Imperial College London

Biology Division
Faculty of Natural Sciences
Imperial College London
Silwood Park Campus
Ascot, Berkshire, SL5 7PY

ROTHAMSTED RESEARCH

Biological Chemistry Division
Rothamsted Research
Harpenden
Hertfordshire
AL5 2JQ

THE USE OF BOTANICAL SYNERGISTS TO INCREASE THE EFFICACY OF NATURAL PYRETHRINS

Anna Khot BSc (Hons)

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Declaration

I hereby declare that I have composed this thesis and the data presented is a record of my own work.

Anna Khot, 2009

Abstract

Insecticide resistance is an important aspect of pest control on both crop pests and vectors of animal diseases. Resistance can be caused by a number of mechanisms, one of which is enhanced detoxification of the insecticide by metabolic enzymes. Synergists may be used in insecticide formulations to inhibit metabolic defences in the insect, allowing the insecticide to reach its target site and kill the insect, thus enhancing the effect of the insecticide.

This PhD project investigated the use of the synergist piperonyl butoxide (PBO) in combination with natural pyrethrins (tank mix) and as a pre-treatment prior to application of pyrethrins, as methods of enhancing the efficacy of the insecticide. The insects studied were *Myzus persicae*, *Bemisia tabaci* and *Musca domestica*. Results showed that the combination treatment (tank mix) was at least as good as, and sometimes better than, the pre-treatment. This is unlike the situation for synthetic pyrethroids where pre-treatments have been shown to be more effective than tank mixes. It is proposed that for natural pyrethrins, PBO aids the penetration of the pyrethrins into the insect, and this enhancement effect is greater than full inhibition of the metabolic enzymes. In some cases, the tank mix enabled less insecticide to be used to achieve 50 % mortality in resistant insects, compared to a susceptible population treated with pyrethrins alone.

A novel laboratory assay was developed to enable the screening of botanical extracts for their ability to inhibit esterase enzymes. This was used to test a range of compounds and those showing esterase inhibition were also screened for their ability to inhibit cytochrome P450 activity. The competency of some of these compounds as synergists was also tested *in vivo* with some showing potential activity both *in vitro* and *in vivo*.

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List of Abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
ATChI	Acetylthiocholine iodide
BRA	Botanical Resources Australia
CL	Confidence limits
DDT	Dichloro-diphenyl-trichloroethane
DEF	S,S,S-tributyl phosphorotrithioate
df	Degrees of freedom
DMC	1,1-bis-(p-chlorophenyl) methyl carbinol
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
E4	Esterase isozyme
EC	Emulsifiable concentrate
EDTA	Ethylenediaminetetracetic acid
ESR	Effective synergistic ratio
FE4	Variant of E4
GABA	Gamma-aminobutyric acid
GSTs	Glutathion S-transferases
HPLC	High performance liquid chromatography
I	Index value: IC_{50} value obtained from Grafit converted into a percentage
IC_{50}	Inhibitor concentration that results in 50 % inhibition of enzyme activity
kdr	Knock down resistance
LC_{50}	Concentration of insecticide required for 50 % mortality
MACE	Modified acetylcholinesterase
MDP	Methylenedioxyphenyl
MFOs	Mixed function oxidases
n	Number of insects tested
Na^+	Sodium ions
NADPH	Dihydronicotinamide adenine dinucleotide phosphate tetrasodium salt
Na_v	'para' voltage-gated sodium channel
NC	Not calculable
OP	Organophosphates
PBO	Piperonyl butoxide
PMSF	Phenylmethanesulfonyl
ppm	Parts per million
PTU	Phenylthiourea
Py	Pyrethrins
Py I	Collective term for pyrethrin I's (esters of Chrysanthemic acid)
Py II	Collective term for pyrethrin II's (esters of Pyrethric acid)
R	Resistant
RF	Resistance Factor
RR	Homozygous resistant
S	Susceptible
SD	Standard deviation (from the mean)
SE	Standard error
SR	Synergistic ratio
v	Volume
VSD	Voltage sensing domains
w	Weight

1 CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Insects become pests in agriculture when they destroy crops and reduce yields thus becoming a nuisance to humans. Some act as disease vectors, often with devastating consequences for crop performance. In animal- and public-health, an insect is considered a pest when it carries disease, is an irritation, or makes living environments unsanitary. Insecticides are used in agricultural farming and animal/public-health to keep the damage and disease caused by insect pests to a minimum. The pyrethrins, the subject of this project, are a naturally occurring insecticide that is obtained from processing the Chrysanthemum flower. Due to its high cost per unit dose and low environmental stability, cheaper and more stable synthetically derived pyrethroid insecticides have been developed using the natural pyrethrins as a template. However, intensive use has meant that resistance to the synthetic pyrethroids is increasing and causing problems for pest-control. This PhD project looks at refocusing on the natural pyrethrins for insect control and examines different methods and approaches to improve their efficacy.

Resistance can be caused by a number of mechanisms which are discussed later in this chapter. One of these mechanisms is enhanced detoxification of the insecticide by metabolic enzymes. In this situation, synergists may be used in insecticide formulations to inhibit the metabolic defences in the insect, allowing the insecticide to reach its target site and kill the insect, thus enhancing the effect of the insecticide. It is upon this principle that this PhD project is based. The study first looks at the use of a well known synergist, Piperonyl Butoxide (PBO), and then moves on to testing other compounds for their ability to synergise natural pyrethrins and increase their efficacy.

Since pyrethrins work by acting on the sodium channel in the nervous system of insects, this chapter begins with a brief overview of insecticide mode of action (section 1.1 and Table 1.1) and a description of the nervous system and how a normal nerve protein functions (section 1.1.1). Resistance is a growing problem in pest control (section 1.2) so the chapter then moves on to describe the different mechanisms that can cause resistance (section 1.2.1 and 1.2.2), focussing on the enzymes capable of detoxifying insecticides (esterases, section 1.2.1.1; cytochrome P450 monooxygenases, section 1.2.1.2; and glutathione S-transferases, section 0), and then a description of different target-site based resistance (acetylcholinesterase, section 1.2.2.1; and knock-down resistance (kdr)/super kdr, section

1.2.2.2). There is then a brief description of cross and multiple resistance which, as explained in section 1.2.3, can increase resistance even further than resistance caused by a single factor.

Over the years there has been much research performed to investigate the use of synergists to increase the efficacy of insecticides. A synergist is a compound that if applied alone, would not exert a noticeable effect on the insect, but when applied with the insecticide, it enhances the effect of the insecticide. Hence the introduction then moves on to describe the use of synergists for enhancing the efficacy of insecticides (section 1.3) and a relatively novel concept which uses a time delay between the application of the synergist and the insecticide (temporal synergism) (section 1.3.1). With this section there is also a description of how the effect of a synergist on an insecticide can be measured (section 1.3.2). The next section focuses on giving an overview of the pyrethrins, the main topic of this study, (section 1.4), looking at their chemistry (section 1.4.1), mode of action (section 1.4.2), residues, persistence and toxicity (section 1.4.3) and then a few examples of when synergists have been used with pyrethrins (section 1.4.4). Since pyrethrins were used as the template for the synthetic pyrethroids, it is important to have an understanding of how the natural pyrethrins were improved upon to create synthetic pyrethroids (section 1.5). The chapter finishes with the aims and objectives of this PhD project (section 1.6).

1.1 Insecticide mode of action

Insecticides are frequently classified by their mode of action. Most affect one of five biological systems in insects: the nervous system; energy production; cuticle production; the endocrine system (insect growth regulators) and water balance. There are several groups of insecticides that act on, and adversely affect, the nervous system and a brief description of each is given in Table 1.1. This project focused on one of those groups of insecticides, the pyrethrins, which affect the sodium channel.

1.1.1 Nervous systems and nerve proteins

The nervous system consists of neurons (single nerve cells) connected to other neurons or muscle fibres through synapses (gaps) at the end of each neuron. Incoming electrical signals are transformed by neurons into an electrical charge that travels the length of the neuron via the movement of ions in/out of the neuron through channels in the membrane of the neurons creating an action potential. There are four main channels that enable different ions to move in and out of the neuron: sodium; potassium; calcium and chloride channels. The

sodium channel has gates which allow the channel to open (to cause stimulation of the nerve) or close (to terminate a nerve signal) in response to a stimulus.

When an electrical charge reaches the end of a neuron, a neurotransmitter (chemical transmitter) is released. It crosses the synapse and binds to a receptor on the post-synaptic membrane of the next neuron. The signal is then converted back into an electrical charge in the second neuron and is transmitted along the length of that neuron. After transmitting its message across the synapse, the neurotransmitter is broken down and reabsorbed back into its originating neuron which is then left in this resting stage until the next signal is received, e.g. the neurotransmitter acetylcholine, is broken down and the choline is reabsorbed.

Table 1.1 Summary of insecticides acting on the nervous system: their modes of action and effects

Primary target site of action	*Chemical subgroup/ exemplifying active ingredient*	*E.g. Active ingredients*	*Mode of action*	*Effect*
Acetylcholine esterase	Carbamates	Aldicarb, Methiocarb	Synaptic poisons: bind to and inhibit the acetylcholinesterase enzyme that is normally responsible for breaking down the ACh	ACh is not broken down and the neurotransmitter continues to cause the neuron to send the electric charge hence preventing termination of the nerve impulse. Continuous stimulation of the nerve leads to tremors, uncoordinated movement and death.
	Organophosphates	Chlorpyrifos, Dimethoate, Malathion		
GABA-gated chloride channel receptor	Cyclodiene organochlorines	Endosulfan, Lindane	Inhibit GABA-receptor	Neurotransmitter is not able to close the chloride channel, thus electrical charge continues down the neuron leading to overstimulation of the nervous system and death.
	Phenylpyrazoles	Fipronil		
Sodium channel	Pyrethroids	Allethrin, Cypermethrin, Deltamethrin, Fenvalerate, Permethrin	Axonic poisons: bind to voltage-gated sodium channel,	Prevent normal closure of the channel, thus continuous nerve firing leading to tremors and uncoordinated movement and death.
	Pyrethrins	Pyrethrins (pyrethrum)		
Nicotinic acetylcholine receptor	Neonicotinoids	Acetamiprid, Imidacloprid, Thiamethoxam	Antagonists of acetylcholine receptor (mimic action of ACh)	Cholinesterase itself is not affected but nerve is continuously stimulated by the neonicotinoid itself which cannot terminate it. Nervous system is overexcited leading to tremors and uncoordinated movement and death.
Chloride channel	Avermectins, Milbemycins	Abamectin	Stimulate GABA receptor thus activating the chloride channel to close	Causes an inhibitory effect, nerve impulses are unable to travel down the chloride channel. Leads to paralysis, insect stops feeding and consequently dies.

1.1.2 Voltage-gated Sodium Channels

The voltage-gated sodium channel, the point of the nervous system where the pyrethrins act, is a large transmembrane protein that regulates the flow of sodium ions across axonal

membranes mediating the rising phase of action potentials. The name of the '*para*' voltage-gated sodium channel (Na_v) was derived from the location of the channel within the paralysis (*para*) locus on the *Drosophila* X chromosome, from which the channel was first cloned by Loughney *et al.* (1989). The *para* channel has been found to be structurally and functionally homologous with the α-subunit of mammalian Na_v channels (reviewed by Catterall, 2000). The structure of the channel can be seen in Figure 1.1.

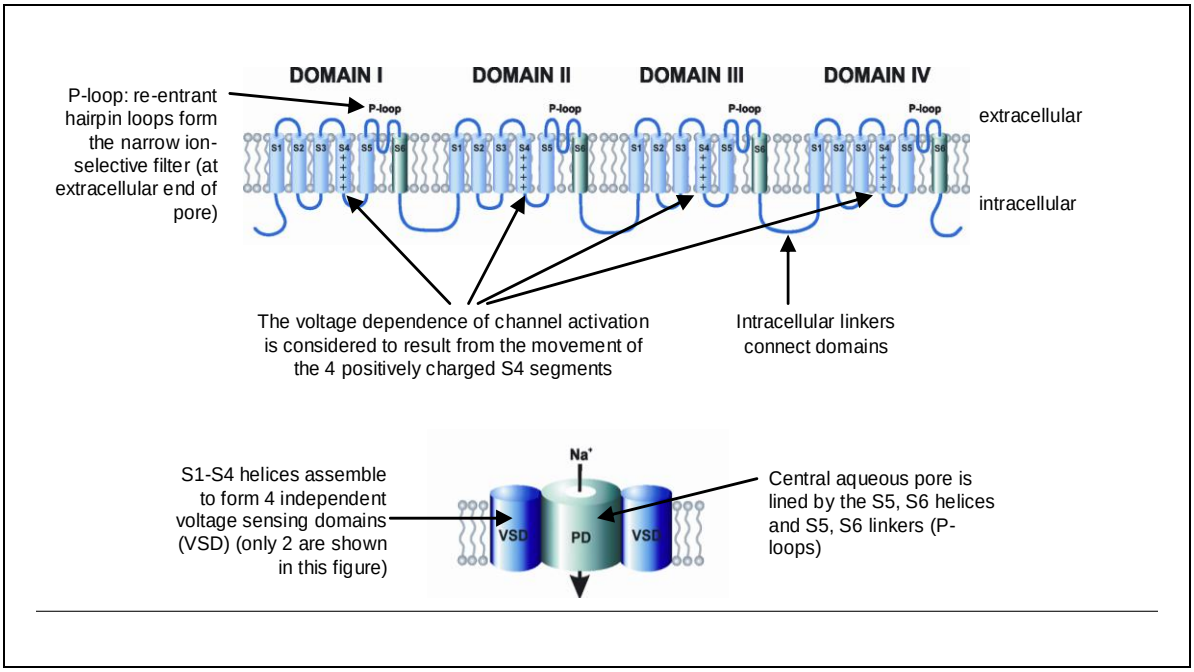


Figure 1.1 The transmembrane structure of the voltage-gated sodium channel

Adapted from Davies *et al.* (2007)

The pore-forming α-subunit consists of a single polypeptide chain with 4 internally homologous domains (I-IV). Each domain has 6 membrane-spanning segments (transmembrane helices) (S1-S6). The 4 domains assemble to form a central aqueous pore (PD). In response to depolarisation the channel undergoes a conformational change which allows a selective influx of sodium ions (Na^+) through the pore. The S1-S4 helices are responsible for the voltage sensitivity of the channel – they assemble to form 4 independent voltage sensing domains (VSD).

1.2 Insecticide resistance

The World Health Organisation (WHO) defines insecticide-resistance as "the inherited ability of a strain or an organism to survive doses of toxicant that would kill the majority of individuals in a normal population of the same species" (WHO, 1957). The extent to which insecticide resistance develops, and the rate at which it occurs, is dependent on a combination of chemical, genetic and biological factors. These include the rate and frequency of application of insecticides used; the mode of action of the applied insecticide; whether resistance is monogenic or polygenic; the frequency of resistant genotypes and the strength (intensity) of resistance associated with each genotype; levels of inherent genetic

variation and the life-cycle and ecology of the insect species in question (Brown, 1990, Roush & Tabashnik, 1990, Hemingway & Ranson, 2000). Short life cycles and production of abundant progeny enable insecticide resistance to evolve and the strong selection pressure of insecticide use results in rapid spread of resistance alleles through pest populations (Hemingway & Ranson, 2000).

There are three main mechanisms by which insects can develop resistance to insecticides. There can be a change in the insects' cuticle which no longer allows the insecticide to penetrate (not discussed), detoxification of the insecticide by metabolic enzymes (section 1.2.1) or target site resistance (section 1.2.2). The most important mechanisms of resistance in insects are metabolic detoxification and target site resistance which can both occur in the same insect. For example, *Anopheles gambiae* (Giles) (Diptera: Culicidae), *Anopheles culicifacies* (Diptera: Culicidae) and *Anopheles subpictus* (Grassi) (Diptera: Culicidae) have all been found with both elevated P450s (section 1.2.1.2) and *kdr* (section 1.2.2) (Karunaratne *et al.*, 2007, Chen *et al.*, 2008).

1.2.1 Metabolic detoxification

All xenobiotics, including naturally occurring plant allelochemicals as well as insecticides and their synergists, are at risk of detoxification (Bernard & Philogene, 1993) involving the transformation of the compound which ultimately reduces its capacity to interact with its target molecule (Figure 1.2). At a biochemical level, detoxification involves three major groups of enzymes: esterases (section 1.2.1.1), cytochrome P-450 monooxygenases (section 1.2.1.2), and glutathione S-transferases (GSTs) (section 0), which can metabolise many pesticides for example organochlorines, organophosphates (OPs), carbamates and pyrethroids. In most, but not all cases, metabolic resistance can be detected in individual insects through increased quantities of enzyme compared to their susceptible counterpart (Brown & Brogdon, 1987, Hemingway, 1989, Hemingway *et al.*, 1995) .

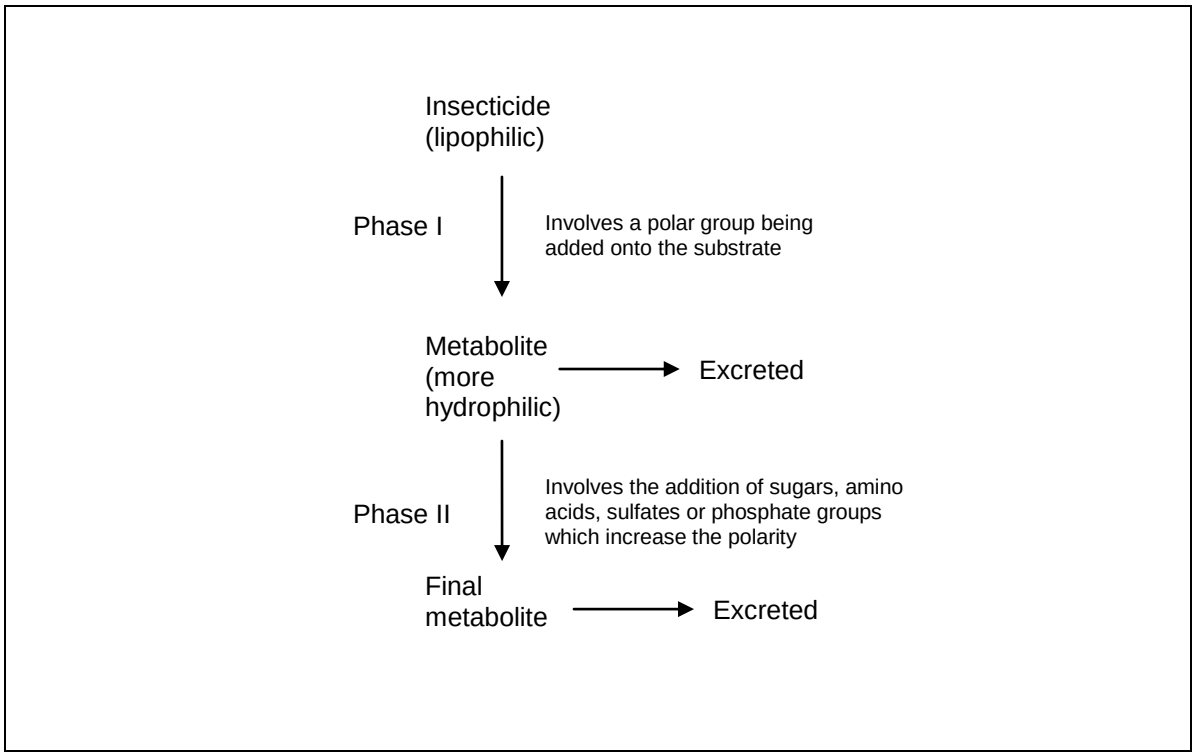


Figure 1.2 The general transformation pathway for most insecticides

(after Hodgson, 1985)
The rate of these metabolic reactions converting a toxic compound into a non-toxic compound involves two steps and is a critical factor in the development of resistance in pest populations.

1.2.1.1 Esterase-based resistance

Esterases are a group of phase I metabolic enzymes that are capable of hydrolysing compounds that contain ester bonds. The increased esterase activity involved in insecticide resistance may be due to an altered enzyme with a higher catalytic rate, or from the esterases being present in elevated levels, through the process of gene amplification (Field *et al.*, 1988, Devonshire *et al.*, 1998, Hemingway *et al.*, 1998, Hemingway, 2000), a spontaneous event which results in an increase in the copy number of one (or more) gene(s) in a genome. This increase in activity was correlated by Field *et al.* (1988) to the level of resistance in *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) and OP resistance in *Culex* species has been associated with increased esterase activity resulting from the amplification of the corresponding structural gene (Mouches *et al.*, 1990, DeSilva *et al.*, 1997).

The esterases involved in insecticide metabolism include carboxylesterases, phosphorotriester hydrolases, carboxylamidases and epoxide hydrolases (Oppenoorth, 1985). Insect carboxylesterases are a large family of enzymes that work by hydrolysis and/or sequestration and have a significant role in insect resistance to many insecticides including OPs, carbamates and pyrethroids (Gupta & Dettbarn, 1993, Devonshire *et al.*, 1998, Casida

& Quistad, 2004, Stok *et al.*, 2004). Esterases hydrolyse ester bonds (on the insecticide) forming a carboxylic acid and an alcohol (Figure 1.3) with the rate of hydrolysis being dependent on the chemical structure of the insecticide (Devonshire & Moores, 1989).

Figure 1.3 Esterase enzymes cleave esters by hydrolysis, forming a carboxylic acid and an alcohol

In the peach-potato aphid, *M. persicae*, detoxification was first characterised biochemically by Devonshire (1977), who carried out enzyme purification studies and demonstrated that a single esterase isozyme (called E4) was highly over expressed in OP resistant clones. E4 can account for as much as 1% of the total proteins in the aphid and confer broad spectrum resistance to OPs, carbamates, and pyrethroid insecticides by ester hydrolysis and sequestration (Devonshire & Moores, 1982). When analysing insecticide-resistant *M. persicae* populations from Italian peach orchards, Mazzoni and Cravedi (2002) found that the over production of E4 and a variant, FE4, was common.

Although many of the esterase studies have focussed on *M. persicae,* high levels of resistance-associated esterases have been reported in other species, including *Culex pipiens* (Linnaeus) (Diptera: Culicidae) (Ben Cheikh *et al.*, 2008, Yan *et al.*, 2008) and *Musca domestica* (Linnaeus) (Diptera: Muscidae) (Zhang *et al.*, 2007).

Changes in the activity of esterases are caused by mutations in the esterase genes. For example OP resistance can occur in *M. domestica* when a single point mutation in an esterase gene confers a single amino acid substitution which results in a reduction in carboxylesterase hydrolysis, but increase in OP hydrolysis, so forming the mutant ali-esterase theory (Oppenoorth & Vanasperen, 1960). Mutations in esterase genes and corresponding changes in the enzymes have also been reported for other insect species, including OP resistant *Culex tarsalis* (Diptera: Culicidae) (Whyard *et al.*, 1995), *M. domestica* (Claudianos *et al.*, 1999) and the sheep blow fly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) (Campbell *et al.*, 1998).

1.2.1.2 Cytochrome P450-based resistance

Detoxification of insecticides has been frequently correlated to the action of mixed-function oxidases (MFOs) that catalyse a reaction that ends with the reduction of molecular oxygen (Casida, 1970). Insects have a complex family of MFOs that are involved in the metabolism of xenobiotics and in endogenous metabolism of insect hormones, pheromones and fatty acids, where they are usually the rate limiting step in the chain (Terriere, 1984, Hodgson *et al.*, 1993).

One group of MFOs are the cytochrome P450s (from here on termed P450s) which are haem-(iron) containing enzymes found in the endoplasmic reticulum of cells and with the ability to carry protons/electrons, thus having oxidative/reductive abilities (Feyereisen, 1999). P450s are phase I metabolic enzymes and are capable of oxidising both endogenous and exogenous compounds (Figure 1.2) and produce a pigment at 450nm which is formed by the absorbance of light at wavelengths near 450nm when the haem-iron is reduced and complexed to form carbon monoxide (Omura & Sato, 1964).

P450s and P450-associated reductases are very diverse and have broad substrate specificity and catalytic versatility enabling them to give some level of resistance to all classes of insecticide (Feyereisen, 2005); metabolising pyrethroids, activating/detoxifying OPs and to a lesser extent, carbamates. There are 25 P450 genes from 4 different gene families which have been found to be overproduced by the up-regulation of genes (reviewed by Li *et al.*, 2007). Up-regulation is a genomic change that increases the production of an enzyme or protein without increasing the number of copies of the gene responsible for producing it.

There are many reports of resistance due to P450s, for example: P450-mediated permethrin resistance that confers limited and larval-specific resistance in *Culex pipiens quinquefasciatus* (Say) (Diptera: Culicidae) (Hardstone *et al.*, 2007) and increased levels of P450s in *An. gambiae* populations in an area of Kenya where permethrin-impregnated bed nets were used (Vulule *et al.*, 1999). In the latter the authors speculated that the use of the impregnated nets selected for higher oxidase and esterase levels. *Anopheles funestus* (Giles) (Diptera: Culicidae), a major vector of malaria in Africa also has P450-based resistance to pyrethroid insecticides. Amenya *et al.* (2008) identified a gene from the P450 CYP6 family, that is highly over-expressed in a pyrethroid-resistant strain of *An. funestus* and is genetically linked to a major locus associated with pyrethroid resistance in the population studied. Later, Wondji *et al.*(2009) identified two

duplicated P450 genes (CYP6P9 and CYP6P4) which are associated with pyrethroid resistance in *An. funestus*.

1.2.1.3 Glutathione-S-transferase based resistance

Glutathione-S-transferases (GSTs) comprise a family of phase II metabolic dimeric multifunctional enzymes that play a role in the detoxification of a wide range of xenobiotics (reviewed by Li *et al.*, 2007). Generally, GSTs catalyse the conjugation of pesticides or their primary metabolites. This conjugation neutralises the electrophilic sites of the lipophilic substrate (e.g. the insecticide) and protects the components of the cell, in particular, the nucleophilic oxygen and nitrogen of DNA from the electrophilic attack of nucleophiles. The conjugation also causes GSTs to increase the water solubility of the conjugation product which then becomes more easily excreted from the cell (Clark, 1989, Enayati *et al.*, 2005).

Although elevated GST activity has been associated with resistance, in many cases the individual GST enzyme(s) involved have not been identified and the role of GSTs has only been established using model substrates (Enayati *et al.*, 2005). Where resistance can be linked to increases in the levels of specific GSTs, it was previously thought to be mainly due to gene amplification or increases in transcription, rather than changes in the enzymes (Grant & Hammock, 1992, Ranson *et al.*, 2001). Amplification or over-expression, have been reported in various insect species including OP-resistant *M. domestica* (Wang *et al.*, 1991, Syvanen *et al.*, 1994); OP-resistant *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (Huang *et al.*, 1998) and DDT-resistant *An. gambiae* (Prapanthadara *et al.*, 1995, Ranson *et al.*, 1997, Ranson *et al.*, 2001). However, more recent studies have suggested the existence of at least one specific GST in conferring resistance. Ding *et al.*(2003) performed expression profiling of the Epsilon class of GSTs which showed that this class is important in conferring insecticide resistance to DDT in *An. gambiae*.

As a phase II metabolic enzyme, elevated GST activity has not been linked to the direct metabolism of pyrethroids but Vontas *et al.* (2001) suggest that they may play a role in conferring resistance to pyrethroids by detoxifying the lipid peroxidation products induced by pyrethoids and Kostaropoulous *et al.* (2001) suggest that GSTs may protect insects against the toxicity of pyrethoids by sequestering the insecticide.

1.2.2 Target-site resistance

A change in an insecticidal target-site protein can lead to a reduction in sensitivity to the inhibiting action of the insecticide. Target-site resistance gives specific resistance profiles, only conferring resistance to insecticides which attack that specific protein. Examples of altered target sites are modified acetylcholinesterase (MACE) and insensitive sodium channels (knock down resistance – *kdr*) (Oppenoorth, 1985, Devonshire *et al.*, 1998). The efficacy of pyrethrins and pyrethroids is decreased by the presence of *kdr* (section 1.2.2.2).

1.2.2.1 Acetylcholinesterase (AChE)

AChE is the target site for OP and carbamate insecticides (Aldridge, 1971). At cholinergic nerve synapses and neuromuscular junctions, the neurotransmitter acetylcholine (ACh) is hydrolysed by AChE following transmission of the impulse so preventing the repeated firing of the postsynaptic nerve. When AChE is inhibited, repeated firing occurs resulting in uncoordinated movements and the eventual death of the insect (Toutant, 1989).

Mutations that result in alterations in the primary structure of AChE reduce the level of inhibition by OPs and carbamates and confer resistance in insects and other arthropod species (Oppenoorth, 1985). It was first found in the *M. persicae - M. nicotianae* complex in 1990 (Moores *et al.*, 1994) and in the UK, aphids with MACE were first identified in 1995 from samples collected in suction traps. In the following year aphids with insecticide-insensitive AChE were found in eastern England, with the insensitivity being specifically to dimethylcarbamates, pirimicarb and triazamate (Foster *et al.*, 1998). Molecular studies have since shown that the reduced sensitivity of AChE is due to one or more point-mutations in the gene which lead to structural changes in the enzyme (Andrews *et al.*, 2004).

1.2.2.2 Knock-down resistance (kdr) and super-kdr

First recognised by Busvine (1951), *kdr* has been found to be the most common form of resistance to DDT and pyrethroids. *Kdr* results from modifications in the axonal sodium channel and is now known to be caused by a recessive allele conferring cross-resistance to both pyrethroids and pyrethrins, as well as DDT (and its analogues). *Kdr*, which has been reported in many insect species, is often accompanied by a second resistance mutation (also recessive) termed super-*kdr* which confers much greater levels of resistance to pyrethroids (Farnham *et al.*, 1987).

Various point mutations in sodium channel genes altering the amino acid sequence of the sodium channel protein have been shown to be responsible for causing DDT and pyrethroid resistance in a wide range of agricultural pests and disease vectors (Davies *et al.*, 2007). In a normal sodium channel, DDT and pyrethroid insecticides bind to sites in the channel, causing it to stay partially open and the nerve to fire continuously, but when *kdr* is present, there is a loss of insecticide binding, resulting in insecticide-insensitvity (Soderlund & Knipple, 2003). Two amino acid substitutions, L1014F (in domain IIS6) and M918T (in domain IIS4-S5 channel linker) were originally identified in pyrethroid resistant housefly strains and were associated with high levels of resistance (Williamson *et al.*, 1996). This *kdr* mechanism involves a mutation in the sodium channel para-type gene which causes the replacement of a leucine by a phenylalanine. The L1014F mutation was also identified cockroaches (Miyazaki *et al.*, 1996) and several other insect species including *M. persicae* (Martinez-Torres *et al.*, 1997). For other *kdr* and super-*kdr* mutations, refer to Davies *et al.* (2007).

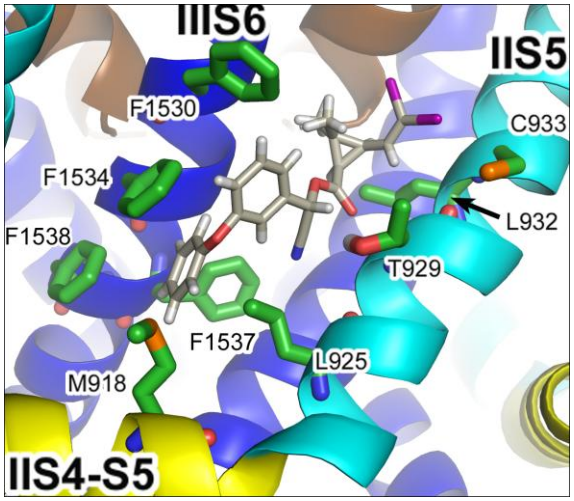


Figure 1.4 Predicted binding sites for pyrethroids in the voltage-gated sodium channel

The O'Reilly model (O'Reilly *et al.,* 2006) shows a hydrophobic cavity formed between the IIS4-S5 linker, the IIS5 helix and the IIIS6 helix. There are several residues, thought to be involved in pyrethroid resistance, which face into this cavity. The acid group is thought to be positioned 'upwards' towards the inner side chains of IIS5 and IIS6. The central ester group is thought to be positioned close to hydrophilic residue T929, and the alcohol groups close to residues of the IIS5 and IIS4-S5 linker (e.g. L925 and M918). The T929 residue on IIS5 helix appears to be a common binding determinant for all pyrethroids, regardless of their structure (O'Reilly *et al.*, 2006).

Williamson *et al.,* (1996) identified the mutations in the voltage-gated sodium channel associated with *kdr* to pyrethroids in houseflies and *kdr* has been found to be a major

mechanism for pyrethroid resistance in field populations of *M. domestica* (Huang *et al.*, 2004). It is now thought that the esterase-based resistance of some *M. persicae* to pyrethroids is of secondary importance to a *kdr*-type mechanism. It is also thought that the modification of the sodium channel which causes *kdr* may not occur at the site at which the insecticide binds, but more likely in a region elsewhere, where the modification affects the conformation of the protein and where ion conductance of the sodium channel is modified (Davies *et al.*, 2007). By contrast modifications of the sodium channel causing *super-kdr* are predicted to be at the actual binding site for pyrethroid insecticides (Figure 1.4) and the change is thought to cause a rejection of the large cyclic side chains in the alcohol component of the pyrethroid molecules (Davies *et al.,* 2007). In normal circumstances, the alcohol component would bind strongly by electrostatic attraction and a number of van der Waals forces (O'Reilly *et al.*, 2006).

1.2.3 Cross-resistance and multiple resistance

Unlike target-site resistance, enzymatic detoxification has the potential to confer cross-resistance to more than one toxin independent of their target site. The occurrence of two or more resistance mechanisms in the same population (multiple resistance) has the potential to produce very high levels of resistance.

The interaction of different mechanisms of resistance can be demonstrated using *M. persicae* where elevated E4 alone can give widespread resistance. However, the combination of enhanced E4 and *kdr* gives an additional resistance to pyrethroids (Devonshire *et al.*, 1998). The combination of enhanced E4 and an insensitive AChE target site gives widespread resistance plus additional high resistance to the insecticide pirimicarb (Moores *et al.*, 1994, Devonshire *et al.*, 1998). The insensitive AChE target site in *M. persicae* is very specific: conferring resistance only to pirimicarb and triazamate. It does not confer resistance to many OP and carbamates (Moores *et al.*, 1994).

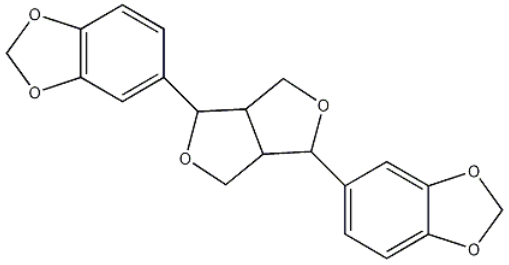
Cross-resistance has also been found in *M. domestica*, for example by Sawicki *et al.* (1984) who demonstrated that very strong pyrethroid resistance could be achieved through the use of non-pyrethroid insecticides. Sawicki reported that the sequential use of two different groups of insecticides (DDT and organophosphates) contributed to a rapid failure of pyrethroid insecticides by the selection for common resistance mechanisms. This work was confirmed

with the production of a pyrethroid-resistant laboratory strain of housefly, by selection with only DDT and trichlophon (Sawicki *et al.*, 1984).

1.3 Synergists and synergism

Metcalf (1967) defined insecticide synergists as non-toxic chemicals that are added to insecticides to increase the insecticidal lethality, or more generally, their effectiveness, against insect pests. When used on its own, a synergist will not produce a notable effect on the insect. However, when it is applied in combination with an insecticide, the synergist enhances the effect of the insecticide. Synergists have been used commercially for over 60 years and have contributed significantly to improve the efficacy of insecticides, especially those to which resistance has occurred (Metcalf, 1967, Bernard & Philogene, 1993). The most efficient synergists are the ones that can interfere with the *in vivo* detoxification of the insecticide (Wilkinson & Hicks, 1969, Raffa & Priester, 1985, Scott, 1990) and the role of a synergist is usually related to its enzyme-inhibiting activity.

a



b

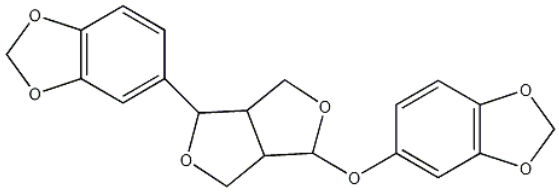


Figure 1.5 Structures of a) sesamin and b) sesamolin

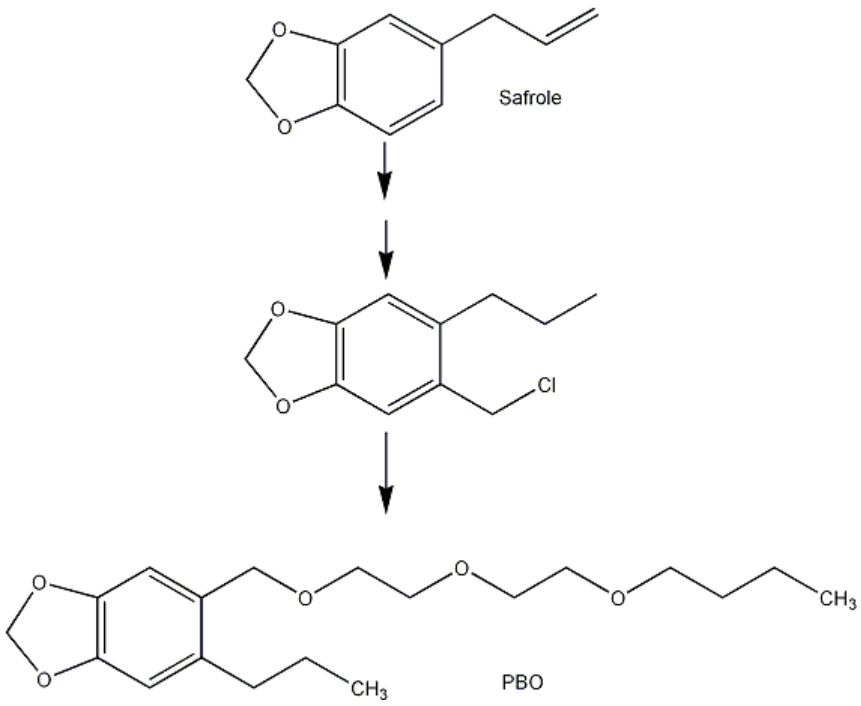


Figure 1.6 Synthesis of PBO from Safrole

(after Casida & Quistad, 1995a)

PBO is synthesised by the hydrogenation of safrole (which is extracted from the root-bark or fruits of sassafras plants, in the form of sassafras oil) chloromethylation, and the addition of the butylcarbityl side chains (Wachs 1947).

Hedenburg investigated the use of compounds containing the methylenedioxyphenyl (MDP) group as insecticides and although it was found that the insecticidal properties were poor, they gave encouraging results when used with pyrethrins. One such compound was piperonyl cycloene. The synergistic activity of sesame oil was due to the sesamin and sesamolin components was also found to be due to the MDP ring (Casida & Quistad, 1995a) (Figure 1.5) and the investigations which followed led to the discovery and production of piperonyl butoxide (PBO). A collaboration between Hendenburg and Wachs found PBO (Figure 1.6) to be the first truly effective and commercially viable synergist with advantages over piperonyl cycloene including complete miscibility with petroleum solvents (Wachs, 1947). It was originally, and still is, regarded as a potent P450 inhibitor (Hodgson & Levi, 1998, Scott *et al.*, 2000). However, PBO has more recently been shown to inhibit resistance-associated esterases in a wide range of agriculturally important pests (Moores *et al.*, 1998a, Moores *et al.*, 1998b, Young *et al.*, 2005, 2006), therefore giving the possibility of enhancing insecticide efficacy in insects where either/both esterase and/or P450-based resistance mechanisms are present.

The mode of action of PBO in the inhibition of P450s is proposed to result from the formation of a stable metabolite (carbene) to form a P450 complex (Philpot & Hodgson, 1972). This is formed between the haem-iron of the P450 and the carbene that is formed when water is cleaved from the hydroxylated methylene carbon of the MDP compound (Dahl & Hodgson, 1979) (Figure 1.7). Little is known about the way in which PBO interacts with esterase enzymes.

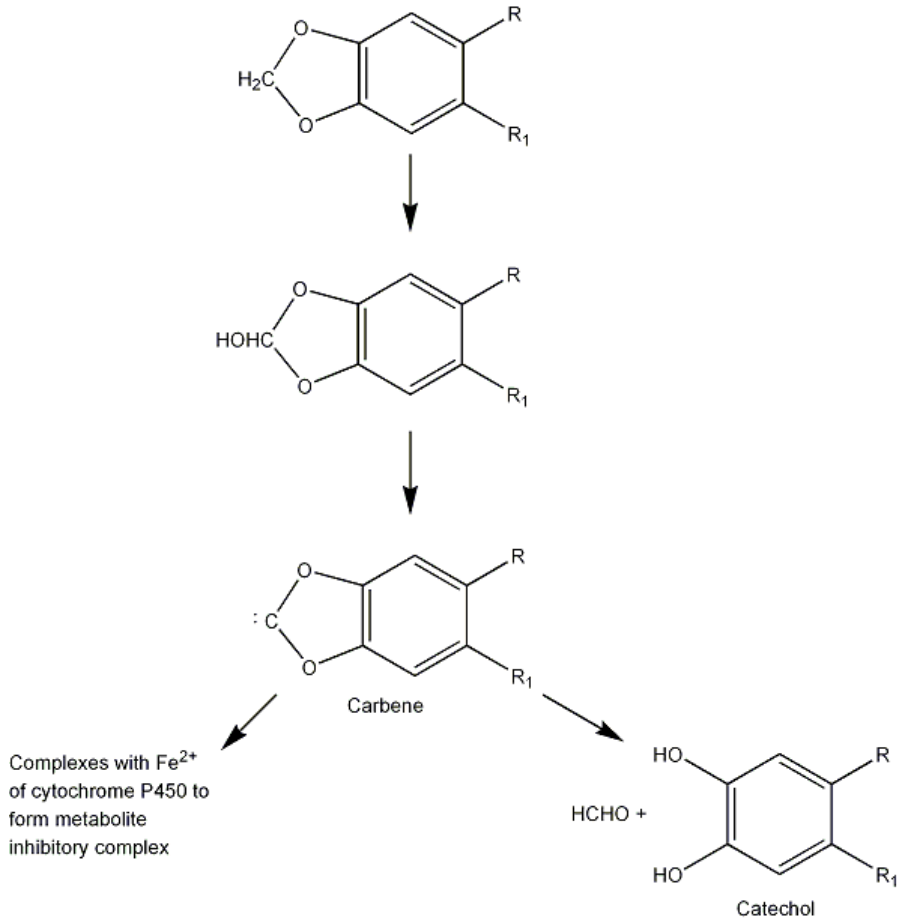


Figure 1.7 Oxidation of MDP compounds by cytochrome P450

(after Hodgson & Levi, 1998)

The ability of PBO to synergise the action of insecticides and overcome resistance has been demonstrated in many systems. For example Vulule *et al.* (1999) found significantly higher mortality rates in a permethrin-tolerant *An. Gambiae* colony in Kenya when the permethrin was synergised with PBO. The colony of mosquitoes studied had increased esterase and oxidase levels and thus the authors speculated the synergism by PBO was due to the suppression of oxidases responsible for permethrin resistance. However it is likely that the PBO was also suppressing the esterases.

PBO has also been found to inhibit AChE and carboxylesterases in *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) (Kang *et al.*, 2006) and it is speculated that the high levels of synergism seen when PBO is used in combination with various insecticides, is due to PBO's multiple effects on esterases and AChE. Gunning (2006) has also found PBO inhibited AChE in the cotton boll worm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and Wu *et al.* (2007) proposed that there is more than one target for PBO as indicated by its high levels of synergism being correlated to its multiple attack on the AChE or detoxification enzymes in various insect species.

Cakir *et al.* (2008) studied the use of PBO as a synergist, with and without tetramethrin as a knock down agent, in combination with synthetic pyrethroids against different housefly populations. They found that PBO promoted the ratio of knockdown to kill with the time taken for the knock down effect decreasing with the addition of tetramethrin, compared to PBO and insecticide, or insecticide only treatment. Synergism studies using PBO and synthetic pyrethroids along with tetramethrin as a knock down agent, have shown PBO to be very beneficial for the biological efficacy of the synthetic pyrethroids, with the PBO and tetramethrin combinations potentially giving a new option for household pest control.

Synergists have also been used to identify the resistance mechanisms present in an insect population. A recent study by Moores *et al.* (2009) investigated the use of a PBO analogue (16/5) to confirm resistance mechanisms. As previously discussed, PBO is known to inhibit both microsomal oxidases and resistance-associated esterases thus making it an ideal synergist. However, it's ability to diagnose resistance mechanisms is limited as it does not clarify which enzyme group is conferring resistance. PBO analogue 16/5 is able to inhibit esterases but due to it's structure it is unable to inhibit microsomal oxidases. By comparing the effect of PBO and the analogue, Moores *et al.* (2009) were able to confirm the identification of the metabolic mechanisms conferring pyrethroid resistance in a clone of *M. persicae* and strain of *B. tabaci*.

When using synergists it is important to note that single inhibitors may not block the activity of all of the toxicologically relevant enzymes, so even if a synergist is applied, the resistance of the insect to a particular insecticide may not be overcome. This is because resistance-associated enzymes may be present but not inhibited by the synergist (Brown & Brogdon, 1987) and a synergist that inhibits a specific metabolic enzyme in one species may not necessarily inhibit the enzymes in another species. For example DEF is commonly used to

inhibit resistance-associated esterases, but does not inhibit those present in *Aphis gossypii* (Glover) (Hemiptera: Aphididae) (Moores *pers. comm.*).

1.3.1 Temporal synergism

PBO is believed to work by binding to the insects' metabolic enzymes that would ordinarily detoxify the insecticide, before the insecticide can act on its target site. With the metabolic enzymes bound to the synergist, the insecticide can act on its target site without being stopped by the enzymes. However, the inactivation of the metabolic enzymes may take time, for example, the topical application of PBO and biochemical analysis has shown that in *B. tabaci* and *H. armigera* PBO-esterase binding is relatively slow *(in vivo)* and a non-permanent process (Young *et al.*, 2005). Temporal synergism (Moores *et al.*, 2005) is the application of a synergist prior to the application of the active insecticide several hours later and it has been shown to enhance the effectiveness of pyrethroids in *B. tabaci*, *H. armigera*, *M. persicae*, and *A. gossypii* (Young *et al.*, 2005, 2006, Bingham *et al.*, 2007) and carbamates and neonicotinoids in *M. persicae*, *B. tabaci* and *A. gossyppi* (Bingham *et al.*, 2008). The work on temporal synergism has led to the development of microencapsulated formulations that deliver an initial burst of PBO followed several hours later by the active insecticide (Bingham *et al.*, 2007).

This idea forms part of the present study, whereby it is determined if the pre-application of a synergist to pyrethrin-resistant insect pests will enable the pyrethrin insecticide, applied several hours later, to act as it would in a susceptible insect and kill the insect rapidly.

1.3.2 Calculating the effect of using a synergist

The synergistsic factor (SF), also known as the synergistic ratio (SR) and the factor of synergism (FOS) can be calculated from the LC_{50} values, where the LC_{50} is the concentration of insecticide required to kill 50% of an insect population.

$$SF = \frac{LC_{50} \text{ insecticide}}{LC_{50} \text{ synergised insecticide}}$$

The SF evaluates the significance of the specific enzymes which are inhibited by the synergist for the detoxification of the insecticide, within a given strain of insects (Bernard & Philogene, 1993). It is helpful to know the SF of a synergist to evaluate its efficacy when used in conjunction with a particular insecticide on a particular insect population. It is important to note however that in calculating the SF certain aspects of the potential activity

of the synergist is overlooked, for example, the insects' behavioural responses (Bernard & Philogene, 1993). The SF, measured *in vivo,* is dependent firstly on the synergist's ability to survive detoxification itself, enabling it to reach its target enzymes, and secondly, upon its inhibitory effect on the detoxifying enzymes which is measurable *in vitro.*

The efficacy of the insecticide normally increases with the relative amount of synergist in the synergist/insecticide mixture (Bingham *et al.*, 2007). As sites on the detoxification protein are occupied by the synergist, the SF reaches a plateau (Brindley & Selim, 1984). At such concentrations the synergist itself becomes toxic, which can interfere with the interpretation of the SFs.

Resistance factors (RF), sometimes known as resistance ratios (RR) (with and without a synergist) can be used to identify the effects of the synergist on the levels of resistance when both a resistant and susceptible strain/clone are treated with the same compounds (Scott, 1990):

$$RF = \frac{LC_{50} \text{ resistant strain}}{LC_{50} \text{ susceptible strain}}$$

Another method of calculating the effect of a synergist, and the one which is used in the present study, is by use of the effective synergism ratio (ESR):

$$\text{ESR (effective synergism ratio)} = \frac{LC_{50} \text{ for any given treatment and clone}}{LC_{50} \text{ susceptible clone, insecticide only}}$$

Calculating the RFs can establish the effect of certain treatments on populations of insects but has a major drawback in that it is only calculable if the treatment is tested against both a resistant and a susceptible clone/strain. Not only is this not always practical but the effect of synergism may not be clear if the synergist also acts on the susceptible clone/strain. However, it does give an indication of how a population is responding to a certain treatment and hence is included in some analyses. The RF describes how many times more insecticide is required on one strain/clone compared to another -normally, a resistant strain/clone compared to a susceptible strain/clone.

The ESR is a useful analysis tool alongside the RF as it gives a better representation of how a population responds to a treatment in relation to how a susceptible population responds to an insecticide only treatment. This means that analysis can show how a treatment responds in relation to field control with a susceptible population. An ESR of 1 means the treatment gives the resistant population of interest the same LC_{50} as the susceptible population after the resistant population has had a given treatment (e.g. synergist plus insecticide). An ESR of less than 1 means the given treatment gives the population of interest a lower LC_{50} than the susceptible treated with insecticide alone and hence comparatively less insecticide is required to kill the resistant population (in the presence of the synergist) than the susceptible population. An ESR greater than 1 means the treatment does not give an LC_{50} as low as the susceptible, ie more/different control would be needed in the field (Moores *et al.*, 2009).

Clearly, neither of the calculations overcome the difficulties where an LC_{50} has not been found in the dose ranges used in the experiments.

1.4 Pyrethrum

Pyrethrum is the generic name for the plant-based insecticide that is derived from the powdered, dried flower heads of the pyrethrum daisy, primarily *Tanacetum cinerariaefolium*, (formerly *Chrysanthemum cinerariaefolium*, of the family Asteracae) but also *Chyrsanthemum coccineum* and *C. marshalli*. Pyrethrum daisies are native to South West Asia with the leading producers being Australia and Kenya (Casida & Quistad, 1995b). The flower is dried and made into pellets and then the pyrethins are extracted using hexane. The solvent is removed to leave a crude oleoresin with a pyrethrin content typically greater than 35 % (Carlson, 1995). The oleoresin is then refined to remove some of the impurities such as vegetable waxes and resins and produce a high quality product.

Pyrethrum is a broad spectrum insecticide consisting of a group of insecticidal compounds with activity against a wide spectrum of insect species which work together to both repel and kill insects. Pyrethrum is made up of six naturally occurring chemical esters, collectively termed pyrethrins (Crombie & Elliot, 1961) and these pyrethrins work as powerful insect nerve agents although they are low in toxicity to humans and other warm blooded animals (Tomlin, 2000), hence making them favourable over other insecticides when human and animal exposure is likely.

Of the hundreds of plants containing chemicals known to be toxic to insects, the pyrethrum daisy is one of the few which has been exploited commercially. Natural pyrethrins have been very important because their chemical constitutions have been used as a template for the development of more cost effective and photostable synthetic pyrethroids (see section 1.5). A major problem with the use of pyrethrins is the high cost per unit dose. Thus, over the years there have been attempts to extend the efficacy of natural pyrethrins and hence provide more economic feasibility, by use of synergists, namely PBO.

1.4.1 Chemistry of pyrethrins

Pyrethrum extract contains three naturally occurring closely related insecticidal esters of chrysanthemic acid (Pyrethrins I) which have a CH_3 group on the acid moiety and three corresponding esters of pyrethric acid (Pyrethrins II) which have a $CH_3OC(O)$ on the acid moiety. The alcohol constituent of the ester has three natural variations: pyrethrolone (in pyrethrin I and II), cinerolone (in cinerin I and II) and jasmolone (in jasmolin I and II) (Elliot & Janes, 1973). All six structures can be seen in Figure 1.8. Collectively, Pyrethrins I and II constitute 45-55% of pyrethrum extract with the remainder of the extract usually being comprised of sterols, triterpenols, alkanes, fatty acids from triglycerides and carotenoids (Maciver, 1995) (Table 1.2). It is known that pyrethrins are altered by heat and light with heat inducing rearrangement and the formation of less active isopyrethrins and light inducing severe degradative changes (Maciver, 1995).

Table 1.2 Chemical formulae and relative proportions of the six naturally occurring pyrethrin esters in a typical 50% extract of pyrethrum

(Casida, 1973)

	Constituent	*Chemical formula*	*Proportion of ester in a typical 50 % extract (%)*	*Molecular weight*
Pyrethrins I (24.7%)	Pyrethrin I	$C_{21}H_{28}O_3$	19.0	328.4
	Cinerin I	$C_{20}H_{28}O_3$	3.7	316.4
	Jasmolin I	$C_{21}H_{30}O_3$	2.0	330.4
Pyrethrins II (25.3%)	Pyrethrin II	$C_{22}H_{28}O_5$	17.5	372.4
	Cinerin II	$C_{21}H_{28}O_5$	5.8	360.4
	Jasmolin II	$C_{22}H_{30}O_5$	2.0	374.4

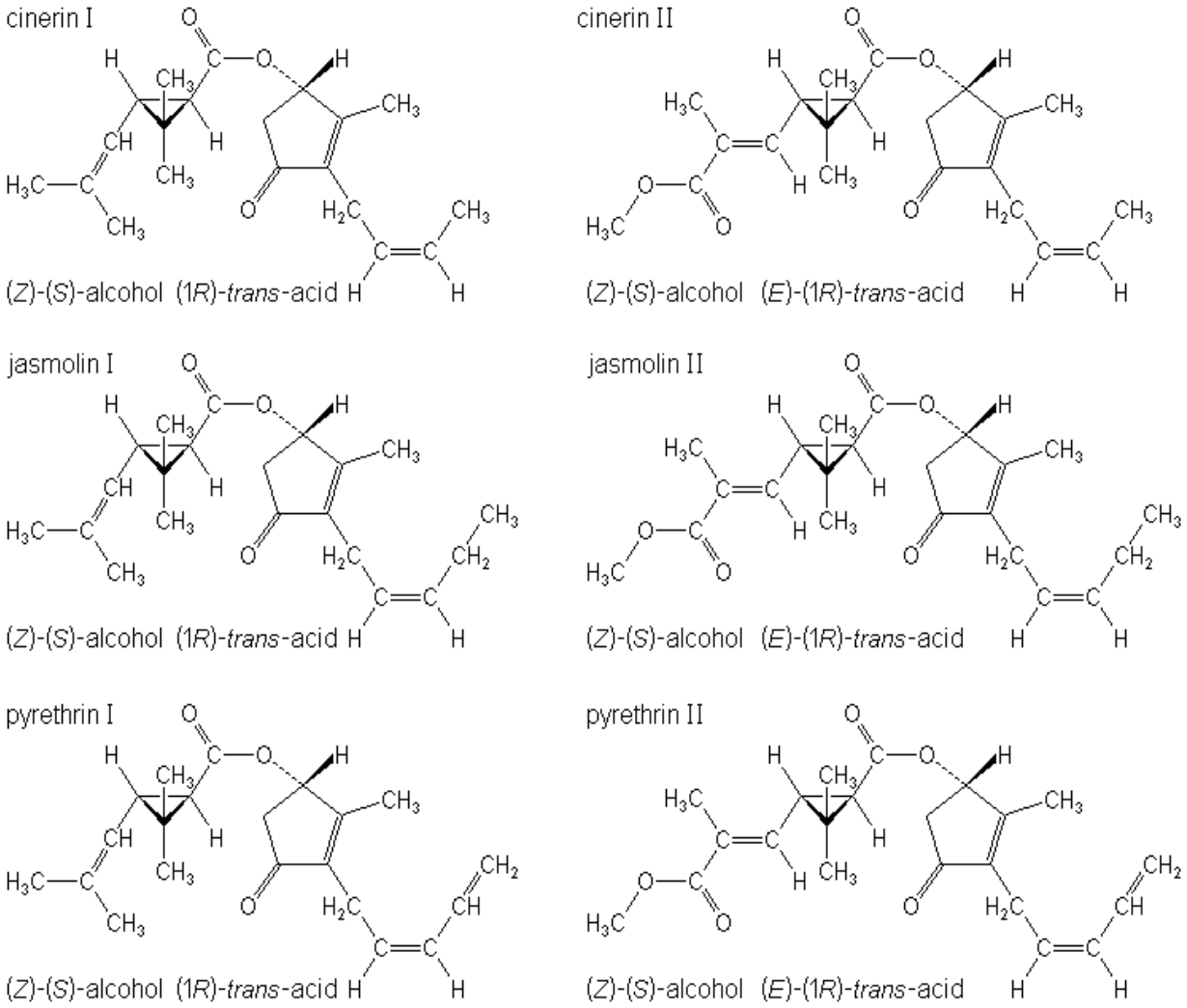


Figure 1.8 Individual pyrethrin esters that together make up 'pyrethrum'

(Casida & Quistad, 1995b)

1.4.2 Mode of action

Pyrethrins are fast acting contact nerve poisons that 'knock down' susceptible insects leaving them paralysed (Klaassen *et al.*, 1996, Tomlin, 2000). The insecticide stimulates repetitive nerve discharges leading to paralysis although some insects can recover from the initial knock down effects if the dose is too low.

The observed effects of the pyrethrins is mediated by binding to the sodium channel (see section 1.1.1). Pyrethrins cause multiple action potentials in the nerve cells by delaying the closure of an ion channel (Costa, 1997). This disruption of the sodium channel leads to repetitive discharges by the nerve cell which causes paralysis and death (Crosby, 1995). Pyrethrins have also been shown to cause female mosquitoes to lose the ability to orientate

themselves thus preventing coordination for feeding by acting as blockers of neurosensing food searching mechanisms in female adult mosquitoes (Maciver, 1963, Maciver, 1964).

1.4.3 Residues, persistence and toxicity

Storage

The storage conditions of pyrethrins are very important. Atkinson *et al.,* (2004) found that prolonged storage of harvested pyrethrum crops in storage sheds results in substantial (ca. 65%) losses of pyrethrin esters (mainly pyrethrin-I and II esters). After an initial rapid loss, pyrethrin content stabilised and it was reported that high temperatures increased the rate of degradation with moisture, oxygen and microbial activity playing minor roles (Atkinson *et al.*, 2004). The authors suggested that *in vivo* the plant structure provides chemical or physical protection to the pyrethrins. This is supported by the findings by Morris *et al.* (2006) that pyrethrins *in planta* do not degrade as rapidly as extracted pyrethrins.

Environmental

The loss of activity of extracted pyrethrins is a disadvantage for their use as an insecticide. They are rapidly degraded in sunlight (Ray, 1991, Crosby, 1995) although use of UV-inhibiting agents can prolong activity. However, the benefits of protecting from pest re-infestation must be weighed up against the concern over impact on beneficial species. Antonious *et al.* (2001) investigated the residues of pyrethrin-I and pyrethrin-II and PBO in soil for a PBO and pyrethrin sprayed potato crop grown in the field. They compared different soil treatments and found that the residues in soil were higher in compost treatments than in no mulch treatments.

Pyrethrin compounds are broken down in water and although their solubility is low they are highly toxic to fish and tadpoles - affecting their skin touch receptors and balance organs (Tomlin, 2000). Pyrethrins are also moderately toxic to birds, including water birds such as ducks and can be toxic to beneficial insects (e.g. honeybees) and many aquatic invertebrates (Tomlin, 2000). Studies by Taiwo and Oso (1997) have shown that treatment of agricultural soils with pyrethrins caused an increase in the abundance of soil bacteria and a decrease in abundance of soil fungi. The number of species present was less in the treated soils than in the untreated soils, with the end result being a reduction in the amount of important soil nitrogen. Another study, conducted by the Central Rice Institute in India, showed that

pyrethrin treatment of rice fields reduced nitrogen fixation in the soil by up to 80% (Nayak *et al.*, 1980).

Animal/Human exposure

After use, commercial preparations of pyrethrins can remain in the air and be deposited onto surfaces giving possible exposure of humans by inhalation or absorbance through the skin (Class & Kintrup, 1991). However, pyrethrin-I can be readily attacked in biological systems and thus, while pyrethrins are highly fat soluble, they are easily metabolised (Ray & Forshaw, 2000) and seem therefore unlikely to accumulate in the body or food chain. Antonious (2004) investigated the half-life of pyrethrins on field-grown peppers and tomatoes and reported that residues were generally higher on the leaves than on the fruits, and that the half-life values on the pepper and tomato fruits did not exceed 2 hours. Thus where concern exists over synthetic pesticide residues on crops intended for human consumption, pyrethrins may be a suitable alternative, reducing the risk of human exposure to synthetic pesticide residues.

1.4.4 Pyrethrins and synergists

Most known synergists of pyrethrins contain an MDP ring (Casida, 1970). Sesame oil was found to increase the activity of pyrethrins and was patented as a synergist by Eagleson (1940). Further studies have shown that it is only the sesamin and sesamolin components that are synergistically active (Figure 1.5) (Haller *et al.*, 1942a, Haller *et al.*, 1942b, Beroza, 1954) with sesamolin being found to be more potent than sesamin (Gersdorff *et al.*, 1954). Limited studies have been performed on these compounds since the 1950s. Sesamex (Figure 1.9), with a structure analogous to sesamolin has been reported to possess very good synergistic activity (Mitchell, 1959).

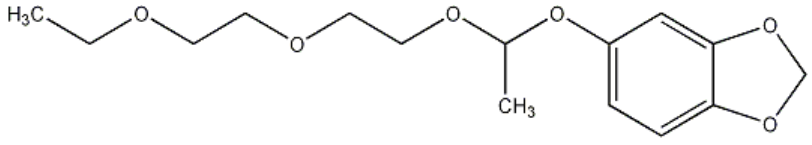


Figure 1.9 Structure of sesamex

The idea that PBO could be used to synergise pyrethrins for the control of pests in stored products was introduced by Dove (1947). Page and Blackith (1950) found that the

insecticidal activity of the pyrethrins, and their stability, could be increased by the addition of PBO. Over the years there has been much research into the synergism of pyrethrins by PBO, some of which was reviewed by Casida and Quistad (1995b). A wide range of PBO/pyrethrin-based insecticidal products have been developed, many of which are licensed for household use including fly sprays and mosquito coils.

1.5 Synthetic pyrethroids

For many decades, scientists have worked on improving the properties of natural pyrethrins by making analogs that are more potent, more stable and less expensive. 'Pyrethroids' is a generic term for the pyrethrins and their synthetic analogs. Many synthetic pyrethroids have been made and the structures of some of those in common use are shown in Figure 1.10. The mode of action of pyrethroids is the same as that of the natural pyrethrins, acting on the sodium channel of the nervous system and causing overstimulation of the nerves and thus a loss of nervous control. Pyrethroids are used against a range of insect pests of ornamentals, fruits, vegetables and other crops, also for household use and control of public health pests as well as being used in animal houses and as an animal ectoparasiticide. Like natural pyrethrins, pyrethroids are generally toxic to birds, fish and bees.

Synthetic pyrethroids are classified into type I or II pyrethroids depending on the presence or absence of a cyano moiety (-CN) at the α-position (Soderlund, 1995), with type II having this group (e.g. cypermethrin, deltamethrin) and type I being without the group (eg permethrin). Type I pyrethroids cause poisoning of the peripheral nerves, seen as hyperexcitation/hyperactivity, loss of muscle coordination and whole body tremors. Type II pyrethroids affect the central nervous system, causing uncontrolled movements and tremors. These have a more prolonged effect and produce higher levels of knock down. Nasuti *et al.* (2003) noted that basic action of the two types of pyrethroids on the sodium channel is similar, although the degree of modification of the sodium currents is different, with single sodium currents being prolonged to a greater extent with type II's than type I's.

A range of synthetic pyrethroids is shown in Figure 1.10 and the modifications for improved performance have been (reviewed by Khambay, 2002). Briefly, some of the key modifications to the alcohol moiety are a benzyl group to replace the cyclopentenone, or an oxygen as a bridge instead of CH_2, or in the acid moiety, dichlorovinyl to replace dimethylvinyl. These are reported to give improved stability in terms of both metabolism

and photodegradation. The volatility of pyrethroids has been improved by the use of polyfluorinated alcohol groups which gives greater control over flying insects. Some synthetic pyrethroids show more persistence and a greater stability in the environment as a result of halides replacing the methyl group of the natural pyrethrins.

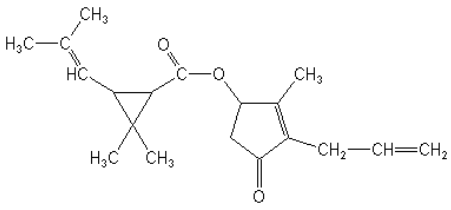
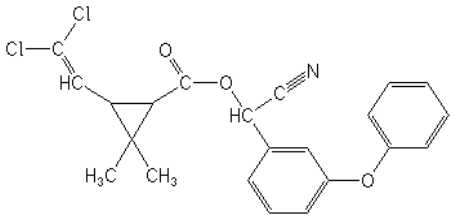
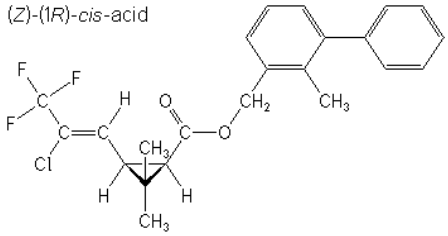
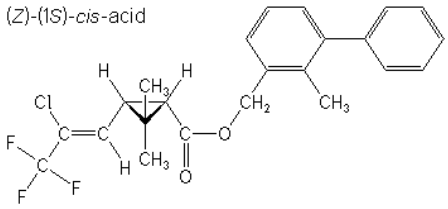
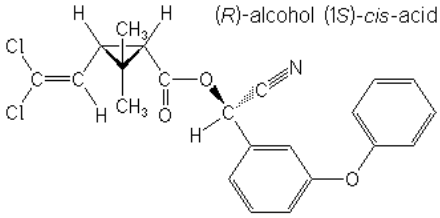
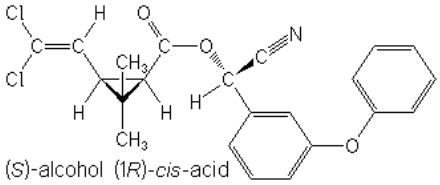
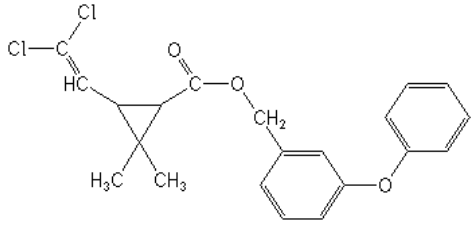
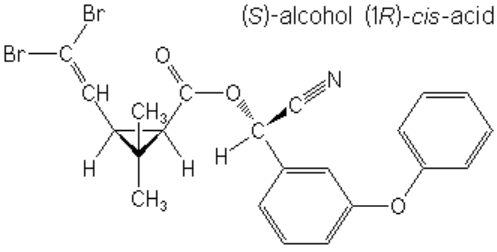
Type I	Type II
 Allethrin	 Cypermethrin
  Bifenthrin	  Alpha- cypermethrin
 Permethrin	 Deltamethrin

Figure 1.10 Structures of some common synthetic pyrethroids

(Tomlin, 2000)

1.6 Aims and objectives of the project

The overall aim of this PhD project was to explore the use of the synergist Piperonyl Butoxide (PBO), and/or alternative botanical synergists, to increase the efficacy of natural pyrethrins against insect-pests.

Specific objectives:

a. To determine if adding PBO as a pre-treatment prior to the application of pyrethrins (Chapter Three) can increase the efficacy of natural pyrethrins against *Myzus persicae*, *Bemisia tabaci* and *Musca domestica*; compared to the more conventional method of using a tank mix treatment (Chapter Three);
b. To optimise the pre-treatment time for PBO on *Myzus persicae* (Chapter Three);
c. To investigate if microencapsulated PBO and pyrethrins enhances the efficacy of natural pyrethrins against *Myzus persicae* (Chapter Three);
d. To develop a new biochemical assay to screen potential synergists *in vitro* for their ability to inhibit esterase activity and to validate this assay by revealing the inhibition of esterases by PBO *in vitro* (Chapter Four);
e. To identify a potential new synergist(s) by first screening different compounds *in vitro* to investigate the putative synergist's ability to inhibit esterases (Chapter Five) and P450s (Chapter Five) and by *in vivo* studies looking at the putative synergists ability to penetrate the insect cuticle and then inhibit resistance associated esterases/P450s thus increasing the efficacy of natural pyrethrins against *Myzus persicae* (Chapter Five) and *Musca domestica* (Chapter Five).

2 CHAPTER TWO: GENERAL MATERIALS AND METHODS

This chapter describes the insects used in the study and their rearing conditions (section 2.1), the details about the insecticide and synergist formulations used (section 2.2) as well as a list of different chemicals used in experiments (section 2.3). Standard experimental methods are described in detail (section 2.4), with specific methods being described in the relevant chapters. Methods of data analysis are also described in this chapter (section 2.5).

2.1 Insects

This section describes the rearing methods for the insects used in the experiments for this PhD project. The insect species used in each experiment were chosen based on the ease of rearing and availability, as well as suitability for the experimental methods used. *Myzus persicae* (section 2.1.1) were chosen because they are easy to rear in large numbers and suitable for both leaf dip (section 2.4.7.1) and topical application bioassays (section 2.4.7.2); *Bemisia tabaci* (section 2.1.2) are easy to rear and suitable for leaf dip bioassay experiments, and *Musca domestica* (section 2.1.3) are relatively straight forward to rear and suitable for topical application bioassays. Where synergist/insecticide solutions were dissolved in acetone, a topical application technique was used, whereas for water soluble formulations, a leaf-dip assay was used.

2.1.1 *Myzus persicae*

A resistant clone (794jz) was originally collected in Worcestershire (UK) in 1982 from a glasshouse. It contains R3 levels of the resistance-associated esterase (E4) that catalogue it as being extremely resistant. This was determined by immunoassay (Devonshire *et al.*, 1986). It is also RR for kdr, based on direct DNA sequencing of PCR-amplified sodium channel gene fragments from aphid genomic DNA i.e. homozygous for the single base change causing an amino acid substitution from a leucine to a phenylalanine in the IIS4-IIS6 region, (mutation: L1014F) (Martinez-Torres *et al.*, 1999). A standard susceptible clone (4106a) was collected in 2000 from a potato crop (determined using the methods described above).

Parthenogenetic stock cultures of the two aphid clones (4106a and 794jz) were reared as described previously (Moores *et al.*, 1994). Briefly, clones were bred without insecticidal selection, and maintained on approximately 2-week-old Chinese Cabbage seedlings (*Brassica rapa* L. var. campestris cv. Wong-Bok) (Brassicaceae) in white fine-netted cages with a plastic base. Cultures were reared in controlled environment rooms at 18 ± 2 °C, 16 h : 8 h L : D

cycle. The seedlings were planted 3 per pot, and 3 pots were used in each cage, watered from a tray below. New cages were set up at approximately 10 day intervals by removing three leaves from infested plants and placing them onto the new seedlings. Clones were then left for 2-3 weeks before the aphids were harvested and frozen at -20 °C for use in the purification of E4 (section 2.4.2). The frequent replacement of plants avoided aphid-overcrowding and the production of winged morphs.

The aphids used for bioassay were reared, without insecticidal selection, in Blackman boxes (Blackman, 1971) as described by Sawicki *et al.* (1980). Two adults were placed in each box and their esterase levels tested four days later, with the nymphs being left to mature. With respect to the resistant clone, boxes containing any nymphs from revertant aphids (Sawicki *et al.*, 1980, Ffrenchconstant *et al.*, 1988, Field *et al.*, 1999) (based on results from esterase tests) were discarded. The aphids were used for bioassay 11-15 days after boxes were set up.

2.1.2 *Bemisia tabaci*

Fours strains of *B. tabaci* were used in this study, with populations being set up from those that had been reared (without insecticidal selection) at Rothamsted Research. Chloraka is a Q-type pyrethroid resistant strain that was collected from cucumber plants in Cyprus in 2003 (Bingham *et al.*, 2007). Pirgos is a B-type pyrethroid resistant strain originally collected in Cyprus in 2003 (Bingham *et al.*, 2007), and Mex-2-GRB is a laboratory cross between a B-type Mexican and a B-type American strain. Sud-S is the standard susceptible strain that was originally collected from the Sudan in 1978 (Bingham *et al.*, 2007).

Stock cultures of three pyrethroid-resistant and one susceptible whitefly strains (Chloraka, Pirgos, Mex2-GRB and Sud-S respectively) were reared on cotton plants (*Gossypium hirsutum* cv. Deltapine 16) (Malvaceae). Strains were bred without insecticidal selection, and maintained on approximately four-week-old cotton seedlings in white fine-netted cages with a plastic base. Cultures were reared in controlled environment rooms at 26 ± 2 °C, 16 h : 8 h, light:dark cycle. The seedlings were planted 1 per pot, and 2 pots were used in each cage, watered from a tray below. New cages were set up at approximately 28 day intervals by collecting a number of individuals from infested plants and placing them onto the new seedlings. Strains were then left to lay eggs on the new plants and the subsequent adults collected approximately 4 weeks later for use in bioassays. To ensure good insect quality, only adults less than 10 days old were used for *in vitro* and *in vivo* studies.

2.1.3 *Musca domestica*

WHO is a susceptible standard reference strain developed and maintained at the WHO Collaborating Laboratory at the Department of Animal Biology, University of Pavia, Italy. The house flies used in the present investigation were progeny of a batch of pupae received at the Danish Pest Infestation Laboratory in 1988 (Kristensen, *pers. comm.*).

The strain 381zb was collected in Denmark in 1978. It is an organophosphate-, carbamate- and pyrethroid-, multiresistant strain. The strain is submitted to periodic selection with topically applied permethrin and topically applied dimethoate. It is postulated that glutathione S-transferase and P450 monooxygenase activities, which are a part of the general detoxification system, are elevated in this strain and that this strain also contains the *super-kdr* mutation of the Na-channel gene causing resistance to pyrethroids (Kristensen, *pers. comm.*). This strain has been reported previously to be 1800-fold resistant to permethrin (Kristensen *et al.*, 2004).

Musca domestica were reared generally following Kristensen *et al.* (2006). Briefly, at all stages the flies were reared at 27 ± 1 °C in a 16:8 h, light:dark cycle. The adults (400-500) were kept in cages with an aluminium frame with a rectangular base (13.5 x 30 cm) and circular ends (24 cm diameter), covered with a clear polythene bag (perforated) (50 cm x 90 cm), closed with an elastic band. On emergence from pupae, flies were fed on water, lump sugar, and a 1:1 mixture of dried milk powder and icing sugar (w/w). Ten days after the pupae were collected (approximately 7 days after emergence as adults), flies were given a milk feed for 24 h with a concertinaed paper towel (surface for egg laying) in the dish containing whole milk. After 24 h the milk was removed and the paper towel placed into a bucket containing larval medium.

Larval medium was prepared in the larvae containers (5 L buckets) and was designed to mimic horse/cow dung. The medium contained fresh yeast (10 g) and malted extract (15 g) mixed with approximately 50 ml warm water before diluting further with 1.25 L warm water. This was mixed into bran (400 g) and lucerne meal (200 g). The buckets containing larvae and medium were covered with a breathable paper sheet and stored at 27 °C. For the first two days they were not touched, after 3 days, the medium was stirred daily with the stirring ceasing when the larvae began to pupate (after approximately 7 days).

Flies reared for bioassay were given only water and cube sugar for the first 4 days post emergence. The milk/icing sugar mixture was supplied after 5 days so that the flies were all at the same stage of sexual development for bioassays. Flies were used for bioassay 9-11 days post-emergence.

2.2 Insecticides and synergists

Three pyrethrum extracts were supplied by Botanical Resources Australia (BRA; Devonport, Tasmania), one of approximately 50 % (w/v) pyrethrins (technical), one of approximately 1 % (w/v) pyrethrins in an emulsifiable concentrate (EC) (dissolvable in water), and the third also an EC formulation of approximately 1 % pyrethrins and 4 % PBO (w/v). A 90 % (w/v) PBO technical grade synergist was purchased from Sigma-Aldrich UK. A separate 4 % PBO EC (w/v) was made up according to a confidential BRA formulation using components provided by BRA with the exception of the PBO which was purchased from Sigma. Two samples of microencapsulated pyrethrins were provided by CNR Istituto di Chimica Biomolecolare (Italy). One sample consisted of ~12.5 % pyrethrins in a γ-cyclodextrin complex, the other consisted of ~10% pyrethrins in a β-cyclodextrin complex.

2.3 Chemicals

All chemicals were stored at room temperature and purchased from Sigma-Aldrich (UK) unless otherwise stated:

- Di-sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$)
- Anhydrous potassium phosphate monobasic (KH_2PO_4)
- Sodium dihydrogen orthophosphate (dihydrate) ($NaH_2PO_4.2H_2O$)
- Ethylenediaminetetracetic acid (EDTA) ($(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$)
- Dithiothreitol (DTT) (stored at 4 °C) ($HSCH_2CH(OH)CH(OH)CH_2SH$)
- Phenylthiourea (PTU) ($C_6H_5NHCSNH_2$)
- Phenylmethanesulfonyl fluoride (PMSF) ($C_7H_7FO_2S$)
- Sucrose ($C_{12}H_{22}O_{11}$)
- 7-ethoxycoumarin ($C_{11}H_{10}O_3$)
- Dihydronicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) (stored at -20 °C) ($C_{21}H_{30}N_7O_{17}P_3.4C_6H_{13}N$)
- Fast Blue RR salt (stored at 4 °C) ($C_{15}H_{14}ClN_3O_3$)
- 1-naphthyl-acetate (stored at -20 °C) ($CH_3CO_2C_{10}H_7$)
- acetylthiocholine iodide (ATChI) (stored at -20 °C) ($(CH_3)_3N(I)CH_2CH_2OCOCH_3$)
- 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) ($[-SC_6H_3(NO_2)CO_2H]_2$)
- Paraoxon (stored at 4°C) ($O_2NC_6H_4OP(O)(OC_2H_5)_2$)
- Eserine (stored at 4°C) ($C_{15}H_{21}N_3O_2$) (> 98 %)

- Azamethiphos (Stored at 4 °C) ($C_9H_{10}ClN_2O_5PS$) (analytical standard)
- Triton X-100 (especially purified for membrane research) (purchased from Roche Diagnostics GmbH)

2.4 Experimental methods

See Appendix 1 for preparation of buffers and substrates.

This sections describes the experimental methods used throughout the PhD project, and are specifically referred to in the relevant chapters of the thesis.

2.4.1 Insect homogenisation

Insects were homogenised in 0.02 M phosphate buffer (pH 7.0) in a tube (1.5 ml) using a plastic pestle. *Myzus persicae* (25), approximately 50 *B. tabaci,* and 10 *M. domestica* heads were homogenised, each in a total of 1.5 ml of buffer.

2.4.2 Purification of carboxylesterase E4

Purification of E4 generally followed the protocol described by Devonshire(1977). Briefly, frozen (-20°C) *M. persicae* (5.8g) were homogenised in 20 ml 0.02 M phosphate buffer (pH 7.0) in a glass homogeniser using a motorised pestle. The homogenate was cooled on ice and centrifuged at 10,000 g for 2 minutes (Eppendorf centrifuge 5810R: in Beckman centrifuge tubes). The supernatant was loaded onto a Sephadex™ G-25 Fine column (Amersham Biosciences) (d:4 x h:15 cm). Protein was eluted from the column using 0.02 M Tris/HCl buffer (pH 8.5) and collected in a conical flask. The elute was then loaded onto an anion exchange column containing a pre-swollen micro-granular anion exchanger (DEAE-Sepharose™ Fast Flow, Amersham Biosciences) (d:3 x h:10 cm) and eluted at 1 ml / min with a linear salt gradient of 0.02 M Tris/HCl (pH 8.5) to 0.02 M Tris/HCl (pH 8.5) plus 0.35 M NaCl (total volume of 400 ml). Fractions (5 ml) were collected and assayed for esterase activity (section 2.4.3), and the fractions with the highest activity were pooled then de-salted and buffer exchanged with 0.02 M phosphate buffer (pH 7.0) and concentrated to approximately 20 ml in a filtration concentrator (Amicon, Hertfordshire UK). The purified E4 was then stored at -20 °C in aliquots until required.

2.4.3 Kinetic assay of esterase activity using 1-naphthyl acetate

Measurement of total esterase activity

Total esterase activity was measured in 96-well NUNC microplates (Fisher Scientific) using a colourimetric assay modified from Grant *et al.* (1989). Esterase activity was determined by

measuring the rate of hydrolysis of the model substrate, 1-naphthyl acetate to 1-naphthol and acetic acid (Figure 2.1) as described by Gunning *et al.* (1998).

A microplate was prepared with 25 µl of 0.02 M phosphate buffer (pH 7.0) in each well, 25 µl insect homogenate and 200 µl substrate. The plate was read on a T_{max} kinetic Microplate Spectrophotometer (Molecular Devices Corporation; Menlo Park, California) at 450 nm at 10 second intervals for 10 minutes. Softmax Pro version 4.6 (Molecular Devices Corporation) was used to provide kinetic plots, the slopes of which were fitted by linear regression. Controls consisting of substrate only were also performed and values achieved were subtracted from those of the samples.

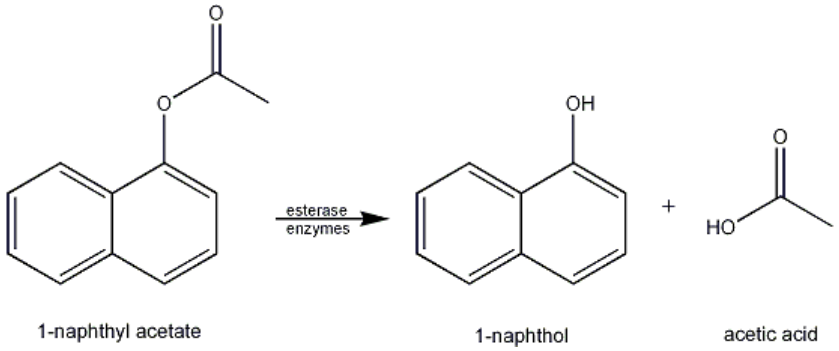


Figure 2.1 The hydrolysis of 1-naphthyl acetate to 1-naphthol and acetate by esterase enzymes

1-naphthol reacts with FBRR (a diazonium salt) to produce a complex. The faster the rate of production of the complex, the higher the level of esterase activity.

The resistance status of aphids reared in Blackman boxes was checked by placing one aphid per well in a microplate and homogenising in 50 µl phosphate buffer using a multi-homogeniser. The homogenate (25 µl / well) was then transferred to a new microplate when the above protocol was then followed.

2.4.4 Assay to measure AChE activity

Kinetic reactions to measure the activity of acetylcholinesterase (AChE) in insect homogenates were performed according to Devonshire and Moores (1984) using a method developed initially by Ellman *et al.* (1961). Acetylthiocholine iodide (ATChI), a synthetic substrate for AChE, is broken down to thiocholine and acetate by AChE (Figure 2.2). The liberated thiocholine then reacts with dithiobisnitrobenzoate (DTNB), to give 2-

nitrobenzoate-5-mercaptothiocholine and 2-nitro-5-thiobenzoate (Figure 2.3). The enzymatic hydrolysis of the ATChI is determined colourimetrically by the characteristic absorbance of 2-nitro-5-thiobenzoate at 405nm for 10 minutes.

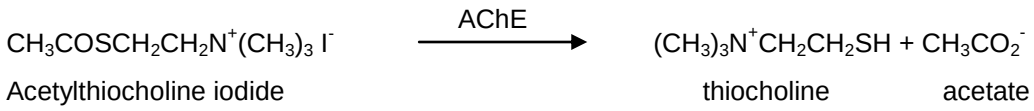


Figure 2.2 The reaction whereby ATChI is broken down by AChE into thiocholine and acetate

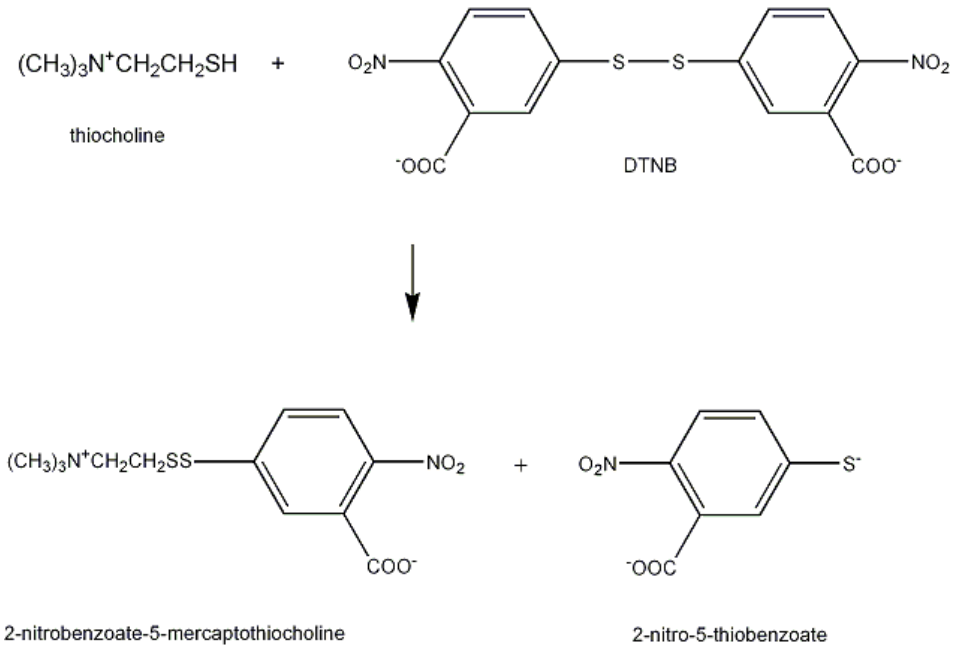


Figure 2.3 The reaction of dithionitrobenzoate (DTNB) with the liberated thiocholine.

To measure AChE activity, a microplate was prepared with 25 µl insect homogenate, 75 µl 0.02M phosphate buffer (pH 7.0), 100 µl DTNB (1.5 mM) and 100 µl ATChI (1.5 mM), unless otherwise stated in the relevant experimental sections, and read on a T_{max} kinetic Microplate Spectrophotometer at 405nm at 10 second intervals for 10 minutes.

2.4.5 'Esterase interference' assay

Purified E4 was incubated with either PBO in acetone (3 mM final concentration, from a 0.3 M stock) or acetone only (to give E4 + 1% acetone), overnight at 4 °C. A 96-well microplate

was prepared as follows: 25 µl 0.02 M phosphate buffer (pH 7.0) was pipetted into every well and 3 µl 10^{-6} M azamethiphos pipetted into the first well, which contained an additional 22 µl of buffer. A 2-fold serial dilution of azamethiphos was performed by pipetting 25 µl from the first column to the next up to the penultimate column, from which the 25 µl was discarded. The last column contained an additional 25 µl buffer only to act as a control. To the wells of row A: 50 µl buffer was added to each well, to row B: 15 µl E4 and 35 µl phosphate buffer were added, and to row C: 15 µl E4 + 3 mM PBO and 35µl phosphate buffer. The E4 was left to stand in the wells for 1 h at room temperature. The resulting azamethiphos concentrations in the wells were (in nM): 20, 10, 5, 2.5, 1.25, 0.62, 0.31, 0.16, 0.78, 0.039, and 0.020. *Musca domestica* homogenate was prepared according to section 2.4.1 with 0.1 % Triton X-100. Homogenate (25 µl) was added to each column and left to stand for 15 minutes at room temperature, followed by 80 µl 1.5 mM DTNB (see appendix 1) and 80 µl 1.5 mM ATChI (see appendix 1). The plates were read as described in Section 2.4.4.

Note: Housefly heads were used, rather than whole bodies, and were homogenised in Triton X-100 to release the enzyme (AChE) from the membrane. In the presence of Triton X-100, AChE behaves kinetically as a single homogeneous enzyme (Devonshire, 1975).

2.4.6 Assay to measure cytochrome P450 activity

This assay involves two different steps: first the P450s from lamb's liver are prepared (section 2.4.6.1), then the assay to measure the activity of the P450s is performed (section 2.4.6.2).

2.4.6.1 Preparation of P450s from lamb's liver

For the screening of putative synergists in chapter five, mixed function oxidases from lamb's liver were used. A 1 g section of fresh lamb's liver was diced and wash in ice-cold buffer (0.1 M sodium phosphate buffer (pH 7.6) containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF, 1.46 M sucrose). The liver was then homogenised in 1 ml of the above buffer (on ice) in a Teflon/glass homogeniser. An aliquot of buffer was then diluted to three-fifths of its original concentration and used to dilute the homogenate to a 10 ml volume. This homogenate was then centrifuged at 10,000 g for 15 minutes. The supernatant was then centrifuged in a Beckman ultracentrifuge at 105,000 g for 1 hour. The supernatant from the ultracentrifugation step was then discarded and the pellet resuspended in 1 ml of the three-fifths diluted sodium phosphate buffer (described above) by using a syringe. This MFO preparation was then stored at -80°C in 100 µl aliquots until required.

2.4.6.2 Activity assay: O-deethylase toward 7-ethoxycoumarin (ECOD)

Substrate (20 mM 7-ethoxycoumarin, Figure 2.4) was prepared by dissolving 7-ethoxycoumarin in 1 ml 100 % ethanol. This was then diluted in 0.1 M sodium phosphate buffer (pH 7.8) to make 0.5 mM 7-ethoxycoumarin. NADPH (dihydronicotinamide adenine dinucleotide phosphate tetrasodium salt, M_r = 833.35) (9.6 mM) was prepared in 0.1 M sodium phosphate buffer (pH 7.8).

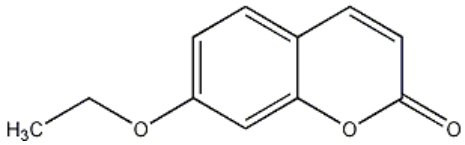


Figure 2.4 7-Ethoxycoumarin

(M_r = 190.20)

To determine the volume of liver homogenate to use in the assay, various volumes of enzyme and diluted sodium phosphate buffer (section 2.4.6.1) were aliquoted onto a white 96-well microplate with three replicates, each giving a final volume of 50 µl. Control wells contained 50 µl diluted sodium phosphate buffer (pH 7.6) only. To each well, 80 µl 0.5 mM 7-ethoxycoumarin was added and the plate was incubated at 30 °C for 3 minutes. To each well, 10 µl 9.6 mM NADPH was then added and the plate read at an excitation wavelength 380 nm, emission wavelength 460 nm using a Victor^2 1420 multilabel counter (Wallac, Milton Keynes, UK) every five minutes, for one hour. The volume of homogenate to use in further assays was chosen based on its ability to give a linear response throughout the assay.

2.4.7 Biological assays

Biological assays were performed by two different methods, either a leaf-dip method (section 2.4.7.1), or a topical application method (section 2.4.7.2). The approach used was dependent upon the insect studied and the compounds being tested. Where compounds were water soluble a leaf-dip approach was performed whereas compounds dissolved in acetone were applied by a topical application technique. For both methods, at least five concentrations of insecticide were tested, plus a control (no insecticide), and each one was replicated three times.

2.4.7.1 Leaf-dip

***Myzus persicae* – EC formulations and microencapsulations**

Chinese cabbage leaf discs (38mm diameter) were treated with dilutions of pyrethrins dissolved in distilled water from an emulsifiable concentrate (EC) (v/v) or a microencapsulated formulation (w/v). Both were dissolved in distilled water and serial dilutions were made using distilled water (v/v). For the EC formulations, a control of the EC formulation minus the active insecticide was also tested at the equivalent of the highest concentration.

The discs were transferred onto 17 ml 1 % agar (made up with boiling tap water) in 30 ml pots (3 cm x 4.5 cm diameter) where they were laid, adaxial surface down to dry in a fume hood. Each treatment was replicated three times. Fluon was applied around the top inner surface of the pots using a cotton bud. Ten aphids were placed onto each leaf disc and the pot was covered with a plastic lid with a hole in the top covered by a fine wire mesh for ventilation. Bioassay dishes were placed in a room with conditions matching those for the insect rearing. Mortality was scored after 72 h and moribund insects were counted as dead.

For a PBO pre-treatment, the aphids were first placed on leaf discs that had been treated with PBO EC (from a stock of 4 %) at a pre-determined concentration (4 times that of the insecticide) for the pre-determined length of time before being transferred to pyrethrin-treated leaf discs.

For a tank mix treatment the PBO and pyrethrins (Py) were premixed by BRA in a ratio of 4:1 (PBO:Py) and applied using the same approaches as for the pyrethrins that were described previously.

***Bemisia tabaci* – EC formulations**

Cotton leaf discs (38 mm diameter) from the same species of plants used for rearing, were treated with pyrethrins from an emulsifiable concentrate (EC) dissolved in distilled water (v/v) and serial dilutions were made using distilled water (v/v). A control of the EC formulation minus the active insecticide was also tested at the equivalent of the highest concentration.

The discs were transferred onto 7.5 ml 1 % agar (made up with boiling tap water) that filled the base of a Petri-dish (0.6 cm x 4 cm diameter) where they were laid, adaxial surface down to dry in a fume hood. Each treatment was replicated three times. Whiteflies were collected by battery operated pooter, briefly anaesthetised with CO_2 and placed onto a freezer block covered by a cloth. Females were collected by mouth pooter and briefly anaesthetised with CO_2. Approximately 10-20 female whiteflies were then distributed onto each leaf disc. The discs were covered with a close fitting lid (0.8 cm x 4 cm diameter) that had 4 small net covered air vents (0.4 cm diameter circles) and the dishes were then placed upside down. Bioassay dishes were placed in a room with conditions matching those for the insect rearing. Mortality was scored after 48 h (moribund insects were also counted as dead).

For a PBO pre-treatment, a four week old cotton plant was sprayed with 500 ppm PBO EC (v/v) (prepared using distilled water) and left to dry in a fume hood for an hour. Female whiteflies were collected as described above and then released onto the plant in a cage for 9 h. The whiteflies were then collected and distributed onto pyrethrin treated leaf discs as described above.

For a tank mix treatment the PBO and pyrethrins were premixed by BRA in a ratio of 4:1 (PBO:Py) and applied using the same approaches as for the pyrethrins that were described previously.

2.4.7.2 Topical application

Myzus persicae

Leaf discs (38 mm diameter) were cut from Chinese Cabbage cv Wong Bok and placed, untreated, onto 17 ml 1 % agar and the pots set up as described above. Ten aphids were placed onto each leaf disc and left to 'settle' for at least 30 min. The aphids were then dosed individually with 0.25 µl of treatment (uncalibrated volume), in acetone, using a Burkard micro-applicator (Burkard Scientific; Uxbridge, Middlesex) and 1 ml glass syringe with a 25 g stainless steel needle. Mortality was scored after 72 h (moribund insects were counted as dead).

Musca domestica

Male and female *M. domestica* were collected and separated for bioassays. *Musca domestica* were collected from rearing cages by battery operated pooter. The flies were then briefly

anaethetised using CO_2 and sexed with males/females being separated for bioassay. Flies (15-25) were put into bioassay dishes which comprised a round, clear plastic tub (10 cm diameter x 3 cm) with a cylinder of paper acting as breathable sides (286 mm x 85 mm, rolled and stapled to fit inside plastic dish). Half of a 10 cm Petri-dish was used as a lid. The flies were given a milk soaked cotton wool pad for 24 h prior to bioassay, and water and cube sugar for the duration of their time in the bioassay pot. Prior to dosing, the flies were chilled at 4 °C for 30 min – 1 h and then briefly anaethetised with CO_2 for dosing. Individual flies were dosed topically with 1 µl compound in acetone (uncalibrated volume) on the dorsal thorax (avoiding the wings), using a Burkard micro-applicator and 1 ml glass syringe with a 25 g stainless steel needle. The control flies were weighed before they were dosed with acetone only. All flies were kept in normal rearing conditions for the bioassay period. The number of dead flies was recorded after 24 h and the LC_{50} in ppm / 20 mg fly calculated from the LC_{50} results achieved using PoloPlus and the weight of the control flies. Mortality was scored after 24 h (moribund insects were counted as dead).

2.5 Analysis of data

Depending upon the experiment performed, different methods were used to analyse the data, as described below.

2.5.1 Novel esterase assay data

Data were analysed using Grafit (Leatherbarrow, R.J., Version 3.09b, Erithacus Software, Horley) to draw graphs of the data, fit curves (4-parameter logistic) and ultimately calculate the IC_{50} for the novel esterase assay (chapter 4). Confidence limits for the novel esterase assay were calculated using the formula shown in Appendix II. Methodology for processing data from the novel esterase assay was developed in accordance with advice from Salvador Gezan (Rothamsted Research, Harpenden, UK).

2.5.2 Cytochrome P450 assay data

The rate of increase in P450 activity over time for each replicate within each compound was calculated using Microsoft Excel (Office XP Edition). Then, the mean and SE of the mean of the rates were calculated for each putative synergist. Methodology for processing data from the P450 assay was developed in accordance with advice from Stephen Powers (Rothamsted Research, Harpenden, UK). Briefly, data were loaded into Excel, and each replicate was plotted, to check for linearity over time. Linear regression was then applied to

estimate the rate of increase for each replicate. The mean rate of the replicates for each putative synergist was calculated, and the efficacy of each one was demonstrated by expressing the mean rate as a percentage of the control (P450 + acetone). The standard errors shown for these values were calculated using the formula for the variance of a ratio (see Appendix III.)

2.5.3 Bioassay data

Raw bioassay data was inputted into PoloPlus (v 1.0, LeOra Software) which converts doses into logarithms and calculates the $LC_{10,\ 50}$ and $_{90,}$ the slope, chi squared and degrees of freedom. Only the LC_{50} and subsequently calculated resistance factors and effective synergism ratios are displayed in data tables in this thesis but the data from the PoloPlus outputs can be found in the tables in the appendices. PoloPlus estimates natural response when control mortality occurs in the bioassays. If no mortality is observed among the controls then the natural response is assumed to be zero (Robertson *et al.*, 2007). The slope gives an indication of the heterogeneity, with steeper slopes being more homogeneous, and also gives an indication of the potency of the insecticide (a more potent insecticide can be seen by a steeper slope). PoloPlus was also used to compare data sets derived from different treatments on the same insect strain/clone. The equality test determines whether the slopes and intercepts of the regression lines are the same. If they are, the treatment effects are deemed not to be significantly different. If the hypothesis of equality is rejected, the treatment response lines are significantly different. Summaries of LC_{50} values (derived from PoloPlus) and corresponding resistance ratios, effective synergism ratios and synergistic factors can be found in relevant sections in relevant experimental chapters. A summary of the PoloPlus outputs can be found in the appendices (Appendix IV for Chapter Three, and Appendix V for Chapter Five).

Resistance factors (RF) and effective synergism ratios (ESR) were calculated using the equations shown below:

$$\text{RF (resistance factor)} = \frac{LC_{50} \text{ (for x treatment) resistant clone}}{LC_{50} \text{ (for x treatment) susceptible clone}}$$

$$\text{ESR (effective synergism ratio)} = \frac{LC_{50} \text{ for any given treatment and clone}}{LC_{50} \text{ susceptible clone, insecticide only}}$$

Because the size of adult *M. domestica* varies, a lethal dose was calculated rather than a lethal concentration. This was calculated for a standard 20 mg fly using the following formula:

$$\begin{array}{c}LC_{50}\text{ (ppm)} \\ \text{(for a standard} \\ \text{20 mg fly)}\end{array} = \frac{LC_{50}\text{ (ppm)}}{\text{Ave. weight (mg)*}} \times 20^{**}$$

*Ave. weight = average weight of one fly based on weight of control flies used in test (mg)
** x20: for a 20 mg standard fly

This calculation was performed as there is a size difference of approximately 25 % between male and female flies, (with females being larger). This is thought to create a similar dilution factor of any chemical substance entering the fly (M. Kristensen *pers. comm.*). Observations in this study, and by Kristensen (*pers. comm.*) indicate that taking the weight of the flies into account usually gives similar LD_{50} values for males and females.

3 CHAPTER THREE: ENHANCING THE EFFICACY OF NATURAL PYRETHRINS USING PIPERONYL BUTOXIDE

3.1 Introduction

Biological assays are important for establishing the *in vivo* effect of a synergist and insecticide on an insect species. Although piperonyl butoxide (PBO) (or an alternative synergist) may give promising results *in vitro*, it is not until these compounds are tested on live insects that their ability to successfully penetrate the insects' cuticle and thus act on their target metabolic enzymes, can be determined.

The bioassay technique using a suitable dose range is very important for establishing the concentration of insecticide required to give 50% mortality in a population of insects (LC_{50}). For each insect species, preliminary experiments were performed to identify the optimal parameters for pyrethrins and PBO/pyrethrin treatments. Various bioassay techniques were tested and the final methods chosen were described in section 2.4.7. The insects studied in this chapter are *M. persicae, B. tabaci* and *M. domestica.*

As discussed in Chapter One, PBO is now known to inhibit non-specific esterases and insecticide action can be enhanced by a mixture of insecticide and synergist. However, studies have shown that it is only by allowing sufficient time for the synergist to penetrate the insect and inhibit the metabolic enzymes, prior to exposure to insecticide (temporal synergism), that maximum control is obtained. This has been demonstrated with various agricultural pests (Young *et al.*, 2005, 2006, Bingham *et al.*, 2007, Bingham *et al.*, 2008). In this chapter the use of PBO, in a combination treatment (tank mix) with pyrethrins, and as a pre-treatment, prior to treatment with pyrethrins are compared.

This chapter describes:

- the determination of the LC_{50} for pyrethrins against *M. persicae*, *B. tabaci* and *M. domestica* to give base-line data for comparisons;
- the testing of the efficacy of a tank mix of PBO and pyrethrins against *M. persicae*, *B. tabaci* and *M. domestica* to assess if a tank mix increases the efficacy of natural pyrethrins;

- the testing of the effect of a PBO pre-treatment prior to treatment with pyrethrins on *M. persicae*, *B. tabaci* and *M. domestica* to assess if a pre-treatment increases the efficacy of natural pyrethrins, compared to a tank mix;
- the use of PBO/pyrethrin microencapsulations on *M. persicae* to assess their effect on the efficacy of natural pyrethrins;
- bioassays using a synthetic pyrethroid (alpha-cypermethrin) to act as a comparison to natural pyrethrins.

The bioassays on *M. domestica* were performed at the Danish Pest Infestation Laboratories (DPIL) in Denmark under the supervision of Michael Kristensen. All other experiments were performed at Rothamsted Research.

3.2 Materials and Methods

3.2.1 Bioassays

Bioassays (pyrethrins only, PBO/pyrethrins tank mix and PBO pre-treatment) were performed on adult insects according to methods described in sections 2.4.7.1 and 2.4.7.2. Important specific details for bioassays, or unique experiments not already described in Chapter Two are described below.

3.2.2 Determining the optimum pre-treatment time for PBO, followed by pyrethrins, for *Myzus persicae*

Bioassays were performed according to section 2.4.7.2. Adult *M. persicae* (794jz clone) were dosed topically with 480 ppm PBO in acetone at intervals to give different pre-treatment times ranging from 30 minutes to 10 hours. All *M. persicae* were then dosed with 120 ppm pyrethrins in acetone after their allotted pre-treatment time. Mortality was scored 72 hours after the pyrethrins treatment. A tank mix treatment (480:120 ppm PBO:pyrethrins) was used as a comparison.

3.2.3 Assessing the effect of a pre-treatment of PBO on the efficacy of natural pyrethrins

Bioassays were performed according to methods described in sections 2.4.7.1 and 2.4.7.2. The pre-treatment times used were 5 hours for *M. persicae*, 9 hours for *B. tabaci* and 3 hours

for *M. domestica*. The ratio of PBO to pyrethrins was 4:1 for all *M. persicae* and *B. tabaci* experiments and 5:1 for *M. domestica*.

3.2.4 Assessing the effect of pyrethrin microencapsulations

Beta- and gamma-microencapsulated pyrethrins were tested on *M. persicae* in a leaf dip bioassay according to methods described in section 2.4.7.1. Where PBO was used, the ratio of PBO to microencapsulated pyrethrins was 4:1.

3.2.5 Bioassays with alpha-cypermethrin

Bioassays were performed on *M. persicae* according to methods described in section 2.4.7.2. The pre-treatment time was 5 hours and for all experiments involving PBO, the ratio of PBO to alpha-cypermethrin was 4:1.

3.3 Results and discussion

Ratios of PBO:pyrethrins of 4:1 for *M. persicae* and *B. tabaci* were chosen based on cost benefits and results of previous studies (Young *et al.*, 2006). A ratio of 5:1 was used for *M. domestica* as this had been used at the Danish Pest Infestation Laboratories and enabled direct comparisons of these experiments with previous work (whilst working in the laboratory in Denmark). Results for *M. domestica* (for a pyrethrins only treatment, and a PBO/pyrethrins tank mix treatment) concurred with those found previously by Kristensen *et al.* (*pers. comm.*) indicating that the insect strains were unchanged.

When pyrethrins were applied at a high concentration, each insect species showed a similar response. Generally, insects were unable to move in a coordinated manner and often lost the ability to feed.

The data shown in Table 3.1 (a-d) are the summaries of LC_{50} values for each insect species studied, and the corresponding RFs and ESRs were calculated using the equations shown in section 2.5.3. The LC_{50} data are derived from the raw data inputted into PoloPlus. The summary of the PoloPlus output can be found in Appendix IV. The data is first discussed in terms of the base-line data (pyrethrins only treatments), then the effect of a tank mix of PBO and pyrethrins on the efficacy of pyrethrins (section 3.3.1), and following that, a discussion of the effect of a PBO pre-treatment in comparison to both pyrethrins alone, and to a tank mix (section 3.3.2). Where a P-value is given, it denotes the result of a test for the hypothesis

of equality based upon the slopes and intercepts of the bioassay data, as explained in section 2.5.3.

Table 3.1 Lethal concentration for 50 % mortality (LC_{50}), resistance factors (RF) and effective synergism ratios (ESR) for a) *Myzus persicae* using a topical application technique; bi) male *Musca domestica* using a topical application technique; bii) female *Musca domestica* using a topical application technique; c) *Myzus persicae* using a leaf-dip technique; d) *Bemisia tabaci* using a leaf-dip technique.

For *M. domestica*, rather than a LC_{50}, the LD_{50} for a 20mg standard fly is given. The corresponding appendix table number is given next to the table sub-headings in brackets. S: susceptible; R: resistant. Clone/strain names are given underneath each sub-table.

a) *Myzus persicae*, topical application bioassay (Table A-IV-Ia)

Treatment	LC_{50} (ppm)		RF		ESR	
	S	R	S	R	S	R
Pyrethrins only	37.2	1630.7	1.0	43.8	1.0	43.8
PBO/pyrethrins tank mix (4:1)	6.8	121.7	1.0	17.9	0.2	3.3
PBO pre-treatment / Pyrethrins (4:1)	46.3	192.7	1.0	4.2	1.2	5.2

S: 4106a clone; R: 794jz clone

bi) *Musca domestica* (male), topical application bioassay (Table A-IV-Ib)

Treatment	LD_{50} (ppm)		RF		ESR	
	S	R	S	R	S	R
Pyrethrins only	659.4	8513.8	1.0	12.9	1.0	12.9
PBO/pyrethrins tank mix (5:1)	46.2	365.3	1.0	7.9	0.07	0.55
PBO pre-treatment / pyrethrins (5:1)	81.3	404.5	1.0	5.0	0.12	0.62

S: $WHOij_2$; R: 381zb

bii) *Musca domestica* (female), topical application bioassay (Table A-IV-Ib)

Treatment	LD_{50} (ppm)		RF		ESR	
	S	R	S	R	S	R
Pyrethrins only	755.7	11251.9	1.0	14.9	1.0	14.9
PBO/pyrethrins tank mix (5:1)	48.6	589.3	1.0	12.1	0.06	0.78
PBO pre-treatment / pyrethrins (5:1)	71.2	570.7	1.0	8.0	0.09	0.76

S: $WHOij_2$; R: 381zb;

c) *Myzus persicae*, leaf-dip bioassay (Table A-IV-Ic)

Treatment	LC_{50} (ppm)		RF		ESR	
	S	R	S	R	S	R
Pyrethrins only	22.7	365.6	1.0	16.1	1.0	16.1
PBO/pyrethrins tank mix (4:1)	-	64.1	-	NC	-	2.8
PBO pre-treatment / pyrethrins (4:1)	-	46.1	-	NC	-	2.1

S: 4106a clone; R: 794jz clone; NC: not calculable

d) *Bemisia tabaci* (female), leaf-dip bioassay (Table A-IV-Id)

Treatment	LC_{50} (ppm)			RF			ESR		
	S	R_1	R_2	S	R_1	R_2	S	R_1	R_2
Pyrethrins only	10.1	629.5	>1000	1.0	62.3	NC	1.0	62.3	NC
PBO/pyrethrins tank mix (4:1)	11.1	173.8	37.4	1.0	15.7	3.4	1.1	17.2	3.7
PBO pre-treatment / pyrethrins (4:1)	10.5	199.8	95.1	1.0	19.0	9.1	1.0	19.8	9.4

S: Sud-S; R_1: Mex2-GRB; R_2: Pirgos; NC: not calculable

Results of the topical application technique of pyrethrins on *M. persicae* can be seen in Table 3.1a. These results show that the susceptible clone (4106a) has a LC_{50} of 37.2 ppm and the resistant clone (794jz) has a LC_{50} of 1630.7 ppm, with a difference of 1593.5 ppm between these two clones. The RF and ESR for the resistant clone are both 43.8 which means that the resistant clone requires 43.8 times more pyrethrins than the susceptible clone for the same level of mortality (50 %).

Results of the topical application of pyrethrins on *M. domestica* can be seen in Table 3.1bi and bii. These results show that the susceptible strain ($WHOij_2$) has a LC_{50} of 659.4 ppm (males) and 755.7 ppm (females). The resistant strain (381zb) has a LC_{50} of 8513.8 ppm (males) and 11251.9 ppm (females). The RF and ESR values are 12.9 for males and 14.9 for females indicating that the LC_{50} for resistant males is 12.9 times greater than for the susceptible males, and for females it is 14.9 times greater.

Results of the leaf dip bioassays on *M. persicae* can be seen in Table 3.1c. The susceptible clone (4106a) has a LC_{50} of 22.7 ppm and the resistant clone has a LC_{50} of 365.6 ppm which is 342.9 ppm greater than the susceptible clone. The RF and ESR for the 794jz clone are 16.1 thus 16.1 times more pyrethrins are required to kill the resistant clone than the susceptible clone using the leaf dip technique. The LC_{50} of the resistant clone using the leaf dip technique is lower than that of the topical application technique.

Results of the leaf dip bioassays on *B. tabaci* can be seen in Table 3.1d. The susceptible strain (Sud-S) has a LC_{50} of 10.1 ppm. One resistant strain (Mex2-GRB) has a LC_{50} of 629.5 ppm which makes it 619.5 ppm greater than the susceptible strain, and with an RF and ESR of 62.3. The other resistant strain (Pirgos) was not killed in the dose range tested and hence the LC_{50} is known to be >1000 ppm and the RF and ESR are not calculable.

3.3.1 The effect of a tank mix of PBO and pyrethrins

The summary of data from bioassays investigating the effect of a tank mix of PBO and pyrethrins can be seen in Table 3.1a-d.

Results for a topical application of a PBO/pyrethrins tank mix to *M. persicae* can be seen in Table 3.1a. Treatment of the susceptible clone (4106a) with the tank mix saw a reduction in LC_{50} from 37.2 ppm to 6.8 ppm which is a 30.4 ppm difference. This indicates that in the apparently susceptible clone, the background metabolic defences are knocked out by the

presence of the PBO. There is a significant difference between the results for the pyrethrins only treatment and the tank mix treatment. The resistant clone (794jz) saw a decrease in LC_{50} from 1630.7 ppm to 121.7 ppm which is a difference of 1509 ppm. This saw a corresponding fall in the RF which was reduced from 43.8 to 17.9, and a decrease in the ESR from 43.8 to 3.3. The pyrethrin only treatment and tank mix treatment results are significantly different ($P<0.05$) (see section 2.5.3) and show a large decrease in LC_{50} when PBO is present. Although this decrease in LC_{50} has occurred, the ESR is greater than 1.0 and this shows that the resistant clone has a higher LC_{50} when treated with the PBO/pyrethrin tank mix, than the susceptible clone treated with pyrethrins alone.

For *M. domestica,* the bioassay data, which can be seen in Table 3.1bi and bii, shows no significant differences between the LC_{50} values for males and females, for either pyrethrins alone or for tank mix treatments, regardless of the strain studied, shown by the overlapping confidence limits. The exception to this was in the resistant strain tank mix treatment which showed a significant difference between males and females ($P<0.05$). Separating flies into sexes before bioassay therefore may not be essential, although it is common practice and the preferred method.

As can be seen in Table 3.1bi and bii, the decrease in LC_{50} comparing pyrethrins alone and the tank mix, was significant for both male and female *M. domestica*, and for both the resistant (381zb) and susceptible ($WHOij_2$) strains ($P<0.05$), indicating that metabolism of pyrethrins was occurring in susceptible *M. domestica*. The resistant strain saw a decrease in LC_{50} from 8513.8 ppm to 365.3 ppm for males, and 11251.9 ppm to 589.3 ppm for females. The decreases in RF were from 12.9 to 7.9 for males and 14.9 to 12.1 for females. These had corresponding decreases in ESR values from 12.9 to 0.55 for males and 14.9 to 0.78 for females which show that with a PBO/pyrethrin tank mix the resistant strain has a lower LC_{50} than the susceptible strain treated with pyrethrins alone.

The susceptible strain of *M. domestica* also saw a decrease in LC_{50} values for both males and females, and corresponding decreases in ESR values so indicating that background metabolic defences were disabled by the presence of PBO in the tank mix treatment, thus enhancing the effect of the pyrethrins.

Results for a tank mix leaf dip treatment on *M. persicae* can be seen in Table 3.1c. Results show a significant reduction ($P<0.05$) in LC_{50} for the resistant clone (794jz) from 365 ppm

with a pyrethrins only treatment to 64 ppm with a tank mix. This had a corresponding ESR decrease from 16.1 to 2.8 which means that the resistant clone still has a higher LC_{50} than the susceptible clone treated with pyrethrins EC only.

The resistant clone of *M. persicae*, treated with pyrethrins EC as a leaf dip application, had a RF of 16.1, which is low compared to 43.8 for technical pyrethrins in a topical application. The susceptible clone also had a lower LC_{50} using the leaf dip of pyrethrins EC compared to technical pyrethrins. The LC_{50} values for topical and leaf dip of pyrethrins EC were very different, 1630 ppm and 365 ppm respectively. Thus it appears that components of the EC formulation reduced the LC_{50} either by inhibiting resistance-associated enzymes and/or aiding penetration of pyrethrins across the cuticle. This is discussed further in Chapter 5.

Results for tank mix treatments as leaf dip bioassays on B. tabaci can be seen in Table 3.1d. Results show that for the susceptible *B. tabaci* (Sud-S) a tank mix of PBO and pyrethrins had no significant effect on the LC_{50} ($P>0.05$) when compared with pyrethrins alone. However, there was a clear difference in LC_{50} values for the Mex2-GRB strain which can be seen as a decrease from 629.5 ppm to 173.8 ppm. This had a corresponding decrease in RF from 62.3 to 15.7, and a decrease in ESR from 62.3 to 17.2.

Unfortunately it was not possible to achieve an LC_{50} for pyrethrins alone for the Pirgos strain of *B. tabaci* using the dose range of these experiments, and higher concentrations could not be used because the pyrethrins EC left the leaf disc with a sticky residue. The Pirgos strain had an ESR of 3.7 when a tank mix was used so although an LC_{50} was not achievable for a pyrethrin only treatment, it is clear that the tank mix has a beneficial effect on the efficacy of the pyrethrins since with the presence of PBO, pyrethrins were able to kill this resistant strain. Thus overall, these results show the benefit of a tank mix on the efficacy of natural pyrethins against both resistant strains of *B. tabaci* tested but the tank mix treatment on the resistant strain does not reduce the LC_{50} values to less than those for the pyrethrin only treatment on the susceptible strain.

Overall the results for all 3 insect species show that for resistant insects a tank mix of PBO and pyrethrins gave a significant reduction in LC_{50} values compared to pyrethrins alone, suggesting that in all cases the tank mix would be a better for field use than pyrethrins only, but this was only better than the susceptible strain treated with pyrethrins only, for *M. domestica*.

3.3.2 The effect of a pre-treatment of PBO prior to treatment with pyrethrins (compared to a tank mix of PBO and pyrethrins)

Bioassays using a range of PBO pre-treatment times showed that for *M. persicae* PBO applied 5 hours prior to treatment with pyrethrins was the most effective at increasing mortality (Figure 3.1). Thus, 5 hours was used as the pre-treatment time for subsequent *M. persicae* experiments. For *B. tabaci* a pre-treatment time of 9 hours was chosen for practical reasons and it is close to the 11 hours found to be the most effective by Young *et al.* (2006). For *M. domestica* a 3 hour pre-treatment time was chosen based on the time available to complete the experiments whilst visiting DPIL in Denmark.

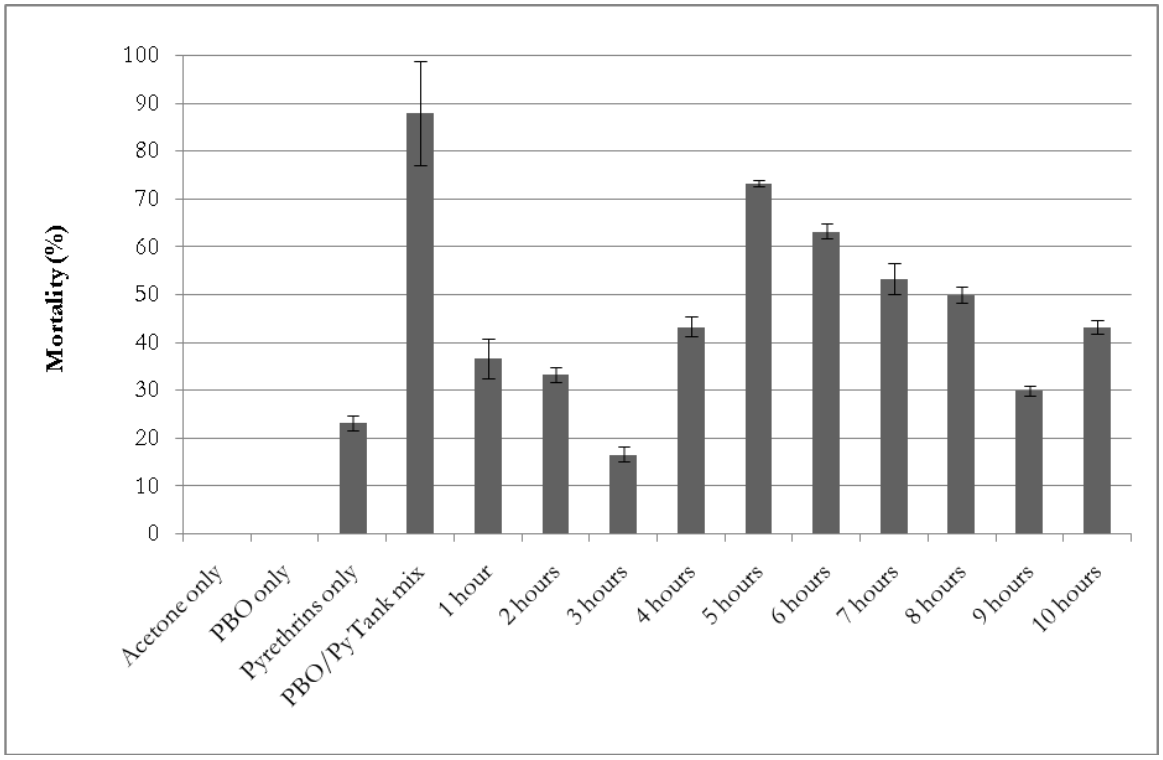


Figure 3.1 The effect of a PBO pre-treatment (prior to application of natural pyrethrins) on the mortality of resistant adult *Myzus persicae* (794jz clone), 72 hours after dosing with pyrethrins

Error bars show the standard deviation from the mean.

Considering both the resistant and susceptible clones of *M. persicae*, the reduction in the LC_{50} using a tank mix was greater than that using a pre-treatment. This was unexpected since previous work had demonstrated that a PBO pre-treatment was more effective for synthetic pyrethroids. It appears that for natural pyrethrins PBO could be enhancing the penetration of the pyrethrins through the insect cuticle and this effect is greatly diminished in the 5hr pre-treatment, i.e., although the optimum time for the inhibition of esterases by PBO is 5

hours, the effect of enhanced penetration through the cuticle is greater than the effect of inhibiting the esterases. The results indicate that the enhanced penetration effect declines rapidly (mortality is reduced by more than 50 % after a 1 hour PBO pre-treatment). The subsequent rise in mortality, to a peak at 5 hours could be due to the inhibitory binding effect that PBO has on the esterases (Figure 3.1).

Results for *M. persicae* with a topical application of technical PBO and pyrethrins can be seen in Table 3.1a. The susceptible clone saw an increase in LC_{50} from 37.2 ppm without a pre-treatment, to 46.3 ppm with a PBO pre-treatment (discussed below). The resistant clone (794jz) showed a decrease in LC_{50} of 1438 ppm, from 1630 ppm to 192.7 ppm with the pre-treatment of PBO followed by application of pyrethrins. The RF decreased from 43.8 to 4.2, and the ESR decreased from 43.8 to 5.2. From the RF, it appears that the pre-treatment was more effective than the tank mix. However, the ESR value for a tank mix is 3.27 thus there is an 8.4-fold difference in the ESR for a pre-treatment compared to pyrethrins alone, and a 13.3-fold difference in the ESR for a tank mix compared to pyrethrins alone, indicating strongly that the tank mix is more effective. However, the ESR is above 1.0 for both tank mix and pre-treatment thus control in the field would require more insecticide than the susceptible clone.

Analysis using PoloPlus revealed a significant difference between the pre-treatment and tank mix on the resistant clone ($P<0.05$) for the topical application treatment. The discrepancy between the RF suggesting that the pre-treatment is more effective, and the ESR showing the tank mix to be more effective, is due to the higher LC_{50} for the susceptible clone after a 5 h pre-treatment with PBO. This is because the RF is calculated using comparable treatments of the resistant and susceptible clones (see section 2.5.3) whereas the ESR looks at the difference between any given treatment and clone compared with the susceptible clone treated only with insecticide (see section 2.5.3).

The higher LC_{50} seen in the susceptible clone of *M. persicae* (4106a) after a 5 hour PBO pre-treatment compared with pyrethrins alone (Table 3.1a) could be due to the recovery of background enzymes, or possibly, an induction of more enzymes due to the PBO pre-treatment. However, statistical analysis showed the difference between the pyrethrins only treatment and a PBO pre-treatment followed by pyrethrins on the susceptible clone to be significant ($P<0.05$).

Induction of P450 activity by PBO has been found in mammals and insects (Skrinjar.M *et al.*, 1971, Yu & Terriere, 1974, Kinsler *et al.*, 1990) but since *Myzus persicae* resistance is established to be due to over-expressed esterases it is felt this is unlikely to be an issue in this case. Similarly, Willoughby *et al.* (2007) found PBO induced expression of glutathione *S*-transferase genes in *Drosophila melanogaster*, but this was only at the gene level and no evidence of increased levels of proteins were given. Nevertheless, the possibility that PBO is inducing one or more detoxification enzymes in the current study cannot be discounted and would require further investigation.

The results of the susceptible clone demonstrate the importance of calculating the ESR rather than RF, as discussed in Chapter Two. For the susceptible clone there are very low levels of metabolic enzymes and therefore the enhanced penetration effect of PBO is more important than its ability to inhibit the enzymes. As a result, the LC_{50} of the susceptible clone is not reduced as much as it is for the resistant clone. Therefore in the susceptible clone with a pre-treatment of PBO prior to application of pyrethrins, the RF decreases but the ESR increases.

Results for pre-treating resistant *M. domestica* (strain 381zb) with PBO prior to application of pyrethrins can be seen in Table 3.1bi and bii. For males there was a decrease in LC_{50} from 8513.8 ppm to 404.5 ppm, and for females the reduction in LC_{50} was from 11251.9 ppm to 570.7 ppm. These LC_{50} values had corresponding reductions in RF, from 12.9 for the pyrethrins only treatment to 5.0 for the PBO pre-treatment followed by pyrethrins for males, and from 14.9 for the pyrethrins only treatment to 8.0 for the PBO pre-treatment followed by pyrethrins for females. Both had corresponding reductions in ESR of over 19-fold. Statistical analysis of the results showed that a pre-treatment reduced the LC_{50} significantly, compared with a pyrethrins only treatment ($P<0.05$).

Results for the susceptible strain of *M. domestica* show that a pre-treatment of PBO decreased the LC_{50} by 578.1 ppm, from 659.4 ppm for pyrethrins only treatment, to 81.3 ppm for a pre-treatment on males (Table 3.1bi). A decrease of 684.5 ppm, from 755.7 ppm for pyrethrins only, to 71.2 ppm for a pre-treatment (Table 3.1bii) was seen for females. When compared to a tank mix, the results showed that in susceptible *M. domestica* the tank mix worked more effectively than the pre-treatment ($P<0.05$).

In the resistant strain of *M. domestica* (381zb), the tank mix and pre-treatment gave similar results with overlapping confidence limits for the LC_{50} values. The pre-treatment time for *M. domestica* was not optimised and if optimised, may improve the pre-treatment result further. However, the results do correspond to those results found in the *M. persicae* bioassays whereby the tank mix was more effective than the pre-treatment at enhancing the efficacy of pyrethrins.

Results for the leaf dip bioassay using a pre-treatment of PBO EC five hours prior to treatment with pyrethrins EC on *M. persicae*, can be seen in Table 3.1c. With a pre-treatment, the LC_{50} was reduced from 365 ppm to 46.7 ppm. Although the RF is not calculable for this experiment (as the susceptible clone was not given a pre-treatment) the ESR showed a reduction from 16.1 to 2.1 which means that although there was nearly an 8-fold decrease in the amount of insecticide required for 50 % mortality, this isn't as low as is required by the susceptible clone with a pyrethrins only treatment. There was no significant difference between results for the tank mix and the PBO pre-treatment.

Results for leaf-dip bioassays on *B. tabaci* can be seen in Table 3.1d. Results show that a pre-treatment of PBO gave no significant effect on Sud-S, the susceptible strain. However, there was a significant difference in LC_{50} values between a pyrethrin only treatment and PBO pre-treatment for the Mex2GRB strain ($P<0.05$) whereby the LC_{50} was reduced by 429.7 ppm, from 629.5 ppm to 199.8 ppm. For this strain, the RF was reduced from 62.3 to 19 and the ESR was reduced more than 3-fold from 62.3 to 19.8. The pre-treatment did therefore improve the efficacy of the pyrethrins, but not to the same extent that the pyrethrin only treatment has on the susceptible strain. The LC_{50} for the Pirgos strain was reduced from >1000 ppm to 95 ppm. For the pyrethrins only treatment the RF and ESR were not calculable but the ESR was reduced from 'uncalculable' down to 9.4 with a pre-treatment. This means that the pre-treatment on the Pirgos strain was more effective than then pre-treatment on the Mex2-GRB strain, perhaps due to different metabolic defences occurring in each strain, and more being eliminated in Pirgos by the pre-treatment than in Mex2-GRB. For both strains, the tank mix was more effective than the pre-treatment.

Overall, the results show that although a PBO pre-treatment can significantly enhance the effect of natural pyrethrins, the effect is either less than, or not significantly different from the effect of a PBO and pyrethrin tank mix. This was in contrast to the results found previously for PBO with synthetic pyrethroids (Young *et al.*, 2006, Bingham *et al.*, 2007).

3.3.3 The effect of pyrethrin microencapsulations

The effect of pyrethrin microencapsulations was tested on resistant *M. persicae* and the results can be seen in Table 3.2. When pyrethrins were incorporated into microencapsulations with either β-cyclodextrin or γ-cyclodextrin formulations, neither could kill the resistant clone of *M. persicae* using the dose range tested. At the highest concentrations tested there was a white chalky residue of the formulation left on the leaf discs. It is highly likely that the actual concentration of pyrethrins on the leaf disc was less than the calculated concentration as the solubility of the compounds is very low. Solubility of the β-cyclodextrin formulation is 0.4 mg/ml but the highest concentration used was 250 times greater than what is realistically soluble. Likewise, solubility of the γ-cyclodextrin formulation is 0.2 mg/ml and the highest concentration used was 625 times greater. As a consequence, the results achieved can only be considered as indicators of the effect of microencapsulated pyrethrins. It would be interesting to look at formulations with improved solubility in the future.

Table 3.2 Lethal concentration for 50 % mortality (LC_{50}), resistance factors (RF) and effective synergism ratios (ESR) for a) *Myzus persicae* treated with β-cyclodextrin microencapsulated pyrethrins (leaf dip bioassay); and b) *Myzus persicae* treated with γ-cyclodextrin microencapsulated pyrethrins (leaf dip bioassay)

The corresponding appendix table number is given next to the table sub-headings in brackets. S: susceptible; R: resistant. Clone/strain names are given underneath each sub-table.

a) *Myzus persicae* treated with β-cyclodextrin microencapsulated pyrethrins (leaf dip bioassay) (Table A-IV-II)

Treatment	LC_{50} (ppm)		RF		ESR	
	S	R	S	R	S	R
Pyrethrins only	583.4	>10000	1.0	NC	1.0	NC
PBO (EC) / pyrethrins tank mix (4:1)	-	50.2	-	NC	-	0.086
PBO (top app) / Pyrethrins (4:1)	-	137.9	-	NC	-	0.236

S: 4106a clone; R: 794jz clone; NC: not calculable

b) *Myzus persicae* treated with γ-cyclodextrin microencapsulated pyrethrins (leaf dip bioassay) (Table A-IV-II)

Treatment	LC_{50} (ppm)		RF		ESR	
	S	R	S	R	S	R
Pyrethrins only	>1000	>10000	NC	NC	NC	NC
PBO (EC) / pyrethrins tank mix (4:1)	-	64.9	-	NC	-	NC
PBO (top app) / Pyrethrins (4:1)	-	261.4	-	NC	-	NC

S: 4106a clone; R: 794jz clone; NC: not calculable

The susceptible clone of *M. persicae* had a LC_{50} of 583 ppm using β-cyclodextrin (Table 3.2a), however with the γ-cyclodextrin formulation, 50 % mortality could not be achieved within the dose range tested (Table 3.2b). The LC_{50} for the susceptible clone using a β-cyclodextrin formulation was 25 times greater than when using BRA recipe pyrethrins in an emulsifiable concentrate and 1.5 times greater than the LC_{50} for the resistant clone (also treated with a

leaf dip of pyrethrins EC) (Table 3.2a). It appears that the pyrethrins are more effectively released from the β-cyclodextrin complex since the susceptible clone survived a γ-cyclodextrin formulation but not the β-cyclodextrin formulation. Likewise, with the addition of PBO (both EC and topical application), the resistant clone had a lower LC_{50} when the β-cyclodextrin formulation was used than with the γ-cyclodextrin formulation.

The addition of PBO (both PBO EC and topical application of technical PBO) to the microencapsulated pyrethrins reduced the LC_{50} for the resistant clone to less than the LC_{50} for the susceptible clone treated with microencapsulated pyrethrins alone. The decrease in the ESR was only calculable for the β-cyclodextrin formulations (as the γ-cyclodextrin formulation did not kill the susceptible clone in the dose range tested) and this gave a dramatic effect for both PBO EC and topical application. Where the LC_{50} was reduced from >10000 ppm down to 50.2 ppm for a PBO EC and microencapsulated treatment, and 137.9 ppm for a topically applied technical PBO treatment with microencapsulated pyrethrins as a leaf dip, the ESR values were reduced from 1.0 to 0.086 and 0.236 respectively. This means that the addition of PBO to a microencapsulated formulation has the ability to make the resistant clone more susceptible than the susceptible clone treated with pyrethrins alone.

Although an ESR was not calculable for the γ-cyclodextrin results (because there was no LC_{50} value for the susceptible clone with γ-cyclodextrin), it was clear that the LC_{50} for the resistant clone was reduced to less than that for the susceptible clone, showing that both formulations have the ability to make the resistant clone of *M. persicae* more susceptible than the laboratory standard susceptible clone. This is probably due to a physical effect whereby the microencapsulations are not releasing the pyrethrins from the complex and into solution, but the addition of PBO can alter the structure of the encapsulation, possibly by breaking up the cyclodextrin complexes. The very low ESR seen for the PBO and β-cyclodextrin complex is not because the LC_{50} values are particularly low, but because the LC_{50} for the susceptible clone with pyrethrins only microencapsulation is high compared to the other results.

Thus, these results suggest that for microencapsulated pyrethrins PBO can help the pyrethrins to be released from the microencapsulation complexes. The LC_{50} values are lower for the PBO EC and microencapsulated pyrethrins treatment (both β- and γ-) compared to a topical application of PBO and microencapsulated pyrethrin treatment, but both methods showed a large reduction in the LC_{50} compared to microencapsulated pyrethrins alone. It is

not possible to give a numerical value to this decrease in LC_{50} since it was not possible to achieve an LC_{50} without PBO. With the PBO EC, the PBO was physically mixed into the microencapsulated formulations, whereas with the topical application of PBO, the PBO was applied directly onto the aphid. For both a leaf dip and a topically applied treatment, PBO may also be acting as a synergist by inhibiting the metabolic defences in the insects but the higher LC_{50} for the topically applied treatment suggests that the main effect is that of a physiochemical reaction upon the encapsulation matrix.

The LC_{50} values obtained in the microencapsulated pyrethrin experiments were very similar to those seen in PBO/pyrethrins EC leaf dip bioassays. Taking into account the enhanced synergism using PBO EC with microencapsulated pyrethrins, the lower LC_{50} values and RFs using pyrethrin EC, as opposed to technical pyrethrins, and the greater levels of synergism when using PBO EC compared to technical, it appears that the components of the EC formulation enhanced the effects of the pyrethrins. This is further discussed in Chapter Five where the components of the EC formulation were tested for their ability to inhibit resistance-associated esterases and P450s, and evaluated as synergists *in vivo* in bioassays.

3.3.4 The effect of a PBO pre-treatment or tank mix with alpha-cypermethrin

The results reported here have shown that a PBO pre-treatment followed by natural pyrethrins was no more effective than a tank mix. This was in contrast to previous studies that involved either a PBO pre-treatment followed by treatment with a synthetic pyrethroid (Young *et al.*, 2006, Bingham *et al.*, 2007), or a microencapsulation of PBO and a synthetic pyrethroid (Bingham *et al.*, 2007). Bioassays using a topical application of PBO and alpha-cypermethrin were therefore performed to confirm the integrity of the technical PBO and can be seen in Table 3.3. These were similar experiments to those reported by Bingham *et al.* (2007) with the same insect species, *Myzus persicae,* and the same resistant clone, where a pre-treatment of PBO reduced the LC_{50} for alpha-cypermethrin more than the tank mix.

Table 3.3 Lethal concentration for 50 % mortality (LC_{50}), resistance factors (RF) and effective synergism ratios (ESR) for *Myzus persicae* treated with α-cypermethrin (topical application bioassay).

(see appendix IV, Table A-IV-III)

Treatment	LC_{50} (ppm)		RF		ESR	
	S	R	S	R	S	R
α-cypermethrin	0.495	3230.2	1.0	6525.7	1.0	6525.7
PBO/α-cypermethrin tank mix (4:1)	-	810.8	-	NC	-	1637.9
PBO pre-treatment/α-cypermethrin (4:1)	-	169.9	-	NC	-	343.2

S: 4106a clone; R: 794jz clone; NC: not calculable

Using a leaf dip approach, Bingham *et al.* (2007) reported that to achieve 50 % mortality the 794jz clone needed 3460 ppm of alpha-cypermethrin when it was applied alone; 62.3 ppm for a tank mix with PBO; and 10.9 ppm for a pre-treatment with PBO. As can be seen in Table 3.3, using a topical application the LC_{50} values were very different to those reported by Bingham *et al.,* (2007) but in both cases there was a similar trend, with the pre-treatment being significantly more effective than a tank mix ($P<0.05$).

These experiments have shown that there are differences in the effect of PBO either as a tank mix with pyrethrins or as a pre-treatment prior to pyrethrins, compared with a PBO tank mix or pre-treatment with the synthetic pyrethroid alpha-cypermethrin. This may be due to PBO giving a greater penetration enhancement with pyrethrins than with the synthetic compound.

3.4 General discussion

General observations from all bioassays correspond with previous findings that pyrethrins have a rapid action whereby insects are knocked down and paralysis occurs, leading to death when the dose is sufficiently high (Stevenson, 1959). This is because pyrethrins have an almost immediate effect of blocking nerve transmission. It has been reported, and was observed in the experiments reported here, that at sub-lethal doses pyrethrins can also cause paralysis but the insect is able to recover, normally within a few hours, and the length of time of the paralysis may reflect the time required for the detoxification of the pyrethrins (Stevenson, 1959).

In the present studies it was shown that with the addition of PBO, the effect of pyrethrins was enhanced for both resistant and susceptible insects. The action on the susceptible

insects is probably due to the background esterases and/or P450s being blocked by PBO. In resistant insects the esterases/P450s which are present in elevated levels are also blocked by PBO. Thus, in the absence of the synergist, the pyrethrins are quickly detoxified and therefore the treatment with the synergist must be made either at the same time, or in advance of the pyrethrins treatment.

The same paralysis effect on the insect was seen for both the pyrethrins and the PBO/pyrethrins mixes, which indicates that PBO is functioning by inhibiting the detoxification of the pyrethrins rather than by forming a complex with the pyrethrins.

The results in this chapter have indicated that there are different levels of synergism occurring in the different insect species. This may be because the level of detoxification mechanisms (esterases/P450s) are different in each species or it may be related to the potency of the pyrethrins in different species.

This work used both topical application and leaf-dip bioassay techniques. It is important to remember that a topical application, where the insecticide is absorbed directly through the insects' cuticle, bypasses any detoxification that would normally take place in the insects' digestive tract. Therefore bioassay results from experiments using a leaf-dip technique cannot be compared directly with those using topical application and *vice versa*.

Although PBO enhanced the effect of pyrethrins in all insects species studied, of the 3 insect species tested, *M. domestica* was the only one where using PBO as a tank mix or pre-treatment reduced the ESR to less than 1, showing that for this strain and species, less insecticide would be required to kill resistant insects than would be required to kill the susceptible strain.

This work has tested the effect of PBO and pyrethrins compared with PBO and a synthetic pyrethroid. Previous results have indicated that target-site insensitivity or knockdown resistance (*kdr*) and super-*kdr* do not affect pyrethrins in the same way, or to the same level, as they affect synthetic pyrethroids (Farnham *et al.*, 1987). Results in this chapter exemplify this by a lower LC_{50} for natural pyrethrins than for synthetic pyrethroids on insects with *kdr*.

Davies *et al.* (2007) suggest *kdr* is caused by a change in the conformation of the target protein, the voltage-gated sodium channel rather than a mutation on the binding site itself. This conformational change, caused by an amino acid substitution (induced at the L1014

residue on domain II, S6) (Davies *et al.*, 2007) means that the protein changes shape and as a result the insecticide may not fit/bind to the protein as well as it would in an unmutated one. The change in the protein caused by *kdr* is more apparent for synthetic pyrethroids than for natural pyrethrins. This is thought to be because many synthetic pyrethroids tend to be much larger molecules, due to the phenoxybenzoyl moiety in the alcohol part, whereas the smaller and less bulky pyrethrins can still fit into the channel (albeit not as well as they might without the mutation) and thus are still able to act on the nervous system. Therefore, in light of results found here, in the absence of target site resistance synthetic pyrethroids would be the insecticides of choice. In the absence of resistance mechanisms, a synthetic pyrethroid will usually be more potent than natural pyrethrins. However, if *kdr* or super-*kdr* are present, there is potential for the pyrethrins to be the more potent xenobiotic. Therefore, if target-site resistance is present, pyrethrins would be a good choice accompanied by PBO if metabolic resistance mechanisms are also present.

This chapter has discussed the use of PBO as a synergist with pyrethrins. On the whole results have shown that a tank mix of PBO and pyrethrins is as effective/more effective than a pre-treatment of PBO followed by treatment with pyrethrins.

4 CHAPTER FOUR: DEVELOPMENT OF A NEW BIOCHEMICAL ASSAY TO DEMONSTRATE THE INHIBITION OF ESTERASES BY PBO *IN VITRO*

4.1 Introduction

Both bioassays and field work have already shown that piperonyl butoxide (PBO) is an effective inhibitor of insect esterases *in vivo* (Young *et al.*, 2005, 2006, Bingham *et al.*, 2007, Bingham *et al.*, 2008). Biochemical assays provide fast and efficient methods of detecting esterase activity in single insects and can, in theory, determine whether or not the esterases can be inhibited by PBO.

Such assays can reduce the need for extensive, time consuming and laborious biological assays by providing an alternative initial screening technique to identify resistance associated with elevated esterases. This chapter considers the robustness of a standard spectrophotometric assay for detecting esterase activity (and the effect of PBO on the esterases), and the subsequent development of a new 'esterase interference assay' better able to demonstrate the inhibition of resistance-associated esterases. The principle behind the new assay is described in Figure 4.1. Esterase activity is measured indirectly: The insecticide acts on the target site and is detected as low AChE activity. With the addition of esterases, they will sequester the insecticide before it is able to act on the target site and this will be indicated by high AChE activity. Incubation of a synergist with the esterases will block their activities, enabling the insecticide to act on its target site. This will be indicated by low AChE activity. The developmental stages of the assay are discussed in section 4.2.

In contrast to this new assay, the standard spectrophotometric assay measures directly how esterase activity is affected by a serial dilution of PBO across a microplate but this is sometimes unable to detect PBO activity (Figure 4.2).

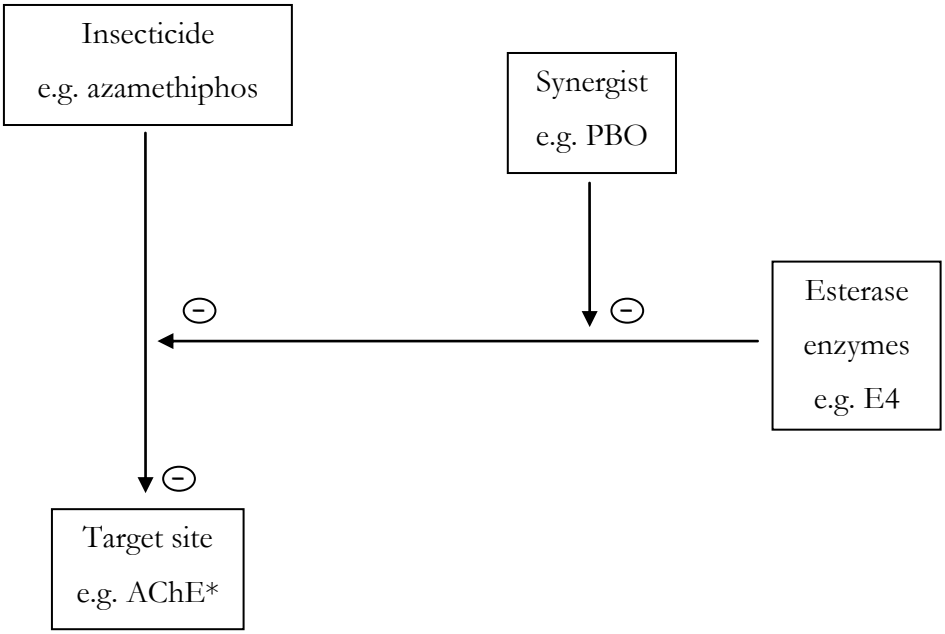


Figure 4.1 The interactions between the insecticide, target site, synergist and esterase enzymes, used as the basis for the 'esterase interference assay'.

In this assay esterase activity is detected indirectly by measuring AChE activity. A negative symbol denotes an inhibitory action.
* measured by activity on a model substrate (ATChI)

In this assay the AChE was supplied as a homogenate of *M. domestica* with Triton X-100 (0.1%) added to solubilise the membrane-bound AChE. The Triton X-100 was only added to the housefly homogenate and was not incubated with any esterases from other sources (e.g. E4) because experiments to investigate the use of 0.1 % Triton X-100 in the phosphate homogenisation buffer (to aid the release of the esterases into solution), had indicated that the Triton X-100 interfered with the binding of PBO to the esterase (p59 Bingham, 2007).

4.1.1 Materials and methods

Unless otherwise stated, the phosphate buffer referred to in the following methods was 0.02 M phosphate buffer (pH 7.0).

4.1.1.1 Standard spectrophotometric assay

A 96-well microplate was prepared as follows: 50 µl phosphate buffer was added to each well, with an additional 22 µl buffer and 3 µl 0.3 M PBO (in acetone) added to the wells of the first column. A three fold serial dilution of PBO was made across the plate by pipetting 25 µl from the first column to the next, up to the penultimate column, from which the 25 µl was discarded. The last column contained an additional 25 µl buffer only to act as a control

(no PBO). A row containing a serial dilution of acetone was used as the acetone control. Insect homogenate (25 µl) was added to each well and the plate left to stand for 15 minutes at room temperature. The resulting concentrations of PBO in the wells were 9000, 3000, 1000, 333.3, 111.1, 37.04, 12.35, 4.115, 1.372, 0.457 and 0.152 µM. After this time, the esterase substrate (200 µl 1 mM 1-naphthyl-acetate: see appendix I) was added to each well. The control well was used to calculate the inhibition of the esterases by PBO. The plates were read as described in section 2.4.3. In addition, pure E4 (see section 2.4.2) was incubated with PBO overnight as described above and the plate was read following the same protocol.

4.1.1.2 Development of the new 'esterase interference assay'

Various initial experiments were performed in the development of this assay. Firstly, the ATChI and DTNB, were examined both together and separately for any absorption at 405 nm without any AChE present and neither showed any activity. A serial dilution of acetone showed that acetone does not affect the level of AChE activity detected and an experiment using three different concentrations of housefly homogenate, diluted with phosphate buffer plus 0.1 % Triton X-100, (undiluted homogenate, 2-fold diluted homogenate, 5-fold diluted homogenate) showed that inhibition of AChE is not affected by the concentration of the housefly homogenate. Acetone was incubated with E4 overnight but did not show any inhibitory effect on the E4. Following these initial experiments, each step in the assay was optimised as follows:

Step 1: Selecting the AChE inhibitor

A 96-well microplate was prepared as follows: 50 µl phosphate buffer was pipetted into every well with a three-fold serial dilution of each AChE inhibitor to be tested across the plate starting with 3 µl in the first well, which contained an additional 22 µl of buffer. A three-fold serial dilution of each inhibitor was then made by pipetting 25 µl from the first column to the next up to the penultimate column, from which the 25 µl was discarded. The last column contained an additional 25 µl buffer only to act as a control (no AChE inhibitor). A further 25 µl phosphate buffer was added to each column. A *M. domestica* homogenate was prepared as described in section 2.4.1 with 0.1% Triton X-100 and 25 µl was added to each column and left to stand for 15 minutes at room temperature and then 100 µl 1.5 mM DTNB and 100 µl 1.5 mM ATChI were added (appendix I). The plates were read as described in Section

2.4.4. The AChE inhibitors tested were azamethiphos (10^{-5} M), eserine (10^{-2} M and 10^{-5}M) and paraoxon (10^{-2} M and 10^{-5} M).

Step 2: Assessing the effect of E4 on AChE inhibition

A 96-well microplate was prepared as follows: 50 µl phosphate buffer was pipetted into every well with a three-fold serial dilution of azamethiphos in acetone (10^{-5} M) starting with 3 µl in the first well, which contained an additional 22 µl of buffer. A three-fold serial dilution of azamethiphos was made by pipetting 25 µl from the first column to the next up to the penultimate column, from which the 25 µl was discarded. The last column contained an additional 25 µl buffer only to act as a control (no azamethiphos). To each row varying volumes of purified E4 (50, 40, 30, 20, 10, and 0 µl) and phosphate buffer (0, 10, 20, 30, 40, 50 µl respectively) were added to give a combined final volume of 50 µl E4+phosphate buffer in each well. The plate was left to stand at room temperature for 15 minutes. A *M. domestica* homogenate was prepared according to section 2.4.1 with 0.1% Triton X-100 and this AChE source was added to each well (25µl) and left to stand for 10 minutes at room temperature, followed by the addition of 100 µl 1.5 mM DTNB and 100 µl 1.5 mM ATChI to each well (appendix I). The plates were read as described in Section 2.4.4. The experiment was repeated with a 2 hour incubation of the E4 and azamethiphos.

Step 3: Assessing the effect of PBO on the E4

Purified E4 was incubated with 3 mM PBO overnight at 4 °C. A 96-well microplate was prepared as described in step 2 above but with a row containing 50 µl E4 in each well, and a row containing 50 µl phosphate buffer, with an additional row on the microplate where each well contained 50 µl E4 + 3 mM PBO and 35 µl phosphate buffer.

Step 4: Optimisation of the assay

From the results of steps 1-3, the whole assay was optimised to use as little E4 as possible. The final protocol was:

Purified E4 was incubated with either PBO in acetone (3 mM final concentration, from a 0.3 M stock) or acetone only (to give E4 + 1% acetone), overnight at 4 °C. A 96-well microplate was prepared as follows: 25 µl 0.02 M phosphate buffer (pH 7.0) was pipetted into every well and 3 µl 10^{-6} M azamethiphos pipetted into the first well, which contained an additional 22 µl of buffer. A 2-fold serial dilution of azamethiphos was performed by pipetting 25 µl from the first column to the next up to the penultimate column, from which the 25 µl was

discarded. The last column contained an additional 25 µl buffer only to act as a control. To the wells of row A: 50 µl buffer was added to each well, to row B: 15 µl E4 and 35 µl phosphate buffer were added, and to row C: 15 µl E4 + 3 mM PBO and 35µl phosphate buffer. The E4 was left to stand in the wells for 1 h at room temperature. The resulting azamethiphos concentrations in the wells were (in nM): 20, 10, 5, 2.5, 1.25, 0.62, 0.31, 0.16, 0.78, 0.039, and 0.020. *Musca domestica* homogenate was prepared according to section 2.4.1 with 0.1 % Triton X-100. Homogenate (25 µl) was added to each column and left to stand for 15 minutes at room temperature, followed by 80 µl 1.5 mM DTNB (see appendix 1) and 80 µl 1.5 mM ATChI (see appendix 1). The plates were read as described in Section 2.4.4. The assay was repeated starting with 0.03 M and 0.003 M stock PBO.

4.2 Results and Discussion

It has been reported that the sensitivity of resistant *M. persicae, B. tabaci* and *H. armigera* to insecticides as diverse as pyrethroids, carbamates and neonicotinoids can be increased by a pre-treatment of PBO (Young *et al.*, 2005, 2006, Bingham *et al.*, 2007, Bingham *et al.*, 2008) This suggests that the synergist was blocking the enhanced metabolic activity, although *in vitro* assays have failed to show its effect (Gunning *et al.*, 1998). This was re-tested and the results of a standard spectrophotometric assay are shown in Figure 4.2. There was no appreciable inhibition of esterase activity for either *M. domestica* or *M. persicae*, although the blockade of esterases from both B- and Q-type *B. tabaci* was seen. This corresponded to previous *in vivo* studies of B-type *B. tabaci* but disagrees with *in vivo* studies on *M. persicae* (Young *et al.*, 2005, 2006, Bingham *et al.*, 2007). Furthermore, an overnight incubation of pure E4 with PBO also failed to show inhibition of the enzyme (results not shown).

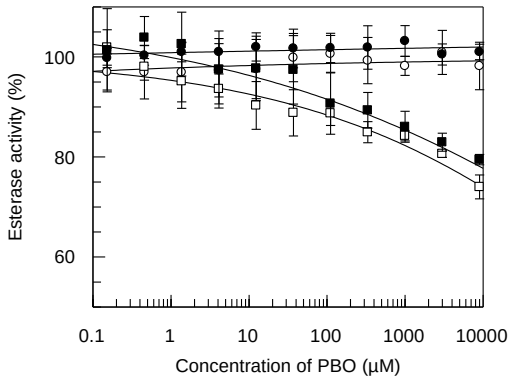


Figure 4.2 Inhibition of esterases from different insect species, by PBO. (Substrate: 1-naphthyl acetate)

Open circle: *M. domestica*, closed circle: *M. persicae*, open square: B-type *B. tabaci*, closed square: Q-type *B. tabaci*.

Error bars shown illustrate the standard deviation derived from three replicates

Thus, the standard spectrophotometric assay is not always suitable for demonstrating PBO-mediated inhibition of esterases since such inhibition cannot always be detected *in vitro* even when inferred from *in vivo* results (see Chapter Three).

One conclusion from this could be that when PBO binds to the esterases of some insects, it does not do so at the hydrolytic active site of the esterase, and the inhibition cannot always be revealed by a conventional *in vitro* spectrophotometric assay (Figure 4.2) (Khot *et al.*, 2008). It may be that different insects have slightly different sized/shaped esterases. It is hypothesised, as illustrated in the conceptual model, (Figure 4.3) that in some insects the spatial distance between the two binding sites allows both the PBO and the substrate to bind (Figure 4.3a) e.g. *M. persicae* and *M. domestica*. In others the spatial distance between the two binding sites is small so that when the PBO is bound, binding of the substrate is blocked (Figure 4.3b) e.g. *B. tabaci*. Only in the latter case would the PBO inhibition be measurable by use of a model substrate. This could explain why a standard esterase assay will reveal PBO-mediated inhibition of esterases from some insects but not others. However, since the esterase/PBO 'complex' has always been found to prevent the insecticide binding (Figure 4.3c) PBO is acting as a synergist *in vivo* regardless of the spatial positions of the two binding sites.

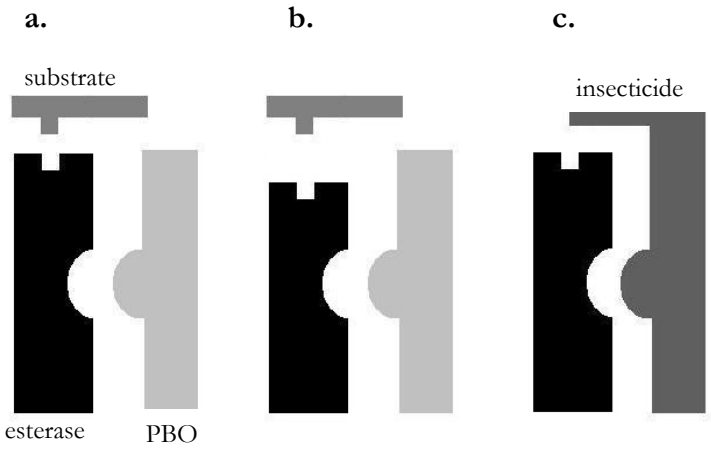


Figure 4.3 Diagrammatic representation of the binding of the substrate, PBO and insecticide to esterase enzymes

a: PBO binds to the esterase but does not cover the active site for the substrate; **b:** PBO binds to the esterase: substrate is unable to bind; **c:** the binding of the insecticide covers both binding sites.

This chapter focussed on the development of an assay to demonstrate the inhibition of esterases by PBO *in vitro* using the approach shown in Figure 4.1 where AChE activity is measured to indirectly detect the level of esterase activity. It had been reported that the

resistance-associated esterase from *M. persicae* (E4) had the ability to both hydrolyse and sequester xenobiotic compounds (Devonshire *et al.*, 1983) hence this was chosen as the esterase for the assay. Initial experiments tested various AChE inhibitors: eserine, paraoxon and azamethiphos (Figure 4.4). They showed that paraoxon was not a suitable candidate for the assay as the concentration required was too high relative to the volume of E4 that would be required to sequester the AChE inhibitor. Eserine showed potential to act as the AChE inhibitor in the assay but on the addition of E4 it was evident that E4 was not able to sequester the eserine (results not shown). Azamethiphos did inhibit the AChE and E4 had the ability to sequester azamethiphos in volumes suitable for use in the assay.

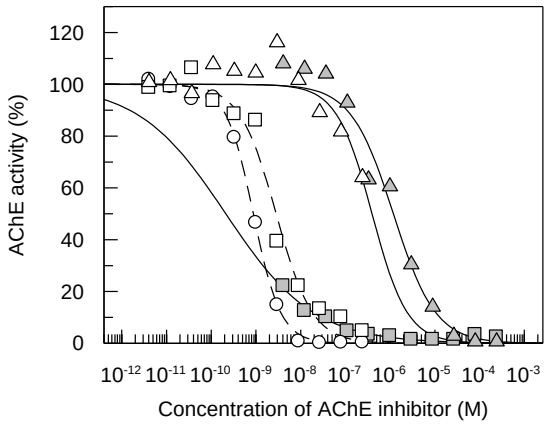


Figure 4.4 Inhibition of AChE by eserine, paraoxon and azamethiphos.

Grey square and open square: eserine from stock concentration of 10^{-2} M and 10^{-5} M respectively, grey triangle and open triangle: paraoxon from stock concentration of 10^{-2} M and 10^{-5} M respectively; open circle: azamethiphos from stock concentration 10^{-6} M.

Thus azamethiphos was selected as the AChE inhibitor. The next stage was to test different volumes of E4 to identify how much would be required to sequester the azamethiphos (rendering it unable to act on the AChE). Five dilutions of pure E4 were tested (Figure 4.5 and Table 4.1) The results showed that the greater the molar proportion of esterase to azamethiphos, the higher the concentration of azamethiphos required to inhibit the AChE, demonstrating directly that E4 sequesters the azamethiphos.

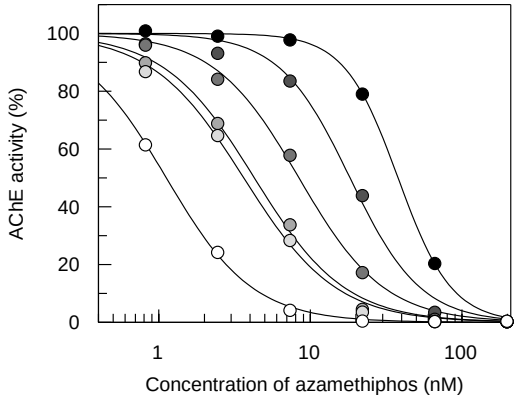


Figure 4.5 E4 protection of the inhibition of *M. domestica* AChE activity by azamethiphos.

Open circle: No E4. Closed circle through to light grey circle (five shades): 50, 40, 30, 20, and 10 µl E4 used in the assay.

Table 4.1 Concentration of azamethiphos required for 50 % inhibition of AChE activity (IC_{50}) in the presence and absence of E4.

Volume of E4 per well (µl)	IC_{50} (nM)	SE
0	1.12	0.02
50	37.89	0.71
40	18.27	1.41
30	8.41	0.48
20	4.27	0.25
10	3.64	0.20

SE refers to the standard error of the IC_{50} value derived from the fitted curve.

The assay was then tested using E4 incubated with different concentrations of PBO (3 mM, 0.3 mM and 0.03 mM) to see how much was needed to get complete inhibition of the E4 (Figure 4.6). Both 0.3 mM and 3 mM PBO gave similar results, whilst with 0.03 mM there was little esterase blockade. Since the primary purpose of the assay was to explain the lack of esterase inhibition seen in a conventional assay it was decided that E4 + 3 mM PBO would provide the optimum concentration to use. Although this may seem high, it is equivalent to 0.1% PBO which is the concentration that has been used for previous *in vivo* experiments and trials (Young *et al.*, 2005, 2006). This part of the assay showed that the addition of PBO to the E4 prevented the esterase from binding to the azamethiphos, so enabling the inhibitor to act on the target site (Figure 4.6).

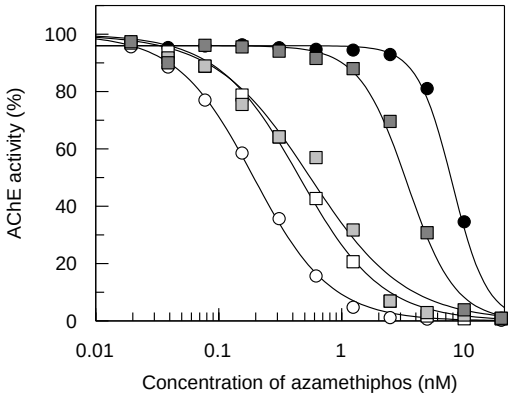


Figure 4.6 Inhibition of *Musca domestica* AChE activity with E4 and E4 plus PBO.

Open circle: no E4 (AChE only); closed circle: E4; open square: E4 + 3 mM PBO; light grey square: E4 + 0.3 mM PBO; dark grey square: E4 + 0.03 mM PBO.

Table 4.2 Concentration of azamethiphos required for 50% inhibition of AChE activity (IC_{50}) in the presence and absence of E4, and in the presence of E4 + PBO.

Corresponds to Figure 4.6. Percentages relate to Figure 4.7.

Sample	IC_{50} (nM)	SE
AChE only (No E4)	0.19	0.01
E4	7.92	0.44
E4+3mM PBO	0.46	0.02
E4+0.3mM PBO	0.55	0.06
E4+0.03mM PBO	3.40	0.25

SE refers to the standard error of the IC_{50} value derived from the fitted curve.

The molarities of the azamethiphos and E4 present in the reactions were calculated to compare the relative quantities required for the inhibition. Calculating the molar amounts of azamethiphos and E4 at the point at which AChE activity is just inhibited, i.e. the point at which azamethiphos has overcome the sequestering effects of the esterase, gave a result of around 5 pmoles of E4 and 1.5 pmoles azamethiphos. This suggests a near-stoichiometric binding between E4 and azamethiphos. The molar amount of PBO present is appreciably higher (4 nmoles). The assay curve and hence blockade of E4 was not significantly different when the assay was repeated with an incubation of E4 + 0.3 mM PBO, but blockade was almost absent using an incubation of 0.03 mM PBO (Figure 4.6). However, to be certain of this inhibitory effect, 3 mM PBO in the incubation was judged to be the most robust concentration to use as it gave an excess of PBO.

Theoretically, a high-throughput method could be created by using a 'diagnostic window' i.e. the concentration at which AChE activity is at a maximum when E4 is present and at a minimum with no E4 or E4 + PBO. This would enable fast screening of potential synergists and reduce the volume of E4 required for the assay by comparing the E4, and E4 + synergist against only one concentration of azamethiphos. The concentration of azamethiphos to be used would be based on the point just before the sigmoid curve approaches zero AChE activity (%) displaying the protection that the E4 gives to the AChE (illustrated by the box in Figure 4.7). Thus, providing the diagnostic window is known (i.e. the concentration of azamethiphos to use) only the three wells highlighted by the box would need to be run in the assay. The AChE activity of the three points highlighted in Figure 4.7 are shown in Figure 4.8. These show that a diagnostic window can be used confidently to accurately determine the efficacy of an unknown synergist.

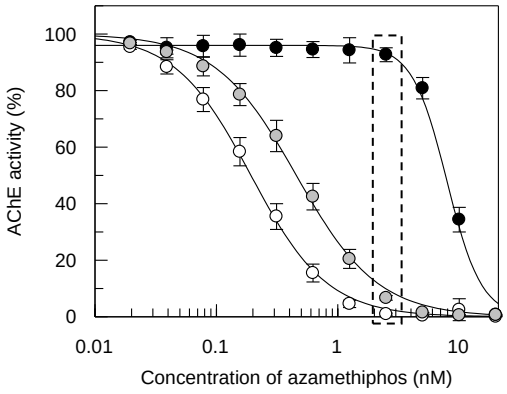


Figure 4.7 The diagnostic window for creating a higher throughput method in order to select a potential synergist.

Open circle: no E4; closed circle: E4; grey circle: E4 + 3 mM PBO. Error bars shown illustrate the standard deviation derived from three replicates.

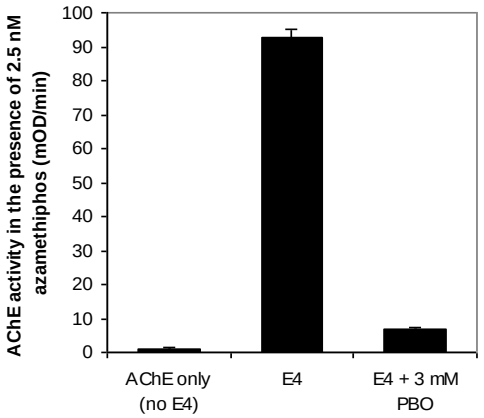


Figure 4.8 AChE activity of the three points from the diagnostic window highlighted in Figure 4.7.

Error bars shown illustrate the standard deviation derived from three replicates.

The development of the novel assay described in this section is used in the next chapter, Chapter Five, to screen a range of putative synergists for their ability to inhibit resistance-associated esterases. In order to use the assay to screen putative synergists, the IC_{50} results must be converted into a percentage (from here on termed the 'Index' value - I) so that synergists could be screened on different days or with different stocks of E4 and still be comparable. This is because although the protocol is standardised, the IC_{50} values achieved will always vary slightly depending on the stock of the E4 and the exact time of the incubation of the E4 with the azamethiphos on the microplate. Comparing results as I values (percentages) (Table 4.3) allows for these variations. The lower the I value for the putative synergist, the better esterase inhibitor it is *in vitro*.

Table 4.3 Concentration of azamethiphos required for 50% inhibition of AChE activity (IC_{50}) in the presence and absence of E4, and in the presence of E4 + 3 mM PBO, with Index values shown, calculated as described in the text.

Corresponds to Figure 4.6. Percentages relate to Figure 4.7.

Sample	IC_{50} (nM)	SE	%
AChE only (No E4)	0.19	0.01	0
E4	7.92	0.44	100
E4+3mM PBO	0.46	0.02	3.49

SE refers to the standard error of the IC_{50} value derived from the fitted curve.

5 CHAPTER FIVE: SCREENING PUTATIVE SYNERGISTS

5.1 Introduction

Using the 'esterase interference' assay developed and optimised in Chapter 4, a range of compounds were screened as potential esterase inhibitors. The compounds were chosen based on their structure or previous reports of insect control properties. This work was carried out in collaboration with Botanical Resources Australia (BRA) who provided the essential oils, and the Chemistry Division at the University of Tasmania, who provided extracts of a number of Tasmanian plants.

As the 3D structure of resistance-associated esterases is unknown, it is not easy to predict the structure of any putative inhibitors/synergists, other than to surmise that it would be beneficial for there to be an ester bond present which the esterase can attack. Most of the putative synergists tested here were essential oils and plant extracts that were selected by BRA (with some suggestions from Rothamsted) based on biological activity and the structures of active components (where known). Some were chosen because they were known for their insecticidal properties (e.g. neem seed oil) and some because they were plants native to Tasmania.

Compounds that inhibited esterases at the same or an equivalent efficacy as PBO were further screened for their ability to inhibit P450 activity. Samples that inhibited both esterase and P450 activity were further tested *in vivo* to assess their effect as a synergist in combination with pyrethrins.

PBO has been used as a synergist for pyrethrins for many years and the aim of these experiments was to find a novel alternative. Most of the compounds tested were of a natural or organic nature to comply with the growing consumer demand for these types of products.

This chapter describes the screening of compounds:

- *In vitro* for their ability to inhibit resistance associated esterases (section 5.3.1) using the assay developed and described previously in Chapter Four;
- *In vitro* for their ability to inhibit cytochrome P450s (section 5.3.2);
- *In vivo* for their ability to penetrate the insect cuticle and inhibit metabolic defences. These experiments assess the effect the putative synergists have on the natural

pyrethrins and thus whether the efficacy of the pyrethrins can be enhanced in *Myzus persicae* (section 5.3.3) and *Musca domestica* (section 5.3.4).

5.2 Materials and Methods

5.2.1 Measuring a putative synergist's ability to inhibit esterases: 'Esterase interference assay'

Purified E4 was incubated with 0.1 % putative synergist (in acetone) overnight at 4 °C. A 96-well microplate was prepared as described in section 4.1.1.2, step 4.

5.2.2 Measuring a putative synergist's ability to inhibit P450s

Lamb's liver P450 preparation (section 2.4.6.1) (7.5 µl) plus 41.5 µl diluted sodium phosphate buffer (pH 7.6) prepared as described in section 2.4.6.1 and 1 µl of 10 % synergist in acetone were added to the wells of a 96-well white microplate. The plate was incubated at room temperature for 15 minutes before the addition of 80 µl of 0.5 mM 7-ethoxycoumarin. The plate was incubated at 30 °C for a further 3 minutes before the addition of 10 µl of 9.6 mM NADPH. The plate was read using an excitation wavelength of 380 nm and an emission wavelength of 460 nm (in a PerkinElmer microfluorometric reader) every 5 minutes for one hour.

Each synergist was tested in triplicate together with controls of buffer only and homogenate plus 1 µl acetone.

5.2.3 Investigating the effect of putative synergists *in vivo*

Putative synergists that performed well as esterase and P450 inhibitors were selected for trial *in vivo* against *M. persicae* and *M. domestica*. These two insect species were selected because the compounds used were dissolved in acetone and thus a topical application technique was required, and both *M. persicae* and *M. domestica* bioassay well using the topical application technique.

Myzus persicae

Bioassays were performed as described in section 2.4.7.2. Briefly, adult insects (794jz clone) were placed onto untreated leaf discs (Chinese cabbage) and dosed by topical application with 0.25 µl compounds in acetone. Mortality was scored after 72 hours. The ratio of

synergist:pyrethrins was 4:1. These experiments were also performed using the putative synergist and alpha-cypermethrin in a ratio of 4:1, and putative synergist, PBO and pyrethrins in a ratio of 4:4:1.

Musca domestica

Bioassays were performed on female flies (381zb strain) as described in section 2.4.7.2. Briefly, insects were dosed (by topical application) with 1 μl treatment in acetone. Mortality was scored after 24 hours. The ratio of synergist:pyrethrins was 5:1. These experiments were also performed using a tank mix of putative synergist, PBO and pyrethrins in a ratio of 5:5:1.

5.2.4 The putative synergists

Table 5.1 shows the properties of the putative synergists used in the experiments described in this chapter. The chemical structures of active components (where known) of the oils and plant extracts tested for synergism of pyrethrins are shown in Figure 5.1.

Table 5.1 Details of oils and plant extracts tested for synergistic potential. Relates to structures in Figure 5.1

(Table continues onto 3 pages and is arranged alphabetically by common name)
Information from (Harbourne, 1996)

Common name *(used throughout chapter)*	***Description of sample (source)***	***Speculated inhibitors of interest*** *(if known)* ***Chemical formula (molecular weight)***	***Type of chemical***
	Extracts of *Correa alba* var alba (Rutaceae) *(BRA/UTas)*		
	Extracts of *Acradenia frankliniae* (Rutaceae) *(BRA/UTas)*		
	Extracts of *Correa stackhousiana* *(BRA/UTas)*		
Angelica	Essential oil of roots of *Angelica archangelica*, *Heracleum* spp. and *Selinum vaginatum* (Umbelliferae) (*Auroma*)	Angelicin $C_{11}H_6O_3$ (186.17) (also known as Isopsoralen)	Furanocoumarin
Aniseed	Essential oil of seeds of Japanese star anise, *Illicium anisatum* (Illiciaceae) (*Bronson and Jacobs*)	Anisatin $C_{15}H_{20}O_8$ (328.32)	Sesquiterpenoid lactone
Azadirachtin	Purified, powder form (*Bhupinder Khambay, Rothamsted Research*)	$C_{35}H_{44}O$ (720.72)	Nortriterpenoid (Limonane)
Bergamot	Essential oil, *Citrus bergamia* (Rutaceae) (*Auroma*)	Bergamottin $C_{21}H_{22}O_4$ (338.40) (also known as Bergaptol geranyl ether; 5-Geranyloxypsoralen; Bergaptin)	Furocoumarin
Citronella java	(*Bronson and Jacobs*)	Citronellal $C_{10}H_8O$ (154.25) (also known as 3,7-dimethyloct-6-enal; Rhodinal)	Monoterpenoid
Cypress	(*Auroma*)		
Dill	Essential oil, *Anethum graveolens* (Umbelliferae) (*Essential Oils of Tasmania Pty.Ltd.*)	Dillapiole $C_{12}H_{14}O_4$ (222.24) (also known as Dill apiole; Dillapiol)	Phenylpropanoid
Ethoxylated castor oil*	(*BRA*)		
Eucalyptus	Essential oil, distilled from fresh leaves of *Eucalyptus globulus* and some other *Eucalyptus* spp. (Myrtaceae) *(Banalasta Lavender Farm)*	1,8-Cineole $C_{10}H_{18}O$ (154.25) (also known as Eucalyptol; Cajeputol; 1,8-Epoxy-*p*-menthane)	Monoterpenoid
Fennel	Essential oil (*Essential Oils of Tasmania Pty.Ltd.*)		

Common name *(used throughout chapter)*	***Description of sample (source)***	***Speculated inhibitors of interest*** *(if known)* ***Chemical formula (molecular weight)***	***Type of chemical***
Garlic	Essential oil, *Allium sativum* (Alliaceae) (*Auroma*)	Diallyl disulfide $C_6H_{10}S_2$ (146.28) (also known as Allyl disulfide; Di-2-propenyl disulfide)	Sulphur compound
Lavender	Essential oil (*Bronson and Jacobs*)		
Lemon-scented Boronia	Extracts of *Boronia citriodora* (Rutaceae) *(BRA/UTas)*		
Linoleic acid	Sigma-Aldrich UK	$C_{18}H_{32}O_2$ (280.45)	Unsaturated omega-6 fatty acid
Manuka	*(BRA)*		
Mountain Correa	Extracts of *Correa lawrenciana* (Rutaceae) *(BRA/UTas)*		
Myristicin	>97% pure *(Sigma-Aldrich UK)*	Myristicin $C_{11}H_{12}O_3$ (192.21)	Phenylpropanoid
Neem (seed oil)	Oil from seeds of neem tree, *Azadirachta indica* (Meliaceae) (*Auroma*)	Azadirachtin $C_{35}H_{44}O$ (720.72) (see, azadirachtin)	Nortriterpenoid (Limonane)
Nutmeg	Essential oil, *Myristica fragrans* (Myristicaeae) (*Auroma*)	Myristicin $C_{11}H_{12}O_3$ (192.21)	Phenylpropanoid
Oleic acid*	2 pure samples: (*BRA* and *Sigma-Aldrich UK*)	Oleic acid (282.46) (also known as *cis*-9-Octadecenoic acid; Elainic acid)	Fatty acid
Parsley (53% and 86% myristicin)	Essential oil from seeds of parsley *Petroselinum crispum* (Umbelliferae) (*Essential Oils of Tasmania Pty.Ltd.*)	Apiole $C_{12}H_{14}O_4$ (222.24) (also known as Apiol; apioline; Parsley camphor) and myristicin (see nutmeg)	Phenylpropanoid
Parsley	Extracts of *Petroselinum crispum* *(BRA/UTas)*	As above	
Pepper	Essential oil from unripe fruit of wild pepper, *Piper cubeba* (Piperaceae) and roots and shoots of *Aristolochia triangularis* (Aristolochiaceae) (*Auroma*)	Cubebin $C_{20}H_{20}O_6$ (356.38)	lignan
Peppermint	Essential peppermint oil, *Mentha piperita* (*Essential Oils of Tasmania Pty.Ltd.*)	Menthol $C_{10}H_{20}O$ (156.27) (also known as Mentol; peppermint camphor; menthacamphor)	monoterpenoid

Common name *(used throughout chapter)*	***Description of sample (source)***	***Speculated inhibitors of interest*** *(if known)* ***Chemical formula (molecular weight)***	***Type of chemical***
Peppermint eucalyptus		See peppermint and eucalyptus	
Piperonyl butoxide (PBO)	Synthetic, 96% pure (*Endura SpA, Italy*)	PBO $C_{19}H_{30}O_5$ (338.4)	
Rose blossom	*Boronia pilosa* (Rutaceae) *(BRA/UTas)*		
Rosemary	Essential oil from leaves of Rosemary (sp. unknown) (*Bronson and Jacobs*)	Palustrol $C_{15}H_{26}O$ (222.37) (also known as 1-aromadendranol)	Sesquiterpenoid
Sesame vegetable oil	(*Auroma*)	Composed of the following fatty acids: Palmitic (7-12 %) $C_{16}H_{32}O_2$ (256.42)	Fatty acid
		palmitoleic (trace – 0.5 %) $C_{16}H_{30}O_2$ (254.41)	Fatty acid
		stearic (3.5 – 6.0 %) $C_{18}H_{36}O_2$ (284.48)	Fatty acid
		oleic (35 – 50 %) (see above)	Fatty acid
		linoleic (35 – 50 %) (see above)	Fatty acid
		linolenic (trace – 1 %)	Fatty acid
		eicosensoic (trace – 1 %)	Fatty acid
Stinkwood	*Zieria arborescens* (Rutaceae) *(BRA/UTas)*		

Sources of samples: Auroma (Victoria, Australia); Bronson & Jacobs (NSW, Australia); Essential oils of Tasmania (Tasmania, Australia); Banalasta lavender farm (Tamworth, Australia); BRA/UTas – provided by Adrian Blackman (University of Tasmania, Hobart, Tasmania, Australia)

* denotes that the compound named is a component of the BRA emulsifiable concentrate formulation.

In the following results and discussion section, the University of Tasmania extractions are referred to using the following abbreviations;

for the sample names:

Af	*Acradenia frankliniae*
Bc	*Boronia citriodora*
Bp	*Boronia pilosa*
Ca	*Correa alba* var *alba*
Cl	*Correa lawrenciana*
Cs	*Correa stackhousiana*
Py	*Petroselinum crispum*
Za	*Zieria arborescens*

for the method of extraction of the sample:

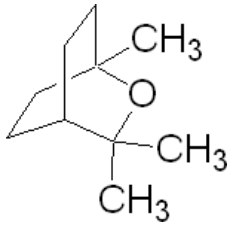
A	steam distilled essential oil
B1	solvent extract – petroleum spirits fraction
B2	solvent extract – dichloromethane fraction
B3	solvent extract – aqueous methanol fraction

For certain samples that were further fractionated to possibly identify which ones gave extracts with highest synergistic activity, 'fr' is used to denote the fraction number.

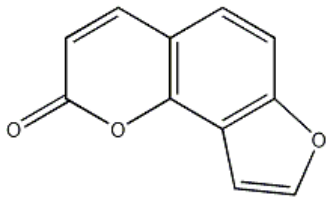
Figure 5.1 Structure of speculated inhibitors of interest for putative synergists listed in Table 5.1

(Arranged alphabetically, continues on 3 pages)

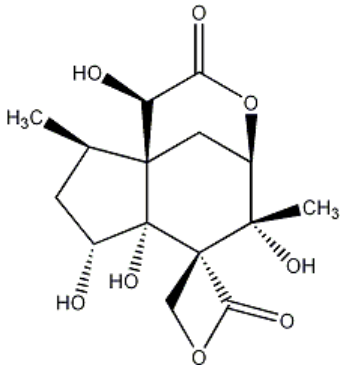
1,8-cineole



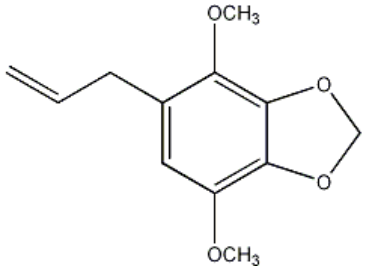
Angelicin



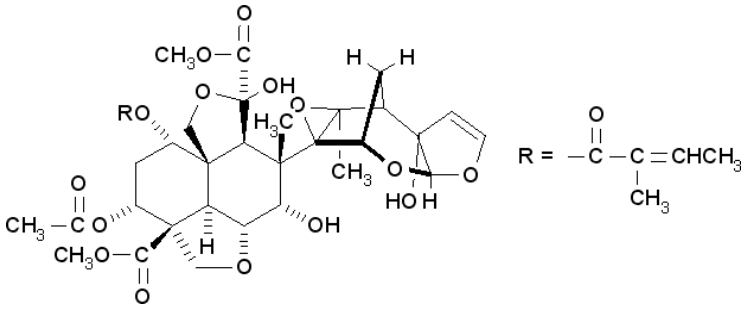
Anisatin



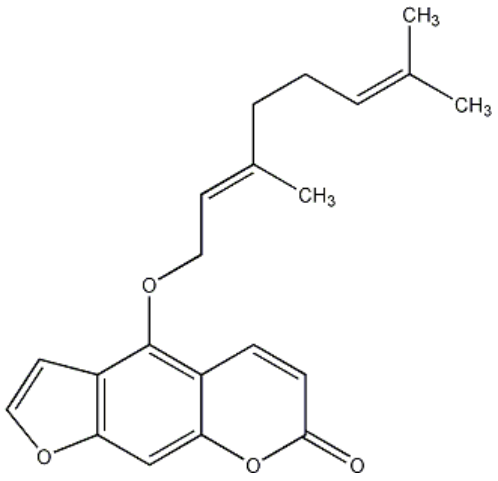
Apiole



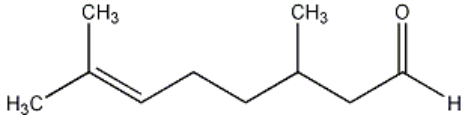
Azadirachtin



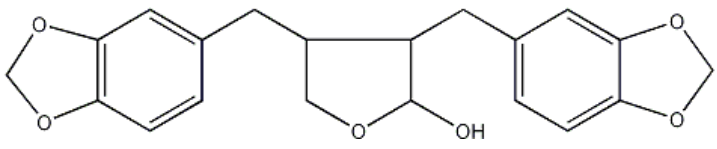
Bergamottin



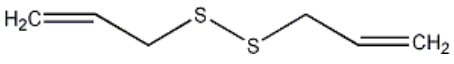
Citronellal



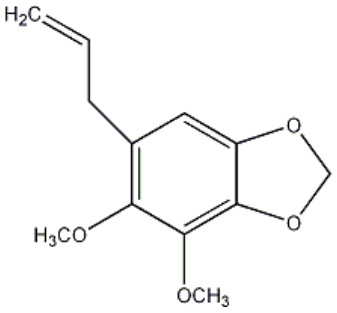
Cubebin



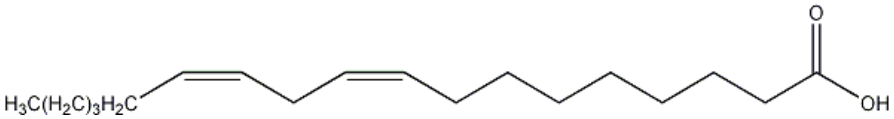
Diallyl disulfide



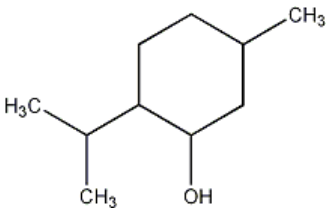
Dillapiole



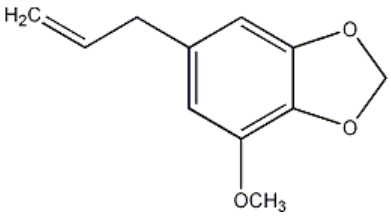
Linoleic acid



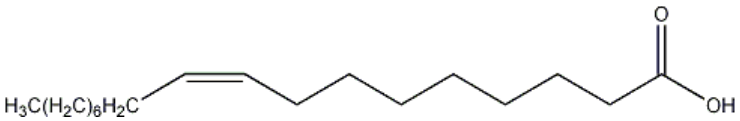
Menthol



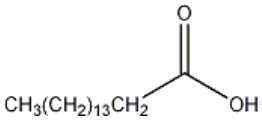
Myristicin



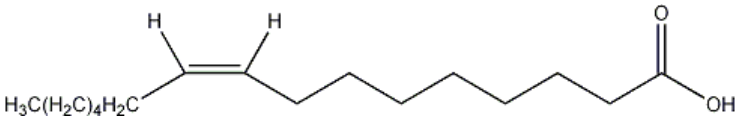
Oleic acid



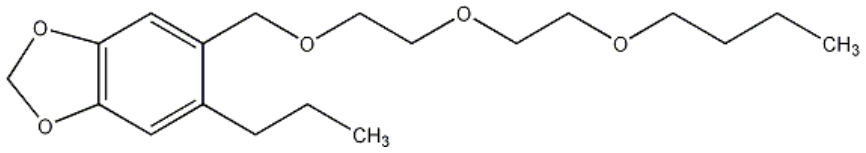
Palmitic acid



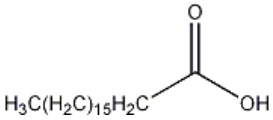
Palmitoleic acid



PBO



Stearic acid



5.2.5 Calculations

Following bioassays, the "synergistic factors" were calculated using the following equation:

$$\text{SF (synergistic factor)} = \frac{LC_{50} \text{ insecticide only (for x clone or strain)}}{LC_{50} \text{ synergised insecticide (for x clone or strain)}}$$

Using the SF enabled a direct comparison of the compounds tested and also had the advantage that studies performed only on a resistant population could be compared.

5.3 Results and discussion

This section is divided into four parts, with a general discussion at the end. The four sections cover the efficacy of the putative synergists: as esterase inhibitors *in vitro* (section 5.3.1); as P450 inhibitors *in vitro* (section 5.3.2); *in vivo* against *M. persicae* (section 5.3.3), and *in vivo* against *M. domestica* (section 5.3.4). A summary of the findings is given before the individual experimental results sections to aid the understanding of the decisions made about which samples should be tried in each of the experiments. Table 5.2 is a summary of all of the putative synergists tested in chapter five. Where a sample performed within the confidence limits of PBO in the esterase interference assay (section 5.3.1), they were selected for their *in vitro* ability to inhibit P450s from lambs liver. Some samples were selected for the P450 assay (and subsequent bioassays) despite not performing as well as PBO because Botanical Resources requested that certain samples be further investigated.

Table 5.2 Summary of the performance of each of the putative synergists tested in chapter five.

✓ indicates that a sample was in the same range as/more potent than PBO; * indicates that a sample was not as effective as PBO; (B) indicates that Botanical Resources requested the sample be looked at further.

In vitro **Esterase Interference assay (section 5.3.1)**	***In vitro*** **P450 assay**	***In vivo Myzus Persicae*** **(4:1 synergist: pyrethrins)**	***In vivo Myzus Persicae*** **(4:1 synergist:alpha-cypermethrin)**	***In vivo Myzus Persicae*** **(4:4:1 synergist:PBO: pyrethrins)**	***In vivo Musca domestica (4:1 synergist: pyrethrins)***	***In vivo Musca domestica (4:4:1 synergist:PBO:pyrethrins)***
(section 5.3.1)	**(section 5.3.2)**	**(section 5.3.3.1)**	**(section 5.3.3.2)**	**(section 5.3.3.3)**	**(section 5.3.4.1)**	**(section 5.3.4.2)**
Af-A						
Af-B1						
Af-B2	✓					
Af-B3						
Angelica						
Aniseed						
Azadirachtin						
Bc-A						
Bc-B1	✓	*				
Bc-B2	✓					
Bc-B3						
Bergamot organic	✓ (B)	*				
Bp-A	✓					
Bp-B1						
Bp-B2						
Bp-B3						
Ca-A						
Ca-B1						
Ca-B2	✓					
Ca-B2-fr1						
Ca-B2-fr10	✓					
Ca-B2-fr2	✓					
Ca-B2-fr3	✓					
Ca-B2-fr4						
Ca-B2-fr5						
Ca-B2-fr6	✓					
Ca-B2-fr7						
Ca-B2-fr8						
Ca-B2-fr9	✓					
Ca-B2-frB2						
Ca-B3						
citronella						
Cl-A						
Cl-B1	✓	*	✓			
Cl-B2	✓					
Cl-B3						

In vitro **Esterase Interference assay (section 5.3.1)**	***In vitro*** **P450 assay**	***In vivo Myzus Persicae*** **(4:1 synergist: pyrethrins)**	***In vivo Myzus Persicae*** **(4:1 synergist:alpha-cypermethrin)**	***In vivo Myzus Persicae*** **(4:4:1 synergist:PBO: pyrethrins)**	***In vivo Musca domestica (4:1 synergist: pyrethrins)***	***In vivo Musca domestica (4:4:1 synergist:PBO:pyrethrins)***
(section 5.3.1)	**(section 5.3.2)**	**(section 5.3.3.1)**	**(section 5.3.3.2)**	**(section 5.3.3.3)**	**(section 5.3.4.1)**	**(section 5.3.4.2)**
Cs-A						
Cs-B1	✓					
Cs-B2						
Cs-B3						
Cypress						
Dill						
Eth. Castor Oil	*	*	✓	✓		
Eucalyptus radiata						
Fennel						
Garlic						
Lavender						
Linoleic acid	✓	*	✓	✓	*	✓
Manuka						
Methyl methoxy butanol						
Myristicin						
Neem seed oil	✓	*	✓	✓	*	✓
Nutmeg						
Oleic acid	✓	*	✓			
parsley (53% myristicin)	✓ (B)	*	✓			
Parsley (86% myristicin)						
Pepper (black)						
Peppermint						
Peppermint Eucalyptus						
Py-A						
Py-B1						
Py-B2						
Py-B3						
Rosemary						
Sesame						
Za-A						
Za-B1	✓					
Za-B2	✓	*				
Za-B2-fr1	✓					
Za-B2-fr2	✓					
Za-B2-fr3	✓					
Za-B2-fr4						
Za-B2-fr5						
Za-B2-fr6	✓					
Za-B2-fr7	✓					
Za-B2-fr8	✓					
Za-B2-fr9						
Za-B3	*					

5.3.1 Esterase inhibition (*in vitro*)

The esterase interference assay was used to indirectly determine how well a sample can inhibit esterase activity (E4) by measuring AChE activity (refer to Chapter 4, Figure 4.1). Results of this assay are shown in Table 5.3, which displays the index value, standard error, lower limits and upper limits for each sample. The index (I) values shown in Table 5.3 represent the samples' potential as an esterase inhibitor (see end of section 4.2). I equates to the percentage AChE activity remaining, with values closest to zero showing the most potent esterase inhibiting action. It is obtained by calculating the IC_{50} values (see end of section 4.2) for each compound (using Grafit) and the putative synergists activity is calculated as a percentage of E4 activity (where the IC_{50} value for E4 = 100 % and for no E4 = 0%). The lower the I value, the better the sample is as an esterase inhibitor *in vitro*.

The results reveal that all except two (methyl methoxy butanol and Bp-B3) of the putative synergists show potential for esteratic synergistic activity (Table 5.3). It is not surprising that methyl methoxy butanol did not perform well as it is an alcohol. It was tested because it is a component of the BRA EC formulation present dissolve the other components.

Many of the samples screened were prepared by the University of Tasmania, and some fractions from each plant used for extractions were as good as/better than PBO apart from those fractionated from extracts of *Boronia pilosa*. Of the University of Tasmania samples, the more potent E4 inhibitors were those in the petroleum spirits fractions (B1) and the dichloromethane fractions (B2). The B2 fractions from *Zieria arborescens* and *Correa alba* were further fractionated by the University of Tasmania into 10 fractions. In the case of *Zieria arborescens*, 7 out of 10 were comparable to PBO at inhibiting esterases *in vitro*. For the *Correa alba* further fractionated B2 samples, 4 out of 10 were comparable to PBO. These are shown in performance order in Figure 5.2.

Each solvent used in the extractions will have extracted chemicals of a certain lipophilicity from the plant samples. Generally, the petroleum spirits fractions (B1) fractions contained an E4 inhibitor(s) regardless of the plant used and this may be an important factor for consideration in the future if further extractions are to be made. It is important to note that the samples extracted from Tasmanian plants (provided by BRA/UTas) were extracted from individual plants. Therefore the samples that look promising would need further evaluation to ensure that extracts from other plants of the same species have the same enzyme inhibiting potential.

Table 5.3 Index value (I) and corresponding standard errors for AChE activity of E4+putative synergists.

I is the IC_{50} value obtained from Grafit, shown as a percentage of E4 and No E4, where E4 = 100% activity and No E4 = 0% activity. (I equates to % AChE activity remaining). SE: standard error of the fit of the curve (calculated by Grafit, then corrected into a percentage thus in the same format as the Index value); LL/UL95%: 95% confidence limits (lower limits/upper limits). Samples with I values or errors less than 4.26 % (the upper limit for PBO) are shown in Figure 5.2. PBO is indicated in bold.

Putative synergist	*I (%)*	*SE (%)*	*LL95%*	*UL95%*
Cl-B2	0.01	0.48	-0.99	1.02
Za-B2-fr3	0.39	0.16	0.06	0.71
Neem	0.41	1.22	-2.14	2.95
Za-B2	0.88	0.35	0.16	1.60
Bc-B2	1.04	0.79	-0.61	2.69
Za-B2-fr9	1.14	0.17	0.79	1.50
Za-B3	1.28	0.35	0.55	2.02
Oleic acid*	1.43	0.32	0.76	2.11
Linoleic acid	1.53	0.36	0.78	2.29
Bc-B1	1.92	1.16	-0.49	4.32
Za-B2-fr1	2.31	0.18	1.94	2.68
Ca-B2-fr3	2.34	0.22	1.89	2.79
Ca-B2-fr2	2.41	0.22	1.95	2.87
Cl-B1	2.50	0.36	1.75	3.24
Za-B2-fr2	2.56	0.28	1.98	3.13
Ca-B2	2.62	0.77	1.02	4.22
Bp-A	2.71	0.24	2.21	3.21
Za-B2-fr6	3.01	0.34	2.30	3.73
Ca-B2-fr10	3.11	0.28	2.52	3.69
Za-B1	3.33	0.45	2.39	4.26
PBO	**3.40**	0.25	**2.89**	**3.92**
Eth. Castor Oil*	3.42	0.32	2.76	4.08
Za-B2-fr8	3.54	0.16	3.22	3.86
Za-B2-fr7	3.76	0.30	3.13	4.38
Ca-B2-fr6	3.96	0.38	3.18	4.74
Ca-B2-fr9	4.74	0.31	4.10	5.38
Ca-B2-fr5	4.76	0.26	4.23	5.30
Ca-B2-fr7	4.93	0.27	4.37	5.50
Cs-B1	4.98	0.94	3.03	6.94
Ca-B2-frB2	5.01	0.29	4.40	5.62
Za-B2-fr4	5.07	0.36	4.31	5.83
Pyrethrins (from 50% extract)	5.29	0.62	4.01	6.57
Py-B1	5.55	0.73	4.02	7.07
Ca-B2-fr8	6.11	0.35	5.38	6.85
Af-B2	6.22	2.02	2.01	10.42
Bp-B1	6.28	0.43	5.38	7.17
Myristicin	6.63	0.36	5.88	7.38
Za-B2-fr5	6.75	0.38	5.95	7.54
Azadirachtin	7.01	0.74	5.48	8.55
Peppermint	7.03	0.92	5.12	8.94
Bc-B3	7.29	0.86	5.50	9.07
Bp-B2	7.31	0.74	5.77	8.86
Cs-B2	7.62	0.97	5.61	9.63

Putative synergist	*I (%)*	*SE (%)*	*LL95%*	*UL95%*
Py-B2	8.74	1.05	6.56	10.92
Af-B1	9.01	0.56	7.85	10.17
Pyrethrins II	9.02	1.49	5.92	12.12
Ca-B1	9.11	1.04	6.96	11.27
Lavender	9.83	1.17	7.40	12.26
Cs-A	9.86	1.09	7.59	12.12
Py-A	10.19	1.39	7.30	13.08
parsley (53% myristicin)	10.41	1.37	7.56	13.27
Ca-B2-fr4	11.41	1.26	8.78	14.04
Cl-B3	11.49	1.16	9.08	13.90
Nutmeg	11.57	1.09	9.30	13.84
Sesame	11.87	1.47	8.81	14.93
Aniseed	11.98	1.61	8.63	15.34
Cypress	12.49	1.45	9.47	15.51
Pyrethrins I	12.69	2.07	8.37	17.00
Manuka	13.43	1.23	10.87	16.00
Garlic	14.44	1.20	11.94	16.95
Parsley (86% myristicin)	14.97	1.24	12.39	17.54
Peppermint Eucalyptus	15.38	1.58	12.09	18.68
Za-A	15.84	1.46	12.80	18.88
citronella	16.60	1.54	13.39	19.81
Ca-B3	17.20	1.63	13.82	20.59
Bergamot organic	21.14	1.65	17.71	24.56
Ca-A	21.21	2.20	16.64	25.79
Af-A	22.42	1.15	20.04	24.80
Fennel	22.43	1.15	20.04	24.83
Bc-A	22.56	1.41	19.64	25.49
Py-B3	27.30	3.12	20.81	33.79
Pepper (black)	31.23	4.26	22.37	40.09
Angelica	31.68	2.12	27.26	36.10
Cs-B3	35.06	2.39	30.08	40.03
Alpha-cypermethrin	35.69	2.52	30.44	40.93
Dill	35.81	2.44	30.74	40.89
Ca-B2-fr1	36.44	2.61	31.01	41.87
Cl-A	37.59	2.88	31.60	43.58
Rosemary	40.83	4.96	30.51	51.15
Af-B3	47.05	2.61	41.62	52.48
Eucalyptus radiata	51.62	3.89	43.54	59.70
Methyl methoxy butanol*	94.18	5.28	83.20	105.15
Bp-B3	98.92	14.58	68.59	129.25

Af is from *Acradenia frankliniae*; Bc is from *Boronia citriodora;* Bp is from *Boronia pilosa;* Ca is from *Correa alba* var *alba;* Cl is from *Correa lawrenciana;* Cs is from Correa stackhousiana; Py is from *Petroselinum crispum*; Za is from *Zieria arborescens;* A: steam distilled essential oil; B1: solvent extract – petroleum spirits fraction; B2: solvent extract – dichloromethane fraction; B3: solvent extract – aqueous methanol fraction; fr denotes a further fractionated sample. * denotes that the compound named is a component of the BRA emulsifiable concentrate formulation.

Samples that showed potent esterase inhibition *in vitro* that were not extracts from Tasmanian plants were neem seed oil, oleic acid, linoleic acid and ethoxylated castor oil. All of these samples were comparable to PBO *in vitro* (Figure 5.2). Oleic acid and ethoxylated castor oil are both components of the BRA EC formulations and their performance in this *in vitro* test, with both samples showing potential for inhibiting esterases, offers an explanation

for the lower LC_{50} values found in Chapter Three when comparing leaf dip bioassays using EC formulations, compared to topical application of technical pyrethrins for *M. persicae*.

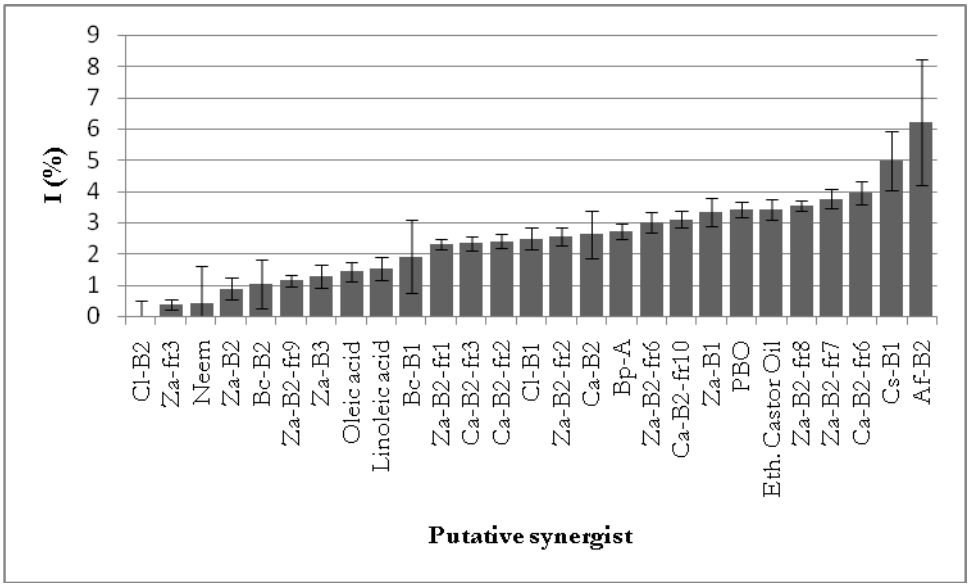


Figure 5.2 Graphical representation of I values (refer toTable 5.3) for esterase inhibition of samples selected for trial in P450 assay.

I is the IC_{50} value obtained from Grafit, shown as a percentage of E4 and No E4, where E4 = 100% activity and No E4 = 0% activity. (I equates to % AChE activity remaining). Error bars indicate the error of the fit of the curve (calculated by Grafit and reformatted to fit the percentage representation of the I value).

Interestingly, azadirachtin (the main component of neem) was not as effective as the neem seed oil. It is possible that there are components, other than azadirachtin, in the neem seed oil that also inhibit esterase activity or work synergistically with azadirachtin.

Generally if the lower confidence limit of a sample was less than or equal to PBO's upper limit (I = 3.92 %) then it was selected for the P450 assay. Samples were also selected if their upper confidence limit was less than or equal to PBO's upper confidence limit. In addition, it was decided that some of the less potent esterase inhibitors would be tested *in vivo*, regardless of *in vitro* results, (for example, nutmeg, parsley and bergamot) (see 5.3.2)

As well as screening putative synergists, the esterase interference assay was also useful to evaluate the esterase inhibiting action of pyrethrins and alpha-cypermethrin. This was of interest because as discussed in Chapter 3, resistance to pyrethrins was less than expected considering resistance factors of the synthetic pyrethroids reported previously (Bingham *et al.*, 2007, Bingham, 2007). Pyrethrins showed a better ability to inhibit esterases than the Pyrethrins I and II extracts. This may be due to the presence of the other components in the combined extract. Collectively, Pyrethrins I and II constitute 45-55% of pyrethrum extract with the remainder usually being comprised of sterols, triterpenols, alkanes, fatty acids from

triglycerides and carotenoids (Maciver, 1995). From these results it can be concluded that esterases bind to pyrethrins, thus confirming esterases as a resistance mechanism for pyrethrins. This also means that pyrethrins, or perhaps more accurately, the constituents of the pyrethrin extract, have the potential to act as their own synergist since the *in vitro* synergism of the pyrethrin extract with E4 is greater than the individual pyrethrin I or II extracts. The synergism between the pyrethrin extract and E4 may contribute to the lower resistance factors compared with synthetic pyrethroids, as well as the difference in response to *kdr* mutations discussed Chapter Three. Alpha-cypermethrin showed some esterase inhibiting potential but this was not in the same range as the pyrethrin samples. Although esterases are a resistance mechanism for alpha-cypermethrin, and this, together with the fact that synthetic pyrethroids are affected by *kdr* mutations helps explain the higher resistance factors for synthetic pyrethroids.

5.3.2 P450 inhibition (*in vitro*)

Compounds were screened for their P450 inhibiting potential by incubating them with P450s from lamb's liver and measuring their activity compared to an uninhibited control. The following samples showed complete inhibition of P450 activity: Bc-B2; Bp-A; Ca-B2; Ca-B2-fr3; Cl-B1; Za-B1; Za-B2; Za-B2-fr1; Za-B2-fr2; Za-B2-fr3; Za-B2-fr6; Za-B2-fr7; Za-B2-fr8. Alpha-cypermethrin did not show any P450 inhibiting potential. Other samples gave intermediate levels of inhibition and results for these samples are shown in Table 5.4 and Figure 5.3. The lower the percentage activity of the control, the better the sample at inhibiting P450 activity and thus the greater potential for being a useful synergist.

The finding that some samples from *Boronia citriodora*, *Boronia pilosa*, *Correa alba* var *alba*, *Correa lawrenciana*, and *Zieria arborescens* gave complete inhibition of P450s suggest that extracts from these plants, and linoleic acid, oleic acid and neem seed oil may be very useful as insecticide synergists, especially where an insects' resistance mechanism is P450-based. Results from these experiments again confirm that components of the BRA EC formulation may be acting as synergists, thus enhancing the efficacy of the pyrethrins in the EC formulations and thus when insects are bioassayed using a leaf dip of pyrethrins EC, the LC_{50} achieved is lower than expected.

The low inhibition of P450 activity by PBO is perhaps surprising, although it has been noted previously that PBO is a more potent esterase inhibitor than oxidase inhibitor (Moores *pers.comm.*).

Table 5.4 Ability of selected putative synergists to inhibit P450s from lamb's liver.

For full list, see text.

Putative synergist	*% activity of the control*	*Standard error*
Bc-B1	1.9	0.38
Ca-B2-fr2	3.1	0.30
Za-B2-fr9	12.5	2.56
Cl-B2	14.7	3.75
Ca-B2-fr10	17.0	5.13
Linoleic acid	19.1	12.46
Oleic acid	25.5	8.59
Neem seed oil	29.0	6.56
Ca-B2-fr6	30.9	3.19
Af-B2	31.0	6.18
Bergamot	36.9	0.39
Parsley (53% myristicin)	47.0	5.59
Cs-B1	54.3	7.87
Pyrethrins II	59.8	7.98
Pyrethrins (from 50 % extract)	61.3	8.71
PBO	63.7	3.85
Eth. Cast. Oil	68.6	3.38
Pyrethrins I	77.7	9.89
Za-B3	93.0	10.98

Bc is from *Boronia citriodora;* Ca is from *Correa alba* var *alba;* Cl is from *Correa lawrenciana;* Cs is from Correa stackhousiana; Za is from *Zieria arborescens;* B1: solvent extract – petroleum spirits fraction; B2: solvent extract – dichloromethane fraction; fr denotes a further fractionated sample

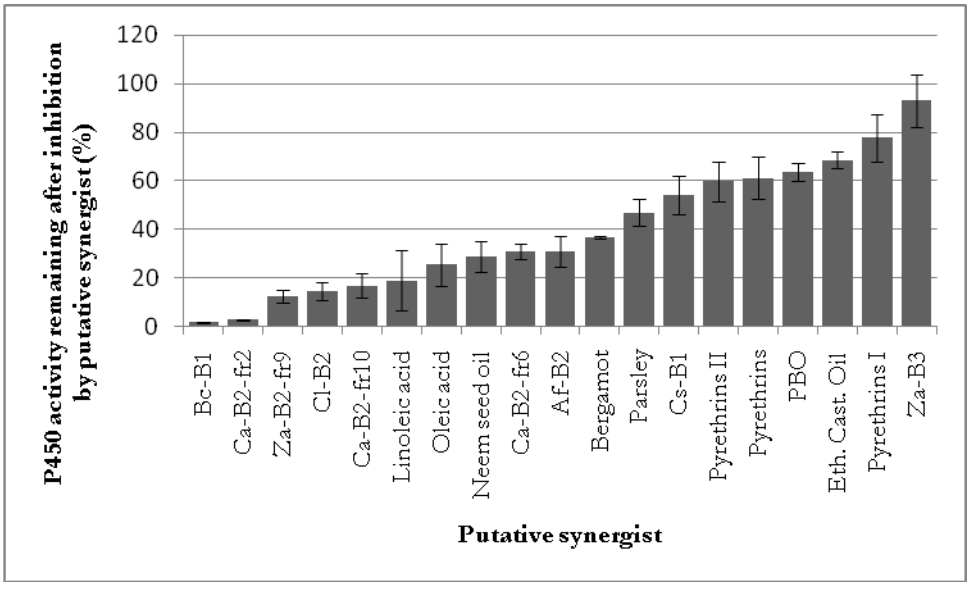


Figure 5.3 Level of inhibition of standard P450 sample (from lamb's liver) by various putative synergists, shown as a percentage activity of P450 sample only

Relates to Table 5.4
Error bars show the standard error

Bc is from *Boronia citriodora;* Ca is from *Correa alba* var *alba;* Cl is from *Correa lawrenciana;* Cs is from *Correa stackhousiana*; Za is from *Zieria arborescens;* B1: solvent extract – petroleum spirits fraction; B2: solvent extract – dichloromethane fraction; fr denotes a further fractionated sample. 'Pyrethrins' sample is from a 50% extract.

5.3.3 The effect of putative synergists against *Myzus persicae* (794jz clone) *in vivo*

As for chapter three, the raw bioassay data was inputted into PoloPlus and a summary of the outputted data is shown here. PoloPlus was used to derive the LC_{50} values in order to calculate ESRs and SFs. The rest of the data from the Polo outputs is shown in Appendix V.

5.3.3.1 Putative synergist:pyrethrins tank mix (4:1)

Following *in vitro* screening of putative synergists for their ability to inhibit esterases and P450s (sections 5.3.1 and 5.3.2), samples were screened *in vivo* against *Myzus persicae* (794jz clone) which have an esterase-based metabolic resistance to pyrethroids/pyrethrins, for their ability to increase the efficacy of pyrethrins. The samples selected for *in vivo* bioassay were those which showed some ability to inhibit esterases and P450s *in vitro* (sections 5.3.1 and 5.3.2) and taking into account Botanical Resources' request for a compound that would not require new licensing, e.g. food products that are already accepted as safe for human exposure.

The *in vivo* assays used a tank mix of putative synergist and pyrethrins (rather than a pre-treatment) because previous results (Chapter 3) showed a tank mix to be at least as effective as a pre-treatment, if not better. Also, it is possible that each compound will have a different pre-treatment time thus using a tank mix treatment oversees the need to optimise this for preliminary testing.

Samples tested in a tank mix with pyrethrins were bergamot essential oil, citronella, ethoxylated castor oil, linoleic acid, neem seed oil, oleic acid, parsley seed oil (53 % myristicin), peppermint oil, and BRA/UTas samples: Bc-B1, Cl-B1, Za-B2. Peppermint and citronella were two of the samples selected for *in vivo* trials as representatives of samples which showed some ability to inhibit esterases *in vitro*, but not in the SE limits of PBO. These *in vivo* trials were performed to demonstrate that any synergism found would not match that of PBO as neither of these compounds was as good as PBO at inhibiting esterases *in vitro*. In both cases tank mixes with pyrethrins did not cause mortality at levels greater than those seen with pyrethrins alone (data not shown) showing that the novel *in vitro* assay described in chapter four is a reliable indicator of a samples' ability to inhibit esterases *in vivo*.

Table 5.5 Lethal concentration for 50 % mortality (LC_{50}), effective synergism ratio (ESR) and synergistic factor (SF) for putative synergists against *Myzus persicae* (794jz clone) as a tank mix with pyrethrins (4:1)

See appendix V, Table A-V-I

Treatment	LC_{50} (ppm)	ESR	SF
Pyrethrins only*	1630.7	43.8	-
PBO*	121.7	3.3	13.4
Ethoxylated castor oil	342.6	9.2	4.8
Za-B2	350.5	9.4	4.7
Linoleic acid	413.0	11.1	3.9
Neem seed oil	463.0	12.4	3.5
Oleic acid	541.0	14.5	3.0
Cl-B1	611.5	16.4	2.7
Parsley seed oil (53)	802.9	21.6	2.0
Bc-B1	822.5	22.1	2.0
Bergamot	1068.2	28.7	1.5

* for reference (data from Chapter Three)

As shown in Table 5.5, none of the compounds tested reduced the LC_{50} to below that achieved with PBO meaning that PBO was still the better synergist with pyrethrins. Ethoxylated castor oil and Za-B2, were the most effective of all the samples tested *in vivo*, with LC_{50} values of 342.6 ppm and 350.5 ppm respectively. However, these both gave an ESR 3-times that for a PBO/pyrethrins tank mix, and a SF 3-times less than a PBO/pyrethrins tank mix. The higher the SF, the higher the synergism thus with the most promising samples having a SF 3-times less than that of PBO, there was still opportunity to increase the efficacy of the pyrethrins.

The finding that some of the putative synergists were not as effective *in vivo* as they were *in vitro* suggests that the *in vivo* activity is not the result of esterase inhibition alone. Thus these results agree with the hypothesis proposed in Chapter Three, that pyrethrins do not penetrate the insect cuticle as readily as many other insecticides. It seems that PBO therefore has a more marked effect than the putative synergists screened here because of its ability to enhance penetration, rather than just inhibit metabolic defences. This hypothesis assumes that the putative synergists do not have the 'penetration enhancement' ability, or that if they do, it is less effective than for PBO.

5.3.3.2 Putative synergist:alpha-cypermethrin tank mix (4:1)

Following the initial studies looking at the effect of the putative synergist with pyrethrins in a tank mix, experiments were repeated using a tank mix of synergist and alpha-cypermethrin

(Table 5.6) to compare both penetration qualities and esterase inhibition qualities of the synergists. Previous results (Chapter Three) indicated that with alpha-cypermethrin, PBO acts by inhibiting metabolic defences (seen by increased synergism with a PBO-pre-treatment compared to a tank mix treatment) rather than by enhanced penetration. It was therefore decided to test the putative synergists with alpha-cypermethrin and thus obtain information about the putative synergists ability to metabolise the esterases. The results are shown in Table 5.6.

Samples tested in a tank mix with alpha-cypermethrin were bergamot, ethoxylated castor oil, linoleic acid, neem seed oil, oleic acid, parsley seed oil (53 % myristicin), peppermint oil and the following BRA/UTas samples: Bc-B1, Cl-B1 and Za-B2. Tank mixes of alpha-cypermethrin with either Bc-B1, Za-B2, bergamot or peppermint oil did not give mortality sufficient for a LC_{50} to be calculated for the dose range used. These results were surprising because the initial *in vivo* trials with pyrethrins had suggested that Za-B2 would give greater synergism with alpha-cypermethrin at the dose range chosen. The reason that it did not perform well *in vitro* is not known.

Table 5.6 Lethal concentration for 50 % mortality (LC_{50}), effective synergism ratio (ESR) and synergistic factor (SF) for putative synergists against *Myzus persicae* (794jz clone) as a tank mix with alpha-cypermethrin (4:1)

See appendix V, Table A-V-II

Treatment	LC_{50} (ppm)	ESR	SF
Alpha-cypermethrin only*	3230.2	6525.2	-
PBO*	810.8	1638.0	4.0
Neem seed oil	177.6	358.8	18.2
Linoleic acid	247.7	500.4	13.0
Parsley seed oil (53)	282.1	569.9	11.5
Ethoxylated castor oil	282.9	571.5	11.4
Cl-B1	386.8	781.4	8.4
Oleic acid	400.2	808.5	8.1

* for reference (data from Chapter Three)

The LC_{50} for alpha-cypermethrin alone was 3230.2 ppm. With PBO in a tank mix, the LC_{50} was reduced to 810.8 ppm. As can be seen from the results displayed in Table 5.6, neem seed oil, linoleic acid, parsley seed oil (53 % myristicin), ethoxylated castor oil, Cl-B1 and oleic acid all synergised alpha-cypermethrin more effectively than PBO, with LC_{50} results ranging from 177.6 ppm to 400.2 ppm. These samples had SFs at least twice as good as

PBO (oleic acid), and up to 4.5-times better than PBO (neem seed oil). Thus these samples all showed the ability to enhance the effect of alpha-cypermethrin when used in a tank mix.

The results of the experiments using a tank mix of putative synergist and alpha-cypermethrin agree with the hypothesis stated previously that the pyrethrins benefit from the ability of PBO to enhance penetration of pyrethrins through the insect cuticle. Compounds that did not show synergism to the same extent as PBO *in vivo* with pyrethrins, show increased levels of synergism when applied as a tank mix with alpha-cypermethrin. This indicates that these compounds are inhibiting esterase activity in the insect, as predicted by the *in vitro* testing.

5.3.3.3 Putative synergist:PBO:pyrethrin tank mix (4:4:1)

Following experiments using putative synergists in tank mixes with pyrethrins and alpha-cypermethrin (independently), the next logical step was to test a mixture of PBO with a putative synergist and pyrethrins to determine if the presence of PBO would help the penetration of the pyrethrins through the cuticle, then the combined synergism from PBO and the other compound would inhibit metabolic defences. Three samples were tested in this way: neem seed oil; linoleic acid; and ethoxylated castor oil. These were chosen because they were each one of the most effective for both the synergist:pyrethrin treatments and the synergist:α-cypermethrin treatments.

Table 5.7 Lethal concentration for 50 % mortality (LC_{50}), effective synergism ratio (ESR) and synergistic factor (SF) for putative synergists against *Myzus persicae* (794jz clone) as a tank mix with PBO and pyrethrins (4:4:1)

See appendix V, Table A-V-III

Treatment	LC_{50} (ppm)	ESR	SF
Pyrethrins only*	1630.7	43.8	-
PBO*	121.7	3.3	3.3
Neem seed oil	65.4	1.8	24.9
Linoleic acid	86.0	2.3	18.9
Ethoxylated castor oil	113.9	3.1	14.3

* for reference (data from Chapter Three)

Results from the multiple combination bioassay treatments (putative synergist, plus PBO, plus pyrethrins) are shown in Table 5.7. These results reveal a dramatic increase in the level of synergism after a synergist:PBO:pyrethrins (4:4:1) treatment for all samples tested, compared to the synergist:pyrethrins (4:1) treatment (Table 5.5) for the same samples.

The LC_{50} for pyrethrins alone was 1630.7 ppm, with an ESR of 43.8 meaning that 43.8-times more insecticide was required to give 50 % mortality in the resistant clone of *M. persicae* compared to the susceptible clone (4106a) (see Chapter Three, Table 3.1a). The tank mix treatment of PBO and pyrethrins reduced the LC_{50} to 121.7, the ESR to 3.3 and gave a SF of 3.3. Where a tank mix of putative synergist was combined with PBO and pyrethrins in a 4:4:1 ratio, all three samples showed a decreased ESR compared to a PBO/pyrethrins tank mix. Neem seed oil gave the highest level of synergism, reducing the LC_{50} to 65.4 ppm which is approximately 50 % of that of a PBO:pyrethrin (4:1) treatment. This had an ESR of 1.8 and a SF of 24.9. This shows that a mixture of neem seed oil, PBO and pyrethrins gives a much higher SF than a PBO/pyrethrins tank mix and thus improves synergism and increases the efficacy of the natural pyrethrins The next most effective putative synergist was linoleic acid which gave a SF of 18.9, and then ethoxylated castor oil which gave a SF of 14.3. In summary, all three samples when combined in a mixture with PBO and pyrethrins show great potential for increasing the efficacy of natural pyrethrins. Further testing to establish financial viability of such mixtures would need to be performed in order to take this result further.

5.3.4 The effect of putative synergists against *Musca domestica* (381zb strain) *in vivo*

Following *in vitro* screening of putative synergists (sections 5.2.2 and 5.3.2), samples were tested against *Musca domestica* with a mixed metabolic resistance to pyrethroids/pyrethrins (see section 2.1.3), for their ability to increase the efficacy of pyrethrins *in vivo*. Samples were selected for *in vivo* trial based on results from sections 5.3.1 and 5.3.2. Two of the more effective P450 inhibitors from *in vitro* assays, neem seed oil and linoleic acid, were selected for screening *in vivo*.

5.3.4.1 Putative synergist:pyrethrins tank mix (5:1)

The results of bioassays using a tank mix of synergist:pyrethrins (5:1) on *M. domestica* are shown in Table 5.8. Results indicate that PBO enhances penetration of pyrethrins through the cuticle corresponding to results found for *M. persicae* (section 5.3.3) as although both neem seed oil and linoleic acid reduced the LC_{50} for pyrethrins, from 11251.9 ppm to 4635.1 ppm and 5657.7 ppm respectively, the reduction of ESR was only 6.1 and 7.5 respectively.

For both compounds the SF was at least 7.9-times less than for PBO thus showing PBO to be more effective at enhancing the efficacy of natural pyrethrins than either of the two putative synergists tested.

Table 5.8 Lethal concentration for 50 % mortality (LC_{50}), effective synergism ratio (ESR) and synergistic factor (SF) for putative synergists against female *Musca domestica* (381zb strain) as a tank mix with pyrethrins (5:1)

See appendix V, Table A-V-IV

Treatment	LC_{50} (ppm/20mg fly)	ESR	SF
Pyrethrins only*	11251.9	14.9	-
PBO*	589.3	0.78	19.1
Neem seed oil	4635.1	6.1	2.4
Linoleic acid	5657.7	7.5	2.0

* for reference (data from Chapter Three)

5.3.4.2 Putative synergist:PBO:pyrethrins tank mix (5:5:1)

The results for these experiments can be seen in Table 5.9. With the addition of PBO to the mixture (synergist:PBO:pyrethrins, 5:5:1) the LC_{50} for each putative synergist was approximately 10-fold less than it was for the synergist:pyrethrin mixture with a LC_{50} of 425.6 ppm for neem seed oil, and 526.5 ppm for linoleic acid. Both of these LC_{50} values were lower than that for PBO:pyrethrin tank mix. However, neither of these mixtures gave an LC_{50} that was significantly different from PBO:pyrethrins alone. Although these results suggest that PBO aids the penetration of neem seed oil, linoleic acid and the pyrethrins, the efficacy of the pyrethrins, compared to a PBO:pyrethrins tank mix, is not increased.

Table 5.9 Lethal concentration for 50 % mortality (LC_{50}), effective synergism ratio (ESR) and synergistic factor (SF) for putative synergists against female *Musca domestica* (381zb strain) as a tank mix with PBO and pyrethrins (5:5:1)

See appendix V, Table A-V-V

Treatment	LC_{50} (ppm/20mg fly)	ESR	SF
Pyrethrins only*	11251.9	14.9	-
PBO*	589.3	0.78	19.1
Neem seed oil	425.6	0.56	26.4
Linoleic acid	526.5	0.70	21.4

* for reference (data from Chapter Three)

5.4 General Discussion for Chapter Five

Methylenedioxyphenyl (MDP) compounds are widespread among plant species (Newman, 1962); and their presence coincides with many classes of secondary compounds that are known to have insecticidal properties and also reported to have the ability to synergise synthetic organic insecticides (Casida, 1970, Lichtenstein *et al.*, 1974, Fuhremann & Lichtenstein, 1979). Berenbaum and Neal (1985) proposed that when insects ingest some plants, MDPs act as natural synergists which interfere with P450 function after ingestion thus leaving the insect more susceptible to the toxic activity of other secondary substances in the plant. Krieger *et al.* (1971) postulated that this mechanism was present in *Chrysanthemum cinerariaefolium* (Compositae) where sesamin (an MDP synergist) co-exists with the insecticidal pyrethrins in the flowerheads. Janzen (1973) proposed that the presence of these synergists may occur to allow plants to reduce the quantity of secondary substances produced without reducing the overall toxicity.

PBO contains an MDP ring (Figure 5.1) which is reported to be responsible for P450 inhibition although it is not thought to play a part in esterase inhibition. It has been shown that the two oxygens on the MDP ring are required for P450 inhibition and the removal of one oxygen (PBO analogue 16-5) affects the ability of the compound to inhibit P450s but not esterases (Moores *et al.*, 2009). If the MDP ring did inhibit esterase activity it would be expected that myristicin (in nutmeg and parsley) and bergamot would be potent esterase inhibitors, although in this study they were less effective than PBO.

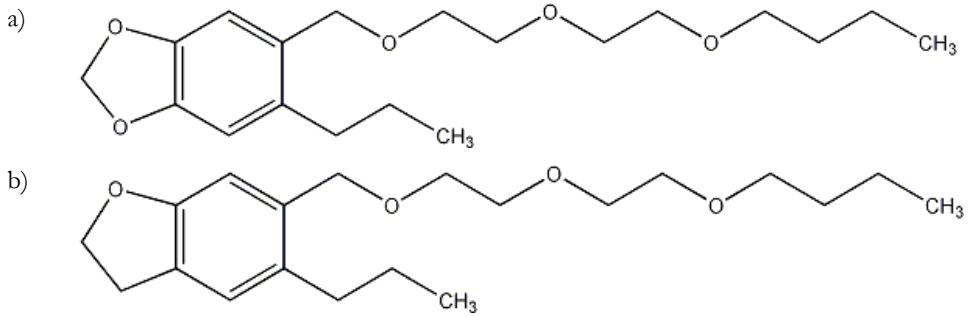


Figure 5.4 Structure of a) PBO; b) PBO analogue 16-5

PBO is a known esterase and P450 inhibitor. This study has shown that PBO inhibits esterases leaving approximately 4 % esterase (E4) activity (ie inhibition of 96 %), but only inhibits P450 activity by approximately 35 % at that same concentration. Other effective esterase inhibitors are oleic acid, ethoxylated castor oil and neem. Oleic acid, ethoxylated

castor oil and PBO all have a long carbon chain that contains oxygens (C-O) or unsaturated oxygens (C=O). Oleic acid and ethoxylated castor oil both have a long carbon chain with an unsaturated portion (C=C). Since neither oleic acid, nor ethoxylated castor oil have an MDP ring it could therefore be postulated that the ethoxy-chain on PBO (and the other structures) is responsible for esterase inhibition.

The bioassay results for *M. persicae* (794jz clone), which has an esterase-based resistance, show that a treatment of PBO plus neem or linoleic acid and pyrethrins, show neem and linoleic acid to be more potent esterase inhibitors *in vivo* than PBO, but without PBO they cannot penetrate with the pyrethrins to exert their potent effect. However, *M. domestica* (381zb strain) are thought to have a more GST- and P450-based resistance profile and from the bioassay results it appears that both neem seed oil and linoleic acid are better P450 inhibitors *in vitro* than *in vivo*. The *in vivo* result is unexpected given the success of the compounds *in vitro* as P450 inhibitors. This may be because the pre-treatment time needs optimising for better metabolic defence inhibition *in vivo*, and investigations into the resistance mechanism would also be beneficial.

Essential oil samples from BRA were originally chosen for synergist screening based on their chemical structures because many contained an MDP ring or a similar group (e.g. bergamot, anise, sage, tea tree, geranium, mint, thyme and rosemary) predicted to give esterase inhibition. Angelica and bergamot were not predicted to be potent P450 inhibitors, as their structures, a single oxygen in the MDP ring, are similar to the PBO analogue which Moores *et al.* (2009) showed to have only esterase activity remaining (no P450 activity).

Most of the samples from BRA/University of Tasmania, including Cl-B1 which performed well in the alpha-cypermethrin bioassay, were extracts produced from single plants and further experiments would be needed to establish if other plants of the same species would give the same results. However, it is encouraging that Cl-B1 has shown synergistic potential as it is an extract from a common Tasmanian garden plant.

Parsley seed oil (53 % myristicin) was selected for trial *in vivo* because BRA were hoping to find a food-based synergist and it had performed well *in vitro* in the esterase inhibition assay and reasonably well in the P450 assay. *In vivo*, parsley showed potential as a synergist for pyrethrins and α-cypermethrin. Apart from being comprised of 53 % myristicin, the other components of the oil are unknown. The sample containing 86 % myristicin did not

perform as well in the esterase inhibition assay as the 53 % sample, perhaps suggesting that the myristicin is not the only component of the oil responsible for esterase inhibition. However, neither parsley sample was as potent as the pure myristicin sample suggesting that pure myristicin is best but in the two parley oil samples there are other compounds present that may be important e.g. apiole. It would be interesting to identify each of the components in the two parsley seed oils, and to evaluate them and myristicin *in vivo* against an insect with a resistance mechanism known to be due to the presence of P450s. Berenbaum and Neal (1985) investigated the synergism between myristicin and xanthotoxin (a furanocoumarin) and suggested that the action of myristicin is via MDP competitive inhibition of P450s. They found myristicin to increase the rate and extent of *Heliothis zea* (Boddie) (Lepidoptera:Noctuidae) mortality at a given xanthotoxin concentration.

Neem seed oil performed well *in vitro* as both an esterase and P450 inhibitor, and also performed well *in vivo* as a synergist with pyrethrins against *Myzus persicae*. This concurs with the findings by Lowery *et al.* (1993) who studied the use of neem for aphid control. They tested pyrethrum because at the time of their study it was the current botanical insecticide of choice by producers of organic crops. The authors used formulated neem seed (expeller) oil (NSO) and ethanolic neem seed extracts (NSE), both with known quantities of azadirachtin and the authors' general conclusions were that the neem-based products were effective against several species of aphids both in the laboratory and in the field, but mixtures of NSO or NSE and pyrethrum did not increase the efficacy of neem. Considering their data from another perspective (i.e. whether neem increases the efficacy of pyrethrum), it can be seen that there was a significant ($P < 0.05$) reduction in the number of strawberry aphids (*Fimbriaphis fimbriata* (Richards) (Homoptera: Aphididae) and *Chaetosiphon fragaefolii* (Cockerell) (Homoptera: Aphididae) present on plants in one of two trials when a NSO and pyrethrum mixture was applied, compared with a pyrethrum treatment alone. The authors hypothesise that the difference in the results between the first and second trials was due to plant size and application rate of the treatment, as well as whether both the top and bottom of the leaf surfaces were covered. Experiments investigating the numbers of *M. persicae* and *A. gossypii* showed a significant ($P < 0.05$) reduction in aphid numbers when a mixture of NSE and pyrethrum was used, compared with pyrethrum alone. Thus the findings of Lowery *et al.,* (1993) although not the focus of their study, confirm those of the present study: that neem has synergistic properties when used in conjunction with pyrethrum.

It is known that neem oil is rich in pre-formed secondary metabolites, e.g. azadirachtin, phenolics and glycosides etc (Singh *et al.*, 2005). These occur constitutively in entire parts of the neem plant and it is thought that they act within the plant as in-built chemical defences to pathogen infection and may protect plants against various insect pests. Properties attributed to neem include insect anti-feedant properties (Govindachari *et al.*, 2000); antibacterial and anti-inflammatory properties (Kraus, 1995). Phenolics have been found to have antifungal properties (Ravn *et al.*, 1989, Osbourn, 1996, Sarma & Singh, 2003), and antifeedant and antibacterial properties (Ravn *et al.*, 1989).

Azadirachtin has an ester bond on the periphery of the molecule which is clearly available for attack by esterases. It is surprising that it was not more potent as an esterase inhibitor *in vitro*. Neem seed oil performed well as an esterase inhibitor both *in vitro* and *in vivo*. As a seed oil it has many components, but there is insufficient data available to conclude which of the components of neem are the active esterase inhibitors.

Singh *et al.* (2005) performed High Performance Liquid Chromotography (HPLC) on different parts of the neem plant and found the seeds to be rich in phenolic acids. Both "raw" and "ripe" seeds were studied and the same four phenolic acids were detected in each, although the level of the acids was greater in "raw" fruit seeds compared with ripe seeds. In order of abundance, the 4 acids identified were Gallic, Chlorogenic, Tannic and Ferulic (Figure 5.5).

Tannic acid is a water soluble polyphenol that is present throughout neem plant and is reported to have anticarcinogenic, antimutagenic and antioxidant properties (Chung *et al.*, 1998). Gallic acid has been reported to have similar properties as tannic acid (Shahrzad & Bitsch, 1998) and ferulic acid is thought to have antifungal (Sarma & Singh, 2003) and antioxidant properties (Graf, 1992). From the results in this study it would be expected that gallic, chlorogenic, tannic and ferulic acids might explain the higher synergism seen when using neem seed oil compared to azadirachtin. In the current experiments, azadirachtin was not tested *in vivo*, however it would be interesting to assess the difference in activity between the purified azadirachtin and the crude neem seed oil *in vivo*. Unfortunately it is not known whether the neem seed oil used in this study was produced from "raw" or "ripe" seeds. HPLC assay of the neem seed oil would determine the relative abundance of the 4 phenolic acids and may provide information as to which are acting as the synergist with pyrethrins.

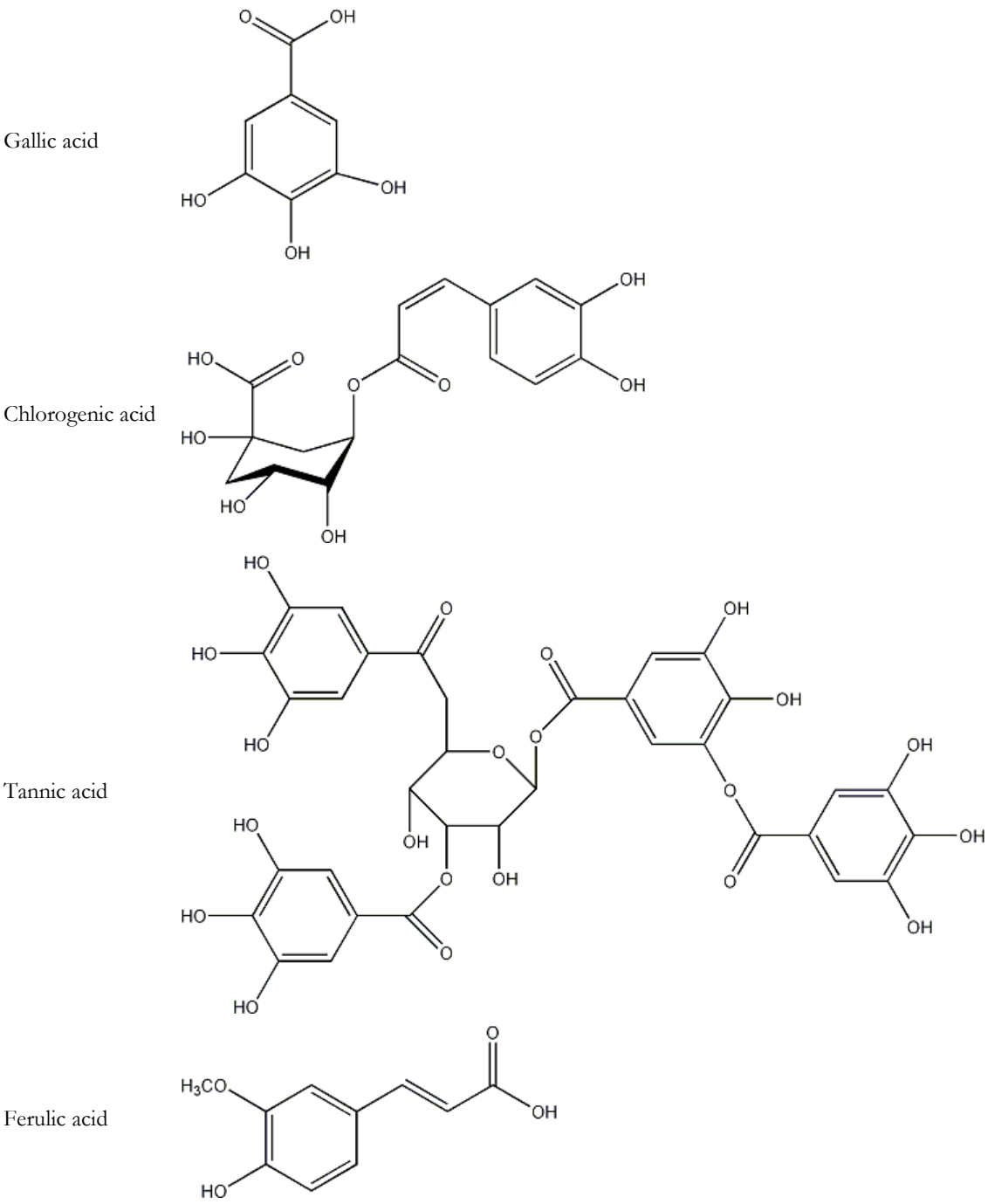


Figure 5.5 The structure of the four phenolic acids (gallic acid; chlorogenic acid; tannic acid and ferulic acid) identified by HPLC by Singh *et al.* (2005) present in "raw" and "ripe" neem seed oil.

Both linoleic acid and oleic acid performed well *in vitro* and *in vivo* in these studies. Although little is known about these compounds, there is a US Patent (5047424) (Puritch & Salloum, 1991) which includes the use of oleic and linoleic acids as part of "an environmentally safe, broad spectrum insecticide". The concentrated formulation proposed comprises a pyrethroid component (0.2-2 % by weight) plus a mixture of monocarboxylic acids (50 % by weight) and the alkali metal salts of the acids. The acids include oleic acid as a major

component and linoleic acid as a minor component (at least 70 % and 6 % by weight of the monocarboxylic acids, respectively). A more recent patent (WO/2007/065026) (Hailu & Anderson, 2007) is for an invention whereby "certain solvent blends of mixtures of fatty acids alkyl esters advantageously possess synergistic solvency and provide improved solubility and uniformity to a pesticide composition". The invention describes a pesticide composition containing a preferred 25-55 % by weight of a blend of C12-C18 alkyl esters corresponding to a formula $R^1CO\text{-}OR^2$, where R^1CO is an aliphatic acyl group (containing 12-18 carbon atoms) and R^2 is a linear or branched alkyl group containing 1-4 carbon atoms, and a biologically active ingredient (the insecticide). The examples given for solvent blends include mixtures of alkyl esters of fatty acids such as lauric, myristic, palmitic, palmitoleic, oleic, stearic, linoleic and linolenic acids, with mixtures of methyl esters of fatty acids containing 12-18 carbon atoms being preferred. Both of these patents are consistent with this study showing that oleic acid and linoleic acid have potential for synergism.

The work discussed in this chapter has highlighted that some plant extracts show promise for use as a synergist with natural pyrethrins. Further experiments are needed to extend these findings. It would be interesting to look at the effect of putative synergists, especially neem seed oil and linoleic acid, on house fly strains that have a different resistance mechanism in addition to testing them on a wider range of insect species.

6 CHAPTER SIX: SUMMARY, DISCUSSION, LIMITATIONS AND RECOMMENDATIONS

6.1 Enhancing the efficacy of pyrethrins using Piperonyl Butoxide (PBO)

- Overall, PBO was found to increase the efficacy of natural pyrethrins against the resistant strains/clones of all insect species studied. It was also found to increase efficacy against the susceptible strains/clones of some species and is it is thought this is because it is blocking the background esterases and/or P450s.
    - A tank mix of PBO and pyrethrins, or a pre-treatment of PBO prior to treatment with pyrethrins, significantly reduced the LC_{50} in resistant *M. persicae*, *M. domestica* and *B. tabaci* compared to a pyrethrins only treatment.
    - The optimum pre-treatment time for *M. persicae* was indicated to be 5 hours.
    - The increased efficacy of natural pyrethrins due to a tank mix of PBO and pyrethrins was generally superior to a pre-treatment.
- The same paralysis effect on the insect was seen for both the pyrethrins and the PBO/pyrethrins mixes.
- Many of the results obtained in this study have indicated that there are different levels of synergism occurring in the different insect species. This may be due to qualitative or quantitative differences in detoxification mechanisms (esterases/P450s).
- Microencapsulations of pyrethrins plus PBO showed potential for increasing the efficacy of pyrethrins. Although the use of microencapsulated formulations requires much more investigation this study did show that there is some potential for increasing the efficacy of pyrethrins, especially if the solubility issues could be overcome. The principle of a modified release formulation, with the addition of compounds capable of stabilising pyrethrins in UV light, would greatly enhance the potential of pyrethrins being used in agriculture.
- Results from these studies have shown that the efficacy of natural pyrethrins can be increased significantly using PBO as a tank mix, but unlike results with synthetic pyrethroids, a pre-treatment of PBO does not improve results further. It appears that for natural pyrethrins PBO could be enhancing the penetration of the pyrethrins through the insect cuticle. Experiments on *M. persicae* (with an esterase-based resistance

mechanism) indicated that the effect of enhanced penetration through the cuticle is greater than the effect of inhibiting the esterases totally, and that the enhanced penetration effect declines rapidly. The rise in mortality (observed over time) with a PBO pre-treatment could be due to the enhanced inhibitory binding effect that PBO has on the esterases.

- Even without a synergist, if *kdr* or super-*kdr* is present, pyrethrins may be more potent than synthetic pyrethroids. Experiments testing the effect of PBO and pyrethrins compared with PBO and a synthetic pyrethroid have supported previous reports that *kdr* does not confer resistance against pyrethrins in the same way, or to the same level, as it does against synthetic pyrethroids. This is shown by a lower LC_{50} for natural pyrethrins than for synthetic pyrethroids on insects with *kdr*. If *kdr* is present in a pest population, pyrethrins could be a good choice for insect control accompanied by PBO if metabolic resistance mechanisms are also present.

6.2 Development and use of a new biochemical assay

- Standard spectrophotometric assays do not always reveal the inhibition of esterases by PBO *in vitro* and the success of the assay to identify the inhibition of esterases *in vitro* by PBO was hypothesised to be dependent upon the spatial distance between the binding sites on the esterase for the PBO and the artificial substrates used in the assays.
- A new assay was successfully developed to test the hypothesis above, and esterase inhibition was detected indirectly by measuring AChE activity in an 'esterase interference assay'.
- The 'esterase interference assay' was used to screen putative synergists *in vitro* for their ability to inhibit esterases. The new assay is a high throughput method of screening compounds for their ability to blockade esterase sequestration. It enables large numbers of compounds to be tested quickly and easily, saving time and effort on *in vivo* testing as only compounds showing potential *in vitro* need be tested *in vivo*.

6.3 Screening putative synergists for use with pyrethrins

- Following bioassays using an emulsifiable concentrate of pyrethrins, oleic acid and ethoxylated castor oil, the two main components of the EC formulations, appeared to enhance the effect of pyrethrins *in vivo* and were found to inhibit esterases and P450s *in vitro*;

- Using the 'esterase interference assay' the following compounds were found to inhibit esterases, *in vitro*, to the same or greater extent than PBO: neem seed oil, oleic acid, linoleic acid, ethoxylated castor oil and extracts of *Correa lawrenciana*, *Correa stackhousiana*, *Correa alba* var *alba*, *Zieria arborescens*, *Boronia citriodora, Boronia pilosa*, *Acradenia frankliniae*.
- Ideally each compound should be tested on purified esterases from the target insect as although the use of E4 gives a good indication about how well the synergist may perform, it was found in Chapter four that esterases can vary and the spatial positions of the binding sites for the synergist and insecticide are important. With some optimisation the esterase interference assay should work with esterases from other insects.
- The following compounds were found to inhibit P450s, *in vitro*, to the same or greater extent than PBO: neem seed oil, oleic acid, linoleic acid, bergamot, parsley and some extracts of *Correa lawrenciana*, *Correa stackhousiana*, *Correa alba* var *alba*, *Zieria arborescens*, *Boronia citriodora, Boronia pilosa*, *Acradenia frankliniae*.
- A limitation of the P450 assay was the use of lamb's liver. During this study P450s were extracted from *M. domestica* (not described) and although some activity was found, it was insufficient to see the differences between in the presence and absence of the synergists. Also, insect P450s do not seem to survive well at -20°C and it is not realistic to try to extract P450s and screen putative synergists all in one day. Although the use of mammalian P450s and the choice of substrate give a more generalised assay, not involving P450s specific to resistance, it was decided that for the purpose of screening putative synergists the use of lamb's liver was acceptable since PBO and other MDP compounds are known to be general P450 inhibitors, effecting insect, vertebrate and plant isoforms by forming a metabolic intermediate with the haem moiety (Scott *et al.*, 2000). Ideally the P450s should have been extracted and used from the insect being studied as variations do occur and future experiments could look at improving the insect P450 extraction process and storage.
- Extracts were chosen on the basis of their structure, or those containing a bioactive compound and many contained an MDP ring. Although the MDP ring is not thought to be important in esterase interactions, those which did prove to be effective against esterases were certain to be P450 inhibitors because of the presence of this moiety.
- As a tank mix with pyrethrins, none of the putative synergists tested *in vivo* performed as well as PBO in a tank mix treatment on resistant *M. persicae*. However, as a tank mix with alpha-cypermethrin, most compounds tested gave better synergism than PBO in treatment on *M. persicae*.

- As a tank mix with PBO and pyrethrins, neem seed oil, linoleic acid and oleic acid all showed the same or better synergism than a PBO and pyrethrins tank mix when used on resistant *M. persicae*.
- As a tank mix with pyrethrins in treatments on resistant *M. domestica*, the efficacy of pyrethrins was improved but not to the same extent as a tank mix of PBO and pyrethrins when using linoleic acid or neem seed oil as the synergist. However, when incorporated into a tank mix with PBO and pyrethrins on *M. domestica*, the putative synergists enhanced the efficacy of pyrethrins to the same extent as a tank mix containing only PBO and pyrethrins.
- In the search for an alternative synergist to use with pyrethrins, various compounds showed potential for inhibiting esterase and P450s *in vitro*. Like PBO, oleic acid, linoleic acid and ethoxylated castor oil all have long carbon chains and it is postulated that their success as synergists results from the presence of the ethoxy-chain, which is perhaps responsible for esterase inhibition. Further investigations would enable a clearer picture of the potential of the more successful synergists *in vivo:* linoleic acid, oleic acid, ethoxylated castor oil and neem seed oil, as synergists for use with pyrethrins or as mixtures with PBO and pyrethrins. The cost of using these compounds would also require consideration.
- Many of the plant extracts produced by Botanical Resources and the University of Tasmania showed, from these preliminary studies, that they have the ability to increase the efficacy of natural pyrethrins. It would be very interesting to extend these studies to see if combinations of putative synergists either with each other, or with PBO, could further enhance pyrethrin activity.
- In addition to the theory proposed in chapter five that the putative synergists may be enhancing the penetration of pyrethrins through the insect cuticle, future work should include bioassays to assess the effects of the synergists alone in bioassays. Some are already known to have insecticidal properties, for example neem (Lowery *et al.*, 1993, Govindachari *et al.*, 2000, Mordue (Luntz) & Nisbet, 2000)and thus may in fact be acting by a joint effort with pyrethrins by means of insecticidal action rather than inhibition of metabolic defences.
- Future work would include purifying the putative synergists which look to have potential and identifying the active components. This would enable a study to be made using molarities rather than percentages (which were used in this study) as the use of percentages for calculating the concentration of insecticide is not ideal. For this study the bioassays had to be performed using percentages as a majority of the compounds to

be tested were plant extracts/essential oils and therefore not pure. If the molecular weight of the compound being tested was known then molarities could be used to ensure that for each synergist being compared, the same number of molecules was being used and would therefore make the study a more fair comparison. Although some compounds were pure and therefore could be calculated in molarities, in order to be consistent throught the study, all compounds were tested by percentage.

6.4 General Limitations

- Although it was decided the most practical approach for the experiments in this project, there are problems associated with keeping a constant ratio of of synergist:insecticide. Despite these problems, it was decided to keep a constant ratio as not only was it more practical but previous studies had shown different ratios to have very different effects on mortality of bioassayed insects (Bingham *et al.*, 2007). The inhibition of metabolic systems may be unequal across the range of doses used in the experiments and the effect of these may become critical if comparisons are made between strains/clones of insects with significantly different dose responses (e.g. resistant and susceptible). As was the case in these experiments, when the susceptible strain/clone was treated, due to the lower level of insecticide required, it would receive a far lower amount of synergist compared to the resistant strain/clone due to the fixed ratio. In the resistant strain/clone the synergist was applied at comparatively high levels compared to the susceptible, and may have been toxic itself at the levels tested. Ideally each putative synergist should have been tested alone in a dose response bioassay to ensure that the effects of the synergist were not overestimated as the synergist dose should be high enough to cause maximal inhibition of the metabolic systems without causing mortality on its own. It is arguable as to whether synergists should be applied at an equal dose to the susceptible and resistant insects since this would change the ratio of synergist:insecticide and as mentioned previously, this has been shown to be important.
- The bioassays used in this study have limitations in how well they represent field use. In the field a plant would be sprayed form above and the inderside of the leaf would not be exposed to much of the treatment. Translaminar flow of insecticide may increase the amount of insecticide that reaches the insects on the undersides of the leaves where insects naturally prefer to live and feed. Given that the insects in this study were either placed onto leaf dics where the both surfaces had been treated with insectide/synergist,

or the treatment was applied directly onto the insects, the result in the field would be different as the insects would receive less treatment than they were exposed to in the bioassays.

6.5 Recommendations

- To increase the efficacy of natural pyrethrins, a tank mix with PBO is recommended over a pre-treatment as efficacy using a tank mix is generally superior, and less labour intensive;
- Improved solubility of pyrethrin microencapsulations with UV stabilisers may enable pyrethrins to be used in agriculture (outdoors) and thus should be investigated, although efficacy testing for comparison to a tank mix would need to be performed.
- The 'esterase interference assay' is a novel and efficient method of screening putative synergists for their ability to inhibit esterases *in vitro* and the P450 assay is an efficient method of screening putative synergists for their ability to inhibit P450s *in vitro.* Used in sequence, these two assays enable a fast and reliable method of screening putative synergists before more time and labour intensive *in vivo* testing.
- Linoleic acid, ethoxylated castor oil and neem seed oil all performed well *in vivo* with pyrethrins and PBO, and thus investigations into the potential of these on different insect species should be continued on insects with different resistance mechanisms and different levels of resistance.
- More detailed investigations using the Tasmanian plant extracts as synergists, in combinations with each other and/or with PBO, would be interesting and may give rise to a novel organic synergist for use with pyrethrins.
- Further experimental work to identify the binding sites on the metabolic enzymes (esterases/P450s) of each of the putative synergists would enable a greater understanding of the synergists' mode of action and thus how to maximise their potential for use in insect control.
- It would be beneficial to identify the 3-dimensional structure of esterases, particularly E4 which is fairly easy to purify, in order to establish the precise characteristics an ideal esterase inhibitor would possess.
- Future work could include identifying the stage of the target insects' life cycle at which it would be most effective to apply the synergist/pyrethrin mixture.

- Overall, this PhD project has found that various compounds have shown potential to increase the efficacy of natural pyrethrins. Further studies may offer improvements in crop protection and public-health by the use of natural pyrethrins in combination with a novel botanical compound as a synergist which may appeal to the organic farming industry.

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Appendix I – Buffers and substrates

0.02M pH7.0 phosphate buffer

3.58g di-sodium hydrogen orthophosphate dodecahydrate

1.36g potassium dihydrogen orthophosphate

made up to one litre with distilled water.

Phosphate buffer plus Triton

0.1% Triton X-100 dissolved in 0.02M pH 7.0 phosphate buffer.

Fast blue RR (FBRR)

0.015g FBRR made up to 25ml with 0.02M pH 7.0 phosphate buffer, then filtered.

1-naphthyl-acetate

0.5ml 30mM 1-naphthyl acetate in 15ml FBRR to give a final concentration of 1mM.

ATChI

0.022g ATChI in 50ml 0.02M pH 7.0 phosphate buffer to give a final concentration of 1.5mM.

DTNB

0.015g DTNB in 25ml 0.02M pH7.0 phosphate buffer to give a final concentration of 1.5mM. The DTNB is then diluted 20-fold to give a final concentration of 75μM.

0.1 M sodium phosphate buffer (pH 7.6) containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF, 1.46 M sucrose

6.230 g di-sodium hydrogen orthophosphate dodecahydrate, 0.406 g sodium di-hydrogen orthophosphate, 0.074 g EDTA, 0.030 g DTT, 0.030 g PThU, 0.034 g PMSF and 100 g sucrose were dissolved in distilled water to make a final volume of 200 ml. The PThU and PMSF were dissolved first in 1ml pure ethanol.

0.1 M sodium phosphate buffer (pH 7.8)

3.277 g di-sodium hydrogen orthophosphate dodecahydrate and 0.133 g sodium di-hydrogen orthophosphate were dissolved in a total of 100 ml distilled water.

Appendix II – Processing novel esterase assay data

The IC50 value found using Grafit was converted into a percentage and given a new terminology: I (for "Index value")

The IC50 for 'E4' was taken as 100 % activity, and for 'no E4' taken as 0% activity.

The IC50 of the putative synergist sample (now termed I) was converted into a percentage by the following formula:

$$I = \frac{I(\text{synergist}) - I(\text{no E4})}{I(\text{E4}) - I(\text{no E4})} \times 100 = \frac{y - x_1}{x_2 - x_1} \times 100$$

Variance of the index (I) was approximated using the following expression:

$$\text{var}(I) = \frac{100^2}{(x_2 - x_1)^2} [\text{var}(y) + \text{var}(x_1)]$$

It is important to note that this calculation often gives an underestimated value for the variance because the denominator is taken as a fixed quantity in the formula.

Here, $\text{var}(y) = (\text{se}(y))^2$ where se(y) is the standard error for the IC50 (for the synergist) as given by the fit provided using Grafit; and similarly for x_1 ('no E4').

Following this,

$$\text{se}(I) = \sqrt{[\text{var}(I)]}$$

$$= \sqrt{\frac{100^2}{(x_2 - x_1)^2} . [\text{var}(y) + \text{var}(x_1)]}$$

The 95% Confidence Interval, denoted CI (95%), is given by

$$I \pm t_{0.05, 21} \times \text{se}(I)$$

where $t_{0.05, 21}$ is the t-value at the $p = 0.05$ level of significance on 21 degrees of freedom (df). These are 21 because there are 33 data points (11 data points for each curve and 3 curves (no E4, E4, E4+synergist), less 12 parameters for fitting the 3 logistic curves.

Appendix III – Processing cytochrome P450 assay data

Given the means and standard errors for all the compounds, the ratio of putative synergists to the control were calculated. The formula for the variance of this ratio is:

$$\mathrm{var}(a/b) = (a^2/b^2)[(\mathrm{var}(a)/a^2)+(\mathrm{var}(b)/b^2)]$$

where a is the mean and var(a) is the square of the standard error of the mean for a putative synergist, and where b is the mean and var(b) is the square of the standard error of the mean for the control.

Appendix IV – Bioassay data for Chapter Three

Data derived from PoloPlus. TM: Tank mix; PT: pre-treatment; Nc: not calculable; R: resistant; S: susceptible; n: number of insects tested; LC_{50} : insecticide concentration required for 50 % mortality in studied population; CL: confidence limits; ppm: parts per million; SE: standard error; χ^2: chi squared value; df: degrees of freedom; RF: resistance factor; ESR: effective synergism ratio.

The number of insects tested is shown in the column marked 'n' and this refers to the number of insects tested in the doses for which results were inputted into PoloPlus. The column labelled 'controls' indicates the number of insects treated with a control solution only (e.g. water or acetone as appropriate). The LC_{50} and LC_{90} are both shown with their corresponding confidence limits (95%). The slope indicates the heterogeneity of the population. The Chi square value indicates the goodness of fit i.e. how well the data fits the underlying probit model.

Table A-IV-I The effect of a tank mix and pre-treatment of PBO on the efficacy of natural pyrethrins against: a)*Myzus persicae* (topical application technique); b)*Musca domestica* (topical application technique); c) *Myzus persicae* (leaf-dip technique); d) *Bemisia tabaci* (leaf-dip technique).

a)*Myzus persicae* (topical application technique)

Treatment	Strain/ Clone	S/R	Sex	n	controls	LC_{50} (ppm)	(95% CL)	LC_{90} (ppm)	(95% CL)	Slope	(+/-SE)	χ^2	df	RF	ESR
Pyrethrins only (topical application)	4106a	S	-	208	60	**37.2**	(23.0-55.2)	150.8	(93.0-396.6)	2.108	0.317	32.5	19	**1.0**	**1.0**
	794jz	R	-	480	90	**1630.7**	(1253.9-2061.1)	6086.7	(4407.7-10092.4)	2.240	0.229	80.8	46	**43.8**	**43.8**
PBO/pyrethrins TM (4:1) (topical application)	4106a	S	-	298	60	**6.8**	(3.6-10.8)	27.2	(16.5-69.7)	2.127	0.264	79.5	28	**1**	**0.2**
	794jz	R	-	330	70	**121.7**	(94.3-156.7)	419.1	(300.4-693.8)	2.387	0.220	57.7	31	**17.9**	**3.3**
PBO PT / pyrethrins (4:1) (topical application)	4106a	S	-	150	30	**46.3**	(35.602-58.332)	86.2	(66.682-146.989)	4.753	1.084	11.6	13	**1.0**	**1.2**
	794jz	R	-	400	90	**192.7**	(129.4-271.3)	750.3	(496.6-1484.5)	2.171	0.188	132.4	38	**4.2**	**5.2**

b) *Musca domestica* (topical application technique)

Treatment	Strain/ Clone	S/R	Sex	n	controls	LC_{50}	(95% CL) (ppm)	LC_{90}	(95% CL) (ppm)	Slope	(+/-SE)	χ^2	df	RF	ESR
Pyrethrins only (topical application)	WHOij2	S	M	361	40	**659.4**	(524.4-834.8)	1851.9	(1372.2-2902.4)	2.86	(0.28)	23.4	16	**1.0**	**1.0**
			F	340	40	**755.7**	(659.4-868.3)	1399.3	(1173.6-1815.4)	4.79	(0.61)	4.6	15	**1.0**	**1.0**
	381zb	R	M	200	40	**8513.8**	(7104.2-9977.6)	17158.5	(14142.4-23121.6)	4.21	(0.62)	6.2	8	**12.9**	**12.9**
			F	200	40	**11251.9**	(9838.2-12685.5)	16342.7	(14189.4-21740.8)	7.91	(1.69)	3.4	8	**14.9**	**14.9**
PBO/pyrethrins TM (5:1) (topical application)	WHOij2	S	M	360	40	**46.2**	(41.2-51.0)	76.2	(67.8-90.4)	5.90	(0.78)	9.3	16	**1.0**	**0.07**
			F	340	40	**48.6**	(44.3-53.2)	73.4	(65.3-87.5)	7.17	(0.86)	16.5	15	**1.0**	**0.06**
	381zb	R	M	240	40	**365.3**	(319.2-418.3)	663.9	(558.4-865.1)	4.94	(0.67)	3.3	10	**7.9**	**0.55**
			F	242	40	**589.3**	(509.9-695.1)	1077.7	(846.9-2176.5)	6.30	(1.66)	15.5	10	**12.1**	**0.78**
PBO PT / pyrethrins (5:1) (topical application)	WHOij2	S	M	278	40	**81.3**	(73.7-88.8)	115.2	(103.5-136.6)	8.47	(1.15)	13.2	12	**1.0**	**0.12**
			F	360	40	**71.2**	(61.8-78.7)	96.2	(86.0-121.3)	9.81	(1.70)	25.2	16	**1.0**	**0.09**
	381zb	R	M	360	40	**404.5**	(358.0-453.6)	552.6	(489.0-665.9)	9.46	(1.55)	8.2	16	**5.0**	**0.62**
			F	360	40	**570.7**	(442.9-756.8)	1220.2	(879.4-2724.5)	3.884	(0.80)	19.2	16	**8.0**	**0.76**

c) *Myzus persicae* (leaf-dip technique)

Treatment	Strain/ Clone	S/R	Sex	n	controls	LC_{50}	(95% CL) (ppm)	LC_{90}	(95% CL) (ppm)	Slope	(+/-SE)	χ^2	df	RF	ESR
Pyrethrins EC (leaf dip)	4106a	S	-	150	30	**22.7**	(10.7-32.5)	74.2	(51.9-157.5)	2.495	0.637	9.4	13	**1.0**	**1.0**
	794jz	R	-	150	30	**365.6**	(259.5-556.9)	1933.4	(1096.7-5194.7)	1.772	0.276	10.5	13	**16.1**	**16.1**
PBO EC/pyrethrins EC TM (4:1) (leaf dip)	794jz	R	-	150	30	**64.1**	(44.7-92.5)	211.1	(136.6-456.0)	2.474	0.349	16.9	13	**Nc**	**2.8**
PBO EC PT / pyrethrins EC (4:1) (leaf dip)	794jz	R	F	150	30	**46.7**	(35.1-59.8)	107.5	(80.5-179.0)	3.538	0.663	7.4	13	**Nc**	**2.1**

d) *Bemisia tabaci* (leaf-dip technique)

Treatment	Strain/ Clone	S/R	Sex	n	controls	LC_{50} (ppm)	(95% CL)	LC_{90} (ppm)	(95% CL)	Slope	(+/-SE)	χ^2	df	RF	ESR
Pyrethrins EC (leaf dip)	Sud-S	S	F	482	73	**10.1**	(7.0-13.4)	28.1	(20.3-47.8)	2.868	0.273	87.7	28	**1.0**	**1.0**
	Mex2-GRB	R	F	493	81	**629.5**	(326.5-1581.3)	4780.4	(1817.4-47871.4)	1.455	0.126	142.1	22	**62.3**	**62.3**
	Pirgos	R	F	214	31	**>1000**								**Nc**	**Nc**
PBO EC/pyrethrins EC TM (4:1) (leaf dip)	Sud-S	S	F	207	40	**11.1**	(8.4-14.9)	27.8	(19.5-53.5)	3.215	0.360	29.7	13	**1.0**	**1.1**
	Mex2-GRB	R	F	195	25	**173.8**	(111.2-316.4)	752.4	(385.9-4038.3)	2.014	0.266	36.2	13	**15.7**	**17.2**
	Pirgos	R	F	265	54	**37.4**	(30.5 – 46.2)	65.2	(51.6 – 103.2)	5.314	0.703	33.8	16	**3.4**	**3.7**
PBO PT / pyrethrins (4:1) (leaf dip)	Sud-S	S	F	175	49	**10.5**	(7.7-14.6)	29.4	(20.0-59.6)	2.874	0.342	25.5	13	**1.0**	**1.0**
	Mex2-GRB	R	F	193	28	**199.8**	(141.1-343.5)	882.2	(464.9-3573.7)	1.987	0.291	20.8	13	**19.0**	**19.8**
	Pirgos	R	F	289	70	**95.1**	(72.9-118.9)	171.1	(134.8-261.3)	5.027	0.586	33.4	13	**9.1**	**9.4**

Table A-IV-II The effect of microencapsulated pyrethrins (plus/minus PBO) on a resistant and susceptible clone of *Myzus persicae*

Treatment	Formulation	Clone	R/S	n	controls	LC_{50} (ppm)	(95% CL)	LC_{90} (ppm)	(95% CL)	Slope	(+/- SE)	χ^2	df	RF	ESR
Microencapsulated pyrethrins	β-cyc	4106a	S	180	30	**583.4**	436.7-871.1	1924.2	1182.3-5151.7	2.473	0.388	20.1	16	**1**	**1**
		794jz	R	90	30	**> 10000**								**Nc**	**Nc**
	γ-cyc	4106a	S	150	30	**> 1000**								**Nc**	**Nc**
		794jz	R	90	30	**> 10000**								**Nc**	**Nc**
PBO (EC) + microencapsulated pyrethrins	β-cyc	794jz	R	150	30	**50.2**	33.7-65.9	103.8	77.4-193.3	4.066	0.759	19.1	13	**Nc**	**0.086**
	γ-cyc	794jz	R	150	30	**64.9**	47.4-88.7	197.7	135.4-374.7	2.648	0.386	13.8	13	**Nc**	**Nc**
PBO (tech.) (top. app.) + microencapsulated pyrethrins	β-cyc	794jz	R	298	60	**137.9**	95.1-184.5	375.0	266.4-720.8	2.950	0.424	52.8	28	**Nc**	**0.236**
	γ-cyc	794jz	R	150	30	**261.4**	184.9-371.8	720.1	478.5-1666.6	2.912	0.391	26.8	13	**Nc**	**Nc**

Table A-IV-III Bioassay results for *Myzus persicae* (72 hours after dosing) using a topical application of technical alpha-cypermethrin

Treatment	Clone	R/S	n	controls	LC_{50} (ppm)	(95% CL)	LC_{90} (ppm)	(95% CL)	Slope	(+/-SE)	χ^2	df	RF	ESR
α-cypermethrin	4106A	S	180	30	**0.495**	0.320-0.729	1.473	0.952-3.540	2.71	0.34	41	16	**1**	**-**
	794jz	R	240	30	**3230.2**	3017.3-3369.9	3769.4	3591.8-4158.1	19.11	3.27	34.3	22	**6525.7**	**6525.7**
PBO + α-cypermethrin (tank mix)	794jz	R	150	30	**810.8**	486.3-1331.7	8309.5	3522.9-100754.9	1.27	0.33	7.1	13	**-**	**1637.9**
PBO (5 hour pre-treatment) + α-cypermethrin	794jz	R	180	30	**169.9**	122.0-257.5	966.3	536.6-2816.2	1.698	0.243	17.8	16	**-**	**343.2**

Appendix V – Bioassay data for Chapter Five

Data derived from PoloPlus. TM: Tank mix; PT: pre-treatment; Nc: not calculable; R: resistant; S: susceptible; n: number of insects tested; LC_{50} : insecticide concentration required for 50 % mortality in studied population; CL: confidence limits; ppm: parts per million; SE: standard error; χ^2: chi squared value; df: degrees of freedom; RF: resistance factor; ESR: effective synergism ratio.

Table A-V-I Bioassays to test putative synergists against *Myzus persicae* (794jz clone) as a tank mix with pyrethrins (4:1)

Treatment	n	controls	LC_{50} (ppm)	(95% FL)	LC_{90} (ppm)	(95% FL)	Slope	(+/- SE)	χ^2	df	ESR	SF
Pyrethrins only*	480	90	**1630.7**	(1253.9-2061.1)	6086.7	(4407.7-10092.4)	2.240	(0.229)	80.8	46	**43.8**	**-**
PBO*	330	70	**121.7**	(94.3-156.7)	419.1	(300.4-693.8)	2.387	(0.220)	57.7	31	**3.3**	**13.4**
Ethoxylated castor oil	140	20	**342.6**	(272.3-434.8)	802.5	(601.5-1279.7)	3.466	(0.532)	9.3	12	**9.2**	**4.8**
Za-B2	140	20	**350.5**	(219.6-583.1)	1334.8	(753.4-4463.8)	2.207	(0.313)	22.9	12	**9.4**	**4.7**
Linoleic acid	140	20	**413.0**	(287.3-625.3)	1350.8	(833.7-3495.4)	2.490	(0.373)	16.8	12	**11.1**	**3.9**
Neem seed oil	80	20	**463.0**	(387.0-552.4)	733.4	(604.0-1073.4)	6.416	(1.353)	2.7	6	**12.4**	**3.5**
Oleic acid	140	20	**541.0**	(336.1-1019.9)	1926.6	(1021.5-9464.4)	2.323	(0.360)	25.2	12	**14.5**	**3.0**
Cl-B1	80	20	**611.5**	(492.1-763.5)	1257.4	(963.8-2042.4)	4.093	(0.751)	3.1	6	**16.4**	**2.7**
Parsley seed oil (53)	60	20	**802.9**	(521.9-990.4)	1340.0	(1073.3-2620.4)	5.761	(1.848)	1.3	4	**21.6**	**2.0**
Bc-B1	80	20	**822.5**	(615.6-1218.0)	2624.7	(1620.5-7774.1)	2.543	(0.547)	3.6	6	**22.1**	**2.0**
Bergamot	100	20	**1068.2**	(784.5-1761.5)	3499.5	(2024.0-12102.9)	2.487	(0.530)	4.0	8	**28.7**	**1.5**

* for reference: data from Chapter Three

Table A-V-II Bioassays to test putative synergists against *Myzus persicae* (794jz clone) as a tank mix with alpha-cypermethrin (4:1)

Treatment	n	controls	LC_{50} (ppm)	(95% FL)	LC_{90} (ppm)	(95% FL)	Slope	(+/- SE)	χ^2	df	ESR	SF
Alpha-cypermethrin only*	240	30	**3230.2**	3017.3-3369.9	3769.4	3591.8-4158.1	19.11	3.27	34.3	22	**6525.2**	**-**
PBO*	150	30	**810.8**	486.3-1331.7	8309.5	3522.9-100754.9	1.27	0.33	7.1	13	**1638.0**	**4.0**
Neem seed oil	180	30	**177.6**	120.1-297.9	986.2	508.3-3719.4	1.722	0.239	25.0	16	**358.8**	**18.2**
Linoleic acid	180	30	**247.7**	190.8-345.1	1012.3	635.3-2261.8	2.096	0.315	11.5	16	**500.4**	**13.0**
Parsley seed oil (53)	180	30	**282.1**	212.7-414.5	1223.7	729.1-3037.0	2.011	0.307	14.5	16	**569.9**	**11.5**
Ethoxylated castor oil	180	30	**282.9**	198.6-475.9	1070.9	594.2-3833.6	2.217	0.332	25.9	16	**571.5**	**11.4**
Cl-B1	120	20	**386.8**	268.4 – 735.4	1603.8	813.6 – 7657.3	2.075	0.444	7.4	10	**781.4**	**8.4**
Oleic acid	90	30	**400.2**	296.3-726.0	878.0	556.7-5560.1	3.756	0.805	11.2	7	**808.5**	**8.1**

* for reference: data from Chapter Three

Table A-V-III Bioassays to test putative synergists against *Myzus persicae* (794jz clone) as a tank mix with PBO and pyrethrins (4:4:1)

Treatment	n	controls	LC_{50} (ppm)	(95% FL)	LC_{90} (ppm)	(95% FL)	Slope	(+/- SE)	χ^2	df	ESR	SF
Pyrethrins only*	480	90	**1630.7**	(1253.9-2061.1)	6086.7	(4407.7-10092.4)	2.240	0.229	80.8	46	**43.8**	**-**
PBO*	330	70	**121.7**	(94.3-156.7)	419.1	(300.4-693.8)	2.387	0.220	57.7	31	**3.3**	**3.3**
Neem seed oil	100	20	**65.4**	(52.0-82.0)	144.2	(109.5-232.7)	3.732	(0.648)	7.0	8	**1.8**	**24.9**
Linoleic acid	140	20	**86.0**	(65.4-114.1)	173.6	(127.9-314.7)	4.200	(0.700)	15.1	12	**2.3**	**18.9**
Ethoxylated castor oil	140	20	**113.9**	(80.0-147.9)	263.5	(196.7-455.0)	3.519	(0.743)	8.2	12	**3.1**	**14.3**

* for reference: data from Chapter Three

Table A-V-IV Bioassays to test putative synergists against female *Musca domestica* (381zb strain) as a tank mix with pyrethrins (5:1)

Treatment	n	controls	LC_{50} (95% FL) (ppm/20mg fly)		LC_{90} (95% FL) (ppm/20mg fly)		Slope	(+/- SE)	χ^2	df	ESR	SF
Pyrethrins only*	200	40	**11251.9**	(9838.2-12685.5)	16342.7	(14189.4-21740.8)	7.91	(1.69)	3.4	8	**14.9**	**-**
PBO*	242	40	**589.3**	(509.9-695.1)	1077.7	(846.9-2176.5)	6.30	(1.66)	15.5	10	**0.78**	**19.1**
Neem seed oil	89	21	**4635.1**	(3335.6-7056.5)	14354.8	(8922.3-34597.4)	2.61	(0.46)	4.2	6	**6.1**	**2.4**
Linoleic acid	89	28	**5657.7**	(3879.0-8146.0)	13832.4	(9312.5-33265.2)	3.30	(0.78)	3.1	6	**7.5**	**2.0**

* for reference: data from Chapter Three

Table A-V-V Bioassays to test putative synergists against female *Musca domestica* (381zb strain) as a tank mix with PBO and pyrethrins (5:5:1)

Treatment	n	controls	LC_{50} (95% FL) (ppm/20mg fly)		LC_{90} (95% FL) (ppm/20mg fly)		Slope	(+/- SE)	χ^2	df	ESR	SF
Pyrethrins only*	200	40	**11251.9**	(9838.2-12685.5)	16342.7	(14189.4-21740.8)	7.91	(1.69)	3.4	8	**14.9**	**-**
PBO*	242	40	**589.3**	(509.9-695.1)	1077.7	(846.9-2176.5)	6.30	(1.66)	15.5	10	**0.78**	**19.1**
Neem seed oil	304	28	**425.6**	(270.9-741.1)	849.5	(544.8-3257.0)	4.27	(0.59)	97.7	22	**0.56**	**26.4**
Linoleic acid	273	28	**526.5**	(441.5-630.7)	982.3	(792.0-1630.7)	4.73	(0.74)	14.6	21	**0.70**	**21.4**

* for reference: data from Chapter Three

Appendix VI – Khot *et al.* (2008)

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A novel assay reveals the blockade of esterases by piperonyl butoxide

Anna C Khot, Georgina Bingham, Linda M Field and Graham D Moores*
Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK

Abstract

BACKGROUND: Conventional *in vitro* assays sometimes fail to reveal esterase inhibition by piperonyl butoxide (PBO), although synergism studies suggest loss of esterase-mediated sequestration of insecticide does take place. A new *in vitro* assay has been devised that routinely reveals binding between PBO and these esterases.

RESULTS: The new 'esterase interference' assay detects the blockade of resistance-associated esterases in a species, *Myzus persicae* Sülzer, where this has not previously been seen. The assay also demonstrates directly the protective effect esterases may confer to target sites of insecticides.

CONCLUSION: The new assay reveals esterase blockade by PBO and thus has the potential to be used as a high-throughput screening method for other potential synergists.
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Keywords: esterase; piperonyl butoxide; inhibitor; synergist

1 INTRODUCTION

Resistance involving heritable changes in a pest population can result in formerly useful pesticides no longer providing adequate control.[1] The extent to which insecticide resistance occurs and the rate at which it spreads are dependent on a combination of chemical, genetic and biological factors. These include: the rate and frequency of application of the insecticide; the mode of action of the insecticide; whether resistance is monogenic or polygenic; the level of inherent genetic variation and the life cycle and ecology of the insect species.[2,3] For example, a short generation time and production of abundant progeny facilitate the rapid development and spread of resistance.[4]

The most important mechanisms of resistance reported in insects are metabolic detoxification and target-site insensitivity. Many xenobiotics, including insecticides, are at risk of detoxification from one or more of the three major groups of enzymes: esterases, microsomal oxidases and glutathione-*S*-transferases. In most cases, such metabolic resistance can be correlated with enhanced enzyme activity compared with that of susceptible insects.[5–7]

Esterases involved in insecticide metabolism include carboxylesterases, phosphorotriester hydrolases, carboxylamidases and epoxide hydrolases.[8] These enzyme systems can confer significant levels of resistance to many diverse groups of insecticides, including organophosphates (OPs), carbamates and pyrethroids.[9–12]

In the peach-potato aphid, *Myzus persicae* Sülzer, detoxification of insecticide was first characterised by Devonshire,[13] who used enzyme purification studies to demonstrate that a single esterase isozyme (E4) was highly overexpressed in OP-resistant strains. In this example, the esterase E4 can account for as much as 1% of the total protein in the aphid and confers broad spectrum resistance by both ester hydrolysis and sequestration.[14]

Synergists have been used commercially for over 60 years to enhance the efficacy of insecticides and have contributed significantly to improving insect control, especially where resistance has occurred.[15,16] Piperonyl butoxide (PBO) was the first effective and commercially viable synergist to be developed.[17] Classically considered a specific inhibitor of oxidases, PBO has more recently been shown to inhibit resistance-associated esterases in a wide range of agriculturally important pests and enhance insecticide efficacy in insects where esterase resistance mechanisms are present.[18–21]

Esterase activity in insects can be detected in simple laboratory assays involving model substrates such as esters of 1-naphthol. These studies have suggested that resistance-associated esterases from some species, e.g. *Aphis gossypii* Glover, are inhibited by PBO, while others, e.g. *M. persicae*, are not.[22] However, the finding that the resistance-associated esterase E4 in *M. persicae* is not inhibited *in vitro* is in conflict with *in vivo* results which show loss of esterase-mediated sequestration.[23,24] This paper describes a novel *in vitro* assay that demonstrates the blockade of E4 by PBO indirectly, by measuring the sequestration of a potent acetylcholinesterase inhibitor (azamethiphos) in the absence and presence of PBO.

* Correspondence to: Graham D Moores, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK
E-mail: Graham.Moores@bbsrc.ac.uk
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2 MATERIALS AND METHODS

2.1 Insects

2.1.1 *Bemisia tabaci* Gennadius

All strains were maintained on cotton plants (*Gossypium hirsutum* L. var. Deltapine 16 and Sicot 189), at 26 ± 2 °C with a 16:8 h light:dark lighting regime. The B-type resistant strain PIRGOS and the Q-type resistant strain CHLORAKA were both collected from Cyprus in 2003.

2.1.2 *Myzus persicae* Sulzer

Clones were reared on three-week-old Chinese cabbage plants (*Brassica campestris* L. var. Pekinensis) maintained at 18 ± 2 °C with a 16:8 h light:dark lighting regime. The highly resistant clone 794JZ was obtained from a glasshouse in Evesham in 1982, and the resistant clone T1V from sugar beet in Bedfordshire in 1975. Both clones contain high levels of the E4 esterase.

2.1.3 *Musca domestica* Linnaeus

WHO, a standard susceptible strain, was maintained at 28 ± 2 °C under a 12:12 h light:dark photoperiod with diet and methods according to Basden.[25] It was obtained from Michael Kristensen (Danish Pest Infestation Laboratory, Denmark).

2.2 Chemicals and insecticides

All standard chemicals and insecticides were purchased from Sigma-Aldrich (UK). Azamethiphos and piperonyl butoxide were both Pestanal, analytical standard grade. Triton X-100 (especially purified for membrane research) was obtained from Boehringer Mannheim.

2.3 Purification of E4

Approximately 6 g of *M. persicae* (mix of clones T1V and 794JZ) was used to purify E4, as described by Devonshire,[13] with the following modifications: aphids were homogenised in 0.02 M phosphate buffer, pH 7.0 (20 mL), containing 0.1% Triton X-100, and the fractions containing the carboxylesterase were desalted and concentrated in a filtration concentrator (Amicon, Hertfordshire, UK).

2.4 Determination of esterase activity

Approximately 100 adult Q-type *B. tabaci*, 100 B-type *B. tabaci*, 30 *M. persicae* (T1V clone) or ten *M. domestica* (WHO strain) heads were homogenised on ice in a total of 1.5 mL of 0.02 M phosphate buffer (pH 7.0). Homogenates were spun at 10 000 × *g* for 2 min, and the supernatant was used for the assays.

Total esterase activity was measured in reactions (250 µL) performed in 96-well microtitre plates using a colorimetric assay modified from Grant *et al.*,[26] with the final concentrations of FBRR and 1-naphthyl acetate being 1.2 and 0.24 mM respectively. Kinetic assays were performed at 450 nm using a T_{max} kinetic microplate spectrophotometer (Molecular Devices Corporation, Menlo Park, California), with readings being taken automatically for 10 min at 10 s intervals. The rate (mOD min^{-1}) was calculated by the integrated software Softmax Pro version 4.6.

2.5 Direct determination of esterase inhibition by PBO in insect homogenates

A stock solution of PBO (0.3 M) was prepared in acetone, and serial dilutions were added to aliquots of insect homogenate and incubated for 30 min. Enzyme solution in 0.02 M phosphate buffer (pH 7.0) and buffer only were positive and negative controls respectively. An acetone control was also used. Esterase activity was measured as described above.

2.6 Preparation of AChE

Homogenates of *M. domestica* heads (ten) in a total of 2 mL of 0.02 M phosphate buffer (pH 7.0) containing 0.1% Triton X-100 were centrifuged at 10 000 × *g* for 2 min, and the supernatant was used as the source of AChE.

2.7 Indirect determination of esterase blockade by PBO, measuring acetylcholinesterase (AChE) activity (esterase interference assay)

Aliquots (15 µL) of E4 or E4 + 3 mM PBO (preincubated for 16 h at 4 °C) were incubated for 1 h with 60 µL of serial dilutions of azamethiphos in 0.02 M phosphate buffer (pH 7.0) in separate wells of a microtitre plate. Housefly head homogenate (25 µL) was added to every well and incubated for 10 min at room temperature. AChE activity was then measured by adding acetylthiocholine iodide (ATChI) substrate and detecting the released thiocholine colorimetrically (405 nm) by its reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[27] The final concentration of both reagents was 0.5 mM. AChE only served as the reference. E4 in buffer and buffer only served as positive and negative controls respectively. An acetone control was also prepared. Kinetic assays to measure AChE activity were performed at 405 nm using a T_{max} kinetic microplate spectrophotometer, with readings being taken automatically for 10 min at 10 s intervals. The rate (mOD min^{-1}) was calculated by Softmax Pro version 4.6. IC_{50} values were calculated using Grafit 3.0 (Ertithacus Software).

3 RESULTS AND DISCUSSION

It has been reported previously that the sensitivity of resistant *M. persicae* and *M. domestica* to insecticides as diverse as pyrethroids, carbamates and neonicotinoids can be increased by a pretreatment of PBO.[23,24] This suggests that the synergist was blocking the enhanced metabolic activity, although *in vitro* assays have failed to show its effect.[22] This is confirmed by results shown in Fig. 1, where no significant inhibition of esterase was seen for either insect, although blockade of esterases from both B- and Q-type *B. tabaci* was

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revealed. Furthermore, an overnight incubation of pure E4 with PBO also failed to reveal inhibition of the enzyme (results not shown). Thus, a new approach is required to measure PBO-mediated blockade. In this 'esterase interference' assay, E4 and E4 plus PBO are incubated with a serial dilution of azamethiphos. AChE is then added, and, following a short incubation, the AChE activity is determined. If PBO binds to the E4, then the E4 cannot bind to the azamethiphos, allowing inhibition of the AChE, as indicated by a reduction in AChE activity.

Using the interference assay, it can be seen that there was a reduction in the ability of E4 to protect the AChE from azamethiphos when the E4 had been preincubated with PBO (Fig. 2; Table 1). This shows that PBO has blocked the E4.

It appears that, when PBO binds to the esterases of insects, it does not do so at the hydrolytic active site, and hence inhibition by PBO cannot always be revealed by a conventional *in vitro* spectrophotometric assay, depending on the insect being studied. The spatial separation of this binding site from the hydrolytic active site will vary from species to species.

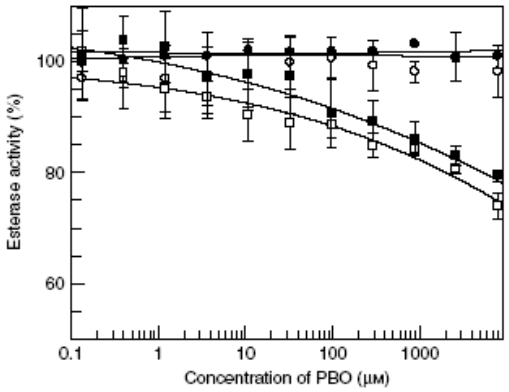

Figure 1. Inhibition of esterases from different insect species by PBO: ○, *Musca domestica*; ●, *Myzus persicae*; □, B-type *Bemisia tabaci*; ■, Q-type *Bemisia tabaci*.

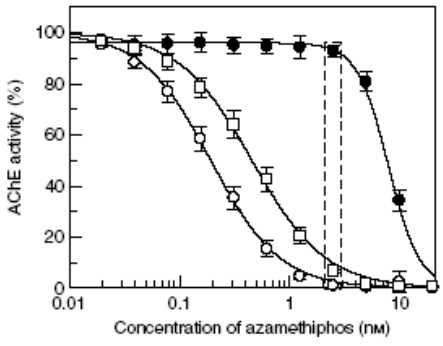

Figure 2. Inhibition of *Musca domestica* AChE activity with E4 and E4 plus PBO: ○, no E4; ●, E4; □, E4 + 3 mM PBO. The 'diagnostic window' is shown within the dashed lines.

Table 1. IC_{50} (concentration of azamethiphos required for 50% inhibition of AChE) values corresponding to Fig. 2

Sample	IC_{50} (nM)	SE
AChE only (no E4)	0.192	0.005
E4	7.915	0.435
E4 + 3 mM PBO	0.455	0.018

Thus, in some cases the bound PBO may occlude entry of the model substrate, while in others it will be hydrolysed. This explains why a standard esterase assay will reveal PBO-mediated inhibition of esterases from some insects but not others. Since the esterase/PBO 'complex' has always been found to prevent the insecticide binding, PBO proved to be a potent synergist *in vivo* regardless of the spatial positions of the two binding sites.

The addition of E4 greatly increased the concentration of azamethiphos required to inhibit AChE activity, demonstrating the ability of E4 to sequester azamethiphos. The addition of E4 plus 3 mM PBO gave a result very close to that without E4, showing that PBO prevents the esterase from binding to azamethiphos, enabling the inhibitor to act on the target site.

Calculating the molar amounts of azamethiphos and E4 at the point at which AChE activity is just inhibited, i.e. the point at which azamethiphos has overcome the sequestering effects of the esterase, gives results of the same order. This suggests a near-stoichiometric binding between E4 and azamethiphos. The assay curve and hence blockade of E4 were not significantly different when the assay was repeated using E4 + 0.3 mM PBO, but blockade was almost absent using E4 + 0.03 mM PBO (results not shown). For this reason, E4 + 3 mM PBO in the incubation (from 0.3 M stock concentration) was judged to be the more robust method.

A higher-throughput screening method could be used by selecting the 'diagnostic window' (Fig. 2). This would enable fast screening of potential synergists by comparing the protective effect of E4 in the presence or absence of the putative synergist against a single diagnostic concentration of azamethiphos. Provided the diagnostic window is known (i.e. the required concentration of azamethiphos), only the three data points highlighted by the outlining box would need to be run in the assay. For example, the AChE activity of the three points selected in Fig. 2 are shown in Fig. 3.

It is important to remember that, although this new assay can provide a means of screening potential synergists *in vitro*, they must subsequently be evaluated *in vivo* to ensure the physicochemical properties of the synergist allow for *in vivo* activity. As sequestration is often the major action of resistance-associated esterases and such binding may leave the hydrolytic active site of the enzyme free, this 'esterase interference' assay should prove invaluable for the screening of putative esterase inhibitors. It also reveals directly the protective effect esterases confer

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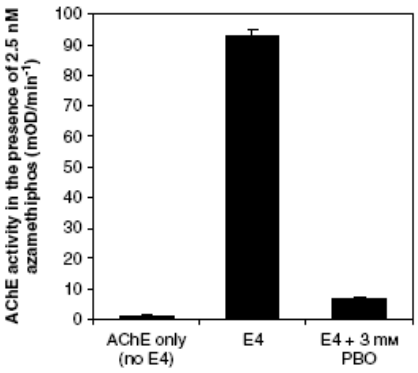

Figure 3. AChE activity of the three assay points in the diagnostic window.

to the target site of an insecticide. A more extensive study could provide additional understanding of the molecular basis of the esterase–piperonyl butoxide interactions.

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