EVALUATION OF POTENTIAL PYRETHRUM SYNERGISTS ON AGRICULTURALLY SIGNIFICANT INSECT SPECIES

by

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Abstract

The indiscriminate use of pesticides in the control of agricultural and public health pests has led to the development of insecticide resistance in many insect pest species, posing a large threat to the continued success of chemical control measures. Resistance towards pesticides can be due to a number of mechanisms, but enhanced detoxification of the insecticide by metabolic enzymes, such as esterases, glutathione-S-transferases (GSTs) or monooxygenases, plays a large role in insecticide resistance. Synergists, such as piperonyl butoxide (PBO) can be used to overcome metabolic insecticide resistance. Synergists are capable of inhibiting enzymes involved in insecticide resistance, thus restoring a level of susceptibility. By virtue of its enzyme inhibitory qualities, PBO is often used to enhance the efficacy of pyrethrum (an organic insecticide). In some countries, however, PBO is no longer certified for organic use and this study explored the use of natural plant oils or extracts as potential organically-certifiable pyrethrum synergists. From a commercial viewpoint, an effective natural synergist could potentially allow development of new pyrethrum formulations into higher value niche markets, such as organic agriculture. Synergised pyrethrum could also offer a new control option against insects resistant to synthetic pesticides in mainstream agriculture.

The efficacy of various natural plant oils and extracts as pyrethrum synergists were examined for synergistic potential towards pyrethrum with several agriculturally significant insect pest species, ***Helicoverpa armigera***, ***Frankliniella occidentalis***, ***Myzus persicae***, ***Meligethes aeneus*** and ***Musca domestica***, a serious public health pest, using discriminating dose bioassays. PBO proved to be the most effective pyrethrum synergist in all species tested, however, a number of natural plant oils also showed efficacy. The most promising compounds were dill apiole oil and parsley seed oil, synergising pyrethrum in all the species tested. Dill apiole oil and parsley seed oil contain the methylenedioxyphenol ring structure that is characteristic of PBO.

Enzyme inhibition studies were used to examine the ability of the natural plant oils and extracts to inhibit the enzymes involved in metabolic insecticide resistance (esterases, GSTs and monooxygenases). In general, inhibition of esterases and GSTs was not correlated to synergism of pyrethrum ***in vivo***. However, in ***H. armigera*** and ***M. persicae***, with esterase mediated pyrethroid resistance, PBO, dill apiole oil and parsley seed oil inhibited non-specific esterases. In ***M. aeneus*** and ***M.***

domestica, where resistance to pyrethroids has been linked to monooxygenases, PBO, dill apiole oil and parsley seed oil showed significant inhibition of monooxygenases. However, not all compounds that inhibited esterases or monooxygenases synergised pyrethrum. The lack of correlation between enzyme inhibition and synergism could be due to a number of factors, including the ability of the synergist to penetrate the insect cuticle, speed of distribution of the synergist through the insect, metabolism of the compounds in the insect and affinity for the target site.

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Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
ATChI	Acetylthiocholine iodide
BRA	Botanical Resources Australia
CDNB	1-Chloro-2,4-dinitrobenzene
CI	Confidence intervals
CP450	Cytochrome P450
D	Dark
DEM	Diethyl maleate
df	Degrees of freedom
DPIL	Danish Pest Infestation Laboratory
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
DTT	Dithiothreitol
E4	Esterase isozyme (***Myzus persicae***)
EC	Emulsifiable concentrate
EDTA	Ethylenediaminetetraacetic acid
EMAI	Elizabeth Macarthur Agricultural Institute
FBRR	Fast blue RR salt
FE4	Esterase isozyme (***M. persicae***)
GABA	gamma-Aminobutyric acid
GLM	Generalized linear model
GLMM	Generalized linear mixed model
GSH	Glutathione reduced form
GST(s)	Glutathione-S-transferase(s)
I	Index value (for the esterase interference assay)
IC_{50}	Concentration of inhibitor at which 50% of enzyme activity is inhibited
I & I NSW	Industry & Investment New South Wales
IPM	Integrated pest management
kdr	Knockdown resistance
L	Light
LSD	Least significant difference
LD_{10}	Lethal dose of a compound estimated to kill 10% of individuals of a test population

LD_{50}	Lethal dose of a compound estimated to kill 50% of individuals of a test population
LD_{90}	Lethal dose of a compound estimated to kill 90% of individuals of a test population
MACE	Modified acetylcholinesterase
MDP	Methylenedioxyphenol
MFOs	Mixed function oxidases
n	Number of insects tested
NS	Not significant
NSW	New South Wales
NADPH	beta-Nicotinamide adenine dinucleotide phosphate tetrasodium salt
OP(s)	Organophosphate(s)
P450(s)	Cytochrome P450(s)
PAGE	Polyacrylamide gel electrophoresis
PBO	Piperonyl butoxide
PMSF	Phenylmethanesulfonyl fluoride
ppm	Parts per million
PTU	Phenylthiourea
RF	Resistance factor
RH	Relative humidity
rpm	Revolutions per minute
SE	Standard error
SF	Synergism factor
TAI	Tamworth Agricultural Institute
TPP	Triphenyl phosphate
v	Volume
w	Weight
WHO	World Health Organisation

Preface

In this thesis, data is presented on the efficacy of potential pyrethrum synergists against a range of agricultural pests (cotton bollworm ***Helicoverpa armigera***, western flower thrips ***Frankliniella occidentalis***, peach-potato aphid ***Myzus persicae*** and pollen beetle ***Meligethes aeneus***) and a public health pest (the house fly ***Musca domestica***). The ability of the potential synergists to inhibit the enzymes involved in detoxification of insecticides (esterases, glutathione-S-transferases and monooxygenases) is also explored since the action of synergists is to inhibit the enzymes involved in the detoxification of an insecticide. Chapter one gives a general introduction into pyrethrum, pyrethroids and their mode of action and a review on insecticide resistance and synergists. Chapter two describes the general materials and methods used throughout this study, including the insects, the chemicals and the synergists used and the basic bioassay and biochemical assay techniques, as well as the general statistical analyses used. Where methodology differed from the general materials and methods, this is discussed in each relevant chapter. Chapters three to seven are the data chapters for each of the respective insect species used in the study, ***H. armigera***, ***F. occidentalis***, ***M. persicae***, ***M. aeneus*** (and includes enzyme assays with dried fruit beetles ***Carpophilus*** spp.) and ***M. domestica***, describing synergism of pyrethrum with the potential synergists and enzyme inhibition in each species. Chapter eight gives a general discussion and the conclusions reached from this study, as well as some suggestions for further research. Appendices on the ***H. armigera*** larval diet and the method of processing data for the esterase interference assay are included at the end of the thesis.

Each of the data chapters have been prepared as stand-alone publishable manuscripts, except for the references to the general materials and methods in chapter two, and the figures and tables have been numbered to fit with the thesis format. Acknowledgements are also at the beginning of the thesis.

1 CHAPTER ONE: GENERAL INTRODUCTION

Pests in agriculture are capable of causing serious economic damage to crops. The over-use of insecticides, coupled with factors such as the short generation time and high fecundity of insects, has led to the development of resistance in many species towards a large variety of insecticides. The growing difficulties of controlling insecticide-resistant pest populations, together with the recent shift towards less harmful methods of controlling pests in agriculture, has led to a renewed interest in the use of pyrethrum as a pest control option. Pyrethrum is a naturally occurring pesticide processed from the flowers of ***Chrysanthemum cinerariaefolium*** (Treviranus) Boccone, showing low toxicity towards mammals and rapid break down of the pesticide in the environment.

Due to the high cost of pyrethrum per unit dose, its efficacy is often enhanced by addition of a synergist, such as piperonyl butoxide (PBO). This lowers the quantity of pyrethrum needed in a pesticide formulation and makes it more cost-effective. A synergist is also capable of enhancing the toxicity of an insecticide in a resistant population of insects, due to its ability to inhibit the enzymes involved in detoxification of that insecticide, allowing the insecticide to still reach its target site. Effective synergists could therefore restore the use of insecticides thought lost to agriculture due to resistance, or can create new pest control options, such as the use of natural pyrethrum.

This PhD looks at testing the efficacy of a variety of natural synergists, to either replace PBO in formulation, or to be added to a formulation containing PBO, to enhance the efficacy of the product. From a commercial viewpoint, an effective natural synergist could potentially allow development of new formulations for retail into higher value niche markets, such as organic agriculture. This project should further our understanding of synergists and the efficacy of pyrethrum against some key agricultural pest species and a public health pest (house flies). Furthermore, it will aid in elaborating resistance mechanisms in these pest species.

In this chapter, pyrethrum and the synthetic pyrethroids are discussed (sections 1.1 and 1.2), together with their mode of action (section 1.3). Insecticide resistance and resistance mechanisms are explained (sections 1.4 and 1.5), with a discussion on insecticide resistance management (section 1.6). Synergists, the main focus of this

PhD project, are discussed (section 1.7), together with a brief description of temporal synergism. Finally, the aims and objectives of this PhD study are given (section 1.8).

1.1 Pyrethrum

Pyrethrum is a naturally occurring insecticide from the flowers of the pyrethrum plant, ***Chrysanthemum cinerariaefolium*** (Synonyms: ***Pyrethrum cinerariaefolium*** (Treviranus) and ***Tanacetum cinerariaefolium*** (Treviranus) Schultz-Bip.) (Asteraceae) (Davies 1985, Narahashi 1982). The first commercial insecticidal use of pyrethrum was possibly in Persia during the Middle Ages (Gnadinger 1936, McLaughlin 1973). The powdered flowers, called "Persian dust", were produced from the dried flowers of ***Chrysanthemum roseum*** (Ray 1991) but this species contained less pyrethrins than ***C. cinerariaefolium***, which is the only species now used commercially (Davies ***et al***. 2007, Gnadinger 1936, McLaughlin 1973). Pyrethrins are usually extracted from the seeds. The achenes (fruits containing the seeds) contain more than 90% of the total pyrethrins, in tiny oil-containing glands. The receptacles and involucral scales contain approximately 5.5% and only traces occur in the disk and ray florets (Ray 1991, Gnadinger & Corl 1930).

Countries producing pyrethrum include Kenya, Uganda, Rwanda, Papua New Guinea, Tanzania and, since 1996, Tasmania. The Tasmanian pyrethrum industry is a major contributor to the local economy, contributing around $A10 million in payments to growers, contractors and employees. Tasmania now produces in excess of 45% of the world's pyrethrum (MGK 2006, BRA 2009), supplying pyrethrum products to the USA, Europe, Japan, Australia and throughout South East Asia.

Pyrethrum contains six insecticidally active esters, pyrethrin I ($C_{21}H_{28}O_3$), pyrethrin II ($C_{22}H_{28}O_5$), jasmolin I ($C_{21}H_{30}O_3$), jasmolin II ($C_{22}H_{30}O_5$), cinerin I ($C_{20}H_{28}O_3$) and cinerin II ($C_{21}H_{28}O_5$), collectively known as the "Pyrethrins" (Kenya Pyrethrum 2001, Casida 1973, Ray 1991) (Fig. 1.1). The Pyrethrins I (pyrethrin I, jasmolin I and cinerin I) are esters of chrysanthemic acid and the Pyrethrins II (pyrethrin II, jasmolin II and cinerin II) are esters of pyrethric acid, with the alcohols pyrethrolone, jasmololone and cinerolone respectively (Head 1973, Kumar ***et al***. 2005, Ray 1991). The greatest component of pyrethrum (67%) is made up of the two esters, pyrethrin I and pyrethrin II (Litchfield 1985). Cinerin I and cinerin II account for 24% of pyrethrum and jasmolin I and jasmolin II comprise the remaining 9%.

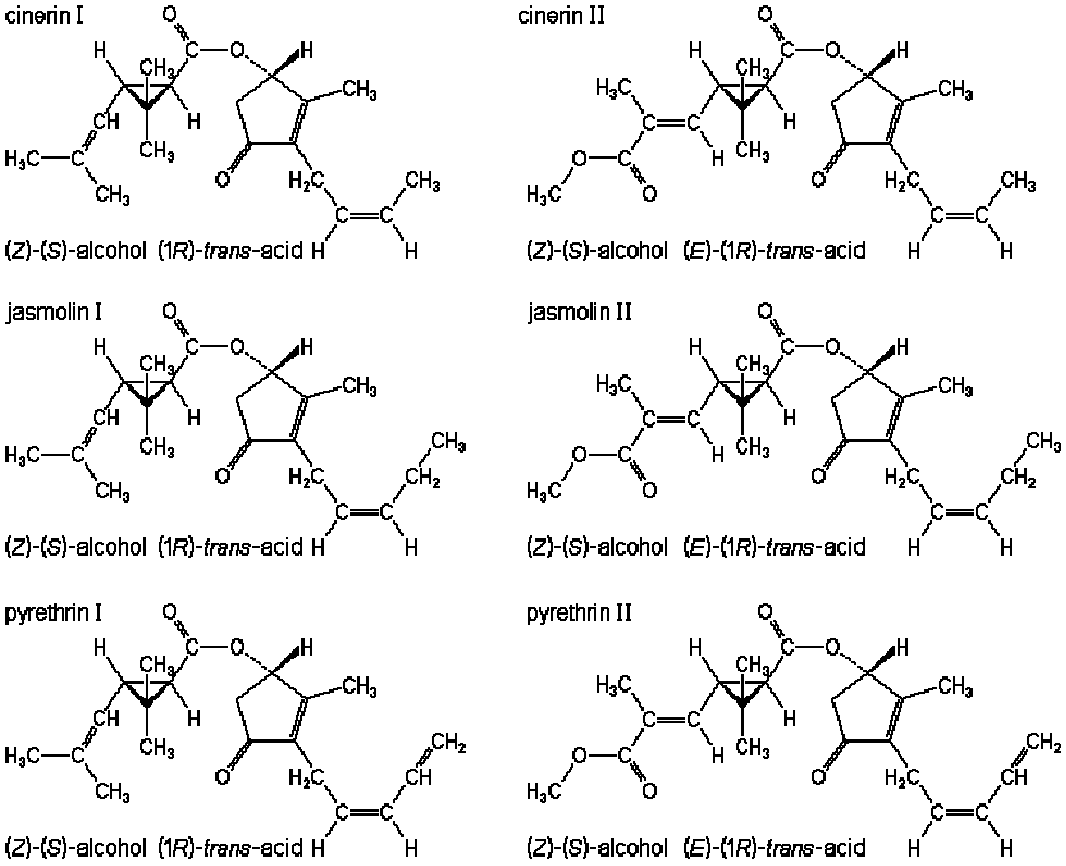

Fig. 1.1. Molecular structure of the six Pyrethrin esters (cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I and pyrethrin II). (Source: Compendium of pesticide common names (Wood 2010)).

Pyrethrum has quick insecticidal action against a broad range of insects, including cockroaches, mosquitoes, fleas, flies and aphids (Mrak 1973), first immediately paralysing the insect, known as “knockdown”, followed later by death (Brown 2006). Pyrethrum also has a good flushing action, causing insects to move out of their hiding places, thereby exposing them to more contact with the insecticidal spray (BRA 2003). At very low dosages, pyrethrum repels insects and can therefore be used in the protection of stored grain, insect resistant packaging and in deterring mosquitoes, either as spray-on repellent or mosquito coils.

Pyrethrum is metabolised rapidly by mammals and its low mammalian toxicity (see for example, Elliott ***et al***. (1972), Ueda ***et al***. (1975), and comprehensive reviews on the subject by Barthel (1973), Gnadinger (1936, 1945) and Litchfield (1985)) makes it safe for use in agriculture, and also around homes and gardens. Pyrethrum is,

however, toxic to fish, frogs and other aquatic organisms, such as clams and crabs (review by Gnadinger (1936, 1945)).

Pyrethrins are photolabile (decomposed by exposure to light) and biodegradable (rapidly oxidized and inactivated by air) and therefore break down quickly in the environment (Mrak 1973), making them useful for fruit and vegetable crops, where residues can be a problem with more persistent chemicals. It can also be used as a post harvest spray to protect crops on their way to the market. Pyrethrum is certified for use on organic farms in Australia due to its rapid breakdown (BRA 2003), and is currently used in horticulture and by organic growers for the control of leaf hoppers and other common pests (Kenya Pyrethrum 2001). Pyrethrum, usually combined with a synergist (discussed in section 1.7), is used in household sprays, in mosquito coils and mats, in shampoos and veterinary flea powders for pets (Ray 1991) and in public health, for the control of mosquitoes and flies. Other uses include sprays and fogs for farms and dairies, protection of food processing and handling, and aerosols for restaurants (Kenya Pyrethrum 2001, Davies ***et al***. 2007).

In contrast to the high number of recorded cases of resistance towards synthetic insecticides, resistance to pyrethrum seems to be very scarce, probably due to the complexity of the pyrethrins and their non-persistence in the environment. A few recorded cases seem to be a result of cross-resistance to other insecticides (BRA 2003).

In the past, pyrethrum was often in short supply, when high demand exceeded world production, due to its wide range of uses and low mammalian toxicity (Klaassen 2001). The instability of pyrethrum in the environment also meant that it would not necessarily remain active long enough to effectively kill pests in agricultural systems (Brown 2006, Davies ***et al***. 2007, Mrak 1973, Ray 1991). These factors, together with the high costs of pyrethrum, led to the development of synthetic pyrethroids.

1.2 Synthetic pyrethroids

For use in agriculture, the demand existed for a product that would be cheaper to produce, had more stability in light and air, had better persistence for longer lasting control, and was more selective for arthropod species but still retained its low toxicity towards mammals. Synthetic pyrethroids were therefore developed using the structure of pyrethrins as a template to fill this demand (Brown 2006, Klaassen 2001).

Research into the development of synthetic pyrethroids began around 1924, with key structure-activity relationship studies on the pyrethrins done by Staudinger & Ruzicka (1924). The first modifications were made to the basic pyrethrin I structure with the aim to obtain more photostability and a higher insecticidal efficacy (Davies ***et al***. 2007, Litchfield 1985). In 1949, allethrin, closely resembling the structure of cinerin I, was the first synthetic pyrethroid available for insecticidal use. Between 1968 and 1974, permethrin, the first photostable compound having high insecticidal activity, low mammalian toxicity and limited soil persistence was produced, followed shortly by cypermethrin and deltamethrin (Davies 1985, Davies ***et al***. 2007).

Pyrethroids can exist in four forms, two optical isomers and two geographical isomers. Pyrethroids usually contain a mixture of these isomeric forms which in turn affects their biological activity, such as mammalian toxicity and insecticidal activity (Elliott 1954, Litchfield 1985, Narahashi 1982). The ***cis*** : ***trans*** ratio seems to play a particularly important role in the activity of synthetic pyrethroids and most of the more important commercially available pyrethroids are produced with consistent isomer content, for example, permethrin with a 40 : 60 ***cis*** : ***trans*** ratio and cypermethrin with a 50 : 50 ***cis*** : ***trans*** ratio (Litchfield 1985).

The advantages of synthetic pyrethroids over natural pyrethrins include photostability and therefore more persistence in the environment, thus having greater insecticidal effect. Pyrethroids are comparatively cheap compared to natural pyrethrins. Pyrethroids are also more toxic to insects than natural pyrethrins and can therefore be applied at lower dosage rates (Thacker 2002). Due to these reasons, as well as their increased availability, the agricultural market became dominated by synthetic pyrethroids, which were used in controlling a wide range of significant insect pest species. Synthetic pyrethroids were also used for many other applications, including household sprays, flea preparations for pets, plant sprays for homes and greenhouse use (Klaassen 2001, Mrak 1973). Currently, a wide range of synthetic pyrethroids are available for use in crop protection, animal health and public health (Davies ***et al***. 2007). A large problem, however, with the widespread use of synthetic pyrethroids was the development of resistance that occurred in many pest species.

With resistance to synthetic pyrethroids becoming a major problem, the focus has once again returned to the use of pyrethrum for controlling pests. Insects have failed to show development of resistance to pyrethrum under normal conditions (McLaughlin 1973), possibly due to the complexity of pyrethrum, with its six active

esters, and its rapid breakdown time. Increasing concern for the environment and the possible negative effects of synthetic pesticides on human health have also contributed to the revival of interest in using natural pyrethrum for the control of agricultural, household and public health pests. Advantages of pyrethrum include its rapid degradation in the environment and its low toxicity towards mammals (Narahashi 1982). The use of a synergist with pyrethrum can help bring down the cost of using pyrethrum. If an effective organic synergist can be found, pyrethrum can also play an important role in the organic niche market in agriculture, as this would be degraded quickly and leave no residues.

1.3 Mode of action of pyrethrum and pyrethroids

Pyrethrum is an axonic poison (poisoning the nerve fibre), acting on the voltage-gated sodium channels in the peripheral and central nervous systems (Miller & Salgado 1985). Pyrethroids have the same mode of action as pyrethrum and both are unique amongst insecticides in having the ability to knock insects down very quickly.

The nervous system of an insect consists of a massive network of cells. In normal nerve cell (neuron) functioning, a stimulus is received that is converted into an electrical signal. The electrical signal is transmitted down the length of the neuron via the movement of ions in and out of the neuron, through channels in the nerve membrane, creating an action potential. The four main channels are sodium, potassium, calcium and chloride channels. The sodium channel is a large gated transmembrane protein that regulates the flow of sodium ions across axonal membranes. These ion-specific channels open or close by varying amounts, determining the permeability of the membrane to each respective ion (Shankland 1976).

When a neuron is inactive, it is at resting potential, which is the electric potential across the cell membrane due to active transport and ion-selective permeability of the plasma membrane, maintaining a slightly negative interior charge relative to outside (-70 mV). The cell is therefore polarised (Shankland 1976). At rest, there are relatively more sodium ions outside the neuron and more potassium ions inside the neuron. When a stimulus is received, sodium channels open and sodium moves into the neuron, due to the neuron being more negative on the inside and also because more sodium ions are present on the outside of the membrane. As sodium moves in,

the neuron becomes more positive and becomes depolarised. When depolarisation reaches a threshold (about -55 mV), it results in an action potential, which is an all-or-none nerve impulse used in the transmission of nerve impulses throughout the nervous system. If the neuron does not reach the threshold level, no action potential will occur, however, if threshold is reached, an action potential of a fixed size will always occur. When an action potential occurs, this causes activation of sodium, immediately followed by sodium inactivation. The diffusion pressure of sodium from outside to inside the neuron allows for a rapid surge of inward sodium current. The positive charges brought into the neuron by sodium decreases or depolarises the membrane potential, producing the rising phase of the action potential. The changed membrane potential results in potassium activation. Potassium moves out of the neuron, resulting in repolarisation of the membrane (starts to go back towards -70 mV). The repolarisation causes a reduction in potassium conductance and the flow of potassium stops. When the potassium current reaches its peak, however, there is still significant sodium conductance and the membrane potential is still below the sodium equilibrium potential, allowing for a delay in the reduction of sodium current, but the repolarisation causes the sodium channels to close. Once sodium channels are closed, there is only potassium current left during the final phase of repolarisation. A hyperpolarisation results (where the action potential goes past -70 mV), due to the potassium channels closing after the sodium channels, but the ion concentrations gradually go back to resting potential (Shankland 1976) (Fig. 1.2).

When an impulse arrives at a nerve terminal, transmission of the impulse across the synapse (the gap between neurons), is by means of a chemical transmitter, such as acetylcholine (ACh), norepinephrine, L-glutamate or gamma-aminobutyric acid (GABA), which bind to receptors on the postsynaptic membrane of the next neuron. The chemical signal received is converted to an electrical response in the neuron, depolarising the postsynaptic membrane and resulting in an action potential. The signal is transmitted along the axon to the next neuron, to eventually reach the target organ. Once the neurotransmitter has transmitted its signal across the synapse, it is hydrolysed by an enzyme specific to that neurotransmitter, such as acetylcholinesterase (AChE), which breaks down ACh, and a portion is reabsorbed by the presynaptic neuron (the choline in the case of ACh) (Narahashi 1976, Shankland 1976).

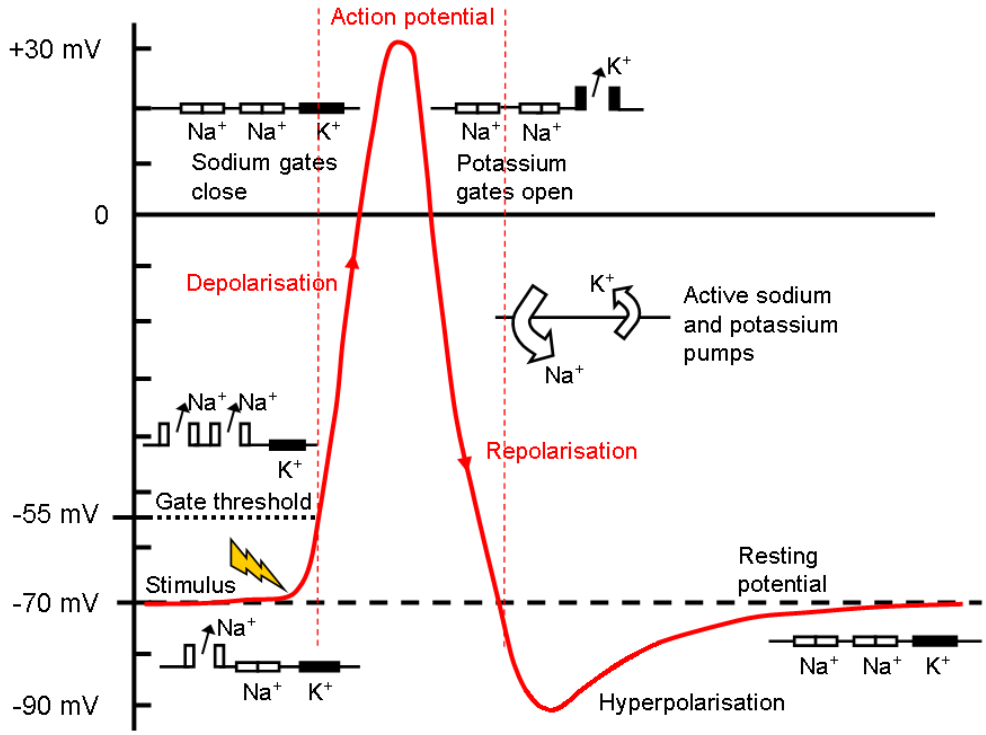

Fig. 1.2. Transmission of a nerve impulse and an action potential. (Source: Charand 2010).

When an insect is poisoned by pyrethrum (or pyrethroids), the insecticide binds to the nerve cell membrane, causing conformational changes to the sodium channels, preventing them from closing. This results in the continuous flow of sodium ions into the neuron, prolonging the sodium current following an action potential. The prolongation of the sodium current after termination of the potassium current causes a depolarisation of the membrane after it was repolarised by the potassium current. When the depolarisation reaches threshold, sodium activation is triggered in the normal way, producing another action potential. This is self-perpetuating and causes repetitive discharge in the poisoned nerve. Repetitive discharges are particularly prominent at the synaptic and neuromuscular junctions (Narahashi 1982). Insects lose control of their nervous systems and are unable to produce coordinated movement. The tremors eventually lead to paralysis, followed by death (Brown 2006, Shankland 1976, Valles & Koehler 2003).

1.4 Insecticide resistance

The intensive use of pesticides has led to the development of resistance in at least 570 arthropod species (listed in the Arthropod Pesticide Resistance Database (Whalon ***et al***. 2010)). Between these species, cases of resistance have been found to all of the major classes of insecticides, including organophosphates, carbamates, pyrethroids, arsenicals, cyanide, DDT and cyclodienes (Brattsen ***et al.*** 1986). Resistance has been defined in a number of ways. The World Health Organisation (WHO) defines resistance as "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species" (WHO 1957). Another definition, by Moores (1997), based on that of Sawicki (1987), states that "resistance results from the presence of genotypes in a population that confer some degree of protection against insecticides. In the presence of a pesticide, individuals possessing such a genotype will be at a selective advantage compared to the rest of the population". With each subsequent exposure to pesticides, the proportion of insects with the resistant genome increases relative to the proportion of susceptible individuals and resistance becomes a problem. Once resistance has developed, an insect population can become very hard or impossible to control, threatening the future success of insecticides in controlling pests. The development of new compounds is slow and expensive and limited control options are available to farmers. Increased application rates result in a higher risk of exposure to pesticides and more environmental contamination. The cost of controlling insects increases dramatically. Ecologically sound pest control strategies are disrupted. Diseases in humans, animals and plants increase where transmission is by insect vectors. Insecticide resistance can also lead to the complete destruction of agricultural production systems (Soderlund & Bloomquist 1990), such as loss of cotton production in the Ord valley (Australia), due to DDT resistance in the cotton bollworm ***Helicoverpa armigera*** (Hübner) (Lepidoptera: Noctuidae) (Castle 2002, Wilson 1974).

The development of resistance in insects depends on a variety of biochemical, genetic and ecological factors, which vary between species and populations (Brattsen ***et al.*** 1986). Factors such as short generation time, high fecundity rate, dispersal ability of the insect, mode of inheritance and fitness costs associated with resistance all play a role in the development of resistance, together with the frequency of insecticide applications, dosage rates applied and persistence of insecticide residues (Tabashnik 1990).

1.5 Insecticide resistance mechanisms

Due to the fact that insects have co-evolved with plants for millions of years, insects already possess a number of mechanisms to cope with often toxic plant allelochemicals, such as alkaloids, terpenes and phenols, which were developed by plants as defence mechanisms against the herbivorous action of insects. These mechanisms in insects include behavioural adaptations, modified physiological processes and biochemical mechanisms. Insects utilize general-purpose defensive enzymes in overcoming the potential toxicity of the plants they feed on. The rapid development of resistance to pesticides could be due to these underlying mechanisms already being in place. Where insecticides are used intensively in areas favouring rapid pest reproduction, such as crop monocultures, the insecticide is the only selecting agent for insecticide resistant genomes (Brattsen ***et al***. 1986).

For an insecticide to reach its target site, it must first penetrate the cuticle of the insect and / or other barrier tissues. Once inside the body, a percentage of the insecticide gets stored in tissues, such as adipose tissue, and the remainder is distributed within the body of the insect. A portion of the transported insecticide is subject to detoxification enzymes and a portion is excreted. The remainder is then available to act on the target site (Brooks 1976). Mutations affecting any of these processes may result in a reduction in the amount of insecticide reaching its target site, or a reduction in the affinity of the insecticide towards its target site, conferring some level of resistance to the insect (Soderlund & Bloomquist 1990).

Despite the large diversity in both available insecticide chemistry and in arthropod species displaying insecticide resistance, few resistance mechanisms have been identified. The four basic mechanisms of resistance are behavioural adaptations (section 1.5.1), physiological processes (section 1.5.2), target site insensitivity (section 1.5.3) and metabolic mechanisms (section 1.5.4). One resistance mechanism is capable of conferring cross-resistance to other insecticides and combinations of these mechanisms can also occur within one insect population (section 1.5.5) (Brattsen ***et al***. 1986, Scott 1990, Soderlund & Bloomquist 1990).

1.5.1 Behavioural adaptations

Behavioural resistance involves an adaptation of the behaviour of an insect in order for it to avoid an insecticide. Behavioural resistance has been found, for example, in the horn fly ***Haematobia irritans*** (L.) (Diptera: Muscidae) in response to pyrethroid-impregnated ear tags on cattle (Lockwood ***et al***. 1985). Malaria mosquitoes ***Anopheles gambiae*** s.l. and ***Anopheles sundaicus*** Rodenwaldt (Diptera: Culicidae) have adapted to the application of DDT to the inside walls of homes by resting on outside walls (Gerold 1977, Sundararaman 1958). A change in adult behaviour regarding oviposition preferences may also be a mechanism of behavioural resistance. DDT-resistant mosquitoes ***Aëdes aegypti*** (L.) (Diptera: Culicidae), for example, tend to choose darker sites for oviposition than susceptible mosquitoes (Wood 1961). Behavioural resistance seems rare compared to biochemical mechanisms of resistance and could involve the ability to learn. It could also involve genetic modifications to peripheral signal receptors or to the central signal-processing system of insects (Brattsen ***et al***. 1986), or the selection of a particular behavioural genotype in the population.

1.5.2 Physiological processes

Physiological processes involved in insecticide resistance include decreased cuticular penetration of insecticides (section 1.5.2.1) and sequestration (section 1.5.2.2).

1.5.2.1 Decreased cuticular penetration

Alterations to the structure of the insect cuticle can result in slower penetration of insecticide through the cuticle, or less insecticide passing through the cuticle. This mechanism confers low levels of resistance on its own, but is usually found in combination with other resistance mechanisms, such as detoxification, which enhances the effect of the mechanism (Soderlund & Bloomquist 1990). Delayed penetration can provide more time for detoxification of the insecticide (Brooks 1976). In the diamondback moth ***Plutella xylostella*** (L.) (Lepidoptera: Plutellidae), resistance to fenvalerate was found to be due to reduced cuticular penetration (Noppun ***et al***. 1989), decreased target site sensitivity (Hama ***et al***. 1987) and increased metabolic detoxification (Yao ***et al***. 1988). In resistant ***H. armigera*** from Thailand, Ahmad & McCaffery (1999) found delayed penetration of cypermethrin through the cuticle to be

a minor mechanism of pyrethroid resistance, functioning together with nerve insensitivity. Decreased cuticular penetration also plays a role in pyrethroid resistance in Australian ***H. armigera*** (Gunning ***et al***. 1991).

1.5.2.2 Sequestration

Sequestration involves the formation of a stable compound with an ion, atom or molecule so that the original compound is no longer available for chemical reactions. Sequestration, although considered to be rare (Scott 1990), has been reported as a resistance mechanism. In the peach-potato aphid ***Myzus persicae*** (Sulzer) (Hemiptera: Aphididae), for example, the esterases responsible for resistance (E4) both sequester and hydrolyse carbamates, organophosphates and pyrethroids (Devonshire & Moores 1982). In Australian ***H. armigera***, esterases responsible for pyrethroid resistance were found to sequester and hydrolyse fenvalerate (Gunning ***et al***. 1996).

1.5.3 Target site insensitivity

Target site insensitivity involves alterations in the target site of an insecticide, preventing insecticides from binding to their target site. Target site modifications include sodium channel modification, known as knockdown resistance (***kdr***) (section 1.5.3.1), modified acetylcholinesterase (section 1.5.3.2) and an altered GABA receptor-ionophore complex (section 1.5.3.3).

1.5.3.1 Modified sodium channels / knockdown resistance (*kdr*)

Insensitivity of the nervous system, or modified sodium channels, is the most common mechanism of resistance towards pyrethroids, DDT and the DDT analogues. Decreased nerve sensitivity was first noted in adult house flies ***Musca domestica*** L. (Diptera: Muscidae) in 1951 (Busvine 1951). Insensitivity of the nervous system confers resistance not only to the lethality of pyrethrins and DDT, but also to their rapid paralytic effect (knockdown), and has therefore been termed “knockdown resistance” or ***kdr***. A form conferring much greater resistance than that of ***kdr*** has also been found, termed ***super-kdr*** (Farnham ***et al***. 1987).

Decreased nerve sensitivity can be recognised in insects by its ability to afford cross-resistance to all the pyrethroids and DDT, and the failure of synergists to increase

toxicity to the insecticides. ***Kdr*** and ***super-kdr*** arise from alterations in the binding site for DDT and pyrethroids on the insect nerve cell membranes (Soderlund & Bloomquist 1990). The mutation causing ***kdr***, L1014F, has been identified as a leucine-to-phenylalanine substitution in transmembrane segment IIS6 on the voltage-gated sodium channel (Williamson ***et al***. 1996). ***Kdr*** has been correlated to pyrethroid resistance in field populations of house flies (DeVries & Georghiou 1981, Huang ***et al***. 2004) and is equally effective against all pyrethroids and DDT, conferring low levels of resistance (10 to 20-fold) across the whole range of structural variation found in pyrethroids (Farnham & Khambay 1995, Farnham ***et al***. 1987). The modification involved may therefore not be at the primary site of insecticide binding, but at a region capable of causing an alteration in the ion conductance properties of the sodium channel (Williamson ***et al***. 1996).

The mutation causing ***super-kdr***, M918T, has been identified in house flies as a methionine-to-threonine replacement in the intracellular IIS4-S5 loop (Williamson ***et al***. 1996). ***Super-kdr*** is highly sensitive to the structure of pyrethroids, conferring a wide range of resistance levels. The highest levels of resistance (up to 500-fold) were found with the more toxic type II pyrethroids (Farnham & Khambay 1995, Farnham ***et al***. 1987). Type II pyrethroids (for example, cypermethrin, deltamethrin and fenvalerate) have an alpha-cyano group not found in the type I pyrethroids (for example, allethrin, tetramethrin and permethrin). S***uper-kdr*** is therefore more likely to be at the actual binding site for pyrethroids, causing a change that would reject the large cyclic side chains in the alcohol component of pyrethroid molecules (Davies ***et al***. 2007, Williamson ***et al***. 1996).

1.5.3.2 Modified acetylcholinesterase (MACE)

Modified acetylcholinesterase (MACE) confers resistance to organophosphates (OPs) and carbamates. OPs and carbamates act by inhibiting the enzyme acetylcholinesterase (AChE), OPs being irreversible, and carbamates, reversible inhibitors of AChE. AChE is responsible for hydrolysing the neurotransmitter acetylcholine (ACh), which transmits nerve impulses across cholinergic synapses. When hydrolysis of ACh does not take place, the accumulation of ACh in the synapse leads to repetitive firing of neurons and eventual death of the insect (Fournier & Mutero 1994, Soderlund & Bloomquist 1990). When AChE is less sensitive to inhibition by OPs and carbamates, the AChE is still able to hydrolyse ACh, providing the insect with some level of resistance to these insecticides.

MACE has been found in a wide variety of insects, including the cotton aphid ***Aphis gossypii*** Glover (Hemiptera: Aphididae) (Moores ***et al***. 1996) and ***M. persicae*** (Moores ***et al***. 1994). AChE of resistant insects shows a decreased affinity for OPs and carbamates (Brattsen ***et al***. 1986). In ***A. gossypii*** and ***M. persicae***, decreased sensitivity of AChE was found to be due to one or more point-mutations in the gene, causing structural changes to the AChE (Andrews ***et al.*** 2004).

1.5.3.3 Altered GABA receptor-ionophore complex

The target site of cyclodiene insecticides, such as endosulfan and dieldrin, is the GABA-gated chloride channel receptor in the insect neuron. GABA is a major inhibitory neurotransmitter in the insect nervous system, producing inhibition of nerve impulses by acting on the membrane-bound GABA receptors (Johnston 2005). Cyclodienes act as receptor antagonists, blocking the $GABA_A$ receptors (ligand-gated ion channels) at the receptor-chloride ionophore complex (Soderlund & Bloomquist 1990, Tanaka ***et al***. 1984). This leaves GABA unable to bind to the blocked receptor, preventing the inhibition of nerve impulses. Nerve impulses continue to fire, leading to overstimulation of the nervous system and death in the insect.

Resistance to cyclodienes seems mainly to be due to insensitivity of the $GABA_A$ receptor in the nervous system, lowering the affinity of the receptor towards cyclodienes (ffrench-Constant ***et al***. 1991, Soderlund & Bloomquist 1990). In ***Drosophila melanogaster*** Meigen and ***Drosophila simulans*** Sturtevant (Diptera: Drosophilidae), resistance to cyclodiene insecticides has been correlated to a single-base-pair mutation, an alanine-to-serine amino acid substitution within the gene ***Resistance to dieldrin*** (***Rdl***) (ffrench-Constant ***et al***. 1993). This gene codes for a novel GABA receptor subunit (ffrench-Constant ***et al***. 1991, Lee ***et al***. 1993).

1.5.4 Metabolic mechanisms

All living organisms possess an array of defence mechanisms to protect themselves from naturally occurring toxins, such as plant allellochemicals. These same enzyme systems involved in detoxification of natural toxins are also utilised in the detoxification of insecticides in resistant insects. Enzymes metabolise toxins or insecticides by changing their molecular structure in such a way so that the product is either rendered less toxic than before, or it can be eliminated more rapidly from the body, or both (Brattsen ***et al***. 1986).

Under natural circumstances, many of the enzymes are temporarily induced by the toxin and persist as long as the toxin is present in sufficient quantities. In metabolic resistance, however, individuals with permanently expressed high enzyme activities survive and reproduce in the presence of insecticides, resulting in resistant populations (Brattsen ***et al***. 1986). The increase in enzyme activity can be due either to an altered enzyme with a higher catalytic rate, or from greater quantities of the enzymes resulting from either increased gene amplification or transcription (Devonshire ***et al.*** 1998, Devonshire & Moores 1982, Field ***et al***. 1988, Hemingway 2000, Puinean ***et al***. 2010).

The three major enzyme groups involved in detoxification of insecticides are esterases (section 1.5.4.1), glutathione-S-transferases (section 1.5.4.2) and microsomal oxidases, or monooxygenases (section 1.5.4.3).

1.5.4.1 Esterases

Esterases are a group of enzymes that act by catalysing the hydrolysis of esters into their component carboxylic acid and alcohol metabolites (Oakeshott ***et al***. 2005). This increases the polarity of the insecticide, allowing it to be excreted more easily from the insect body. Esterases are capable of hydrolysing ester insecticides such as pyrethroids, organophosphates and carbamates, and can also sequester these insecticides by binding to the insecticide and releasing it at a rate whereby the insect is able to excrete or detoxify the compound (Devonshire & Moores 1982). Esterases involved in resistance to insecticides include carboxylesterases and phosphatases (Oppenoorth & Welling 1976).

Total esterase activity can be measured using artificial substrate techniques such as polyacrylamide gel electrophoresis (PAGE), by staining for esterase bands, or by spectrophotometric assays using simple substrates such as the acetate and butyrate esters of para-nitrophenol and the acetate esters of alpha- and beta-naphthol (Soderlund & Bloomquist 1990). Esterases exist in multiple forms. Maa & Terriere (1983) detected 17 different esterase isozymes in adult susceptible and resistant house flies. Only a few of these esterases are likely to be involved in the hydrolysis of insecticides. Comparisons of total esterase activity may therefore not reveal true differences in the levels of insecticide-hydrolysing esterases (Soderlund & Bloomquist 1990).

A further way to determine whether esterases may be involved in resistance is by the use of synergists. If an esterase-inhibiting synergist can increase the toxicity of an insecticide in a resistant population, esterases can be inferred to be involved in resistance. Some relatively non-toxic organophosphate esters, such as TPP (***O,O,O***-triphenyl phosphate) and DEF (***S,S,S***-tributyl phosphorothioate), act as irreversible or slowly reversible inhibitors of esterases and have therefore been used as synergists in esterase studies. Metabolism studies using resistant insects can also be undertaken to detect high levels of insecticide hydrolysis products (Oppenoorth & Welling 1976, Soderlund & Bloomquist 1990). Binding studies between the insecticide and enzymes can also be used as a means of determining the enzymes involved in resistance.

No one particular approach provides complete evidence on its own as to the involvement of a specific enzyme pathway in resistance to insecticides. The best approach for determining the involvement of an enzyme system is to do a combination of all approaches, including esterase assays, metabolism studies and synergism studies (Oppenoorth & Welling 1976, Scott 1990).

Increased esterase activity has been associated with insecticide resistance in many insect species. Insects exhibiting increased esterase activity include pyrethroid-resistant Australian ***H. armigera*** (Gunning ***et al***. 1996), azinphosmethyl-resistant obliquebanded leafroller ***Choristoneura rosaceana*** Harris (Lepidoptera: Tortricidae) (Smirle ***et al***. 1998) and pyrethroid-, organophosphate- and carbamate-resistant ***M. persicae*** (Devonshire & Moores 1982). The sweetpotato whitefly ***Bemisia tabaci*** (Gennadius) (Hemiptera: Aleyrodidae) showed increased esterase activity in methidathion-resistant populations (Bloch & Wool 1994).

1.5.4.2 Glutathione-S-transferases

Glutathione-S-transferases (GSTs) are a group of multifunctional detoxification enzymes catalysing the conjugation of reduced glutathione (GSH) with electrophilic substrates, such as plant allelochemicals or insecticides. GSTs convert these reactive lipophilic molecules into water soluble, non-toxic conjugates (mercapturic acids) which are excreted from the insect (Habig ***et al***. 1974, Hollingworth 1976, Yu 1996). The formation of mercapturic acid involves a four-step process. The substrate initially forms a conjugate with GSH. This conjugate is transformed to a cysteine

conjugate through stepwise removal of a glutamyl residue and a glycinyl residue. The cystiene conjugate is then acetylated to give a mercapturic acid (Yang 1976).

GSTs are important in the detoxification of organophosphates, involving the cleavage of either the alkyl or aryl groups on the phosphate (Oppenoorth & Welling 1976, Yang 1976). GSTs also provide metabolic resistance to DDT, through dehydrochlorination to DDE, known as "DDT-dehydrochlorinase" or "DDTase" (Brattsen ***et al***. 1986, Soderlund & Bloomquist 1990).

As with esterases, GSTs exist in multiple forms and correlations of enzyme activity with insecticide resistance can therefore be complicated. To elucidate GSTs as the mechanism of resistance involves a similar series of studies, using GST assays, metabolism studies and synergism studies (Oppenoorth & Welling 1976, Soderlund & Bloomquist 1990). The synergist, diethyl maleate (DEM) is commonly used for GST studies (for example, Kao & Sun 1991, Wu ***et al***. 2007). Model substrates frequently used in spectrophotometric assays of total GST activity include 1-chloro-2,4-dinitrobenzene (CDNB) and 3,4-dichloronitrobenzene (DCNB) (Habig ***et al***. 1974).

Insecticide resistance has been shown to be due to GSTs in a number of insect species. GSTs are involved in resistance to parathion and methyl parathion in ***P. xylostella*** (Kao & Sun 1991). Reidy ***et al***. (1990) showed that an increase in both GSH concentration and GST activity was correlated to cyfluthrin resistance in rust-red flour beetles ***Tribolium castaneum*** (Herbst) (Coleoptera: Tenebrionidae). In ***M. domestica***, resistance to azinphosmethyl has also been associated with GSTs (Motoyama & Dauterman 1972).

1.5.4.3 Monooxygenases

Mixed function oxidases (MFOs), or microsomal oxidases, are a diverse group of enzymes involved in the detoxification of xenobiotics (substances foreign to an organism, such as insecticides or allellochemicals), as well as in the metabolism of endogenous substances, such as hormones, pheromones and fatty acids (Feyereisen 2005, Mansuy 1998). The main function of these enzymes, as with the previous enzyme systems, is to convert lipophilic substances into more polar substances, which are more readily eliminated from the body. The respiratory apparatus, the integument and the digestive tract, especially the insect midgut,

contain the highest concentration of MFOs, since these are the first insect tissues to come into contact with xenobiotics (Nakatsugawa & Morelli 1976).

Microsomal oxidases are membrane-bound enzymes found in the microsomal fraction of cell homogenates, but are also found in mitochondria. The cytochrome P450s (CP450s or P450s) are a group of microsomal oxidases that are hemethiolate enzymes with a characteristic absorbance peak near 450 nm of their hemeprotein-carbon monoxide complex. The overall process of microsomal oxidation requires the transfer of two electrons from NADPH through a series of redox components to cytochrome P450. P450s are involved in the monooxygenase reaction, catalysing the transfer of one atom of molecular oxygen to a substrate and reducing the other to water. The monooxygenase reaction is commonly described as:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

P450s are not only involved in oxygen atom transfer, but also in other activities, such as oxidases, reductases, desaturases and isomerases, catalysing at least 60 distinct reactions (Feyereisen 2005, Mansuy 1998, Nakatsugawa & Morelli 1976).

P450s are involved in resistance to many insecticide classes, including carbamates, OPs, pyrethroids and DDT (Brattsen ***et al***. 1986). The use of synergists, monooxygenase assays and metabolism studies have been used to infer involvement in insecticide resistance. Measurement of P450s is difficult due to the large number of P450 enzymes and their broad substrate specificity. A large number of assays therefore exist for measuring total monooxygenase activity, including aldrin epoxidation, ***p***-nitroanisole O-demethylation and 7-ethoxycoumarin O-deethylation (Feyereisen 2005). Progress with insect preparations is also limited due to the difficulty in obtaining high enough yields of P450s after solubilisation and purification, and by the instability of the P450s (Soderlund & Bloomquist 1990). PBO, a well known inhibitor of monooxygenases (Wilkinson 1976), is the main synergist used in bioassays for monooxygenase studies, which can increase the toxicity of insecticides in resistant populations. However, PBO has also been shown to be an inhibitor of insecticide sequestering esterases (Gunning ***et al***. 1998, Young ***et al.*** 2005, 2006). Kennaugh ***et al***. (1993) found, in ***H. armigera***, that synergism of permethrin by PBO did not correlate with P450s and suggested that the increase in mortality could be due to increased penetration of the insecticide through the insect cuticle. A similar result was found by Sun & Johnson (1972), where the synergist was acting as a

carrier, increasing the cuticular penetration of carbamates into house flies. The same conclusion was reached by Gunning ***et al***. (1995), where PBO was thought to enhance the penetration of esfenvalerate through the cuticle of pyrethroid-resistant ***H. armigera.*** Subsequent biochemical studies are therefore essential when inferring resistance mechanisms (Soderlund & Bloomquist 1990).

Many cases of insecticide resistance have been ascribed to the involvement of monooxygenases. Resistance to deltamethrin in ***H. armigera*** in West Africa has been associated with an increase in monooxygenase activity (Martin ***et al***. 2002). Chen ***et al***. (2005) and Yang ***et al.*** (2004) found elevated cytochrome P450 monooxygenases to be important in pyrethroid resistance in ***H. armigera*** larvae from Asia. In ***M. domestica***, pyrethroid resistance has been correlated with an increase in monooxygenase activity (Lee & Scott 1989). Daborn ***et al***. (2002) found that the overtranscription of a single P450 gene, ***Cyp6g1***, was responsible for resistance to DDT in ***D. melanogaster***.

1.5.5 Cross-resistance and multiple resistance

Cross-resistance is found in a population of insects when resistance to one insecticide confers resistance towards other insecticides to which they have had no previous exposure, due to the same resistance mechanism. Cross-resistance is not restricted to a chemical class and can occur towards insecticides with a different mode of action (Castle 2002, Oppenoorth & Welling 1976, Scott 1990).

Multiple resistance occurs when different resistance mechanisms coexist within a population, conferring resistance to the same insecticide or class of insecticides (Oppenoorth & Welling 1976, Scott 1990). Multiple resistance may, for example, involve decreased cuticular penetration and an insensitive target site, combined with a metabolic resistance factor. Multiple resistance can confer cross-resistance to other classes of insecticides that have a similar mode of action or to insecticides that are detoxified by the same enzyme (Brattsen ***et al***. 1986).

The occurrence of both cross-resistance and multiple resistance is of particular importance in the control of pests, due to the additive effect caused by their presence. Cross-resistance leads to control failures with a much broader range of insecticides than those initially used for their control, eliminating these additional insecticides from possible use in the future. The presence of multiple resistance

mechanisms can result in populations with extreme resistance to a pesticide, making control extremely difficult (Soderlund & Bloomquist 1990).

An example of multiple resistance occurs in demeton-S-methyl-resistant ***A. gossypii***, where resistance is due to insensitive AChE coupled with enhanced esterase activity (Han ***et al.*** 1998). Other examples of multiple resistance are mentioned in section 1.5.2.1 (Decreased cuticular penetration).

Cross resistance between pyrethroids has been found in pyrethroid-resistant tobacco budworm ***Heliothis virescens*** (F.) (Lepidoptera: Noctuidae) (Leonard ***et al***. 1988), and similarly in pyrethroid-resistant ***H. irritans*** (Byford ***et al***. 1985). In spotted bollworm ***Earias vittella*** (F.) (Lepidoptera: Noctuidae), cross resistance was found in a fenvalerate-resistant population towards endosulfan and carbaryl, whereas a cypermethrin-resistant population developed cross-resistance to endosulfan, fenvalerate and carbaryl (Saini ***et al***. 1989).

Cross-resistance between the synthetic pyrethroids and pyrethrum could potentially be a problem for pyrethrum use due to similarities in structure and mode of action. Knockdown resistance tends not to affect pyrethrum to a similar degree as found with synthetic pyrethroids (Dr. Graham D. Moores, ***Pers. comm.***), however, metabolic factors could still potentially play a role in the detoxification of pyrethrum in insect strains possessing pyrethroid-resistance.

1.6 Metabolic resistance and resistance management

Insecticide resistance has led to many pesticides being forced out of the market place, reducing the choices available to farmers for control of insect pests. Resistance is not only a problem in agriculture, but also in the control of household pests, such as cockroaches, and in public health, where insect vectors spread diseases, such as mosquitoes that transmit malaria (Tabashnik & Roush 1990). In the past, resistance was managed by either increasing pesticide dosages or by applying new compounds as alternative methods for control. This approach is, however, no longer viable, due to increasing concerns about environmental contamination by pesticides. The high cost of developing new pesticides has also led to a decline in their rate of development. The available pesticide chemistries are limited and need to be conserved for their extended use. It is therefore essential to

prevent the development of resistance, or to reduce its impact, as much as possible (Castle 2002, Soderlund & Bloomquist 1990).

Resistance management involves the use of rational and informed strategies to delay, prevent or reverse the development of resistance in pests. Resistance management can also include an increase in the development of resistance in beneficial species, such as natural enemies, which would contribute to controlling pests. An understanding of the resistance mechanisms in insects is essential for the development of a successful resistance management program (Soderlund & Bloomquist 1990, Tabashnik 1990).

Resistance monitoring would help decrease the number of ineffective sprays made, which is costly to the farmer and damaging to the environment. Resistance monitoring tends to often be done using full dose response bioassays, where insects are exposed to a range of insecticide dosages that cause between 5% and 100% mortality, and a probit analysis (Finney 1971) is used to obtain the lethal dose required to kill 50% of the test population (LD_{50}). The LD_{50} can then be compared to standard susceptible laboratory populations and a resistance factor (RF) calculated, by dividing the LD_{50} of the resistant strain by the LD_{50} of the susceptible strain, to obtain the level of resistance in the population. The population can then be monitored over time to determine whether resistance is increasing or decreasing in a population. These bioassays are time consuming and require large numbers of insects reared in the laboratory. If the mechanisms of insecticide resistance are known, highly sensitive monitoring techniques could be developed that rapidly detect resistance in populations, using biochemical techniques.

Knowledge of the resistance mechanism can help in making an informed decision regarding a replacement chemical, for example, with a different mode of action. Knowledge of the patterns of cross-resistance would also be important in this regard. Determining ways of overcoming resistance mechanisms can also be devised when the mechanism is known, for example, by using insecticide synergists (discussed in the next section) (Scott 1990, Tabashnik & Roush 1990). The use of PBO was recommended for control of severely pyrethroid-resistant ***H. armigera*** in Australia (Forrester 1988).

An increased understanding of the physiology and biochemistry of resistant insects is essential in the search for effective synergists (Brattsen ***et al***. 1986). Synergists may

also play a role in delaying the development of resistance, since insects with resistant genomes would be killed in equal proportion to susceptible insects, removing the selective advantage otherwise conferred by metabolic resistance mechanisms (Ranasinghe & Georghiou 1979). Another advantage of synergists could include if a difference existed in the resistance mechanisms of the pest species and its natural enemies. A synergist could be selected that was specifically targeted at the mechanism of resistance present in the pest species but not in the natural enemies. In this way, the efficacy of an insecticide could be increased against the target pest and still keep natural enemy populations high (Ishaaya & Casida 1981, Plapp & Vinson 1977).

1.7 Synergists and synergism

Synergists are compounds that are either negligibly toxic or non-toxic to insects when applied on their own, but when used in combination with an insecticide, enhance the efficacy of that insecticide. Synergists can be used in combination with pesticides against insects possessing metabolic insecticide resistance mechanisms, since synergists act primarily by inhibiting the metabolic pathway involved in detoxification of an insecticide, and are thus able to temporarily restore a level of susceptibility in the resistant insects (Casida 1970, Metcalf 1967). Synergism would, however, still be found in susceptible insect strains since the detoxification enzymes inhibited by the synergists are still present in baseline amounts. The use of a synergist with pyrethrum thus enhances its efficacy in both resistant and susceptible insect strains, allowing for a more cost-effective formulation of pyrethrum. Processes other than enzyme inhibition, such as increasing the penetration of an insecticide through the cuticle or preventing the deterioration of the insecticide are not considered as classical synergism (Metcalf 1967).

Synergism has particularly been associated with the use of pyrethrum because although pyrethrum is a potent knockdown agent, it is only moderately toxic to most insects without the addition of a synergist. Many insects are able to efficiently detoxify the pyrethrins and thus still survive the application (Casida & Quistad 1995, Scott 1990). A synergist used in combination with pyrethrum inhibits the detoxification of the pyrethrins, and therefore enhances the efficacy of pyrethrum (Casida 1970). For this reason, and due to the high cost of pyrethrum, a search was made for a compound having insecticidal properties to replace pyrethrum, or to use in combination with pyrethrum, in order to enhance its efficacy.

In the search for a replacement for pyrethrum, it was discovered that sesame oil synergised pyrethrum, having no insecticidal activity on its own. The active compounds were shown to be sesamin, and the more potent, sesamolin (Beroza 1954, Haller ***et al***. 1942a, 1942b). The synergistic effects were attributed to the methylenedioxyphenol (MDP) ring, and the nature of the substituents on the benzene ring was considered important (Haller ***et al***. 1942a). Sesame oil became one of the first commercially available synergists, but its use was limited due to the difficulty in preparing it in suitable quantities, and its low solubility in freon and petroleum hydrocarbons (used in insecticidal sprays). Piperonyl cyclohexenone was subsequently commercialised as a synergist, but an even more active compound was soon found, piperonyl butoxide (Fig. 1.3), which was completely soluble in freon and petroleum hydrocarbons, and relatively non-toxic to mammals (Wachs 1947). Although a number of synergists with the MDP ring have since been commercialised, for example, sulfoxide, propyl isome and Tropital, none have found as much practical use as PBO. PBO is synthesised from the natural product, safrole (Casida 1970). A rich source of safrole originates from trees in Brazil of the genus ***Ocotea***, however, safrole is also produced synthetically (Casida & Quistad 1995).

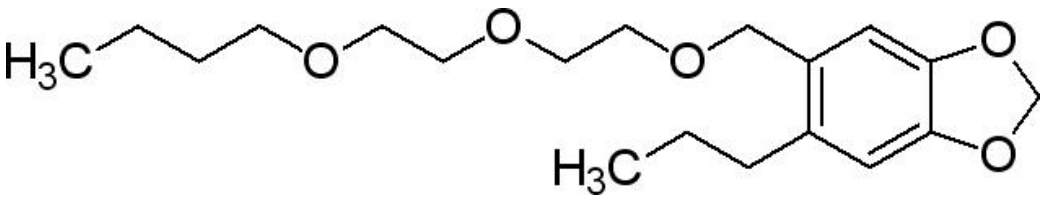

Fig. 1.3. Structure of piperonyl butoxide (PBO). (Source: Sigma-Aldrich 2010).

The efficacy of the MDP compounds as synergists seems to be influenced by the length of the side chains, the functional groups and the position of the double bond in the side chain attached to the benzene ring (Wen ***et al***. 2006). A long, polyether or oxygen-containing side chain seems to be involved in synergism (Casida 1970). Moore & Hewlett (1958) found that a side chain length of six to ten carbon atoms showed pronounced synergistic activity, whereas fewer or more carbon atoms had less activity. The MDP ring is not a necessity for an effective synergist. A number of synergists without the MDP ring have been commercialised, including SKF 525A, MGK 264 and Synepyrin 500 (Casida 1970).

The action of synergists may not necessarily be restricted to one specific metabolic pathway but can be involved in the inhibition of several enzymes. DEF, for example, acts on both P450s and esterases (Scott 1990). PBO has long been known to inhibit P450s (Wilkinson 1976) but has also been found to inhibit esterases (Gunning ***et al***. 1998, Young ***et al***. 2005, 2006). PBO has also been shown to inhibit AChE (Gunning 2006, Kang ***et al.*** 2006). The effects of PBO therefore seem to be multiple, which could explain the high efficacy of PBO.

The efficacy of a synergist is commonly expressed as the synergism factor (SF), which is the ratio of the LD_{50} of insecticide alone to the LD_{50} of insecticide with the synergist (Yamamoto 1973). PBO has been shown to increase the toxicity of many insecticides in resistant insects, resulting in high SF values. In ***B. tabaci***, for example, PBO significantly synergised methamidophos, chlorpyrifos, fenvalerate, avermectin, emamectin benzoate, spinosads, fipronil and imidacloprid (Kang ***et al.*** 2006). In pyrethroid-resistant ***H. virescens***, PBO mixed with amitraz enhanced the toxicity of cypermethrin (Bagwell & Plapp 1992). Significant synergistic effects were found using PBO with methamidophos, fenvalerate, fipronil and avermectin in ***P. xylostella***, ***Phyllotreta striolata*** (F.) (Coleoptera: Chrysomelidae), ***Liriomyza sativae*** Blanchard (Diptera: Agromyzidae), ***Propylea japonica*** Thunberg (Coleoptera: Coccinellidae) and ***Cotesia plutellae*** Kurdjumov (Hymenoptera: Braconidae) (Wu ***et al***. 2007).

There seems to be no one particular best general combination or ratio at which to administer a synergist and an insecticide. The synergistic effect is greatly influenced by the synergist itself, the insecticide used, and the insect species involved. In order to be effective, a synergist should penetrate the insect and be transported to the target enzyme at least equally, but preferably more rapidly, than the insecticide. Once at the target site, the synergist should have a higher affinity for the target enzyme and a lower metabolism rate than the insecticide. The specificity of a synergist can be greatly influenced by any of these processes (Casida 1970, Yamamoto 1973).

A factor known as temporal synergism has also been found to be important when administering synergists in combination with an insecticide. This refers to the amount of time between the application of the synergist and the insecticide (Gunning ***et al***. 1999, Moores ***et al***. 2005, Scott 1990). Pre-treatment with a synergist can increase the amount of synergism found, due to the considerable time it can take for the synergist to maximally inhibit the specific metabolic enzymes involved in resistance

(Bingham ***et al***. 2007, Sawicki 1962, Young ***et al.*** 2005, 2006). The concept of temporal synergism has led to the development of a novel, microencapsulated insecticide with PBO, where PBO is released initially, followed several hours later by release of the insecticide (Patent: Gunning, R.V & Moores, G.D. ***Method and Composition for Combating Pesticide Resistance*** PCT/GB2003/001861). A microencapsulated formulation of PBO with alpha-cypermethrin was found to be effective against a few important agricultural insect pest species, ***H. armigera***, ***M. persicae***, ***A. gossypii*** and ***B. tabaci*** (Bingham ***et al***. 2007).

With the current focus on decreasing environmental contamination and the increasing demand for organic products, a natural, organically-certifiable compound for use as a synergist would be ideal. PBO, although effective as a synergist, is not classified as an organic product in many countries and an organic compound would therefore be required to replace PBO in formulation with pyrethrum. Natural compounds that have been tested for synergistic activity with pyrethrum include myristicin, safrole, isosafrole, sesamin, sesamolin, piperine, dillapiole, croweacin, elemicin and pongapin (Attri ***et al***. 1973, Brooker ***et al***. ***unpublished***). Searches for effective synergists have not yielded many compounds with potential, however, dill apiole was found to be relatively effective as a synergist (Saxena ***et al***. 1977, Singh ***et al***. 1976).

An appropriate combination of pyrethrum with a non-toxic, environmentally benign synergist constitutes one of the best and safest means of insect control (Casida 1970) and is the focus of this PhD study. Important qualities for a good synergist would include low mammalian toxicity, effectiveness against a variety of insects, a rapid rate of absorption into the insect body (to reach the target enzyme before the insecticide), stability in storage, good solubility characteristics and a low cost of production or extraction (Beroza & Barthel 1957).

1.8 Aims and objectives of this PhD

This project aimed to investigate the synergistic qualities of natural plant oils and extracts with pyrethrum against a number of pests of agriculture, cotton bollworm ***H. armigera***, western flower thrips ***Frankliniellla occidentalis*** (Pergande) (Thysanoptera: Thripidae), peach-potato aphid ***M. persicae***, pollen beetle ***Meligethes aeneus*** F. (Coleoptera: Nitidulidae) and a public health pest and pest in livestock and poultry, the house fly ***M. domestica***. The study aimed to identify effective synergists to

potentially replace PBO in formulation with pyrethrum, for possible use in the organic agricultural market.

Specific objectives:

1. To determine ***in vivo*** whether a number of natural plant oils or extracts were capable of synergising pyrethrum (chapters three, four, five, six and seven).
2. To determine ***in vivo*** whether a number of natural plant oils or extracts showed temporal synergism (chapter three).
3. To determine ***in vitro*** whether any of the natural plant oils or extracts were capable of inhibiting the esterase detoxificative enzyme system (chapters three, four, five, six and seven).
4. To determine ***in vitro*** whether any of the natural plant oils or extracts were capable of inhibiting the glutathione-S-transferase detoxificative enzyme system (chapters three, four and six).
5. To determine ***in vitro*** whether any of the natural plant oils or extracts were capable of inhibiting the monooxygenase detoxificative enzyme system (chapters six and seven).
6. To determine whether a correlation existed between the ***in vivo*** and ***in vitro*** results, in order to find a quick assay technique useful for screening the potential of future synergists. This would be useful in initially finding synergists which show potential before doing time-consuming bioassays, which require large numbers of insects all in the same stage of development (chapters three, four, five, six and seven).

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2 CHAPTER TWO: GENERAL MATERIALS AND METHODS

Chapter two gives a description of the insects used in this study and the conditions under which they were reared (section 2.1), details about the insecticides and compounds used as synergists (section 2.2) and the laboratory chemicals used during experiments (section 2.3). Standard bioassay methods are described for each insect species (section 2.4) and procedures for the enzymatic assays are explained (section 2.5). The methods used in analysing data are also described in this chapter (section 2.6). Where any differences from the standard methods occurred, these are described in the relevant chapters.

2.1 Insects

The insects chosen for this study are serious agricultural pests world-wide. Species studied were the cotton bollworm ***Helicoverpa armigera***, western flower thrips ***Frankliniella occidentalis***, peach-potato aphid ***Myzus persicae***, pollen beetle ***Meligethes aeneus*** and dried fruit or sap beetles ***Carpophilus*** spp. (***Carpophilus*** spp. were only used for enzyme inhibition studies with the various synergists). The house fly ***Musca domestica*** is an important pest in poultry and livestock and in public health and was also studied for this project. Synergised pyrethrum could play an important role in an integrated pest management program as an additional means of ***M. domestica*** control, especially around areas where humans, livestock or poultry could be exposed to sprays, since pyrethrum shows low mammalian toxicity and rapid break down in the environment.

No records could be found for insecticide resistance in ***Carpophilus*** spp. but the other species all have well-documented resistance to insecticides. Resistant insect strains, mostly with known metabolic resistance mechanisms and enhanced enzyme activity (***H. armigera*** and ***M. persicae***, for example, with elevated levels of esterases and ***M. aeneus*** with high levels of oxidase activity) were chosen for this study to determine the synergistic potential of various natural compounds as pyrethrum synergists. When greater quantities of the detoxification enzymes are present, the synergistic activity of a compound should be easier to define since the synergistic effect should be more pronounced than when less enzyme is present. Susceptible insects should still show synergistic activity, however, due to the enzymes being present in baseline amounts, but the differences in efficacy of potential synergists could be more difficult to elucidate if synergistic effects were smaller. A susceptible strain of house fly

(WHO) was also included in this study to examine synergistic activity in a susceptible strain.

2.1.1 *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae Heliothinae)

The cotton bollworm ***Helicoverpa armigera*** is a highly polyphagous pest species and occurs world-wide, with the exception of the Americas. ***H. armigera*** is responsible for great economic losses to a variety of crops, including sunflower, tobacco, maize, cotton, soybean, lucerne and cabbage (Firempong & Zalucki 1990). ***H. armigera*** has developed resistance to a number of insecticides, including pyrethroids (Gunning ***et al***. 1984, Kranthi ***et al***. 2001, van Jaarsveld 1994), organophosphates (Gunning & Easton 1993, Gunning ***et al***. 1998) and carbamates (Gunning ***et al***. 1992).

The multiresistant HaC strain of ***H. armigera*** was used for this study due to its resistance status and elevated esterase levels. The HaC strain was established from eggs collected during 2002 and 2003 off cotton grown in New South Wales (NSW) and Queensland (Australia). The bollworms were subjected to heavy exposure of insecticides, including pyrethroids, in the field. The HaC strain was reared in the laboratory at Industry & Investment New South Wales (I & I NSW), Tamworth Agricultural Institute (TAI) (Australia).

H. armigera moths and pupae were kept in 'moth cages' in an insectary at 25 ± 2 °C, 60-80% relative humidity (RH) and natural daylight. Approximately 20 to 30 pupae were placed in each moth cage. Moth cages contained some shredded paper for moths to rest on and a small plastic cup containing two cotton wool balls soaked in a 5% (w/v) sugar solution for moths to feed on. Cloth lids were secured over the top of each cage with an elastic band, for moths to lay eggs on. Cloth lids with eggs were removed three times per week and replaced with new ones. Cloth lids with eggs were put into containers with a small amount of larval medium. Larvae were fed a modified Shorey Hale artificial diet (Gunning ***et al***. 1984, Shorey & Hale 1965) (the recipe is given in Appendix I). When eggs hatched, larvae were transferred to individual wells in a 32-well rearing tray (CD International) with a block of larval medium to feed on. Wells were closed with vented adhesive lids (CD International) for ventilation. Larvae were kept at 25 °C in natural daylight. When larvae in the rearing trays had pupated, pupae were removed from the rearing trays, surface sterilised with disinfectant, sexed and placed into moth cages.

2.1.2 *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae)

Western flower thrips ***Frankliniella occidentalis*** are a serious pest on a number of fruit, vegetable and ornamental crops, especially in greenhouses (López-Soler ***et al***. 2008) and are capable of spreading a number of devastating viral diseases, such as tomato spotted wilt virus (Roselló ***et al***. 1996). Western flower thrips have built up resistance to a number of insecticides, including pyrethroids (Thalavaisundaram ***et al***. 2008), organophosphates (Zhao ***et al***. 1995), carbamates (Jensen 1998), endosulfan (an organochlorine) (Brødsgaard 1994), fipronil (a phenylpyrazole) and spinosad (spinosyns) (Herron & James 2005). Resistance develops relatively quickly in thrips due to their short generation time, haplodiploidy, high fecundity and high polyphagy. Western flower thrips are a particular problem in greenhouses, where favourable climatic conditions lead to continuous availability of hosts and rapid population growth, and where immigration and escape are limited, coupled with heavy use of insecticides to minimise damage to crops by both feeding and viral diseases (Denholm ***et al***. 1998).

A pyrethroid resistant strain of ***F. occidentalis***, Leppington Rose, was obtained from I & I NSW (Dr. Grant A. Herron, Elizabeth Macarthur Agricultural Institute (EMAI), Australia) and maintained in an incubator (at TAI) at 25 ± 1 °C, 65% RH and a 12 : 12 h Light : Dark (L : D) cycle (light intensity at 60 µE). The strain was collected in Leppington (NSW, Australia) off of commercial roses in 2005 and was also resistant to spinosad (Dr. Grant A. Herron, ***Pers. comm.***). Thrips containers had a thin layer of vermiculite at the bottom, covered by a layer of laboratory roll paper and a wire rack over the paper. Each container had two to three ventilation holes drilled through at the sides and lid, covered by a thrips-proof fine mesh (105 µm nylon mesh) (Sefar filter specialists, Blacktown, NSW). The colony was fed on green beans (***Phaseolus vulgaris*** L.), pollen and honey. Five green beans (purchased from the supermarket and rinsed in 2% (w/v) Pyroneg and 1% (v/v) bleach, to sterilise and remove insecticide residues) were placed alongside one another on the wire rack, twice per week. Cumbungi pollen (***Typha*** spp.), collected in Tamworth (Australia) and stored in the freezer at -20 °C, was sprinkled over the green beans. Fresh pollen was collected during December 2009. Honey was spread lightly onto the mesh of the ventilation hole in the lid of the thrips container. Clean thrips containers were set up every two to three weeks, by adding beans infested with thrips from the previous containers.

2.1.3 *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

The peach-potato aphid ***Myzus persicae*** is a polyphagous pest species causing direct feeding damage to a large variety of crops and also transmits a number of viral diseases (Martinez-Torres ***et al.*** 1999). ***M. persicae*** have evolved resistance to organophosphates, carbamates and pyrethroids by overproduction of the closely related carboxylesterases, E4 and FE4, which both hydrolyse and sequester these insecticides (Devonshire & Moores 1982), knockdown resistance ***kdr*** (Martinez-Torres ***et al.*** 1999) and modified acetylcholinesterase (MACE) (Moores ***et al***. 1994).

Two clones, 794JZ and 5191A, were reared in the laboratory at Rothamsted Research (Harpenden, United Kingdom). Clone 794JZ, originally collected from a glasshouse in Evesham, Worcestershire (UK) in 1982, is highly resistant and contains R_3 levels of the resistance-associated esterase, E4. Clone 794JZ is also homozygous for ***kdr***, containing the mutation of a leucine-to-phenylalanine substitution in transmembrane segment IIS6 on the voltage-gated sodium channel (Martinez-Torres ***et al.*** 1999).

Clone 5191A, a neonicotinoid-resistant strain, was collected off tobacco in Greece in 2007. The strain contains the FE4 variant of enhanced esterase at R_3 levels, although resistance to imidacloprid seems rather to be due to enhanced oxidase activity (Philippou ***et al***. 2010) as a result of amplification of a single cytochrome P450 gene (Puinean ***et al***. 2010).

Aphids were reared in cages and in small plastic box-cages (Blackman boxes) at 18 ± 2 °C with a 16 : 8 h L : D cycle. Fine net cages with plastic bases were used, into which a water tray was placed. Two pots were placed into the water tray, each pot containing three seedlings of approximately 2-week-old Chinese cabbage (***Brassica rapa*** L. var. ***pekinensis*** cv. Wong-Bok) (Brassicaceae). To avoid overcrowding, which led to the production of winged (alate) aphid morphs, cages were replaced at approximately 10 day intervals, by setting up a new cage with cabbage seedlings and placing two to three leaves with aphids out of the old cage onto the new seedlings.

Aphids used for bioassays were reared according to the method of Blackman (1971). Small plastic box-cages were set up to contain a sponge in the bottom section of the box and a Chinese cabbage leaf ***B. rapa*** var ***pekinensis*** in the top section, with its petiole extending into the sponge at the bottom (Fig. 2.1). Boxes were placed in trays

of water, with the water level high enough to cover the top of the sponges to keep leaves alive. Two winged aphids were placed from cages onto each cabbage leaf. Each winged aphid would produce unwinged (apterous) morphs. The nymphs were left to mature into adults, which took approximately 10 to 12 days after boxes were set up. Adults (unwinged females) were taken directly from the boxes and used for bioassays.

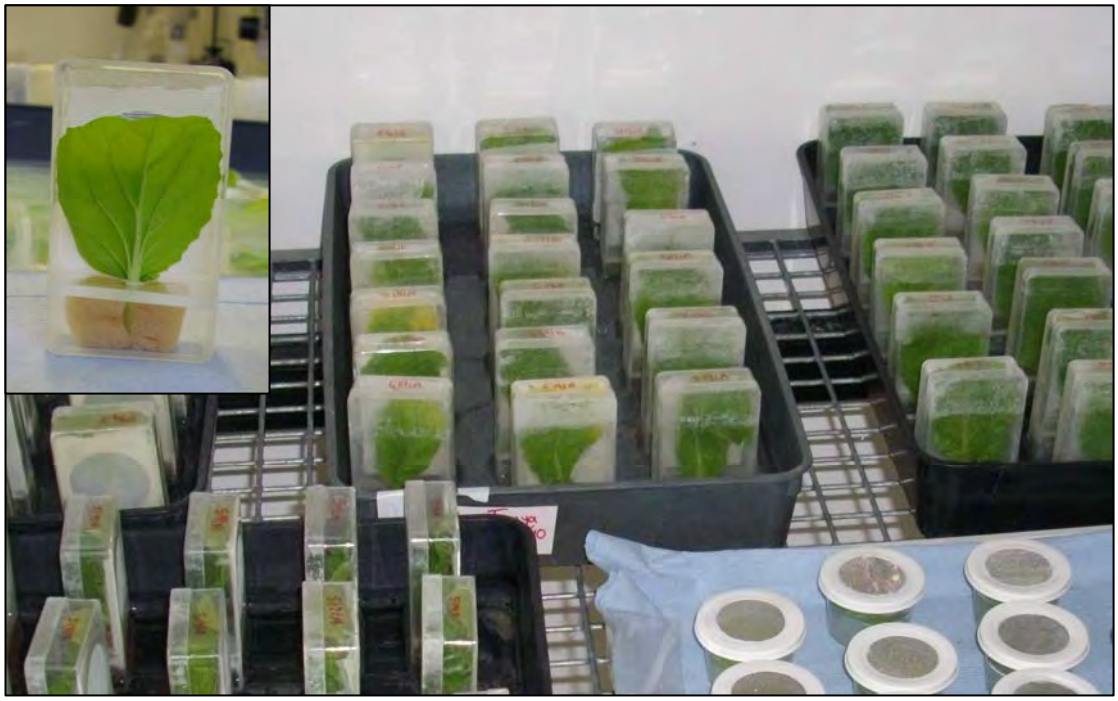

Fig. 2.1. Small plastic box-cages for rearing *Myzus persicae*. (*M. persicae* bioassay pots in the bottom right-hand corner).

2.1.4 *Meligethes aeneus* F. and *Carpophilus* spp. (Coleoptera: Nitidulidae)

The pollen beetle ***Meligethes aeneus*** is a major pest of oilseed rape (***Brassica napus*** L. (Brassicaceae) in Europe, causing great damage to the buds and flowers. Pollen beetles have developed resistance to a variety of insecticides, including pyrethroids, organophosphates and neonicotinoids (Hansen 2003, Węgorek & Zamoyska 2008). Pyrethroid resistance in ***M. aeneus*** has been ascribed to monooxygenase activity (Philippou ***et al***. 2011).

For this study, ***M. aeneus*** were collected from an unsprayed oilseed rape field on the experimental farm at Rothamsted Research (United Kingdom) throughout April and May 2010. Beetles occurred in large numbers on the crop and were used for bioassays and biochemical work within two to three days after collection. No colonies were maintained in the laboratory.

Carpophilus spp. (***Carpophilus davidsoni*** Dobson, ***C. mutilatus*** Erichson and ***C. hemipterus*** (L.)) are known as dried fruit or sap beetles in Australia and are serious pests on ripening fruit, especially berries and stone fruit, such as peaches, plums, nectarines and cherries (Hely ***et al***. 1982, James ***et al***. 1994). ***Carpophilus*** spp. also transfer spores of the fungus ***Monilinia fructicola*** (Wint.) Honey, causing brown rot in apricots and peaches (Kable 1969). The ***Carpophilus*** spp. complex also occurs in cotton flowers when the crop is flowering, where adults and larvae appear to feed on pollen but do not cause any damage to the cotton crop (Bailey 2007).

Carpophilus spp. used for this study were collected off cotton grown at the Liverpool Plains Field Station (I & I NSW, Breeza, Australia) from November 2009 to March 2010. The beetles occurred in large numbers in the cotton flowers and colonies were not maintained in the laboratory. Enzyme assays were done using the field-collected individuals, within four days after collection.

2.1.5 *Musca domestica* L. (Diptera: Muscidae)

The house fly ***Musca domestica*** L. (Diptera: Muscidae) is a serious public health pest and is also a pest in poultry and livestock, capable of transmitting a variety of diseases to both humans and animals (Kristensen ***et al***. 2006).

Two strains of ***M. domestica*** were used for this study, World Health Organisation (WHO) and 381zb. The WHO strain is an insecticide-susceptible reference strain developed and maintained at the University of Pavia (Italy). The colony used for this work was maintained at Rothamsted Research. The resistant strain 381zb was collected in 1978 in Denmark from a multiresistant population of house flies. It is resistant to organophosphates, carbamates and pyrethroids and the resistance is maintained by periodic selection with permethrin and dimethoate. The strain has elevated glutathione-S-transferase (GST) activity (Kristensen 2005) and also contains MACE. Strain 381zb may also have the ***super-kdr*** double mutation of the sodium channel gene that confers resistance to pyrethroids (Kristensen ***unpublished***,

In: Kristensen 2005). The colony used in this study was obtained from the Danish Pest Infestation Laboratory (DPIL) (Denmark) during April 2010 and maintained at Rothamsted Research for one generation before being used for bioassays and biochemical assays.

The house fly colonies were kept at 28 °C, 65% RH and a 16 : 8 h L : D photoperiod with diet and methods adapted from the DPIL rearing protocol (Basden 1946, Kristensen ***et al***. 2006). The adult flies were kept in BugDorm-4090 Insect Rearing Cages (47.5 x 47.5 x 47.5 cm) (Megaview, Taiwan). Each cage contained a 12 oz food container with five white sugar cubes and a 1:1 (w/w) dried milk powder : icing sugar mixture, and had a constant supply of water through ten small test tubes, each filled with water and a cotton wool bung, kept together with an elastic band. Each cage received two bundles of water tubes every third day. An oviposition towel (paper tissue folded into a concertina) soaked in full fat UHT milk was provided (inside a 12 oz container), onto which the adults could lay eggs. After three days, the oviposition towel was transferred to a bucket (5 L) filled with larval diet and a new container with milk and concertinaed towel was placed in the cage with the adult flies (again for removal after three days and transference of the eggs to larval mix). The larval diet was made up of wheat bran (300 g), one sachet of baker's yeast (7 g) and molasses sugar (15 g) (purchased from Sainsbury's, Harpenden, UK). The yeast and molasses sugar were first mixed in 500 ml of hand-hot water (200 ml boiling water plus 300 ml cold water) before being mixed into the bran. The buckets were covered with paper towel secured in place with an elastic band and left at 28 °C for three days for the larvae to develop. The bucket was then transferred to a cage for adult flies and the paper towel removed until adult flies emerged from pupae after seven to 10 days.

Flies reared for bioassays were only given the milk powder : icing sugar mix five days after emergence to ensure all flies were at the same stage of sexual development for bioassays, but water and sugar cubes were available from emergence. Flies (males and females) were used for bioassays nine to 11 days post-emergence.

2.2 Insecticides and synergists

The insecticides used during this study are described in section 2.2.1. Details of the compounds tested as potential synergists and of the standard synergists are given in section 2.2.2.

2.2.1 Insecticides

Six pyrethrum extracts were supplied by Botanical Resources Australia (BRA) Pty Ltd (Devonport, Australia), two technical grade solutions of 50% (w/w) and 75% (w/w) refined pyrethrum extract; an aqueous emulsifiable concentrate (EC) formulation of 4% (w/v) pyrethrins; PyZap, an EC formulation of 4% (w/v) pyrethrins and 16% (w/v) piperonyl butoxide (PBO); PyGanic, an EC formulation of 1.3% (w/v) pyrethrins; and a microencapsulated formulation of 8% (w/v) pyrethrins and 16% (w/v) PBO. Alkamuls® OR36, a surfactant, or emulsifying agent that holds pyrethrum, PBO or other oils in suspension in water, was also supplied by BRA to emulsify oil-based synergists. Technical grade Curacron® (active ingredient: profenofos), an organophosphate (OP) insecticide, was provided by Syngenta. Technical grade imidacloprid, a neonicotinoid insecticide (analytical standard 99.7%), was obtained from Promochem Ltd (Welwyn Garden City, UK). Analytical standard azamethiphos (an OP) was purchased from Sigma (UK).

2.2.2 Synergists

Natural plant oils and extracts tested for synergism of pyrethrum and / or enzyme inhibition in the test insect species, together with the standard synergists used in this PhD, are listed in Table 2.1. (The potential synergists are referred to as ‘synergist’ or ‘compound’ interchangeably throughout this thesis, even though compounds may not necessarily have shown synergistic activity). Potential synergists were chosen on the basis of known insecticidal activity (for example, neem oil), or insect repellence properties (for example, citronella oil), or because of the presence of the methylenedioxyphenol (MDP) ring structure (such as is found in sassafras oil and PBO), or based on the presence of plant metabolites as natural plant defence chemicals against insect herbivory (for example, eucalyptus oil). Dill apiole is well known from the literature as possessing synergistic activity towards pyrethrum. Dill apiole has shown synergistic activity towards pyrethrum against adult house flies (Saxena ***et al***. 1977) and red flour beetle ***Tribolium castaneum*** (Herbst) (Singh ***et al***.

1976). Dill seed oil, from the same plant species as the dill apiole oil used in this study (***Anethum graveolens***) was also investigated for synergistic potential, since different constituents were more prevalent in dill seed oil (carvone and limonene) than dill apiole. Differences in synergistic activity may therefore be observed between the two oils. Preliminary studies with ***H. armigera*** indicated efficacy of parsley seed oil as a pyrethrum synergist (Dr. Robin V. Gunning, ***Pers. comm.***). Myristicin, a constituent of parsley seed oil was tested only for enzyme inhibition (in some of the ***H. armigera***, ***F. occidentalis*** and ***Carpophilus*** spp. enzyme inhibition assays) due to very limited available quantity, which was not sufficient for insect bioassays.

The idea of using Australian or Tasmanian native plants as novel pyrethrum synergists was examined by studying plant extracts from ***Boronia citriodora, Correa*** spp. and ***Zieria arborescens*** (Table 2.1). A literature search was initially undertaken to determine plant species or families that may possess similar chemistry to safrole or PBO, with constituents containing the MDP ring (Brooker ***et al***. ***unpublished***) and extracts were prepared for use in this study (Dr Adrian Blackman, School of Chemistry, University of Tasmania, Hobart, Australia) to examine their synergistic potential and enzyme-inhibiting capabilities.

Table 2.1. Details of the standard synergists and the plant oils and extracts tested for synergistic potential throughout this study*.

Common name (used throughout thesis) (Biological activity) (If any and if known)	Description of sample ***(Source)***[¥]	Speculated inhibitors of interest (if known) Chemical formula (molecular weight)	Type of chemical
Piperonyl butoxide (PBO) (Standard synergist) (used at TAI)	Synthetic, 90% pure ***(Aldrich, Australia)***	PBO $C_{19}H_{30}O_5$ (338.44)	Methylenedioxyphenol ring
Piperonyl butoxide (PBO) (Standard synergist) (used at Rothamsted)	Synthetic, 94% pure ***(Endura SpA, Italy)***	PBO $C_{19}H_{30}O_5$ (338.44)	Methylenedioxyphenol ring
Triphenyl phosphate (TPP) (Standard synergist)	≥99% pure ***(EMAI)***	TPP $(C_6H_5O)_3PO$ (326.28)	Ester (triester)
Diethyl maleate (DEM)	97% pure ***(Sigma-Aldrich, Australia)***	DEM $C_8H_{12}O_4$	Ester

(Standard synergist)		(172.18)	
BcB1 extract[§]	A solvent extract, petroleum spirits fraction from lemon-scented Boronia ***Boronia citriodora*** Gunn ex Hook. f. (Rutaceae) ***(UTas)***		
BcB2 extract[§]	A solvent extract, dichloromethane fraction from ***B. citriodora*** ***(UTas)***		
CaB2 extract[§]	A solvent extract, dichloromethane fraction from ***Correa alba*** Andrews var ***alba*** (Rutaceae) ***(UTas)***	Elemicin $C_{12}H_{16}O_3$ (208.25)	Phenylpropene
CaB2 frac3 extract[§]	A fractionated extract of CaB2 ***(UTas)***	Elemicin $C_{12}H_{16}O_3$ (208.25)	Phenylpropene
ClB1 extract[§]	A solvent extract, petroleum spirits fraction from mountain Correa ***Correa lawrenceana*** Hook. (Rutaceae) ***(UTas)***	Limonene $C_{10}H_{16}$ (136.23)	Cyclic terpene
ClB2 extract[§]	A solvent extract, dichloromethane fraction from ***C. lawrenceana*** ***(UTas)***	Limonene $C_{10}H_{16}$ (136.23)	Cyclic terpene
ZaB2 extract[§]	A solvent extract, dichloromethane fraction from stinkwood ***Zieria arborescens*** Sims (Rutaceae) ***(UTas)***	Zierone $C_{15}H_{22}O$ (218.34)	Sesquiterpenoid
Angelica root oil (Antifeedant properties) (Pavela 2010)	Essential oil from roots of ***Angelica archangelica*** L., ***Heracleum*** spp. and ***Selinum vaginatum*** (Edgew.) C.B. Clarke (Apiaceae) ***(Auroma)***	Angelicin $C_{11}H_6O_3$ (186.16) alpha-Pinene $C_{10}H_{16}$ (136.23)	Angular furocoumarin Bicyclic monoterpene
Aniseed oil (Insect repellence properties) (Erler ***et al***. 2006)	A 100% (v/v) pure essential oil from the dry ripe fruits of ***Pimpinella anisum*** L. (Apiaceae) ***(Sunspirit Aromatherapy)***	***trans***-Anethole $C_{10}H_{12}O$ (148.20)	Unsaturated ether / phenylpropene derivative
Bergamot oil (Insecticidal activity) (Papachristos ***et al***. 2009)	A 100% (v/v) pure essential oil, cold pressed, from the fresh pericarp of the fruit of ***Citrus aurantium*** L. var ***bergamia*** (Risso) Wright & Arnott. (Rutaceae) ***(Sunspirit Aromatherapy)***	Limonene $C_{10}H_{16}$ (136.23) Linalyl acetate $C_{12}H_{20}O_2$ (196.29)	Cyclic terpene Acetate ester
Black pepper oil (Synergistic properties due to methylenedioxyphe-nol ring) (Jensen ***et al***. 2006)	Essential oil from unripe fruit of the wild pepper ***Piper cubeba*** L. (Piperaceae) and roots and shoots of ***Aristolochia triangularis*** Cham. (Aristolochiaceae) ***(Auroma)***	Safrole $C_{10}H_{10}O_2$ (162.19) Cubebin $C_{20}H_{20}O_6$ (356.38) Piperine	Methylenedioxyphenol ring / phenylpropene Methylenedioxyphenol ring / lignan Methylenedioxyphenol

		$C_{17}H_{19}NO_3$ (285.34)	ring / alkaloid
Canola oil	Oil from ***Brassica napus*** L. (Brassicaceae) ***(BRA)***	Oleic acid $C_{18}H_{34}O_2$ (282.46) Linoleic acid $C_{18}H_{32}O_2$ (280.45)	Mono-unsaturated omega-9 fatty acid Unsaturated omega-6 fatty acid
Citronella oil (Insect repellence properties) (Barnard & Xue 2004)	A 100% (v/v) pure essential oil from the aerial parts, fresh or partially dried, of ***Cymbopogon nardus*** (L.) W. Watson (Poaceae) ***(Sunspirit Aromatherapy)***	Geraniol $C_{10}H_{18}O$ (154.25) Limonene $C_{10}H_{16}$ (136.23)	Monoterpenoid / alcohol Cyclic terpene
Citronella java oil (Insect repellence properties)	Essential oil from leaves of ***Cymbopogon winterianus*** Jowitt (Poaceae) ***(Bronson and Jacobs)***	Citronellal $C_{10}H_{18}O$ (154.25) Geraniol $C_{10}H_{18}O$ (154.25)	Monoterpenoid Monoterpenoid / alcohol
Cypress oil (Insecticidal activity) (Kanat & Alma 2004)	Essential oil from needles and terminal branchlets of ***Cupressus sempervirens*** L. (Cupressaceae) ***(Auroma)***	alpha-Pinene $C_{10}H_{16}$ (136.23)	Bicyclic monoterpene
Dill apiole oil (Synergistic properties) (Saxena ***et al***. 1977, Singh ***et al***. 1976)	Oil from ***Anethum graveolens*** L. (Apiaceae) ***(BRA)***	Dill apiole $C_{12}H_{14}O_4$ (222.24)	Methylenedioxyphenol ring / phenylpropanoid
Dill seed oil (Possible synergistic properties)	Oil from the seeds of ***Anethum graveolens*** L. (Apiaceae) ***(BRA)***	Carvone $C_{10}H_{14}O$ (150.22) Limonene $C_{10}H_{16}$ (136.23)	Terpenoid Cyclic terpene
Eucalyptus oil (Insect repellence properties (Erler ***et al***. 2006); Plant metabolites; Insecticidal activity) (Kanat & Alma 2004)	A 100% pure, naturally sourced essential oil from ***Eucalyptus*** spp. (Myrtaceae) ***(Thursday Plantation Essential Care)***	Eucalyptol $C_{10}H_{18}O$ (154.25)	Monoterpenoid / ether
Fennel oil (Insect repellence properties) (Kim ***et al***. 2002)	An essential oil from ***Foeniculum vulgare*** Miller (Apiaceae) ***(Essential Oils of Tasmania Pty Ltd)***	***trans***-Anethole $C_{10}H_{12}O$ (148.20) Fenchone $C_{10}H_{16}O$ (152.23)	Unsaturated ether / phenylpropene derivative Monoterpene / ketone
Garlic oil (Insecticidal activity	Essential oil from cloves of ***Allium sativum*** L. (Alliaceae)	Diallyl trisulfide $C_6H_{10}S_3$	Sulphur compound

(Machial ***et al***. 2010) and insect repellence properties) (Rahman & Motoyama 2000)	***(Auroma)***	(178.34) Diallyl sulfide $C_6H_{10}S$ (114.21)	 Sulphur compound
Geranium oil (Insect repellence properties) (Barnard & Xue 2004)	A 100% (v/v) pure essential oil from herbaceous parts of ***Pelargonium graveolens*** L'Hértier ex Aiton and ***Pelargonium roseum*** Willdenow. (Geraniaceae) ***(Sunspirit Aromatherapy)***	Citronellol $C_{10}H_{20}O$ (156.27) Geraniol $C_{10}H_{18}O$ (154.25)	Monoterpenoid / alcohol Monoterpenoid / alcohol
Grapefruit oil (Insect repellence properties) (Zhu ***et al***. 2010)	A 100% (v/v) pure essential oil, cold pressed, from fruit peel of ***Citrus x paradisi*** Macfadyen (Rutaceae) ***(Sunspirit Aromatherapy)***	Limonene $C_{10}H_{16}$ (136.23)	Cyclic terpene
Lavender oil (Insecticidal activity) (Kanat & Alma 2004)	Essential oil from ***Lavandula angustifolia*** Miller (Lamiaceae) ***(Bronson and Jacobs)***	Linalool $C_{10}H_{18}O$ (154.25) Linalyl acetate $C_{12}H_{20}O_2$ (196.29)	Terpene / Monoterpenol Acetate ester
Manuka oil (Plant metabolites; Insecticidal activity) (George ***et al***. 2010)	Essential oil from ***Leptospermum scoparium*** J.R. Forster & G. Forster (Myrtaceae) ***(BRA)***	trans-Calamenene $C_{15}H_{22}$ (202.34) Leptospermone $C_{15}H_{22}O_4$ (266.33)	Sesquiterpene hydrocarbon Triketone derivative
Melaleuca oil (Anti-septic, anti-bacterial, anti-fungal, anti-viral properties)	Tea tree oil from ***Melaleuca alternifolia*** (Maiden & Betche) Cheel. (Myrtaceae) ***(Thursday Plantation essential oil)***	Terpinen-4-ol $C_{10}H_{18}O$ (154.25) gamma-Terpinene $C_{10}H_{16}$ (136.23)	Terpene Terpene
Myristicin oil (Synergistic properties) (Berenbaum & Neal 1985, Lichtenstein & Casida 1963)	From parsley leaf oil (≥85%) ***(Sigma-Aldrich, Australia)***	Myristicin $C_{11}H_{12}O_3$ (192.21)	Methylenedioxyphenol ring / phenylpropene
Neem oil (Insecticidal activity (Adarkwah ***et al***. 2010) and insect repellence properties) (Mikami & Ventura 2008, Xie ***et al***. 1995)	Oil from seeds of the neem tree ***Azadirachta indica*** Adr. Juss. (Meliaceae) ***(Auroma)***	Azadirachtin $C_{35}H_{44}O_{16}$ (720.71)	Tetranortriterpenoid / liminoid
Nutmeg oil (Possible synergistic properties due to	Essential oil from seeds of ***Myristica fragrans*** Houtt. (Myristicaceae) ***(Auroma)***	alpha-Pinene $C_{10}H_{16}$ (136.23)	Bicyclic monoterpene

methylenedioxyphe-nol ring)		Safrole $C_{10}H_{10}O_2$ (162.19) Myristicin $C_{11}H_{12}O_3$ (192.21)	Methylenedioxyphenol ring / phenylpropene Methylenedioxyphenol ring / phenylpropene
Oleic acid (Component of the BRA emulsifiable concentrate (EC) formulation of pyrethrum)	A pure sample from an unspecified plant or animal source. Oleic acid is found in a variety of plant oils, such as olive oil (Dabbou ***et al***. 2010), peanut oil (Singkham ***et al***. 2010), cactus pear seed oil (Labuschagne & Hugo 2010) and sesame oil (Bahkali ***et al***. 1998). (***BRA***)	Oleic acid $C_{18}H_{34}O_2$ (282.46)	Mono-unsaturated omega-9 fatty acid
Parsley seed oil (Synergistic properties) (Berenbaum & Neal 1985, Lichtenstein & Casida 1963, Lichtenstein ***et al***. 1974)	Oil from seeds of ***Petroselinum crispum*** (Mill.) Nyman ex A.W. Hill (Apiaceae) ***(BRA)***	Myristicin $C_{11}H_{12}O_3$ (192.21) alpha-Pinene $C_{10}H_{16}$ (136.23) Apiole $C_{12}H_{14}O_4$ (222.24)	Methylenedioxyphenol ring / phenylpropene Bicyclic monoterpene Methylenedioxyphenol ring / phenylpropanoid
Peppermint oil (Insecticidal activity) (Samarasekera ***et al***. 2008)	Essential oil from ***Mentha piperita*** L. (Lamiaceae) ***(Essential Oils of Tasmania Pty Ltd)***	Menthol $C_{10}H_{20}O$ (156.27) Menthone $C_{10}H_{18}O$ (154.25)	Monoterpenoid Monoterpene / ketone
Platyphyllol oil (Insect repellence properties)	Oil from the cajuput tree ***Melaleuca cajuputi*** Powell subsp. ***platyphylla*** Barlow (Myrtaceae) ***(BRA)***	Platyphyllol (6,6-dimethyl-2-acetyl-5-methoxycyclohex-4-ene-1,3-dione)	Oxygenated sesquiterpene / Sesquiterpenol
Propyl gallate (antioxidant)	An antioxidant that protects against oxidation by hydrogen peroxide and oxygen free radicals ***(Sigma-Aldrich, Australia)***	Propyl gallate $C_{10}H_{12}O_5$ (212.20)	Ester
Rosemary oil (Insect repellence properties) (Momen ***et al***. 2001)	A 100% (v/v) pure essential oil from the flowering aerial parts of ***Rosmarinus officinalis*** L. (Lamiaceae) ***(Sunspirit Aromatherapy)***	Eucalyptol $C_{10}H_{18}O$ (154.25) alpha-Pinene $C_{10}H_{16}$ (136.23) Camphor $C_{10}H_{16}O$ (152.23)	Monoterpenoid / ether Bicyclic monoterpene Terpenoid
Sassafras oil (Synergistic	Unspecified plant source ***(BRA)***	Safrole $C_{10}H_{10}O_2$	Methylenedioxyphenol ring / phenylpropene

properties)		(162.19)	
Sesame oil (Synergistic properties) (Beroza 1954, Haller ***et al***. 1942a, 1942b)	Oil from ***Sesamum indicum*** L. (Pedaliaceae) ***(BRA)***	Sesamin $C_{20}H_{18}O_6$ (354.35) Sesamolin $C_{20}H_{18}O_7$ (370.35)	Methylenedioxyphenol ring / lignan Methylenedioxyphenol ring / lignan
White cypress oil (Insecticidal activity)	Essential oil from the leaves and twigs of ***Callitris columellaris*** F.Muell. (Synonym: ***C. glaucophylla*** Thompson & Johnson) (Cupressaceae) ***(BRA)***	alpha-Pinene $C_{10}H_{16}$ (136.23) Myrcene $C_{10}H_{16}$ (136.23) Limonene $C_{10}H_{16}$ (136.23)	Bicyclic monoterpene Monoterpene Cyclic terpene

* Information from the chromatographic profiles of each oil (from the essential oil suppliers) and from Sigma-Aldrich (2010).

[¥] Sources of samples: Auroma (Victoria, Australia); Bronson and Jacobs (NSW, Australia); Essential oils of Tasmania Pty Ltd (Tasmania, Australia); Sunspirit Aromatherapy essential oils (Good Price Pharmacy Warehouse, Tamworth, Australia); Elizabeth Macarthur Agricultural Institute (EMAI) (Australia) (kindly donated by Dr Martin McLoon); Botanical Resources Australia (BRA) Pty Ltd; The University of Tasmania (UTas) (provided by Dr Adrian Blackman, School of Chemistry, University of Tasmania, Hobart, Australia); and Sigma-Aldrich (Australia).

[§] Novel potential synergists from Australian or Tasmanian native plants.

2.3 Laboratory chemicals

All chemicals were stored at room temperature and purchased from Sigma-Aldrich Australia (for chemicals used at TAI) or UK (for chemicals used at Rothamsted) unless otherwise stated:

- Di-sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$) (AnalaR BDH, purchased from Bacto laboratories (TAI) and Sigma (Rothamsted)).
- Potassium dihydrogen orthophosphate (KH_2PO_4) (AnalaR BDH, purchased from Bacto laboratories (TAI) and Sigma (Rothamsted)).
- Ethylenediaminetetraacetic acid (EDTA) ($(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$).
- Dithiothreitol (DTT) ($HSCH_2CH(OH)CH(OH)CH_2SH$), (stored at 4 °C).
- Phenylthiourea (PTU) ($C_6H_5NHCSNH_2$).
- Phenylmethanesulfonyl fluoride (PMSF) ($C_7H_7FO_2S$).

- 7-Ethoxycoumarin ($C_{11}H_{10}O_3$).
- beta-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH) ($C_{21}H_{26}N_7Na_4O_{17}P_3.xH_2O$), (stored at -20 °C).
- Fast Blue RR salt ($C_{15}H_{14}ClN_3O_3.1/2\ ZnCl_2$), (stored at 4 °C).
- alpha-Naphthyl acetate ($CH_3CO_2C_{10}H_7$), (stored at -20 °C).
- para-Nitrophenyl acetate ($CH_3CO_2C_6H_4NO_2$), (stored at 4 °C).
- Acetylthiocholine iodide (ATChI) ($CH_3COSCH_2CH_2N(CH_3)_3I$), (stored at -20 °C).
- 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) ($[-SC_6H_3(NO_2)CO_2H]_2$).
- 1-Chloro-2,4-dinitrobenzene (CDNB) ($Cl_2C_6H_3NO_2$).
- L-Glutathione reduced (GSH) ($H_2NCH(CO_2H)CH_2CH_2CONHCH(CH_2SH)CONH CH_2CO_2H$), (stored at 4 °C).
- Glutathione reductase from bakers yeast (***Saccharomyces cerevisiae***) (200 units per mg protein) (ammonium sulfate suspension), (stored at 4 °C).
- L-Glutathione oxidised ($C_{20}H_{32}N_6O_{12}S_2$), (stored at 4 °C).
- Triton X-100 (especially purified for membrane research) (***t***-Oct-C_6H_4-$(OCH_2CH_2)_xOH$, x= 9-10) (purchased from Roche Diagnostics GmbH).
- G-25 Sephadex™ fine (purchased from Amersham Biosciences).

2.4 Bioassays

Two types of bioassays were performed during this study, full dose response and discriminating dose bioassays (ffrench-Constant & Roush 1990). For dose response bioassays, insects were exposed to a range of dosages of an insecticide causing between 5% and 100% mortality. Probit analysis was used to calculate lethal dose (LD_{10}, LD_{50} and LD_{90}) values, giving the dosage required to kill 10%, 50% and 90% of the test population respectively (Busvine 1971, Finney 1971). From this data, the LD_{10} was generally used for discriminating dose bioassays as a fixed concentration of insecticide in combination with potential synergists, to compare the relative efficacy of the synergists to one another and to unsynergised insecticide.

Bioassays were undertaken on ***H. armigera***, ***F. occidentalis***, ***M. persicae***, ***M. aeneus*** and ***M. domestica***.

2.4.1 *Helicoverpa armigera*

H. armigera larval bioassays were done by topical application, using a microapplicator (fixed needle syringe, 50 µl volume) (Hamilton co., Reno, Nevada) to apply 1 µl of treatment on the dorsal thorax of each larva. Diet incorporation bioassays were done by mixing synergists into the standard larval-rearing diet. The diet containing synergist was fed to larvae for five hours, after which, larvae were returned back to the standard diet. Pyrethrum was applied topically, immediately after larvae were placed back onto the standard diet. A potential problem could lie with using diet incorporation bioassays, however, when insects may not want to feed on the manipulated diet, which could influence results. Technical grade (75% (w/w)) pyrethrum was diluted with acetone to make serial dilutions of pyrethrum for bioassays. All ***H. armigera*** bioassays were done in standard 128-well bioassay trays (CD International), using vented adhesive lids. Larvae within the weight range of 25 to 40 mg (third instar) were used. Acetone controls were included in all trials. After treatment, larvae were left on sufficient quantity of food at a constant temperature of 25 °C with normal daylight hours. Mortality was assessed after 72 hours. Larvae incapable of acting in a coordinated way after being probed with forceps were counted as dead.

2.4.2 *Frankliniella occidentalis*

F. occidentalis bioassays were done using a leaf dip technique. A 4% (w/v) aqueous EC pyrethrum solution was used to make a serial dilution of pyrethrum, using distilled water. Distilled water was used as a control. Alkamuls® diluted in distilled water was also used as a control to determine whether it showed any toxicity on its own, as the oil-based synergists were diluted in Alkamuls for the discriminating dose bioassays. Leaf discs (25 mm diameter) were cut from the cotyledons of cotton ***Gossypium hirsutum*** L. (Solanaceae) (Cultivar: Sicot 189) and dipped into each treatment for 20 seconds. Leaf discs were left to dry in a laminar flow cabinet. Thrips (adult females, approximately two weeks old) were collected using an electric suction pump, to suck approximately twenty to thirty thrips into vials. When leaf discs were dry, leaves were placed adaxial surface down into petri dishes (50 x 9 mm, Falcon 35-1006) with close-fitting lids. Before lids were closed, thrips from one vial were emptied onto the leaf surface and petri dish lids were quickly closed to prevent thrips from escaping. Petri dishes were left at 25 °C with normal daylight hours after treatment. Mortality

was assessed after 48 hours using a stereomicroscope. Moribund thrips were scored as dead.

2.4.3 *Myzus persicae*

Bioassays on ***M. persicae*** were done by topical application using a Rothamsted standard technique. Technical grade pyrethrum (50% (w/w)) was serially diluted with acetone to obtain a range of dosages. Plastic 30 ml containers (30 mm x 45 mm diameter) were filled with approximately 20 ml of a boiled, aqueous 1% agar solution. A cotton bud was used to apply Fluon (Whitford Plastics, UK) around the top inner surface of the pots, above the agar to prevent aphids from climbing up onto the lids. Leaf discs (38 mm diameter) were cut from Chinese cabbage cv Wong Bok and placed, untreated onto the agar, abaxial surface up. Ten aphids (adult unwinged females, approximately 10 to 12 days old) were transferred from Blackman boxes onto each leaf disc and left to settle for approximately one hour before being treated. Aphids were treated topically with 0.25 µl of treatment, using a Burkard micro-applicator (Burkard Scientific; Uxbridge, Middlesex) and 1 ml glass syringe with a 25 g stainless steel needle. Plastic lids with holes covered with a fine wire mesh for ventilation were placed over pots to keep aphids inside. Treated aphids were left at 18 ± 2 °C with a 16 : 8 h L : D cycle. Mortality was assessed after 72 hours using a stereomicroscope. Moribund aphids were scored as dead.

2.4.4 *Meligethes aeneus*

M. aeneus bioassays were done using a coated vial technique. Pyrethrum (technical grade 50% (w/w)) was diluted with acetone into a range of concentrations. Into each vial, 500 µl of solution was added and vials were placed directly onto a blood roller (Luckham Multimix major) until all acetone had evaporated (approximately 30 minutes). An electric suction pump was used to suck approximately ten pollen beetles (field-collected adult males and females, unsexed) into each vial. Lids were closed loosely for ventilation. Vials were left at 18 ± 2 °C with a 16 : 8 h L : D cycle. Mortality was assessed after 24 hours. Beetles unable to walk in a coordinated manner after being probed with forceps were scored as dead.

2.4.5 *Musca domestica*

M. domestica bioassays were done by topical application, using a Burkard micro-applicator with 1 ml glass syringe and a 25 g stainless steel needle. Technical grade pyrethrum (50% (w/w)) was serially diluted with acetone to obtain a range of dosages. A battery operated aspirator was used to collect adult flies from rearing cages. Male and female flies (unsexed, nine to 11 days post-emergence) were used. Flies were briefly anaesthetised with CO_2 and 1 µl of a treatment solution was applied to the dorsal thorax of each fly, avoiding the wings. Approximately ten flies were placed into plastic cups (200 ml) after treatment and netting was secured over the top with an elastic band to prevent flies from escaping. A dental roll soaked in 50% (w/v) sugar solution was left on the netting of each bioassay cup for the flies to feed on. Flies were left at 25 °C, with normal daylight hours after treatment. Mortality was assessed after 24 hours. Moribund flies were scored as dead.

2.5 Microplate assay enzymatic techniques

Metabolic mechanisms conferring resistance to insecticides have been shown to be mainly due to three enzyme systems in insects, esterases, glutathione-S-transferases (GSTs) and monooxygenases, the latter also referred to as oxidases or cytochrome P450's (CP450s). In some resistant insects, these enzyme systems are responsible for breaking down the insecticide or its active metabolites, rendering the insecticide ineffective. Synergists, such as PBO, work by inhibiting specific pathways involved in detoxification of an insecticide or its active metabolites so that the insecticide is still able to reach its target site and kill the insect. The inhibitory effects of the natural plant compounds on insect detoxificative enzyme systems were therefore studied. Each of the methods used to detect inhibition are described below, for esterases (section 2.5.1), GSTs (section 2.5.2) and monooxygenases (section 2.5.3). Details specific to each insect are discussed in the relevant chapters.

2.5.1 Esterase assays

Assays were done to measure total esterase activity directly, using two different substrates, alpha-naphthyl acetate (section 2.5.1.1) and para-nitrophenyl acetate (section 2.5.1.2). The esterase interference assay was used to measure esterase activity indirectly (section 2.5.1.3).

2.5.1.1 Esterase assay with alpha-naphthyl acetate as a substrate

Total esterase activity was measured using a colourimetric assay modified from Grant ***et al.*** (1989). Esterase activity was determined by measuring the rate of hydrolysis of alpha-naphthyl acetate to alpha-naphthol and acetate, according to Gunning ***et al.*** (1996). All wells in a 96-well microplate (transparent, Titertek®) were filled with 25 µl of 0.02 M phosphate buffer, pH 7. An additional 22 µl of 0.02 M phosphate buffer, pH 7, was added to the first column of the plate. Acetone (as control) (3 µl) or 100 000 ppm synergist solution (3 µl) was added to the first column, and a two-fold serial dilution was made across the plate until well 11 (with a different synergist per row). The last well was left as an uninhibited control (Fig. 2.2).

Into each well, 25 µl of freshly prepared insect homogenate was added and the plate was left to incubate at 25 °C for 30 minutes. After incubation, 200 µl of a 1 mM alpha-naphthyl acetate solution containing 1.5 mM Fast blue RR (FBRR) salt (diluted in 0.2 M phosphate buffer, pH 6) was added to each well and a kinetic assay was run on a ThermoMax (Rothamsted) or SpectraMax M2 (TAI) microplate reader (Molecular Devices) at 450 nm for 10 minutes with 18 second intervals between readings, using the Software SoftMax Pro version 5.2 (TAI) and SoftMax Pro version 5.4 (Rothamsted) to monitor the Vmax values. The reaction produces a complex with a characteristic absorbance at 450 nm. To determine if any of the synergists themselves interfered with the assay, buffer blanks were run for each synergist in the same way as above, by adding 25 µl of 0.02 M phosphate buffer, pH 7 into each well instead of insect homogenate.

Well:	1	2	3	4	5	6	7	8	9	10	11	12
Row A	25 µl P containing 300 ppm synergist	25 µl P containing 150 ppm synergist	25 µl P containing 75 ppm synergist	25 µl P containing 37.5 ppm synergist	25 µl P containing 18.8 ppm synergist	25 µl P containing 9.4 ppm synergist	25 µl P containing 4.7 ppm synergist	25 µl P containing 2.3 ppm synergist	25 µl P containing 1.2 ppm synergist	25 µl P containing 0.6 ppm synergist	25 µl P containing 0.3 ppm synergist	25 µl P

Fig. 2.2. Setup of one row in a 96-well microplate containing a serial dilution of a synergist for the esterase assay with alpha-naphthyl acetate as a substrate. (P = 0.02 M phosphate buffer, pH 7).

2.5.1.2 Esterase assay with para-nitrophenyl acetate as a substrate

Total esterase activity was determined by measuring the rate of hydrolysis of para-nitrophenyl acetate towards para-nitrophenol and acetate, based on a method by Pocker & Stone (1967). All wells in a 96-well microplate (transparent, NUNC) were filled with 25 µl of 0.02 M phosphate buffer, pH 7. An additional 22 µl of 0.02 M phosphate buffer, pH 7, was added to the first and seventh column of the plate. Acetone (as control) (3 µl) or 10 000 ppm synergist solution (3 µl) was added to the first or seventh column and a two-fold serial dilution was made across the next four wells. The sixth well was left without inhibitor as a control. Into each well, 25 µl of freshly prepared insect homogenate was added and the plate was left to incubate for 30 minutes at room temperature. After incubation, 200 µl of 1 mM para-nitrophenyl acetate (diluted in 0.02 M phosphate buffer, pH 7) was added to each well and a kinetic assay was run on a ThermoMax microplate reader (Molecular Devices) at 405 nm for 10 minutes with 10 second intervals between readings, using the Software SoftMax Pro version 5.4 (Rothamsted) to monitor the Vmax values. To determine if any of the synergists interfered with the assay, buffer blanks were run for each synergist in the same way as above, by adding 25 µl of 0.02 M phosphate buffer, pH 7 into each well instead of insect homogenate.

2.5.1.3 Esterase interference assay

Esterase activity was measured indirectly using the method of Khot ***et al***. (2008). This assay measures the sequestration of azamethiphos (a potent acetylcholinesterase (AChE) inhibitor) in the absence and presence of a synergist. In some insects it would seem that a synergist does not always bind to the hydrolytic active site of an enzyme but may bind in such a way so as to occlude entry of the model substrate, whereas in other insects it could still be hydrolysed. Inhibition may therefore not always be detected using the conventional total esterase assay methods described above. In the esterase interference assay, AChE activity is measured as a way of determining the ability of the synergist to bind to esterases. When no esterases are present, the azamethiphos inhibits AChE activity. In the presence of esterases, the esterases bind to azamethiphos and the azamethiphos is unable to inhibit AChE activity. When a synergist binds to the esterases, the esterases are unable to bind to azamethiphos and the azamethiphos remains available to inhibit AChE activity. A reduction in AChE activity is thus observed in this assay when a synergist binds to the esterases. The ability of the esterases to protect AChE from inhibition by

azamethiphos is reduced when the esterase is blocked by a synergist, as has been found with PBO, blocking E4 of ***M. persicae*** (Khot ***et al***. 2008).

For the interference assay, synergists were incubated at 4 °C for 16 h with purified ***M. persicae*** E4 or with ***H. armigera*** esterases that had been run through a G-25 column. All wells in a 96-well microplate (transparent, NUNC) were filled with 25 µl of 0.02 M phosphate buffer, pH 7. An additional 22 µl of 0.02 M phosphate buffer, pH 7, was added to the first column of the plate. Into every well in the first column, 3 µl of $1x10^{-6}$ M azamethiphos was added and a two-fold serial dilution was made across the plate, until well 11. The last well was left as an uninhibited control. To each well in the first row (the azamethiphos only row), 50 µl of 0.02 M phosphate buffer, pH 7 was added. To each well in the second row (the esterase only row), 25 µl of the enzyme:acetone solution was added across the row, plus 25 µl of 0.02 M phosphate buffer, pH 7. To each well in subsequent rows, 25 µl of the enzyme:synergist solution was added across the row, plus 25 µl of 0.02 M phosphate buffer, pH 7. Plates were incubated for one hour at room temperature. House fly head homogenate (house flies not sexed or aged) was made up as a source of AChE using ***M. domestica*** heads. House fly heads were homogenised in 0.02 M phosphate buffer, pH 7 containing 0.1% (w/v) Triton X-100. Homogenate was centrifuged at 10 000 rpm for one minute and supernatant was used for the assay. After the incubation, 25 µl of house fly head homogenate was added to each well and the plate was left to incubate for 15 minutes at room temperature. After incubation, 100 µl of 1.5 mM ATChI and 100 µl of 1.5 mM DTNB were added to each well. A kinetic assay was run immediately on a ThermoMax microplate reader (Molecular Devices) at 405 nm for 10 minutes at 10 second intervals, using the Software SoftMax Pro version 5.4 (Rothamsted) to calculate the Vmax values.

2.5.2 Glutathione-S-transferase assay

Glutathione-S-transferase assays were done based on methods by Booth ***et al***. (1961) and Zhu ***et al***. (2007). All wells in a 96-well microplate (transparent, Titertek®) were filled with 25 µl of 0.2 M phosphate buffer, pH 6.5. An additional 22 µl of 0.2 M phosphate buffer, pH 6.5, was added to the first column of the plate. Acetone (as control) (3 µl) or 100 000 ppm synergist solution (3 µl) was added to the first column and a two-fold serial dilution made across the plate, until well 11. The 12th well was left without inhibitor as a control. Into each well, 25 µl of freshly prepared insect homogenate was added and the plate was left to incubate at 25 °C for 30 minutes.

After incubation, 200 µl of a 9.5 mM glutathione solution containing 3 mM CDNB (diluted in 0.2 M phosphate buffer, pH 6.5) was added to each well and a kinetic assay was run on a SpectraMax M2 microplate reader (Molecular Devices) at 340 nm for five minutes with 18 second intervals between readings, using the Software SoftMax Pro 5.2 to calculate the Vmax values. To determine if any of the synergists interfered with the assay, buffer blanks were run for each synergist in the same way as above, by adding 25 µl of 0.2 M phosphate buffer, pH 6.5 into each well instead of insect homogenate.

2.5.3 Monooxygenase assay

O-Deethylation of 7-ethoxycoumarin was measured using the method of Ullrich & Weber (1972), adapted for the microplate by de Sousa ***et al***. (1995). Insects were homogenised in homogenisation buffer (0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU and 1 mM PMSF). Homogenates were centrifuged for five minutes at 10 000 rpm. Microplates used were 96-well black and white or plain white, PerkinElmer plates. Into a well, either 50 µl of homogenisation buffer was added or 10 µl of homogenisation buffer with 40 µl of insect homogenate. Into all wells, 3 µl of 1000 ppm synergist was added and plates were left to incubate for 10 minutes at 25 °C. After incubation, 80 µl of 0.5 mM 7-ethoxycoumarin (diluted in 0.1 M phosphate buffer, pH 7.8) was added to each well and left to incubate for five minutes at 30 °C. After incubation, 10 µl of 9.6 mM NADPH (diluted in 0.1 M phosphate buffer, pH 7.8) was added to each well to start the reaction. A fluorometric kinetic assay was run immediately on a SpectraMax Gemini XPS (Rothamsted) or SpectraMax M2 (TAI) microplate reader (Molecular Devices) for one hour with readings at 18 second intervals, at an excitation wavelength of 380 nm, an emission wavelength of 450 nm and a cutoff of 435 nm, using the Software SoftMax Pro 5.2 (TAI) or version 5.4 (Rothamsted) to calculate the Vmax values. Buffer blanks were run in the same way for each synergist, adding homogenising buffer instead of insect homogenate.

M. domestica monooxygenase assays used an adapted methodology, described in chapter seven (section 7.2.2.2).

2.6 Statistical analyses

Full dose response bioassays were analysed using probit analysis (Finney 1971), with the program PoloPlus (version 1.0, LeOra Software). Abbott's formula (Abbott 1925) was used to correct for control mortality, where this occurred. Discriminating dose bioassays were analysed using a generalized linear mixed model (GLMM) with an assumed binomial distribution and a logit link function, using the program Genstat (version 12.1) or ASREML. Significant differences were determined using Fisher's (protected) least significant difference (LSD) at the 5% level.

For total esterase assays using alpha-naphthyl acetate and para-nitrophenyl acetate as substrates, and for the GST data, the percentage enzyme activity remaining across the dilution series was calculated for each compound tested. The enzyme (esterase or GST) activity obtained in each buffer blank well was subtracted from the corresponding well containing insect homogenate. The percentage enzyme activity remaining was calculated by dividing the remaining activity in each inhibited well across the dilution series by the activity in the uninhibited control and multiplying by 100. Grafit (Leatherbarrow, R.J., Version 5.0.10, Erithacus Software Limited) was used to draw graphs of the data, fit curves (four parameter logistic or, when appropriate, the two parameter logistic with the lower and upper limits of 0% and 100%) and calculate the IC_{50} and Y-range values.

For the esterase interference assay, the percentage AChE activity across the dilution series was calculated by dividing the AChE activity in the wells as a percentage of the activity in the uninhibited control. Data were analysed using Grafit (Version 5.0.10) to draw graphs of the data, fit curves (four parameter logistic) and calculate IC_{50} values. Data were processed according to methods given in Appendix II.

The percentage monooxygenase activity was calculated for each synergist after subtracting the activity in the buffer blank wells from the wells containing enzyme. The remaining activity was calculated as a percentage of the uninhibited acetone control. Data were analysed with a one-way analysis of variance (ANOVA) using the program Genstat (version 12.1). Significant differences were determined using Fisher's (protected) LSD at the 5% level.

The correlations made throughout this study were done by calculating correlation coefficients using Microsoft Excel (2003) (Analysis Toolpak). A table of correlation

coefficients (significant values of ***r*** and ***R***) was used to determine if the correlations were significant at the 1 or 5% level.

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3 CHAPTER THREE: THE EVALUATION OF VARIOUS NATURAL COMPOUNDS AS POTENTIAL PYRETHRUM SYNERGISTS AGAINST THE COTTON BOLLWORM *HELICOVERPA ARMIGERA*

Abstract

The cotton bollworm ***Helicoverpa armigera*** is a devastating pest of many agricultural crops. The injudicious use of pesticides in controlling ***H. armigera*** has led to insecticide resistance and pyrethrum is being considered as an additional control option. The synergist, piperonyl butoxide (PBO), is often used in combination with pyrethrum in order to enhance its efficacy, especially in products for house and garden use. In Australia, PBO is not certified for organic use and a potentially organically-certifiable synergist would be beneficial in developing a pyrethrum product suitable for the organic crop market. This chapter explored the efficacy of various natural compounds in synergising pyrethrum against ***H. armigera***. Using ***in vivo*** bioassays (discriminating dose, diet incorporation and full dose response), PBO was found to be the most effective pyrethrum synergist. However, some natural compounds also showed significant synergism of pyrethrum, with bergamot oil, parsley seed oil, dill apiole oil, aniseed oil and sassafras oil being the most effective.

In vitro biochemical esterase, glutathione-S-transferase (GST) and monooxygenase assays were used to examine the enzyme systems involved in synergism of pyrethrum within ***H. armigera***. Monooxygenase activity was not detected in ***H. armigera*** during this study but activity was detected for esterases and GSTs. Compounds that gave good inhibition of esterases and GSTs, such as neem oil and propyl gallate, did not synergise pyrethrum, whereas compounds that synergised pyrethrum (PBO, dill apiole oil and parsley seed oil) gave no or little inhibition of esterases and GSTs. It is concluded that the use of esterase or GST assays in predicting pyrethrum synergists may not necessarily prove useful and bioassays would still be recommended for screening of potential pyrethrum synergists with ***H. armigera***.

3.1 Introduction

The cotton bollworm ***Helicoverpa armigera*** is a serious pest in agriculture, attacking a large variety of crops, including cotton, sunflowers, maize, soybeans, wheat and vegetables (Wu ***et al***. 2008). Pesticide applications aimed at controlling ***H. armigera*** have led to the development of resistance against many of the available insecticides, including pyrethroids (Gunning ***et al***. 1984, Kranthi ***et al***. 2001, van Jaarsveld 1994) and organophosphates (Gunning & Easton 1993). Even with the introduction of transgenic crop varieties, such as cotton containing the ***Bacillus thuringiensis*** (Bt) toxin, the development of resistance to the Bt toxin still remains a serious threat to the control of ***H. armigera***. The pink bollworm has already shown resistance in the field to Bt cotton in India (Bagla 2010).

Insecticide resistance is not the only factor contributing to the limitation of available control options, but also the withdrawal of registered products from the market and the limited registration of new pesticides, together with increasing public concern over pesticide residues and contamination of the environment. Future control options need to focus on the development of products that are less harmful to the environment while still remaining effective (Erler ***et al***. 2006). Pyrethrum, a naturally organic insecticide that degrades quickly in the environment, has the potential to play a significant role in controlling pests, but the addition of an effective synergist is vital in making pyrethrum a cost-effective option, even against susceptible insects. PBO, the synergist used most often with pyrethrum, no longer has organic certification in some countries and a suitable natural synergist would be required to replace PBO in order for it to be a marketable organic product.

Synergists enhance the efficacy of an insecticide by inhibiting enzymes responsible for degradation of the pesticide in the insect. In insecticide-resistant insects with metabolic mechanisms of resistance, where esterases, glutathione-S-transferases (GSTs) and / or monooxygenases are effectively able to detoxify pesticides before they reach their target site, synergists can play an important role in restoring susceptibility to an insecticide, and thus restore the usefulness of that pesticide, by inhibiting the enzymes responsible for resistance (Bingham ***et al.*** 2007, Gunning ***et al***. 1999, Young ***et al***. 2005, 2006). ***H. armigera*** has been shown to have metabolic resistance against pyrethroids, with esterases being an important mechanism of resistance in Australian ***H. armigera*** (Gunning ***et al***. 1996). Monooxygenases have been shown to play a role in resistance in ***H. armigera*** from West Africa and Asia (Chen ***et al***. 2005, Martin ***et al***. 2002, Yang ***et al.*** 2004).

This study aims to identify effective natural synergists to potentially replace PBO in formulation with pyrethrum. ***H. armigera*** was used to study the potential of a variety of natural compounds for use as synergists with pyrethrum. Bioassays and a field trial were undertaken to determine the efficacy of the compounds ***in vivo***. The capacity of the compounds to inhibit the enzymes involved in metabolic insecticide resistance was determined ***in vitro***, using enzyme inhibition assays. Since PBO is known to effectively synergise pyrethrum and PBO is an inhibitor of both esterases and monooxygenases, the bioassay data was correlated with enzyme inhibition data to determine which enzyme system could be identified as more important in identifying potential synergists in ***H. armigera***. It would be a useful tool if an ***in vitro***

enzyme assay could be used to screen for potential pyrethrum synergists. Specific compounds showing high potential ***in vitro*** could then be tested ***in vivo***, using bioassays.

3.2 Materials and Methods

3.2.1 Bioassays

H. armigera bioassays were done by topical application using third instar larvae (25-40 mg, HaC strain) according to the general methods given in chapter two (section 2.4.1). (The HaC strain of ***H. armigera*** is described in chapter two, section 2.1.1). A full dose response bioassay was used to determine the dose-response curve and obtain the lethal dose (LD) values for pyrethrum. Temporal synergism was examined using temporal bioassays, to determine whether an optimal pre-treatment time could be established for application of synergists prior to the application of pyrethrum. Discriminating dose bioassays were undertaken to compare the efficacy of the natural plant oils and extracts in synergising pyrethrum, using each synergist on its own, but also using each synergist in a mixture with PBO, since the surfactant properties of PBO might facilitate synergist penetration through the insect cuticle. Diet incorporation bioassays were performed to determine whether ingestion of synergists could increase their synergistic efficacy towards pyrethrum. Full dose response bioassays were undertaken using a range of synergists to obtain LD values and calculate the synergism factor (SF) to compare the relative efficacies of the compounds as pyrethrum synergists.

3.2.1.1 Full dose response bioassay

A topical application bioassay was conducted using five pyrethrum dosages, ranging from 937.5 ppm to 10 000 ppm (diluted in acetone), to determine a dose-response curve for the pyrethroid-resistant HaC laboratory strain of ***H. armigera***. Acetone was applied as the control. Each treatment contained ten larvae and the bioassay was performed in triplicate. A probit analysis was done using PoloPlus (version 1.0, LeOra Software) to determine the concentration-mortality regression and to calculate the lethal dose required to kill 50% of the test population (LD_{50}) (Finney 1971).

3.2.1.2 Temporal synergism

Temporal synergism refers to the amount of time between the application of the synergist and the subsequent application of the insecticide. Pre-treatment with a synergist can increase synergism, due to the time it may take for a synergist to maximally inhibit the resistance-associated metabolic enzymes (Bingham ***et al***. 2007, Gunning ***et al***. 1999, Moores ***et al***. 2005, Young ***et al.*** 2005, 2006). An optimal concentration of synergist was established initially, for use with the temporal synergism bioassays, to determine a pre-treatment time that would be optimal for synergism.

3.2.1.2.1 Synergist concentration range bioassays

From the dose-response curve obtained for pyrethrum, the LD_{50} was 2536 ppm (section 3.3.1.1). A fixed dose of 2500 ppm pyrethrum was thus chosen for determining whether any synergist concentration would be more effective at synergising pyrethrum. A 4-hour pre-treatment time was chosen for this bioassay, since research has shown four hours to be the most effective pre-treatment time for synergism of pyrethroids (zeta-cypermethrin and fenvalerate) with PBO against ***H. armigera*** (Young ***et al***. 2005).

A summary of the potential synergists (plant oils and extracts) tested with each bioassay and enzyme assay throughout this chapter is given in Table 3.1. (Details for each synergist are given in chapter two, section 2.2.2, Table 2.1). The following compounds were tested for synergism, with their applied concentration ranges:

1. ZaB2 extract at 100, 1000, 10 000, 50 000 and 100 000 ppm;
2. ClB2 extract at 100, 1000, 10 000, 50 000 and 100 000 ppm;
3. BcB1 extract at 100, 1000, 10 000, 50 000 and 100 000 ppm;
4. CaB2 extract at 100, 1000, 10 000 and 50 000 ppm;
5. CaB2 frac3 extract at 100, 1000, 10 000 and 50 000 ppm;
6. ClB1 extract at 100, 1000, 10 000 and 50 000 ppm;
7. BcB2 extract at 100, 1000, 10 000 and 50 000 ppm;
8. Neem oil at 100, 1000, 10 000 and 50 000 ppm;
9. Parsley seed oil at 100, 1000, 10 000 and 50 000 ppm.

Synergists (in acetone) were applied to the dorsal thorax of each larva and after four hours, 2500 ppm pyrethrum (in acetone) was applied, again to the dorsal thorax of each larva. The highest synergist concentration (100 000 ppm) with pyrethrum was not included for the latter six synergists as mortality tended to decrease with an increasing concentration of synergist. The inherent toxicity of each synergist at 1000 ppm was determined by applying acetone (instead of pyrethrum) to the dorsal thorax of larvae after the 4-hour pre-treatment time. Toxicity was tested at 1000 ppm since the highest mortality with pyrethrum tended to be found at this concentration. The treatments each contained ten larvae and the bioassay was performed in triplicate.

Results were analysed with the program Genstat (version 12.1), using a generalized linear model (GLM) with an assumed binomial distribution and a logit link function. Data were calculated as proportions (number of dead larvae out of the total). Pairwise comparisons were done to determine significant differences between every pair of predictions using Fisher's (protected) least significant difference (LSD) at the 5% level.

3.2.1.2.2 Temporal bioassays

Temporal bioassays were used to determine whether an increase in synergism occurred with an increasing synergist pre-treatment time. This could indicate if there was an optimum pre-treatment time where the inhibition of enzymes involved in the detoxification of pyrethrum was at a maximum. In ***H. armigera***, Young ***et al***. (2005) showed that inhibition of resistance-associated esterases by PBO reached a maximum at four hours in Australian ***H. armigera***. Using the results from the synergist concentration range bioassays (section 3.3.1.2.1), a trend showed that 1000 ppm synergist appeared to be most effective. Synergists were therefore applied at 1000 ppm (in acetone) for determining temporal synergism. PBO was applied at a standard concentration of 50 000 ppm (in acetone). The concentration of pyrethrum was lowered from 2500 ppm to 1250 ppm after initial pilot trials with application at 2500 ppm pyrethrum with PBO caused 100% mortality in ***H. armigera*** larvae. Differences in the efficacy of synergists could not be compared if mortalities were to reach 100%.

Table 3.1. Summary of the compounds tested for synergism of pyrethrum and enzyme inhibition with *Helicoverpa armigera* (HaC strain). (α-NA = alpha-naphthyl acetate, p-NPA = para-nitrophenyl acetate, ✓ = tested, x = not tested).

Compound	Synergist concentration range bioassay	Temporal bioassay	Discriminating dose bioassay	Discriminating dose with PBO bioassay	Diet incorporation bioassay	Full dose response bioassay	Esterase assay (α-NA)	Esterase assay (p-NPA)	Esterase interference assay	GST assay
Acetone	x	✓	✓	x	x	✓	✓	✓	✓	✓
Aniseed oil	x	x	✓	✓	✓	✓	✓	✓	✓	✓
BcB1 extract	✓	x	✓	✓	x	x	✓	✓	✓	✓
BcB2 extract	✓	x	✓	✓	x	x	✓	✓	✓	✓
Bergamot oil	x	x	✓	✓	✓	✓	✓	✓	✓	✓
CaB2 extract	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
CaB2 frac3 extract	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
Canola oil	x	x	✓	✓	✓	x	✓	✓	✓	✓
Citronella oil	x	x	x	x	x	x	✓	✓	✓	✓
ClB1 extract	✓	x	✓	✓	x	x	✓	✓	✓	✓
ClB2 extract	✓	✓	✓	✓	x	x	✓	✓	✓	✓
Dill apiole oil	x	x	✓	✓	x	✓	✓	✓	✓	✓
Dill seed oil	x	x	✓	✓	x	✓	✓	✓	✓	✓
Geranium oil	x	x	x	x	x	x	✓	✓	✓	✓
Grapefruit oil	x	x	✓	✓	x	✓	✓	✓	✓	✓
Neem oil	✓	x	✓	✓	✓	✓	✓	✓	✓	✓
Oleic acid	x	x	✓	✓	✓	✓	✓	✓	✓	✓
Parsley seed oil	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
PBO	x	✓	✓	✓	✓	✓	✓	✓	✓	✓
Propyl gallate	x	x	✓	✓	✓	✓	✓	✓	✓	✓
Pyrethrum	-	-	-	-	-	-	✓	✓	✓	✓
Rosemary oil	x	x	✓	✓	✓	x	✓	✓	✓	✓
ZaB2 extract	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
Angelica root oil	x	x	✓	✓	x	x	✓	x	x	✓

Black pepper oil	x	x	✓	✓	x	x	✓	x	x	✓
Citronella java oil	x	x	✓	✓	x	x	✓	x	x	✓
Cypress oil	x	x	✓	✓	x	x	✓	x	x	✓
DEM	x	x	✓	✓	x	x	✓	x	x	✓
Eucalyptus oil	x	x	✓	✓	x	x	✓	x	x	✓
Fennel oil	x	x	✓	✓	x	x	✓	x	x	✓
Garlic oil	x	x	✓	✓	x	x	✓	x	x	✓
Lavender oil	x	x	✓	✓	x	x	✓	x	x	✓
Manuka oil	x	x	✓	✓	x	✓	✓	x	x	✓
Melaleuca oil	x	x	✓	✓	x	x	✓	x	x	✓
Myristicin oil	x	x	x	x	x	x	✓	x	x	✓
Nutmeg oil	x	x	✓	✓	x	x	✓	x	x	✓
Peppermint oil	x	x	✓	✓	x	x	✓	x	x	✓
Profenofos	x	x	✓	x	x	x	✓	x	x	✓
Platyphyllol oil	x	x	✓	✓	x	✓	✓	x	x	✓
Sassafras oil	x	x	✓	✓	x	x	✓	x	x	✓
Sesame oil	x	x	✓	✓	x	x	✓	x	x	✓
TPP	x	x	✓	✓	x	x	✓	x	x	✓
White cypress oil	x	x	✓	✓	x	✓	✓	x	x	✓

The following synergists (described in chapter two, Table 2.1) and acetone (as a control) were used for temporal bioassays (listed in Table 3.1), at each pre-treatment time:

1. PBO (50 000 ppm) at 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h and 8 h;
2. ZaB2 extract (1000 ppm) at 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h and 8 h;
3. CaB2 extract (1000 ppm) at 30 min, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h;
4. Parsley seed oil (1000 ppm) at 30 min, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h;
5. CaB2 frac3 extract (1000 ppm) at 30 min, 1 h, 2 h, 3 h, 4 h, 5 h and 8 h;
6. ClB2 extract (1000 ppm) at 30 min, 1 h, 2 h, 3 h, 4 h and 5 h;
7. Acetone at 30 min, 1 h, 2 h, 3 h, 4 h and 5 h.

Synergists or acetone were applied to the dorsal thorax of each larva and after the specified pre-treatment time, 1250 ppm pyrethrum (in acetone) was applied to each larva, again on the dorsal thorax. Ten larvae were treated at each pre-treatment time and each treatment was performed in triplicate. Results were analysed as for the synergist concentration range bioassays (section 3.2.1.2.1).

3.2.1.3 Discriminating dose bioassays

A discriminating dose bioassay was undertaken to compare the relative synergistic efficacies of the potential pyrethrum synergists. A discriminating dose bioassay using the potential synergists mixed with PBO was also performed, to assess whether PBO could improve cuticular penetration of the synergists, increasing mortality to above what would be expected for either the synergist or PBO alone.

3.2.1.3.1 Discriminating dose bioassay - synergists

A dose of approximately the LD_{20} (lethal dose required to kill 20% of the test population; obtained from the dose-response curve for pyrethrum, section 3.3.1.1) was initially chosen as the discriminating dose (1250 ppm pyrethrum) in a ratio of 4:1 synergist:pyrethrum (thus 5000 ppm synergist concentration). Pyrethrum is not registered for agricultural use against ***H. armigera***, however, formulated pyrethrum products, such as for home and garden use, often contain PBO in the ratio of 4:1 PBO:pyrethrum and this ratio was therefore chosen for the bioassay. PBO with 1250 ppm pyrethrum, however, caused almost 100% mortality and the pyrethrum dosage

was lowered to approximately the LD_{10} (937.5 ppm pyrethrum) (section 3.3.1.1), since differences between synergists could not be quantified if mortalities were to reach 100%. Using the LD_{10} would ensure low mortality with pyrethrum alone and would allow for any synergistic activity to be observed. With the lowered pyrethrum concentration, the ratio of synergist:pyrethrum became 5.3:1.

The compounds used as standard or potential synergists in this study are described in detail in chapter two (section 2.2.2, Table 2.1). All compounds tested for the discriminating dose bioassay with ***H. armigera*** were at a concentration of 5000 ppm (in acetone) and are listed in Table 3.1. Natural plant oils tested were angelica root, aniseed, bergamot, black pepper, canola, citronella java, cypress, dill apiole, dill seed, eucalyptus, fennel, garlic, grapefruit, lavender, manuka, melaleuca, neem, nutmeg, parsley seed, peppermint, platyphyllol, rosemary, sassafras, sesame and white cypress oils. Propyl gallate, an anti-oxidant, and oleic acid, a mono-unsaturated omega-9 fatty acid, were also tested for synergistic activity. Oleic acid is a component of the BRA emulsifiable concentrate (EC) formulation of pyrethrum.

Plant extracts from the University of Tasmania tested for synergism were ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2 (details regarding each plant extract are described in chapter two, section 2.2.2, Table 2.1).

An organophosphate (OP), Curacron® (active ingredient: profenofos), was included because organophosphates are known esterase inhibitors and pyrethroid synergists in Australian ***H. armigera*** (Gunning ***et al***. 1999). PBO, TPP and DEM were used as standard synergists. PBO is an inhibitor of both monooxygenases (Wilkinson 1976) and esterases (Gunning ***et al***. 1998, Young ***et al.*** 2005, 2006). TPP is an esterase inhibitor and DEM is often used as a standard for glutathione-S-transferase conferred resistance (Soderlund & Bloomquist 1990, Wu ***et al***. 2007).

A 30-minute pre-treatment time was used throughout since no significant differences were found between pre-treatment times in the temporal bioassays (section 3.3.1.2.2). Synergists (5000 ppm) (1 µl) were applied to the dorsal thorax of each larva and after 30 minutes, 937.5 ppm pyrethrum (in acetone) (1 µl) was applied to the dorsal thorax of each larva. The inherent toxicity of each synergist at 5000 ppm was also determined, by applying acetone (1 µl) (instead of pyrethrum) after the 30-minute pre-treatment time to each of the larvae. Ten larvae were treated with each

synergist and the trial was replicated five times for pyrethrum treatments and twice for acetone controls. Data were analysed using a generalized linear mixed model (GLMM) with an assumed binomial distribution and a logit link function, using the program ASREML. Significant differences at the 5% level were determined using Fisher's (protected) least significant difference (LSD).

Comparison between mouthparts and thorax for topical application with neem oil

An alternative method of testing for synergism was examined to determine whether a difference occurred due to the location of the synergist application, as a result of potential differences in cuticular penetration at different localities. Sun & Johnson (1972) found great differences in synergism of carbamates when applying synergists either in an oil spray, topically or by injection.

Using neem oil, topical application was used to determine whether there was any improvement in synergism if the compound was applied on the mouthparts instead of on the thorax. Neem oil was chosen as it showed good inhibition ***in vitro*** of both esterases (section 3.3.2.1) and GSTs (section 3.3.2.2) in ***H. armigera***, as well as moderate inhibition of monooxygenases (45%) using ***Carpophilus*** spp. homogenate (chapter six, section 6.3.2.3). Fifty larvae were treated with neem oil (5000 ppm in acetone) on the dorsal thorax and fifty larvae were treated on the mouthparts. After 30 minutes, pyrethrum (937.5 ppm in acetone) was applied on the dorsal thorax of both groups. Acetone controls were included on the mouthparts and dorsal thorax instead of the pyrethrum application. The data were analysed with Genstat (version 12.1) using a Chi-square test.

3.2.1.3.2 Discriminating dose bioassay - synergists mixed with PBO

A discriminating dose bioassay was undertaken as above but with each synergist mixed in a 1:1 ratio with PBO. Not all of the natural plant oils and extracts may have been efficient in penetrating the insect cuticle and the surfactant properties of PBO might have allowed for easier penetration into the insect body.

Compounds applied in a mixture of 5000 ppm synergist:5000 ppm PBO (in acetone) are listed in Table 3.1. A 30-minute pre-treatment time was used for the bioassay. The synergist:PBO mixtures (1 µl) were applied topically to the dorsal thorax of each

larva. After 30 minutes, 937.5 ppm pyrethrum (in acetone) (1 µl) was applied to the dorsal thorax of each larva. The toxicity of each synergist:PBO mixture alone was also determined, by applying 1 µl of acetone to each of the larvae after the 30-minute pre-treatment time. The bioassay was replicated five times for pyrethrum treatments and twice for the acetone controls, using ten larvae per replicate. Data were analysed as for the discriminating dose bioassay above (section 3.2.1.3.1).

3.2.1.4 Diet incorporation bioassay

To determine whether synergists showed more efficacy when ingested than through topical application, due to possible lack of cuticular penetration by the synergist, the standard bollworm diet was made up to a concentration of 5000 ppm synergist diet, and larvae were placed on the diet for five hours. To determine whether any of the synergists repelled the larvae, the number of larvae either on or off the synergist diet was noted at five hours. After five hours, larvae were removed from the synergist diet, placed on standard diet and immediately treated topically with either 937.5 ppm pyrethrum, or acetone (as a control), on the dorsal thorax.

Diets were incorporated with PBO, aniseed oil, bergamot oil, canola oil, neem oil, oleic acid, parsley seed oil, propyl gallate and rosemary oil (listed in Table 3.1). For each treatment, ten larvae were placed on individual blocks of diet. The bioassay was replicated six times for pyrethrum treatments and twice for acetone controls. Mortality data were analysed as for the discriminating dose bioassays, using the program Genstat (version 12.1). For the number of larvae off the diet, the data contained a large number of zeroes and Genstat could not be used to analyse the data. The same analysis was run using the program, ASREML. Significant differences for both analyses were determined using Fisher's (protected) LSD at the 5% level.

3.2.1.5 Full dose response bioassays with synergists

To compare the efficacies of a variety of potential pyrethrum synergists, dose-response curves were obtained for each synergist and their corresponding $LD_{50/90}$ values were used to calculate a synergism factor (SF). The SF gives an indication of the relative efficacy of a synergist compared to unsynergised insecticide.

Full dose response bioassays were undertaken using the synergists listed in Table 3.1. PBO was tested at both 10 000 and 50 000 ppm and all remaining compounds were tested at 10 000 ppm (in acetone). Acetone was used as the pre-treatment for unsynergised pyrethrum. A 30-minute pre-treatment time was used for the bioassays. The synergist or acetone (1 µl) was applied to the dorsal thorax of each larva and after thirty minutes, pyrethrum (1 µl) was applied to each larva. Between five and eight pyrethrum dosages were applied, within the range of 78.13 ppm to 20 000 ppm pyrethrum (in acetone). The toxicity of each synergist was determined by applying acetone (1 µl) after the 30-minute pre-treatment time. Ten larvae were used for each replicate and the bioassays were performed in triplicate.

Probit analyses were performed using PoloPlus (version 1.0) to determine the concentration-mortality regression lines and to calculate the lethal dose required to kill 50% and 90% of the test population (LD_{50} and LD_{90} respectively) with each synergist. Statistical comparisons using LD_{50} and LD_{90} values were based on non-overlap of the 95% confidence intervals. The SF was calculated by dividing the $LD_{50/90}$ value obtained without the synergist (the acetone pre-treatment) by the $LD_{50/90}$ value with the synergist. Statistical comparisons for the SF values at the LD_{50} and LD_{90} levels were based on non-overlap of the 95% confidence intervals.

3.2.2 Biochemical assays

Esterase, glutathione-S-transferase (GST) and monooxygenase inhibition assays were undertaken to investigate the possible enzyme systems involved with pyrethrum resistance and synergism in ***H armigera***. General methodology is described in chapter two but details specific to ***H. armigera*** are described in this section. The HaC strain of ***H. armigera***, used for all biochemical assays, is described in chapter two (section 2.1.1).

3.2.2.1 Esterase assays

In ***H. armigera***, total esterase assays were undertaken using two esterase substrates, alpha-naphthyl acetate and para-nitrophenyl acetate. An indirect measure of esterase activity, using the esterase interference assay, was also performed. With ***Myzus persicae*** (chapter five), esterase inhibition was not revealed using the total esterase assay with alpha-naphthyl acetate as the substrate, as was also found by

Khot ***et al***. (2008), who developed the esterase interference assay for this reason. The esterase interference assay measures inhibition of house fly acetylcholinesterase (AChE) by free azamethiphos. In the presence of non-specific esterase, for example, ***M. persicae*** esterase, the amount of free azamethiphos is reduced by sequestration. Pre-incubation of the esterase with a synergist may reduce the sequestration, resulting in inhibition of AChE similar to that seen in the absence of the esterase. With ***M. persicae*** (chapter five), when no inhibition was found, the total esterase assay was repeated using a different esterase substrate, para-nitrophenyl acetate, to try detect inhibition, and the esterase interference assay was also undertaken. Due to the differences found between the total esterase assays with the two substrates, and a significant correlation between the total esterase assay with para-nitrophenyl acetate and the esterase interference assay, the three different esterase assays were also undertaken with ***H. armigera***. This would reveal whether differences in esterase inhibition occurred between the different assays in ***H. armigera*** and would give an indication of whether any one assay correlated more closely with the bioassay results, to find the enzyme system and assay that would be most useful in predicting possible future pyrethrum synergists.

3.2.2.1.1 Esterase assay with alpha-naphthyl acetate as a substrate

Total esterase assays using alpha-naphthyl acetate as a substrate were run according to the method described in chapter two (section 2.5.1.1). ***H. armigera*** homogenate was made up using 20 larvae (HaC strain) (weighing 3 to 4 mg each) per 1 ml of 0.02 M phosphate buffer, pH 7. Larvae were homogenised in eppendorf tubes with a plastic handheld homogeniser and centrifuged at 10 000 rpm for 4 minutes. The supernatant was used immediately for esterase assays.

Compounds tested for inhibition of total esterase activity (alpha-naphthyl acetate as a substrate) are listed in Table 3.1. The stock solution of profenofos used for the assay was 10 000 ppm (in acetone) whereas all other compounds were 100 000 ppm stock solutions (in acetone). Acetone was used as a control.

The ability of pyrethrum (100 000 ppm stock solution in acetone) to inhibit total esterase activity was also examined. This would give an indication of whether esterases were interacting with pyrethrum and thus potentially playing a role in detoxification of pyrethrum.

Data for the esterase assay were processed according to the method described in chapter two (section 2.6), by calculating activity as a percentage of the uninhibited control. Inhibition curves were determined by fitting a four parameter logistic to the data, using the program Grafit (Version 5.0.10). The IC_{50} and Y-range, calculated by Grafit, were used to compare the esterase-inhibiting capabilities of the compounds. The Y-range describes the percentage of the total esterases that are susceptible to inhibition by the synergist and gives an indication of how much of the total esterases have been inhibited. The IC_{50} describes the concentration of inhibitor at which inhibition (of the inhibited esterases indicated by the Y-range) is at 50%. Synergists with small IC_{50} values require lower concentrations of synergist to inhibit 50% of esterases and are therefore more potent esterase inhibitors than those with larger IC_{50} values. A synergist with a low IC_{50} value and high Y-range could potentially make a better synergist than one with a small Y-range, since a broader spectrum of the total esterases are inhibited at a lower dosage of synergist. The greater the Y-range, the more likely a synergist is to have also inhibited the resistance-associated esterase(s). Many factors, however, influence the ***in vivo*** efficacy of a synergist, including its ability to penetrate the insect cuticle, affinity for the target site, and whether or not it targets the specific esterases associated with insecticide detoxification.

For some synergists where inhibition occurred only at the highest concentrations of the dilution series, not all the information was available to accurately predict the fit of the data with a full inhibition curve (four parameter logistic). The Y-range in these cases would exceed 100% and the data were fitted with fully range corrected inhibition curves (a two parameter logistic with the lower and upper limits of 0% and 100% respectively). For the esterase assay, garlic oil and BcB2 extract were fitted with fully range corrected inhibition curves. Assays were run with 100 000 ppm stock solutions (with the exception of profenofos) to enable comparisons to be made regarding the potency of the compounds as enzyme inhibitors. When solutions much greater than 100 000 ppm are required for enzyme inhibition, the compounds become impractical as inhibitors and may cause interference with the assay.

Correlations were tested for between the percentage mortality found in the discriminating dose bioassay for synergists with pyrethrum (section 3.3.1.3.1) and the inhibition of esterases from the total esterase assay (using the percentage esterase

activity at the highest concentration of the synergist dilution series, the IC_{50} values and the Y-ranges). The correlation analyses were performed according to the methods described in chapter two (section 2.6), by calculating correlation coefficients with Excel (2003) and using a table of correlation coefficients (significant values of ***r*** and ***R***) to determine if the correlations were significant at the 1 or 5% level.

3.2.2.1.2 Esterase assay with para-nitrophenyl acetate as a substrate

Total esterase assays using para-nitrophenyl acetate as a substrate were run according to the method described in chapter two (section 2.5.1.2). ***H. armigera*** homogenate was made up using two third instar larvae (HaC strain) (weighing between 30 and 40 mg each) in 4 ml of 0.02 M phosphate buffer, pH 7. Larvae were homogenised in an eppendorf tube with a plastic handheld homogeniser and centrifuged at 10 000 rpm for 3 minutes. The supernatant was used immediately for esterase assays.

Compounds tested for inhibition of total esterase activity (para-nitrophenyl acetate as a substrate) are listed in Table 3.1. All compounds used were 10 000 ppm stock solutions (in acetone). Acetone was used as a control. The ability of pyrethrum (10 000 ppm stock solution in acetone) to inhibit total esterase activity was also examined as a way to determine whether pyrethrum was binding to esterases.

Data were processed and analysed as for the esterase assay (alpha-naphthyl acetate as a substrate) above (section 3.2.2.1.1). Correlations between pyrethrum synergism and esterase inhibition were tested for as described above and analysed according to the methods in chapter two (section 2.6).

3.2.2.1.3 Esterase interference assay

An esterase interference assay was run using ***H. armigera*** homogenate according to methods explained in chapter two (section 2.5.1.3). ***H. armigera*** homogenate was prepared using approximately 40 frozen third instar larvae (HaC strain) (with a total frozen weight of 9.29 g) in 10 ml of 0.02 M phosphate buffer, pH 7. (Larvae were transported alive from Tamworth Agricultural Institute (TAI) (Tamworth, Australia) and frozen (at -80 °C) at Rothamsted Research (Harpenden, UK) since no rearing facilities were available for ***H. armigera*** at Rothamsted Research). The esterase

activity is not expected to be significantly decreased since the insects were transported and kept intact. Only when homogenised and compartmentalisation broken would protease activity be expected to decrease the esterase activity. Esterase activity obtained from frozen ***M. persicae*** is the same as that from fresh ***M. persicae*** (Dr. Graham D. Moores, ***Pers. comm.***). ***H. armigera*** larvae were homogenised with a glass homogeniser and the mixture was put through cheesecloth to filter the homogenate. The filtered homogenate was transferred to eppendorf tubes and centrifuged at 10 000 rpm for two minutes. The enzyme solution was partially purified by running the supernatant through a G-25 (Sephadex^{TM} fine) column (adding 0.02 M phosphate buffer, pH 7) into five separate test tubes. From each test tube, a 25 µl aliquot was taken and added to a microplate. Esterase activity was measured by adding 200 µl of 1 mM para-nitrophenyl acetate (diluted in 0.02 M phosphate buffer, pH 7) to each well and running a kinetic assay on a ThermoMax microplate reader (Molecular Devices) at 405 nm for 10 minutes with 10 second intervals between readings. The fraction with the highest esterase activity was used for the interference assay. A matrix was set up to determine the most suitable concentration of esterase (homogenate) to use for the assay by running the interference assay without any synergist (only the azamethiphos and esterase rows (as explained in chapter two)). Three rows for esterase were compared, using 5 µl, 25 µl and 50 µl of ***H. armigera*** homogenate respectively. The most suitable concentration was found to be 25 µl of homogenate. Incubations were set up by adding 1.7 µl of 10 000 ppm synergist to 170 µl of homogenate. Acetone (1.7 µl) was added to 170 µl of homogenate for the esterase row. The incubations were left for 16 hours at 4 °C before being used for the interference assay.

Incubations were set up with the compounds listed in Table 3.1. All synergists used were 10 000 ppm stock solutions in acetone. Acetone was used as the positive control. Pyrethrum (a 10 000 ppm stock solution) was also incubated with ***H. armigera*** esterases to determine the level of binding of pyrethrum to esterases.

Data for the esterase interference assay were processed according to the methods described in chapter two (section 2.6) and Appendix II. The IC_{50} value for each synergist obtained from Grafit was converted to an index value (termed I), and was calculated as a percentage of esterase and no esterase, where esterase was equivalent to 100% esterase activity and no esterase was equivalent to 0% activity. The index value (I) equates to the percentage house fly AChE activity remaining after

addition of the synergists. The SE values were corrected in the same way to equate to the I values. Upper and lower confidence intervals (95%) were also calculated for each synergist.

A correlation was tested for between the percentage mortality found in the discriminating dose bioassay for synergists with pyrethrum (section 3.3.1.3.1) and the esterase interference assay (using index values). In addition, correlations were also tested for between the esterase assays (alpha-naphthyl acetate and para-nitrophenyl acetate as substrates) (percentage esterase activity remaining at the highest concentration of the synergist dilution series) and the esterase interference assay (index values). Correlations were analysed as described in chapter two (section 2.6).

3.2.2.2 Glutathione-S-transferase assay

GST assays were run using CDNB as a substrate according to the method described in chapter two (section 2.5.2). ***H. armigera*** homogenate was prepared by homogenising 40 larvae (HaC strain) (weighing 3 to 4 mg each) per 2.5 ml of 0.2 M phosphate buffer, pH 6.5. Larvae were homogenised in eppendorf tubes with a plastic handheld homogeniser and centrifuged at 10 000 rpm for 4 minutes. The supernatant was used immediately for GST assays.

Compounds tested for inhibition of GST activity are listed in Table 3.1. The stock solution of profenofos was 10 000 ppm (in acetone) whereas all other compounds were 100 000 ppm stock solutions (in acetone). Acetone was used as a control. Pyrethrum (100 000 ppm stock solution in acetone) was also tested as a GST inhibitor.

The GST data were analysed and processed as for the esterase assay (alpha-naphthyl acetate as a substrate) (section 3.2.2.1.1). For the GST assay, platyphyllol, sassafras, dill apiole, nutmeg and white cypress oils, CaB2 frac3, CaB2, ZaB2 and ClB2 extracts and pyrethrum were fitted with fully range corrected inhibition curves (two parameter logistic with the lower and upper limits of 0% and 100% respectively) since not enough information was available with the dilution series tested to fit a full inhibition curve (four parameter logistic) to the data accurately. For all remaining compounds, lines were fitted with a four parameter logistic curve.

Correlations were tested for between the percentage mortality found in the discriminating dose bioassay for synergists with pyrethrum (section 3.3.1.3.1) and inhibition of GST activity (percentage GST activity at the highest concentration of the synergist dilution series, the IC_{50} values and the Y-ranges). The correlation analyses were performed as above (section 3.2.2.1.1).

3.2.2.3 Monooxygenase assay

Monooxygenase assays were run using ***H. armigera*** homogenate (HaC strain) according to the method described in chapter two (section 2.5.3). Initially, homogenate was prepared by homogenising 65 whole larvae (3-4 mg each) in 2 ml of homogenisation buffer (0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU and 1 mM PMSF). When no detectable monooxygenase activity was found, an ultracentrifuge was used to prepare homogenate. Homogenate was prepared using 112 whole bollworm larvae (80 larvae of 3-4 mg, 11 larvae of 30-40 mg and 21 larvae of greater than 40 mg (total weight of 4.64 g)). Larvae were homogenised in 4 ml homogenisation buffer containing 1.46 M sucrose and centrifuged at 10 000 rpm for 5 minutes. Homogenate was filtered through cheesecloth and placed in an ultracentrifuge for 1 hour at 40 000 rpm (equivalent to 100 000 g) at 4 °C. The supernatant was removed and the pellet was resuspended in 500 µl homogenisation buffer and run immediately according to methods described in chapter two (section 2.5.3). Still no activity was found and the method above with the ultracentrifuge was repeated using 15.16 g of ***H. armigera*** larvae (made up of 55 third and fourth instar larvae and 12 fifth instar larvae) homogenised in 10 ml homogenisation buffer with 1.46 M sucrose. After ultracentrifugation, the pellet was resuspended in 1 ml of homogenisation buffer and used immediately for the assay.

3.2.3 Field trial

A field trial was undertaken at Liverpool Plains Field Station (I & I NSW, Breeza, Australia) to determine the efficacy of pyrethrum against ***H. armigera*** on cotton, using neem oil, parsley seed oil and canola oil as potential synergists. The trial was laid out in a randomized block design, with eight treatments and four repetitions. The treatments are listed in Table 3.2.

Table 3.2. Treatments applied on the cotton field trial using synergised and unsynergised pyrethrum against *Helicoverpa armigera* larvae at the Liverpool Plains Field Station (I & I NSW, Breeza, Australia). (EC = emulsifiable concentrate formulation).

Treatment		Dosage (product / 100 ℓ water) (Active ingredient (a.i.))	Volume product added to 2 ℓ solution (a.i.)	Volume synergist added to 2 ℓ solution
1.	Untreated control	-------	-------	-------
2.	Pyrethrum plus PBO (PyZap) (EC)	300 ml (12.0 g pyrethrins; 48.0 g PBO)	6 ml (0.24 g pyrethrins; 0.96 g PBO)	-------
3.	Pyrethrum only (EC)	300 ml (12.0 g pyrethrins)	6 ml (0.24 g pyrethrins)	-------
4.	Pyrethrum (EC) plus parsley seed oil	300 ml (12.0 g pyrethrins; 49.0 g parsley seed oil)	6 ml (0.24 g pyrethrins; 0.98 g parsley seed oil)	0.96 ml*
5.	Pyrethrum (EC) plus neem oil	300 ml (12.0 g pyrethrins; 49.5 g neem oil)	6 ml (0.24 g pyrethrins; 0.99 g neem oil)	1.05 ml¥
6.	Pyrethrum (EC) plus canola oil	300 ml (12.0 g pyrethrins; 49.0 g canola oil)	6 ml (0.24 g pyrethrins; 0.98 g canola oil)	1.13 ml§
7.	PyGanic (EC)	300 ml (12.0 g pyrethrins; 48.0 g PBO)	6 ml (0.24 g pyrethrins; 0.96 g PBO)	-------
8.	Microencapsulated formulation of pyrethrum plus PBO (EC)	150 ml (12.0 g pyrethrins; 24.0 g PBO)	3 ml (0.24 g pyrethrins; 0.48 g PBO)	-------

* Density of parsley seed oil: 1.02 g ml^{-1}.

¥ Density of neem oil: 0.94 g ml^{-1}.

§ Density of canola oil: 0.87 g ml^{-1}.

Cotton (Delta 18 RF RoundupReady Flex, Monsanto herbicide-tolerant variety) was planted at the I & I NSW Liverpool Plains Field Station (Co-ordinates: 30° 15' South, 150° 28' East; Elevation: 436.5 m above mean sea level) in mid to late October 2007. Seed was obtained from DeltaPine (Narrabri, NSW, Australia). The trial was planted by tractor in a heavy black clay soil and irrigated by flood irrigation soon after planting. The trial was irrigated again a few weeks after germination but sufficient rain

fell throughout the rest of the season so as not to require further irrigation before the trial was completed.

Each plot for the trial consisted of one row of cotton 5 m in length (approximately 33 plants per plot). Each plot was thus 1 m x 5 m, or 0.0005 ha and each treatment (of 4 plots) thus measured 0.002 ha. The first two replications were laid out within one row of cotton and the third and fourth replications were laid out within one row, which were separated by an untreated buffer row in between the two rows.

A single full cover spray application was made on 21 February 2008 between 12 00 h and 14 00 h. Spray volume applied was 1.6 ℓ water / treatment (equivalent to 800 ℓ water / ha). (A total of 2 ℓ solution was made up but only 1.6 ℓ was sprayed out per treatment). The trial was sprayed with a 14 litre battery operated knapsack sprayer with a hollow cone nozzle.

3.2.3.1 Weather conditions during the trial

21 February 2008 (Addition of larvae and spray application): Temperature approximately 31 °C; Very little to no cloud cover; Wind-still conditions.
22 February 2008 (Bollworm count): Temperature approximately 34 °C; Very little to no cloud cover; Wind-still conditions.

3.2.3.2 Insect evaluation methods and sampling area

Forty pyrethroid resistant ***H. armigera*** larvae (HaC strain) (second instar) were placed within each plot before spraying was undertaken. The trial was evaluated 24 hours after spraying. The number of living ***H. armigera*** larvae were recorded from six whole plants per plot. Plants monitored for bollworm larvae were chosen at random from within the middle 3 m of each plot, thus excluding 1 m from each end of the plot. The mean number of ***H. armigera*** larvae per six plants per treatment was calculated.

3.2.3.3 Statistical analysis of results

Results were analysed using a one-way analysis of variance (ANOVA) for a randomized block design, with the program Genstat (version 12.2). Significant differences were determined using Fisher's (protected) LSD at the 5% level.

3.2.3.4 Percentage control of *H. armigera* larvae

Abbott's formula (Abbott 1925) was used to calculate the percentage control of ***H. armigera*** larvae for each treatment:

Percentage control = (X-Y)/X x 100

where X is the number of living ***H. armigera*** larvae from the control plots and Y is the number of living ***H. armigera*** larvae from the treated plots.

Table 3.3. The lethal dose (LD) values with 95% confidence intervals (CI) for pyrethrum (calculated by probit analysis) from topical application bioassays against a pyrethroid-resistant laboratory strain (strain HaC) of *Helicoverpa armigera* larvae (25-40 mg larvae).

LD value	Pyrethrum dose (ppm)	95% CI
LD_{10}	941	541 – 1293
LD_{20}	1323	873 – 1724
LD_{30}	1690	1213 – 2157
LD_{40}	2085	1577 – 2661
LD_{50}	2536	1976 – 3303
LD_{60}	3086	2424 – 4186
LD_{70}	3806	2960 – 5498
LD_{80}	4864	3675 – 7696
LD_{90}	6837	4874 – 12 489
LD_{95}	9056	6102 – 18 786
LD_{99}	15 344	9207 – 40 816
Number of larvae exposed = 180		
Slope ± SE = 2.976 ± 0.400		
Degrees of freedom (df) = 13		
$\chi^2 = 17.235$		

3.3 Results

3.3.1 Bioassays

3.3.1.1 Full dose response bioassay

The dose-response curve for unsynergised pyrethrum using ***H. armigera*** larvae was calculated by probit analysis (Table 3.3). No mortality was found with the acetone controls. The LD_{50} was 2536 ppm pyrethrum.

3.3.1.2 Temporal synergism

3.3.1.2.1 Synergist concentration range bioassays

A range of synergist concentrations were applied to ***H. armigera*** larvae with the LD_{50} of pyrethrum (2500 ppm), using nine synergists, to determine whether any specific concentration of synergist was the most effective. For ZaB2 extract, ClB2 extract and parsley seed oil, no significant differences were found between synergist concentrations (Table 3.4). However, several compounds had significantly higher mortality at lower concentrations than at higher concentrations, including neem oil and extracts BcB1, CaB2, ClB1 and BcB2. Mortality for CaB2 frac3 extract varied, when applied at 50 000 ppm it resulted in significantly lower mortality than the 100, 1000 and 10 000 ppm applications but at 1000 ppm showed significantly higher mortality than at 100 ppm. No mortality was found in ***H. armigera*** pre-treated with any of the synergists at 1000 ppm with the acetone controls.

The data tended to show an overall trend of giving the highest mortality with pyrethrum when using the synergists at 1000 ppm, and significantly so for CaB2 frac3 extract. A concentration of 1000 ppm was therefore used for determining whether temporal synergism occurred. An increase in the synergist dosage above 1000 ppm tended to correspond with a decrease in mortality.

Table 3.4. The mean percentage mortality (± SE) of *Helicoverpa armigera* larvae (25-40 mg larvae) with pyrethrum over a range of synergist concentrations.

	Mean Mortality (%)* (± SE)								
Synergist concentration	ZaB2 extract	ClB2 extract	BcB1 extract	CaB2 extract	CaB2 frac3 extract	ClB1 extract	BcB2 extract	Neem oil	Parsley seed oil
100 ppm	50.00 [a] (± 10.19)	30.00 [a] (± 10.05)	46.67 [a] (± 8.35)	50.00 [a] (± 5.64)	56.67 [b] (± 4.35)	43.33 [a] (± 5.16)	53.33 [a] (± 5.83)	46.67 [a] (± 5.43)	63.33 [a] (± 8.66)
1000 ppm	66.67 [a] (± 9.59)	66.67 [a] (± 10.33)	50.00 [a] (± 8.36)	60.00 [a] (± 5.52)	73.33 [a] (± 3.88)	53.33 [a] (± 5.20)	46.67 [a] (± 5.83)	46.67 [a] (± 5.43)	90.00 [a] (± 5.39)
10 000 ppm	43.33 [a] (± 10.10)	40.00 [a] (± 10.75)	33.33 [ab] (± 7.88)	46.67 [ab] (± 5.63)	70.00 [ab] (± 4.02)	20.00 [b] (± 4.17)	20.00 [b] (± 4.67)	23.33 [b] (± 4.60)	80.00 [a] (± 7.19)
50 000 ppm	43.33 [a] (± 10.10)	40.00 [a] (± 10.75)	16.67 [b] (± 6.23)	30.00 [b] (± 5.16)	33.33 [c] (± 4.14)	13.33 [b] (± 3.54)	20.00 [b] (± 4.67)	36.67 [ab] (± 5.24)	86.67 [a] (± 6.11)
100 000 ppm	26.67 [a] (± 9.01)	30.00 [a] (± 10.05)	16.67 [b] (± 6.22)	-	-	-	-	-	-
$P_{(0.05)}$value	NS[¥]	NS[¥]	0.029	0.030	<0.001	0.001	0.004	0.039	NS[¥]

* Means within the same column having the same symbols do not differ significantly from one another at the p=0.05 test level.

[¥] NS = not significant.

Table 3.5. The mean percentage mortality (± SE) of *Helicoverpa armigera* larvae (25-40 mg larvae) with pyrethrum over a range of synergist or acetone pre-treatment times.

Pre-treatment time	Mean Mortality (%) (± SE)						
	PBO	ZaB2 extract	CaB2 extract	Parsley seed oil	CaB2 frac3 extract	ClB2 extract	Acetone
30 min	83.33 (± 7.38)	6.67 (± 6.13)	23.33 (± 9.74)	36.67 (± 11.91)	20.00 (± 7.65)	20.00 (± 8.94)	10.00 (± 5.87)
1 h	96.67 (± 3.53)	13.33 (± 8.45)	30.00 (± 10.56)	26.67 (± 10.93)	13.33 (± 6.48)	13.33 (± 7.60)	33.33 (± 9.23)
2 h	93.33 (± 4.94)	13.33 (± 8.46)	30.00 (± 10.55)	20.00 (± 9.88)	16.67 (± 7.12)	10.00 (± 6.71)	20.00 (± 7.83)
3 h	90.00 (± 5.94)	10.00 (± 7.47)	30.00 (± 10.56)	33.33 (± 11.65)	20.00 (± 7.65)	3.33 (± 4.01)	16.67 (± 7.30)
4 h	86.67 (± 6.73)	20.00 (± 9.96)	26.67 (± 10.18)	26.67 (± 10.93)	20.00 (± 7.64)	19.23 (± 9.47)	10.00 (± 5.86)
5 h	93.33 (± 4.94)	10.00 (± 7.46)	16.67 (± 8.57)	36.59 (± 10.18)	30.00 (± 8.76)	11.11 (± 12.83)	20.00 (± 7.82)
6 h	83.33 (± 7.38)	16.67 (± 9.27)	30.43 (± 12.10)	40.00 (± 12.11)	-	-	-
7 h	83.33 (± 7.38)	26.67 (± 11.02)	-	-	-	-	-
8 h	86.67 (± 6.73)	23.33 (± 10.52)	-	-	16.67 (± 7.13)	-	-
$P_{(0.05)}$value	NS*	NS*	NS*	NS*	NS*	NS*	NS*

* NS = not significant.

3.3.1.2.2 Temporal bioassays

A fixed dose of pyrethrum (1250 ppm) was used with six synergists and acetone to determine whether differences occurred depending on pre-treatment time with the synergist before the application of pyrethrum. No significant differences were found between any of the pre-treatment times for pyrethrum with any of the synergists or for acetone (Table 3.5). A 30-minute pre-treatment time was therefore used for all discriminating dose and full dose response bioassays.

3.3.1.3 Discriminating dose bioassays

3.3.1.3.1 Discriminating dose bioassay - synergists

Profenofos was the only compound tested for synergism that caused mortality with the acetone control, killing 100% of larvae. Results with profenofos were therefore omitted from the statistical analysis. Profenofos is registered for use on cotton as a broad-spectrum organophosphate for control of ***H. armigera*** and ***Helicoverpa punctigera*** eggs and larvae. Due to its high toxicity, profenofos does not make an ideal synergist at this concentration. No other synergist showed toxicity towards ***H. armigera***, with no mortality found in any of the acetone controls pre-treated with each synergist.

Pyrethrum synergised by PBO gave significantly higher mortality (86.00%) than all of the other compounds ($p<0.001$) (Fig. 3.1). In addition to PBO, bergamot, parsley seed, dill apiole, aniseed, sassafras and canola oils also gave significantly higher mortality (59.00%, 52.00%, 44.00%, 43.00%, 42.00% and 32.00% respectively) than pyrethrum alone (16.67%). Neem and platyphyllol oils appeared antagonistic, with mortalities significantly lower (both 4.00% mortality) than pyrethrum alone. No other treatment differed significantly from pyrethrum alone.

Comparison between mouthparts and thorax for topical application with neem oil

Neem oil applied to the mouthparts of ***H. armigera*** larvae resulted in 12% mortality with pyrethrum, compared to 4% mortality when neem oil was applied on the dorsal thorax with pyrethrum. Although this was a small increase, the difference was not statistically significant ($X^2 = 2.27$, df = 1, $p=0.132$). No mortality was found with the acetone controls.

3.3.1.3.2 Discriminating dose bioassay - synergists mixed with PBO

When PBO was mixed with each synergist and treated with pyrethrum, none of the compounds resulted in mortality significantly higher than that of pyrethrum with PBO alone (Fig. 3.2).

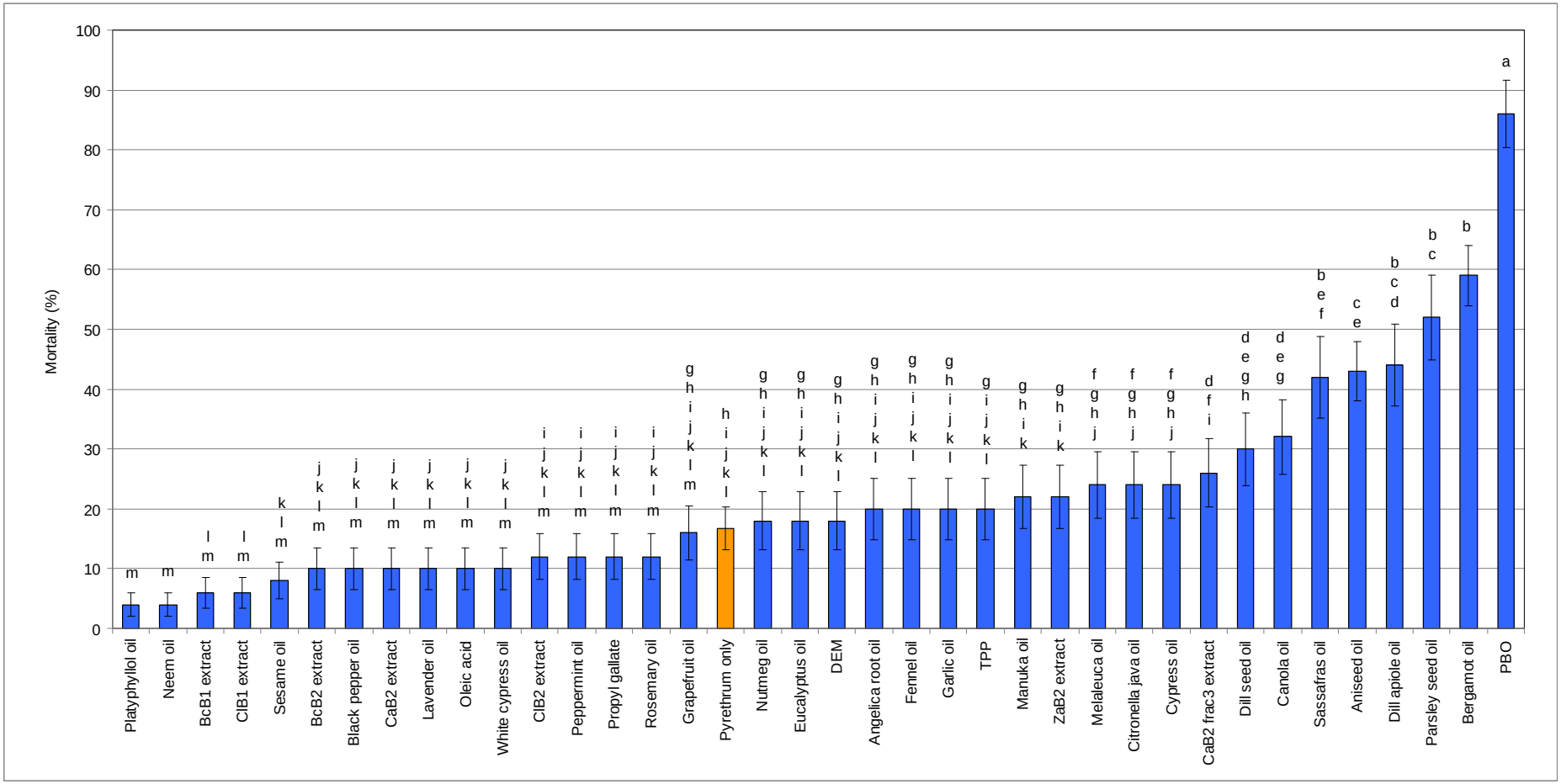


Fig. 3.1. The mean percentage mortality (± SE) of *Helicoverpa armigera* larvae (25-40 mg larvae) by topical application of 937.5 ppm pyrethrum with various pre-applied synergists (5000 ppm) (30-minute pre-treatment). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

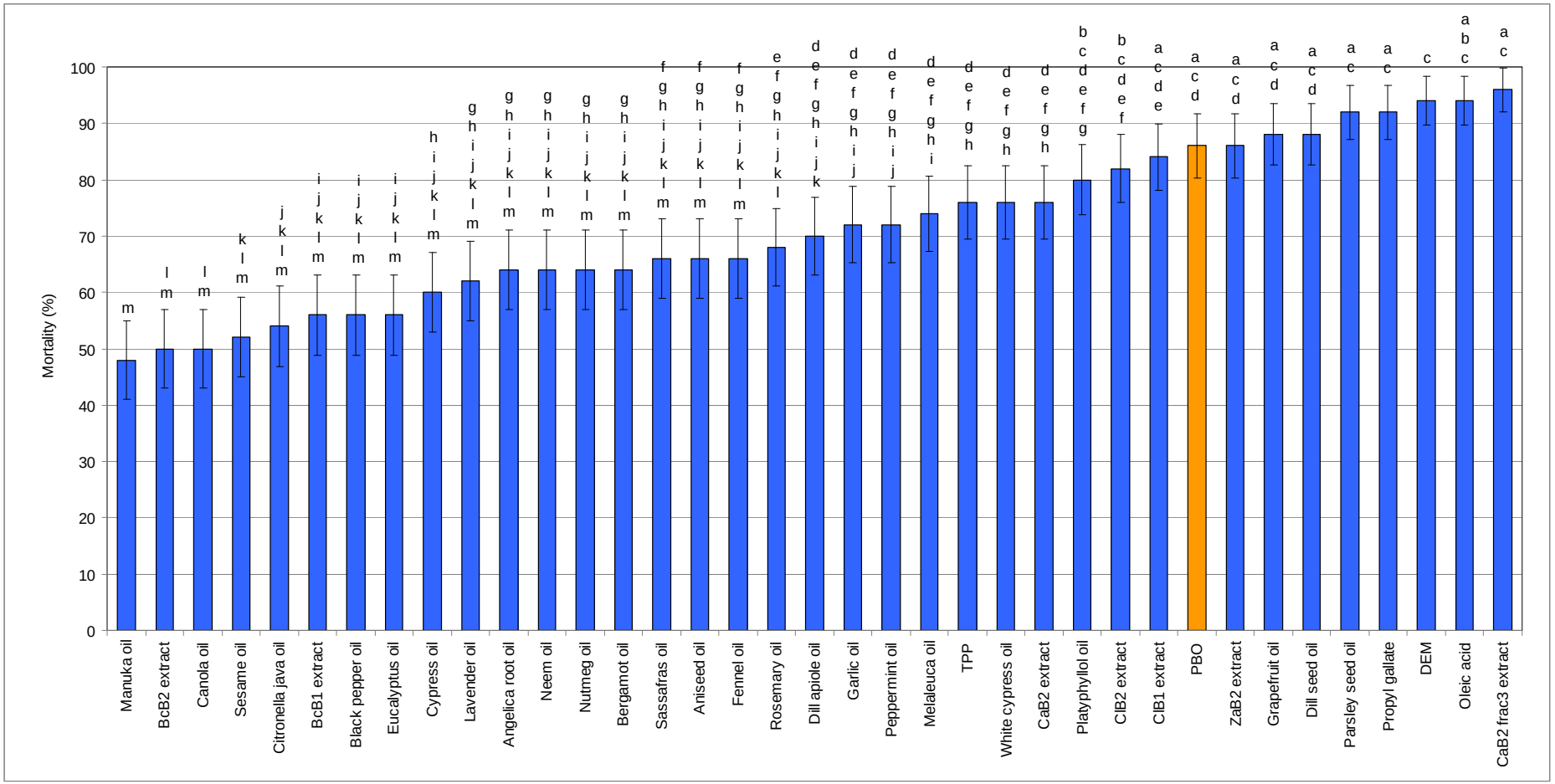


Fig. 3.2. The mean percentage mortality (± SE) of *Helicoverpa armigera* larvae (25-40 mg larvae) by topical application of 937.5 ppm pyrethrum with various pre-applied synergist:PBO solutions (in a 1:1 ratio) of synergist (5000 ppm):PBO (5000 ppm) (30-minute pre-treatment). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

Some mixtures appeared antagonistic (rosemary, fennel, aniseed, sassafras, bergamot, nutmeg, neem, angelica root, lavender, cypress, eucalyptus and black pepper oils, BcB1 extract, citronella java, sesame and canola oils, BcB2 extract and manuka oil), with mortalities significantly lower than pyrethrum with PBO alone ($p<0.001$). No toxicity was shown by any of the synergist:PBO mixtures towards ***H. armigera***, with no mortality in the acetone controls pre-treated with each mixture.

3.3.1.4 Diet incorporation bioassay

The highest percentage mortality was found with ***H. armigera*** larvae treated with pyrethrum after being fed the PBO diet (53.33%), which was significantly higher than for larvae treated with pyrethrum after being fed the standard diet (pyrethrum only) and the oleic acid, rosemary oil, propyl gallate, canola oil, neem oil and aniseed oil diets ($p<0.001$) (Fig. 3.3). The percentage mortality for larvae treated with pyrethrum after being fed the PBO, parsley seed oil and bergamot oil diets did not differ significantly from one another (53.33%, 33.33% and 18.33% respectively) and were the only compounds with mortalities higher (though not always significantly) than larvae fed the standard diet (pyrethrum only) (16.67%). No synergism was shown by any of the other compounds. Neem and aniseed oils appeared antagonistic, with significantly lower mortalities (6.67% and 5.00% respectively) than for larvae fed the parsley seed oil and PBO diets ***(***33.33% and 53.33% respectively). None of the diets incorporated with synergist showed toxicity towards ***H. armigera***, with no mortality in the acetone controls.

The aniseed oil diet appeared to repel ***H. armigera*** larvae, with significantly more larvae avoiding the aniseed oil diet (26.25%) than the standard diet (0%, $p<0.001$) (Fig. 3.4). Both rosemary oil and PBO diets showed non-significant increases in avoidance, with 11.25% and 13.75% of larvae avoiding each diet respectively.

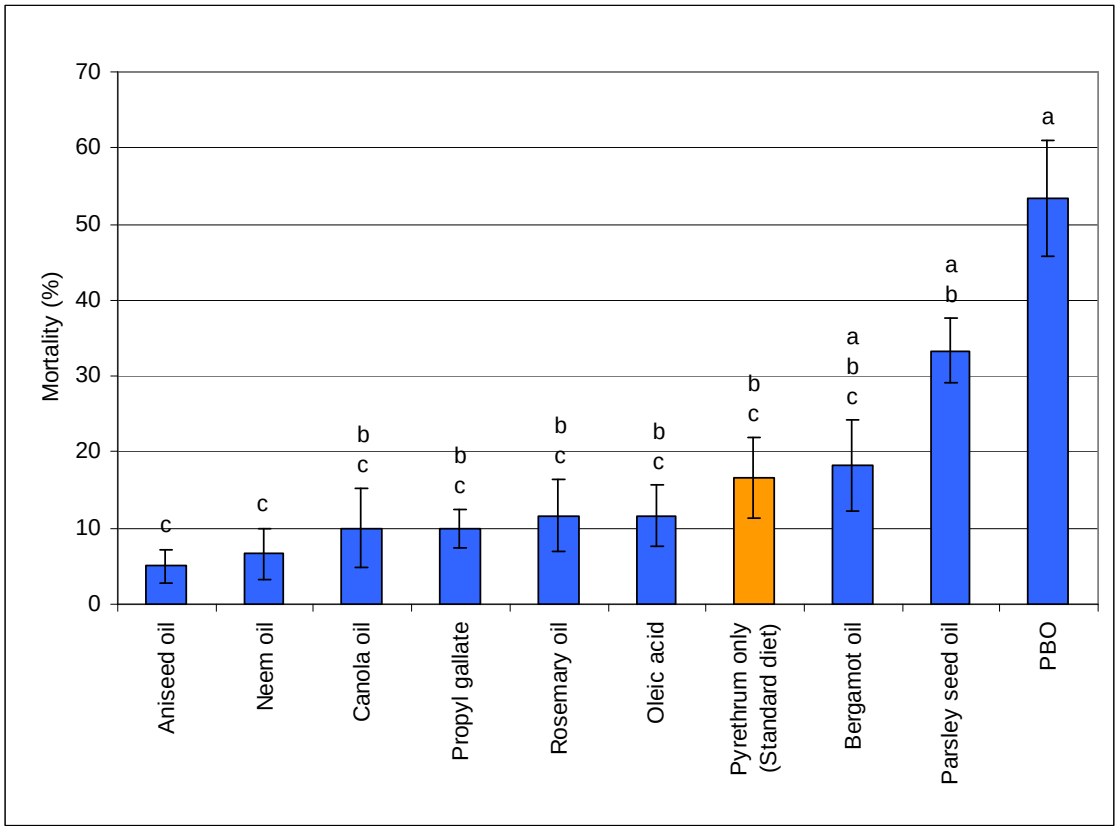


Fig. 3.3. The mean percentage mortality (± SE) of *Helicoverpa armigera* larvae (25-40 mg larvae) treated with 937.5 ppm pyrethrum after feeding on diet incorporated with various synergists (5000 ppm) or the standard diet (pyrethrum only). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

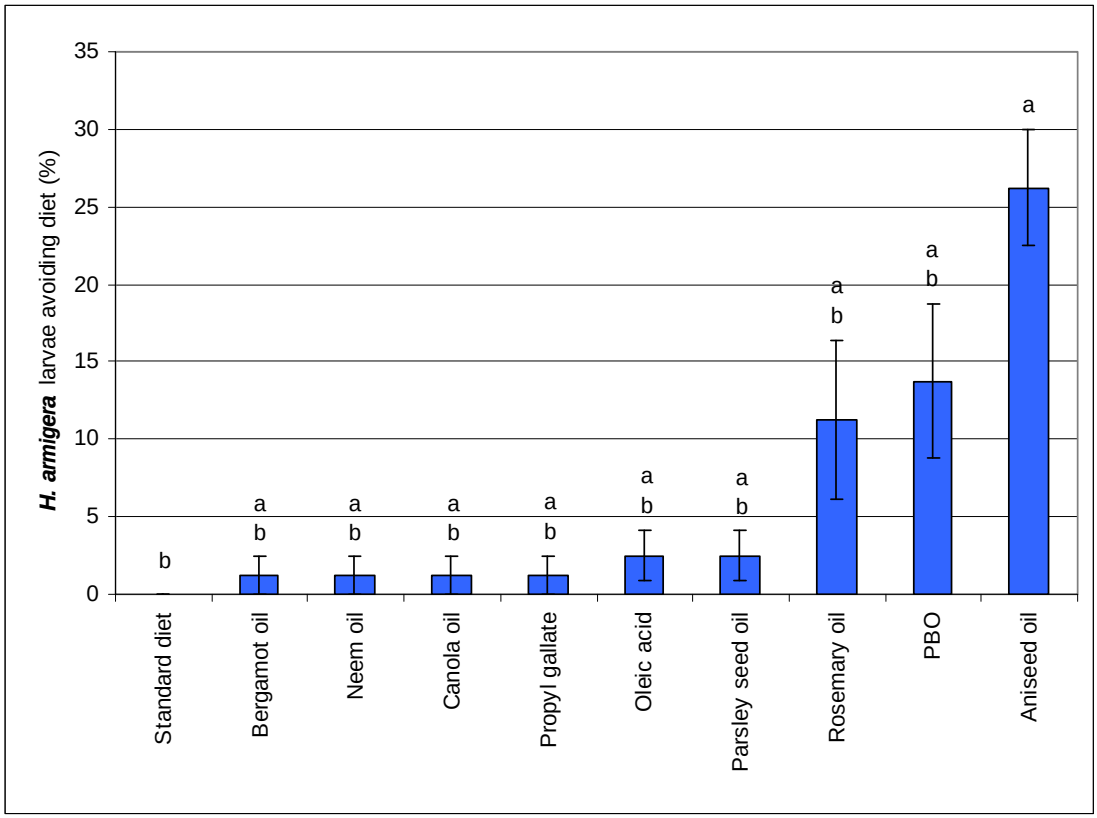


Fig. 3.4. The mean percentage (± SE) of *Helicoverpa armigera* larvae (25-40 mg larvae) avoiding the standard diet and each of the synergist-incorporated diets (5000 ppm). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

3.3.1.5 Full dose response bioassays with synergists

Full dose response bioassays were used to compare the relative synergistic efficacy of synergists with pyrethrum. None of the synergists showed toxicity towards ***H. armigera*** at the applied dosage, with no mortality found in any of the synergist-only controls (with acetone).

The LD_{50} values of pyrethrum synergised by PBO at 10 000 ppm and at 50 000 ppm did not differ significantly from one another (297 ppm and 311 ppm respectively) but both were significantly lower than the LD_{50} values for all the other treatments (Table 3.6), making PBO the most effective pyrethrum synergist. Pyrethrum synergised by parsley seed oil, dill apiole oil and bergamot oil had significantly lower LD_{50} values (1197 ppm, 1630 ppm and 1637 ppm respectively) than pyrethrum with acetone

(unsynergised) (2561 ppm). All other synergists had LD_{50} values equivalent to unsynergised pyrethrum, with the exception of pyrethrum synergised by CaB2 extract, which had a significantly higher LD_{50} (4442 ppm). Pyrethrum synergised by ZaB2 extract also tended toward a higher LD_{50} than the other treatments (3889 ppm), with both CaB2 and ZaB2 seeming to antagonise rather than synergise pyrethrum and could possibly be due to the synergists blocking or slowing the penetration of pyrethrum through the cuticle.

The LD_{90} for pyrethrum synergised by 10 000 ppm PBO (866 ppm) was significantly lower than the LD_{90} values for all the other treatments (Table 3.6). The LD_{90} of pyrethrum synergised by 50 000 ppm PBO (1442 ppm) did not differ significantly from the LD_{90} of pyrethrum synergised by 10 000 ppm PBO (866 ppm) or from pyrethrum synergised by dill apiole oil (3413 ppm), however, it had a significantly lower LD_{90} than the other treatments. PBO was therefore the most effective pyrethrum synergist, followed by dill apiole oil, at the LD_{90} level. The LD_{90} for pyrethrum synergised by parsley seed oil (3974 ppm) did not differ significantly from the LD_{90} of pyrethrum with dill apiole oil but was significantly lower than LD_{90} values for many synergists, including unsynergised pyrethrum (acetone). The LD_{90} values of pyrethrum with plant extracts CaB2 and ZaB2 were the highest, with ZaB2 extract being significantly higher than unsynergised pyrethrum, showing antagonism rather than synergism of pyrethrum with these two compounds.

Synergism factors calculated for the LD_{50} and LD_{90} values showed PBO (10 000 and 50 000 ppm) to have significantly higher (3 fold or greater) synergism of pyrethrum than all the other treatments (Table 3.6). Parsley seed oil had significantly higher synergism of pyrethrum for the LD_{50} values than all other remaining synergists but that of dill apiole oil and bergamot oil. Synergism for LD_{90} values ranked dill apiole oil as producing significantly higher synergism of pyrethrum than most of the remaining synergists, with parsley seed oil producing the next highest synergism. CaB2 and ZaB2 extracts at both LD_{50} and LD_{90} values had the lowest ranked synergism factors.

Table 3.6. Probit analysis of pyrethrum bioassay data with *Helicoverpa armigera* larvae (25-40 mg larvae) using a variety of synergists with a 30-minute pre-treatment time. (n = number of larvae exposed, CI = Confidence Intervals, SF = Synergism Factor, df = degrees of freedom and χ^2 = Chi squared).

Pre-treatment	n	LD_{50} (ppm)*	95% CI for LD_{50}	SF (LD_{50}) (95% CI)¥	LD_{90} (ppm)*	95% CI for LD_{90}	SF (LD_{90}) (95% CI)¥	Slope ± SE	df	χ^2
Acetone	610	2561 ^{dfg}	2260 – 2907	-	9243 ^{ej}	7540 – 11 979	-	2.299 ± 0.168	15	6.952
PBO (10 000 ppm)	498	297 ^{a}	254 – 347	8.63 ^{a} (7.27 – 10.25)	866 ^{a}	686 – 1199	10.68 ^{a} (7.84 – 14.54)	2.756 ± 0.211	19	30.215
PBO (50 000 ppm)	270	311 ^{a}	218 – 407	8.22 ^{a} (6.28 – 10.77)	1442 ^{ab}	982 – 2900	6.41 ^{a} (4.04 – 10.17)	1.925 ± 0.269	11	13.514
Parsley seed oil (10 000 ppm)	170	1197 ^{b}	852 – 1518	2.14 ^{b} (1.58 – 2.89)	3974 ^{cd}	2969 – 6592	2.33 ^{bc} (1.51 – 3.60)	2.459 ± 0.429	11	9.743
Dill apiole oil (10 000 ppm)	180	1630 ^{bc}	1383 – 1924	1.57 ^{bc} (1.28 – 1.93)	3413 ^{bc}	2738 – 4896	2.71 ^{b} (1.89 – 3.88)	3.994 ± 0.598	11	4.946
Bergamot oil (10 000 ppm)	350	1637 ^{bc}	1324 – 2053	1.56 ^{bcd} (1.25 – 1.96)	7755 ^{deg}	5320 – 13 902	1.19 ^{bfh} (0.75 – 1.90)	1.897 ± 0.203	15	16.880
Grapefruit oil (10 000 ppm)	420	2138 ^{cd}	1882 – 2427	1.20 ^{cde} (1.00 – 1.43)	6700 ^{de}	5447 – 8877	1.38 ^{bcde} (0.99 – 1.93)	2.583 ± 0.235	17	12.186
CaB2 frac3 extract (10 000 ppm)	282	2182 ^{cde}	1834 – 2575	1.17 ^{cdef} (0.95 – 1.45)	7410 ^{def}	5769 – 10 600	1.25 ^{cdefg} (0.86 – 1.82)	2.413 ± 0.262	18	12.268
White cypress oil (10 000 ppm)	180	2275 ^{cde}	1886 – 2758	1.13 ^{cdefg} (0.90 – 1.41)	5785 ^{cde}	4459 – 8647	1.60 ^{bcd} (1.08 – 2.37)	3.162 ± 0.421	11	7.532

Oleic acid (10 000 ppm)	379	2425 ^{df}	2030 – 2861	1.06 ^{defg} (0.88 – 1.26)	6752 ^{de}	5327 – 9662	1.37 ^{cdef} (1.00 – 1.87)	2.882 ± 0.266	14	21.939
Propyl gallate (10 000 ppm)	370	2646 ^{dfg}	2257 – 3164	0.97 ^{efg} (0.80 – 1.18)	8543 ^{dhij}	6443 – 12 910	1.08 ^{ch} (0.74 – 1.58)	2.517 ± 0.241	17	18.868
Manuka oil (10 000 ppm)	330	2777 ^{dfg}	2400 – 3224	0.92 ^{efgh} (0.76 – 1.12)	8321 ^{eh}	6647 – 11 317	1.11 ^{ch} (0.78 – 1.58)	2.689 ± 0.257	11	8.017
Platyphyllol oil (10 000 ppm)	180	2955 ^{dfgh}	2320 – 3818	0.87 ^{efgh} (0.68 – 1.10)	8537 ^{dhij}	6094 – 15 009	1.08 ^{ch} (0.71 – 1.66)	2.781 ± 0.364	12	13.429
Dill seed oil (10 000 ppm)	180	3003 ^{dfgh}	2371 – 3861	0.85 ^{efgh} (0.65 – 1.12)	11 055 ^{eij}	7617 – 20 569	0.84 ^{dh} (0.50 – 1.41)	2.264 ± 0.331	12	8.799
Neem oil (10 000 ppm)	210	3136 ^{efgh}	2491 – 4098	0.82 ^{efgh} (0.64 – 1.05)	10 905 ^{eij}	7411 – 21 062	0.85 ^{dh} (0.52 – 1.39)	2.368 ± 0.316	12	12.204
Aniseed oil (10 000 ppm)	420	3275 ^{fgh}	2819 – 3846	0.78 ^{fgh} (0.64 – 0.96)	12 694 ^{fghj}	9719 – 18 274	0.73 ^{eh} (0.49 – 1.08)	2.178 ± 0.195	17	14.434
ZaB2 extract (10 000 ppm)	180	3889 ^{gh}	2881 – 5681	0.66 ^{gh} (0.47 – 0.93)	22 709 ^{gi}	12 516 – 74 894	0.41 ^{gh} (0.18 – 0.94)	1.672 ± 0.305	10	7.394
CaB2 extract (10 000 ppm)	180	4442 ^{h}	3307 – 6664	0.58 ^{h} (0.42 – 0.80)	20 598 ^{gij}	11 746 – 62 101	0.45 ^{gh} (0.22 – 0.92)	1.924 ± 0.309	12	12.074

HYPOTHESIS OF EQUALITY (equal slopes, equal intercepts): REJECTED ($p<0.05$) (χ^2 = 0.102E+04, df = 34, tail probability = 0.000).

HYPOTHESIS OF PARALLELISM (equal slopes): REJECTED ($p<0.05$) (χ^2 = 40.92, df = 17, tail probability = 0.001).

* LD_{50} and LD_{90} values with differing symbols within the same column were significantly different from one another (failure of the 95% confidence intervals to overlap).

¥ SF values with differing symbols within the same column were significantly different from one another (failure of the 95% confidence intervals to overlap).

3.3.2 Biochemical assays

3.3.2.1 Esterase assays

3.3.2.1.1 Esterase assay with alpha-naphthyl acetate as a substrate

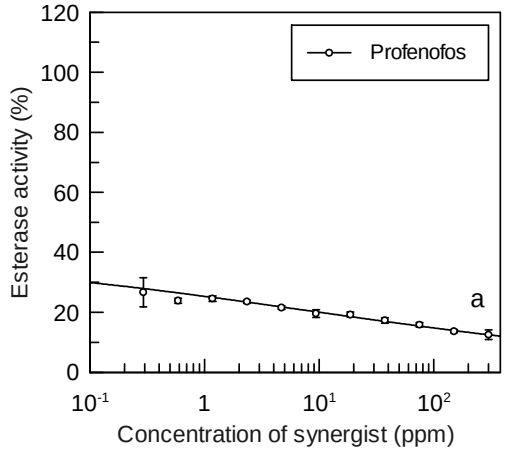
Inhibition curves for compounds showing inhibition of esterases using the esterase assay (alpha-naphthyl acetate as a substrate) are illustrated in Fig. 3.5a-n. The IC_{50} and Y-ranges (± SE) for each synergist that showed inhibition of esterases are presented in Table 3.7. Synergists showing no inhibition of esterases were excluded from the table since the data did not fit the four (or two) parameter logistic used for inhibition data and the IC_{50} and Y-ranges could therefore not be calculated.

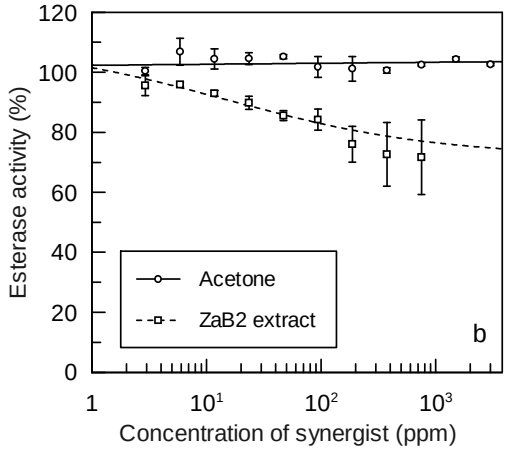
Profenofos, ZaB2 extract and propyl gallate were the most potent inhibitors of ***H. armigera*** esterases, with the lowest IC_{50} values (Table 3.7). Profenofos at the concentration tested gave very high inhibition of esterases (Fig. 3.5a) and the solution should have been diluted further until initial inhibition was observed, in order to obtain a more complete inhibition curve. Compounds in addition to profenofos with an IC_{50} lower than PBO (the standard synergist) were neem oil, BcB1 extract, CaB2 frac3 extract, CaB2 extract, rosemary oil, fennel oil and nutmeg oil. BcB2 extract, dill apiole oil and myristicin oil had the highest IC_{50} values, showing the least potency as esterase inhibitors. PBO had the lowest Y-range of all the synergists (22%) and myristicin oil the highest (100%).

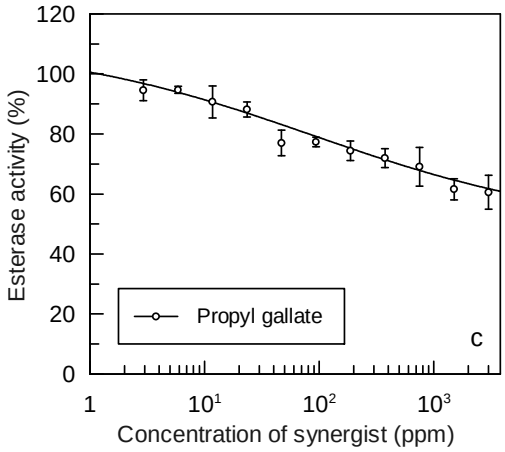
No inhibition of total esterase activity was found with acetone (control) (Fig. 3.5b), canola, dill seed, eucalyptus, grapefruit, melaleuca, platyphyllol, sesame, angelica root, bergamot, black pepper, lavender and peppermint oils, DEM and ClB1 extract. Geranium, citronella and citronella java oils gave apparent esterase activity higher than the uninhibited control, due to a probable anomaly with the assay, where some compounds may bind to the esterase in such a manner so as to speed up the substrate (Dr. Graham D. Moores, ***Pers. comm.***).

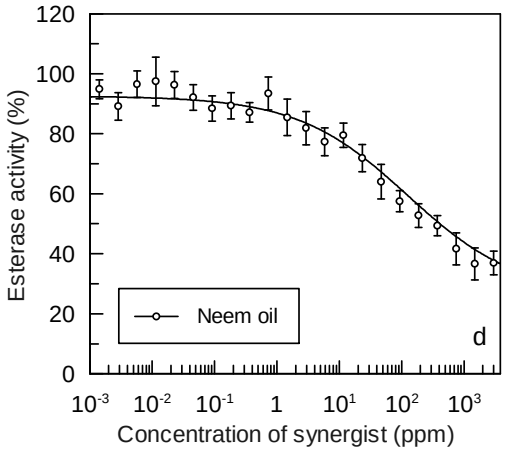
Table 3.7. Inhibition of *Helicoverpa armigera* (3-4 mg larvae) total esterase activity (alpha-naphthyl acetate as the substrate) by potential pyrethrum synergists, profenofos, TPP and PBO. The IC_{50} and Y-range values (± SE) are tabulated for each synergist. (Relates to Fig. 3.5a-n; Synergists ordered from lowest to highest IC_{50}).

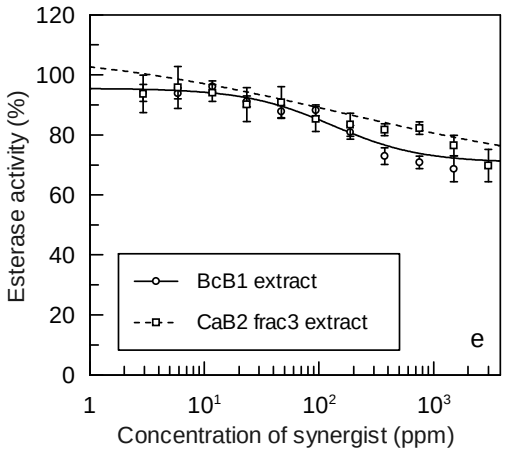
Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
Profenofos	Organophosphate	8.61 (± 13.68)	87.16 (± 13.52)
ZaB2 extract	Sesquiterpenoid	15.07 (± 71.07)	37.74 (± 46.87)
Propyl gallate	Ester	87.45 (± 65.77)	61.58 (± 8.99)
Neem oil	Tetranortriterpenoid / liminoid	125 (± 70.83)	66.17 (± 8.43)
BcB1 extract	Unknown	131 (± 54.09)	24.79 (± 5.13)
CaB2 frac3 extract	Phenylpropene	159 (± 72.42)	43.21 (± 3.45)
CaB2 extract	Phenylpropene	159 (± 57.25)	28.07 (± 5.35)
Rosemary oil	Monoterpenoid / ether; Bicyclic monoterpene; Terpenoid	237 (± 124)	35.58 (± 9.93)
Fennel oil	Unsaturated ether / phenylpropene derivative Monoterpene / ketone	242 (± 181)	47.12 (± 5.78)
Nutmeg oil	Bicyclic monoterpene; Methylenedioxyphenol ring	658 (± 227)	29.21 (± 5.39)
PBO	Methylenedioxyphenol ring	695 (± 383)	22.06 (± 5.66)
ClB2 extract	Cyclic terpene	1296 (± 577)	36.81 (± 12.42)
Aniseed oil	Unsaturated ether / phenylpropene derivative	2173 (± 965)	42.57 (± 23.09)
Cypress oil	Bicyclic monoterpene	2255 (± 683)	76.13 (± 7.49)
Garlic oil	Sulphur compound	2776 (± 720)	100
Parsley seed oil	Methylenedioxyphenol ring; Bicyclic monoterpene; Methylenedioxyphenol ring / phenylpropanoid	2840 (± 6299)	41.17 (± 48.09)
TPP	Ester (triester)	3255 (± 1098)	65.88 (± 8.95)
Manuka oil	Sesquiterpene hydrocarbon; Triketone derivative	3593 (± 3710)	33.27 (± 7.77)
White cypress oil	Bicyclic monoterpene; Monoterpene; Cyclic terpene	5317 (± 1583)	77.42 (± 10.52)
Oleic acid	Mono-unsaturated omega-9 fatty acid	5870 (± 2615)	67.20 (± 14.62)
Sassafras oil	Methylenedioxyphenol ring	8257 (± 40 453)	46.88 (± 108)
Myristicin oil	Methylenedioxyphenol ring	11 676 (± 11 862)	101 (± 48.13)
Dill apiole oil	Methylenedioxyphenol ring / phenylpropanoid	11 756 (± 15 671)	55.08 (± 35.98)
BcB2 extract	Unknown	-	100

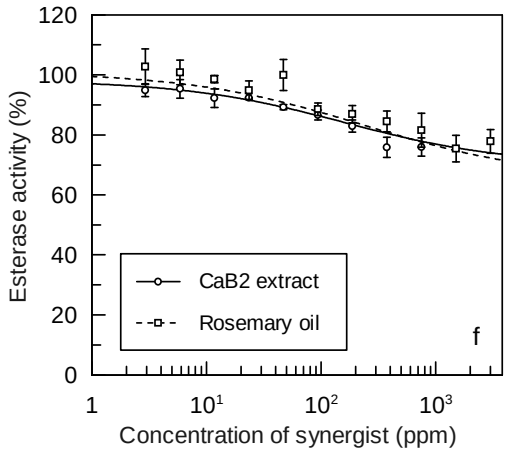

Profenofos
a
Esterase activity (%)
Concentration of synergist (ppm)

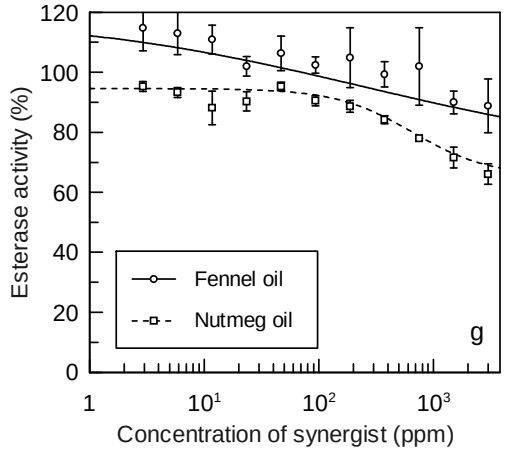

Acetone
ZaB2 extract
b
Esterase activity (%)
Concentration of synergist (ppm)

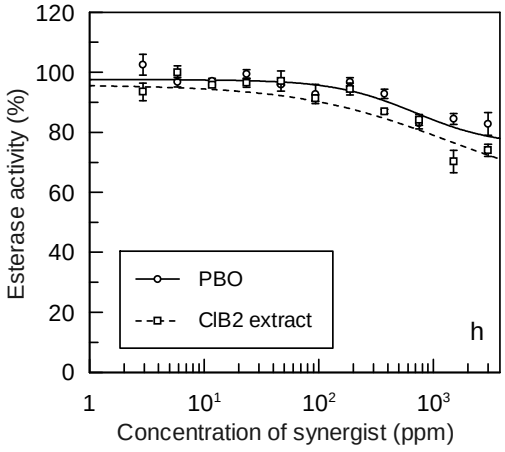

Propyl gallate
c
Esterase activity (%)
Concentration of synergist (ppm)

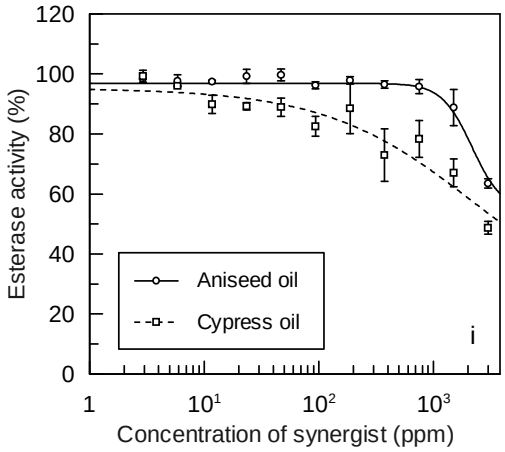

Neem oil
d
Esterase activity (%)
Concentration of synergist (ppm)

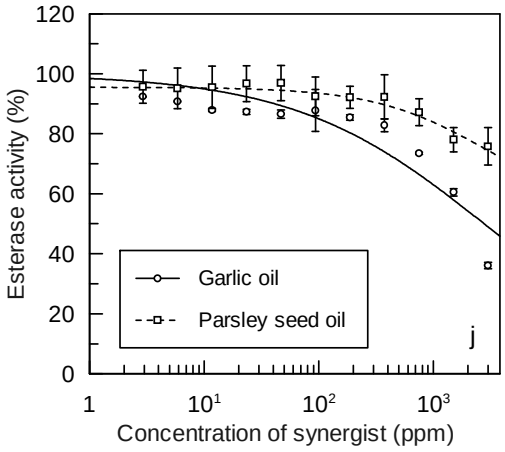

BcB1 extract
CaB2 frac3 extract
e
Esterase activity (%)
Concentration of synergist (ppm)

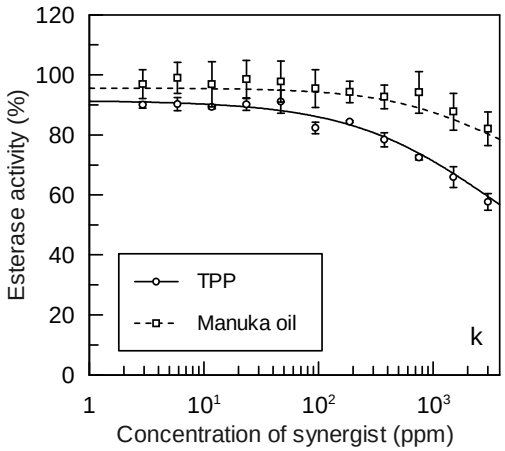

CaB2 extract
Rosemary oil
f
Esterase activity (%)
Concentration of synergist (ppm)

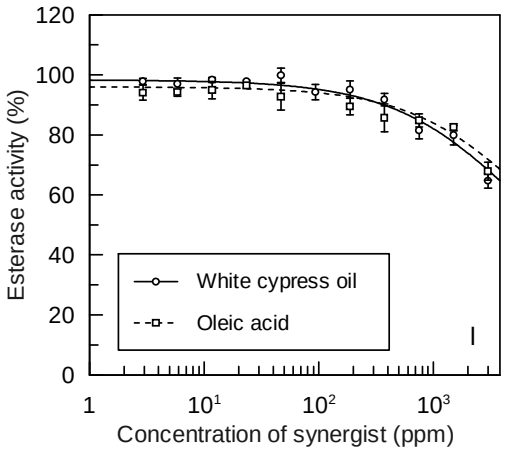

Esterase activity (%)
Concentration of synergist (ppm)
Fennel oil
Nutmeg oil
g


Esterase activity (%)
Concentration of synergist (ppm)
PBO
ClB2 extract
h


Esterase activity (%)
Concentration of synergist (ppm)
Aniseed oil
Cypress oil
i


Esterase activity (%)
Concentration of synergist (ppm)
Garlic oil
Parsley seed oil
j


Esterase activity (%)
Concentration of synergist (ppm)
TPP
Manuka oil
k


Esterase activity (%)
Concentration of synergist (ppm)
White cypress oil
Oleic acid
l

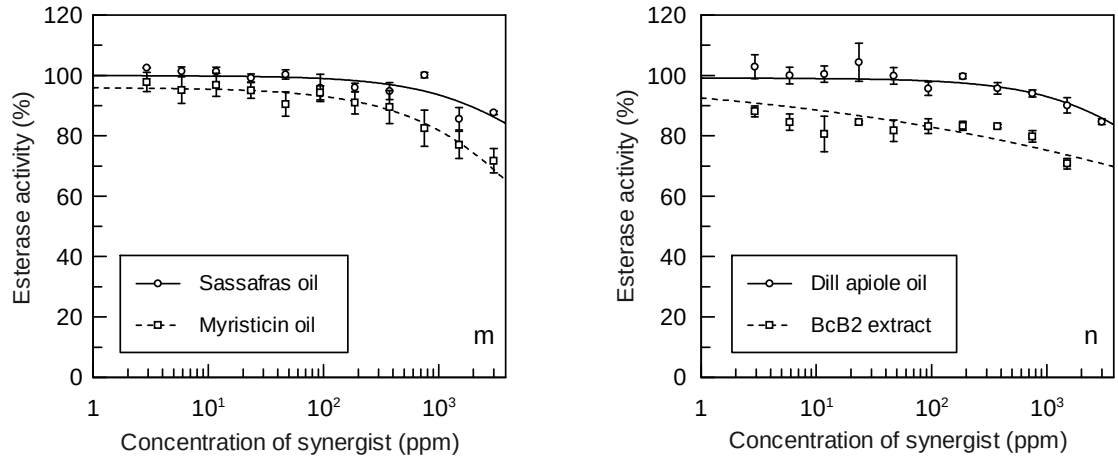


Fig. 3.5a-n. Inhibition of *Helicoverpa armigera* (3-4 mg larvae) total esterase activity (alpha-naphthyl acetate as the substrate) by potential pyrethrum synergists, profenofos, TPP, PBO and acetone (control). Error bars represent standard deviation. For garlic oil and BcB2 extract, lines were fitted with two parameter logistic curves (lower and upper limits of 0% and 100% respectively). For all remaining compounds, lines were fitted with a four parameter logistic curve. (Relates to Table 3.7; Synergists ordered from lowest to highest IC_{50}).

No significant correlations were found between ***in vivo*** synergism of pyrethrum and inhibition of esterase activity (Table 3.8). PBO, bergamot oil, parsley seed oil, dill apiole oil, aniseed oil and sassafras oil showed the highest synergism of pyrethrum. However, bergamot oil did not inhibit esterases whilst the other compounds showed varying degrees of esterase inhibition (Table 3.7, Fig. 3.5a-n). Propyl gallate and neem oil, which were good esterase inhibitors, did not synergise pyrethrum.

3.3.2.1.2 Esterase assay with para-nitrophenyl acetate as a substrate

When para-nitrophenyl acetate was used as the esterase substrate, only eight compounds inhibited ***H. armigera*** esterases, with ZaB2 extract, propyl gallate and neem oil showing the highest potency (Table 3.9, Fig. 3.6a-e). The majority of compounds gave no inhibition of esterases with this assay (Fig 3.6e-k).

Table 3.8. Correlations in *Helicoverpa armigera* between pyrethrum synergism (discriminating dose bioassay) and esterase inhibition (alpha-naphthyl acetate (α-NA) and para-nitrophenyl acetate (p-NPA) as substrates and the esterase interference assay) and glutathione-S-transferase (GST) inhibition.

Bioassay / enzyme assay	Enzyme assay	n[¥]	Correlation coefficient	Significance[§] (p-value) (two-tailed)
H. armigera discriminating dose	Highest concentration of synergist (α-NA)	37	0.162	NS (0.338)
	IC_{50} (α-NA)	21	0.250	NS (0.274)
	Y range (α-NA)	20	-0.295	NS (0.207)
	Highest concentration of synergist (p-NPA)	19	0.419	NS (0.074)
	IC_{50} (p-NPA)	8	0.105	NS (0.805)
	Y range (p-NPA)	8	-0.011	NS (0.979)
	Interference assay	19	0.429	NS (0.067)
	Highest concentration of synergist (GST)	37	0.230	NS (0.171)
	IC_{50} (GST)	22	0.334	NS (0.129)
	Y range (GST)	14	-0.340	NS (0.234)
Highest concentration of synergist (α-NA)	Highest concentration of synergist (p-NPA)	19	0.446	NS (0.056)
	Interference assay	19	0.400	NS (0.090)
Highest concentration of synergist (p-NPA)	Interference assay	19	0.702	** (0.001)

[¥] n = Number of synergists used in the correlation analysis.

[§] NS = not significant, ** Significant correlation (p<0.01).

No significant correlations were found between ***in vivo*** synergism of pyrethrum and esterase inhibition using the total esterase assay (para-nitrophenyl acetate as a substrate) (Table 3.8). PBO, bergamot oil, parsley seed oil, dill apiole oil and aniseed oil gave no inhibition of esterases, whereas all showed synergism of pyrethrum. Compounds that inhibited esterases, including neem oil and propyl gallate, did not synergise pyrethrum.

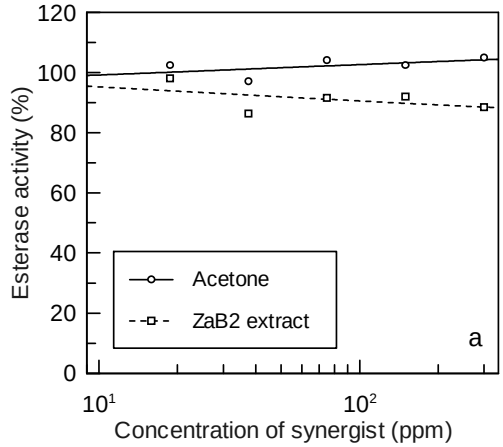
Table 3.9. Inhibition of *Helicoverpa armigera* (30-40 mg larvae) total esterase activity (para-nitrophenyl acetate as the substrate) by potential pyrethrum synergists. The IC_{50} and Y-range values (± SE) are tabulated for each synergist. (Relates to Fig. 3.6a-k; IC_{50} and Y-range values could not be accurately calculated for compounds showing very little or no inhibition of esterases; Compounds ordered from lowest to highest IC_{50}).

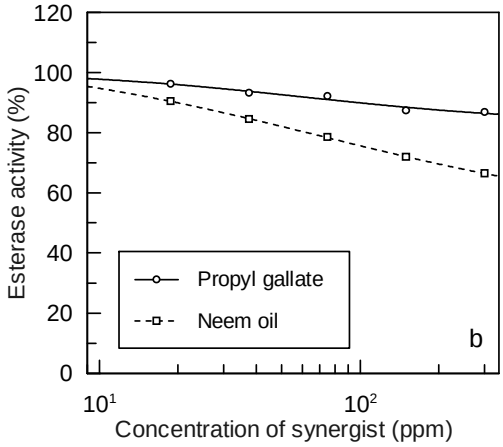
Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
ZaB2 extract	Sesquiterpenoid	29.64 (± 160)	21.48 (± 19.74)
Propyl gallate	Ester	60.45 (± 77.12)	17.55 (± 11.49)
Neem oil	Tetranortriterpenoid / liminoid	71.03 (± 12.93)	54.47 (± 18.38)
ClB1 extract	Cyclic terpene	149 (± 805)	56.59 (± 85.30)
CaB2 extract	Phenylpropene	209 (± 39.15)	31.73 (± 4.27)
CaB2 frac3 extract	Phenylpropene	412 (± 368)	66.51 (± 27.54)
ClB2 extract	Cyclic terpene	418 (± 396)	54.30 (± 18.98)
BcB1 extract	Unknown	451 (± 403)	36.09 (± 19.81)

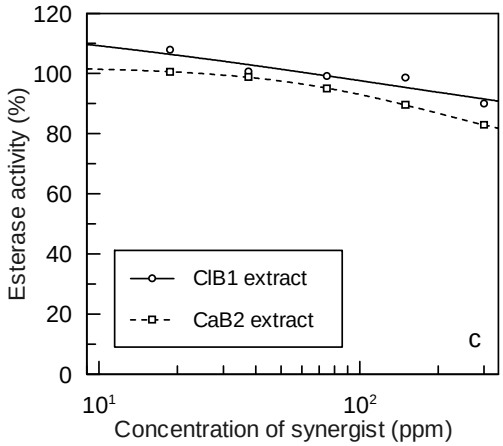
3.3.2.1.3 Esterase interference assay

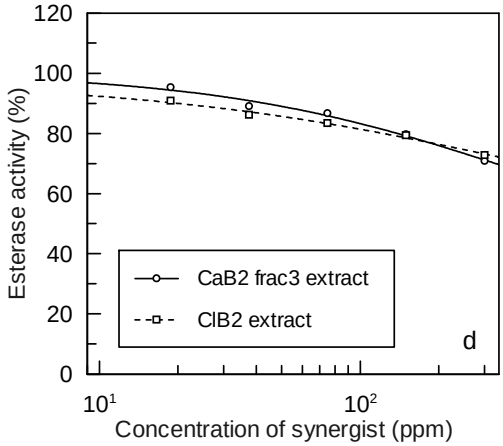
Neem oil, CaB2 frac3 extract and propyl gallate showed the highest inhibition (or "blockade") of esterases with the esterase interference assay (index values = 55.88%, 56.96% and 65.94% respectively) (Table 3.10). The least effective compounds were bergamot oil, dill apiole oil and oleic acid (index values = 91.46%, 90.92% and 88.40% respectively). No inhibition of esterases was found with dill seed oil, geranium oil, aniseed oil, citronella oil, PBO and grapefruit oil.

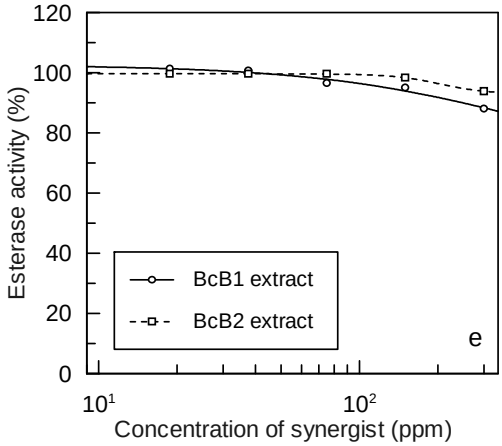
The correlation tested for between ***in vivo*** synergism of pyrethrum in ***H. armigera*** and the inhibition of esterases using the esterase interference assay was not significant (Table 3.8). Of the compounds that synergised pyrethrum, PBO and aniseed oil showed no inhibition of esterases, bergamot and dill apiole oils showed slight inhibition and parsley seed oil gave the most inhibition of esterases. Neem oil, CaB2 frac3 extract and propyl gallate exhibited the highest inhibition in the esterase interference assay but failed to synergise pyrethrum.

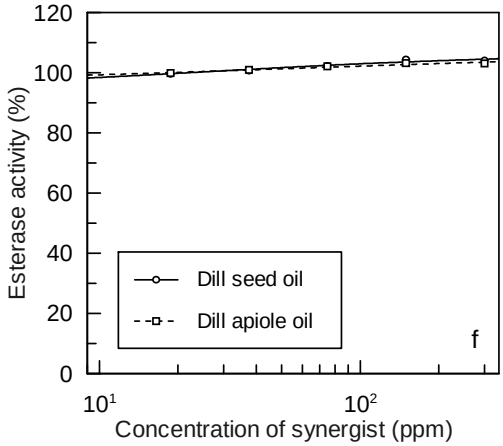

Esterase activity (%)
Acetone
ZaB2 extract
a
Concentration of synergist (ppm)


Esterase activity (%)
Propyl gallate
Neem oil
b
Concentration of synergist (ppm)


Esterase activity (%)
ClB1 extract
CaB2 extract
c
Concentration of synergist (ppm)


Esterase activity (%)
CaB2 frac3 extract
ClB2 extract
d
Concentration of synergist (ppm)


Esterase activity (%)
BcB1 extract
BcB2 extract
e
Concentration of synergist (ppm)


Esterase activity (%)
Dill seed oil
Dill apiole oil
f
Concentration of synergist (ppm)

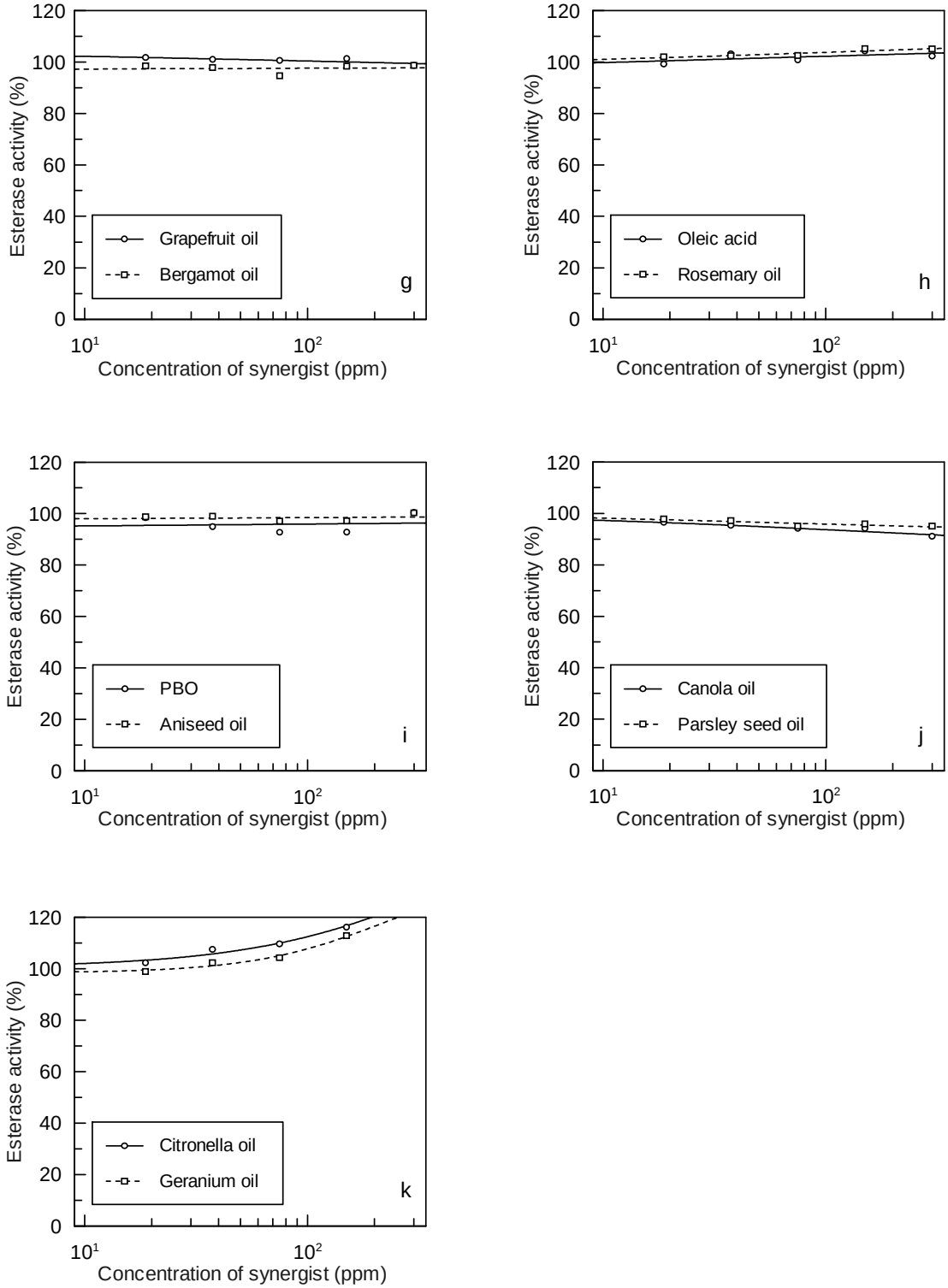


Fig. 3.6a-k. Inhibition of *Helicoverpa armigera* (30-40 mg larvae) total esterase activity (para-nitrophenyl acetate as the substrate) by potential pyrethrum synergists, PBO and acetone (control). All lines were fitted with a four parameter logistic curve. (Relates to Table 3.9; Synergists ordered from lowest to highest IC_{50} until BcB1 extract (showing inhibition), after which compounds are in no particular order).

Table 3.10. The index value (I), standard error (SE) and 95% confidence intervals (CI) for *Helicoverpa armigera* esterase (30-40 mg larvae) with potential pyrethrum synergists and PBO with the esterase interference assay*. (Synergists ordered from lowest to highest I value).

Compound / esterase / no esterase	Type of chemical	Index value (I) %	SE (%)	95% CI for I
No esterase	-	0	0.52	-1.08 - 1.08
Neem oil	Tetranortriterpenoid / liminoid	55.88	6.45	42.46 - 69.30
CaB2 frac3 extract	Phenylpropene	56.96	4.58	47.44 - 66.48
Propyl gallate	Ester	65.94	4.92	55.70 - 76.17
ClB2 extract	Cyclic terpene	68.37	5.10	57.77 - 78.97
Canola oil	Mono-unsaturated omega-9 fatty acid; Unsaturated omega-6 fatty acid	77.90	2.93	71.80 - 84.00
Parsley seed oil	Methylenedioxyphenol ring; Bicyclic monoterpene; Methylenedioxyphenol ring / phenylpropanoid	79.26	9.91	58.65 - 99.88
ClB1 extract	Cyclic terpene	83.56	5.27	72.59 - 94.52
Rosemary oil	Monoterpenoid / ether; Bicyclic monoterpene; Terpenoid	83.81	2.17	79.30 - 88.32
BcB2 extract	Unknown	85.49	1.39	82.61 - 88.37
BcB1 extract	Unknown	85.56	4.92	75.33 - 95.78
ZaB2 extract	Sesquiterpenoid	87.34	4.46	78.06 - 96.61
CaB2 extract	Phenylpropene	87.75	5.08	77.20 - 98.31
Oleic acid	Mono-unsaturated omega-9 fatty acid	88.40	4.12	79.83 - 96.97
Dill apiole oil	Methylenedioxyphenol ring / phenylpropanoid	90.92	6.48	77.44 - 104
Bergamot oil	Cyclic terpene; Acetate ester	91.46	5.58	79.85 - 103
Dill seed oil	Terpenoid; Cyclic terpene	99.09	3.44	91.94 - 106
Esterase	-	100	8.05	83.26 - 117
Geranium oil	Monoterpenoid / alcohol	105	5.29	93.76 - 116
Aniseed oil	Unsaturated ether / phenylpropene derivative	106	5.65	94.46 - 118
Citronella oil	Monoterpenoid / alcohol; Cyclic terpene	109	5.89	96.69 - 121
PBO	Methylenedioxyphenol ring	122	6.08	109 -135
Grapefruit oil	Cyclic terpene	135	2.16	130 - 139

* I is the IC_{50} value calculated from Grafit for the house fly acetylcholinesterase (AChE) activity remaining, shown as a percentage of Esterase and No esterase, where Esterase = 100% activity and No esterase = 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 I.

The esterase assay (alpha-naphthyl acetate as the substrate) did not correlate significantly to either the esterase assay (para-nitrophenyl acetate as a substrate) or the esterase interference assay (Table 3.8).

A significant positive correlation was found (correlation coefficient = 0.702, n=19, p=0.001) between the esterase assay (para-nitrophenyl acetate as the substrate) and the esterase interference assay (index values) (Table 3.8, Fig. 3.7). Synergists showing inhibition of esterases with the esterase assay (para-nitrophenyl acetate as a substrate) also showed inhibition of esterases with the esterase interference assay, these included, amongst others, BcB1, BcB2 and CaB2 extracts (Table 3.11).

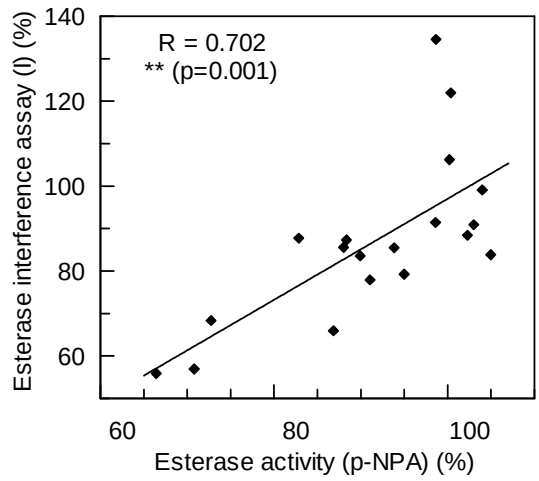


Fig. 3.7. Correlation between the esterase assay (para-nitrophenyl acetate as a substrate (p-NPA)) (percentage esterase activity remaining at the highest concentration of the synergist dilution series) and the esterase interference assay (index values (I)) with the synergists tested for inhibition of *Helicoverpa armigera* esterases. Each point indicates a different synergist (n=19), lines were fitted by linear regression, R is the correlation coefficient and ** indicates a significant correlation (p<0.01). (Relates to Table 3.8).

Table 3.11. Esterase inhibition by potential pyrethrum synergists and acetone (control) (alpha-naphthyl acetate (α-NA) and para-nitrophenyl acetate (p-NPA) as substrates, and the esterase interference assay) and GST inhibition in *Helicoverpa armigera* larvae. (✓ = inhibition, x = no inhibition, NA = not applicable (the assay was not run)).

	Inhibition			
Compound	α-NA	p-NPA	Int. assay	GST
Acetone (control)	x	x	NA	x
Aniseed oil	✓	x	x	✓
BcB1 extract	✓	✓	✓	✓
BcB2 extract	✓	✓	✓	✓
Bergamot oil	x	x	✓	x
CaB2 extract	✓	✓	✓	✓
CaB2 frac3 extract	✓	✓	✓	✓
Canola oil	x	✓	✓	x
Citronella oil	x	x	x	x
ClB1 extract	x	✓	✓	✓
ClB2 extract	✓	✓	✓	✓
Dill apiole oil	✓	x	✓	✓
Dill seed oil	x	x	x	✓
Geranium oil	x	x	x	x
Grapefruit oil	x	x	x	✓
Neem oil	✓	✓	✓	✓
Oleic acid	✓	x	✓	x
Parsley seed oil	✓	x	✓	x
PBO	✓	x	x	x
Propyl gallate	✓	✓	✓	✓
Rosemary oil	✓	x	✓	x
ZaB2 extract	✓	✓	✓	✓
Angelica root oil	x	NA	NA	✓
Black pepper oil	x	NA	NA	✓
Citronella java oil	x	NA	NA	✓
Cypress oil	✓	NA	NA	✓
DEM	x	NA	NA	x
Eucalyptus oil	x	NA	NA	x
Fennel oil	✓	NA	NA	✓
Garlic oil	✓	NA	NA	✓
Lavender oil	x	NA	NA	x
Manuka oil	✓	NA	NA	x
Melaleuca oil	x	NA	NA	x
Myristicin oil	✓	NA	NA	✓
Nutmeg oil	✓	NA	NA	✓
Peppermint oil	x	NA	NA	x
Platyphyllol oil	x	NA	NA	✓
Profenofos	✓	NA	NA	✓
Sassafras oil	✓	NA	NA	✓
Sesame oil	x	NA	NA	x
TPP	✓	NA	NA	x
White cypress oil	✓	NA	NA	✓

3.3.2.2 Glutathione-S-transferase assay

For the synergists showing inhibition of GST activity, profenofos and BcB1 extract had the lowest IC_{50} values, whereas dill seed and white cypress oils had the highest IC_{50} values (Table 3.12, Fig. 3.8a-n). Black pepper, aniseed and fennel oils had the smallest Y-ranges whereas neem oil and propyl gallate had the largest Y-ranges (both 100% using the four parameter logistic fit) (Table 3.12).

The acetone control showed no inhibition of GSTs (Fig. 3.8b), along with bergamot, canola, citronella, eucalyptus, geranium, lavender, manuka, melaleuca, parsley seed, peppermint, rosemary and sesame oils, oleic acid, PBO, DEM and TPP. The standard synergist commonly used to indicate GST inhibition, DEM, did not inhibit ***H. armigera*** GSTs.

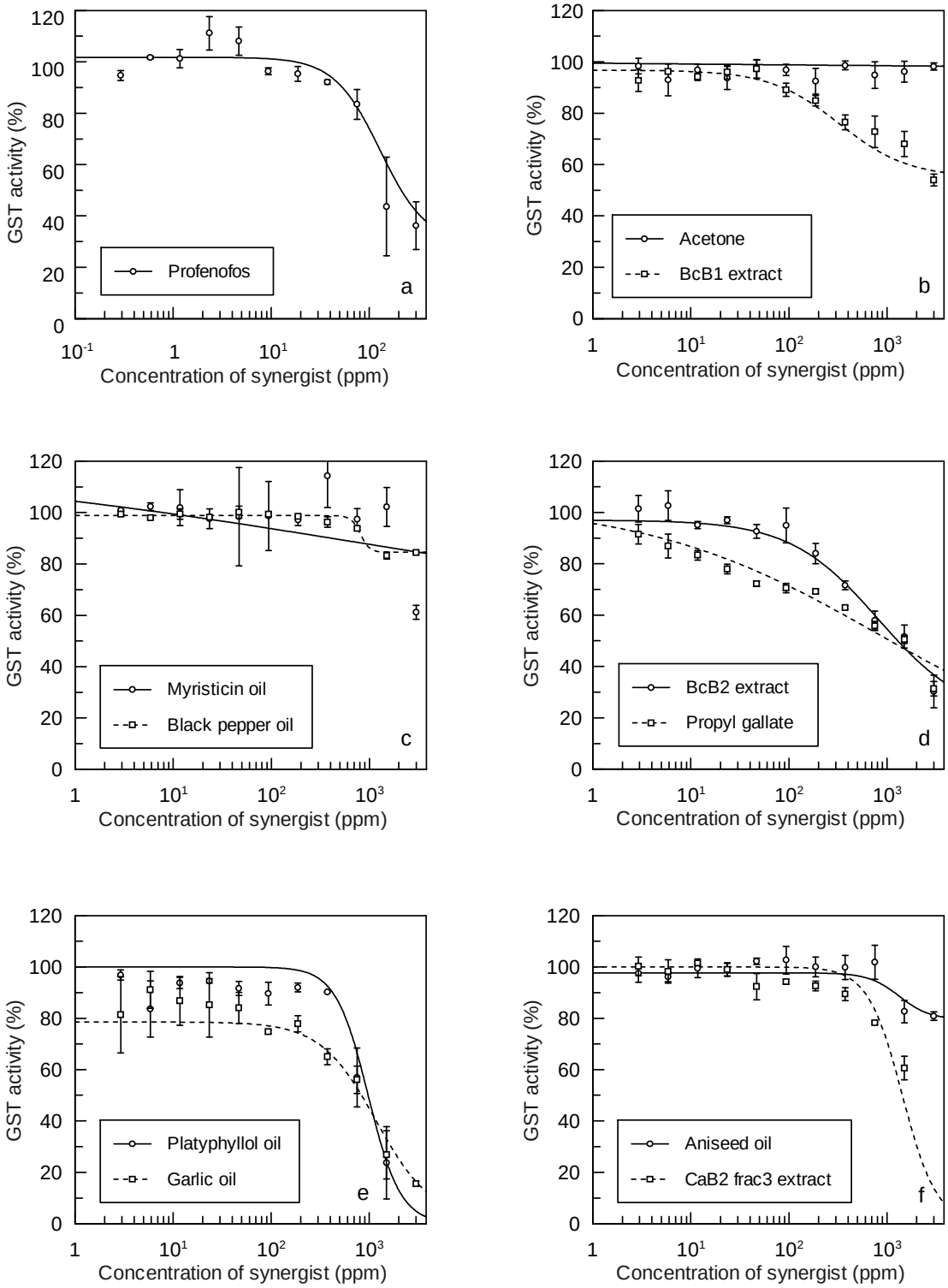
No significant correlations were found between synergism of pyrethrum and inhibition of GST activity (Table 3.8). PBO, bergamot oil and parsley seed oil showed no inhibition of GSTs whereas dill apiole, sassafras and aniseed oils showed varying degrees of GST inhibition. BcB1 and BcB2 extracts and black pepper oil showed good inhibition of GSTs but were not effective as pyrethrum synergists.

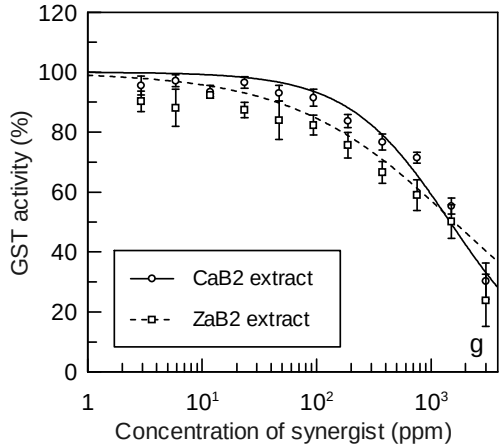
3.3.2.3 Monooxygenase assay

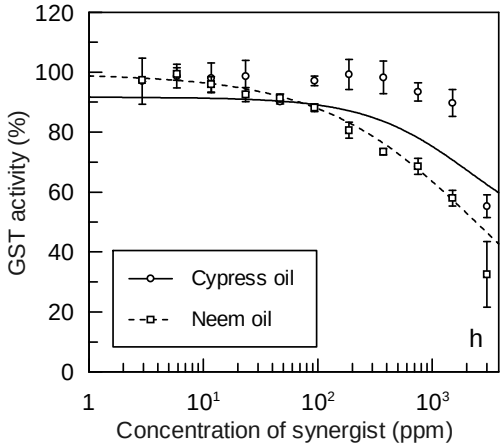
Monooxygenase activity was not detected in ***H. armigera*** during this study and the synergists could therefore not be compared regarding their ability to inhibit ***H. armigera*** monooxygenases. A correlation was tested for between ***in vivo*** synergism of pyrethrum in ***H. armigera*** and the monooxygenase inhibition data found with the synergists using ***Meligethes aeneus*** and ***Carpophilus*** spp. in order to get an indication of how synergism might compare to ***in vitro*** inhibition of monooxygenase activity (chapter six, section 6.3.2.3), however, the use of inter-taxon comparisons may potentially be limited due to differences between taxa.

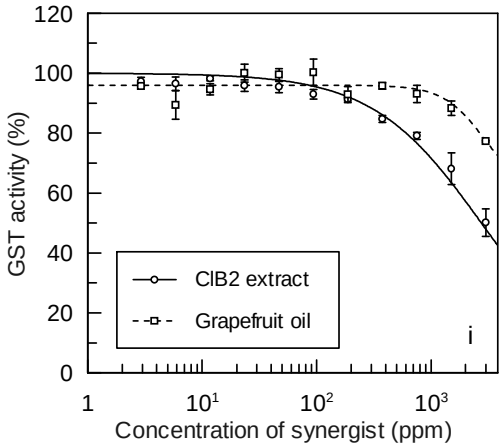
Table 3.12. Inhibition of *Helicoverpa armigera* glutathione-S-transferase activity (3-4 mg larvae) (CDNB as the substrate) by potential pyrethrum synergists. The IC_{50} and Y-range values (± SE) are tabulated for each synergist. (Relates to Fig. 3.8a-n; Synergists ordered from lowest to highest IC_{50}).

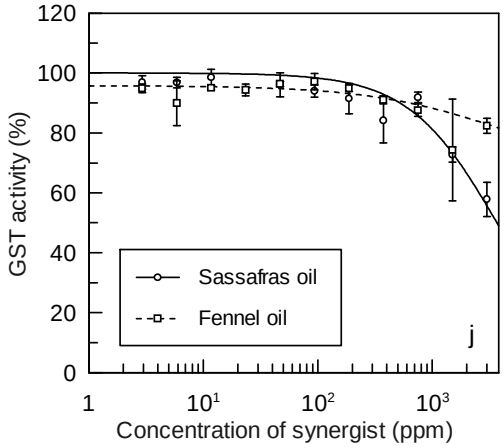
Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
Profenofos	Organophosphate	130 (± 69.74)	72.66 (± 27.17)
BcB1 extract	Unknown	326 (± 58.84)	41.80 (± 3.93)
Myristicin oil	Methylenedioxyphenol ring	717 (± 1401)	61.07 (± 12.54)
Black pepper oil	Methylenedioxyphenol ring	807 (± 137)	14.31 (± 1.21)
BcB2 extract	Unknown	820 (± 287)	77.57 (± 12.51)
Propyl gallate	Ester	848 (± 314)	104 (± 7.10)
Platyphyllol oil	Oxygenated sesquiterpene	974 (± 95.20)	100
Garlic oil	Sulphur compound	1176 (± 545)	77.04 (± 22.58)
Aniseed oil	Unsaturated ether / phenylpropene derivative	1331 (± 693)	18.19 (± 7.91)
CaB2 frac3 extract	Phenylpropene	1434 (± 79.52)	100
CaB2 extract	Phenylpropene	1462 (± 118)	100
ZaB2 extract	Sesquiterpenoid	1590 (± 192)	100
Cypress oil	Bicyclic monoterpene	2151 (± 5371)	50.42 (± 6.03)
Neem oil	Tetranortriterpenoid / liminoid	2610 (± 981)	102 (± 14.17)
ClB2 extract	Cyclic terpene	2742 (± 123)	100
Grapefruit oil	Cyclic terpene	2939 (± 8867)	36.25 (± 129)
Sassafras oil	Methylenedioxyphenol ring	3674 (± 518)	100
Fennel oil	Unsaturated ether / phenylpropene derivative Monoterpene / ketone	3979 (± 5673)	28.74 (± 7.53)
ClB1 extract	Cyclic terpene	4144 (± 10 153)	52.15 (± 79.41)
Dill apiole oil	Methylenedioxyphenol ring / phenylpropanoid	4144 (± 665)	100
Angelica root oil	Angular furocoumarin	4265 (± 4451)	57.55 (± 25.89)
Nutmeg oil	Bicyclic monoterpene; Methylenedioxyphenol ring	6325 (± 981)	100
Citronella java oil	Monoterpenoid / alcohol	7528 (± 7342)	65.11 (± 43.03)
Dill seed oil	Terpenoid; Cyclic terpene	9814 (± 8904)	65.93 (± 39.17)
White cypress oil	Bicyclic monoterpene; Monoterpene; Cyclic terpene	-	100

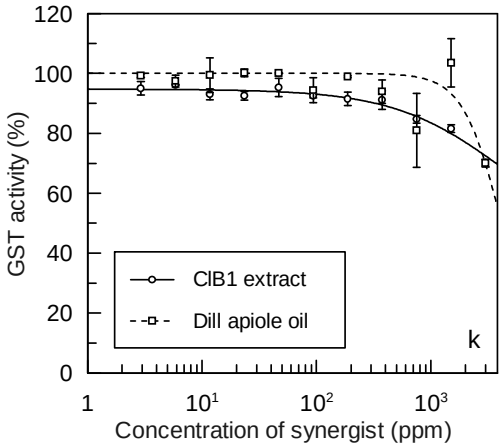

GST activity (%)
Concentration of synergist (ppm)
Profenofos
a
Acetone
BcB1 extract
b
Myristicin oil
Black pepper oil
c
BcB2 extract
Propyl gallate
d
Platyphyllol oil
Garlic oil
e
Aniseed oil
CaB2 frac3 extract
f

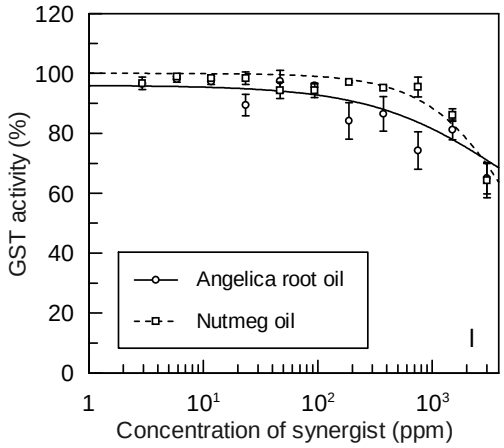

GST activity (%)
Concentration of synergist (ppm)
CaB2 extract
ZaB2 extract
g


GST activity (%)
Concentration of synergist (ppm)
Cypress oil
Neem oil
h


GST activity (%)
Concentration of synergist (ppm)
ClB2 extract
Grapefruit oil
i


GST activity (%)
Concentration of synergist (ppm)
Sassafras oil
Fennel oil
j


GST activity (%)
Concentration of synergist (ppm)
ClB1 extract
Dill apiole oil
k


GST activity (%)
Concentration of synergist (ppm)
Angelica root oil
Nutmeg oil
l

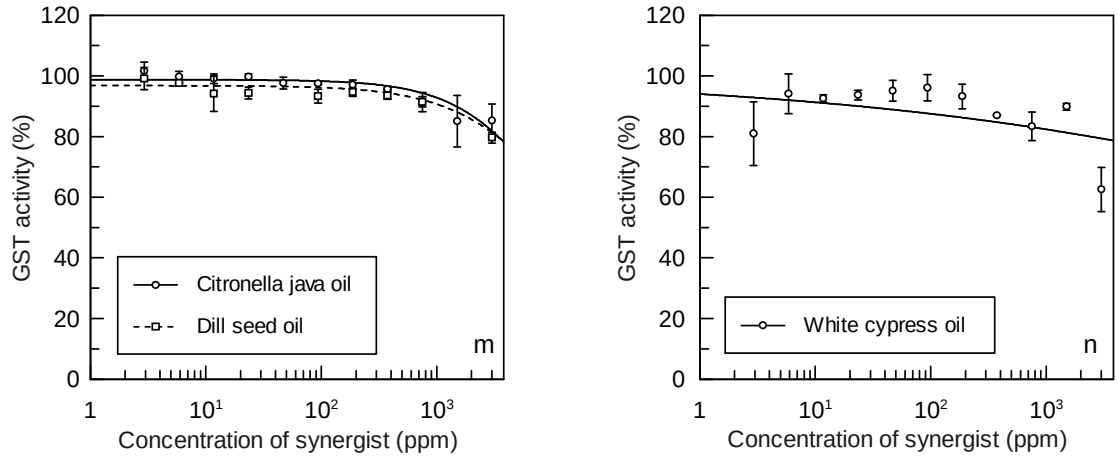


Fig. 3.8a-n. Inhibition of *Helicoverpa armigera* (3-4 mg larvae) glutathione-S-transferase (GST) activity (CDNB as the substrate) by potential pyrethrum synergists, profenofos and acetone (control). Error bars represent standard deviation. For platyphyllol, sassafras, dill apiole, nutmeg and white cypress oils, CaB2 frac3, CaB2, ZaB2 and ClB2 extracts, lines were fitted with two parameter logistic curves (lower and upper limits of 0% and 100% respectively). For all remaining compounds, lines were fitted with a four parameter logistic curve. (Relates to Table 3.12; Synergists ordered from lowest to highest IC_{50}).

3.3.2.4 Enzyme inhibition by pyrethrum

Some inhibition of ***H. armigera*** esterases by pyrethrum was found using the total esterase assay (alpha-naphthyl acetate as a substrate) (Fig. 3.9a), with an IC_{50} (± SE) of 1994.88 ppm (± 1167.92) and a Y-range (± SE) of 30.68% (± 6.72). No inhibition of esterases by pyrethrum was found with the alternative substrate (para-nitrophenyl acetate) (Fig. 3.9a) or with the esterase interference assay (index value (± SE) of 104.81% (± 10.17) (95% confidence intervals of 83.66 - 125.95)).

Pyrethrum gave some inhibition of GST activity in ***H. armigera***, mostly only with the highest concentration of pyrethrum in the dilution series, giving an IC_{50} (± SE) of 12 630.67 ppm (± 14 483.20) (Fig. 3.9b).

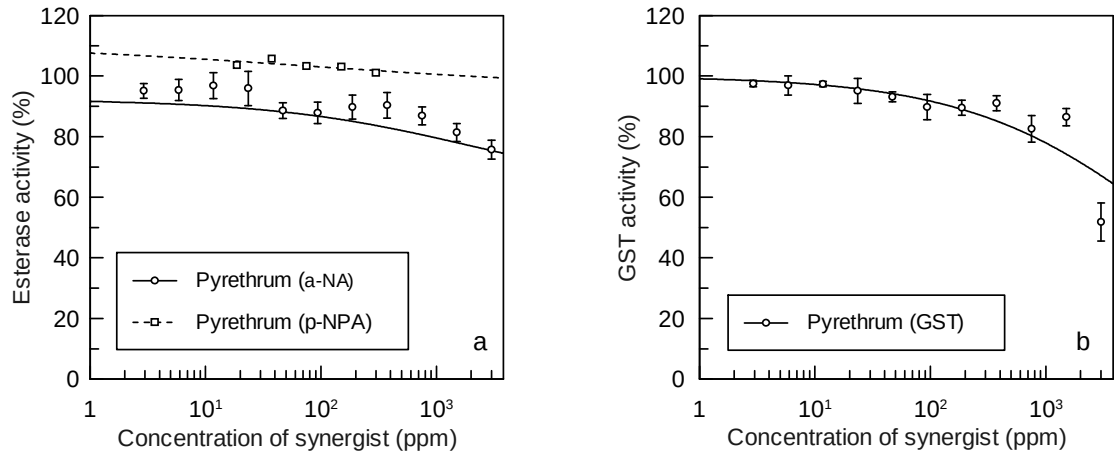


Fig. 3.9a-b. Inhibition of *Helicoverpa armigera* enzyme activity by pyrethrum. Fig. 3.9a represents inhibition of total esterase activity (alpha-naphthyl acetate (a-NA) and para-nitrophenyl acetate (p-NPA) as substrates); Fig. 3.9b represents inhibition of glutathione-S-transferase activity (CDNB as a substrate). Error bars represent standard deviation. Lines for Fig. 3.9a were fitted with a four parameter logistic curve. In Fig. 3.9b, the line was fitted with a two parameter logistic (lower and upper limits of 0% and 100% respectively).

3.3.3 Field trial

3.3.3.1 The mean number and percentage control of *H. armigera* larvae

The mean number of ***H. armigera*** larvae per six plants in the unsprayed control was significantly higher (18.50 larvae, $p<0.001$) than all the other treatments (Fig. 3.10). The microencapsulated treatment of pyrethrum plus PBO had significantly fewer larvae per six plants (2.25 larvae) than the unsprayed control, the pyrethrum only, pyrethrum plus parsley seed oil and pyrethrum plus canola oil treatments (18.50, 8.50, 8.00 and 7.75 larvae respectively). Percentage control (calculated using Abbott's formula) ranged from 87.84% in the microencapsulated treatment of pyrethrum plus PBO to 54.05% with pyrethrum only.

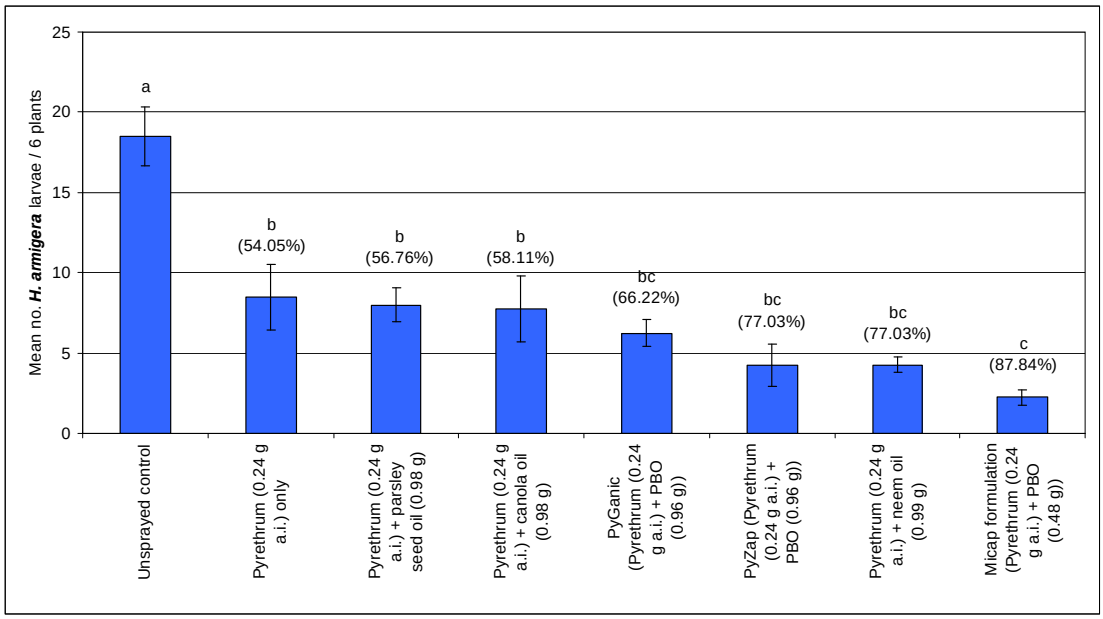


Fig. 3.10. The mean number of *Helicoverpa armigera* larvae (± SE) (per six plants per treatment) on cotton, 24 hours after spraying. (All pyrethrum formulations were emulsifiable concentrate (EC) solutions; a.i. = active ingredient; Micap = Microencapsulated). ($P_{(0.05)}$value <0.001, $LSD_{(p=0.05)}$ = 4.256, CV = 38.8%). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level). Percentage values in parentheses are the percentage control for each treatment, calculated from Abbott's formula.

3.4 Discussion and Conclusions

Temporal synergism was examined by first determining an optimal synergist concentration from a range of dosages for application in the temporal bioassays. The synergists at 1000 ppm with pyrethrum tended to give the highest mortalities (and significantly so for CaB2 frac3 extract) and this concentration was therefore chosen for the temporal bioassay. An increase in the synergist concentration above 1000 ppm tended to correspond with a decrease in mortality and may have to do with penetration through the cuticle, with an increasing amount of synergist perhaps blocking the penetration of pyrethrum through the cuticle. Some synergists have been shown to reduce insecticide penetration through the cuticle. Piperonyl cyclonene, for example, significantly reduced the absorption of topically applied pyrethroids in house flies ***Musca domestica*** L. (Diptera: Muscidae) (Winteringham ***et al***. 1955). Sesamex reduced the absorption of both labelled C^{14}-pyrethrin I and C^{14}-

cinerin I by approximately half in house flies, suggesting that sesamex, a viscous oil, possibly competed with the house fly epicuticle for any lipophilic compound (Chang & Kearns 1964). An alternative explanation to high synergist concentrations reducing synergism could be that the mechanism of metabolism of an insecticide is not altered by a synergist but that the synergist can influence the reaction rate, or shift significant detoxification reactions to non-oxidative mechanisms (Casida 1970). Oxidation or hydroxylation reactions form products of either reduced potency (detoxification) or enhanced potency (activation) and a synergist might thus increase or decrease insecticide toxicity depending on the shift in balance of the competing activation or detoxification reactions.

Temporal synergism bioassays gave no significant differences between any of the pre-treatment times for pyrethrum with any of the synergists or for acetone. In ***H. armigera***, a 4-hour pre-treatment time with PBO has previously been shown to be optimal for the inhibition of metabolic enzymes and the highest resultant synergism with pyrethroids (Young ***et al***. 2005), however, this was not found during this study. The concentration of PBO may have been too high for temporal differences to be elucidated, since high mortalities were found with each pre-treatment time. Parsley seed oil showed no significant increase in mortality over time. The remaining compounds used to test for temporal synergism did not show any synergistic activity and were thus perhaps not ideal synergists for use in testing of temporal synergism with pyrethrum. A difference may also potentially exist in the ability of natural pyrethrum to cross the insect cuticle compared to synthetic pyrethroids, and the possible enhancement or hindrance by the synergists in penetration of the different pesticides.

Discriminating dose bioassays were used to compare the efficacy of a number of natural compounds in synergising pyrethrum. PBO showed the highest efficacy as a pyrethrum synergist against ***H. armigera***, followed by bergamot oil, parsley seed oil, dill apiole oil, aniseed oil and sassafras oil. PBO is the synergist most often used in formulation with pyrethrum, although not available for use on ***H. armigera***. Parsley seed oil, containing myristicin and apiole, and sassafras oil, containing safrole, are all compounds with the methylenedioxyphenol (MDP) ring structure characteristic of PBO and dill apiole, which has commonly been associated with synergism of pyrethrum (Beroza 1954, Casida 1970, Haller ***et al***. 1942). PBO is synthesised by hydrogenation of the safrole molecule found in sassafras oil (extracted from the root,

bark or fruits of the ocotea tree), chloromethylation and the addition of the butylcarbityl side chains (Casida & Quistad 1995, Wachs 1947). Safrole also occurs naturally in a number of other plant species, including black pepper (Russell & Jennings 1969) and nutmeg (Power & Salway 1907). Even though PBO is synthesised from the safrole molecule from sassafras oil, mortality with PBO in combination with pyrethrum was found to be double that of pyrethrum with sassafras oil. Mortality with black pepper and nutmeg oils with pyrethrum did not differ significantly from pyrethrum alone. Since the plant extracts and oils used in this study were not pure, safrole could make up too small a percentage of the total composition of the oils to contribute greatly to synergism of pyrethrum, or the presence of any of the other constituents may potentially have antagonistic effects. PBO has been found to be an inhibitor of monooxygenases (Wilkinson 1976) and esterases (Bingham ***et al***. 2007, Gunning ***et al***. 1999, Young ***et al***. 2005, 2006), which could account for its efficacy as a synergist, most likely enhanced by its surfactant properties, increasing the passage of pyrethrum across the insect cuticle. In Australia, PBO is not certified for organic use and the present data show that the efficacy of pyrethrum may be increased to a degree using some natural and potentially organically-certifiable compounds, namely bergamot oil, parsley seed oil, dill apiole oil, aniseed oil or sassafras oil.

The involvement of the MDP ring, although tending to be present in most of the effective pyrethrum synergists, seemed not to be crucial for synergism of pyrethrum. Aniseed oil contains ***trans***-anethole (an unsaturated ether) and bergamot oil contains limonene (a cyclic terpene) and linalyl acetate (an acetate ester). The ester in bergamot oil could play a role in synergism of pyrethrum if it was able to bind to the esterase and act as an inhibitor. An ester could be considered as an inhibitor if its hydrolysis was very slow, thus leaving the active site of the esterase unavailable to other substrates for an extended amount of time (Dr. Graham D. Moores, ***Pers. comm.***).

Application of platyphyllol oil and neem oil with pyrethrum resulted in the lowest mortalities of ***H. armigera*** larvae in the discriminating dose bioassay, showing antagonism of pyrethrum. This could indicate that these compounds may be unsuitable for use in combination with pyrethrum against ***H. armigera***.

The ability of PBO to act as a surfactant may allow it to improve the penetration of compounds across the insect cuticle. In ***H. armigera***, PBO has been thought to enhance the penetration of esfenvalerate (Gunning ***et al***. 1995) and permethrin (Kennaugh ***et al***. 1993) through the cuticle. Compounds were therefore applied in a mixture with PBO in an attempt to increase cuticular penetration of the potential synergists where synergists were perhaps efficient at enzyme inhibition but may not have been efficient at crossing the insect cuticle. The efficacy of none of the compounds was enhanced to have significantly higher mortality than the synergistic effects of pyrethrum with PBO alone. A number of compounds with pyrethrum did, however, have significantly lower mortality than with pyrethrum with PBO alone, including manuka oil, BcB2 extract, canola oil and sesame oil, indicating a possible antagonistic effect when the compounds were applied in combination.

A diet incorporation bioassay with ***H. armigera*** larvae showed PBO to be the most effective pyrethrum synergist and the only synergist-containing diet that resulted in mortality with pyrethrum significantly higher than the standard diet. The parsley seed oil diet showed the most efficacy as a potentially organically-certifiable synergist to synergise pyrethrum, followed by bergamot oil, though results were not significant. This bioassay confirmed the efficacy of synergists even through ingestion of the synergist. However, synergism may also be due to topical exposure to the synergists through the mouthparts or with larvae being in direct contact with the diet. In the corn earworm ***Heliothis zea*** (Boddie) (Lepidoptera: Noctuidae), diets incorporated with myristicin and PBO have been shown to effectively synergise xanthotoxin, a naturally occurring furanocoumarin with insecticidal properties (Berenbaum & Neal 1985). The diets containing aniseed oil and neem oil resulted in the lowest mortalities with ***H. armigera*** larvae, showing no synergism of pyrethrum.

The aniseed oil diet showed significant repellency of ***H. armigera*** larvae compared to the standard diet. This could explain the lower mortality with aniseed oil in the diet incorporation bioassay when synergism was found with the discriminating dose bioassay, since larvae may not have fed as much on the aniseed oil diet, therefore ingesting less of the synergist. Larvae may have also had less exposure to aniseed oil topically by avoiding the diet. Repellency has been found by aniseed, basil and eucalyptus oils against a common mosquito pest ***Culex pipiens*** L. (Diptera: Culicidae) in Antalya, Turkey, by Erler ***et al***. (2006) (only adult female ***C. pipiens*** were tested). Rosemary was also shown to have repellency properties against spider mites

Tetranychus urticae and ***Eutetranychus orientalis*** (Acari: Tetranychidae) (Momen ***et al***. 2001). Neem, which is known to have repellent properties, showed no significant repellency of ***H. armigera*** larvae. Mikami & Ventura (2008) found neem to repel only male leaf beetles ***Microtheca punctigera*** (Coleoptera: Chrysomelidae). Xie ***et al***. (1995) found that neem repelled three stored-product beetles, the rusty grain beetle ***Cryptolestes ferrugineus*** (Stephens) (Coleoptera: Laemophloeidae), the rice weevil ***Sitophilus oryzae*** (L.) (Coleoptera: Curculionidae) and the red flour beetle ***Tribolium castaneum*** (Herbst) (Coleoptera: Tenebrionidae).

Full dose response bioassays were undertaken to determine the LD_{50} and LD_{90} values and calculate synergism factor (SF) values for a range of the synergists. PBO was the most effective pyrethrum synergist, followed by parsley seed oil, dill apiole oil and bergamot oil, confirming the results of the discriminating dose bioassays. The compounds with the lowest SF values were neem oil, aniseed oil, CaB2 extract and ZaB2 extract, resulting in a decrease in the efficacy of pyrethrum and thus showing a possible antagonistic effect. The reason why aniseed oil failed to show effective synergism in the full dose response bioassay is unclear, since synergism was found with aniseed oil in the discriminating dose bioassay, however, aniseed oil also never showed synergism with the diet incorporation bioassay. This, however, could have been due to the repellence shown by aniseed oil, with larvae ingesting less synergist or with larvae having less contact with the oil. The results may be due to bioassay anomaly. Neem oil showed low mortality towards ***H. armigera*** larvae with pyrethrum using all three bioassays, discriminating dose, full dose response and diet incorporation bioassays, confirming the ineffectiveness of neem as a pyrethrum synergist against ***H. armigera***.

A comparison between topical application of a synergist on the dorsal thorax and application to the mouthparts was made using neem oil with pyrethrum. No significant differences were found in this bioassay. Neem oil consistently failed to show synergism of pyrethrum in the bioassays, despite showing good ***in vitro*** inhibition of ***H. armigera*** esterases and GSTs and ***Carpophilus*** spp. monooxygenases. Neem oil contains the tetranortriterpenoid, azadirachtin, a large molecule (molecular weight of 720.71), which might slow or block the penetration of pyrethrum into the insect body. Neem oil may also potentially cause a change in the reactions involved with detoxification of pyrethrum, resulting in products of reduced potency (Casida 1970).

A field trial showed that treatments containing pyrethrum had significantly fewer ***H. armigera*** larvae compared to the unsprayed control. The microencapsulated treatment of pyrethrum plus PBO showed the highest percentage control of all the treatments and was the only pyrethrum-containing treatment to have significantly fewer ***H. armigera*** larvae than pyrethrum only. The microencapsulated formulation releases PBO first, followed by a release of pyrethrum approximately four hours later, showing that temporal synergism might play a role in a field situation, compared to the other treatments where synergists and pyrethrum were applied in a mixture and not formulated to be released at different times. A microencapsulated formulation of PBO with alpha-cypermethrin has been shown to be effective against ***H. armigera***, ***M. persicae***, ***Aphis gossypii*** Glover (Hemiptera: Aphididae) and ***Bemisia tabaci*** (Gennadius) (Hemiptera: Aleyrodidae) (Bingham ***et al***. 2007).

The synergists were compared with regard to their ability to inhibit the enzymes involved in metabolic resistance (esterases, GSTs and monooxygenases), which may give an indication of the enzyme systems involved in synergism of pyrethrum. The total esterase assay using alpha-naphthyl acetate as a substrate revealed differences in inhibition compared to the inhibition found with the esterase assay using para-nitrophenyl acetate as the substrate and the esterase interference assay. A significant positive correlation was found between inhibition with the esterase assay (para-nitrophenyl acetate as the substrate) and the esterase interference assay, with compounds such as BcB1, BcB2 and CaB2 extracts showing good inhibition in both assays. However, since very little inhibition was revealed with para-nitrophenyl acetate as the substrate, and both the esterase assay (para-nitrophenyl acetate as a substrate) and the esterase interference assay failed to show inhibition of esterases by PBO, alpha-naphthyl acetate seemed thus to be a more suitable substrate for showing inhibition by synergists of ***H. armigera*** esterases. Esterase inhibition was found using PBO in the total esterase assay with alpha-naphthyl acetate as the substrate and since PBO was the most efficient pyrethrum synergist ***in vivo***, this would (at least in part, since synergism by PBO may be facilitated further by its ability to increase the passage of insecticides through the cuticle) be due to inhibition of the resistance-associated esterases in ***H. armigera***. Resistance to pyrethroids in Australian ***H. armigera*** has been found to be esterase mediated (Gunning ***et al***. 1996). PBO has previously been shown to inhibit the esterase activity related to pyrethroid resistance in Australian ***H. armigera*** using alpha-naphthyl

acetate as the substrate for the esterase assay (Gunning ***et al***. 1998). It would seem synergists are binding to the esterases in such a way so as to prevent the active site from hydrolysing alpha-naphthyl acetate, but still allow the binding of both para-nitrophenyl acetate and azamethiphos.

No significant correlations were found between esterase inhibition and synergism of pyrethrum in ***H. armigera***. Neem oil, propyl gallate and the plant extracts BcB1, BcB2, CaB2, CaB2 frac3, ClB2 and ZaB2 were the only compounds showing inhibition in all three esterase assays (Table 3.11), however, none of these compounds showed synergistic activity towards pyrethrum. All three esterase assays may therefore not necessarily be able to accurately predict compounds that synergise pyrethrum successfully.

No significant correlations were detected between inhibition of ***H. armigera*** GST activity and synergism of pyrethrum. PBO, bergamot oil and parsley seed oil, showing the highest synergism of pyrethrum, gave no inhibition of GSTs, whereas the most effective GST inhibitors, including propyl gallate and neem oil, did not show synergism of pyrethrum.

Monooxygenase assays failed to give any detectable activity. Using whole insects for the homogenate may result in loss of monooxygenase activity and assays should perhaps be undertaken using only ***H. armigera*** midgut. Kennaugh ***et al***. (1993) found it essential that only the midgut (thoroughly washed with saline solution prior to homogenisation) of ***H. armigera*** was used to obtain activity, since any incorporation of foregut or hindgut tissue caused a loss of monooxygenase activity. A lack of monooxygenase activity could be due to the presence of proteases, which destroy the enzyme, as has been found with ***M. persicae*** (Philippou ***et al***. 2010). The proteases would need to be neutralized, such as by clearing the gut tissue or by using stronger chemicals than DTT, PTU and PMSF. A problem with monooxygenases compared with esterases and GSTs is that the former are membrane bound enzymes while the latter are soluble. With monooxygenases their integrity appears to be completely changed during homogenisation. When assays are undertaken with vertebrates, the enzymes come from “clean” tissue, such as liver, whereas with insects most preparations are from whole individuals, which could explain why little or no activity is measurable (Dr. Harley A. Rose, ***Pers. comm.***).

The inhibition of enzymes by pyrethrum was determined as a way of examining the interaction of the enzymes with pyrethrum and their possible involvement in detoxification of pyrethrum and pyrethrum resistance. The total esterase assay using alpha-naphthyl acetate as a substrate revealed some inhibition of esterases by pyrethrum. However, no inhibition of ***H. armigera*** esterases was found when para-nitrophenyl acetate was used as the esterase substrate or with the esterase interference assay. When examining inhibition of esterases by the synergists, it was discovered that alpha-naphthyl acetate was the more suitable substrate for detecting inhibition of ***H. armigera*** esterases. Inhibition of esterases by PBO was also only detected with the total esterase assay using alpha-naphthyl acetate as the substrate. PBO showed the highest synergistic activity towards pyrethrum ***in vivo***. Pyrethrum could thus potentially be binding to ***H. armigera*** esterases in a manner that prevents hydrolysis of alpha-naphthyl acetate but the binding of esterases to para-nitrophenyl acetate and azamethiphos is still possible. Some inhibition of ***H. armigera*** GST activity was found by pyrethrum, suggesting binding to GSTs and thus the possible involvement of GSTs in the detoxification process of pyrethrum in ***H. armigera***.

In conclusion, PBO was found to be the most effective synergist of pyrethrum ***in vivo***, whereas the most effective potentially organically-certifiable synergists were bergamot, parsley seed, dill apiole, aniseed and sassafras oils. Aniseed oil, however, showed variable results. Parsley seed oil and sassafras oil contain components with the MDP ring structure found in dill apiole and PBO, confirming its importance in pyrethrum synergism. The most effective synergists from bioassay data did not correlate well with either esterase or GST enzyme inhibition data. This data suggests that the use of either the esterase or GST assays may not necessarily prove useful as predictors of potentially effective pyrethrum synergists for ***H. armigera***.

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4 CHAPTER FOUR: THE EVALUATION OF VARIOUS NATURAL COMPOUNDS AS POTENTIAL PYRETHRUM SYNERGISTS AGAINST WESTERN FLOWER THRIPS *FRANKLINIELLA OCCIDENTALIS*

Abstract

Western flower thrips ***Frankliniella occidentalis*** are a serious pest on a large range of agricultural crops, including fruit, vegetables and ornamental plants, causing severe damage by feeding but especially due to the transmission of viral diseases. The development of insecticide resistance in ***F. occidentalis*** has hampered the successful control of this pest. In this research, pyrethrum is being considered as an additional control option. PBO is a synergist often used to enhance the efficacy of pyrethrum, however in some countries, including Australia, PBO is no longer certified for organic use. Several natural plant oils and extracts were thus tested, using a discriminating dose bioassay, to assess their synergistic potential towards pyrethrum against ***F. occidentalis***. Parsley seed oil, PBO, dill apiole oil and sassafras oil showed the highest efficacy as pyrethrum synergists. Oleic acid also significantly enhanced the toxicity of pyrethrum but to a lesser extent. When PBO was added to synergists in a mixture, only the mixture with parsley seed oil showed higher mortality with pyrethrum than PBO alone. However, the synergist mixture itself was toxic and mortality was thus likely caused by a combination of toxicity and synergism rather than by synergism alone.

Compounds tested for synergism of pyrethrum against ***F. occidentalis*** were examined for their capacity to inhibit the enzymes involved in insecticide resistance (esterases, GSTs and monooxygenases). No detectable monooxygenase activity was found in ***F. occidentalis*** and inhibition data could therefore not be obtained. However, parsley seed oil and dill apiole oil inhibited esterases and GSTs to varying degrees. PBO and sassafras oil never inhibited GSTs but inhibited esterases to a small degree. Oleic acid gave no inhibition of esterases or GSTs. Propyl gallate and neem oil gave good inhibition of esterases, and neem oil also showed good inhibition of GSTs, but neither gave synergism in the discriminating dose bioassay. No correlation could thus be established between synergism and inhibition of esterases or GSTs, suggesting that the specific assays used may not be reliable in predicting potential pyrethrum synergists in this strain of ***F. occidentalis***.

4.1 Introduction

Western flower thrips ***Frankliniella occidentalis*** are a devastating pest world-wide, feeding on a wide range of crop species, including tomatoes, cotton, cucurbits, ornamental plants and fruits. ***F. occidentalis*** cause substantial damage to crops, especially to those grown in greenhouses, due to direct feeding damage and by transmission of viral diseases (López-Soler ***et al***. 2008, Roselló ***et al***. 1996). Due to the nature of crops grown in greenhouses, where the presence of any disfigurement or damage to fruit, vegetable or ornamental plants significantly lowers the quality of the crop, the use of pesticides has been very heavy, resulting in the rapid

development of resistance against a wide range of pesticides, including pyrethroids, organophosphates and carbamates (Jensen 1998, Thalavaisundaram ***et al***. 2008, Zhao ***et al***. 1995).

In this chapter, the main aim of the project, to determine whether a number of natural plant oils and extracts would be effective as pyrethrum synergists, was examined using ***F. occidentalis*** as the test insect. Different taxa were studied throughout the project since the enzymes responsible for pyrethroid resistance, and their affinity in binding with the synergistic compounds, may vary between taxa. Pyrethroid resistance in cotton bollworm ***Helicoverpa armigera*** from Australia, for example, has been ascribed to an increase in esterase activity (Gunning ***et al***. 1996), whereas pyrethroid resistance in the pollen beetle ***Meligethes aeneus*** was found to be monooxygenase mediated (Philippou ***et al***. 2011). In ***F. occidentalis*** from Spain, pyrethroid resistance was related to monooxygenase activity (Espinosa ***et al***. 2005). Resistance to acrinathrin and methiocarb in Spanish ***F. occidentalis*** has been associated with esterase activity (López-Soler ***et al***. 2008, Maymó ***et al***. 2006). The nature of penetration of the synergists into the insect body may also vary between taxa, due to differences in the characteristics of the cuticle. Amphiphylic analogs of insect pyrokinin neuropeptides, for example, were found to penetrate the cuticle of the American cockroach ***Periplaneta americana*** (L.) in smaller quantities and at a much slower rate than through the cuticle of the tobacco budworm moth ***Heliothis virescens*** (F.). Differences were ascribed to the cockroach cuticle being thicker and more dense than that of ***H. virescens***, and also due to the protein composition of the cuticular layers of the cockroach containing fewer polar amino acids than that of moths (Teal ***et al***. 1999). Compounds could therefore show efficacy as pyrethrum synergists against some insect taxa but not against others.

F. occidentalis discriminating dose bioassays were undertaken with a pyrethroid-resistant strain of thrips to determine the efficacy of the plant oils and extracts as synergists of pyrethrum ***in vivo***, followed by biochemical enzyme assays (esterases, glutathione-S-transferases (GSTs) and monooxygenases) to study the ***in vitro*** enzyme-inhibiting capacity of the synergists. Bioassay data were correlated with enzyme inhibition data to determine which enzyme system could be identified as more important in identifying potential synergists and could suggest the possible involvement of that specific enzyme system in ***F. occidentalis*** pyrethroid or pyrethrum resistance.

4.2 Materials and Methods

4.2.1 Bioassays

Leaf-dip bioassays were used to assess the efficacy of a number of natural synergists with pyrethrum using a pyrethroid-resistant strain of ***F. occidentalis*** (Leppington Rose). (***F. occidentalis*** (Leppington Rose) is described in chapter two, section 2.1.2). The general bioassay methods are explained in chapter two (section 2.4.2). The dose-response curve for pyrethrum was determined to obtain the lethal dose values for use in the discriminating dose bioassays.

4.2.1.1 Full dose response bioassay

A dose-response curve for pyrethrum was determined for the Leppington Rose strain of ***F. occidentalis***. Leaf dip bioassays were undertaken using eight pyrethrum dosages, ranging from 3 ppm to 10 000 ppm (an emulsifiable concentrate formulation of pyrethrum was used, diluted in distilled water). Distilled water was used as the control. The dose-response curve and LD values were calculated by probit analysis (Finney 1971), using PoloPlus (version 1.0, LeOra Software), taking control mortality into consideration (Abbott 1925).

4.2.1.2 Discriminating dose bioassays

Discriminating dose bioassays were undertaken using a variety of synergists with pyrethrum to determine their synergistic potential against ***F. occidentalis.*** A pyrethrum discriminating dose bioassay was also performed using synergists mixed with PBO, to determine whether PBO enhanced the synergistic efficacy of any of the compounds to a degree greater than would be expected from either the synergist or PBO alone. The surfactant properties of PBO might give enhanced penetration of the synergist through the insect cuticle if any of the natural plant oils or extracts are perhaps not efficient in crossing the insect cuticle.

4.2.1.2.1 Discriminating dose bioassay - synergists

F. occidentalis discriminating dose bioassays were done by leaf dip using mixtures of pyrethrum plus synergist. A concentration of 1300 ppm pyrethrum was chosen as

discriminating dose, equivalent to approximately the LD_{20} of pyrethrum (1313 ppm) (determined in section 4.3.1.1). A low LD value was chosen to give low mortality with pyrethrum alone so that the synergistic abilities of the compounds could be compared.

Synergists were chosen for the discriminating dose bioassay based on known bio-activity (such as insecticidal activity, insect repellence properties, or possible or known synergistic activity). Synergists tested with pyrethrum against ***F. occidentalis*** were aniseed, bergamot, canola, citronella, dill apiole, manuka, neem, parsley seed, rosemary and sassafras oils, oleic acid and propyl gallate. (The plant extracts from the University of Tasmania (ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2) were not tested for synergism of pyrethrum in ***F. occidentalis*** due to insufficient quantities available for use with leaf dip bioassays). PBO was used as the standard synergist. (All synergists are described in detail in chapter two, Table 2.1). The concentration of each synergist was 5000 ppm, in a ratio of 4:1 synergist:pyrethrum. Pyrethrum was used alone as the unsynergised treatment. Synergists were also tested alone to determine if any compound showed inherent toxicity towards ***F. occidentalis***. All solutions were in distilled water and distilled water was used as a control. Parsley seed oil showed inherent toxicity at 5000 ppm and was thus also tested at 2500 ppm. PBO was also tested at a concentration of 2500 ppm, to compare pyrethrum synergism with parsley seed oil.

Data for the discriminating dose bioassay were analysed with a logistic regression, with an assumed binomial distribution, using logit-transformed data. The program, ASREML, was used to analyse data. The model incorporated both compounds treated with pyrethrum and compounds alone into the same analysis. Pairwise comparisons were done to determine significant differences using Fisher's (protected) least significant difference (LSD) at the 5% level.

4.2.1.2.2 Discriminating dose bioassay - synergists mixed with PBO

F. occidentalis discriminating dose bioassays were undertaken by leaf dip to determine whether the addition of PBO in a mixture with another synergist enhanced the efficacy of any of the synergists towards pyrethrum against ***F. occidentalis***. PBO was added in a 1:1 ratio to each of the synergists used above for the discriminating dose bioassay (section 4.2.1.2.1) and mixtures were tested for synergism towards

pyrethrum. PBO and synergists were used at a concentration of 5000 ppm each and pyrethrum was 1300 ppm. PBO at 2500 ppm was also included. The synergist:PBO mixtures were also tested alone, to determine if any of the mixtures showed inherent toxicity towards ***F. occidentalis***.

The discriminating dose bioassay data were analysed as for the discriminating dose bioassay above (section 4.2.1.2.1).

4.2.2 Biochemical assays

The enzyme systems potentially involved in synergism of pyrethrum were investigated by examining the inhibition of esterase, GST and monooxygenase activity by each synergist and correlating inhibition data to bioassay data. General methods for each assay are described in chapter two but details specific to ***F. occidentalis*** are mentioned below. All assays were undertaken with the Leppington Rose strain of ***F. occidentalis*** (described in chapter two, section 2.1.2).

4.2.2.1 Esterase assay with alpha-naphthyl acetate as a substrate

Total esterase assays using alpha-naphthyl acetate as a substrate were run according to the method described in chapter two (section 2.5.1.1). ***F. occidentalis*** homogenate was made up using 0.0125 g of thrips (male and female nymphs and adults) per 1.5 ml of 0.02 M phosphate buffer, pH 7. Thrips were homogenised in eppendorf tubes with a cordless motor tissue grinder (Kontes® pellet pestle, Quantum Scientific, Australia) and centrifuged at 10 000 rpm for 4 minutes. The supernatant was used immediately for esterase assays.

Compounds tested for inhibition of ***F. occidentalis*** total esterase activity were aniseed, bergamot, canola, citronella, dill apiole, dill seed, eucalyptus, geranium, grapefruit, manuka, melaleuca, myristicin, neem, parsley seed, platyphyllol, rosemary, sassafras, sesame and white cypress oils, propyl gallate, oleic acid and PBO. Plant extracts tested were ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2. (All synergists are described in detail in chapter two, Table 2.1). Profenofos, as a known esterase inhibitor, was also tested for its ability to inhibit ***F. occidentalis*** esterases. Profenofos was in a 10 000 ppm stock solution (in acetone) whereas all

other compounds were 100 000 ppm stock solutions (in acetone). Acetone was used as the control.

The ability of pyrethrum (100 000 ppm stock solution in acetone) to inhibit total esterase activity was also examined. This could give an indication of whether esterases were interacting with pyrethrum, and therefore may potentially play a role in its possible detoxification.

Data for the esterase assay were processed as described in chapter two (section 2.6), by calculating activity as a percentage of the uninhibited control. The program Grafit (Version 5.0.10) was used to determine inhibition curves for each compound and calculate the IC_{50} and Y-range values (as described in chapter three, section 3.2.2.1.1). The inhibition curve for sassafras oil was fitted with a two parameter logistic curve (lower and upper limits of 0% and 100% respectively) (as explained in chapter three, section 3.2.2.1.1), since insufficient data was obtained to accurately fit a four parameter logistic curve. The inhibition curves for all remaining compounds were fitted with a four parameter logistic curve.

Correlations were tested for between the ***F. occidentalis*** discriminating dose bioassay mortality data for synergists with pyrethrum and esterase inhibition (using the IC_{50} values, Y-ranges and the percentage esterase activity remaining at the highest concentration of the synergist dilution series). Correlation analyses were performed as described in chapter two (section 2.6).

4.2.2.2 Glutathione-S-transferase assay

GST assays were run using CDNB as a substrate, as described in chapter two (section 2.5.2). ***F. occidentalis*** homogenate was prepared by homogenising 0.0125 g of thrips (male and female nymphs and adults) per 1.5 ml of 0.2 M phosphate buffer, pH 6.5. Thrips were homogenised in eppendorf tubes with a cordless motor tissue grinder and centrifuged at 10 000 rpm for 4 minutes. The supernatant was used immediately for GST assays.

Compounds tested for inhibition of ***F. occidentalis*** GST activity were aniseed, bergamot, canola, citronella, dill apiole, dill seed, geranium, manuka, neem, parsley seed, rosemary, sassafras and sesame oils, propyl gallate, oleic acid, PBO, DEM

and the plant extracts ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2. (All synergists are described in chapter two, Table 2.1). All compounds were 100 000 ppm stock solutions (in acetone). Acetone was used as the control.

The ability of pyrethrum (100 000 ppm stock solution in acetone) to inhibit GST activity was also determined, to give an indication of potential pyrethrum resistance mechanisms in the Leppington Rose strain of ***F. occidentalis***.

Data for the GST assay were processed in the same way as for the esterase assays (section 4.2.2.1). A two parameter logistic curve (lower and upper limits of 0% and 100% respectively) was used to fit lines for dill apiole oil, CaB2 frac3 extract, CaB2 extract, BcB1 extract, propyl gallate and pyrethrum. Data from all remaining compounds were fitted with a four parameter logistic curve.

Correlations were tested for between the ***F. occidentalis*** discriminating dose bioassay mortality data for synergists with pyrethrum and GST inhibition (using the IC_{50} values, Y-ranges and the percentage GST activity remaining at the highest concentration of the synergist dilution series). Correlation analyses were performed as described in chapter two (section 2.6).

4.2.2.3 Monooxygenase assay

F. occidentalis homogenate was prepared for the monooxygenase assay using 0.0429 g of ***F. occidentalis*** (male and female nymphs and adults) in 2.8 ml of homogenisation buffer (0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU and 1 mM PMSF). Thrips were homogenised in an eppendorf tube with a cordless motor tissue grinder and centrifuged for 30 minutes at 10 000 rpm. The supernatant was used immediately for the assays, run as described in chapter two (section 2.5.3). Monooxygenase activity in ***F. occidentalis*** was not detected with this assay. This method of monooxygenase detection in insect homogenate was verified using ***Carpophilus*** spp. and ***M. aeneus*** (chapter six).

4.3 Results

4.3.1 Bioassays

4.3.1.1 Full dose response bioassay

The dose-response curve for unsynergised pyrethrum using ***F. occidentalis*** was calculated by probit analysis. The LD_{20} was 1313 ppm pyrethrum (Table 4.1) and a concentration of 1300 ppm was chosen for the discriminating dose bioassays.

Table 4.1. The lethal dose (LD) values with 95% confidence intervals (CI) for pyrethrum (calculated by probit analysis) from leaf dip bioassays against a pyrethroid-resistant laboratory strain of *Frankliniella occidentalis* (Leppington Rose) (adult females).

LD value	Pyrethrum dose (ppm)	95% CI
LD_{10}	743	89.26 – 1512
LD_{20}	1313	290 – 2274
LD_{30}	1981	661 – 3125
LD_{40}	2813	1288 – 4258
LD_{50}	3906	2246 – 6086
LD_{60}	5422	3515 – 9688
LD_{70}	7701	5069 – 17 841
LD_{80}	11 613	7172 – 39 551
LD_{90}	20 528	10 955 – 126 354
LD_{95}	32 858	15 238 – 336 372
LD_{99}	79 409	27 768 – 2 151 249
Number of ***F. occidentalis*** exposed = 714		
Slope ± SE = 1.778 ± 0.337		
Degrees of freedom (df) = 30		
$\chi^2 = 56.213$		

4.3.1.2 Discriminating dose bioassays

4.3.1.2.1 Discriminating dose bioassay - synergists

Compounds applied with pyrethrum that gave significantly higher mortality than unsynergised pyrethrum (25.00% mortality) were parsley seed oil (5000 ppm), PBO (2500 and 5000 ppm), parsley seed oil (2500 ppm), manuka oil, dill apiole oil, sassafras oil and oleic acid (96.72%, 89.91%, 88.07%, 78.70%, 61.17%, 51.71%, 45.89% and 33.47% mortality respectively, $p<0.001$) (Fig. 4.1). Canola oil seemed to have an antagonistic effect, as mortality from pyrethrum with canola oil was significantly lower (14.92%) than mortality with unsynergised pyrethrum (25.00%). No other compound plus pyrethrum differed significantly from unsynergised pyrethrum.

Parsley seed oil showed significant inherent toxicity towards ***F. occidentalis*** at both 5000 ppm and 2500 ppm (50.71% and 23.37% mortality respectively) compared to the distilled water control (14.16% mortality, $p<0.001$) (Fig. 4.1). The mortality thus found with parsley seed oil plus pyrethrum would partly have been due to toxicity of the compound itself. However, in both the parsley seed oil treatments (2500 and 5000 ppm), the mortality found with parsley seed oil plus pyrethrum was significantly higher than with parsley seed oil itself, and mortality could therefore, at least in part, be ascribed to synergistic effects. PBO at 5000 ppm showed inherent toxicity, with mortality (24.40%) significantly higher than the distilled water control. However, mortality with PBO plus pyrethrum could still be attributed to synergistic effects, since PBO plus pyrethrum was significantly higher than PBO alone. PBO alone at 2500 ppm did not show significant toxicity towards ***F. occidentalis***. Manuka oil showed significant inherent toxicity (45.16% mortality), which would have contributed greatly to the mortality observed with pyrethrum plus manuka oil (61.17%). The additional mortality was therefore most likely not due to synergism but to the actual toxicity of manuka oil itself. No other compound tested showed significant toxicity towards ***F. occidentalis***.

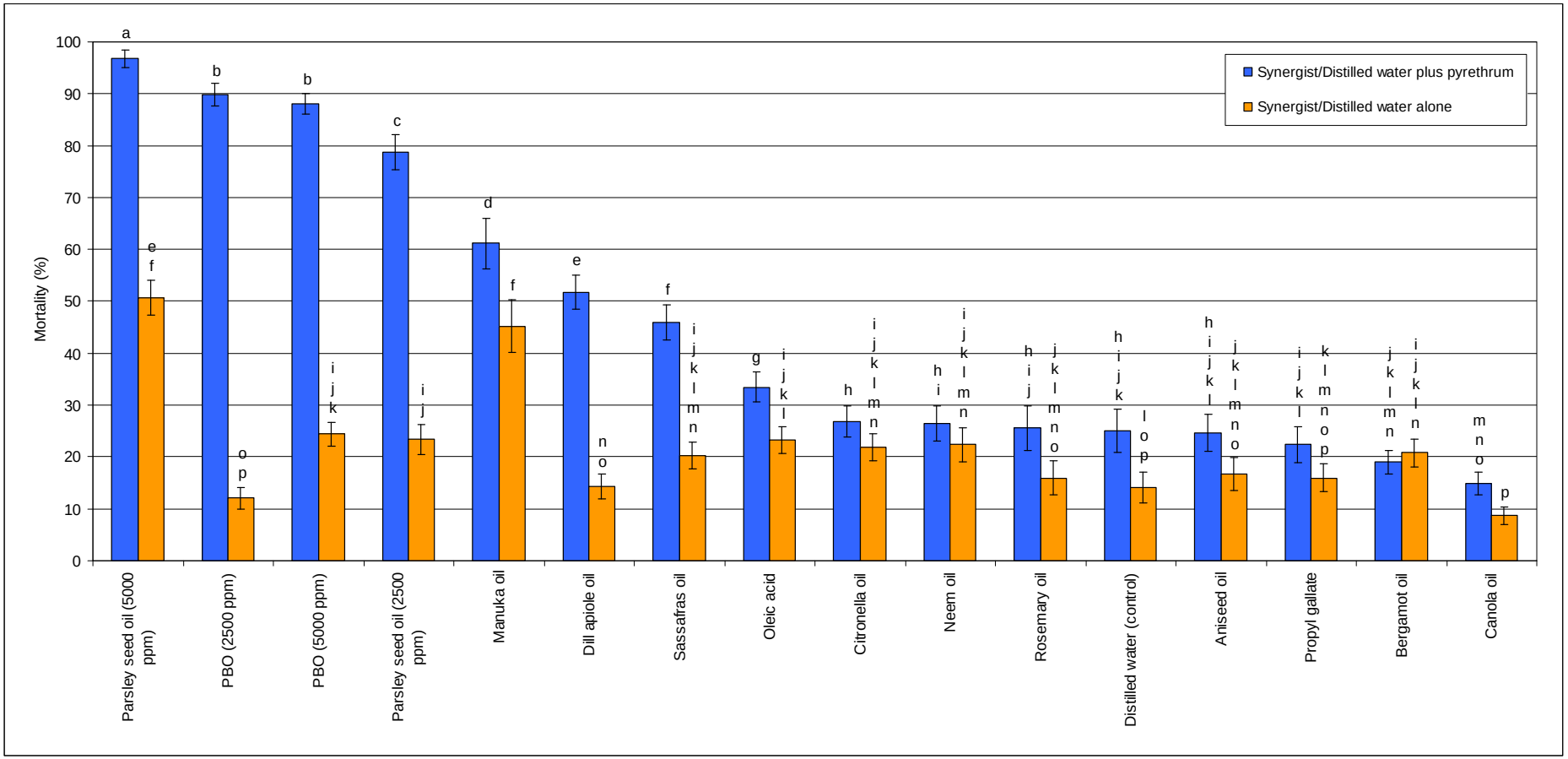


Fig. 4.1. The mean percentage mortality (± SE) of *Frankliniella occidentalis* (adult females) calculated from leaf dip bioassays with mixtures of 1300 ppm pyrethrum:5000 ppm (or 2500 ppm if specified) synergist, or with 5000 ppm (or 2500 ppm if specified) synergist alone. Distilled water was used as the control (for unsynergised pyrethrum and distilled water alone). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

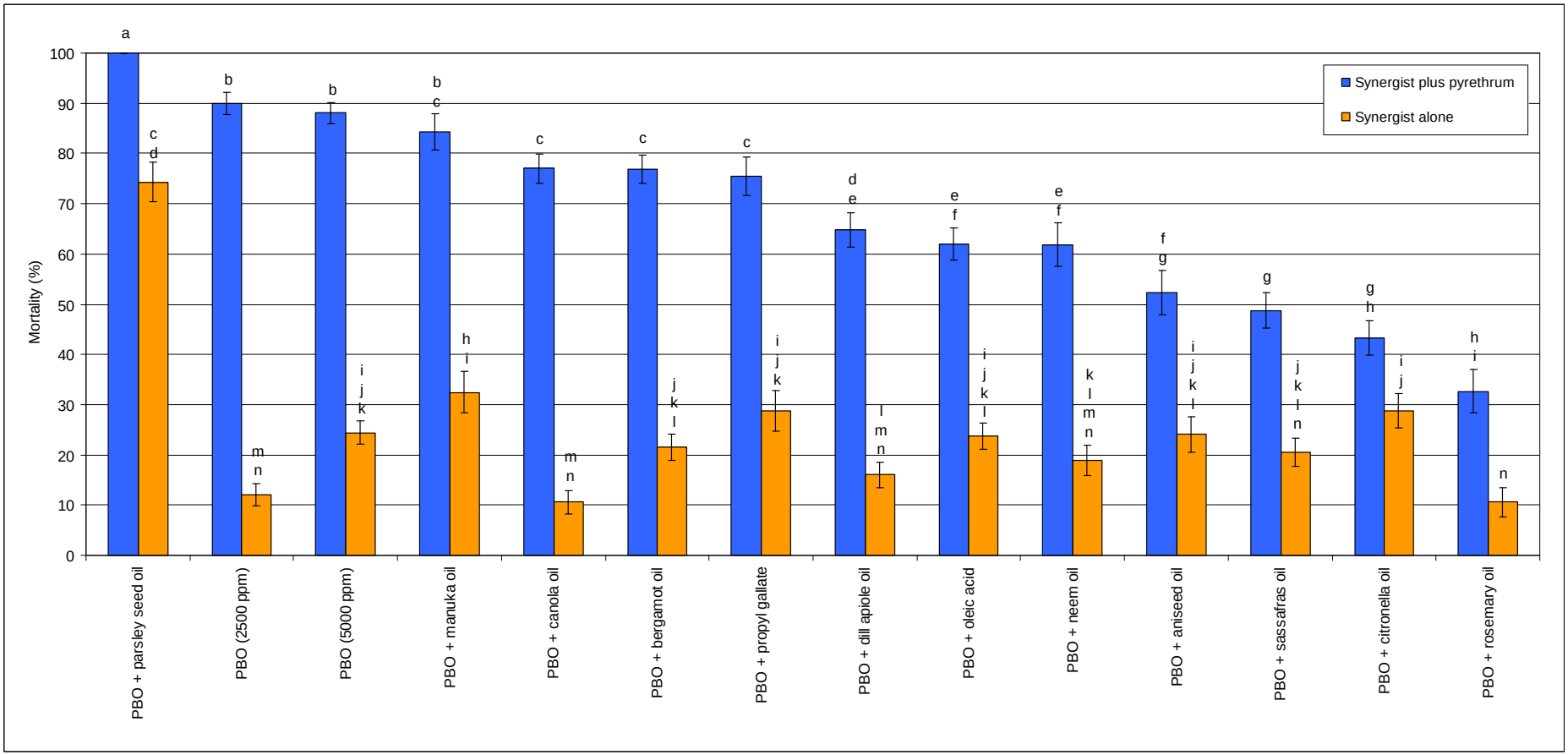


Fig. 4.2. The mean percentage mortality (± SE) of *Frankliniella occidentalis* (adult females) calculated from leaf dip bioassays with 1300 ppm pyrethrum applied with a mixture of synergist (5000 ppm) and PBO (5000 ppm) (or 2500 ppm if specified), or with a mixture of synergist (5000 ppm) and PBO (5000 ppm) (or 2500 ppm if specified) alone. (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

4.3.1.2.2 Discriminating dose bioassay - synergists mixed with PBO

Discriminating dose bioassays with synergists mixed with PBO showed that the parsley seed oil:PBO mixture plus pyrethrum caused 100% mortality in ***F. occidentalis***, which was significantly higher than all the other treatments ($p<0.001$) (Fig. 4.2). However, the parsley seed oil:PBO mixture itself caused high mortality (74.26%), which would have contributed to the high mortality found with the mixture with pyrethrum. The PBO plus pyrethrum controls (PBO at both 2500 ppm and 5000 ppm) caused high mortalities in ***F. occidentalis*** (89.91% and 88.07% mortality respectively), which did not differ significantly from one another, nor from the mortality caused by pyrethrum plus the manuka oil:PBO mixture (84.30% mortality), but had significantly higher mortalities than all the remaining treatments. The results suggest that the addition of PBO to the compounds did not enhance potential efficacy as a synergist.

4.3.2 Biochemical assays

4.3.2.1 Esterase assay with alpha-naphthyl acetate as a substrate

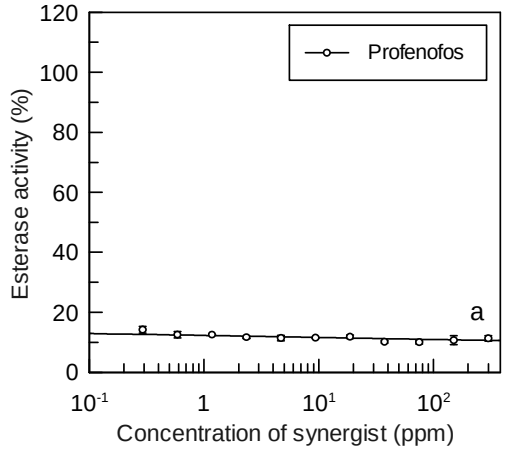
Esterase assays revealed varying degrees of ***F. occidentalis*** esterase inhibition by the synergists (Table 4.2, Fig. 4.3a-k). The most potent ***F. occidentalis*** esterase inhibitors, with the lowest IC_{50} values, were profenofos, propyl gallate, neem oil and BcB2 extract. Profenofos gave the highest inhibition of ***F. occidentalis*** esterases, with high inhibition (12.93% esterase activity remaining) even at the lowest concentration in the serial dilution (Fig. 4.3a). The profenofos should have been more dilute to observe initial inhibition and obtain a complete inhibition curve. The least potent, with the highest IC_{50} values, were sassafras oil and PBO. The higher the IC_{50}, the higher the concentration of synergist required to inhibit 50% of esterases. Synergists with lower IC_{50} values are therefore more potent inhibitors than those with higher IC_{50} values and could potentially make better synergists. This is not necessarily the case, however, as the specific esterases involved with insecticide detoxification would still need to be inhibited.

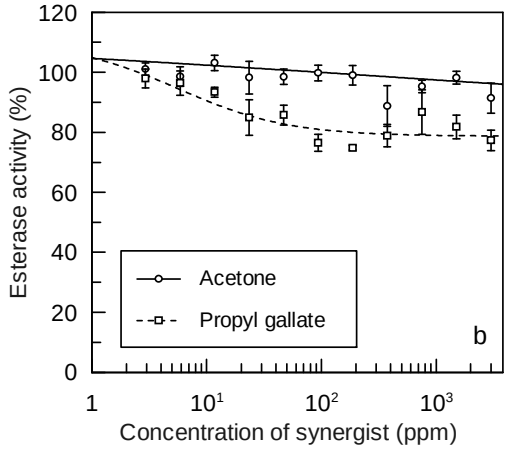
Propyl gallate had the smallest Y-range, inhibiting the most limited range of esterases. Manuka oil and CaB2 frac3 extract had the largest Y-ranges, inhibiting the broadest range of total esterases (Table 4.2).

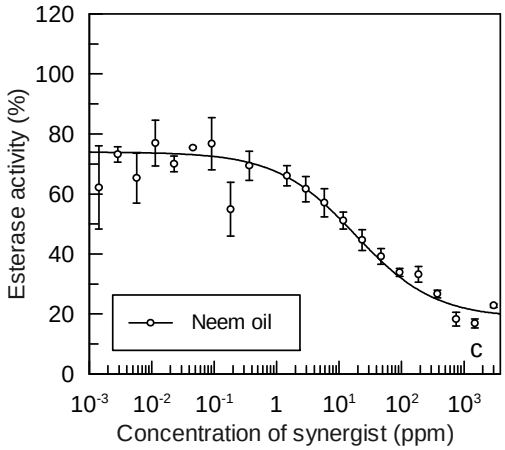
Compounds that showed no inhibition of esterases were acetone (Fig. 4.3b), oleic acid, aniseed, bergamot, canola, dill seed, eucalyptus, grapefruit, melaleuca, platyphyllol and rosemary oils (not illustrated). Citronella and geranium oils showed esterase activity that was higher than in the uninhibited control (not illustrated) (due to a possible anomaly with the assay, as described in chapter three, section 3.3.2.1.1).

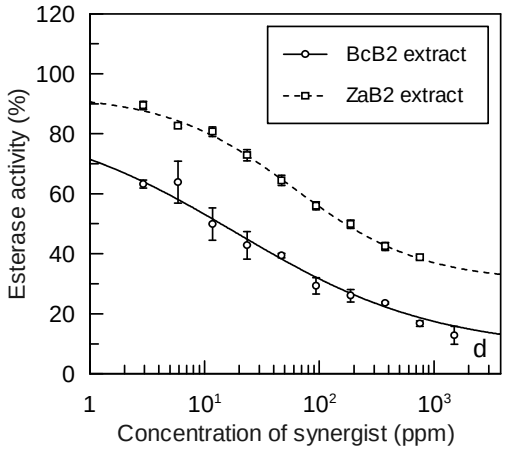
Table 4.2. Inhibition of *Frankliniella occidentalis* (male and female nymphs and adults) total esterase activity (alpha-naphthyl acetate as the substrate) by potential pyrethrum synergists, PBO and profenofos. The IC_{50} and Y-range values (± SE) are tabulated for each compound. (Relates to Fig. 4.3a-k; Compounds ordered from lowest to highest IC_{50}).

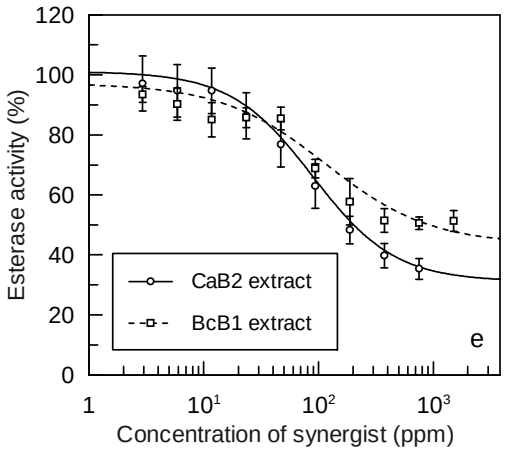
Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
Profenofos	Organophosphate	4.28 (± 8.44)	87.79 (± 0.85)
Propyl gallate	Ester	5.65 (± 4.18)	31.67 (± 22.89)
Neem oil	Tetranortriterpenoid / liminoid	18.32 (± 4.11)	55.34 (± 3.26)
BcB2 extract	Unknown	18.59 (± 8.08)	78.22 (± 9.50)
ZaB2 extract	Sesquiterpenoid	59.44 (± 6.95)	62.20 (± 5.20)
CaB2 extract	Phenylpropene	89.08 (± 19.16)	69.99 (± 9.12)
BcB1 extract	Unknown	115 (± 25.64)	53.62 (± 6.71)
ClB2 extract	Cyclic terpene	145 (± 15.60)	50.39 (± 2.63)
ClB1 extract	Cyclic terpene	357 (± 83.27)	57.00 (± 6.47)
CaB2 frac3 extract	Phenylpropene	3002 (± 854)	94.70 (± 12.05)
Myristicin oil	Methylenedioxyphenol ring	3274 (± 1311)	63.83 (± 42.68)
White cypress oil	Bicyclic monoterpene; Monoterpene; Cyclic terpene	5088 (± 4157)	73.77 (± 38.77)
Parsley seed oil	Methylenedioxyphenol ring; Bicyclic monoterpene; Methylenedioxyphenol ring / phenylpropanoid	5298 (± 1950)	83.13 (± 13.58)
Sesame oil	Methylenedioxyphenol ring	5703 (± 10 086)	49.32 (± 36.88)
Manuka oil	Sesquiterpene hydrocarbon; Triketone derivative	6534 (± 3855)	93.18 (± 33.02)
Dill apiole oil	Methylenedioxyphenol ring	8588 (± 19 725)	52.92 (± 66.82)
PBO	Methylenedioxyphenol ring	10 418 (± 8111)	68.15 (± 29.60)
Sassafras oil	Methylenedioxyphenol ring	576 321 (± 1 095 995)	100

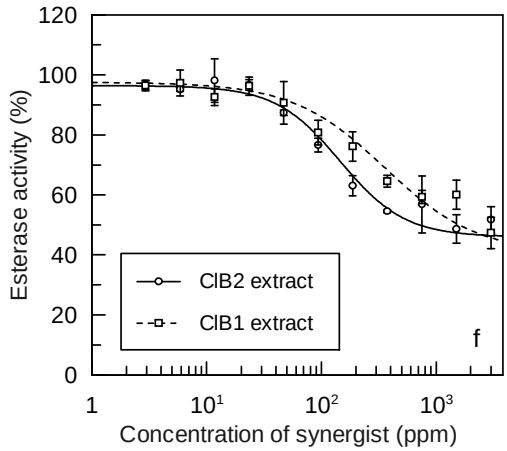

Profenofos
a
Esterase activity (%)
Concentration of synergist (ppm)


Acetone
Propyl gallate
b
Esterase activity (%)
Concentration of synergist (ppm)


Neem oil
c
Esterase activity (%)
Concentration of synergist (ppm)


BcB2 extract
ZaB2 extract
d
Esterase activity (%)
Concentration of synergist (ppm)


CaB2 extract
BcB1 extract
e
Esterase activity (%)
Concentration of synergist (ppm)


ClB2 extract
ClB1 extract
f
Esterase activity (%)
Concentration of synergist (ppm)

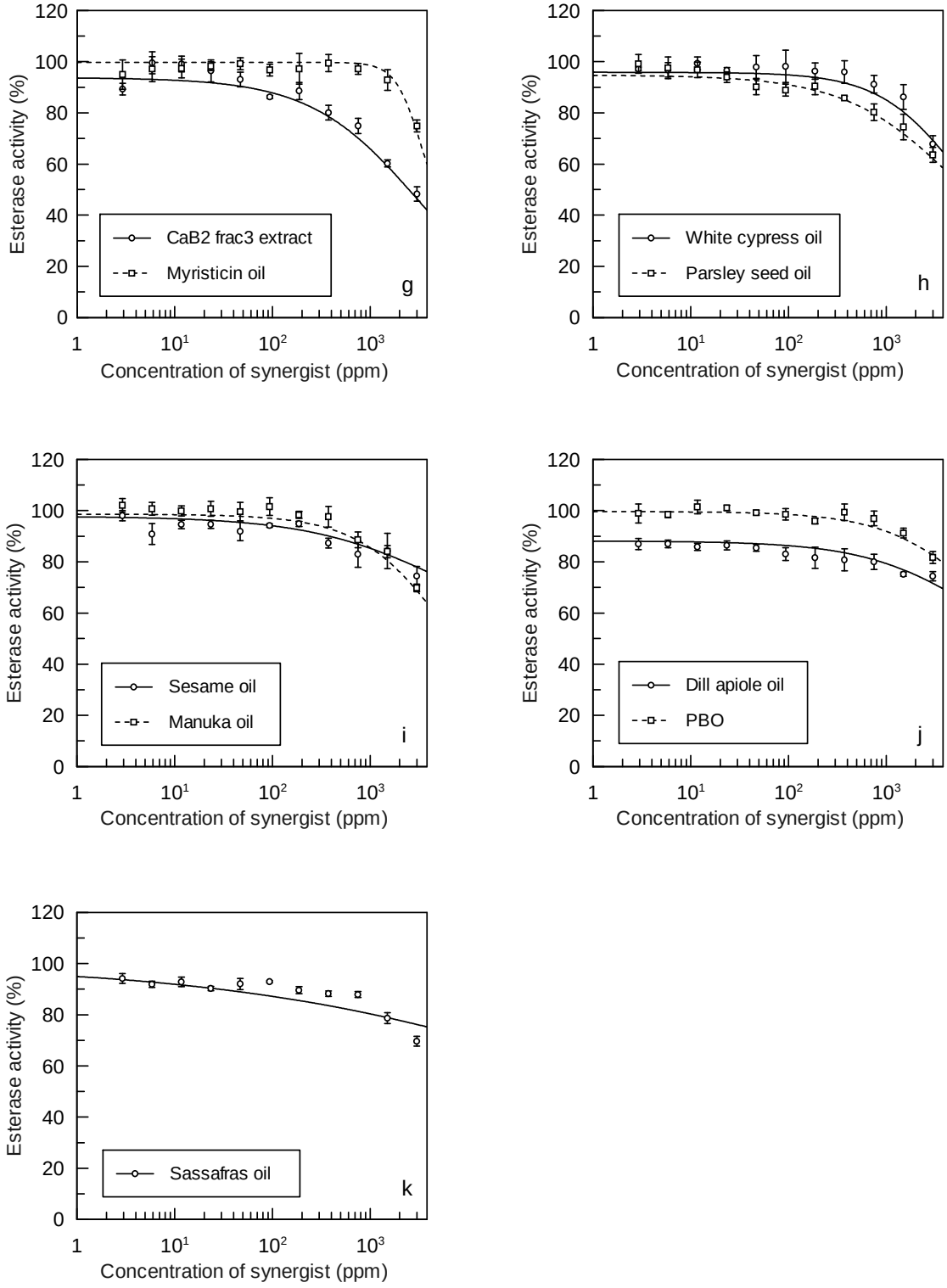


Fig. 4.3a-k. Inhibition of *Frankliniella occidentalis* (male and female nymphs and adults) total esterase activity (alpha-naphthyl acetate as the substrate) by potential pyrethrum synergists, PBO, profenofos and acetone (control). Error bars represent standard deviation. For sassafras oil, the line was fitted with a two parameter logistic curve (lower and upper limits of 0% and 100% respectively). For all remaining compounds, lines were fitted with a four parameter logistic curve. (Relates to Table 4.2; Compounds ordered from lowest to highest IC_{50}).

No significant correlations were found between synergism of pyrethrum and inhibition of esterases (percentage esterase activity remaining at the highest concentration of the synergist dilution series, IC_{50} and Y-range values) (Table 4.3). Discriminating dose bioassays showed that parsley seed oil, PBO, dill apiole oil, sassafras oil and, to a lesser extent, oleic acid, were most effective at synergising pyrethrum ***in vivo*** (section 4.3.1.2.1, Fig. 4.1). Oleic acid did not inhibit esterases ***in vitro***. Parsley seed oil, PBO, dill apiole oil and sassafras oil showed some inhibition of esterases, but with relatively high IC_{50} values (Table 4.2, Fig. 4.3h, j & k). Propyl gallate and neem oil (Fig. 4.3b & c), with low IC_{50} values, did not significantly synergise pyrethrum.

Table 4.3. Analysis of the correlations between the *Frankliniella occidentalis* discriminating dose bioassay mortality data for synergists with pyrethrum and inhibition data from the *F. occidentalis* esterase assay (alpha-naphthyl acetate as a substrate).

Bioassay	Esterase assay parameter	n[¥]	Correlation coefficient	Significance[§] (p-value) (two-tailed)
F. occidentalis discriminating dose	Highest concentration of synergist	12	-0.299	NS (0.345)
	IC_{50}	5	0.752	NS (0.143)
	Y-range	6	0.698	NS (0.123)

[¥] n = Number of synergists used in the correlation analysis.

[§] NS = not significant.

4.3.2.2 Glutathione-S-transferase assay

The GST-inhibiting capabilities of the potential synergists were studied using CDNB as a substrate. The most potent inhibitors of ***F. occidentalis*** GST activity, with the lowest IC_{50} values, were ClB2 extract, ZaB2 extract and neem oil (Table 4.4, Fig. 4.4a-f). Propyl gallate and parsley seed oil were the least potent, with the highest IC_{50} values. Synergists with the smallest Y-ranges, inhibiting only a small range of GSTs, were parsley seed oil and manuka oil. BcB2 extract had the largest Y-range, inhibiting 100% of GSTs (Fig. 4.4c).

No inhibition of GSTs was found with acetone (Fig. 4.4a), oleic acid, PBO, DEM, ClB1 extract, aniseed, bergamot, canola, citronella, dill seed, geranium, rosemary, sassafras and sesame oils (not illustrated).

Table 4.4. Inhibition of *Frankliniella occidentalis* (male and female nymphs and adults) glutathione-S-transferases (CDNB as the substrate) by potential pyrethrum synergists. The IC_{50} and Y-range values (± SE) are tabulated for each compound. (Relates to Fig. 4.4a-f; Compounds ordered from lowest to highest IC_{50}).

Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
ClB2 extract	Cyclic terpene	1007 (± 398)	71.49 (± 16.06)
ZaB2 extract	Sesquiterpenoid	1242 (± 915)	69.11 (± 23.30)
Neem oil	Tetranortriterpenoid / liminoid	1846 (± 4929)	74.63 (± 64.64)
BcB2 extract	Unknown	2132 (± 807)	102 (± 18.88)
Dill apiole oil	Methylenedioxyphenol ring	2305 (± 123)	100
CaB2 frac3 extract	Phenylpropene	2510 (± 126)	100
CaB2 extract	Phenylpropene	2790 (± 264)	100
BcB1 extract	Unknown	2981 (± 309)	100
Manuka oil	Sesquiterpene hydrocarbon; Triketone derivative	3556 (± 2651)	47.60 (± 21.90)
Propyl gallate	Ester	5161 (± 573)	100
Parsley seed oil	Methylenedioxyphenol ring; Bicyclic monoterpene; Methylenedioxyphenol ring / phenylpropanoid	16 669 (± 33 063)	46.30 (± 34.57)

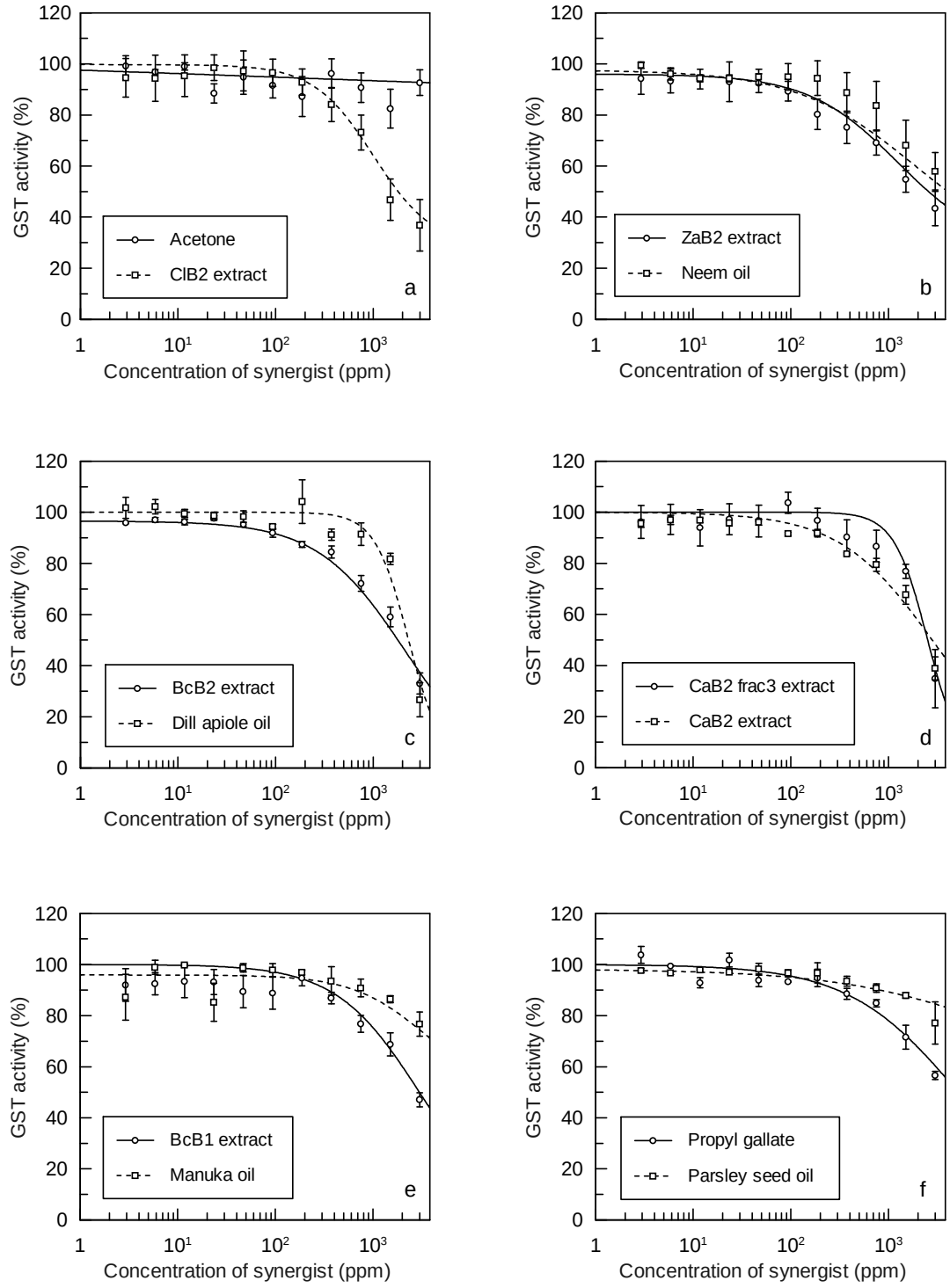


Fig. 4.4a-f. Inhibition of *Frankliniella occidentalis* (male and female nymphs and adults) glutathione-S-transferase (GST) activity (CDNB as the substrate) by potential pyrethrum synergists and acetone (control). Error bars represent standard deviation. For dill apiole oil, CaB2 frac3 extract, CaB2 extract, BcB1 extract and propyl gallate, lines were fitted with two parameter logistic curves (lower and upper limits of 0% and 100% respectively). For all remaining compounds, lines were fitted with a four parameter logistic curve. (Relates to Table 4.4; Compounds ordered from lowest to highest IC_{50}).

No significant correlations were found between synergism of pyrethrum and the inhibition of GSTs (percentage GST activity remaining at the highest concentration of the synergist dilution series, IC_{50} and Y-range values) (Table 4.5). The most effective pyrethrum synergists were parsley seed oil, PBO, dill apiole oil, sassafras oil and oleic acid (Fig. 4.1). PBO, sassafras oil and oleic acid, however, showed no inhibition of GST activity. Parsley seed oil had the highest IC_{50} value of the compounds showing GST inhibition (Fig. 4.4f) and dill apiole oil showed inhibition only at the highest concentrations in the dilution series (Fig. 4.4c). Considering only the compounds that were tested for synergism of pyrethrum in the discriminating dose bioassay (section 4.3.1.2.1), the highest inhibition of GSTs was by neem oil (Fig. 4.4b), however, neem oil did not synergise pyrethrum.

Table 4.5. Analysis of the correlations between the *Frankliniella occidentalis* discriminating dose bioassay mortality data for synergists with pyrethrum and inhibition data from the *F. occidentalis* glutathione-S-transferase (GST) assay.

Bioassay	GST assay parameter	n[¥]	Correlation coefficient	Significance[§] (p-value) (two-tailed)
F. occidentalis discriminating dose	Highest concentration of synergist	13	0.050	NS (0.871)
	IC_{50}	4	-0.226	NS (0.774)
	Y-range	3	-0.957	NS (0.187)

[¥] n = Number of synergists used in the correlation analysis.

[§] NS = not significant.

4.3.2.3 Monooxygenase assay

Monooxygenase activity was not detected using ***F. occidentalis*** homogenate with the monooxygenase assay. Synergists could thus not be compared in their ability to inhibit ***F. occidentalis*** monooxygenase activity.

4.3.2.4 Enzyme inhibition by pyrethrum

In ***F. occidentalis***, pyrethrum gave little inhibition of total esterase activity (Fig. 4.5a), with an IC_{50} (± SE) of 9993.58 ppm (± 4938.26) and a Y-range (± SE) of 103.74% (± 32.08). Pyrethrum showed inhibition of GSTs in ***F. occidentalis*** only in the very highest concentration of pyrethrum tested, giving an IC_{50} (± SE) of 2910.30 ppm (± 328.29) (Fig. 4.5b).

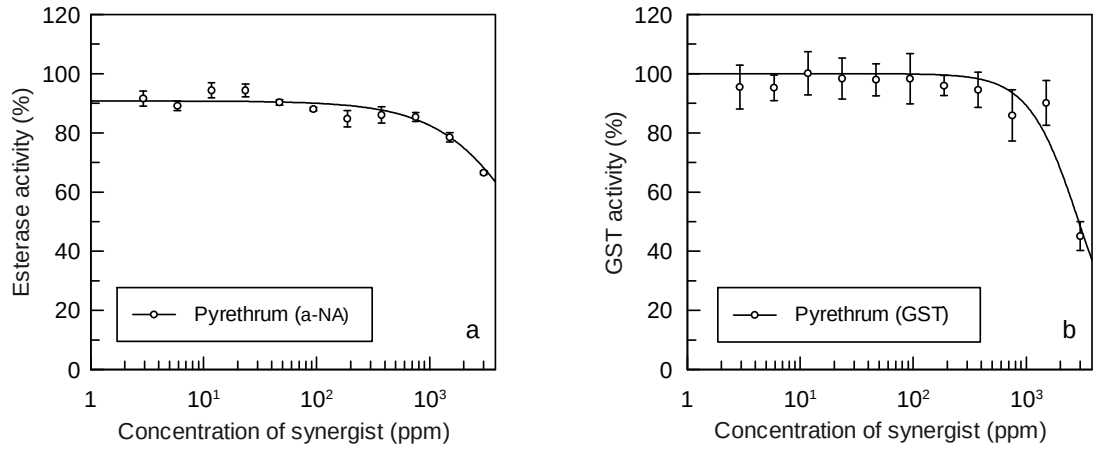


Fig. 4.5a-b. Inhibition of *Frankliniella occidentalis* (male and female nymphs and adults) enzyme activity by pyrethrum. Fig. 4.5a represents inhibition of total esterase activity (alpha-naphthyl acetate (a-NA) as the substrate); Fig. 4.5b represents inhibition of glutathione-S-transferase (GST) activity (CDNB as a substrate). Error bars represent standard deviation. In Fig. 4.5a, the line was fitted with a four parameter logistic curve. In Fig. 4.5b, the line was fitted with a two parameter logistic (lower and upper limits of 0% and 100% respectively).

4.4 Discussion and Conclusions

Discriminating dose bioassays were used to determine whether a number of natural plant oils and extracts showed effective synergism of pyrethrum against ***F. occidentalis***. Parsley seed oil, PBO, dill apiole oil, sassafras oil and oleic acid were found to significantly synergise pyrethrum. Parsley seed oil contains myristicin and apiole, both containing the methylenedioxyphenol (MDP) ring structure found in dill apiole and PBO. Myristicin and apiole have both previously been shown to demonstrate synergistic activity (Berenbaum & Neal 1985, Lichtenstein & Casida

1963, Lichtenstein ***et al***. 1974). Myristicin, for example, effectively synergised pyrethrum and carbamates against house flies ***Musca domestica*** L. (Diptera: Muscidae) and vinegar flies ***Drosophila melanogaster*** Meigen. (Diptera: Drosophilidae) (Lichtenstein & Casida 1963). Dill apiole was found to synergise pyrethrum against red flour beetles ***Tribolium castaneum*** (Herbst) (Coleoptera: Tenebrionidae) (Tomar ***et al***. 1979a,b) and adult ***M. domestica*** (Saxena ***et al***. 1977). Sassafras oil contains safrole, the compound from which PBO is synthesised, also containing the MDP ring important for synergism of pyrethrum (Casida 1970, Casida & Quistad 1995). PBO is well recognised to effectively synergise a wide range of insecticides. In resistant strains of ***F. occidentalis*** in Spain, PBO has shown synergistic activity towards pyrethroids (acrinathrin and deltamethrin), carbamates (formetanate and methiocarb) and the organophosphate, methamidophos (Espinosa ***et al***. 2005). PBO has been shown to synergise diazinon (organophosphate), bendiocarb (carbamate) and fenvalerate (pyrethroid) in a diazinon-resistant strain of ***F. occidentalis*** in the USA (Zhao ***et al***. 1995). PBO has also been shown to synergise tau-fluvalinate in pyrethroid-resistant strains of ***F. occidentalis*** in Australia (Thalavaisundaram ***et al***. 2008). Oleic acid, a mono-unsaturated omega-9 fatty acid, also showed significant synergism of pyrethrum in the discriminating dose bioassay, although to a much lesser extent than the afore-mentioned compounds. Oleic acid has shown low levels of synergism towards esfenvalerate (2.25 fold) and methomyl (2.65 fold) in the cotton leafworm ***Spodoptera littoralis*** (Boisduval) (Lepidoptera: Noctuidae) (Hatem ***et al***. 2009). In this study, the most effective compounds thus found synergising pyrethrum in ***F. occidentalis*** tended to contain the MDP ring. However, it was not exclusively required for synergistic activity, since oleic acid (without a MDP ring) also showed synergistic activity.

The surfactant properties of PBO allows it to enhance the penetration of lipophilic compounds through the insect cuticle. PBO has, for example, been thought to increase the penetration of esfenvalerate (Gunning ***et al***. 1995) and permethrin (Kennaugh ***et al***. 1993) through the cuticle of ***H. armigera.*** PBO could therefore potentially act to enhance the penetration of other synergists through the insect cuticle. The synergistic action of compounds that might be good enzyme inhibitors but are perhaps not efficient at crossing the insect cuticle may be enhanced when used in mixture with PBO. PBO was therefore added to the synergists to compare synergistic efficacy towards pyrethrum against ***F. occidentalis***. The PBO and parsley seed oil mixture with pyrethrum resulted in 100% mortality but a large proportion of

mortality was most likely due to the toxicity of the parsley seed oil:PBO mixture itself (74% mortality). Parsley seed oil at 5000 ppm on its own also caused high mortality (51%) in ***F. occidentalis***. The addition of PBO to the potential synergists did not result in an increase in mortality in ***F. occidentalis*** for any of the compounds with pyrethrum compared to the mortality caused by pyrethrum with PBO alone. Since PBO is already a highly efficient pyrethrum synergist, mortality may not have been increased significantly with the addition of another synergist. The presence of another synergist with PBO tended to rather have an antagonistic effect, with lower mortalities than with pyrethrum plus PBO alone.

The synergistic abilities of the compounds tested with pyrethrum in the discriminating dose bioassay were compared regarding their abilities to inhibit the resistance-associated enzymes (esterases, GSTs and monooxygenases). ***F. occidentalis*** esterase assays (alpha-naphthyl acetate as a substrate) showed profenofos, propyl gallate, neem oil and BcB2 extract to be the most potent inhibitors of esterases. Profenofos was tested as an esterase inhibitor control in this study. Organophosphates are known esterase inhibitors and profenofos has been shown to inhibit esterases in Australian ***H. armigera*** (Gunning ***et al***. 1999). Neem oil has previously been shown to depress esterase activity in the obliquebanded leafroller ***Choristoneura rosaceana*** (Harris) (Lepidoptera: Tortricidae), with significantly lower esterase activity in male and female moths reared on larval diet containing neem oil until larvae reached pupation (Smirle ***et al***. 1996). Azadirachtin, the main component of neem oil (a tetranortriterpenoid), was found to be a reversible competitive inhibitor of esterase activity in the diamondback moth ***Plutella xylostella*** (L.) (Lepidoptera: Plutellidae) (He 2003).

No significant correlations were found between inhibition of ***F. occidentalis*** esterases by the various potential synergists and mortality of ***F. occidentalis*** in the discriminating dose bioassay. Parsley seed oil, PBO, dill apiole oil, sassafras oil and oleic acid showed synergistic activity towards pyrethrum in the discriminating dose bioassay. Oleic acid, however, failed to inhibit ***F. occidentalis*** esterases and PBO, dill apiole oil and sassafras oil gave very little inhibition of esterases. Parsley seed oil showed slightly more inhibition of esterases than PBO, dill apiole oil and sassafras oil. Neem oil and propyl gallate showed good esterase inhibition but neither synergised pyrethrum ***in vivo***. Acrinathrin and methiocarb resistance in ***F. occidentalis*** from Spain has been correlated to esterase enzymes (López-Soler ***et al***.

2008, Maymó ***et al***. 2006). Using two pyrethroid-resistant strains of ***F. occidentalis*** in Australia, profenofos was found to synergise tau-fluvalinate in one strain but not in the other, suggesting the involvement of esterases as a resistance mechanism only in the strain where synergism was observed (Thalavaisundaram ***et al***. 2008). The involvement of monooxygenases was implicated as the main metabolic resistance mechanism in ***F. occidentalis*** from south-eastern Spain (Espinosa ***et al***. 2005) using synergism by PBO. However, PBO is also known to inhibit esterase activity (Gunning ***et al***. 1998, Young ***et al.*** 2005, 2006) and observed synergism due to PBO might possibly have been due to esterase inhibition in some strains. A lack of correlation between ***in vivo*** synergism and ***in vitro*** esterase inhibition could be due to a number of reasons, including penetration of the synergist through the insect cuticle, metabolism of the synergist and affinity for the target site (Casida 1970, Yamamoto 1973), or the esterase assay may fail to properly quantify inhibition of the esterases directly involved in resistance by the synergists.

GST assays done with ***F. occidentalis*** showed ClB2 extract, ZaB2 extract and neem oil to be the most potent inhibitors of GST activity. A decrease in GST activity has been observed in female ***C. rosaceana*** moths (but not in the male moths) for larvae fed on diet containing neem oil until pupation and subsequently reared through to adults (Smirle ***et al***. 1996). ClB2 extract contains limonene, a cyclic terpene, ZaB2 extract contains zierone, a sesquiterpenoid, and the tetranortriterpenoid, azadirachtin, is the main component of neem oil. In general, no trends were evident between the main chemical structure of the synergists and inhibition of both ***F. occidentalis*** esterases and GSTs. This could be due to the presence of any of the other constituents present in the natural plant oils and extracts that could contribute to inhibition of enzyme activity.

No significant correlations were found between inhibition of GST activity and the ***in vivo*** synergism of pyrethrum against ***F. occidentalis***. Parsley seed oil and dill apiole oil showed some inhibition of GSTs, whereas PBO, sassafras oil and oleic acid did not inhibit GST activity. While neem oil was one of the most effective inhibitors of GSTs, it failed to show synergism of pyrethrum ***in vivo***. An increase in both GST and esterase activity has previously been reported in multiresistant field strains of ***F. occidentalis*** (Maymó ***et al***. 2002). A lack of correlation could suggest that GSTs are not involved in resistance in the Leppington Rose strain of ***F. occidentalis***, or could

be due to a number of other reasons, as mentioned above for the lack of correlation to esterases (Casida 1970, Yamamoto 1973).

Monooxygenase activity was not detected during this study using ***F. occidentalis***. Lack of monooxygenase activity could be due to the presence of proteases destroying the enzyme, as has been found with ***Myzus persicae*** (Philippou ***et al***. 2010). The assay might have to be repeated using an ultracentrifuge to prepare ***F. occidentalis*** homogenate, or with a different quantity of ***F. occidentalis***. A monooxygenase assay with a different substrate could also possibly be required to detect monooxygenase activity in ***F. occidentalis***. Monooxygenase assays on insects as small as ***F. occidentalis*** tend to be difficult to implement (Jensen 2000).

The ability of pyrethrum to inhibit esterases and GSTs was determined to examine whether the enzyme systems were playing a role in the possible detoxification of pyrethrum. Pyrethrum showed a small amount of inhibition of both esterases and GSTs, suggesting that pyrethrum is binding to both esterases and GSTs. Both enzyme systems may thus be involved in the detoxification of pyrethrum in the Leppington Rose strain of ***F. occidentalis***. Since inhibition was only slight with both enzyme systems, however, it would be ideal to examine the inhibition of ***F. occidentalis*** monooxygenase activity by pyrethrum to assess their possible role in pyrethroid or pyrethrum resistance in ***F. occidentalis***. A significant correlation was detected between ***in vivo*** synergism of pyrethrum in ***F. occidentalis*** and inhibition of ***Carpophilus*** spp. monooxygenase activity (chapter six, section 6.3.2.3), and although a strict comparison between taxonomic orders is not ideal, it might suggest, due to the high similarity between P450s in terms of structure, that monooxygenases could potentially be playing a role in synergism of pyrethrum in ***F. occidentalis***, and thus also perhaps in pyrethroid resistance.

In conclusion, the most effective pyrethrum synergists against ***F. occidentalis in vivo*** were PBO, parsley seed oil, dill apiole oil and sassafras oil, all containing the MDP ring structure important for synergism of pyrethrum. Oleic acid, a fatty acid (not containing the MDP ring), however, also showed synergistic activity towards pyrethrum. The addition of PBO to the synergists never enhanced the synergistic activity of any of the synergists tested. The most potent esterase inhibitors were propyl gallate, neem oil and BcB2 extract, whereas the most potent GST inhibitors were ClB2 extract, ZaB2 extract and neem oil. ***In vitro*** inhibition of esterases and

GSTs could not be correlated to pyrethrum synergism ***in vivo***, suggesting that the specific assays used may not be effective for screening of potential pyrethrum synergists against ***F. occidentalis***.

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5 CHAPTER FIVE: THE EVALUATION OF VARIOUS NATURAL COMPOUNDS AS POTENTIAL PYRETHRUM SYNERGISTS AGAINST THE PEACH-POTATO APHID *MYZUS PERSICAE*

Abstract

The peach-potato aphid ***Myzus persicae*** is a devastating polyphagous pest worldwide that has developed high levels of insecticide resistance due to pesticide over-use. Synergists such as PBO can increase insecticide efficacy in insects with metabolic mechanisms of resistance, since synergists inhibit the enzymes involved in detoxification of insecticides. The efficacy of a number of natural, potentially organically-certifiable compounds as pyrethrum synergists was assessed using a pyrethroid-resistant clone of ***M. persicae*** with an esterase-based mechanism of resistance (clone 794JZ) and a neonicotinoid-resistant clone of ***M. persicae*** (5191A) with a monooxygenase-based resistance mechanism, using imidacloprid, with the intention of finding a synergist capable of acting across both enzyme systems. Discriminating dose bioassays showed a significant positive correlation between synergism with pyrethrum and synergism with imidacloprid. PBO was the most effective synergist of both pyrethrum and imidacloprid. Natural compounds showing the highest efficacy as synergists in both clones of ***M. persicae*** were parsley seed oil, dill apiole oil, bergamot oil and neem oil. Parsley seed oil and dill apiole oil contain the methylenedioxyphenol (MDP) ring chemical structure also present in PBO that is considered important for synergism of pyrethrum. The plant oils and extracts, however, are not pure and any of the constituents present, even in small quantities, could be contributing to synergism.

In vitro total esterase assays using alpha-naphthyl acetate as a substrate showed only neem oil to inhibit esterases. However, a significant positive correlation was found between inhibition of esterases when para-nitrophenyl acetate was used as the substrate and a further ***in vitro*** assay, the esterase interference assay. In both these assays, good inhibition of esterases was found by PBO, BcB1 extract, oleic acid, BcB2 extract, neem oil and ClB2 extract and little inhibition by rosemary oil, dill seed oil, bergamot oil and grapefruit oil. Synergism found ***in vivo*** with both pyrethrum and imidacloprid did not correlate significantly with any of the three ***in vitro*** esterase assays.

5.1 Introduction

The peach-potato aphid ***Myzus persicae*** is a serious pest of many crop species, including peaches (Grechi ***et al***. 2010), cabbage (Kalule & Wright 2002), tobacco (Margaritopoulos ***et al***. 2010) and potatoes (Srinivasan & Alvarez 2007). ***M. persicae*** also acts as a vector of viral diseases (Martinez-Torres ***et al.*** 1999), such as ***potato virus Y*** and ***potato leafroll virus***, causing great economic loss to potato crops (Srinivasan & Alvarez 2007). The ongoing use of pesticides against ***M. persicae*** has led to the development of resistance towards many pesticides, including organophosphates, carbamates, pyrethroids (Devonshire & Moores 1982) and neonicotinoids (Philippou ***et al***. 2010, Puinean ***et al***. 2010). Synergists, such as PBO,

may be an important tool in overcoming metabolic insecticide resistance, by inhibiting the enzymes involved in metabolic resistance (esterases, glutathione-S-transferases and monooxygenases) and thus restoring a level of susceptibility in resistant insects.

The main aim of this project was to identify effective natural synergists that could potentially be certified for organic use and formulated with pyrethrum in order to enhance its efficacy. In this chapter, ***M. persicae*** was used as the test insect. Bioassays were performed to determine whether a variety of natural compounds showed synergism of pyrethrum against a pyrethroid-resistant clone of ***M. persicae*** (clone 794JZ). Clone 794JZ has a well-characterized esterase-based mechanism of resistance, containing R_3 (the highest) levels of the resistance-associated carboxylesterase, E4. In resistant aphids, an increase in E4 has been correlated to an increase in resistance. Aphids are classified either as susceptible, R_1, R_2 or R_3 based on the amount of E4 present (Devonshire & Moores 1982, Martinez-Torres ***et al.*** 1999).

Since synergists act directly on the enzymes responsible for insecticide resistance, a neonicotinoid-resistant clone of ***M. persicae*** (5191A) with a known monooxygenase-based resistance mechanism (Philippou ***et al***. 2010, Puinean ***et al***. 2010) was also used for bioassays, using imidacloprid. The efficacy of the potential synergists in the presence of a monooxygenase-based resistance mechanism could thus be determined and compared to synergism in insects with an esterase-based mechanism of resistance. Synergists that show efficacy in both enzyme systems could make for more practical synergists. The same synergist could potentially be used for a wider variety of purposes, across taxa with different metabolic insecticide-resistance mechanisms, or within one species possessing different metabolic mechanisms of insecticide-resistance. Insects from different taxonomic orders were used in this study to determine the efficacy of synergists between taxa (see other chapters). An effective synergist for one species may not necessarily synergise pyrethrum in another since the enzymes responsible for insecticide resistance and their affinity in binding with the synergistic compounds may vary between taxa. The nature of penetration of the synergists into the insect body may also differ.

This study aimed to also investigate the enzyme-inhibiting capacity of the synergists using biochemical assays, since synergists inhibit the enzymes involved in detoxification of insecticides. ***M. persicae*** clone 794JZ has a well-known esterase-

based resistance mechanism and esterase assays were therefore undertaken with this clone to study the ability of the synergists to inhibit ***M. persicae*** esterases ***in vitro***.

5.2 Materials and Methods

5.2.1 Bioassays

M. persicae bioassays were done by topical application using adult female apterous morphs, according to the methods described in chapter two (section 2.4.3). Two ***M. persicae*** clones were used to examine the efficacy of potential synergists. Clone 794JZ, a highly pyrethroid-resistant clone with R_3 levels of the resistance-associated carboxylesterase, E4 (Martinez-Torres ***et al.*** 1999), and clone 5191A, a neonicotinoid-resistant clone with monooxygenase-based insecticide resistance (Philippou ***et al***. 2010, Puinean ***et al***. 2010). (More detail on each clone is given in chapter two, section 2.1.3). The efficacy of synergists was determined for clone 794JZ using pyrethrum and for clone 5191A, using imidacloprid. A dose-response curve was determined for each clone, with pyrethrum (clone 794JZ) and imidacloprid (clone 5191A), to calculate the respective lethal dose (LD) values. The LD_{10} for each clone with each insecticide respectively was used as the discriminating dose in the discriminating dose bioassays as their low mortality with unsynergised pyrethrum and imidacloprid would allow any increase in efficacy with synergists to be compared.

5.2.1.1 Full dose response bioassay

The dose-response curve for pyrethrum using ***M. persicae*** clone 794JZ was determined using six pyrethrum dosages, ranging from 30 ppm to 7500 ppm (diluted in acetone). Acetone was applied as the control. The dose-response curve for imidacloprid using clone 5191A was determined using six imidacloprid dosages, from 0.1 ppm to 30 ppm (diluted in acetone), with acetone as the control. For both clones, each treatment contained ten aphids and the bioassay was replicated three times. Data were analysed with a probit analysis (Finney 1971), using PoloPlus (version 1.0, LeOra Software) taking control mortality into account (Abbott 1925).

5.2.1.2 Discriminating dose bioassays

5.2.1.2.1 Discriminating dose bioassay with pyrethrum (clone 794JZ)

The LD_{10} of pyrethrum with clone 794JZ was 108 ppm (section 5.3.1.1). A dose of 100 ppm pyrethrum was therefore chosen as the discriminating dose for the bioassay. The concentration of synergist chosen for the bioassay was 1000 ppm. The ratio was thus 10:1 synergist:pyrethrum. Treatments were applied as mixtures since temporal application (applying a pre-treatment of synergist followed later by an application of insecticide) resulted in high aphid control mortality, due to an increased handling of aphids and two doses of acetone (since pyrethrum and synergists were both dissolved in acetone).

The plant oils, extracts and compounds used as synergists or potential synergists in this study are described in chapter two (section 2.2.2, Table 2.1). Compounds were chosen based on insecticidal activity or insect repellence properties, plant metabolites or possible or known synergistic potential (for example, the presence of compounds containing the methylenedioxyphenol ring present in PBO). Natural plant oils tested for synergism with pyrethrum with aphid clone 794JZ were aniseed, bergamot, canola, citronella, dill apiole, dill seed, geranium, grapefruit, neem, parsley seed and rosemary oils. Propyl gallate, an anti-oxidant, and oleic acid, a mono-unsaturated omega-9 fatty acid, were also tested. Oleic acid is a component of the BRA emulsifiable concentrate (EC) formulation of pyrethrum. Plant extracts from the University of Tasmania tested for synergism were ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2. PBO was used as the standard synergist. Acetone applied with pyrethrum was used as the unsynergised pyrethrum (or pyrethrum only) treatment. Each synergist was also applied alone, without pyrethrum, to test for inherent toxicity of the synergists towards ***M. persicae*** clone 794JZ. Acetone was used as the control. Each treatment with pyrethrum was replicated from three to five times, with each replicate consisting of ten aphids. Each treatment without pyrethrum was replicated from two to five times, with each replicate consisting of ten aphids.

Discriminating dose bioassay data for ***M. persicae*** clone 794JZ were analysed with a logistic regression, with an assumed binomial distribution, using logit-transformed data. The program, ASREML, was used to analyse data. The model incorporated both compounds treated with pyrethrum and compounds alone into one analysis.

Pairwise comparisons were done to determine significant differences using Fisher's (protected) least significant difference (LSD) at the 5% level.

5.2.1.2.2 Discriminating dose bioassay with imidacloprid (clone 5191A)

The LD_{10} of imidacloprid with clone 5191A was 0.81 ppm (section 5.3.1.1). A discriminating dose of 1 ppm imidacloprid was therefore chosen as the discriminating dose and used together with each synergist at a concentration of 1000 ppm (thus a ratio of 1000:1 synergist:imidacloprid). If the synergists were used in too low a concentration, no synergism may be seen. Since imidacloprid is intrinsically much more potent than pyrethrum, a higher ratio of imidacloprid to synergist was used than for the pyrethrum discriminating dose bioassays with clone 794JZ. Treatments were applied as mixtures since high aphid control mortalities were found with a temporal application of synergist followed by insecticide, as explained above with clone 794JZ.

The plant oils, extracts and compounds used as synergists or potential synergists in the discriminating dose bioassay with clone 5191A were the same as those used above with clone 794JZ (section 5.2.1.2.1). Acetone applied with imidacloprid was used as the unsynergised imidacloprid (or imidacloprid only) treatment. Inherent toxicity of the synergists towards ***M. persicae*** clone 5191A was determined by applying each synergist alone, without imidacloprid. Acetone was applied as the control. Each replicate contained ten aphids. Treatments with imidacloprid were replicated from three to seven times. Treatments without imidacloprid were replicated from two to six times.

The discriminating dose bioassay with ***M. persicae*** clone 5191A was analysed in the same way as for clone 794JZ. In addition, a correlation was tested for between the percentage mortality found in the discriminating dose bioassay for ***M. persicae*** clone 794JZ with the synergists plus pyrethrum and the percentage mortality found in the discriminating dose bioassay for ***M. persicae*** clone 5191A with the synergists plus imidacloprid. Correlation analyses were performed according to the methods in chapter two (section 2.6).

5.2.2 Biochemical assays

Esterase assays were undertaken using ***M. persicae*** clone 794JZ, which has R_3 levels of the resistance-associated esterase, E4, to determine the esterase-inhibiting capabilities of the compounds tested for synergism in the discriminating dose bioassays. Resistance to pyrethroids in clone 794JZ is a well-characterized esterase-based mechanism and only esterase assays were therefore performed during this study. Biochemical assays with clone 5191A were not undertaken due to limited numbers of 5191A aphids and limited research time at Rothamsted Research.

5.2.2.1 Esterase assays

Total esterase assays using alpha-naphthyl acetate as the substrate were done to determine the esterase-inhibiting capabilities of the synergists. With alpha-naphthyl acetate as the substrate, however, neem oil was the only compound to show inhibition of ***M. persicae*** esterases. Alpha-naphthyl acetate was therefore not necessarily a suitable substrate to detect esterase inhibition by the synergists. The assay was thus repeated using para-nitrophenyl acetate as the substrate to determine whether further inhibition of esterases could be revealed. Inhibition of esterases by synergists using the total esterase assay has previously not been detected with ***M. persicae***, leading to the development of the esterase interference assay (Khot ***et al***. 2008) (explained in chapter two, section 2.5.1.3). The esterase interference assay was thus undertaken to indirectly determine binding of the synergists used in this study to esterases.

Since esterases are known to be the important enzyme system involved in resistance to pyrethroids in ***M. persicae*** clone 794JZ, using the different esterase assays could give an indication of which assay more closely correlated to synergism observed ***in vivo*** with the discriminating dose bioassay. A biochemical assay that identified effective synergists would be useful for replacing the screening of potential synergists with bioassays, which are often difficult to implement, require a large number of insects reared in the laboratory, and are time consuming.

5.2.2.1.1 Esterase assay with alpha-naphthyl acetate as a substrate

Total esterase assays using alpha-naphthyl acetate as a substrate were run using E4 purified from ***M. persicae*** clone 794JZ, according to methods by Devonshire (1977). The assays were run according to the methods described in chapter two (section 2.5.1.1), adding 25 µl of E4 into each well of the microplate.

The compounds used in the discriminating dose bioassays with ***M. persicae*** (section 5.2.1.2.1) were tested for their ability to inhibit esterases using the total esterase assay (alpha-naphthyl acetate as the substrate). PBO was used as the standard synergist. All compounds tested were 10 000 ppm stock solutions (in acetone) and acetone was used as the control. The ability of pyrethrum (10 000 ppm stock solution in acetone) to inhibit total esterase activity was also examined as a way of confirming whether esterases were interacting with pyrethrum and thus playing a role in detoxification of pyrethrum, and therefore contributing to insecticide resistance.

Data for the esterase assay (alpha-naphthyl acetate as a substrate) were processed according to the method described in chapter two (section 2.6), by calculating activity as a percentage of the uninhibited control. The program Grafit (Version 5.0.10) was used to determine inhibition curves for each compound (by fitting a four parameter logistic to the data) and calculating the IC_{50} and Y-range values (as described in chapter three, section 3.2.2.1.1). There were no correlations made between the esterase assay (alpha-naphthyl acetate as the substrate) and the discriminating dose bioassay as only neem oil inhibited esterases with this assay.

5.2.2.1.2 Esterase assay with para-nitrophenyl acetate as a substrate

Total esterase assays using para-nitrophenyl acetate as a substrate were run with purified E4 (clone 794JZ) according to the methods described in chapter two (section 2.5.1.2), by adding 25 µl of E4 into each well of the microplate.

The compounds used in the ***M. persicae*** discriminating dose bioassays were tested for their ability to inhibit esterases using the total esterase assay with para-nitrophenyl acetate as the substrate. All compounds were diluted and controls as for the esterase assay (alpha-naphthyl acetate as a substrate). The ability of pyrethrum (10 000 ppm stock solution in acetone) to inhibit esterases was also examined.

Data for this esterase assay were processed as for the esterase assay above. For ZaB2 extract, propyl gallate and aniseed oil, lines were fitted with two parameter logistic curves with the lower and upper limits of 0% and 100% respectively (as explained in chapter three, section 3.2.2.1.1). The inhibition curves for all remaining compounds were fitted with a four parameter logistic curve.

Correlations were tested for between the percentage mortality in the discriminating dose bioassays for synergists with pyrethrum (***M. persicae*** clone 794JZ) and synergists with imidacloprid (clone 5191A) and esterase inhibition (total esterase assay with para-nitrophenyl acetate as the substrate), using the percentage esterase activity remaining at the highest concentration of the synergist dilution series, the IC_{50} values and the Y-ranges. Correlation analyses were performed as described in chapter two (section 2.6).

5.2.2.1.3 Esterase interference assay

The ability of the synergistic compounds to bind to esterases was examined using the esterase interference assay, using purified E4 (clone 794JZ), according to the methods in chapter two (section 2.5.1.3).

For the esterase interference assay, the same compounds were tested as with the total esterase assays above. All compounds were 10 000 ppm stock solutions (in acetone). Each synergist (2.3 µl) was incubated with purified E4 (230 µl) for 16 h at 4 °C. Purified E4 (230 µl) incubated with acetone (2.3 µl) was used as the positive control (esterase only treatment) because all synergist stock solutions were dissolved in acetone. Pyrethrum was also included in the esterase interference assay to determine whether esterases were binding to pyrethrum and thus possibly playing a role in detoxification of pyrethrum. Pyrethrum (10 000 ppm stock solution in acetone) (2.3 µl) was incubated with purified E4 (230 µl) and the assay run in the same way as for the synergists.

Data for the esterase interference assay were processed according to the methods described in chapter two (section 2.6) and Appendix II. The IC_{50} value for each synergist obtained from Grafit (version 5.0.10) was converted to an index value (termed I), and was calculated as a percentage of esterase and no esterase, where

esterase was equivalent to 100% esterase activity and no esterase was equivalent to 0% activity. The index value (I) equates to the percentage house fly AChE activity remaining after addition of the synergists. The SE values were corrected in the same way to equate to the I values. Upper and lower confidence intervals (95%) were also calculated for each synergist.

Correlations were tested for between the percentage mortality in the discriminating dose bioassays for synergists with pyrethrum and for synergists with imidacloprid for ***M. persicae*** clones 794JZ and 5191A respectively and the esterase interference assay (index values). In addition, a correlation was tested for between the esterase assay (para-nitrophenyl acetate as a substrate) (percentage esterase activity remaining at the highest concentration of the synergist dilution series) and the esterase interference assay (index values) with the various synergists (and pyrethrum) tested for esterase inhibition in each assay. The correlation analyses were performed as described in chapter two (section 2.6).

5.3 Results

5.3.1 Bioassays

5.3.1.1 Full dose response bioassay

The dose-response curves for unsynergised pyrethrum and imidacloprid using ***M. persicae*** clones 794JZ and 5191A were calculated by probit analysis. The LD_{10} values of pyrethrum (clone 794JZ) and imidacloprid (clone 5191A) were 108 ppm and 0.81 ppm respectively (Table 5.1). Discriminating doses chosen were 100 ppm pyrethrum and 1 ppm imidacloprid. For both aphid clones, synergists were applied at 1000 ppm.

Table 5.1. The lethal dose (LD) values with 95% confidence intervals (CI) (calculated by probit analysis) from topical application bioassays for pyrethrum against *Myzus persicae* clone 794JZ, and imidacloprid against clone 5191A respectively (apterous adult females).

LD value	Pyrethrum dose (ppm) (794JZ)	95% CI (794JZ)	Imidacloprid dose (ppm) (5191A)	95% CI (5191A)
LD_{10}	108	35.19 – 206	0.81	0.01 – 2.78
LD_{20}	197	82.73 – 346	1.72	0.05 – 4.67
LD_{30}	304	149 – 517	2.94	0.18 – 7.01
LD_{40}	440	237 – 754	4.65	0.57 – 10.36
LD_{50}	621	356 – 1107	7.15	1.54 – 16.10
LD_{60}	878	516 – 1683	10.99	3.63 – 28.63
LD_{70}	1270	742 – 2722	17.41	7.39 – 65.31
LD_{80}	1956	1099 – 4937	29.81	13.46 – 216
LD_{90}	3563	1827 – 11 703	62.89	25.20 – 1396
LD_{95}	5846	2726 – 24 327	116	39.41 – 6991
LD_{99}	14 798	5640 – 98 339	370	85.66 – 152 720
Number of 794JZ aphids exposed = 209			Number of 5191A aphids exposed = 213	
Slope ± SE (794JZ) = 1.690 ± 0.194			Slope ± SE (5191A) = 1.357 ± 0.365	
Degrees of freedom (df) (794JZ) = 16			Degrees of freedom (df) (5191A) = 16	
χ^2 (794JZ) = 33.762			χ^2 (5191A) = 19.771	

5.3.1.2 Discriminating dose bioassays

5.3.1.2.1 Discriminating dose bioassay with pyrethrum (clone 794JZ)

The discriminating dose bioassay with ***M. persicae*** clone 794JZ showed PBO to be the most effective pyrethrum synergist, with significantly higher mortality when applied with pyrethrum than all the other treatments (88.89% mortality, p=0.05) (Fig. 5.1). After PBO, pyrethrum plus parsley seed oil showed the next highest efficacy as a pyrethrum synergist (67.74% mortality), followed by dill apiole oil, bergamot oil, CaB2 extract, aniseed oil, neem oil, ClB2 extract and oleic acid (65.31%, 58.33%, 51.72%, 51.52%, 50.00%, 48.39% and 43.33% mortality respectively), all of which never differed significantly from one another and all of which had mortalities

significantly higher than unsynergised pyrethrum (18.02%). No other synergist plus pyrethrum treatment differed significantly from unsynergised pyrethrum.

Compounds that showed inherent toxicity towards aphids were bergamot oil, CaB2 extract, aniseed oil, oleic acid, ZaB2 extract and CaB2 frac3 extract, with significantly higher mortalities (29.09%, 25.00%, 32.26%, 24.24%, 35.00% and 31.03% respectively) than the acetone control (3.33% mortality, p=0.05) (Fig. 5.1). When applied with pyrethrum, bergamot oil, CaB2 extract, aniseed oil and oleic acid had significantly higher mortalities than in the unsynergised pyrethrum treatment. However, due to the significant inherent toxicity shown by each of these compounds, this mortality was thus, at least in part, due to toxicity of the compound itself and not due to synergistic effects. Bergamot oil plus pyrethrum, however, had significantly higher mortality than bergamot alone. Some of the mortality for bergamot oil plus pyrethrum could therefore be ascribed to synergistic effects.

No other compound showed inherent toxicity towards aphids at the applied dosage, including PBO, parsley seed oil, dill apiole oil, neem oil and ClB2 extract, all of which showed significant synergism of pyrethrum.

5.3.1.2.2 Discriminating dose bioassay with imidacloprid (clone 5191A)

The discriminating dose bioassay with ***M. persicae*** clone 5191A showed PBO to be the most effective synergist of imidacloprid (90.32% mortality), not differing significantly from imidacloprid plus bergamot and canola oils (82.26% and 73.77% mortality respectively), but significantly higher than all the remaining treatments ($p<0.001$) (Fig. 5.2). Imidacloprid plus oleic acid showed the next highest efficacy as an imidacloprid synergist (66.67% mortality), followed by imidacloprid plus aniseed oil, BcB2 extract, grapefruit oil, neem oil, ZaB2 extract, parsley seed oil, dill apiole oil, ClB1 extract, dill seed oil, CaB2 extract, ClB2 extract and citronella oil (56.67%, 54.84%, 52.46%, 48.39%, 46.67%, 45.16%, 43.66%, 36.67%, 35.00%, 31.03%, 26.67% and 25.00% mortality respectively), all with mortalities significantly higher than unsynergised imidacloprid (13.64% mortality). No other synergist plus imidacloprid treatment differed significantly from unsynergised imidacloprid.

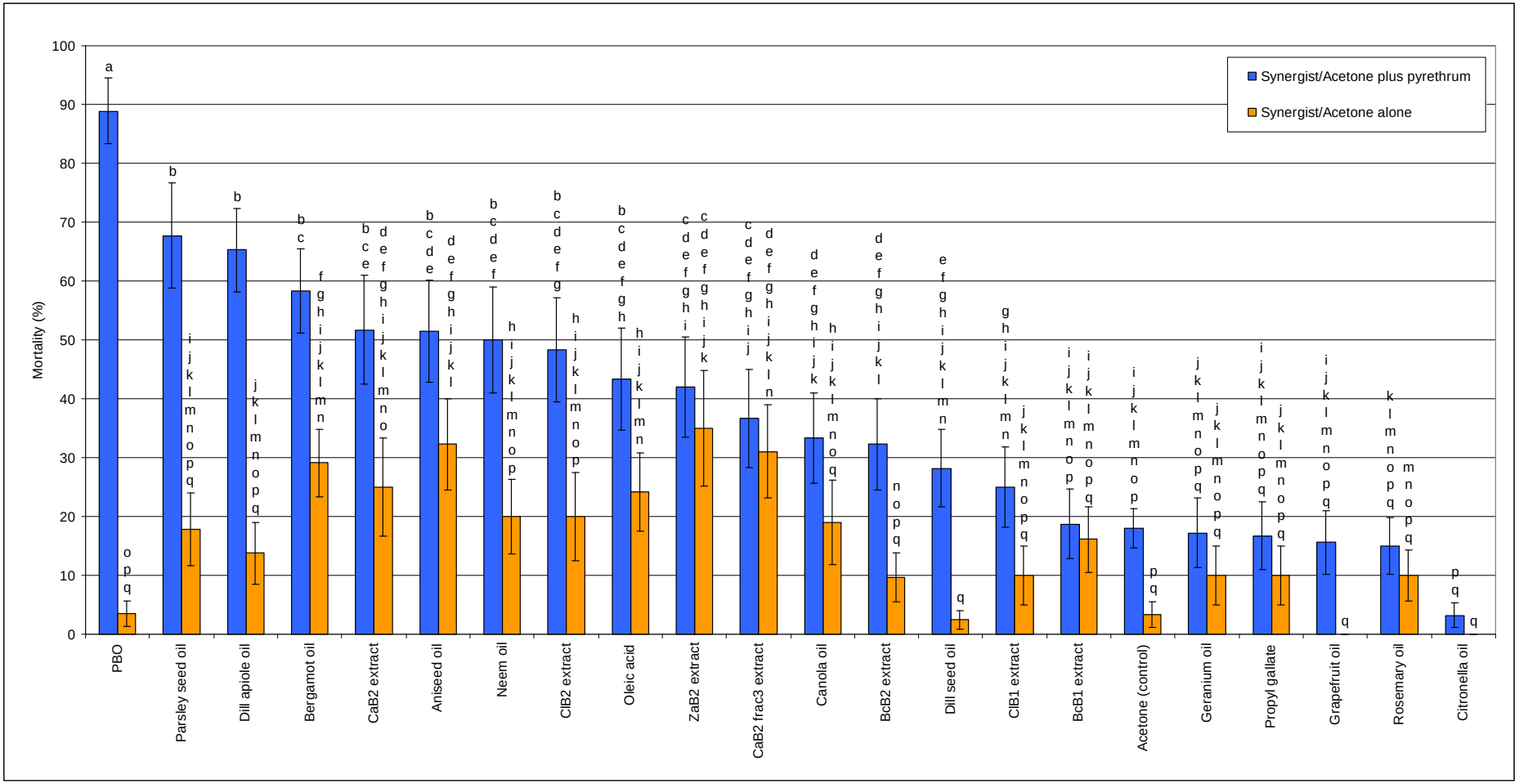


Fig. 5.1. The mean percentage mortality (± SE) of *Myzus persicae* clone 794JZ (apterous adult females) by topical application of 100 ppm pyrethrum:1000 ppm synergist solutions, or with 1000 ppm synergists alone. Acetone was used as the control. (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

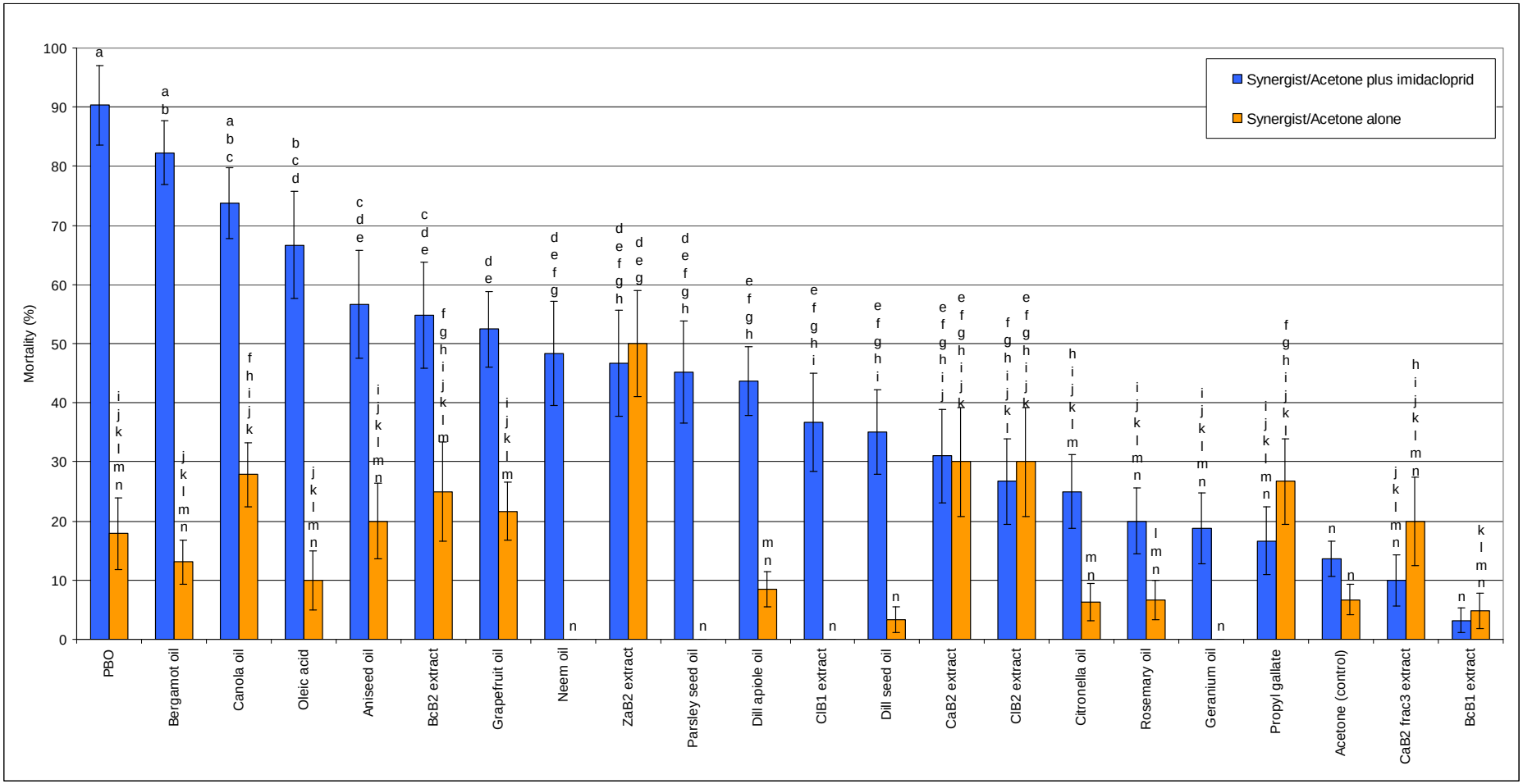


Fig. 5.2. The mean percentage mortality (± SE) of *Myzus persicae* clone 5191A (apterous adult females) by topical application of 1 ppm imidacloprid:1000 ppm synergist solutions, or with 1000 ppm synergists alone. Acetone was used as the control. (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

Compounds that showed inherent toxicity towards aphids at the applied dosage were canola oil, BcB2 extract, grapefruit oil, ZaB2 extract, CaB2 extract, ClB2 extract and propyl gallate, with significantly higher mortalities (27.87%, 25.00%, 21.67%, 50.00%, 30.00%, 30.00% and 26.67% respectively) than the acetone control (6.67% mortality, $p<0.001$) (Fig. 5.2). When applied with imidacloprid, canola oil, BcB2 extract, grapefruit oil, ZaB2 extract, CaB2 extract and ClB2 extract had significantly higher mortalities than the unsynergised imidacloprid treatment. This mortality was therefore, at least in part, due to toxicity of the compound itself and not due to synergistic effects. Despite having significant inherent toxicity, however, the mortalities of imidacloprid plus canola oil, BcB2 extract and grapefruit oil were significantly higher than the mortalities found with each of these compounds on their own. Some of the mortality for imidacloprid plus canola oil, BcB2 extract and grapefruit oil could therefore be ascribed to synergistic effects.

No other compound showed inherent toxicity towards aphids at the applied dosage, including PBO, bergamot oil, oleic acid, aniseed oil, neem oil, parsley seed oil, dill apiole oil, ClB1 extract, dill seed oil and citronella oil, all of which showed significant synergism of imidacloprid.

A significant positive correlation was found between the percentage mortality in the discriminating dose bioassay for ***M. persicae*** clone 794JZ with synergists plus pyrethrum and the percentage mortality in the discriminating dose bioassay for clone 5191A with synergists plus imidacloprid (correlation coefficient = 0.610, $n=21$, $p=0.003$) (Fig. 5.3). Similar compounds were thus effective as synergists for both imidacloprid and pyrethrum against aphid clones 794JZ and 5191A respectively, with PBO being the most effective synergist of both pyrethrum and imidacloprid.

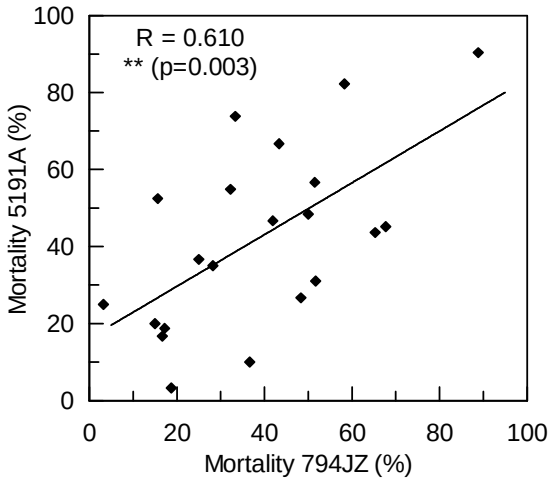


Fig. 5.3. The correlation between the percentage mortality in the discriminating dose bioassay for *M. persicae* clone 794JZ with the synergists with pyrethrum and the percentage mortality in the discriminating dose bioassay for *M. persicae* clone 5191A with the synergists with imidacloprid. Each point indicates a different synergist (n=21), lines were fitted by linear regression, R is the correlation coefficient and ** indicates a significant correlation (p<0.01) (two-tailed).

5.3.2 Biochemical assays

5.3.2.1 Esterase assays

5.3.2.1.1 Esterase assay with alpha-naphthyl acetate as a substrate

Neem oil was the only compound that showed inhibition of esterases using alpha-naphthyl acetate as an esterase substrate (Fig. 5.4a). The IC_{50} (± SE) for neem oil was 88.26 ppm (± 4.16) and the Y-range (± SE) was 90.27% (± 4.69). All the remaining compounds showed activity that was higher than in the uninhibited control (illustrated by PBO and parsley seed oil as examples in Fig. 5.4b), which may be due to interference with the assay, where the compounds possibly bound to the esterase in such a way so as to facilitate a speeding up of the substrate (Dr. Graham D. Moores, ***Pers. comm.***). Khot ***et al***. (2008) also found that the total esterase assay with ***M. persicae*** using alpha-naphthyl acetate as a substrate failed to identify the esterase-inhibiting capabilities of synergists.

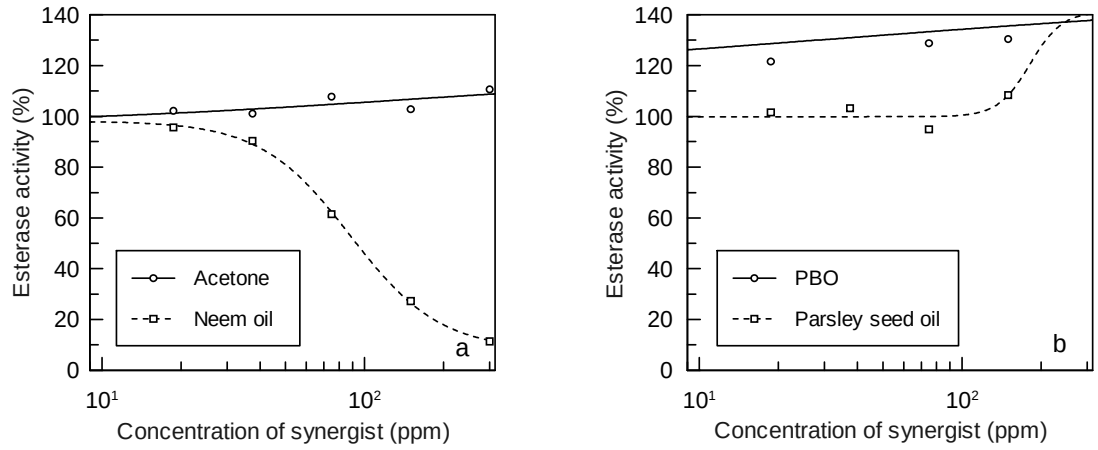


Fig. 5.4a-b. Inhibition of *Myzus persicae* (clone 794JZ) total esterase activity (alpha-naphthyl acetate as the substrate) by acetone (control), neem oil, PBO and parsley seed oil. Lines were fitted with a four parameter logistic curve.

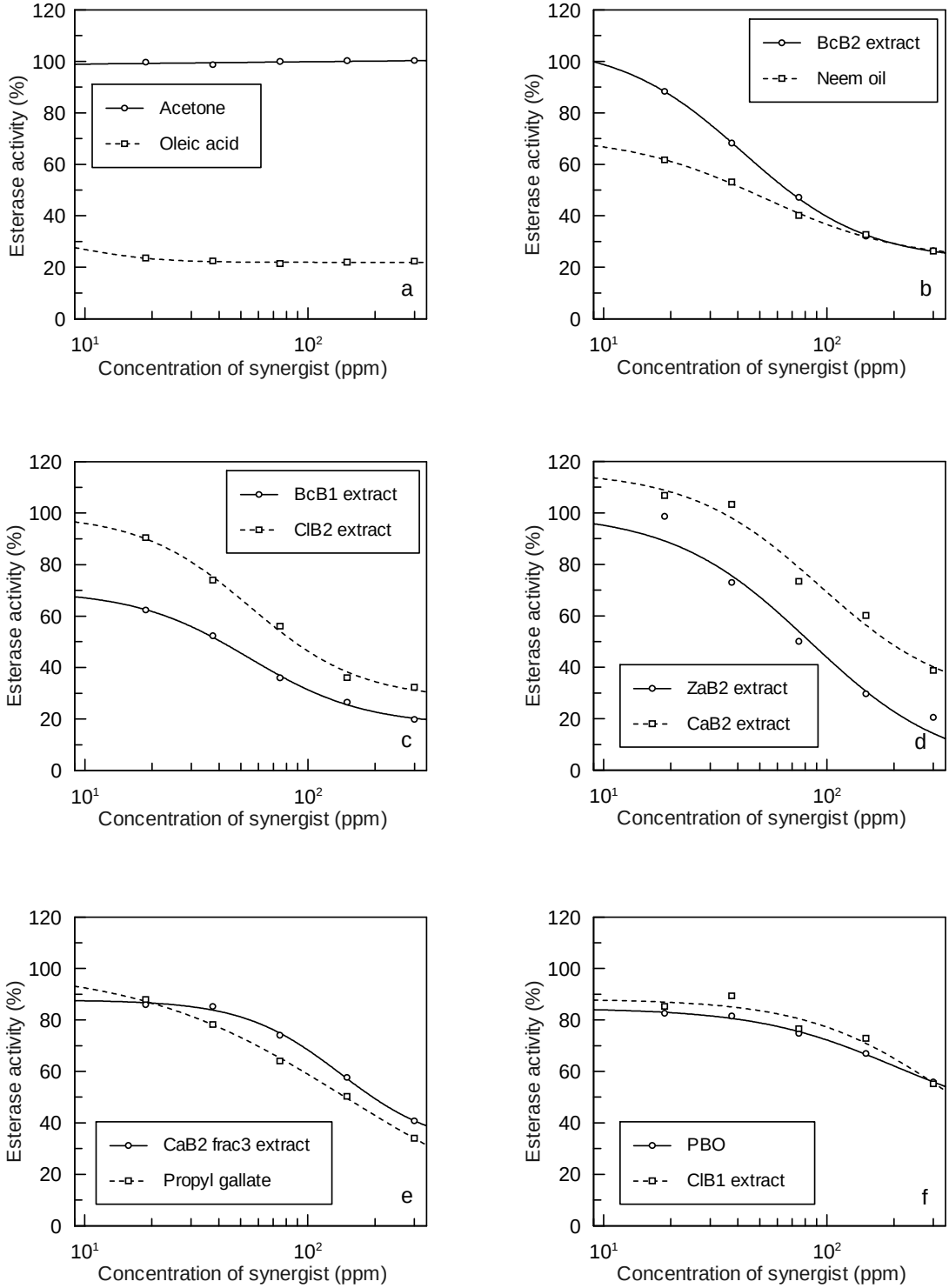
5.3.2.1.2 Esterase assay with para-nitrophenyl acetate as a substrate

Esterase assays using para-nitrophenyl acetate as a substrate revealed several compounds that showed inhibition of ***M. persicae*** E4 (Table 5.2, Fig. 5.5a-k). Oleic acid was the most potent esterase inhibitor, inhibiting the esterases to approximately 22% activity across the entire dilution series (Fig. 5.5a). Oleic acid had the smallest IC_{50} value of all the compounds tested (with 10.00 ppm oleic acid required to inhibit 50% of the esterases). BcB2 extract, neem oil, BcB1, ClB2, ZaB2, CaB2, CaB2 frac3 extracts and propyl gallate all also showed good inhibition of esterases, with IC_{50} values smaller than PBO. PBO had the smallest Y-range, indicating that PBO inhibited the smallest range of the total esterases, however, 45% was still inhibited (Table 5.2). CaB2 and BcB2 extracts showed the widest range of inhibition of total esterases, with the largest Y-ranges (using the four parameter logistic fit).

Compounds showing little to no inhibition of esterases were citronella, geranium, parsley seed, bergamot, rosemary, canola, dill seed, dill apiole and grapefruit oils (Fig. 5.5g-k), of which parsley seed, bergamot and rosemary oils showed the least inhibition (Fig. 5.5h & i). Acetone showed no inhibition of esterases (Fig. 5.5a).

Table 5.2. Inhibition of *Myzus persicae* (clone 794JZ) total esterase activity (para-nitrophenyl acetate as the substrate) by potential pyrethrum synergists and PBO. The IC_{50} and Y-range values (± SE) are tabulated for each synergist. (Relates to Fig. 5.5a-k; IC_{50} and Y-range values could not be accurately calculated for compounds showing very little or no inhibition of esterases; Compounds ordered from lowest to highest IC_{50}).

Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
Oleic acid	Mono-unsaturated omega-9 fatty acid	10.00 (± 13.97)	77.69 (± 31.49)
BcB2 extract	Unknown	42.08 (± 3.52)	84.25 (± 6.89)
Neem oil	Tetranortriterpenoid / liminoid	52.45 (± 12.44)	48.24 (± 13.06)
BcB1 extract	Unknown	54.59 (± 8.04)	52.38 (± 8.95)
ClB2 extract	Cyclic terpene	54.99 (± 11.86)	71.78 (± 17.59)
ZaB2 extract	Sesquiterpenoid	83.87 (± 10.66)	100
CaB2 extract	Phenylpropene	93.22 (± 46.94)	89.46 (± 56.66)
CaB2 frac3 extract	Phenylpropene	140 (± 26.95)	57.35 (± 11.30)
Propyl gallate	Ester	147 (± 3.34)	100
PBO	Methylenedioxyphenol ring	203 (± 153)	45.37 (± 26.59)
ClB1 extract	Cyclic terpene	324 (± 402)	68.91 (± 67.24)
Aniseed oil	Unsaturated ether / phenylpropene derivative	374 (± 30.98)	100


Esterase activity (%)
Concentration of synergist (ppm)
Acetone
Oleic acid
a
BcB2 extract
Neem oil
b
BcB1 extract
ClB2 extract
c
ZaB2 extract
CaB2 extract
d
CaB2 frac3 extract
Propyl gallate
e
PBO
ClB1 extract
f

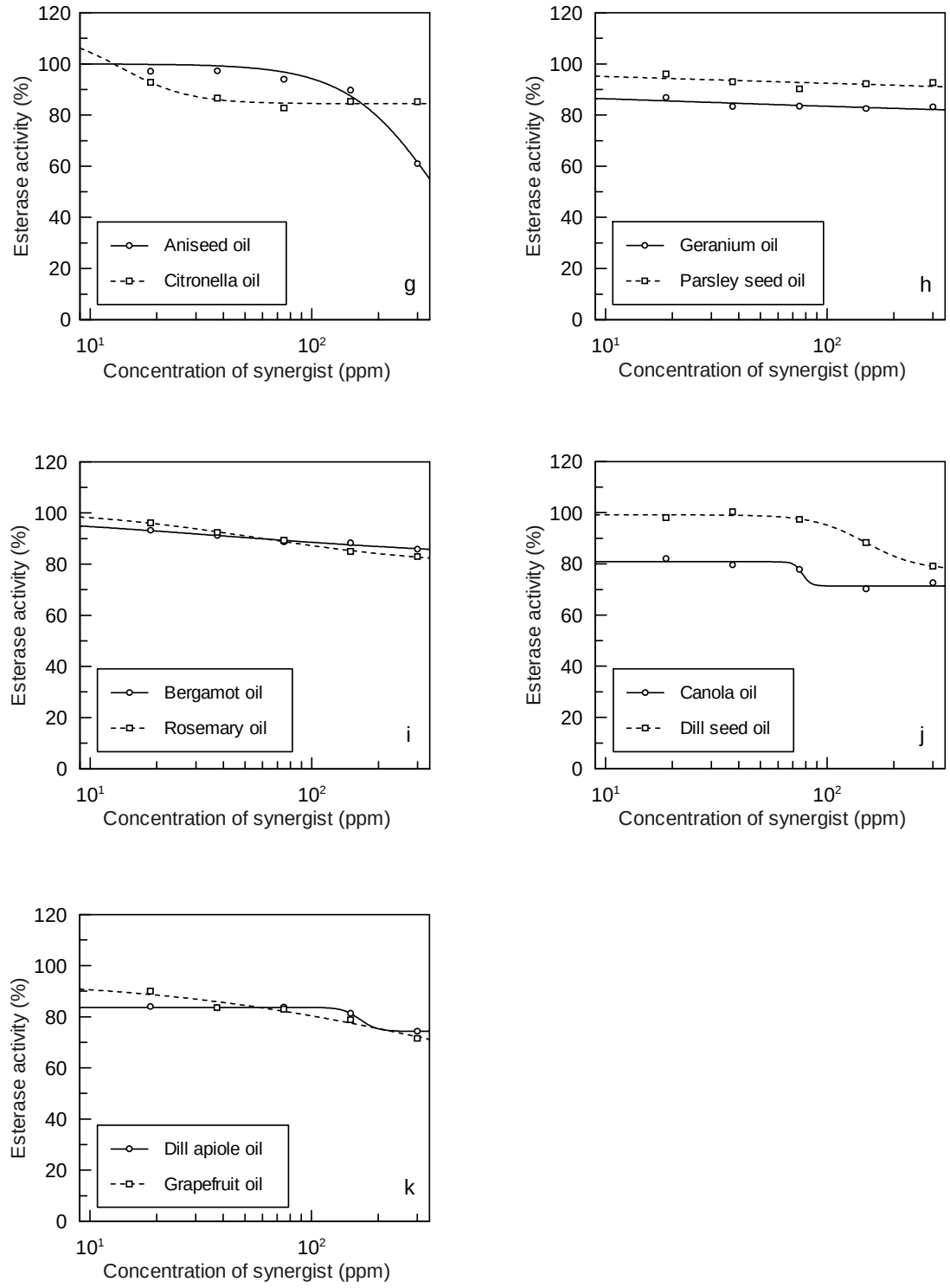


Fig. 5.5a-k. Inhibition of *Myzus persicae* (clone 794JZ) total esterase activity (para-nitrophenyl acetate as the substrate) by various potential pyrethrum synergists, PBO and acetone (control). For ZaB2 extract, propyl gallate and aniseed oil, lines were fitted with two parameter logistic curves (lower and upper limits of 0% and 100% respectively). For all remaining compounds, lines were fitted with a four parameter logistic curve. (Relates to Table 5.2; Synergists ordered from lowest to highest IC_{50} until aniseed oil (showing inhibition), after which, compounds in no particular order).

None of the correlations tested for between synergism of pyrethrum (discriminating dose bioassay against ***M. persicae*** clone 794JZ) and esterase inhibition (para-nitrophenyl acetate as a substrate) (percentage esterase activity remaining at the highest concentration of the synergist dilution series, the IC_{50} values and the Y-ranges respectively) were significant (Fig. 5.6a-c). In the ***M. persicae*** (clone 794JZ) discriminating dose bioassay, PBO showed the highest efficacy as a pyrethrum synergist, followed by parsley seed oil, dill apiole oil, bergamot oil, neem oil and ClB2 extract (Fig. 5.1, section 5.3.1.2.1). Considering the esterase-inhibiting capabilities of the compounds showing synergism, neem oil, ClB2 extract and PBO gave good inhibition of esterases (Fig. 5.5b, c & f), whereas parsley seed oil, bergamot oil and dill apiole oil showed little inhibition of esterases (Fig. 5.5h, i & k). Oleic acid, which showed the most inhibition of esterases (Fig. 5.5a), also showed significant synergism of pyrethrum, however, oleic acid displayed significant toxicity towards ***M. persicae*** as well. Plant extracts BcB2 and BcB1, which showed good inhibition of esterases (Fig. 5.5b & c) did not show significant synergism in the discriminating dose bioassays.

Correlations tested for between synergism of imidacloprid (discriminating dose bioassay against ***M. persicae*** clone 5191A) and esterase inhibition (para-nitrophenyl acetate as a substrate) were not significant (Fig. 5.6d-f). In the discriminating dose bioassay, PBO showed the most efficacy as an imidacloprid synergist, followed by bergamot oil, canola oil, oleic acid, aniseed oil, BcB2 extract, grapefruit oil, neem oil, parsley seed oil, dill apiole oil, ClB1 extract, dill seed oil and citronella oil (Fig. 5.2, section 5.3.1.2.2). The esterase assays were run using E4 from ***M. persicae*** clone 794JZ, but the compounds that showed both significant synergism of imidacloprid (clone 5191A) and good inhibition of esterases (clone 794JZ E4) were oleic acid, BcB2 extract, neem oil and PBO (Fig. 5.5a, b & f). Compounds showing significant synergism with imidacloprid (clone 5191A) that gave a medium amount of esterase inhibition (clone 794JZ E4) were ClB1 extract and aniseed oil (Fig. 5.5f & g). Compounds showing significant synergism of imidacloprid (clone 5191A) but that gave little inhibition of esterases (clone 794JZ E4) were citronella oil, parsley seed oil, bergamot oil, canola oil, dill seed oil, dill apiole oil and grapefruit oil (Fig. 5.5g-k). BcB1 extract showed good inhibition of esterases (Fig. 5.5c) but did not show any synergism, causing the lowest observed mortality with imidacloprid, not significantly different from unsynergised imidacloprid (acetone plus imidacloprid).

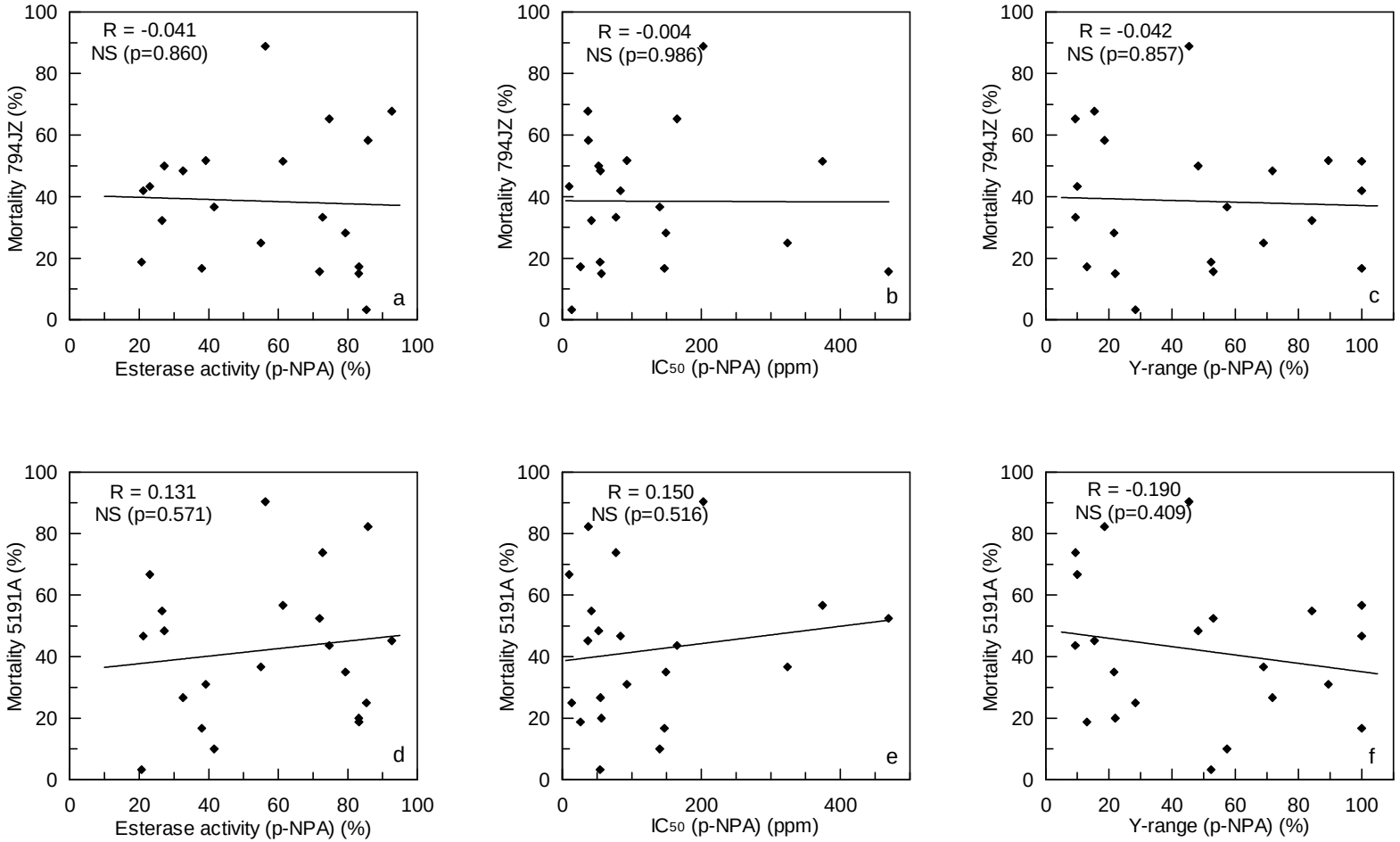


Fig. 5.6a-f. The correlations between the esterase assay (para-nitrophenyl acetate as a substrate (p-NPA)) and the discriminating dose bioassay mortality data with various potential synergists for *Myzus persicae* clones 794JZ and 5191A. Each point indicates a different synergist (n=21), lines were fitted by linear regression, R is the correlation coefficient and NS = not significant (two-tailed).

Table 5.3. The index value (I), standard error (SE) and 95% confidence intervals (CI) for *Myzus persicae* (clone 794JZ) E4 with various potential pyrethrum synergists and PBO with the esterase interference assay*. (Synergists ordered from lowest to highest Index value).

Compound / esterase / no esterase	Type of chemical	Index value (I) %	SE (%)	95% CI for I
No esterase	-	0	1.24	-2.58 – 2.58
BcB1 extract	Unknown	0.51	1.48	-2.57 – 3.58
Oleic acid	Mono-unsaturated omega-9 fatty acid	2.38	1.38	-0.49 – 5.26
BcB2 extract	Unknown	3.10	1.22	0.57 – 5.63
Neem oil	Tetranortriterpenoid / liminoid	3.55	2.00	-0.62 – 7.71
ClB2 extract	Cyclic terpene	4.77	1.51	1.63 – 7.92
PBO	Methylenedioxyphenol ring	7.98	1.98	3.85 – 12.11
CaB2 frac3 extract	Phenylpropene	10.16	1.52	6.99 – 13.32
ClB1 extract	Cyclic terpene	11.60	3.12	5.11 – 18.09
CaB2 extract	Phenylpropene	11.70	2.71	6.07 – 17.33
ZaB2 extract	Sesquiterpenoid	11.76	1.49	8.65 – 14.87
Canola oil	Mono-unsaturated omega-9 fatty acid; Unsaturated omega-6 fatty acid	19.94	2.22	15.31 – 24.56
Dill apiole oil	Methylenedioxyphenol ring / phenylpropanoid	30.58	2.73	24.91 – 36.24
Citronella oil	Monoterpenoid / alcohol; Cyclic terpene	32.38	3.07	25.99 – 38.77
Propyl gallate	Ester	36.26	3.16	29.69 – 42.83
Parsley seed oil	Methylenedioxyphenol ring; Bicyclic monoterpene; Methylenedioxyphenol ring / phenylpropanoid	38.07	3.67	30.43 – 45.70
Aniseed oil	Unsaturated ether / phenylpropene derivative	40.04	3.45	32.86 – 47.22
Geranium oil	Monoterpenoid / alcohol	45.35	3.93	37.17 – 53.53
Grapefruit oil	Cyclic terpene	61.83	3.63	54.29 – 69.37
Bergamot oil	Cyclic terpene; Acetate ester	63.39	3.43	56.26 – 70.52
Dill seed oil	Terpenoid; Cyclic terpene	78.69	4.58	69.16 – 88.22
Rosemary oil	Monoterpenoid / ether; Bicyclic monoterpene; Terpenoid	99.18	5.47	87.82 – 111
Esterase	-	100	6.35	86.79 – 113

* I is the IC_{50} value calculated from Grafit for the house fly acetylcholinesterase (AChE) activity remaining, shown as a percentage of E4 (esterase) and No E4 (no esterase), 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 I.

5.3.2.1.3 Esterase interference assay

The ability of the potential synergists and PBO were compared regarding their ability to bind to esterases using the esterase interference assay. BcB1 extract showed the highest "blockade" of esterases, followed by oleic acid, BcB2 extract, neem oil, ClB2 extract and PBO (Table 5.3). Dill seed, bergamot and grapefruit oils gave the least inhibition (or "blockade") of esterases whereas rosemary oil showed no inhibition of esterases.

The correlations tested for between inhibition of esterases with the esterase interference assay (index values) and mortality in the discriminating dose bioassays for synergists with pyrethrum (***M. persicae*** clone 794JZ) and synergists with imidacloprid (***M. persicae*** clone 5191A) were not significant (Fig. 5.7a-b).

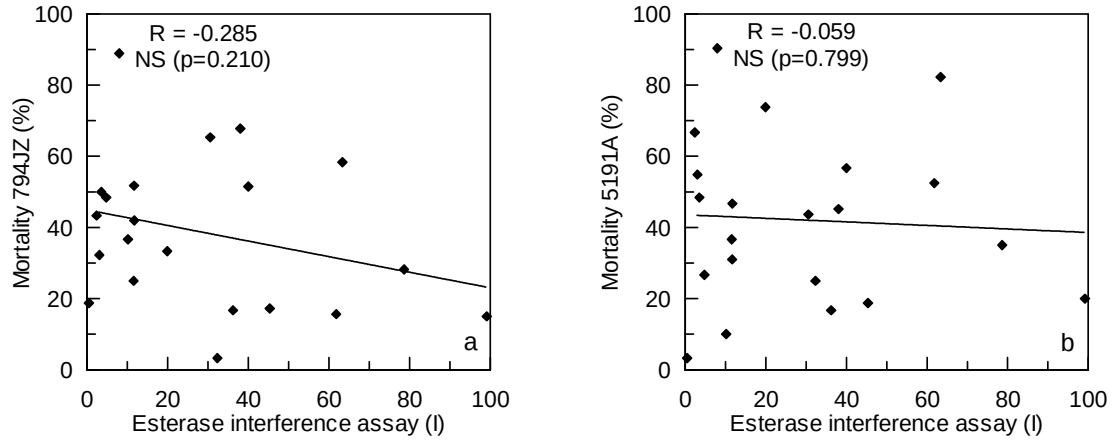


Fig. 5.7a-b. The correlations between the esterase interference assay (index values (I)) and the discriminating dose bioassay mortality data with various potential synergists for *Myzus persicae* clones 794JZ and 5191A. Each point indicates a different synergist (n=21), lines were fitted by linear regression, R is the correlation coefficient and NS = not significant (two-tailed).

A significant positive correlation was found between the esterase assay (para-nitrophenyl acetate as a substrate) (percentage esterase activity remaining at the highest concentration of the synergist dilution series) and the esterase interference assay (index values) with the various synergists and pyrethrum tested for esterase inhibition (correlation coefficient = 0.749, n=22, p=0.00006) (Fig. 5.8). The most

effective esterase inhibitors in the esterase interference assay (oleic acid, BcB1 extract, BcB2 extract, neem oil and ClB2 extract) were also the most potent inhibitors in the total esterase assay (para-nitrophenyl acetate as a substrate). Rosemary oil and dill seed oil were amongst the compounds showing little esterase inhibition in both assays.

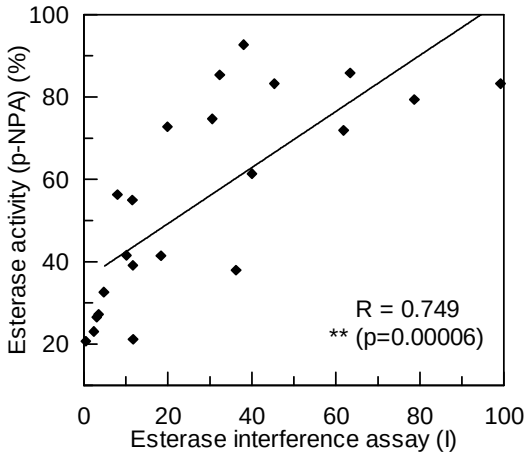


Fig. 5.8. The correlation between the esterase assay (para-nitrophenyl acetate as a substrate (p-NPA)) (percentage esterase activity remaining at the highest concentration of the synergist dilution series) and the esterase interference assay (index values (I)) with the synergists tested for esterase inhibition using *Myzus persicae* E4 (clone 794JZ). Each point indicates a different synergist or pyrethrum (n=22), lines were fitted by linear regression, R is the correlation coefficient and ** indicates a significant correlation (p<0.01) (two-tailed).

5.3.2.2 Esterase inhibition by pyrethrum

Pyrethrum caused a similar interference to the total esterase assay (alpha-naphthyl acetate as a substrate) as was found with the potential synergists (section 5.3.2.1.1) (Fig. 5.9). alpha-Naphthyl acetate may thus not be a suitable substrate for detecting inhibition of esterases in ***M. persicae***.

The total esterase assay was repeated using para-nitrophenyl acetate as an esterase substrate and pyrethrum gave good inhibition of esterases (Fig. 5.9). Pyrethrum had a small IC_{50} (± SE) (55.41 ppm (± 10.30)) and a Y-range (± SE) of 44.70% (± 10.12).

The good inhibition of esterases by pyrethrum indicates that pyrethrum is binding to the esterases and confirms the importance of esterases as a resistance mechanism in ***Myzus persicae*** clone 794JZ.

Using the esterase interference assay, pyrethrum gave good inhibition (or "blockade") of esterases, with an index value (± SE) of 18.34% (± 3.30) (95% confidence intervals of 11.48% – 25.21%). This is in agreement with the total esterase assay (para-nitrophenyl acetate as a substrate), confirming the involvement of esterases in pyrethroid resistance in ***M. persicae*** clone 794JZ.

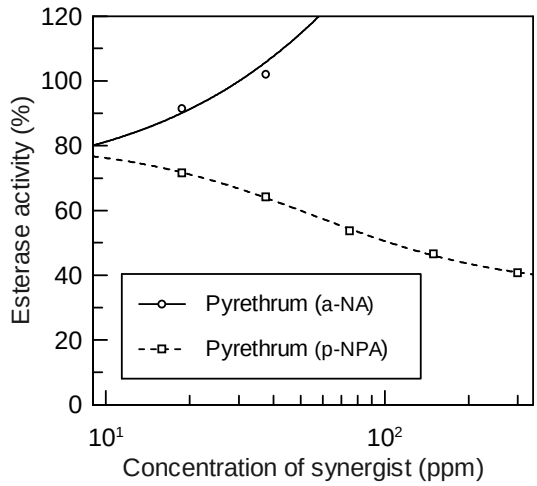


Fig. 5.9. Inhibition of *Myzus persicae* (clone 794JZ) total esterase activity (alpha-naphthyl acetate (a-NA) and para-nitrophenyl acetate (p-NPA) as substrates) by pyrethrum. Lines were fitted with a four parameter logistic curve.

5.4 Discussion and Conclusions

Discriminating dose bioassays were done by topical application with pyrethrum, using ***M. persicae*** clone 794JZ, and with imidacloprid, using clone 5191A, to examine the potential of a number of natural compounds as synergists. Using ***M. persicae*** clone 794JZ, a clone with the highest (R_3) levels of the resistance-associated carboxylesterase, E4, PBO was found to be the most effective pyrethrum synergist. The potentially organically-certifiable compounds showing efficacy as pyrethrum

synergists were parsley seed oil, dill apiole oil, bergamot oil, neem oil and ClB2 extract.

Parsley seed oil contains the compounds myristicin and apiole, both of which have the methylenedioxyphenol (MDP) ring also present in dill apiole oil and PBO. The MDP ring has long been considered important in synergism of pyrethrum. The synergistic activity of sesame oil was attributed to the compounds, sesamin and sesamolin, each containing the MDP ring (Beroza 1954, Haller ***et al***. 1942), leading to the subsequent development of many synergists with the MDP ring, such as piperonyl cyclohexenone and PBO (Wachs 1947). The cyclic terpene, limonene, is present in high quantities in both bergamot oil and ClB2 extract, whereas bergamot oil also contains high quantities of the ester, linalyl acetate. Since both ClB2 and bergamot oil showed significant synergism, limonene might play a role in synergism of pyrethrum in ***M. persicae***. Linalyl acetate could, however, also be contributing to synergism of pyrethrum by bergamot oil. The ester could act as a substrate for the esterase, and could also potentially act as an inhibitor, depending on how easily the ester is hydrolysed. (If the process is very slow, thus tying up the active site of the esterase for a long time, the ester could be considered to be an inhibitor). The natural plant oils and extracts used in this study, however, were not pure compounds, and synergism could thus be due to any of the constituents present in the oils or extracts even in small amounts. In sesame oil, Beroza (1954) found sesamolin to be about five times more active as a pyrethrum synergist than sesamin, and even though sesamolin was present in smaller amounts, it accounted for most of the synergistic activity of sesame oil. The active ingredient of neem oil, azadirachtin, is a tetranortriterpenoid, and has been shown to possess insecticidal activity, for example, against red flour beetle ***Tribolium castaneum*** (Herbst) (Coleoptera: Tenebrionidae) (Adarkwah ***et al***. 2010). Neem has also shown synergistic activity when mixed with other botanical oils, for example, with karanj oil, showing synergistic effects against red spider mites ***Tetranychus*** spp. and the chrysanthemum aphid ***Macrosiphoniella sanborni*** (Gillette) (Hemiptera: Aphididae) (Kumar ***et al***. 2007), or when mixed with other insecticides. Neem, for example, has shown pronounced synergism of fenpropathrin against the cotton aphid ***Aphis gossypii*** Glover (Hemiptera: Aphididae) (Ghoneim 2002), and against the lesser grain borer ***Rhyzopertha dominica*** (F.) (Coleoptera: Bostrichidae) when applied with malathion (El-Lakwah 1997).

Synergism of imidacloprid was examined using ***M. persicae*** clone 5191A, a clone with monooxygenase-based resistance (Philippou ***et al***. 2010) due to the amplification of a single cytochrome P450 gene (Puinean ***et al***. 2010). PBO was found to be the most effective imidacloprid synergist. Potentially organically-certifiable compounds that showed significant synergism of imidacloprid were bergamot oil, canola oil, oleic acid, aniseed oil, BcB2 extract, grapefruit oil, neem oil, parsley seed oil, dill apiole oil, ClB1 extract, dill seed oil and citronella oil.

PBO, parsley seed oil and dill apiole oil all contain the MDP ring, which is known to contribute greatly to synergistic activity. Canola oil contains oleic acid, a mono-unsaturated omega-9 fatty acid, as well as linoleic acid, an unsaturated omega-6 fatty acid. The presence of at least oleic acid could therefore possibly contribute to imidacloprid synergism in ***M. persicae*** clone 5191A, since pure oleic acid also showed synergism of imidacloprid. Aniseed oil contains ***trans***-anethole, an unsaturated ether. Bergamot oil, grapefruit oil, ClB1 extract, dill seed oil and citronella oil all contain limonene. Carvone (a terpenoid) is also present in dill seed oil, whereas bergamot also contains linalyl acetate. Citronella and geranium oils contain geraniol (a monoterpenoid) in similar quantities, however, geranium oil did not show any synergism of imidacloprid. The synergistic effects of bergamot oil, grapefruit oil, ClB1 extract, dill seed oil and citronella oil against imidacloprid in ***M. persicae*** could thus potentially be due to the limonene common to all of these plant oils and extracts. The plant oils and extracts, however, were not all pure and any of the constituents could be adding to the observed synergism.

A significant positive correlation was found between pyrethrum synergism in ***M. persicae*** clone 794JZ and imidacloprid synergism in clone 5191A. Similar compounds were therefore found to be effective as synergists with the two insecticides (pyrethrum and imidacloprid) with each clone of aphid, and thus showing effective synergism in both esterase and monooxygenase-based insecticide resistance. PBO was the most effective synergist of both pyrethrum and imidacloprid. PBO is a known inhibitor of both esterases (Gunning ***et al***. 1998, Young ***et al.*** 2005, 2006) and monooxygenases (Wilkinson 1976) and would therefore be an effective synergist in both cases. The natural, potentially organically-certifiable compounds showing significant synergism in both clones for both insecticides were parsley seed oil, dill apiole oil, bergamot oil and neem oil. PBO, dill apiole oil and parsley seed oil all contain the MDP ring, confirming the importance of the MDP ring in synergism of

pyrethrum, but also showing their efficacy as synergists of imidacloprid. Compounds without the MDP ring, however, can also make effective synergists, with bergamot oil and neem oil showing significant synergism. Compounds that showed no synergism with both insecticides were rosemary oil, geranium oil, propyl gallate, CaB2 frac3 extract and BcB1 extract.

Esterase assays were undertaken with ***M. persicae*** clone 794JZ to determine the esterase-inhibiting capabilities of the synergists used in the discriminating dose bioassays. Total esterase assays were performed using alpha-naphthyl acetate as a substrate, however, neem oil was the only compound to show inhibition of esterases with this assay. In ***M. persicae***, Khot ***et al***. (2008) also found that the total esterase assay with alpha-naphthyl acetate as a substrate was not able to identify synergists that inhibited esterases. The total esterase assay was therefore undertaken using para-nitrophenyl acetate as an esterase substrate. The highest inhibition of esterases was found with oleic acid (fatty acid), BcB2 extract, neem oil (azadirachtin (tetranortriterpenoid)), BcB1 extract and ClB2 extract (limonene (terpene)), whereas parsley seed oil (MDP ring), bergamot oil (limonene and linalyl acetate (ester)) and rosemary oil (eucalyptol (monoterpenoid), alpha-pinene (monoterpene) and camphor (terpenoid)) gave the poorest inhibition. No noticeable trends could be observed regarding inhibition of esterases and the chemical classes of compounds found in the plant oils and extracts. However, since not all the plant oils and extracts were pure, it might be possible that compounds present in smaller quantities may be more potent esterase inhibitors and thus play a role in esterase inhibition.

An esterase interference assay, developed by Khot ***et al***. (2008) as a means to indirectly measure the binding of potential synergists to esterases was also undertaken. A significant positive correlation was found between inhibition of esterases using the total esterase assay (para-nitrophenyl acetate as a substrate) and the index values obtained in the esterase interference assay. Synergists were thus showing similar inhibition, or lack of inhibition, with both esterase assays. Compounds that showed good inhibition of esterases in both assays included PBO, BcB1 extract, oleic acid, BcB2 extract, neem oil and ClB2 extract. These results suggest that the synergists are binding to the esterase in a position, either at the active site itself, or at an allosteric binding site, in such a way so as to prevent the active site of the esterase from hydrolysing para-nitrophenyl acetate, and similarly, are also binding in a position that prevents binding of the esterase to azamethiphos.

When compounds bind at an allosteric binding site, they may either cause a conformational change to the enzyme resulting in a lower affinity for the substrate (allosteric inhibition), or the compound may block the active site and thus prevent the substrate from entering the active site and binding to the enzyme (Garrett & Grisham 2005). Compounds showing little inhibition in both assays included rosemary oil, dill seed oil, bergamot oil and grapefruit oil.

Correlations were tested for between the percentage mortality in the discriminating dose bioassays for synergists with pyrethrum and imidacloprid (for clones 794JZ and 5191A respectively) and esterase inhibition found in both the total esterase assay (para-nitrophenyl acetate as a substrate) and the esterase interference assay. No significant correlations were found. These results could be due to the assays possibly not being able to properly quantify inhibition by the synergists of the esterases directly involved in resistance. However, differences in synergistic efficacy ***in vivo*** could be influenced by a number of factors, including penetration of the synergist through the cuticle, speed of distribution and metabolism of the compounds in the insect body and their affinity for the target site (Casida 1970, Yamamoto 1973), which could explain the lack of correlation between synergism observed ***in vivo*** and the ***in vitro*** inhibition of esterases.

The involvement of esterases as a pyrethrum resistance mechanism in ***M. persicae*** clone 794JZ was examined by studying the esterase inhibiting capacity of pyrethrum, using the total esterase assay (alpha-naphthyl acetate and para-nitrophenyl acetate as substrates) and the esterase interference assay. Alpha-naphthyl acetate seemed to not be a useful substrate for detecting inhibition of esterases in ***M. persicae***, as interference with the assay was detected, which was also found when examining inhibition by the potential synergists with this assay. Pyrethrum, however, showed good inhibition of esterases in the total esterase assay (para-nitrophenyl acetate as a substrate) and in the esterase interference assay. This indicates that pyrethrum is binding to the esterases and confirms the importance of esterases as a resistance mechanism in ***Myzus persicae*** clone 794JZ. As with the synergists, pyrethrum may be binding to the esterase in such a manner so as to prevent the active site of the esterase from hydrolysing para-nitrophenyl acetate, and also preventing binding of the esterase to azamethiphos. PBO, the most effective pyrethrum synergist found in this study, also showed good inhibition of esterases in both esterase assays,

indicating that the inhibition of esterases by PBO does likely play an important role in synergism in ***Myzus persicae*** clone 794JZ.

In conclusion, a significant correlation was found between synergism with pyrethrum and synergism with imidacloprid in ***M. persicae*** clones 794JZ and 5191A. PBO exhibited the highest efficacy as a synergist of both pyrethrum and imidacloprid. The most effective natural compounds showing efficacy as synergists in both clones of ***M. persicae***, 794JZ and 5191A, were parsley seed oil, dill apiole oil, bergamot oil and neem oil. The importance of the MDP ring in synergism of pyrethrum was confirmed, with PBO, dill apiole and parsley seed oil all containing the MDP ring, and this also seemed to play a role in synergism of imidacloprid. Synergism did not correlate significantly with inhibition found in either the esterase assay (para-nitrophenyl acetate as a substrate) or the esterase interference assay, and inhibition was not effectively detected with the esterase assay (alpha-naphthyl acetate as a substrate). These esterase assays do therefore not seem to prove useful for predicting potential pyrethrum synergists ***in vitro***.

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6 CHAPTER SIX: THE EVALUATION OF VARIOUS NATURAL COMPOUNDS AS POTENTIAL PYRETHRUM SYNERGISTS AGAINST POLLEN BEETLES *MELIGETHES AENEUS* AND ENZYME INHIBITION STUDIES IN DRIED FRUIT BEETLES *CARPOPHILUS* SPP. AND *M. AENEUS*

Abstract

This chapter explores the potential of various natural plant oils and extracts as organically-certifiable pyrethrum synergists against the pollen beetle ***Meligethes aeneus***. ***M. aeneus*** discriminating dose bioassays using coated vials showed PBO, dill apiole oil, parsley seed oil and CaB2 frac3 extract to significantly synergise pyrethrum when synergists were applied at 100 ppm. When the synergist concentration was lowered to 10 ppm, only PBO synergised pyrethrum significantly. PBO, dill apiole oil and parsley seed oil contain the methylenedioxyphenol (MDP) ring considered important for pyrethrum synergism, whereas CaB2 frac3 extract contains elemicin, a phenylpropene compound similar in structure to PBO.

Enzyme inhibition assays were used to assess the ability of the plant oils and extracts to inhibit the insecticide resistance associated enzymes (esterases, glutathione-S-transferases (GSTs) and monooxygenases). With the exception of BcB2, the most potent esterase inhibitors varied between beetle species, with BcB2, CaB2 frac3 and BcB1 extracts most potent for ***Carpophilus*** spp. esterases whereas neem oil, ClB1 and BcB2 extracts were most potent for ***M. aeneus***. Esterase inhibition did not correlate with pyrethrum synergism. Neem oil, BcB2 and ClB2 extracts were the most potent GST inhibitors in ***Carpophilus*** spp., however, this too did not correlate to pyrethrum synergism. PBO, dill apiole oil, parsley seed oil and CaB2 frac3 extract, all showing pyrethrum synergism, significantly inhibited ***Carpophilus*** spp. and ***M. aeneus*** monooxygenases. Since dill apiole oil, parsley seed oil and CaB2 frac3 extract were more potent inhibitors of ***M. aeneus*** monooxygenases than PBO, the high efficacy of PBO as a pyrethrum synergist against ***M. aeneus*** may possibly be due to an additional factor, perhaps its ability to enhance the passage of pyrethrum across the insect cuticle. These results tend to confirm the involvement of monooxygenases in ***M. aeneus*** pyrethroid resistance, as well as the importance of the MDP ring in synergism of pyrethrum.

6.1 Introduction

The pollen beetle ***Meligethes aeneus*** is a major pest of oilseed rape in Europe, causing great damage to the buds and flowers of the crop. As a result of insecticide applications to control pollen beetles, resistance has built up in populations across Europe towards insecticides, including pyrethroids, organophosphates and neonicotinoids (Hansen 2003, Węgorek & Zamoyska 2008). Resistance towards pyrethroids in ***M. aeneus*** was found to be due to an increase in oxidative metabolism (Philippou ***et al***. 2011).

The dried fruit beetle species complex (***Carpophilus*** spp.) in Australia consists mainly of ***Carpophilus davidsoni***, ***C. mutilatus*** and ***C. hemipterus*** and are serious pests of many fruit crops, including peaches, plums and nectarines (Hely ***et al***. 1982, James ***et al***. 1994), also transferring the devastating fungal disease, brown rot, in peaches and apricots (Kable 1969). When cotton is flowering, ***Carpophilus*** spp. are often present in large numbers inside the flowers. Adults and larvae appear to feed on pollen but do not cause any damage to the cotton crop (Bailey 2007). No accounts of insecticide resistance have been recorded for ***Carpophilus*** spp. but they would often be exposed to pesticide applications applied for control of cotton pest species such as the cotton bollworm ***Helicoverpa armigera*** or the cotton aphid ***Aphis gossypii***.

The main aims of this chapter were to identify effective natural, organically-certifiable synergists that could be formulated with pyrethrum to increase its efficacy; and to investigate the ability of the synergists in inhibiting the insecticide resistance associated metabolic enzymes (esterases, glutathione-S-transferases (GSTs) and monooxygenases). Discriminating dose bioassays were used to study synergism of pyrethrum against ***M. aeneus in vivo***. Biochemical assays were undertaken with ***M. aeneus*** and ***Carpophilus*** spp. to examine enzyme inhibition by the synergists. ***M. aeneus*** was known to have high levels of monooxygenase activity (Dr. Graham D. Moores, ***Pers. comm.***) and pyrethroid resistance in ***M. aeneus*** has previously been linked to monooxygenases (Philippou ***et al***. 2011). ***M. aeneus*** was therefore chosen for this study to examine pyrethrum synergism in a species with a known monooxygenase-based resistance mechanism. Due to similar feeding behaviour by ***Carpophilus*** spp., it was thought they might also possess high levels of monooxygenase activity and were therefore used to examine the inhibition of monooxygenases by the synergists in another insect species, to determine whether similarities may exist.

6.2 Materials and Methods

6.2.1 Bioassays

M. aeneus bioassays were performed at Rothamsted Research using coated vials according to the methods described in chapter two (section 2.4.4), using field-collected ***M. aeneus*** off oilseed rape. (More detail on ***M. aeneus*** is given in chapter two, section 2.1.4). A dose-response curve was determined to obtain the lethal dose

of pyrethrum required to kill 10% of the population (LD_{10}), for use in the discriminating dose bioassays. Discriminating dose bioassays were undertaken to assess the efficacy of various natural synergists in synergising pyrethrum against ***M. aeneus***.

6.2.1.1 Full dose response bioassay for *Meligethes aeneus*

The dose-response curve for pyrethrum with ***M. aeneus*** was determined using five pyrethrum dosages, ranging from 1 ppm to 100 ppm (diluted in acetone). Acetone was used as the control. Approximately ten adult pollen beetles (males and females) were used per treatment and the bioassay was replicated three times. The data were analysed with a probit analysis (Finney 1971) using PoloPlus (version 1.0, LeOra Software), correcting for control mortality with Abbott's formula (Abbott 1925).

6.2.1.2 Discriminating dose bioassay with *Meligethes aeneus*

The synergistic potential of a variety of natural plant oils and extracts in synergising pyrethrum against ***M. aeneus*** was examined with a discriminating dose bioassay using the LD_{10} of pyrethrum. The LD_{10} was 4.49 ppm pyrethrum (section 6.3.1.1) and a discriminating dose of 3 ppm pyrethrum was chosen for the bioassay to ensure low mortality with unsynergised pyrethrum. Synergists at a concentration of 100 ppm were mixed with the discriminating dose of pyrethrum (in acetone). The discriminating dose in acetone was used as the unsynergised treatment. For each synergist plus pyrethrum mixture that showed mortality higher than unsynergised pyrethrum, toxicity of the synergist alone was determined at 100 ppm. For the synergists showing significant toxicity at 100 ppm, the bioassay was repeated using synergists at 10 ppm mixed with the discriminating dose of pyrethrum. Synergist toxicity was also tested at 10 ppm. Acetone was used as the control. Each treatment contained approximately ten adult pollen beetles (males and females) per vial and the bioassays were replicated three times.

The plant oils and extracts tested as synergists are described in detail in chapter two (section 2.2.2, Table 2.1). Potential synergists were chosen based on similarity to the structure of PBO, or known insecticidal activity, insect repellence properties or the presence of allelochemicals as defence against insect herbivory. The plant oils used in the discriminating dose bioassay were aniseed, bergamot, canola, citronella, dill apiole, dill seed, geranium, grapefruit, neem, parsley seed and rosemary oils. Plant

extracts from the University of Tasmania tested for synergism were ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2. Propyl gallate, an anti-oxidant, and oleic acid, a mono-unsaturated omega-9 fatty acid, were also tested. PBO was used as the standard synergist.

Discriminating dose bioassay data were analysed with a logistic regression, with an assumed binomial distribution, using logit-transformed data. The program, ASREML, was used to analyse the data. For bioassays with synergists plus pyrethrum and synergists alone, the model incorporated all treatments into one analysis. Pairwise comparisons were done to determine significant differences using Fisher's (protected) least significant difference (LSD) at the 5% level.

6.2.2 Biochemical assays

The enzyme inhibiting-capabilities of the various compounds tested for synergism of pyrethrum were examined using esterase, GST and monooxygenase assays. Inhibition of enzyme activity could give an indication of which enzyme system played more of a role in synergism and thus possibly in resistance towards pyrethroids. Since ***M. aeneus*** was known to have high levels of monooxygenase activity, ***Carpophilus*** spp. were also examined for monooxygenase activity, due to their similar feeding behaviour. ***Carpophilus*** spp. were collected off cotton flowers. (More detail on ***Carpophilus*** spp. is given in chapter two, section 2.1.4). ***Carpophilus*** spp. were not separated to species level due to the high morphological similarities between species and their co-occurrence in cotton flowers. Preliminary investigations revealed high monooxygenase activity in ***Carpophilus*** spp. and were thus used to examine monooxygenase inhibition by the potential synergists. High levels of esterase and GST activity was also found in ***Carpophilus*** spp. and assays were subsequently undertaken for exploratory purposes. Esterase and monooxygenase inhibition by the synergists was also examined in ***M. aeneus***.

6.2.2.1 Esterase assays

Total esterase assays were performed to determine the inhibition of non-specific esterases by the synergists. The inhibition of esterases in ***Carpophilus*** spp. was examined using alpha-naphthyl acetate as the esterase substrate, whereas in ***M. aeneus***, para-nitrophenyl acetate was used as the esterase substrate. The substrate,

para-nitrophenyl acetate, was chosen for ***M. aeneus*** assays since in ***H. armigera*** (chapter three) and ***Myzus persicae*** (chapter five) a significant correlation was found between esterase inhibition in the esterase interference assay and the total esterase assay using para-nitrophenyl acetate as the substrate. The esterase interference assay was developed by Khot ***et al***. (2008) when esterase inhibition was not revealed by synergists in ***M. persicae*** using alpha-naphthyl acetate as the esterase substrate, as an indirect method of determining synergist binding to esterases.

6.2.2.1.1 *Carpophilus* spp. esterase assay (alpha-naphthyl acetate)

Total esterase assays (alpha-naphthyl acetate as a substrate) were run according to the methods described in chapter two (section 2.5.1.1). Homogenate was prepared from field-collected ***Carpophilus*** spp. by homogenising 20 beetles (approximately 0.065 g) (adult males and females) per 2.7 ml of 0.02 M phosphate buffer, pH 7. Beetles were homogenised in eppendorf tubes with a plastic handheld homogeniser and centrifuged at 10 000 rpm for 4 minutes. The supernatant was used immediately for esterase assays.

Compounds tested for inhibition of total esterase activity in ***Carpophilus*** spp. were aniseed, bergamot, canola, citronella, dill apiole, geranium, grapefruit, myristicin, neem, parsley seed, rosemary, sassafras and sesame oils; ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2 extracts; oleic acid and propyl gallate. PBO was used as the standard synergist. (Detailed descriptions for each are in chapter two, Table 2.1). All compounds tested were 100 000 ppm stock solutions (in acetone). Acetone was used as a control.

The ability of pyrethrum (100 000 ppm stock solution in acetone) to inhibit total esterase activity was also examined to obtain an indication of whether ***Carpophilus*** spp. esterases were interacting with pyrethrum, and therefore may potentially play a role in its detoxification.

Data for the esterase assays were processed according to the method described in chapter two (section 2.6), by calculating activity as a percentage of the uninhibited control. The program Grafit (Version 5.0.10) was used to determine inhibition curves for each compound (by fitting a four parameter logistic to the data), and calculating the IC_{50} and Y-range values (as described in chapter three, section 3.2.2.1.1).

6.2.2.1.2 *M. aeneus* esterase assay (para-nitrophenyl acetate)

Homogenate for the total esterase assays (para-nitrophenyl acetate as a substrate) was prepared by homogenising 50 field-collected ***M. aeneus*** (approximately 0.048 g) (adult males and females) in 4 ml of 0.02 M phosphate buffer, pH 7, using a plastic handheld homogeniser. Homogenate was centrifuged at 10 000 rpm for 3 minutes. The supernatant was used immediately for the esterase assays, which were run according to the methods described in chapter two (section 2.5.1.2).

Compounds tested for inhibition of ***M. aeneus*** total esterase activity were as for the discriminating dose bioassay (section 6.2.1.2). All compounds were in 10 000 ppm stock solutions (in acetone) and acetone was used as a control. The ability of pyrethrum (10 000 ppm stock solution in acetone) to inhibit total esterase activity was also assessed to determine whether ***M. aeneus*** esterases were interacting with pyrethrum. Data for the esterase assays were analysed as for the ***Carpophilus*** spp. esterase assay (section 6.2.2.1.1).

6.2.2.2 Glutathione-S-transferase assays

GST assays were run using CDNB as a substrate according to the method described in chapter two (section 2.5.2). ***Carpophilus*** spp. homogenate was prepared by homogenising 15 field-collected beetles (approximately 0.051 g) (adult males and females) per 2.7 ml of 0.2 M phosphate buffer, pH 6.5. Beetles were homogenised in eppendorf tubes with a plastic handheld homogeniser and centrifuged at 10 000 rpm for 4 minutes. The supernatant was used immediately for GST assays.

The compounds tested for inhibition of GST activity, and their concentrations, were as for the ***Carpophilus*** spp. total esterase assay (section 6.2.2.1.1). Acetone was used as the control. The ability of pyrethrum (100 000 ppm stock solution in acetone) to inhibit GST activity was also examined to determine whether GSTs were interacting with pyrethrum.

Data for the GST assay were processed and analysed as for the esterase assays (section 6.2.2.1). Inhibition curves for BcB1, BcB2, ClB1, ClB2, CaB2, CaB2 frac3 and ZaB2 extracts, neem, sassafras and grapefruit oils, oleic acid and pyrethrum

were fitted with two parameter logistic curves with the lower and upper limits of 0% and 100% respectively, as explained in chapter three (section 3.2.2.1.1), since not enough inhibition data was obtained within the tested concentration range to accurately fit a four parameter logistic curve. The inhibition curves for all remaining compounds were fitted with a four parameter logistic curve.

6.2.2.3 Monooxygenase assays

Meligethes aeneus

M. aeneus homogenate was prepared by homogenising 55 field-collected ***M. aeneus*** (approximately 0.052 g) (adult males and females) per 1 ml of homogenisation buffer (0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU and 1 mM PMSF), using a plastic handheld homogeniser. Homogenate was centrifuged at 10 000 rpm for 2 minutes. The supernatant was used immediately for the monooxygenase assay, run according to the methods described in chapter two (section 2.5.3).

Compounds tested for inhibition of ***M. aeneus*** monooxygenase activity were the same as those used in the discriminating dose bioassay (section 6.2.1.2). All compounds tested were 1000 ppm stock solutions (in acetone). Acetone was used as the control. The ability of pyrethrum (1000 ppm stock solution in acetone) to inhibit monooxygenase activity was also examined.

Carpophilus spp.

Homogenate from field-collected ***Carpophilus*** spp. was prepared by homogenising 50 beetles (approximately 0.175 g) (adult males and females) per 1.4 ml of homogenisation buffer (0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU and 1 mM PMSF), using a plastic handheld homogeniser. Homogenate was centrifuged at 10 000 rpm for 5 minutes. The supernatant was used immediately for the monooxygenase assay and run according to the methods described in chapter two (section 2.5.3).

Compounds tested for inhibition of ***Carpophilus*** spp. monooxygenases were angelica root, aniseed, bergamot, black pepper, canola, citronella, citronella java, cypress, dill apiole, dill seed, eucalyptus, fennel, garlic, geranium, grapefruit, lavender, manuka, melaleuca, myristicin, neem, nutmeg, parsley seed, peppermint, platyphyllol, rosemary, sassafras, sesame and white cypress oils; ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2 extracts; oleic acid, propyl gallate and the standard synergist, PBO (detailed descriptions for each in chapter two, Table 2.1). All compounds used were 1000 ppm stock solutions in acetone. Acetone was used as the control. Pyrethrum (1000 ppm stock solution in acetone) was also tested for its ability to inhibit monooxygenases.

Data for the ***M. aeneus*** and ***Carpophilus*** spp. monooxygenase assays were processed according to the methods described in chapter two (section 2.6), by calculating activity as a percentage of the uninhibited acetone control and using an analysis of variance (ANOVA) to analyse data. Significant differences were determined using Fisher's (protected) LSD at the 5% level.

Correlations between monooxygenase activity (between M. aeneus ***and*** Carpophilus ***spp.) and with the discriminating dose bioassays for*** Helicoverpa armigera***,*** Frankliniella occidentalis ***and*** Myzus persicae

A correlation was tested for between inhibition of ***M. aeneus*** monooxygenases and inhibition of ***Carpophilus*** spp. monooxygenases by synergists, to determine whether similar compounds showed inhibition in the different taxa. In addition, correlations were tested for between the percentage mortality found in the discriminating dose bioassays with ***H. armigera*** (chapter three), ***Frankliniella occidentalis*** (chapter four) and ***M. persicae*** (chapter five) and inhibition of ***M. aeneus*** and ***Carpophilus*** spp. monooxygenases by synergists, to determine whether synergism of pyrethrum could be correlated to the inhibition of monooxygenase activity. Inter-taxon comparisons may not be ideal, however, they might offer a potential insight into the enzyme systems possibly playing a role in synergism of pyrethrum. Correlations were made by calculating correlation coefficients using Microsoft Excel (2003) (Analysis Toolpak). A table of correlation coefficients (significant values of ***r*** and ***R***) was used to determine if the correlations were significant at the 1 or 5% level.

6.3 Results

6.3.1 Bioassays

6.3.1.1 Full dose response bioassay for *Meligethes aeneus*

In ***M. aeneus***, the LD_{10} of pyrethrum was 4.49 ppm pyrethrum (Table 6.1). For the discriminating dose bioassay, 3 ppm pyrethrum was chosen as the discriminating dose to ensure a low percentage mortality with unsynergised pyrethrum, to allow for the observation of synergistic activity by the potential synergists.

Table 6.1. The lethal dose (LD) values with 95% confidence intervals (CI) for pyrethrum (calculated by probit analysis) from coated vial bioassays against field-collected *Meligethes aeneus* (male and female adults).

LD value	Pyrethrum dose (ppm)	95% CI
LD_{10}	4.49	1.12 – 7.58
LD_{20}	6.37	2.16 – 9.76
LD_{30}	8.20	3.45 – 11.82
LD_{40}	10.17	5.10 – 14.06
LD_{50}	12.43	7.24 – 16.79
LD_{60}	15.20	10.00 – 20.57
LD_{70}	18.85	13.52 – 26.74
LD_{80}	24.25	18.01 – 38.83
LD_{90}	34.39	24.70 – 70.68
LD_{95}	45.89	31.00 – 120
LD_{99}	78.83	46.05 – 333
Number of ***M. aeneus*** exposed = 179		
Slope ± SE = 2.900 ± 0.719		
Degrees of freedom (df) = 13		
$\chi^2 = 4.528$		

6.3.1.2 Discriminating dose bioassay with *Meligethes aeneus*

The discriminating dose bioassay with synergists at 100 ppm showed that pyrethrum synergised by both PBO and dill apiole oil caused 100% mortality in ***M. aeneus***, which was not significantly higher than the mortality found with pyrethrum plus CaB2 frac3 extract (96.87%, $p<0.001$) (Fig. 6.1). The mortalities in these three treatments were significantly higher than all the remaining treatments. Pyrethrum plus parsley seed oil caused mortality significantly lower than pyrethrum with PBO, dill apiole oil and CaB2 frac3 extract, but significantly higher than all the remaining treatments (63.64% mortality). No other potential synergist had mortality significantly different from pyrethrum alone (19.40% mortality).

The toxicity of PBO, dill apiole oil, CaB2 frac3 extract, parsley seed oil, oleic acid, propyl gallate and rosemary oil was assessed at 100 ppm (Fig. 6.2). PBO, dill apiole oil, CaB2 frac3 extract and parsley seed oil showed significant toxicity at this concentration, with mortalities significantly higher (61.76%, 73.53%, 42.42% and 30.56% respectively) than acetone alone (4.21%, $p<0.001$). The mortality caused by PBO, dill apiole oil, CaB2 frac3 extract and parsley seed oil plus pyrethrum however, could at least in part be ascribed to synergistic effects, since each had significantly higher mortality than the mortality caused by the synergist itself.

The bioassay was repeated for PBO, dill apiole oil, CaB2 frac3 extract and parsley seed oil at 10 ppm (Fig. 6.3). At this concentration, PBO was the only compound that showed significant synergism of pyrethrum, with mortality significantly higher than all the other treatments (83.87% mortality, $p=0.008$).

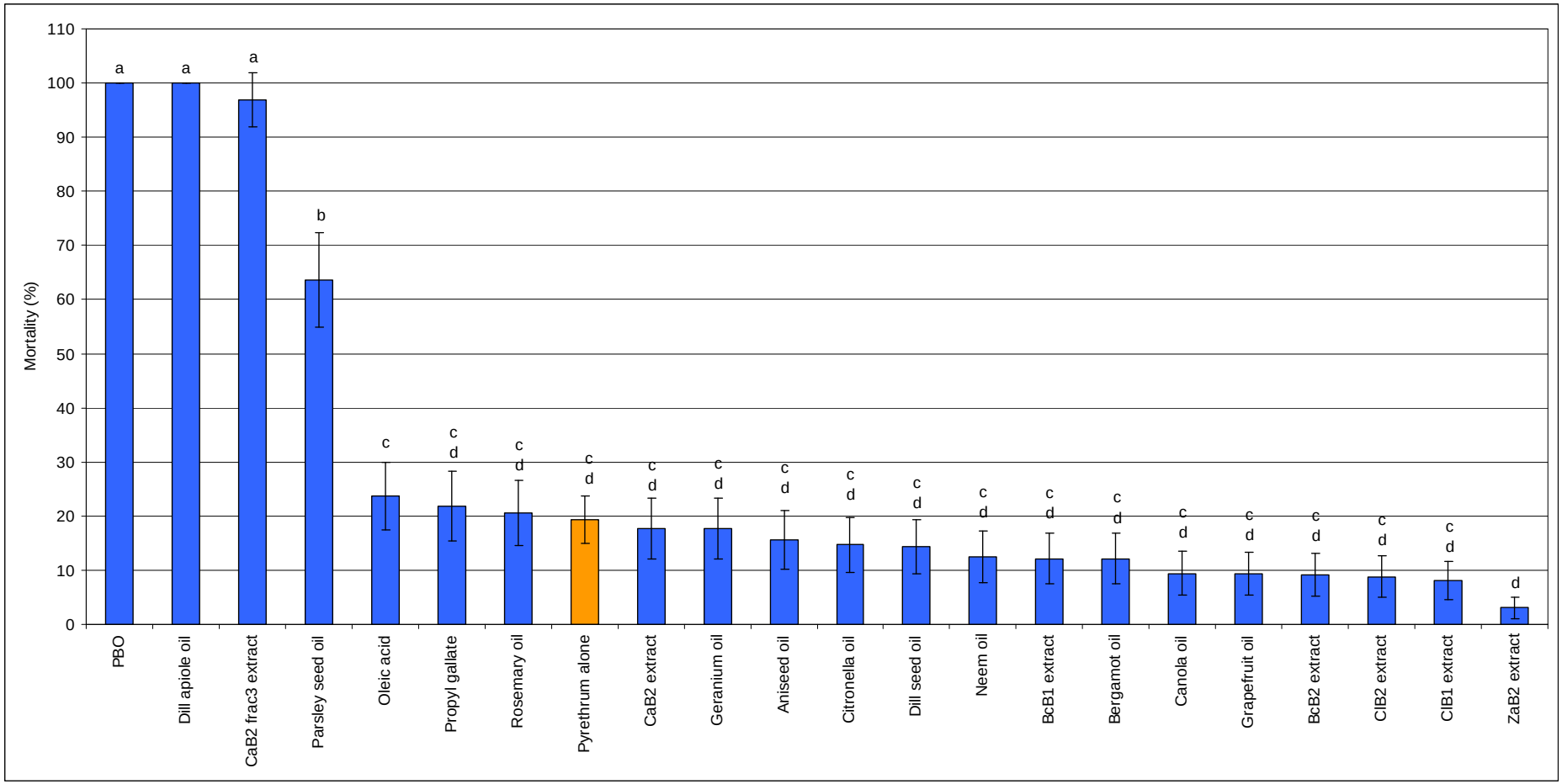


Fig. 6.1. The mean percentage mortality (± SE) of *Meligethes aeneus* (male and female adults) using coated vials with 3 ppm pyrethrum:100 ppm synergist solutions. Pyrethrum alone (3 ppm) was used as the unsynergised pyrethrum treatment. (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

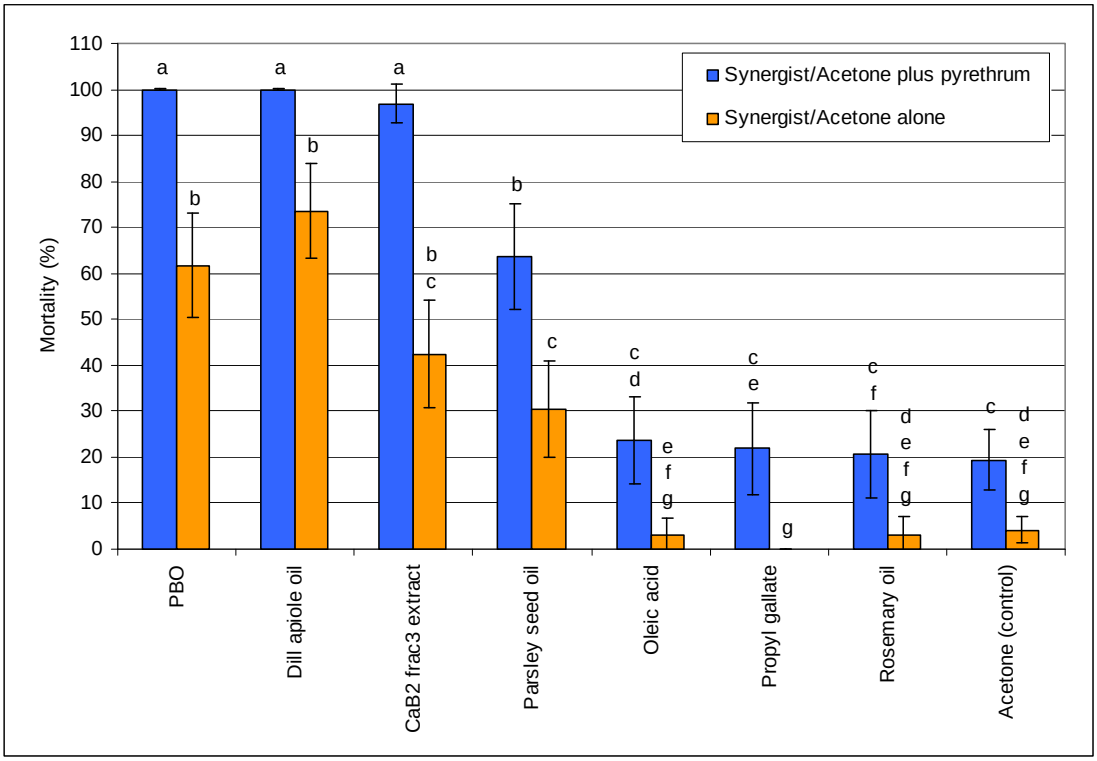


Fig. 6.2. The mean percentage mortality (± SE) of *Meligethes aeneus* (male and female adults) using coated vials with 3 ppm pyrethrum:100 ppm synergist solutions, or with 100 ppm synergists alone. Pyrethrum in acetone (3 ppm) was used as the unsynergised pyrethrum treatment. Acetone was used as the control. (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

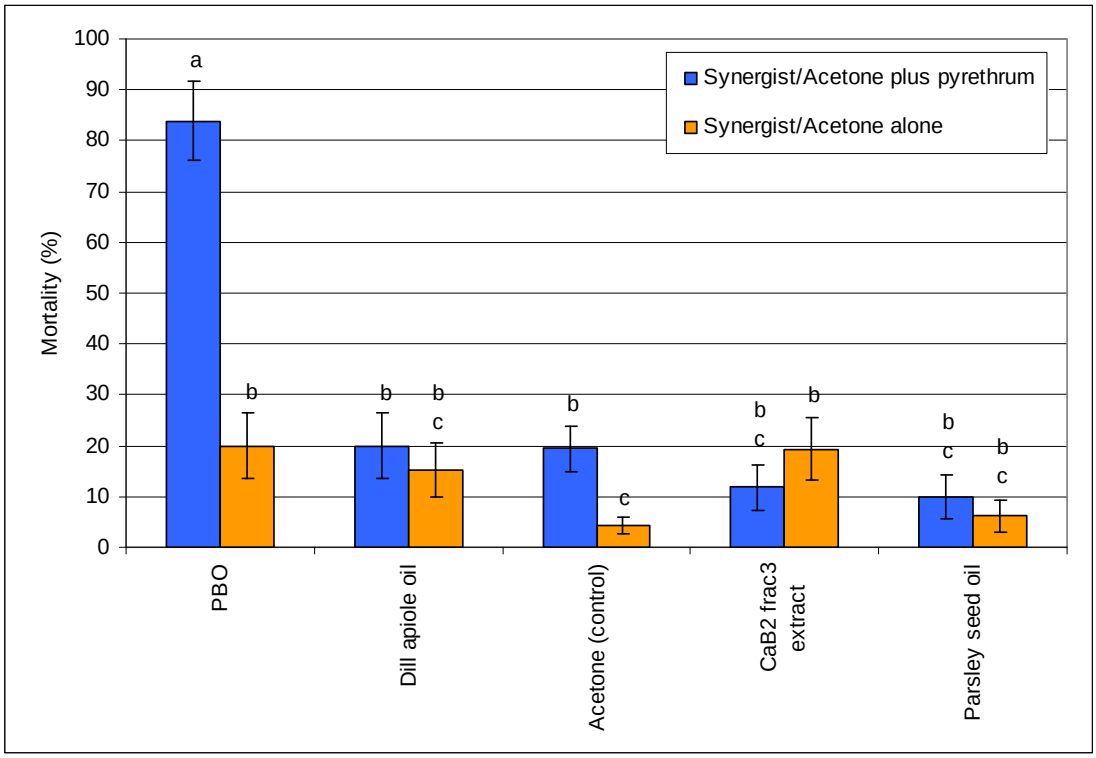


Fig. 6.3. The mean percentage mortality (± SE) of *Meligethes aeneus* (male and female adults) using coated vials with 3 ppm pyrethrum:10 ppm synergist solutions, or with 10 ppm synergists alone. Pyrethrum in acetone (3 ppm) was used as the unsynergised pyrethrum treatment. Acetone was used as the control. (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

6.3.2 Biochemical assays

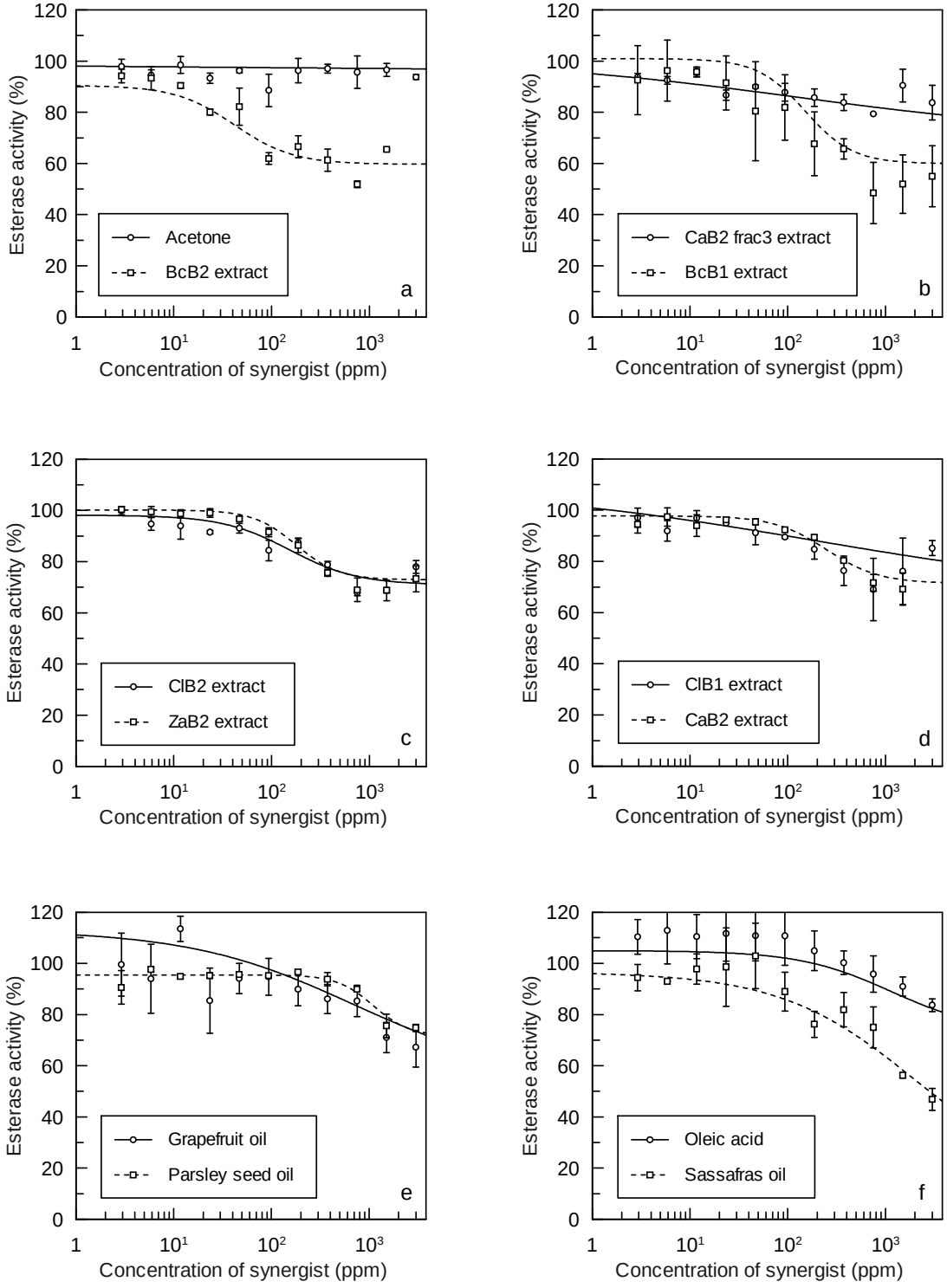
6.3.2.1 Esterase assays

6.3.2.1.1 *Carpophilus* spp. esterase assay (alpha-naphthyl acetate)

The most potent inhibitor of ***Carpophilus*** spp. esterases was BcB2, followed by CaB2 frac3 and BcB1 extracts (Table 6.2, Fig. 6.4a-i). PBO and dill apiole oil, which showed significant pyrethrum synergism against ***M. aeneus in vivo***, did not inhibit ***Carpophilus*** spp. esterases (Fig. 6.4i). No inhibition of esterases was also found with acetone (Fig. 6.4a), aniseed, bergamot, citronella, geranium, myristicin, rosemary and sesame oils (not illustrated).

Table 6.2. Inhibition of *Carpophilus* spp. (male and female adults) total esterase activity (alpha-naphthyl acetate as the substrate) by potential pyrethrum synergists. The IC_{50} and Y-range values (± SE) are tabulated for each synergist. (Relates to Fig. 6.4a-i; IC_{50} and Y-range values could not be calculated for compounds showing no inhibition of esterases; Synergists ordered from lowest to highest IC_{50}).

Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
BcB2 extract	Unknown	41.60 (± 14.30)	30.78 (± 8.72)
CaB2 frac3 extract	Phenylpropene	124 (± 138)	30.11 (± 3.82)
BcB1 extract	Unknown	151 (± 63.16)	41.03 (± 8.17)
ClB2 extract	Cyclic terpene	154 (± 57.40)	27.20 (± 4.95)
ZaB2 extract	Sesquiterpenoid	178 (± 23.93)	27.26 (± 1.92)
ClB1 extract	Cyclic terpene	190 (± 321)	42.89 (± 6.24)
CaB2 extract	Phenylpropene	249 (± 61.26)	26.32 (± 3.72)
Grapefruit oil	Cyclic terpene	678 (± 389)	57.81 (± 13.99)
Parsley seed oil	Methylenedioxyphenol ring; Bicyclic monoterpene; Methylenedioxyphenol ring / phenylpropanoid	1095 (± 255)	23.88 (± 4.23)
Oleic acid	Mono-unsaturated omega-9 fatty acid	1139 (± 2710)	31.87 (± 35.03)
Sassafras oil	Methylenedioxyphenol ring	1959 (± 2009)	83.72 (± 32.43)
Neem oil	Tetranortriterpenoid / liminoid	4602 (± 3044)	95.46 (± 24.61)
Propyl gallate	Ester	5464 (± 2088)	105 (± 18.59)
Canola oil	Mono-unsaturated omega-9 fatty acid; Unsaturated omega-6 fatty acid	7632 (± 11 678)	44.88 (± 40.39)


Esterase activity (%)
Concentration of synergist (ppm)
Acetone
BcB2 extract
a
CaB2 frac3 extract
BcB1 extract
b
ClB2 extract
ZaB2 extract
c
ClB1 extract
CaB2 extract
d
Grapefruit oil
Parsley seed oil
e
Oleic acid
Sassafras oil
f

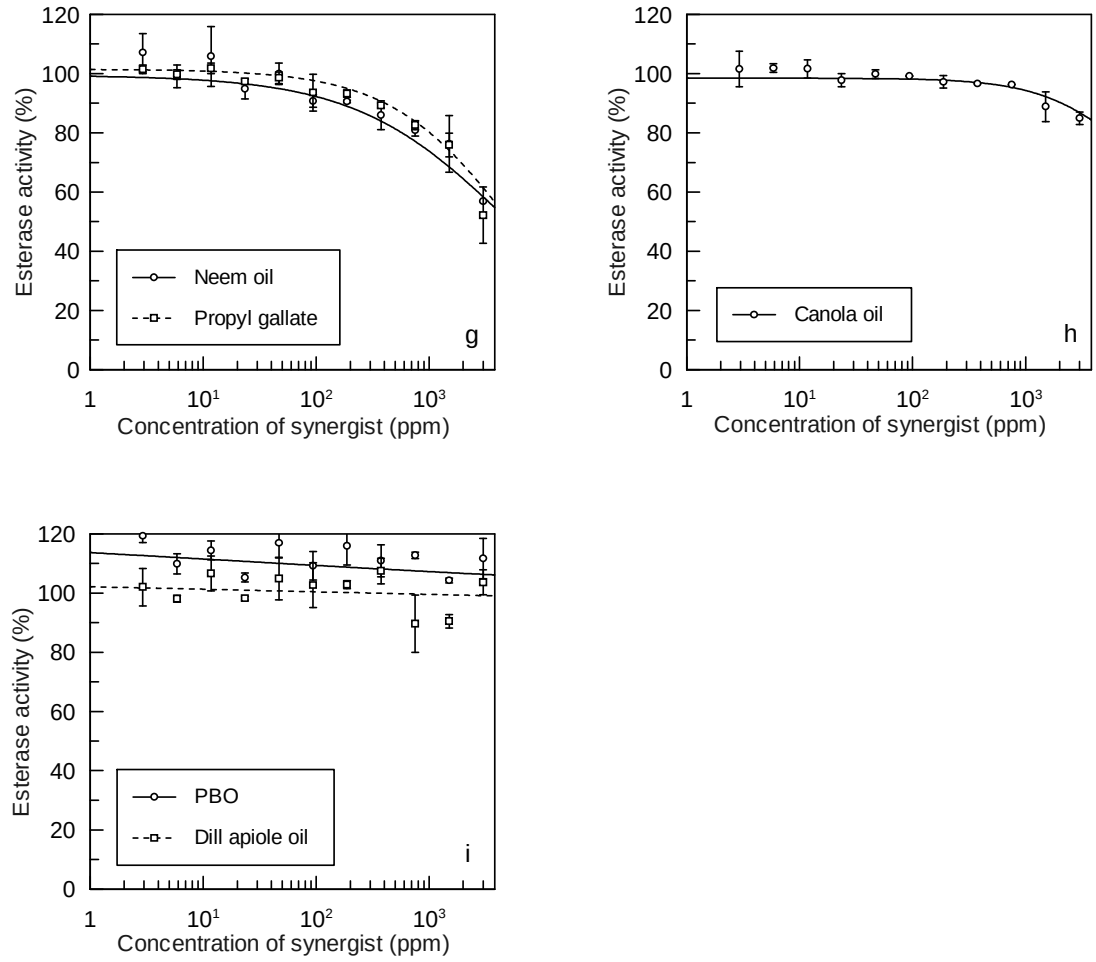


Fig. 6.4a-i. Inhibition of *Carpophilus* spp. (male and female adults) total esterase activity (alpha-naphthyl acetate as the substrate) by potential pyrethrum synergists, PBO and acetone (control). Error bars represent standard deviation. Lines were fitted with a four parameter logistic curve. (Relates to Table 6.2; Synergists ordered from lowest to highest IC_{50}).

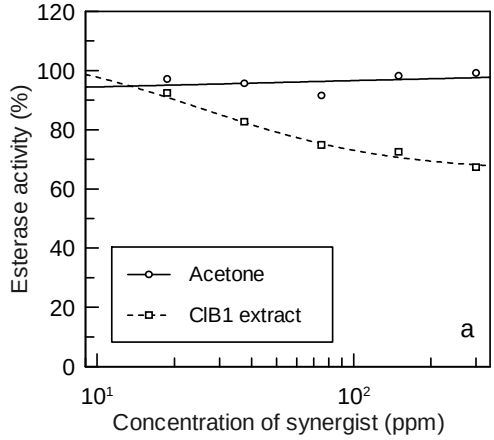
6.3.2.1.2 *M. aeneus* esterase assay (para-nitrophenyl acetate)

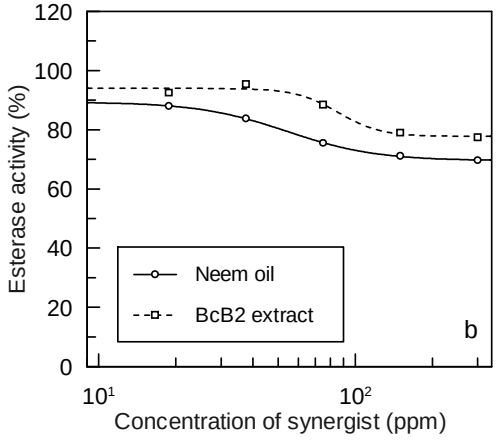
The total esterase assay with ***M. aeneus*** revealed only nine of the tested compounds to show any esterase inhibition (Table 6.3, Fig. 6.5a-g). The most potent of the synergists showing inhibition of esterases was ClB1 extract, followed by neem oil, whereas the least potent was BcB1 extract.

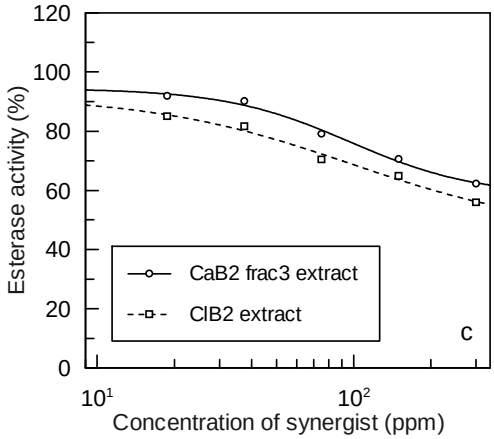
From the compounds that showed synergism of pyrethrum against ***M. aeneus***, PBO, dill apiole oil and parsley seed oil did not inhibit ***M. aeneus*** esterases (Fig. 6.5f & g). ***M. aeneus*** esterases were also not inhibited by acetone (Fig. 6.5a), propyl gallate, aniseed, bergamot, canola, citronella, dill seed, geranium, grapefruit and rosemary oils (not illustrated).

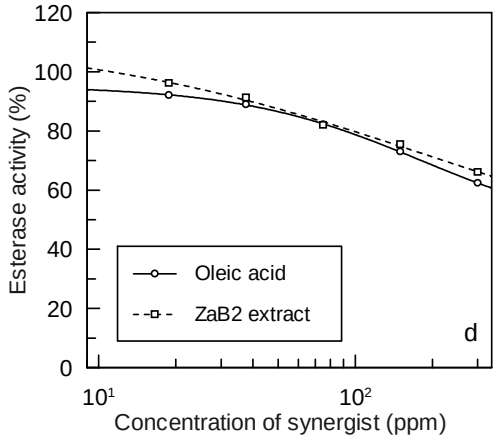
Table 6.3. Inhibition of *Meligethes aeneus* (male and female adults) total esterase activity (para-nitrophenyl acetate as the substrate) by potential pyrethrum synergists. The IC_{50} and Y-range values (± SE) are tabulated for each synergist. (Relates to Fig. 6.5a-g; IC_{50} and Y-range values could not be calculated for compounds showing no inhibition of esterases; Synergists ordered from lowest to highest IC_{50}).

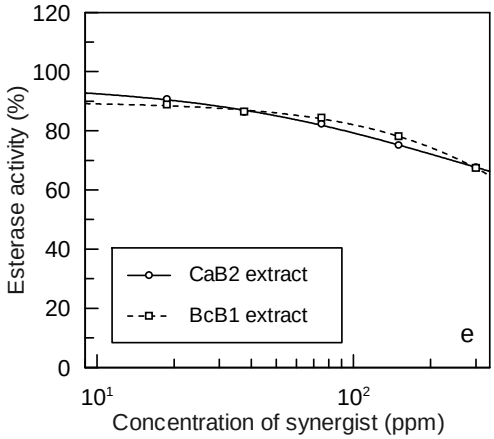
Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
ClB1 extract	Cyclic terpene	25.66 (± 24.16)	42.34 (± 18.69)
Neem oil	Tetranortriterpenoid / liminoid	54.26 (± 1.77)	19.85 (± 0.69)
BcB2 extract	Unknown	85.72 (± 17.26)	16.25 (± 3.05)
CaB2 frac3 extract	Phenylpropene	97.05 (± 26.39)	36.27 (± 11.86)
ClB2 extract	Cyclic terpene	98.49 (± 74.08)	46.11 (± 44.94)
Oleic acid	Mono-unsaturated omega-9 fatty acid	182 (± 31.10)	50.50 (± 6.54)
ZaB2 extract	Sesquiterpenoid	201 (± 643)	81.33 (± 155)
CaB2 extract	Phenylpropene	253 (± 90.17)	52.49 (± 9.90)
BcB1 extract	Unknown	969 (± 937)	105 (± 76.86)

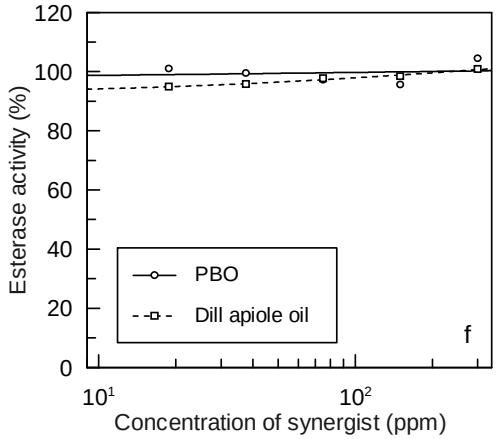

Esterase activity (%)
120
100
80
60
40
20
0
Acetone
ClB1 extract
a
10^1
10^2
Concentration of synergist (ppm)


Esterase activity (%)
120
100
80
60
40
20
0
Neem oil
BcB2 extract
b
10^1
10^2
Concentration of synergist (ppm)


Esterase activity (%)
120
100
80
60
40
20
0
CaB2 frac3 extract
ClB2 extract
c
10^1
10^2
Concentration of synergist (ppm)


Esterase activity (%)
120
100
80
60
40
20
0
Oleic acid
ZaB2 extract
d
10^1
10^2
Concentration of synergist (ppm)


Esterase activity (%)
120
100
80
60
40
20
0
CaB2 extract
BcB1 extract
e
10^1
10^2
Concentration of synergist (ppm)


Esterase activity (%)
120
100
80
60
40
20
0
PBO
Dill apiole oil
f
10^1
10^2
Concentration of synergist (ppm)

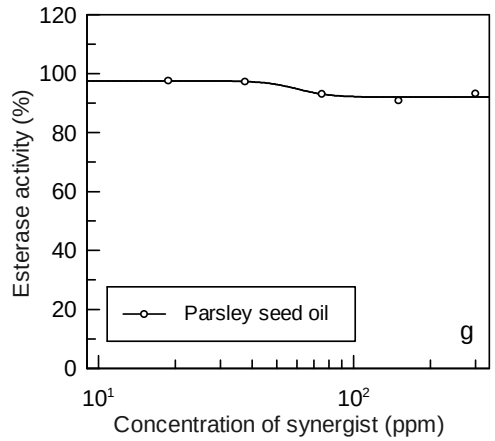


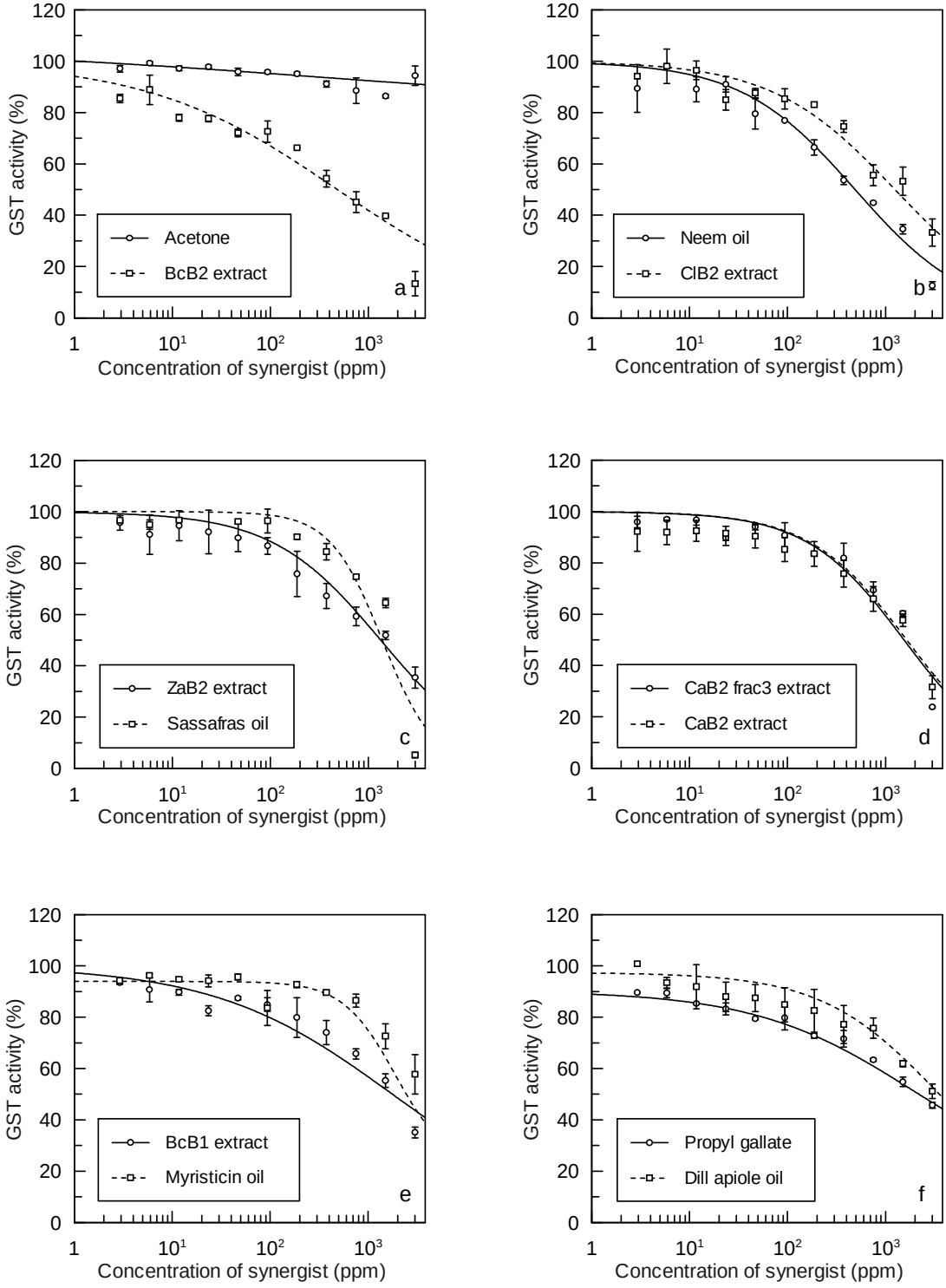
Fig. 6.5a-g. Inhibition of *Meligethes aeneus* (male and female adults) total esterase activity (para-nitrophenyl acetate as the substrate) by potential pyrethrum synergists, PBO and acetone (control). Lines were fitted with a four parameter logistic curve. (Relates to Table 6.3; Synergists ordered from lowest to highest IC_{50}).

6.3.2.2 Glutathione-S-transferase assays

GST assays with ***Carpophilus*** spp. showed BcB2 extract and neem oil to be the most potent inhibitors of GST activity (Table 6.4, Fig. 6.6a-i). Grapefruit oil and oleic acid showed the least inhibition out of the compounds showing any inhibition of GSTs. For the compounds that showed pyrethrum synergism against ***M. aeneus***, PBO gave no inhibition of ***Carpophilus*** spp. GST activity (Fig. 6.6i), whereas CaB2 frac3 extract, dill apiole oil and parsley seed oil showed inhibition of GSTs (Fig. 6.6d, f & g). In addition to PBO, acetone (Fig. 6.6a) and aniseed, bergamot, canola, citronella, rosemary and sesame oils gave no inhibition of GSTs (not illustrated).

Table 6.4. Inhibition of *Carpophilus* spp. (male and female adults) glutathione-S-transferases (CDNB as the substrate) by potential pyrethrum synergists. The IC_{50} and Y-range values (± SE) are tabulated for each synergist. (Relates to Fig. 6.6a-i; IC_{50} and Y-range values could not be calculated for compounds showing no inhibition of esterases; Synergists ordered from lowest to highest IC_{50}).

Compound	Type of chemical	IC_{50} (ppm) (± SE)	Y-range (%) (± SE)
BcB2 extract	Unknown	483 (± 85.75)	100
Neem oil	Tetranortriterpenoid / liminoid	489 (± 35.35)	100
ClB2 extract	Cyclic terpene	1248 (± 143)	100
ZaB2 extract	Sesquiterpenoid	1333 (± 118)	100
Sassafras oil	Methylenedioxyphenol ring	1386 (± 191)	100
CaB2 frac3 extract	Phenylpropene	1541 (± 188)	100
CaB2 extract	Phenylpropene	1645 (± 189)	100
BcB1 extract	Unknown	1762 (± 292)	100
Myristicin oil	Methylenedioxyphenol ring	1881 (± 1813)	72.55 (± 25.42)
Propyl gallate	Ester	1934 (± 387)	78.07 (± 4.12)
Dill apiole oil	Methylenedioxyphenol ring / phenylpropanoid	3301 (± 2132)	92.45 (± 25.16)
ClB1 extract	Cyclic terpene	3746 (± 633)	100
Parsley seed oil	Methylenedioxyphenol ring; Bicyclic monoterpene; Methylenedioxyphenol ring / phenylpropanoid	5499 (± 1381)	100 (± 12.26)
Geranium oil	Monoterpenoid / alcohol	10 903 (± 10 308)	58.93 (± 17.34)
Grapefruit oil	Cyclic terpene	11 106 (± 3602)	100
Oleic acid	Mono-unsaturated omega-9 fatty acid	73 640 (± 89 602)	100


GST activity (%)
Concentration of synergist (ppm)
Acetone
BcB2 extract
a
Neem oil
ClB2 extract
b
ZaB2 extract
Sassafras oil
c
CaB2 frac3 extract
CaB2 extract
d
BcB1 extract
Myristicin oil
e
Propyl gallate
Dill apiole oil
f

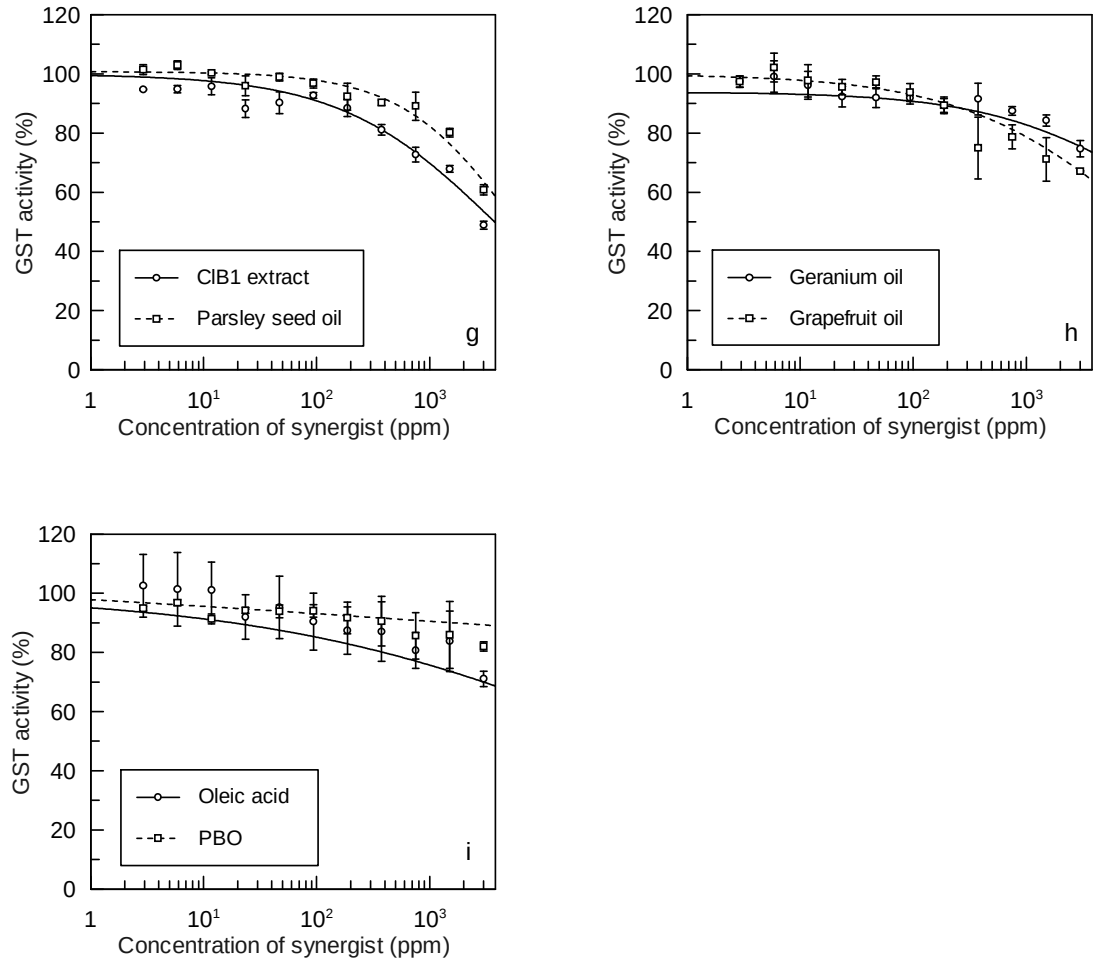


Fig. 6.6a-i. Inhibition of *Carpophilus* spp. (male and female adults) glutathione-S-transferase (GST) activity (CDNB as the substrate) by potential pyrethrum synergists, PBO and acetone (control). Error bars represent standard deviation. For BcB2, ClB2, ZaB2, CaB2 frac3, CaB2, BcB1 and ClB1 extracts, neem, sassafras and grapefruit oils and oleic acid, lines were fitted with two parameter logistic curves (lower and upper limits of 0% and 100% respectively). For all remaining compounds, lines were fitted with a four parameter logistic curve. (Relates to Table 6.4; Synergists ordered from lowest to highest IC_{50}).

6.3.2.3 Monooxygenase assays

Meligethes aeneus

In ***M. aeneus***, oleic acid showed the highest efficacy as a monooxygenase inhibitor, with almost complete inhibition (0.11% monooxygenase activity remaining), inhibiting significantly more monooxygenase activity than all of the other compounds ($p<0.001$) (Fig. 6.7). Dill apiole oil followed, with 17.34% monooxygenase activity, which was significantly different to all the remaining compounds. Parsley seed oil was the next most effective monooxygenase inhibitor and did not differ significantly from ZaB2 extract (35.57% and 47.69% monooxygenase activity respectively) but was significantly different from all the remaining treatments. CaB2 frac3 extract followed (57.65% monooxygenase activity) and was not significantly different from the inhibition found with PBO (67.46% monooxygenase activity).

PBO, dill apiole oil, parsley seed oil and CaB2 frac3, all of which showed pyrethrum synergism against ***M. aeneus***, were good monooxygenase inhibitors, with all having significantly higher inhibition of monooxygenases than the acetone control. With the exception of oleic acid, dill apiole and parsley seed oil were more potent monooxygenase inhibitors than all the remaining compounds (Fig. 6.7).

Carpophilus spp.

In ***Carpophilus*** spp., BcB1 extract, myristicin oil and parsley seed oil inhibited the highest amount of monooxygenase activity, significantly more than all of the other compounds (with 0%, 0% and 0.85% monooxygenase activity remaining respectively, $p<0.001$) (Fig. 6.8). Dill apiole oil followed (13.74% monooxygenase activity) and did not differ significantly from inhibition shown by parsley seed oil, nor from inhibition by sassafras oil, BcB2 extract, angelica root oil and PBO (20.50%, 22.04%, 23.34% and 25.09% monooxygenase activity respectively). CaB2 frac3 extract, which synergised pyrethrum in ***M. aeneus in vivo***, (along with PBO, dill apiole oil and parsley seed oil), also showed significant inhibition of monooxygenase activity, with 39.25% of monooxygenase activity remaining.

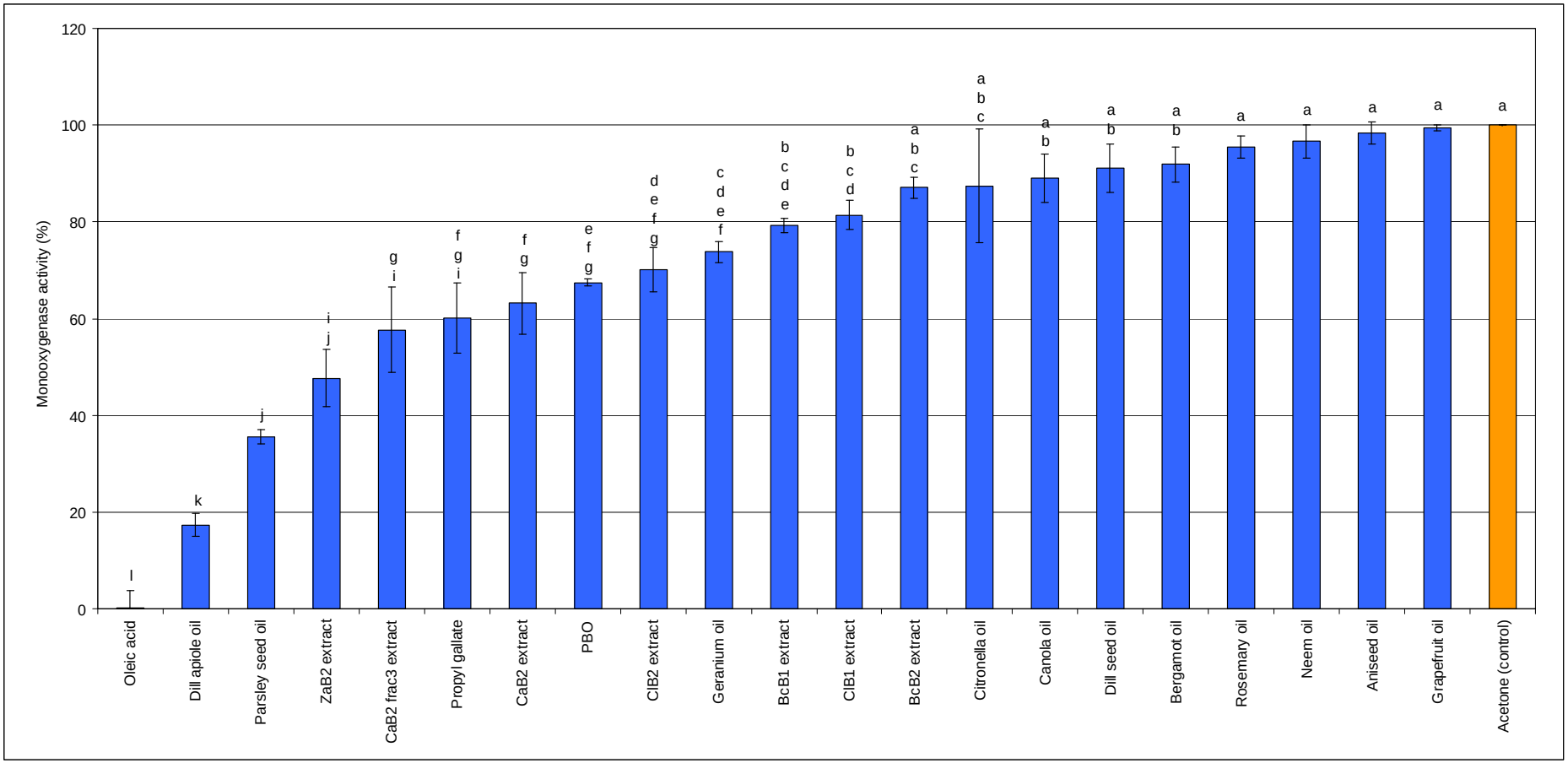


Fig. 6.7. The mean percentage monooxygenase activity (± SE) with *Meligethes aeneus* (male and female adults) by potential pyrethrum synergists, PBO and acetone (control). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

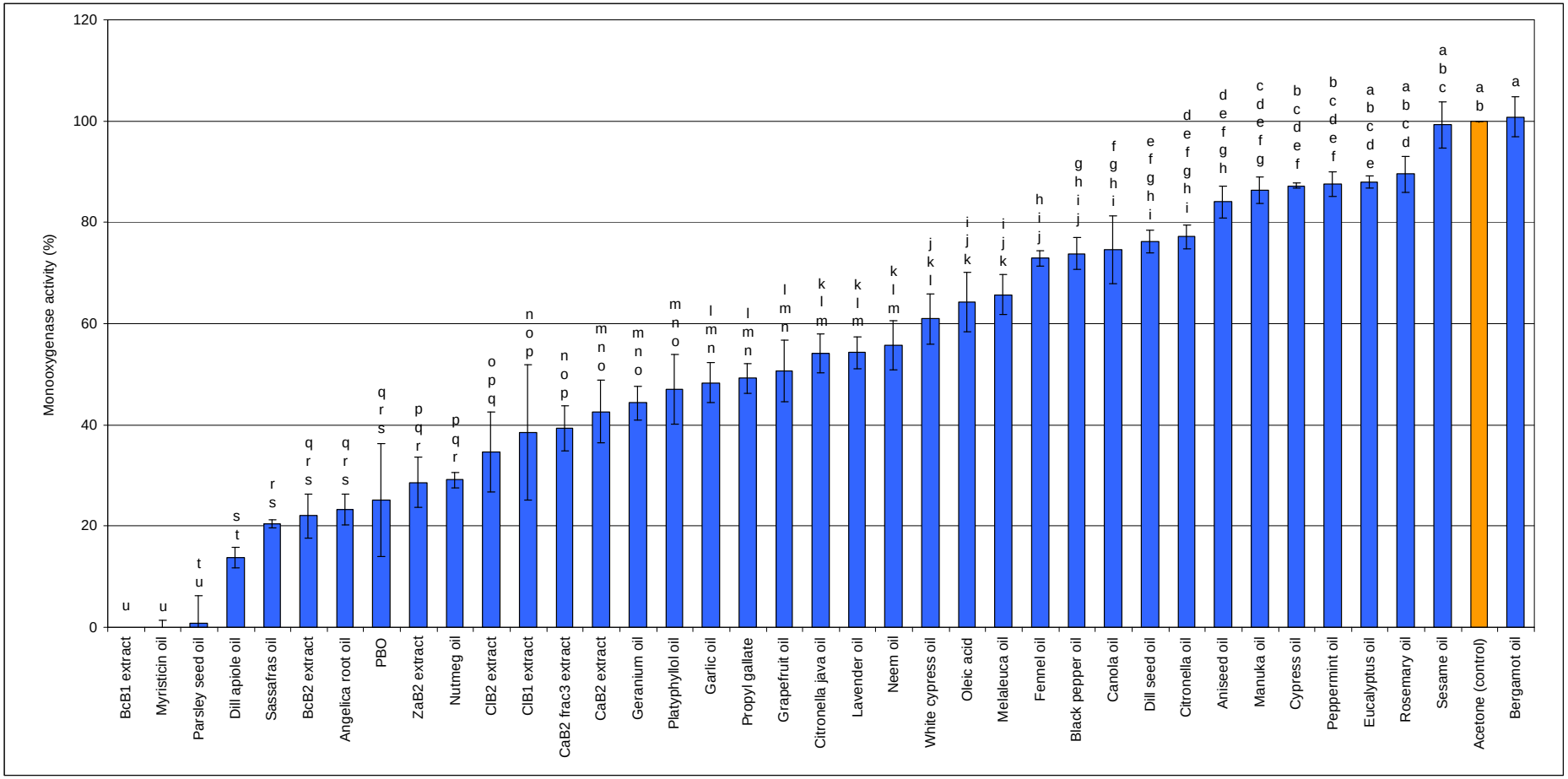


Fig. 6.8. The mean percentage monooxygenase activity (± SE) with *Carpophilus* spp. (male and female adults) by potential pyrethrum synergists, PBO and acetone (control). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

Correlations between monooxygenase activity (between M. aeneus ***and*** Carpophilus ***spp.) and with the discriminating dose bioassays for*** Helicoverpa armigera***,*** Frankliniella occidentalis ***and*** Myzus persicae

A significant positive correlation was found between monooxygenase inhibition by synergists in ***M. aeneus*** and monooxygenase inhibition by synergists in ***Carpophilus*** spp. (correlation coefficient = 0.435, n=21, p=0.049) (Table 6.5, Fig. 6.9). This showed that similar compounds were showing inhibition of monooxygenase activity in both species.

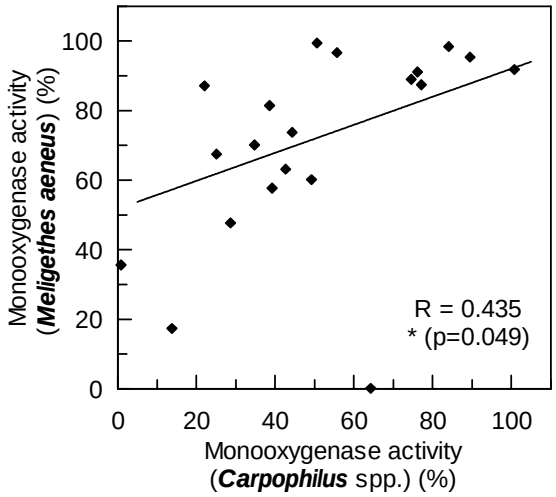


Fig. 6.9. The correlation between monooxygenase inhibition by synergists in *Carpophilus* spp. and monooxygenase inhibition by synergists in *Meligethes aeneus*. Each point indicates a different synergist (n=21), lines were fitted by linear regression, R is the correlation coefficient and * indicates a significant correlation (p<0.05).

Correlations were not tested for between synergism in the ***M. aeneus*** discriminating dose bioassay and enzyme inhibition since so few compounds showed pyrethrum synergism against ***M. aeneus*** at 100 ppm synergist, and PBO was the only compound to still show significant synergism at 10 ppm synergist.

A significant negative correlation was found between pyrethrum synergism in ***F. occidentalis*** (mortality in the discriminating dose bioassay) and monooxygenase activity remaining with the different synergists in ***Carpophilus*** spp. (correlation coefficient = -0.671, n=13, p=0.012) (Table 6.5, Fig. 6.10). This correlation showed that synergism in ***F. occidentalis*** correlated to inhibition of monooxygenases by synergists. No other significant correlations were found.

Table 6.5. Analysis of the correlations between monooxygenase activity (between *Meligethes aeneus* and *Carpophilus* spp.) and with the discriminating dose bioassays for *Helicoverpa armigera*, *Frankliniella occidentalis* and *Myzus persicae*. (Significant correlations illustrated in Figs 6.9 & 6.10).

Monooxygenase assay	Bioassay / monooxygenase assay	n[¥]	Correlation coefficient	Significance[§] (p-value) (two-tailed)
Carpophilus spp. monooxygenase	***M. aeneus*** monooxygenase	21	0.435	* (0.049)
	H. armigera discriminating dose	36	-0.112	NS (0.515)
	F. occidentalis discriminating dose	13	-0.671	* (0.012)
	M. persicae 794JZ discriminating dose	21	-0.242	NS (0.291)
	M. persicae 5191A discriminating dose	21	0.256	NS (0.263)
M. aeneus monooxygenase	***H. armigera*** discriminating dose	19	-0.094	NS (0.702)
	F. occidentalis discriminating dose	11	-0.440	NS (0.176)
	M. persicae 794JZ discriminating dose	21	-0.382	NS (0.087)
	M. persicae 5191A discriminating dose	21	-0.027	NS (0.908)

[¥] n = Number of synergists used in the correlation analysis.

[§] NS = not significant, * Significant correlation ($p<0.05$).

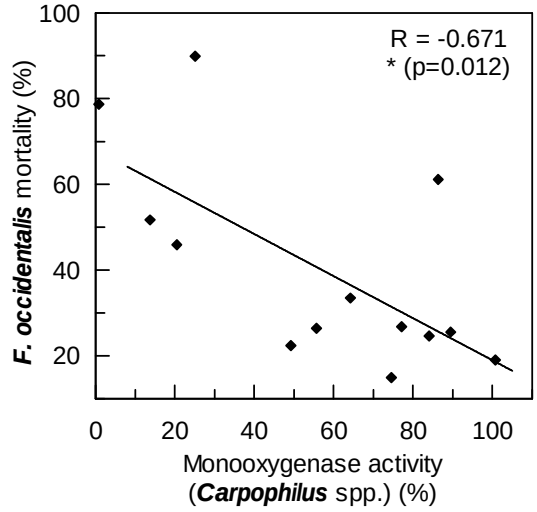


Fig. 6.10. The correlation between monooxygenase activity with synergists in *Carpophilus* spp. and the synergism of pyrethrum in the discriminating dose bioassay with *Frankliniella occidentalis* (chapter four). Each point indicates a different synergist (n=13), lines were fitted by linear regression, R is the correlation coefficient and * indicates a significant correlation (p<0.05).

6.3.2.4 Enzyme inhibition by pyrethrum

In ***Carpophilus*** spp., pyrethrum showed some esterase inhibition (Fig. 6.11a), with a large IC_{50} (± SE) (9276.87 ppm (± 12 769.39)) and a Y-range (± SE) of 100.76% (± 88.36). In ***M. aeneus***, pyrethrum gave no inhibition of esterases (Fig. 6.11a). Pyrethrum gave good inhibition of GSTs in ***Carpophilus*** spp., with an IC_{50} (± SE) of 1101.05 ppm (± 178.52) and a Y-range of 100% (Fig. 6.11b).

Pyrethrum showed some inhibition of ***M. aeneus*** monooxygenases, with monooxygenase activity (± SE) of 88.84% (± 0.27). Inhibition of monooxygenase activity by pyrethrum in ***Carpophilus*** spp., however, was very little, with monooxygenase activity (± SE) of 97.22% (± 4.50).

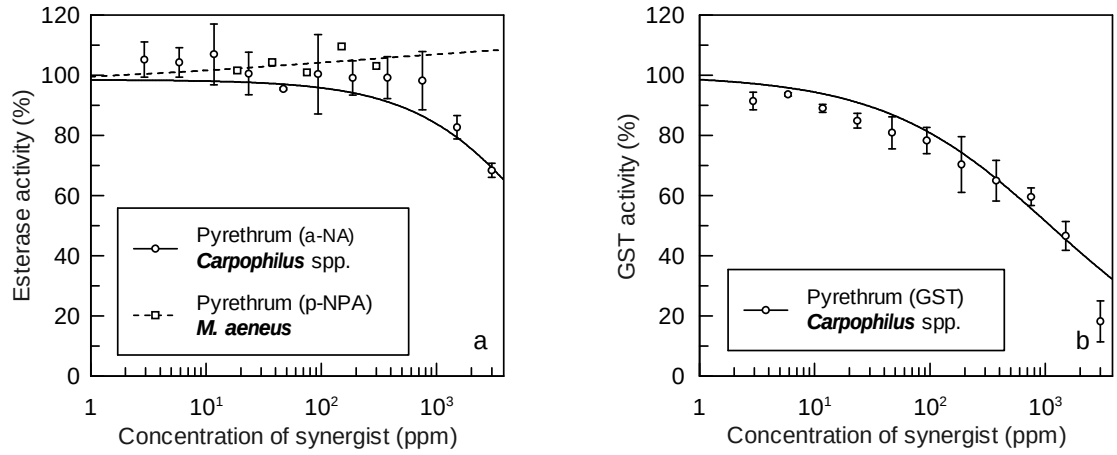


Fig. 6.11a-b. Inhibition of *Carpophilus* spp. and *Meligethes aeneus* (male and female adults) enzyme activity by pyrethrum. Fig. 6.11a represents inhibition of total esterase activity (alpha-naphthyl acetate (a-NA) (*Carpophilus* spp.) and para-nitrophenyl acetate (p-NPA) (*M. aeneus*) as substrates); Fig. 6.11b represents inhibition of glutathione-S-transferase activity (CDNB as a substrate) (*Carpophilus* spp.). Error bars represent standard deviation. Lines for Fig. 6.11a were fitted with a four parameter logistic curve. In Fig. 6.11b, the line was fitted with a two parameter logistic (lower and upper limits of 0% and 100% respectively).

6.4 Discussion and Conclusions

In ***M. aeneus***, PBO, dill apiole oil, CaB2 frac3 extract and parsley seed oil (at 100 ppm) showed significant synergism of pyrethrum using coated vial discriminating dose bioassays, although there was some inherent toxicity observed that may have been due to synergist residues in the coated vials. The methylenedioxyphenol (MDP) ring structure considered important for synergism of pyrethrum (Beroza 1954, Casida 1970, Haller ***et al***. 1942) is present in PBO, dill apiole oil and some constituents of parsley seed oil (myristicin and apiole). PBO is a well known insecticide synergist, with many examples demonstrating its synergistic efficacy. PBO has, for example, been shown to effectively synergise deltamethrin in pyrethroid-resistant strains of bed bug ***Cimex lectularius*** L. (Heteroptera: Cimicidae) (Romero ***et al***. 2009). PBO has also been found to effectively synergise spinosad in spinosad-resistant beet armyworm ***Spodoptera exigua*** (Hübner) (Lepidoptera: Noctuidae) (Wang ***et al***. 2006). Dill apiole has previously shown synergism of pyrethrum against red flour beetles ***Tribolium castaneum*** (Herbst) (Coleoptera: Tenebrionidae) (Tomar ***et al***. 1979a,b)

and adult house flies ***Musca domestica*** L. (Diptera: Muscidae) (Saxena ***et al***. 1977). Myristicin was found to have synergistic properties towards pyrethrum and carbamates against ***M. domestica*** and ***Drosophila melanogaster*** Meigen. (Diptera: Drosophilidae) (Lichtenstein & Casida 1963). Myristicin has also effectively synergised xanthotoxin, a naturally occurring insect toxicant, against the corn earworm ***Heliothis zea*** (Boddie) (Lepidoptera: Noctuidae) (Berenbaum & Neal 1985). Apiole was shown to effectively synergise parathion against ***D. melanogaster*** (Lichtenstein ***et al***. 1974). The plant extract CaB2 frac3 contains elemicin, a phenylpropene. In elemicin, the methoxy groups attached to the benzene ring are important, since elemicin is a trimethoxybenzyl compound compared to PBO, which is a dimethoxyphenyl, making these compounds structurally very similar to one another and could account for the synergistic activity found with CaB2 frac3 extract.

In the discriminating dose bioassay with ***M. aeneus*** at the lowered concentration of 10 ppm synergist, PBO was the only compound to still show significant synergism of pyrethrum. Since dill apiole oil, parsley seed oil and CaB2 frac3 extract were all more potent inhibitors of ***M. aeneus*** monooxygenases than PBO in the monooxygenase assay, the high efficacy of PBO as a synergist could additionally possibly be ascribed to its surfactant action, enhancing the penetration of pyrethrum across the insect cuticle. In ***H. armigera***, PBO has been thought to increase the penetration of esfenvalerate (Gunning ***et al***. 1995) and permethrin (Kennaugh ***et al***. 1993) through the cuticle.

Esterase assays with ***Carpophilus*** spp. showed BcB2, CaB2 frac3 and BcB1 extracts to be the most potent inhibitors of esterases ***in vitro***. Of these compounds, only CaB2 frac3 extract showed any efficacy as a synergist of pyrethrum in ***M. aeneus***. PBO and dill apiole oil, which showed the highest efficacy as pyrethrum synergists with ***M. aeneus***, failed to inhibit ***Carpophilus*** spp. esterases, however, this could also be due to differences between the esterases of different taxa.

Esterase assays undertaken with ***M. aeneus*** revealed inhibition of esterases only by very few of the tested compounds, including ClB1 extract, neem oil and BcB2 extract. None of these compounds, however, showed efficacy as pyrethrum synergists with ***M. aeneus***. PBO, dill apiole oil and parsley seed oil, which showed efficacy as pyrethrum synergists with ***M. aeneus***, did not inhibit esterases ***in vitro***. Resistance to pyrethroids in ***M. aeneus*** has been found to be due to an oxidative-based mechanism

(Philippou ***et al***. 2011) and esterases may thus have little involvement in synergism of pyrethrum in ***M. aeneus***.

The chemical structures of the most common constituents in each plant oil and extract was studied to determine whether a trend could be found regarding chemical structure and inhibition of esterases. The most potent esterase inhibitors (in both the esterase assays) included BcB1, BcB2, ClB1 and ClB2 extracts. The composition of BcB1 and BcB2 extracts are unknown, however, ClB1 and ClB2 contain limonene, a cyclic terpene. ZaB2, which also showed inhibition of esterases in both assays, contains zierone, a sesquiterpenoid. Parsley seed oil and sassafras oil contain constituents with the MDP ring (found in PBO) and showed inhibition of ***Carpophilus*** spp. esterases, however, no compounds containing the MDP ring showed inhibition of ***M. aeneus*** esterases. In general, no trend was apparent regarding chemical class and esterase inhibition, however, the plant oils and extracts used for the study were not pure and a number of constituents could have contributed towards inhibition of esterases.

Inhibition of GSTs examined using ***Carpophilus*** spp. showed BcB2 extract, neem oil and ClB2 extract to be the most potent inhibitors of GST activity. None of these compounds, however, showed pyrethrum synergism in ***M. aeneus***. PBO, which had the highest efficacy as a pyrethrum synergist, did not inhibit GSTs. Inhibition of GST activity in ***Carpophilus*** spp. did thus not correlate to observed synergism in ***M. aeneus***. This lack of a correlation could be due to species differences, despite similarities in feeding habits. The resistance mechanism in pyrethroid-resistant ***M. aeneus*** has been found to be due to an increase in oxidase activity (Philippou ***et al***. 2011) and GSTs may therefore play little role in pyrethroid or pyrethrum resistance.

Plant compounds that inhibited GSTs showed no general trend with regard to structure. The composition of BcB2 extract, which showed the highest inhibition of GSTs, is unknown, neem oil contains azadirachtin, a tetranortriterpenoid, and ClB2 extract contains limonene, a cyclic terpene. Most of the plant oils and extracts used in this study were not pure, however, but consisted of a number of compounds, any of which could contribute to the observed GST inhibition.

In ***M. aeneus***, oleic acid, a fatty acid, showed the highest efficacy as a monooxygenase inhibitor, followed by dill apiole oil and parsley seed oil (both of

which contain the MDP ring). In ***Carpophilus*** spp., BcB1 extract showed the highest efficacy as a monooxygenase inhibitor, followed by myristicin, parsley seed, dill apiole and sassafras oils, all of which contain the MDP ring. PBO and nutmeg oil (both containing the MDP ring) also showed good inhibition of ***Carpophilus*** spp. monooxygenases, and PBO also inhibited monooxygenases in ***M. aeneus***. These data revealed that the most inhibition of monooxygenases was by compounds containing an MDP ring. Compounds with other chemical structures however, also showed significant inhibition of monooxygenases, including oleic acid (a fatty acid), ZaB2 extract (a sesquiterpenoid) and CaB2 frac3 extract (a phenylpropene).

In bioassays with ***M. aeneus***, PBO, dill apiole oil, parsley seed oil and CaB2 frac3 extract were the only compounds showing significant synergism of pyrethrum. All of these compounds also gave good inhibition of ***M. aeneus*** monooxygenase activity ***in vitro***. These results support those of Philippou ***et al***. (2011), implicating the involvement of monooxygenases in pyrethroid resistance in ***M. aeneus***. Dill apiole oil and parsley seed oil showed the highest inhibition of monooxygenases out of the four compounds, followed by CaB2 frac3 extract. PBO, which was the most effective pyrethrum synergist against ***M. aeneus***, even at the lowest concentration of synergist tested (10 ppm), was less effective in inhibiting monooxygenases than dill apiole oil, parsley seed oil and CaB2 frac3 extract. The efficacy of PBO as a synergist may therefore, at least in part, be attributed to an extra facet other than its enzyme inhibition properties, perhaps its ability to enhance the penetration of lipophilic compounds such as pyrethrum through the insect cuticle.

Inhibition of monooxygenase activity by the synergists in ***M. aeneus*** and ***Carpophilus*** spp. gave a significant positive correlation. This may indicate similarities in monooxygenases between the two taxa. Dill apiole oil and parsley seed oil showed high inhibition of monooxygenases in both taxa, whereas compounds such as bergamot, aniseed and rosemary oils gave little or no monooxygenase inhibition in both taxa. Dill apiole has been found to be a highly potent inhibitor of human cytochrome P450 3A4 (CYP3A4) (Budzinski ***et al***. 2000). Grapefruit and black pepper oils, showing significant inhibition of ***Carpophilus*** spp. monooxygenases, have also been found to inhibit monooxygenase activity. Piperine from black pepper oil inhibited human CYP3A4 (Bhardwaj ***et al***. 2002) whereas grapefruit oil has been shown to inhibit human CYP3A4 (Bailey ***et al***. 1998, Kane & Lipsky 2000) and the human

cytochrome P450 isoform CYP1A2 (Fuhr ***et al***. 1993), thus indicating at least some degree of conservatism between monooxygenases from different species.

Synergism in ***F. occidentalis*** (chapter four) was found to correlate significantly to inhibition of ***Carpophilus*** spp. monooxygenases. Although a strict comparison may not be ideal between two different taxonomic orders, the high similarity between monooxygenases in terms of structure suggests that a strong inhibitor to one will also inhibit others, since the haem moiety is retained in all P450s, and if the inhibitor is interfering with the action of this moiety it is likely to do so throughout (Dr. Graham D. Moores, ***Pers. comm.***). This correlation could suggest that resistance to pyrethroids in ***F. occidentalis*** is at least partly monooxygenase-based. In ***F. occidentalis*** in Spain, resistance to most insecticides, including pyrethroids, has been attributed to monooxygenases as the major resistance mechanism (Espinosa ***et al***. 2005). Synergists that showed high inhibition of monooxygenases in ***Carpophilus*** spp. also showed significant synergism in ***F. occidentalis*** (chapter four), including parsley seed oil, dill apiole oil, sassafras oil and PBO. As was found with ***M. aeneus***, PBO, which gave the least inhibition of these compounds ***in vitro***, showed the highest synergism, which could again signify the importance of the surfactant properties of PBO in improving the cuticular penetration of pyrethrum.

The inhibition of esterases, GSTs and monooxygenases by pyrethrum was examined in ***Carpophilus*** spp. and ***M. aeneus***. In ***Carpophilus*** spp., pyrethrum gave slight inhibition of esterases and very good inhibition of GSTs, but tended not to inhibit monooxygenase activity. No insecticide resistance has been recorded in ***Carpophilus*** spp., however, and the inhibition can not be compared to a resistance mechanism but GSTs, and to a much lesser degree, esterases, seem to be binding to pyrethrum, and would thus possibly play a role in the detoxification of pyrethrum in ***Carpophilus*** spp. In ***M. aeneus***, pyrethrum did not inhibit any esterase activity but showed inhibition of monooxygenase activity. This tends to confirm the involvement of monooxygenases in detoxification of pyrethrum in ***M. aeneus***, and thus as a possible resistance mechanism in pyrethroid resistance in ***M. aeneus***. GST activity was not studied in ***M. aeneus***, but should possibly be examined in the future since in ***Carpophilus*** spp., pyrethrum was showing good inhibition of GST activity.

In conclusion, PBO showed the highest efficacy as a pyrethrum synergist against ***M. aeneus***, even at very low synergist concentrations. At a higher concentration of

synergist, dill apiole oil, parsley seed oil and CaB2 frac3 extract also synergised pyrethrum. No statistical correlations were tested for between pyrethrum synergism in ***M. aeneus*** and enzyme inhibition due to only few compounds showing synergism, however, the results were compared to inhibition found in each enzyme assay. PBO and dill apiole oil did not inhibit esterases ***in vitro***. PBO also showed no inhibition of GSTs. All four synergists inhibited ***M. aeneus*** monooxygenases ***in vitro***, with dill apiole oil and parsley seed oil showing the highest inhibition of monooxygenases. Since PBO showed the least inhibition of monooxygenases of the four compounds exhibiting pyrethrum synergism, the high efficacy of PBO as a synergist could be attributed to an additional factor other than its ability to inhibit monooxygenases, likely its surfactant properties, which could facilitate the penetration of pyrethrum through the insect cuticle. These results support the involvement of monooxygenases in ***M. aeneus*** pyrethroid resistance, as well as the importance of the MDP ring in synergism of pyrethrum.

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7 CHAPTER SEVEN: THE EVALUATION OF VARIOUS NATURAL COMPOUNDS AS POTENTIAL PYRETHRUM SYNERGISTS AGAINST HOUSE FLIES *MUSCA DOMESTICA*

Abstract

House flies ***Musca domestica*** are a serious public health pest, transmitting diseases such as cholera and typhoid fever, and also present a health and sanitation problem around poultry and livestock. The use of pyrethroids against house flies and baits containing organophosphates has led to insecticide resistance and subsequent difficulties in house fly control. Pyrethrum with a synergist such as PBO is effective in controlling house flies and no resistance has developed towards pyrethrum, however, PBO is no longer available for organic use in many countries. An organically-certifiable synergist would be beneficial in developing an organic product for use around humans, poultry and livestock. Discriminating dose bioassays revealed that PBO was the most effective pyrethrum synergist against house flies. However, dill apiole and parsley seed oils showed potential as possible organic synergists in the insecticide-susceptible WHO and resistant 381zb strains of house flies. Grapefruit oil and CaB2 frac3 extract showed additional synergistic activity towards pyrethrum in the WHO strain of house flies.

The ability of the synergists to inhibit ***M. domestica*** esterase and monooxygenase activity was examined, since synergists act by inhibiting the resistance-associated enzymes. Only neem oil and oleic acid showed esterase inhibition, however, neither synergised pyrethrum. Dill apiole oil, PBO and parsley seed oil gave the highest inhibition of monooxygenase activity, followed by CaB2 frac3 extract and grapefruit oil, all of which showed pyrethrum synergism against ***M. domestica***. The methylenedioxyphenol ring structure is found in PBO, dill apiole oil and parsley seed oil, highlighting its importance in pyrethrum synergism. Correlations tested for between monooxygenase inhibition and pyrethrum synergism were not significant when PBO was included in the correlation analysis. However, the high efficacy of PBO as a pyrethrum synergist resulted in 100% mortality in both house fly strains. PBO was thus removed from the analysis and correlations became significant for both house fly strains. This suggests the possible involvement of monooxygenases in house fly pyrethroid resistance and in synergism of pyrethrum, whilst an additional factor could be contributing to the high efficacy of PBO as a synergist, likely its surfactant action, enhancing the penetration of pyrethrum through the insect cuticle.

7.1 Introduction

The house fly ***Musca domestica*** L. (Diptera: Muscidae) is a serious pest of public health, poultry and livestock, responsible for transmitting a variety of diseases, such as typhoid fever (Cirillo 2006), cholera and ***Escherichia coli*** (Echeverria ***et al***. 1983). In the past, pyrethroids have been the chemical control agent of choice (Liu & Yue 2001), but this has led to serious resistance problems (Kristensen ***et al***. 2001, MacDonald ***et al***. 1983). More recent control methods include the use of sanitation, combined with waste management and insecticides for larvae and adult flies. Traps

are another control method, as well as natural enemies for controlling house flies. Baits containing organophosphates such as azamethiphos, dimethoate and methomyl have also been used but following the development of resistance to these chemicals, neonicotinoids have recently been introduced (Kristensen ***et al***. 2006).

Insecticide resistance can be due to a number of mechanisms, including reduced penetration of insecticides (Noppun ***et al***. 1989) and target site insensitivity, such as modified acetylcholinesterase (MACE) (Moores ***et al***. 1996) or reduced sensitivity of the nervous system (knockdown resistance or ***kdr***) (DeVries & Georghiou 1981). Metabolic mechanisms of resistance involve detoxification of insecticides by monooxygenases, esterases or glutathione-S-transferases (GSTs), either due to an altered enzyme with a higher catalytic rate, or from enzymes being present in elevated levels (Devonshire ***et al.*** 1998, Devonshire & Moores 1982, Field ***et al***. 1988, Hemingway 2000, Puinean ***et al***. 2010). In house flies, a number of these resistance mechanisms have been reported. The ***kdr*** mutation (L1014F), conferring approximately 10 to 20-fold resistance to pyrethroids (Farnham & Khambay 1995) has been correlated to pyrethroid resistance in field populations of house flies (DeVries & Georghiou 1981, Huang ***et al***. 2004). The ***super-kdr*** mutation (M918T), conferring up to 500-fold resistance to Type II pyrethroids (Farnham & Khambay 1995, Sawicki 1978), has also been found in house flies (Williamson ***et al***. 1996). Reduced penetration of insecticides has been demonstrated in pyrethroid-resistant house flies (DeVries & Georghiou 1981, Liu & Yue 2001). Elevated GSTs have been correlated to azamethiphos resistance in resistant house fly populations (Kristensen 2005). MACE has also been found in organophosphate-resistant house flies (Devonshire 1975).

Pyrethrum, with its rapid breakdown time, would be ideal for use in an Integrated Pest Management (IPM) program as an additional option in the control of house flies. Little resistance has developed in insects towards natural pyrethrum (McLaughlin 1973), however, natural pyrethrum tends to be expensive and available in limited quantities. For these reasons, piperonyl butoxide (PBO) has been used as a synergist, allowing a smaller quantity of pyrethrum to be used in an effective formulation. Synergists act primarily by inhibiting the metabolic pathway involved in detoxification of an insecticide in resistant insects, thus temporarily restoring a level of susceptibility in the resistant insects (Casida 1970, Metcalf 1967). The addition of an inexpensive compound functioning as an effective synergist would benefit both

the pyrethrum industry and consumers alike, as it expands the use of pyrethrum whilst making it a more viable and affordable method of control (Casida & Quistad 1995).

This chapter examines the potential of various natural compounds in synergising pyrethrum using ***Musca domestica*** as the test insect. Since it is known that PBO synergises pyrethrum successfully, and that PBO is capable of inhibiting both esterases and monooxygenases, the bioassay data were correlated with enzyme inhibition data to determine which enzyme system could be identified as more important in identifying potential synergists. It would be a useful tool if an ***in vitro*** enzyme assay could be used to screen for potential pyrethrum synergists. Specific compounds showing high potential ***in vitro*** could then be tested ***in vivo***, using bioassays.

7.2 Materials and Methods

7.2.1 Bioassays

M. domestica bioassays were done by topical application according to the methods described in chapter two (section 2.4.5). A susceptible house fly strain (WHO) and a multiresistant strain (381zb) were used to compare the synergistic activity of various natural plant oils and extracts towards pyrethrum. (More detail on each strain is given in chapter two, section 2.1.5). In a resistant strain, synergism would be expected to be more pronounced, due to an increased presence of the resistance-associated enzymes. Using both strains would thus allow for a comparison between pyrethrum synergism in a susceptible and a resistant strain. A dose-response curve was obtained for pyrethrum with each strain and the respective LD_{10} values for pyrethrum with each strain were used to compare the efficacy of synergists in a discriminating dose bioassay. The LD_{10} was chosen to obtain low mortality with house flies in the absence of a synergist.

7.2.1.1 Full dose response bioassays

To determine a dose-response curve for pyrethrum, five pyrethrum dosages were tested against the WHO strain of house flies, ranging from 30 ppm to 3000 ppm (diluted in acetone). Eight pyrethrum dosages were used against strain 381zb,

ranging from 30 ppm to 10 000 ppm (diluted in acetone). House flies treated with acetone only were included as controls. Each treatment consisted of approximately ten adult house flies (male and female) and the bioassays were performed in triplicate. Data were analysed with a probit analysis (Finney 1971), using PoloPlus (version 1.0, LeOra Software), taking control mortality into account (Abbott 1925).

Significant differences were determined at the LD_{50} level (lethal dose required to kill 50% of the population) between the two house fly strains (WHO and 381zb) based on failure of the 95% confidence intervals to overlap. If no overlap of the confidence intervals occurred, the LD_{50} values were considered significantly different. To determine the level of resistance in the 381zb strain compared to the WHO strain, a resistance factor (RF) was calculated at the LD_{50} level, by dividing the LD_{50} of the resistant strain (381zb) by the LD_{50} of the susceptible strain (WHO).

7.2.1.2 Discriminating dose bioassay

Discriminating dose bioassays were undertaken to compare the efficacy of various natural compounds in synergising pyrethrum against house fly strains WHO and 381zb using the respective LD_{10} values for each strain as the discriminating dose of pyrethrum. The LD_{10} for the WHO strain was 42.74 ppm pyrethrum and for strain 381zb was 779.02 ppm (determined in section 7.3.1.1). The concentrations of 40 ppm and 800 ppm pyrethrum were therefore chosen as the discriminating dose for strains WHO and 381zb respectively. Compounds were applied topically as mixtures of 1000 ppm synergist (for both strains) with the LD_{10} of pyrethrum for each strain.

The potential synergists used for this study were chosen based on bio-activity properties, for example, insecticidal activity or insect repellence qualities, or known or possible synergistic activity. Natural plant oils tested for pyrethrum synergism against the WHO strain of house flies were aniseed, bergamot, canola, citronella, dill apiole, dill seed, geranium, grapefruit, neem, parsley seed and rosemary oils. Plant extracts tested for synergism were ZaB2, CaB2, CaB2 frac3, ClB1, ClB2, BcB1 and BcB2. Propyl gallate, an anti-oxidant, and oleic acid, a mono-unsaturated omega-9 fatty acid, were also tested. PBO was used as the standard synergist. All of the compounds are described in detail in chapter two (Table 2.1).

Due to the limited availability of 381zb house flies, many fewer compounds could be tested with this strain. The compounds generally showing the highest mortality with the WHO strain discriminating dose bioassay were chosen for the bioassay. These compounds were bergamot, dill apiole, grapefruit, neem and parsley seed oils, CaB2 frac3 extract, oleic acid and PBO.

The LD_{10} for each strain was mixed with acetone as the unsynergised pyrethrum treatment. Compounds were applied alone at 1000 ppm to determine inherent toxicity of the compounds towards house flies at the applied concentration, with acetone as the control. Approximately ten house flies (male and female adults) were used per treatment and each treatment was replicated three times.

Data for the discriminating dose bioassays were analysed using a logistic regression, with an assumed binomial distribution and logit-transformed data, using the programs ASREML and Genstat (version 12.2). The model incorporated both compounds treated with pyrethrum and compounds alone into one analysis. Pairwise comparisons were done to determine significant differences using Fisher's (protected) least significant difference (LSD) at the 5% level.

A correlation was tested for between mortality with synergists in the discriminating dose bioassay with pyrethrum against WHO house flies and mortality in the discriminating dose bioassay against 381zb house flies. A correlation coefficient was calculated by Excel (2003) and a table of correlation coefficients (significant values of ***r*** and ***R***) was used to determine if the correlation was significant at the 1 or 5% level.

7.2.2 Biochemical assays

Enzyme inhibition assays were used to assess the ability of the various natural plant oils and extracts to inhibit house fly esterases and monooxygenases. Inhibition of enzyme activity could give an indication of which enzyme system played more of a role in synergism and thus possibly in resistance towards pyrethrum and / or pyrethroids.

7.2.2.1 Esterase assay with para-nitrophenyl acetate as a substrate

The esterase-inhibiting capabilities of the potential synergists were compared using total esterase assays with para-nitrophenyl acetate as the substrate. Homogenate was prepared by homogenising 40 house flies (of each strain separately) in 4 ml of 0.02 M phosphate buffer, pH 7. Homogenates were centrifuged at 10 000 rpm for three minutes and supernatant was used immediately for the assays, which were run as described in chapter two (section 2.5.1.2).

Compounds tested for inhibition of esterase activity with the WHO strain of house flies were the same as those used in the WHO strain discriminating dose bioassay (section 7.2.1.2). All compounds were 10 000 ppm stock solutions (in acetone) and acetone was used as a control. The ability of pyrethrum (10 000 ppm stock solution in acetone) to inhibit total esterase activity was also examined. If pyrethrum was binding to esterases, it could indicate the potential involvement of esterases in detoxification of pyrethrum.

For house fly strain 381zb, the compounds used in the discriminating dose bioassay with that strain were also examined for inhibition of esterase activity (section 7.2.1.2). Compounds used were 10 000 ppm stock solutions (in acetone).

Data for the total esterase assays were processed according to the methods in chapter two (section 2.6), by calculating activity as a percentage of the uninhibited control, and using Grafit to fit a four parameter logistic and calculate the IC_{50} and Y-range values for inhibition curves (as described in chapter three, section 3.2.2.1.1).

Because so few of the compounds gave significant inhibition of esterase activity using the total esterase assay, no correlations against mortality in the discriminating dose bioassays were attempted.

7.2.2.2 Monooxygenase assay

O-deethylation of 7-ethoxycoumarin by house fly strain 381zb was measured according to Stumpf & Nauen (2001) and adapted to the microplate format as described by de Sousa ***et al***. (1995). (The monooxygenase assay was only run using strain 381zb due to time contraints at Rothamsted Research).

Fifty microlitres of a 50 000 rpm supernatant of house fly abdomen homogenate in buffer (0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF and 1.46 M sucrose) were added to the wells of a 96-well microplate (Costar, black). Acetone (3 µl) (as control) or 10 000 ppm synergist (in acetone) (3 µl) were added and left to incubate for 10 minutes at 25 °C (using the same synergists as for the strain 381zb discriminating dose bioassay). Subsequently, 80 µl of 0.5 mM 7-ethoxycoumarin and 10 µl of 9.6 mM NADPH (both diluted in 0.1 M phosphate buffer, pH 7.8) were added to each well to give final concentrations of 0.4 and 1 mM respectively. The plate was incubated for 30 minutes at 30 °C while shaking. After incubation, the reaction was terminated by the addition of 100 µl of stopping solution (0.05 M Tris/HCl buffer, pH 10) containing 5 µl of 100 mM glutathione oxidised and 0.88 µl glutathione reductase (200 U/mg protein) to remove NADPH, as described by Chauret ***et al***. (1999). The plate was left to incubate for 15 minutes at 25 °C. Quantification was provided by a SpectraMax Gemini XPS microplate reader (Molecular Devices) at an excitation wavelength of 370 nm and an emission wavelength of 460 nm (with 435 nm cut-off), using the Software SoftMax Pro version 5.4.

Data for the monooxygenase assay were processed as described in chapter two (section 2.6), by calculating activity as a percentage of the uninhibited acetone control. Inhibition of monooxygenase activity was analysed using a linear mixed model with the program Genstat (version 12.2). Pairwise comparisons were used to determine significant differences with Fisher's (protected) least significant difference (LSD) at the 5% level.

Correlations were tested for between mortality in the discriminating dose bioassay for synergists with pyrethrum against WHO and 381zb house flies and inhibition of monooxygenase activity (strain 381zb). The correlation analysis was performed as for the discriminating dose bioassays (section 7.2.1.2).

7.3 Results

7.3.1 Bioassays

7.3.1.1 Full dose response bioassays

Probit analysis was used to determine dose-response curves for both house fly strains, WHO and 381zb (Table 7.1). Based on overlap of the 95% confidence intervals at the LD_{50} level, the susceptible WHO strain had a significantly lower LD_{50} (213.42 ppm pyrethrum) than the resistant 381zb strain (1929.77 ppm pyrethrum). The resistance factor (RF) for 381zb at the LD_{50} level was 9.042.

Table 7.1. The lethal dose (LD) values with 95% confidence intervals (CI) for pyrethrum (calculated by probit analysis) from topical application bioassays against *Musca domestica* strains WHO (susceptible) and 381zb (multiresistant) (male and female adults).

LD value	Pyrethrum dose (ppm) (WHO)	95% CI (WHO)	Pyrethrum dose (ppm) (381zb)	95% CI (381zb)
LD_{10}	42.74	21.60 – 66.26	779	250 – 1278
LD_{20}	74.23	44.08 – 106	1064	422 – 1609
LD_{30}	111	72.52 – 152	1331	613 – 1907
LD_{40}	155	109 – 210	1613	840 – 2214
LD_{50}*	213	156 – 290	1930	1121 – 2559
LD_{60}	293	217 – 412	2309	1485 – 2984
LD_{70}	412	302 – 614	2797	1973 – 3573
LD_{80}	614	434 – 1005	3501	2661 – 4562
LD_{90}	1066	699 – 2043	4780	3740 – 6895
LD_{95}	1681	1023 – 3715	6182	4719 – 10 184
LD_{99}	3953	2059 – 11 574	10 015	6932 – 22 281
Number of WHO flies exposed = 257			Number of 381zb flies exposed = 287	
Slope ± SE (WHO) = 1.835 ± 0.212			Slope ± SE (381zb) = 3.253 ± 0.692	
Degrees of freedom (df) (WHO) = 15			Degrees of freedom (df) (381zb) = 22	
χ^2 (WHO) = 16.883			χ^2 (381zb) = 12.041	
			RF¥ (LD_{50}) (CI) = 9.042 (5.766 – 14.179)	

* Significant differences based on failure of the 95% confidence intervals to overlap.

¥ Resistance Factor (RF) = LD_{50} of the resistant strain (381zb) / LD_{50} of the susceptible strain (WHO).

The LD_{10} values for strains WHO and 381zb were 42.74 ppm and 779.02 ppm pyrethrum respectively (Table 7.1). The concentrations of 40 ppm and 800 ppm pyrethrum were therefore chosen for use in the discriminating dose bioassays with strains WHO and 381zb respectively.

7.3.1.2 Discriminating dose bioassay

The discriminating dose bioassay (WHO strain of house flies) showed pyrethrum with PBO, dill apiole oil, grapefruit oil, parsley seed oil, CaB2 frac3 extract and dill seed oil (100%, 59.09%, 50.00%, 41.38%, 37.04% and 36.67% mortality respectively) to have significantly higher mortalities than unsynergised pyrethrum (18.46% mortality, p=0.004) (Fig. 7.1). PBO was the most effective pyrethrum synergist, with mortality significantly higher than all of the other treatments. PBO itself showed no inherent toxicity towards WHO house flies, with the mortality caused by PBO alone (11.43%) not significantly different from acetone alone (10.39%). Dill apiole oil was the most effective potentially organically-certifiable compound tested to synergise pyrethrum and did not differ significantly from the mortality caused by pyrethrum plus grapefruit oil, parsley seed oil, CaB2 frac3 extract or dill seed oil. Dill seed oil, however, could not be considered as a synergist, due to significant toxicity caused by dill seed oil alone (causing 40.00% mortality in WHO house flies compared to 36.67% mortality for dill seed oil plus pyrethrum). Dill apiole oil, grapefruit oil, parsley seed oil and CaB2 frac3 extract showed no inherent toxicity towards house flies, with mortalities not significantly different from acetone alone.

Aniseed oil and geranium oil were the only compounds in addition to dill seed oil that showed significant toxicity towards WHO house flies, with mortalities (28.57% and 35.00% respectively) significantly higher than acetone alone (10.39% mortality).

PBO was the only synergist to show significant pyrethrum synergism (100% mortality) in the discriminating dose bioassay with house fly strain 381zb compared to unsynergised pyrethrum (29.63% mortality, p<0.001) (Fig. 7.2). Parsley seed and dill apiole oils tended to show the most synergism of pyrethrum after PBO (however, not significantly so). None of the compounds showed inherent toxicity towards 381zb house flies.

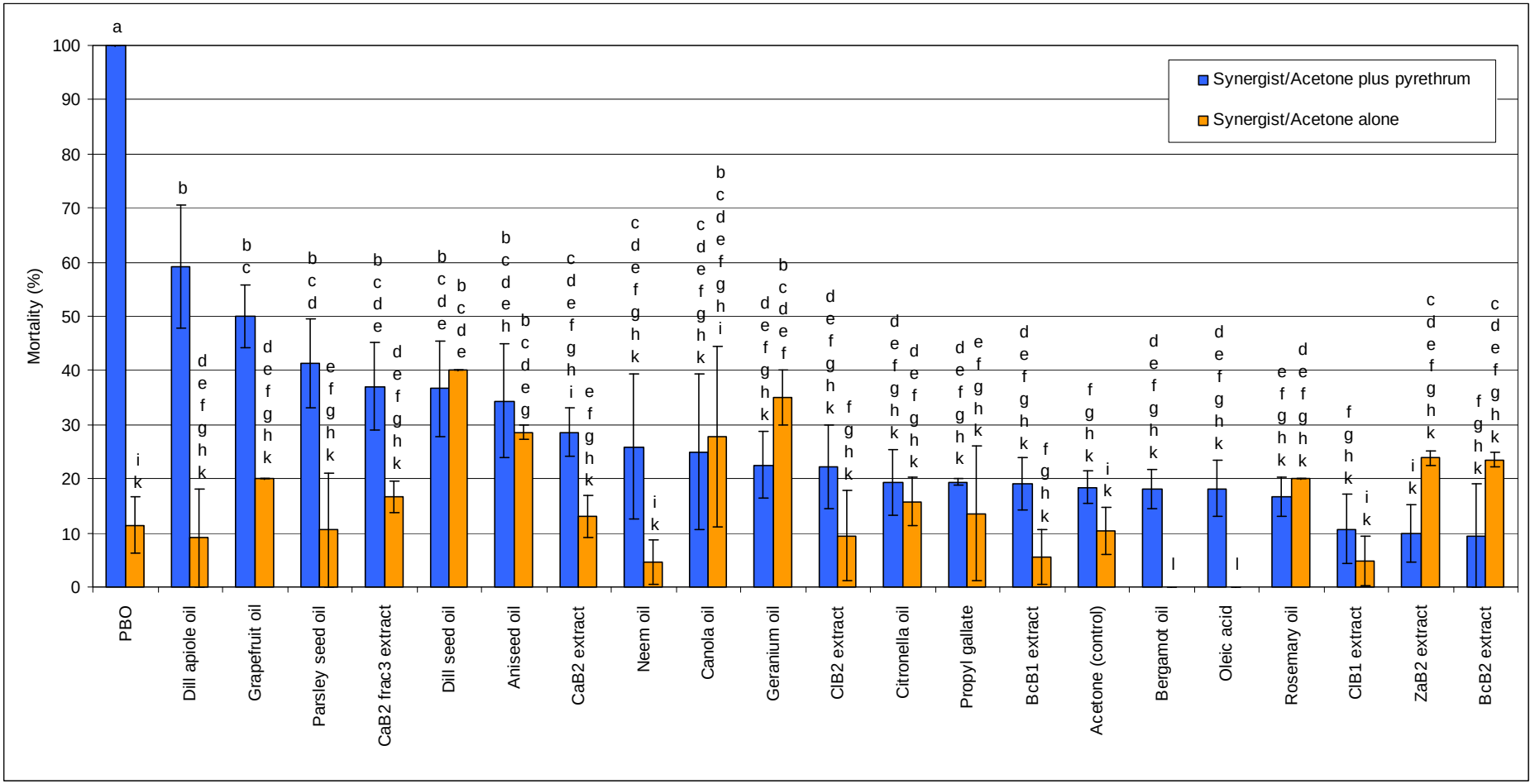


Fig. 7.1. The mean percentage mortality (± SE) of *Musca domestica* WHO strain (male and female adults) treated topically with synergists mixed with pyrethrum or with synergists alone. Acetone was used as the control. (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

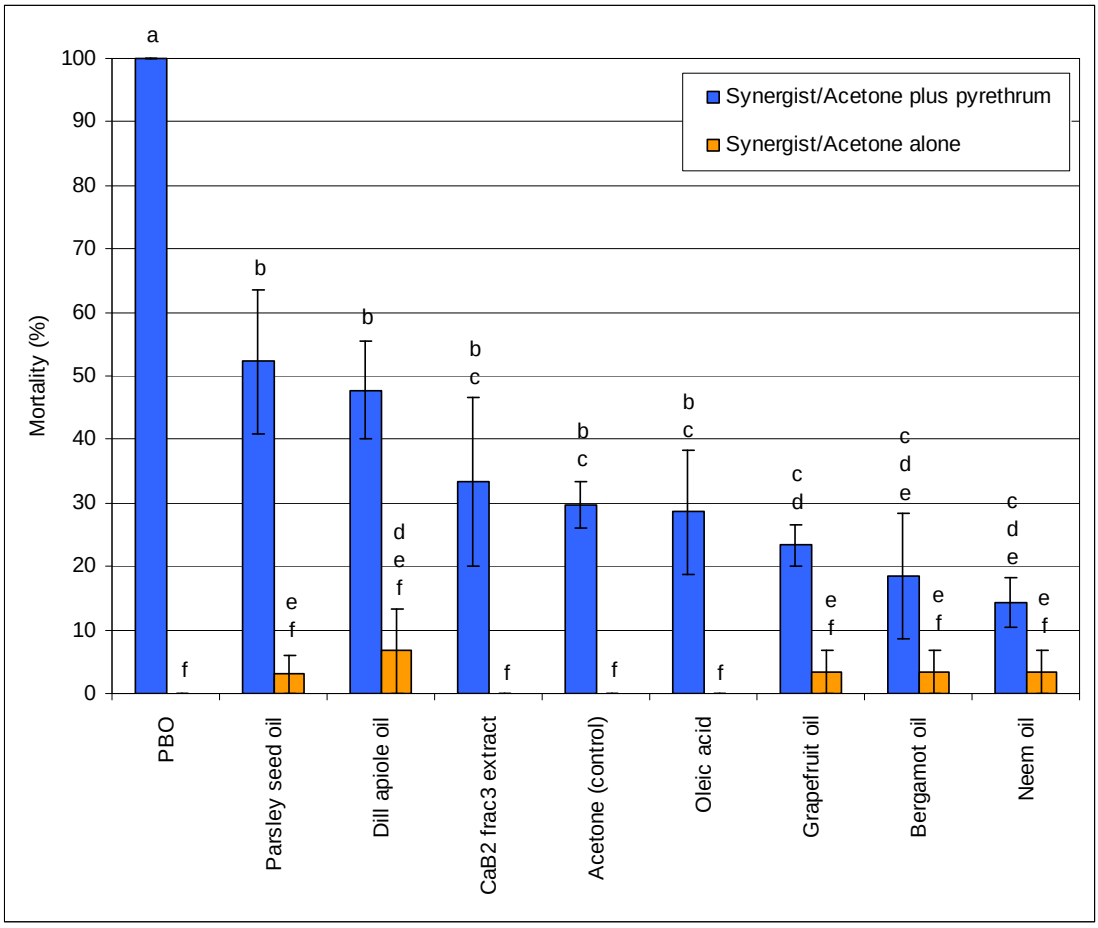


Fig. 7.2. The mean percentage mortality (± SE) of *Musca domestica* strain 381zb (male and female adults) treated topically with synergists mixed with pyrethrum or with synergists alone. Acetone was used as the control. (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

A significant positive correlation was found between the discriminating dose bioassay data (% mortality with pyrethrum) of the two strains of house flies, WHO and 381zb (correlation coefficient = 0.897, n=8, p=0.003) (Fig. 7.3), showing that similar compounds showed efficacy as pyrethrum synergists in both the resistant and the susceptible strains.

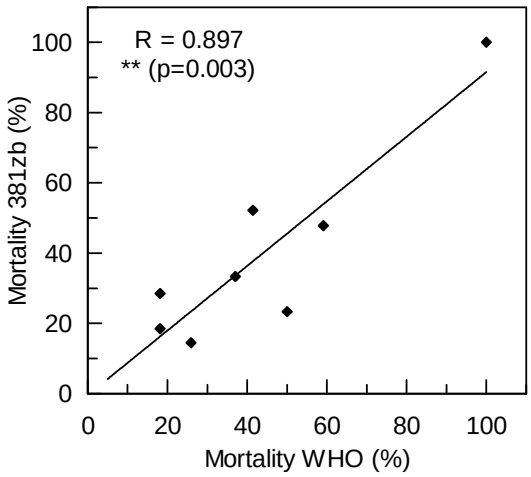


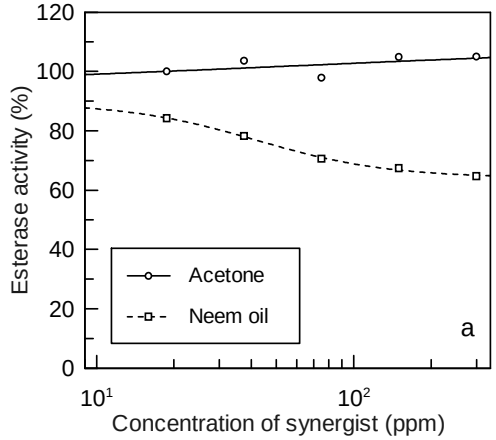
Fig. 7.3. Correlation between the percentage mortality in the discriminating dose bioassays with the two strains of *Musca domestica*, WHO and 381zb, for synergists with pyrethrum. Each point indicates a different synergist (n=8), lines were fitted by linear regression, R is the correlation coefficient and ** indicates a significant correlation (p<0.01) (two-tailed).

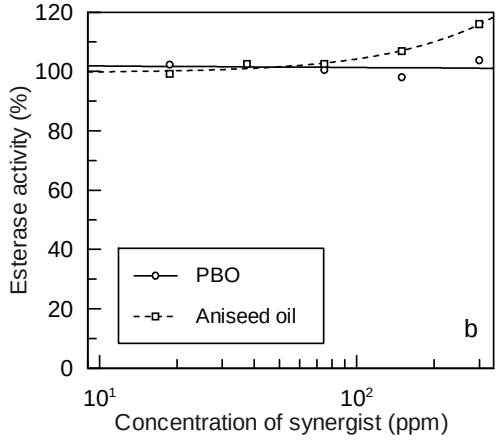
7.3.2 Biochemical assays

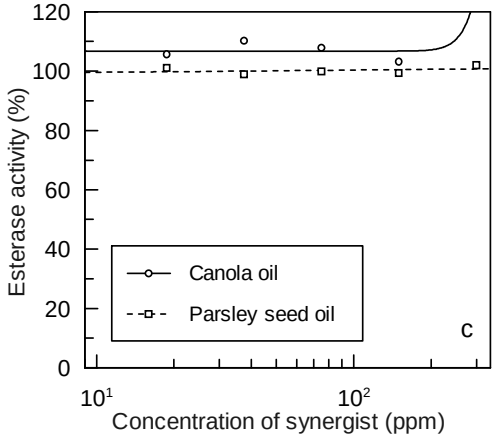
7.3.2.1 Esterase assay with para-nitrophenyl acetate as a substrate

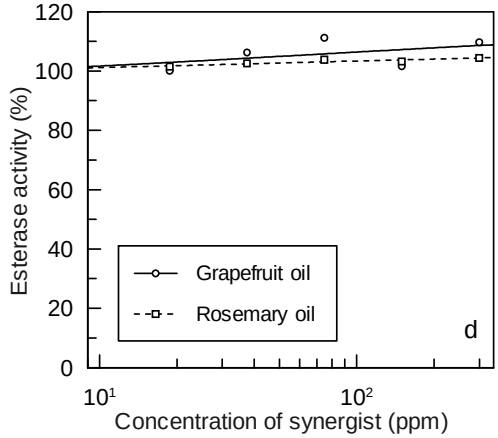
In both strains of house flies (WHO and 381zb), inhibition of esterases was very poor and most compounds gave no esterase inhibition (Fig. 7.4a-k and Fig. 7.5a-d). A control assay using acetone only showed no inhibition of esterases (Fig. 7.4a). Neem oil was the only compound to inhibit esterases in the WHO strain of ***M. domestica***, with an IC_{50} (± SE) of 42.01 ppm (± 11.06) and a Y-range (± SE) of 25.19% (± 6.65) (Fig. 7.4a).

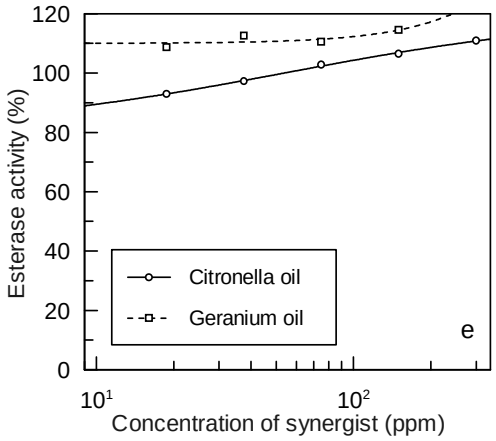
With ***M. domestica*** strain 381zb, neem oil and oleic acid showed inhibition of esterases, with neem oil showing more potency as an esterase inhibitor than oleic acid (Fig. 7.5a). Neem oil had an IC_{50} (± SE) of 50.73 ppm (± 24.01) and a Y-range (± SE) of 8.35% (± 7.66), whereas oleic acid had an IC_{50} (± SE) of 744.75 ppm (± 542.14) and a Y-range (± SE) of 85.97% (± 57.24).

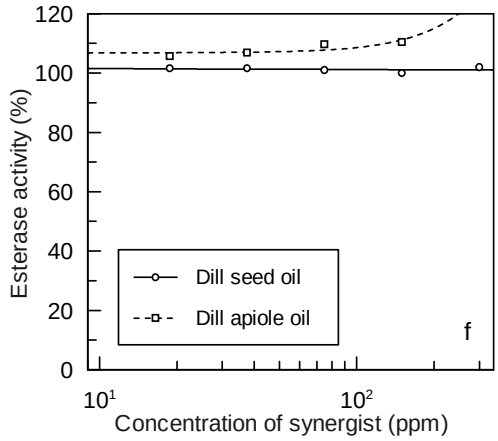

Esterase activity (%)
Concentration of synergist (ppm)
Acetone
Neem oil
a


Esterase activity (%)
Concentration of synergist (ppm)
PBO
Aniseed oil
b


Esterase activity (%)
Concentration of synergist (ppm)
Canola oil
Parsley seed oil
c


Esterase activity (%)
Concentration of synergist (ppm)
Grapefruit oil
Rosemary oil
d


Esterase activity (%)
Concentration of synergist (ppm)
Citronella oil
Geranium oil
e


Esterase activity (%)
Concentration of synergist (ppm)
Dill seed oil
Dill apiole oil
f

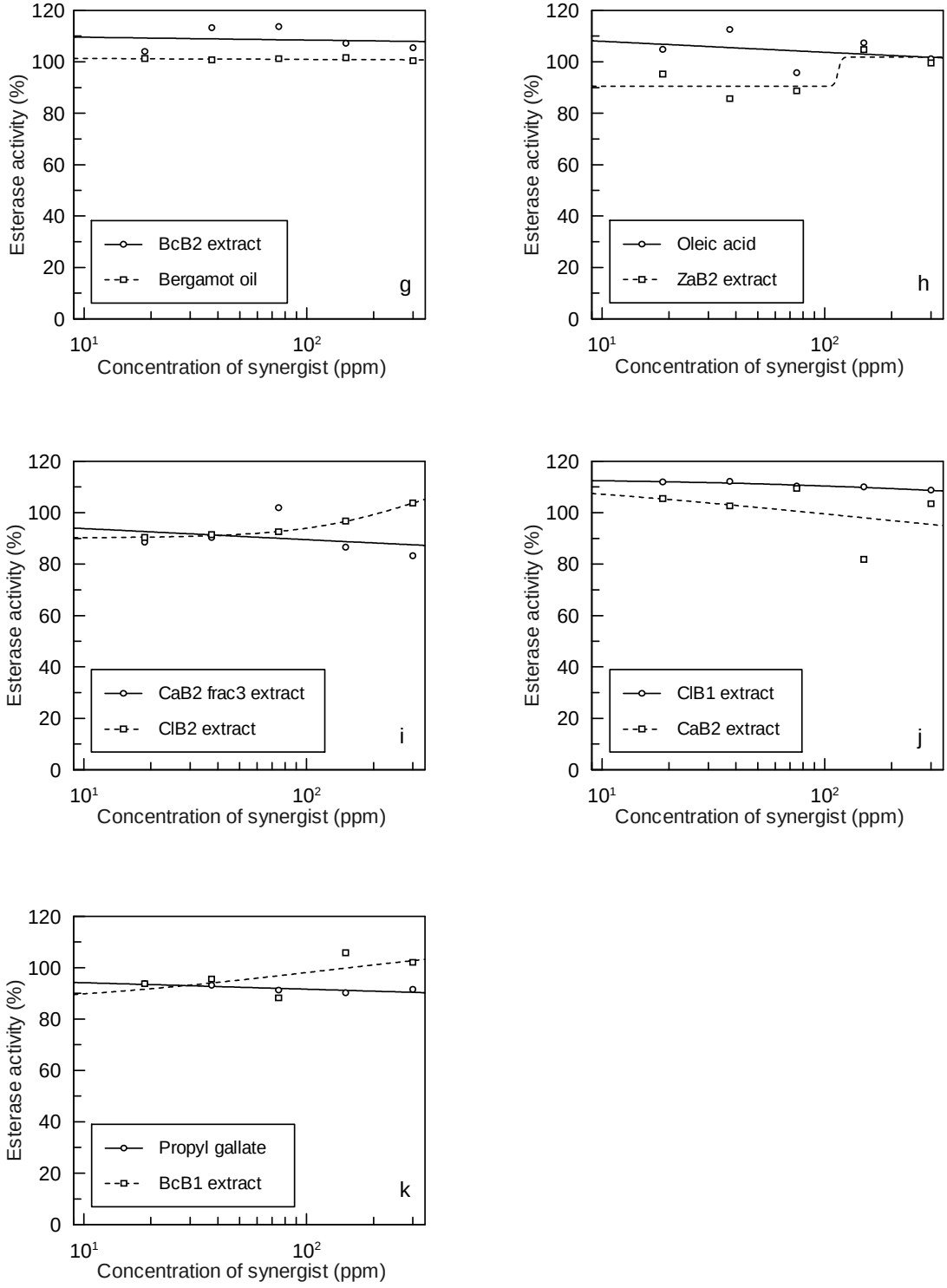


Fig. 7.4a-k. Inhibition of *Musca domestica* (WHO strain) (male and female adults) total esterase activity (para-nitrophenyl acetate as the substrate) by potential pyrethrum synergists, PBO and acetone (control). Lines were fitted with a four parameter logistic curve. (After neem oil (showing inhibition), synergists in no particular order).

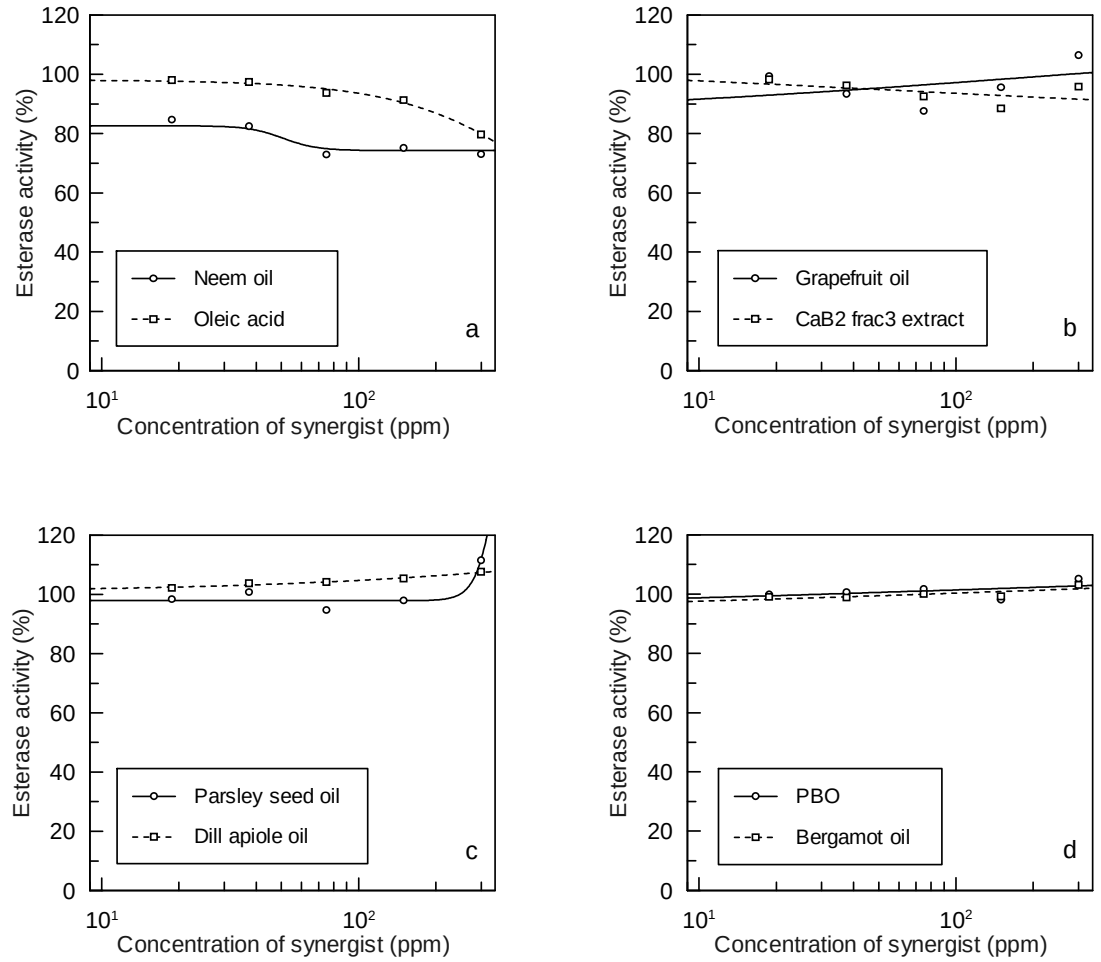


Fig. 7.5a-d. Inhibition of *Musca domestica* (strain 381zb) (male and female adults) total esterase activity (para-nitrophenyl acetate as the substrate) by potential pyrethrum synergists and PBO. Lines were fitted with a four parameter logistic curve. (After neem oil and oleic acid (showing inhibition), synergists in no particular order).

7.3.2.2 Monooxygenase assay

Using house fly strain 381zb, all compounds showed significant inhibition of monooxygenase activity compared to the acetone control ($p<0.001$) (Fig. 7.6). Dill apiole oil gave the highest inhibition of monooxygenases, neem oil the least. Dill apiole oil inhibited monooxygenase activity to a significantly higher degree than neem oil, bergamot oil and oleic acid. There was no significant difference in the inhibitory potency of dill apiole oil, PBO, parsley seed oil, CaB2 frac3 extract and grapefruit oil.

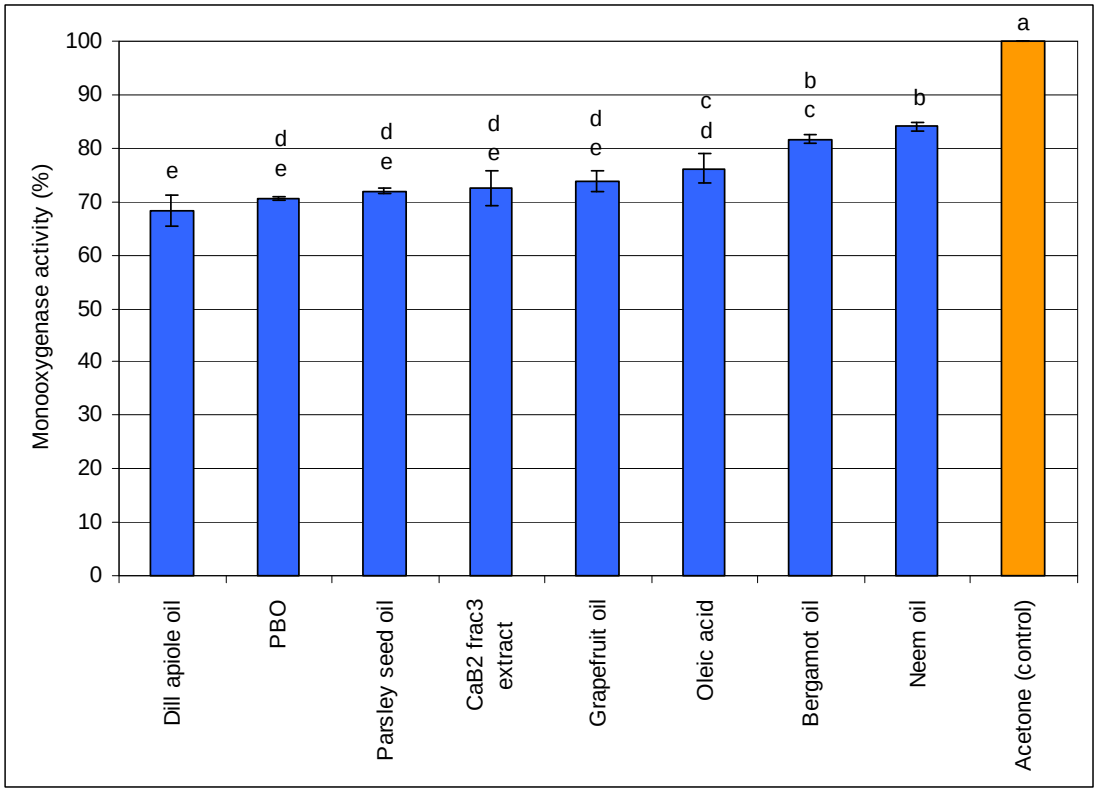


Fig. 7.6. The mean percentage monooxygenase activity (± SE) with *Musca domestica* (strain 381zb) (male and female adults) by potential pyrethrum synergists, PBO and acetone (control). (Means having the same symbols above the bar do not differ significantly from one another at the p=0.05 test level).

The correlations tested for between monooxygenase activity (%) and the discriminating dose bioassay data (% mortality with pyrethrum) for house fly strains WHO and 381zb respectively were not significant with PBO in the correlation analysis (Fig. 7.7a & b) but when PBO was excluded, both correlations became significant (correlation coefficients = -0.792 and -0.851; p=0.034 and 0.015 respectively) (Fig. 7.7c & d). (The high efficacy of PBO as a pyrethrum synergist caused 100% mortality in both strains, WHO and 381zb, and was therefore excluded from the analysis). All compounds showing ***in vivo*** synergism of pyrethrum with the WHO strain (PBO, dill apiole oil, parsley seed oil, grapefruit oil and CaB2 frac3 extract) and with strain 381zb (PBO, dill apiole oil and parsley seed oil) also showed the highest inhibition of monooxygenases.

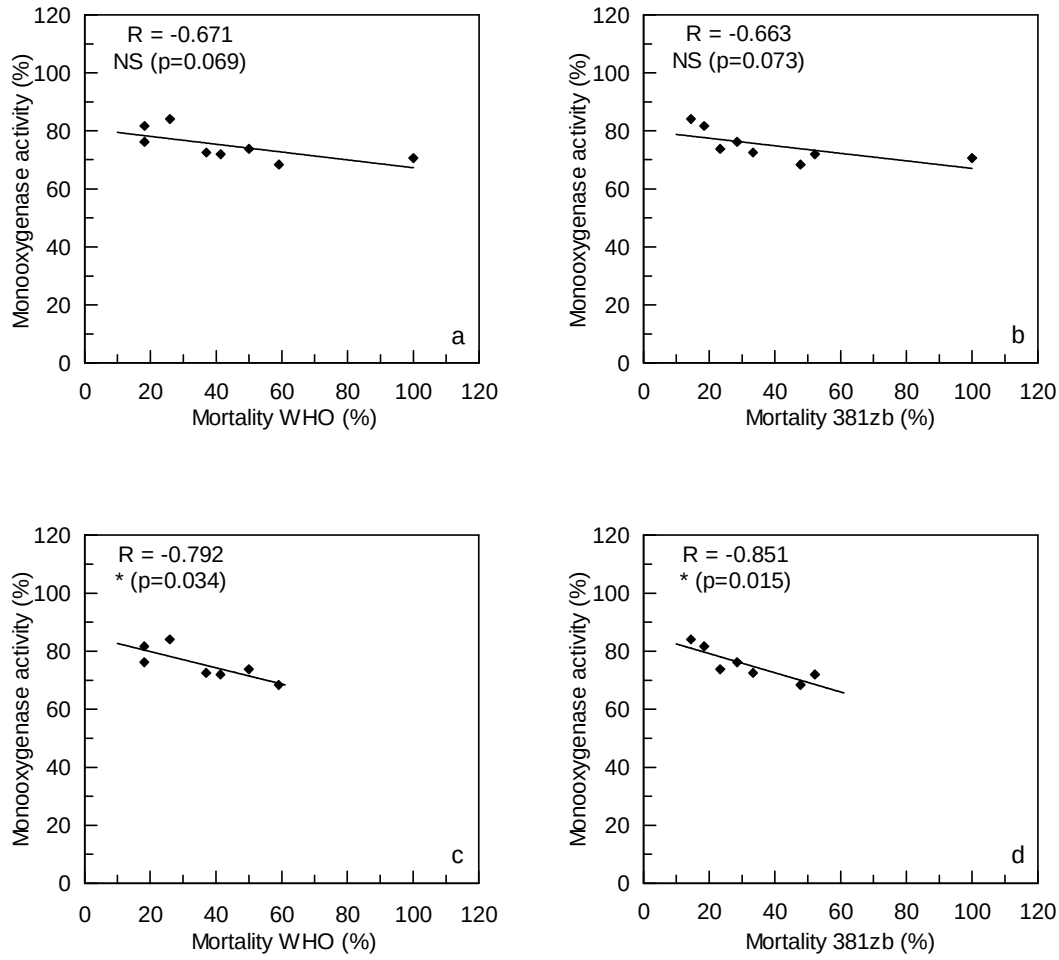


Fig. 7.7a-d. Correlations between the percentage mortality in the discriminating dose bioassays for synergists with pyrethrum with each strain of *Musca domestica* (WHO and 381zb) and monooxygenase activity (381zb). Fig. 7.7a & b represent monooxygenase activity (%) against mortality (%) from the discriminating dose bioassays with WHO and 381zb respectively (including PBO in the correlation; n=8); Fig. 7.7c & d represent monooxygenase activity (%) against mortality (%) from the discriminating dose bioassays with WHO and 381zb respectively (excluding PBO from the correlation; n=7). Each point indicates a different synergist, lines were fitted by linear regression, R is the correlation coefficient, NS = not significant and * indicates a significant correlation ($p<0.05$) (two-tailed).

7.3.2.3 Esterase inhibition by pyrethrum

In ***M. domestica*** (WHO strain), pyrethrum gave no inhibition of esterases until the highest pyrethrum dosage, where slight inhibition could be observed, using the total esterase assay (para-nitrophenyl acetate as a substrate) (Fig. 7.8). Pyrethrum had an IC_{50} (± SE) of 303.37 ppm (± 56.10) and a Y-range (± SE) of 23.90% (± 7.49).

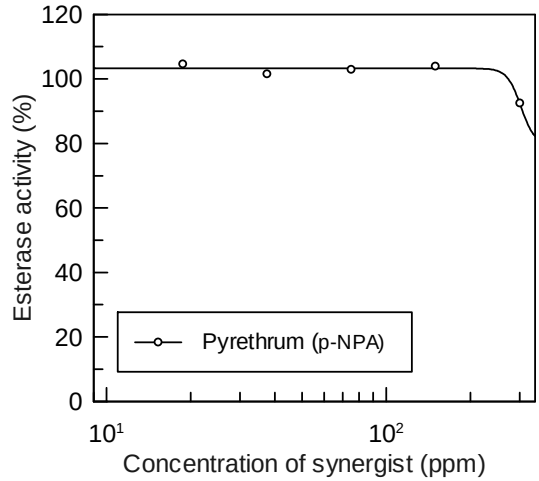


Fig. 7.8. Inhibition of *Musca domestica* (WHO strain) (male and female adults) total esterase activity (para-nitrophenyl acetate as the substrate) by pyrethrum. Line was fitted with a four parameter logistic curve.

7.4 Discussion and Conclusions

Using discriminating dose bioassays, PBO showed the highest efficacy as a pyrethrum synergist, followed by dill apiole oil and parsley seed oil in both WHO and 381zb house flies. Grapefruit oil and CaB2 frac3 extract also showed significant synergism in the WHO strain, but not in strain 381zb. PBO is known to be a very effective synergist. In ***Bemisia tabaci*** (Gennadius) (Hemiptera: Aleyrodidae), for example, PBO significantly synergised methamidophos, chlorpyrifos, fenvalerate, avermectin, emamectin benzoate, spinosads, fipronil and imidacloprid (Kang ***et al.*** 2006). Significant synergism was also found using PBO with methamidophos, fenvalerate, fipronil and avermectin in ***Plutella xylostella*** (L.) (Lepidoptera: Plutellidae), ***Phyllotreta striolata*** (F.) (Coleoptera: Chrysomelidae), ***Liriomyza sativae*** Blanchard (Diptera: Agromyzidae), ***Propylea japonica*** Thunberg (Coleoptera:

Coccinellidae) and ***Cotesia plutellae*** Kurdjumov (Hymenoptera: Braconidae) (Wu ***et al***. 2007). PBO, however, is not universally classified as acceptable for organic agriculture and a natural compound is therefore required to enhance pyrethrum activity and meet the requirements of a certified organic product. Dill apiole oil, although not as effective as PBO, does show potential as an organic synergist. Dill apiole oil has previously been found to be an effective synergist of pyrethrum against adult house flies (Saxena ***et al***. 1977) and red flour beetle ***Tribolium castaneum*** (Herbst) (Coleoptera: Tenebrionidae) (Singh ***et al***. 1976). Parsley seed oil contains myristicin and apiole, both compounds with the methylenedioxyphenol (MDP) ring structure characteristic of dill apiole and PBO. Discovery of the importance of the MDP ring in synergism of pyrethrum led to the development of synergists, such as PBO, that contain the MDP ring (Beroza 1954, Haller ***et al***. 1942, Wachs 1947). Myristicin and apiole could therefore be contributing to the synergistic activity seen by parsley seed oil. Both myristicin and apiole have previously been shown to possess synergistic activity (Berenbaum & Neal 1985, Lichtenstein & Casida 1963, Lichtenstein ***et al***. 1974).

The main constituent of grapefruit oil, which showed significant synergism of pyrethrum in the WHO strain of house flies, is limonene, a cyclic terpene, whilst CaB2 frac3 extract contains elemicin, a phenylpropene. The methoxy groups attached to the benzene ring of elemicin appear to be the most important structures for synergistic activity, since elemicin is a trimethoxybenzyl and thus structurally very similar to PBO, which is a dimethoxyphenyl compound.

Testing for inhibition of esterases using a conventional total esterase assay (with para-nitrophenyl acetate as the substrate) failed to identify those compounds interacting with the esterase activity. This has also been found in ***Myzus persicae*** (Sulzer) (Hemiptera: Aphididae) using alpha-naphthyl acetate as a substrate (Khot ***et al***. 2008), where total esterase assays failed to identify esterase inhibitors. Only neem oil reduced esterase activity in both strains, whilst oleic acid also reduced activity in strain 381zb. However, in the discriminating dose bioassays, neem oil and oleic acid both failed to show significant synergism of pyrethrum. The active component of neem, azadirachtin (a tetranortriterpenoid), is a large molecule, and it is probable that when bound to the esterase this causes steric hindrance to the substrate molecule whilst other smaller chemicals do not. Thus the result is either an indication of synergist size rather than synergist esterase inhibition potency, or

esterase activity plays little or no part in synergism of pyrethrum in ***M. domestica*** and therefore in pyrethroid resistance in strain 381zb of ***M. domestica***. Neem oil has, however, been shown to inhibit esterase activity in moths of the obliquebanded leafroller ***Choristoneura rosaceana*** (Harris) (Lepidoptera: Tortricidae) when larvae were fed diets containing neem oil (Smirle ***et al***. 1996). Azadirachtin has also been reported to act as a reversible competitive inhibitor of esterase activity in the diamondback moth ***P. xylostella*** (He 2003).

The monooxygenase assay showed the highest inhibition of monooxygenases by dill apiole oil, PBO, parsley seed oil, CaB2 frac3 extract and grapefruit oil. Budzinski ***et al***. (2000) studied the oxidase-inhibiting capability of 13 pure plant compounds and found dill apiole to be the most potent oxidase inhibitor of human cytochrome P450 3A4 (CYP3A4), revealing a degree of conservatism between P450s from different species. Significant correlations were found between the percentage mortality in the discriminating dose bioassays for both the house fly strains, WHO and 381zb, and the inhibition of monooxygenase activity when PBO was removed from the correlation analysis. This revealed that the compounds with the highest efficacy as monooxygenase inhibitors were also the most effective synergists of pyrethrum ***in vivo***. Since PBO showed significantly higher pyrethrum synergism ***in vivo*** compared to the other compounds but did not differ significantly in its monooxygenase-inhibiting capabilities, the additional synergistic efficacy could be ascribed to its ability to act as a surfactant. PBO is able to cross the insect cuticle and increase the passage of lipophilic compounds (such as insecticides) through the cuticle, thus likely improving the penetration of pyrethrum into the insect.

The MDP ring-containing compounds, dill apiole oil, PBO and parsley seed oil, showed the highest inhibition of ***M. domestica*** strain 381zb monooxygenase activity, signifying its importance in pyrethrum synergism in ***M. domestica*** and suggesting the possible involvement of monooxygenases in pyrethroid resistance in house flies. The ability of pyrethrum to inhibit ***M. domestica*** esterases was very slight, with only very little inhibition observed at the highest concentration of pyrethrum used in the total esterase assay. House fly esterases were also not inhibited by PBO, parsley seed oil or dill apiole oil, suggesting little involvement of esterases in synergism of pyrethrum and pyrethroid resistance.

In conclusion, the results suggest that in these strains of house flies, the efficacy of the pyrethrum synergists was more closely associated with inhibition of monooxygenases than with esterases. PBO, dill apiole oil and parsley seed oil, all containing the MPD ring structure, showed the highest efficacy as pyrethrum synergists and also inhibited the most monooxygenase activity. Dill apiole oil showed the most potential as an organically-certifiable pyrethrum synergist, followed closely by parsley seed oil. Neither parsley seed oil nor dill apiole oil were as effective as PBO, but could possibly be used to enhance the efficacy of pyrethrum for use as an organic product. The data indicate that the high synergistic efficacy of PBO could be due to its surfactant properties, potentially enhancing the penetration of pyrethrum through the insect cuticle. Penetration by the natural plant oils and extracts should be examined further. The involvement of glutathione-S-transferases in the synergism of pyrethrum was not investigated in this study and could be an area for further research, together with testing a wider range of organic compounds for more effective pyrethrum synergists ***in vivo***.

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8 CHAPTER EIGHT: GENERAL DISCUSSION AND CONCLUSIONS

8.1 General Discussion

8.1.1 Synergism of pyrethrum

The efficacy of a variety of natural and potentially organically-certifiable plant oils and extracts were tested for synergism of pyrethrum against ***Helicoverpa armigera***, ***Frankliniella occidentalis***, ***Myzus persicae***, ***Meligethes aeneus*** and ***Musca domestica***, compared to the standard synergist, PBO. The potential synergists were also tested for synergism of imidacloprid against ***M. persicae*** clone 5191A. Table 8.1 summarizes the plant oils and extracts that showed significant synergism in each insect species, together with PBO. PBO showed the highest efficacy as a pyrethrum synergist against all the species tested (and towards imidacloprid in ***M. persicae*** clone 5191A). In ***F. occidentalis***, pyrethrum plus parsley seed oil gave higher mortality than pyrethrum plus PBO, however, parsley seed oil at this concentration was significantly toxic towards ***F. occidentalis***. When the concentration of parsley seed oil was halved, toxicity towards ***F. occidentalis*** was significantly lower while significant synergism was still observed with parsley seed oil plus pyrethrum. The synergism was, however, significantly less than with PBO plus pyrethrum. PBO was therefore still the most effective pyrethrum synergist against ***F. occidentalis***. A synergist by definition is a non-toxic compound that enhances the efficacy of an insecticide when used in combination with that insecticide (Casida 1970, Metcalf 1967). The high efficacy of PBO as a synergist could be ascribed to its ability to inhibit monooxygenases (Wilkinson 1976) and esterases (Bingham ***et al***. 2007, Gunning ***et al***. 1999, Young ***et al***. 2005, 2006) and further enhanced by an additional factor, likely its surfactant properties, facilitating the penetration of insecticide through the insect cuticle.

Parsley seed oil and dill apiole oil showed significant synergism of pyrethrum in all species tested (however not significant only for strain 381zb of ***M. domestica***), and showed significant synergism of imidacloprid in ***M. persicae*** clone 5191A (Table 8.1). Myristicin and apiole are constituents of parsley seed oil containing the methylenedioxyphenol (MDP) ring structure characteristic of dill apiole and PBO and commonly associated with pyrethrum synergism (Beroza 1954, Casida 1970, Haller ***et al***. 1942). Myristicin and apiole have previously been shown to possess synergistic activity. Myristicin has shown synergism towards pyrethrum and carbamates against

M. domestica and ***Drosophila melanogaster*** Meigen. (Diptera: Drosophilidae) (Lichtenstein & Casida 1963), and towards xanthotoxin against the corn earworm ***Heliothis zea*** (Boddie) (Lepidoptera: Noctuidae) (Berenbaum & Neal 1985). Apiole was found to synergise parathion against ***D. melanogaster*** (Lichtenstein ***et al***. 1974). Dill apiole has also been reported to have synergistic activity, effectively synergising pyrethrum against red flour beetles ***Tribolium castaneum*** (Herbst) (Coleoptera: Tenebrionidae) (Tomar ***et al***. 1979a,b) and adult house flies ***M. domestica*** (Saxena ***et al***. 1977).

Sassafras oil, containing safrole, also with the MDP ring structure, showed significant synergism of pyrethrum against ***H. armigera*** and ***F. occidentalis*** (Table 8.1). (Sassafras oil was not tested for synergism against the other species). PBO is synthesised from the safrole molecule (Casida & Quistad 1995, Wachs 1947). The plant oils and extracts used in this study were not pure and safrole may therefore not have been present in large enough quantities within the sassafras oil to show the high synergistic activity found with PBO, which was a pure, synthesised compound. The presence of other constituents could also potentially act as antagonists, decreasing the efficacy as a synergist.

Bergamot oil showed significant synergism of pyrethrum in ***H. armigera*** and ***M. persicae*** clone 794JZ (and significant synergism of imidacloprid against ***M. persicae*** clone 5191A) (Table 8.1). Bergamot oil contains limonene (a cyclic terpene) and linalyl acetate (an acetate ester). The ester in bergamot oil could play a role in synergism of pyrethrum if it was able to bind to the esterase involved in pyrethroid resistance and act as an inhibitor. An ester could be considered as an inhibitor if its hydrolysis was very slow, thus leaving the active site of the esterase unavailable to other substrates for an extended amount of time (Dr. Graham D. Moores, ***Pers. comm.***). Limonene, however, was also found in a number of other plant oils and extracts that showed synergism of pyrethrum and / or imidacloprid, namely grapefruit oil, ClB1 extract, ClB2 extract, dill seed oil and citronella oil (mentioned below). Limonene could therefore potentially be playing a role in synergism by compounds that do not contain the MDP ring. Since the plant oils and extracts were not pure, the composition of the oils and extracts could be having an effect on the relative efficacy of the plant oil or extract as a synergist, since constituents could be acting either synergistically or antagonistically, or the relative quantity of the synergistic component could not be great enough in the plant oil or extract.

Compounds that were generally less effective as synergists but that still showed significant pyrethrum synergism were aniseed and canola oils against ***H. armigera***, oleic acid against ***F. occidentalis***, neem oil and ClB2 extract against ***M. persicae*** clone 794JZ, CaB2 frac3 extract against ***M. aeneus***, and grapefruit oil and CaB2 frac3 extract against ***M. domestica*** (WHO strain) (Table 8.1). Additional compounds showing significant synergism of imidacloprid against ***M. persicae*** clone 5191A were canola oil, oleic acid, aniseed oil, BcB2 extract, grapefruit oil, neem oil, ClB1 extract, dill seed oil and citronella oil. Oleic acid is a mono-unsaturated omega-9 fatty acid also found in canola oil, which could thus be contributing to the synergism given by canola oil as well. Oleic acid has previously been reported to synergise esfenvalerate and methomyl against the cotton leafworm ***Spodoptera littoralis*** (Boisduval) (Lepidoptera: Noctuidae) (Hatem ***et al***. 2009). CaB2 frac3 extract contains elemicin, a phenylpropene with a benzene ring. However, it is the methoxy groups attached to the benzene ring that are considered important for synergistic activity, where elemicin is a trimethoxybenzyl compound, PBO is a dimethoxyphenyl, making these two compounds structurally very similar to one another.

The feasibility of using pyrethrum as a synergised insecticide in agriculture may require identification of the active fractions of the plant oils or extracts in order to increase synergistic efficacy. A pyrethrum product for use in organic agriculture or in niche markets could offer high returns for farmers. In addition, pyrethrum is accepted as non-residual, which allows insect management immediately prior to harvest or post harvest. The niche market is important as the use of pyrethrum in agriculture would rarely compete on a cost basis against synthetic off-patent insecticides. The average cost of off-patent products such as the synthetic pyrethroids, bifenthrin, cypermethrin, deltamethrin and fenvalerate, is around $10 per hectare, whereas the average cost of pyrethrum is around $50 to $100 per hectare at the accepted application rate of between 20 and 40 g/ha. Patent-protected synthetic insecticides, however, or insecticides that are newer to the market, such as imidacloprid and pymetrozine, demand a much higher price, with a cost per hectare similar to that of pyrethrum. Pyrethrum with PBO competes with synthetic insecticides in selected high value markets such as horticulture, protected cropping (hydroponics and greenhouse production) and organic agriculture. However, with no synergist, the effective cost per hectare would double and the motivation for "conventional" farmers to use pyrethrum is less. The cost of PBO is low, at around $20/kg, so any alternative organically-certifiable synergist would need to be within a similar price range.

Table 8.1. Compounds showing significant pyrethrum synergism against *Helicoverpa armigera*, *Frankliniella occidentalis*, *Myzus persicae* (clone 794JZ), *Meligethes aeneus* and *Musca domestica*, and imidacloprid synergism in *M. persicae* (clone 5191A), using discriminating dose bioassays. (Synergists ordered from highest to lowest percentage mortality; from each respective chapter).

H. armigera	***F. occidentalis***	***M. persicae*** (clone 794JZ)	***M. persicae*** (clone 5191A) (Imidacloprid)	***M. aeneus***	***M. domestica*** (WHO strain)	***M. domestica*** (strain 381zb)
PBO[1]	Parsley seed oil[2]	PBO[1]	PBO[1]	PBO[1]	PBO[1]	PBO[1]
Bergamot oil[3]	PBO[1]	Parsley seed oil[2]	Bergamot oil[3]	Dill apiole oil[4]	Dill apiole oil[4]	Parsley seed oil[2] (NS[13])
Parsley seed oil[2]	Parsley seed oil[2,14]	Dill apiole oil[4]	Canola oil[5]	CaB2 frac3 extract[6]	Grapefruit oil[7]	Dill apiole oil[4] (NS[13])
Dill apiole oil[4]	Dill apiole oil[4]	Bergamot oil[3]	Oleic acid[8]	Parsley seed oil[2]	Parsley seed oil[2]	
Aniseed oil[9]	Sassafras oil[1,6]	Neem oil[10]	Aniseed oil[9]		CaB2 frac3 extract[6]	
Sassafras oil[1,6]	Oleic acid[8]	ClB2 extract[7]	BcB2 extract			
Canola oil[5]			Grapefruit oil[7]			
			Neem oil[10]			
			Parsley seed oil[2]			
			Dill apiole oil[4]			
			ClB1 extract[7]			
			Dill seed oil[7,11]			
			Citronella oil[7,12]			

[1] Methylenedioxyphenol (MDP) ring.
[2] MDP ring / phenylpropanoid / phenylpropene; bicyclic monoterpene.
[3] Cyclic terpene; acetate ester.
[4] MDP ring / phenylpropanoid.
[5] Mono-unsaturated omega-9 fatty acid; unsaturated omega-6 fatty acid.
[6] Phenylpropene.
[7] Cyclic terpene.
[8] Mono-unsaturated omega-9 fatty acid.
[9] Unsaturated ether / phenylpropene derivative.
[10] Tetranortriterpenoid / liminoid.
[11] Terpenoid.
[12] Monoterpenoid / alcohol.
[13] NS = not significant.
[14] Lowered concentration of parsley seed oil.

8.1.2 Enzyme inhibition

8.1.2.1 Esterase inhibition

The inhibition of esterases by the potential synergists was examined using three esterase assays, the esterase assay (alpha-naphthyl acetate as a substrate), the esterase assay (para-nitrophenyl acetate as a substrate) and the esterase interference assay. (Not all of the assays were run with all of the insect species). For the specific assays used with each of the different test insects, ***H. armigera***, ***F. occidentalis***, ***M. persicae***, ***Carpophilus*** spp., ***M. aeneus*** and ***M. domestica***, the five most potent esterase inhibitors are listed in Table 8.2, together with the ranking for PBO. The plant extracts from the University of Tasmania, BcB1, BcB2, ClB1, ClB2, CaB2, CaB2 frac3 and ZaB2, generally tended to rank amongst the most potent esterase inhibitors throughout. ClB1 and ClB2 extracts contain the cyclic terpene, limonene, whereas CaB2 and CaB2 frac3 extracts contain elemicin (a phenylpropene) and ZaB2 contains a sesquiterpenoid (zierone). Some compounds showing a small degree of synergism of pyrethrum and imidacloprid ***in vivo*** contained limonene, which could thus have been due to its ability to inhibit esterases. With the exception of ***Carpophilus*** spp., neem oil ranked amongst the five most potent esterase inhibitors for all the other species tested and with all the assays. Neem oil has previously been shown to lower esterase activity in the male and female moths of obliquebanded leafroller ***Choristoneura rosaceana*** (Harris) (Lepidoptera: Tortricidae) larvae reared on larval diet containing neem oil until pupation (Smirle ***et al***. 1996). Azadirachtin, the main component of neem oil (a tetranortriterpenoid), has been shown to be a reversible competitive esterase inhibitor in the diamondback moth ***Plutella xylostella*** (L.) (Lepidoptera: Plutellidae) (He 2003). Propyl gallate (an ester) and oleic acid (a mono-unsaturated omega-9 fatty acid) tended to also often show good inhibition of esterases with some of the species. Profenofos was only tested as an esterase inhibitor with ***H. armigera*** and ***F. occidentalis*** using the esterase assay (alpha-naphthyl acetate as a substrate) and showed the highest inhibition of esterases in both cases. Organophosphates are known esterase inhibitors and profenofos has been shown to inhibit esterases in Australian ***H. armigera*** (Gunning ***et al***. 1999).

PBO showed relatively efficient inhibition of esterase activity only with ***M. persicae*** clone 794JZ using the esterase assay (para-nitrophenyl acetate as a substrate) and the esterase interference assay; and with ***H. armigera***, using the esterase assay with

alpha-naphthyl acetate as a substrate (Table 8.2). PBO was otherwise either non-inhibitory or showed very little inhibition of esterases. ***M. persicae*** clone 794JZ and pyrethroid-resistant ***H. armigera*** (from Australia) are known to have esterase-based resistance (Devonshire & Moores 1982, Gunning ***et al***. 1996) and the inhibition by PBO of the esterases involved with resistance would thus likely contribute towards synergism of pyrethrum observed in these two species. In Australian ***H. armigera***, inhibition by PBO of the esterases related to pyrethroid resistance has previously been found (Gunning ***et al***. 1998).

Esterase inhibition was not always observed with the total esterase assays using model substrates. This may be due to synergists binding at areas other than the active site while still allowing for hydrolysis of the substrate at the active site. Use of the esterase interference assay may have revealed this binding in some species, as was found with ***M. persicae*** (clone 794JZ) (Table 8.2), since the interference assay measures indirectly synergist binding to esterases.

8.1.2.2 Glutathione-S-transferase inhibition

The inhibition of glutathione-S-transferase (GST) activity by the potential synergistic compounds was studied using ***H. armigera***, ***F. occidentalis*** and ***Carpophilus*** spp. The five most potent inhibitors of GSTs for each species are listed in Table 8.3. The plant extracts BcB1, BcB2, ClB2 and ZaB2 tended to rank amongst the most potent inhibitors of GSTs between all the species tested. Profenofos, a potent esterase inhibitor, was also found to be the most potent inhibitor of GST activity in ***H. armigera***. (Profenofos was only tested with ***H. armigera***, for the sake of comparative purposes, to compare compounds tested for ***in vivo*** synergism of pyrethrum with enzyme inhibition). Neem oil was amongst the five most potent GST inhibitors in ***F. occidentalis*** and ***Carpophilus*** spp. Myristicin (not tested in ***F. occidentalis***), black pepper (not tested in ***F. occidentalis*** and ***Carpophilus*** spp.), dill apiole and sassafras oils, all containing the MDP ring, ranked amongst the five most potent GST inhibitors between the three species, however PBO, another MDP ring-containing compound, was found to be non-inhibitory towards GST activity in all three species tested. PBO was the most effective synergist of pyrethrum ***in vivo*** against ***H. armigera*** and ***F. occidentalis*** (no bioassays were undertaken with ***Carpophilus*** spp.) and it would thus suggest that GSTs may play little or no role in synergism of pyrethrum in the pyrethroid-resistant strains of ***H. armigera*** and ***F. occidentalis*** tested in this study.

Table 8.2. The five most potent esterase inhibitors in each esterase assay for *Helicoverpa armigera*, *Frankliniella occidentalis*, *Myzus persicae*, *Carpophilus* spp., *Meligethes aeneus* and *Musca domestica*, compared to PBO. (α-NA = esterase assay (alpha-naphthyl acetate as a substrate); p-NPA = esterase assay (para-nitrophenyl acetate as a substrate)). (Compounds ordered from lowest to highest IC_{50}or index value (interference assay); from each respective chapter).

H. armigera α-NA	***H. armigera*** p-NPA	***H. armigera*** Interference assay	***F. occidentalis*** α-NA	***M. persicae*** (clone 794JZ) α-NA	***M. persicae*** (clone 794JZ) p-NPA	***M. persicae*** (clone 794JZ) Interference assay	***Carpophilus*** spp. α-NA	***M. aeneus*** p-NPA	***M. domestica*** (WHO strain) p-NPA	***M. domestica*** (strain 381zb) p-NPA
Profenofos[1]	ZaB2 extract[2]	Neem oil[3]	Profenofos[1]	Neem oil[3]	Oleic acid[4]	BcB1 extract[5]	BcB2 extract[5]	ClB1 extract[6]	Neem oil[3]	Neem oil[3]
ZaB2 extract[2]	Propyl gallate[7]	CaB2 frac3 extract[8]	Propyl gallate[7]	-	BcB2 extract[5]	Oleic acid[4]	CaB2 frac3 extract[8]	Neem oil[3]	-	Oleic acid[4]
Propyl gallate[7]	Neem oil[3]	Propyl gallate[7]	Neem oil[3]	-	Neem oil[3]	BcB2 extract[5]	BcB1 extract[5]	BcB2 extract[5]	-	-
Neem oil[3]	ClB1 extract[6]	ClB2 extract[6]	BcB2 extract[5]	-	BcB1 extract[5]	Neem oil[3]	ClB2 extract[6]	CaB2 frac3 extract[8]	-	-
BcB1 extract[5]	CaB2 extract[8]	Canola oil[9]	ZaB2 extract[2]	-	ClB2 extract[6]	ClB2 extract[6]	ZaB2 extract[2]	ClB2 extract[6]	-	-
PBO[10] ranked as 11th most effective inhibitor	PBO[10] non-inhibitory	PBO[10] non-inhibitory	PBO[10] ranked as 17th most effective inhibitor	PBO[10] non-inhibitory	PBO[10] ranked as 10th most effective inhibitor	PBO[10] ranked as 6th most effective inhibitor	PBO[10] non-inhibitory	PBO[10] non-inhibitory	PBO[10] non-inhibitory	PBO[10] non-inhibitory

[1] Organophosphate.
[2] Sesquiterpenoid.
[3] Tetranortriterpenoid / liminoid.
[4] Mono-unsaturated omega-9 fatty acid.
[5] Unknown.
[6] Cyclic terpene.
[7] Ester.
[8] Phenylpropene.
[9] Mono-unsaturated omega-9 fatty acid; unsaturated omega-6 fatty acid.
[10] Methylenedioxyphenol (MDP) ring.

Table 8.3. The five most potent glutathione-S-transferase (GST) inhibitors in *Helicoverpa armigera*, *Frankliniella occidentalis* and *Carpophilus* spp., compared to PBO. (Compounds ordered from lowest to highest IC_{50}; from each respective chapter).

H. armigera	***F. occidentalis***	***Carpophilus*** spp.
Profenofos[1]	ClB2 extract[2]	BcB2 extract[3]
BcB1 extract[3]	ZaB2 extract[4]	Neem oil[5]
Myristicin oil[6]	Neem oil[5]	ClB2 extract[2]
Black pepper oil[6]	BcB2 extract[3]	ZaB2 extract[4]
BcB2 extract[3]	Dill apiole oil[7]	Sassafras oil[6]
PBO[8]	PBO[8]	PBO[8]
non-inhibitory	non-inhibitory	non-inhibitory

[1] Organophosphate.
[2] Cyclic terpene.
[3] Unknown.
[4] Sesquiterpenoid.
[5] Tetranortriterpenoid / liminoid.
[6] Methylenedioxyphenol (MDP) ring / phenylpropene.
[7] MDP ring / phenylpropanoid.
[8] MDP ring.

8.1.2.3 Monooxygenase inhibition

The inhibition of monooxygenase activity by the potential synergistic compounds was studied using ***Carpophilus*** spp., ***M. aeneus*** and ***M. domestica*** (strain 381zb). The five most potent monooxygenase inhibitors for each species are listed in Table 8.4. Myristicin (a component of parsley seed oil), parsley seed oil, dill apiole oil, sassafras oil and PBO, all containing the MDP ring, were amongst the five compounds showing the highest potency as monooxygenase inhibitors between the three species tested. PBO ranked as the eighth most potent monooxygenase inhibitor in both ***Carpophilus*** spp. and ***M. aeneus*** and the second most potent in ***M. domestica*** (strain 381zb). CaB2 frac3 extract contains elemicin, a phenylpropene compound similar in structure to PBO (as well as safrole and myristicin, which are also classed as phenylpropene compounds). Dill apiole has been found to be a highly potent inhibitor of human cytochrome P450 3A4 (CYP3A4) (Budzinski ***et al***. 2000). Grapefruit juice has also been shown to inhibit human CYP3A4 (Bailey ***et al***. 1998, Kane & Lipsky 2000) as well as the human cytochrome P450 isoform CYP1A2 (Fuhr ***et al***. 1993), revealing a possible degree of similarity between P450s from different species. Parsley seed oil, dill apiole oil and PBO were the only compounds tested that showed synergism of pyrethrum in all the insect species tested in this study. (Sassafras and myristicin oils

were only tested against ***Carpophilus*** spp.). These results would confirm the involvement of inhibition of monooxygenase activity in synergism of pyrethrum at least in ***M. aeneus*** and ***M. domestica*** (strain 381zb), but could also suggest a possible involvement, at least in part, in the other species tested, ***H. armigera***, ***F. occidentalis*** and ***M. persicae***.

Table 8.4. The five most potent monooxygenase inhibitors in *Carpophilus* spp., *Meligethes aeneus* and *Musca domestica*, compared to PBO. (Compounds ordered from lowest to highest percentage monooxygenase activity; from each respective chapter).

Carpophilus spp.	***M. aeneus***	***M. domestica*** (strain 381zb)
BcB1 extract[1]	Oleic acid[2]	Dill apiole oil[3]
Myristicin oil[4]	Dill apiole oil[3]	PBO[5]
Parsley seed oil[3,4,6]	Parsley seed oil[3,4,6]	Parsley seed oil[3,4,6]
Dill apiole oil[3]	ZaB2 extract[7]	CaB2 frac3 extract[8]
Sassafras oil[4]	CaB2 frac3 extract[8]	Grapefruit oil[9]
PBO[5] ranked as 8th most effective inhibitor	PBO[5] ranked as 8th most effective inhibitor	

[1] Unknown.
[2] Mono-unsaturated omega-9 fatty acid.
[3] Methylenedioxyphenol (MDP) ring / phenylpropanoid.
[4] MDP ring / phenylpropene.
[5] MDP ring.
[6] Bicyclic monoterpene.
[7] Sesquiterpenoid.
[8] Phenylpropene.
[9] Cyclic terpene.

8.2 Conclusions

This project aimed to investigate the potential of a number of natural plant oils and extracts as pyrethrum synergists against some agricultural pest species, ***H. armigera***, ***F. occidentalis***, ***M. persicae*** and ***M. aeneus*** and a public health and veterinary pest, ***M. domestica***. The specific objectives were outlined in chapter one (section 1.8).

1. PBO (the standard synergist) showed the highest efficacy as a pyrethrum synergist in all insect species tested in this study.
2. Parsley seed oil and dill apiole oil showed significant synergism of pyrethrum in all species tested (though less effective than PBO), and could be useful as organically-certifiable pyrethrum synergists due to their ability to act as synergists across taxa.

3. Other compounds showing significant synergism of pyrethrum, but not across all the taxa, included bergamot oil, grapefruit oil and CaB2 frac3 extract and may thus be useful for more species-specific synergism of pyrethrum.
4. Most of the synergists showing significant pyrethrum synergism were compounds containing an MDP ring, however, compounds such as limonene and elemicin may also possibly be implicated in synergism of pyrethrum.
5. PBO showed the highest efficacy as an imidacloprid synergist in ***M. persicae*** clone 5191A, with bergamot oil, parsley seed oil and dill apiole oil also showing significant synergism of imidacloprid in ***M. persicae*** clone 5191A.
6. Temporal bioassays with ***H. armigera*** did not reveal any significant differences in pre-treatment times when synergists were applied initially followed by a later application of pyrethrum. Differences may exist between the penetration abilities of pyrethrum compared to synthetic pyrethroids into the insect body.
7. The plant extracts BcB1, BcB2, ClB1, ClB2, CaB2, CaB2 frac3 and ZaB2 tended to show the highest potency as esterase inhibitors across taxa, together with neem oil, propyl gallate and oleic acid.
8. PBO showed inhibition of ***M. persicae*** (clone 794JZ) and ***H. armigera*** esterases, but tended to be non-inhibitory towards esterases of the strains of the other species tested.
9. The plant extracts BcB1, BcB2, ClB2 and ZaB2, together with neem oil gave the highest inhibition of glutathione-S-transferase (GST) activity, with the phenylpropene-containing compounds, myristicin, black pepper and sassafras oils, and the phenylpropanoid, dill apiole oil, also showing GST inhibition.
10. PBO showed no inhibition of GST activity in all three species tested, ***H. armigera***, ***F. occidentalis*** and ***Carpophilus*** spp.
11. Myristicin (a component of parsley seed oil), parsley seed oil, dill apiole oil, sassafras oil and PBO, all containing the MDP ring, or containing constituents with the MDP ring, were amongst the compounds showing the highest potency as monooxygenase inhibitors between the three species tested, ***Carpophilus*** spp., ***M. aeneus*** and ***M. domestica*** (strain 381zb), with PBO, parsley seed oil and dill apiole oil showing high potency in all three species.
12. CaB2 frac3 extract, containing elemicin, a phenylpropene (similar in structure to safrole, myristicin and PBO) also ranked amongst the highest inhibitors of monooxygenase activity in ***M. aeneus*** and ***M. domestica*** (strain 381zb).
13. Correlations tested for between ***in vivo*** pyrethrum synergism and ***in vitro*** inhibition of esterases and GSTs were not significant with all assays and all the species tested.

14. A statistical correlation could not be made between the ***in vivo*** synergism of pyrethrum against ***M. aeneus*** and inhibition of ***M. aeneus*** monooxygenases (due to too few compounds showing pyrethrum synergism), however, all compounds showing significant synergism (PBO, dill apiole oil, CaB2 frac3 extract and parsley seed oil) were also potent monooxygenase inhibitors.
15. Significant correlations were found between ***in vivo*** synergism of pyrethrum against ***M. domestica*** (strains WHO and 381zb) and inhibition of ***M. domestica*** monooxygenases (strain 381zb) when PBO was removed from the analysis, since PBO resulted in total mortality with pyrethrum in both strains. Since PBO showed significantly higher efficacy as a pyrethrum synergist compared to all the other compounds, but not significantly higher monooxygenase-inhibiting capabilities, the high efficacy of PBO as a pyrethrum synergist could at least in part thus likely be attributed to its surfactant properties, enhancing the penetration of pyrethrum through the insect cuticle.
16. Results tended to indicate that the use of biochemical screening techniques to identify potential pyrethrum synergists may not prove particularly useful until more specific assays could be identified which would correlate more closely to bioassay results. The use of bioassays may thus still remain essential for testing synergism.

8.3 Possible areas of further research

- The potential and efficacy as pyrethrum synergists of more refined constituents from the natural plant oils and extracts showing synergistic activity, such as limonene, elemicin and myristicin.
- Studies into the relative toxicity of the six individual pyrethrin esters in different insect taxa, to determine which of the pyrethrins are more important in each taxon, and whether different esters require specific synergists as this may contribute to variability in pyrethrum synergism.
- Penetration studies to determine the ability of the synergists to penetrate the insect cuticle. Penetration studies could, for example, be conducted similarly to those for the penetration of insecticides into the insect body, such as done by Ahmad ***et al***. (2006) for deltamethrin in pyrethroid-resistant strains of ***H. armigera***, Wang ***et al***. (2005) for abamectin in ***H. armigera***, or Bull & Pryor (1990) for malathion and permethrin in ***M. domestica***. The use of radioisotopic labelling of synergists could be useful for synergism penetration studies.

Penetration studies could be done using radiolabelled pyrethrins in the presence and absence of a synergist and results compared. Insecticide and insecticide-synergist mixtures could be applied topically and at various time periods there-after, the cuticle could be washed and the radiolabel left over counted (indicating how much did not penetrate the insect cuticle). Alternately, the cuticle could be washed and liquid chromatography-mass spectrometry (LC-MS) used to measure insecticide left on the outside of the insect.

- Use of the esterase interference assay with a greater number of species, to by-pass problems using model substrates. In some insects, synergists may not always bind to the hydrolytic active site of an enzyme but may bind allosterically in a way that prevents the hydrolysis of the model substrate, whereas in other insects, the allosteric binding could still allow hydrolysis of the model substrate to occur and inhibition is not detected. A way to by-pass this would be to use the esterase interference assay, which indirectly measures binding to esterases by measuring the inhibition of acetylcholinesterase activity by azamethiphos in the presence and absence of esterases and synergists.
- Study the possible role of glutathione-S-transferase activity in pyrethroid resistance and in synergism of pyrethrum in ***M. aeneus*** and ***M. domestica***.
- Study the possible role of monooxygenase activity in pyrethroid resistance and in synergism of pyrethrum in ***H. armigera*** and ***F. occidentalis***.
- Studies into the chemical structures important for pyrethrum synergism and perhaps particularly the involvement of the MDP ring.
- Studies to determine three dimensional structures of the enzymes and synergists and the binding sites on the enzymes by the synergists and pyrethrum and how they are interacting or binding, for example, at the active or allosteric binding sites.
- Binding studies, to determine direct binding between pyrethrum, the synergists and enzymes using surface plasmon resonance, to study real time binding between natural extracts and detoxificative enzymes.
- Molecular studies to determine more specifically the enzymes involved in pyrethrum resistance, as has been done, for example, by Puinean ***et al***. (2010), studying the amplification of a cytochrome P450 gene in neonicotinoid-resistant ***M. persicae***, using microarray analysis and real-time quantitative polymerase chain reaction (PCR).

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Appendix I. The Modified Shorey Hale diet used for bollworm colonies.

Modified Shorey Hale Diet (Flour)

1.	Soy bean flour	450 g
	Wheat germ	120 g
	Brewer's yeast	105 g
	Ascorbic acid	10.5 g
	Methyl para-hydroxybenzoate (Nipagin)	6.8 g
	Sorbic acid	3.3 g
	Streptomycin sulphate	0.2 g
2.	Agar (Grade J3)	45 g + 1200 ml H_2O
3.	Formaldehyde (40% w/v)	6 ml
	H_2O	1500 ml
4.	Mould inhibitor*	8 ml

Microwave soy bean flour for 4 min on HIGH (Setting at 8 on microwave).
Blend ingredients 1, 3 and 4 together.
Mix agar and cold water and bring to boil, let cool to about 70 ^{o}C then blend with other ingredients till smooth.
Pour and set into suitable (shallow) tray.

* Mould inhibitor: 42% Propanoic acid and 4% Phosphoric acid diluted in distilled water

Appendix II. Processing data for the esterase interference assay.

(Analysis methods from Dr. Graham D. Moores, ***Pers. comm.***)

The IC_{50} value found using Grafit was converted into a percentage and given a new terminology: I (for "Index value").

The IC_{50} for 'E4 (or esterase)' was taken as 100% activity, and for 'No E4 (or esterase)' taken as 0% activity.

The IC_{50} 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 IC_{50} (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

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

where $t_{0.05,\ 21}$ is the t-value at the $\boldsymbol{p}$ = 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).