Dedicated to:

My Father and Mother for all their love and support

"If Darwin were alive today the insect world would delight and astound him with its impressive verification of his theories of survival of the fittest. Under the stress of intensive chemical spraying the weaker members of the insect populations are being weeded out."

Rachel Carson

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BIOCHEMICAL AND MOLECULAR MECHANISMS OF ACARICIDE RESISTANCE IN *TETRANYCHUS URTICAE*

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Front cover: Clockwise from top left, a schematic diagram of the *para* voltage-gated sodium channel, two-spotted spider mites on roses, adult spider mites on their webs, schematic illustration of a cholinergic synapse

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Jahangir, November 2010

LIST OF ABBREVIATIONS

1-NA	1-naphthyl acetate
3D	three-dimensional structure
4-NPA	4-nitrophenyl acetate
4-NPB	4-nitrophenyl butyrate
4-NPP	4-nitrophenyl propionate
7-EFC	7-ethoxy-4-trifluoromethylcoumarin
aa	amino acid
AcSCh	acetylthiocholine
a.i.	active ingredient
ACCase	acetyl-coenzyme A carboxylase
ACh	acetylcholine
AChE	acetylcholinesterase
ANOVA	analysis of variance
APRD	Arthropod pesticide resistance database
ATHRos-Bf	bifenthrin resistant strain
ATHRos-Pm	organophosphate resistant strain
BC	biotin carboxylase
BCC	biotin carboxyl carrier
CARB	Carbamate insecticide/acaricide
CDNB	1-chloro-2,4-dinitrobenzene
CL	confidence limit
CNS	central nervous system
COE	carboxylesterase
CREVeg-Bf	bifenthrin resistant strain
CT	carboxyl transferase
D	degree of dominance
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAT	day(s) after treatment
DCNB	1,2-dichloro-4-nitrobenzene
DDT	dichlorodiphenyltrichloroethane
DEF	*S*,*S*,*S*-tributyl phosphorotrithioate
DEM	diethylmaleate

DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTNB	5,5- dithiobis 2-nitrobenzoic acid
EC	emulsifiable concentrate
FD	Field dose
GSH	glutathione
GSS	German susceptible strain
GST	glutathione *S*-transferase
IC_{50}	concentration for 50% inhibition
IEF	isoelectric focusing
IPM	integrated pest management
IRAC	Insecticide Resistance Action Committee
IRM	Insecticide Resistance Management
LC_{50}	lethal concentration that kills 50%
LS-VL	laboratory susceptible strain
M	mortality
MCB	monochlorobimane
METI	mitochondrial electron transport inhibitor
MO	mono-oxygenase
MOI	multiplicity of infection
MR-VL	multi resistant strain
nACh	nicotinic acetylcholine
OP	organophosphate insecticide/acaricide
P450	cytochrome P450 mono-oxygenases
para	voltage-gated sodium channel gene
PBO	piperonyl butoxide
PCR	Polymerase chain reaction
pNP	*p*-nitrophenol
PVDF	polyvinylidine fluoride
Rdl	resistance to dieldrin
RFU	relative fluorescence unit
RH	relative humidity
R_0	net reproductive rate
r_c	capacity of increase

r_m	intrinsic rate of increase
RNA	ribonucleic acid
RR	resistance ratio
SAMB	laboratory susceptible strain
SC	suspension concentrate
SEM	standard error of the mean
SR	synergism ratio
SR-TK	spirodiclofen resistant strain (Tina Kramer, Bayer CropScience)
SR-VP	spirodiclofen resistant strain (Van Pottelberge, Agrozoology)
T	generation time
T_c	cohort generation time
TP	transit peptide
TSSM	two-spotted spider mite
VGSC	voltage-gated sodium channels
WI	organophosphate resistant strain
WP	wettable powder

TABLE OF CONTENTS

SCOPE AND THESIS OUTLINE

The aim of the present thesis was to get more insight in a number of aspects dealing with acaricide resistance in the two-spotted spider mite, *Tetranychus urticae*. The problems associated with the widespread occurrence of acaricide resistance in this species stimulated us to investigate this phenomenon. Out of the wide range of research topics that could have been interesting to study, we have chosen to focus on the biochemical and molecular mechanisms involved in acaricide resistance. We therefore chose three important groups of acaricides, two of which have been used for decades (i.e. pyrethroids and organophosphates) and a recently introduced group (tetronic acid derivatives). Next to a number of more fundamental topics, we also wanted to study some more practical aspects by trying to determine the acaricide resistance status and cross-resistance patterns in this species in several strains collected in the field.

Chapter I provides an overview of the taxonomy, biology and pest status of *T. urticae*. The known mechanisms of resistance to insecticide and acaricides are discussed with emphasis on mechanisms conferring resistance in the two-spotted spider mite.

Pyrethroids are currently widely used insecticides/acaricides and many pest species have developed resistance to them. Therefore, the resistance mechanisms responsible for resistance to an important pyrethroid acaricide, bifenthrin, in *T. urticae* were investigated. **Chapter II** focuses on the resistance-associated mutations in the *T. urticae para* sodium channel, the target site of pyrethroids. Sequence characteristics and phylogeny of a partial cDNA fragment of the *T. urticae* sodium channel and its alternative splicing variants are presented.

Next to pyrethroids, organophosphates still occupy a dominant position as crop protection chemicals. Despite the fact that organophosphates are usually not used directly as acaricides, spider mites are frequently exposed to them while crops are treated against insect pests. The mechanism of resistance to organophosphates in two-spotted spider mite and its consequences are presented in **Chapter III**. Several point mutations in the *T. urticae* acetylcholinesterase conferring resistance to organophosphates and carbamates and also a highly developed sensitive monitoring technique based on the PCR-RFLP are discussed.

To overcome the problem of resistance in spider mites, one of the best approaches is the judicious use of new chemistries with novel mode of actions. If it were possible to predict likely resistance mechanisms to novel acaricides before they evolve, it might lead to more effective management strategies. Spirodiclofen differs from other acaricides in its chemical structure and biochemical mode of action. It inhibits lipid biosynthesis, possibly through

inhibition of acetyl-CoA carboxylase (ACCase). No field resistance to spirodiclofen has been reported up till now. The results of a large number of experiments trying to elucidate spirodiclofen resistance, using a strain which has been selected for spirodiclofen resistance in the laboratory, are presented in **Chapter IV.** First, the results of crossing experiments, biochemical assays and cDNA sequences in spirodiclofen resistant and susceptible strains are discussed, followed by results on the *in vitro* expression of the spirodiclofen target site, ACCase, in insect cell lines and bacteria. Expression of the target genes in model systems can be an essential step in elucidating resistance mechanisms at the molecular level, since the functionality of reported field mutations can be mimicked by site directed mutagenesis *in vitro* and their effect on acaricide binding can be quantified.

Results of the survey on the acaricide resistance status in *T. urticae* populations collected from cut roses in greenhouses were presented in **Chapter V**. Details on the toxicity of 10 commercially important acaricides on a large number of *T. urticae* strains, the activities of detoxifying enzymes and the presence of resistance-associated point mutations in the collected strains are discussed and practical advice is given concerning the future use of some of the tested compounds.

CHAPTER I

INTRODUCTION

1 TWO-SPOTTED SPIDER MITE

1.1 Classification

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Chelicerata
Class: Arachnida
Subclass: Acari
Superorder: Acariformes
Order: Trombidiformes
Suborder: Prostigmata
Superfamily: Tetranychoidea
Family: Tetranychidae
Subfamily: Tetranychinae
Genus: *Tetranychus*
Species: *T. urticae*
(Lindquist et al. 2009)

The subclass of the Acari, or mites and ticks, are a very diverse group of arthropods which form the bigger part of the class Arachnida (which also comprises scorpions, spiders and harvestman) (Zhang 2003, Lindquist et al. 2009). They have colonized any terrestrial and aquatic habitat capable of supporting life. Up till now, approximately 55,000 acarine species have been described but the estimated numbers of extant species vary from 500,000 to 1,000,000. Described acarine species are classified in 5500 genera, 540 families, 124 superfamilies and 6 orders (Krantz 2009).

The two-spotted spider mite (TSSM), *Tetranychus urticae*, is a member of the family Tetranychidae or spider mites. Tetranychids are obligatory phytophagous mites and are unique in having greatly elongated movable cheliceral stylets which are used to puncture individual cells of their host plants (Walter et al. 2009). The family Tetranychidae is a large family, including 1250 species belonging to 73 genera. It also is undoubtedly the most important family of mites attacking plants (Zhang 2003, Vacante 2010). Well known economically important pests belonging to this group are *T. urticae*, the fruit tree red spider mite *Panonychus ulmi* and the citrus red spider mite *Panonychus citri*. TSSM is also known

as the red spider mite because of the red/ orange colour of the overwintering form, and as the glasshouse spider mite, due to its importance in protected crops (Jeppson et al. 1975, Zhang 2003).

The taxonomic placing of *T. urticae* has been an issue of debate for the past few decades and as many as 44 synonymous names are used for this species (Bolland et al. 1998). Although interspecific matings have been reported between *T. urticae* and some closely related species, some of which resulted in viable F1 females, F2 eggs cannot hatch possibly due to a post-zygotic reproductive barrier (Ben-David et al. 2009).

1.2 Developmental biology

The TSSM has five life stages i.e. egg, larva, protonymph, deutonymph and adult. The spherical, semi-translucent eggs are often deposited on the underside of leaves. As the embryo develops, the eggs become opaque and yellow. Prior to hatching, the red eye spots of the larva are visible. The yellowish six-legged larval stage is followed by two yellowish green eight-legged nymphal stages, the protonymph and deutonymph. Adult females are about 0.5 mm long, pale yellow, greenish or straw-coloured, with two characteristic lateral dark green to black spots which are the result of an accumulation of food material in the digestive tract (summer form). Only female adults overwinter. In autumn, due to shorter photoperiod, lower temperatures and declining food supply, they become orange or red and lose their dark body spots (non-feeding overwintering form). In spring, females leave their overwintering places, start to feed, loose their red colour and start egg deposition. Males are about 0.3 mm long, with a narrower, more pointed abdomen. At the end of each active larval and nymphal stage, there are quiescent phases called nymphochrysalis (=protochrysalis), deutochrysalis and teliochrysalis. During these periods, mites anchor to a leaf or to their webbing, to prepare moulting (Crooker 1985).

Reproduction is arrhenotokous parthenogenesis. Males are haploid and develop from unfertilized eggs, whereas females are diploid and develop from fertilized eggs. Unmated females give rise only to males, while mated females can produce both female or male progeny. The sex ratio is three females: one male (Helle and Sabelis 1985). Male adults emerge earlier than females. The males await the emergence of the female adult and often help them to free themselves from exuvia. Immediately after emergence, copulation takes place (Mitchell 1973, Potter et al. 1976).

The life cycle of spider mites is driven by temperature, as is the case in most arthropods. Table 1.1 provides a summary of the effect of temperature on a number of biological traits of TSSM. Females and males have a similar total developmental time at 15 and 20°C, but at

higher temperatures males develop faster. The duration of the life stages decreases with increasing temperature up to 30°C (Bounfour and Tanigoshi 2001).

1.3 Host plants and damage

Among spider mites, *T. urticae* is the most polyphagous species. It causes significant yield losses globally in many cultivated crops, both in the field and greenhouses (Jeppson et al. 1975). A recent checklist of host plants of TSSM includes more than 1000 species (Bolland et al. 1998, Zhang 2003, Migeon and Dorkeld 2010). TSSM is an economically important pest in protected vegetable crops, especially in *Solanaceae* (e.g. tomatoes, eggplant, pepper) and *Cucurbitaceae* (e.g. cucumbers, squashes), and greenhouse ornamentals (e.g. roses, chrysanthemum, carnations, azalea). It is also a major pest in annual field crops such as maize, soybean and sugar beet, and in perennial cultures like alfalfa, strawberries, grapevines, citrus, apples, pears, almonds, plums, maples and elms.

Table 1.1 Developmental duration, sex ratio, life table parameters, longevity and oviposition rates for *T. urticae* at four constant temperatures (mean± SEM) (Bounfour and Tanigoshi 2001)

Life stages or parameters	Temperature, °C			
	15	20	25	30
Developmental duration in days				
egg	10.18±0.28	6.56±0.17	3.06±0.08	2.7±0.08
larva	2.52±0.09	1.63±0.07	1.14±0.09	0.75±0.06
nymphochrysalis	2.65±0.07	1.32±0.09	0.98±0.07	0.59±0.03
protonymph	2.57±0.11	1.36±0.09	1.63±0.11	0.82±0.04
deutochrysalis	2.41±0.11	1.37±0.09	1.85±0.12	0.54±0.04
deutonymph	2.38±0.06	1.69±0.11	1.97±0.11	0.78±0.09
teliochrysalis	2.47±0.07	1.94±0.11	2.22±0.12	0.64±0.04
total - female	25.3±0.55	16.0±0.44	13.9±0.37	7.4±0.27
total - male	24.9±0.64	15.9±0.49	10.7±0.45	6.0±0.30
Sex ratio				
proportion of female	65%	63%	65%	63%
Life table parameters				
net reproductive rate R_o	24.66	80.99	54.86	86.01
capacity of increase r_c	0.082	0.149	0.174	0.259
intrinsic rate of increase r_m (day^{-1})	0.084	0.166	0.188	0.321
cohort generation time, T_c (days)	39.28	29.53	22.97	17.19
mean generation time T (days)	38.29	26.48	21.25	13.86
Longevity and oviposition				
female longevity	22±1.4	26.9±0.8	20.9±1.1	17.1±1.63
total eggs per female	38.1±2.9	124.7±4.6	92.8±6.4	121.2±12.4
eggs per female per day	1.7±1.08	4.6±0.12	4.4±0.17	7.1±0.18

All moving stages of the TSSM feed actively on the lower leaf surface by piercing epidermal cells with their cheliceral stylets, after which they consume the protruding cell fluid. The upper surface of the leaf develops a characteristic whitish or yellowish speckling. These speckles may join and become patches and turn reddish-brown as the damage intensifies. Heavy damage results in complete defoliation, and the plant may die prematurely (Devine et al. 2001).

TSSM has been an important agricultural pest since 1900 (van de Vrie et al. 1972). Recently dramatic crop losses caused by TSSM have been described in Europe, as exemplified by the outbreak in Belgium sugar beet fields in 1996 (Legrand et al. 1997). In heavy infestations the crop loss reached to 60 percent (AGRIS 1997, Carbonelle et al. 2007).

Considering that TSSM becomes more destructive during dry and hot weather conditions, due to its increase in feeding activity and reproductive rate (Weidhaas 1979), global warming and climate change may lead to an increase in outbreaks and host plant damage and a wider geographical distribution. There are already some reports on the northward expansion of TSSM distribution area in Western Europe (Carbonelle et al. 2007).

The control of TSSM is achieved mainly through the use of biological and chemical methods in integrated pest management programs. Today biological control, for the bigger part by means of predatory phytoseiid mites, is widely used and successful in protected crops. However, the use of acaricide based chemical control still is a necessity, especially in field crops where biological control has not been adopted yet and for correcting biological control failures in protected crops (Zhang 2003, Vacante 2010).

Managing TSSM and other spider mites is becoming more and more difficult due to the decreasing number of registered acaricides and the quick development of resistance to many important acaricides.

2 PESTICIDE RESISTANCE

2.1 Magnitude and definition

Arthropod pests cause enormous annual losses in crop production worldwide and are able to transmit a number of infectious diseases. Pesticides play a key role in their management. However, frequent application of pesticides on the same pest population may lead to pesticide resistance, one of the most serious obstacles and challenges for effective pest control today. Since the first report on resistance development to lime sulphur in 1908 (Melander 1914), pesticide resistance in arthropods has occurred and is still occurring with an ever increasing frequency, both in numbers of species and pesticides affected. While in the early 1950s

resistance was still a relatively rare phenomenon, since the late 1980s, on the contrary, fully susceptible insect populations are becoming hard to find. The database on arthropod pesticide resistance (http://www.pesticideresistance.org) indicates that currently over 570 species of insects and mites have developed resistance to one or more insecticides and acaricides, often belonging to the major classes of active ingredients (Whalon et al. 2010). Pesticide resistance poses serious economic and public health problems because it compromises agricultural production and causes outbreaks of vector-borne diseases. It also results in an increased pesticide use, higher environmental contamination and disruption of wildlife and natural enemies, leading to disturbances in ecosystems (Georghiou 1986).

Pesticide resistance has been defined as "the inherited ability in a target population to tolerate doses of toxicant that would prove lethal to a majority of individuals in a normal population of that species" (National Research Council 1986). Tabashnik et al. (2009) defined resistance as "a heritable decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin". These definitions do not imply loss of economic efficacy in the field, yet another definition provided by Whalon and McGaughey (1998) states that resistance "is a microevolutionary process of genetic adaptation in response to pesticides that can result in unique and often more difficult management challenges".

Arthropods commonly exhibit different levels of resistance to a range of insecticides. A situation where resistance to a pesticide by a pest population results in resistance to a second pesticide or group of pesticides without having been exposed to them is called cross-resistance. In this phenomenon a single gene or mechanism confers resistance to more than one pesticide. When two or more different resistance mechanisms (each affecting different groups of chemicals) are involved in the same individual or pest population, it is called multiple resistance (Georghiou 1986, Stenersen 2004). Biochemical tests are required to determine whether or not a mechanism is conferring cross-resistance to the tested insecticides or several mechanisms are conferring multiple-resistance (Perez et al. 2009).

2.2 Resistance mechanisms

Although there are thousands of arthropod pest species and a wide variety of pesticides, the number of processes known to cause resistance to pesticides is limited to a very few types. These mechanisms involve either the reduction of the effective dose of the pesticide that reaches the target site (pharmacokinetically derived resistance) or the modification of the target site itself (pharmacodynamically derived resistance) or a combination of the two. Well demonstrated mechanisms such as behavioural avoidance, reduced penetration through the cuticle, enhanced excretion, sequestration and detoxification all contribute to decrease the

dose of the pesticide that finally reaches the target site. Alternatively, target site modifications, will contribute to render a dose of pesticide ineffective through reduced sensitivity of the target site by point mutations or by reducing or increasing the quantity of target site (Feyereisen 1995). These two types of resistance mechanisms are generally referred to as metabolic resistance (increased pesticide detoxification) and target site insensitivity (alterations to the pesticide target site). In both, several biochemical mechanisms are involved. Other mechanisms are generally considered to play a minor role or to arise only under very specialized conditions (Denholm and Devine 2001). Depending on the mechanism involved, three general types of genetic changes can result in resistance: a gene may be amplified, the expression of a gene may be altered, or a structural alteration in a gene encoding target site proteins or detoxifying enzymes can result in resistance (Scott 1995).

2.2.1 Metabolic resistance

Metabolic resistance can be caused by interference with three major enzyme systems: cytochrome P450 mono-oxygenases (P450s), esterases, and glutathione S-transferases (GSTs). These enzymes act by actively degrading the pesticide to non-toxic products, or by binding rapidly to the pesticide (sequestration), combined with very low degradation activity. Metabolically resistant strains possess enzymes that are quantitatively and/or qualitatively different from the enzymes of the susceptible strains (Hemingway et al. 1998).

Cytochrome P450 enzymes are best known for their mono-oxygenase activity, catalyzing the transfer of one atom of molecular oxygen to a substrate and reducing the other to water. P450s catalyse a variety of detoxification reactions in virtually all living organisms and can potentially confer resistance to most chemical classes. Biochemical changes in P450 structure or activity can lead to changes in insecticide activation or inactivation. To date, in a few instances gene amplification contributes to P450-mediated resistance (Wondji et al. 2009, Puinean et al. 2010), but other molecular mutations (structural, up- or down-regulation) are frequently involved. The first indirect lines of investigation for this mechanism have been the use of mono-oxygenase inhibitors (e.g. piperonyl butoxide, PBO) and the measurement of metabolic breakdown of selected model substrates. However, metabolic studies with the insecticide itself in the resistant strain are more conclusive. Recently, genomic information has been used to search for differences in the P450 gene structures and expression between susceptible and resistance strains (Feyereisen 2005, Li et al. 2007, Hollingworth and Dong 2008).

Esterases are a large group of enzymes able to hydrolyze ester bonds in the presence of water. Many widely used insecticides contain ester bonds, including virtually all organophosphates

and carbamates, most pyrethroids, and other compounds belonging to different chemical groups (i.e. acequinocyl, spiromesifen, fluacrypyrim and bifenazate). Esterases are frequently implicated in insecticide resistance through gene amplification, upregulation, coding sequence mutations, or a combination of these mechanisms (Li et al. 2007, Hollingworth and Dong 2008). The majority of esterases which function by sequestration are overproduced through gene amplification. Point mutations within structural genes produce esterases with an enhanced capability for increased metabolism. Finally, mutations in regulatory genes may also cause the over-expression of these enzymes resulting in an enhanced capacity for metabolizing insecticides (Oakeshott et al. 2005a, Wheelock et al. 2005, Li et al. 2007, Hollingworth and Dong 2008).

Glutathione S-transferases are a diverse family of multifunctional enzymes that enhance the conjugation of electrophilic sites of xenobiotics with the thiol group of reduced glutathione (GSH), generally resulting in GSH conjugates which are less toxic and more water soluble and excretable than the non-GSH conjugated substrates. Increases in the amount of one or more GST enzymes have been implicated in resistance to all the major classes of insecticides, either as a result of gene amplification or more commonly through increases in transcriptional rate, rather than through qualitative changes in individual enzymes. GSTs are also involved in other reactions besides conjugation. Dehydrochlorination of DDT to the noninsecticidal metabolite DDE is catalyzed by GSTs as a cofactor (Enayati et al. 2005, Ranson and Hemingway 2005, Li et al. 2007). GSTs also play a very important role in conferring resistance to pyrethroid insecticides in *Nilaparvata lugens* through detoxifying lipid peroxidation products induced by pyrethroids and protecting tissues from oxidative damage (Vontas et al. 2001).

2.2.2 Target site resistance

A change in the target protein that decreases the pesticide binding or activity results in target site resistance. Point mutations leading to alterations of critical amino acids responsible for insecticide binding cause the insecticide to be less effective or even ineffective. Overproduction of target molecules, as is sometimes recorded for herbicides or drugs, is not commonly reported in arthropods. Despite the potential complexity and/or large size of the target site receptors or enzymes, a limited number of resistance associated amino acid substitutions can cause a sufficient resistance while retaining an adequate physiological function of the receptor or enzyme. Hence, identical amino acid substitutions are commonly found across diverged taxa (ffrench-Constant et al. 1998, Hollingworth and Dong 2008).

Generally, insecticide target molecules are functionally important and therefore knockout mutations in target molecules will tend to be lethal. However, there are few reports that the insecticide target site is not essential for the insect's survival and a knockout mutation in a target molecule results in insecticide resistance (Baxter et al. 2010). For example, mutations resulting in a non-functional cadherin protein are an important mechanism for Cry1Ac resistance in some Bt resistant lepidopteran pests (Gahan et al. 2001, Zhao et al. 2010).

The genes encoding for the historically important targets of conventional insecticides are: *Rdl*, which encodes a γ-aminobutyric acid receptor subunit (RDL) as the target site of cyclodiene insecticides; *para*, which encodes for a voltage-gated sodium channel, target site of DDT and pyrethroids; and *Ace*, which encodes for insect acetylcholinesterase (AChE), target site of organophosphorus and carbamate insecticides. An alanine 302 to serine replacement within RDL confers resistance to cyclodienes in a wide range of insect species (ffrench-Constant et al. 2000). So far, about 26 mutations in sodium channel genes have been found to be responsible for pyrethroid resistance in different insect and arachnid pest species (Soderlund 2005, Dong 2007). Several point mutations in the AChE genes are known to provide decreased sensitivity to inhibition of the enzyme by organophosphate and carbamate insecticides. Each mutation in the AChE provides a specific resistance pattern, sometimes conferring resistance to one insecticide while increasing sensitivity to another (Fournier 2005).

2.3 Detecting and characterizing resistance

Detecting and monitoring resistance and understanding its biochemical and molecular mechanisms have a practical use both in resistance management and the search for new pesticides. Although several laboratory methods are available for detecting and characterizing resistance, bioassays still remain the main component of most resistance monitoring programs, certainly where resistance mechanisms have not been identified. Since bioassays are resource-intensive, rapid and more incisive biochemical and molecular assays can be used in monitoring and also diagnosing the type of resistance mechanism present. A variety of biochemical methods have been adopted for unravelling the mechanisms and detection of resistance, including electrophoretic or immunological detection of detoxifying enzymes, kinetic and end-point assays for quantifying the activity of enzymes or their inhibition by insecticides. In cases where the molecular mechanisms of resistance are known and the underlying DNA changes identified, DNA-based diagnostics for mutant resistant alleles is an available option. Point mutations, deletions, and inversions can all be detected through designing allele-specific probes and PCR primers (Denholm and Devine 2001, Hollomon

2002, Black and Vontas 2007). Although most of the genes encoding for the targets of conventional insecticides have been identified in important pest species, recent advances in insect genomics now facilitate the identification of new resistance genes and also (whole genome) expression microarray data further unravels complex detoxification pathways (Hemingway et al. 2002, ffrench-Constant et al. 2004). The discovery of resistance-associated mutations can be also facilitated by new generation genome/transcriptome sequencing technologies (Vontas et al. 2010).

2.4 Genetics of insecticide resistance

Resistance is a heritable trait and under genetic control. In population genetics of resistance some aspects are critical for the formulation of resistance management strategies such as the expression of resistance (i.e. dominant vs. recessive), whether the resistance is monogenic or polygenic, and how many independent origins they have.

Generally crossing experiments are used to determine whether the resistance is sex-linked or autosomal and whether the resistance trait is dominant, recessive or intermediate. Such procedures are carried out using crosses between the resistant and susceptible strains in reciprocal directions. Bioassays on offspring resulting from backcrosses between F1 progeny and the most phenotypically different parent can provide insights in the number of genes involved. Economically important cases of insecticide and acaricide resistance have been usually due to a single dominant or semi-dominant gene. However, the number of resistance genes depends on whether selection acts within or outside of the phenotypic distribution of the susceptible population. The doses applied in the field are designed to kill every individual (selection outside the normal range of the physiological distribution), whereas repeated selection in the laboratory necessitates at least some survivors and uses doses at 80-90% mortality (selecting within the normal physiological distribution). Most laboratory strains selected continuously from small genetically-uniform populations, show polygenically inherited resistance, whereas most resistant field strains show monogenic resistance (Roush and McKenzie 1987, ffrench-Constant et al. 2004). Although polygenic resistance in field situations may be less common than monogenic resistance, there are some well known cases of polygenic resistance. Resistance through a high frequency of major resistance genes in field populations may result rather from the very strong selection than from an inherent bias in genetic potential. The response observed in the field depends on how the variation is screened. The intent of new methods of pesticide application to lower the effective intensity of selection may increase the incidence of polygenic resistance (Via 1986, McKenzie 2000). Furthermore, when a gradual selection is operating in the range of an existing genetic

variation, a series of resistance genes may evolve, and will lead to polygenic resistance (Gassmann et al. 2009).

Insecticide resistance is believed to be the result of the natural selection of pre-existing forms that possess genetically controlled mechanisms conferring some degree of resistance. Insecticides, at recommended application rates, often are not mutagenic; therefore it is supposed that resistance associated mutations occur independently of insecticide application. Despite that, the pre-adaptative nature of resistance evolution is a well established hypothesis (Crow 1957, Hartley et al. 2006), it was also stated that insecticides might increase mutation rates (post-adaptive), especially with regard to amplified resistance genes. However, no substantial data have been provided to test this hypothesis (Devonshire and Field 1991, ffrench-Constant 2007). Resistance alleles may arise once and spread globally or arise several times independently at different locations. Both hypotheses, single or multiple origins, have been supported by DNA sequence data flanking the insecticide associated mutations (Andreev et al. 1999, Daborn et al. 2002, ffrench-Constant et al. 2004).

2.5 Selection of resistance genes

Resistance does not evolve at the same rate for all organisms; it may develop rapidly in one species and more slowly in another. The rate at which resistance genes are selected reflects the combined influences of many factors which, for convenience, can be classified into factors relating to the intrinsic properties of pests and resistance mechanisms within these pests (genetic and ecological) and factors relating to the chemical itself and how it is applied (operational). Georghiou and Taylor (1986) classified these influencing factors as shown in Table 1.2.

One of the important factors influencing the strength of selection and stability of resistance is the relative fitness of susceptible and resistant genotypes. Despite the advantages they confer under exposure to insecticides, with few exceptions, resistance genes confer lower fitness than their susceptible counterparts. This could lead to counterselection when insecticides are not applied. Continued selection may improve fitness through coadaptation of the resistant genome, i.e., the integration of resistance genes with other modifier loci that ameliorate fitness costs, resulting in more stable resistance (Georghiou and Taylor 1986, Denholm and Devine 2001). The fitness estimates have to be obtained for resistant and susceptible genotypes as functions of stage in the life cycle and concentrations of pesticides and it is necessary to check for a variety of other environmental and genetic factors such as temperature, season, physiological state, population density, and genetic background (Georghiou and Taylor 1986). In many cases the strength of the selection pressure of

insecticides is far greater than the counterbalancing selection imposed by fitness costs (Gassmann et al. 2009).

Table 1.2 Known or suggested factors influencing the selection of resistance to insecticides in field populations (Georghiou and Taylor 1986)

A. Genetic	**B. Biological/Ecological**	**C. Operational**
a. Frequency of R alleles	**1.** Biotic	**1.** The chemical
b. Number of R alleles	a. Generation turnover	a. Chemical nature of pesticide
c. Dominance of R alleles	b. Offspring per generation	b. Relationship to earlier-used chemicals
d. Penetrance, expressivity, interactions of R alleles	c. Monogamy/polygamy, parthenogenesis	c. Persistence of residues, formulation
e. Past selection by other chemicals	**2.** Behavioural/Ecological	**2.** The application
f. Extent of integration of R genome with fitness factors	a. Isolation, mobility, migration	a. Application threshold
	b. Monophagy/polyphagy	b. Selection threshold
	c. Fortuitous survival, refugia	c. Life stage(s) selected
		d. Mode of application
		e. Space-limited selection
		f. Alternating selection

2.6 Managing insecticide resistance in arthropods

The aim of insecticide resistance management (IRM) is to reduce the deleterious impact of resistance on pest management through the implementation of strategies for overcoming resistance to currently used compounds or preventing its development to existing or new compounds. Resistance management tactics should be aimed at reducing allele frequencies, reducing dominance, and minimizing the fitness of resistance genotypes. Between the factors influencing resistance selection, only operational factors can be manipulated. There are several practical, economic, and political constraints on the choice of possible IRM tactics (Leeper et al. 1986, Denholm and Rowland 1992). Different tactics have yet been suggested, such as the use of insecticide mixtures or high doses, and alternation of insecticides, while keeping in mind the mode of action of the compounds. However, all strategies depend first and foremost on methods to reduce the selection pressure of the insecticide on the target pests. In theory, resistance development can be delayed either by reducing the mortality of susceptible homozygotes or by overpowering resistant insects before they become too common. The first can be achieved by decreasing overall exposure or by lowering application rates. Simulations performed based on this theory do not usually predict significant delays. The second can be applied using doses of insecticides that are at least high enough to kill heterozygotes, but this theory is only applicable when heterozygotes are not highly resistant and the resistant genes are rare. Susceptible migrants also are essential to dilute resistance

among any individuals that survived the high dose (Roush and McKenzie 1987, Denholm and Rowland 1992).

On cotton in Israel and southwestern USA, a dramatic reduction in the number of insecticide applications was achieved after implementing resistance management programs for *Bemisia tabaci* based on a combination of increased chemical diversity, voluntary or mandatory restrictions on the use of key insecticides, and integration of non-chemical pest-management options (Denholm et al. 1998). In the Australian cotton industry, a large-scale IRM strategy was introduced in 1983, when pyrethroid resistance in *H. armigera* resulted in unsatisfactory control in the field. The program was primarily based on insecticide rotation and resulted in the delay of the rate of pyrethroid resistance evolution but not in its prevention (Andow et al. 2008).

3 ACARICIDE RESISTANCE IN THE TWO-SPOTTED SPIDER MITE

Resistance of mites and ticks to pesticides is a widespread and growing problem. The intensive use of acaricides has resulted in a rapid selection of resistant populations - more than 70 mite and tick species have now shown resistance to the chemicals used for their control, summing up to 1102 documented resistance cases in the Arthropod Pesticide Resistance Database (APRD) (Whalon et al. 2010). *T. urticae* has developed resistance to over 91 different acaricides and can be considered as the most resistant species among all arthropod pests. A high level of resistance can develop as fast as one to four years after introduction of a new chemical. This resistance is often accompanied with a high degree of cross-resistance. The high reproductive potential, their extremely short life cycle and arrhenotokous reproduction, combined with the frequent acaricide applications usually required to maintain the population below economic threshold, facilitates rapid resistance build-up. Its ability to develop resistance to nearly all acaricides used for its control and its global distribution, make it one of the most notorious agricultural pests in the world (Cranham and Helle 1985, Croft and van de Baan 1988).

3.1 Effect of arrhenotoky on TSSM resistance

The arrhenotokous reproduction is a special feature which can help in accelerating resistance evolution no matter whether it is dominant or recessive (Croft and van de Baan 1988). Because of male haploidy, the situation occurs that mutations are immediately expressed in the male, without respect to whether they are dominant or recessive. These characteristics can be added quickly to the population through natural selection (Helle and Overmeer 1973,

Denholm et al. 1998). Therefore the potential for development of genetic resistance to insecticides and acaricides in TSSM can be greatly enhanced by this method of reproduction.
The resistance development rate is much lower in diploid populations than haplodiploid populations when the resistance trait is recessive or partially recessive (Denholm et al. 1998). However, this theory was based on the assumption that R males and RR females are equally tolerant to pesticides. Using simulations considering between-sex differences in pesticide tolerance, Carriere (2003) concluded that resistance evolves at a slower rate in haplodiploid populations, if resistant diploid females are more tolerant to a pesticide than resistant haploid males. For resistant strains of TSSM, this author reported a ratio of male to female tolerance of 0.002 to 0.85 against the tested acaricides such as dicofol, oxydemeton-methyl and propargite.

3.2 Acaricide chemistry and mode of action

Acaricides, which are chemical compounds used to control Acari, belong to different chemical groups, with different modes of action (Figure 1.1). Among the oldest recorded are sulfur, lime sulfur and petroleum oils. Dinitrophenol was the first synthetic organic compound, widely used between 1930 and 1950. From 1940 on, a large number of acaricides belonging to different chemical categories, such as dithiocarbamates, diphenyl carbinols, organochlorines, the sulfur-bridged, organotins, formamidines, organophosphates, carbamates, pyrethroids, benzoylphenylureas, abamectin, tetronic acid derivatives and other substances were developed, registered and commercialized (Knowles 1997, Vacante 2010).
Historically, comparable to insecticides and antihelmintics, the vast majority of acaricides were neurotoxic compounds, such as organophosphates, pyrethroids and avermectins (Lees and Bowman 2007). A number of these neurotoxins are not used anymore at the moment and have been taken out of the market. More recently, several new and structurally diverse acaricides were developed which inhibit mitochondrial respiration. These include acaricides belonging to a wide range of chemistries: pyridazines, pyrazoles, quinazolines, naphthoquinones, pyrroles, thioureas and pyrimidines. Other compounds, like benzoylphenylureas, tetrazines, tetronic acids and oxazolines, effectively affect growth and development (Dekeyser and Downer 1994, Dekeyser 2005, Lees and Bowman 2007).
Based on the biological mode of action, acaricides can be divided into three classes: chemicals that are primarily toxic to eggs (ovicides), those showing high toxicity to larvae (larvicides), and others having toxicity to all motile stages.

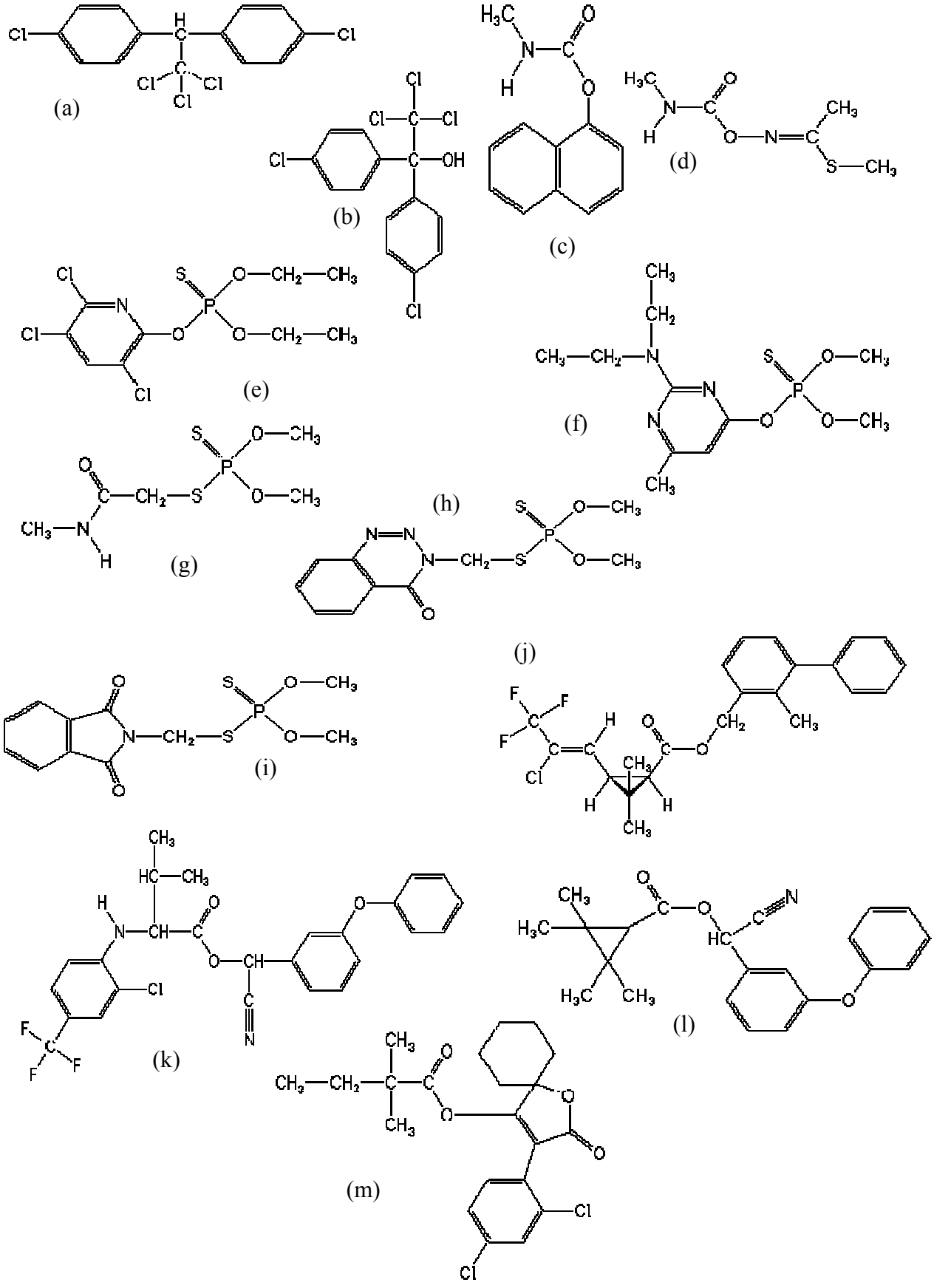

Figure 1.1 Chemical structure of some acaricides, DDT (a), dicofol (b), carbaryl (c), methomyl (d), chlorpyrifos (e), pirimiphos-methyl (f), dimethoate (g), azinphos-methyl (h), phosmet (i), bifenthrin (j), fluvalinate (k), fenpropathrin (l), spirodiclofen (m)

3.3 Resistance mechanisms in the *T. urticae*

Cranham and Helle (1985), Knowles (1997), Van Leeuwen et al. (2009) and more recently Van Leeuwen et al. (2010a) have reviewed acaricide resistance in spider mites. Organophosphates and carbamates (acetylcholinesterase inhibitors) on one hand and pyrethroids on the other hand were widely used insecticides/ acaricides and in this thesis major emphasis is placed on those, together with the new class of tetronic acids.

3.3.1 Resistance to acetylcholinesterase inhibitors

Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme in the insect central nervous system (CNS) which terminates nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine (Ach) in the synapse. Irreversible inhibitors of AChE like organophosphates and carbamates block this enzyme, causing a disruption of the hydrolysis, resulting in over-excitation of the nervous system and finally death (Menozzi et al. 2004). They phosphorylate or carbamoylate the active-site serine leading to inhibition of the enzyme. This results in an accumulation of acetylcholine at nerve synapses which in turn leaves the acetylcholine receptors permanently open, resulting in the paralysis and death of the insect (Oakeshott et al. 2005a).

Metabolic resistance to organophosphate and carbamate insecticides has been associated with an elevation in the activity of a number of detoxification systems. Most frequently, resistance to these insecticides has been correlated with elevated esterase activity. The esterase-mediated metabolic resistance involves degradation or sequestration of the pesticides by mutant or overexpressed carboxylesterases (Oakeshott et al. 2005b). Glutathione S-transferases and P450 mono-oxygenases have also been frequently associated with OP resistance. However, modified AChE has been reported as the main mechanism of resistance since the first report of insensitive AChE to organophosphates in *T. urticae* (Smissaert 1964). Most insects and acarines have two AChEs encoded by two genes, but only one is responsible for neural acetylcholine hydrolysis and its inhibition causes the death of the organism. It is also this one in which one mutation or a combination of mutations are linked to the resistance to organophosphate and carbamate insecticides (Fournier 2005, Oakeshott et al. 2005a). Increased AChE production was also found to have contributed in resistance (Charpentier and Fournier 2001). The role of AChE gene duplication in organophosphate resistance and the reduction of the fitness cost associated with the resistant allele have been documented in the mosquito *Culex pipiens* (Labbe et al. 2007).

Spider mites, like insects, can become resistant to organophosphates and carbamates by insensitive AChE (Stumpf et al. 2001) caused by several amino acid substitutions that will be

discussed in the chapter 3. Recently, Kwon et al. (2010a) reported that resistance to organophosphates has evolved through a combination of mutation accumulation and extensive gene duplication which is actively involved in the increasing level of resistance in *T. urticae* field populations. Enhanced detoxification enzyme activity may also be involved in organophosphate resistance in spider mites. Enhanced carboxylesterase activity was reported to confer organophosphate resistance in two American strains of *T. urticae*, the Blauvelt strain (Matsumura and Voss 1964) and Niagara strain (Herne and Brown 1969). Kwon et al. (2010d) reported a significant increase in P450 activity, suggesting that oxidative metabolism is likely involved in monocrotophos resistance in the two-spotted spider mite. Ay and Yorulmaz (2010) suggested that esterase enzymes may be playing a minor role in chlorpyrifos resistance in a laboratory-selected strain of *T. urticae*, while glutathione S-transferase and P450 enzymes had no effect on chlorpyrifos resistance.

3.3.2 Resistance to sodium channel modulators

Voltage-gated sodium channels (VGSC) are well known as the primary target of DDT, pyrethrins and pyrethroids. They are integral transmembrane proteins responsible for the initiation and propagation of action potentials in almost all excitable cells (Soderlund 2005). Pyrethroids, one of the most widely used group of insecticides, cause prolonged opening of the channels upon binding to VSCGs primarily by inhibiting channel deactivation, thereby stabilizing the open configuration of the activation gate. VGSCs also possess at least nine independent toxin-binding sites for a variety of neurotoxins and some insecticides such as indoxacarb (Silver et al. 2010). Insect's VGSC are structurally and functionally homologous with the α-subunit of mammalian VGSCs, containing 4 internally homologous domains (named I–IV) connected by intracellular linkers, with each domain consisting of 6 membrane-spanning segments (named S1–S6) joined by intracellular or extracellular loops (Figure 1.2) (Davies et al. 2007a, Davies et al. 2007b). Although several (at least nine) different sodium channel α -subunits have been identified in mammals, it seems that only one gene encodes for the sodium channel in *Drosophila* and presumably in other insect species (Dong 2007). VGSC sequence comparisons between susceptible and pyrethroid-resistant insects and mites and functional expression of cloned sodium channels in *Xenopus laevis* oocytes has provided convincing evidence that point mutations in insect voltage-sensitive sodium channel genes are the primary cause of pyrethroid resistance (Figure 1.2) (Soderlund and Knipple 2003).

Involvement of an amino acid substitution (phenylalanine to isoleucine) in domain IIIS6 (F1538I according to *Musca domestica* numbering), has been documented in pyrethroid resistance in *T. urticae* (Chapter 2). Kwon et al. (2010b) recently proposed that a leucine to

valine (L1025V, *Musca domestica* numbering) mutation may confer sodium channel insensitivity to pyrethroids in a fenpropathrin-resistant *T. urticae* strain from Korea (Figure 1.2). Besides target site modification, enzyme-mediated metabolic detoxification through elevated levels of mono-oxygenases (Yang et al. 2001, Van Leeuwen et al. 2005), esterases (Ay and Gürkan 2005, Van Leeuwen and Tirry 2007) and glutathione-S-transferases (Yang et al. 2002, Lin et al. 2009) have been found in pyrethroid resistant spider mites.

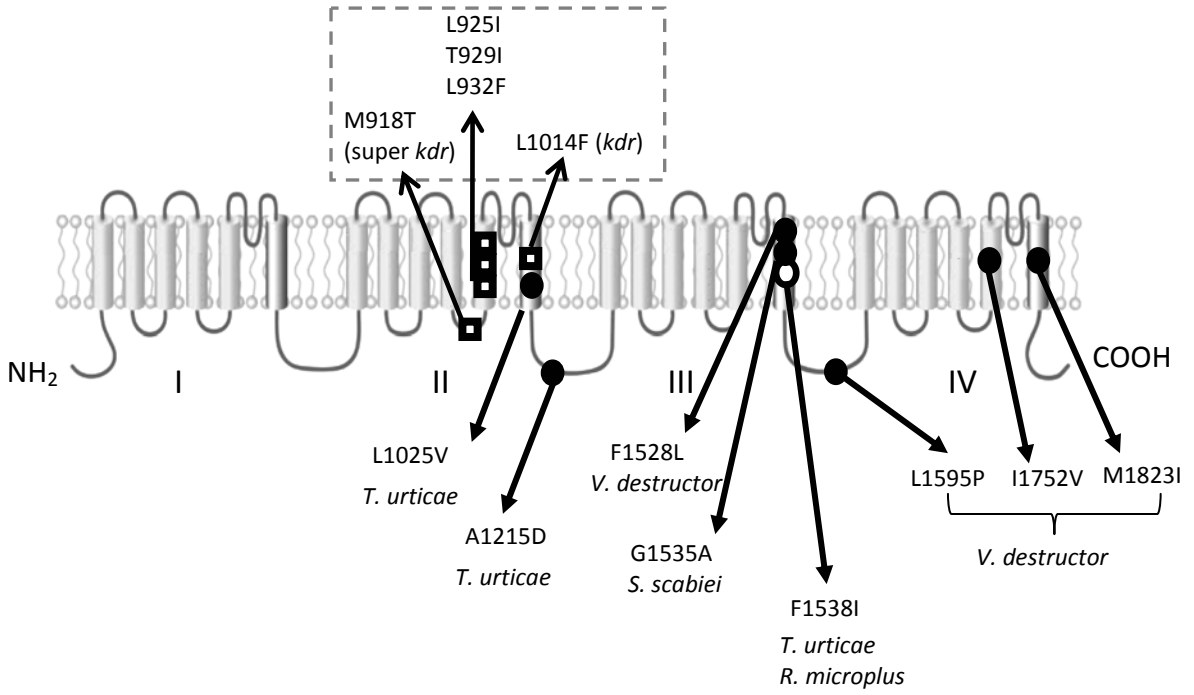

Figure 1.2 The schematic diagram of the sodium channel protein indicating four homologous domains (I–IV), each having six transmembrane segments. The location of amino acid substitutions occurring in the para-sodium channel gene of pyrethroid resistant insects, mites and ticks. Mutations that have been documented in insects are indicated by squares and described in the upper part of the figure. Mutations that have been documented in Acari are indicated by circles and described below. Open squares and circles indicate mutations that have been functionally characterized and confirmed to reduce the sodium channel sensitivity to pyrethroids (Adapted from Van Leeuwen T, Vontas J, Tsagkarakou A, Dermauw W, Tirry L. 2010. Acaricide resistance mechanisms in the two-spotted spider mite *Tetranychus urticae* and other important Acari: A review. *Insect Biochemistry and Molecular Biology* 40: 563-572).

3.3.2 Resistance to acetyl-coenzyme A carboxylase inhibitors

Inhibitors of acetyl-coenzyme A carboxylase (ACCase), the enzyme responsible for the fatty acid biosynthesis, are effective pharmaceuticals, herbicides, fungicides and insecticides. Three classes of herbicides including aryloxyphenoxypropionates, cyclohexanediones and phenylpyrazolines, target ACCase and have a specific graminicide activity (Muehlebach et al.

2007). Based on the IRAC mode of action classification (version 6.3, July 2009) tetronic and tetramic acid derivative insecticides such as spirodiclofen, spiromesifen and spirotetramat interfere with lipid biosynthesis by inhibiting ACCase. Soraphen A, an inhibitor of ACCase, has demonstrated strong promise as a broad-spectrum fungicide against various plant pathogenic fungi (Shen et al. 2004).

ACCase (E.C. 6.4.1.2) is composed of a biotin carboxyl carrier (BCC), biotin carboxylase (BC), and carboxyl transferase (CT), and catalyses the synthesis of malonyl-coenzyme A which is the substrate required for fat synthesis (Incledon and Hall 1997). Two distinct types of this enzyme, a heteromeric and a homomeric, are found in nature. The heteromeric form is composed out of four distinct subunits, BCC, BC and two α and β subunits of CT, and usually is present in prokaryotes. In contrast, the homomeric form is composed of a large polypeptide carrying all functional domains (Sasaki and Nagano 2004). Most plants have both forms, the heteromeric form in plastids and the homomeric form in the cytosol. Poaceae (grasses) are an exception, since they have the homomeric form in both cytosol and plastids (Figure 1.2). Addition of a transit peptide (TP) at the N-terminal end of plastidic and mitochondrial homomeric isoform of ACCase (Figure 1.3) likely enables this isoform to be directed to chloroplasts and mitochondria (Delye 2005).

Frequent use of ACCase-inhibiting herbicides has resulted in the development of resistance in at least 35 grass species worldwide (Wenger and Niderman 2007). Mechanisms of this resistance can be divided in two ACCase-related and metabolism-based mechanisms. In most cases, mutations within the CT domain of homomeric plastidic ACCase are involved in altered sensitivity to ACCase inhibitors. An L1781I was the first identified resistance endowing mutation in the ACCase of resistant grass weeds. Since then, seven other resistance substitutions have been identified in ACCase of various grass species (W1999C, W2027C, I2041N, I2041V, D2078G, C2088A and G2096A). Of these mutations, L1781I is the most common. However, depending on the weed species and type of applied herbicide, particular mutations result in high-level to quite low-level resistance (Powles and Yu 2010). Metabolism-based resistance involves detoxifying enzymes such as cytochrome P450 mono-oxygenases, glutathione-S-transferases and glucosyltransferases (Delye 2005, Wenger and Niderman 2007). It has been found that a mutation in the BC domain of yeast ACCase renders the enzyme resistant to soraphen A (Shen et al. 2004).

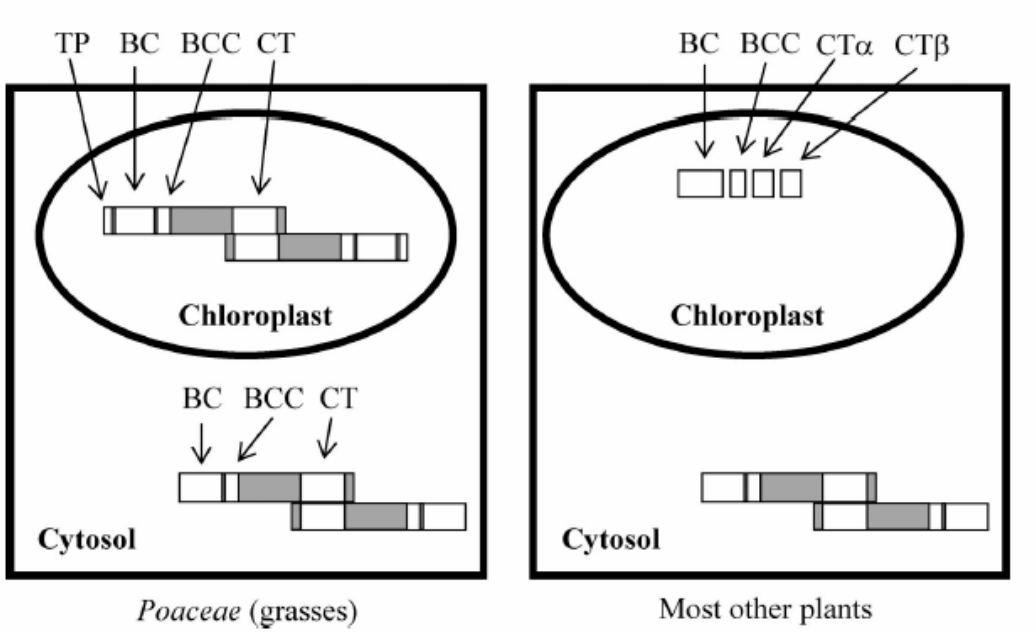

Figure 1.3 ACCase isoforms in plants. TP, transit peptide; ACC functional domains: BCC, biotin carboxyl-carrier; BC, biotin carboxylase; CT, carboxyl transferase. Note that the TP could be cleaved after the plastidic ACCase isoform has been imported into the chloroplast (Adapted from Delye C. 2005. Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. *Weed Science* 53: 728-746).

3.4 Cross/multi-resistance in acaricide-resistant *T. urticae* strains

Selection for resistance to an acaricide may lead to cross-resistance to others of the same class or even other chemical classes (Nauen et al. 2001). Development of cross and/or multi-resistance to acaricides is a commonly found problem in spider mites on several crops (Van Leeuwen et al. 2004, Osakabe et al. 2009). Adequate control of spider mites was impossible in 1966 in the Aalsmeer, The Netherlands, because of resistance to organophosphates, tetradifon, chlorobenzilate and dicofol. Even 7 years after interrupting the treatments, a high proportion of the population was still resistant to dicofol and tetradifon (Overmeer et al. 1975). Kono (1985) reported that a Japanese dicofol-resistant *T. urticae* strain showed cross-resistance to organophosphates and structurally dicofol-resembling acaricides. A dicofol-resistant *T. urticae* strain showed positive cross-resistance to amitraz, bromopropylate, and chlorobenzilate (Fergusson-Kolmes et al. 1991). Cross-resistance between METI (mitochondrial electron transport inhibitor) acaricides is common and can occur very rapidly (Stumpf and Nauen 2001, Van Pottelberge et al. 2009a). A strain of *T. urticae*, collected from hops in England with a short history of tebufenpyrad exposure, exhibited high levels of resistance to four METI acaricides (tebufenpyrad, pyridaben, fenazaquin and fenpyroximate). Similar patterns of resistance inheritance were found and suggested a common mechanism of resistance to all the METI acaricides (Devine et al. 2001). However, genetic analysis of a

Belgian field strain exhibiting different levels of resistance by Van Pottelberge et al. (2009a) revealed differences in the mode of inheritance to these four METI acaricides.

Cross-resistance was detected in a chlorfenapyr laboratory selected strain to amitraz, bifenthrin, bromopropylate, clofentezine and dimethoate (Van Leeuwen et al. 2004). A field-collected strain from Belgium with resistance to bifenthrin, dicofol and fenbutatin oxide showed cross-resistance with clofentezine, dimethoate, chlorfenapyr, bromopropylate, amitraz, flucycloxuron and azocyclotin (Van Leeuwen et al. 2005). Nicastro et al. (2010) found a significant correlation between the resistance frequencies for milbemectin and abamectin in 25 field populations of *T. urticae*, and concluded there was a positive cross-resistance for these acaricides. In Australia, clofentezine resistance conferred high levels of cross-resistance to hexythiazox (Herron et al. 1993). An etoxazole resistant population collected from rose greenhouses in Korea and further selected exhibited resistance to acequinocyl, emamectin benzoates, milbemectin, amitraz and pyridaben (Lee et al. 2004). Some mutations in the mitochondrial cytb gene can confer cross-resistance between bifenazate and acequinocyl, although not all bifenazate resistance-associated mutations resulted in acequinocyl cross-resistance (Van Nieuwenhuyse et al. 2009).

Negative cross-resistance, i.e. the fact that resistance to one compound causes greater susceptibility to another, has been reported in some *T. urticae* resistant strains. Negative cross-resistance to chlorpyrifos in a dicofol resistant strain was linked to increased oxidative activation of chlorpyrifos (Hatano et al. 1992). In a strain collected from apple orchards in New Zealand, it was demonstrated that increasing azinphos-methyl (an organophosphate) resistance conferred an increase in fenvalerate (a pyrethroid) susceptibility (Chapman and Penman 1979). The etoxazole resistant strain from Korea, which was selected further (Lee et al. 2004) showed negative cross resistance to bifenazate.

CHAPTER II

IDENTIFICATION OF PYRETHROID RESISTANCE ASSOCIATED MUTATIONS IN THE *PARA* SODIUM CHANNEL OF THE TWO-SPOTTED SPIDER MITE *TETRANYCHUS URTICAE* (ACARI: TETRANYCHIDAE)

This chapter has been redrafted from:

Tsagkarakou A, Van Leeuwen T, Khajehali J, Ilias A, Grispou M, Williamson MS, Tirry L, Vontas J (2009) Identification of pyrethroid resistance associated mutations in the para sodium channel of the two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae). Insect Molecular Biology 18: 583-593

1 INTRODUCTION

Tetranychus urticae Koch is an economically important phytophagous and polyphagous mite with a worldwide distribution. The species has been heavily exposed to insecticides and acaricides, and insecticide-resistant populations have been reported in more than 40 countries in both greenhouses and open field crops (Georghiou and Lagunes-Tejeda 1991, Stumpf and Nauen 2001, Van Leeuwen et al. 2008). The rapid development of resistance in *T. urticae* is favoured by its high reproductive potential, extremely short life cycle and arrhenotokous reproduction. In greenhouses resistance develops even faster due to the relative isolation of the mite populations and the extended growing season (Cranham and Helle 1985).

The pyrethroid bifenthrin, an interesting development in pyrethroid chemistry after the initial success of allethrin synthesis, as a result of the replacement of 3-phenoxybenzyl alcohol by 3-phenylbenzyl alcohol and the introduction of a methyl group at position 2 (Khambay and Jewess 2005), is particularly active against mites and has been used extensively for *T. urticae* control in several countries. Bifenthrin resistance was first detected by Farnham et al. (1992), in *T. urticae* collected from an apple orchard in New York. A number of resistance case reports followed, such as the field-caught populations from cotton in New South Wales (Resistance Ratio (RR) up to 109 fold (Herron et al. 2001)), the laboratory strains originating from cotton plants in Turkey (RR up to 650 fold, Ay and Gürkan 2005) and a strain isolated from a greenhouse nursery at Ghent University (RR > 1850 fold, Van Leeuwen et al., 2005).

Insecticide resistance in insects and mites has been attributed to increased rates of insecticide detoxification or mutations in the target sites (Bloomquist 1996, Zlotkin 1999, Soderlund and Knipple 2003, Li et al. 2007). Metabolic resistance to pyrethroids has been associated with P450 oxidation (Bergé et al. 1998) and carboxylesterase (COE)-mediated hydrolytic degradation (Wheelock et al. 2005). Glutathione S-transferases (GST) have been also shown to contribute to the resistant phenotype (Vontas et al. 2001). The majority of *T. urticae* resistance reports implicate COE hydrolysis in pyrethroid detoxification (Ay and Gürkan 2005, Van Leeuwen et al. 2005, Van Leeuwen and Tirry 2007). Increased mono-oxygenase (MO) activities were also detected in a pyrethroid resistant strain from Belgium (Van Leeuwen et al. 2005). A small number of conserved voltage gated sodium channel (VGSC) resistance mutations have also been reported to confer target site insensitivity to pyrethroids. Most mutations have been found in the transmembrane segments 4 to 6 of the domain II (IIS4-IIS6) region of this large membrane protein, and include changes at residues such as M918 in the IIS4-IIS5 linker, L925, T929 and L932 in IIS5 and L1014 in IIS6 (Figure 1.2) (Soderlund

and Knipple 2003, Davies et al. 2008). A resistance mutation, F1538I, has also been reported within the S6 transmembrane segment of domain III, which is predicted to form part of the pyrethroid binding site, based on a recent computer modelling study of pyrethroid/sodium channel interactions (O'Reilly et al. 2006). This mutation has been confirmed to confer strong insensitivity to a number of pyrethroids by functional assays (He et al. 1999, Tan et al. 2005).
Target-site resistance has been also associated with sodium channel alternate transcriptional variation and/or post transcriptional modifications in some species (Tan et al. 2002b, Du et al. 2006), although the possible broader role of such changes in other species remains unclear.
In this chapter the resistance mechanism responsible for high levels of bifenthrin resistance in two *T. urticae* strains from Greece was investigated. Also, cDNA sequences which encode a large part of the *T. urticae para* sodium channel were isolated and identified mutations were associated with the resistance phenotype.

2 Materials and Methods

2.1 Strains

The pyrethroid susceptible strain SAMB was initially collected on *Sambucus nigra* (L) in the Netherlands and obtained from the Centre de Biologie et de Gestion des Populations, Montpellier France. The German susceptible strain GSS was obtained from Bayer CropScience.
Two pyrethroid resistant *T. urticae* strains were used in this study. The ATHRos-Bf strain was isolated from a heavily sprayed rose plant greenhouse in 2007 near Athens, and the CREVeg-Bf strain was derived from field populations collected from several vegetable crops from the island of Crete in 2006. Before collection, these field strains had been heavily treated with several insecticides and acaricides. Both strains were selected with concentrations causing ~ 70% mortality every two generations, until homogeneity in bioassay response was obtained. Mites were maintained on detached bean leaves that were deposited on a piece of moist cotton placed on trays filled with water to prevent contamination and reared at 25 ± 2°C, 70 ± 5% relative humidity with a photoperiod of 16:8 h (L:D). Under these conditions, a new generation was obtained every 2 weeks and mites were transferred to new bean leaves every week.

2.2 Bioassays

Commercial formulations of bifenthrin 100 g L^{-1} EC (Talstar, FMC Greece), fluvalinate 240 g L^{-1} SC (Mavrik, Alpha Greece) and of dicofol 35% WP (Kelthane, Protex, Belgium) were used. DDT and fenpropathrin were of analytical grade (Chem Service) and formulated by

preparing stock solutions in a 3:1 (v/v) mixture of *N*,*N*-dimethylformamide and alkylarylpolyglycolether (emulsifier) and subsequently diluted with de-ionised water (Van Leeuwen et al. 2007). Bean leaves were placed on wet cotton in plastic Petri dishes for conducting bioassays. Twenty five adult females were transferred onto a leaf with a fine brush inside a 35 mm disc delimitated by insect glue. Individual Petri dishes were then sprayed with a precision Potter Spray Tower (Burkard, Rickmansworth, Hertfordshire, UK) (Potter 1952) with 1 ml aqueous solutions of insecticide at 1 bar pressure in order to obtain a homogenous spray film (2.4 mg ± 0.3 aqueous acaricide deposit cm^{-2}). Four replicates of at least 4 concentrations causing between 20% and 80% mortality 48 hours after treatment were tested with each insecticide. The synergists piperonyl butoxide (PBO) (Sigma-Aldrich, UK), an inhibitor of cytochrome P450 mono-oxygenases and carboxylesterases, and S,S,S-tributyl phosphorotrithioate (DEF, Sigma, UK), an inhibitor of esterases and glutathione-S-transferases (GST) were used to investigate the effect of inhibition of detoxifying enzymes on bifenthrin resistance. Female mites were sprayed with PBO and DEF at the highest concentration causing no mortality (30 mg L^{-1} and 100 mg L^{-1} respectively), 4 h prior to bifenthrin application as described above. Before use, PBO and DEF were dissolved in a mixture of *N*,*N*-dimethylformamide and emulsifier W (3:1 by weight) and subsequently diluted with de-ionised water (1000-fold). Mites sprayed with synergist only served as control. LC_{50} values and their 95% confidence limits were calculated from probit regression analysis (Raymond et al. 1993) based on Finney (1971). Resistance ratios (RR) or synergism ratios (SR) were considered to be significantly different from 1 when their 95% confidence limits (95% CL) did not include this value.

For selections, the two resistant strains were exposed to successive applications of bifenthrin every ~ 2 generations. Adult females were placed on bean leaf discs and sprayed as described above with concentrations causing 70% mortality. The surviving mites were transferred to new leaves after 48 hours.

2.3 Crossing experiments

To estimate the dominance of the resistance, the SAMB and CREVeg-Bf strains were reciprocally crossed to produce hybrid F1 females as described by Van Leeuwen et al. (2004). Briefly, 10 female teleiochrysalis of one strain and 30 adult males of the other strain were placed together on the upper side of a primary bean leaf on wet cotton in a Petri dish. Directly after molting the diploid females were fertilized by the haploid males. After 3 days, fertilized females were collected and placed on fresh bean leaves and were allowed to lay eggs for 14 days. Every day, the egg laying females were collected and placed on a fresh leaf. The

resulting F1 females were collected 10 days after hatching and were used after maturation (1–3 days) in a bioassay with the appropriate concentrations of bifenthrin, as described in paragraph 2.2. The following formula was used to calculate the degree of dominance (Stone 1968).

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

in which X_1 is the log of the LC_{50} of the resistant strain, X_2 is the log of the LC_{50} of the heterogenous strain and X_3 is the log of the LC_{50} of the susceptible strain. This formula will result in a value of -1 if resistance is fully recessive, a value of 0 if there is no dominance, and a value of +1 if resistance is fully dominant.

2.4 Biochemical assays

COE, GST and MO activities of mass homogenates of female mites were determined on a SpectraMax M2e microplate reader (Molecular Devices, UK). COE activities were determined with the substrates α-naphthyl acetate, p-nitrophenyl acetate (pNPA) and the more specific 1-naphthyl 2,2-dimethylbutyrates, as detailed in Van Leeuwen et al. (2005) and Rauch and Nauen (2002). GST activities were measured with the substrates 1-chloro-2,4 dinitrobenzene (CDNB) and monochlorobimane as detailed in Stumpf and Nauen (2002). The O-deethylation of 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) by *T. urticae* P450 mono-oxygenases was measured according to Van Leeuwen et al. (2005). The protein concentration in the enzyme source was determined according to Bradford (1976) using bovine serum albumin as a standard, to normalise activities for protein concentration. All enzymatic assays were repeated at least 3 times. The mean activity values were compared between resistant and each of the susceptible strains, by one-way analysis of variance (ANOVA) using the SPSS statistical software version 13.0 (LSTM, UK).

2.5 Extraction of gDNA and RNA, cDNA synthesis, cloning, and sequencing

Total RNA was extracted from mass homogenates of *T. urticae* mites using TRI reagent (Sigma-Aldrich, Belgium) according to the manufacturer's instructions. The RNA was treated with DNase RQ1 (Promega, USA) to remove genomic DNA and 2 µg RNA for each sample was reverse transcribed into cDNA by using the High Fidelity cDNA synthesis kit (Roche, Belgium) and random hexamer primers. Genomic DNA (gDNA) was extracted using the DNeasy Kit (Qiagen, Germany) following the manufacturer's protocol. A number of degenerate primers (Table 2.1) designed against conserved coding sequences of Acari sodium channel genes in domains II, III and IV, were used to PCR amplify partial sequences of the *T. urticae para* sodium channel gene.

Amplifications from approximately 40 ng gDNA or 0.5 µl cDNA were performed in 1x Taq reaction buffer (Promega, Greece) with a final concentration of 2 mM $MgCl_2$ and 0.5 µM each primer, and cycling conditions 94 ^oC for 5 min, then 30 cycles of 50 ^oC for 30 s, 72 ^oC for 2 min, 95 ^oC for 15 s, followed by a final cycle of 72 ^oC for 5 min. On the basis of partial domain II and IV sequences that were obtained with primer pairs aIIS3-F1/aIIS5-R6 and aIIIS5-F2/ aIVS6-R3 (Table 2.1), a single pair of specific primers (GAP2F and GAP2R) were designed to amplify the entire fragment using a long PCR approach (Expand Long Range Kit, Roche, Belgium) with cDNA as template. Fragments were purified with QIAEX II (Qiagen, the Netherlands) and sequenced by primer walking. To ensure allele representations, fragments were also cloned into the pGEM-T vector (Promega, the Netherlands). PCR fragments and plasmids were sequenced by Agowa Sequencing Service (Germany). Sequence data was analyzed using BIOEDIT 7.0.1 (Hall 1999).

Table 2.1. Primers used for the amplification of the domain IIS4-IVS6 of the *T. urticae para* sodium channel gene.

Primers	Sequence (5' – 3')
aIIS3-F1	GAAGGNTGGAAYATNTTYGA
aIIS5-R6	ATCCAYTCKCCRCAHARMACHCGRAA
aIIIS5-F2	CATCTTCAAYGTRYTRYTGGT
aIVS6-R3	TGCCARATYTCRTAGTACAT
aIVS6-F3	TGCTGTTYGCSYTNGCYATG
aIIS4-F4	TTYAARYTDGCMAARTCDTGG
aIIS5-F5	GGMGARTGGATMSARTCBATGTGG
aIIS5-F6	TAYTTCACVAAYGYBTGGTGYTGG
aIIS6-R1	CYTCTKSWANYTTNTTKGTRT
aIIIS6-R2	GAKCCCATYTTYTTCATNGC
aIIIS3-R4	ATVACRAARTCVAGCCARCACCA
aIIIS4-R5	TTGAAGATGGCNGGNATNGCTTG
GAP2F	ATCGTGGCCCACTCTTAACC
GAP2R	GACCCACAATCTGACACTTCG
KdrR1	TCTTCCGTCATCAACATCTCC
KdrR2	GTTTCTTCCAGGCAACATGG
SGF3B	TAAAGAACGTCCCTGGTTGG
SGR6	GCCAAAAATGATGAAAAATACG

2.6 Phylogenetic analysis

A phylogenetic analysis was performed as in Davies et al. (2007a) using the *para* sodium channel sequences of 17 arthropod (5 Arachnida and 12 Insecta) species. Amino acid sequences were aligned by CLUSTAL W (Thompson et al. 1994) as implemented in BIOEDIT 7.0.1. Ambiguously aligned parts were omitted from the analysis by making use of GBLOCKS 0.91b (Castresana 2000), using default block parameters except for changing "allowed gap positions" to "with half". Model selection was done with PROTTEST 1.4 (Abascal et al. 2005). According to the Akaike information criterion the WAG + I + G + F model was optimum for phylogenetic analysis with amino acid alignments and a Bayesian inference (BI) was used. (Mr. Bayes ver. 3.1.2, (Huelsenbeck and Ronquist 2001)). Four chains ran for 100,000 generations and tree sampling was carried out every 100 generations. The first 250 out of 1000 trees were discarded as burn-in and the remaining 750 were used to calculate Bayesian posterior probabilities.

3 RESULTS

3.1 Characterization of bifenthrin resistance in the resistant strains

The ATHRo-Bf and CREVeg-Bf strains exhibited high levels of resistance to bifenthrin (RR, 2495 and 1026, respectively), compared to the susceptible SAMB strain (Table 2.2). ATHRo-Bf and CREVeg-Bf had high levels of cross resistance to another halogenated pyrethroid, fluvalinate (RR 1251 and 968, respectively), but lower levels of cross resistance to fenpropathrin (RR 86 and 47) and the organochlorine dichlorodiphenyltrichloromethyl-methane (DDT) (RR 6.76 and 2.33). Cross resistance to dicofol was high in ATHRos-Bf strain (200-fold resistance) but only twofold in CREVeg-Bf.

Inhibitors of COE, GSTs and P450 MO only slightly synergized bifenthrin toxicity in the ATHRos-Bf strain (Table 2.3), whilst COE and GST activities with several substrates were not statistically different ($P > 0.01$) in the resistant strains compared to the susceptible ones (Table 2.4). P450 activities with the substrate 7-ethoxy-4-trifluoromethylcoumarin was statistically significantly elevated ($P < 0.01$) in the ATHRos-Bf and the CREVeg-Bf compared to GSS (nine and six fold, respectively) and the SAMB (three and twofold, respectively).

Table 2.2 Concentration probit mortality data of several acaricides on the *T. urticae* strains

Treatment	n^a	LC_{50} [b]	Slope ± SE	x^{2} [c]	*df*	RR_{50} [d]
SAMB						
Bifenthrin	317	9 (6 – 12)	1.88 ± 0.29	5.05	3	
Fluvalinate	266	26 (13 – 39)	1.58 ± 0.30	3.61	2	
Fenpropathrin	342	44 (36 – 54)	2.62 ± 0.27	0.09	2	
Dicofol	568	10 (9 – 11)	5.10 ± 0.45	13	7	
DDT	487	2940 (1975 – 4386)	3.10 ± 0.77	15***	2	
GSS						
Bifenthrin	257	13 (4.38 – 41)	1.79 ± 0.92	18***	2	1.49 (0.68 – 32)
Fluvalinate	225	55 (16 – 190)	2.49 ± 1.86	40***	2	2.05 (0.59 – 7.10)
Fenpropathrin	238	63 (49 – 82)	3.45 ± 0.57	5	2	1.43 (1.04 – 1.97)
Dicofol	534	15 (13 – 18)	5.62 ± 1.03	25***	6	1.54 (1.14 – 2.09)
DDT	305	6994 (5927 – 8059)	3.62 ± 0.52	0.50	2	2.38 (1.40 – 4.03)
CREVeg-Bf						
Bifenthrin	356	9162 (7014 – 11242)	2.13 ± 0.30	3.02	2	1026 (807 – 1304)
Fluvalinate	351	26072 (12626 – 54421)	1.22 ± 0.38	7.83*	2	968 (614 – 1524)
Fenpropathrin	368	2078 (1749 – 2497)	3.19 ± 0.38	1.44	2	47 (37 – 60)
Dicofol	598	25 (21 – 30)	4.33 ± 0.83	26***	7	2.56 (1.95 – 3.37)
DDT	432	6851 (5165 – 9072)	3.47 ± 0.83	13**	3	2.33 (1.29 – 4.21)
ATHRos-Bf						
Bifenthrin	400	22269 (17285 – 33211)	2.02 ± 0.31	2.20	2	2495 (1905- 3266)
Fluvalinate	283	33711 (22237– 132277)	0.98 ± 0.17	4.27	2	1251 (949 – 1649)
Fenpropathrin	308	3786 (3050 – 4400)	3.34 ± 0.78	3.92	2	86 (75 – 97)
Dicofol	516	2212 (1996 – 2456)	3.61 ± 0.36	9.95	7	225 (189 – 267)
DDT	472	19893 (12358 – 32022)	4.17 ± 1.44	47.84***	3	6.76 (2.81 – 16)

[a] number of mites tested
[b] LC, lethal concentration expressed in mg L^{-1}
[c] Chi-square testing linearity, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
[d] RR, resistance ratio = LC/LC_{SAMB}

Table 2.3 Effect of pre-exposure to S,S,S-tributyl phosphorotrithioate (DEF) and piperonyl butoxide (PBO) on mortality as a result of bifenthrin treatment

Treatment	n^a	LC_{50}[b]	Slope ± SE	χ^{2}[c]	*df*	RR_{50}[d]	SR_{50}[e]
SAMB							
Bifenthrin	317	9 (6 - 12)	1.88 ± 0.29	5.05	3	-	-
Bifenthrin + DEF	262	3.17 (2.09 - 5.3)	1.42 ± 0.23	5.68	3	-	2.82 (2.15 - 3.69)
Bifenthrin + PBO	252	4.37 (0.03 - 545)	2.13 ± 3.9	214***	2	-	2.04 (0.04 - 7.48)
CREVeg-Bf							
Bifenthrin	356	9162 (7014 - 11242)	2.13 ± 0.30	3.02	2	1026 (807 - 1304)	-
Bifenthrin + DEF	464	12441 (8941 - 17469)	1.81 ± 0.39	6.27*	2	3928 (2840 - 5432)	0.76 (0.62 - 0.92)
Bifenthrin + PBO	365	6950 (5562 - 8479)	1.93 ± 0.26	1.89	2	1590 (28 - 90514)	1.36 (1.00 - 1.84)
ATHRos-Bf							
Bifenthrin	400	22269 (17285 - 33211)	2.02 ± 0.31	2.20	2	2495 (1905 - 3266)	-
Bifenthrin + DEF	299	8473 (7283 - 9871)	2.87 ± 0.38	1.86	2	2675 (2107 - 3395)	2.63 (2.07 - 3.33)
Bifenthrin + PBO	306	8458 (7243 - 10017)	2.87 ± 0.37	3.10	2	1934 (344 - 110255)	2.63 (2.07 - 3.36)

[a] number of mites tested
[b]LC, lethal concentration expressed in mg L^{-1}
[c]Chi-square testing linearity, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
[d]RR, resistance ratio = LC/LC_{SAMB}
[e]SR, synergism ratio = LC observed in absence of synergist / LC observed in presence of synergist

Table 2.4 Resistance ratios and detoxifying enzyme activities in the two susceptible SAMB and GSS and the two resistant CREVeg-Bf and ATHRos-Bf strains.

Strain	Bifenthrin	Mean esterase activity			Mean GSTs activity		Mean P450 activity
	RR	α-Naphthyl-acetate[a]	p-NPA[b]	1-Naphthyl-2,2 dimethylbutyrate[a]	CDNB[c]	Monochlorobimane[d]	7-EFC[e]
SAMB		131 ± 60	2.91 ± 0.57	1465 ± 173	1.30 ± 0.20	67122 ± 2812	248 ± 20
GSS	1.49 (0.68 - 32)	120 ± 66	2.91 ± 1.2	1099 ± 269	1.28 ± 0.83	88902 ± 48105	92 ± 12
CREVeg-Bf	1026 (807 - 1304)	315 ± 174	5.02 ± 0.36	1181 ± 293	0.99 ± 0.14	69068 ± 67155	564 ± 25
ATHRos-Bf	2495 (1905 - 3266)	167 ± 38	4.97 ± 2.77	1251 ± 267	0.92 ± 0.28	151712 ± 89427	844 ± 24

[a]nmole α naphthol/min/mg protein
[b]µmole/min/mg
[c]µmole glutathione conjugated/min/mg protein
[d] relative fluorescent units/min/mg protein
[e]pmol 7-hydroxycoumarin/(30min)/mg protein

3.2 Cloning of the *T. urticae* voltage gated sodium channel gene (domains II-IV)

A 3324 bp fragment encoding an 1108 amino acid sequence of the *T. urticae para* sodium channel across domains II, III and IV (IIS4-IVS6, see Figure 2.1) was amplified from *T. urticae* cDNA using a degenerate primer and long-PCR strategy. Sequences were deposited in GenBank under the accession numbers FJ906804-FJ906811.

The *T. urticae* sodium channel gene fragment showed highest similarity to other species of the same subphylum: 56 % with the other Acariformes mite *Sarcoptes scabiei*, 51.7% and 48.9% with the two Parasitiformes *Boophilus microplus* and *Varroa destructor* and 49.4% with the scorpion *Mesobuthus martensii.*

```
                                                    IIS5
M. domestica   VFKLAKSWPT LNLLISIMGR TMGALGNLTF VLCIIIFIFA VMGMQLFGKN YIDHKDRFKD  960
B. microplus   .......... .........K .I........ ..G....... ........... .EES.HK...  99
S. scabiei     .......... .........K .I.D...... ..V....... ........... .TEES--.GG  159
T. urticae     ---------. .....T...K .L.D...... ..A..V.... ........A. .SKKVYL.PN  51
                                                                  IIS6
M. domestica   HELPRWNFTD FMHSFMIVFR VLCGEWIESM WDCMYVGDVS CIPFFLATVV IGNLVVLNLF  1020
B. microplus   NMV.....V. .......... .......Q.. ....W.SGWP .......... ...L......  159
S. scabiei     K.I.....K. .......... .......... ....R.SGAA .V........ ...L......  219
T. urticae     A.I.....K. .......... .......... .S..L.CGFV .V.......I ..HL.M....  111

M. domestica   LALLLSNFGS SSLSAPTADN -DTNKIAEAF NRIARFKNWV KRNIADCFKL IRNKLTNQIS  1079
B. microplus   ....S..A .N..QANP.S G..K.LQ..I D.FH.ASR.I .S.SMKL..S F.R.PR...G  219
S. scabiei     ....S..A .N.....SES A..K.LQ... D.FS.GGK.I .HRTLSLL.R MSP.TR...R  279
T. urticae     ....S..A .N..S..SES A..K.LQ... D.FG.AHK.L .NR.LTGL.Q L.S.TR...R  171

M. domestica   D--QPSEH-- --GDNELELG ----HDEIMG DG---LIKKG MKG------- ----------  1109
B. microplus   .--.TTDIRG GGAGE...AD PGVAGEVVLL ..RVPMRDRK PQH------- ----------  260
S. scabiei     .--.ICD.S- -ISYE.MLKT ----SNK.DQ TSEII.VENE KNQ------- ----------  314
T. urticae     .TA.WPGRGG ASSGGMVGRS MMLGSESVLD E.DIIMMDGL NASGLIRDKK LLAAAAYGET  231

M. domestica   ---------- ---------- ---------- ---------- ---------- ---ETQLEVA  1116
B. microplus   ---------- ---------- ---------- ---------- ---------- ---NND...V  267
S. scabiei     ---------- ---------- ---------- ---------- ---------- ---QISPTIS  321
T. urticae     VVGPDGLEYS LTESGKVTLK SSAQTVLNSV KLSQAVKDLS SDKLILTESA LIEKLSPS.T  291

M. domestica   IGDGMEFTIH GD---MKNNK PKKSKFINNT TMIGNSINHQ DNRLEHELNH RG--------  1165
B. microplus   V...LDIA.Q ..GKAV.MKL KNN..PVM.S VWV.PM.EPK NKQ..KDNKE KEKEAQGNKV  327
S. scabiei     .PNANNMKAS TILPNVNLEM VHLIQNQ.DL NK.NGTNSQT KSIINRKESN DAQ-------  374
T. urticae     NHSDEGNRL. ..VSTVN..N VNDRSTSL.N .NFN..L.SN NLLVSASTIN NNNVNKVKPS  351

M. domestica   LSIQD----- ---------- ---------- ---------- --------DD TASINSYGSH  1182
B. microplus   YPQK.----- ---------- ---------- ---------- --------E. .L.EK.AS.P  344
S. scabiei     I.RKN----- ---------- ---------- ---------- --------SM NVNFE.DLTR  391
T. urticae     .HDANGCSPF PLDDNHHHHH LVQQQPHGGD YHGESGYYGS SESQQHLVNN NV.N.NDS.A  411
                                                                          ↓
M. domestica   K------NRP FKDESHKGS- AETIEGEEKR DVSKEDLG-- ---------- ----LDEELD  1219
B. microplus   .EKVLLG.K. S..L.NSSLY LGNNLE...K .A.....-- ---------- ----TK.GEE  388
S. scabiei     .IS-MDTEII V...KFSSLD SN..Q..SRS ..I.S----- ---------- ----------  425
T. urticae     QQSSSLSVT. SHHLN.PS.H PTAHHYNASQ LSKVHPA.GP VPISFQHSYS SLNR.APSPL  471

M. domestica   EEAEG----D EGQLDGDIII HAQNDDEIID DYPADCFPDS YYKKFPILA- GDEDSPFWQG  1274
B. microplus   APT.EPINP. TEDV.T.KLE T.TS-.I..P EM....C..W C.TR.AFACF F..NKI...R  447
S. scabiei     ---------- ---IEVNGKL D.ATA.VV.N E..SE.W.EP M.QYC.WCL- --DETT..AR  469
T. urticae     NHFN.PGEDV M.EEMNANKM VTVTA.VN.N .H.D..L.EY W.HR..CCL- --.ETA..IK  528

                          IIIS1                                   IIIS2
M. domestica   WGNLRLKTFQ LIENKYFETA VITMILMSSL ALALEDVHLP DRPVMQDILY YMDRIFTVIF  1334
B. microplus   YKIV.T.AYA .V.H.....I .VVL..T... ..........N.K ...TLKAV.T ...KT.....  507
S. scabiei     .KTI.S.CYK IV.H.....L ...L..I..M ..........NFK KD.LFMEY.N .V.KF..I..  529
T. urticae     .REV.S.CYK .V.D.....L ...L..I..M T.........N.K E..WLEYS.K .I.QF..I..  588

                                  IIIS3                                   IIIS4
M. domestica   FLEMLIKWLA LGFKVYFTNA WCWLDFVIVM LSLINLVAVW SGLNDIAVFR SMRTLRALRP  1394
B. microplus   .F..ML.... F...K..... .........L V.FF.MAVAM M.YGR.PA.K T.........  567
S. scabiei     IC........ F..YG....V .......... V.IM.IIVGI A..GN.PA.K T.........  589
T. urticae     TC...L..F. Y...S..S.. ......I... V....FTVGQ L.FSN.PA.K T.........  648
                                               ******************************
```

Figure 2.1

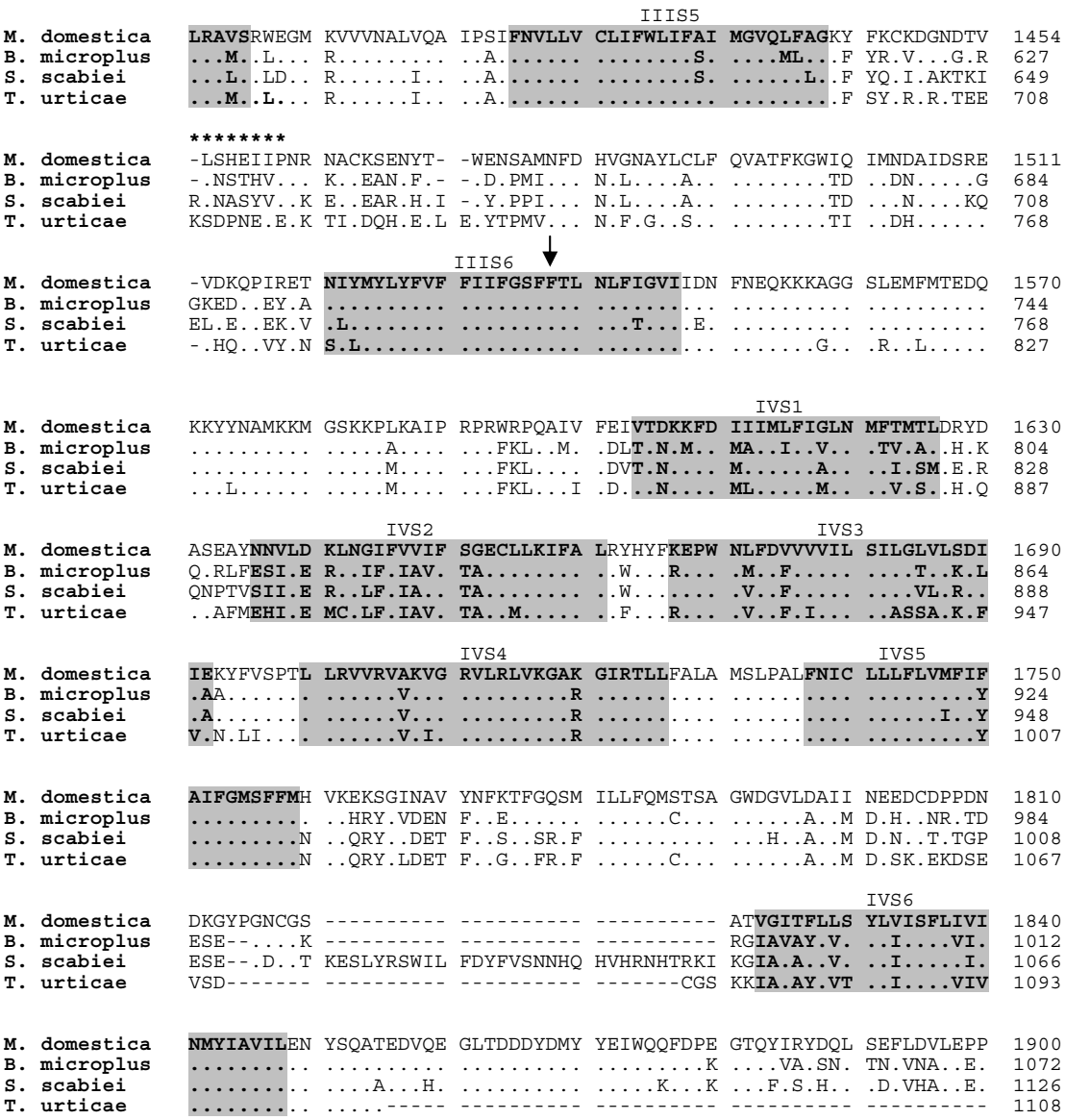

Figure 2.1 Continued Alignment of the deduced amino acid sequence of *T. urticae* sodium channel (FJ906808) with susceptible *M. domestica* (U38813), *S. scabiei* (DQ077148) and *Boophilus* (=*Rhipicephalus*) *microplus* (AF134216). Dots represent identical residues, and dashes indicate gaps introduced to obtain optimal alignment. Numbers on the right indicate the amino acid positions in each protein. The IIS5 to IVS6 transmenbrane segments are highlighted. Arrows indicate the position of the two mutations found only in the two resistant *T. urticae* strains. Stars indicate the location of the transcript variants in *T. urticae.* Here the transcript variant v2 corresponding to the exon k is presented.

The overall similarity to insect species (subphylum Hexapoda) was slightly lower (for example 47.7 % for *Musca domestica*) and against all species the greatest similarities (57.7% to 96.3%) were found in the transmembrane segments of domains II, III, IV as well as the intracellular linker connecting domains III and IV (91%). The *T. urticae* sequence also contains the conserved residues of the channel selectivity filter from the P-loop regions of domains II-IV, including residues referred to as the 'DEKA motif', which is important for

determining sodium selectivity (these are E76, K754 and A1047 in Figure 2.1) (Heinemann et al. 1992). In contrast, much greater variation was observed in the cytoplasmic linker between domain II and III (similarity 25%), due to the differences in both sequence and length of this highly divergent region (422 residues in *T. urticae* compared to 256 and 270 in *S. scabiei* and *M. domestic*a respectively, Figure 2.1).

The phylogenetic relationship of the *T. urticae* voltage gated sodium channel with Insecta and Arachnida sequences is presented in an unrooted phylogenetic tree in Figure 2.2 As the cytoplasmic loop between domain II and domain III varies extensively in length and sequence amongst species, we excluded this region from the phylogenetic analyses. The overall topology of the tree shows that voltage gated sodium channel from Insecta form a more homogenous group compared to those of the Arachnida. The voltage gated sodium channel of the Arachnida is most divergent, as within group distances are equivalent to those between Arachnida and Insecta species (Figure 2.2).

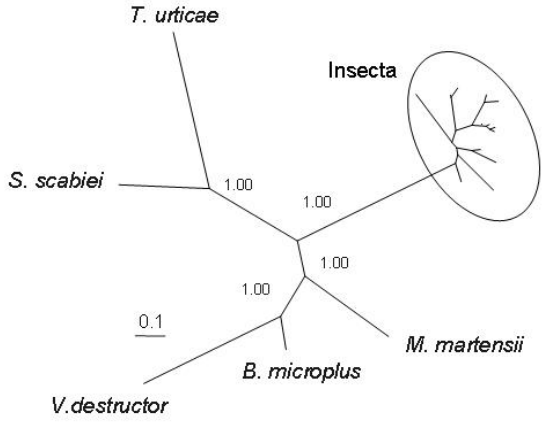

Figure 2.2 Phylogenetic tree of insect and arachnid species voltage-gated sodium channel cDNAs. Sequences were aligned and a bootstrapped unrooted tree was constructed as described in the materials and methods. Bootstrap values are indicated on branches.

3.3 Identification of mutations within the sodium channel gene of *T. urticae*

The cloned region contains the majority of mutation sites previously implicated in conferring target site resistance to pyrethroids across a range of insect and mite species (Davies et al. 2007b). Residues associated with the susceptible phenotype were present at most of these positions in both the resistant and susceptible *T. urticae* strains analysed, including M918, T929, L925 and L1014 (numbering according to *M. domestica*). An interesting exception is the presence of methionine at residue 1016 in *T. urticae*, as this is normally a valine in all

other known insect and mite sequences, and replacement of this valine by methionine has been identified as a possible resistance mutation in *Aedes aegypti* mosquito populations from Central America (Saavedra-Rodriguez et al. 2007). The M1016 is present in all the *T. urticae* strains tested here, so although not associated solely with the resistant phenotype, it may nevertheless contribute towards the general reduced sensitivity of this species for most pyrethroids (Khambay and Jewess 2005).

Comparison of the cDNA sequences between the resistant and susceptible *T. urticae* strains revealed a total of 29 nucleotide substitutions. Most of these were silent, although 7 were found to result in amino acid replacements, and of these 2 were found in both ATHRos-Bf and CREVeg-Bf resistant strains but not in the susceptible SAMB and GSS strains. The first, a phenylalanine (TTC) to isoleucine (ATC) replacement at amino acid 1538 (*M. domestica* numbering) is located within domain III segment 6 (IIIS6) and has been previously reported in the southern cattle tick *B. microplus* (He et al. 1999). The role of this mutation has been established by site directed mutagenesis and functional expression studies in *Xenopus laevis* oocytes (Tan et al. 2005) and shown to confer high levels of insensitivity to a number of pyrethroids (Tan et al. 2005). The second is an alanine (GCT) to aspartic acid (GAT) substitution at amino acid residue 467 of the *T. urticae* sequence (Figure 2.1). This residue corresponds to the amino acid D1215 in housefly, which is located in the intracellular linker between domains II and III and has not been previously reported to be associated with resistance in any species. The remaining 5 amino acid replacements are found only in the resistance strain CREVeg-Bf. Following the numbering of *T. urticae* in Figure 2.1, these amino acid changes are L151V, G178S, A182G, G189D and S253T and are all found within the II/III linker, a highly variable region.

3.4 Splicing variants

The sequence alignment of several cDNA clones from the resistant and susceptible strains revealed the presence of a variable region overlapping segments S3-S4 of domain III with three variants (v1, v2 and v3 Figure 2.3). The two variants (v1 and v2) differed from each other in 14 /40 amino acids (35%) and correspond to the mutually exclusive k/l exon pair already identified in insects (Davies et al. 2007b, Dong 2007). A larger variant (v3) containing both v1 and v2 sequences was observed in some rare plasmids. In order to investigate the frequency of these variants in resistant and susceptible strains, we analyzed a larger number of colonies with the 3.3 kb insert. We therefore amplified the region around the two variants (primers SGF3b and SGR6) with a colony-PCR based method, followed by a *Dra*I

(Fermentas) restriction digest to distinguish between colonies with the transcript v1 or v2 (l or k respectively) or v1 + v2 transcript variant. This digest generates fragments of 465+108+103 or 573 +103, or 465+231+103 bp for the v1 (exon trancript l), v2 (exon transcript k) or v3 (exons k+l) respectively. The v2 transcript (Figure 2.3) was found at a frequency of 63% and the v1 transcript was present in 36% of the plasmids analyzed, whereas the v3 was present in only 1%. No association between splicing variant frequencies and resistance phenotype was observed, as the before-mentioned frequencies were not significantly different between the resistant and susceptible strains.

In order to investigate any possible association between putative resistance point mutations identified and transcript variants, we sequenced several plasmids containing the 3.3 kb fragment with the internal primers KdrR1 and KdrR2. Sequencing reads with these primers covered both the mutation-regions and transcript variants. The phenylalanine with isoleucine and the alanine with aspartic acid replacements were absent from all the 10 colonies of the susceptible strains. They were found together in all the 10 colonies from the resistant strains (five of each strain) independently of the transcript v1 or v2.

3.5 Inheritance of resistance

Reciprocal crosses of CREVeg × SAMB were performed in order to examine the degree of dominance. Crossing female CREVeg-Bf with male SAMB resulted in 100% male offspring indicating cytoplasmic incompatibility, since in the arrhenotokous reproduction mode of *T. urticae* unfertilised eggs result in male progeny. SAMB was strongly infected by *Wolbachia* as determined by PCR with universal wsp primers (Zhou et al. 1998), while infection could not be detected in CREVeg-Bf, suggesting that interference in reproduction by this endosymbiont is one possible explanation of the observed incompatibility (results not shown). The dominance was hence calculated by the mortality response of the female offsprings of the cross f SAMB × m CREVeg-Bf. In Table 2.5, the pesticide concentrations giving 50% mortality (LC50s) of the two parental strains and F1 females are presented. The resistance was almost fully recessive with degree of dominance D = -0.57.

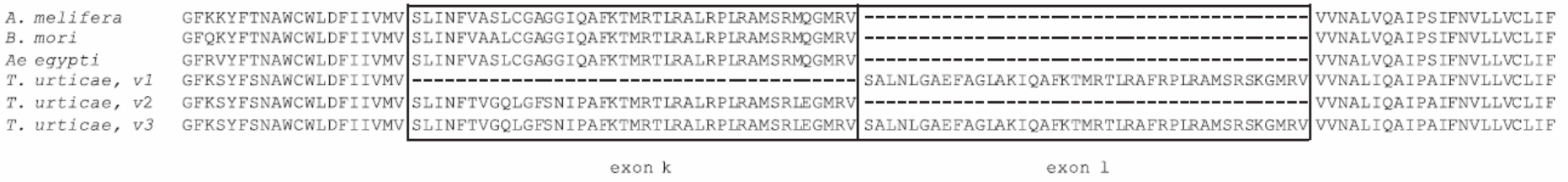

Figure 2.3 The three transcript variants (v1, v2, v3) in *T. urticae* VGSC corresponding to the mutually exclusive exons k and l found in insects.

Table 2.5 Mortality data of the F1 progeny

Strain	n^a	LC_{50} [b]	Slope ± SE	χ^2 [c]	*df*	RR_{50} [d]
SAMB	565	11 (12 - 14)	3.19 ± 0.27	5.46	4	-
CREVeg-Bf	540	3397 (2733 - 4219)	4.35 ± 0.91	16.44***	3	311 (218 - 442)
F1 *SAMB* × *CREVeg-Bf*	329	37 (29 - 47)	1.46 ± 0.18	1.70	5	3.38 (2.92 - 3.92)

[a]number of mites tested
[b]LC, lethal concentration expressed in mg L^{-1}
[c]Chi-square testing linearity, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
[d]RR, resistance ratio = LC/LC_{SAMB}

4 DISCUSSION

We have investigated pyrethroid resistance in two strains of *T. urticae* from Greece (ATHRos-Bf and CREVeg-Bf) exhibiting strong resistance to bifenthrin (2495- and 1026-fold) and fluvalinate (867- and 1132-fold). Combined bioassays (cross resistance pattern), biochemical and synergistic data indicated that enhanced detoxification was not a major component of resistance, suggesting target-site resistance as the main mechanism. A 3.3-kb cDNA fragment (domains II-IV) of the *T. urticae para* sodium channel gene was obtained by a degenerate PCR strategy. A comparison of the sequences between susceptible and resistant mites revealed two changes that were found only in the resistant strains: a phenylalanine to isoleucine substitution (equivalent to F1538I) in domain IIIS6 and an alanine to aspartic acid substitution (equivalent to A1215D) within the II/III intracellular linker region. The F1538I mutation seems to be the most important in determining the resistance phenotype because 1) this same mutation has previously been identified in several pyrethroid-resistant strains of the southern cattle tick *B. microplus* (He et al. 1999); 2) it has been shown to confer strong insensitivity to a range of pyrethroids when functionally expressed in an *in vitro* assay system (Tan et al. 2005); 3) F1538 is one of several aromatic residues in the IIIS6 helix that form part of a putative hydrophobic binding site for pyrethroids in a computer-generated 3D model of the housefly sodium channel (O'Reilly et al. 2006, Davies et al. 2008). In this model, which is based on crystal structures of closely-related potassium channels, a high-affinity site for pyrethroid binding is formed during channel activation as the IIS4-S5 linker is pulled closer to the IIS5 and IIIS6 helices, with the bound pyrethroid then acting as a molecular ‘wedge’ to hold the channel in its open conformation and thereby delaying channel de-activation. F1538 is one of several aromatic residues that are predicted to interact with the alcohol groups of double-ring cyclic pyrethroids and so it is not surprising that mutations at this residue will destabilise this high affinity binding to give resistance (O'Reilly et al. 2006, Davies et al. 2008), although the exact nature of these interactions and the reason(s) why that certain pyrethroids (e.g. bifenthrin and fluvalinate) are more affected than others (e.g. fenpropathrin) are not clear at present, but are likely to reflect other sequence differences between the insect and mite sodium channel within the proposed binding site (O'Reilly et al. 2006) and can be explored in more detail by continued site-directed mutagenesis and expression studies of insect/mite sodium channels in *Xenopus oocytes* (Tan et al. 2005).

At this stage, it is not possible to predict whether the second substitution (A1215D) is likely to

be contributing to the resistance phenotype. This residue lies within the domain II-III intracellular linker which is one of the most variable regions of the protein amongst species and the role of this region in determining channel structure and function is currently not known. It is of course possible that this is a simple polymorphism that happened to be present in the resistance allele when F1538I was selected, and so makes no contribution towards resistance. Likewise, the five amino acid replacements (L151V, G178S, A182G G189D and S253T) found only in the resistant strain from Crete (CREVeg-Bf) are similarly located in the highly variable region of the domain II/III linker and so may also be polymorphisms that are related to the origin of the strain rather than to the resistant phenotype. Nevertheless, the possibility that these mutations also contribute to the resistance, in line with I/II linker mutations of the cockroach channel (Tan et al. 2002a) can not be excluded completely.

Finally, the possibility of the occurrence of additional mutation(s) outside the IIS4-IVS6 gene region analysed in this study, associated or contributing to the resistance phenotype can not be discounted.

In contrast to previous studies (Van Leeuwen et al. 2005), biochemical and bioassay (synergistic) data indicated that detoxifying enzymes are probably not the primary resistance component in the ATHRo-Bf and the CREVeg-Bf strains. However, the overexpression of P450s in both resistant strains, and notably (up to 9 fold) in the ATHRo-Bf with the highest resistance levels requires further investigation, as it is possible (but not clear, given that the *in vivo* data with PBO do not support this hypothesis) that the P450 based detoxification supplements the main target site resistance mechanism.

The putative 1108 amino acid sequence isolated in this study had highest similarities to the remarkably heterogeneous class Arachnida and less than 50% compared to the class Insecta. The distinct sequence characteristics and variation of the *T. urticae* sodium channel gene compared to insects might be related to the pharmacologically different response of this mite to several pyrethroids in comparison to insects (Khambay and Jewess 2005).

Alternative splicing is a major mechanism by which sodium channels increase their functional diversity in several species, including the physiological response to pyrethroid insecticides (Tan et al. 2002b, Davies et al. 2007a). Three alternative splicing variants were identified in this study. Two of them differed dramatically in sequence variation and correspond to the mutually exclusive k/l exon pair already identified in insects. A larger variant that includes both k and l exons was also observed in some rare plasmids and probably results from incomplete RNA processing. The frequency of variants was not different between resistant and susceptible strains and the occurrence of resistant mutations was not affected by the

transcript variation. The functional importance of this transcript variation in *T. urticae* (if any) remains to be investigated.

Mortality data with bifenthrin in the F1 progeny from crosses between SAMB and CREVeg-Bf indicated that the mode of inheritance of the target insensitivity-based bifenthrin resistance was almost completely recessive, consistent with studies of this mechanism in other species (Halliday and Georghiou 1985, Sayyed et al. 2005). The resistance in a *B. microplus* strain with the F1538I mutation displayed various levels of incomplete dominance to pyrethroids, although this was probably a maternal effect due to additional mechanism(s) involved in pyrethroid resistance (Aguilar-Tipacamú et al. 2008). In *T. urticae* it was not possible to detect the involvement of such maternal effects since one of our reciprocal crosses resulted only in male offspring due to possible *Wolbachia* mediated reproductive incompatibility (Breeuwer 1997).

The recessive mode of inheritance means that the trait will be mainly expressed in homozygous individuals. Therefore, the ability to detect heterozygotes is of paramount importance for the early detection of resistance and the application of resistance management strategies.

In conclusion, we characterized the pyrethroid resistance mechanism in two *T. urticae* strains isolated from Greece. The substitution F1538I in III6 of the voltage gated sodium channel, one of the most highly effective resistance loci for synthetic pyrethroids, has been found for the first time in an agricultural pest. The mutation appears to be a prime target for developing a DNA-based screening method for pyrethroid resistance in field populations of *T. urticae*.

CHAPTER III

MECHANISMS AND CONSEQUENCES OF RESISTANCE TO ORGANOPHOSPHATES AND CARBAMATES IN *TETRANYCHUS URTICAE* (ACARI: TETRANYCHIDAE)

This chapter has been redrafted from:

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

Khajehali J, Van Leeuwen T, Tirry L (2009) Susceptibility of an organophosphate resistant strain of the two-spotted spider mite (*Tetranychus urticae*) to mixtures of bifenazate with organophosphate and carbamate insecticides. Experimental and Applied Acarology 49: 185-192

1 INTRODUCTION

Organophosphates (OPs), and, to a lesser extent, carbamates (CARB), are both chemical groups used to control the two-spotted spider mite *T. urticae,* an economically important agricultural pest with a wide range of host plants and geographic distribution. They act by inhibiting acetylcholinesterase (EC 3.1.1.7, AChE), a key enzyme in the nervous system of both vertebrates and invertebrates.

Since the first reported cases of OP resistance in the late 40's (Helle 1962), *T. urticae* has developed resistance towards almost every acaricide used for its control. Within the OP family, cross-resistance is a general rule in *T. urticae* (Voss and Matsumura 1964, Cranham 1974, Brun et al. 1983, Tsagkarakou et al. 1996, Herron et al. 1998, Stumpf et al. 2001, Van Leeuwen et al. 2005). This can result from enhanced detoxification (Voss and Matsumura 1964, Van Leeuwen et al. 2005) and/or target site insensitivity. The insensitivity of *T. urticae* AChE towards OPs was first reported in 1964 (Smissaert 1964) suggesting the presence of some modifications at the active site of the enzyme (Voss and Matsumura 1964). Since then, AChE insensitivity to OPs has been demonstrated in various strains of *T. urticae* from the Netherlands, Germany, USA, New Zealand, Israel, Egypt, Greece and Japan (Zahavi and Tahori 1970, Helle 1984, Cranham and Helle 1985, Eldin 1990, Stumpf et al. 2001, Tsagkarakou et al. 2002, Anazawa et al. 2003).

Many insects and ticks have at least two genes, termed *ace-1* and *ace-2,* encoding for AChE1 and AChE2, respectively. These genes correspond to the *Drosophila* paralogous and orthologous genes (Weill et al. 2002). In the dipteran order of the Cyclorrhapha (true flies), only the *ace-2* gene is present (Huchard et al. 2006). In insects that have both genes, the main synaptic function is encoded by *ace-1* (Weill et al. 2002, Weill et al. 2003). In this gene, seven point mutations associated with resistance to OPs and CARBs have been reported, resulting in the following amino acid substitutions: G119S, A201S, F290V, G227A, S331F, and F331W/C; numbering based on *Torpedo californica* AChE nomenclature (Massoulié et al. 1992, Anazawa et al. 2003, Nabeshima et al. 2003, Weill et al. 2003, Li and Han 2004, Nabeshima et al. 2004, Baek et al. 2005, Cassanelli et al. 2006, Alout et al. 2007b, Alout et al. 2007a). However, only G119S, F290V, S331F and F331W were demonstrated to confer insensitivity by means of *in vitro* experiments. In true flies, one or a combination of mutations in the *ace-2* gene, including substitutions F78S, I129V/T, V150L, G227A/V, F290Y, G328A and G396S conferred *in vitro* insensitivity to OP insecticides (Mutero et al. 1994, Walsh et al. 2001, Vontas et al. 2002, Menozzi et al. 2004, Aiki et al. 2005, Magana et al. 2008).

Although biochemical evidence of AChE insensitivity in *T. urticae* was already revealed in 1964 by Smissaert (1964), the exploration of the molecular basis of AChE1 insensitivity in Tetranychidae started only quite recently. Among phytophagous mites, *ace-1* gene sequences encoding for the enzyme AChE1 have been determined in two tetranychid species, i.e. *T. urticae* and *T. kanzawai* (Anazawa et al. 2003, Aiki et al. 2005). The comparison of the AChE1 amino acid sequence from susceptible and OP resistant *T. urticae* from Japan revealed the presence of a F331C substitution (Anazawa et al. 2003). In OP resistant *T. kanzawai* a tryptophan was detected at position 331 (W331) (Aiki et al. 2005). In a first part of this chapter the presence of mutations in the ACHE1 of several OP- and CARB-resistant *T. urticae* strains was examined.

Bifenazate, a hydrazine derivative with acaricidal activity, was discovered in 1990 by Uniroyal Chemical and commercialised in 1999 (Dekeyser et al. 1994, 2003, Grosscurt and Avella 2005). It was thought to be a neurotoxin, acting on the post-synaptic GABA-receptor in the insect/mite nervous system (Dekeyser 2005). However, a different mode of action as a mitochondrial Q_o inhibitor has recently been proposed mainly based on genetic and cross-resistance evidence (Van Leeuwen et al. 2008, Van Nieuwenhuyse et al. 2009). In addition, while investigating possible detoxification routes, Van Leeuwen et al. (2006a) discovered the pro-acaricide nature of bifenazate, i.e. it must be metabolized first in order to become toxic. They proposed an activation pathway where hydrolysis of the ester bond by a *S,S,S*-tributyl phosphorotrithioate (DEF) sensitive esterase is the first step.

Bifenazate mainly shows excellent activity against many phytophagous mite species including *T. urticae*, providing quick knockdown through contact activity and long residual control (four weeks) (Ochiai et al. 2007). Bifenazate can be included in integrated management programs because of its low toxicity to predatory mites (Ochiai et al. 2007).

The current trend in pesticide development is to release less broad-spectrum and more specific pesticides like bifenazate, in order to avoid negative effects on non-target organisms, while still providing effective control of the target pest. However, the two-spotted spider mite is typically not the only arthropod pest in fields and greenhouses. Consequently, growers can encounter a range of insect and mite pests simultaneously (Warnock and Cloyd 2005). Therefore, mixtures of different pesticides are used to manage the diversity of arthropod pests present in a crop (Bynum et al. 1997). Although there are concerns associated with pesticide mixtures such as enhanced resistance to one or more pesticides, phytotoxicity and pesticide incompatibility and antagonism (Warnock and Cloyd 2005), they are widely used.

Recently, it was reported that OP and CARB insecticides can interfere with bifenazate toxicity (Van Leeuwen et al. 2007). Experiments in which a susceptible reference strain of *T. urticae* was pre-treated with sublethal doses of OPs and CARBs, revealed a clear antagonistic effect on bifenazate toxicity. It was argued that antagonism could compromise bifenazate efficacy under field conditions. This antagonism may be explained by the proposed esterase-mediated activation pathway of the bifenazate carboxylester bond (Van Leeuwen et al. 2007). Although OP and CARB compounds target acetylcholinesterase (AChE), they can also inhibit general hydrolytic esterase activity (Gunning et al. 1999), and hence interfere with bifenazate activation and toxicity.

So, in the second part of this chapter, the risk of antagonism between bifenazate and OPs and CARBs in mixtures was estimated, using two OP resistant strains, in contrast to the pre-treatment experiments previously reported (Van Leeuwen et al. 2007). OPs and CARBs are ineffective against these mites, allowing high uptake and inhibition of the general esterase activity without inhibiting AChE. Since many strains worldwide have developed resistance to OPs, the aim of this study was to evaluate whether these resistant strains pose a threat to antagonism based control failure of bifenazate, when OPs or CARBs are mixed with bifenazate to control other pests.

2 MATERIALS AND METHODS

2.1 Strain characteristics

In this study we characterized three laboratory *T. urticae* strains which have been commonly used as reference strains (SAMB, GSS and LS-VL) and three resistant strains maintained in the laboratory under continuous OP selection (OP strains: WI, MR-VL and ATHRos-Pm). SAMB and GSS were both maintained in the laboratory without treatment since 1965. SAMB was initially collected on *Sambucus nigra* (L) in the Netherlands and obtained from the Centre de Biologie et de Gestion des Populations, Montpellier, France. GSS was obtained from Bayer CropScience (Monheim, Germany) and was originally collected from an unknown host in Germany. LS-VL was collected in 2000 from roses in a garden near Ghent, Belgium (Van Leeuwen et al. 2004) and since then it had been maintained in the laboratory without treatment.

The historical German resistant strain WI has a well studied target site resistance to OPs and CARBs (Stumpf et al. 2001). It was obtained from Bayer CropScience where it has been maintained in the laboratory under biannual selection with the OP oxydemeton-methyl since 1954 (Nauen et al. 2001). Biochemical characterization has shown that the main resistance

mechanism was an altered acetylcholinesterase with a 110-fold and 340-fold lower sensitivity to inhibition by chlorpyrifos oxon and ethyl paraoxon, respectively (Stumpf et al. 2001).

The OP strains MR-VL (from Belgium) and ATHRos-Pm (from Greece) were more recently collected from fields on which failure of all insecticide treatments, including OPs, was claimed by the farmers. MR-VL was collected in 2003 from a greenhouse nursery where poplar cuttings, beans and ornamentals were grown, while ATHRos-Pm was collected in 2007 on roses in a greenhouse near Athens. Both strains developed resistance to many acaricides currently used to control spider mites (Van Leeuwen et al. 2005, Tsagkarakou et al. 2009b). ATHRos-Pm was maintained in the laboratory under pirimiphos-methyl selection every two generations with concentrations causing ~ 80% mortality before its characterization after eight selections.

All mites were reared continuously on potted kidney bean plants, *Phaseolus vulgaris* L. cv. "Prelude", in a climatically controlled room at 26 °C $\pm$ 1°C, 50-60 % RH and 16/8 h (L/D) photoperiod.

2.2 Chemicals

Commercial formulations of pirimiphos-methyl 500 g L^{-1} EC (Actellic, Syngenta, UK), dimethoate 400 g L^{-1} EC (Dimethoate, Lapafarm, Greece), chlorpyrifos 480 g L^{-1} EC (Dursban Dow Agrosciences, USA), bifenazate 240 g L^{-1} SC (Floramite, Crompton Crop Protection, UK), azinphosmethyl 500 g L^{-1} WP (Guthion, Bayer CropScience, Germany), phosmet 700 g L^{-1} WP (Imidan, Gowan Company, USA), carbaryl 500 g L^{-1} WP (Sevin, Southern Agricultural Insecticides, USA) and methomyl 200 g L^{-1} SL (Alpha, Greece) were used.

2.3 Bioassays

Toxicity bioassays using 6 strains and 4 compounds (pirimiphos-methyl, chlorpyrifos, dimethoate and methomyl) were conducted on detached bean leaves with a precision Potter Spray Tower (Burkard, Rickmansworth, Hertfordshire, UK (Potter 1952)) as described by Tsagkarakou et al (2009b). LC_{50} values and 95% confidence limits were calculated by the log-probit program (Raymond et al. 1993) based on Finney (Finney 1971). Resistance ratios at the LC_{50} (RR_{50}) were considered to be significantly different from 1 when their 95% confidence limits did not include this value.

To evaluate antagonistic effects of OPs and CARBs on bifenazate toxicity, first, toxicity of the OP and CARB insecticides (methomyl, azinphosmethyl, phosmet and carbaryl) to the WI and MR-VL strains was assessed using a dose-screening test (between 50-2000 mg active ingredient (a.i.) L^{-1}). Second, mixtures were prepared by adding a constant dose of 100 mg a.i.

L^{-1} (when LC_{10} is higher than 100 mg a.i. L^{-1}) or LC_{10} of OP and CARB insecticides to varying concentrations (125, 250, 500, 1000 mg a.i. L^{-1}) of bifenazate. Also a water treatment as control, a 125 mg a.i. L^{-1} bifenazate and 100 mg a.i. L^{-1} or LC_{10} of the OP and CARB alone were used. Each treatment was replicated at least 3 times. Mortality, defined by the inability of mites to move when prodded by a camel-hair pencil, was scored every 24 hours for 5 consecutive days after treatment.

2.4 Acetylcholinesterase activity and inhibition studies

For determining the AChE1 activity, 1000 adult females were mass homogenized in 1 ml extraction buffer containing 10 mM Tris, pH 7.0, 1 M NaCl and 0.4% Triton X-100. The supernatant obtained after centrifugation (10000 g, 4°C, 10 min) was used as the enzyme source. The reaction was conducted in 1ml substrate-reagent solution containing 30-50 μg of protein, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and acetylthiocholine (AcSCh) each in a final concentration of 0.5mM. The mean activity values were compared between strains by one-way analysis of variance (ANOVA) using the SPSS statistical software version 13.0 (LSTM, UK). Residual AChE1 activities were measured with increasing concentrations of the analytical grade inhibitors chlorpyrifos-oxon (varying between 10^{-9} M to 10^{-3} M), paraoxon (10^{-9} M - 10^{-3} M), carbaryl (10^{-10} M - 10^{-3} M), pirimiphos methyl (10^{-7} M - 10^{-3} M) and omethoate (10^{-7} M - 10^{-3} M). Briefly, the enzyme source was incubated for 10 min with the inhibitor solution at a given concentration before adding the substrate-reagent solution. Residual activity was estimated by kinetically measuring the variation in optical density at 412 nm with a spectrophotometer (M2e, Molecular Device, UK). Blanks without homogenate or substrate were used to correct for non-enzymatic activity. Enzyme inhibition was expressed as the mean percentage of activity remaining at different inhibitor concentrations and the inhibitor concentration inducing 50% inhibition (IC_{50}) was determined using the Microcal Origin 6.0 data analysis program.

2.5 Detoxifying enzyme assays

The *in vitro* activity of cytochrome P450 mono-oxygenases was quantified by measuring the O-deethylation of 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) and esterase activity was measured with the substrate 4-nitrophenylacetate (4-NPA), as outlined by Van Leeuwen et al. (2005). Glutathione-S-tranferase (GST) activity was measured with substrates 1-chloro-2,4-dinitrobenzene (CDNB) and monochlorobimane as described by Van Pottelberge et al. (2009b). Enzyme activities were compared by analysis of variance (ANOVA) and significant differences were assessed using Tukey's method (S-Plus 8.0 for Windows, Insightful Corp)

2.6 Extraction of DNA and RNA, cDNA synthesis, cloning, and sequencing

Total DNA was extracted from mass homogenates as described by Van Leeuwen et al. (2008). Total RNA was extracted from mass homogenates of *T. urticae* mites using TRI reagent (Sigma-Aldrich, Belgium) according to the manufacturer's instructions. The RNA was treated with DNase RQ1 (Promega, Madison, WI) to remove genomic DNA. Two µg total RNA for each sample was reverse transcribed into cDNA by using the High Fidelity cDNA synthesis kit (Roche, Belgium) and random hexamer primers. Primers (Table 3.1) were designed based on the published sequence of *T. urticae* AChE1 (AY188448). For cDNA amplification, a long PCR was performed using Acetrur F and Acetrur R primers (Expand Long Range Kit, Roche, Belgium), according to the manufacturer's instructions. 2.5 kb fragments were purified with QIAEX II (Qiagen), cloned into the pGEM-T vector (Promega), and sequenced with primer walking. To ensure allele representations, three clones were sequenced for each strain. Plasmids were sequenced by Agowa Sequencing Service (Berlin). Sequence data were analyzed using BioEdit 7.0.1. (Hall 1999).

Table 3.1 Primers used for the amplification of the *Tetranychus urticae* AChE1.

Primers	Sequence (5' – 3')
Acetrur F	AAAGGGAGAAGGCAAAAGTGT
Acetrur R	TGCTCATGTTCAGTTGATCG
AcETuSeqF1	AATGCCACCTCATTTTCAGG
AcETuSeqF2	TATCACGCAACGTTTTCAGC
AcETuSeqF3	ATGAAGATCCCGAGGTTTCC
AcETuSeqF4	TGGGAGTTATTCATGGTGAGG
AcETuSeqR1	CATGAATCGATGGGCTTAGG
AcETuSeqR2	AGCTGATTCACCGAAAATGG
AcETuSeqR3	AACTGCTTCTTGGGCTAAAGG
AcETuSeqR4	CAGTGTACTCAGGCCACACG
AchEDISCF2	AAGCAATCGTGATGAGGGGCCCTATT
AchEDISCR2	CTAAAGGACTCAGATGGGGATAAAT

2.7 Three-dimensional modelling

The modelled three-dimensional structure (3D) of *T. urticae* AChE1 was created by automated homology modelling as described by Weill et al. (2004). The structural templates used were AChE from *Torpedo californica* (PDB: 1EA5) (Sussman et al. 1991) and *Drosophila melanogaster* (PDB: 1DX4) (Harel et al. 2000). The alpha-carbon skeleton of the modelled 3D structure of AChE1 was superimposed on that of the AChE of *T. californica*.

RMS deviation is 1.1 Å on 532 carbon atoms (from Dali pairwise comparison webserver [http://ekhidna.biocenter.helsinki.fi/dali_lite/start]).

2.8 Diagnostic PCR-RFLP for W331

Two primers were designed (AcheDISCF2, AcheDISCR2, Table 3.1) around position 331 that amplified a 137 bp fragment using genomic DNA as template. Amplifications from approximately 40 ng gDNA were performed in 1xTaq reaction buffer (Invitrogen) with a final concentration of 1.5 mM $MgCl_2$ and 0.5 µM of each primer, with cycling conditions 94 ^oC for 3 min, 35 cycles of 94 ^oC for 15 s, 55 ^oC for 30 s, 72 ^oC for 40 s, followed by 72 ^oC for 2 min. A restriction digest using 10 µl PCR product was performed in a total volume of 30 µl using *Bgl* I according to the manufacturer's instructions (Fermentas). Approximately 20µl was loaded onto a 3% agarose gel and after 1.5 hours at 50V the separated restriction fragments were visualized by ethidium bromide staining.

3 RESULTS

3.1 Resistance characteristics of the *T. urticae* strains

Among the reference strains, variable levels of sensitivity were found (Table 3.2). LS-VL proved to be the most susceptible to all insecticides tested: LC_{50} of pirimiphos methyl, chlorpyrifos, methomyl and dimethoate were 16, 11, 28 and 8 mg L^{-1}, respectively. Therefore this strain was used as a susceptible reference to calculate the RR_{50} of the other strains. SAMB exhibited a 31 and 29- fold resistance to chlorpyrifos and dimethoate, respectively. GSS was 21 and 1411- fold more resistant to pirimiphos-methyl and dimethoate, respectively. The OP strains ATHRos-Pm, MR-VL and WI were resistant to all insecticides tested, with RR_{50} of at least 8.4 (Table 3.2). Among the three OP strains, the highest levels of resistance to pirimiphos methyl (RR_{50} = 43), dimethoate (RR_{50} = 4164) and methomyl (RR_{50} = 333) were exhibited by ATHRos-Pm, and the weaker levels by WI (RR = 8.9, 137 and 8.4 to pirimiphos-methyl, dimethoate and methomyl, respectively). For chlorpyrifos, the resistance ratio was higher in the WI and MR-VL strain (RR_{50} = 421 and 586, respectively).

3.2 Biochemical characterization of the AChE1 enzyme

AChE1 activity was highest in SAMB strain (63 ± 11 mOD/min/mg protein), followed by MR-VL strain (37 ± 1 mOD/min/mg protein) ($P < 0.01$) (Table 3.3). The four other strains showed lower activities, which did not differ significantly from each other.

As is shown by the IC_{50} values (Table 3.3), AChE1 from the OP strains WI, MR-VL and ATHRos-Pm were much less sensitive to inhibition by pirimiphos methyl, chlorpyrifos oxon, paraoxon and carbaryl than the three reference strains. WI and MR-VL were less sensitive to

inhibition by omethoate than LS-VL and SAMB (omethoate was not tested for AChE1 from GSS and ATHRos-Pm strains and no comparison is possible). AChE1 from SAMB and LS-VL were more sensitive to inhibition by chlorpyrifos-oxon but less sensitive to inhibition by carbaryl than the AChE1 from GSS. For paraoxon, IC_{50} values for SAMB and GSS were very similar and lower than that of LS-VL. Among the OP strains, IC_{50} values were similar for the OPs and the CARBs tested.

Table 3.2 Toxicity data of the OP selected and the reference *Tetranychus urticae* strains treated with several OP and one CARB insecticides.

Treatment	n[a]	LC_{50}[b]	Slope ± SE	χ^2[c]	df	RR_{50}[d]
LS-VL						
Pirimiphos methyl	522	16 (12 – 21)	2.66 ± 0.39	11*	4	
Chlorpyrifos	435	11 (4.8 – 24)	0.98 ± 0.27	14***	3	
Methomyl	435	28 (14 – 57)	1.20 ± 0.30	24***	4	
Dimethoate	214	8 (2 – 29)	0.88 ± 0.27	12**	3	
SAMB						
Pirimiphos methyl	254	18 (16 – 21)	4.50 ± 0.14	0.4	1	1.1 (0.8 – 1.7)
Chlorpyrifos	315	333 (173 - 646)	2.75 ±1.17	16***	2	31 (15 – 64)
Methomyl	378	40 (9.34 – 167)	2.48 ± 1.36	52***	2	1.4 (0.4 – 5.4)
Dimethoate	226	226 (182 - 282)	4.97 ± 0.14	5	2	29 (17 – 47)
GSS						
Pirimiphos methyl	577	332 (166 - 665)	6.56 ± 6.85	135***	2	21 (3.9 – 113)
Chlorpyrifos	328	63 (19 – 215)	5.67 ± 7.05	136***	2	5.8 (0.4-79)
Methomyl	279	50 (26 – 94)	2.11 ± 0.50	6.7*	2	1.8 (1.0 – 3.2)
Dimethoate	281	11164 (7418 - 16836)	3.80 ± 1.43	8.8*	2	1411 (686 – 2904)
WI						
Pirimiphos methyl	463	142 (96 – 209)	4.70 ± 1.81	37***	3	8.9 (4.4 – 18)
Chlorpyrifos	424	4584 (2786 – 7530)	3.93 ± 1.23	28***	3	421 (196 – 907)
Methomyl	373	236 (151 – 373)	3.86 ± 1.26	17***	3	8.4 (4.2 – 17)
Dimethoate	394	1502 (915 – 2467)	2.24 ± 0.64	18***	3	137 (84 – 224)
MR-VL						
Pirimiphos methyl	329	314 (266 – 374)	3.75 ± 0.41	3.9	3	20 (14 – 28)
Chlorpyrifos	392	6386 (4261 – 9567)	4.05 ± 1.08	21***	3	586 (302 – 1137)
Methomyl	419	3759 (3178 – 4370)	3.08 ± 0.32	4.2	3	134 (95 – 189)
Dimethoate	334	5878 (2218 – 15579)	3.16 ± 2.02	59***	3	743 (223 – 2465)
ATHRos-Pm						
Pirimiphos methyl	337	685 (497 – 944)	2.81 ± 0.73	12**	3	43 (28 - 66)
Chlorpyrifos	366	895 (624 - 1289)	2.62 ±0.57	9.4*	3	78 (39 - 156)
Methomyl	301	9338 (4421 - 19649)	3.11 ± 0.95	16***	2	333 (136 - 818)
Dimethoate	319	32950 (23977 - 45249)	6.09 ± 3.28	8.5***	1	4164 (1728 – 10036)

[a] n, number of mites tested
[b] LC, lethal concentration expressed in mg L^{-1}
[c] χ^2, Chi-square testing linearity, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
[d] RR, resistance ratio = $LC/LC_{LS\text{-}VL}$
Confidence Intervals of LC_{50} and RR_{50} are given in parenthesis

3.3 Comparison of the AChE1 sequence of the reference and the OP selected strains

Using specific primers and long PCR strategy, the cDNA sequence of a 2.5 kb fragment was determined in the six strains (GenBank accession nos. GQ461336-GQ461353). This fragment contains an open reading frame encoding for an AChE1 precursor of 687 amino acid residues. Three cDNA clones were sequenced from each strain resulting in a total of 18 clones which revealed 88 variable nucleotide sites including inter- and intra-strain polymorphisms. Forty four of these resulted in amino acid substitutions. By comparing these substitutions in the AChE1 primary sequence from the different strains, we identified six mutations in the OP strains that were not present in the reference strains. Only four of them were present in the mature protein (numbering based on *Torpedo californica* AChE nomenclature, Massoulié et al. 1992): 1) at position 201, an alanine residue (GCT) is replaced by a serine (TCT) (A201S) in the three clones of the OP resistant strain ATHRos-Pm, 2) at position 280, a threonine residue (ACA) is replaced by an alanine (GCA) (T280A) in all the three OP strains except for one clone of the ATHRos-Pm strain, 3) at position 328, a glycine (GGA) is replaced by an alanine (GCA) (G328A) in the ATHRos-Pm and MR-VL strains and 4) at position 331, a phenylalanine (TTT) is replaced by a tryptophan (TGG) (F331W) in all clones of the three OP resistant strains. Interestingly, the F331 residue is replaced by a tyrosine (TAT) (F331Y) in two clones of reference strains (one in SAMB and one in GSS strain). Moreover, two amino acid substitutions, a valine (GTT) to isoleucine (ATT) at position 7 (V7I) and a glycine (GGC) to serine (AGC) at position 119 (G119S) were found specifically in the reference strain SAMB (Table 3.4).

3.4 Three-dimensional modelling

The positions of the mutations in the structural model of *T. urticae* AChE1 are shown in Figure 3.1. The active site of cholinesterases is subdivided into functional groups important for the hydrolysing process: the catalytic triad (S200, E327 and H440), the peripheral anionic site (D72, Y121, W279 and Y334), the choline binding site (W84, Y130, Y330 and F331), the acyl binding pocket (F288 and F290) and the oxyanion hole (G118, G119, A201) (Gibney et al. 1990, Sussman et al. 1991, Harel et al. 1992, Ordentlich et al. 1993, Vellom et al. 1993).

Among the two substitutions specifically found in the susceptible strain SAMB the first one, V7I, is located in the extremity of the N-terminal of the enzyme structure and may not influence the catalytic properties of AChE1. The second substitution, G119S, is located in the active site that belongs to the oxyanion hole and affects an important residue close to the catalytic serine residue (S200) thus it may modify the catalytic properties of the enzyme.

Table 3.3 Specific activities and IC_{50} of various OP and CARB inhibitors on AChE1 extracted from OP selected and reference *Tetranychus urticae* strains

		IC_{50} (M)				
Strain	AChE activity mOD/min/mg	Pirimiphos methyl	Chlorpyrifos-oxon	Paraoxon	Carbaryl	Omethoate
LS-VL	22 ± 1.5 a	$3.7 \pm 0.7 \times 10^{-4}$	$7.0 \pm 2 \times 10^{-8}$	$4.3 \pm 1.2 \times 10^{-6}$	$6.0 \pm 1.8 \times 10^{-6}$	$7.4 \pm 2.7 \times 10^{-6}$
SAMB	63 ± 11 b	$2.7 \pm 0.4 \times 10^{-4}$	$2.5 \pm 0.3 \times 10^{-8}$	$1.0 \pm 0.4 \times 10^{-7}$	$8.7 \pm 4.3 \times 10^{-6}$	$5.2 \pm 3.4 \times 10^{-6}$
GSS	24 ± 4 a	$3.8 \pm 1.2 \times 10^{-4}$	$1.8 \pm 0.2 \times 10^{-7}$	$1.2 \pm 0.1 \times 10^{-7}$	$8.2 \pm 3.8 \times 10^{-7}$	
WI	23 ± 2 a	> 10-^{3}	$1.8 \pm 0.6 \times 10^{-6}$	$8.0 \pm 3 \times 10^{-4}$	$7.6 \pm 4.2 \times 10^{-5}$	$2.3 \pm 1.5 \times 10^{-4}$
MR-VL	37 ± 1 c	> 10-^{3}	$1.1 \pm 0.9 \times 10^{-6}$	$4.3 \pm 1.8 \times 10^{-4}$	$4.4 \pm 2.2 \times 10^{-4}$	$8.7 \pm 5.2 \times 10^{-4}$
ATHRos-Pm	26 ± 6 a	> 10-^{3}	$1.0 \pm 0.5 \times 10^{-6}$	$2.5 \pm 0.3 \times 10^{-4}$	$6.2 \pm 3.2 \times 10^{-4}$	

Table 3.4 Amino acid polymorphism between reference laboratory and OP selected strains of *Tetranychus urticae*. Numbers refer to the position in mature AChE1 of *Torpedo californica* (Massoulié et al. 1992). Numbers in brackets refer to the position in *T. urticae* AChE1 precursor.

Strains-Clones (genbank accession)	Position					
	7 (115)	119 (228)	201 (309)	280 (391)	328 (436)	331 (439)
LS-VL-1 (GQ461336)	V (GTT)	G (GGC)	A (GCT)	T (ACA)	G (GGA)	F (TTT)
LS-VL-2 (GQ461337)	V	G	A	T	G	F
LS-VL-3 (GQ461338)	V	G	A	T	G	F
SAMB-1 (GQ461342)	I (ATT)	S (AGC)	A	T	G	Y (TAT)
SAMB-2 (GQ461343)	I	S	A	T	G	F
SAMB-3 (GQ461344)	I	S	A	T	G	F
GSS-1 (GQ461348)	V	G	A	T	G	F
GSS-2 (GQ461349)	V	G	A	T	G	Y
GSS-3 (GQ461350)	V	G	A	T	G	F
WI-1 (GQ4613451)	V	G	A	A(GCA)	G	W (TGG)
WI-2 (GQ461352)	V	G	A	A	G	W
WI-3 (GQ461353)	V	G	A	A	G	W
ATHRos-Pm-1 (GQ461345)	V	G	S (TCT)	A	A (GCA)	W
ATHRos-Pm-2 (GQ461346)	V	G	S	A	A	W
ATHRos-Pm-3 (GQ461347)	V	G	S	T	A	W
MR-VL-1 (GQ461339)	V	G	A	A	A	W
MR-VL-2 (GQ461340)	V	G	A	A	A	W
MR-VL-3 (GQ461341)	V	G	A	A	A	W

The T280A mutation was found in all OP strains and the structural model reveals its position on the surface of the enzyme near the gorge entrance. However, the T280 residue is not involved in the catalytic process and was never shown to confer AChE insensitivity (neither AChE1 nor AChE2). Hence, this mutation might be neutral. In contrast, the F331W mutation that is also found in all OP strains is at a residue which is conserved among AChE1 from both vertebrates and invertebrates, and is a component of the choline-binding site involved in the catalytic process. Thus, it is probably responsible, at least partially, for the modification of the catalytic properties of *T. urticae* AChE1 in the three OP resistant strains. In addition to the F331W, a G328A substitution was found in the ATHRos-Pm and the MR-VL strains. This position is not located in the active site but is in the same α-helix as the 331 position. Therefore this substitution may indirectly influence the catalytic process. In the ATHRos-Pm strain only, besides the 3 above substitutions (T280A, G328A and F331W), also the A201S substitution was detected. This substitution is close to the catalytic serine (S200), therefore it may modify some of the biochemical properties of the enzyme.

3.5 Diagnostic PCR-RFLP for W331

We have developed a PCR-RFLP assay, based on primer induced mutagenesis that directly revealed the presence of W at codon 331. Three (CAA to GGC in MR-VL and ATHRos-Pm) or two (GAA to GGC in LS-VL, GSS, SAMB and WI) mutations were introduced by the forward primer by site directed mutagenesis, resulting in the generation of a *Bgl* I restriction site (5'-GCCNNNNNGGC), specifically in the PCR product of the resistant allele carrying W331. Digestion of the PCR products (137 bp) with *Bgl* I, consequently allowed for the discrimination between the resistance-associated W331 and the F or Y 331 alleles (Figure 3). The PCR-RFLP assay with male (haploid) *T. urticae* displays two different patterns: one with two fragments (112 bp and 25 bp) in 331W males and one with one fragment (137 bp), the original PCR product, in F331 or Y331 males (Figure 3.2). When pooled DNA from F331 and W331 males was used as template, a third pattern with three fragments (137 bp, 112 bp and 25 bp) was obtained, suggesting that the assay can be used to detect heterozygous females (Figure 3.2). The fragment of 25 bp is not detected as it is too small to be visualized by electrophoresis in a routine agarose gel. It is important to keep in mind that sequence conservation and the presence of other resistant alleles should be confirmed by sequencing in any future studies of *T. urticae* of other geographic origins before this diagnostic test is used, as it will not identify the C331 allele previously suggested to be associated with OP resistance in a Japanese *T. urticae* strain by Anazawa et al. (2003).

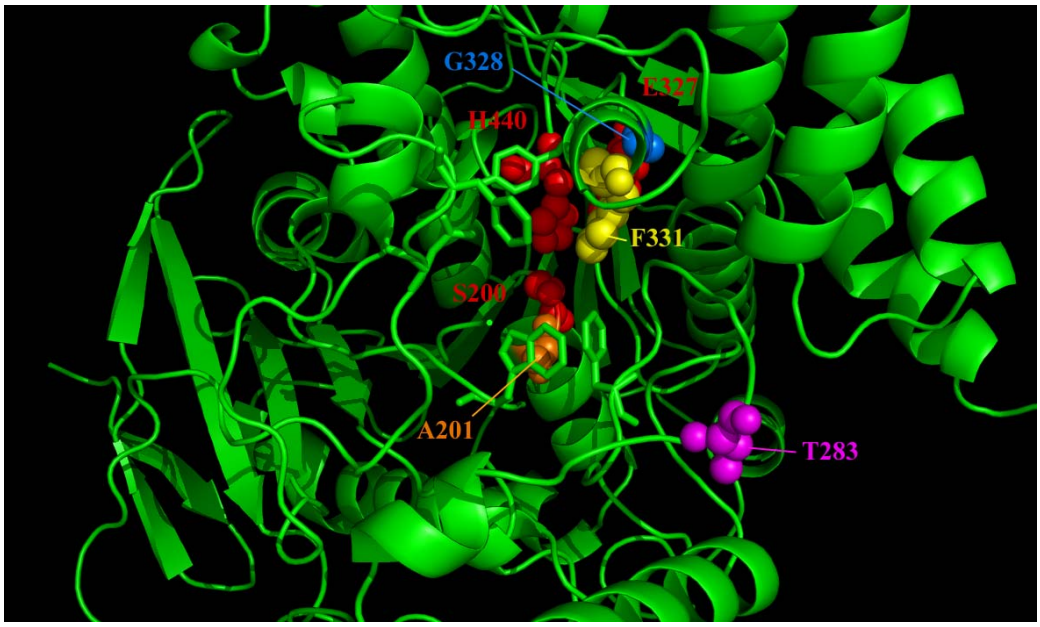

Figure 3.1 Three dimensional structure model of *Tetranychus urticae* AChE1. The backbone of the enzyme structure is rendered as green ribbon with secondary structure. The catalytic triad (S200, E327 and H440) appears as van der Waals red spheres. The different amino acid substitutions are shown as van der Waals coloured spheres. The view points to the entrance of the catalytic gorge.

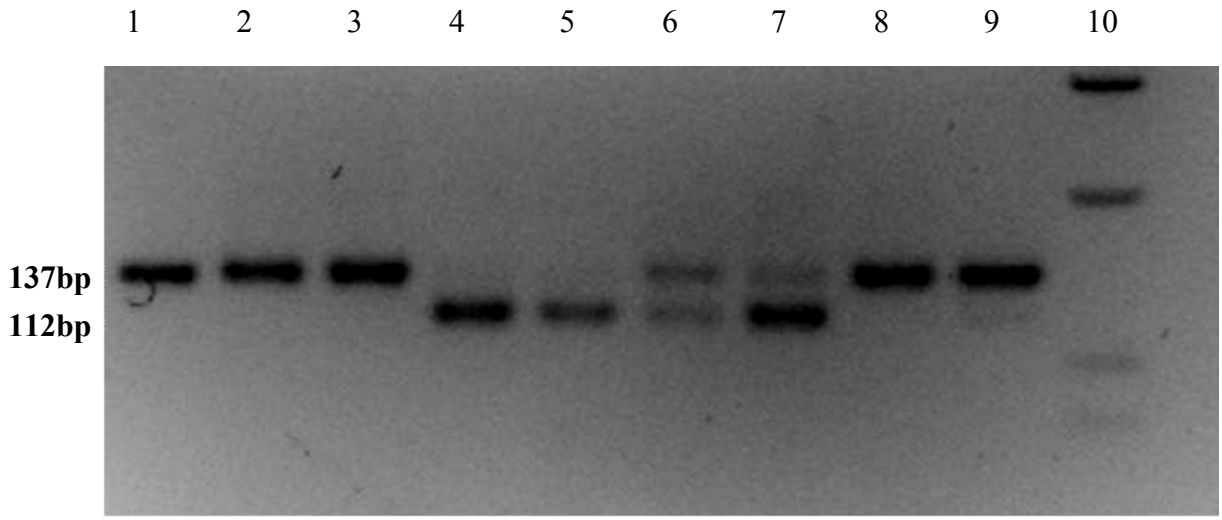

Figure 3.2 Diagnostic PCR-RFLP for 331W after primer induced mutagenesis.
Lane 1: uncut PCR product
Lane 2: PCR was performed on plasmid DNA containing GSS 331 Y clone
Lane 3: PCR on plasmid DNA containing LS-VL 331F clone
Lane 4: PCR on plasmid DNA containing clone WI 331W clone
Lane 5: PCR was performed on DNA extracted from a single male of strain MR-VL (containing 331W)
Lane 6: PCR on pooled plasmids DNA containing clones GSS and WI strains
Lane 7: PCR on pooled g DNA from two males from GSS and MR-VL strains
Lane 8: PCR product on pooled mites GSS
Lane 9: PCR products on pooled mites form LS-VL
The PCR-RFLP assay on heterozygote females (F,Y331/W331) is expected to display pattern of lanes 6 and 7 whereas patterns of lanes 1,2,3,8, and 9 are expected from homozygote (F or Y331) females and lanes 4,5 from homozygote (W331) females.
Lane 10: MR DNA ladder (Invitrogen, Belgium).

3.6 Effects of mixtures of bifenazate with OP and CARB insecticides

Both the WI and MR-VL strains were resistant to chlorpyrifos, carbaryl, phosmet and azinphosmethyl, and the estimated LC_{10} exceeded 100 mg a.i. L^{-1} (data not shown). Only methomyl was toxic to the WI strain, and the LC_{10} (50 mg L^{-1}) was selected for mixing with bifenazate. A concentration of 100 mg a.i. L^{-1} was used in the mixtures with all other compounds, since this concentration did not cause mortality in both strains.

Mixing chlorpyrifos with bifenazate decreased bifenazate toxicity in the WI strain. Mortality remained low even five days after treatment with the highest bifenazate concentrations (500 and 1000 mg a.i. L^{-1}) (Figure 3.3a). The maximum mortality obtained by a mixture after 5 days was 16.2%. A similar effect was also observed in the MR-VL strain during two DAT. However, three days after exposure mortality increased, and after five days reached 44.5% at 125 mg a.i. L^{-1} and 94% at 250 mg a.i. L^{-1} (Figure 3.3b). Since the antagonistic effect of chlorpyrifos was most prominent in WI strain, other OPs and CARBs were tested only on this strain. Methomyl induced a significant antagonistic effect ($\alpha<0.05$) one DAT, which disappeared two DAT (Table 3.5). Azinphos methyl, phosmet, carbaryl and methomyl did not have an impact on bifenazate toxicity.

Quantitative analyses of the enzyme activities of three tested strains are presented in Table 3.6. No significant difference was observed in P450 O-deethylation activity measured with 7-EFC. In contrast, a significant difference in esterase activity was found between strains. The activity in MR-VL strain was 1.81-fold higher in comparison to that in the WI strain. GST activity measured with CDNB and MCB also were significantly higher in MR-VL strain in comparison to GSS and WI.

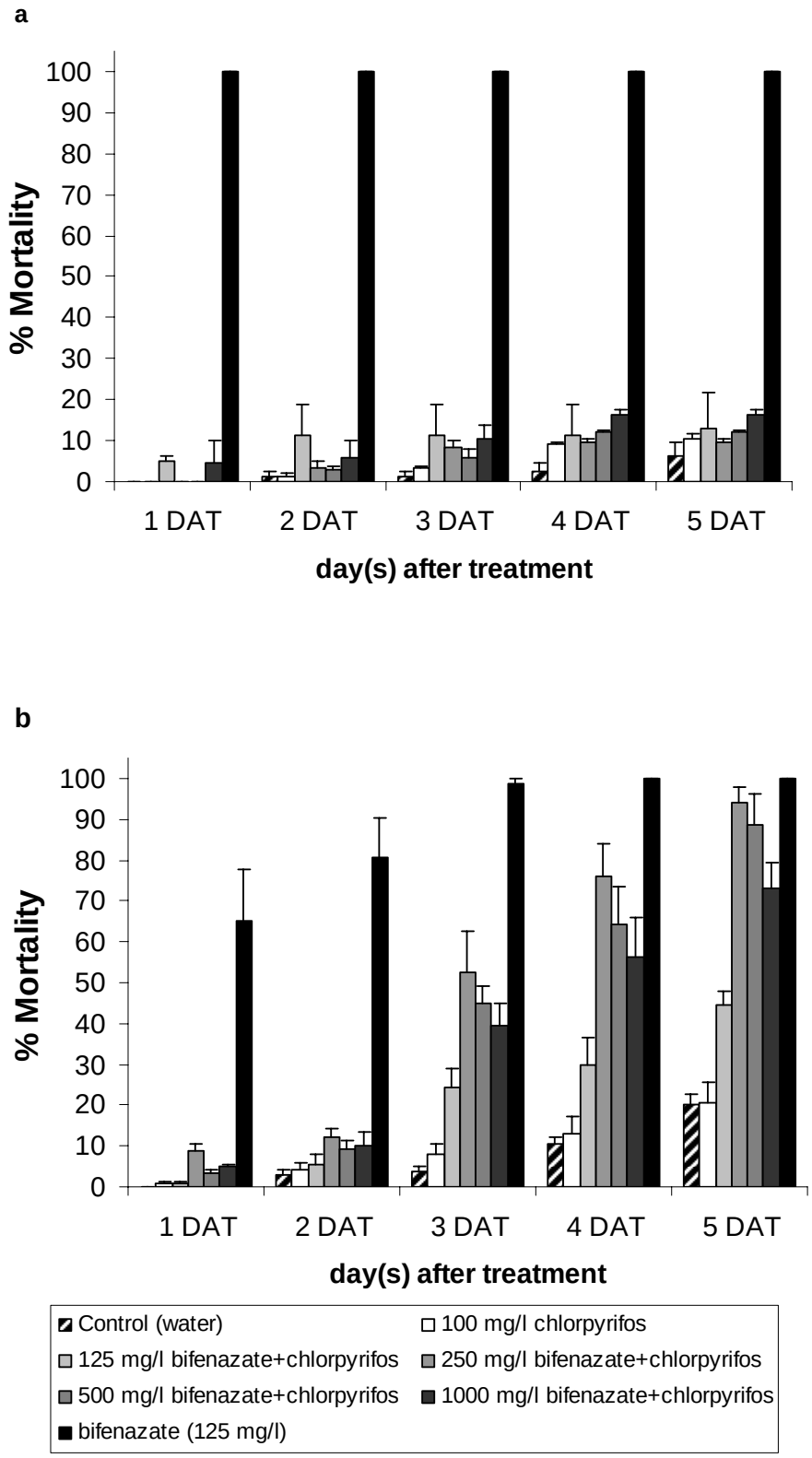

Figure 3.3 Control percentage of *Tetranychus urticae* strain WI (a) and strain MR-VL (b) with chlorpyrifos, different doses of bifenazate and their mixture (mean values + SEM).

Table 3.5 Percent mortality (mean± SEM) of *Tetranychus urticae* strain WI with four OPs and CARBs, different doses of bifenazate and their mixture.

	1 DAT	2 DAT	3 DAT	4 DAT	5 DAT
methomyl (50 mg a.i. L^{-1})					
Control (water)	0	3.1±1.2	6.2±2.7	11.3±3.1	21.7±4.6
50 mg L^{-1} methomyl	17.3±8.2	22.1±9.9	30.8±12.5	37.5±9.8	46.2±7.9
125 mg L^{-1} bifenazate	95.7±6.3	100	100	100	100
125 mg L^{-1} bifenazate + methomyl	75±10.6	99.1±0.7	100	100	100
250 mg L^{-1} bifenazate + methomyl	77.7±7.9	93.6±4.4	100	100	100
500 mg L^{-1} bifenazate + methomyl	91.5±2.4	100	100	100	100
1000 mg L^{-1} bifenazate+ methomyl	100	100	100	100	100
azinphosmethyl (100 mg a.i. L^{-1})					
Control (water)	2.1±1.2	5.3±2.6	10.6±3.3	18.1±4.5	27.66±7.6
100 mg L^{-1} azinphosmethyl	1±0.7	3±1.1	5±1.6	12±1.7	16±2.3
125 mg L^{-1} bifenazate	98±1.7	100	100	100	100
125 mg L^{-1} bifenazate + azinphos methyl	99.1±0.7	100	100	100	100
250 mg L^{-1} bifenazate + azinphos methyl	99.3±0.8	100	100	100	100
500 mg L^{-1} bifenazate + azinphos methyl	99.1±1.1	100	100	100	100
1000 mg L^{-1} bifenazate + azinphos methyl	100	100	100	100	100
phosmet (100 mg a.i. L^{-1})					
Control (water)	0	1.4±0.8	12.4±1.4	15.9±1.1	17.9±2.1
100 mg L^{-1} phosmet	0.9±1.3	0.9±1.3	5.5±1.4	7.3±2	9.1±1.8
125 mg L^{-1} bifenazate	100	100	100	100	100
125 mg L^{-1} bifenazate + phosmet	99.1±0.8	100	100	100	100
250 mg L^{-1} bifenazate + phosmet	97.4±0.9	100	100	100	100
500 mg L^{-1} bifenazate + phosmet	99.1±0.9	100	100	100	100
1000 mg L^{-1} bifenazate + phosmet	100	100	100	100	100
carbaryl (100 mg a.i. L^{-1})					
Control (water)	0	4±1.1	12.6±3.3	14.3±3.4	20.6±1.5
100 mg L^{-1} carbaryl	0	4±.7	10.7±2.4	16±1.9	22.7±2.6
125 mg L^{-1} bifenazate	100	100	100	100	100
125 mg L^{-1} bifenazate + carbaryl	99.4±1	100	100	100	100
250 mg L^{-1} bifenazate + carbaryl	98.6±0.7	100	100	100	100
500 mg L^{-1} bifenazate + carbaryl	100	100	100	100	100
1000 mg L^{-1} bifenazate + carbaryl	100	100	100	100	100

DAT, day(s) after treatment

Table 3.6 Detoxification enzyme activities in different strains of *Tetranychus urticae* (mean ± SEM)

Strain	P450 mono-oxygenases	Esterases	Glutathione-S-transferases	
	O-de-ethylation of EFC[A]	4-NPA activity[B]	CDNB conjugation[C]	MCB conjugation[D]
GSS	225.5 (± 34.2)a	107.2 (± 5.8)b	694.3 (± 17.3)b	805.4 (± 70.3)b
WI	214.5 (± 23.4)a	88.0 (± 2.5)b	595.2 (± 14.4)c	1398.0 (± 144.6)a
MR-VL	215.7 (± 96.8)a	159.2 (± 6.9)a	870.9 (± 17.0)a	1695.5 (± 39.5)a

Means (± SEM) within the same column followed by the same letter are not significantly different (Tukey method, $\alpha = 0.05$).

[A] pmol 7-hydroxy-4-trifluoromethylcoumarin $(30min)^{-1}$ mg^{-1} protein (± SEM)
[B] nmol 4-nitrophenol min^{-1} mg^{-1} protein (± SEM)
[C] nmol glutathione conjugated min^{-1} mg^{-1} protein (± SEM)
[D] relative fluorescence units (RFU) μg^{-1} protein (± SEM)

4 DISCUSSION

Since the development of OP resistance in *T. urticae* in the late 1960s, the use of these compounds to control spider mites has largely been abandoned in many parts of the world. However, OPs and CARBs which account for more than 35 % of the total global insecticide sales (McCaffery and Nauen 2006) are still amongst the most widely used insecticides to control a broad range of arthropod pests of agricultural importance. This keeps spider mites like *T. urticae* under continuous selection pressure in many field and greenhouse crops. This is why OP resistance in *T. urticae* remains a not to be neglected economic factor.

In an attempt to investigate the molecular basis of OP resistance and its relationship to the observed phenotype, a study was set up to elucidate the characteristics of resistance in 3 three reference laboratory strains (LS-VL, SAMB and GSS) and three OP resistant strains of *T. urticae* which were continuously kept under OP selection: a strain that historically developed OP resistance in the 1960s (WI) and 2 recently collected field strains (ATHRos-Pm from Greece and MR-VL from Belgium). Toxicity bioassays on the OP resistant strains revealed varying but high levels of resistance to most of the tested insecticides (Table 3.2). The WI strain proved to be more resistant to chlorpyrifos whereas the recently collected strains MR-VL and ATHRos-Pm were particularly resistant to dimethoate. Compared to the reference strains, the biochemical experiments confirmed the presence of a more insensitive AChE1 in all OP resistant strains (Table 3.3). The high variation found between LC_{50}s of OP resistant strains was not reflected in a high variation between IC_{50}s. Thus, an insensitive AChE1 is

probably not the only mechanism involved in the observed resistant phenotype and other mechanisms such as metabolic mediated resistance (carboxylesterase, gluthathione-S-transferase or P450 mono-oxygenase-activity) are likely also involved.

Target site insensitivity as a mechanism of OP resistance in *T. urticae* was the first case ever reported, and provided a proof of principle for resistance development in arthropods in the early 1960s (Smissaert 1964, Voss and Matsumura 1964). In this study, the amino acid sequence of AChE1 in three reference strains SAMB, GSS, LS-VL and three OP resistant strains (WI, MR-VL and ATHRos-Pm) was determined. Also four amino acid substitutions in the mature AChE1 that are present in one or more resistant strains and absent in the susceptible ones were identified.

Two substitutions (F331W and T280A) were consistently present in all three OP strains. The position of the T280A substitution in the AChE1 structural model indicates that this mutation does not seem to be responsible for the modification of the biochemical properties of AChE1 (Figure 3.1). This is further supported by the facts that this residue is not conserved among species and that both resistant and susceptible strains of *T. urticae* from Japan have a threonine at position 280 (Anazawa et al. 2003). In contrast, the phenylalanine 331 residue is involved in substrate guidance and in the hydrolyzing process by interacting with the catalytic histidine (H440) (Millard et al. 1999). A substitution at this position could lead to the modification of the catalytic properties of AChE1 (i.e. by altering catalytic efficiency and/or inhibitor sensitivity). In *T. urticae* from Japan, a F331C substitution was found to be associated with OP resistance (Anazawa et al. 2003). In *T. kanzawai,* a species closely related to *T. urticae*, a tryptophan at position 331 has been detected in resistant strains (Aiki et al. 2005). The F331W substitution has also been shown to be responsible for AChE1 insensitivity to OP insecticides in the mosquito *Culex tritaeniorhynchus* by functional characterization after site directed mutagenesis and *in vitro* expression (Nabeshima et al. 2004, Oh et al. 2006). In addition, in the aphid species *Aphis gossypii* and *Myzus persicae,* a S331F was shown to confer insensitivity to AChE1, especially to the CARB pirimicarb (Nabeshima et al. 2003, Andrews et al. 2004, Benting and Nauen 2004). More recently, the same substitution was associated with OP resistance in the B and Q biotype of *Bemisia tabaci* from Israel and Greece (Alon et al. 2008, Tsagkarakou et al. 2009a). In conclusion, the 331 position in AChE1 has been associated with OP-resistance in many species, and the functional expression has clearly illustrated the effect of the substitutions on AChE1 sensitivity. Hence, the F331W substitution is most probably the major factor in many OP resistant spider mite strains. This is further emphasized by the fact that the WI-strain which developed resistance in the 1960s, and the

recently collected strains from geographically distant regions (MR-VL and ATHPros-Pm) carry this same F331W substitution.

An allele with a F331Y substitution was sequenced in one clone of the reference strains (SAMB and GSS strain). The F331Y substitution may play a role in the carbaryl sensitivity since AChE1 of the GSS strain is more sensitive to carbaryl than the AChE1 of SAMB strain. Genotyping of single mites displayed that in GSS the F331Y is predominant whereas in the SAMB strain the F331 is predominant (Tsagkarakou and Van Leeuwen unpublished data). However, it is not known how this substitution influences the catalytic parameters of AChE1 in these two strains.

In the recently collected strains ATHRos-Pm and MR-VL strains, another substitution, G328A, was found in addition to F331W and T280A. The position 328 in the *T. urticae* AChE1 model suggests that this substitution may indirectly influence the catalytic process by affecting position 331 (located in the same α-helix). However, when we compared AChE1 sensitivity of the MR-VL and ATHRos-Pm strains to that of the WI strain, the additional G328A substitution does not seem to alter the sensitivity to the tested insecticides, except for carbaryl. This substitution has been also found in the AChE2 of *Ceratitis capitata* (Magana et al. 2008), *D. melanogaster* (Menozzi et al. 2004) and *Musca domestica* (Walsh et al. 2001). Site directed mutagenesis followed by *in vitro* expression of *D. melanogaster* and *M. domestica* AChE2 showed that this mutation conferred insensitivity to chlorpyrifos-oxon, omethoate, malaoxon, methamidophos, paraoxon, methyl-paraoxon, and pirimicarb but increased the sensitivity to coumaphos and carbaryl (Walsh et al. 2001, Menozzi et al. 2004).

In the ATHRos-Pm strain a fourth substitution (A201S) was found in addition to T280A, G328A and F331W. The proximity of this position to the catalytic serine (S200) may lead to some modifications in AChE1 biochemical properties. However, when IC_{50} values were compared between MR-VL and ATHRos-Pm strains (differing only by the presence of the A201S), no effect on AChE1 sensitivity toward the tested inhibitors was detected. This mutation was also found in other arthropod species, such as a resistant clone of *A. gossypii* (Andrews et al. 2004, Li and Han 2004) and a resistant strain of *Plutella xylostella* (Baek et al. 2005, Lee et al. 2007). In *A. gossypii* the A201S substitution was always found in tandem with S331F and in *P. xylostella* in tandem with G227A. In both cases it was suggested that its presence increases resistance levels, playing a supplementary role to the main mutations (Andrews et al. 2004, Baek et al. 2005, Lee et al. 2007). In *A. gossypii* it was shown that AChE1 from insects carrying both mutations possess a better substrate affinity than that carrying the S331F mutation alone. The role of A201S mutation in resistance was

characterized by site directed mutagenesis in AChE1 of *Culex tritaeniorhynchus* (Oh et al. 2007), but no functional expression of recombinant A201S AChE1 of *A. gossypii* or *P. xylostella* has been performed until now.

Among the two substitutions specifically found in the reference SAMB strain (i.e. V7I and G119S), only G119S is located in the active site. This substitution is known to be responsible for AChE1 insensitivity (Weill et al. 2002) or reduced AChE1 activity in mosquitoes (Bourguet et al. 1996, Alout et al. 2008). The 119 position is close to the catalytic serine (S200) and the G-to-S substitution would reduce the accessibility to inhibitors and substrates by steric hindrance. Recombinant *Culex pipiens* G119S AChE1 was similarly or more resistant than the recombinant F331W AChE1 to most of the OPs and CARBs tested, including chlorpyrifos-oxon and paraoxon (Alout et al. 2007a). SAMB exhibited moderate levels of resistance towards chlorpyrifos and dimethoate and the involvement of an AChE mutation in resistance cannot be ruled out. However the biochemical data do not support the involvement of G119S mutation in resistance towards the insecticides tested in this study, which is in line with previous reports on Tetranychidae (Anazawa et al. 2003, Aiki et al. 2005). Moreover, the AChE1 specific activity of the SAMB strain is higher than that of all other strains. However, before any sound conclusions can be made about the role of this mutation in *T. urticae* OP resistance, the frequency of this mutation should be determined by genotyping single mites of different strains, combined with a functional analysis of this mutation.

Although it is presumed that the F331W substitution reported in this study is essential in determining the insensitivity of AChE1 towards the OP and the CARB, the A201S and G328A mutations might have been selected by exposure to other unknown OPs in the population's history. These substitutions may confer an advantage to other inhibitors. Different mutated AChE1 could cause a strong insensitivity to specific insecticides since a good relationship has been found between the highest insensitivities and the insecticide(s) used locally in mosquito control programs (Alout and Weill 2008). Thus it is possible that in *T. urticae* also different mutations are selected and maintained in the populations, each one conferring advantage against particular OP or CARB insecticides. Alternatively, substitutions A201S and G328A might be selected to reduce a possible fitness cost associated with the F331W substitution in *T. urticae.* Valuable information on the importance of the different substitutions in the development and maintenance of an OP-resistant phenotype could be obtained by following their dynamics with molecular diagnostic tests under different OP spraying protocols, aimed at controlling other arthropod pests in the field. The development of such a diagnostic test was

illustrated for F331W mutation, since it was consistently present in recently resistant spider mite strains from different geographical regions, as well as in a strain that developed resistance in the late 1960s.

Different mutations and their combinations can result in varying levels of resistance to many OP and CARB insecticides (Mutero et al. 1994). Some of them have essentially an additive effect, both in terms of reducing the sensitivity of the enzyme to inhibition by the insecticide as well as in terms of increasing the stability of the enzyme and its ability to turn over acetylcholine (Shi et al. 2004). Functional analysis should be performed to test the influence of each mutation (alone or in combination) in OP and CARB insensitivity and in the specific activity of the recombinant mutated AChE1 in *T. urticae*.

Recently, Van Leeuwen et al. (2007) revealed that OP and CARB insecticides, when applied prior to bifenazate, antagonised bifenazate toxicity. This antagonistic effect occurred at very low, non-toxic concentrations of the antagonists in an OP susceptible strain, and was correlated with a decrease in general esterase activity towards model substrates, as p-nitrophenyl acetate. However, the presence of low concentrations of OPs and CARBs applied before bifenazate treatment on OP susceptible strains is not realistic for field conditions.

In this study we tested whether mixtures (not simultaneous applications) of OP and CARB compounds with bifenazate, when applied on OP resistant strains, which can tolerate higher doses of OP and CARB insecticides, are a major risk for bifenazate control failure due to antagonism. Based on percent mortality, mixtures of bifenazate and chlorpyrifos revealed an immediate and high antagonism in both strains (Figure 3.3). In these experiments, bifenazate was not toxic, even at concentrations exceeding 10 times the recommended field rate (96 mg a.i. L^{-1}). The effect was more long-lasting in the WI strain, compared to the MR-VL strain, in which antagonism decreased two DAT. Possibly, the detoxifying capacity of the MR-VL strain resulted in degradation of the internalised OP and CARB insecticides, hence counteracting in time the observed antagonism. When the OP concentration within the body decreases, inhibition of the general hydrolytic esterase activity decreases and bifenazate activation by esterases can reoccur in time. This theory is supported by enzymatic assays revealing differences in esterase activity between strains (Table 3.5). A higher esterase activity in the MR-VL strain may also be associated with a more rapid recuperation of esterase inhibition (Wheelock et al. 2005).

With the exception of chlorpyrifos, all other tested chemicals did not cause any antagonism when mixed with bifenazate. This is in contrast with a previous study in which pre-treatment of mites with all tested OPs (acephate, chlorpyrifos, dichlorvos, dimethoate, ethion,

monocrotophos, paraoxon, profenofos and trichlorfon) and CARBs (carbaryl and oxamyl) antagonised bifenazate toxicity with ratios between 2.8 to 169 (Van Leeuwen et al. 2007). It is possible that inhibition of esterase activity is not initiated after treatment with OPs (Gunning et al. 1999), while bifenazate exerts quick knockdown activity. In our experiments bifenazate intoxication (paralysis) occurred rapidly in the first few hours after treatment, thereby limiting further OP and CARB uptake through tarsal contact. The difference in antagonistic effect between chlorpyrifos and the other insecticides may be related to differences in speed of penetration and rate of conversion to the active oxon-form.

Several studies evaluating the effect of mixing pesticides for control of *T. urticae* have reported improved control (Bynum et al. 1997) or in a control which was not negatively affected by mixture (Cloyd et al. 2007). It can be concluded that mixing OP and CARB insecticides with bifenazate in some cases can completely inhibit bifenazate efficacy. However, the antagonistic effect depends on the OP and CARB type, as well as on the mite strain. As has become clear from this study, OP resistant mites are a greater risk for antagonism-based control failure, since they survive OP treatments targeting other pests, while the OP insecticides compromise bifenazate activation. In practice, greenhouse experiments should be conducted before recommending a specific mixture to growers.

CHAPTER IV

GENETIC, BIOCHEMICAL AND MOLECULAR ANALYSIS OF A LABORATORY-SELECTED SPIRODICLOFEN RESISTANT STRAIN OF *TETRANYCHUS URTICAE* KOCH

This chapter has been redrafted from:

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Van Pottelberge S, Khajehali J, Van Leeuwen T, Tirry L (2009) Effects of spirodiclofen on reproduction in a susceptible and resistant strain of *Tetranychus urticae* (Acari: Tetranychidae). Experimental and Applied Acarology 47: 301-309

1 INTRODUCTION

Spirodiclofen is a persistent non-systemic selective acaricide which chemically belongs to the group of spirocyclic tetronic acid derivatives. It was discovered by Bayer CropScience and was first registered in 2002 with the trade name Envidor® (Bretschneider et al. 2007). It has been commercialized for the control of economically important phytophagous mite species including *T. urticae*, *Panonychus ulmi*, *Panonychus citri*, *Aculus schlechtendali*, *Phyllocoptruta oleivora* and *Brevipalpus phoenicis* (Rauch and Nauen 2002). Spirodiclofen has no neurotoxic activity, but mainly acts on mite development, hereby eliciting unique symptoms of poisoning in spider mites, like flabby legs, immatures which are trapped in the quiescent stage and inhibition of molting. Spirodiclofen is active against all developmental stages but shows the highest acute toxicity to eggs and immature stages. Treated immature mobile stages die in the subsequent quiescent stage. Female adults prove to be less susceptible and it takes a few days after treatment before they die. This may be due to the fact that these females are unable to deposit eggs, causing a high and lethal number of accumulated eggs in their body. Also, fertility is highly affected. It was observed that a treatment with 200 mg L^{-1} spirodiclofen significantly decreased the lipid content in female adults of *T. urticae*, suggesting an interference with lipid biosynthesis (Wachendorff et al. 2002). This interference seemed to be based on a selective and potent inhibition of acetyl-CoA carboxylase (ACCase). ACCase (E.C. 6.4.1.2) is a member of the biotin-dependent enzyme family which, in short, catalyses the synthesis of malonyl-coenzyme A which is the substrate required for fat synthesis (Incledon and Hall 1997).

Fatty acids are important molecules in biological systems. They are key building blocks for the synthesis of the phospholipids, important components of the cell membrane. They act as hormones and as sources of metabolic energy that can be mobilized to meet the energy requirements of the insect. They constitute a large part of the lipids of the cuticular wax layer that protects terrestrial arthropods from desiccation. Many of the sex pheromones synthesized by insects are derived from fatty acids as well as some compounds within defensive secretions such as quinones, phenols, and carboxylic acids (Klowden 2007, Hanin et al. 2008). Due to their involvement in many processes, the biosynthesis of fatty acids is a promising target for the development of novel chemotherapeutics. For instance, the enzymes involved in fatty acid biosynthesis provide excellent targets for the design of novel antibiotics (Campbell and Cronan 2001), therapeutics (Tong 2005) and pesticides (Wenger and Niderman 2007).

ACCase catalyzes the first committed step in the fatty acid biosynthetic pathway in all animals, plants, and bacteria, i.e. the carboxylation of acetyl-CoA to produce malonyl-CoA.

This generally involves two separate reactions and depends on the tightly-bound cofactor (prosthetic group) biotin (Figure 4.1). The first reaction involves the ATP-dependent carboxylation of the biotin moiety of the biotin carboxyl carrier protein (BCC), catalyzed by the biotin carboxylase (BC). In the second reaction, the activated carboxyl group is transferred from the biotin moiety to acetyl-CoA, thereby generating malonyl-CoA. This reaction is catalyzed by carboxyltransferase (CT) (Barber et al. 2005). Malonyl-CoA is used for *de novo* fatty acid biosynthesis as well as in fatty acid elongation.

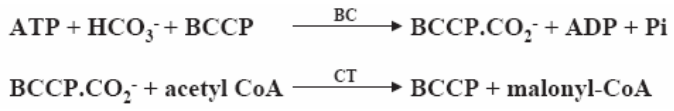


Figure 4.1 Reaction mechanisms for ACCase. Biotin carboxylase (BC) catalyses the ATP-dependent phosphorylation of bicarbonate and formation of carboxyphosphate-BCCP (biotin carboxyl carrier protein). Acetyl-CoA carboxytransferase catalyses the transfer of the carboxyl group from carboxybiotin-BCCP to an acetyl-CoA acceptor, resulting in malonyl-CoA (Barber et al. 2005).

In prokaryotes, as well as in the chloroplasts of many plants, ACCase is a multi-subunit enzyme consisting of BC, BCC, and CT as separate proteins. In contrast, in most eurokaryotes, ACCase is a large (>200 kD), multi-domain enzyme containing all three of these components within a single polypeptide chain (a BC, BCC and CT domain). The active form of animal ACCase is a linear polymer with a molecular weight of about 8000 kD, made up of 10-20 protomers, which are dimers of the enzyme (Tong 2005).

In humans and other mammals as well as in birds and fish and some other animals there are two isoforms of the ACCase, while some animal classes appear to have only one ACCase gene. Although only one ACCase gene is present in the *Drosophila melanogaster* genome (Pan and Hardie 2002), this gene generates two transcripts through alternative splicing, resulting in a normal ACCase and an ACCase with an extended N-terminus (Barber et al. 2005).

Again, due to its central role in fatty acid biosynthesis, ACCase would be an excellent target for the design of new pesticides. Shutting down the catalytic activities of either BC or CT should be sufficient to inhibit the overall reaction of the enzyme.

Almost all known ACCase inhibitors such as two classes of commercial herbicides, the aryloxyphenoxypropionates and the cyclohexanediones, inhibit the CT, while soraphen A, a broad-spectrum fungicide, is the only known potent inhibitor of the BC (Weatherly et al. 2004). In animals, it might be expected that inhibitors could be discovered that disrupt the

enzyme polymerization (Tong 2005). The action of aryloxyphenoxypropionates and cyclohexanediones on weeds (Delye 2005) and *Toxoplasma* (Jelenska et al. 2002) indicates that, in all these systems, inhibition of ACCase activity leads to a complete inhibition of the organism's growth.

Despite its economic importance, knowledge about arthropod ACCase structure and molecular interactions with inhibitors is lacking. One reason for the scarcity of information is the difficulty in obtaining sufficient quantities of pure protein. Eukaryotic ACCase is difficult to purify in high enough quantity from endogenous sources, and their size and complexity make recombinant expression difficult as well (Weatherly et al. 2004). To fill this gap, efforts should be directed towards the expression and purification of recombinant ACCase enzymes in high yield, purity, and activity. Joachimiak et al. (1997) established a yeast expression system to investigate the wheat ACCases. The mode of action of ACCase inhibiting herbicides have been studied using heterologous expression of chimeric ACCase. It was shown that some determinants of sensitivity to these herbicides were located on a 400–amino acid fragment of wheat plastidic ACCase CT domain (Nikolskaya et al. 1999). There are only a few reports of the recombinant expression of a full-length eukaryotic ACCase. Jelenska et al. (2002) tried to develop an expression system for *Toxoplasma gondii* ACCase, but the expressed protein was highly proteolysed, the bulk of it lacked bound biotin, and the preparation had very low specific activity. Weatherly et al. (2004) succeeded in the expression of enzymatically active fungal (*Ustilago maydis*) ACCase in *Escherichia coli*. Cheng et al. (2007) developed an efficient protocol to express recombinant human ACCase2, human ACCase1, and rat ACCase2 in high yield and activity in baculovirus/Sf9 cell expression system.

The application of acaricides is usually needed to maintain mite populations below economic thresholds. However, *T. urticae* has the ability to rapidly develop resistance after only a few applications. To manage resistance development, it is necessary to gather information on the genetics, modes and mechanisms of resistance to each compound (Leeper et al. 1986).

Up till now, most information on resistance against inhibitors of ACCase has been gathered from studies on commercially available herbicides belonging to the aryloxyphenoxypropionates and the cyclohexanediones, and to a lesser extent, the cyclic 1,3-diketones (2-aryl-1,3-diones), the latter being structurally related to spirodiclofen (Dekeyser 2005). Graminicide herbicides targeting ACCase have been widely used for the control of annual and perennial grasses in broadleaf crops and even in certain cereals. Resistance to ACCase-inhibiting herbicides has developed in at least 35 grass species worldwide (Wenger

and Niderman 2007). Mechanisms of this resistance can be divided in two groups: ACCase-related and metabolism-based mechanisms. In most cases, mutations within CT domain of homomeric plastidic ACCase are involved in altered sensitivity to ACCase inhibitors. Metabolism-based resistance involves detoxifying enzymes such as cytochrome P450 mono-oxygenases, glutathione-S-transferases and glucosyltransferases (Delye 2005, Wenger and Niderman 2007).

The present study reports on a spirodiclofen resistant *T. urticae* strain that was selected from a susceptible *T. urticae* strain of under laboratory conditions. We determined the resistance ratio and the cross-resistance to several important acaricides. Metabolic detoxification was studied by using synergists, and by determining the *in vitro* enzyme activities. The total lipid content of the susceptible and resistant female mites was also measured. Possible target site modification was investigated by sequencing and analysing the cDNA encoding the ACCase from the susceptible and resistant *T. urticae* strains. To design and screen effective and specific new pesticides and to manage resistance to currently used ACCase inhibiting pesticides, it will be necessary to study the ACCase structure and function from each pest species, because each may show a different sensitivity and a different mechanism of resistance to inhibitors. Here, we tried to develop an optimal protocol to express mite (in casu *T. urticae*) recombinant ACCase. The recombinant expression system can be used as the foundation for the subsequent site-directed mutagenesis experiments in order to investigate the function of specific residues involved in the enzyme activity or inhibitor binding.

2 MATERIALS AND METHODS

2.1 Acaricides and chemicals

Commercial formulations of abamectin 18 g L^{-1} SC (Vertimec®), pyridaben 150 g L^{-1} SC (Sanmite®) and tebufenpyrad 200 g L^{-1} SC (Pyranica®) were purchased in a store (Fyto Vanhulle, Belgium). Formulated acequinocyl 150 g L^{-1} SC (Kanemite®) and bifenazate 240 g L^{-1} SC (Floramite®) were kindly provided by W. Petersen (Bayer CropScience) and A. Grosscurt (Crompton Crop Protection), respectively. Formulated etoxazole 110 g L^{-1} SC (Borneo®), spirodiclofen 240 g L^{-1} SC (Envidor®), spiromesifen 240 g L^{-1} SC (Oberon®) and the esterase substrate 1-naphthyl 2,2-dimethylbutyrate were provided by R. Nauen (Bayer Cropscience). All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Belgium).

2.2 Spider mites

The reference laboratory susceptible strain (LS-VL) of *T. urticae* was originally collected in October 2000 from roses in a garden near Ghent (Belgium), where pesticides had not been used for at least 10 years (Van Leeuwen et al. 2004).

To select for a spirodiclofen-resistant strain, LS-VL was exposed to successive applications of a gradually increasing concentration of spirodiclofen, starting from 10 mg L^{-1} (approximately the LC_{90} of LS-VL) in January 2007. Roughly 5000 larvae were used to start the selection. During the first selection cycles, spirodiclofen was sprayed on larvae living on detached bean leaf discs (as described below). Selection pressure was maintained by varying the concentration so that 10 to 20 % of the larvae survived for succeeding generations. Once the concentration of 500 mg L^{-1} was reached, subsequent selections with spirodiclofen were done on all life stages on bean plants which were sprayed with a hand-pressurised sprayer (Birchmeier, Switzerland) until runoff. Selection was stopped in December 2007, when a concentration of 5000 mg L^{-1} was used. The laboratory selected strain was named SR-VP and maintained on bean plants sprayed with 5000 mg L^{-1} spirodiclofen containing the wetting agent Citowet at 0.025%. Mites were used in the experiments for the first time in January 2008.

The SR-TK strain was a field strain obtained from Bayer CropScience and was maintained under selection pressure using 1200 mg L^{-1} spirodiclofen. The London strain was provided by M. Grbic (University of Western Ontario). This strain has been used in the genome sequencing project of *T. urticae* (Grbic et al. 2008) (http://bioinformatics.psb.ugent.be/webtools/bogas/users/login).

Mites of the LS-VL, London, SR-TK and SR-VP strain were reared on potted kidney bean (*Phaseolus vulgaris* L. cv. Prelude) plants in a climatically controlled room at 26 (±0.5)°C, 60 % RH and 16/8 h L/D photoperiod.

2.3 Larval and egg bioassays

For larval and egg bioassays, 20-30 adult females were transferred to the upper side of 9 cm^2 square-cut kidney bean leaf discs on wet cotton wool, and permitted to lay eggs for 6 hours, after which they were removed. The plates were then placed in a climatically controlled room at 26 (±0.5) °C, 60 % RH and 16/8 h L/D photoperiod. Immediately after removal of the adults (egg bioassay) or after hatching of the eggs (larval bioassay), the eggs or larvae were sprayed with 0.75 ml spray fluid at 1 bar pressure using a Cornelis spray tower, resulting in 1.58 ± 0.06 mg aqueous acaricide deposit cm^{-2} (Van Laecke and Degheele 1993). Four replicates of six concentrations of spirodiclofen plus a control (de-ionised water) were tested.

Mortality was assessed when adults appeared in the control. Mortality percentages were calculated by dividing the number of mites reaching adulthood by the number of total eggs (in the egg bioassay) or larvae and nymphochrysalis (in the larval bioassay) counted one day after application. In all bioassays, control mortality was lower than 10 %. LC_{50}-values, slopes and 95% confidence limits were calculated by probit analysis (POLO, LeOra Software, Berkeley, USA) (Robertson and Preisler 1992a). Resistance ratios (RR) were calculated by dividing the LC_{50} value of the resistant strain by the LC_{50}-value of the susceptible strain.

2.4 Screening for cross-resistance

Cross-resistance between spirodiclofen and eight other commonly used or new acaricides were evaluated using the selected resistant (SR-VP) and susceptible (LS-VL) populations. The pesticides used were abamectin, acequinocyl, bifenazate, etoxazole, pyridaben, tebufenpyrad and spiromesifen. The bioassay used for etoxazole and spiromesifen was similar to that described for spirodiclofen in paragraph 2.3 (larval bioassays). For all other acaricides, adult females were used. Adulticidal bioassays were conducted using a standard method (Van Leeuwen et al. 2004). Mortality was assessed after 2 days. Mites were scored as 'alive' if they could walk normally after they were prodded with a camel's hair brush; all others were scored as dead including those that had run off the leaf disk.

2.5 Synergism studies

In order to check for metabolic resistance by means of synergists, newly hatched larvae were sprayed with PBO, DEF or DEM, 3-4 h prior to spirodiclofen application as described in paragraph 2.3. These compounds inhibit cytochrome P450 mono-oxygenases, esterases and glutathione-*S*-transferases, respectively. Based on preliminary tests, the doses of PBO, DEF and DEM were chosen as the highest dose that caused maximum 5 % larval mortality (200 mg L^{-1} PBO, 10 mg L^{-1} DEF, 200 mg L^{-1} DEM). Before use, PBO (~90% purity), DEF (98% purity) and DEM (97% purity) were dissolved in a mixture of *N*,*N*-dimethylformamide and emulsifier W (3:1 by weight) and subsequently diluted with de-ionised water (1000-fold). Mites sprayed with synergist only served as control. Synergism ratios (SR) and their confidence limits were calculated using the formula and statistics of dose ratios (Robertson and Preisler 1992b). If the 95% confidence interval includes 1, then the LC_{50} of the acaricide alone is not significantly different from the LC_{50} of the acaricide + synergist.

In order to measure the effect of synergists on the inhibitory effect of spirodiclofen on female fertility and fecundity, gravid adult female mites of the LS-VL and SR-VP strains were placed on leaf discs sprayed with PBO, DEF or DEM. Based on preliminary tests, the doses of 2000 mg L^{-1} PBO, 20 mg L^{-1} DEF, 2000 mg L^{-1} DEM were chosen. These doses caused maximum

5 % mortality and did not inhibit fecundity. After 24 h, living mites were collected and transferred to leaf discs that had been sprayed with different concentrations of spirodiclofen or with distilled water (control). After 24 h exposure, 4 replicates of 10 mites from each spirodiclofen concentration were transferred to unsprayed leaf discs for oviposition. The day after, the female mites were removed. The eggs were left to develop to adults, which were finally counted.

Inhibition by spirodiclofen (the difference between the number of offspring from mites from leaves treated with distilled water and those treated with spirodiclofen) was expressed as a percentage of the number of offspring in mites treated with water. Spirodiclofen concentrations inhibiting reproductivity by 50% (IC_{50}-values) for LS-VL were calculated using the logistic equation of the fitted curves. The concentration-inhibition data for SR-VP were fitted with a second order regression curve.

2.6 Total lipid content in *T. urticae*

For the determination of the total lipid content, procedures based on the method of Van Handel (1985) and described previously (Wachendorff et al. 2002) were used. Two hours, 2 days and 5 days after spirodiclofen treatment, groups of ten living female adults (respectively 4, 6 and 9 days old) were randomly selected from (untreated) leaf disks with mites that had been exposed for 12 h to spirodiclofen (200 mg L^{-1}, 1000 mg L^{-1}) or distilled water (control), as described in 2.3. Additionally, groups of 100 adult females were sampled from the LS-VL and SR-VP strain and were pre-treated for 12 h with spirodiclofen. Surviving females were transferred to a non-sprayed leaf disc and lipid content was measured after two days. A lipid standard curve was obtained using corn oil (analytical grade). Data were analysed by ANOVA with means separated by Tukey ($p < 0.05$). If the assumption of normality or equality of variance was not met, a non-parametric Kruskal-Wallis test was used.

2.7 Crossing experiments

2.7.1 Bioassay – reciprocal crossing LS-VL♀ × SR-VP♂ and SR-VP♀ × LS-VL♂

In an attempt to estimate the dominance of the resistance, individuals of the susceptible (LS-VL) and resistant (SR-VP) strain were reciprocally crossed to produce hybrid F_1 females, as described previously (Herron and Rophail 1993, Van Leeuwen et al. 2004). This was achieved by placing 50 teleiochrysalid females of one strain and 100 adult males of the other strain on the upper side of a primary bean leaf on wet cotton wool in a Petridish (2 replicates). Directly after moulting the diploid females were fertilised by the haploid males. These cultures were left for three days to produce two-day-old cross-fertilized females. From these reciprocal-cross-strains, 25 females were transferred to 9 cm^2 square-cut bean leaf discs and

left to oviposit for 5-6 hours. Four times a day, the egg laying females were collected and placed on fresh discs. When larvae hatched, the discs of each of the reciprocal-cross-strains were sprayed with several concentrations of spirodiclofen covering the range of 0 to 100 % mortality. Each concentration was replicated at least 4 times and a water only sprayed control was included daily. The assays were assessed by the protocol of Overmeer (1967), which requires male and female progeny to be counted separately. The unsprayed control discs were used to calculate the average number of males in the test population (sex-ratio) in order to correct the reciprocal-cross assays for the haploid male offspring, which always show the genotype of the mother, due to the arrhenotokous method of reproduction (haplodiploidy - males result from unfertilised haploid eggs, females result from fertilised diploid eggs).

The formula of Stone (1968) was used to calculate the degree of dominance (*D*), as described in Chapter II, 2.3.

2.7.2 Bioassay–backcrosses (LS-VL×SR-VP)♀×LS-VL♂ and (SR-VP×LS-VL)♀×SR-VP♂

To obtain F_2 progeny, teleiochrysalid females of the reciprocal crosses and males were allowed to mate in a similar manner as described above. The resulting F_2 larvae were treated with several concentrations of spirodiclofen, covering the range of 0 to 100 % mortality. Mortalities were also corrected for the haploid male offspring. Analysis of the concentration-mortality data for the F_2 females was done to determine whether the resistance to spirodiclofen is under the control of one major gene or more than one gene. Single major gene inheritance is characterized graphically by a plateau at 50% mortality of the concentration-mortality lines (Georghiou 1969). The expected responses of the F2 generation for each concentration tested can be obtained with the following formula (Georghiou 1969):

$c = (0.5)\ W(\text{parent 1}) + (0.5)\ W(\text{parent 2})$

where *c* is the expected mortality at a given concentration and W is the observed mortality of the parental types at a given concentration. A χ^2 goodness-of-fit analysis was then used to determine how well the observed responses fit expected responses.

2.7.3 Bioassay – haploid F1 progeny of strains LS-VL and SR-VP

Analogous to Herron (1994), one hundred and fifty teleiochrysalid females were removed from the LS-VL and SR-VP cohort cultures and transferred to new detached leaf cultures for three days, by which time they were young adults laying haploid eggs. Twenty five females from each culture were transferred to 9 cm^2 square-cut bean leaf discs and left to oviposit for 5-6 hours. Four times a day, the egg laying females were collected and placed on fresh discs.

Male haploid offspring in the larval stage were subsequently used in a bioassay as described above.

2.7.4 Bioassay–haploid F2 progeny of strains LS-VL♀ × SR-VP♂ and SR-VP♀ × LS-VL♂

Haploid reciprocal crosses were initiated as above. Eggs laid by young adult females from the reciprocal crosses were allowed to develop to the teleiochrysalid stage. The female teleiochrysalids were then transferred to a detached leaf culture for three days where they developed into young adults producing haploid progeny. Twenty five females from each culture were transferred to 9 cm^2 square-cut bean leaf discs and left to oviposit for 5-6 hours. Four times a day, the adult females were removed to leave haploid progeny that were used in the described bioassay. Similar to the F_2 females, the concentration-mortality data for the F_2 males was analysed to determine whether resistance to spirodiclofen was under the control of one major gene or more than one gene.

2.8 Enzymatic assays

For the determination of the *O*-deethylation of 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) by P450 mono-oxygenases and the esterase activity towards the substrates 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl propionate (4-NPP) and 4-nitrophenyl butyrate (4-NPB), procedures were used as previously described (Van Leeuwen et al. 2005). To measure the 7-EFC-*O*-deethylation activity in larvae, 500 larvae were homogenised. Esterase activity detected with the substrate 1-naphthyl 2,2-dimethylbutyrate (Nauen et al. 2003) was measured in a similar way as with 1-naphthyl acetate (1-NA), according to Van Leeuwen et al. (2005) The final concentration of all the different substrates in the esterase reaction mixtures was 0.3 mM.

Glutathion-*S*-transferase (GST) activity was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, based on the method of Habig and Jakoby (1981), and also previously described (Van Leeuwen et al., 2005). GST activity was also determined using monochlorobimane (MCB) as substrate, based on the method of Stumpf and Nauen (2002). Thirty fresh adult females were homogenized in 500 µl Tris-HCl buffer (0.05 M, pH 7.5). The homogenate was centrifuged for 10 min at 10,000 g and 4 °C. The total reaction volume per well of a 96-well microtiter plate was 300 µl, consisting of 50 µl supernatant, 50 µl buffer, 100 µl MCB (0.9 mM, dissolved in buffer with 1% ethanol) and 100 µl reduced glutathione (9 mM, dissolved in buffer). The plate was incubated for 20 min at 22°C and fluorescence was measured with a Tecan Infinite M200 microtitre plate spectrofluorometer at 465 nm while

exciting at 390 nm. The nonenzymatic reaction of MCB without homogenate served as control.

All protein concentrations were measured with a Coomassie protein assay (Perbio Science, Belgium). All enzymatic assays were repeated independently at least three times.

2.9 Separation of esterases by native isoelectric focusing (IEF)

IEF was performed on extracted proteins from adults and nymphs (mixed protonymphs and deutonymphs of variable age) of two spirodiclofen resistant (SR-VP and SR-TK) and two susceptible (LS-VL and London) strains as described by Van Leeuwen et al. (2006b). Briefly, the enzyme samples (10,000*g* supernatant, 12 µg protein) with 5 µl IEF marker (Serva Liquid Mix, Serva, Germany) were loaded into each well of a Novex pH 3–7 precast gel (Invitrogen, USA). IEF gels were run according to the manufacturer's instructions (1 h at 100 V, 1 h at 200 V and 30 min at 500 V). The gel was stained for esterase activity with 1-NA as a substrate and Fast blue RR (Van Leeuwen et al. 2006b). To visualise the marker, the gel also was stained using GelCode Blue Stain Reagent (Pierce, USA) as described by the manufaturer.

2.10 Cloning of a *T. urticae* cDNA encoding ACCase

Total RNA was extracted from mass homogenates of *T. urticae* strains GSS, LS-VL, SR-VP and SR-TK using TRI reagent (Sigma-Aldrich, Belgium) according to the manufacturer's instructions. A quantity of 2 µg total RNA for each sample was reverse transcribed into cDNA by using the High Fidelity cDNA Synthesis Kit (Roche, Belgium) and anchored-oligo (dT) primers. Several conserved regions on the basis of the published ACCase sequences of several insect species were used as the targets of six degenerate primers. Different primer (Table 4.1) sets and different PCR conditions were tested. However, with a nested PCR strategy we succeeded to amplify a part of cDNA encoding ACCase. First the cDNA of LS-VL strain was amplified with 2 µM each of TurACCF2 and TurACCR1 degenerate primers using Taq polymerase (Invitrogen, USA) at 94°C 30 s, 54°C 30 s, and 72°C 60 s for 35 cycles. Then the reaction mix was amplified with TurACCF1 and TurACCR1degenerate primers in the same conditions. An amplified fragment with 325 bp (5975- 6299 *Drosophila melanogaster* numbering) was gel extracted using the QIAEX II kit (Qiagen, USA), and cloned into a pGEM-T vector (Promega, USA) and sequenced by Agowa Sequencing Service (Berlin, Germany). Sequence data were analysed using BioEdit 7.0.1 (Hall 1999).

Using this 325 bp fragment and by examining the annotated ACCase gene retrieved from the *T. urticae* genome database (http://bioinformatics.psb.ugent.be/webtools/bogas/users/login), the putative ACCase gene was inferred. For cDNA amplification several primer sets were

designed (Table 4.1, from TurACCF3a to TurACCR11; Figure 4.2). One µl of cDNA was used as a template for PCR carried out in a Biometra TProfessional Thermocycler (Biometra, Germany). PCR reactions were performed in 50 µl containing 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM deoxynucleotide triphosphate (dNTP) mix (Invitrogen, USA), 5 µl 10× PCR-buffer (Invitrogen, USA) and 1 U Taq DNA polymerase (Invitrogen), under the following conditions: 2 min at 94 °C, 35 cycles of 20 s at 94 °C, 30 s at 54 °C, 90 s at 72 °C and a final extension of 3 min at 72 °C. Long PCRs (>1.5kb) were performed using Expand Long Range Kit (Roche, Belgium), according to the manufacturer's instructions. PCR products were subjected to direct sequencing after purification using E.Z.N.A Cycle-Pure Kit (Omega Biotek, USA). To search for possible mutations in the *T. urticae* ACCase, we aligned the nucleotide and deduced amino acid sequences of spirodiclofen resistant and susceptible strains.

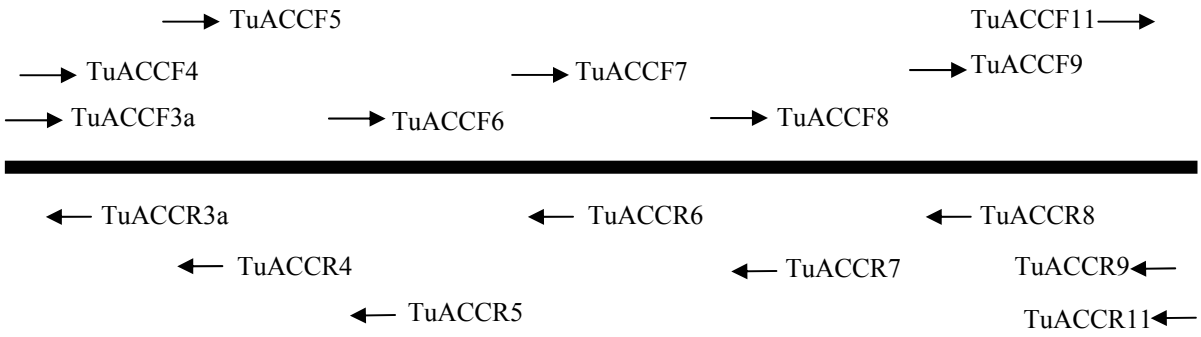


Figure 4.2 Location of the primer binding sites for cDNA amplification of *T. urticae* ACCase

Table 4.1 Primer sequences used for cloning and genotyping

Primers[a]	Sequences (5´–3´)[b]
TurACCF1	TGATIGARGAYRARGGIGAR
TurACCR1	CCRTTRTTRWICATRATYTG
TurACCF2	CCDGAIAARGGITTYAARTA
TurACCF3a	ATGAGTGGACCAAATTTATCTTCAC
TurACCR3a	GATGAGGTGGACCCATGAAC
TurACCF4	CACAAGATCAAGATGCAGCAA
TurACCR4	GATTCGGGCTGCAATAACAT
TurACCF5	GTCAATTTGCCTGCTTGTCA
TurACCR5	ATATCGCGAGCCATCAATTC
TurACCF6	TTCTGGAAGCGGGTTGTATC
TurACCR6	TGATGGATGTGACGAAGGAA
TurACCF7	CCGGGCGTATATTTGCTATG
TurACCR7	CCTGAAGCAGGATCACGAGT
TurACCF8	ATGCCTCTCGTTTGAGGAAA
TurACCR8	GGAGCGTATGAAAGCCAATC
TurACCF9	CACTGAACAAGGTGCTTGGA
TurACCR9	TTTTTGGTCCGCATAGAACC
TuACCF11	TTAACAAAACAACCGATCCTG
TuACCR11	GATTGTCGAATCACAACTTTGTCC
Rp49_qpcrF	CTTCAAGCGGCATCAGAGC
Rp49_qpcrR	CGCATCTGACCCTTGAACTTC
ACC_qpcrF	TAACAATGGTAACGTGTCGTGCAAT
ACC_qpcrR	ACCTTGTTCAGTGATGCATGTCC
TuACC-untF3	TTCTATGCCTTTTTGTGTTACCG
TuACC-untR3	TGGTGACATCTCTGATTTTTATGG
TuACC-untR2	TCTGCCCGCTACTAACTTTCA
TurACC-ExpF1	**GCGGCCGC**AGTGGACCAAATTTATCTTC
TurACC-ExpR1	**TCTAGAA**ACTTTGACGATCCATTTCCA
TurAcc-endF	ATGAGTGGACCAAATTTATC
TurAcc-endR	TCACTTTGACGATCCATTTCC
OpIE2R	GACAATACAAACTAAGATTTAGTCAG
pEIAF1	**CAACATG**AGTGGACCAAATTTATCTTCACA
pEIAF2	**CCCGGG**CAACATGAGTGGACCAAAT
pEIAR1	**GCGGCCGC**GACAATACAAACTAAGATTTAG
pUC/M13F	CCCAGTCACGACGTTGTAAAACG
pUC/M13R	AGCGGATAACAATTTCACACAGG
OpIE2R	GACAATACAAACTAAGATTTAGTCAG
pET-ACCF	**GTCGAC**AGTGGACCAAATTTATCTTCACAAGATCAAG
pET-ACCR	**GCGGCCGCTCA**AACTTTGACGATCCATTTCCAACACC

a The suffixes F and R in primer names indicate forward and reverse primers, respectively.
b Mixed base code letter used in degenerate primers: R (A+G), S (C+G), Y (C+T), W (A+T), D (G+A+T), I (inosine). Mutations are bold.

2.11 Quantitative reverse transcription PCR

Levels of specific mRNA species were measured by comparative quantitative real-time PCR amplification using the isolated RNA from pools of 300-400 deutonymphs of each *T. urticae* strain (SR-TK, SR-VP, London). Extracted RNAs were treated with TurboDNAse (Ambion, USA) to remove any genomic DNA contamination, and were then used with Superscript III reverse transcriptase (Invitrogen, USA) to make first strand cDNA using oligo-dT primers. Quantitative reverse transcriptase PCR reactions were performed in triplicate on a MiniOpticon Two-Color Real-Time PCR Detection System (BioRad, USA) using 30 ng of cDNA, 0.15 µM primers, and GoTaq qPCR Master Mix (Promega, USA). Cycling conditions and dissociation curve analyses were performed according to the manufacturer's instructions. To amplify the ACCase gene the primers ACCqpcrF1 and ACCqpcrR1 were used (Table 4.1). The ribosomal protein gene Rp49 (Tetur18g03590, 12/07/2010, BOGAS) was used as the reference gene to which the level of other transcripts were standardized (for primers see Table 4.1). Quantification of the transcript level or relative copy number of a gene was conducted according to the $2^{-\Delta Ct}$ method (Pfaffl 2001). All experiments were repeated three times.

2.12 Construction of Expression Plasmids

2.12.1 Construction of vector pMIB-ACC

In order to amplify the whole coding sequence of ACCase, six primers were designed inside the untranslated regions. First cDNA of LS-VL was amplified using TuACC-untF3 and TuACC-untR3 primers. Then, by nested PCR the reaction mix was amplified using TuACC-untF2/ TuACC-untR2 and TuACC-F3a/TuACC-untR2. All long PCRs were performed using the Expand Long Range Kit (Roche, Belgium), according to the manufacturer's instructions. The PCR products were cloned using the CloneJET™ PCR Cloning Kit (Fermentas, Germany) following the recommendations of the supplier. *Escherichia coli* DH5α were transformed by heat-shock and positive clones were picked and grown overnight. Plasmid Mini Kit I (Omega, USA) was used to extract the recombinant plasmids. Recombinant plasmids, pACCunt and pACCF3, containing inserts of about 7 kb, were sequenced. The ACCase coding region of sequenced and correct recombinant plasmids (pACCF3) was re-amplified by PCR using TurACC-ExpF1/ TurACC-ExpR1 oligonucleotides to introduce the restriction site for *Not*I and delete the initiation codon, and the *Xba*I restriction site and delete the terminator codon. The PCR fragment was ligated into the vector pMIB/V5-His B (Invitrogen, USA) (Appendix 1) after digestion of both ends with *Not*I –HF and *Xba*I (New England BioLabs), heat shocked in *E. coli* and correct recombinant pMIB/V5-His clones

identified. Plasmid DNA for sequencing and transfection was prepared using an EndoFree Plasmid Maxi kit (Qiagen, USA) to avoid possible endotoxin contamination. Since the result of the sequencing showed that there was a 39bp insert between *Not*I site and the (deleted) initiation codon, it was decided to first re-clone the appropriate fragment into the CloneJET™ vector. In order to do this, first a PCR was performed with TurAcc-endF/ TurAcc-endR primers and the double digested (*Not*I –HF and *Xba*I) recombinant CloneJET plasmid (pACCF3) as template. This reaction mix was used as a template for the next PCR using TurACC-ExpF1/ TurACC-ExpR1 primers. The PCR product was ligated into the CloneJET™ vector (pJetExp1) and sequenced. The digested pJetExp1 with *Not*I –HF, *Xba*I and *Bgl*I was ligated into the digested (*Not*I –HF and *Xba*I) vector pMIB/V5-His B. The result of sequencing showed that the cloned gene was not in frame with the N-terminal HBM secretion signal and the C-terminal peptide for detection with the V5 or His(C-term) antibodies. To make the gene in frame with the N-terminal signal, the recombinant pMIB was digested with *Not*I and then the protruding ends were conversed by Klenow DNA polymerase (Fermentas, Germany) to blunt ends and finally recircularized with T4 ligase to finally generate the correct construct pMIB-ACC.

2.12.2 Construction of vector pEIA+ACC21

Next to the expression plasmid pMIB, we ligated the ACCase in a second expression plasmid, pEIA (formerly pIE1/153A) (Lu et al. 1997) (Appendix 2), provided by Kostas Iatrou, Institute of Biology, National Centre for Scientific Research Demokritos, Athens, Greece.

The ACCase coding region was amplified by two PCR reactions. In the first PCR the recombinant pMIB plasmid was chosen as template and pEIAF1/ OpIE2R oligonucleotides were used to introduce a Kozak sequence (CAAC) and the initiator codon (ATG) immediately upstream of the deleted initiation codon. The second PCR using pEIAF2/ pEIAR1 primers and the first PCR as template added a restriction site for *Sma*I upstream of the Kozak sequence and *Not*I restriction site following the V5 epitope and polyhistidine (6xHis) region of pMIB and OpIE2 reverse priming site. To keep the possibility of further use of the *Sac*II restriction site upstream of the V5 epitope and polyhistidine region in future experiments with this vector, we first deleted *Sac*II restriction site of the original pEIA vector using *Sac*II digestion and treatment by Klenow fragment and generated pEIA+ vector. The product of the second PCR was ligated into the CloneJET™ vector (pJetpEIA7). The digested pJetpEIA7 with *Not*I –HF, *Sma*I and *Bgl*I was ligated into the digested (*Not*I –HF and *Sma*I) vector pEIA+ to generate pEIA+ACC21. The extra digestion with *Bgl*I cuts the vector, but not the

insert, and assured rare re-ligation of the CloneJET vector and the ACCase insert, making cloning into pEIA+ more efficient.

2.12.3 Construction of recombinant pFastBac and production of recombinant bacmids

ACCase recombinant baculoviruses were generated using the Bac-to-Bac baculovirus expression system (Invitrogen, USA) after subcloning the ACCase coding sequence into the pFastBac shuttle vector, according to the Bac-to-Bac® protocol (Invitrogen, USA). The digested pJetExp1 (*Not*I –HF, *Xba*I and *Bgl*I) was ligated in frame into the digested (*Not*I –HF and *Xba*I) vector pFastBac HT C (Appendix 3) downstream from the polyhedron promoter. A positive pFastBac clone (pFast HTC12) was sequenced. The plasmid was recombined into the bacmid backbone following the Bac-to-Bac® Baculovirus Expression system recommendations (Invitrogen, USA). Briefly, recombinant pFastBac HTC12 was transformed into DH10Bac *E. coli* competent cells containing the bacmid and helper plasmid in a Bac-to-Bac® Expression system (Invitrogen, USA). The ACCase fragment was transposed to the bacmid. Insertion of the foreign gene caused disruption of lac Zα gene, and thus colonies containing the recombinant bacmid were white on Luria Bertani (LB) plates in the presence of blue-gal, isopropyl-L-thio-b-galactoside, and antibiotics (kanamycin, gentamicin, and tetracycline). The recombinant bacmids (bacmids 1 to 5) were purified and analysed by PCR amplification using pUC/M13F and pUC/M13R primers to verify the presence and site of insertion of ACCase in the bacmid. Recombinant viruses were produced in Sf9 insect cells (Invitrogen, USA) and stored at 4°C. A second positive pFastBac clone (pFast HTC17) originating from another pJetExp clone (pJetExp32) was also transformed into competent DH10Bac cells, and recombinant bacmids (bacmids 12 and 16) were produced.

2.12.4. Construction of vector pET-ACC

A PCR (Expand Long Range Kit) fragment was amplified using a positive pFastBac clone (pFast HTC12) as template and the primers pET-ACCF to introduce a *Sal*I restriction site and primer pET-ACCR to insert a stop codon and a *Not*I restriction site, immediately upstream and downstream the ACCase CDS, respectively. This PCR fragment was cloned into the pJet 1.2 vector using the CloneJET™ PCR Cloning Kit. Positive clones were checked by colony PCR. A positive clone with the correct insert was digested with *Sal*I/*Not*I and ligated into the pETDuet-1 vector (Novagen, USA) (Appendix 4) resulting in pET-ACC.

2.13 Transfection, virus generation and protein production in Sf9 and High Five cells

Spodoptera frugiperda Sf9 or *Trichoplusia ni* High Five cells (Invitrogen, USA) were seeded at 1×10^6 per 2 ml of medium (Sf900 II SFM or complete IPL41 with 10 % FBS) in each well of a 6-well plate. For each well, Cellfectin (Invitrogen, USA) solution was prepared at 15 µl

of Cellfectin per 250 µl of Grace's Insect Medum or basal IPL41 medium in a clear Falcon tube and was incubated at room temperature for 30-45 min. Plasmid DNA of expression plasmids (pMIB-ACC or pEIA+ACC21) for transfection of each well was dissolved at 1.5-2.5 µg per 250 µl of basal IPL41 medium in a clear Falcon tube and incubated on ice. After removing the medium from seeded cells, 1 ml of basal IPL41 medium was gently added to wash the cells and remove proteins that could interfere with transfection. Then 250 µl of Cellfectin solution was added to each 250 µl of plasmid DNA solution in basal IPL41 to make the transfection mixture and was incubated on ice for 15 min. The medium was removed from seeded cells and replaced with 500 µl of transfection mixture in each well and was rocked gently. The 6-well plate was wrapped in a plastic bag to prevent desiccation and was incubated at 28ºC for 4-6 hours. After removing the transfection mixture from the cells in each well, 2 ml of medium with 50 µg/ml gentamycin was added to each well and rocked gently. The plate was wrapped in plastic and incubated at 27ºC. Transfected cells were incubated in an incubator for an additional 48, 72, 96 or 120 h and were harvested by centrifugation at 3000 g (5 min) for Western blot analysis.

Recombinant bacmids were transfected into Sf9 insect cells following the the Bac-to-Bac® Baculovirus Expression system recommendations (Invitrogen, USA). For each transfection, 500 ng of recombinant bacmid was used. Transfected cells were incubated in a 27°C incubator. Cells showed signs of viral infection after 4 days. Once the cells appeared infected, they were harvested and cell culture medium was saved as a master virus stock. The virus titer was determined using the BacPak Baculovirus Rapid Titer Kit (Clontech, USA) according to the manufacturer's protocol. The titer of virus stock P1 was 8×10^4 ml^{-1}. Because of the low virus titer, it was decided to further propagate the virus to a higher titer by infection of Sf9 cells in serum-free medium. After a 4-day-incubation, a titer of 5.7×10^8 ml^{-1} was reached (P2). The virus stock P2 (of bacmid 4) was used for expression studies in Sf9 cells. Exponentially growing Sf9 cells in suspension culture in serum-free SF-900 II SFM medium (300 ml per flask; 2.1×10^6 cells per ml) were infected with baculovirus stock at a multiplicity of infection (MOI) of 0.9. At 24 h, 48 h and 72 h after infection, 100 ml of cell suspension were harvested by centrifugation at 500 g (10 min). Cell pellets were resuspended in lysis buffer (10 mM HEPES pH 7.4, 20 mM KCl, complete EDTA-free protease inhibitors (Roche, Belgium); 5 ml per g fresh weight). Cells were broken using a Potter homogenizer (10 × 1000 rpm) and lysed by sonication for 5 × 20 sec (70% amplitude). The cell lysate was clarified by centrifugation (100000 g for 60 min) and the supernatant (soluble proteins) was normalized to

a protein concentration of 1.0 mg ml^{-1} and used further for enzyme activity and Western blotting assays.

2.14 Expression of pET-ACC in *E. coli* strains

E. coli strains "Codon Rare" and "BL21 DE3" (Novagen, USA) were heat-shock transformed with pET-ACC. Single colonies of transformed cells were grown in LB medium overnight. One ml of overnight culture was diluted into 100 ml LB medium and vigorously shaken at 37°C for at least 6 hours. Thereafter, 0.25 mM IPTG was added to the medium to induce protein synthesis. At 1, 2 and 3 h post-induction, cells were harvested and resuspended in SDS-lysis buffer. Cellular debris was pelleted at 10,000*g*, and supernatant containing soluble protein was diluted with an equal volume of NuPAGE LDS sample buffer (Invitrogen, USA) and detected by Western blot analysis using anti-His antibodies and streptavidin-HRP.

2.15 Analysis of transcript levels of recombinant ACCase by RT-PCR

Harvested cells (by centrifugation for 5 min at 3000*g*) were subjected to RNA extraction, DNase treatment and reverse transcription using the High Fidelity cDNA Synthesis Kit (Roche, Belgium). ACCase expression was confirmed by PCR amplification of cDNA using TurACCF6 and TurACCR6 (for plasmids) and pUC/M13F and pUC/M13R (for virus expression) from transfected cells and non-transfected cells, and virus and mock-infected cells (empty virus). The absence of contaminating genomic DNA in the RNA samples was verified by running PCR directly without reverse transcription.

2.16 Stable expression of pMIB-ACC

Adherent Sf9 or High Five cell monolayers transfected with plasmid DNA pMIB-ACC were subjected to selection with 30 and 60 µg/ml blasticidin–HCl, respectively, for 4 weeks.

2.17 SDS-PAGE and Western blotting

Individual plasmid transfected cell pellets were resuspended in 50 µl of lysis buffer [125 mM Tris (pH 6.8), 5% β-mercaptoethanol, 2% SDS and 4 M urea] or were disrupted by sonication. Cell lysate was centrifuged at 3000 *g* for 5 min to remove cell debris. Supernatant preparation from baculovirus infected Sf9 cells was explained in 2.14. Supernatant from each lysate was transferred to a fresh tube to make similar dilutions of total proteins. Protein concentration was determined with a commercially available Coomassie protein assay kit (Perbio Science, Belgium). Twelve to 20 µg of supernatant was used for SDS-PAGE and Western blot analysis to examine protein expression levels. Supernatant from each cell line infected with recombinant plasmid or virus and supernatant of uninfected control cells, Sf9 or High Five (Invitrogen, USA), were heated at 70°C for 10 min with NuPAGE LDS sample buffer (Invitrogen, USA). Total protein samples were separated using 10% Bis-Tris (with MOPS

SDS Running Buffer) or 7% Tris-Acetate (with Tris-Acetate SDS Running Buffer) polyacrylamide gel electrophoresis (Invitrogen, USA), and then transferred to a PVDF membrane. Blotting also was carried out on cellular debris containing insoluble proteins. The gels were in parallel stained with Coomassie blue or GelCode Blue Stain Reagent (Pierce, USA). Membranes were blocked with 5% non-fat dry milk, and then incubated with Anti-His (Qiagen, USA) or Anti-V5 antibodies (Invitrogen, USA) followed by goat-anti-mouse-HRP-conjugated secondary antibody (Dako Cytomation, Denmark) or with Streptavidin−Peroxidase Polymer (Sigma Aldrich, Belgium) according to the manufacturer's instructions (proteins that bind biotin can be visualised using a streptavidin linked peroxidase). Immunodetection was achieved by a colorimetric assay using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, Belgium). A precision protein standard (SeeBlue® Plus2 Pre-Stained Standard, Invitrogen, USA) was included for estimating molecular weight of the protein.

Western blotting was also used to compare the ACCase protein level in two susceptible and two resistant strains of *T. urticae*. From each strain 500 deutonymphs were aspirated and crushed in 100 µL sodium phosphate buffer (0.1M, pH 7.6) supplemented with protease inhibitors (Halt protease inhibitor cocktail kit, Pierce, USA) with a motorised Teflon pestle in glass tubes. The homogenate was centrifuged at 10000g and 4° C for 10 min. The resulting supernatant was diluted to 800 µg protein mL^{-1} and used as a protein source.

2.18 ACCase activity assay

Preliminary assays of ACCase enzymatic activity were carried out by Peter Lümmen (Bayer Cropscience, Germany) according to Tanabe et al. (1981) with some modifications. BSA solution was made using 0.1 mg ml^{-1} BSA in HKM assay buffer (100 mM HEPES, pH 7.4, 15 mM $MgCl_2 \times 6\ H_20$, 15 mM K-citrate). Equal volumes of ATP solution (4 mM ATP in HKM buffer) and Acetyl-Co A stock (8 mM in HKM buffer) were mixed (mixture A) prior to the assay. Assay mixtures in Micronic glass tubes (Micronic System, The Netherlands) were prepared using 100 µl protein solution (= 50 µg protein per assay mix), 36 µl BSA solution, 30 µl mixture A (ATP, acetyl-CoA), 4 µl DMSO or inhibitor solution in DMSO (10 µM final inhibitor concentration) and 30 µl Na-bicarbonate solution (16.7 mM Na-bicarbonate in 10 mM Tris/HCl, pH 9.0, including 10 µM ^{14}C-bicarbonate as a radioactive tracer). The reaction mixtures were incubated for 30 min at 25°C and were then stopped by adding 35 µl 3 M HCl. Addition of the acid liberates CO_2 from the unreacted bicarbonate, which evaporated into the gas phase for 10 min. Reaction mixtures were combined with 5.0 ml of scintillation cocktail

and the amount of radioactive malonyl-CoA was measured by liquid scintillation counting. Enzymatic rates were calculated using the specific activity of 2.04 GBq $mmol^{-1}$.

3 RESULTS

3.1 Spirodiclofen resistance

The LS-VL strain proved to be very susceptible to spirodiclofen prior to selection (Table 4.2). The LC_{50}-value of this strain, derived from log-dose probit-mortality data, was 4.9 mg L^{-1}. In addition, the response of LS-VL to spirodiclofen appeared to be very homogeneous (slope of probit line = 5.1).

Artificial selection of the LS-VL strain was carried out as described in 2.2, and resulted in a slow increase of the resistance. One month after the final selection (5000 mg spirodiclofen L^{-1}), the LC_{50} of spirodiclofen for the laboratory selected SR-VP was 675 mg L^{-1}, which is far above the recommended field-application rate for spirodiclofen (50-200 g a.i./1000 L) (Wachendorff et al. 2000). Consequently, the resistance ratio was 138, indicating strong resistance in the SR-VP strain. A continuous selection pressure of 5000 mg L^{-1} during the next 5 months even doubled the resistance ratio to 274 (Table 4.2). At that time, an LC_{90}-value of 5931 mg L^{-1} (95% CI 4234-9564) was obtained. The slope of the concentration-mortality curve decreased to 2.0, indicating a more heterogeneous response in the laboratory-selected resistant strain. The LC_{50} of spirodiclofen for the SR-TK strain, just before it was used for RNA extraction, was 441 mg L^{-1}.

Furthermore, the egg toxicity bioassay data indicated that the eggs of the SR-VP strain, contrary to the eggs of LS-VL, remained far more susceptible to spirodiclofen than the larvae. LC_{50}s and slopes were respectively 8.7 mg L^{-1} (95% CI 7.9-9.4) and 5.5 ± 0.3 for LS-VL, and 17.9 mg L^{-1} (14.6-21.1) and 2.5 ± 0.2 for SR-VP, resulting in a resistance ratio of only 2.0 (95% CI 1.8-2.4). The LC_{90} for SR-VP eggs is only 60 mg L^{-1} (95% CI 50-76) (results not shown).

3.2 Cross-resistance and synergism studies

With the laboratory susceptible strain as control, the resistant strain was tested for cross-resistance to different acaricides. The results are presented in Table 4.2. The SR-VP strain exhibited no cross-resistance to abamectine, etoxazole and pyridaben. In the case of acequinocyl, bifenazate and tebufenpyrad, the spirodiclofen resistant population presented only a slightly higher LC_{50} than the susceptible population, indicating that this possible associated resistance is of minor importance. The resistance ratios for acequinocyl, bifenazate

and tebufenpyrad were 3.8, 2.0 and 2.2, respectively. The highest cross-resistance was observed with the tetronic acid derivate spiromesifen (RR = 18).

Table 4.2 Concentration probit-mortality data of several acaricides on the LS-VL and SR-VP strain with calculated resistance ratios (RR)

Acaricide	Life stage tested	LS-VL		SR-VP		RR (95%CI)
		LC_{50} (95%CI) (mg L^{-1})	Slope ± SE	LC_{50} (95%CI) (mg L^{-1})	Slope ± SE	
Spirodiclofen	larvae	4.9 (4.5-5.3)	5.1 ± 0.2	675 (574-781) [a]	2.3 ± 0.2	138 (116-165)
				1343 (1122-1650) [b]	2.0 ± 0.1	274 (235-322)
	eggs	8.7 (7.9-9.4)	5.5 ± 0.3	17.9 (14.6-21.1)	2.5 ± 0.2	2.0 (1.8-2.4)
Abamectine	adults	0.40 (0.36-0.45)	5.2 ± 0.7	0.52 (0.46-0.58)	4.4 ± 0.5	1.3 (1.1-1.5)
Acequinocyl	adults	7.7 (6.7-8.9)	4.1 ± 0.4	29.6 (24.3-35.7)	3.6 ± 0.5	3.8 (3.2-4.6)
Bifenazate	adults	1.2 (1.0-1.4)	4.1 ± 0.5	2.4 (1.7-3.1)	2.8 ± 0.2	2.0 (1.6-2.5)
Etoxazole	larvae	0.76 (0.65-0.87)	3.9 ± 0.3	0.61 (0.52-0.69)	4.2 ± 0.3	0.8 (0.7-0.9)
Pyridaben	adults	156 (96-271)	0.8 ± 0.1	135 (84-200)	1.0 ± 0.1	0.9 (0.7-1.1)
Spiromesifen	larvae	0.79 (0.63-0.94)	3.5 ± 0.3	14.5 (9.8-18.9)	2.2 ± 0.2	18 (15-23)
Tebufenpyrad	adults	63.5 (52.3-75.7)	2.4 ± 0.2	137.5 (108.1-165.8)	2.6 ± 0.3	2.2 (1.7-2.7)

[a] January, 2008
[b] May, 2008

The effect of pre-treatment with PBO, DEF and DEM on the toxicity of spirodiclofen is presented in Table 4.3. Synergistic effects on the selected resistant strain were tested using mites that have been reared under a continuous pressure of 5000 mg spirodiclofen L^{-1} for 5 months. PBO and DEF clearly enhanced the toxicity of spirodiclofen to SR-VP by 3.5- and 3.3-fold, respectively, and decreased the resistance ratio from 275 to 97 and 64 compared to LS-VL. DEM did not synergize the toxicity of spirodiclofen to SR-VP at all. However, DEM had a small antagonistic effect on the spirodiclofen toxicity to LS-VL, resulting in a 1.5-fold reduction of the resistance ratio.

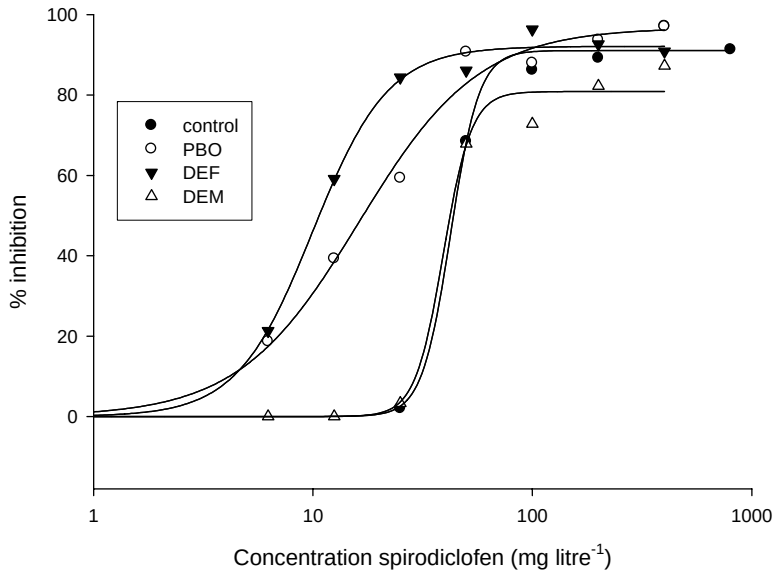
The effect of spirodiclofen on reproduction after pre-treatment with PBO, DEF and DEM is presented in Figure 4.3. Without synergists, the IC_{50} value for the LS-VL strain was 43.7 mg L^{-1} spirodiclofen. A similar value (42.2 mg L^{-1}) was obtained when the susceptible strain was pre-treated with DEM. Pre-treatment with PBO and DEF increased the inhibitory effect of spirodiclofen on reproduction. The IC_{50} values decreased to 10.7 (DEF) and 16.9 mg L^{-1} (PBO). It can be noted that the slopes of these curves are less steep than that of the control, indicating a less homogeneous response. In SR-VP mites, spirodiclofen could not inhibit the reproductive capacity more than 20%, even at highest concentrations, and the IC_{50} value could not be calculated. Pre-treatment with DEM even reduced the inhibitory effect of spirodiclofen. However, as observed in LS-VL, the synergistic effect of PBO and DEF treatment was clear. To get an estimation of the IC_{50} values, a regression curve was added. The estimated theoretical IC_{50} values obtained by extrapolation were 8×10^3 (PBO) and 59×10^3 mg L^{-1} (DEF).

Table 4.3 Effect of pre-exposure to PBO, DEF and DEM on spirodiclofen mortality in the LS-VL and SR-VP strains, with synergism ratios (SR)

	LS-VL			SR-VP			
	LC_{50} (95%CI) (mg L^{-1})	Slope ± SE	SR [a] (95%CI)	LC_{50} (95%CI) (mg L^{-1})	Slope ± SE	SR (95%CI)	RR (95%CI)
-	4.9 (4.5-5.3)	5.1 ± 0.2	1	1343 (1122-1650)	2.0 ± 0.1	1	275 (235-322)
PBO	3.9 (3.4-4.5)	3.9 ± 0.2	1.2 (1.1-1.3)	380 (321-449)	1.9 ± 0.1	3.5 (3.0-4.1)	97 (84-110)
DEF	6.3 (5.3-7.1)	3.7 ± 0.3	0.8 (0.7-0.9)	402 (346-467)	1.8 ± 0.1	3.3 (2.9-3.9)	64 (56-73)
DEM	6.9 (5.8-8.3)	4.0 ± 0.2	0.7 (0.7-0.8)	1306 (1119-1549)	1.3 ± 0.1	1.0 (0.9-1.2)	189 (162-221)

[a] SR = LC_{50} of spirodiclofen alone / LC_{50} of spirodiclofen + synergist

a



b

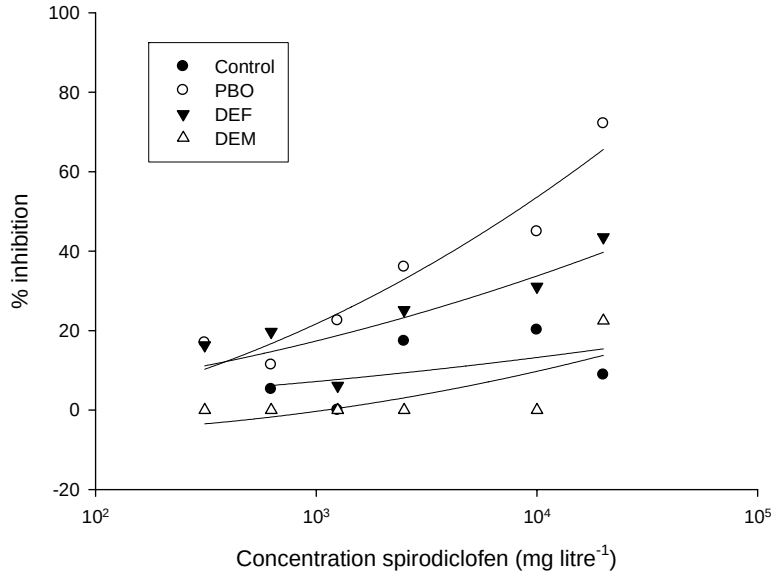


Figure 4.3 Inhibition of reproductivity of LS-VL (a) and SR-VP (b) females after 24 h of spirodiclofen pre-treatment without (●) or with prior application of synergists PBO (○), DEF (▼) or DEM (∇)

3.3 Detoxification enzymes

A significant difference in mono-oxygenase-mediated 7-EFC deethylation activity was found between adult females of LS-VL and SR-VP. After 5 months of selection with spirodiclofen, the activity was increased 11-fold in the resistant strain (Table 4.4). The mono-oxygenase activity in larvae of the susceptible strain did not differ from the activity in female adults. Activity in larvae collected from the SR-VP strain was enhanced 6-fold compared to the LS-VL larvae (Table 4.4). Finally, the mono-oxygenase activities in the females from the reciprocal crosses were determined (Table 4.4). The activities of both reciprocal crosses were significantly different from their parental strains (*t*-test, $P<0.05$), but not significantly different from each other (*t*-test, $P>0.05$). A drop in activity by 2.9- and 4.0-fold compared to SR-VP was measured for LS-VL♀ × SR-VP♂ and SR-VP♀ × LS-VL♂ respectively.

Table 4.4 Detoxifying enzyme activities in the LS-VL and SR-VP strain [a]

	LS-VL	SR-VP	Ratio
Mono-oxygenases			
O-deethylation of 7-EFC [b]			
→ adult ♀	316 ± 26^{A}	3437 ± 132^{B}	11
→ larvae	336 ± 92^{A}	1962 ± 312^{B}	5.8
→ LS-VL♀ × SR-VP♂	1250 ± 91		
→ SR-VP♀ × LS-VL♂	903 ± 105		
Esterases			
4-nitrophenyl acetate[c]	82.3 ± 1.8^{A}	110.3 ± 2.6^{B}	1.3
4-nitrophenyl propionate[c]	201.3 ± 1.6^{A}	275.7 ± 7.1^{B}	1.4
4-nitrophenyl butyrate[c]	195.2 ± 3.8^{A}	271.9 ± 5.4^{B}	1.4
1-naphthyl acetate[d]	271.0 ± 5.1^{A}	395.7 ± 8.5^{B}	1.5
1-naphthyl 2,2-dimethyl butyrate[d]	16.7 ± 0.9^{A}	33.7 ± 1.2^{B}	2.0
Glutathione-*S*-transferases			
CDNB conjugation[e]	762 ± 78^{A}	1438 ± 72^{B}	1.9
MCB[f]	955 ± 78^{A}	2388 ± 57^{B}	2.5

[a] Means (±SEM) within a row followed by the same capital letter are not significantly different (*t*-test, $P>0.05$)
[b] pmol 7-hydroxy-4-(trifluoromethyl)-coumarin (30 min)$^{-1}$ mg^{-1} protein (±SEM, n=3)
[c] nmol 4-nitrophenol min^{-1} mg^{-1} protein (±SEM, n=3)
[d] nmol 1-naphthol min^{-1} mg^{-1} protein (±SEM, n=3)
[e] nmol glutathione conjugated min^{-1} mg^{-1} protein (±SEM, n=3)
[f] relative fluorescence units (RFU) µg^{-1} protein (±SEM, n=3)

The esterase activities determined with the common substrates 4-NPA, 4-NPP, 4-NPB and 1-NA are shown in Table 4.4 and reveal a significant, but not very high, increase in SR-VP. Interestingly, the esterase activity measured with the new 1-naphthyl derivative, 1-naphthyl 2,2 dimethylbutyrate, clearly showed a 2-fold increase in SR-VP compared to LS-VL, though the absolute values were much lower than those measured with 1-NA.

The conjugation of glutathione with the artificial substrate CDNB was significantly higher in the selected resistant compared to the sensitive strain (Table 4.4). Additionally, the GST activity was measured in a fluorometric assay with MCB. Compared to strain LS-VL, SR-VP exhibited a 2.5-fold increase in GST activity.

3.4 Esterase activity gel profiles

The esterase isozymes of adults and nymphs of both susceptible (LS-VL and London) and resistant (SR-VP and SR-TK) strains of *T. urticae* after separation with p*I* 3–7 IEF gels, were visualised with 1-NA activity staining (Figure 4.4). The overall banding pattern was similar, however, adults and deutonymphs of the resistant strains showed a highly intensified band at an estimated pH of about 5.12. This band also seems to be present in the susceptible strains but with a much lower activity. These results suggest that one esterase is mainly responsible for the enhanced esterase activity in the resistant strains, and although the increase in esterase activity measured by artificial substrates in the resistant strains is not very high, it might possibly be associated with spirodiclofen resistance.

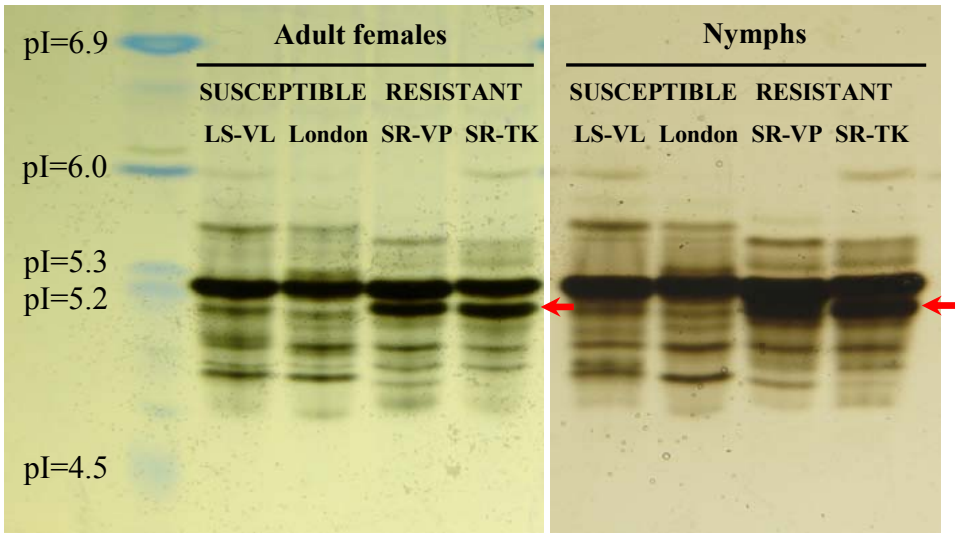


Figure 4.4 Esterase isozymes of adults and nymphs of two spirodiclofen resistant (SR-VP and SR-TK) and two susceptible (LS-VL and London) strains of *T. urticae* after separation with IEF pH 3–7, visualised with 1-NA activity-staining. The marker was stained using GelCode Blue Stain Reagent. Arrows indicate the intensified band in the resistant strains.

3.5 Effects of spirodiclofen on lipid content

Mites of the LS-VL or the SR-VP strain that were exposed to different concentrations of spirodiclofen did not show any significant difference in total lipid content compared with the controls (Table 4.5). Also, no significant differences could be observed between different measurements in time. Only the lipid content in SR-VP mites treated with 1000 mg L^{-1} measured after 5 days was significantly different from the lipid content 2 hours after treatment (Table 4.5). When the lipid content of 100 mites (instead of 10) was measured, again no significant differences were found between treatments within each strain (results not shown). However, comparing both strains, the lipid content in SR-VP mites appeared to be significantly higher than that in LS-VL mites, as can also be observed in most measurements in Table 4.5.

Table 4.5 Total lipid content in female adults of LS-VL and SR-VP strain (µg/10 spider mites) after a 12 h pre-treatment with 200 and 1000 mg L^{-1} spirodiclofen. Means (±SEM, n=3) within a column or row (and same strain) followed by the same small letter or capital letter, respectively, are not significantly different (non-parametric test, Kruskal-Wallis, $p > 0.05$).

strain	conc (mg L^{-1})	female adult age (days) 4	6	9
LS-VL	0	21.7 ± 2.3 *a A*	21.2 ± 0.2 *a A*	25.1 ± 2.1 *a A*
	200	21.4 ± 0.2 *a A*	23.5 ± 1.9 *a A*	24.5 ± 2.4 *a A*
	1000	19.0 ± 0.9 *a A*	19.6 ± 0.5 *a A*	21.4 ± 0.8 *a A*
SR-VP	0	25.9 ± 1.4 *a A* *	33.7 ± 4.6 *a A*	34.1 ± 5.2 *a A* *
	200	34.7 ± 3.1 *a A*	35.9 ± 1.9 *a A*	36.1 ± 2.3 *a A*
	1000	25.9 ± 0.7 *a A*	29.0 ± 1.6 *a AB*	32.0 ± 0.7 *a B*

* not significantly different with LS-VL (Kruskal-Wallis, $p > 0.05$)

3.6 Mode of inheritance of spirodiclofen resistance

The concentration-mortality curves of the parental strains and actual data points of the reciprocal crosses are shown in Figure 4.5. The fitted curves of the corrected results for the haploid male offspring are shown in the same figure. Without this correction there would be an overestimation of mortality at the lower concentrations for LS-VL♀ × SR-VP♂ and an underestimation of mortality at the higher concentrations for the other reciprocal cross. At the moment of the crossing experiments, the LC_{50} of the resistant SR-VP mites was 675. The (corrected) LS-VL♀ × SR-VP♂ and SR-VP♀ × LS-VL♂ reciprocal crosses had an LC_{50}

(95% CI) of 68 mg L^{-1} (63-74) and 85 mg L^{-1} (69-103), respectively. Since the concentration-mortality curves for the reciprocal F_1 crosses were nearly identical (the 95% confidence intervals at LC_{50} overlapped), there was no pronounced maternal effect involved in the inheritance of resistance. Only at higher concentrations, differences in mortality could be detected. The LC_{90} values of both crosses were significantly different to each other (150 mg L^{-1} (136-170) for LS-VL♀ × SR-VP♂ and 236 mg L^{-1} (187-323) for SR-VP♀ × LS-VL♂) (results not shown). A visual examination of Figure 4.5 suggests spirodiclofen resistance is intermediate. This is confirmed by substituting the LC_{50} values of the LS-VL, SR-VP and reciprocal crosses into the formula of Stone (1968), resulting in degrees of dominance close to zero (Table 4.6). Figure 4.6 shows the observed concentration-mortality lines of spirodiclofen for the backcrosses, corrected for haploid male offspring. The dotted lines indicate the expected values for F_2 females if spirodiclofen resistance was determined by a single major gene. Because 1) there is no plateau seen at the LC_{50} level for both crosses, and 2) the observed mortality for (LS-VL × SR-VP)♀ × LS-VL♂ and (SR-VP × LS-VL)♀× SR-VP♂ was significantly different (χ^2=67.15, df=20 and χ^2=62.70, df=16, respectively, p<0.001) from that expected on the basis of monogenic inheritance, spirodiclofen resistance in SR-VP appears to be polygenic.

The mortality curves of the haploid F_1 progeny of strains LS-VL and SR-VP, and the haploid F_2 progeny of the virgin F_1 heterozygous females are represented in Figure 4.7. The dotted line indicates the expected mortality if a single gene conferred the spirodiclofen resistance. The intermediate and polygenic inheritance of resistance is also confirmed by the dominance factors calculated for the haploid F_2 progeny (Table 4.6) and the absence of a plateau at 50% mortality for both crosses (Figure 4.7). A χ^2 goodness-of-fit analysis of the observed mortality to the expected line indicated significant departure from the one major gene model (χ^2=155.27, df=12 for LS-VL♀ × SR-VP♂, and χ^2=133.14, df=13 for SR-VP♀ × LS-VL♂, p<<0.001).

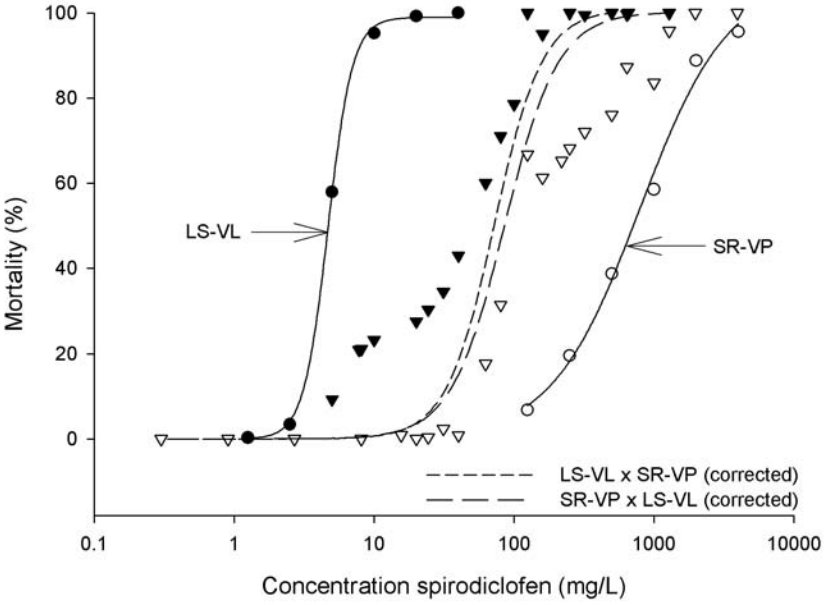


Figure 4.5 Spirodiclofen concentration-mortality curves for LS-VL, SR-VP and reciprocal crosses. The triangles represent actual data points of the haploid and diploid progeny (▼ = LS-VL x SR-VP; ∇ = SR-VP x LS-VL). The dashed lines represent the reciprocal crosses corrected for haploid males.

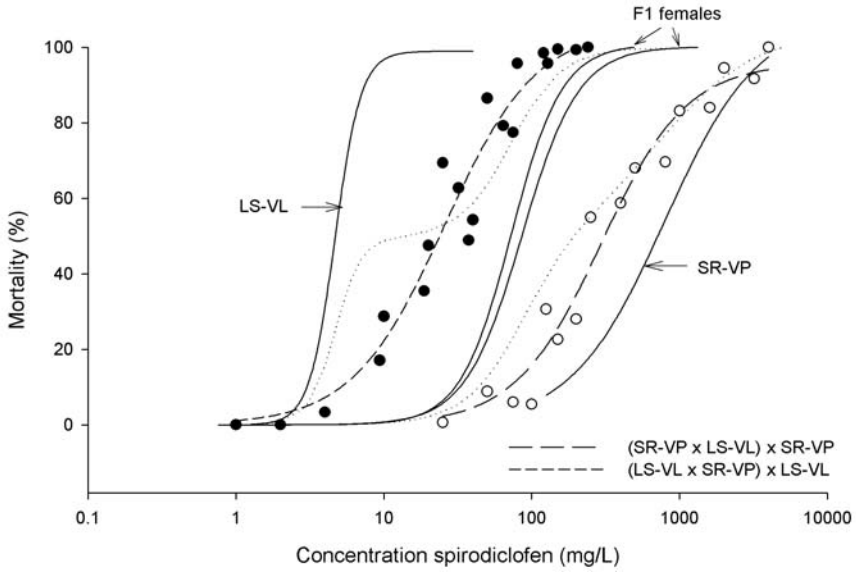


Figure 4.6 Spirodiclofen concentration-mortality curves for the diploid progeny of the backcrosses (LS-VL × SR-VP)♀ × LS-VL♂ (●) and (SR-VP × LS-VL)♀× SR-VP♂(○). Circles are actual data points (corrected for haploid male progeny), the dashed lines are fitted curves. The dotted lines represent the response expected if spirodiclofen resistance were determined by a single major gene.

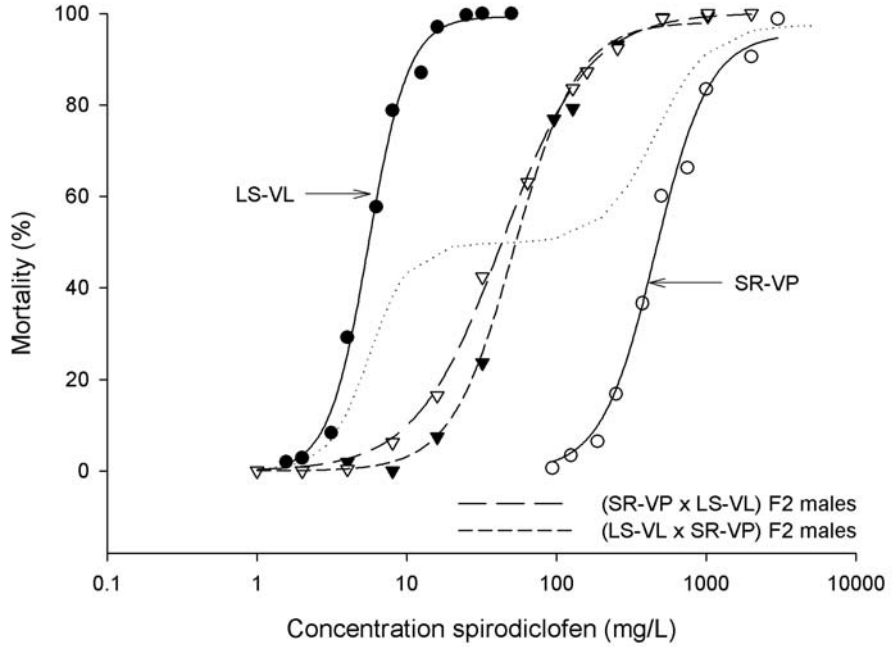


Figure 4.7 Spirodiclofen concentration-mortality curves for parental males and F_2 males derived from virgin F_1 females (▼ = LS-VL x SR-VP; ∇ = SR-VP x LS-VL). The dotted line represents the response expected if spirodiclofen resistance were determined by a single major gene.

Table 4.6 Probit statistics for the reciprocal crosses tested against spirodiclofen (n = number of mites and *D* = degree of dominance)

	Strain	n	Slope ± SE	LC_{50} (95%CI) (mg L^{-1})	*D*
diploid F_1 progeny	LS-VL♀ × SR-VP♂	3843	3.7 ± 0.1	68 (63-74)	0.07
	SR-VP♀ × LS-VL♂	1812	2.9 ± 0.1	85 (69-103)	0.16
haploid F_2 progeny	LS-VL♀ × SR-VP♂	3887	2.5 ± 0.1	56 (50-62)	0.004
	SR-VP♀ × LS-VL♂	3737	2.0 ± 0.1	44 (40-49)	-0.10

3.7 Sequence characterization of ACCase

The cDNA sequence was 6957-bp long with an open reading frame and deduced amino acid sequence (AA) of 2318 amino acids (AA), giving an estimated molecular weight of 261.8 kDa. A BlastP search of the GenBank database revealed that the translated cDNA sequence displayed a moderate level of similarity with ACCase of *Pediculus humanus corporis* (60%), *Acyrthosiphon pisum* (60%) and *Tribolium castaneum* (59%). Analysis of the amino acid sequence with Scan Prosite (http://www.expasy.org/prosite) revealed the existence of several structural motifs typical of this enzyme (Appendix 5). These motifs include: 1) the BCC domain (residues 662-728), which contains a biotin group covalently linked to a conserved lysine (residue 696); 2) the BC domain (residues 31-528), that catalyzes the ATP-dependent carboxylation of this biotin group; and 3) the CT domain (residues 1639- 2143) that catalyzes the transfer of the carboxyl group from biotin to acetyl-CoA to produce malonyl-CoA. The amino acid sequence of binding sites for ATP (residues 220-235; Figure 4.8a), biotin (residues 689-701; biotin at 696; Figure 4.8b), carboxybiotin (residues 1615-1664; Figure 4.8c), and acetyl-CoA (residues 1918-1929; Figure 4.8d) were highly conserved in animals and plants.

To investigate whether spirodiclofen resistance could be mediated by target site insensitivity, full-length cDNA fragments of *T. urticae* ACCase gene were cloned from the spirodiclofen-resistant (SR-VP and SR-TK) and susceptible strains (LS-VL and London). We compared their full length amino acid sequences and despite of several synonymous mutations, we could not detect any fixed non-synonymous mutations in the resistant strains. However, the electropherograms of the SR-VP strain showed the presence of two peaks at nucleotide position 3235, results in an alanine to threonine (A1079T) substitution.

3.8 Association between ACCase expression and spirodiclofen resistance

Real-time PCR was used to determine whether ACCase expression in the resistant strains is altered when compared to the susceptible strain. No change in expression levels was found in the spirodiclofen resistant strains.

A Western blot assay of total protein extracts from spirodiclofen resistant and susceptible deutonymphs using streptavidin-HRP showed no difference in the biotin-binding protein pattern between strains, indicating that the resistance to spirodiclofen might not be due to an increase in the ACCase expression.

Figure 4.8 Multiple sequence alignment of selected ACCase homologs from *Tetranychus urticae* (*Tu*), *Pediculus humanus corporis* (*Phc*, XP_002429216.1), *Tribolium castaneum* (*Tc*, XP_969851.2), *Aedes aegypti* (*Aa*, XP_001651879.1), *Drosophila melanogaster* ACCase isoform A (*Dm*, NP_610342.1), *Homo sapiens* ACCase1 (*Hs*, NP_942133.1), *Gallus gallus* (*Gg*, NP_990836.1), *Triticum aestivum* cytosolic ACCase (*Ta*C, ACD46674), *Triticum aestivum* plastid ACCase (*Ta*P, ACD46685), *Zea mays* (*Zm*, AAA80214), *Oryza sativa* (*Os*, AAM18728), *Arabidopsis thaliana* (*At*, AAC41645), *Glycine max* (*Gm*, AAA75528), *Brassica napus* (*Bn*, CAA54683). **a** ATP binding sites; **b** biotin binding sites; **c** acetyl-CoA binding sites; **d** carboxybiotin binding sites. Protein sequences were aligned using the CLUSTALW alignment algorithm. Sequences were shaded using the BoxShade program. Identical and conserved residues are shaded black and gray, respectively.

3.9 ACCase functional expression

3.9.1 Constructs

Restriction analysis and sequencing of recombinant expression plasmid pMIB-ACC (after Klenow DNA polymerase treatment) revealed that the ACCase insert is in frame with the N-terminal HBM secretion signal and His tag at the C-terminal.

Also, sequencing of pEIA+ACC21 showed that the ACCase was correctly inserted and that a Kozak sequence and C-terminal polyhistidin tag for detection is present.

The constructed pFast HTC12 and 17 were in frame with the N-Terminal 6×His tag and the stop codon was located in the vector polyadenylation signal. pFast HTC12 showed an N444D polymorphism that was possibly the result of a PCR error.

Sequencing and double digestion using *Sal*I/*Not*I showed that the pET-ACC construct, was in frame and correct.

3.9.2 Expression using expression plasmids pMIB and pEIA+ in insect cells

Transcription levels after transfection were analysed by RT-PCR using gene-specific primers. The gene was transcribed in both Sf9 and High Five cell lines, transfected with either one of the expression vectors. All RNA samples extracted from transfected cells were subjected to PCR without reverse transcription as control. The negative result indicated that RNA samples were free of genomic DNA contamination. Designed primers proved to be specific for *T. urticae* ACCase gene, since no amplification of the endogenous SF9 and High Five cell ACCase was detected in the non-transfected samples.

Sf9 and High Five cells transfected with pMIB-ACC showed no ACCase expression after blotting with Anti-His or Anti-V5 antibodies. Contrary to Sf9-cells, High Five cells showed limited ACCase expression two days after transfection with pEIA+ACC21 (results not shown). However, the ACCase protein was mainly found in the insoluble fraction (cell-debris fraction), and these experiments need to be repeated.

3.9.3 Baculovirus expression

Transformation of recombinant pFast HTC12 and HTC17 into DH10Bac competent cells resulted in the construction of the recombinant bacmids and transfection of these bacmids generated recombinant viruses. RT-PCR confirmed transcription of the recombinant ACCase after infection of Sf9 cells by the baculoviruses. Western blots revealed detectable protein 2 and 3 days after virus infection (Figure 4.9).

Activity of the recombinant ACCase was analysed by monitoring the ACCase- and ATP-dependent incorporation of radioactivity from acid labile $H[^{14}C]O_3^-$ into acetyl-CoA to form the acid-stable malonyl-CoA product. Comparison of test results in the presence and absence

of 10 μM spirotetramate-enol showed that more than 90% of the carbon dioxide incorporation into buffer-soluble, acid-resistant material can be readily inhibited. This means that the assay method specifically measured ACCase activity in the cell extracts. 24 h after infection there was no difference between the ACCase activities of infected (virus constructed using bacmid 4) and mock-infected cells. The control activity can be attributed to the endogeneous ACCase activity of Sf9 cells. At 48 h after infection an increase in ACCase activity was observed in the infected cells (7.3 nmol min^{-1} mg^{-1}) in comparison with the mock-infected cells. At 72 h after infection, almost no activity could be measured in the infected as well as in the mock-infected cells, probably due to advanced cell damage (Figure 4.10).

3.9.4 Expression in *E. coli*

Promising results were also achieved by heat-shocking "Codon Rare" *E. coli* cells transfected with the pET-ACC vector. A Western blot probed with streptavidin-HRP (Figure 4.11) showed there was an approximately equal amount of protein of each treatment (1, 2 and 3 hours after induction with IPTG and without IPTG induction) loaded on the Bis-Tris gel, while the same blot probed with anti-His antibody revealed there was ACCase expression 2 and 3 hours after induction with IPTG (small band at 260 kDa). However, besides the ACCase protein band several other smaller protein bands were detected, indicating that the expressed ACCase protein had been degraded by proteases after expression.

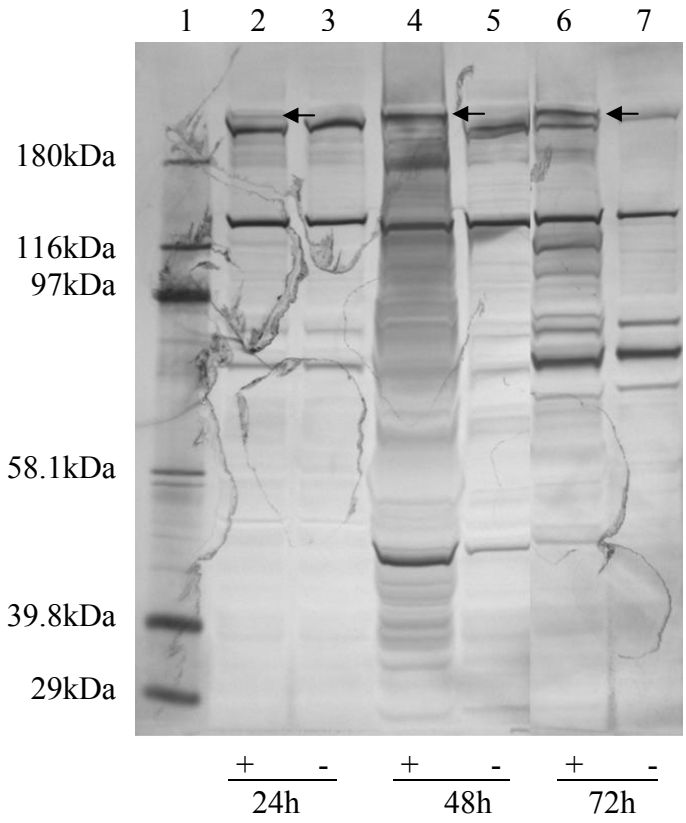


Figure 4.9 Western blot analysis of the supernatant cell extracts of baculovirus infected Sf9 cells 1 (lane 2), 2 (lane 4) and 3 (lane 6) days after infection and cell extracts from mock-infected Sf9 cells l (lane 3), 2 (lane 5) and 3 (lane 7) days after infection. The blots were probed with Streptavidin-HRP antibody. Biotinylated molecular weight markers (lane 1) was used to control the electrophoresis and the electrotransfer of proteins. The arrows indicate the expressed recombinant ACCase.

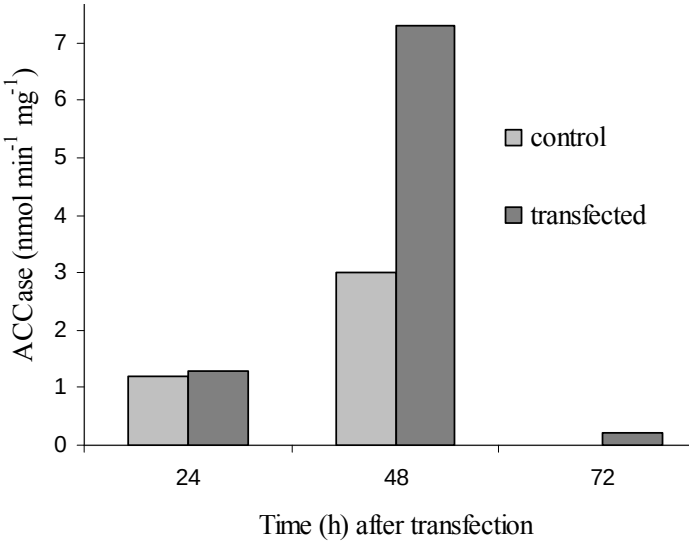


Figure 4.10 ACCase activity in the cell extracts. 24, 48 and 72 h after infection vs. control (mock-infected cells).

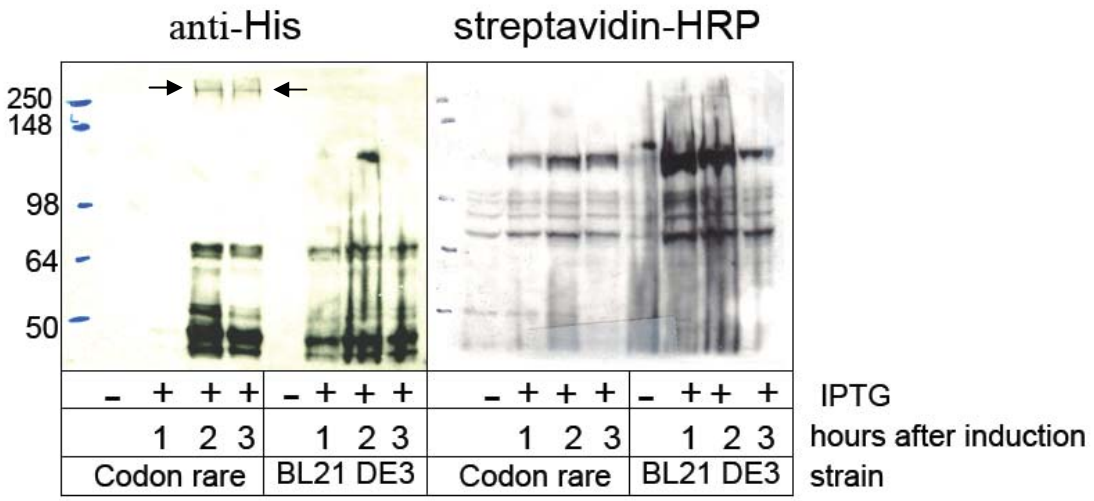


Figure 4.11 Western blot of supernatant cell extract of pET-ACC heat-shocked "Codon Rare" and BL21 DE3 *E. coli* cells 1, 2 and 3 hours after induction with IPTG. The blots were probed with Streptavidin-HRP- (right panel) or with anti-His (B) antibodies (left panel). The arrows indicate the expressed recombinant ACCase.

4 DISCUSSION

The main objective of this study was to make a biochemical, genetic and molecular analysis of a laboratory selected spirodiclofen resistant strain (SR-VP) of *T. urticae*. Selecting an acaricide-susceptible field strain of *T. urticae* (LS-VL) with increasing concentrations of spirodiclofen resulted in a gradual increase of the resistance ratio. The slow increase of resistance may be partly due to the low initial frequency of resistance alleles in the parental homogeneous LS-VL strain. It is possible that during selection an accumulation of resistance genes with minor effects occurred, creating a more heterogeneous population, of which the decreasing slopes of the concentration-mortality lines could be an indication (Robertson et al. 2007). After 17 months of selection, the strain (SR-VP) reached a stable and high resistance ratio of 274. This is considerably higher than the previously reported resistance ratio (13 fold) after selecting an Italian field collected strain for 21 months (Rauch and Nauen 2002).

Cross-resistance experiments revealed that the resistant SR-VP strain showed considerable cross resistance towards spiromesifen, another acaricide within the same chemical class of tetronic acid derivatives. The existence of cross-resistance among tetronic acid derivative acaricides has also been documented in the citrus red mite, *Panonychus citri*: a spirodiclofen

resistant population collected from Chinese citrus orchards, which had no previous history of spirotetramat application, proved to be resistant to spirotetramat (Hu et al. 2010).

Our study could indicate that spirodiclofen and spiromesifen share a common detoxification route, but with a higher specificity towards spirodiclofen. Alternatively, it could also mean that SR-VP has an altered target site affecting binding of both compounds possibly in combination with a specific spirodiclofen detoxification mechanism.

However, amino acid sequence alignment of ACCase revealed no consistent amino acid polymorphisms between the spirodiclofen resistant (SR-VP) and susceptible strain of *T. urticae*. The documented A1079T polymorphism was not fixed in this strain, and possibly not associated with spirodiclofen resistance. Another spirodiclofen resistant strain SR-TK (RR=90), obtained from Bayer CropScience, with a similar esterase isozyme profile and ACCase expression level, did not exhibit this mutation. Also, this residue is not located within any of the conserved functional domains. Previous findings in weeds revealed that variable mutations in the CT domain of homomeric plastidic ACCase within grass species were associated with resistance to ACCase-inhibiting herbicides. Also it has been found that a mutation in the BC domain of yeast ACCase can render the enzyme resistant to soraphen A, a potent inhibitor of eukaryotic ACCase (Shen et al. 2004). Next to sequence polymorphisms, we also analysed expression levels with RT-PCR, and no up- or down regulation in the resistant strains were detected. The experiment needs to be repeated since at least two reference genes are recommended. Using only one reference gene can result in errors. These results were confirmed on the protein level, since Western blots using streptavidin-HRP also showed no difference in the biotin-binding proteins in *T. urticae* deutonymphs of resistance and susceptible strains. Although not detected here, altered expression levels of ACCase have been linked with resistant in plants. A johnsongrass, *Sorghum halepense*, biotype resistant to ACCase inhibiting-herbicides showed an ACCase that was still sensitive to the herbicides, but the specific activity of ACCase in the resistant biotype was two to three times higher than that of the susceptible biotype. It was suggested that resistance is conferred by an overproduction of ACCase in the resistant johnsongrass biotype (Bradley et al. 2001).

Since target-site resistance is probably not involved, we conducted synergism studies and biochemical assays with model substrates to identify metabolic resistance mechanisms. Pre-treating SR-VP larvae with PBO prior to spirodiclofen application resulted in a clear decrease of resistance (synergism ratio = 3.5), while the susceptibility of the LS-VL strain was hardly affected. This indicated that P450 mono-oxygenases might play an important role in spirodiclofen detoxification. Determination of detoxifying enzyme activities *in vitro*

confirmed this hypothesis, since the mono-oxygenase activity, measured by *O*-deethylation of 7-EFC in adults and larvae of the resistant strain was increased 11 and 6-fold, respectively. In addition, pre-treatment with PBO increased the inhibitory effect of spirodiclofen on reproduction: pre-treatment of resistant females could partially restore the inhibition of the reproductive capacity caused by spirodiclofen. Rauch and Nauen (2002) also found P450 mono-oxygenases to be important in spirodiclofen resistance in this species.

Next, *in vivo* synergism assays revealed that the esterase inhibitor DEF, despite the low concentration (10 mg L^{-1}), synergized spirodiclofen in the SR-VP strain to the same extent as PBO (3.3-fold), indicating the importance of esterases in the detoxification of spirodiclofen. DEF also synergized the effect of spirodiclofen on reproduction in females of the resistant strain. Further evidence was provided by the increase in mean esterase activity towards different substrates in the SR-VP strain. The increase was most prominent when measured with the purpose-designed substrate 1-naphthyl 2,2-dimethylbutyrate. The metabolism of this substrate, which mimics the acyl-ester side chain of spirodiclofen, might be a good representative for imitating the hydrolytic degradation of spirodiclofen (Rauch and Nauen 2002). Probably, among the esterases of *T. urticae* only one is more expressed in spirodiclofen resistant strains, and this one apparently prefers 1-naphthyl 2,2-dimethylbutyrate. This was confirmed when esterase isozymes were separated by native isoelectric focusing. A clearly enhanced esterase near pH 5 in the resistant strains was responsible for the higher esterase activity associated with spirodiclofen resistance. Using radiolabelled [^{14}C]spirodiclofen Rauch and Nauen (2002) revealed that hydrolytic and oxidative degradation of spirodiclofen occurred more rapidly in the resistant strain.

Although enzymatic assays with CDNB and MCB indicated that the resistant strain had a higher GST activity, *in vivo* synergism assays revealed that DEM did not synergize the spirodiclofen efficacy in the resistant strain. GST activity was also determined to be significantly higher by Rauch and Nauen (2002) in the resistant compared to the parental strain. These results make the roles of GSTs in spirodiclofen resistance in *T. urticae* unclear, but there is no reason to exclude their roles completely.

There are also some reports that PBO is able to inhibit some esterases as well (Young et al. 2005). In theory, a synergistic effect of PBO can hence also be attributed to the inhibition of certain esterases. However, considering the highly increased P450 MO activity, this is most likely not the case. Similarly, the esterase inhibitor DEF is also able to inhibit GSTs. So, caution must be taken when interpreting the results from tests with synergistic.

In conclusion, in contrast to our attempts to reveal an altered target-site, we found several indications that enhanced detoxification might be the major cause of resistance.

We were not able to find a link between spirodiclofen treatment and lipid biosynthesis. However, the increase in lipid content over time, although not statistically significant, was more pronounced in resistant mites. Based on this experiment, it was not really possible to make conclusive remarks about the impact of spirodiclofen on the *de novo* lipid biosynthesis. However, total lipid content in the SR-VP strain was higher compared to the LS-VL strain, which could be an essential condition for spirodiclofen resistance. Higher lipid contents have been found in the resistant strains of *M. domestica* (Patil and Guthrie 1979) and in the cuticles of resistant tobacco budworm larvae (Vinson and Law 1971). A more detailed study of the different lipids may generate more relevant information to elucidate the spirodiclofen mode of action and the role of specific lipids in this process.

An important observation from a resistance management perspective was the very low resistance in SR-VP eggs. Life stage variations in insecticidal susceptibility are not unusual and were previously reported (Brattsten 1988, Gunning et al. 1992, Sato et al. 2006, Nauen et al. 2008). This lack of relationship between ovicidal and larvicidal resistance may be related to a differential expression of the detoxifying enzymes causing variations in metabolic detoxification capacity between eggs and larvae. This is also consistent with the lack of an altered target-site, that would probably also protect eggs at least partially from spirodiclofen intoxication. Considering this developmental stage-dependent response to spirodiclofen in *T. urticae*, treatment of plants infested with resistant mites with the recommended field dose of spirodiclofen, will only kill eggs that come in direct contact with the acaricide, while others, e.g. eggs deposited on young, newly grown leaves or in the webbing after spraying, will hatch and larvae will still develop. To guarantee sustainable control of spider mite populations, it is important to spray at early infestation, at the very beginning of the population build up. Spirodiclofen might be used best as an ovicide, mixed with or in close succession with another acaricide with a different mode of action active against motile stages, in order to prevent migration of the adults and the outbreak of a new infestation.

Our results regarding the resistance profile in female progeny of reciprocal crosses, both by visual examination of concentration-mortality curves and by calculating degrees of dominance suggest that spirodiclofen resistance in SR-VP strain has no substantial dominance. Similarity in dose-response curves for these reciprocal crosses, indicating that no apparent extranuclear inheritance was involved. The measurement of 7-EFC *O*-deethylation activity of these reciprocal crosses supports the role of metabolic detoxification via

cytochrome P450 mono-oxygenase activity. Although resistance was much lower when resistant and susceptible individuals mate, i.e. in heterozygous progeny, compared to the resistant strain, it would be expected that typical field rates of spirodiclofen (200 mg L^{-1}) allow some heterogeneous individuals to survive. Models predict that resistance will be delayed substantially if the field doses of insecticide kill heterozygotes (Roush and McKenzie 1987). In spirodiclofen resistance, the resistance genes cannot be effectively swamped out by susceptible immigrants, as would be the case with a recessive inheritance mode (Yamamoto et al. 1995). Data for the backcrossing of F1 progeny to parental strains (F2 female progenies) fit a polygenic model, in which multiple loci contribute equally and additively to the resistance. To complement and support analyses of the responses of F_1 female progeny, haploid reciprocal crosses were established. Due to arrhenotoky, haploid F2 males produced by virgin heterozygous F1 females would respond in a 1:1 ratio of phenotypes if resistance was conferred by a single major gene and they inherited resistance genes from their mothers (Robertson et al. 2007). However, the response of such F2 males to spirodiclofen showed no 1:1 segregation of susceptible and resistant haplotypes, and confirmed that resistance was under control of more than one gene. This result, polygenic inheritance, is also consistent with *in vitro* and *in vivo* enzyme activity bioassays which showed that both an increased P450 mono-oxygenase and specific esterase activity are associated with the resistance trait. Most laboratory selection programs may accumulate many altered genes, each having a small effect, resulting in polygenic resistance. In contrast, field treatments are thought to usually select a single, major resistance gene that can confer large increases in resistance immediately. Therefore, laboratory selection may not represent the real situation (Roush and McKenzie 1987, Cluck et al. 1990). However, in the case of spider mites, it has often been illustrated that both field collected and laboratory strains can acquire resistance by the combination of more than one factor, often the joint effect of different groups of detoxifying enzymes (Stumpf and Nauen 2002, Van Leeuwen et al. 2005, 2006b, Van Pottelberge et al. 2009a), or by the same target site (Van Leeuwen et al. 2008). Moreover, laboratory selection is often the only practical means to develop resistant strains before widespread use of pesticide in the field.

We assembled a full-length cDNA clone encoding the *T. urticae* ACCase inside four vectors, pMIB/V5-His B and pEIA+ for direct plasmid expression in insect cells, pETduet for *E. coli* expression and pFastBac shuttle vector for expression by baculovirus in insect cells. Sequencing results showed that all constructs were correct. Successful transfection and mRNA ACCase expression with insect cell systems was confirmed by RT-PCR.

Transfection of pMIB-ACC into Sf9 or High Five cells did not change growth of the transformed cells. Blasticidin treatment caused non-transfected cells to enlarge and detach from the plate while pMIB-ACC transfected cells continued to grow normally. Although we detected expression, we could not reproducibly detect ACCase protein with the tested expression plasmids (pMIB-ACC and pEIA+ACC21) in different insect cell lines. Transformed insect cells and transient expression by pMIB/V5-His (Gouveia et al. 2007) and pEIA vectors (Douris et al. 2006) have been used for the production of recombinant proteins but not for proteins with high molecular weight, like the eukaryotic ACCase.

A Western blot probed with anti-His antibody on heat-shocking "Codon Rare" *E. coli* cells transfected with the pETduet-ACC vector revealed ACCase expression 2 and 3 hours after induction with IPTG. However, the expressed ACCase protein had been partly degraded by proteases after expression. Using lower temperatures during growing of transformed *E. coli* colonies and using other modified *E. coli* strains could maybe improve recovery of complete non degraded protein. Unexpectedly, the bacterial expression looked very promising after a first attempt, since a limited culture could produce detectable levels of full length protein.

More exciting, we were able to detect expression in SF9 cells by Western blots with the baculovirus system. Moreover, enzyme activity assays revealed that the recombinant protein is active, and this activity can be fully inhibited by spirotetramate-enol. Using this baculovirus-insect cell system it will be possible to investigate whether mutations associated with resistance to ACCase-inhibiting herbicides in various weed species can also change biochemical properties of *T. urticae* ACCase and confer resistance to ACCase inhibiting insecticides.

Looking back, the negative results of pMIB-ACC transfected cells could be related to the Western blot analysis sensitivity that possibly was not enough to detect small amounts of produced recombinant ACCase or to the fact that the ACCase was completely proteolysed. Since the mRNA production (transcription) was confirmed by RT-PCR, it is also possible that protein translation or post-translational modification failed. For the production of recombinant ACCase proteins, the baculovirus expression vector system and *Escherichia coli* expression have been previously used successfully (Weatherly et al. 2004, Cheng et al. 2007, Kim et al. 2007). However, the big size and structural complexity of ACCase make its expression difficult and also, the high protease susceptibility of recombinant ACCase has been reported as well (Weatherly et al. 2004).

In conclusion, selecting for spirodiclofen resistance yielded a strain with a very high resistance in larvae, which proved to be controlled by more than one gene, as was indicated

by the crossing experiments. However, spirodiclofen was still toxic to eggs of the resistant strain. The spirodiclofen resistant strain did not show any cross-resistance against other frequently used acaricides, except against spiromesifen. Synergism studies and biochemical assays suggested the involvement of the three detoxification enzyme systems in the metabolic detoxification of spirodiclofen, of which the P450 mono-oxygenases and esterases play significant roles. When esterase isozymes were separated by isoelectric focusing, only one esterase located near pH 5 was clearly enhanced in resistant strains. No evidence was found to support the existence of other resistance mechanisms, like mutations in the target site (ACCase) or overproduction of the target enzyme. In order to facilitate designing new ACCase inhibitor insecticides and understanding the mechanism of resistance to currently used ACCase inhibitors, we tried to clone and express *T. urticae* ACCase using different expression systems for further site-specific mutagenesis combined with functional characterization. The most promising results were achieved by a baculovirus expression system and also by heat-shocking Codon Rare *E. coli* cells with the pET-ACC vector. More efforts should be done to overcome several problems in expressing this enzyme to optimize conditions for higher levels of expression, protein solubility, activity and stability.

CHAPTER V

ACARICIDE RESISTANCE AND RESISTANCE MECHANISMS IN *TETRANYCHUS URTICAE* POPULATIONS FROM ROSE GREENHOUSES IN THE NETHERLANDS

This chapter has been submitted as:

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1 INTRODUCTION

Control of the two-spotted spider mite, *Tetranychus urticae* Koch populations mostly relies on the use of acaricides. However, this species rapidly develops resistance to acaricides due to its high reproductive potential, extreme short lifecycle and arrhenotokous reproduction. Genetically fixed resistance mechanisms in spider mites are thought to be similar with those documented in insects and involve enhanced detoxification through the enzymatic activity of esterases, glutathione-S-transferases and P450 mono-oxygenases and/or modification of the acaricide target-site (reviewed in Van Leeuwen et al. 2010a). Especially in greenhouses, the risk of developing resistance is particularly high because of the relative isolation of mite populations, the extended growing season and the intensive use of pesticides (Cranham and Helle 1985). The most threatening situation probably occurs in ornamental horticultural crops like potted plants and cut flowers, where biological control is not well established due to a zero pest tolerance.

Rose is the top cut flower species sold at the Dutch auctions, with more than 0.8 billion euro turnover in 2008 (http://www.flowercouncil.org). In worldwide retail cut flower sales are worth approximately 11 billion US dollar per year (Short and Roberts 1991). Next to the fact that cut flowers are supposed to be pest free, they are also not edible and thus excluded from regulations on pesticide residues, resulting in intensive pesticide use. In rose greenhouses, this pesticide use poses a threat to occupational safety, which is as important as the enhanced risk of resistance development (Tenenbaum 2002). *T. urticae* is one of the economically most important pests on rose. Its quick resistance build up to acaricides is threatening proper rose production in greenhouses.

In the present study the efficacy of 10 commercially important acaricides, applied at different rates to 15 *T. urticae* strains collected at different locations in the Dutch rose cultivation areas was investigated. The selected acaricides included some older compounds with a long history of widespread use (such as pyrethroids and organophosphates), some currently registered and frequently used compounds, and a potential candidate for European registration (cyflumetofen). Many studies in the past have linked an increase in detoxifying enzyme activities to a certain resistant phenotype. In this study we determined the activity of esterases, glutathione-S-transferases (GST) and cytochrome P450 mono-oxygenases (MFO) in 10 selected strains to determine whether or not a correlation exists between the general detoxifying activity and the observed resistance status.

Although the molecular elucidation of target-site resistance in mites has not keep pace with that in insects, recently a number of studies have documented resistance mutations in genes of

major acaricide targets such as acetylcholine esterease (Khajehali et al. 2010, Kwon et al. 2010a), the *para* sodium channel (Tsagkarakou et al. 2009b, Kwon et al. 2010b), glutamate-gated chloride channel (Kwon et al. 2010c) and cytochrome b (Van Leeuwen et al. 2008, Van Nieuwenhuyse et al. 2009, Van Leeuwen et al. 2010b). These genes and their proteins are the target sites of organophosphates, pyrethroids, avermectins and mitochondrial complex III Qo inhibitors, respectively (reviewed in Van Leeuwen et al. 2010a). To estimate the importance of these previously reported mutations, we also amplified and sequenced the relevant gene regions of 10 selected Dutch field strains.

2 MATERIAL AND METHODS

2.1 Strains

The susceptible SAMB strain was initially collected on *Sambucus nigra* (L.) in the Netherlands and obtained from the Centre de Biologie et de Gestion des Populations, Montpellier, France. The German susceptible strain GSS was obtained from Bayer CropScience and originates from Schering AG (Berlin, Germany). It was originally collected from an unknown host in Germany.

Fifteen field strains were collected from rose greenhouses in the Dutch provinces of North Holland, South Holland and Limburg from September 2008 till July 2009 (Figure 5.1). In every location, samples were taken throughout the greenhouse in hot-spots of mite infestation and put together. In order to have sufficient mites to conduct bioassays, the collected mites were manually transferred from the sampled leaves to non-treated potted kidney bean (*Phaseolus vulgaris* L. cv. Prelude) plants and reared for 2-4 weeks in a climatically controlled room at 26±0.5 °C, 60% RH and 16/8 h (L/D) photoperiod. Each strain was tested within 2-4 months after arrival at the laboratory. To test the stability of resistance, four strains were re-examined approximately one year after their arrival in the laboratory.

Species identification and genetic variability between strains was analysed using a large partial sequence of mitochondrial cytochrome oxidase subunit I gene (COI), based on the complete genome sequence (Van Leeuwen et al. 2008). A 1257-bp COI fragment was amplified by PCR using the primers TuCOIF1 and TuCOIR1 (Table 5.1) and sequenced directly with the original PCR primers on both strands at Agowa Sequencing Service (Berlin, Germany).

Figure 5.1 Map of collection sites of *T. urticae* strains from rose greenhouses in the Netherlands

2.2 Chemicals

All tested acaricides were commercial formulations that were kindly provided by Dennis Eekhof (Certis Europe, The Netherlands) and Wim Petersen (Bayer Cropscience, The Netherlands) (Table 5.2). Bifenazate, acequinocyl, tebufenpyrad, milbemectin, abamectin, cyflumetofen, bifenthrin and chlorpyrifos were tested on adult female mites, whereas spiromesifen, hexythiazox and etoxazole were tested on larvae (Table 5.2).

2.3 Bioassays

Toxicity bioassays on adult female mites or newly hatched larvae were performed as previously described (Van Leeuwen et al. 2004) with some modifications. Briefly, 30-40 young adult female mites were introduced to the upper side of 9 cm^2 square cut kidney bean leaf discs placed on wet cotton, after which the disc with mites was sprayed in a Cornelis spray-tower (1.5 ± 0.05 mg aqueous acaricide deposit cm^{-2}). For larval bioassays, 20-30 adult female mites were allowed to lay eggs for 24 hours on the leaf discs, which were sprayed directly after the hatching of the larvae. The treated discs were subsequently transferred to a climatically controlled room and kept at 26±0.5 °C; 60% RH; 16/8 (L/D) photoperiod. Mortality was assessed after 4 days.

For all tested acaricides the field dose (FD), one fifth of field dose (FD/5) and five times of field dose (5FD) were used to discriminate between susceptible and resistant mites (Table 5.2). Three replicates were used per concentration. Concentrations were prepared in de-ionized water. Blank controls were sprayed with de-ionized water only. The mortality data were corrected using Abbott's formula (Abbott 1925). Resistance levels were classified based on the percent mortality (M) as follows: **susceptible** (M > 90% in FD), **intermediate** (90% in FD > M >90% in 5FD) and **resistant** (M < 90% in 5FD). Cross-resistance amongst acaricides was analysed by calculating the correlation coefficient between percentage mortality at the FD. The correlation coefficient was calculated using PROC CORR (SAS Institute 1996).

Table 5.1 Primers and amplified PCR products used for the analysis of the genetic variability and the detection of resistance associated mutations in *T. urticae* strains.

Gene	Fragment size	Mutation	Primers	Sequence (5' – 3')
COI	1306		TuCOIF1	TCAACAAATCATAAAAATATTGGAAC
			TuCOIR1	AAAAATCAGAATATCGTCGAGGT
AChE	579	F or Y331W [A]	AchEdiaF2	TACCTGCCTCTGAATTGGTTGC
			AchEdiaR2	GAACTTCTGCAGGCGTATAACC
Para SC	226	F1538I [B]	KdrF4	CAACATTCAAAGGTTGGACAAT
			KdrR1	TCTTCCGTCATCAACATCTCC
	255	A1215D [B]	KdrF2	TGCATCTCAATTGTCCAAGG
			KdrR2	GTTTCTTCCAGG CAACATGG
	292	L1025V [B]	KdrF5	TGATTGTTTTCCGTGTCCTG
			KdrR5	CTGCGAAGCTGCTTAAGTCC
Glutamate channel	522	G323D [C]	TuGluF1	TGTGCCCTGTTGTATGTTGG
			TuGluR1	AAAATGGCGAAAAGGAAAGG
Cytb	1577	all mutations in Qo site	Cytbdia2F	TTAAGAACTCCTAAAACTTTTCGTTC
			Cytbdia2R	GAAACAAAAATTATTATTCCCCCAAC
Cytb	Sequencing primers		cytbWTF	CGGAATAATTTTACAAATAACTCATGC
			cytbWTR	TGGTACAGATCGTAGAATTGCG
			PEWYF1	AAAGGCTCATCTAACCAAATAGG
			PEWYR2	AATGAAATTTCTGTAAAAGGGTATTC

[A,B,C] *Torpedo* numbering, *Musca domestica* numbering and *T. urticae* numbering, respectively.

Table 5.2 List of tested acaricides tested with their trade names, active ingredient, IRAC[1] classification, tested mite stages, recommended field rates and registration date of the compound.

Product	Active Ingredient	IRAC MOA[2]	Tested Mite Stage	Formulated Field Rate L^{-1}	a.i. Field Rate (ppm)	Registration date
Masai 25%WG	Tebufenpyrad	group 21A	Adults	0.125 g	31.25	January, 1997
Milbeknock 1%EC	Milbemectin	group 6	Adults	0.5 ml	5	September, 2002
Vertimec 1.8%EC	Abamectin	group 6	Adults	0.25 ml	4.5	April, 1988
Danisaraba 20%SC	Cyflumetofen	unknown	Adults	1 ml	200	not registered
Talstar 8%SC	Bifenthrin	group 3A	Adults	0.5 ml	40	August, 1999
Oberon 24%SC	Spiromesifen	group 23	Larvae	0.5 ml	120	October, 2004
Nissorun 25%SC	Hexythiazox	group 10A	Larvae	0.2 ml	50	September, 1987
Borneo 11%SC	Etoxazole	group 10B	Larvae	1 ml	110	September, 2009
Floramite 24%SC	Bifenazate	unknown	Adults	0.4 ml	96	April, 2003
Cantack 16.4%SC	Acequinocyl	group 20B	Adults	1 ml	164	June, 2007
Dursban 48%EC	Chlorpyrifos	group 1B	Adults	1 ml	480	-

[1] Insecticide Resistance Action Committee (www.irac-online.org)
[2] IRAC mode of action classification

2.4 Enzyme assays

The *in vitro* activity of cytochrome P450 mono-oxygenases was quantified by measuring the O-deethylation of 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) while esterase activity was measured with the substrate 4-nitrophenylacetate (4-NPA), as outlined by Van Leeuwen et al. (2005). Glutathione-S-tranferase (GST) activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as described by Van Pottelberge et al.(2009b). Enzyme activities were compared by analysis of variance (ANOVA). Significant differences were assessed using Tukey's method and SAS 6.12 (SAS Institute 1996).

2.5 Detection of resistance associated mutations

Genomic DNA was extracted from approximately 400 female mites per strain as described by Van Leeuwen et al. (2008). Briefly, adult mites were homogenised in a 2 ml Eppendorf tube containing 800 µl SDS buffer (2% SDS, 200 mM Tris-HCl, 400 mM NaCl, 10 mM EDTA, pH = 8.33) followed by DNA extraction using the phenol-chloroform extraction method (Sambrook and Russell 2001). The resulting DNA solution was used as template for PCR carried out in a Biometra TProfessional Thermocycler (Biometra, Germany). Primers for PCR amplification of regions with reported mutation in several target-sites are listed in Table 5.1. PCR reactions were performed in 50 µl containing 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM dNTP mix (Invitrogen, USA), 5 µl 10× PCR-buffer (Invitrogen, USA), 1 µl template

(between 100 and 200 ng/µl) and 1 U Taq DNA polymerase (Invitrogen, USA). PCR was performed under the following conditions for all primer pairs: 2 min at 94 °C, 35 cycles of 20 s at 94 °C, 30 s at 54 °C, 60 s at 72 °C and a final extension of 3 min at 72 °C. Cytb sequences of the strains were obtained as described by Van Leeuwen et al. (2008) performing long-PCR (Expand Long Range PCR kit, Roche, Belgium). All PCR products were purified using the EZNA Cycle-Pure Kit (Omega Biotek, USA) according to the manufacturer's instructions and sequenced directly on PCR product with the original PCR primers at Agowa Sequencing Service (Berlin, Germany). For complete cytb sequencing four additional internal primers were used: cytbWTF, cytbWTR, PEWYF1, PEWYR2 (Table 5.1). The obtained sequence data were analyzed with BioEdit 7.0.1 software (Hall 1999) and mutation frequency was estimated with proportional sequencing (Van Leeuwen et al. 2008). The occurrence of mutations was categorised based on a visual inspection of sequencing chromatographs as: not detected, present and fixed (when no background was detected at the investigated position).

3 RESULTS

3.1 COI amplification, sequencing and analysis

A partial COI sequence of 1257 nucleotides was amplified, sequenced and analysed in 10 of the collected *T. urticae strain*s. Comparison with available COI sequences in public databases (NCBI) identified all strains as *T. urticae*. A total of 6 haplotypes was detected. No insertion or deletions were found in the sequenced region. The number of nucleotide substitutions and nucleotide divergence percentage are presented in Table 5.3. Strain 003 differed from all other strains by 9-12 substitutions (0.72-0.95% divergence), while the sequences of the other strains displayed only zero to four substitutions.

Table 5.3 Number of nucleotide substitutions (below the diagonal) and nucleotide divergence percentage (above the diagonal) calculated from the COI sequences of *T. urticae* strains

	GSS	001	003	005	006	007	008	011	013	020
GSS		0,32	0,80	0,32	0,24	0,24	0,16	0,16	0,24	0,16
001	4,00		0,95	0,00	0,08	0,08	0,16	0,16	0,08	0,16
003	10,00	12,00		0,95	0,88	0,88	0,80	0,72	0,88	0,72
005	4,00	0,00	12,00		0,08	0,08	0,16	0,16	0,08	0,16
006	3,00	1,00	11,00	1,00		0,16	0,08	0,08	0,16	0,08
007	3,00	1,00	11,00	1,00	2,00		0,08	0,24	0,00	0,24
008	2,00	2,00	10,00	2,00	1,00	1,00		0,16	0,08	0,16
011	2,00	2,00	9,00	2,00	1,00	3,00	2,00		0,24	0,00
013	3,00	1,00	11,00	1,00	2,00	0,00	1,00	3,00		0,24
020	2,00	2,00	9,00	2,00	1,00	3,00	2,00	0,00	3,00	

3.2 Resistance levels

The mortality percentages at FD/5, FD and 5FD of 15 strains from rose and two susceptible laboratory strains of *T. urticae* are presented in Table 5.4. The strains originating from different rose greenhouses exhibited various levels of susceptibility to each acaricide. Most strains showed moderate to high levels of resistance to several acaricides. Strains 007, 008 and 032 were highly resistant to at least six acaricides. Hexythiazox resistance was the most widespread since 12 out of 15 strains were highly resistant, followed by tebufenpyrad resistance and bifenthrin resistance (9 out of 15 strains). Only nine strains were treated with chlorpyrifos. They all showed a moderate level of resistance. Strains 003, 007, 008, 013, 028 and 032 appeared to be resistant to the recently introduced acaricide bifenazate while strains 028 and 029 were resistant to acequinocyl.

3.3 Stability of resistance

Table 5.5 displays the susceptibility of strains 007, 008, 022 and 028 to the tested acaricides after one year of laboratory rearing without acaricide exposure. The resistance level was stable for tebufenpyrad and bifenthrin in these four strains, but dropped for most other tested acaricides. In strain 008, resistance levels to all compounds seemed to be stable after one year.

3.4 Resistance correlation

Correlation coefficients between percentage corrected mortality at the FD amongst the tested acaricides are presented in Table 5.6. Except for acequinocyl and chlorpyrifos, there was a significant correlation amongst the tested acaricides. The highest correlation was found between abamectin and milbemectin. Next, mortality percentages at FD between tebufenpyrad, abamectin, milbemectin, bifenthrin and hexythiazox were highly correlated.

3.5 Detoxifying enzyme activities

Detoxifying enzyme activities of 9 field strains and a laboratory susceptible strain are presented in Table 5.7. A significant difference was observed in P450 O-deethylation activity measured by 7- EFC between susceptible and resistant strains. P 450 mono-oxygenase activity in the field strains increased 1.33 (strain 006) to 7.29 (strain 003) times compared to the susceptible GSS. In addition, significant differences in esterase activity were detected in 5 out of 9 strains, while in GST activity was found in 6 out of 9 strains (Table 5.7). Mean general esterase activity peaked in strain 007. GST activity was highest in strain 008.

Table 5.4 Percent corrected mortality (mean ± SEM) at FD/5, FD and 5FD of different acaricides for strains of *T. urticae*. Mortality assessed 2-4 months after populations arrived, White, light grey and dark grey boxes indicate susceptible, intermediate and resistant population(s) to the tested acaricide, respectively.

	strains of *T. urticae*																
Acaricides	**GSS**	**SAMB**	**001**	**003**	**005**	**006**	**007**	**008**	**011**	**013**	**020**	**022**	**025**	**028**	**029**	**032**	**033**
	43±5.2	82±1.6	37±3.1	10±3.1	6±0.7	10±2.7	5±1.4	9±1.5	16±3.3	1±1.2	74±10.3	26±3.5	17±3.9	5±3.4	2±4.9	27±2.3	10±2.3
Tebufenpyrad	100	89±0.9	97±3.5	44±2.2	5±1.5	18±6.7	8±2.8	18±5.2	80±2.7	20±1.9	98±1.3	45±4.6	54±3.7	57±6.1	5±2.6	35±8	29±5.4
	100	100	100	93±1.6	8±2	63±4.7	86±7.1	23±2.9	97±1.4	84±2.5	100	92±6.6	74±0.4	96±1.8	18±9.8	64±3.1	77±7.7
	24±1.4	51±3.8	31±0.6	13±4.8	3±2.4	18±2.3	3±2.3	15±5.4	41±4.6	16±1.6	93±2.1	0	61±3.9	9±0.8	19±1.4	0.1±1.7	60±3.6
Milbemectin	88±0.2	98±1.8	93±1.1	64±1.2	26±4.9	74±4.1	21±3.7	17±1.8	84±1.1	32±3.7	100	74±2.6	92±0.7	60±1.1	76±5.7	73±2.7	95±1.4
	100	100	100	98±1.1	96±2.3	91±3.3	95±0.3	98±1.6	100	94±1.7	100	100±	100	100	100	86±1.2	100
	100	100	98±1.6	43±3.0	4±2.3	46±1.8	5±1.6	14±3	88±1.2	6±0.9	100	35±3.3	81±2.2	19±4.9	2±2.0	42±2.4	64±3.1
Abamectin	100	100	100	57±11.1	4±2	72±5.6	8±1.8	24±4.0	96±0.7	45±2.5	100	80±3.0	95±1.2	100	29±3.9	69±1.2	97±1.9
	100	100	100	91±2.5	34±6.4	95±0.9	60±7.6	61±1.6	100	81±3.7	100	99±1.4	100	100	95±2.9	91±1.1	100
	100	100	100	69±1.4	72±5.3	72±4.0	4±1.8	19±4.9	92±1.5	94±1.3	82±7.4	26±4.7	95±2.9	19±3.1	87±4.6	80±6.7	97±1.3
Cyflumetofen	100	100	100	92±3.9	92±1.3	91±1.3	57±3.4	35±1.1	100	100	98±2.4	56±6.9	98±2.3	47±5.1	97±1.5	95±0.8	98±2.3
	100	100	100	99±1.2	100	98±1.0	89±4.4	84±2.3	100	100	100	96±2.5	100	100	100	97±3.1	100
	28±3.9	93±1.4	23±2.5	14±1.1	4±2.2	8±1.2	12±4.8	13±0.8	48±2.0	6±1.7	84±4.7	16±3.2	10±2.5	51±9.1	27±3.2	0	7±2.5
Bifenthrin	84±3.3	100	72±2.9	26±2.6	7±1.3	11±1.5	26±7.2	16±3.0	87±4.7	27±2.4	97±3.3	41±7.2	52±4.4	94±1.0	39±2.3	12±3.0	44±5.0
	100	100	85±5.7	56±3.6	23±2.3	22±3.1	46±3.1	23±2.2	92±2.4	47±2.6	100	91±2.4	94±1.7	99±1.2	62±3.7	26±4.8	83±2.1
	100	100	100	71±8.8	100	88±2.9	14±3.8	46±5.3	100	71±3.5	100	75±3.9	100	95±1.2	100	97±1.4	84±1.5
Spiromesifen	100	100	100	97±0.5	100	97±0.6	31±3.2	61±7.1	100	88±4.5	100	97±0.9	100	100	100	99±0.9	97±1.7
	100	100	100	99±0.5	100	99±0.7	58±11.8	74±11.6	100	98±2.2	100	99±0.6	100	97±1.9	100	98±2.5	99±1
	100	100	98±1.0	25±4.6	00	13±3.0	00	17±2.5	40±5.3	24±5.3	100	26±6.2	82±2.9	4±1.8	32±1.4	27±3.7	68±1.6
Hexythiazox	100	100	99±1.2	46±5.1	11±3.2	18±5.1	00	26±1.9	70±0.7	29±5.0	100	52±8.6	90±2.3	4±2.2	41±6.5	60±2.3	73±0.5
	100	100	100	64±6.4	11±5.3	13±4.3	7±4.1	24±4.3	64±3.9	28±11.1	100	66±2.7	92±0.8	15±2.6	47±6.0	66±3.3	81±2.0
	100	100	100	96±0.4	89±0.7	93±0.8	96±0.9	4±8.1	95±0.6	99±1.1	100	99±0.8	100	100	100	74±2.1	100
Etoxazole	100	100	100	94±1.0	97±0.9	94±1.1	97±0.6	2±3.5	95±1.4	99±1.3	100	99±0.4	100	100	100	79±3.8	100
	100	100	100	97±2.0	99±0.6	98±1.8	98±0.8	31±11.4	98±1.1	100	100	100	100	100	100	83±2.2	100
	100	100	100	75±7.9	20±1.6	75±9.5	6±0.2	3±3.0	96±1.9	8±6.3.0	99±0.8	40±13.5	100	7±2.1	4±1.8	49±17.2	93±3.8
Bifenazate	100	100	100	90±5.3	99±1.3	97±2.6	50±25.2	10±1.1	100	26±2.0	99±0.7	97±1.8	100	57±8.9	42±15.6	90±2.9	99±0.9
	100	100	100	89±5.7	100	100	18±5.3	7±1.4	100	76±5.2	97±0.7	100	100	74±12.6	92±0.7	87±4.4	99±1
	100	100	100	61±6	30±2.2	27±2.7	3±0.4	28±5.0	100	38±3.5	100	57±12.7	100	1±1.2	11±1.7	69±7.7	85±5.6
Acequinocyl	100	100	100	96±2.1	97±1.3	83±3.1	66±6.9	90±1.6	100	97±1.7	100	73±2.3	100	5±1.1	26±6.9	91±4.8	89±2.8
	100	100	100	100	100	100	99±0.9	100	100	100	100	94±1.6	100	6±1.0	50±4.2	98±1.8	100
	100	83±1.6	45±5.2	19±2.6	53±8.1	25±2.4	15±3.5	50±3.5	25±6.8	8±1.5	0						
Chlorpyrifos	100	100	85±1.3	29±7.7	83±6.0	49±3.0	35±3.2	63±4.8	38±7.3	24±0.8	1±0.5						
	100	100	100	100	100	99±1.2	96±2.3	100	100	100	100						

Table 5.5 Percent corrected mortality (mean ± SEM) at FD/5, FD and 5FD of different acaricides for strains of *T. urticae*. Mortality assessed approximately one year after populations arrived, White, light grey and dark grey boxes indicate susceptible, intermediate and resistant population(s) to the tested acaricide, respectively.

	strains of *T. urticae*			
Acaricides	**007**	**008**	**022**	**028**
Tebufenpyrad	8±4.0	2±2.2	24±2.8	59±3.7
	21±8.9	20±1.6	59±4.0	58±1.62
	62±3.3	57±1.9	71±7.6	92±3.1
Milbemectin	86±4.0	85±1.5	37±4.7	57±3.9
	100	99±1.4	97±2.9	100
	100	100	100	100
Abamectin	100	100	100	100
	100	100	100	100
	100	100	100	100
Cyflumetofen	100	100	100	100
	100	100	100	100
	100	100	100	100
Bifenthrin	12±1.8	6±1.6	58±4.9	13±2.5
	86±3.6	39±4.1	92±1.6	89±3.9
	96±2.1	61±2.2	100	100
Spiromesifen	93±6.8	97±2.1	85±4.2	89±6.3
	99±0.7	97±1.5	91±7.2	85±1.7
	100	100	96±1.8	94±3.5
Hexythiazox	87±1.3	29±1.4	95±1.3	99±0.6
	99±0.3	61±2.3	96±0.4	100
	100	68±2.1	100	100
Etoxazole	100	89±0.9	100	100
	100	89±0.7	100	100
	100	94±1.1	100	100
Bifenazate	100	10±1.8	100	100
	100	49±9.0	100	100
	100	27±10.9	100	100
Acequinocyl	100	38±7.1	100	100
	100	99±0.9	100	100
	100	100	100	100

Table 5.6 Correlation coefficient (r) between percentages of the field dose corrected mortality (field strains) of tested acaricides

	Tebufenpyrad	Milbemectin	Abamectine	Cyflumetofen	Bifenthrin	Spiromesifen	Hexythiazox	Etoxazole	Bifenazate	Acequinocyl	Chlorpyrifos
Tebufenpyrad	1.00										
Milbemectin	0.67*	1.00									
Abamectine	0.80*	0.84*	1.00								
Cyflumetofen	0.23	0.55*	0.26	1.00							
Bifenthrin	0.83*	0.57*	0.71*	0.07	1.00						
Spiromesifen	0.41	0.68*	0.59*	0.58*	0.32	1.00					
Hexythiazox	0.75*	0.82*	0.68*	0.56*	0.50	0.48	1.00				
Etoxazole	0.22	0.46	0.33	0.56*	0.33	0.41	0.17	1.00			
Bifenazate	0.50	0.69*	0.56*	0.52*	0.24	0.57*	0.55*	0.55*	1.00		
Acequinocyl	0.25	0.10	0.07	0.46	-0.20	0.05	0.49	-0.12	0.39	1.00	
Chlorpyrifos	-0.19	-0.23	-0.26	-0.15	-0.36	0.06	-0.16	-0.24	0.08	0.06	1.00

* Values are significantly different from zero ($p<0.05$)

Table 5.7 Detoxification enzyme activities in different strains (mean ± SEM)

Strain	P450 mono-oxygenases		Esterases		Glutathione-S-transferases	
	O-de-ethylation of EFC[A]	Ratio to GSS	4-NPA activity[B]	Ratio to GSS	CDNB conjugation[C]	Ratio to GSS
GSS	134.66 (± 15.01)c	-	79.23 (± 0.51)d	-	444.12 (± 49.31)e	-
001	314.91 (± 10.57)bc	2.34	79.62 (± 10.23)d	1.00	572.87 (± 16.34)cde	1.29
003	982.22 (± 187.28)a	7.29	116.29 (± 3.22)ab	1.47	532.75 (± 19.62)de	1.20
005	325.20 (± 25.56)bc	2.41	83.11 (± 4.72)cd	1.05	719.03 (± 21.74)ab	1.62
006	179.74 (± 11.70)c	1.33	110.43 (± 1.95)ab	1.39	705.00 (± 17.24)abc	1.59
007	451.72 (± 4.15)bc	3.35	120.61 (± 4.38)a	1.52	783.74 (± 34.61)a	1.76
008	359.65 (± 52.46)bc	2.67	86.60 (± 2.98)cd	1.09	794.47 (± 18.05)a	1.79
011	257.66 (± 34.99)bc	1.91	95.28 (± 3.15)bcd	1.20	636.06 (± 23.09)bcd	1.43
013	571.01 (± 90.42)b	4.24	103.20 (± 3.07)abc	1.30	758.83 (± 25.70)ab	1.71
020	585.76 (± 75.71)b	4.35	115.20 (± 3.70)ab	1.45	514.43 (± 20,77)de	1.16

Means (± SEM) within the same column followed by the same letter are not significantly different (Tukey method, $\alpha = 0.05$).
[A] pmol 7-hydroxy-4-trifluoromethylcoumarin $(30min)^{-1}$ mg^{-1} protein (± SEM)
[B] nmol 4-nitrophenol min^{-1} mg^{-1} protein (± SEM)
[C] nmol glutathione conjugated min^{-1} mg^{-1} protein (± SEM)

3.6 AChE, para SC, Glutamate-gated chloride channel and cytb genotypes

An overview of the detected genotypes is presented in Table 5.8.

The organophosphate resistance allele F/Y331W was detected in all tested field strains. In the *para* sodium channel of *T. urticae*, the F1538I substitution, known to confer strong resistance to pyrethroids, was only detected in stains 005 and 008. Another reported substitution in *para* SC with an unknown function (A1215D) was present in 6 out of 9 tested field strains, and already fixed in 4 strains (003, 005, 007 and 013). None of the tested strains harboured L1025V (according to *Musca domestica* numbering) mutation reported by Kwon et al (2010b).

The putative abamectin resistance mutation, G323D, in the *T. urticae* glutamate-gated chloride channel was not detected in any strain.

Several point mutations at conserved regions in the mitochondrial cytb previously linked with bifenazate and acequinocyl resistance were detected (Van Nieuwenhuyse et al. 2009). Strains 001, 003, 005, 007, 008 and 013 harboured the G126S mutation and this mutation was fixed in the strains 007, 008 and 013. In strain 008 mutation G126S was combined with mutation I136T. This combination, which was fixed, is known to confer high resistance to bifenazate.

A P262T mutation was found in strain 003, but was not fixed. No amino acid substitutions in the cytb at positions 141 and 161 were detected in any strain.

Table 5.8 Amino acid substitutions in different strains of *T. urticae*

Strain	Acetylcholine esterase Genotype[A]	*para* sodium channel Genotype[B]			Glutamate-gated chloride channel[C]	cytochrome b residues at different positions[C]				
	331	1538	1215	1025	323	126	136	141	161	262
GSS	F(Y)	F	A	L	G	G	I	S	D	P
001	F(Y)/W	F	A	L	G	G/S	I	S	D	P
003	F(Y)/W	F	D	L	G	G/S	I	S	D	P/T
005	W	F/I	D	L	G	G/S	I	S	D	P
006	F/W	F	A	L	G	G	I	S	D	P
007	F/W	F	D	L	G	S	I	S	D	P
008	F/W	F/I	A/D	L	G	S	T	S	D	P
011	F(Y)/W	F	A/D	L	G	G	I	S	D	P
013	F/W	F	D	L	G	S	I	S	D	P
020	F/W	F	A	L	G	G	I	S	D	P

[A,B,C] *Torpedo* numbering, *Musca domestica* numbering and *T. urticae* numbering, respectively.

4 DISCUSSION

4.1 Resistance status

Since about 5 years, many Dutch rose growers have reported to be unsatisfied with the decreased efficacy of the acaricides they use for spider mite control in their greenhouses. Since problems in the chemical control of mites can be caused by many reasons which are not related to resistance, including operational factors such as an incorrect spray technique, the use of tank mixes and a wrong application time, the main aim of this study was to separate facts from fiction regarding spider mite resistance in Dutch rose culture. Unfortunately, the results revealed high levels of resistance to some acaricides in almost all tested strains collected on cut roses from different locations in the Netherlands (Table 5.4). Although monitoring of resistance is usually based on calculating resistance ratios or by comparing LD_{50}'s or LD_{90}'s, mainly due to the heavy workload, we surveyed the resistance status in the Dutch rose greenhouse strains of *T. urticae* using three discriminating doses. This type of

diagnostic test can be more efficient than dose-response curves, especially for detecting an initial resistance outbreak (Roush and Miller 1986), since the presence of resistant individuals at low frequency will not greatly influence calculated LC50 values, but can be detected easily when testing a discriminating dose such as FD or 5FD.

The most striking and prominent result is the presence of resistance to hexythiazox (12 out of 15 strains), bifenthrin (9 out of 15 strains) and tebufenpyrad (9 out of 15 strains), which is now confirmed in the laboratory. These acaricides have been used since long (at least 10 years) in Dutch horticulture (Table 5.2), and it is clear that at the time of collection for this study, their efficacy has become very low. The observed intermediate and (mainly) high resistance to these compounds probably reflects prolonged selection on isolated greenhouse populations. Also, the activity of abamectin and milbemectin seemed to have decreased in many strains and might become problematic in the near future. Abamectin has also been registered for more than 20 years, and many strains that developed resistance to abamectine might show cross resistance to the more recently introduced milbemectin. Bifenazate is probably one of the most popular acaricides amongst rose growers at this time, due to its compatibility with natural enemies of spider mites and the complete lack of phytotoxicity. Hence, the occurrence of resistance in Dutch horticulture has been previously documented (Van Leeuwen et al. 2008, Van Nieuwenhuyse et al. 2009). In the present survey, 6 out of 15 strains proved to be resistant to bifenazate, while most other strains remained fully susceptible. The quite recently introduced compounds spiromesifen, etoxazole and acequinocyl showed the best activity in our study, although resistance was also already detected in some strains. Next to the differences in resistance amongst acaricides, we could also observe significant differences between strains were in place. Two strains (007 and 008) were resistant to almost all tested acaricides, including cyflumetofen, a new acaricide with new mode of action that has never been used in Europe. Similarly, these two strains were also resistant to spiromesifen, a recently registered molecule in Dutch rose culture, and possibly a common mechanism is responsible for such a broad cross-resistance in these two strains. Finally, also strain 032 was found to be resistant to 6 out of 15 compounds, including both older (tebufenpyrad, bifenthrin and hexythiazox) and more recent (etoxazole, bifenazate) compounds.

4.2 Resistance mechanisms

In previous studies, the occurrence of resistance in *T. urticae* has been linked with various resistance mechanisms, for the bigger part with an increased detoxification by metabolising enzymes such as P450 mono-oxygenases, esterases and glutathione-S-transferases and, more

recently, with point mutations in acaricide target proteins (reviewed in Van Leeuwen et al. 2010a). The elucidation of resistance mechanisms might also help in explaining cross-resistance or the observed correlation between acaricide resistance patterns. In this study, we first quantified the activity of detoxifying enzymes in a selection of 10 strains, in an attempt to establish a link between the activity of these enzymes and the observed resistance status of a certain strain. The results in Table 5.7 indeed reveal that all field strains have an increased metabolic activity when compared to the susceptible strain. However, there was no clear correlation between overall activity and resistance status. Of all tested strains, strain 020 was the most susceptible, but it shows one of the most prominent increased P450 mono-oxygenase and esterase activities, comparable to or higher than the activities measured in the multi-resistant strains 007 and 008. The lack of clear correlation between detoxifying enzyme activity and resistance levels yet again clearly stresses the need for synergism tests. Only controlled experiments assessing the effects of synergists on metabolic enzymes carried out on both susceptible and resistant strains, can correlate a certain resistant phenotype to the involvement of one or several of these enzyme systems. Our results reveal that the determination of detoxifying enzyme activities without synergism tests has probably limited predictive value for resistance development and monitoring in spider mites.

The molecular mechanisms of organophosphate target-site resistance are well understood in insects and several point mutations resulting in an insensitive AChE have been documented (reviewed in Oakeshott et al. 2005a). In the spider mite *T. urticae*, the F331W substitution of AChE1 has been detected in strains from Japan and Europe. Although other mutations might play a role in resistance, such as G119S, A201S, A280T and G328A (Khajehali et al. 2010, Kwon et al. 2010d), the F331W mutation has been functionally characterized in insects and was shown to drastically reduce the sensitivity of OP compounds to AChE1 (Oh et al. 2006). We screened 10 *strain*s for the presence of this substitution on genomic DNA and found that all field strains harboured this mutation, while it was not present in the susceptible GSS (Table 5.8). The presence of this mutation probably attributes to the general low activity of the OP compound chlorpyrifos in all strains. However, the situation might be more complex, since extensive gene duplication, with each copy carrying a different mutation, has also been reported (Kwon et al. 2010a). OPs are currently not registered anymore for spider mite control, but until recently, they played an important role in insect control, so mites can still get into contact with the compounds. An insensitive AChE might also have more indirect consequences in current mite control. It was shown that the use of OPs can antagonize

bifenazate efficacy in laboratory conditions, and that this risk increases when an insensitive AChE is present (Van Leeuwen et al. 2007, Khajehali et al. 2009).

Recently, a F1538I mutation in domain III segment 6 of the *para* sodium channel was found to be associated with high bifenthrin resistance levels in *T. urticae* (Tsagkarakou et al. 2009b). The same mutation was previously identified in the sodium channel of pyrethroid resistant *Boophilus microplus* (He et al. 1999) and has been functionally characterized and expressed in *Xenopus laevis* oocytes. It is considered as one of the most effective resistance loci for synthetic pyrethroids up to date (Tan et al. 2005). Another point mutation, L1025V, was reported to elicit high resistance to fenpropathrin in a South-Korean strain of *T. urticae* (Kwon et al. 2010b). Remarkably, both reported mutations were accompanied by an A1215D substitution located in the intracellular linker between domains I and II, but its role is still unknown. We amplified and sequenced the corresponding regions for these mutations in 9 Dutch field strains resistant to bifenthrin. Only two strains harboured the F1538I resistance mutation, but it was not fixed, while the L1025V mutation was not detected in any strain. These two strains (005 and 008) showed, together with strain 006, the highest levels of resistance to bifenthrin. Selection with bifenthrin under field conditions could increase the frequency of this mutation and lead to a more pronounced resistance phenotype. Interestingly, the A1215D mutation was detected in 4 out of 10 strains, fixed in 2 strains and it occurred in combination with the F1538I substitution. Although this might be a coincidence, the involvement of this substitution should be better studied. However, since we were not able to detect any of the reported *para* sodium channel mutations in the majority of the strains, it seems unlikely that an altered target-site is the main cause of bifenthrin resistance in Dutch field strains. High bifenthrin resistance was previously linked to an increased esterase hydrolysis mechanism in a Belgian field collected strain (Van Leeuwen et al. 2005, Van Leeuwen and Tirry 2007), and this could well be the most common mechanism of bifenthrin resistance.

The molecular mechanisms of abamectin resistance were recently investigated by Kwon et al. (2010c). It was shown using isogenic lines that a G323D mutation in one of the transmembrane regions of the glutamate gated chloride channel was tightly linked to resistance. Although most strains under investigation in this study displayed a decreased abamectin and milbemectin susceptibility, and 4 strains were found to be highly resistant, the reported G323D mutation was not detected in these strains. This indicates that alternative mechanisms are in place, possibly including increased metabolism as was previously reported (Stumpf and Nauen 2002).

Bifenazate resistance has been linked to mutations in the mitochondrial cytochrome b gene in a number of studies (Van Leeuwen et al. 2008, Van Nieuwenhuyse et al. 2009, Van Leeuwen et al. 2010b), which led to the hypothesis that bifenazate acts as a Qo inhibitor of complex III (Van Leeuwen et al. 2008). We amplified and sequenced the complete cytochrome b of 10 strains, of which 4 displayed resistance to bifenazate. These four strains (003, 007, 008 and 013) all contained point mutations at the suspected positions: G126S + P262T (non fixed), G126S (fixed), G126S + I136T (fixed) and G126S (fixed), respectively. All other strains were found to be susceptible to bifenazate and showed the wild-type cytb genotype. Strain 008 was clearly the most resistant (Table 5.4) and the only strain where the combination of G126S + I136T was fixed, as no background in sequencing chromatographs was detected. It was previously reported that a combination of at least two mutations in the cd1-helix is necessary for a potent resistant phenotype (Van Leeuwen et al. 2008) and this seems to be reconfirmed in this study. Although it is clear that there is a correlation between mutations in the cytb and bifenazate resistance, our dataset also supports the presence of additional mechanisms responsible for decreased bifenazate efficacy. Bifenazate toxicity at 5FD in strain 007 is very low, while this strain harbours a single G126S (without S141F) mutation that confers only limited resistance to bifenazate, as was previously shown (Van Leeuwen et al. 2008).

4.3 Resistance correlation, cross-resistance and stability of resistance

For an effective resistance management strategy, insights into cross-resistance amongst compounds are essential. Several reports document potential cross-resistance amongst acaricides in *T. urticae* (reviewed in Van Leeuwen et al. 2009). Cross-resistance can have different underlying mechanisms. Most common is a shared target-site, or cross reactivity of an increased detoxifying enzyme. A possible approach to detect cross-resistance patterns is to study the correlation between acaricide resistance patterns. It is then assumed that the possibility of cross-resistance is higher when the correlation coefficient between acaricides is higher, as stated by Koh et al. (2009). High correlation coefficients were found between tebufenpyrad and abamectin (0.80), bifenthrin (0.83), and hexythiazox (0.75). Although cross-resistance between some of these compounds has been previously reported, such as cross-resistance between METI acaricides and pyrethroids (Devine et al. 2001, Koh et al. 2009), the underlying mechanism has not been found. A partial explanation of the high correlation coefficients may be that all these compounds have been in use for 10-15 years in rose greenhouses. The observed correlation might reflect the long period for resistance selection of each chemical separately. Hexythiazox is an ovo-larvicide and growers have mixed it frequently with other acaricides for more than 10 years, which has apparently led to the

complete loss of efficacy. More puzzling is the pronounced resistance to tebufenpyrad in most strains, since this acaricide has never been popular amongst rose growers. However, several other studies have documented that a limited use of tebufenpyrad can lead very quickly to resistance, e.g. in apple orchards in Australia (Herron and Rophail 1998) and hops in England (Devine et al. 2001). This resistance is usually based on metabolic detoxification and linked to an increased P450 activity (Van Pottelberge et al. 2009a). It gives broad cross-resistance to other METIs such as pyridaben, fenpyroximate and fenazaquin. Remarkably, we found that after laboratory cultivation for approximately one year without selection pressure, tebufenpyrad resistance seemed to be stable in all strains, in contrast to hexythiazox, abamectin and bifenthrin resistance. Tested rose strains of *T. urticae* showed the highest and lowest resistance to hexythiazox and the recently registered etoxazole respectively. Despite a nearly similar mode of action, cross-resistance between hexythiazox and etoxazole has not been reported, except for one Japanese population (Asahara et al. 2008). In this study FD corrected mortality of hexythiazox showed the highest correlation coefficient with milbemectin and tebufenpyrad and the lowest with chlorpyrifos and etoxazole. Hexythiazox resistance was reported to be stable by Herron et al. (1993), however, in this study, it appeared to be stable in only one of four tested strains in the absence of selection pressure.

The highest correlation amongst acaricides was found between abamectin and milbemectin. Abamectin was first registered in 1988 and is one of the oldest compounds used in Dutch horticulture. It has remarkably retained some of its efficacy on several strains, although 4 strains proved to be resistant. Milbemectin, a chemically similar molecule belonging to the same mode of action (group 6, IRAC classification) was more recently registered in 2002. The high correlation found between these compounds can be due to similar resistance mechanisms. We did not find evidence that the previously documented resistance mutation G323D in the glutamate gated sodium channel is present in Dutch *strain*s, and it is also currently unknown whether this mutation provides cross-resistance to milbemectin. However, cross-resistance between abamectin and milbemectin has been previously reported (Nicastro et al. 2010) and might be linked to a joint detoxification mechanism, explaining the high correlation between both compounds that were registered with a 15 years interval. In our study, strains resistant to abamectin showed significantly higher GST activities. Also, metabolic resistance has already been documented for abamectin (Stumpf and Nauen 2002).

Another interesting point is the lack of correlation between bifenazate and acequinocyl, both compounds that are thought to act as Qo inhibitors. This study showed that the previously reported resistance mutations do occur in Dutch field strains resistant to bifenazate. However,

the exact combination of two cd-1 helix mutations is important, since it determines the potential cross-resistance risk with acequinocyl. The combination of mutations G126S and S141F that was reported in a laboratory selected resistant strain (Van Leeuwen et al. 2008) leads to a 100-fold cross resistance to acequinocyl (Van Nieuwenhuyse et al. 2009), but this combination was not found in the field yet. In contrast, the combination G126S and I136T previously found in Dutch field strains has no effect on acequinocyl toxicity. This observation is confirmed in this study, since strain 008 is fully susceptible to acequinocyl. The same study revealed that a single P262T substitution in the ef-helix region of the cytb can lead to a limited (50-fold) cross resistance to acequinocyl, on condition that this mutation is fixed in the population. Although this mutation was detected in one strain with decreased bifenazate activity, it was not fixed and it had no effect on the field performance of acequinocyl. Given the low cross-resistance ratios between bifenazate and acequinocyl that were reported for this mutation (Van Nieuwenhuyse et al. 2009), it is doubtful that it could lead to acequinocyl resistance under field conditions. Bifenazate resistance seemed to be unstable in 2 out of 3 resistant strains. Only strain 008 retained bifenazate resistance (Table 5.5) and in this strain the combination of two bifenazate resistance mutations in the cd1 helix were fixed at the time of analysis (Table 5.8). From a resistance management point of view, this might be an important observation, since resistance mutations seem to dilute quickly in an unselected population, possibly due to a fitness cost. Hence, one or two applications of bifenazate in the field per season could probably safeguard the efficacy of bifenazate for a longer period. Given the potential cross-resistance risk with acequinocyl, a combined anti-resistance strategy should be put forward. However, as stated previously, bifenazate is very popular amongst growers that sometimes apply bifenazate more than 10 times in one growing season. The resistance seemed to be generally stable in strain 008, which can be characterized as problematic. It is not clear which mechanism gives such broad cross-resistance, even to chemically not related and not registered acaricides with a new mode of action such as cyflumetofen (Sasama et al. 2007). When looking at the genetic distance between strains expressed as nucleotide divergence of COI sequence, there is no correlation between COI polymorphism, geographical location of sampling and resistance status. The COI gene was shown to be well suited for intraspecific analysis in spider mites because of its high polymorphism within the species in several studies (Navajas et al. 1998, Xie et al. 2006). In comparison to results obtained from a study on the genetic structure of *T. urticae* in China, rather low percentage of divergence was found (< 0.3), except for strain 003 (between 0.72-0.95) which might be recently introduced in the region, possibly by imported plant material.

One could expect that bifenazate resistance could fix a single mitochondrial haplotype resulting in a selective sweep, and that should be detectable in the population and studied by examining the COI polymorphism variation in single mites form different locations.

4.4 Conclusions

Our study revealed that mite populations present in the Dutch rose cultivation area have acquired resistance to many of the acaricides that were used for control of *T. urticae*. Traditional acaricides such as tebufenpyrad, bifenthrin, hexythiazox and abamectin have been in use for a long time, resulting in the presence of high levels or resistance in many strains. Tebufenpyrad resistance was stable up to 1 year after collection from the field (in the absence of selection pressure). Based on these results, it could be advised not to use this compound anymore in the field. Resistance to hexythiazox, abamectin and bifenthrin proved to be generally unstable. These compounds might still remain useful for mite control when integrated in a proper resistance management strategy. The efficacy of the recently introduced acaricides spiromesifen and etoxazole was good, except for a few strains that seemed to be multi-resistant to almost all tested molecules, including cyflumetofen, an acaricide with new mode of action that was never used in Europe. The mechanisms behind such a broad resistance are currently unclear. Bifenazate resistance was reported in some strains and correlated with point mutations in the mitochondrial cytb, but stability tests revealed that reversion to susceptibility is possible when resistance mutations are not fixed in the population, leaving a great opportunity for a well thought-out resistance management program. Although some strains were resistant to the recently introduced active ingredient acequinocyl, which has a similar mode of action as bifenazate, no evidence was found for cross-resistance between these compounds under field conditions. Resistance to abamectin was detected in several populations, and was linked to a decreased activity of milbemectin, a compound with a similar mode of action. Care should be taken when including both compounds in control programs. The activity of detoxifying enzymes was increased in all strains, but our results indicate they are not necessarily predictive for the resistance status of a certain strain.

CHAPTER VI

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Acaricide resistance

The evolution of pesticide resistance in insects and mites has become a major problem worldwide, since it has a major impact on crop yield. Unravelling the resistance mechanisms both at the biochemical and molecular level is useful and necessary to provide essential information for the development of insecticide resistance management (IRM) strategies. Identification of the resistance mechanism may also facilitate the development of alternative chemistry which can lead to insecticides which are not affected by a given resistance mechanism. The main objective of this study was the characterization of mechanisms of acaricide resistance in *T. urticae.* While the original idea was to focus on the most common mechanisms of target site resistance, with time it grew towards a more in-depth approach, including, amongst others, the *in vitro* expression of a target site protein.

Pyrethroids and point mutations in the *para* sodium channel

The widespread development of resistance to pyrethroids **(Chapters II and V)** in the two-spotted spider mite is rendering some of the important and widely used acaricides, such as bifenthrin, ineffective. Pyrethroid resistance mechanisms were investigated in two strains of *T. urticae* from Greece. Both strains were highly resistant to bifenthrin and showed a high degree of cross-resistance to another pyrethroid, fluvalinate. Synergism studies in which bifenthrin and inhibitors of detoxifying enzymes were applied in susceptible and resistant strains suggested that the resistance might be due to a mutation within the target site of pyrethroids rather than to enhanced metabolic detoxification.

In many arthropod pests, pyrethroid resistance due to reduced sensitivity of the neurons is linked to point mutations in the sodium channel genes **(Chapter I)**. Since the *T. urticae para* sodium channel sequence was not available at the time this work was initiated, a number of degenerate primers was chosen based on conserved regions of Acari sodium channel genes. A large cDNA fragment encoding for an 1108 amino acid sequence of the *T. urticae para* sodium channel gene (domains II-IV) was amplified for the first time using a long PCR strategy. The overall similarity of the deduced amino acid sequence from this cDNA fragment to insect species was lower than 50%. This might be a partial explanation for the fact that insects and mites have different sensitivities to pyrethroids (Khambay and Jewess 2005, Dong 2007).

The amino acid sequence comparison between susceptible and resistant strains revealed two amino acid substitutions, F1538I in domain IIIS6 and A1215D within the II/III intracellular

linker region. The F1538I substitution was proposed to play a major role in bifenthrin resistance because this mutation has previously been associated with pyrethroid resistance in the southern cattle tick, *B. microplus* (He et al. 1999) and it has been shown to confer strong insensitivity to a range of pyrethroids when functionally expressed in an *in vitro* assay system (Tan et al. 2005). In addition, F1538I is one of several aromatic residues in the IIIS6 helix that form part of a putative hydrophobic binding site for pyrethroids in a computer-generated 3D model of the housefly sodium channel (O'Reilly et al. 2006, Davies et al. 2008).

The second substitution (A1215D) lies within the domain II-III intracellular linker which is one of the most variable regions of the protein amongst species. There has been no report on any mutation in this intracellular loop that is associated with pyrethroid resistance. Although Kwon et al. (2010b) also found this substitution in a fenpropathrin resistant strain of *T. urticae*, they concluded that it may not be directly involved in resistance. Also, when testing a range of *T. urticae* strains collected from cut roses, this mutation was present in some bifenthrin resistant strains. It is possible that it is a simple polymorphism that is fixed in the resistance allele. Nevertheless, the possibility that this mutation also contributes to the resistance, comparable to the mutations in the I/II linker region in the cockroach sodium channel (Tan et al. 2002a), can not be excluded completely. Also, there is the possibility of the occurrence of additional mutation(s) outside the IIS4-IVS6 gene region which we have analysed in this study, associated with or contributing to the resistance phenotype. Kwon et al. (2010b) reported an L1025V mutation could be linked to pyethroid resistance. However, this mutation was not found in the bifenthrin resistant strains from Greece, nor in the bifenthrin resistant strains collected from cut roses **(Chapter V)**.

The mode of inheritance of bifenthrin resistance was determined to be incompletely recessive, which is in agreement with studies of sodium channel insensitivity in other species (Halliday and Georghiou 1985, Sayyed et al. 2005). The recessive mode of inheritance means that the trait will be mainly expressed in homozygous individuals. Our results on the mechanism and inheritance of the pyrethroid resistance in the two-spotted spider mite allow the early detection of heterozygotes, using the F15381I substitution as a prime target for developing a DNA-based screening method for pyrethroid resistance in field populations of *T. urticae*. This could of course be important in pyrethroid resistance management.

Resistance to organophosphates and carbamates

In 2007, Van Leeuwen et al. reported on the antagonism between OP and bifenazate in laboratory experiments. These findings encouraged us to evaluate this antagonism in a more realistic field situation. We found that mixtures of the OP chlorpyrifos with bifenazate, when applied on OP resistant strains (which can tolerate higher doses of OP and CARB insecticides), were a major risk for bifenazate control failure, due to antagonism. However, all other tested OP and CARB insecticides did not cause any antagonism when mixed with bifenazate. It could be concluded that mixing OP and CARB insecticides with bifenazate can result in an antagonistic effect, depending on the OP and CARB type, as well as the mite strain. OP resistant mites seem to be a greater risk for antagonism-based control failure, since they survive OP treatments targeting other pests, while the OP insecticides in the meanwhile compromise bifenazate activation.

In order to elucidate the molecular basis of OP resistance, the characteristics of resistance were studied in three OP resistant strains of *T. urticae.* Biochemical experiments confirmed that the AChE insensitivity is probably the major resistance mechanism, since detoxification metabolism mediated resistance only plays a minor role in OP resistance. The acetylcholinesterase gene AChE1 from both organophosphate-resistant and susceptible strains has been fully cloned and sequenced. Comparison of the deduced amino acid sequences revealed four amino acid substitutions in the mature AChE1 that are present in one or more resistant strains and absent in the susceptible ones. The F331W substitution occurred consistently in all resistant strains. Since the 331 position in AChE1 has been associated with OP-resistance in many species and since the functional expression has clearly illustrated the effect of the substitutions on AChE sensitivity (Oh et al. 2006), it seems plausible that the F331W substitution reduces the enzyme sensitivity and results in OP resistance. This was further supported by the presence of this mutation in all strains collected from roses **(Chapter V)** that showed a moderate resistance level to chlorpyrifos. Two other substitutions, A201S and G328A, may have been selected by the application of other unknown OPs in the history of the tested populations. It is possible that different mutations are selected and maintained in populations of *T. urticae*, each one conferring an advantage against one or more OP or CARB insecticides. One hypothesis can be that these mutations might reduce the fitness cost associated with the F331W substitution, thereby allowing its persistence even in the absence of OP pressure. However, the situation might be more complex, since extensive gene duplication, with each copy carrying a different mutation, has also been reported in *T. urticae* AChE (Kwon et al. 2010a). Since the early detection of insecticide resistance is a prerequisite

for resistance management strategies, and since this mutation was consistently present in all resistant spider mite strains from different geographical regions, including the strains collected from rose greenhouses, we set up a simple and rapid molecular diagnostic method for the detection of the F331W mutation. This tool can support us in the quick and early detection of OP resistance in *T. urticae* in the field

Resistance to spirodiclofen and ACCase expression

In order to investigate the inheritance, biochemical and molecular mechanisms of resistance to the recently introduced compound spirodiclofen in *T. urticae*, a laboratory selection was set up, resulting in the SR-VP strain, which showed a resistance ratio of 274 **(Chapter IV)**. Although it has been hypothesized that selection for resistance in the laboratory and in the field would result in different forms of pesticide resistance, in reality there are several cases where similar resistance mechanisms occurred in laboratory- and field-selected strains (Pedra et al. 2004, Van Leeuwen et al. 2008, Van Leeuwen et al. 2010a).

Biochemical analysis using synergist experiments and *in vitro* enzyme assays showed that the elevated levels of P450s, esterases and glutathione *S*-transferases in the resistant strain could be involved in the metabolic detoxification of spirodiclofen. The higher level of esterases in the resistant strain, when measured with the purpose-designed substrate 1-naphthyl 2,2-dimethylbutyrate, indicated that possibly only a few esterases of *T. urticae* are more expressed in spirodiclofen resistant strains. This finding was further supported when esterase isozymes were separated by native isoelectric focusing. Since in the resistant strains only the mobile stages were resistant, while the eggs remained more susceptible, expression of the detoxifying genes should be different in eggs, compared to other stages. In order to find candidate genes whose expression is different between these stages, the next goal is to construct a microarray containing fragments from several hundreds of genes which are putatively involved in insecticide metabolism.

The results of the crossing experiments suggested that resistance to spirodiclofen in this *T. urticae* strain is controlled by polygenic and intermediate inherited factors. Further research on the genetics of spirodiclofen resistance in field strains and its possible fitness costs is essential to achieve a better understanding of this phenomenon and its implications in spider mite control.

Next to the metabolic detoxification, the existence of an altered target site (acetyl-CoA carboxylase, ACCase) was also investigated. We therefore used a resistant field strain as well as the laboratory selected strain. Sequence analysis of ACCase showed no amino acid

substitution between the spirodiclofen resistant and susceptible strains of *T. urticae*. Moreover, RT-PCR analysis of expression of this gene revealed no up-regulation in the resistant strains. Western blot analysis also showed no difference in the biotin-binding proteins in *T. urticae* deutonymphs of resistance and susceptible strains. It can be concluded that the resistance in the tested strains was not due to an overexpression or structural change in *T. urticae* ACCase, the suggested target site for spirodiclofen.

Since the ACCase is a new and interesting target, a complete understanding of the structure and function of this enzyme in *T. urticae* is necessary to screen effective and specific acaricides and to prevent or manage resistance to currently used ACCase inhibiting acaricides. Obtaining sufficient quantities of purified *T. urticae* ACCase from living mites for enzymatic studies proved to be very difficult, so there was a need to produce recombinant enzymes. To develop an optimal protocol to express *T. urticae* recombinant ACCase, we assembled a full-length cDNA inside three vectors, pMIB/V5-His B, pEIA and pET and also into pFastBac shuttle vector for expression in baculovirus. The most promising results were achieved using a baculovirus expression system and also the heat-shocking Codon Rare *E. coli* cells with the pET vector. Preliminary enzyme assays confirmed that the ACCase expressed in Sf9 cells infected with recombinant baculovirus proved to be active. More efforts are needed for high-level recombinant expression of active full-length *T. urticae* ACCase. When the enzyme is available in higher quantities, it will be possible to characterize it in terms of its kinetic properties and its inhibition by spirodiclofen. Also by using these expression systems it will be possible to investigate whether mutations associated with resistance to ACCase-inhibiting herbicides in various weed species can also change biochemical properties of *T. urticae* ACCase and confer resistance to ACCase-inhibiting insecticides/acaricides.

Acaricide resistance in rose greenhouses

The effectiveness of 11 acaricides was tested on 15 Dutch rose greenhouse strains of the two-spotted spider mite. Instead of dose-response bioassays, we used three doses: FD/5, FD and 5FD. This approach can be more efficient than dose-response curves for detecting an initial resistance outbreak (Roush and Miller 1986). The selected doses were useful to create a general overview of the resistance status since they provided more than 99% mortality in the susceptible strains.

Toxicity tests revealed that most strains have different levels of resistance to tested chemicals. The quantification of detoxifying enzyme activities in a selection of 10 strains showed that all field strains have an increased metabolic activity compared to the susceptible strains.

However, when using only enzyme activities, no clear correlations were found between overall activity and the resistance status of the strains **(Chapter V)**. This fact clearly points out the need that synergism experiments are needed to be able to draw sound conclusions.

Two strains showed high levels of resistance to most tested acaricides, even against cyflumetofen, a novel selective acaricide which has not been registered in Europe. The majority of the tested strains showed resistance to acaricides which have been in use for more than a decade, including hexythiazox (12 out of 15 strains), bifenthrin (9 out of 15 strains) and tebufenpyrad (9 out of 15 strains). In order to check for target site resistance, we looked for amino acid substitutions known to be involved in resistance to different groups, in different strains. The corresponding regions for three mutations in the *T. urticae para* sodium channel, the target site of pyrethroids, were amplified and sequenced in nine Dutch field strains. Only two bifenthrin resistant strains harboured the F1538I resistance mutation **(Chapter II)**, but it was not fixed yet. The L1025V mutation reported by Kwon et al. (2010b) was not detected in any strain. Although the role of A1215D mutation in the resistance to pyrethroids has not been confirmed (Tsagkarakou et al. 2009b, Kwon et al. 2010b), it was detected in 4 out of 10 Dutch field strains and showing that the involvement of this substitution in resistance should be better studied.

All tested strains exhibited reduced susceptibility to chlorpyrifos. This could easily be linked to the presence of F331W mutation in all these strains. Although other mutations might play a role in resistance (i.e. G119S, A201S, A280T and G328A), the F331W mutation **(Chapter III)**has been functionally characterized in insects and was shown to drastically reduce the sensitivity of AChE to OP compounds (Oh et al. 2006). As was already mentioned, the use of OP compounds can antagonize bifenazate efficacy in laboratory conditions, and this risk increases when an insensitive AChE is present **(Chapter III)**.

Abamectin and milbemectin exhibited reduced activity in many strains and it can lead to future problems. Abamectin resistance has been reported to be linked to a G323D mutation in the glutamate gated chloride channel but this mutation was not detected in the abamectin resistant strains.

Six out of 15 strains were resistant to bifenazate, while nine strains remained fully susceptible. All tested bifenazate resistant strains contained point mutations in the mitochondrial cytochrome b gene, while bifenazate susceptible strains showed the wild-type cytb genotype. This data again show the correlation between mutations in the cytb and bifenazate resistance, as described earlier (Van Leeuwen et al. 2008, Van Nieuwenhuyse et al. 2009). It also supports the presence of additional mechanisms responsible for decreased

bifenazate efficacy. Although some strains were resistant to the recently introduced acequinocyl, a compound which has a similar mode of action as bifenazate, there was no evidence for cross-resistance between these compounds under field conditions.

The efficacy of the recently introduced acaricides spiromesifen and etoxazole was still good, with the exception of a few strains that seemed to be multi-resistant to almost all tested molecules including cyflumetofen. The mechanisms behind such a broad resistance are currently unclear.

Widespread occurrence of acaricide resistance and also cross/multiple resistance in *T. urticae* strains collected from rose greenhouses in the Netherlands, demands for accurate resistance monitoring and thoughtful and systematic resistance management programs to prevent the loss of effective acaricides that can contribute to controlling spider mites.

Perspectives for future research

To elucidate mechanism of resistance to pyrethroids, organophosphate and carbamate acaricides, sequence characteristics of the target genes of susceptible and resistant strains were compared and several amino acid substitutions were identified. Some substitutions have previously been proved to confer resistance, but the role of other mutations in the resistance trait is not clear and needs to be clarified. Functional expression of these substitutions in an *in vitro* assay system can demonstrate their possible role in the *para* sodium channel insensivity or AChE insensivity. Determination of the number of origins of the resistance-associated mutations will be useful in modeling their spread and in designing resistance managing programs. Sequence phylogeny from an exon (which contains the resistance associated mutation) and part of a flanking intron of a wide range of globally collected resistant and susceptible strains can be explored in finding the number of origins.

Spirodiclofen resistance in *T. urticae* was documented to be mediated primarily by increased detoxifying enzyme activities. Since the *T. urticae* genome data now are available, the specific detoxifying genes can be identified and their expression followed with microarray analysis. A pharmacokinetic approach can also be explored to clarify exclusively the spirodiclofen metabolism in susceptible and resistant strains. Further investigation into field resistant strains, with possible target site mutation(s), will be helpful in the clarification of spirodiclofen resistance. The baculovirus expression system for *T. urticae* ACCase developed in this study needs to be optimized (time after infection, cell density at infection, MOI, extraction protocol) but can possibly in the future be used to check the effect of field reported mutations on inhibitor binding.

In conclusion …

The results obtained in this research raise some more tips of the veil and may collectively lead to better understanding of acaricide resistance mechanisms in *T. urticae*, leading to more efficient resistance management programs.

Future investigations should focus on the biochemical and molecular mechanisms of multiple resistance commonly observed in *T. urticae* field strains coming from glasshouse growing areas. Utilizing high-throughput genomic strategies, further dissecting the whole genome sequence and proteomic data of susceptible and resistant *T. urticae* strains will further elucidate mechanisms of resistance to acaricides in the two-spotted spider mite that hopefully will result in a long-lasting resistance management strategy for the established and new acaricides.

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SUMMARY

The two-spotted spider mite *Tetranychus urticae* is an economically important pest in many agricultural crops worldwide. Its high reproductive potential and extremely short life cycle, combined with the frequent acaricide applications usually required to maintain the population below economic threshold, facilitates rapid resistance build-up. This has led to the development of resistance against almost all commercially used compounds. In this study we tried to unravel the mechanisms behind the resistance against a number of acaricides with different mode of action at the toxicological, biochemical, genetic and molecular level.

In a first series of experiments, we investigated pyrethroid resistance in Greek *T. urticae* strains. Combined data from toxicological bioassays and biochemical and synergistic studies indicated that although enhanced P450 mono-oxygenase activities were associated with resistance, target site insensitivity proved to be the major resistance component. In order to get a better insight in the latter, a 3.3 kb cDNA fragment of the *T. urticae para* sodium channel gene encompassing segment 4 of domain II to segment 6 of domain IV was obtained by a degenerate PCR strategy. The *T. urticae* sequence showed highest identity (56%) to the scabies mite, *Sarcoptes scabiei*, and was phylogenetically classified within the divergent group of Arachnida. Comparison of resistant and susceptible strains identified the point mutation F1538I in segment 6 of domain III, which is known to confer strong resistance to pyrethroids, along with a second mutation (A1215D) in the intracellular linker connecting domains II and III, which still has an unknown role. Also, three alternative splicing variants were identified, of which two correspond to the mutually exclusive k/l exon pair already identified in insects. The mode of inheritance of resistance was confirmed to be almost completely recessive, which is consistent with studies on the target site mechanism for pyrethroids in other organisms.

In *T. urticae*, organophosphate and carbamate resistance is often caused by acetylcholinesterase insensitivity. By combining toxicological, biochemical and molecular data from three reference laboratory and three organophosphate resistant (OP) strains, the AChE1 mutations associated with resistance in *T. urticae* were characterised. The resistance ratios of the OP strains varied from 9 to 43 for pirimiphos-methyl, from 78 to 586 for chlorpyrifos, from 8 to 333 for methomyl and from 137 to 4164 for dimethoate. Compared to the reference strains, the insecticide concentration needed to inhibit 50% of the AChE1 activity in the OP strains was at least 2.7, 55, 58 and 31 times higher for the OP pirimiphos-methyl, chlorpyrifos oxon, paraoxon and omethoate respectively, and 87 times higher for the carbamate carbaryl. By comparing the AChE1 sequence between laboratory and OP strains, four amino acid substitutions were detected: (1) F331W which was present in all three OP

strains; (2) T280A found in the three OP strains but not in all clones; (3) G328A, found in two OP strains; (4) A201S found in only one OP strain. F331W, G328A and A201S are possibly involved in resistance to organophosphate and carbamate insecticides. These findings were also confirmed by means of a structural model. F331W is probably the most important and the most common in *T. urticae*. It can be easily detected by a diagnostic PCR-RFLP assay developed in this study.

We also evaluated the possible antagonism of organophosphate and carbamate insecticides on the toxicity of bifenazate in *T. urticae* when applied in mixtures. Bifenazate, a new and frequently used carbazate, is a pro-acaricide which needs to be activated by carboxylesterases. Two OP strains were used and several organophosphate (chlorpyrifos, azinphos methyl and phosmet) and carbamate (carbaryl and methomyl) insecticides were evaluated. Mixing chlorpyrifos with bifenazate strongly decreased bifenazate toxicity in both tested strains. However, in the strain with a higher esterase activity, antagonism decreased after 2 days. Of all other tested chemicals, only methomyl displayed an antagonistic effect 1 day after treatment. These findings indicate that mixing organophosphate and carbamate insecticides with bifenazate may inhibit bifenazate efficacy under field conditions, especially when resistant strains are present.

Spirodiclofen, a recently developed and commercialized acaricide, is a selective, non-systemic tetronic acid derivative. In order to develop strategies to minimise resistance in the field to spirodiclofen, we selected a *T. urticae* population for spirodiclofen resistance in the laboratory. This selection yielded a strain with a resistance ratio of 274, determined on the larval stage. This strain was used to determine genetic, toxicological, biochemical and cross-resistance data. The egg stage of the resistant strain remained far more susceptible than the mobile stages. No cross-resistance was found against other acaricides, except for spiromesifen, another tetronic acid derivative. Based on synergist experiments and enzyme assays, it appeared that especially P450 mono-oxygenases, but also esterases and glutathione-*S*-transferases, could be involved in the metabolic detoxification of spirodiclofen. Pre-treatment of the resistant females with the synergists PBO or DEF could increase the inhibitory effect of spirodiclofen on reproduction, again demonstrating the possible involvement of mono-oxygenases and esterases. Among the esterases of *T. urticae*, probably only a few are more expressed in spirodiclofen resistant strains, and these apparently prefer the purpose-designed substrate 1-naphthyl 2,2-dimethylbutyrate. This was also confirmed by separating esterase isozymes by native isoelectric focusing. Because spirodiclofen interferes with lipid biosynthesis, total lipid content was measured in female adults. No significant

differences between treated and non-treated female adults were found, both in the susceptible and resistant strain. However, the total lipid content in the resistant females was significantly higher than in susceptible females. The genetic analysis using crossing experiments showed that the resistance is inherited as an intermediate trait under control of more than one gene. Resistance to spirodiclofen in the laboratory selected strain exceeded by far the recommended field rate, showing the capacity for quick resistance development in the field. A good acaricide resistance management programme is necessary to prevent fast resistance build-up in the field. However, without selection pressure, resistance tends to be unstable and can decrease in the presence of susceptible individuals owing to the intermediate, polygenic inheritance mode.

No evidence was found to support the existence of other resistance mechanisms, like mutations in the target site (ACCase) or overproduction of the target enzyme. In order to facilitate designing new ACCase inhibitor insecticides and understanding mechanism of resistance to currently used ACCase inhibitors, we tried to clone and express *T. urticae* ACCase using different expression systems. The most promising results were achieved by a baculovirus expression system and also by heat-shocking Codon Rare *E. coli* cells with the pET-ACC vector. More efforts should be done to overcome several problems in expressing this enzyme e.g. by optimizing conditions to get higher levels of expression, protein solubility, activity and stability.

We also performed a practical screening in Dutch cut rose glasshouses due the fact that growers complained on failing spider mite control. In order to check whether control failure was caused by resistance, the susceptibility of 15 strains, sampled from infested roses, was tested to 11 acaricides: tebufenpyrad, milbemectin, abamectin, cyflumetofen, bifenthrin, spiromesifen, hexythiazox, etoxazole, bifenazate, acequinocyl and chlorpyrifos. Three doses were used to discern between resistance and susceptibility: the recommended field dose (FD), FD/5 and FD x 5. Ten of these strains were screened for known resistance mechanism such as elevated detoxifying enzyme activities and previously reported resistance mutations using biochemical and molecular diagnostics. Potential cross-resistance between acaricides was estimated by correlation analysis. The strains showed different levels of resistance to tested acaricides. Twelve out of 15 strains showed resistance against hexythiazox, and 9 out of 15 against bifenthrin and tebufenpyrad. Two strains were found which showed high levels of resistance to most tested insecticides, even against one which has not been registered in Europe. A high correlation was found between some acaricides, e.g. between abamectin and milbemectin. Without selection pressure, resistance seemed to be unstable in most cases, after

one year. All field strains had an increased metabolic activity, compared to the susceptible strains. A number of amino acid substitutions known to be involved in resistance to different groups were present in different strains. The best example was the presence of the F331W substitution in the acetylcholine esterase, which was present in all field strains tested.

The results obtained in this research may all together lead to a better understanding of acaricide resistance in *T. urticae*, which, in turn, may lead to more efficient resistance management programs.

SAMENVATTING

De bonenspintmijt *Tetranychus urticae* veroorzaakt wereldwijd economisch belangrijke schade in een groot aantal land- en tuinbouwgewassen. De combinatie van een groot vermenigvuldigingsvermogen, een zeer korte levenscyclus en frequente acariciden-toepassingen, die noodzakelijk zijn om de populatie onder de economische schadedrempel te houden, werkt een snelle resistentie-opbouw in de hand. Dit heeft geleid tot de ontwikkeling van resistentie tegen bijna alle gewasbeschermingsmiddelen die tegen deze mijten worden gebruikt. In deze studie hebben we geprobeerd om de mechanismen te ontrafelen die leiden tot resistentie tegen een aantal acariciden met verschillende werkingswijze, en dit op biochemisch, genetisch en moleculair niveau.

In een eerste reeks experimenten werd de pyrethroïdenresistentie in Griekse stammen van *T. urticae* onderzocht. De combinatie van toxiciteitsdata, biochemische en synergistische testen toonden aan dat, ondanks het feit dat er een verhoogde P450 mono-oxygenase-activiteit werd gedetecteerd, de belangrijkste oorzaak van de resistentie toch de ongevoeligheid van de inwerkingsplaats was. Om hierin een beter inzicht te krijgen werd met behulp van een gedegenereerde PCR methode uiteindelijk de sequentie van een cDNA fragment van 3.3 kb van het para-natriumkanaal bepaald. Deze sequentie omvat een gebied lopende van segment 4 van domein II tot segment 6 van domein IV. Het partieel geamplificeerde para-natriumkanaal van *T. urticae* vertoonde de hoogste gelijkenis (56%) met de schurftmijt *Sarcoptes scabiei*, en werd fylogenetisch ingedeeld binnen de divergente groep van *Arachnida*. Door vergelijking van resistente en gevoelige stammen werd de puntmutatie F1538I in segment 6 van domein III geïdentificeerd. Van deze mutatie is bekend dat ze een hoge resistentie tegen pyrethroïden veroorzaakt. De functie van een tweede mutatie (A1215D) in de intracellulaire verbindingsdomeinen II en III is tot op heden onbekend. Ook werden alternatieve transcripten geïdentificeerd, waarvan er twee overeen komen met het "mutually exclusive" k/l exonpaar, dat reeds in insecten werd vastgesteld. De wijze van overerving van de resistentie bleek bijna volledig recessief, wat overeenkomt met onderzoeken omtrent de dominantie van natriumkanaal in andere organismen.

Resistentie tegen organofosfaten en carbamaten wordt in *T. urticae* dikwijls veroorzaakt door ongevoeligheid van acetylcholinesterase. Door het combineren van toxicologische, biochemische en moleculaire gegevens van 3 referentielaboratoriumstammen en 3 organofosfaatresistente (OPR) stammen werden de mutaties in het AChE1 gen, die geassocieerd zijn met resistentie bij *T. urticae*, gekarakteriseerd. De resistentieratio's van de OPR stammen varieerden van 9 tot 43 voor pirimifos-methyl, van 78 tot 586 voor chloorpyrifos, van 8 tot 333 voor methomyl en van 137 tot 4164 voor dimethoaat. Vergeleken

met de referentiestammen was de insecticideconcentratie nodig om 50 % van de AchE1 activiteit te onderdrukken in de OPR stammen minstens 2.7, 55, 58 en 31 keer hoger voor respectievelijk pirimifos-methyl, chloorpyrifos-oxon, paraoxon en omethoaat, en 87 keer hoger voor carbamaat-carbaryl. Bij het vergelijken van de AChE1 sequentie van laboratoriumstammen met OP stammen kwamen 4 aminozuursubstituties aan het licht: (1) F331W, aanwezig in alle 3 de OP stammen; (2) T280A, gevonden in 3 OP stammen, maar niet in alle klonen; (3) G328A, gevonden in 2 OP stammen; (4) A201S, gevonden in slechts 1 OP stam. F331W, G328A en A201S zijn mogelijk betrokken bij resistentie tegen organofosfaat- en carbamaatinsecticiden. Deze bevindingen werden ook bevestigd met behulp van een structureel model. De F331W substitutie is waarschijnlijk de belangrijkste en meest voorkomende in *T. urticae*. Ze kan eenvoudig opgespoord worden met een diagnostische PCR-RFLP test, ontwikkeld in deze studie.

We onderzochten ook het mogelijk antagonistisch effect van organofosfaat- en carbamaatinsecticiden op de toxiciteit van bifenazaat in *T. urticae* bij toepassing in mengsels. Bifenazaat, een nieuw en veelvuldig gebruikt carbazaat, is een pro-acaricide dat geactiveerd moet worden door carboxylesterasen. Twee OPR stammen werden gebruikt om het effect van verschillende organofosfaat- (chloorpyrifos, azinfos-methyl en fosmet) en carbamaat-insecticiden (carbaryl en methomyl) te evalueren. Menging van chloorpyrifos met bifenazaat leidde tot een sterke daling van de bifenazaattoxiciteit in beide onderzochte stammen. Bij de stam met een hogere esterase-activiteit verminderde het antagonisme echter al na 2 dagen. Van alle geteste producten vertoonde enkel methomyl een antagonistisch effect 1 dag na behandeling. Deze bevindingen tonen aan dat menging van organofosfaat- en carbamaatinsecticiden met bifenazaat de werkzaamheid van deze laatste onder veldomstandigheden kunnen inhiberen, vooral wanneer resistente stammen aanwezig zijn.

Spirodiclofen, een recent ontwikkeld en gecommercialiseerd acaricide, is een selectieve, niet-systemische verbinding, afgeleid van tetronzuur, die de werking van ACC-ase inhibeert. We selecteerden een spiridiclofen-resistente *T. urticae* stam in het laboratorium, met de bedoeling om informatie te verzamelen die nuttig kon zijn om resistentie-ontwikkeling in het veld te vermijden. De resistentieratio van deze stam, bepaald op het larvaal stadium, was 274. Deze stam werd gebruikt om genetische, toxicologische, biochemische en kruisresistentie-gegevens te bepalen. De eitjes van de resistente stam bleken veel gevoeliger dan de mobiele stadia. Er werd geen kruisresistentie tegen andere acariciden gevonden, behalve tegen spiromesifen, een andere afgeleide van tetronzuur. Op basis van synergist- en enzymtesten bleek dat vooral P450 mono-oxygenasen, maar ook esterasen and glutathione-*S*-transferasen, betrokken waren

bij de metabolische detoxificatie van spirodiclofen. Voorbehandeling van resistente wijfjes met de synergisten PBO of DEF konden het inhibitie-effect van spirodiclofen op reproductie verhogen, wat opnieuw een aanwijzing was voor een mogelijke betrokkenheid van mono-oxygenasen en esterasen. Binnen de groep van de esterasen van *T. urticae* zijn er waarschijnlijk maar enkele die meer tot expressie gebracht worden in spirodiclofenresistente stammen. Precies deze esterasen hebben een sterke voorkeur voor het voor dit doel ontworpen substraat 1-naftyl-2,2-dimethylbutyraat. Dit werd ook bevestigd door scheiding van de esterase-isozymen met behulp van natieve IEF. Aangezien spirodiclofen interfereert met de lipidensynthese, werd de totale hoeveelheid lipiden van volwassen wijfjes gemeten. Zowel in de gevoelige als de resistente stam werden geen significante verschillen tussen behandelde en niet-behandelde volwassen wijfjes gevonden. De totale hoeveelheid lipiden in de resistente wijfjes was echter beduidend hoger dan deze in de gevoelige wijfjes. De genetische analyse via kruisingsexperimenten toonde aan dat resistentie overerft als een intermediair kenmerk dat gestuurd wordt door meer dan één gen. De laboratoriumgeselecteerde stam was totaal ongevoelig voor een behandeling met de aanbevolen velddosis van spirodiclofen, waardoor men er mag van uitgaan dat ook in het veld het risico voor een snelle resistentie-ontwikkeling groot is. Een goede strategie voor het beheer van de acaricideresistentier is noodzakelijk om snelle resistentie-opbouw in het veld te voorkomen. Zonder selectiedruk blijkt de resistentie niet stabiel te zijn. Ze kan snel verminderen in aanwezigheid van gevoelige individuen, door de intermediaire, polygene overerving.

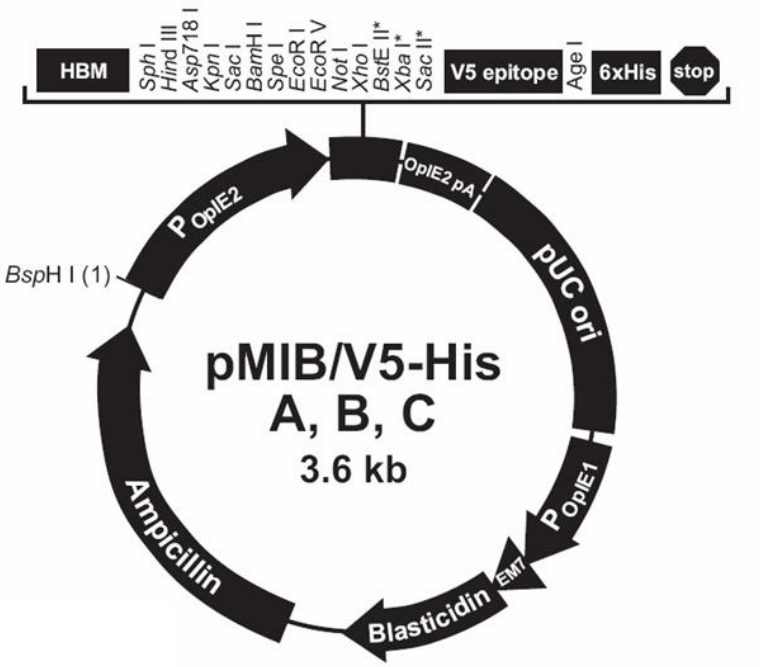
Er werd geen bewijs gevonden dat er buiten de metabolische detoxificatie andere resistentiemechanismen, zoals mutaties in de inwerkingsplaats (ACCase) of de overproductie van het doelenzym, aanwezig waren. Om het ontwerp van nieuwe ACCase-inhiberende insecticiden te vereenvoudigen en om het resistentiemechanisme van de op dit moment gebruikte ACCase inhibitoren beter te begrijpen, probeerden we *T. urticae* ACCase te kloneren en tot expressie te brengen met verschillende expressiemethoden De beste resultaten tot nu toe werden bereikt met een baculovirus expressiesysteem en expressie in E. Coli Codon Rare cellen via de vector pETDuet-1. Deze methode kan echter nog verbeterd worden, bv. door het optimaliseren van omstandigheden om hogere expressieniveaus en een betere eiwitoplosbaarheid, activiteit en stabiliteit te verkrijgen.

In een laatste deel was het de bedoeling om de verworven kennis te toetsen aan de praktijk. We contacteerde daarvoor een aantal Nederlandse snijrozenkwekers die geconfronteerd werden met een falende spintmijtbestrijding. Om na te gaan of dit falen veroorzaakt werd door resistentie, werden 15 stammen, verzameld op snijrozen in serres, getest met 11

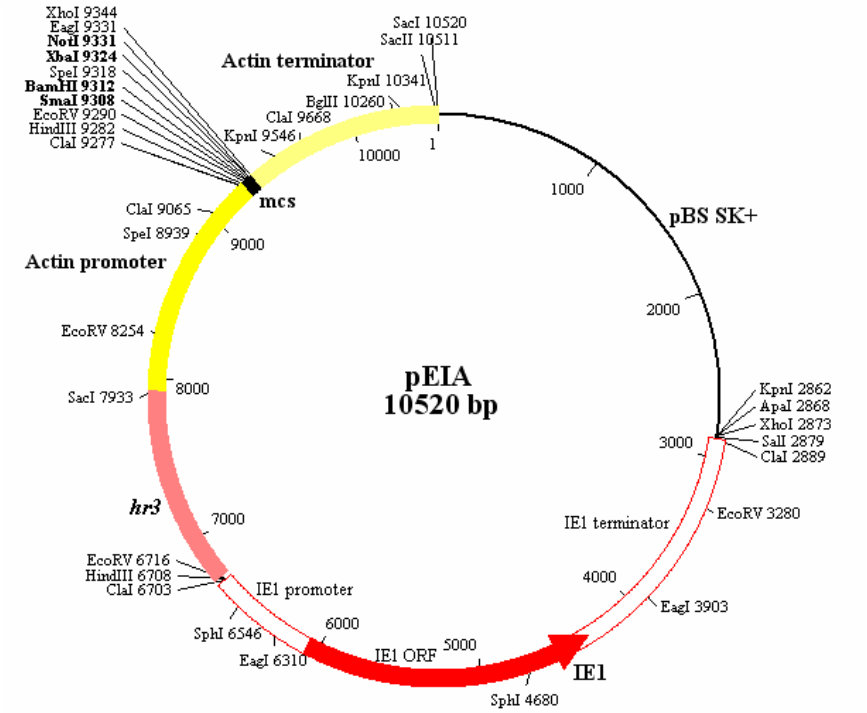
acariciden: tebufenpyrad, milbemectine, abamectine, cyflumetofen, bifenthrin, spiromesifen, hexythiazox, etoxazole, bifenazaat, acequinocyl and chloorpyrifos. Om duidelijk resistentie van gevoeligheid te onderscheiden werden drie dosissen gebruikt: de aanbevolen velddosis (FD), FD/5 en FD x 5. Met het gebruik van biochemische en moleculaire technieken werden tien stammen onderzocht op een gekend resistentiemechanisme, zoals verhoogde detoxificatie of mutaties die leidden tot resistentie. Mogelijke kruisresistentie tussen acariciden werd ingeschat door middel van een correlatie-analyse. De stammen vertoonden verschillende niveaus van resistentie tegen de geteste acariciden. Twaalf van de 15 stammen waren resistent tegen hexythiazox en 9 van de 15 tegen bifenthrin en tebufenpyrad. Er werden twee stammen gevonden die een hoge resistentie vertoonden tegen de meeste geteste insecticiden, zelfs tegen een product dat nog niet in Europa geregistreerd werd. Tussen sommige acariciden werd een hoge kruisresistentie gevonden, bv. tussen abamectine and milbemectine. Zonder selectiedruk bleek de resistentie in de meeste gevallen onstabiel na één jaar. Alle veldstammen vertoonden een verhoogde metabolische activiteit in vergelijking met de gevoelige stammen. Een aantal aminozuursubstituties, waarvan bekend is dat ze betrokken zijn bij resistentie tegen producten met verschillende werkingswijzen, werden teruggevonden. Het duidelijkste voorbeeld was de F331W substitutie in acetylcholine-esterase, dat in alle veldstammen werd teruggevonden.

De resultaten van dit onderzoek dragen bij tot een beter begrip van acaricideresistentie bij *T. urticae*, en bij uitbreiding tot efficiëntere strategieën voor resistentiebeheer.

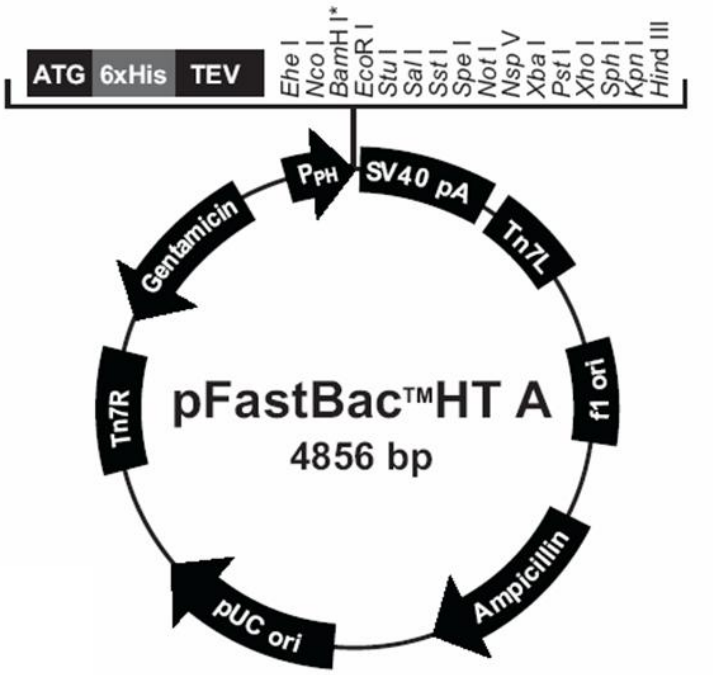
APPENDICES



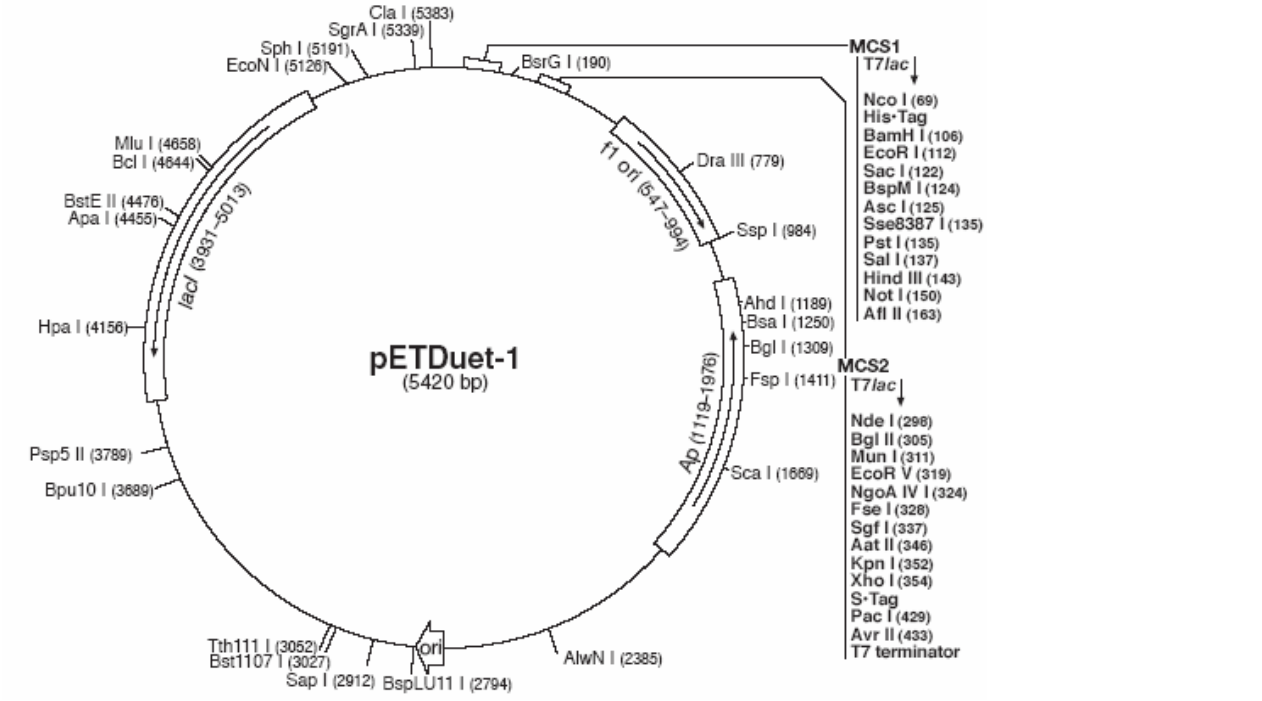
Appendix 1 The map and features of the pMIB/V5-His A, B, and C vectors, contains *Op*IE2 promoter, OpIE2 Forward priming site, Honeybee melittin secretion site, Multiple cloning site, V5 epitope, Polyhistidine (6xHis) region, OpIE2 Reverse priming site, *Op*IE*2* polyadenylation sequence, pUC origin, *Op*IE1 promoter, EM7 promoter, Blasticidin resistance gene (*bsd*), Ampicillin resistance gene (*bla*).



Appendix 2 The map and features of the pEIA contains actin promoter (*B. mori* A3 cytoplasmic actin promoter), baculoviral (BmNPV) homologous region 3 enhancer (*hr3*), baculoviral (BmNPV) DNA fragment containing the IE1 transactivator gene under the control of its native viral promoter (*IE1*), The black box downstream of the promoter indicates the position of the cloning sites

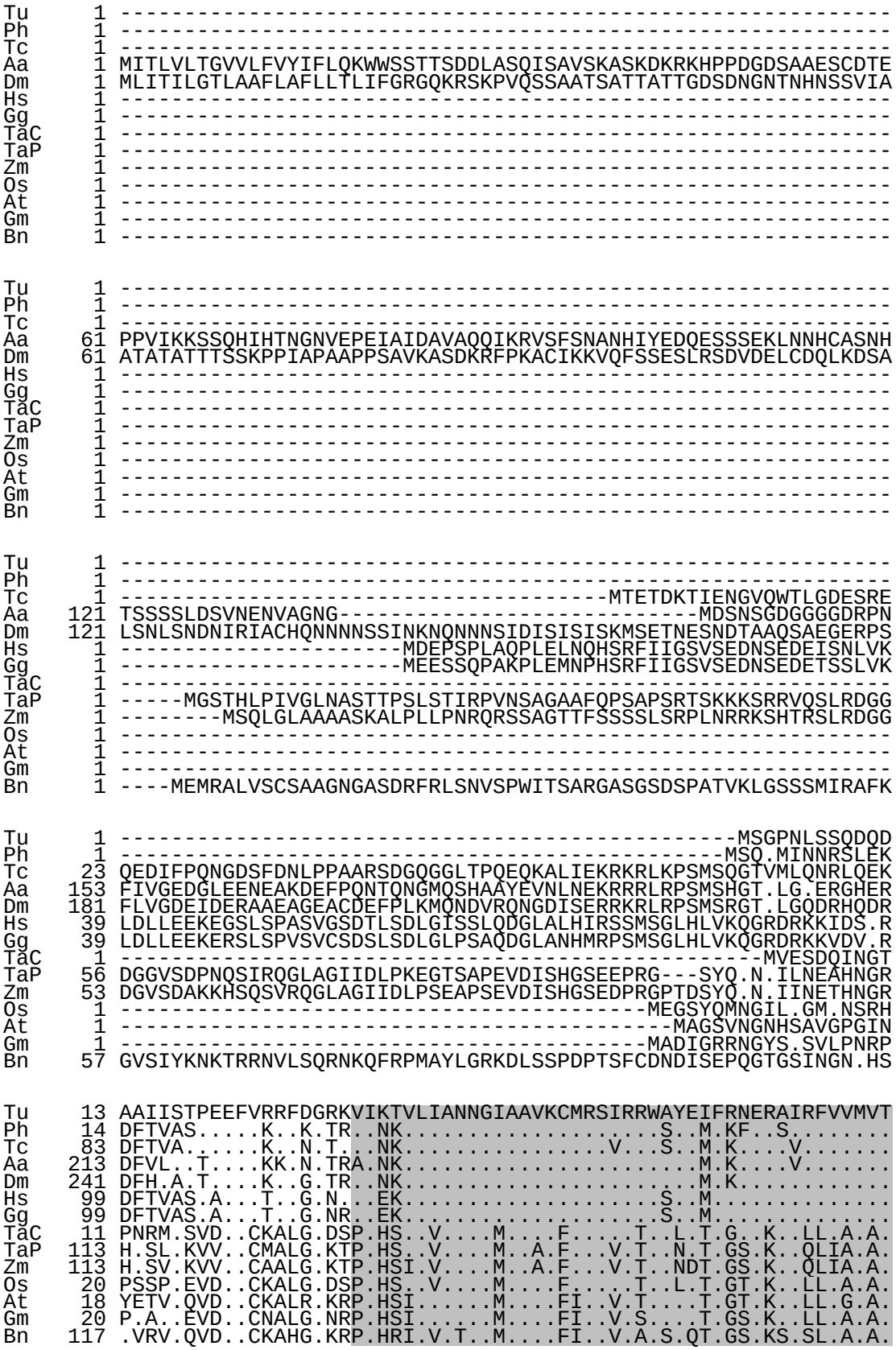


Appendix 3 The map and features of the pFastBac HT A contains f1 origin, Ampicillin resistance gene, pUC origin, Tn7R, Gentamicin resistance gene, Polyhedrin promoter (PPH), Initiation ATG, 6xHis tag, TEV recognition site, Multiple cloning site, SV40 polyadenylation signal, Tn7L



Appendix 4 The map and features of the pETDuet-1 vector contains the pBR322-derived ColE1 replicon, *lacI* gene and ampicillin resistance gene, two multiple cloning sites (MCS), each of which is preceded by a T7 promoter/*lac* operator and a ribosome binding site (rbs)

Appendix 5 Sequence alignment of selected ACCase homologs



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Tu     73 PEDLKANAEYIKMADHYVPVPGGPNNNNYANVDLIVDIAIRMKAQGVWAGWGHASEYPRL
Ph     74 ........................A........E..L...L.CQV.A..........N.K.
Tc    143 ........................T........E......T.LQV.A..........N.K.
Aa    273 ........................S........E......L.TQV.A..........N.K.
Dm    301 ........................S........E......L.TQV.A..........N.K.
Hs    159 ................................E..L...K.IPV.A..........N.K.
Gg    159 ................................E..L...K.IPV.A..........N.K.
TaC    71 ....RI...H.RI..QFLE....T........Q...E..E.TRVSA..P.......N.E.
TaP   173 ...MRI...H.RI..QF.E....T........Q...E..V.TGVSA..P.......N.E.
Zm    173 ...MRI...H.RI..QF.E....T........Q...GM.QKLGVSA..P.......N.E.
Os     80 .....I...H.RI..QF.E....T........Q...E..E.THVSA..P.......N.E.
At     78 ...MRI...H.RI..QF.E....T........Q...EM.EVTRVDA..P.......N.E.
Gm     80 ...MRI...H.RI..QF.E....T........Q..LEM.EITHVDA..P.......N.E.
Bn    177 ...MRI...H.RI..QFMQ....T........H...EM.EATGVDA..P.......N.E.

Tu    133 PELLNKAGIAFMGPPHHAMWALGDKIASSIVAQTARVPTLSWSGSGLVDSNYVE---GKP
Ph    134 ....H.NN.V.I...EK...T..............EI...P......IATFSER---N.R
Tc    203 ....H.N....I...DK...................DI...P....D.KAQYTG-----.K
Aa    333 ....H.K.LV.L...ER........V.........EI...P....E.KAQYSG-----.K
Dm    361 ....H.E.LV.L...ER........V.........EI...P....D.KAQYSG-----.K
Hs    219 ....L.N........SQ.....................GI...P......RVDWQENDFSKRI
Gg    219 ....H.N........SQ.....................GI...P.N....RVDWQENDLQKRI
TaC   131 .DA.MEK..I.L...SA..G.....G..LI..A.G....P....HVKVPQET------C
TaP   233 .DA..AN..V.L...SSS.N.....VG.ALI..A.G....P....QVEIPLE.C-----L
Zm    233 .DA.TAK..V.L...ASS.N.....VG.ALI..A.G....A....HVEVPLECC-----L
Os    140 .DA.KEK..I.L...SA..A......G..LI..A.G....P....HVKIPPES------C
At    138 .DA.DAK..I.L...ASS.A......G..LI..A.D....P....HVKIPPNSN-----L
Gm    140 .DA.KAK..V.L...AIS.A......G..LI..A.E....P....HVKIPPESS-----L
Bn    237 .DA.KAK.VI.L..TAAS.L......G..LI..A.D....P....HVKIPPGSS-----L

Tu    190 IKISADLYRKGCVSDEVEGLEASYKIGFPVMIKASEGGGGKGIRKCESADEFSIKFRQVQ
Ph    191 ....SE..KE...QTVED..A.AQ.....................V..GE..PNQ.....
Tc    258 ....S..FAR...QTAEQ..A.AQ.....................V.N..D.PAA.....
Aa    388 ....S..FAR...TTSEQ..I.AG.....................RVDH....PAL.....
Dm    416 ....SE.FAR...TNVEQ..A.VN.....................RVDTTE..PGL.....
Hs    279 LNVPQE..E..Y.K.VDD..Q.AEEV.Y.................VNN..D.PNL.....
Gg    279 LNVPQE..E..Y.K.ADD..R.AEEV.Y.................VNN..D.PNL.....
TaC   185 HS.PEEI.KNA...TTD.AVASCQVV.Y.A.....W.........VHND..VRAL.K...
TaP   288 DS.P.EM...A...TTE.A.ASCQM..Y.A.....W.........VNND.DVRAL.K...
Zm    288 DA.PEEM...A..TTTE.AVASCQVV.Y.A.....W.........VHND..VRAL.K...
Os    194 NS.PEEM..SA...TTE.AVASCQVV.Y.A.....W.........VHND..VRAL.K...
At    193 VT.PEEI..QA..YTTE.AIASCQVV.Y.A.....W.........VHND..VRAL.K...
Gm    195 .T.PDEI..EA..YTTE.AVASCQVV.Y.A.....W.........VHND..VRAL.K...
Bn    292 VT.PEEM..QA..YTTE.AVASCQVV.Y.A.....W.........VHDD..VRAL.K...

Tu    250 NEVPGSPIFIMKLATCARHLEVQLLADQYGNAISLFGRDCSIQRRHQKIIEEAPCVIANE
Ph    251 A.............R.....................................AI..KL
Tc    318 T.I......V....K.....................................A...AS
Aa    448 A........V....RG....................................AI..EP
Dm    476 A........V....RG.....................................AIV.QP
Hs    339 A........V.R..KQS......I..................V.........AT..TP
Gg    339 A........V.R..KQS......I..................V.........AS..TS
TaC   245 G..........V.SQS........C.KH..VAA.HS....V.........G.ITV.PP
TaP   348 G.........R..SQS........C.....VAA.HS....V.........G.VTV.PR
Zm    348 G........V.R..SQS........C.....VAA.HS....V.........G.VTV.PR
Os    254 G..........V.SQS........C.KH..VAA.HS....V.........G.ITV.PS
At    253 G..........V.SQS........C.KH..VSA.HS....V.........G.ITV.PP
Gm    255 G..........V.SQS........C.....VAA.HS....V.........G.ITV.TI
Bn    352 G..........V.SQS........C.....VSA.HS....V.........G.ITV.PR

Tu    310 EAFAEMEEAAVRLAKMVGYESTGTVEYLYDPAENKFYFLELNPRLQVEHPCSEMVSDVNL
Ph    311 .V.ED..R...........V.A.........-TNGNY...............T........
Tc    378 DV.ED..K...........V.A.........-TSGHY...............T........
Aa    508 .V.ED..K...........V.A.........-..GRYF..............T...AE...
Dm    536 .V.ED..K...........V.A.........-P.GRYF..............T...A....
Hs    399 AV.EH..QC..K.......V.A.......S-QDGS................T...A....
Gg    399 VV.EH..QC..K.......V.A.......S-QDGS................T...A....
TaC   305 .TIK.L.Q..R....C.Q.QGAA......SMETGEY..............VT.WIAEI..
TaP   408 .TVK.L.Q..R....A...VGAA......SMETGEY..............VT.WIAE...
Zm    408 .TVKAL.Q..R....A...VGAA......SMETGDY..............VT.WIAE...
Os    314 .TVK.L.Q..R....C.H.VGAA......SMETGEY..............VT.WIAEI..
At    313 .TVKKL.Q..R....S.N.VGAA.I....SMDTGEY..............VT.WIAEI..
Gm    315 .TVKKL.Q..R...IS.N.VGAA......SMETGEY..............VT.WIAEI..
Bn    412 DTVKKL.Q..R....S.N.VGAA...F..SMDTGDYF.............VT.RIA.I..
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Tu   370 PACQLQIAMGLPLYRIKDIRLLYGEPPLNDNPID----------FKLG--RAQPWGHVIA
Ph   370 ..A...V.......K..........S.WG..E..----------.DQPRHKP........
Tc   437 ..A.........HY..........S.WGNSE..----------.DQPRHKP........
Aa   567 .......G..I.............N.WGSTV..----------.DVPTTKPR.R.....
Dm   595 ..A....G..I....L.........S.WGSSV..----------.ENPPNKPR.S.....
Hs   458 ..A.......I.........MM..VS.WG.S..----------.EDSAHVPC.R.....
Gg   458 ..A.......I..H.....VM..VS.WG.GS..----------.ENSAHVPC.R.....
TaC  365 ..S.VVVG..I...N.PE..RF..IEHGGGYHAWKEISAVATKFDLDKAQSVK.K..CV.
TaP  468 ..A.VAVG..I..WQVPE..RF..MDNGGGYD.WRKTAALATPFNFDEVDSQW.K..CV.
Zm   468 ..A.VAVG..I..WQ.PE..RF..MDYGGGYD.WRKTAALATPFNFDEVDSQW.K..CV.
Os   374 ..A.VVVG..V...N.PE..RF..MEHGGGYDAWRKISAVATKFDLDNAQSVK.K..CV.
At   373 ..A.VAVG..I..WQ.PE..RF..IEHGGGYDSWRKTSVVAFPFDFDKAQSIR.K..CV.
Gm   375 ..A.VA.G..V..WQ.PE..RF..VEHGGGYDAWRKTSVLATPFDFDKAQSTR.K..CV.
Bn   472 ..A.VAVG..I..WQ.PE..RF..IEQ---YDSWRTTSLLASPFNFDKAESVR.K..CV.

Tu   418 ARITSENPDEGFKPSSGTVQELNFRSNKNVWGYFSVGASGGLHEFADSQFGHCFSWGEDR
Ph   420 ..........................S.........A.....................T.
Tc   487 ..........................S.........A.....................N.
Aa   617 ..........................SQ........A.....................N.
Dm   645 ..........................S.........A.....................N.
Hs   508 ..................................A.A.....................N.
Gg   508 ..................................A.A.....................N.
TaC  425 V.V...D..D....T..R.E....K.KP...A....KSG.AI...S......V.AF..S.
TaP  528 V.....D..D....TG.K.K.IS.K.KP...A....KSG..I..........V.AY.VS.
Zm   528 V.....D..D....TG.K.K.IS.K.KP...A....KSG..I..........A.AY.LS.
Os   434 V.V...D..D....T..R.E....K.KP...A....KSG.AI...S......V.AF..S.
At   433 V.V...D..D....T..R....S.K.KP...A....KSG..I...S......V.AF..S.
Gm   435 V.V...D..D....T..K.....K.KP...A....KSG..I...S......V.AF..S.
Bn   529 V.V...D..D....T..Q....S.K..P........KSG..I...S......V.AF..S.

Tu   478 EQARENLVIALKELSIRGDFRTTVEYLITLLETEAFQKNEIDTGWLDKLISERVQSDKPK
Ph   480 ..................................KS..E.S...A...I..A..M.AE..N
Tc   547 ..................................KS..E.T...A...I...K.E.E..N
Aa   677 Q.................................NS.LE.T...A...A..A...KA...D
Dm   705 Q.................................NR.LD.S...A...A..A.....E..D
Hs   568 .E.IS.M.V.....................K.....S..M.R.......R..A.K..AER.D
Gg   568 .E.IS.M.V.....................K.....S..Q.R.......R..A.K..AER.D
TaC  485 SL.IA.M.LG...IQ...EI..N.D.TVD..NAAEYRE.K.H.....SR.AM..RAER.P
TaP  588 AA.IT.MSL....IQ...EIHSN.D.TVD..NASD.KE.R.H.....NR.AM...AER.P
Zm   588 PA.IT.MSL....IQ...EIHSN.D.TVD..NASD.RE.K.H.....TR.AM...AER.P
Os   494 SL.IA.M.LG...IQ...EI..N.D.TVD..NAAEYRE.K.H.....SR.AM..RAER.P
At   493 AL.IA.M.LG...IQ...EI..N.D.T.D..HASDYRD.K.H.....SR.AM..RAER.P
Gm   495 AL.IA.M.LG...IQ...EI..N.DNT.D..NASDYRE.K.H.....SR.AM..RAER.A
Bn   589 AM.IA.M.L....IQ...EV..N.D.T.D..HAFDYRE.K.H.....SR.AM..RAER.P

Tu   538 TSLALICGSIHVADAQFSGNWQNFESSLQKGQIPHATTLTNSVNVELIYENVKYLFNVAK
Ph   540 VLVGV.S.AL....RAITN.F.S.QQA.ER...QGSN.IDHT.S....N.GL..KVQ.T.
Tc   607 VM.GV....L.I..KTI.TAF.E.QN..ER...QGSN....V.D....HGGN..KVQAT.
Aa   737 II.GV...AL.I..RKITEAFTS.KT.ME....QA.N....V.D....S.GIR.KVQP..
Dm   765 IL.GVM...L.I..R.ITESFSS.QT..E....QA.N....V.D....NDGIR.KVQA..
Hs   628 .M.GVV..AL....VSLRNSVS..LH..ER..VLP.H..L.T.D......G...VLK.TR
Gg   628 .M.GVV..AL....VS.RNSVS..LH..ER..VLP.H..L.T.D......GR..VLK.TR
TaC  545 WY.SVVG.ALYE.SSRS.SVVTDYVGY.S.....PKHISLVNLT.T.NIDGS..TIETVR
TaP  648 WYISVVG.ALYKTITSNTDTVSEYV.Y.V.....PKHISLVHST.S.NI.ES..TIETIR
Zm   648 WYISVVG.ALYKTVTTNAATVSEYV.Y.T..H..PKHISLVNST.N.NI.GS..TIETVR
Os   554 WY.SVVG.ALYE.SSRS.SVVTDYVGY.S.....PKHISLVNLT.T.NI.GS..TIETVR
At   553 WY.SVVG.ALYK.S.TSAAVVSDYVGY.E.....PKHISLVHSQ.S.NI.GS..TID.VR
Gm   555 WY.SVVG.ALYK.SDSSAALVTDYVGY.E.....PKHISLVHSQ.S.NI.GS..TIDMIR
Bn   649 WY.SVVS.ALYK.S.TGAAVVSDYIAY.D.....PKHISLVHSQ.S.NI.GS..TID.VR

Tu   598 SGPNSYFVVLNDSYKEVEVHQMSDSGLLLSVDGSSIVTYMKEEIDQYRVIVANQTCVFSK
Ph   600 ...T...L.M.GHF..I...RL..G........A.CI......V.R..IVIG......E.
Tc   667 ....T..LMM.GTF..I...RLT.G.I.......FT......V.R..IVIG...V..E.
Aa   797 T..ST..L.M.G.F.....RL..G.M.I.L...YT......V.R..IVIG......D.
Dm   825 ..A....LLM.S.F..I...RL..G...I.LE.A.YT......V.R..IVIG......E.
Hs   688 QS....V.IM.G.CV..D..RL..G.....Y....YT......V.R..ITIG.K....E.
Gg   688 QS....V.IM.S.CV..D..RL..G.....Y....YT......V.R..ITIG.K....E.
TaC  605 G..R..KLRI.E.EV.A.I.SLR.G...MQL..N.H.I.AET.AAGT.LLINGR..LLQ.
TaP  708 ..QG..RLRM.G.VI.AN.QTLC.G...MQL..N.H.I.AE..AGGT.LLIDGK..LLQN
Zm   708 T.HG..RLRM...TV.AN.QSLC.G...MQL..N.H.I.AE..AGGT.LQIDGK..LLQN
Os   614 R..R..TLRM.G.EI.A.I.SLR.G...MQL..N.H.I.AET.AAGT.LLINGR..LLQ.
At   613 G.SGT.RLRM.K.EVVA.I.TLR.G...MQL..K.H.I.AE..AAGT.LLIDGR..LLQN
Gm   615 G.SG..RLRM.Q.EI.A.I.TLR.G...MQL..N.H.I.AEG.AAGT.LLIDGR..LLQN
Bn   709 ..SG..KLRM.N.EVAA.I.TLR.G...MQL..K.H.I.AQ..TSGT.LLIDGK..LLQN
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Tu    658 ENDPTILRSPSPGKLQQFLVEDGSHVSAGQPYAEIEVMKMIMTLTTSESGIIRHAKRFGS
Ph    660 ....SL.....A...LN..ID..G.LE...A.........V...SAT.T.TLVYV..P.A
Tc    727 ..........A...IS..ID..G..DK..A.........V....S...T.FYV..P.A
Aa    857 ....SL.....A...INL.I...A..VK...........V....AG.T.TVSFVR.P.A
Dm    885 ....SL.....A...INMI....A...K..A.........V....SQ.A.TVTFVR.P.A
Hs    748 ....SVM....A...I.YI....G..F...C.........V....AV...C.HYV..P.A
Gg    748 ....S......A...I.YV....G..F...CF.......V....AG...C.HYV..P.A
TaC   665 .H..SR.LADT.C..LR...A.....V.DT....V.....C.P.LLPA..V.HFVMPE.Q
TaP   768 DH..SR.LAET.C..LR...A..A..E.DV....V.....C.P.LSPAA.V.NVLLSE.Q
Zm    768 DH..SK.LAET.C..LR...A..A..D.DV....V.....C.P.LSPA..V.HCMMSE.Q
Os    674 .H..SK.LADT.C..LR...A.....D.DT....V.....C.P.LLPA..V.HFVMPE.Q
At    673 DH..SK.MAET.C..MRY.IS.N.NID.DT....V.....C.P.LSPA..V.HFKMSE.Q
Gm    675 DH..SK.VAET.C..LRY..A.D...D.DT....V.....C.P.LSPA....HFTMSE.Q
Bn    769 .H..SK.MAET.C..LRY..S.D.SID.DT....V.....C.P.LSPA..V.HFKMCE.Q

Tu    718 VLEAGCILARLDLDDPTKVRRAELYSEGWQELMARDIVGKNPSETDSAHPQKLNVTFSSV
Ph    720 ..D..SLI.H.E...ASLITK.LD.KG.FPDADVSAPIVG----------E...QLHN.Y
Tc    787 .....STI.S.E....SL.TK.M..KGPFP..DVSQPIVS----------E...HIHN.Y
Aa    917 ..D..SL.GH.E....SL.TK.QP.KNP.PLTGDSVQ.P----------E...RVH..Y
Dm    945 ..D..SL.GH.E....SL.TK.QPFKGQFLQP-ENAP.P----------E...RVHNTY
Hs    808 A.DP..V..KMQ..N.S..QQ...HTGSLPRIQSTALR.----------E..HRV.HY.
Gg    808 ..DP..VI.K.Q....SR.QQ...HTGTLPQIQSTALR.----------E..HRI.HY.
TaC   725 AMQ.SDLI.........SS.....PFHGTFPK.GPPTAISG----------------KVH
TaP   828 PMQ..DLI.........SA.K...PFNGSFP.MSLPIAASG----------------QVH
Zm    828 A.Q..DLI.........SA.K...PFDGIFPQMELPVA.SS----------------QVH
Os    734 AMQ.ADLI.........SS.....PFHGTFPK.GPPTA.SG----------------KVH
At    733 AMQ..ELI.N......SA..K..PFHGSFPR.GLPTAISG----------------RVH
Gm    735 AMQ..ELI.........SA..K..PFTGSFPV.GPPTAISV----------------KVH
Bn    829 .MLP.ELI.N..A..ST..K..PFHG.FPR.GLPTEISA----------------KVH

Tu    778 KSHLENFLAGYVLPEPFYTTKMQENVTLFMRCLKDPQLPLLELQDIISSQSGRIPPSVES
Ph    770 RCM.D.I....C..D.YHEPRVR.VIEK..TS.R..S...M...EV...I.....I...K
Tc    837 .AV...T....C..D.YNAPRLR.VIEK..SS.R..S.......QVMA.I......A..K
Aa    966 .TI...T....C..D.YNAPRLR.IIEK..QS.R..S.......EV.A.I.....L...K
Dm    993 ..I...T....C.....NAQRLRDIIEK..QS.R..S.......EV.A.I.....I...K
Hs    857 LDN.V.VMN..C..D..FSS.VKDW.ERL.KT.R..S.........MT.V......N..K
Gg    857 LDN.V.VMN..C....YFSS.VKGW.ERL.KT.R..S.........MT.V......N..K
TaC   768 QKFAASVNSAHMILAGYEHNINHVVQD.LN-..DS.E..F.QW.ELM.VLAT.L.KDLRN
TaP   871 .RCATSLN.ARMVLAGYDHPINKVVQD.VS-..DA.E..F.QWEELM.VLAT.L.RLLK.
Zm    871 .RYAASLN.ARMVLAGYEHNINEVVQD.VC-..DN.E..F.QWDELM.VLAT.L.RNLK.
Os    777 QKFAASVNSAHMILAGYEHNINEVVQD.LN-..DS.E..F.QW.ELM.VLAT.L.KDLRN
At    776 QRCAATLN.ARMILAGYEHKVDEVVQD.LN-..DS.E..F.QW.ECFAVLAT.L.KNLRN
Gm    778 QKCAASLN.ARMILSGYEHNIDEVV.QSLLN..DS.E..F.QW.ECLAVLAT.L.KELKN
Bn    872 QRCAATLD.ARMILAGYEHQVDEVVQDFVS-..DS.E..F.QW.ECFAVLAT.L.KDLRI

Tu    838 SIKKLMQKYAKNITAVLQIFPSQEIASVIDSYAATLEKRSDRDVFFLNTQGIVQLVQRYR
Ph    830 K.R...SL.ER...S..AQ....Q.............Q..T.......T...........
Tc    897 K.RR..SL.ER...S..AQ....Q.......H..SMQ..AE..G...A.ES..........
Aa   1026 K.R....L.ER...S..AQ....Q.......MH....Q..T.......T...........
Dm   1053 K.R...TL.ER...S..AQ....Q.......H....Q..A.......T..S.........
Hs    917 ....E.AQ..S...S..CQ....Q..NIL..H....NRK.E.E...M...S.........
Gg    917 ....E.AQ..S...S..CQ....Q..NIL..H....NRK.E.E...M...S.........
TaC   827 ELDAKYKE.EL.ADFRKSKDFPAKLLRGVIEANLAYCSEK..VTSERLVEPLMS..KS.E
TaP   930 ELEGKYSE.KL.VGHGKSKDFPSKMLRE.IEENLAHGSEKEIATNERLVEPLMS.LKS.E
Zm    930 ELEDKYKE.KL.FYHGKNEDFPSKLLRD.IEENLSYGSEKEKATNERLVEPLMN.LKS.E
Os    836 ELDGKYKE.EL.SDFRKNKDFPAKLLRG.IEANLAYCSEK..VTNERLVEPLMS..KS.E
At    835 MLESKYREFESISRNS.TTDFPAKLLKG.LEAHLSSCDEKE.GALERLIEPLMS.AKS.E
Gm    838 ELESKY.EFEGIS-SSQIVDFPAKLLKG.IEAHLSSCPDKEKGAQERLVEPLLS..KS.E
Bn    931 MLESKYME.ECISRNS.TADFPAKLLKG.LEAHVASCDETE.GALARLIEPLMS.AKC.E

Tu    898 NGIRGRLRSCVQDLLKNYIDVEQHFQAGDYDKCVSALTEKYKDEGMSKAVANIFSHLNVA
Ph    890 ......MK.V.HE...Q.YA..SQ..L.H.......IR..H..D-.AAVTSL....GQ.S
Tc    957 ......MKVA..E..RQ.Y...SQ..Q.S.....AL.R..H..D-.QSVSNT....SQ..
Aa   1086 ......MKAA.HE..RQ.YA..SQ..H.H.....A.IR..H..N-.DVV.GT....SQ..
Dm   1113 ......MKAA.HE..RQ.Y...SQ..Y.H.....GLVR.HN..D-.QTV.NT....SQ..
Hs    977 S....HMKAV.M...RQ.LR..TQ..N.H.....F..R.EN.SD-.NTVLNY....AQ.T
Gg    977 S....HMKAV.M...RQ.LK..TQ..H.H.....F..R.EN.SD-.NAVLNY....AQ.T
TaC   887 G.RESHA.AV.KS.FEE.LS..EL.SDDIQSDVIER.RLQHAKD-LE.V.YIV...QG.K
TaP   990 G.RESHAHFI.KS.FED.LS..EL.SD.IQSDVIER.RQQHSKD-LQ.V.DIVL..QG.R
Zm    990 G.RESHAHFV.KS.FEE.LT..EL.SD.IQSDVIET.RHQHSKD-LQ.V.DIVL..QG.R
Os    896 G.RESHA.VV.KS.FEE.LS..EL.SDNIQSDVIER.RLQHAKD-LE.V.YIV...QG.R
At    895 G.RESHA.VI.HS.FEE.LS..EL.NDNMLADVIERMRQL..KD-LL.I.DIVL..QGIK
Gm    897 G.RESHAHII..S.FDE.LS..EL.SDNIQADVIER.RLQ..KD-LL.I.DIVL..QGIK
Bn    991 G.RESHACVI.RS.FEE.LS..EL.NNNMLADVIERMRHE..KD-R..I.DIVL..QGLK
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Tu   958 KKNQLIIKLIDHLAGQEPGLTEELSNILNALTALNRSENVKVALRARQVLISAHQPPYAL
Ph   949 ...F.VT.....IWDN.....D..AS...E..S...Q.HSR..........A....A.E.
Tc  1016 ...L.VTM.....WSN.....D..APA.TE..S.....HSR..........A....A.E.
Aa  1145 ...L.VTL.V...WAN.....D..AAT.SE..S...A.HSR..........A....A.E.
Dm  1172 ...L.VTL.....WAN.....D..A.T.SE..S...A.HSR....S.....A....A.E.
Hs  1036 ...L.VTM...Q.C.RD.T..D..L...TE..Q.SKTT.A...........AS.L.S.E.
Gg  1036 ...L.VTM...Q.C.RD.T..D..I...TE..Q.SKTT.A...........AS.L.S.E.
TaC  946 S..K..LR.MEA.VYPN.---SAYRDQ.IRFS...HTAYSGL..K.S.L.EHTKLSELRT
TaP 1049 N.TK..LT.MEK.VYPN.---AAYKDQ.TRFSS..HKRYY.L..K.SEL.EQTKLSELRT
Zm  1049 N.AK.VTA.MEK.VYPN.---GGYRDL.VRFSS..HKRYY.L..K.SEL.EQTKLSELRA
Os   955 T..K..LR.MEA.VYPN.---SAYRDQ.IRFSG..NTVYSEL..K.S.L.EHTKLSELRT
At   954 N..K.VLR.MEQ.VYPN.---AAYRDK.IRFST..HTNYSEL..K.S.L.EQTKLSELRS
Gm   956 S..K..LQ.M.K.VYPN.---VAYRDQ.IRFSL.HHTNYSEL..K.S.L.EQTKLSELRS
Bn  1050 S..K.VLG.ME.FVNAN.---AMYRDK.IRFLK..HTNYSE.V.K.S.L.KQRKLSELRS

Tu  1018 RFSQMEAMFLSVIDMYS-------HEFDANNMNKLIISETSIFDVLHQFFYHSNIVVRKA
Ph  1009 .HN...SI...AV...G---------.HPE.IQ...Q.......I..D......KA.CN.
Tc  1076 .HN...SI...AV...G---------.HPE.LQ...V.......I..D....A.RA.CN.
Aa  1205 .HN...SI...AV...G-------.D.HPE.LQR..L.......I..D......RA.CN.
Dm  1232 .HN...SI...AV...G-------.D.HPE.LQR..L.......I..D......RA.CN.
Hs  1096 .HN.V.SI...A....G-------.Q.CIE.LQ...L.........PN......Q...M.
Gg  1096 .HN.V.SI...A....G-------.Q.CIE.LQ...L.........PN......Q...M.
TaC 1003 SIARSLSELEMFTEEGERISTPRRKMAINER.ED.VCAPVAVE.A.VAL.D..DPTLQRR
TaP 1106 SIARSLSELEMFTE---------ERTAISEI.GD.VTAPLPVE.A.VSL.DC.DQTLQQR
Zm  1106 SVARSLSDLGMHKG---------EMS-IKD..ED.VSAPLPVE.A.ISL.DY.DRT.QQK
Os  1012 SIARSLSELEMFTEEGERVSTPRRKMAINER.ED.VGAPLAVE.A.VAL.D..DPTLQRR
At  1011 NIARSLSELEMFTEDGENMDTPKRKSAINERIED.VSASLAVE.A.VGL.D..DHTLQRR
Gm  1013 NIARSLSELEMFTEDGENIDTPKRKSAINDR.ED.VSAPFAVE.A.VGL.D..DHTLQRR
Bn  1107 SILSELE..AEDGEN-------MDTPKRKCA.ED.VSAPLAVE.A.VGL.D..DRTLQRL

Tu  1071 SLEVYVRRAYICYEMVALQHLFIRNSPNDSGSIELSNSYAEQNDSVIPTAVFHFILPSSH
Ph  1062 A.......T..S.DLTC....ELSG--------------------EVSLVH.Q.L.....
Tc  1129 A.........TS.DITC....ELSG--------------------EV.VVH.Q.L..PT.
Aa  1258 A.........TS..LTC....ELSG--------------------EV.LVH.Q.L..TA.
Dm  1285 A.........TS..LTC....ELSG--------------------GL.LVH.Q.L..TA.
Hs  1149 A..........A..LNSV..RQLKD--------------------NTCVVE.Q.M..T..
Gg  1149 A..........A..LNSV..RQLKD--------------------NTCVVE.Q.M..T..
TaC 1063 VV.T.I..L.QH.LVRGSVRMQWHR--------------------SGLI.LWE.SEEHIE
TaP 1157 VI.T.IS.L.QPHLVKDSIQ.KYQE--------------------SGVI.LWE.AEAH.E
Zm  1156 VI.T.IS.L.QPHLVKDSIQMKFKE--------------------SGAITFWE.YEGHVD
Os  1072 VV.T.I..L.QP.LVKGSVRMQWHR--------------------SGLI.LWE.SEEHIK
At  1071 VV.T.I..L.QP.VVKDSVRMQWHR--------------------SGLL.SWE.LEEHME
Gm  1073 VV.S.I..L.QP.LVKGSARMQWHR--------------------SGLI.TWE.YDEYIE
Bn  1160 VV.T.I..L.QP.VVKESVRMQWHQ--------------------YGFI.SWE.LDDHME

Tu  1131 PSIAS------------QPQSQNTEGLSNNFSGESSAHLHSNYRIGVIAAFKNLEHVEEH
Ph  1102 .N---------------RIPINKLNHD..DLECSL.FSPQNWF.T..MT..QSF.QF.MY
Tc  1169 .N---------------RLIKLDDFKD.EDGPK----VVDTFQ.T.CM...Q.FQEF.QY
Aa  1298 .N---------------RYKLLPDG------TETDN-IFD.FM.T.CM...DSF..FTQY
Dm  1325 .N---------------RLF.RMSSPDGLDQAAAE.-LGN.FV.T.A....DSF..F.MY
Hs  1189 .NRGNIPTLNRMSFSSNLNHYGM.HVA.VSDVLLDNSFTPPCQ.M.GMVS.RTF.DFVRI
Gg  1189 .N--------RMSFSSNLNHYGMVHVA.VSDVLLDNSFTPPCQ.M.GMVS.RTF.DFVRI
TaC 1103 QRNG----------------------QSASLLKPQVEDPIGR.W..MVVI.S.QLLSTA
TaP 1197 KR-----------------------------------------L.AMVIV.S..S.SAA
Zm  1196 TRNG-----------------------------HGAIIGGK.W.AMVVL.S..SASTA
Os  1112 QRNG----------------------QDAM.LKQQVEDPEEK.W..MVVI.S.QYLSSA
At  1111 RKNIG--------------------LDDPDT.EKGLVEKR.KRKW.AMVII.S.QFLPSI
Gm  1113 RKNG---------------------VEDQSLSKTVEEK..EKKW..MVII.S.QFLPAI
Bn  1200 RKNSEG-------------------SDDQET.EKVFVEKRRKKKK.FMVII.S..FLPNI

Tu  1179 FDSLLNALTDFDKDLAAEHFDAPESNHQSSSPSDKGSSD---MNGYNGPDTEESQNKESN
Ph  1147 A.EI.DLVK.SLRNVNVSNKVLEAVENA.E.RNSGSN.----VSLNL.DAIRIEEQNQKS
Tc  1210 A.EI.DLIE..ANPATISAK.LIAVESG.E.RNSTSIN----VSLSLEGQRGGEE-SCMH
Aa  1336 S.EI.DL.E..ASPSFVNAKVLEAVEAGD.DRRMST.INVSISDPISR.AETDGIATHPA
Dm  1369 S.EI.DL.E..VSPAMVNAKVLEAVEAAD.ISDSRH.TSINVSLSDPVTRANAAEEAK.T
Hs  1249 ..EVMGCFS.SPPQSPTFPEAGHT.-------------------------LYDEDKVPRD
Gg  1241 ..EVMSCFC.SPPQSPTFPEAGHA.-------------------------LYDEDKAARE
TaC 1140 IEAA.KETSHYGAGVGSVSNGN.INLNG.NMLHIALVGI----.NQMSTLQDSGDEDQAQ
TaP 1215 IGAA.KGTSRYA-------------SSEGNIMHIALLGA----DNQMHGTEDSGD.DQAQ
Zm  1225 IVAA.KDSAQ.N-------------SSEGNMMHIALL.A----ENESNISGIS.D-DQAQ
Os  1149 I.AA.KETSHYKAGAGNVSNGNSA.SSHGNMLHIALVGI----.NQMSTLQDSGDEDQAQ
At  1151 ISAA.RETKHN--.YETAG-----APLSGNMMHIAIVGI----.NQMSLLQDSGDEDQAQ
Gm  1151 ITAA.REA.NN--PHE.LTSGSV.PVNYGNMMHIGLVGI----.NQMSLLQDSGDEDQAQ
Bn  1241 ITAAALTE.NHIDYGESAG-----.PLSGNIMHIAVLDS----ENE------------ED
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Tu  1236 EPIHIINIAIKPEG-ADDEILSKKCEAFCQSRSKLLIERGVRRVTFIVCNLY--------
Ph  1203 .....LS..V.DHNKS..ST...MFGD..RTNLEE.TD..I..I..L.L.K---------
Tc  1265 .....LHVG..DK.DT..SSM..IFGG..VRHKEE..S..I..I..AALKH---------
Aa  1396 .A...LS..VRDM.DM..LQMEHVFGS..AQHREE.LS.R...I..AALKK---------
Dm  1429 .....VSV.VRET.EL..LQMAQIFGNY..EHNEE.FQ.RI..I..AALKK---------
Hs  1284 .....L.V...TDCDIE.DR.AAMFRE.T.QNKAT.VDH.I..L..L.AQKDFRKQVNYE
Gg  1276 .....L.V...TD.DV..DG.AAMFRE.T..KKSV...H.I..L..L.AQK---------
TaC 1196 .R.NKLSKIL.DNTITSHLNGAGVRVVS.IIQRDEGRSPMRHSFKWSSDK.---------
TaP 1258 VR.DKLSATLEQNTVTA.LRAAGVKVIS.IVQRDGALMPMRHTFLLSDEK.---------
Zm  1267 HKMEKLSKIL.DTSV.S.LQAAGLKVIS.IVQRDEARMPMRHTFLWLDDKS---------
Os  1205 .R.NK.SKIL.DSTVTSHLNGAGVRVVS.IIQRDEGRPPMRHSFQWS.DKI---------
At  1200 .RVNKLAKIL.E.EVSSSLCSAGVGVIS.IIQRDEGRTPMRHSFHWSLEKQ---------
Gm  1205 .R.NKLAKIL.EQEVGSTIRAAGVGVIS.IIQRDEGRAPMRHSFHWSEEK.---------
Bn  1280 QAQERVEKIL.E.EVSSSLCSAGVGVVS.IIERDEGRTPIRHSFHWSMEKQ---------

Tu  1287 -------CFPRYFTYRQRDGFTEDRIYRHLEPALAFQLEINRLRNYDLEAIPTANQRMHL
Ph  1254 ------KQ...F........Q..........GM.....L..M.T.....LA...RK.Y.
Tc  1316 ------KQS.KF..F.S..D.K..K.........C.....L..M...N...L..S..K...
Aa  1447 ------RQ..KF....A..N.E...........C.....L..M.T.....L.....K...
Dm  1480 ------RQ..KF..F.A..K.............S..H..L..MKT.....L.....K...
Hs  1344 VDRRFHRE..KF..F.A..K.E.................L..M..F..T...C..HK...
Gg  1327 ------RE..KF..F.A..K.E.................L..M..F..T...C..HK...
TaC 1247 --------------------YYE..PML..V..P.STF..LDKVNLEGYNDAKYTPS.DRQ
TaP 1309 --------------------CYE.EPVL..V..P.SAL..LGK.KVKGYNEVKYTPS.DRQ
Zm  1318 --------------------CYE.EQ.L..V..P.STL..LDK.KVKGYNEMKYTPS.DRQ
Os  1256 --------------------YYE..PML..V..P.STF..L.KVNLDGYNEVKYTPS.DRQ
At  1251 --------------------YYV.EPLL.....P.SIY..LDK.KG.SN--.QYTPS.DRQ
Gm  1256 --------------------YYA.EPLL.....P.SIY..LDK.KA.EN--.RYTPS.DRQ
Bn  1331 --------------------YYA.EPML.....P.SIY..LDK..G.EN--.QYTPT.DRQ

Tu  1340 YLGKAKSTHTK--QLIDYRFFIRTIIRHS--------------DLITKEASYEFLQNEGE
Ph  1308 ......VAKGQ--EVT.......S.....................F.Y.....
Tc  1370 ......GIGG.--EVT.......S.....................F.Y.....
Aa  1501 ...R..VPKGQ--EVT.F.....S.....................F.Y.....
Dm  1534 ......VSKGQ--EVT......S.....................F.Y.....
Hs  1404 ...A..VEVGT--EVT.....V.A.....................V.....F.Y.....
Gg  1381 ...A..VEVGT--EVT....V.A.....................V.....F.Y.....
TaC 1288 WHMYTLVKNK.DPRSN.Q.M.L...V.QPSVTNGFLFG-SIDNEV---Q..SS.TS.SIL
TaP 1350 WNIYTLRNTEN--PKMLH.V.F..LV.QPGASNKFTSGNISDVEVGGA.E.LS.TSSSIL
Zm  1359 WHIYTLRNTEN--PKMLH.V.F...V.QPNAGNKFRSAQISDAEVGCP.E.LS.TS.SIL
Os  1297 WHIYTLIKNK.DQRSN.Q.L.L...V.QPGVTNGFLSG-NVDNEVGRAQ..SSYTSSSIL
At  1290 WHLYTVTDK----PVPIK.M.L.SLV.QATMNDGFILQQGQDKQ.SQTLI.MA.TSKCVL
Gm  1295 WHLYTVVD.K---PQPIQ.M.L..LV.QPTTNEGFSSYQRLDAETSRTQLAMS.TSRSIF
Bn  1370 WHLYTVTDNKP---VPVR.M.L.SLV.QGQDNQ---------------------.SQTLV

Tu  1384 RFLLEALDELEVAFTHPAAKQTDCNHIFLNFVP--------------------KVTMDPL
Ph  1352 .V....M.......S..HS.R..........--------------------T.I...A
Tc  1414 .V....M.......S..HSRR.........I.--------------------T.I...A
Aa  1545 .V....M.......S..Q..R..........--------------------T.I...A
Dm  1578 .V....M.......S..H..R..........--------------------T.I...A
Hs  1448 .L....M.......NN-TNVR..........--------------------T.I...S
Gg  1425 .L....M.......NN-TNVR..........--------------------T.I...S
TaC 1344 .S.MA..E.I.LRAHSET-GMSGHS.MY.CIMREQRLFDLIPS-SRMTNEVGQDEKTACT
TaP 1408 .S.MT.IE...LHAIRT-----GHS.M..CILKEQKLLDLVPVSGNKVVDIGQDEATAC.
Zm  1417 .S.MT.IE...LHAIRT-----GHS.MY.CILKEQKLLDLIPFSGSTIVDVGQDEATACS
Os  1356 .S.MA..E.I.LHAHNET-VRSSYS.MY.CILRVQQLFDLIPF-SRTIDNVGQDEATACT
At  1346 .S.MD.ME...LNAHNA.-MKP.HA.M..CILRDEQIDDLVPFPRRVEVNAEDEE.TVEM
Gm  1352 .S.MA.ME...LNAHNVN-IKSGHA.MY.YIIREQQIDDLVPYPKRINIEAGKEEITVEA
Bn  1406 .S.MD.ME...LNAHKEDGAKP.HA.M..CLLREQHINDLVPYPRRVEVNAEVEE.GIEM

Tu  1424 RIAENVKNTVKNYASRLRKLRVLQAELRMRIRMTTNGKCTPFRLYVSYESGYSLAIHLYK
Ph  1392 K.E.S.T.M.MR.GP..W.........K.T..T.P.S.TETI..CLAND...Y.D.CM..
Tc  1454 K.E.A.TSM.MR.GP..W.........K.I..LAP.APTQTI..CLAND...Y.D.NM.T
Aa  1585 K.E.S.TKM.MR.GP..W.........K.V..Q..QSPT.SV..CIAND...F.D.AM.T
Dm  1618 K.E.S.TKMIMR.GP..W.........K.V..QSPQSPTQAV..CIAND...F.D.SM.T
Hs  1487 K.E.S.RSM.MR.G...W.........KIN..L.PT..AI.I..FLTN....Y.D.S...
Gg  1464 K.E.S.RSM.MR.G...W.........KIN..L.PT..AI.I..FLTN....Y.D.S...
TaC 1402 LLKHM.M.IYEHVGV.MHR.S.C.W.VKLWLDSDGQA-NGAW.VV.TSVT.HTCTVDI.R
TaP 1463 LLK.MALQIHELVGA.MHH.S.C.W.VKLKLDSDGPA-SGTW.VVTTNVTSHTCTVDI.R
Zm  1472 LLKSMALKIHELVGA.MHH.S.C.W.VKLKLDCDGPA-SGTW.VVTTNVT.HTCT.DI.R
Os  1414 LLKNMAL.IYEHVGV.MHR.S.C.W.VKLWLDCDGQA-SGAW.VV.TNVT.HTCTVDI.R
At  1405 ILE.AAREIHRSVGV.MHR.G.CEW.V.LWLVSSGLA-.GAW.VV.ANVT.RTCTV.I.R
Gm  1411 VLE.LAREIHSSVGV.MHR.G.VVW.IKLWMAACGQA-NGAW.VI.NNVA.HTCTV...R
Bn  1466 ILE.T.REIH.SAGV.MHR.G.CEW.V.L.LAS------GVW.AV.TNVT.RTCTV.I.R
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Tu  1484 ETRDPASGLTKFEAWGAPYYEPGPLNDQPISSPYRTKDYLQSKRFLAQSNGTTYIYDFPV
Ph  1452 .IT..KT.TI....Y.S---KQ..MHGL..TV..V......Q...Q...T....V..I.D
Tc  1514 .EV..DT.II..R.Y.K---KQ..MHGL...T..LA.....Q........A....V..Y.D
Aa  1645 .VT..ETHVI..Q.Y.N---RQ...HGL.....M.....Q...Q........V..I.D
Dm  1678 .QTE.ET.II..K.Y.E---KQ.S.HGH...T..M...F..Q...Q........V..V.D
Hs  1547 .VT.SRTAQIM.Q.Y.D---KQ...HGML.NT..V...L......Q...L.......I.E
Gg  1524 .VT.SRT.QIM.Q.Y.D---KQ...HGML.NT..V...L......Q...L..S.V..I.E
TaC 1461 .VE..NTHKLFYRSATP---TA...HGIALHE..KPL.AIDL..AA.RK.E...C....L
TaP 1522 .VE.TE.QKLVYHSAPS---SS...HGVALNT..QPLSVIDL..CS.RN.R...C....L
Zm  1531 .VEEIE.QKLVYHSASS---SA...HGVALNN..QPLSVIDL..CS.RN.R...C....L
Os  1473 .VE.SNTHKLFYHSVTP---SL...HGIVLDE..KPL.AIDL..YS.RK.E...C....L
At  1464 .VET.GRNSLIYHSITK---KG-..HET...DQ.KPLG..DRQ.LA.RRSN...C....L
Gm  1470 .KE.TITHKVVYSSVSV---KG-..HGVAVNEN.QPLGVIDR..LS.RK.S...C....L
Bn  1520 .VEATG.NSLIYQSITK---KG-..HGT..NDQ.KPLGH.VRQ.LA.RRSN...C....L

Tu  1544 MFQTALTQIWREYLKSKEDYELE--MPKTVLSYVELVVDFS-TGKMTEQNRQPGENECGM
Ph  1509 ..RQMTDKL..D.MDENPQEDIS--I.SQLMN.....L.--DDN.LI.TK.L....DV..
Tc  1571 ..RQMVDLQ.KQ.MNQRISEHIQ--P.DKLMDF...IL.PETESRLI..K.V....NV..
Aa  1702 ..RQMTERL.K.FS.ARPTEDIR--I.EKI.LVCNEL.LK--GDTLE.IQ.L....NV..
Dm  1735 ..RQMTERH...FS.ARPTVDIR--T.DKI.IECKEL.LE--GDNLV.MQ.L....N...
Hs  1604 ..RQS.IKL.ESMSTQAFLPSPP--L.SDM.T.T...L.D--Q.QLVHM..L..G..I..
Gg  1581 ..RQS.IKL.DSMNEHAFLPTPP--L.SDI.T.T...L.D--Q.QLVHM..L..G..I..
TaC 1518 A.E...KKS.ESGISHVAESNEHNQRYAE.TELIFADSTG.WGTPLVPVE.P..S.NF.V
TaP 1579 A.E..VQKS.SNISSDTN------RCYVKATEL.FAHKNG.WGTPVIPME.PA.L.DI..
Zm  1588 A.E...QKS.QSNGSTVSEGNENSKSYVKATEL.FAEKHG.WGTPIIPME.PA.L.DI..
Os  1530 A.E...KRS.KST.SVVAEANEHNKSYAK.TELMFADSTG.WGTPLVPVE.S..I.DI.I
At  1520 A.G...ELL.ASQHPGVKKPYKD--TLIN.KEL.FSKPEG.SGTSLDLVE.P..L.DF..
Gm  1526 A.E...E.S.AIQQPGFQRAKDK--NLLK.TELKFADKEG.WGTPLVPVENY..L.DV..
Bn  1576 A.E...EEL.ESQHPGVKKPFKN--TLTN.EEL.FSTPEG.----LIPVK.P..L.D...

Tu  1601 VAWHFTLFTPEYPEGRDIIVIGNDITHQLGIFGPREDIAFNLASVRARELGIPRIYISAN
Ph  1565 ...KVS.N.....T..E....A..L.YLI.S......KV.C...EM..K.K.......V.
Tc  1629 ...RL..Y.....D...V...A.....MI.S......KV.G...EI..CMK.....LA..
Aa  1758 ...RIV.A...F.D..E.V..A..L.YFI.S...Q..ML.CK..ELS.QRKC......V.
Dm  1791 ...RIV.A.....N..E....A..L.YLI.S...IK..VL.AK..QL..Q.KV......V.
Hs  1660 ...KM.FKS.................YRI.S...Q..LL.LR..EL..AE......V...
Gg  1637 ...KM..K.................YRI.S...Q..VL.LR..EL..TH......VA..
TaC 1578 ...NMK.S...F.G..E...VA..V.FKA.S......AF.DAVTNL.C.RK..L..L..T
TaP 1633 ...ILDMS.....N..Q.V..A....FRA.S......AF.ETVTNL.C.RKL.L..LA..
Zm  1648 ...IMEMS...F.N..Q...VA....FRA.S......AF.ETVTNL.C.RKL.L..LA..
Os  1590 ...IMK.S...F.S..E...VS..V.FKA.S......AF.DAVTNL.C.RKL.L..L..T
At  1578 ...CLDMS...F.M..KLL..A..V.FKA.S......AF.LAVTEL.CAKKL.L..LA..
Gm  1584 ...FMEMC...F.S..T.L.VA..V.FKA.S......AF.RAVTDL.CTKKL.L..LA..
Bn  1630 ...TLEMS...F.K..K..IVA..V.FKA.S......AF.LAVTEL.CAEKL.L..LA..

Tu  1661 SGAKIGLAEEVKSIFEICWIDKNIPDKGFHYLYLSPENYKKIEKS--INAELIEDEGEQR
Ph  1625 ...R......I.AL.K.A.E.PDE.....K....TT.D.A.VSALNSVKTV.......R.
Tc  1689 ...R........NSYK.A.E.N.E.....R....T..D.A.VSAQNSVR.V.......S.
Aa  1818 ...R.........L.KVA.E.PDE.E...K....TT.D.S..ANTNSVR.I.......P.
Dm  1851 ...R........AM.K.A.E.PEE.....K.....T.D.AQVANLNSVR.I........
Hs  1720 ...R.......IRHM.HVA.V.PED.Y..YR....T.QD..RVSALNSVHC.HV.....S.
Gg  1697 ...R.......IRHM.HVA.E.PDD.Y..YK....T.QD...VSALNSVHC.HV..N..S.
TaC 1638 A..RL.V...I.AC.HVG.S.DQS.ER....I..TEQD.SRLSS.VIAHELKVPES..T.
TaP 1693 ...R..I.D....C.RVG.S.DGS.ER..Q.I..TE.DHAR.SA.VIAHKMQLD-N..I.
Zm  1708 ...R..I.D....C.RVG.S.EGS.ER..Q.I..TE.D.AR.SS.VIAHKLELD-S..I.
Os  1650 A..RL.V...I.AC.NVG.S.DES.ER....I..TEQD.SRLSS.VIAHELKL.-S..T.
At  1638 ...RL.V.....AC.KVG.S.EIS.EN..Q.I.....DHER.GS.VIAHEVKLS-S..T.
Gm  1644 ...RL.V......C.RVG.SEESN.EN..Q.V..T..DNAR.GS.VIAHELKL.-S..T.
Bn  1690 ...RL.V.....AC.KVG.S.EVS.EN..Q.I.....DHER.GS.VIAHEIKLP-S..T.

Tu  1719 YKILDIVGKEEGLGVENLKYAGLIAGETSRAYQEVITITMVTCRAIGIGSYLARLSQRIV
Ph  1685 ...T..I..DD.......R...M......E..K.IV..S.............V..G..VI
Tc  1749 ..LT..I..DD.......R...M.........D.IV..S..S...........V..G..VI
Aa  1878 ...T..I..TD.......R...M.........ED.V..S.....T.......V..G..VI
Dm  1911 ...T..I..DD.......R...........Q..E.IV..A.....T......VV..G..VI
Hs  1780 ...T..I.....I.P...RGS.M....S.L..N.I...SL.........A..V..G..TI
Gg  1757 ...T..I...D...I...RGS.M....S.L..ESI...NL.........A..V..G..TI
TaC 1698 WVVDT.....D...C...HGS.A..SAY.K..R.TF.L.F..G......A.....GM.CI
TaP 1752 WV.DSV....D......IHGSAA..SAY....E.TF.L.F..G.TV...A.....GI.CI
Zm  1767 WI.DSV....D......IHGSAA..SAY....E.TF.L.F..G.TV...A.....GI.CI
Os  1709 WVVDT.....D...C...HGS.A..SAY.K..K.TF.L.F..G..V...A.....GM.CI
At  1697 WV.DT.....D.I.....TGS.A...AY.K..N.TF.L.F.SG.TV...A.....GM.CI
Gm  1703 WV.DT.....D.......SGS.A...AY....K.TF.L.Y..G.TV...A.....GM.CI
Bn  1749 WV.DT.....D.I.....TGS.A...AY..G.R.TF.L.F.SG.TV...A.....GM.CI
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Tu  1779 QLESSHIILTGHASLNKVLGTEVYSSNNQLGGTQIMFANGVSHAKAKDDLEAILVILDWL
Ph  1745 .I.N.......YQA...L..R...A.......I...YN.....KTEPH..DG.YT..K..
Tc  1809 .I.N.......YSA...L..RQ..A.......I...YN.....KVEQG..DG.ATV.K..
Aa  1938 .IDN.......F.A...L..RK..A.......I...YN...T.KTEAL..DGVYT..Y..
Dm  1971 .IDN.......Y.A...L..RK..A.............N...T.KTEAI..DGVYT.....
Hs  1840 .V.N..L....AGA......R...T.......I...HN...T.CTVC..F.GVFTV.H..
Gg  1817 .V.N.......CGA......R...T.......I...HN...T.GTVC..F.GVYT..L..
TaC 1758 .RLDQP.....YSA...L..R.....QM....PK..AT...V.LTVS....GVSA..K..
TaP 1812 .RTDQP.....FSA...L..R.....HM....PK..AT...V.LTVS....GVSN..R..
Zm  1827 .RLDQP.....FSA...L..R.....HM....PK..AT...V.LTVP.V..GVSN..R..
Os  1769 .RLDQP.....FSA...L..R.....HM....PK..AT...V.LTVS....GVSA..K..
At  1757 .RLDQP.....FST...L..R.....HM....PK..GT...V.LTVS....GVSA..N..
Gm  1763 .RLDQP.....FSA...L..R.....HM....PK..AT...V.LTVS....GVSS..K..
Bn  1809 .RLDQP.....FST...L..R...T.HM....PK..G....V.LTVS....GVSA..N..

Tu  1839 SYAPKKREKATKLVNLTKIKPAPLPIMEPTDIIDRDVVYCPTSSPYDPRWILNGR--ESP
Ph  1805 ..I..DKY------------SIV..IKVS.P...EIS.M..KT......M.A..IINDG
Tc  1869 ..V.RDKN------------S...VIK.I.P.E.E.DFI..KA......M.E..ASPLD
Aa  1998 ..I.DV.G------------GL...VSAS.S.N.PIDFM..KA......M.A..VNP.N
Dm  2031 ..I.AYIG------------CD...VL.N.R.E.P.DFM..K.......M.G..VNPVN
Hs  1900 ..M..SVH------------SSV.LLNSK.P...IIEFV..KT......M.A..PHPTQ
Gg  1877 ..M..SVY------------S.V..LKVK.P...TIDFV..KT......M.A..PNP.Q
TaC 1818 ..V.PYVGG----------....VKSL.PPE.A.T.F.EN.-C.A.AAIC.IQ--DT
TaP 1872 ..V.ANIGG----------....TKSL.PP..P.A.I.ENT-C...AAIS.ID--DS
Zm  1887 ..V.ANIGG----------....TK.L.PP..P.A.I.ENT-C...AAIC.VD--DS
Os  1829 ..V.PYVGG----------.....K.L.PP..P.T.F.EN.-C.A.AAIC.VQ--DS
At  1817 ..I.AYVGG----------...VLA.L.PPE.I.E.V.EN.-C...AAIA.VK--DN
Gm  1823 ..I.SHVGG----------A...VK.L.PPE.P.E.F.EN.-C...AAIS.TL--DG
Bn  1869 ..V.AHAGGP---------L...LLS.L.PPE.T.E.V.EN.-C...AAIA.VN--DN

Tu  1897 QGVWEEGFFDKGSFHEIMAGWAKTVVVGRARLGGIPVGVISVEVRKTEVIIPADPANPTS
Ph  1852 -TD..S....R..WD....P..Q...C.............A..T.TV.LNL.......LE.
Tc  1916 PKN..S....RD.WS...KP..Q...A.............A..T.TV.LNL......LD.
Aa  2045 PSE..T....R.TWS.V.EP..........K.........A..T.TV.LT.......LD.
Dm  2078 AND..N....RD.WS....S.....T.......V.....A..T.TV..EM......LD.
Hs  1947 K.Q.LS....Y...S...QP..Q.................VA..T.TV.LS......LD.
Gg  1924 K.Q.QS....N...L...QP..Q.................VA..T.TV.LS......LD.
TaC 1862 ..K.LS.M..RE..V.TLE......IT...K.......I.A..TETVMQV.....GQLD.
TaP 1916 ..K.LG.M...D..V.TFE....S..T...K.........A..TQTMMQL.....GQLD.
Zm  1931 ..K.LG.M...D..V.TFE.......T...K.........A..TQTMMQ......GQLD.
Os  1873 ..K.MG.M..RE..V.TLE.......T...K.........A..TQTMMQV.....GQLD.
At  1861 T.K.LG.I...N..I.TLE...R...T...K.........VA..TQTVMQ......GQLD.
Gm  1867 N.R.LG.I...D..V.TLE...R...T...K.........VA..TQTVMQ......GQLD.
Bn  1915 A.K.LG.I...N..M.TLE...R...T...K...V....VA..TQTVTQ......GQVD.

Tu  1957 ETKVQSQAGQVWFPDSAFKTAQAINDFNREGLPLMIFANWRGFSGGMKDMYDQILKFGAQ
Ph  1911 .A.TV.............Y......Q..SK.....F.................E.VI....Y
Tc  1976 .A.TV.............Y....S.A..S..D...F.................E..M....Y
Aa  2105 .A.TFQ...........SY......K..G..E...I.L..........Q....E..V....Y
Dm  2138 .A.TLQ......Y...SY......K..G..E...IV................E..V....Y
Hs  2007 .A.IIQ..............Y...K..........V..................V.....Y
Gg  1984 .A.IIQ.....................V..................V.....Y
TaC 1922 AER.VP............A.....LL.....E...F.L.........QR.LFEG..QA.SM
TaP 1976 HERSVPR...........T.....ML.........F.L.........QR.LFEG..QA.ST
Zm  1991 HERSVPR...........T.....LL.........F.L.........QR.LFEG..QA.ST
Os  1933 AER.VP............T.....LL.....E...F.L.........QR.LFEG..QA.SN
At  1921 HER.VP............A.....LM.....E...F.L.........QR.LFEG..QA.ST
Gm  1927 HER.VP............T......L.....E...F.L.........QR.LFEG..QA.ST
Bn  1975 HER.VP........G..A.....LM.........F.L.........QR.LFEG..QA.SA

Tu  2017 IVDALVAYNQPIIVYIPPYAELRGGAWAVVDTTINPRHMEMYADPDSRGGVIEPEGSVEI
Ph  1971 ...G.RE.T...I.L..NG............M.....Y.......E.....L..Q.I...
Tc  2036 ...G.RQ.RK.V.I....NG...........P...S.Y.......E.....L....I...
Aa  2165 ...G.RE.K..V.I.L..N..........L.P.....Y..T....E..A..L....I..V
Dm  2198 ...G.RE.KK.VLI.L..N..........L.SL....Y..T....EA....L....I...
Hs  2067 ...G.RECC..VL.....Q......S.V.I.SS............RE...S.L....T...
Gg  2044 ...G.RE.R..VLI....Q......S...I.P............RE....IL....T...
TaC 1982 ..EN.RT.K..AF....KAG.......V...SK...E.I....ERTA..N.L.AP.LI..
TaP 2036 ..EN.RT....AF....KA........V.I.SK...DRI.F..ERTAK.N.L..Q.LI..
Zm  2051 ..EN.RT....AF....MAG.......V...SK...DRI.C..ERTAK.N.L..Q.LI..
Os  1993 ..EN.RT....AF....MGG.......V...SK...E.I....ERTAK.N.L....L...
At  1981 ..EN.RT.R..VF....MMG.......V...SQ..SDYV.....ETA..N.L....TI..
Gm  1987 ..EN.RT.K...F....MMG.......V..VSR..SD.I.....RTDK.N.L....MI..
Bn  2035 ..EN.RT.G..VF....KMG.......V...SQ..SDYV.....ET...N.L....MI..
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Tu   2077 RFRIKDQIKAMHRNDVICKKIIAEINKTTDPAVK--------KKLERQLNDREQQIKGAY
Ph   2031 K..MR.LL.T...I.PVILS.KN.MMSKGEMLPE------QKAAY.KKIKA..TFLAPM.
Tc   2096 KY.K..LL.T...I.PVLRELDEK.V.FNQTRPMGER--TSIVD..AKITE..NFLMPM.
Aa   2225 K.KE..LV.SIQ.L.PVVLELKKKQAEAAGNKD-------ALAE..N.IKM.VNSLLHV.
Dm   2258 KYKE..LV.TI..L.PTTIALKK.LDEANASGD.-----VRAAQVDEKIKA.IAVLMHV.
Hs   2127 K..R..LV.T.R.V.PVYIHLAERLGTPELSTAE-------R.E..NK.KE..EFLIPI.
Gg   2104 K..R..LV.T.R.V.PVYMRLAERLGTPELS.AD-------R.D..SK.KE..EFLIPI.
TaC 2042 K.KPNELEES.L.L.PELISLN.KLL.E.SASPSPWETAAAAETIR.SMAA.RK.LMPI.
TaP 2096 K..SEELQEC.G.L.PELINLK.KLLGAKHENGS----LSESES.QKSIEA.KK.LLPL.
Zm   2111 K..SEELQDC.G.L.PELINLK.KLQDVNHGNGS----LPDIEGIRKSIEA.TK.LLPL.
Os   2053 K..P.ELEEC.L.L.PELI.LSTRLREMKKENAG----LSEMDTTR.SIIA.MK.LMPI.
At   2041 K..T.ELLEC.G.L.QKLISLK.KLQDAKQ-----SEAYANIEL.QQ.IKA..K.LLPV.
Gm   2047 K..TRELLES.G.L.QQLITLK.PLQEAKS-----SRNIVAFVS.QQ.IKS..R.LLPV.
Bn   2095 K..KREMLEC.G.L.QKLISLK.KLRDAKQ---------SEMELIKQ.IKA.KK.LLPL.

Tu   2129 HIVATYFADLHDKPYNMLMRKAIRGIIPWHRSRYYFYWRLRRLSVEEILKCDLESIDG-S
Ph   2085 .Q..VQ......T.ER.QEKGC.LD.V..RN..KF.....K..VL.NEV.NK.LD.QP-D
Tc   2154 .Q.SVH......T.ER..EKG..ND.V..RK..SIL.......LLQDKVVTA.LDAQP-M
Aa   2278 .T..VH......T.ER..EKGC.SE.V..RS..SF....M...LL..HFIKQILDAQD-.
Dm   2313 .T..VH......T.ER..EKEC.SE.V..RD..RWL.......LL.DAYIKKILRAQD-N
Hs   2180 .Q..VQ......T.GR.QEKGV.SD.LD.KT..TF........LL.DLV.KKIHNANP-E
Gg   2157 .Q..MQ......T.GR.QEKG..TD.LD.KT..TF........LL.DVV.KKIHDANP-E
TaC 2102 TQ...R..E...TSAR.AAKGV.SKVVD.EE..AF..R..L.RLA.DS.AKQVREAA.EQ
TaP 2152 TQI.VR..E...TSLR.AAKGV.KKVVD.ED..SF..K....RIS.DV.AKEIRGVS.KQ
Zm   2167 TQI.IR..E...TSLR.AAKGV.KKVVD.EE..SF..K....RIA.DV.AKEIRQ.V.DK
Os   2109 TQ...R..E...TSAR.AAKGV.GKVVD.EE..SF..R....RVT.DA.AKEIREAA.EQ
At   2096 IQI..K..E...TSMR.AAKGV.KSVVE.SG..SF..KK.N.RIA.SS.VKNVREAS.DN
Gm   2102 TQI..K..E...TSLR.AAKGV..EVLD.RN..SV..Q..H.RIG.QS.INSVRDAA.DQ
Bn   2146 IQI..K......TSMR.AAKGV.KSVVE.SG..SF..KK.I.RIA.SS.VKNVIEAS.DN

Tu   2188 MTIEQINNLMEKWFYADQ-KNTQTQLWNDDKFVAEWLQMQLDDPASLVNENLKELRLNNF
Ph   2144 L.VG.ASSMLRR..TEQE-GPAKVYM.DNNEE.I...ENPE-----TKMKLEENMEAVKR
Tc   2213 LGVG.GEAMLRR..IE.K-GS.EGYK.DNNET.VH..ED..RK.P-SQSIINYN.HCVKK
Aa   2337 LSVG.AKSMLRR..VE.K-GA.EAY..ENNEP.V...EN.KK----SDSTVSRNIYAVKK
Dm   2372 LSVG.AKQMLRR.LVEEK-GA.EAY..DKNEEMVS.YEE.IN----AESIVSRNVNSVRR
Hs   2239 L.DG..QAMLRR..VEVE-GTVKAYV.DNN.DL....EK..TEEDGVHSVIEENIKCISR
Gg   2216 L.DG..QAMLRR..VEVE-GTVKAY..DSN.DLV...EK..MEEEGVRSVVDENIKYISR
TaC 2162 QMPTHRAA.ECIKKWYLASQGGDGEK.S..EAFFA.KDDPDK-----YGKY.E..KAERA
TaP 2212 FSHQSAIE.IQ..YL.SKGAE.GNTE.D..DAFVA.RENPEN-----YQ.YI....AQRV
Zm   2227 F.HQLAME.IKE.YL.S.ATTG-STG.D..DAFVA.KDSPEN-----YKGHIQK..AQKV
Os   2169 LSQKSALDYIK..YLSSNGSDGNSEK..N.EAFFA.KDDPTN-----YENQ.E..KAERV
At   2156 LAYKSSMR.IQD..CNSDIAKGKEEA.T..QVFFT.KDNVSN-----YELK.S...AQKL
Gm   2162 LSHASAM..LKE.YLNSDIAKGREDA.L..EAFFR.KDIPSN-----YENK.....VQKV
Bn   2206 LSYKSAMG.IKD..CKSDVAKGKEEA.T..QVFFT.KENVGN-----YESE.S...AQKL

Tu   2247 SQVVESVCEQFLPKGLTSEDSLRVADCLRTLTDKLKTKVVFSNKHSDIEKEATNDGQSVE
Ph   2198 NSI..QISHKVKECPQAAM...IGIFEILSPSQRVESHAHLESLLQKL.S.QHP------
Tc   2271 DA.INQINVALEDCPDVAL.AVVQIIQKLSDNQ.AEVIRTL.QLG.VENID---------
Aa   2392 DAIISQIQKALEECPEIAL.AVVGLCQALSPAHRGEVVKTL.QLEFTEKEH.KQHKYNPR
Dm   2427 DAIISTISKMLEDCPDVAL.AVVGLCQGL.PVNRGVVVRTLAQMQLNE.TSNS.Q.----
Hs   2298 DY.LKQIRSLVQANPEVAM..IIHMTQHISP.QRAEVIRIL.TMD.PST-----------
Gg   2275 DYILKQIRSLVQANPEVAM..IVHMTQHISP.QRAEIVRIL.TMD.PSST----------
TaC 2217 .TLLSNLA.-TSDAKALPNG-.SLLLSKMDPAKREQVMDGLRQLLG--------------
TaP 2267 ..LLSD.ADSSPDLEALPQG-.SMLLEKMDPSRRAQFVEEVKKVLK--------------
Zm   2281 .HSLSDLADSSSDLQAF.QG-.STLLDKMDPSQRA.FVQEVKKVLD--------------
Os   2224 .KWLSRLA.-SPDVKALPNG-.SIVLNKMNPSKREQVIDGLRQLLG--------------
At   2211 LNQLAEIGN-SSDLQALPQG-.ANLLNKVEPSKREELVAAIRKVLG--------------
Gm   2217 LLQLTNIGDSA.DLQALPQG-.AALLSKLEPLGRV.LTDELRKVLG--------------
Bn   2261 LKQLAEIGN-SSDLQALPQG-.ANILHKVEPLKREQLVDALRKVL.--------------

Tu   2307 STSNGVGNGSSK----------------------------------------------------
Ph        ----------------------------------------------------------------
Tc        ----------------------------------------------------------------
Aa   2452 RAKRLAE.L.VDLRDLVKRCGFERYRVLAVVTVGDRNSQDFKSVLRFVWDAEKDGYVNVT
Dm        ----------------------------------------------------------------
Hs        ----------------------------------------------------------------
Gg        ----------------------------------------------------------------
TaC       ----------------------------------------------------------------
TaP       ----------------------------------------------------------------
Zm        ----------------------------------------------------------------
Os        ----------------------------------------------------------------
At        ----------------------------------------------------------------
Gm        ----------------------------------------------------------------
Bn        ----------------------------------------------------------------
```

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Tu        ------------------
Ph        ------------------
Tc        ------------------
Aa  2512 YETPTYFIVATVFAVYYE
Dm        ------------------
Hs        ------------------
Gg        ------------------
TaC       ------------------
TaP       ------------------
Zm        ------------------
Os        ------------------
At        ------------------
Gm        ------------------
Bn        ------------------
```

Appendix 5 Sequence alignment of selected ACCase homologs from *Tetranychus urticae* (*Tu*), *Pediculus humanus corporis* (*Phc*, XP_002429216.1), *Tribolium castaneum* (*Tc*, XP_969851.2), *Aedes aegypti* (*Aa*, XP_001651879.1), *Drosophila melanogaster* ACCase isoform A (*Dm*, NP_610342.1), *Homo sapiens* ACCase1 (*Hs*, NP_942133.1), *Gallus gallus* (*Gg*, NP_990836.1), *Triticum aestivum* cytosolic ACCase (*Ta*C, ACD46674), *Triticum aestivum* plastid ACCase (*Ta*P, ACD46685), *Zea mays* (*Zm*, AAA80214), *Oryza sativa* (*Os*, AAM18728), *Arabidopsis thaliana* (*At*, AAC41645), *Glycine max* (*Gm*, AAA75528), *Brassica napus* (*Bn*, CAA54683). Protein sequences were aligned using the CLUSTALW alignment algorithm. Sequences were shaded using the BoxShade program. Structural motifs typical of this enzyme are shaded gray, the BC domain (31-528 AA), the BCC domain (662- 728 AA) and the CT domain (1639- 2143 AA).

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4 PUBLICATIONS (in peer-reviewed international journals)

- **Publications in peer-reviewed international journals**

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