated with pyrethroids metabolic resistance (Chiu *et al.,* 2008; Wondji *et al.,* 2009; Matambo *et al.,* 2010; Stevenson *et al.,* 2011). Puinean *et al.,* (2010) reported that a cytochrome P450 close to the CYP6CY3 of *Acyrthosiphon pisum* (Harris) was related to neonicotinoids resistance in *Myzus percicae* (Sulzer). The same Cytochrome P450 has been previously reported to be implicated in neonicotinoid resistance in *Bemisia tabaci* (Gennadius) (Karunker *et al.,* 2008). A CYP6ER1 was highly over-expressed in resistant strains of *Nilaparvata lugens* (Stål) (Bass *et al.,* 2011).

Studying the mechanism of metabolic resistance in *Tuta absoluta* is still at its start. Up to date, no published information is available on P450 genes in this species. This study aimed at obtaining preliminary information on P450 gene families in *T. absoluta* but neither investigating their association with insecticides resistance nor giving them any official names. The identification of the particular P450 gene(s) associated with resistance in *T. absoluta* is important in understanding their role in insecticide resistance.

This information is therefore important because of the absence of cloned CYP genes for this insect, the impact of *T. absoluta* as an economic pest, and the different reports of insecticide control failures from different countries.

9 – General discussion and recommendations

The present study aimed to characterise mechanisms related to insecticide resistance in *Tuta absoluta* (Meyrick). Firstly, the susceptibility to insecticides of different strains has been investigated. Secondly two major insecticide targets were examined, using a variety of molecular and biochemical techniques, and thirdly a diagnosis tool has been developed and tested for early insecticide resistance detection.

The work has focused on different classes of insecticides, targeting different proteins in the insect nervous system, with special emphasis to two proteins widely targeted by pyrethroids and organophosphates: the voltage-gated sodium channel and the acetylcholinesterase enzyme, which were examined for possible mutations related to insecticide resistance.

This study demonstrated that insecticides resistance in *T. absoluta* may be caused by mutations similar to residue changes previously reported in different insecticide resistant insects.

A leaf dip methodology was used in this study. The method was recently recommended by Insecticide Resistance Action Committee (IRAC) and validated by Roditakis *et al.* (2011). The method proved to be robust with high levels of repeatability and reproducibility.

Although a known characterized susceptible strain was not available, the results of bioassays from this study showed high probability of occurrence of insecticide resistance to organophosphates, pyrethroids and more interestingly neonicotinoids, while the more recent classes of oxidiazine and anthranilic diamides are still providing good susceptibility of the pest.

Organophosphates and pyrethroids have been widely used for controlling different pests and many insect species have been reported as resistant for these two classes. They have been intensively used to control *T. absoluta* in its area of origin; hence the widespread

occurrence of resistance to these classes in *T. absoluta* populations seems to be predictable and even unavoidable.

The neonicotinoids are rarely recommended for use to control *Tuta absoluta* (Mallia, 2009), but have been intensively used to control a different pest, *Bemisia tabaci* (Gennadius), sharing tomato as host plant with *T. absoluta*. Resistance to neonicotinoids compounds in *B. tabaci* and other species has been reported (Nauen *et al.,* 2008). The occurrence of the same resistance in *T. absoluta*, yet to be confirmed, can be considered as a side effect result of the intensive use of this class of insecticides against *B. tabaci*.

The oxidiazine and anthranilic diamides are very recent classes of insecticides. They still provide high levels of control of *T. absoluta*. But recent reports (Gerson *et al.,* 2011; Roditakis *et al.*, 2011) indicated cases of a lesser efficacy to control *T. absoluta* with Indoxacarb, an oxidiazine targeting also sodium channel.

In this study, three amino acid replacements have been identified in the *para*-type sodium channel gene of five laboratory strains *T. absoluta*. The mutations L1014F, M918T and T929I have been previously reported to be implicated in pyrethroids resistance of several other insect species. A rare novel mutation (L925M) has also been found in two individuals. In addition, five laboratory strains carried a mutation A201S in the acetylcholinesterase gene. The highly conserved occurrence of the same mutations in a same single gene controlling the target site resistant phenotypes in different insect species refers to a monogenic resistance, where the insecticide target protein primarily needs to maintain its original function while carrying a limited number of mutations to decrease the efficacy of the insecticide. These mutations should bear very low fitness costs for the survival and normal functioning of the wildtype (Ffrench-Constant *et al.*, 2004).

Although the GA strain has been kept in the laboratory for few years without any insecticide selection pressure, it is still carrying the three mutations in the sodium channel and the one in the Ace gene, strongly confirming that these mutations have a low fitness cost at least in the laboratory conditions.

This study described two resistance mechanisms based on target site and no cross-resistance to other classes of insecticides with different target sites should be expected. But

it is possible that this two resistance mechanisms could act with other metabolic resistance mechanisms, including P450-dependent mono-oxygenase or esterase mediated detoxification, to give higher levels of resistance to organophosphate, pyrethroid or both. Obviously, more detailed investigation is needed to determine both the exact levels of resistance caused by mutations and the consequences of their high incidence in field populations of *Tuta absoluta*.

In this study, fluorescence PCR based TaqMan was designed for *T. absoluta* in order to be used for early detection of alleles conferring resistance in filed populations at low frequencies. After optimization, it proved to be a reliable, sensitive and robust tool for quick SNPs genotyping of a large number of individual carrying kdr type mutations. The ideal assay should be able to detect alleles conferring resistance in the heterozygous form in single insects (Soderlund, 1997). The developed TaqMan showed high ability to discriminate the different forms of alleles.

The results confirmed the widespread of the mutations within populations of *T. absoluta* from very varied geographic origins, encompassing the area of origin and the newly invaded European and Mediterranean area. It confirms also the hypothesis of a low cost fitness of the mutations. The results suggest either the possibility of original occurrence of the mutation in a single location, followed by a progressive invasion of neighbouring locations, or several independent occurrences in different locations in different dates. The later seems to be more likely, considering the high potential of reproduction and adaptation of the insect.

Although the TaqMan screening for the found Ace mutation was not yet complete when writing this thesis, the frequency patterns seem to be similar to the three kdr type mutations, suggesting a similar pathway of occurrence and spread of the mutation across different areas.

The low cost fitness and the widespread of the found residues substitutions coupled with the high reproductive potential and high propensity of adaptation of *T. absoluta* are serious concerns for pest control strategies, because not only the resistance alleles may persist in insect populations for long periods, even in the absence of strong insecticide

selection, but also the risks of appearance and development of new mutations and mechanism are very high.

It is very plausible that the continued use of insecticides may, and surely will, select for additional resistance related mutations and mechanisms, which may act in combination with the already found mutations to confer even higher levels of resistance.

To extend the life of insecticides that are currently providing high levels of efficacy in controlling *T. absoluta,* it is important to both investigate and characterise different target site mutations and other mechanisms related to resistance and monitor insect susceptibility to the insecticide, so that a prompt and rapid action can be taken when new tolerance or resistance is detected.

The early and sensitive detection of resistance is a prerequisite for the development of effective chemical use strategies aiming to minimise the selection pressure on *T. absoluta* and enhance the life of the different insecticides still providing good control of this insect pest. The use of mixes of two or more insecticides with different modes of action and/or rotation of several insecticides with different activities should be strictly respected for an effective management of *T. absoluta.*

A holistic approach in the management of T. absoluta is the only way to overcome the disadvantage of intensive use of insecticides. This approach should be based on Integrated Pest Management (IPM) principles, as promoted by the DIRECTIVE 2009/128/EC of the European parliament, where it is stated that:" *Member States shall take all necessary measures to promote low pesticide-input pest management, giving wherever possible priority to non-chemical methods, so that professional users of pesticides switch to practices and products with the lowest risk to human health and the environment among those available for the same pest problem. Low pesticide-input pest management includes integrated pest management as well as organic farming*".

IPM strategy should include all available practices and technologies starting with a good sanitation, the use of insect proof nets, the use of pheromones and light traps for both

monitoring and mass trapping, the use of biological agents and natural enemies and finally an adequate use of available insecticides.

This study aimed to characterise some of the aspects of insecticide resistance in *T. absoluta*. However, there are still a number of questions that could be addressed in future investigations:

1. More bioassays should be conducted on *T. absoluta* strains with different frequencies of the kdr and *Ace* mutations using a variety of pyrethroids, organophosphates and finding a susceptible strain is of crucial importance to validate all the findings. The resistance levels conferred by each mutation should be examined singly, then in combination with other mutations and mechanisms of resistance.

   The validity of the bioassay should be strengthened by conducting more bioassays in different conditions and using larger number of insecticides with different modes of action. The actual leaf dip method is labour intensive and time consuming; different variant of this method should be developed and investigated. Alternative methods are also to be considered particularly when the resistance mechanism studied is not age dependent and requiring the use of a particular stage of life of the insect. In this order, feeding assays and topical application may prove to be good alternatives.

2. The TaqMan assays developed in this study for all mutations in sodium channel and Ace genes are a useful tool for future studies examining the frequency of these mutations in field populations of *T. absoluta* and they could be extended to look at further populations, especially in areas where resistant populations to OP and/or pyrethroids have been reported.

3. The nicotinic receptors (nAChR) targeted by the nenicotinoids are worthy to investigate even though this class of insecticide is not widely used against *T. absoluta*.

4. This PhD study has focused on target site resistance in *T. absoluta* but future works need to examine possible metabolic mechanisms singly and in combination with the target site mechanisms characterized in this study. The work on the partial P450s can

be considered as a starting point for a more in depth investigations of monooxygenases related resistance in *T. absoluta*. Other works may investigate other metabolic mechanisms such as esterases and glutathione-S-transferases (GSTs).

In this order the transcriptome analysis of *T. absoluta* being carried out in Rothamsted Research (UK) is of particular interest to understand many of the resistance mechanisms.

References

Abd el-latif, A. O. Subrahmanyam, B. (2010). Pyrethroid resistance and esterase activity in three strains of the cotton bollworm, *Helicoverpa armigera* (Hübner). *Pesticide Biochemistry and Physiology*, 96, 155-159.

Abolmaaty S.M., Hassanein M.K., Khalil A.A., Abou-Hadid A.F. (2010) Impact of Climatic Changes in Egypt on Degree Day's Units and Generation Number for Tomato Leaf miner Moth *Tuta absoluta*, (Meyrick) (Lepidoptera gelechiidae). *Nature and Science*, 8, 11.

Acevedo, G. R., Zapater, M., Toloza, A. C. (2009) Insecticide resistance of house fly, *Musca domestica* (L.) from Argentina. *Parasitology research*, 105, 489-93.

Aguiar A. (2011) Pest management in vegetable production in "masseira" fields region. *IOBC/WPRS Bulletin,* 65, 33-36.

Ahmad, A. and arif, M. I. (2010) Resistance of beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae) to endosulfan, organophosphorus and pyrethroid insecticides in Pakistan. *Crop Protection*, 29, 1428-1433.

Ahmad, M. and Iqbal Arif, M. (2009) Resistance of Pakistani field populations of spotted bollworm *Earias vittella* (Lepidoptera: Noctuidae) to pyrethroid, organophosphorus and new chemical insecticides. *Pest management science*, 65, 433-9.

Ahmad, M., saleem, M. A. , sayyed, A. H. (2009) Efficacy of insecticide mixtures against pyrethroid- and organophosphate-resistant populations of *Spodoptera litura* (Lepidoptera: Noctuidae). *Pest management science*, 65, 266-74.

Ai J., Zhu, Y., Duan, J., Yu, Q., Zhang, G., wan, F., Xiang, Z. H. (2011) Genome-wide analysis of cytochrome P450 monooxygenase genes in the silkworm, *Bombyx mori*. *Gene*, 480, 42-50.

Aldridge W.N., (1950) Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl pnitrophenyl thiophosphate (E 605) and analogues, *J. Biochem*, 46, 451–460.

Alim Y. (2008) Les champs de tomates décimés par une maladie. El Watan. *Disponible sur Internet (2/12/08) algerie-infos-actualites.com/articles-109020.html.*

Alon M., Alon F., Nauen R., Morin S. (2008). Organophosphates resistance in the B-biotype of *Bemisia tabaci* (Hemiptera: Aleyrodidae) is associated with a point mutation in an ace1-type acetylcholinesterase and overexpression of carboxylesterase. *Insect Biochemistry and Molecular Biology*, 38, 940–949.

Alout H., Djogbenou, L., Berticat, C., Chandre, F. , Weill, M. (2008) Comparison of *Anopheles gambiae* and *Culex pipiens* acetycholinesterase 1 biochemical properties. Comparative biochemistry and physiology. *Part B, Biochemistry and molecular biology*, 150, 271-7.

Álvaro H., Siqueira A., Guedes R.N.C., Picanço M. C. (2000) Insecticide resistance in populations of *Tuta absoluta* (Lepidoptera: Gelechiidae), *Agricultural and Forest Entomology* , 2, 2, 147-153

Al-Zaidi S. (2009) Recommendations for the detection and monitoring of *Tuta absoluta*. American tomato moth. Available at: Available at: *http://www.fera.defra.gov.uk/showNews.cfm?id=402.*

Anazawa Y., Tomita, T., Aiki, Y., Kozaki, T., Kono, Y. (2003) Sequence of a cDNA

encoding acetylcholinesterase from susceptible and resistant two-spotted spider mite, *Tetranychus urticae*. *Insect biochemistry and molecular biology*, 33, 509-514.

Andrews M. C., Callaghan A., Field L. M., Williamson M. S., Moores G. D. (2004) Identification of mutations conferring insecticide-insensitive AChE in the cotton-melon aphid, *Aphis gossypii* Glover. *Insect Molecular Biology,* 13, 555-561.

Anstead J. A., Williamson M. S., Eleftherianos L., Denholm I. (2004) High-throughput detection of knockdown resistance in *Myzus persicae* using allelic discriminating quantitative PCR. *Insect Biochemistry and Molecular Biology,* 34, 871-877.

Apablaza J. (1992) La polilla del tomate y su manejo. *Tattersal 79*, 12-13.

Araujo R. A., Williamson M. S., Bass C., Field L. M., Duce I. R. (2011) Pyrethroid resistance in *Sitophilus zeamais* is associated with a mutation (T929I) in the voltage-gated sodium channel. *Insect Molecular Biology,* 20, 437-445.

Argentina S. (2009) Control of tomato moth. *Horticultura, Revista de Industria, Distribucion y Socioeconomia Horticola*, 54-56.

Arno J., Sorribas R., Prat M., Matas M., Pozo C., Rodriguez D., Garreta A., Gomez A., Gabarra R. (2009) *Tuta absoluta*, a new pest in IPM tomatoes in the northeast of Spain. *IOBC/WPRS Bulletin,* 49, 203-208.

Arno J., Sorribas R., Prat M., Montse M., Pozo C., Rodriguez D., Garreta A., Gomez A., Gabarra R. (2009) *Tuta absoluta*, a new pest in IPM tomatoes in the northeast of Spain. *IOBC/WPRSBull,* 49, 203–208.

Astor E., Scals D. D., de Scals D. (2009) The control of *Tuta absoluta* with insecticides compatible with integrated pest management programmes and the prevention of resistance. *Agricola Vergel: Fruticultura, Horticultura, Floricultura, Citricultura, Vid, Arroz,* 28, 492-495.

Avilla C., and González-Zamora, J. E. (2010) Monitoring resistance of *Helicoverpa armigera* to different insecticides used in cotton in Spain. *Crop Protection*, 29, 100-103.

Bacci L., Picando M. C., Sousa F. F., Silva E. M., Campos M. R., Tome´ H. V. T. (2008) Inimigos naturais da traca do tomateiro. *Hortic Bras* 26, 2808–2812.

Bahamondes L. A., Mallea A. R. (1969) Biologıa en Mendoza de *Scrobipalpula absoluta* (Meyrick) Povolny (Lepidoptera: Gelechiidae), especie nueva para la Repu´blica Argentina. *Rev Fac Cs Agrarias* 15, 96–104.

Bai D. L., Lummis S. C. R., Leicht W., Breer H., Sattelle D. B. (1991) Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor-neuron. *Pesticide Science*, 33, 197-204.

Baniameri V(2011) The current status of *Tuta absoluta* in Iran in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of Tuta absoluta (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18

Barrientos Z .R., Apablaza H .J., Norero S .A., Estay P. P. (1998) Threshold temperature and thermal constant for development of the South american tomato moth, *Tuta absoluta* (Lepidoptera: Gelechiidae). *Ciencia e Investigacion Agraria,* 25, 133-137.

Barrientos Z. R., Apablaza H. J., Norero S. A., Estay P. P. (1998) Threshold temperature and thermal constant for development of the South American tomato moth, *Tuta absoluta* (Lepidoptera, Gelechiidae). *Ciencia e Investigacion Agraria* 25, 133-137.

Bass C., carvalho, R. A., oliphant, L., puinean, A. M., field, L. M., nauen, R., williamson, M. S., moores, G. , gorman, K. (2011) Overexpression of a cytochrome P450 monooxygenase, CYP6ER1, is associated with resistance to imidacloprid in the

brown planthopper, *Nilaparvata lugens*. *Insect molecular biology*.

Bass C., D. Nikou., J. Vontas., M. S.Williamson., Field L.M. (2010) Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (ace −1R) in *Anopheles gambiae*, *Pesticide Biochemistry and Physiology*, 96, 2, 80–85.

Bass C., Lansdell, S. J., Millar, N. S., Schroeder, I., Turberg, A., Field, l., Williamson, M. S. (2006) Molecular characterisation of nicotinic acetylcholine receptor subunits from the cat flea, *Ctenocephalides felis* (Siphonaptera : Pulicidae). *Insect Biochemistry and Molecular Biology*, 36, 86-96.

Bass C., Nikou D., Blagborough A. M. (2008) PCR-based detection of Plasmodium in *Anopheles* mosquitoes: a comparison of a new high-throughput assay with existing methods, *Malaria Journal*, vol. 7, article no. 177.

Bass C., Nikou D., Donnelly M. J. (2007) Detection of knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods, *Malaria Journal*, vol. 6, article no. 111.

Bass C., Nikou D., Donnelly M. J., Williamson M. S., Ranson H., Ball A., Vontas J., Field L. M. (2007) Detection of knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. *Malaria Journal,* 6.

Bass C., Nikou, D., Vontas, J., Williamson, M. S., Field, L. M. (2010) Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (ace-1R) in *Anopheles gambiae*. *Pesticide Biochemistry and Physiology,* 96, 80-85.

Bass C., Schroeder I., Turberg A., Field L. M., Williamson M. S. (2004) Identification of mutations associated with pyrethroid resistance in the para-type sodium channel of the cat flea, *Ctenocephalides felis*. *Insect Biochemistry and Molecular Biology,* 34, 1305-1313.

Bass C., Schroeder, I., Turberg, A., Field, L. M., Williamson, M. S. (2004b) Identification of the Rdl mutation in laboratory and field strains of the cat flea, *Ctenocephalides felis* (Siphonaptera : Pulicidae). *Pest Management Science,* 60, 1157-1162.

Bass C., Williamson M. S., Field L. M. (2008) Development of a multiplex real-time PCR assay for identification of members of the *Anopheles gambiae* species complex, *Acta Tropica*, vol. 107, 1, 50–53,.

Bass C., Williamson M. S., Wilding C. S., Donnelly M. J., Field L. M. (2007) Identification of the main malaria vectors in the *Anopheles gambiae* species complex using a TaqMan real-time PCR assay, *Malaria Journal*, vol. 6, 155.

Baxter G. D., Barker S.C. (1998) Acetylcholinesterase cDNA of the cattle tick, *Boophilus microplus*: characterisation and role in organophosphate resistance. *Insect Biochem. Mol. Biol*. 28, 581–589.

Becerra V. C., Lombardich J., Gonzalez Maldonado D. J. (1999) *Scrobipalpuloides absoluta* (Meyrick) Povolny: Efficiency of insecticides in its control. *Revista de la Facultad de Ciencias Agrarias Universidad Nacional de Cuyo,* 31, 1-6.

Bell C. A., Williamson M. S., Denholm I., Devonshire A. L. (1995) Restriction fragment length polymorphism analysis of a sodium-channel gene locus in susceptible and knockdown-resistant house flies, *Musca domestica*. *In: ACS Symposium Series; Molecular action of insecticides on ion channels*, 86-96.

Benddine F. (2011) IPM Strategy: A case of *Tuta absoluta* in Algeria in

EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of Tuta absoluta (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18

Bentancourt C. M., Scatoni I. B. (1995) Description of the development stages of the "tomato borer", *Scrobipalpuloides absoluta* (Meyrick) (Lep., Gelechiidae). *Boletin de Investigacion - Facultad de Agronomia, Universidad de la Republica*, 1-14.

Benting J. and Nauen, R. (2004) Biochemical evidence that an S431F mutation in acetylcholinesterase-1 of *Aphis gossypii* mediates resistance to pirimicarb and omethoate. *Pest management science*, 60, 1051-5.

Benvenga S. R., Fernandes O. A., Gravena S. (2007) Decision making for integrated pest management of the South American tomato pinworm based on sexual pheromone traps. *Hortic Bras* 25, 164–169.

Berge J. B., Feyereisen R., Amichot M. (1998) Cytochrome P450 monooxygenases and insecticide resistance in insects. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences,* 353, 1701-1705.

Betancourt C. M., Scatoni I. B., Rodriguez J. J. (1996) Influencia de la temperatura sobre la reproduccion y el desarrollo de Scrobipalpuloides absoluta (Meyrick) (Lepidoptera: Gelechiidae). *Rev Bras Biol* 56, 661–670.

Branco M. C., Franca F. H. (1996) Interference of sunlight and precipitation on the efficacy of abamectin and cartap in controlling *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Anais da Sociedade Entomologica do Brasil,* 25, 489-494.

Branco M. C., Franca F. H., Fontes R. R. (1996) Relative efficacy of insecticides in mixture with mineral oil on the economic threshold of the Brazilian tomato pinworm. *Horticultura Brasileira,* 14, 36-38.

Branco M. C., Pontes L. A., Amaral P. S. T., Mesquita Filho M. V. (2003) Insecticides for the control of the South American tomato pinworm and the corn earworm and impact of those products on *Trichogramma pretiosum. Horticultura Brasileira,* 21, 652-654.

Brooke B. D. (2008) kdr: can a single mutation produce an entire insecticide resistance phenotype? *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 102, 524-5.

Brooke B. D., Kloke G., Hunt R. H., Koekemoer L. L., Temu E. A., Taylor M. E., Small G., Hemingway J., Coetzee M. (2001) Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). *Bull Entomol Res*, 91, 265–272.

Brooke B. D., Kloke, G., Hunt, R. H., Koekemoer, L. L., Tem, E. A., Taylor, M. E., Small, G., Hemingway, J. and Coetzee, M. (2007) Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). *Bulletin of Entomological Research,* 91, 265-272.

Brown A. W. A. (1971) Pest resistance to pesticides, in "Pesticides in the Environment" (R. White- Stevens, Ed.), Vol. 1, Part II. pp. 457-552. Dekker, New York.

Brun-Barale A., Hema O., Martin T., Suraporn S., Audant P., Sezutsu H., Feyereisen R. (2010) Multiple P450 genes overexpressed in deltamethrin-resistant strains of *Helicoverpa armigera*. *Pest Management Science,* 66, 900-909.

Bues R., Bouvier, J. , Boudinhon, L. (2005) Insecticide resistance and mechanisms of resistance to selected strains of (Lepidoptera: Noctuidae) in the south of France. *Crop Protection,* 24, 814-820.

Buhl O., Falck P., Karsholt O., Larsen K., Vilhelmsen F. (2010) Records of

microlepidoptera from Denmark in 2009 (Lepidoptera). *Entomologiske Meddelelser,* 78, 101-116.

Burton M. J., Mellor I. R., Duce I. R., Davies T. G. E., Field L. M., Williamson M. S. (2011) Differential resistance of insect sodium channels with kdr mutations to deltamethrin, permethrin and DDT. *Insect Biochemistry and Molecular Biology,* 41, 723-732.

Busvine, J. R. (1951) Mechanism of resistance to insecticide in houseflies. *Nature*, 168, 193-195.

Byrne F.J. and Devonshire A.L. (1993) Insensitive acetylcholinesterase and esterase polymorphism in susceptible and resistant populations of the tobacco whitefly *Bemisia tabaci* (Genn). *Pest. Biochem. Physi*, 45, 34-42.

Cabello, T., Gallego, J.R., Vila, E., Soler, A., Pino, M. del., Carnero, A., Hernández-Suárez, E.and Polaszek A. (2009) Biological control of the South American Tomato Pinworm,*Tuta absoluta* (Lep.: Gelechiidae), with releases of *Trichogramma achaeae* (Hym.:Trichogrammatidae) on tomato greenhouse of Spain. *Published in http://www.tutacontrol.com/ accessed 25 of September.*

Caceres S. (1992) La polilla del tomate en Corrientes. Biologıa y control. *Estacion Experimental Agropecuaria Bella Vista, INTA,* 19.

Cáceres S. (2000) La polilla del tomate: Manejo químicocultural. Hoja de Divulgación 15. *Estación Experimental Agropecuaria Bella Vista, INTA*, 5.

Caceres S. (2007) Manejo de la polilla del tomate en Corrientes. Hoja de Divulgacion No 32. Estacion Experimental Agropecuaria Bella Vista. *Instituto Nacional de Tecnologı´a Agropecuaria, Bella Vista, Argentina.*

Calvo F. J., Belda J. E., Gimenez A. (2010) Una estrategia para el control biologico de mosca blanca y *Tuta absoluta* en tomate. *Phytoma Espana* 216, 46–52.

Caponero A. (2009) Solanacee, rischio in serre. Resta alta l'attenzione alla tignola del pomodoro nelle colture protette. *Colture Protette* 10, 96–97.

Cassanelli S., Reyes M., Rault M., Manicardi G. C., Sauphanor B. (2006) Acetyleholinesterase mutation in an insecticide-resistant population of the codling moth *Cydia pomonella* (L.). *Insect Biochemistry and Molecular Biology,* 36, 642-653.

Castelo Branco M., Franca F. H., Medeiros M. A., Leal J. G. T. (2001) Use of insecticides for controlling the South American tomato pinworm and the diamondback moth: a case study. *Horticultura Brasileira,* 19, 60-63.

Castrillón M. G., and G. V. Cambra. (2009) *Tuta absoluta*, Tomato leaf miner moth or South American tomato moth. Ministry of Agriculture, Nature and Food Quality (LVN) Plant Protection Service of the Netherlands.

Cestele S. and Catterall. W.A. (2000) Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie*, 82:883–892.

Chang C., Shen, W. K., Wang, T. T., Lin, Y. H., Hsu, E. L. , Dai, S. M. (2009). A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in *Aedes aegypti*. *Insect biochemistry and molecular biol*ogy, 39, 272-8.

Changeux J. P. and Edelstein S . J. (1998) Allosteric receptors after 30 years. *Neuron*, 21, 959-980.

Chen Z.Z., Newcomb. R., Forbes. E., McKenzie J., and Batterham P. (2001) The acetylcholinesterase gene and organophosphorous resistance in the Australian sheep

blowfly, *Lucilia cuprina*. *Insect Biochem Mol Biol*, 31: 805–816.
Cifuentes D., Chynoweth R., Bielza P. (2011) Genetic study of Mediterranean and South American populations of tomato leafminer *Tuta absoluta* (Povolny, 1994) (Lepidoptera: Gelechiidae) using ribosomal and mitochondrial markers. *Pest Management Science,* 67, 1155-1162.
CIP. (1996) Major Potato Diseases, Insects, and Nematodes , 3rd edn. Centro Internacionalde la Papa, Lima (PE).
Clarke J. F. (1962) New species of microlepidoptera from Japan. *Entomol News* 73, 102.
Coelho M. C. F., França F. H. (1987) Biologia e quemotaxia da larva e descricao da pupa e adulto da trac¸a-do-tomateiro. *Pesqui Agropecu Bras* 22, 129–135.
Collavino M. D., Gimenez R. A. (2008) Efficacy of imidacloprid to control the tomato borer *Tuta absoluta* (Meyrick). *Idesia,* 26, 65-72.
Cordova, D., Benner, E., Sacher, M., Rauh, J., Sopa, J., Lahm, G., Selby, T., Stevenson, T., Flexner, L. , Gutteridge, S. (2006) Anthranilic diamides: A new class of insecticides with a novel mode of action, ryanodine receptor activation. *Pesticide Biochemistry and Physiology,* 84, 196-214.
Corringer P. J., Le Novere N., Changeux J. P. (2000) Nicotinic receptors at the amino acid level. *Annual Review of Pharmacology and Toxicology*, 40, 431-458.
Cousin X., Strahle U., Chatonnet A. (2005) Are there non-catalytic functions of acetylcholinesterases? Lessons from mutant animal models. *Bioessays*, 27, 189–200.
Cui F., Qu H., Cong J., Liu X. L., Qiao C. L. (2007a) Do mosquitoes acquire organophosphate resistance by functional changes in carboxylesterases? *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 21, 3584-91.
Cui F., Raymond M., Qiao C. L. (2006) Insecticide resistance in vector mosquitoes in China. *Pest management science*, 62, 1013-22.
Cui F., Weill M., Berthomieu A., Raymond M., Qiao C. L. (2007b) Characterization of novel esterases in insecticide-resistant mosquitoes. *Insect biochemistry and molecular biology,* 37, 1131-7.
Daborn P. J., Lumb, C., Boey, A., Wong, W., Ffrench-Constant, R. H. , Batterham, P. (2007) Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic over-expression. *Insect biochemistry and molecular biology,* 37, 512-9.
Dandria D., Catania A. (2009) *Tuta absoluta* (Povolny, 1994), an important agricultural pest in Malta (Lepidoptera: Gelechiidae). *Bulletin of the Entomological Society of Malta,* 2, 57-60.
Danielson P.B., MacIntyre R.J., Fogleman J.C. (1997) Molecular cloning of a family of xenobiotic-inducible drosophilid cytochrome P450s: evidence for involvement in host-plant allelochemical resistance. *Proc. Natl. Acad. Sci*, 94, 10797–10802.
Darbon P.J., Yen J.L., Bogwitz M.R., Le Goff G., Feil E., Jeffers S., Tijet N., Perry T., Heckel D., Batterham P., Feyereisen R., Wilson T.G., Ffrench-Constant R.H. (2002) A single P450 allele associated with insecticide resistance in *Drosophila*. *Science*, 27: 2253-2256.
Davies T. G. E., Field L. M., Usherwood P. N. R., Williamson M. S. (2007a) A comparative study of voltage-gated sodium channels in the Insecta: implications for pyrethroid resistance in *anopheline* and other neopteran species. *Insect Molecular Biology,* 16, 361-375.

Davies T. G. E., Field L. M., Usherwood P. N. R., Williamson M. S. (2007b) DDT, pyrethrins, pyrethroids and insect sodium channels. *Iubmb Life,* 59, 151-162.

Decoin M. (2010) Tomate, *Tuta absoluta* touche a` l'Atlantique. *Phytoma* 631, 44–45.

Desneux N., Wajnberg E., Wyckhuys K. A. G., Burgio G., Arpaia S., Narvaez-Vasquez C. A., Gonzalez-Cabrera J., Catalan Ruescas D., Tabone E., Frandon J., Pizzol J., Poncet C., Cabello T., Urbaneja A. (2010) Biological invasion of European tomato crops by *Tuta absoluta*: ecology, geographic expansion and prospects for biological control. *Journal of Pest Science,* 83, 197-215.

Devine G. J., and Denholm I. (2009) Insecticide and Acaricide Resistance in *Encyclopedia of Insects* (Second Edition), 505-511.

Devonshire A. L., and Moores G. D. (1984) Different forms of insensitive acetylcholinesterase in insecticide-resistant houseflies (*Musca domestica*). *Pestic. Biochem. Physiol*, 21, 341-348.

Devonshire A. L., Field L. M., Foster S. P., Moores G. D., Williamson M. S., Blackman R. L. (1998) The evolution of insecticide resistance in the peach-potato aphid, *Myzus persicae*. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 353, 1677-1684.

Devonshire, A. (2003) Kinetic efficiency of mutant carboxylesterases implicated in organophosphate insecticide resistance. *Pesticide Biochemistry and Physiology,* 76, 1-13.

Diabate A., Baldet T., Chandre F., Akogbeto M., Guiguemde T. R., Darriet F., Brengues C., Guillet P., Hemingway J., Small G. J., Hougard J. M. (2002) The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae* SL in Burkina Faso. *American Journal of Tropical Medicine and Hygiene,* 67, 617-622.

Dolores Rodriguez M. (2007) *Tuta absoluta* (Meyrick), an enemy that wants to conquer the Old Continent. *Horticultura Internacional,* 14, 22-23.

Dong K. (2007). Insect sodium channels and insecticide resistance. Invertebrate *neuroscience : IN,* 7, 17-30.

Dong K., Valles S. M., Scharf M. E., Zeichner B., Bennett G. W. (1998) The knockdown resistance (kdr) mutation in pyrethroid-resistant German cockroaches. *Pesticide Biochemistry and Physiology,* 60, 195-204.

Dunkov B. C., RodriguezArnaiz R., Pittendrigh B., FfrenchConstant R. H., Feyereisen R. (1996) Cytochrome P450 gene clusters in *Drosophila melanogaster*. *Molecular and General Genetics,* 251, 290-297.

Dunkov B.C., Guzov V.M., Mocelin G., Shotkoski F., Brun A., Amichot M., Ffrench-Constant R.H., Feyereisen R. (1997) The Drosophila cytochrome P450 gene Cyp6a2: structure, localization, heterologous expression, and induction by phenobarbital. *DNA Cell Biol* 16, 1345–1356.

Elbert A., Haas, M., Springer, B., Thielert, W. *,* Nauen R. (2008) Applied aspects of neonicotinoid uses in crop protection. *Pest management science,* 64, 1099-105.

Eldefrawi A.T. (1985) Acetylcholinesterases and anticholinesterases.Vol. 12, Pergamon, New York.

Eldefrawi M.E., Miskus R., Sutcher V. (1960) Methylenedioxyphenyl derivatives as synergists for carbamate insecticides on susceptible, DDT- and parathion-resistant house flies. *J. Econ. Entomol*, 53, 231–234.

Eleftherianos I., Foster, S. P., Williamson, M. S. *,* Denholm I. (2008) Characterization of the M918T sodium channel gene mutation associated with strong resistance to

pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer). *Bulletin of Entomological Research,* 98, 183-91.
Elliot M., Farnham A.W., Janes N.F., Needham P.H., Pulman D.A. (1973) Potent pyrethroid insecticides from modified cyclopropan acids. *Natur*e, 244, 456.
Ellmann G.L., Courtney K.D., Anders V.Jr., Featherstone R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*, 7, 88–95.
Enayati A. A., Vatandoost H., Ladonni H., Townson H., Hemingway J. (2003) Molecular evidence for a kdr-like pyrethroid resistance mechanism in the malaria vector mosquito *Anopheles stephensi*. *Medical and Veterinary Entomology,* 17, 138-144.
Enayati A.,Hemingway J. (2006) Pyrethroid insecticide resistance and treated bednets efficacy in malaria control. *Pesticide Biochemistry and Physiology,* 84, 116-126.
EPPO (2005) Data sheets on quarentine pests: *Tuta absoluta* .
EPPO (2008b) Additional information provided by Spain on EPPO A1 pests.
EPPO (2008c) First record of *Tuta absoluta* in Algeria. EPPO reporting service 2008/135.
EPPO (2008d) First record of *Tuta absoluta* in Morocco. EPPO reporting service 2008/174.
EPPO (2009) EPPO *Reporting service—Pest and Diseases. No 8, Paris, 2009-08-01.*
EPPO (2009a) First report of *Tuta absoluta* in France. EPPO reporting service 2009/003.
EPPO (2009b) First report of *Tuta absoluta* in Tunisia. EPPO reporting service 2009/042.
EPPO (2009c) *Tuta absoluta* reported for first time from Lazio region Italy.
EPPO (2009d) *Tuta absoluta* reported from Abruzzo, Liguria and Umbria regions Italy.
EPPO (2010) EPPO *Reporting service—Pest and Diseases. No 1, Paris, 2010-01-01.*
Erler F., Can M., Erdogan M., Ates A. O., Pradier T. (2010) New Record of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) on Greenhouse-Grown Tomato in Southwestern Turkey (Antalya). *Journal of Entomological Science,* 45, 392-393.
Espinosa B., Sannino L. (2009) *Tuta, Keiferia and Phthorimaea*, grape berry moths which need to be controlled. *Informatore Agrario,* 65, 56-58.
Estay P. (2000) Polilla del tomate *Tuta absoluta* (Meyrick). *Informativo La Platina 9,* 1-4.
Farenhorst, M. Mouatcho, J. C. Kikankie, C. K. Brooke, B. D. Hunt, R. H. Thomas, M. B. Koekemoer, L. L. Knols, B. G., Coetzee M. (2009) Fungal infection counters insecticide resistance in African malaria mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America,* 106, 17443-7.
Fernandez S. and Montagne A. (1990a) Preferencia de oviposición de las hembras y duración, crecimiento y sobrevivencia de las larvas de *Scrobipalpula absoluta* (Meyrick) (Lepidoptera:Gelechiidae) en diferentes solanáceas. Boletín de Entomología Venezolana 5(13), 100-106.
Fernandez S. and Montagne A. (1990b) Biología del minador del tomate, Scrobipalpula absoluta(Meyrick) (Lepidoptera: Gelechiidae). *Boletín de Entomología Venezolana* 5(12), 89-99.
Fernandez S., Montagne A. (1990) Biologıa del minador del tomate, *Scrobipalpula absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Bol Entomol Venez* 5, 89–99.
Festucci-Buselli R.A., Carvalho-Dias A.S., de Oliveira-Andrade M., Caixeta- Nunes C., Li H.M., Stuart J.J., Muir W., Scharf M.E., Pittendrigh B.R. (2005) Expression of Cyp6g1 and Cyp12d1 in DDT resistant and susceptible strains of *Drosophila melanogaster*. *Insect Molecular Biology*,14, 69-77.
Feyereisen R. (1995a) Molecular biology of insecticide resistance. *Toxicology Letters,* 82-3, 83-90.

Feyereisen R. (1995b) P450 mediated resistance in the house fly. *Journal of Cellular Biochemistry Supplement,* 0, 192.
Feyereisen R. (1996) Insect cytochrome P450: Pesticides' boon, bane and bounty. *Abstracts of Papers American Chemical Society,* 211, 19.
Feyereisen R. (1999) Insect P450 enzymes. *Ann. Rev. Entomol*, 44, 507–533.
Feyereisen R. (2006) Evolution of insect P450, *Biochem Soc Trans*, 34, 1252–1255.
Feyereisen R. (2011) Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochimica Et Biophysica Acta-Proteins and Proteomics,* 1814, 19-28.
Feyereisen R., Andersen J. F., Carino F. A., Cohen M. B., Koener J. F., Longbottom J. M., Snyder M. J., Unnithan G. C. (1993) Insect cytochrome P450 on the pathway to pesticide discovery. *Abstracts of Papers American Chemical Society,* 206, 72.
Feyereisen R., Andersen J.F., Carino F.A., Cohen M.B., Koener J.F. (1995) Cytochrome P450 in the house fly: structure, catalytic activity and regulation of expression of CYP6A1 in an insecticide resistant strain. *Pestic. Sci*, 43, 233–239.
Feyereisen R., Baldridge G.D., Farnsworth D.E. (1985) A rapid method for preparing insect microsomes. Comp. Biochem. Physiol, 32B, 559–562.
Feyereisen R., Koener J.F., Farnsworth D.E., Nebert D.W. (1989) Isolation and sequence of cDNA encoding a cytochrome P-450 from an insecticide-resistant strain of the house fly, *Musca domestica*. *Proc. Natl. Acad. Sci* USA, 86, 1465–1469.
Feyereisen, R. (2011) Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochimica et biophysica acta,* 1814, 19-28.
Ffrench-constant R.H., Daborn P.J. Le Goff G. (2004) The genetics and genomics of insecticide resistance. Trends in Genetics, 20, 163-170.
Ffrench-Constant R.H., Pittendrigh B., Vaughan A., Anthony N. (1998) Why are there so few resistance-associated mutations in insecticide target genes? *Philos. Trans. Roy. Soc.* Lond, B 353,1685–1693.
Ffrench-Constant, R. H. (2007) Which came first: insecticides or resistance? *Trends in genetics : TIG,* 23, 1-4.
Field L. M., Devonshire A. L., and Forde B. G. (1988) Molecular evidence that insecticide resistance in peachpotato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochem. J*, 251, 309-312.
Flores L. V., Gilardon E., Gardenal C. N. (2003) Genetic structure of populations of *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae). *BAG Journal of Basic and Applied Genetics,* 15, 47-54.
Forgash A. J. (1984) History, Evolution, and Consequences of Insecticide Resistance, *Pesticide Biochemistry and Physiology*, 22, 178-186.
Fournier D. (2005) Mutations of acetylcholinesterase which confer insecticide resistance in insect populations. *Chemico-Biological Interactions,* 157, 257-261.
Fournier D., and Mutero A. (1994) Modification of acetylcholinesterase as a mechanism of resistance to insecticides, *Comp. Biochem. Physiol*. 108C, 19–31.
Fournier D., Bride J.M., Hoffmann F., Karch F. (1992) Acetylcholinesterase. Two types of modifications confer resistance to insecticide, *J. Biol. Chem*, 267, 14270–14274.
Fournier D., Karch F., Bride J. M., Hall L. M. C., Berge J. B., Spierer P. (1989) *Drosophila melanogaster* acetylcholinesterase gene, structure evolution and mutations. *J. Mol. Evol*,210, 15–22.

Gabarra R. and Arno´ J. (2010) Resultados de las experiencias de control biologico de la polilla del tomate en cultivo de invernadero y aire libre en Catalun˜a. *Phytoma Espana* 217, 65–68.

Galzi J. L. and Changeux J. P. (1994) Neurotransmitter-gated ion channels as unconventional allosteric proteins. *Current Opinion in Structural Biology*, 4, 554-565.

Garcia M. F., Espul J. C. (1982) Bioecologıa de la polilla del tomate (Scrobipalpula absoluta) en Mendoza, Republica Argentina. *Rev Invest Agropecuarias INTA (Argentina)* 18, 135–146.

Georghiou G. P. (1980) Insecticide resistance and prospects for its management, *Residue Rev,* 48, 131.

Georghiou G. P., and Lagunes-Tejeda A. (1991) The Occurrence of Resistance to Pesticides in Arthropods. FAO UN, Rome, p. 318.

Georghiou G. P., Taylor C. E. (1977) Genetic and biological influences in the evolution of insecticide resistance. *J. Econ. Entomol*, 70, 319–323.

Germain J. F., Lacordaire A. I., Cocquempot C., Ramel J. M., Oudard E. (2009) Un nouveau ravageur de la tomate en France: *Tuta absoluta* . *PHM-Revue Horticole* 512, 37–41.

Germain J. F., Lacordaire A.I., Cocquempot C., Ramel J.M., Oudard E. (2009) Un nouveau ravageur de la tomate en France: *Tuta absoluta*. *PHM-Revue Horticole* 512, 37–41.

Gershater M., Sharples K., Edwards R. (2006) Carboxylesterase activities toward pesticide esters in crops and weeds. *Phytochemistry,* 67, 2561-7.

Giraudo M., Unnithan G. C., Le Goff G. , Feyereisen R. (2010) Regulation of cytochrome P450 expression in Drosophila: Genomic insights. *Pesticide Biochemistry and Physiology,* 97, 115-122.

Giustolin T. A., Vendramim J. D. (1996) Biology of *Scrobipalpuloides absoluta* (Meyrick) on tomato leaves in the laboratory. *Ecossistema,* 21, 11-15.

Giustolin T. A., Vendramim J. D., Alves S. B., Vieira S. A. (2001b) Susceptibility of *Tuta absoluta* (Meyrick) (Lep, Gelechiidae) reared on two species of Lycopersicon to Bacillus thuringiensis var. kurstaki. *J Appl Entomol* 125, 551–556.

Giustolin T. A., Vendramim J. D., Parra J. R. P. (2002) Number of larval instars of *Tuta absoluta* (Meyrick) in tomato genotypes. *Scientia Agricola,* 59, 393-396.

Goncalves-Gervasio R. D. R., Vendramim J. D. (2007) Bioactivity of aqueous neem seeds extract on the *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) in three ways of application.

Gorman K. (2011) Incidence of tomato borer, *Tuta absoluta*, in the UK in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of Tuta absoluta (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-

Gorman K., Hewitt F., Denholm I., Devine G.J. (2001) New developments in insecticide resistance in the glasshouse whitefly (*Trialeurodes vaporariorum*) and the two-spotted spider mite (*Tetranychus urticae*) in the UK. *Pest Manag Sci*, 58, 123–130.

Guedes R.N.C., Picanco M.C., Matioli A.L., Rocha D.M. (1994) Efeito de insecticides e sistemas de conduc¸ ˜ao do tomateiro no control de *Scrobipalpuloides absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *AnSoc Entomol Bras*, 23,321–325.

Guenaoui Y., Ghelamallah A. (2008) *Tuta absoluta (*Meyrick) (Lep: Gelechiidae) a new pest on tomato in Algeria. First data on its development time at different temperatures.

Guerrero F.D., Jamroz R.C., Kammlah D., Kunz S.E. (1997) Toxicological and molecular

characterization of pyrethroidresistant horn flies, *Haematobia irritans*: Identification of kdr and super-kdr point mutations. *Insect Biochem Mol Biol*, 27, 745–755.

Haji F. N., Oliveira C. A. V., Amorim M. S., Batista J. G. S. (1988) Flutuação populacional da traça do tomateiro, no submédio São Francisco. *Pesquisa Agropecuária Brasileira* 23, 7-14.

Hanck D. A., Fozzard H. A. (2007) Voltage-gated sodium channels. *Biological and Medical Physics, Biomedical Engineering*, 219-239.

Hardstone, M. C., Leichter, C. A. , Scott, J. G. (2009) Multiplicative interaction between the two major mechanisms of permethrin resistance, kdr and cytochrome P450-monooxygenase detoxification, in mosquitoes. *Journal of evolutionary biology,* 22, 416-23.

Hemingway J., Hawkes N.J., McCarroll L., Ranson H. (2004) The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology*, 34, 653–665.

Hemingway J., and Ranson H. (2000) Insecticide resistance in insect vectors of human disease. *Annu Rev Entomol*, 45, 371–391.

Hemingway J., Karunarantne S.H.P.P. (1998) Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med. Vet. Entomol*, 12, 1–12.

Hemingway J., Smith C., Jayawardena K.G.I., Herath P.R.J. (1986) Field and laboratory detection of the altered acetylcholinesterase resistance genes which confer organophosphate and carbamate resistance in mosquitoes (Diptera: Culicidae). *Bull. Ent. Res*, 76, 559–565.

Hernandez-Martinez P., Ferre J., Escriche B. (2009) Broad-spectrum cross-resistance in *Spodoptera exigua* from selection with a marginally toxic Cry protein. *Pest management science,* 65, 645-50.

Hille B. (1992) Ionic channels of excitable membranes. Sinaver, Boston.

Holt, R. A., Subramanian, G. M., Halpern, A., Sutton, G. G., Charlab, R., Nusskern, D. R., Wincker, P., Clark, A. G., Ribeiro, J. M., Wides, R., Salzberg, S. L., Loftus, B., Yandell, M., Majoros, W. H., Rusch, D. B., Lai, Z., Kraft, C. L., Abril, J. F., Anthouard, V., Arensburger, P., Atkinson, P. W., Baden, H., De Berardinis, V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, S., Center, A., Chaturverdi, K., Christophides, G. K., Chrystal, M. A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C. A., Flanigan, M., Grundschober-Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M. E., Hladun, S. L., Hogan, J. R., Hong, Y. S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J. J., Lobo, N. F., Lopez, J. R., Malek, J. A., Mcintosh, T. C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Murphy, S. D., O'brochta, D. A., Pfannkoch, C., Qi, R., Regier, M. A., Remington, K., Shao, H., Sharakhova, M. V., Sitter, C. D., Shetty, J., Smith, T. J., Strong, R., Sun, J., Thomasova, D., Ton, L. Q., Topalis, P., Tu, Z., Unger, M. F., Walenz, B., Wang, A., Wang, J., Wang, M., Wang, X., Woodford, K. J., Wortman, J. R., Wu, M., Yao, A., Zdobnov, E. M., Zhang, H., Zhao, Q., Et Al. (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science,* 298, 129-49.

Homan R. (2010) *Tuta absoluta* (Meyrick, 1917: Lep: Gelechiidae): an adventive species new to Britain. *Entomologist's Record and Journal of Variation,* 122, 31-32.

Hosie A.M., Aronstein K., Sattelle D.B., Ffrench-Constant R.H. (1997) Molecular biology of insect neuronal GABA receptors. *Trends Neurosci*, 20, 578–583.

Hu Z., Du Y., Nomura Y., Dong K. (2011) A sodium channel mutation identified in Aedes aegypti selectively reduces cockroach sodium channel sensitivity to type I, but not type II pyrethroids. *Insect Biochemistry and Molecular Biology,* 41, 9-13.

Huang H.S., Hu N.T., Yao Y.E., Wu C.Y., Chiang S.W., Sun, C.N. (1998) Molecular cloning and heterologous expression of a glutathione S160 transferase involved in insecticide resistance from the diamondback moth *Plutella xylostella*. *Insect Biochemistry and Molecular Biology* 28, 651- 658.

Hucho F., Tsetlin V.I., Machold J. (1996) The emerging three-dimensional structure of a receptor. The nicotinic acetylcholine receptor. *European Journal of Biochemistry*, 239, 539-557.

Hui L., DelMonte T., Ranade K. (2008) Genotyping Using the TaqMan Assay,*Current Protocols in Human Genetics* 2.10.1-2.10.8.

Ishaaya I., Barazani A., Kontsedalov S., Horowitz A. R. (2007) Insecticides with novel modes of action: Mechanism, selectivity and cross-resistance. *Entomological Research,* 37, 148-152.

Jacobson R., (2011) *Tuta absoluta*: A season-long IPM strategy based on predatory bugs (UK) in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of *Tuta absoluta* (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18.

Javed N., Viner R., Williamson M. S., Field L. M., Devonshire A. L., Moores G. D. (2003) Characterization of acetylcholinesterases, and their genes, from the hemipteran species *Myzus persicae* (Sulzer), *Aphis gossypii* (Glover), *Bemisia tabaci* (Gennadius) and *Trialeurodes vaporariorum* (Westwood). *Insect Molecular Biology,* 12, 613-620.

Jiang Y.X., Lee A., CHen J.Y., Ruta V., Cadene M., Chait B.T., Mackinnon R. (2003) X-ray structure of a voltage-dependent K+ channel. *Nature*, 423, 33-41.

Jimenez M., Bobadilla D., Vargas H., Taco E., Mendoza R. (1998) Nivel de dano de *Tuta absoluta* (Meyrick), (Lepidoptera: Gelechiidae), en cultivos experimentales de tomate sin aplicacion de insecticidas convencionales. Sociedad Chilena de Entomologıa Universidad de Concepcio´n. XX Congreso Nacional de Entomologı´a. *Concepcio´n, 11, 12 y 13 de noviembre 1998,* 43–51.

Karunker I., Benting J., Lueke B., Ponge T., Nauen R., Roditakis E., Vontas J., Gorman K., Denholm I., Morin S. (2008) Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera : Aleyrodidae). *Insect Biochemistry and Molecular Biology,* 38, 634-644.

Karunker I., Morou E., Nikou D., Nauen R., Sertchook R., Stevenson B. J., Paine M. J. I., Morin S., Vontas J. (2009) Structural model and functional characterization of the *Bemisia tabaci* CYP6CM1vQ, a cytochrome P450 associated with high levels of imidacloprid resistance. *Insect Biochemistry and Molecular Biology,* 39, 697-706.

Kasai S., Scott J.G. (2000) Over expression of cytochrome P450 CYP6D1 is associated with monooxygenase-mediated pyrethroids resistance in houseflies from Georgia. *Pestic. Biochem. Physiol*, 68, 34–41.

Kaufman, P. E., Nunez, S. C., Mann, R. S., Geden, C. J. , Scharf, M. E. (2010) Nicotinoid and pyrethroid insecticide resistance in houseflies (Diptera: Muscidae) collected

from Florida dairies. *Pest management science*, 66, 290-4.

Khajehali J., Van Leeuwen T., Grispou M., Morou E., Alout H., Weill M.Tirry L., Vontas J. Tsagkarakoub A. (2010). Acetylcholinesterase point mutations in European strains of *Tetranychus urticae* (Acari: Tetranychidae) resistant to organophosphates. *Pest Management Science*, 66,220–228.

Kharroubi A. (2008) Agriculture: *Tuta absoluta threatens the Moroccan tomato.*

Kilic T. (2010) First record of *Tuta absoluta* in Turkey. Phytoparasitica.

Knipple D.C., Doyle K.E., Marsella-Merrick P.A., Soderlund D.M. (1994) Tight genetic linkage between the kdr insecticide resistance trait and a voltage-sensitive sodium channel gene in the house fly. *Proc Natl Acad Sci* USA, 91, 2483–2487

Kolaczinski J., and Curtis C. (2001) Laboratory evaluation of fipronil, a phenylpyrazole insecticide, against adult *Anopheles* (Diptera: Culicidae) and investigation of its possible cross- resistance with dieldrin in *Anopheles stephensi*. *Pest Manag. Sci*, 57, 41–45.

Kono Y., Tomita T. (2006) Amino acid substitutions conferring insecticide insensitivity in Ace-paralogous acetylcholinesterase. *Pesticide Biochemistry and Physiology,* 85, 123-132.

Kostaropoulos I., Papadopoulos A.I., Metaxakis A., Boukouvala E., Papadopoulou-Mourkidou E. (2001) Glutathione S-transferase in the defence against pyrethroids in insects. *Insect Biochem. Mol. Biol*, 31, 313–319.

Kozaki T., Shono T., Tomita T., Kono Y. (2001) Fenitroxon insensitive acetylcholinesterases of the housefly, *Musca domestica* associated with point mutations, *Insect Biochem. Mol. Biol*, 31, 991–997.

Kulkarni M.A., Rowland M., Alifrangis M., Mosha F.W., Matowo J., Malima R., Peter J., Kweka E., Lyimo I., Magesa S., Salanti A., Rau M.E., Drakeley C. Occurrence of the leucine-to-phenylalanine knockdown resistance (kdr) mutation in *Anopheles arabiensis* populations in Tanzania, detected by a simplified high-throughput SSOPELISA method. *Malar J* 2006, 5,56.

Labinas A. M., Crocomo W. B., Raetano C. G. (1999) Evaluation of insecticides efficiency in the *Tuta absoluta* (Meyrick, 1917) (Lepidoptera - Gelechiidae) control in tomato crop. *Cientifica (Jaboticabal),* 27, 129-136.

Lacoudiere A.I., and Feuvrier E. (2010) Tomate, traquer *Tuta absoluta*. *Phytoma, la defence des vegertaux*, 632, 40-44

Lahm G. P., Selby T. P., Freudenberger J. H., Stevenson T. M., Myers, B. J., Seburyamo, G., Smith, B. K., Flexner, L., Clark, C. E. , Cordova, D. (2005) Insecticidal anthranilic diamides: a new class of potent ryanodine receptor activators. *Bioorganic and medicinal chemistry letters, 15,* 4898-906.

Lahm G. P., Stevenson T. M., Selby T. P., Freudenberger J. H., Cordova D., Flexner L., Bellin C. A., Dubas C. M., Smith B. K., Hughes K. A., Hollingshaus J. G., Clark C. E. , Benner E. A. (2007) Rynaxypyr: a new insecticidal anthranilic diamide that acts as a potent and selective ryanodine receptor activator. *Bioorganic and medicinal chemistry letters,* 17, 6274-9.

Le Corronc H., Alix P., Hue B., (2002) Differential sensitivity of two insect GABA-gated chloride channels to dieldrin, fipronil and picrotoxinin. *J. Insect Physiol*. 48, 419–431.

Leahey J.P. (1985) The Pyrethroid Insecticides. ed. Taylor & Francis, London.

Lebdi-Grissa K., Skander M., Ben Jbara R., Bel Hadj R., Mhafdhi M., Loussaief (2009*)* Note technique sur les travaux de recherche sur *Tuta absoluta* . *Ministère de l'Agriculture, DGPCQPA,* 14.

Lee S. H., Dunn J. B., Clark J. M., Soderlund D. M. (1999a) Molecular analysis of kdr-like resistance in a permethrin-resistant strain of Colorado potato beetle. *Pesticide Biochemistry and Physiology,* 63, 63-75.

Lee S. H., Smith T. J., Knipple D. C., Soderlund D. M. (1999b) Mutations in the house fly Vssc1 sodium channel gene associated with super-kdr resistance abolish the pyrethroid sensitivity of Vssc1/tipE sodium channels expressed in Xenopus oocytes. *Insect Biochemistry and Molecular Biology,* 29, 185-194.

Li F., and Han Z. (2004) Mutations in acetylcholinesterase associated with insecticide resistance in the cotton aphid, *Aphis gossypii* Glover. *Insect biochemistry and molecular biology,* 34, 397-405.

Li F., and Han Z. J. (2005) Mutations in acetylcholinesterase associated with insecticide resistance in the cotton aphid, *Aphis gossypii* Glover (vol 34, pg 397, 2004). *Insect Biochemistry and Molecular Biology,* 35, 1309-1309.

Lietti M. M. M., Botto E., Alzogaray R. A. (2005) Insecticide resistance in Argentine populations of *Tuta absoluta* (Meyrick) (Lepidoptera : Gelechiidae). *Neotropical Entomology,* 34, 113-119.

Lima J. O. G. D., Machado W. A., De Lima J. O. G. (1996) Greenhouse evaluation of insecticides for control of the tomato leafminer, *Scrobipalpuloides absoluta* (Meyrick). *Revista Ceres,* 48, 337-345.

Li-Ming H., John Troiano., Albert Wang., Kean Goh D.M. (2008) Environmental Chemistry, Ecotoxicity, and Fate of Lambda-Cyhalothrin, Reviews of Environmental Contamination and Toxicology, Whitacre (ed).

Linden A. V. D., Staaij M. V. D., der Staaij M. V., van der Linden A., van der Staaij M. (2011) Effectiveness of pesticides and potential for biological control of the tomato leaf miner *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in Europe. *IOBC/WPRS Bulletin,* 68, 97-100.

Littleton J.T., Ganetzky B. (2000) Ion channels and synaptic organisation: analysis of the Drosophila genome. *Neuron*, 26, 35–43.

Liu M.Y. and Casida J.E. (1993) High affinity binding of (3H)imidacloprid in the insect acetylcholine receptor. *Pesticide Biochemistry and Physiology*, 46, 40-46.

Liu Z., Tan J., Huang Z. Y., Dong K. (2006) Effect of a fluvalinate-resistance-associated sodium channel mutation from varroa mites on cockroach sodium channel sensitivity to fluvalinate, a pyrethroid insecticide. *Insect Biochemistry and Molecular Biology,* 36, 885-889.

Livak K. J. (1999) Allelic discrimination using fluorogenic probes and the 5' nuclease assay, *Genetic Analysis-Biomolecular Engineering*, 14, 5-6, 143–149.

Lopez E. (1991) Polilla del tomate: Problema crıtico para la rentabilidad del cultivo de verano. *Empresa y Avance Agrıcola* 1, 6–7.

Lynd A., Ranson H., McCall P.J., Randle N.P., Black W.C., Walker E.D., Donnelly M.J (2005) A simplified high-throughput method for pyrethroids knock-down resistance (kdr) detection in *Anopheles gambiae. Malar J*, 4,16.

Magana C., Hernandez-Crespo P., Brun-Barale A., Couso-Ferrer F., Bride J. M., Castanera P., Feyereisen R. *,* Ortego, F. (2008). Mechanisms of resistance to malathion in the medfly *Ceratitis capitata*. *Insect biochemistry and molecular biology,* 38, 756-62.

Mallea A. R., Macola G. S., Garcia S. J. G., Bahamondes L. A. , Suarez J. H. (1972) Nicotiana tabacum var. Virginica, a new host of Scrobipalpula absoluta. *Revista de la Facultad de Ciencias Agrarias 18,* 13-15, Universidad Nacional de Cuyo.Marcano R., (2008). Minador Pequeño de la hoja del tomate; Palomilla pequeña, Minador del tomate *Tuta absoluta* (Meyrick) 1917. Plagas Agrícolas de Venezuela.

Mansuy D., (1998) The great diversity of reactions catalyzed by cytochromes P450. In: Comparative Biochemistry and Physiology C Pharmacology Toxicology and Endocrinology.

Marchiori C. H., Silva C. G., Lobo A. P. (2004) Parasitoids of *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) collected on tomato plants in Lavras, state of Minas Gerais, Brazil. *Braz J Biol* 64, 551–552.

Marin M. S., Diaz Bruno E. A., Quercetti M. J., Caballero A. E. (2002) *Tuta absoluta*: Rearing in laboratory conditions. *Revista de la Facultad de Ciencias Agrarias Universidad Nacional de Cuyo,* 34, 1-6.

Martinez-Torres D., Chandre F., Williamson M. S., Darriet F., Berge J. B., Devonshire A. L., Guillet P., Pasteur N., Pauron D. (1998) Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* S.S. *Insect Molecular Biology,* 7, 179-184.

MartinezTorres D., Devonshire A. L., Williamson M. S. (1997) Molecular studies of knockdown resistance to pyrethroids: Cloning of domain II sodium channel gene sequences from insects. *Pesticide Science,* 51, 265-270.

Martinez-Torres D., Foster S. P., Field L. M., Devonshire A. L., Williamson M. S. (1999) A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera : Aphididae). *Insect Molecular Biology,* 8, 339-346.

Matambo T. S., Paine M. J. I., Coetzee M., Koekemoer L. L. (2010) Sequence characterization of cytochrome P450 CYP6P9 in pyrethroid resistant and susceptible *Anopheles funestus* (Diptera: Culicidae). *Genetics and Molecular Research,* 9, 554-564.

Maxwell D. M. and Brecht K. M. (2001) Carboxylesterase: specificity and spontaneous reactivation of an endogenous scavenger for organophosphorus compounds. *Journal of applied toxicology : JAT,* 21 Suppl 1, S103-7.

Mcabee R. D., Kang K. D., Stanich M. A., Christiansen J. A., Wheelock C. E., Inman A. D., Hammock B. D. and Cornel A. J. (2004) Pyrethroid tolerance in *Culex pipiens pipiens var molestus* from Marin County, California. *Pest management science,* 60, 359-68.

Medeiros M. A. (2007) The role of biodiversity in managing the tomato leafminer *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae). *Doctoral Dissertation, University of Brasilia,* 145.

Medeiros M. A., Sujii E. R., Rasi G. C., Liz R. S., de Morais H. C. (2009b) Oviposition pattern and life table of South American tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera, Gelechiidae).

Menozzi P., Shi M.A., Lougarre A., Tang Z.H., Fournier D. (2004) Mutations of acetylcholinesterase which confer insecticide resistance in *Drosophila melanogaster* populations, *BMC Evol. Biol*, 4, 4.

Michereff F. M. and Vilela E. F. (2001) Traça-dotomateiro, *Tuta absoluta* (Lepidoptera: Gelechiidae), p. 81-84. *In*: E.F. Vilela, R.A. Zucchi , F. Cantor (eds.), Histórico e impacto das pragas.

Michereff M., Vilela E. F., Attygalle A. B., Meinwald J., Svatos A., Jham G. N. (2000) Field trapping of tomato moth, Tuta absoluta with pheromone traps. *Journal of Chemical Ecology,* 26, 875-881.

Mihsfeldt L. H., Parra J. R. P. (1999) Biology of *Tuta absoluta* (Meyrick, 1917) reared on an artificial diet. *Scientia Agricola,* 56, 769-776.

Miles K., Audigier S. S. M., Greengard P., Huganir R. L. (1994) Autoregulation of phosphorylation of the nicotinic acetylcholine receptor. *Journal of Neuroscience*, 14, 3271-3279.

Miranda M. M. M., Picanço M. C., Zanuncio J. C., Bacci L., da Silva E. M. (2005) Impact of integrated pest management on the population of leafminers, fruit borers, and natural enemies in tomato. *Cienc Rural* 35, 204–208.

Miranda M. M. M., Picanco M., Zanuncio J. C., Guedes R. N. C. (1998) Ecological life table of *Tuta absoluta* (Meyrick) (Lepidoptera : Gelechiidae). *Biocontrol Science and Technology,* 8, 597-606.

Miranda M. M. M., Picanco M., Zanuncio J. C., Guedes R. N. C. (1998) Ecological Life Table of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Biocontrol Sci Technol* 8, 597–606.

Miyazaki N., Ohyama K., Dunlap D.Y., Matsumara F. (1996) Cloning and sequencing of the para-type sodium channel gene from susceptible and kdr resistant German cockroaches (*Blattela germanica*) and the house fly (*Musca domestica*). *Mol. Gen. Genet*, 252,61–68.

Miyazawa A., Fujiyoshi Y., Unwin, N. (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature*, 423, 949-955.

Molla O., Monton H., Beitia Crespo F. J., Urbaneja A. (2008) La polilla del tomate *Tuta absoluta* (Meyrick), una nueva plaga invasora. *Terralia* 69, 36–42.

Monserrat A. (2009a) La polilla del tomate *Tuta absoluta* en la Region de Murcia: bases para su control. Serie Tecnica y de Estudios No. 34. *Conserjeıa de Agricultura y Agua.*

Monserrat A. (2009b) Basic measures for the management of the tomato moth *Tuta absoluta*. *Agricola Vergel: Fruticultura, Horticultura, Floricultura, Citricultura, Vid, Arroz,* 28, 481-491.

Monserrat A. (2010) Estrategias globales en el manejo de *Tuta absoluta* en Murcia. *Phytoma Espana 217:*81–86.

Moore J.E. (1983) Control of tomato leafminer (*Scrobipalpula absoluta*) in Bolivia. *Trop. Pest Manage*, 29, 231-238.

Moores G. D., Philippou D., Borzatta V., Trincia P., Jewess P., Gunning R., Bingham G. (2008) An analogue of piperonyl butoxide facilitates the characterisation of metabolic resistance *Pest Manag Sci*, 65, 150–154

Moreno, M., Vicente, J. L., Cano, J., Berzosa, P. J., De Lucio, A., Nzambo, S., Bobuakasi, L., Buatiche, J. N., Ondo, M., Micha, F., Do Rosario, V. E., Pinto, J. , Benito, A. (2008) Knockdown resistance mutations (kdr) and insecticide susceptibility to DDT and pyrethroids in *Anopheles gambiae* from Equatorial Guinea. *Tropical medicine and international health : TM and IH,* 13, 430-3.

Morin S., Williamson M. S., Goodson S. J., Brown J. K., Tabashnik B. E., Dennehy T. J.

(2002) Mutations in the *Bemisia tabaci* para sodium channel gene associated with resistance to a pyrethroid plus organophosphate mixture. *Insect Biochemistry and Molecular Biology,* 32, 1781-1791.

Mota-Sanchez D., Bills P. S., Whalon M. E. (2002) Arthropod resistance to pesticides: status and overview, Chapter 8. In Wheeler, W. B. (ed.), Pesticides in Agriculture and the Environment. Marcel Dekker, Inc., New York.

Mota-Sanchez D., Hollingworth R.M., Grafius E.J. (2003) Mechanisms of resistance to imidacloprid in Colorado potato beetles. *Abstracts of Papers of the American Chemical Society*, 225, 0034-AGRO

Mota-Sanchez D., Wise J. C., Poppen R. V., Gut L. J. , Hollingworth R. M. (2008) Resistance of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), larvae in Michigan to insecticides with different modes of action and the impact on field residual activity. *Pest management science,* 64, 881-90.

Mutero A., Pralavorio M., Bride J.M., Fournier D. (1994) Resistance associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc. Natl. Acad. Sci*. USA, 91, 5922–5926.

Nabeshima T., Kozaki T., Tomita T., Kono Y. (2003) An amino acid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach-potato aphid, *Myzus persicae*. *Biochem. Biophys. Res. Commun*, 307, 15–22.

Nakao H. K., Funasaki G. Y. (1979) Introductions for biological control in Hawaii: 1975 and 1976. *Proc Hawaiian Entomol Soc* 13, 125–128.

Nasraoui B. (2011) Tuta absoluta in Tunisia: three years later? in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of *Tuta absoluta* (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18

Nauen R. and Bretschneider T. (2002) New modes of action of insecticides. *Pesticide Outlook,* 13, 241-245.

Nauen R., Bielza P., Denholm I., Gorman K. (2008) Age-specific expression of resistance to a neonicotinoid insecticide in the whitefly *Bemisia tabaci Pest Manag Sci*, 64,1106–1110

Nauen, R. (2007) Insecticide resistance in disease vectors of public health importance. *Pest management science,* 63, 628-33.

Nelson D. R., *http://drnelson.utmem.edu/nelsonhomepage.html*

Nelson D. R., Kamataki T., Waxman D. J., Guengerich F. P., Estabrook R.W., Feyereisen R., Gonzalez F. J., Coon M. J., Gunsalus I. C., Gotoh O., Okuda K., Nebert D.W. (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol*, 12, 1–51.

Nelson D. R., Koymans L., Kamataki T., Stegeman J. J., Feyereise R., Waxman D. J., Waterman M. R., Gotoh O., Coon M. J., Estabrook R.W., Gunsalus I. C., Nebert D. W. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, 6, 1–42.

Nikou D., Ranson H., Hemingway J. (2003) An adult-specific CYP6 P450 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, *Anopheles gambiae. Gene*, 318, 91-102.

Notz A. P. (1992) Distribution of eggs and larvae of *Scrobipalpula absoluta* in potato plants. *Rev Facultad de Agronomıa Maracay* 18, 425–432.

Nunez P., Zignago A., Paullier J., Nunez S. (2009) Sex pheromones to control tomato moth

Tuta absoluta (Meyrick) (Lep., Gelechiidae). *Agrociencia (Montevideo),* 13, 27-35.
Nyoni B. N., Gorman K., Mzilahowa T., Williamson M. S., Navajas M., Field L. M. , Bass C. (2011). Pyrethroid resistance in the tomato red spider mite, *Tetranychus evansi*, is associated with mutation of the para-type sodium channel. *Pest Management Science,* 67, 891-897.
Oakeshott J. G., Devonshire A. L., Claudianos C., Sutherland T. D., Horne I., Campbell P. M., Ollis D. L. , Russell R. J. (2005) Comparing the organophosphorus and carbamate insecticide resistance mutations in cholin- and carboxyl-esterases. *Chemico-biological interactions,* 157-158, 269-75.
Oliveira F. A., da Silva D. J. H., Leite G. L. D., Jham G. N., Picanco M. (2009) Resistance of 57 greenhouse-grown accessions of Lycopersicon esculentum and three cultivars to *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae). *Sci Hortic* 119, 182–187.
Onstad D. W. (2008) Insect Resistance Management . Academic Press, London, U.K .
Oppenoorth F.J. (1982) Two different paraoxon-resistant acetylcholinesterase mutants in the housefly. *Pesticide Biochemistry and Physiology*, 18, 26-27.
Oppenoorth F.J. (1984) Biochemistry of insecticide resistance. *Pestic. Biochem. Physiol*, 22, 187–193.
O'Reilly A. O., Khambay B. P. S., Williamson M. S., Field L. M., Wallace B. A., Davies T. G. E. (2006) Modelling insecticide-binding sites in the voltage-gated sodium channel. *Biochemical Journal,* 396, 255-263.
Ortega F., Astor E., Scals D. d., de Scals D. (2008) Control of the tomato fruit moth (*Tuta absoluta*). *Horticultura Internacional,* 15, 30-31.
Ottea J. (2000) Mechanisms of Pyrethroid Resistance in Larvae and Adults from a Cypermethrin-Selected Strain of *Heliothis virescens* (F.). *Pesticide Biochemistry and Physiology,* 66, 20-32.
Ouardi K. (2011) La stratégie nationale de lutte contre *Tuta absoluta* in Maroc in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of Tuta absoluta (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18
Park Y., and Taylor M.F.J. (1997) A novel mutation L1029H in sodium channel gene hscp associated with pyrethroid resistance for *Heliothis virescens* (Lepidoptera: Noctuidae). *Insect Biochemistry and Molecular Biology*, 27, 9-13.
Paupy C., Delatte H., Bagny L., Corbel V., Fontenille D. (2009) *Aedes albopictus*, an arbovirus vector: from the darkness to the light. *Microbes and infection / Institut Pasteur,* 11, 1177-85.
Pereyra P. C., Sanchez N. E. (2006) Effect of two solanaceous plants on developmental and population parameters of the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae). *Neotrop Entomol* 30,277–282.
Picancëo M. C., Guedes R. N. C., Leite G. L. D., Fontes P. C. R., Silva E. A. (1995) Incidencia de *Scrobipalpuloides absoluta* (Meyrick) (Lepidoptera: Gelechiidae) em tomateiro sob diferentes sistemas de tutoramento e controle quõÂmico de pragas. *Horticultura Brasileira*, 13, 180 -183.
Pires L. M., Marques I. M. R. (2002) Biological data of the tomato pinworm and storage of its eggs and pupae at low temperature. *Revista de Agricultura (Piracicaba),* 77, 357-368.
Polack A. (2007) Perspectivas para el control biologico de la polilla de tomate. *Horticom News, 8 October 2009.*

Polack L. A. (1999) Ensayos de eficacia de plaguicidas empleados contra la polilla del tomate *Tuta absoluta* (Meyrick). Buenos Aires, Centro Agrícola El Pato, INTA. 2.

Polaszek (2009) Biological control of the South American tomato pinworm, *Tuta absoluta* (Lep.: Gelechiidae), with releases of *Trichogramma achaeae* (Hym.: Trichogrammatidae) in tomato greenhouses of Spain. *Meeting of the IOBC/WPRS Working Group-Integrated Control in Protected Crops, Mediterranean Climate.* Mediterranean Agronomic Institute of Chania (MAICh), Crete, Greece.

Potter P. M., and Wadkins R. M. (2006) Carboxylesterases – Detoxifying Enzymes and Targets for Drug Therapy, *Current Medicinal Chemistry*, 2006, 13, 1045-1054.

Potting R. (2009) Pest risk analysis, *Tuta absoluta* , tomato leaf miner moth. *Plant protection service of the Netherlands,* 24. www.minlnv.nl

Price N. R. (1991) Insect Resistance to Insecticides: Mechanisms and Diagnosis*, Comp. Biochem. Physiol*. Vol. IOOC, 3, 319-326,

Pridgeon J.W., Zhang L., Liu N. (2003) Overexpression of CYP4G19 associated with a pyrethroid-resistant strain of the German cockroach, *Blattella germanica* (L.). *Gene*, 314, 157–163.

Puinean A. M., Foster S. P., Oliphant L., Denholm I., Field L. M., Millar N. S., Williamson M. S., Bass C. (2010) Amplification of a Cytochrome P450 Gene Is Associated with Resistance to Neonicotinoid Insecticides in the Aphid Myzus persicae. *Plos Genetics,* 6.

Quiroz E. C. (1976) Nuevos antecedentes sobre la biologia de la polilla del tomate, *Scrobipalpula absoluta* (Meyrick). *Agricultura Técnica* 36(2), 82-86.

Rached Iost C. A., Ferreira M. d. C., Martinelli N. M., Bottura Maccagnan D. H. (2008) Evaluation of diferent application volumes on the control of *Tuta absoluta* (Meirick, 1917) (Lepidoptera: Gelechiidae) in tomato crop. *Acta Scientiarum-Agronomy,* 30, 619-624.

Raffa K. F., Priester T. M. (1985) Synergists As Research Tools And Control Agents In Agriculture. *Journal of Agricultural Entomology,* 2, 27-45.

Ranson H., Jensen B., Vulule J.M., Wang X., Hemingway J., Collins F.H. (2000a) Identification of point mutation in the voltage-gated sodium channel gene in Kenya *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Mol Biol*, 9, 491–497.

Ranson H., Jensen B., Wang X., Prapanthadara L., Hemingway J., Collins F.H. (2000b) Genetic mapping of two loci affecting DDT resistance in the malaria vector, *Anopheles gambiae*. *Insect Mol Biol*, 9, 499-507.

Raymond-Delpech V., Matsuda K., Sattelle B. M., Rauh J. J. , Sattelle D. B. (2005) Ion channels: molecular targets of neuroactive insecticides. *Invertebrate neuroscience : IN,* 5, 119-33.

Razuri V. and Vargas E. (1975) Biología y comportamiento de *Scrobipalpula absoluta* (Meyrick) (Lepidoptera:Gelechiidae) en tomatera. *Rev. Peruana Entomol.* 18(1), 84-89.

Reis P. R., Souza J. C. D. (1998) Chemical control of *Tuta absoluta* (Meyrick) in staked tomato plants. *Ciencia e Agrotecnologia,* 22, 13-21.

Rendic S., Di Carlo F. J., (1997) Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab. Rev*. 29, 413–580.

Reyes M., Franck P., Charmillot P. J., Ioriatti C., Olivares J., Pasqualini E. *,* Sauphanor B.

(2007) Diversity of insecticide resistance mechanisms and spectrum in European populations of the codling moth, *Cydia pomonella*. *Pest management science*, 63, 890-902.

Reyes M., Sauphanor B. (2008) Resistance monitoring in codling moth: a need for standardization. *Pest management science*, 64, 945-53.

Rinkevich F. D., Hamm R. L., Geden C. J. , Scott J. G. (2007) Dynamics of insecticide resistance alleles in house fly populations from New York and Florida. *Insect biochemistry and molecular biology,* 37, 550-8.

Riquelme V. M. B., Botto E. N., Lafalce C. (2006) Efficacy of insecticides against the "tomato moth" *Tuta absoluta* (Lepidoptera : Gelechiidae) and their residual effects on the parasitoid Trichogrammatoidea bactrae (Hymenoptera : Trichogrammatidae). *Revista de la Sociedad Entomologica Argentina,* 65, 57-65.

Robredo Junco F., Cardenoso Herrero J. M. (2008) Strategies for control of the tomato moth, *Tuta absoluta* Meyrick. *Agricultura, Revista Agropecuaria,* 77, 70-74.

Robredo Junco F., Cardenoso Herrero J. M., Junco Alonso J. (2008) *Tuta absoluta* Meyrick: number of potential generations a year. *Agricultura, Revista Agropecuaria,* 78, 474-480.

Roditakis E. and Seraphides N. (2011) The current status of *Tuta absoluta* in Greece and Cyprus in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of Tuta absoluta (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-

Roditakis E., Papachristos D., Roditakis N. E. (2010) Current status of the tomato leafminer *Tuta absoluta* in Greece. *Bulletin OEPP,* 40, 163-166.

Rodpradit P., Boonsuepsakul S., Chareonviriyaphap T., Bangs M.J., Rongnoparut P. (2005) Cytochrome P450 genes: molecular cloning and overexpression in a pyrethroid-resistant strain of *Anopheles minimus* mosquito. *Journal of the American Mosquito Control Association*, 21, 71-79.

Rodríguez M. A., Bosch D., Sauphanor B. , Avilla J. (2010) Susceptibility to Organophosphate Insecticides and Activity of Detoxifying Enzymes in Spanish Populations ofCydia pomonella(Lepidoptera: Tortricidae). *Journal of Economic Entomology, 103*, 482-491.

Roe R. M., Young H. P., Iwasa T., Wyss C. F., Stumpf C. F., Sparks T. C., Watson G. B., Sheets J. J. , Thompson G. D. (2010) Mechanism of resistance to spinosyn in the tobacco budworm, *Heliothis virescens*. *Pesticide Biochemistry and Physiology,* 96, 8-13.

Rohan T. R., and Brown T. (2005) Fluorescence based strategies for genetic analysis,The Royal Society of Chemistry *Chem. Commun*., 5487–5502

Rojas S. (1981) Control de la polilla del tomate: enemigos naturales y patogenos. *IPA La Platina* 8, 18–20.

Rose R.L., Goh D., Thompson D.M., Verma K.D., Heckel D.G. (1997) Cytochrome P450 (CYP)9A1 in *Heliothis virescens*: the first member of a new CYP family. *Insect. Biochem. Mol. Biol*, 27,605–15.

Roush R. T. (1989) Designing resistance management programs: how can you choose? *Pestic. Sci,* 26, 423–441.

Roush R. T. (1994) Managing pests and their resistance to *Bacillus thuringiensis*: can transgenic crops be better than sprays?, *Biocontrol Sci*. Tech, 4, 501–516.

Royo J. L., Hidalgo M. , Ruiz A. (2007) Pyrosequencing protocol using a universal

biotinylated primer for mutation detection and SNP genotyping. *Nature protocols*, 2, 1734-9.

Russell R. J., Claudianos C., Campbell P. M., Horne I., Sutherland T. D., Oakeshott J. G. (2004) Two major classes of target site insensitivity mutations confer resistance to organophosphate and carbamate insecticides. *Pesticide Biochemistry and Physiology,* 79, 84-93.

Salazar E. R., Araya J. E. (1997) Deteccion de resistencia a insecticidas en la polilla del tomate. *Simiente* 67, 8–22.

Salazar E. R., Araya J. E. (2001) Tomato moth, *Tuta absoluta* (Meyrick), response to insecticides in Arica, Chile. *Agricultura Tecnica,* 61, 429-435.

Salazar E.R., and Araya J.E. (1997) Deteccion de resistencia a insecticidas en la polilla del tomate. *Simiente*, 67, 8–22.

Sannino L., Espinosa B. (2009) Keiferia lycopersicella, una nuova tignola su pomodoro. *Informat Agr 4:*69–70.

Sardegna L., (2010) Tignola o falena del pomodoro Riconoscimento e lotta alla *Tuta absoluta*. Laore Sardegna.

Schuler T. H., Martinez-Torres D., Thompson A. J., Denholm I., Devonshire A. L., Duce I. R., Williamson M. S. (1998) Toxicological, electrophysiological, and molecular characterisation of knockdown resistance to pyrethroid insecticides in the diamondback moth, *Plutella xylostella* (L.). *Pesticide Biochemistry and Physiology,* 59, 169-182.

Scott J. G. (1996) Cytochrome P450 monooxygenase-mediated resistance to insecticides. *Journal of Pesticide Science,* 21, 241-245.

Scott J. G. (1999) Cytochromes P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology,* 29, 757-777.

Scott J. G. (2001) *Cytochrome P450 monooxygenases and insecticide resistance: Lessons from CYP6D1*.

Scott J. G. (2008) Insect cytochrome P450s: thinking beyond detoxification. *Recent advances in insect physiology, toxicology and molecular biology*, 117-124.

Scott J. G., Liu N., Wen Z. M. (1998) Insect cytochromes P450: diversity, insecticide resistance and tolerance to plant toxins. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology and Endocrinology,* 121, 147-155.

Scott J. G., Wen Z. M. (2001) Cytochromes P450 of insects: the tip of the iceberg. *Pest Management Science,* 57, 958-967.

Seino A., Kazuma T., Tan A. J., Tanaka H., Kono Y., Mita K., Shiotsuki T. (2007) Analysis of two acetylcholinesterase genes in *Bombyx mori*. *Pesticide Biochemistry and Physiology,* 88, 92-101.

Seplyarsky V., Weiss M., Haberman A. (2010) *Tuta absoluta* Povolny (Lepidoptera: Gelechiidae), a new invasive species in Israel. *Phytoparasitica,* 38, 445-446.

Shi M. A., Lougarre A., Alies C., Fremaux I., Tang Z. H., Stojan J., Fournier D. (2004) Acetylcholinesterase alterations reveal the fitness cost of mutations conferring insecticide resistance. *Bmc Evolutionary Biology,* 4.

Shono T. (1986) Genetical and biochemical studies on resistance to pyrethroid and organophosphorus insecticides. *J. Pesticide Sci*. II, 275-285.

Shono T., Zhang L. , Scott J. (2004) Indoxacarb resistance in the house fly. *Pesticide Biochemistry and Physiology,* 80, 106-112.

Silman I. , Sussman J. L. (2008) Acetylcholinesterase: how is structure related to function?

Chemico-biological interactions, 175, 3-10.
Silva G. A., Picanco M. C., Bacci L., Crespo A. L. B., Rosado J. F., Guedes R. N. C. (2011) Control failure likelihood and spatial dependence of insecticide resistance in the tomato pinworm, *Tuta absoluta*. *Pest Management Science,* 67, 913-920.
Silver K., and Soderlund D.M. (2005b) State-dependent block of rat Nav1.4 sodium channels expressed in *Xenopus oocytes* by pyrazoline-type insecticides. *Neuro.Toxicol*, 26, 397– 406.
Silver K., and Soderlund D.M. (2005a) Action of pyrezoline-type insecticides at neuronal target sites. *Pestic Biochem Physiol*, 81,136–143.
Silverio F. O., de Alvarenga E. S., Moreno S. C., Picanco M. C. (2009) Synthesis and insecticidal activity of new pyrethroids. *Pest Management Science,* 65, 900-905.
Singh O. P., Bali P., Hemingway J., Subbarao S. K., Dash A. P., Adak T. (2009) PCR-based methods for the detection of L1014 kdr mutation in *Anopheles culicifacies* sensu lato. *Malaria Journal,* 8.
Singh O. P., Dykes C. L., Das M. K., Pradhan S., Bhatt R. M., Agrawal O. P., Adak T. (2010) Presence of two alternative kdr-like mutations, L1014F and L1014S, and a novel mutation, V1010L, in the voltage gated Na(+) channel of *Anopheles culicifacies* from Orissa, India. *Malaria Journal,* 9.
Singh O. P., Dykes C. L., Lather M., Agrawal O. P., Adak T. (2011) Knockdown resistance (kdr)-like mutations in the voltage-gated sodium channel of a malaria vector *Anopheles stephensi* and PCR assays for their detection. *Malaria journal,* 10, 59.
Siqueira H. A. A., Guedes R. N. C., Fragoso D. B., Magalhaes L. C. (2001) Abamectin resistance and synergism in Brazilian populations of *Tuta absoluta* (Meyrick) (Lepidoptera : Gelechiidae). *International Journal of Pest Management,* 47, 247-251.
Siqueira H. A. A., Guedes R. N. C., Fragoso D. B., Magalhaes L. C. (2001) Abamectin resistance and synergism in Brazilian populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Int J Pest Manage* 47, 247–251.
Siqueira H. A. A., Guedes R. N. C., Picanco M. C. (2000a) Cartap resistance and synergism in populations of *Tuta absoluta* (Lep., Gelechiidae). *Journal of Applied Entomology,* 124, 233-238.
Siqueira H. A. A., Guedes R. N. C., Picanco M. C. (2000b) Insecticide resistance in populations of *Tuta absoluta* (Lepidoptera: Gelechiidae). *Agricultural and Forest Entomology,* 2, 147-153.
Siqueira H. A., Guedes R. N., Picanço M. C. (2000) Insecticide resistance in populations of *Tuta absoluta* (Lepidoptera: Gelechiidae). *Agric For Entomol* 2, 147–153.
Smissaert H. R. (1964) Cholinesterase inhibition in spider mites susceptible and resistant to organophosphate, *Science*, 143, 129–131.
Snyder M. J., Andersen J. F., Feyereisen R. (1993) Diversity of cytochrome P450 enzymes in single tissues of the tobacco hornworm, *Manduca sexta*. *American Zoologist,* 33, 42A.
Soderlund D. M. (2007) AGRO 135-Pyrethroids, knockdown resistance, and sodium channels. *Abstracts of Papers of the American Chemical Society,* 233, 319-319.
Soderlund D. M. (2008) Pyrethroids, knockdown resistance and sodium channels. *Pest management science,* 64, 610-6.
Soderlund D. M., Knipple D. C. (2003) The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochemistry and Molecular Biology,* 33, 563-577.

Soderlund D.M., Bloomquist J.R. (1989) Neurotoxic action of pyrethroid insecticides. *Annu. Rev. Entomol*, 34,77–96.

Song W., Liu Z., Dong K. (2006) Molecular basis of differential sensitivity of insect sodium channels to DCJW, a bioactive metabolite of the oxadiazine insecticide indoxacarb. *Neurotoxicology,* 27, 237-44.

Soreq H., Gnat A., Loewenstein Y., Neville L.F. (1992) Excavations into the active-site gorge of cholinesterases. TIBS, 17, 353–358.

Souza J. C. , Reis P. R. (1986) Controle da traça do tomateiro em minas Gerais. *Pesquisa Agropecuária Brasileira* 21, 343-354.

Souza J. C., and Reis P. R. (1986) Controle da traca-do-tomateiro em Minas Gerais. *Pesquisa Agropecuaria Brasileira*, 21, 343-354.

Souza J. C., Reis P. R., Salgado L. O. (1992) Tracea do Tomateiro: Historico, Reconhecimento, Biologia, Prejuõazos e Controle (*Belo Horizonte: EPAMIG*), pp. 20.

Srinivas R., Udikeri S. S., Jayalakshmi S. K. , Sreeramulu K. (2004) Identification of factors responsible for insecticide resistance in *Helicoverpa armigera*. Comparative biochemistry and physiology. *Toxicology , pharmacology : CBP,* 137, 261-9.

Steitz T.A., Shulman R.G., (1982) Crystallographic and NMR studies of the serine proteases, *Ann. Rev. Biophys. Bioeng*. 11, 419–444.

Stevenson B. J., Bibby J., Pignatelli P., Muangnoicharoen S., O'Neill P. M., Lian L. Y., Muller P., Nikou D., Steven A., Hemingway J., Sutcliffe M. J. , Paine M. J. (2011) Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed. *Insect biochemistry and molecular biology,* 41, 492-502.

Suinaga F. A., Casali V. W. D., Picanco M., Foster J. (2004) Genetic divergence among tomato leafminer populations based on AFLP analysis. *Pesquisa Agropecuaria Brasileira,* 39, 645-651.

Taco E., Quispe R., Bobadilla D., Vargas H., Jimenez M., Morales A. (1998) Resultados preliminares de un ensayo de control biologico de la polilla del tomate, *Tuta absoluta* (Meyrick).

Tan J., Liu Z., Tsai T.D., Valles S.M., Goldin A.L., Dong K. (2002) Novel sodium channel gene mutations in *Blattella germanica* reduce the sensitivity of expressed channels to deltamethrin. *Insect Biochem Mol Biol*, 32,445–454.

Tantely M. L., Tortosa P., Alout H., Berticat C., Berthomieu A., Rutee A., Dehecq J. S., Makoundou P., Labbe P., Pasteur N. , Weill M. (2010) Insecticide resistance in *Culex pipiens quinquefasciatus* and *Aedes albopictus* mosquitoes from La Reunion Island. *Insect biochemistry and molecular biology,* 40, 317-24.

Temerak S. (2011) The status of Tuta absoluta in Egypt in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of *Tuta absoluta* (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18

Temeyer K.B., Li A.Y., Lohmeyer K.H., Chen A.C., Olafson P.U., Sanson D.W., Foil L.D., (2008) Acetylcholinesterase mutation in diazinon-resistant *Haematobia irritans* (L.) (Diptera: Muscidae). *Vet. Parasitol*. 154, 300-310.

Tijet N., Helvig C., Feyereisen R. (2001) The cytochrome P450 gene superfamily in *Drosophila melanogaster*: Annotation, intron-exon organization and phylogeny. *Gene,* 262, 189-198.

Toda S., Komazaki S., Tomita T., Kono Y. (2004) Two amino acid substitutions in

acetylcholinesterase associated with pirimicarb and organophosphorous insecticide resistance in the cotton aphid, *Aphis gossypii* Glover (Homoptera: Aphididae), *Insect Mol. Biol*, 13, 549–553.

Tomita T., Itokawa K., Komagata O., Kasai S. (2010) Overexpression of cytochrome P450 genes in insecticide-resistant mosquitoes. *Journal of Pesticide Science,* 35, 562-568.

Tomita T., Yaguchi N., Mihara M., Takahashi M., Agui N., Kasai S. (2003) Molecular analysis of a para sodium channel gene from pyrethroid-resistant head lice, *Pediculus humanus capitis* (Anoplura : Pediculidae). *Journal of Medical Entomology,* 40, 468-474.

Tripet F., Wright J., Lanzaro G.(2006) A new high-performance PCR diagnostic for the detection of pyrethroid knockdown resistance kdr in *Anopheles gambiae*. *Am J Trop Med Hyg*, 74:658-662.

Tropea Garzia G. (2009) Physalis peruviana L. (Solanaceae) a host plant of *Tuta absoluta* in Italy. *IOBC/WPRS Bull* 49, 231–232.

Tropea Garzia G. (2011) Biology, distribution and damage of *Tuta absoluta*, an exotic invasive pest from South America (Italy) in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of *Tuta absoluta* (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18.

Tropea Garzia G., Siscaro G., Colombo A., Campo G. (2009) Rinvenuta in Sicilia, *Tuta absoluta.- L'Informatore Agrario,* 4, 71

Trottin Y. (2011) The current status of *Tuta absoluta* in France in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of Tuta absoluta (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18

Twyman R. M. (2005) Single Nucleotide Polymorphism (SNP) Genotyping Techniques An Overview in Encyclopedia of Diagnostic Genomics and Proteomics DOI: 10.1081/E-EDGP 120020761

Uchoa-Fernandes M. A., Della Lucia T. M. C., Vilela E. F. (1995) Mating, oviposition and pupation of *Scrobipalpula absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Anais da Sociedade Entomologica do Brasil* 24, 159–164 *Universidade de São Paulo,* 87.

Ugurlu S. (2011) The current status of *Tuta absoluta* in Turkey and toxicity of some insecticides in EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of *Tuta absoluta* (tomato borer, Lepidoptera: Gelechiidae), Agadir, Morocco, Nov 16-18

Urbaneja A., Monton H., Molla O. (2009) Suitability of the tomato borer *Tuta absoluta* as prey for Macrolophus caliginosus and Nesidiocoris tenuis. *J Appl Entomol* 133, 292–296.

Urbaneja A., Vercher R., Garcia Mari F. and Porcuna J.L. (2007) La popilla del tomate, *Tuta absoluta* . *Phytoma España* 194(12), 16-23.

Vais H., Williamson M. S., Devonshire A. L., Usherwood P. N. R. (2001) The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. *Pest Management Science,* 57, 877-888.

Vais H., Williamson M.S., Goodson S.J., Devonshire A.L., Warmke J.W., Usherwood P.N.R., Cohen C.J. (2000) Activation of Drosophila sodium channels promotes modification by deltamethrin – Reductions in affinity caused by knock-down resistance mutations. *J. Gen. Physiol*, 115, 305–318.

Vargas H.C. (1970) Observaciones sobre la biología y enemigos naturales de la polilla del tomate, *Gnorimoschema absoluta* (Meyrick) (Lepidoptera: Gelechiidae). Idesia, 1,

75-110.
Vercher R., Calabuig A., Felipe C. (2010) Ecologıa, muestreos y umbrales de *Tuta absoluta* (Meyrick). *Phytoma Espana*, 217, 23–26.
Verhaeghen K., Van Bortel W., Roelants P., Backeljau T., Coosemans M. (2006) Detection of the East and West Africankdr mutation in *Anopheles gambiae* and *Anopheles arabiensis* from Uganda using a new assay based on FRET/Melt Curve analysis, *Malaria Journal*, 5, 16,
Viggiani G., Filella F., Delrio G., Ramassini W., Foxi C. (2009) *Tuta absoluta*, a new Lepidoptera now reported in Italy. *Informatore Agrario,* 65, 66-68.
Vontas J.G., Hejazi M.J., Hawkes N.J., Cosmidis N., Loukas M., Janes R.W., Hemingway J. (2002) Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*, *Insect Mol. Biol*. 11 329–336.
Vontas J.G., Hejazi M.J., Hawkes N.J., Cosmidis N., Loukas M.,. Janes R.W, Hemingway J. (2002) Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*, *Insect Mol. Biol*, 11, 329–336.
Vontas J.G., Small G.J., Hemingway J. (2001) Glutathione S-transferases as antioxidant defense agents confer pyrethroid resistance in *Nilaparvata lugens. Biochem, J*, 357, 65-71.
Voudouris C. C., Sauphanor B., Franck P., Reyes M., Mamuris Z., Tsitsipis J. A., Vontas J. , Margaritopoulos J. T. (2011). Insecticide resistance status of the codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) from Greece. *Pesticide Biochemistry and Physiology,* 100, 229-238.
Walsh S. B., Dolden T. A., Moores G. D., Kristensen M., Lewis T., Devonshire A. L., Williamson M.S. (2001) Identification and characterization of mutations in housefly (*Musca domestica*) acetylcholinesterase involved in insecticide resistance, *Biochem. J*, 359,175–181.
Wang W., Mo J., Cheng J., Zhuang P., Tang Z. (2006) Selection and characterization of spinosad resistance in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). *Pesticide Biochemistry and Physiology,* 84, 180-187.
Weill M., Berthomieu A., Berticat C., Lutfalla G., Negre V., Pasteur N., Philips A., Leonetti J. P., Fort P. , Raymond, M. (2004) *Insecticide resistance: a silent base prediction. Current biology : CB,* 14, R552-3.
Weill M., Lutfalla G., Mogensen K., Chandre F., Berthomieu A., Berticat C., Pasteur N., Philips A., Fort P., Raymond M. (2003) Comparative genomics: insecticide resistance in mosquito vectors, *Nature*, 423, 136–137.
Weill M., Malcolm C., Chandre F., Mogensen K., Berthomieu A., Marquine M., Raymond M. (2004) The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors, *Insect Mol. Biol*, 13, 1–7.
Werck-Reichhart D., and Feyereisen R. (2000) Cytochromes P450: a success story. *Genome Biology*, 3003, 1-9.
Williamson M. S., Anstead J. A., Devine G. J., Devonshire A. L., Field L. M., Foster S. P., Moores G. D., Denholm I., (2003) *Insecticide resistance: from science to practice*.
Williamson M. S., Denholm I., Bell C. A., Devonshire A. L. (1993) knockdown resistance (kdr) to ddt and pyrethroid insecticides maps to a sodium-channel gene locus in the housefly (musca-domestica). *Molecular and General Genetics,* 240, 17-22.

Williamson M. S., Martinez Torres D., Hick C. A., Devonshire A. L. (1996b) Analysis of sodium channel gene sequences in pyrethroid-resistant houseflies - Progress toward a molecular diagnostic for knockdown resistance (kdr). *In: Molecular Genetics and Evolution of Pesticide Resistance*, 52-61 Ed T. M. Brown.

Williamson M. S., Martinez Torres D., Hick C. A., Devonshire A. L. (1996c) Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. *Molecular and General Genetics,* 252, 51-60.

Williamson M. S., Martinez-Torres D., Hick C. A., Devonshire A. L. (1996a) The molecular genetics of knockdown resistance to pyrethroids. *Abstracts of Papers American Chemical Society,* 212, 16.

Wing K. D., Andaloro J. T., McCann S. F., Salgado V. L. (2005) Indoxacarb and the sodium channel blocker insecticides: chemistry, physiology and biology in insects. In: Gilbert L. I., Iatrou K., Gill S.S. (eds) Comprehensive insect science. Insect control, vol 6. Elsevier B.V., Amsterdam, 30–53.

Wing K. D., Schnee M. E., Sacher M., Connair M. (1998) A novel oxadiazine insecticide is bioactivated in lepidopteran larvae. *Arch Insect Biochem Physiol*, 37, 91–103.

Wondji C. S., Irving H., Morgan J., Lobo N. F. (2009) Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus*, a major malaria vector. *Genome Res*, 19, 452-459.

Yu F. H., Catterall W. A. (2003) Overview of the voltage-gated sodium channel family. *Genome Biology,* 4.

Zhang H., Tang T., Cheng Y., Shui R., Zhang W., Qiu, L. (2010) Cloning and expression of cytochrome P450 CYP6B7 in fenvalerate-resistant and susceptible *Helicoverpa armigera* (Hübner) from China. *Journal of Applied Entomology,* 134, 754-761.

Zhang L., Shi J., Shi X., Liang P., Gao J., Gao X. (2010) Quantitative and qualitative changes of the carboxylesterase associated with beta-cypermethrin resistance in the housefly, *Musca domestica* (Diptera: Muscidae). Comparative biochemistry and physiology. *Part B, Biochemistry and molecular biology,* 156, 6-11.

Zhang Y., Kua J., McCammon J.A. (2002) Role of the catalytic triad and oxyanion hole in acetylcholinesterase catalysis: An ab initio QM/MM study. *J. Am. Chem. Soc*, 124, 10572–10577.

Zhao X. and Salgado V. L. (2010) The role of GABA and glutamate receptors in susceptibility and resistance to chloride channel blocker insecticides. *Pesticide Biochemistry and Physiology,* 97, 153-160.

Zhou X., Sheng C., Li M., Wan H., Liu D., Qiu X. (2010) Expression responses of nine cytochrome P450 genes to xenobiotics in the cotton bollworm *Helicoverpa armigera*. *Pesticide Biochemistry and Physiology,* 97, 209-213.

Zhu K. Y., Lee S. H., Clark J. M. (1996) A point mutation of acetylcholinesterase associated with azinphosmethyl resistance and reduced fitness in Colorado potato beetle, *Pest. Biochem Physiol*, 55, 100–108.

Zlotkin E. (1999) The insect voltage-gated sodium channel as target of insecticides. *Annual Review of Entomology,* 44, 429-455.

Zlotkin E. (2001) Insecticides affecting voltage-gated ion channels. *Biochemical sites of insecticide action and resistance*, 43-76.

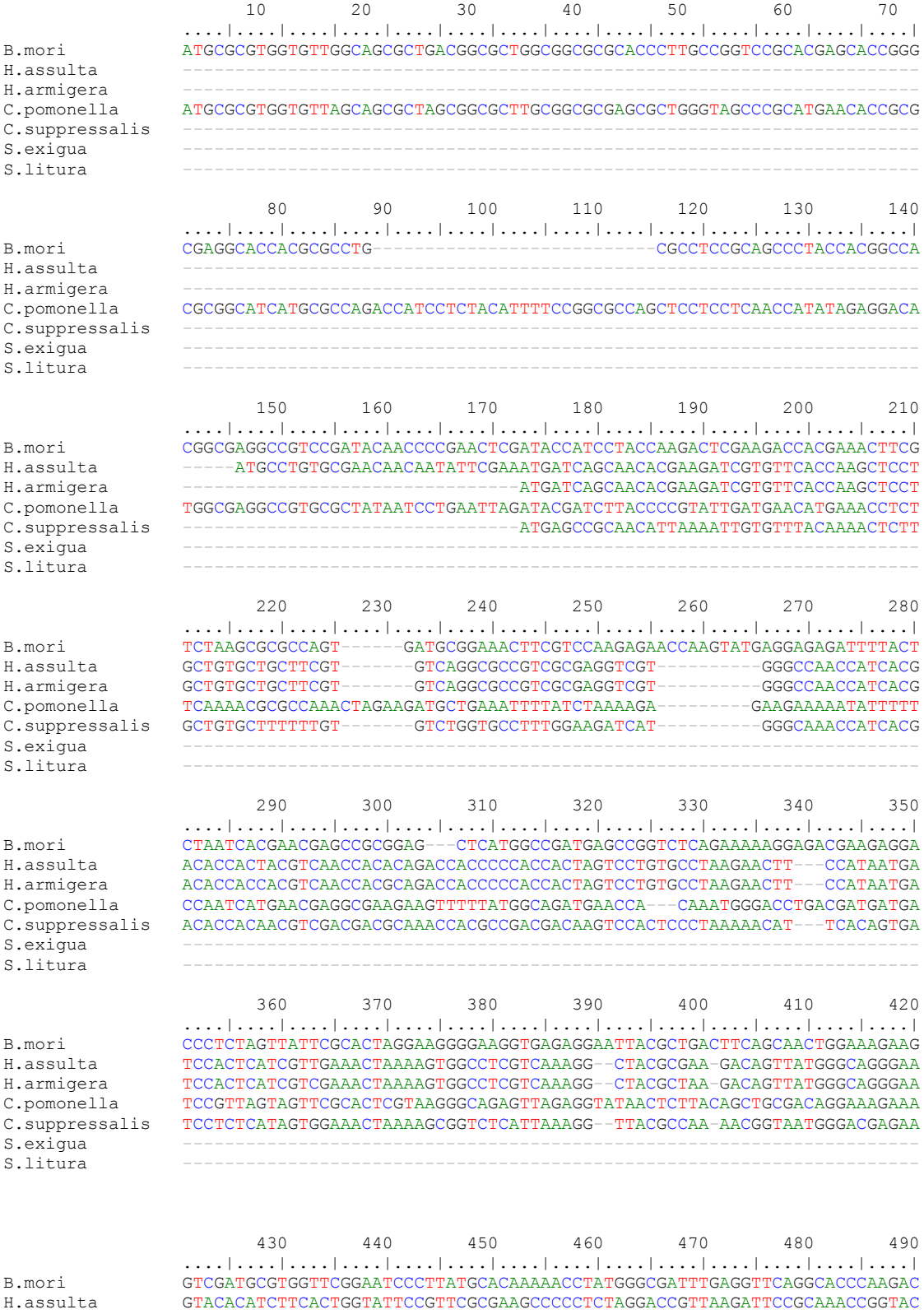
Zlotkin E., Fishman Y., Elazar M. (2000) AaIT: From neurotoxin to insecticide. *Biochimie,* 82, 869-881.

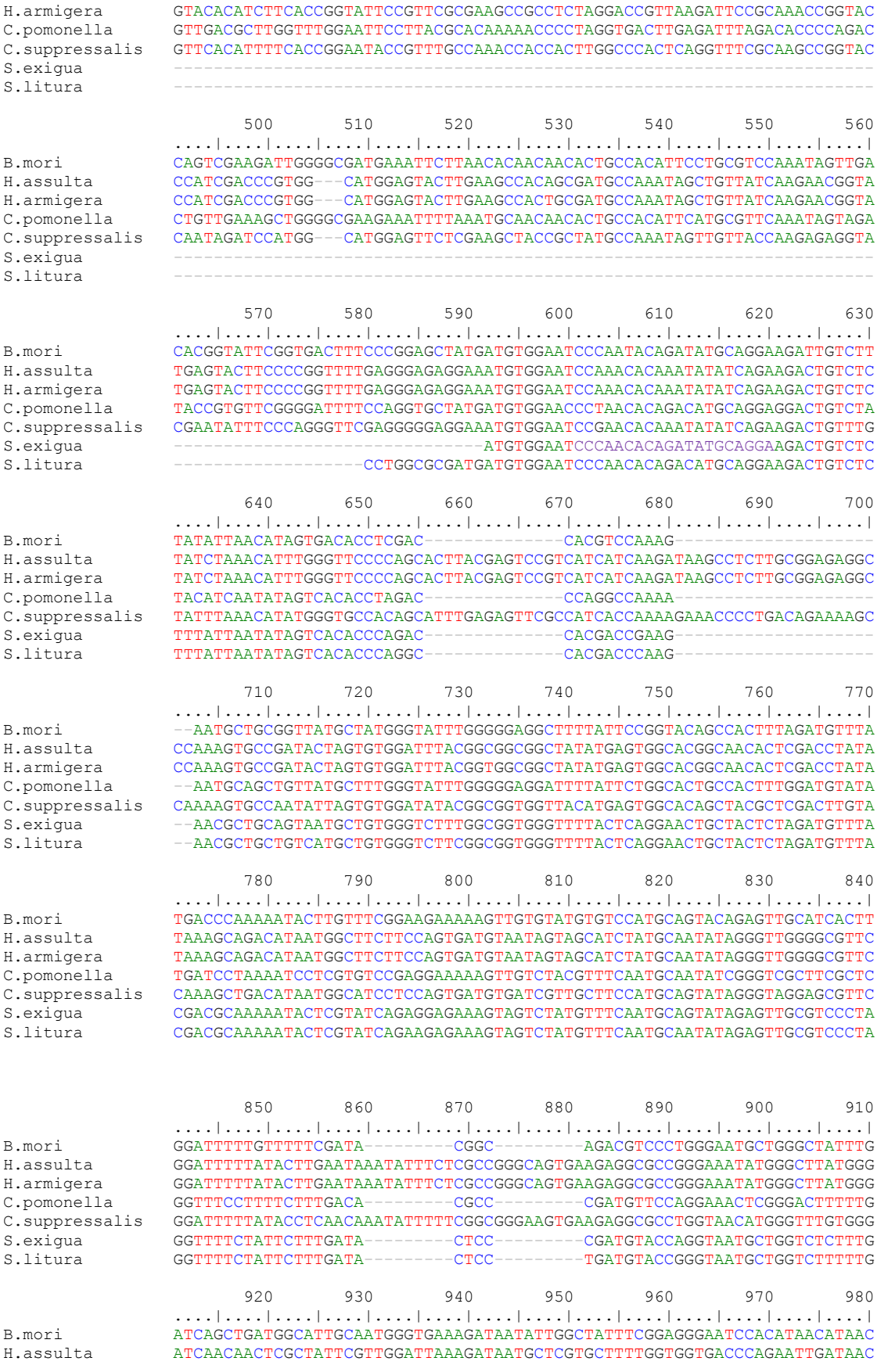
Zlotkin E., Moskowitz H., Pelhate M., Gordon D. (1994) the insect sodium-channel as the target for insect selective neurotoxins. *Journal of Neurochemistry,* 63, S4-S4.

Websites:
www.tutaabsoluta.com
www.pesticideresistance.org
www.irac-online.org

Appendix A

The alignment of Ache gene from different lipedopterea insects used to design primers.

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C.pomonella GTTGACGCTTGGTTTGGAATTCCTTACGCACAAAAACCCCTAGGTGACTTGAGATTTAGACACCCCAGAC
C.suppressalis GTTCACATTTTCACCGGAATACCGTTTGCCAAACCACCACTTGGCCCACTCAGGTTTCGCAAGCCGGTAC
S.exigua
S.litura
500 510 520 530 540 550 560
B.mori CAGTCGAAGATTGGGGCGATGAAATTCTTAACACAACAACACTGCCACATTCCTGCGTCCAAATAGTTGA
H.assulta CCATCGACCCGTGG---CATGGAGTACTTGAAGCCACAGCGATGCCAAATAGCTGTTATCAAGAACGGTA
H.armigera CCATCGACCCGTGG---CATGGAGTACTTGAAGCCACTGCGATGCCAAATAGCTGTTATCAAGAACGGTA
C.pomonella CTGTTGAAAGCTGGGGCGAAGAAATTTTAAATGCAACAACACTGCCACATTCATGCGTTCAAATAGTAGA
C.suppressalis CAATAGATCCATGG---CATGGAGTTCTCGAAGCTACCGCTATGCCAAATAGTTGTTACCAAGAGAGGTA
S.exigua
S.litura
570 580 590 600 610 620 630
B.mori CACGGTATTCGGTGACTTTCCCGGAGCTATGATGTGGAATCCCAATACAGATATGCAGGAAGATTGTCTT
H.assulta TGAGTACTTCCCCGGTTTTGAGGGAGAGGAAATGTGGAATCCAAACACAAATATATCAGAAGACTGTCTC
H.armigera TGAGTACTTCCCCGGTTTTGAGGGAGAGGAAATGTGGAATCCAAACACAAATATATCAGAAGACTGTCTC
C.pomonella TACCGTGTTCGGGGATTTTCCAGGTGCTATGATGTGGAACCCTAACACAGACATGCAGGAGGACTGTCTA
C.suppressalis CGAATATTTCCCAGGGTTCGAGGGGGAGGAAATGTGGAATCCGAACACAAATATATCAGAAGACTGTTTG
S.exigua ATGTGGAATCCCAACACAGATATGCAGGAAGACTGTCTC
S.litura CCTGGCGCGATGATGTGGAATCCCAACACAGACATGCAGGAAGACTGTCTC
640 650 660 670 680 690 700
B.mori TATATTAACATAGTGACACCTCGAC CACGTCCAAAG
H.assulta TATCTAAACATTTGGGTTCCCCAGCACTTACGAGTCCGTCATCATCAAGATAAGCCTCTTGCGGAGAGGC
H.armigera TATCTAAACATTTGGGTTCCCCAGCACTTACGAGTCCGTCATCATCAAGATAAGCCTCTTGCGGAGAGGC
C.pomonella TACATCAATATAGTCACACCTAGAC CCAGGCCAAAA
C.suppressalis TATTTAAACATATGGGTGCCACAGCATTTGAGAGTTCGCCATCACCAAAAGAAACCCCTGACAGAAAAGC
S.exigua TTTATTAATATAGTCACACCCAGAC CACGACCGAAG
S.litura TTTATTAATATAGTCACACCCAGGC CACGACCCAAG
710 720 730 740 750 760 770
B.mori --AATGCTGCGGTTATGCTATGGGTATTTGGGGGAGGCTTTTATTCCGGTACAGCCACTTTAGATGTTTA
H.assulta CCAAAGTGCCGATACTAGTGTGGATTTACGGCGGCGGCTATATGAGTGGCACGGCAACACTCGACCTATA
H.armigera CCAAAGTGCCGATACTAGTGTGGATTTACGGTGGCGGCTATATGAGTGGCACGGCAACACTCGACCTATA
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C.suppressalis CAAAAGTGCCAATATTAGTGTGGATATACGGCGGTGGTTACATGAGTGGCACAGCTACGCTCGACTTGTA
S.exigua --AACGCTGCAGTAATGCTGTGGGTCTTTGGCGGTGGGTTTTACTCAGGAACTGCTACTCTAGATGTTTA
S.litura --AACGCTGCTGTCATGCTGTGGGTCTTCGGCGGTGGGTTTTACTCAGGAACTGCTACTCTAGATGTTTA
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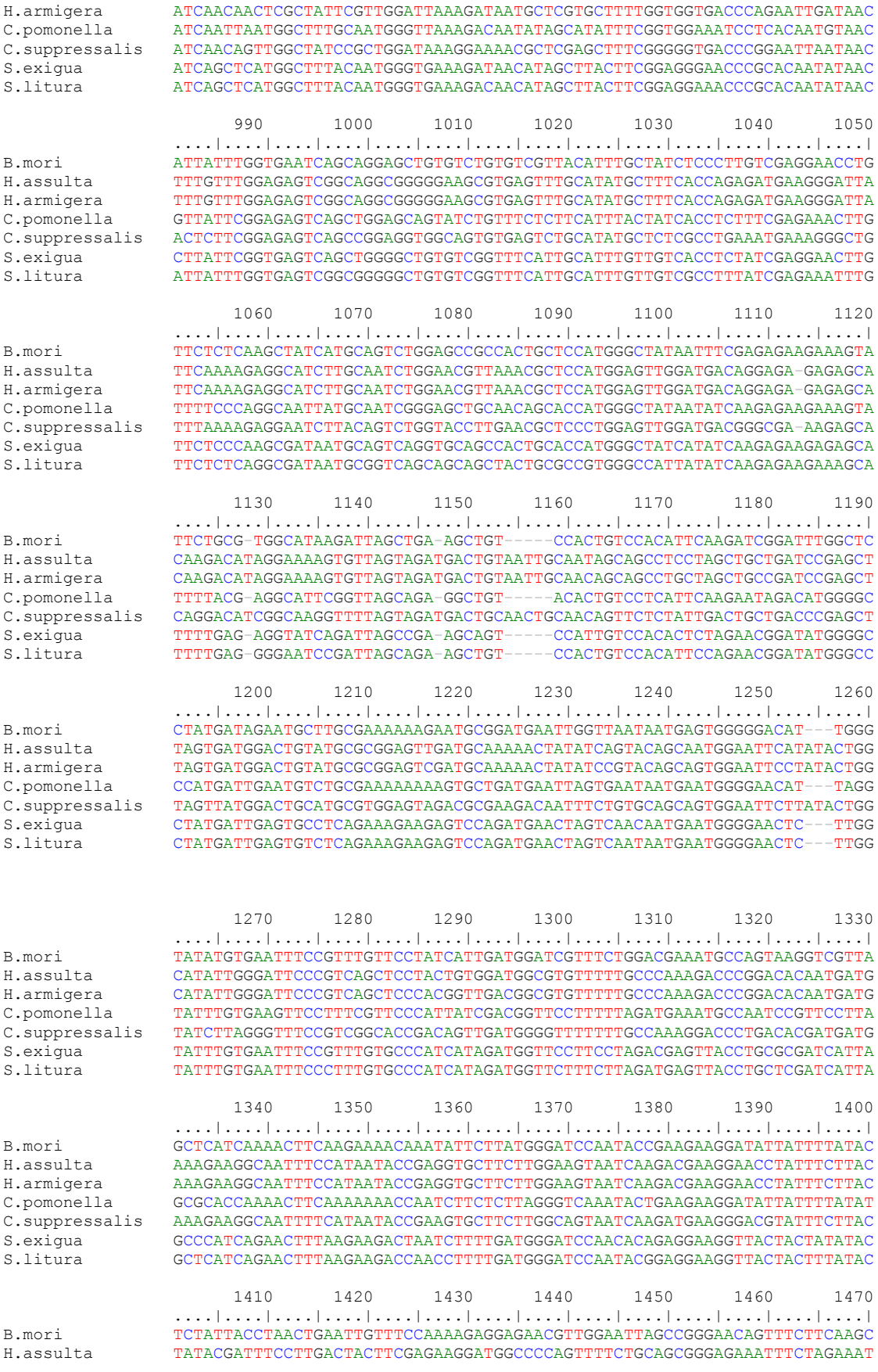
B.mori TGACCCAAAAATACTTGTTTCGGAAGAAAAAGTTGTGTATGTGTCCATGCAGTACAGAGTTGCATCACTT
H.assulta TAAAGCAGACATAATGGCTTCTTCCAGTGATGTAATAGTAGCATCTATGCAATATAGGGTTGGGGCGTTC
H.armigera TAAAGCAGACATAATGGCTTCTTCCAGTGATGTAATAGTAGCATCTATGCAATATAGGGTTGGGGCGTTC
C.pomonella TGATCCTAAAATCCTCGTGTCCGAGGAAAAAGTTGTCTACGTTTCAATGCAATATCGGGTCGCTTCGCTC
C.suppressalis CAAAGCTGACATAATGGCATCCTCCAGTGATGTGATCGTTGCTTCCATGCAGTATAGGGTAGGAGCGTTC
S.exigua CGACGCAAAAATACTCGTATCAGAGGAGAAAGTAGTCTATGTTTCAATGCAGTATAGAGTTGCGTCCCTA
S.litura CGACGCAAAAATACTCGTATCAGAAGAGAAAGTAGTCTATGTTTCAATGCAATATAGAGTTGCGTCCCTA
850 860 870 880 890 900 910
B.mori GGATTTTTGTTTTTCGATA---------CGGC---------AGACGTCCCTGGGAATGCTGGGCTATTTG
H.assulta GGATTTTTATACTTGAATAAATATTTCTCGCCGGGCAGTGAAGAGGCGCCGGGAAATATGGGCTTATGGG
H.armigera GGATTTTTATACTTGAATAAATATTTCTCGCCGGGCAGTGAAGAGGCGCCGGGAAATATGGGCTTATGGG
C.pomonella GGTTTCCTTTTCTTTGACA---------CGCC---------CGATGTTCCAGGAAACTCGGGACTTTTTG
C.suppressalis GGATTTTTATACCTCAACAAATATTTTTCGGCGGGAAGTGAAGAGGCGCCTGGTAACATGGGTTTGTGGG
S.exigua GGTTTTCTATTCTTTGATA---------CTCC---------CGATGTACCAGGTAATGCTGGTCTCTTTG
S.litura GGTTTTCTATTCTTTGATA---------CTCC---------TGATGTACCGGGTAATGCTGGTCTTTTTG
920 930 940 950 960 970 980
B.mori ATCAGCTGATGGCATTGCAATGGGTGAAAGATAATATTGGCTATTTCGGAGGGAATCCACATAACATAAC
H.assulta ATCAACAACTCGCTATTCGTTGGATTAAAGATAATGCTCGTGCTTTTGGTGGTGACCCAGAATTGATAAC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S.litura ATCAGCTCATGGCTTTACAATGGGTGAAAGACAACATAGCTTACTTCGGAGGAAACCCGCACAATATAAC
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B.mori ATTATTTGGTGAATCAGCAGGAGCTGTGTCTGTGTCGTTACATTTGCTATCTCCCTTGTCGAGGAACCTG
H.assulta TTTGTTTGGAGAGTCGGCAGGCGGGGGAAGCGTGAGTTTGCATATGCTTTCACCAGAGATGAAGGGATTA
H.armigera TTTGTTTGGAGAGTCGGCAGGCGGGGGAAGCGTGAGTTTGCATATGCTTTCACCAGAGATGAAGGGATTA
C.pomonella GTTATTCGGAGAGTCAGCTGGAGCAGTATCTGTTTCTCTTCATTTACTATCACCTCTTTCGAGAAACTTG
C.suppressalis ACTCTTCGGAGAGTCAGCCGGAGGTGGCAGTGTGAGTCTGCATATGCTCTCGCCTGAAATGAAAGGGCTG
S.exigua CTTATTCGGTGAGTCAGCTGGGGCTGTGTCGGTTTCATTGCATTTGTTGTCACCTCTATCGAGGAACTTG
S.litura ATTATTTGGTGAGTCGGCGGGGGCTGTGTCGGTTTCATTGCATTTGTTGTCGCCTTTATCGAGAAATTTG
1060 1070 1080 1090 1100 1110 1120
B.mori TTCTCTCAAGCTATCATGCAGTCTGGAGCCGCCACTGCTCCATGGGCTATAATTTCGAGAGAAGAAAGTA
H.assulta TTCAAAAGAGGCATCTTGCAATCTGGAACGTTAAACGCTCCATGGAGTTGGATGACAGGAGA-GAGAGCA
H.armigera TTCAAAAGAGGCATCTTGCAATCTGGAACGTTAAACGCTCCATGGAGTTGGATGACAGGAGA-GAGAGCA
C.pomonella TTTTCCCAGGCAATTATGCAATCGGGAGCTGCAACAGCACCATGGGCTATAATATCAAGAGAAGAAAGTA
C.suppressalis TTTAAAAGAGGAATCTTACAGTCTGGTACCTTGAACGCTCCCTGGAGTTGGATGACGGGCGA-AAGAGCA
S.exigua TTCTCCCAAGCGATAATGCAGTCAGGTGCAGCCACTGCACCATGGGCTATCATATCAAGAGAAGAGAGCA
S.litura TTCTCTCAGGCGATAATGCGGTCAGCAGCAGCTACTGCGCCGTGGGCCATTATATCAAGAGAAGAAAGCA
1130 1140 1150 1160 1170 1180 1190
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C.pomonella TTTTACG-AGGCATTCGGTTAGCAGA-GGCTGT-----ACACTGTCCTCATTCAAGAATAGACATGGGGC
C.suppressalis CAGGACATCGGCAAGGTTTTAGTAGATGACTGCAACTGCAACAGTTCTCTATTGACTGCTGACCCGAGCT
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S.litura TTTTGAG-GGGAATCCGATTAGCAGA-AGCTGT-----CCACTGTCCACATTCCAGAACGGATATGGGCC
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H.armigera TAGTGATGGACTGTATGCGCGGAGTCGATGCAAAAACTATATCCGTACAGCAGTGGAATTCCTATACTGG
C.pomonella CCATGATTGAATGTCTGCGAAAAAAAAGTGCTGATGAATTAGTGAATAATGAATGGGGAACAT---TAGG
C.suppressalis TAGTTATGGACTGCATGCGTGGAGTAGACGCGAAGACAATTTCTGTGCAGCAGTGGAATTCTTATACTGG
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S.litura CTATGATTGAGTGTCTCAGAAAGAAGAGTCCAGATGAACTAGTCAATAATGAATGGGGAACTC---TTGG
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H.armigera CATATTGGGATTCCCGTCAGCTCCCACGGTTGACGGCGTGTTTTTGCCCAAAGACCCGGACACAATGATG
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S.litura TATTTGTGAATTTCCCTTTGTGCCCATCATAGATGGTTCTTTCTTAGATGAGTTACCTGCTCGATCATTA
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C.pomonella GCGCACCAAAACTTCAAAAAAACCAATCTTCTCTTAGGGTCAAATACTGAAGAAGGATATTATTTTATAT
C.suppressalis AAAGAAGGCAATTTTCATAATACCGAAGTGCTTCTTGGCAGTAATCAAGATGAAGGGACGTATTTCTTAC
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S.litura GCTCATCAGAACTTTAAGAAGACCAACCTTTTGATGGGATCCAATACGGAGGAAGGTTACTACTTTATAC
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C.suppressalis   TCTACGACTTTCTTGACTACTTCGAAAAAGATGGGCCCAGTTTTCTACAGCGAGAGAAGTTCTTGGAGAT
S.exigua         TTTATTATCTTACTGAGTTATTCCCCAAGGAAGAGAATGTAGGGATAAGTAGGGAGCAGTATTTGCAGGC
S.litura         TTTATTATCTTACTGAACTATTCCCCAGGGAAGAGAATGTAGGGATAAGTAGGGAGCAGTACTTGCAGGC

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H.armigera       TGTCGACACTATATTCAAGGATTTCTCCAAAATAAAGAGGGAAGCTATCGTATTCCAATATACGGACTGG
C.pomonella      CGTAAGGGAGTTAAATCCATATGTCACTGATGTAGCTCGTCAAGCAATAGTGTTTGAGTACACTGATTGG
C.suppressalis   CGTCGACACGATTTTCAAGGAATTTTCCAAAATCAAAAGGGAAGCCATAGTTTTTCAATACACTGACTGG
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S.litura         CGTCAGAGAGTTGAATCCGTACGTGAATGACGTTGGCAGACAGGCTATTGTGTTCGAGTACACGGACTGG

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H.armigera       GAGGAAATTACCGATGGCTATCTGAACCAGAAAATGATAGCTGACGTGGTGGGCGACTATTTCTTCGTGT
C.pomonella      TTAAACCCTGATGATCCGATCAAAAATCGTAATGCATTGGATAAAATGGTGGGTGACTACCATTTTACTT
C.suppressalis   GAGGAAATCACAGATGGCTATCTAAACCAGAAGATGATAGCTGACGTGGTTGGCGATTATTTCTTCGTGT
S.exigua         TTGAACCCAGACGATCCAATTAGGAACCGTAATGCTTTGGATAAAATGGTGGGCGACTATCACTTCACGT
S.litura         TTGAACCCAGACGATCCAATAAGGAACCGAAATGCTTTGGATAAGATGGTTGGAGACTATCACTTTACTT

                       1620      1630      1640      1650      1660      1670      1680
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H.armigera       GCCCTACTAACTACTTCGCGGAAGTGCTGGCCGATTCAGGAGTTGATGTTTACTACTACTACTTCACACA
C.pomonella      GTGGTGTAAATGAGTTAGCACATCGTTACGCGGAGACTGGCAATAACGTGTATACATATTATTATAAACA
C.suppressalis   GCCCCACCAACTACTTCGCGGAGATCTTGGCCGATGCTGGGGTGGATGTCTACTATTACTACTTCACCCA
S.exigua         GC--------------------------------------------------------------------
S.litura         GTGGAGTGAATGAAATGGCGCATCGTTATGCAGAAACTGGAAACAACGTCTTTACATATTACTACAAGCA


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H.armigera       TCGCACCAGCACGAGCCTCTGGGGTGAATGGATGGGGGTGATGCACGGCGATGAGATGGAGTATGTCTTC
C.pomonella      CCGTAGTAAGAATAACCCATGGCCCTCATGGACGGGTGTCATGCATGCTGATGAAATCAATTATGTATTT
C.suppressalis   CCGCACCAGCACGAGTCTTTGGGGCGAATGGATGGGCGTGATGCACGGCGATGAGATGGAGTACGTGTTT
S.exigua         --------------------------------------------------------------------
S.litura         TCGCAGCAAGAACAACCCCTGGCCATCATGGACAGGAGTGATGCATGCTGATGAAATCAATTATGTATTC

                       1760      1770      1780      1790      1800      1810      1820
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C.pomonella      GGCGAGCCTCTGAATCCCGGAAAAAATTATTCACCGGAAGAAGTAGAATTCAGCAAACGAATGATGAGAT
C.suppressalis   GGCCACCCGCTAAACATGTCGCTGCAGTACCACACTCGCGAGCGGGACTTGGCCGCACACATCATGCAGT
S.exigua         --------------------------------------------------------------------
S.litura         GGGGAGCCATTGAATCCAGGGAAGAACTATTCGCCGGAGGAGGTGGAATTCAGCAAGCGCCTAATGAGAT

                       1830      1840      1850      1860      1870      1880      1890
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H.armigera       CTTTCACGAGATTCGCTTTGACTGGCAAACCACAC---AAGCCGGACG-AGAAATGGCCGCTGTACTCTC
C.pomonella      ACTGGGCAAACTTTGCGAAAACTGGAAATCCATCCTTAAGTCCGAATGGAGAAATGACTAAAGTGCACTG
C.suppressalis   CCTTCACCAGATTCGCTCTGACCGGTAAGCCACAC---AAGCCGGATG-AGAAATGGCCCCTGTACTCCA
S.exigua         --------------------------------------------------------------------
S.litura         ATTGGGCCAACTTA--------------------------------------------------------

                       1900      1910      1920      1930      1940      1950      1960
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C.suppressalis    GATCCT-CGCCGCACTACTATACGTACACAGCCGATGGCACCAGCGGCCCC----GCAGGACCCCGGGGA
S.exigua          ------------------------------------------------------------------------
S.litura          ------------------------------------------------------------------------

                        1970      1980      1990      2000      2010      2020      2030
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H.armigera        CCTAGGGCGTCCGCTTGCGCTTTCTGGAATGATTTCCTTAACAAGCTTAATGAGCTGGAACACATGCCGT
C.pomonella       CTGCGAGTTAAACAATGTGCTTTTTGGCAAAAATATCTCCCCCAA-TTAATATCAGCAACAAAAAAAACG
C.suppressalis    CCGAGGGCCTCCGCCTGTGCCTTCTGGAACGACTTCCTGAACAAACTCAACGAATTGGAGCACGTCCCCT
S.exigua          ------------------------------------------------------------------------
S.litura          ------------------------------------------------------------------------

                        2040      2050      2060      2070      2080      2090      2100
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H.assulta         GTGACGGGGCTGTGACCGGCCCGTACAGCAGCGTTGCCGGCACCACCCTGCCGATAGTTCTGCTGACCAC
H.armigera        GTGACGGCGCCGTGACCGGCCCGTACAGCAGCGTCGCCGGCACCACACTGCCGATAGTGCTGCTGACCAC
C.pomonella       GAACCACCCCGGAATTGCACTGGCAGTAGTTCCTTACTGCGCCCGTCGCTGCATACGCTAGGACTGGGCG
C.suppressalis    GTGATAGAGCAGTGACGGGCCCCTACAGTAGCGTAGCAGGCACTACCCTGCCCATACTGCTGCTCACTGC
S.exigua          ------------------------------------------------------------------------
S.litura          ------------------------------------------------------------------------


                        2110      2120      2130      2140      2150      2160      2170
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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H.armigera        GCTCGCCACCACCGTCGCACTCTAA------------------------------------------------
C.pomonella       TCGCGGCGGCGGCGGCGCCCTTCACACACCACTTGTTGTTGACACATTTAGTGTCCATGTTCATAAACAT
C.suppressalis    CCTCGCTACCAGCGTAGCCCTGTAA------------------------------------------------
S.exigua          ------------------------------------------------------------------------
S.litura          ------------------------------------------------------------------------
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Appendix B

Genetic code and amino acid abbreviations