Buprofezin Resistance in B-biotype *Bemisia tabaci*

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Ellen Cottage 1909 - 2002

This thesis is dedicated to the memory of my beloved grandmother Nelly Cottage, who passed away eleven days before its submission.

She was one of my greatest supporters and an inspiration to all that knew her.

Acknowledgements

First and foremost, I thank my supervisor Dr Robin Gunning for her invaluable assistance and guidance during the course of this thesis. Without her, this PhD would not have been possible. Merci beaucoup!!

Nous ne sommes nous qu'aux yeux des autres et c'est à partir du regard des autres que nous nous assumons comme nous. Jean-Paul Sartre, *L'Être et le néant*

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I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

Emma Louise Ariel Cottage

In forming an ideal we may assume what we wish, but should avoid impossibilities.

Aristotle, 4th Century BC.

Abstract

Laboratory selection of an Australian B-biotype *Bemisia tabaci* population with the insect growth regulator buprofezin rapidly produced high levels of buprofezin resistance (in excess of 2000-fold) after 4 applications. An unselected B-biotype *B. tabaci* population was originally 11-fold resistant to buprofezin. Resistance mechanism studies showed two mechanisms of resistance.

Buprofezin resistant B-biotype *B. tabaci* had higher esterase activity and additional esterase bands compared to a buprofezin unselected B-biotype and native Australian non B-biotype *B. tabaci* strains. Esterase from buprofezin resistant B-biotype *B. tabaci* was inhibited both *in vitro* and *in vivo* by buprofezin to a far greater degree (2-fold) than in the non buprofezin selected *B. tabaci* strain. Esterase from insecticide susceptible native non B-biotype *B. tabaci* and SUD-S strains was uninhibited by buprofezin. Esterase mediated sequestration of buprofezin is proposed as a mechanism of resistance to buprofezin in *B. tabaci*. This is the first report of a resistance mechanism to buprofezin in *B. tabaci*.

Other insecticides with novel modes of action were investigated for effects on *B. tabaci* esterases. Pyriproxyfen inhibited esterase activity *in vitro* in both resistant and non selected B-biotype strains and this implied cross-resistance was confirmed by bioassay. Novaluron and pymetrozine also inhibited esterase in buprofezin resistant *B. tabaci*, but not in the unselected population. Fenoxycarb did not inhibit esterase activity in either strain.

Acetylcholinesterase (AChE) studies showed a new mode of action for IGRs and a second buprofezin resistance mechanism. Buprofezin resistant B-biotype *B. tabaci* AChE had a different electrophoretic mobility to both the unselected and non B-biotype strains. Buprofezin inhibited AChE activity in the non selected B-biotype population, but not in resistant *B. tabaci*. The mutant form of AChE in B-biotype *B. tabaci* appeared to be unrelated to previously discovered AChE variants.

Results also showed that another IGR (novaluron) inhibited AChE activity in non-buprofezin selected strain, but buprofezin resistant AChE was unaffected. Experiments indicated no significant differences in mono-oxygenase activity in either the buprofezin unselected or resistant *B. tabaci* strains.

The speed with which *B. tabaci* were selected for high levels of buprofezin resistance, and the potential for cross-resistance between insect growth regulators is a warning that buprofezin should be used sparingly against *B. tabaci* and in rotation with other insecticides. The results have already provided essential information for the whitefly insecticide resistance management strategy in central Queensland. With the escalating problem of B-biotype *B. tabaci* in many Australian crop production systems, including cotton, an effective management strategy is vital.

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Chapter 1: General Introduction

Modern day agriculture, particularly cotton growing, involves the extensive application of insecticides against pests. These chemicals can be detrimental to the environment, with many concerns arising from residues in soils and water, and spray drift into non-crop environments. Repeated use of insecticides has often led to insecticide resistance, reduced effectiveness, ever-increasing insecticide use and cost of insect control. Insecticides are a considerable component of the production expenses of cotton, with costs ranging from $A300-$A600 per hectare in Australia (Fitt, 2000).

There is an urgent need to reduce the use of environmentally destructive pesticides, although it seems probable that insecticides will be required for cotton production in the foreseeable future. Over recent years there has been considerable interest in insecticides with novel modes of action, such as chitin inhibitors, juvenile hormone analogues and feeding inhibitors. These insecticides are selective and thus act against specific pests with minimal effects on beneficial insects. With an increasing focus upon environmental issues and a need to establish a balance between agricultural production and conservation, insecticides with novel modes of action are playing an important role in reducing the use of more traditional insecticides, such as organochlorines, organophosphates, carbamates and synthetic pyrethroids (Fitt, 2000).

The whitefly *Bemisia tabaci* (Gennadius) (Fig. 1.1, Fig. 1.2) belongs to the order Hemiptera, family Aleyrodidae. It is a serious pest of many agricultural, horticultural and ornamental crops in many areas of the world (Gerling & May, 1996). World-wide populations of *B. tabaci* comprise a number of biotypes, of which one of the most damaging is the B-biotype (silverleaf whitefly). It is difficult for farmers to detect *B. tabaci* in the field because of its small size (Fig. 1.2) and rapid movement. Immature *B. tabaci* are even smaller. When whiteflies exceed treatment thresholds in the field, such as during population explosions in the US in 1992 and 1995 (Dennehy & Williams, 1997; T.J. Dennehy, pers. comm. 2002), it is already a plague situation (Fig. 1.2) and control is very difficult.

B-biotype *B. tabaci* was first detected in Australia in October 1994 (Gunning *et al.*, 1995a), and now presents a problem in cotton and many horticultural crops in Australia. Since 1994, B-biotype *B. tabaci* numbers have progressively increased in cotton in Australia (R.V. Gunning, pers. comm., 2001) and reached epidemic numbers in locations such as Emerald in Queensland in early 2002. B-biotype *B. tabaci* has been a major problem for horticulture in Queensland, northern NSW and in the Darwin area of the Northern Territory for some years (Adamson, 1999; R.V. Gunning, pers. comm., 2000). The silverleaf whitefly poses a great threat to the Australian cotton and horticultural industries (Gunning *et al.*, 1997). An economic study predicted an annual production loss in Queensland fruit and vegetable industries in 1999 of $A86 million from B-biotype *B. tabaci* infestations for that year (Adamson, 1999), which is in addition to potential losses in the cotton industry, or crop damage in other parts of Australia. The total cost of

Figure 1.1 *Bemisia tabaci* on a cotton leaf. (Photo: G. Garradd).

Figure 1.2 Large infestation of *B. tabaci* (arrowed) in cotton in Emerald, Qld., Feb. 2002. (Photo: R.V. Gunning).

B-biotype *B. tabaci* to Australian plant production in later years is likely to be much higher than $A86 million.

B. tabaci causes damage to plants through direct feeding, excreting honeydew, transmission of plant viruses and inflicting plant physiological disorders. B-biotype *B. tabaci* is difficult to control because it is highly resistant to many conventional insecticides, such as organophosphates, carbamates and pyrethroids. Thus, insecticides with novel modes of action, such as insect growth regulators (IGRs), are playing an increasingly important role in managing B-biotype *B. tabaci* throughout the world. Buprofezin is an example of an IGR. Buprofezin is a potent agent against *B. tabaci*, affecting larval stages and suppressing embryogenesis through direct contact and vapour toxicity (Ishaaya *et al.*, 1988; De Cock *et al.*, 1990; Ishaaya, 1990, Horowitz & Ishaaya, 1992). The IGRs are promising whitefly control agents for use against B-biotype *B. tabaci* in Australia.

Very little is known, however, about the mode of action of buprofezin, or other insect growth regulators, on *B. tabaci*, the potential for resistance development, or how IGRs would fit into a resistance management program in Australia. Therefore, the objective of this PhD study was to investigate insect growth regulators and in particular, buprofezin, which may be used in Australia. This project studied the potential of B-biotype *B. tabaci* to develop resistance, identified resistance mechanisms and the potential for cross-resistance within the IGR group.

1.1 General background on *Bemisia tabaci*

Bemisia tabaci was first described in 1889 as a tobacco pest in Greece (Gennadius, 1889 cited by Brown *et al.*, 1996). Since this time, the pest status of *B. tabaci* has greatly increased. It now causes many hundreds of millions of dollars of plant damage worldwide through lost production per year. The geographic range of *B. tabaci* includes most of the world's tropical and subtropical countries, and colder climatic countries with greenhouse crops such as the Netherlands, Japan and Canada.

Taxonomy

Bemisia tabaci has the common names of the sweetpotato, cotton or tobacco whitefly. Worldwide, there are many different biotypes of *B. tabaci*. Biotypes were proposed in the 1950s to describe *B. tabaci* populations with definitive host associations and specific virus-vector capabilities (Brown *et al.*, 1995b). Biotypes of *B. tabaci* were identified using esterase banding patterns on polyacrylamide gels (PAGE) (Costa & Brown, 1991). B-biotype *B. tabaci* can be distinguished from other biotypes by its banding patterns following hydrolysis of naphthyl butyrate. They show the presence of a "B band" with an electrophoretic mobility of 0.14 (Byrne & Devonshire, 1993; Byrne *et al.*, 1995a; Byrne *et al.*, 2000). The presence of this esterase band initially defined the B-biotype (Costa & Brown, 1991).

There was some taxonomic controversy regarding the status of *B. tabaci* B-biotype, and in the United States, it was described as a new species, *Bemisia argentifolia* Bellows and Perring (Bellows *et al.*, 1994), with the common name of silverleaf whitefly. *B.*

argentifolia was differentiated from *B. tabaci* by several morphological features of the pupal case, such as the absence of anterior submarginal (dorsal) seta 4 (Bellows *et al.*, 1994). There are also biological differences between the B-biotype and other *B. tabaci* strains such as increased fecundity, excretion of more honeydew and induction of plant physiological disorders like squash silverleaf (Brown *et al.*, 1995a). The concept of *B. argentifolia* as a new species has not been widely accepted and the majority of whitefly researchers still regard it as a biotype of *B. tabaci*. Arguments against a new species are that the diagnostic dorsal seta can be damaged or fall off (J. Martin, pers. comm., 2000) and that populations of *B. tabaci* can, in any case, vary in characteristics such as pupal case morphology from location to location (Brown *et al.*, 1995b). Moreover, the diagnostic E_{14} esterase band is not genetically linked to biological characteristics such as the ability to cause squash silverleaf (Byrne *et al.*, 1995a). Also, B-biotype *B. tabaci* can interbreed with other biotypes to produce fertile offspring, and this does not support a new species argument (Byrne *et al.*, 1995b; Gunning *et al.*, 1997).

Pest status

The pest status of *B. tabaci* has become more acute due to the rise of damaging biotypes such as the 'B' strain. The change of *B. tabaci* from a minor to a major pest was gradual. In 1970-1979, *B. tabaci* outbreaks were recorded in India, Sudan and Israel. In 1986 it was identified as a serious pest in Florida (Price *et al.*, 1987). B-biotype *B. tabaci* has spread globally, largely through the world-wide trade in ornamentals such as poinsettia (*Euphorbia pulcherrima* Willd *ex.* Klotzsch) (Cheek & Macdonald, 1994). In 1995, a B-

biotype *B. tabaci* outbreak caused $US64 million in damage to cotton crops in Arizona alone (Ellsworth & Jones, 2002).

Biology

B-biotype *B. tabaci* is an extremely virulent whitefly which has a host range of over 500 plants from 63 families including lucerne, carrots, melons, onions, tomatoes, zucchini and cotton (USDA, 2000). Fecundity is high, with up to 300 eggs per female (Mau & Kessing, 2002). Mated females can lay both haploid (male) and diploid (female) eggs, while unmated females lay only haploid eggs, a process known as arrhenotoky or arrhenotokous parthogenesis (Van Lenteren & Noldus, 1990; Byrne *et al.*, 1995b; Isaacs, 1996). Adult male longevity is 10-22 days, whereas females live for approximately 60 days (Bower, 1995).

B-biotype *B. tabaci* can damage the plant through direct feeding, excessive production of honeydew, transmission of viral diseases and induction of physiological disorders. Direct feeding at high population levels can defoliate and kill the host. In cotton, damage can result in a large reduction in yield (Ernst, 1994). Indirect damage to plants can result from the secretion of honeydew by the insect. Honeydew is a sticky solution formed from the phloem sap of its host plant, mainly comprised of sugars and sugar components (Hendrix *et al.*, 1996). In processing honeydew contaminated cotton ("sticky cotton"), sugary deposits adhere to machinery, slowing or preventing processing of the fibre. B-biotype *B. tabaci* produces more honeydew than other *B. tabaci* biotypes, perhaps due to having greater access to phloem sap (Byrne & Miller, 1990). The honeydew also

provides a medium for black sooty mould fungus, *Capnodium* spp. (Byrne & Draeger, 1989; Horowitz & Ishaaya, 1994; Ernst, 1994; USDA, 2000). Black sooty mould fungus reduces photosynthetic activity in the leaf (Ernst, 1994; USDA, 2000).

B-biotype *B. tabaci* transmits many plant viral diseases, mainly from the geminivirus group (Cohen & Berlinger, 1986; Markham *et al.*, 1994). Some cause serious economic losses in crops worldwide (Markham *et al.*, 1994) and limit agricultural production (Mehta *et al.*, 1994). Virus transmission can occur even at low whitefly population densities. Of particular concern is the cotton leaf curl virus geminivirus, which caused many millions of US dollars in lost revenue in Pakistan (Markham *et al.*, 1996). If this virus was to be introduced into Australian cotton crops, losses could be very serious.

Feeding by B-biotype *B. tabaci* can also induce physiological disorders in horticultural produce, such as irregular ripening of tomatoes, silvering of squash foliage (Brown *et al.*, 1991; Paris *et al.*, 1993) and 'white stem' in cole crops (Brown *et al.,* 1991). The silvering of squash leaves gave rise to the common name of "silverleaf whitefly".

B. tabaci biotypes in Australia comprise the exotic B-biotype and an indigenous non-B biotype, which is found in tropical northern Australia. The northern Australian native *B. tabaci* is uncommon and not generally considered to be a pest (Gunning *et al.*, 1997), although it can transmit Australian tomato leaf curl virus (Condé & Connelly, 1994). This virus can cause losses in tomato crops in northern Australia.

B-biotype *B. tabaci* was first reported in Australia in 1994, with the initial records from Darwin, Northern Territory and Tamworth, New South Wales (Gunning *et al.*, 1995a). Its introduction was attributed to importation of infested poinsettia cuttings into Australia. The biotype is now widespread in Australia, and is established in the Northern Territory, Queensland, Western Australia, South Australia and New South Wales (Gunning *et al.*, 1997; R.V. Gunning, pers. comm. 2000).

B-biotype *B. tabaci* entered Australia with resistance to a wide range of insecticides (Gunning *et al.*, 1995a; M. Cahill, pers. comm. 1999; Gunning, 1999). These include most pyrethroids (permethrin, cypermethrin, deltamethrin, esfenvalerate), organophosphates (profenofos, methyl parathion, methamidphos, fenthion, sulprofos, dimethoate) and carbamates (methomyl, methiocarb) (Gunning, 1999). B-biotype *B. tabaci* were initially susceptible to endosulfan, imidacloprid, bifenthrin and amitraz. However, field use of these insecticides in Queensland horticulture resulted in rapid selection of resistance (Gunning, 1999). Native non-B-biotype *B. tabaci* are susceptible to all insecticides tested (Gunning, 1999). However, since B-biotype *B. tabaci* can interbreed with native non B-biotype (Byrne *et al.*, 1995b; Gunning *et al.*, 1997) there is a possibility of insecticide resistance genes being spread into indigenous *B. tabaci* populations. Development of a resistance management program is imperative.

1.2 Insect Growth Regulators and other Novel Insecticides

Insect growth regulators (IGRs) differ from traditional organic insecticides in both structure and mode of action. IGRs interfere with normal growth and development of insects. Insect growth and development are primarily regulated by the ecdysteroids and juvenile hormone, the actions of which are mimicked by chitin synthesis inhibitors and juvenile hormone analogues (JHA) respectively (Riddiford, 1985). JHAs interfere with normal metamorphic changes in insects, whereas the chitin inhibitors inhibit *in vivo* chitin synthesis, resulting in moulting disruption (Retnakaran *et al.*, 1985).

Commercial IGRs such as buprofezin, novaluron (chitin inhibitors) and pyriproxyfen (JHA) play an important role in *B. tabaci* control regimes in the USA (Dennehy *et al.*, 1996: Dennehy & Williams, 1997) and Israel (Horowitz & Ishaaya, 1996) due to resistance to other types of insecticides. Other insecticide groups with novel modes of action include feeding inhibitors such as pymetrozine.

The name "third generation insecticides" was coined by Carroll Williams (Williams, 1967) to describe insecticides which mimicked insect hormones. The first generation of insecticides was exemplified by inorganic substances such as arsenate of lead and botanical extracts like pyrethrum. The second generation included broad-spectrum synthetic organic insecticides such as organochlorines, organophosphates, carbamates and synthetic pyrethroids. Control of insects with third generation insecticides such as insect growth regulators is still under-utilised, due to the cheapness and ready availability of older chemistry, but IGRs offer many opportunities. Today, with problems of

resistance to broad-spectrum synthetic organic pesticides and environmental pollution due to insecticide residues, it is important to explore alternative insecticide options. Insect growth regulators are generally considered to be less harmful to the environment and beneficial insects. The "soft" qualities of insect growth regulators are an advantage to pest control considering the adverse environmental and public health issues associated with many other insecticides.

1.2.1 Juvenile hormone analogues (JHAs)

Juvenile hormone (JH) is a hormone required for development of immature insects and is secreted by the corpora allata (Chapman, 1971). Juvenile hormone, which is only secreted by immature insects, controls the development of the early stages of insects. Metamorphosis into a sexually mature adult requires cessation of the flow of juvenile hormone. Juvenile hormone related insecticides can be a highly desirable alternative to conventional insecticides because of their focus on developmental processes exclusive to insects (Bowers, 1985).

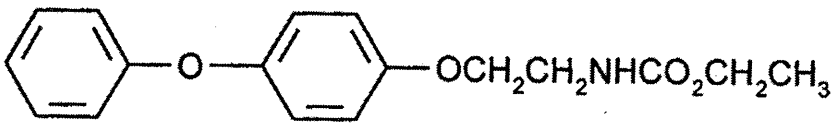
The potential of juvenile hormones and synthetic hormone analogues to manage insect populations was first recognised by Professor Carroll Williams in 1956 (Williams, 1967). Applied uses of juvenile hormone include prevention of successful adult ecdysis and reproduction, as well as ovicidal effects (Schneiderman, 1972; Sehnal, 1976). JHAs are synthetic juvenile hormones, which mimic effects of juvenile hormones on growth, development and reproduction in insects. They can be used as pesticides by disturbing the course of insect morphogenesis, thus preventing normal development (Novak, 1975).

Commercial JHAs used against *B. tabaci* include pyriproxyfen and fenoxycarb, depicted in Fig. 1.3.

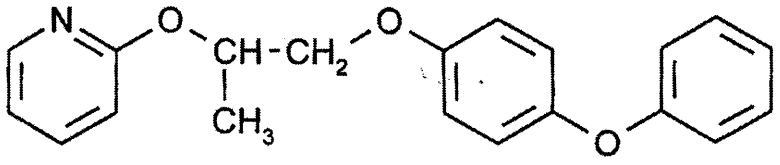
1.2.2 Chitin inhibitors

The insect cuticle is composed of both protein and a polysaccharide called chitin. Chitin occurs only in arthropods. During growth, immature insects moult, shedding their cuticle at intervals. Moulting is always preceded by the deposition of a new cuticle under the old one. The moulting cycle is initiated by an increase in the titre of moulting hormone, which causes the larvae to stop feeding. The insect's epidermis separates from the old cuticle (apolysis), and the ecdysial space that results is filled with moulting fluid, containing chitinolytic enzymes to be used later for digestion of the old cuticle. The epidermal cells then secrete a new epicuticle and cuticle. Moulting hormone levels begin to decline, stimulating digestion of old cuticle. Moulting fluid is resorbed and post-ecdysial tanning of new cuticle takes place. Moulting hormone titre then decreases to basal levels, and the insect escapes from old cuticle (ecdysis) and feeding resumes (Locke, 1974; Reynolds, 1987).

Compounds of the benzoylphenylurea (BPU) group are known to inhibit chitin synthesis, resulting in a disruption of the moulting process in insects (Grosscurt, 1978; Hajjar & Casida, 1979; Retnakaran *et al.*, 1985). Benzoylphenylureas prevent synthesis and deposition of new cuticle during the preparatory period of the moulting cycle prior to ecdysis (Reynolds, 1987).



Fenoxycarb



Pyriproxyfen

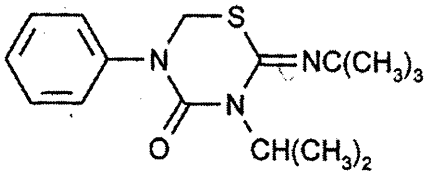
Figure 1.3 Chemical structures of commercial JHA insecticides fenoxycarb and pyriproxyfen (from Thomson, 1998).

Benzoylphenylureas consist of two substituted benzene ring structures connected by a urea bridge. The substitutions are usually halogens, though other insecticidally active groups containing methyl, methoxy, trifluoromethoxy or pentafluoroethoxy groups have been used (Retnakaran *et al.*, 1985). Structures of the chitin inhibiting insecticides buprofezin, novaluron and flufenoxuron are shown in Fig. 1.4. Benzoylphenylureas do not affect the insect until ecdysis, when it fails to shed the old cuticle properly (Reynolds, 1987). According to Retnakaran *et al.* (1985) there are levels of severity of this disruption. Either ecdysis is prevented completely so that the insect dies within its old cuticle, or ecdysis is initiated but not finished successfully so portions of the old cuticle are retained, disrupting pupal formation.

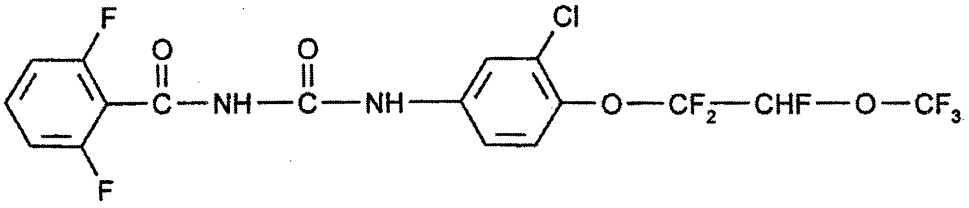
Chitin inhibitors can act very slowly on immature insects. Insects may not show any effects until adult emergence, when the adult may not completely escape from the pupal case, or may be moribund (Retnakaran *et al.,* 1985). F_1 ovicidal effects have also been shown after treatment of the parents with chitin inhibitors. Feeding failure due to displaced mouthparts can also be induced by chitin inhibitors (Retnakaran *et al.,* 1985). Buprofezin is another chitin synthesis inhibitor, acting specifically against sucking pests. Buprofezin inhibits incorporation of N-acetyl-D [1-H_3] glucosamine into chitin, thus suppressing chitin formation (Ishaaya *et al.*, 1989).

1.2.3 Other insecticides with novel modes of action

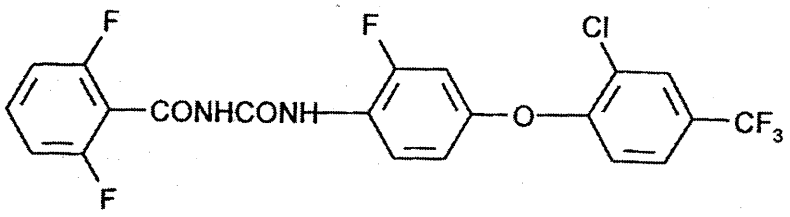
Pymetrozine (Fig. 1.5) is an asymmetric triazone compound. It is not directly toxic to sucking pests, but irreversibly affects feeding ability by acting on the salivary pump,



Buprofezin



Novaluron



Flufenoxuron

Figure 1.4 Chemical structures of commercial chitin inhibiting insecticides (from Thomson, 1998).

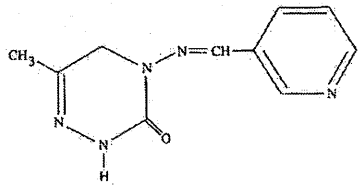


Figure 1.5 Structure of pymetrozine (from Thomson, 1998).

causing death by starvation within two to four days (Horowitz & Ishaaya, 1996). Impaired feeding ability of insects is also a means to reduce virus transmission from insects to plants.

The neo-nicotinoids are a new class of neurotoxins that act on the central nervous system of insects, causing irreversible blockage of post-synaptic nicotinergic acetylcholine receptors (Bai *et al.*, 1991; Liu & Casida, 1993). Imidacloprid (Fig. 1.6) is highly effective for the control of sucking pests such as *B. tabaci* and can be applied to soil as well as to foliage.

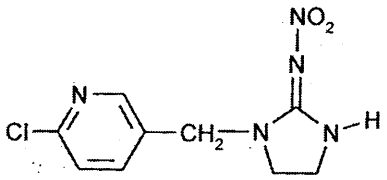


Figure 1.6 Structure of imidacloprid (from Thomson, 1998).

Diafenthiuron (Fig. 1.7) is a thiourea derivative which acts on sucking pests such as *B. tabaci*. Diafenthiuron is photochemically converted within a few hours of application to an insecticidally active carbodiimide derivative and is a potent acaricide and insecticide.

Carbodiimide inhibits mitochondrial ATP synthesis and reacts with ATPase and porin (Ruder *et al.*, 1992).

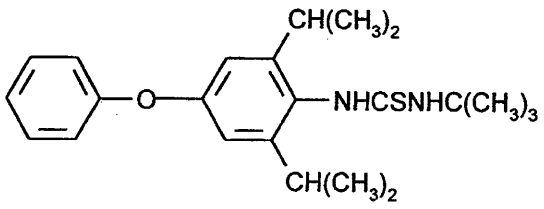


Figure 1.7 Structure of diafenthiuron (from Thomson, 1998).

Pyridaben (Fig. 1.8) is a pyridazinone compound which acts as an acaricide and it belongs to the mitochondrial electron transport inhibitor (METI) group. These inhibit complex I (NADH: ubiquinone oxidoreductase) compounds of the mitochondrial respiratory pathway (Devine *et al.*, 2001).

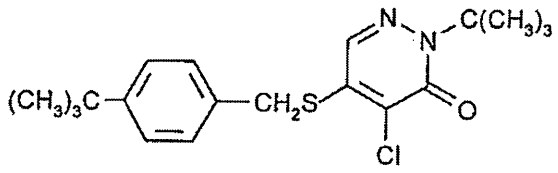


Figure 1.8 Structure of pyridaben (from Thomson, 1998).

1.3 Insecticide Resistance

The development of insecticide resistance is a major problem for agriculture, human and animal health. In 1971 the World Health Organisation defined resistance as "the developed 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" (Brown & Pal, 1971). A more pragmatic definition is "resistance marks a genetic change in response to selection by toxicants that may impair control in the field" (Sawicki, 1987). Insecticides are not mutagenic and select for genetic predispositions for resistance which already exist in populations which have not been exposed to insecticides, albeit at very low frequencies (Rees, 1977). Thus resistance is a genetic trait and is inherited. When a population of insects is placed under insecticide selection pressure, susceptible individuals are culled and thus the proportion of resistant insects increases. Within a few generations the majority, if not all, of a population can be resistant. Resistance is an extreme example of evolutionary selection.

Once resistance is established in a population, its increase is dependent upon factors such as insect fecundity, dispersal power of the insect, level of gene dominance and insecticide selection pressure and any fitness costs of resistance (Tabashnik, 1990). Insect species which produce many generations per year can build up resistant populations rapidly (Way & Cammell, 1985) of which B-biotype *B. tabaci* is a prime example (Mau & Kessing, 2002).

Insects that have greater dispersive ability are more likely to remain susceptible to insecticides, due to continual migration from susceptible populations diluting the resistant gene pool. An example in Australia is *Helicoverpa punctigera* (Wallengren), which despite intense insecticide selection pressure, has remained relatively insecticide susceptible compared to *Helicoverpa armigera* (Hübner) (Gunning & Easton, 1994). *H.*

punctigera is generally thought to be a more migratory species than *H. armigera* (Gregg, 1995). Geographical isolation can, however, increase the rate of resistance development if insects have little dispersive capacity. A greenhouse is thus an ideal environment for resistance problems and this is often the situation with whiteflies such as *B. tabaci.*

Resistance may be inherited as a recessive, partially dominant or dominant character. The level of dominance is a measure of the relative expression of the resistance gene in the heterozygote compared to the two corresponding homozygotes (Bourget *et al.*, 2000). When a resistance gene is established in a population, its rate of increase is also dependent on its dominance characteristics. A more dominant gene will increase the resistance phenotype more rapidly in a population than one that is recessive.

Insecticide selection pressure is accentuated when insecticides with the same mode of action are applied repeatedly. Resistance to one insecticide can be associated with a simultaneous development of resistance to another, even if one of these insecticides has not been used. The term 'cross-resistance' is used to describe this phenomenon. An example of cross-resistance between IGRs occurs in the pyriproxyfen resistant housefly (*Musca domestica)* (L.) to fenoxycarb and methoprene (Zhang *et al.*, 1998). Insecticide mixtures facilitate development of multiple resistance to a number of insecticides.

1.4 Insecticide resistance mechanisms

Insecticide resistance may evolve in a number of ways. There are three major mechanisms of insect resistance to insecticides: (a) metabolic mechanisms which detoxify insecticides, (b) reduced sensitivity of target sites to insecticides and (c) decreased penetration of insecticides (Sawicki, 1979; Oppenoorth, 1985). Resistance mechanisms are schematically represented in Fig. 1.9. Pests may also become functionally resistant by avoiding the insecticide, which is known as behavioural resistance or avoidance. True behavioural resistance is rare, and is of lesser importance compared to the other mechanisms described. In practice, the evolution of resistance by an insect to a particular insecticide may involve a number of mechanisms.

1.4.1 Metabolic mechanisms

All living organisms possess defence mechanisms intended to protect them from the deleterious effects of many toxic compounds. Enzymes that metabolise foreign compounds such as insecticides can serve two related functions; by changing the molecular structure so the product is less harmful than the original substance and/or by rendering the substances more polar thus increasing their rate of elimination from the body (Hassell, 1990). The three major insect enzyme groups involved in this process are mono-oxygenases, glutathione-*S*-transferases and esterases.

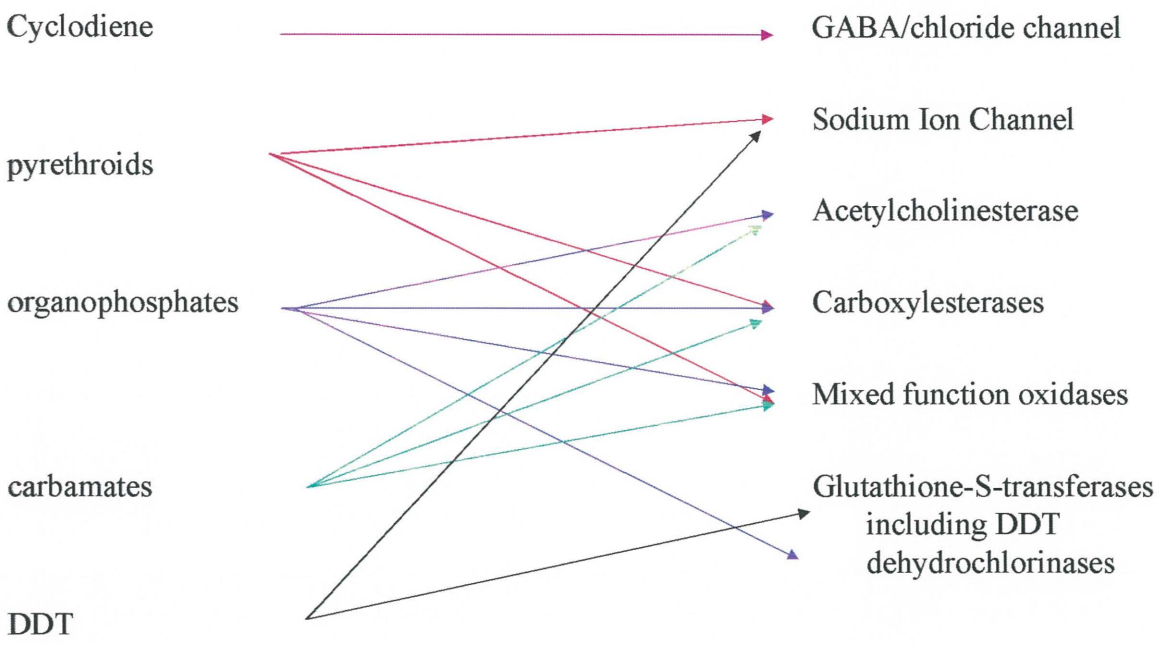


Figure 1.9 Mechanisms of resistance to major insecticide groups (after McKenzie, 1996).

Mono-oxygenases

Insect P-450 enzymes (mixed function oxidases, cytochrome P-450 mono-oxygenases) are a diverse class of enzymes. They not only provide protection against natural and synthetic toxicants, but are also involved in the biosynthesis and degradation of ecdysteroids and juvenile hormones (Wilkinson, 1985; Wheelock & Scott, 1992; Scott, 1996; Feyereisen, 1999). Insects may well have been pre-adapted to selective pressures of synthetic insecticides by cytochrome P-450 mono-oxygenases which metabolise plant defence chemicals (Hodgson & Tate, 1976; Rosenheim *et al.,* 1996). Mono-oxygenases are associated with the microsomal fraction of cells (Wilkinson, 1983). The "P-450" refers to the peak position (450nm) in the optical difference spectrum of the carbon monoxide complex with reduced cytochrome (Hodgson, 1983).

In vitro studies from both insects and mammals indicate that mono-oxygenases require both NADPH and O_2. The reaction involves oxidation, that is, incorporating one atom of molecular oxygen into a substrate while the other is reduced to water. Monooxgenases are dependent on the electron transport system (Fig. 1.10) which transfers reducing equivalents (electrons) from NADPH through a flavoprotein (NADPH-cytochrome *c* reductase, shown in Fig. 1.10 as F_{P1}) (Wilkinson, 1983). The toxin first forms a complex with the oxidised form of cytochrome P-450, then this complex is reduced by an electron passing down the chain from NADPH. The reduced cytochrome P-450/toxin complex reacts with, and activates molecular oxygen. The resulting oxygenated complex breaks down to yield a product and water. Therefore, reduction occurs in two separate stages involving one electron each, the first of these arising from NADPH (F_{P1} in Fig 1.10).

However, it is uncertain if this is true of the second electron stage, which may pass down another microsomal electron transport pathway involving cytochrome b_5 and the flavoprotein NADH-cytochrome b_5 reductase (F_{P2} in Fig. 1.10).

The general reaction equation is shown as follows:

$$NADPH + H^+ + AH_2 + O_2 \rightarrow NADP^+ + AHOH + H_2O$$

Where A= toxin

Insecticide resistance in insects can be due, at least in part, to increased levels of mono-oxygenase activity (Hodgson & Tate, 1976; Kasai *et al.,* 1998). In a fenitrothion-resistant strain of *Oryzaephilus surinamensis* (L.), cytochrome P-450 levels were 3- and 10-fold greater in larvae and adults respectively, compared with a susceptible strain (Rose & Wallbank, 1986). Other examples of mono-oxygenase activity include the CYP6A1 protein that epoxidizes the cyclodiene insecticides aldrin and heptachlor in *Musca domestica* (Schuler, 1996).

Mono-oxygenase oxidase mediated resistance is not always associated with increased levels of cytochrome P-450s (Hodgson, 1983) and may be due to the other components of the system, such as cytochrome *b*5 and NADPH-cytochrome *c* reductase (P-450 reductase). In pyrethroid resistant strains of *M. domestica*, cytochrome *b*5 and P-450 reductase levels were elevated by 1.2- to 2.2-fold and 1.5 to 2.3-fold respectively, compared to susceptible strains (Scott *et al.,* 1990). While *B. tabaci* has developed

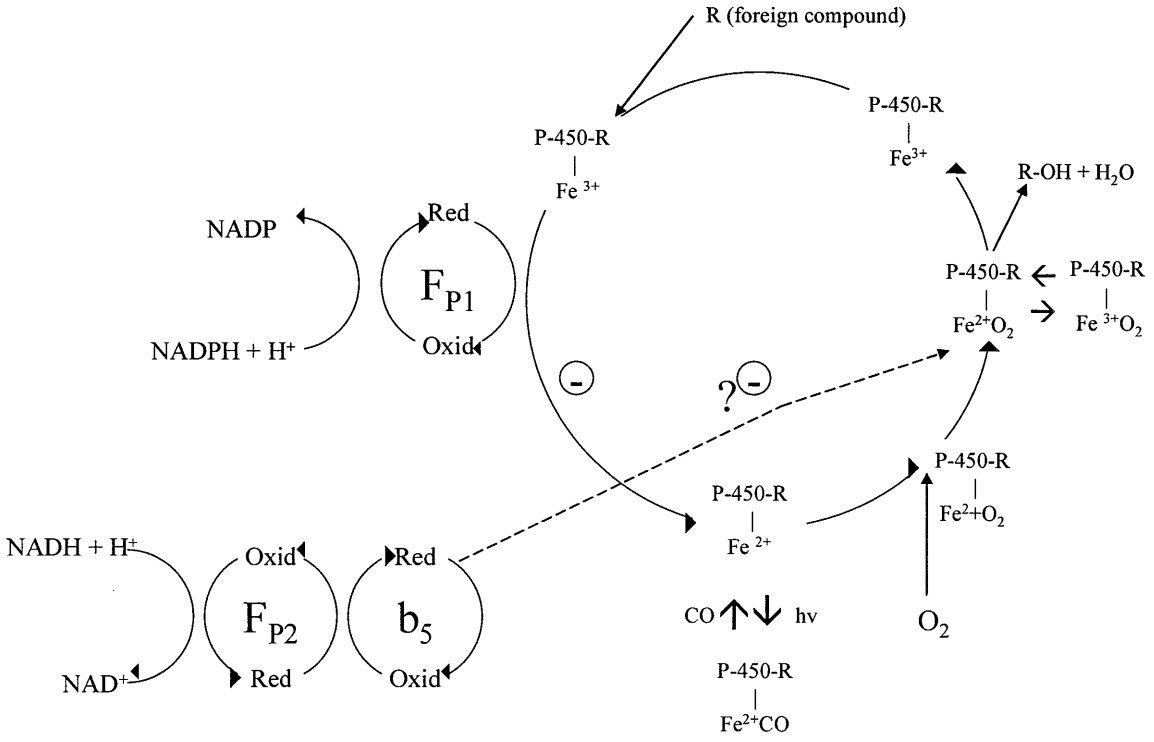


Figure 1.10 Microsomal electron pathway (after Wilkinson, 1983).

The key to the system is a common electron transport pathway that transfers reducing equivalents from NADPH through a flavoprotein (NADPH-cytochrome *c* reductase, F_{P1}). The toxin first forms a complex with the oxidised form of cytochrome P-450 and the complex is reduced by one electron passing down the chain from NADPH. The reduced cytochrome P-450/substrate complex then reacts with and activates molecular oxygen and the resulting oxygenated complex breaks down to yield the product and water.

resistance to many synthetic insecticides, there is no evidence from the literature to suggest that mono-oxygenases are involved (Chapter 7).

Glutathione-*S*-transferases

The family of glutathione-*S*-transferases comprises a general detoxifying system of enzymes in insects. It was the last discovered class of detoxifying enzymes involved in insecticide resistance. Glutathione-*S*-transferases catalyse endogenously reduced glutathione with insecticides. Insecticides are then further metabolised to mercapturic acids and excreted from the insect (Hollingworth, 1976; Dauterman & Hodgson, 1978; Oppenoorth, 1985; Kotze & Rose, 1987).

Glutathione conjugation, that is, *O*-dealkylation or *O*-dearylation (Fig. 1.11a & 1.11b) is a detoxifying reaction (Dauterman, 1985). Insecticides which can be metabolised by insect glutathione-*S*-transferases include many organophosphates; ethyl parathion (Fig. 1.11a), methyl paraoxon, dimethoate, azinphosmethyl, dipterex, methyl parathion (Fig. 1.11b), diazinon, diazoxon, isopropyl diazinon, N-propyl diazinon, diazoxon, isopropyl diazinon, *N*-propyl diazinon and ethyl chlorthion (Dauterman, 1985). For example, glutathione activity was increased compared to a susceptible population in azinphosmethyl resistant strains of Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Ahammad-Sahib *et al.*, 1994). There are no reports of glutathione-*S*-transferase involvement in *B. tabaci* resistance.

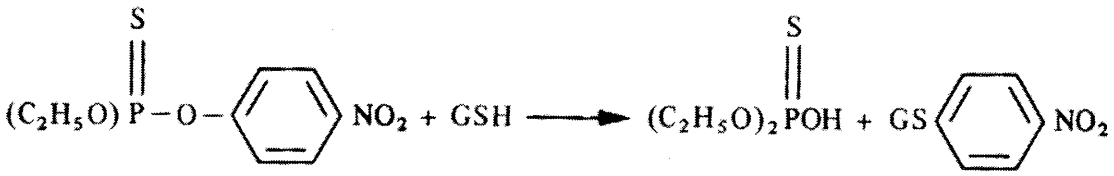


Figure 1.11a Metabolism of ethyl parathion by glutathione-*S*-transferases: Methyl parathion is demethylated to form desmethyl parathion and methyl glutathione (Dauterman, 1985).

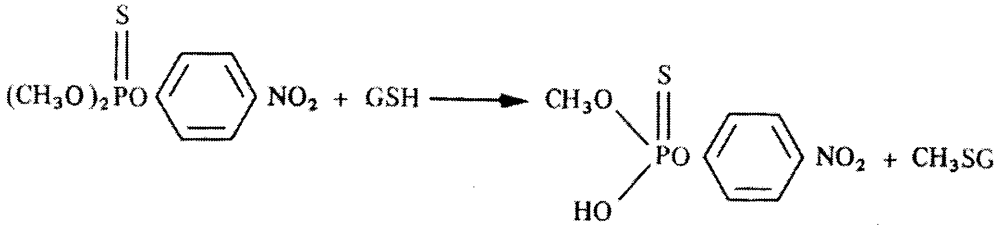


Figure 1.11b Metabolism of methyl parathion by glutathione-*S*-transferases: Dearylation of parathion to form diethyl phosphorothioic acid and *S*-(*p*-nitrophenyl) glutathione (Dauterman, 1985).

Esterases

Esterases are a group of enzymes that primarily act by hydrolysing ester insecticides such as pyrethroids, organophosphates and carbamates. Resistance may occur as a result of more active enzymes or increased amounts of enzyme. However, esterases have also been shown to sequester insecticides by binding the toxicant and slowly releasing it at a rate that the insect can excrete or detoxify (Devonshire & Moores, 1982; Devonshire, 1991). Esterases catalyse the hydrolysis of ester insecticides. Hydrolysis involves the cleavage of esters to alcohol and carboxylic acid metabolites with a consequent increase in polarity so that the toxicant can be excreted from the insect. Ester hydrolysis in the mosquitoes *Culex* spp. required increased amounts of esterase, due to the 1:1 stoichiometry of the hydrolysis reaction and slow metabolism times (Hemingway & Karunaratne, 1998).

The best known example of sequestration by esterases is that of the E4 esterase in organophosphate and carbamate resistant peach aphid *Myzus persicae* (Sulzer) (Devonshire, 1977; Devonshire, 1979; Devonshire & Moores, 1982; Devonshire, 1989). The most carbamate and organophosphate resistant *M. persicae* clone had ten picomoles of E4 esterase per aphid, which equated to approximately 1% of total protein, thus E4 esterase could detoxify large amounts of insecticide simply by sequestration (Devonshire, 1987; Devonshire, 1989; Devonshire & Moores, 1989).

Increased esterase activity is commonly associated with insecticide resistance in insects (Devonshire & Field, 1991; Rosario-Cruz *et al.,* 1997). This may be due to an altered

enzyme with a higher catalytic rate or to more enzyme being produced. Increased quantity of enzyme may be the result of gene amplification. Esterases can be detected by techniques such as polyacrylamide gel electrophoresis and staining for esterase bands or by total esterase assays, usually with α-naphthyl acetate or butyrate as the substrate (Devonshire *et al.,* 1986; Byrne & Devonshire, 1991; Gunning *et al.,* 1996a; Hemingway & Karunaratne, 1998).

M. persicae is one of the best studied examples of esterase mediated insecticide resistance. Other insects with esterase mediated resistance mechanisms include organophosphate resistant *Culex pipiens* (L.) (Hemingway & Karunaratne, 1998; Devonshire & Field, 1991), profenofos resistant *Heliothis virescens* (Fabricius) (Harold & Ottea, 1997) and pyrethroid resistant Australian *Helicoverpa armigera* (Gunning *et al.,* 1996a).

Esterase activity has been shown to be associated with a number of insecticide resistances in B-biotype *B. tabaci* such as organophosphate and carbamates (Dittrich *et al.,* 1991; Byrne & Devonshire, 1991). Pyrethroid resistance was also positively correlated with esterase activity in a Sudan field strain of *B. tabaci* (Dittrich *et al.,* 1985). Furthermore, in some B-biotype *B. tabaci* populations, pyrethroid resistance (permethrin and cypermethrin) was found to be primarily due to increased ester hydrolysis (Ishaaya *et al.,* 1987; Dittrich *et al.,* 1991; Byrne *et al.,* 2000). Detoxification of bifenthrin by ester hydrolysis in B-biotype *B. tabaci* has also been correlated to esterase activity (Riley *et al.,* 1999).

1.4.2 Target site modification

All insecticides have target sites. Some resistant insects have evolved target sites which are insensitive to insecticide attack, such as altered acetylcholinesterase and modified sodium channels.

Acetylcholinesterase

Acetylcholinesterase (AChE) is responsible for synapse re-activation via hydrolysis of the chemical neurotransmitter acetylcholine to choline and acetate in insect and vertebrate nervous systems (Fig. 1.12). Acetylcholinesterase is the target site for ester organophosphate and carbamate insecticides and inhibition, which is usually irreversible and fatal, leading to the failure of the nervous system (Fournier & Mutero, 1994). This process is shown schematically in Fig. 1.13.

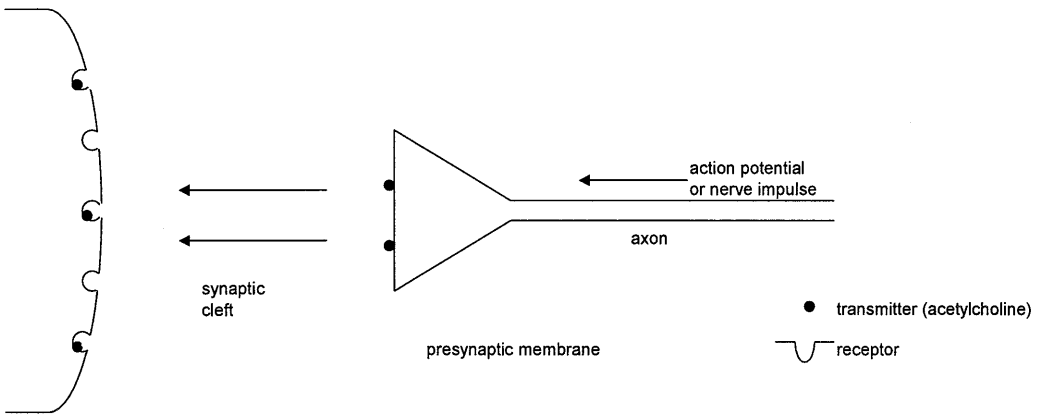


Figure 1.12 Diagram of the synapse (after Corbett, 1975).

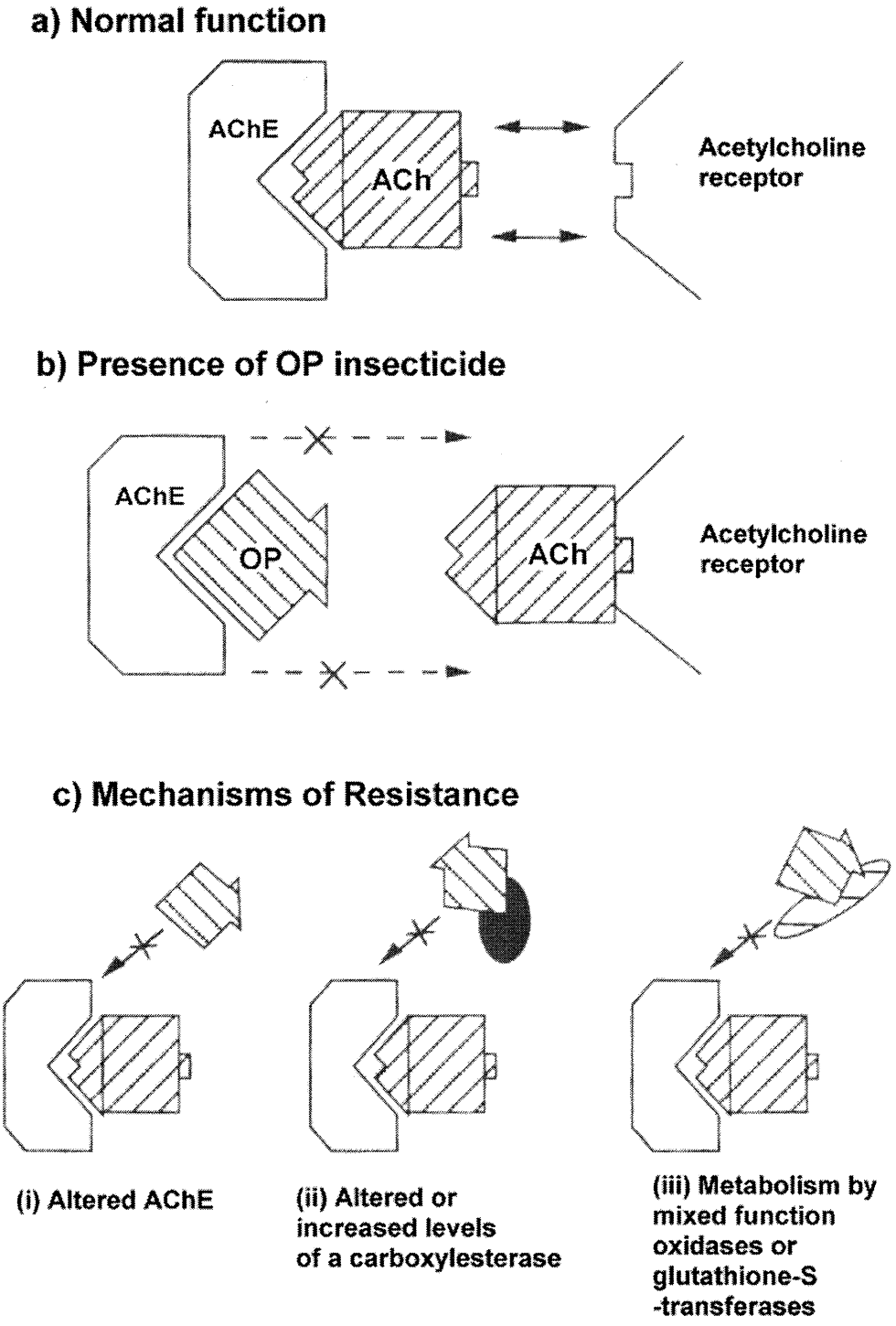


Figure 1.13 Schematic diagram showing target site of organophosphate insecticides (after McKenzie, 1996).

Acetylcholinesterase is inhibited by some ester insecticides, resulting in a build up of acetylcholine in the synaptic region, causing hyperexcitation of the nervous system. Hydrolysis of organophosphate and carbamate esters by AChE results in the phosphorylation or carbamylation of acetylcholinesterase. Enzyme inhibition is usually irreversible and dephosphorylation or decarbamylation steps (k_3) either do not occur, or are very slow. This process is shown in Fig. 1.14.

$$E + AX \underset{}{\overset{k_d}{\longleftrightarrow}} E\text{-}AX \overset{k_2}{\rightarrow} EA + X \overset{k_3}{\rightarrow} E + A$$

Key
E = free enzyme
AX = organophosphate insecticide
k_d = dissociation constant of the enzyme inhibitor complex
EA= phosphorylated/carbamylated enzyme
k_2 = phosphorylation rate
E-AX = Michaelis complex of enzyme and organophosphate
k_3 = rate at which enzyme reactivates

Figure 1.14 Phosphorylation/carbamylation of acetylcholinesterase.

The biomolecular rate constant, k_i, is a measure of the sensitivity of AChE to inhibition – the lower the k_i, the more sensitive AChE is to inhibition. The Michaelis constant (k_m) is a measure of the affinity of an enzyme for its substrate – the lower the K_m, the greater the affinity. A high affinity of AChE and acetylcholine can protect AChE from insecticide inhibition because substrate and inhibitor compete to bind to the catalytic centre.

Insects can have modified forms of AChE which confer insecticide resistance. Thus, if two forms of AChE had the same k_i, but different k_m, the form with the lower k_m would exhibit greater insensitivity towards an insecticide. A major mechanism of organophosphate resistance in both B-biotype and non B-biotype *B. tabaci* involves a modification of acetylcholinesterase conferring insensitivity to organophosphates (Dittrich *et al.*, 1991; Byrne & Devonshire, 1993). Three mutant forms of *B. tabaci* AChE showing reduced sensitivity to organophosphate inhibition have been diagnosed (Byrne & Devonshire, 1993; Moores *et al.*, 2000). The susceptible and three organophosphate resistant forms of AChE can be distinguished using paraoxon and azamethiphos as inhibitors (Byrne *et al.*, 1992; Moores *et al.*, 2000).

Modified sodium channels

Sodium channels (Fig. 1.15) are located in neuronal cell membranes. Sodium channels are involved in transmitting action potentials (nerve impulses), by conveying electrical nerve currents. Active transport of sodium and calcium ions results in the ions being more concentrated outside the cell. These ions have a strong tendency to leak into the cell, and must be continually pumped out by a gating mechanism. Stimuli that increase normal low permeability to either calcium or sodium ions depolarise the cell interior, resulting in nerve impulses.

The sodium channel is the major target site of type I and II pyrethroids and DDT (Narahashi, 1987). Type I pyrethroids (eg. allethrin, tetramethrin, phenothrin and

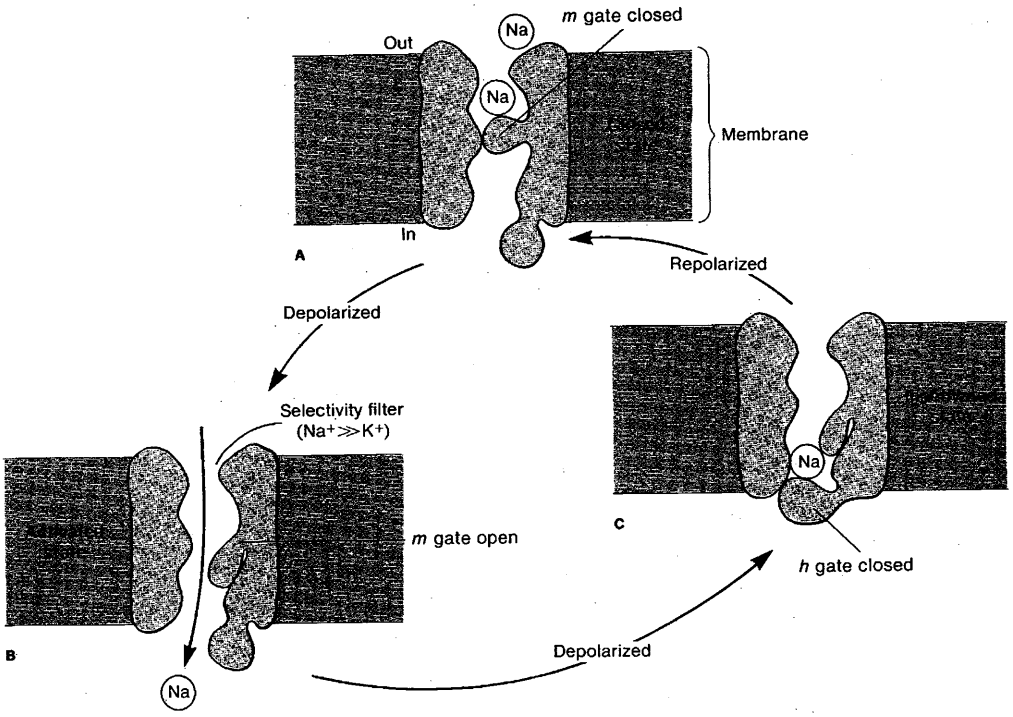


Figure 1.15 Major states of the sodium channel (A) before depolarisation, (B) depolarisation and (C) closing of the gate (after Eckert *et al.,* 1988).

permethrin) do not have an alpha-cyano group, unlike type II pyrethroids (eg. cypermethrin, cyphenothrin, deltamethrin and fenvalerate). The mode of action of DDT and pyrethroids involves prolonging the sodium current of voltage sensitive sodium channels, causing membrane depolarisation by interfering with the gating mechanism. Symptoms are repetitive discharges, membrane depolarisation and conduction block.

Kdr ('knockdown resistance') is the term applied to a nervous system insensitive to the paralytic effects of DDT and pyrethroids showing low sensitivity in the voltage-dependent sodium channel. *Kdr* was the first target site resistance mechanism to be identified in *M. domestica*, when a field population from Italy was found to show knockdown resistance towards DDT, DDT analogues and pyrethrins (Busvine, 1951). Knockdown resistance to DDT and pyrethroids is an intractable resistance mechanism that threatens the effectiveness of pyrethroid insecticides in many pest species (Soderlund & Knipple, 1999). An example of pyrethroid resistance attributed to *Kdr* is *Heliothis virescens* (Fabricius) from the Imperial Valley in California (Soderlund & Bloomquist, 1990). *Kdr* was also responsible for the first dramatic field failure of pyrethroids on Australian *Helicoverpa armigera* in 1984, but is now less important (Gunning *et al.*, 1990).

Super kdr is an exaggerated form of nerve insensitivity found in *M. domestica*. *Super kdr* confers very high levels of resistance to pyrethroids which have an α-cyano-3-phenoxybenzyl alcohol moiety, such as deltamethrin, cypermethrin and fenvalerate (Sawicki, 1978; Farnham *et al.*, 1987; Farnham & Khambay, 1995).

1.4.3 Insecticide penetration resistance

Some insects have become resistant to cuticular insecticide penetration. While the resistance effects of reduced insecticide penetration are usually small, multiplying effects in combination with other resistance mechanisms, such as detoxification, can be very important (Sawicki & Lord, 1970). Insecticide penetration is usually measured by tracking the progress of radiolabelled insecticides through the integument.

In *M. domestica*, penetration resistance was dosage dependent (dieldrin, diazinon, parathion, chlorthion-ethyl), with little or no effect at large doses (20µg/fly) (Sawicki & Lord, 1970). Reduced cuticular penetration of *S*-[C14]fenvalerate was found to be an important mechanism of fenvalerate resistance in the diamondback moth, *Plutella xylostella* (L.) (Noppun *et al.*, 1989). Penetration resistance is also a minor pyrethroid resistance mechanism in Australian *H. armigera* (Gunning *et al.*, 1990). Penetration resistance has not been recorded as a resistance mechanism in *B. tabaci*.

1.4.4 Avoidance and behavioural resistance

Avoidance and behavioural resistance involves an insect altering behaviour or protective habits, to lessen or avoid contact with insecticides. Mechanisms of behavioural resistance are not well understood and many perceived behavioural mechanisms are probably as a result of physiological changes in the peripheral nervous system. Georghiou (1972) considered a behavioural pattern to be a resistance mechanism only if it had been enhanced by insecticide selection. Behaviours not influenced by insecticide selection were considered to be avoidance behaviour.

Bifenthrin susceptible two-spotted spider mite (*Tetranychus urticae* Koch) had increased locomotor activity when exposed to bifenthrin, while the bifenthrin resistant strain showed less activity. Increased movement may have increased the exposure to bifenthrin in the susceptible strain (Kolmes *et al.,* 1994).

1.5 Insecticide resistance management

Effective resistance management requires an effective resistance monitoring program and an understanding of the underlying mechanisms of resistance. It requires the ability to detect resistance at very low allele frequencies in a population, so that action can be taken before insects become homozygous for resistance (Ahammad-Sahib *et al.,* 1994). The aim of resistance management is reduce or prevent resistance selection by insecticides.

Goals of a resistance management strategy should be:-

- avoiding/retarding resistance to effective available insecticides
- prolonging the life span of new insecticides by optimising use and delaying resistance development.

Tactics useful in resistance management include mixtures, rotations and the use of synergists. Insecticides can be used as mixtures or in single use, although it is important that compounds with identical modes of action not be used in mixtures or consecutively. Mixtures also can select for multiple resistance mechanisms. In a resistance management strategy, it is better to rotate insecticides with differing modes of action. Rotation is based upon the theory that the frequency of resistance declines in the absence of a selection pressure. Rotation aims to delay resistance by restricting exposure to each

selecting agent. Insecticides with novel modes of action, such as IGRs, should be important components of insecticide rotations. Synergists, such as piperonyl butoxide (PBO), can act as an enzyme inhibitor to enzymes involved in insecticide detoxification, thus rendering such insects susceptible.

B. tabaci has been the subject of a number of resistance management strategies. An example of such a strategy is one formulated for cotton in Israel, as shown in Table 1.1. This strategy involved rotation of insecticides with different modes of action so that a different insecticide was used against each generation of *B. tabaci* (Horowitz & Ishaaya, 1996). Another example of a resistance management strategy for *B. tabaci* was one implemented in Arizona, prompted by a resistance crisis in 1995. This strategy was modified from the Israeli program to include the annual use of pyriproxyfen and buprofezin once only, and delaying application of pyrethroids as long as possible (Dennehy & Williams, 1997).

Effective resistance management requires reliable and accurate resistance monitoring techniques. Resistance detection methods can involve bioassays of responses to insecticides, or techniques that can detect resistance mechanisms or resistance genes. Bioassay methods must be tailored to the type of insecticide used, for example, ovicides, IGRs, contact or stomach poisons, and the test insect. Bioassays give dosage mortality data, such as the LD_{50} or LD_{99}, which are the insecticide doses needed to kill 50% and 99% of a population respectively. Populations that are more resistant to an insecticide have higher LD values than their relatively susceptible counterparts.

Table 1.1 *B. tabaci* resistance management strategy in Israeli cotton, 1987-1994 (Horowitz & Ishaaya, 1996).

Years	Period 1 April-Mid May	Period II Mid May-Mid July	Period III Mid July-Mid Aug.	Period IV Mid Aug.-Mid Sep.
1987-1988	not applied	aldicarb endosufan	**Pyrethroids** cypermethrin fenpropathin bifenthrin	**Organophosphates** pirimiphos-methyl profenofos methidathion **Carbamates** methomyl
1989-1990	not applied	aldicarb* endosulfan	buprofezin **Organophosphates** pirimiphos-methyl profenofos methidathion **Carbamates** methomyl	**Pyrethroids** cypermethrin fenpropathrin bifenthrin
1991-1994	not applied	(endosulfan)**	pyriproxyfen*** diafenthiuron (Pyrethroids) (OPs and Carbamates)	(OPs and Carbamates)

*Aldicarb was removed with the introduction of the IGRs; ** Insecticides in parentheses may be applied if a high population of adults develop; ***This insecticide was registered in 1991, and is more effective than the others against *B. tabaci.*

In a resistance monitoring programme, prior baseline susceptibility data are required, as it provides a yardstick against which to measure future changes in susceptibility. Collection of baseline susceptibility data also allows the reliable determination of a single discriminating dose that will kill 100% of susceptible insects. Discriminating doses are often used in resistance monitoring programs, as they provide a more cost and time efficient alternative to a full dose response study.

A variety of insecticide bioassay techniques have been used against *B. tabaci*. The most common bioassay method for contact insecticides used against *B. tabaci* are leaf dip techniques, where insects are confined on insecticide dipped leaves and mortality assessed after 48 hours or longer. Other bioassay methods include using live plants and assessing insect mortality during development, as in the case of IGRs such as buprofezin.

Direct detection of resistance mechanisms can also be used for resistance monitoring. Resistance monitoring methods which detect actual resistance mechanisms include assays for resistant enzymes, immunoassays for resistant enzymes and detection of specific resistant DNA sequences (Brown and Brogdon, 1987). In *B. tabaci*, biochemical resistance detection methods have been used to detect organophosphate insensitive AChE (Moores *et al.*, 1988; Byrne and Devonshire, 1991). Enzyme (E4) specific antiserum has been used to detect organophosphate resistance in *M. persicae* (Devonshire *et al.*, 1986). Specific E4 DNA sequences, in a dot blot test have also been used to detect organophosphate resistance in *M. persicae* (Field *et al.*, 1989). These novel methods to detect resistance are very rapid compared to conventional bioassays, which can take days

or even weeks to complete. Results are more informative than discriminating dose bioassays, because individuals heterozygous and homozygous for resistance can be detected. Such methods can also be adapted for resistance detection in the field. For example, biochemical kits were developed to detect pyrethroid and carbamate resistance in *H. armigera* in Australia (Gunning *et al.,* 1997). Naturally, all biochemical resistance detection methods require verification by conventional bioassay techniques.

1.6 Conclusions of the literature review, and aims of this PhD thesis

B-biotype *B. tabaci* is a severe pest of many plant production industries overseas. It was first discovered in Australia in 1994, and outbreaks in the tomato industry of northern Queensland and horticultural crops of northern NSW have occurred in recent years. B-biotype *B. tabaci* numbers have progressively increased in cotton in Australia and are now reaching epidemic numbers in some areas.

The biological parameters of high fecundity, quick reproduction time and wide host range, combined with the ability to rapidly evolve resistance to insecticides, mean that *B. tabaci* can be a severe pest in the wide area of Australia that is climatically suited. It is difficult for farmers to detect *B. tabaci* in the field because of their small size and rapid movement. Immature *B. tabaci* are even smaller. When whiteflies are readily detectable in the field, control is very difficult. The best way to manage B-biotype *B. tabaci* is by a pre-emptive approach, rather than waiting until a major outbreak occurs. This research project is a part of a pre-emptive strategy to gather insecticide resistance information on new insecticides in regards to *B. tabaci*. The use of chemical insecticides against insect

pests remains essential for the economic production of cotton in Australia. As yet, there are not a great enough variety of insecticides registered in Australia capable of controlling B-biotype *B. tabaci.*

Insect growth regulators such as buprofezin and other insecticides with novel modes of action will be a valuable tool for control of whiteflies resistant to conventional insecticides. Buprofezin is one of the insecticides being considered for registration. It is essential that use of these insecticides be managed in order to avoid or delay the development of resistance to them. To this end, the aims of this study include:-

- to develop suitable techniques to bioassay insect growth regulators against B-biotype *B. tabaci*
- to examine the potential for IGR resistance selection
- to investigate resistance mechanisms to IGRS in B-biotype *B. tabaci*, and
- to look at the potential for cross-resistance between buprofezin and other novel insecticides.

Chapter 2: General Materials and Methods

2.1 Insects

B-biotype *B. tabaci* were originally collected from horticultural crops from a variety of sites in north Queensland (Ayr, Bowen, Cairns) and then cultured on poinsettia plants (*Euphorbia pulcherrima* Willd.). Native, insecticide susceptible non B-biotype *B. tabaci* from the Darwin area (12°33' 131°18') were cultured on painted spurge (*Euphorbia cyathophora* Murr.). Whitefly cultures were kept in cages, segregated in a glasshouse and held at 27°C in natural daylight.

2.2 Insecticides

Insecticides used throughout this thesis include the insect growth regulators buprofezin (2-[(1,1-dimethylethyl)imino]tetrahydro-3-(1-methylethyl)-5-=phenyl-4H-1,3,5-thiadiazi n-4-one) (Dow AgroSciences Ltd), novaluron (N-[[[3-chloro-4-(1,1,2-trifluro-2-trifluromethoxyethoxy)=phenyl]amino]carbonyl]-2,6-difluorobenzamide) (NuFarm Pty. Ltd.), pyriproxyfen (2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine) (Sumitomo Australia Ltd), fenoxycarb (ethyl [2-(4-phenoxyphenoxy)ethyl]carbamate) (Novartis), flufenoxuron (N-[[[4-[2-chloro-4-(trifluromethyl)phenoxy]-2-fluorophenyl]=amino] carbonyl]-2,6-difluorobenzamide) (BASF Australia), pymetrozine (E)-4,5-dihydro-6-methyl-4-[(3-pyridinylmethylene)amino]-1,2,4-triazin-3(2H)-one (Novartis) and pyridaben (4-chloro-2-(1,1-dimethylethyl)-5-[[[4-(1,1-dimethylethyl)=phenyl]methyl]

thio]-3(2H)-pyridazinone (BASF Australia). Addresses of insecticide suppliers are listed in Appendix 1.

2.3 Laboratory chemicals and equipment

Addresses of suppliers of laboratory chemicals and equipment used throughout this thesis are listed in Appendix 2.

2.4 Insecticide bioassays

2.4.1 "Leaf dip" bioassay technique for contact insecticides

The leaf dip bioassay method used for testing contact insecticides against *B. tabaci* was similar to that described by Cahill *et al.* (1995). Cotton plants (*Gossypium hirsutum* L.) were grown in the glasshouse without any exposure to insecticides. Leaf squares (40mm x 40mm) were cut and dipped into aqueous solutions of insecticide containing 0.01% Agral® surfactant (Crop Care Australiasia, Appendix 1) and allowed to dry at 25°C. Control leaves were dipped in Agral® and distilled water only. Leaf squares were placed adaxial side down on a bed of agar gel (0.5%) (Leiner Davis Gelatin, Appendix 2) in plastic bioassay trays (40mm diameter, 30mm high) (Rearing Tray BIO-RT-32, C-D International, Inc., Pitman, NJ, USA).

Female adult whiteflies of required strains were captured using an aspirator, temporarily anaesthetised with carbon dioxide and placed on the cotton leaf squares. Twenty whiteflies were placed on each cotton leaf square and bioassay trays were sealed with a breathable clear plastic cover (BIO-CV-4, C-D International, Inc.). The whiteflies were

allowed to feed on the leaf squares and were assessed at maximum mortality (24 or 48 hours).

2.4.2 Bioassay technique for insect growth regulators

Bioassay methods must take into account the targeted life stage and the most appropriate insecticide application method. It was not possible to use leaf dip bioassay techniques with excised leaves to bioassay IGRs due to leaf senescence before bioassay completion. IGRs are aimed at immature insects and bioassays take some time to complete, therefore other methods had to be used. The bioassay technique used was identical to the methods of Cahill *et al.* (1996) and Simmons *et al.* (1997). Poinsettia 'V10 Red' cultivars (Oasis Horticulture Pty Ltd, Appendix 2) were chosen as host plants for B-biotype *B. tabaci* bioassay as they were able to withstand large whitefly infestations. The stem of the cutting was coated with root hormone striking powder (Yates™ Striking Powder 3000 No. 2; active ingredient indole-butyric acid (3g/kg) Arthur Yates and Co., Ltd., Appendix 2) and placed in a wet Jiffy pot N-9 containing a mix of peat (50%), perlite (40%) and vermiculite (10%). Cuttings were held under low light intensity and regular water misting to encourage roots to strike. Six poinsettia cuttings were placed for whitefly oviposition in large rearing cages containing adult *B. tabaci* of the strains chosen for bioassay.

Small leaf cages, adapted from the design of Melamed-Madjar *et al.* (1984), containing 20 adult *B. tabaci*, were attached to poinsettia cuttings. A diagram of the leaf cage is shown in Fig. 2.1. Leaf cages were used to encourage oviposition in a concentrated area

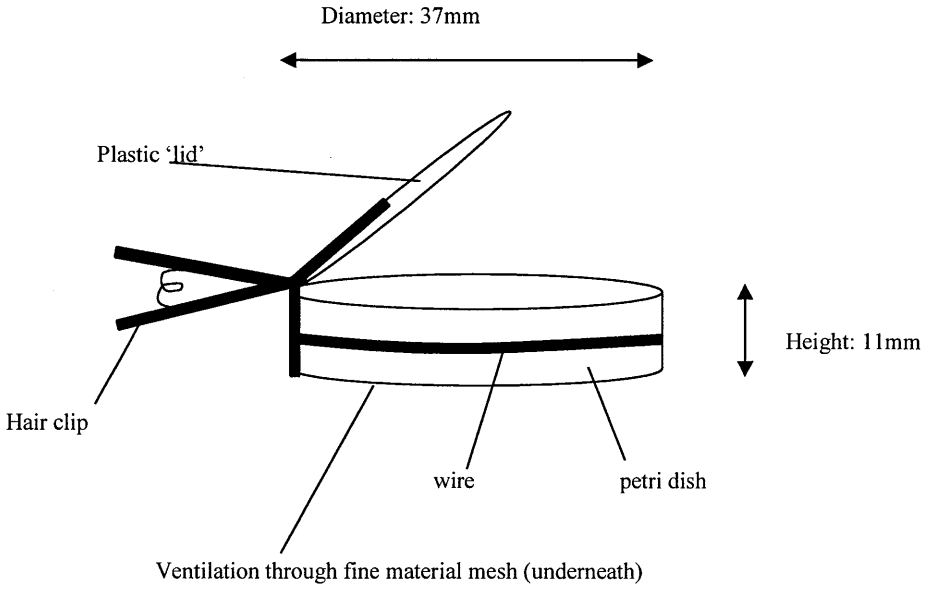


Figure 2.1 Leaf cage used in IGR bioassays.

of the leaf and give a better distribution of eggs on each cutting. The cuttings were placed in the larger leaf cages to increase the total egg lay. After oviposition, adult *B. tabaci* and leaf cages were removed from the cuttings so that no more eggs would be laid, thus ensuring that resultant nymphs were at the same developmental stage. Cuttings were put in cotton mesh whitefly proof cages (Australian Entomological Supplies, Appendix 2). Immature *B. tabaci* on leaves were counted and then cuttings were dipped into formulated insecticide and Agral® solutions (to ensure wetting). Mortality was assessed 20 days after oviposition, by counting the number of living nymphs.

2.5 Statistical analysis

Bioassay data were analysed by probit analysis (Finney, 1971). Control mortality was corrected for using Abbott's formula (Abbott, 1925). Abbott's formula is usually applied as:

$$Pt = \frac{Po - Pc}{100 - Pc} \times 100$$

where

Pt = corrected mortality (%)
Po = observed mortality (%), and
Pc = control mortality (%) (Busvine, 1971).

Percentage kills from bioassay data were plotted against the logarithm of the dose and analysed by probit analysis (Finney, 1971). Probit analysis is a tranformation to facilitate computation, which converts the data to a straight line on probit graph paper. The method is to replace each percentage by its corresponding probit. The line which gives the best fit of the experimental data ($y = ax+b$) is computed from the transformed data, using a modified regression technique. In the equation $y = ax+b$, y represents the probit

kill and x the log dosage. The calculations also give the slope of the line, confidence limits for the estimated doses corresponding to percent mortality and a means for testing the homogeneity of the population used in the bioassay.

The Probit program used in these studies was P-A Mod (A. Woods, C. Orton & C. Virgona, CERIT, Appendix 2) for Macintosh computers.

Chapter 3: Buprofezin Bioassays

3.1 Introduction

The synthetic insect growth regulator buprofezin (Applaud®, Dow Agrochemicals Ltd.) was used as a 25% active ingredient (w/w) wettable powder formulation. The chemical structure of buprofezin is shown in Fig. 1.4. Buprofezin is a thiadiazine chitin synthesis inhibitor that has been used overseas against *B. tabaci*. It is used against *B. tabaci* at field rates between 100 and 250 mg/L (25ppm - 62.5ppm) (De Cock & Degheele, 1998). Buprofezin affects nymphal stages and embryogenesis through contact and vapour action (Horowitz *et al.*, 1994). It suppresses the formation of chitin, in a similar way to benzoylphenyl ureas (Cohen, 1987), although its mode of action is not fully understood.

Treatment of adult whiteflies with buprofezin reduces longevity, suppresses oviposition, and causes some egg sterility (Horowitz & Ishaaya, 1996; Simmons *et al.*, 1997). Hatch rate of eggs laid by *B. tabaci* females treated repeatedly with buprofezin was extremely low (De Cock & Degheele, 1998). The LD_{50} for suppression of egg hatch was approximately 15mg/L (Horowitz & Ishaaya, 1996).

The aim of the present experiments was to select for, and document, buprofezin resistance in laboratory populations of Australian B-biotype *B. tabaci* by repeated exposure to buprofezin.

3.2 Materials and methods

3.2.1 Nymphal leaf dip bioassay

The bioassay method used for buprofezin was described in Section 2.4.2. *B. tabaci* were allowed to oviposit on poinsettia cuttings for 48 hours before being removed. Eight days after oviposition, 1st/2nd instar nymphs were counted and cuttings were dipped for 20 seconds in six buprofezin solutions, ranging from 0.5ppm to 5000ppm. Leaves dipped in Agral® and water only were designated as controls. Twenty days after oviposition, a final count of live 3rd/4th instar was made and the percentage mortality at each dose calculated. Data were corrected for control mortality and analysed by probit analysis.

3.2.2 Selection for buprofezin resistance

B-biotype *B. tabaci* populations were selected for resistance by spraying with 50ppm buprofezin (approximate LD_{80}) three times, leaving the population to recover between sprays for a generation before respraying. This concentration was selected as it fell in the higher range of the field rates suggested by De Cock & Degheele (1998). The objective of these insecticide applications was to select for resistance by progressively decreasing the proportion of susceptible insects. Buprofezin unselected B-biotype *B. tabaci* from the original strain were kept segregated from the selected strain. Bioassays were conducted after each buprofezin selection to determine the rate of resistance development.

3.3 Results

The unselected B-biotype *B. tabaci* strain had an LD_{50} of 3.0ppm and a slope of 0.76, the slope value indicating considerable heterogeneity of response towards buprofezin.

Bioassay data are shown in Table 3.1. The selection experiment produced increases in LD_{50}'s from 3 to 595 ppm (Table 3.1), and great increases in slope (0.76 to 3.7) (Table 3.1). This suggested that if only one locus was involved, a strain had been selected that was virtually homozygous for buprofezin resistance.

The laboratory population of a native non B-biotype *B. tabaci* population from Darwin was lost from culture and could not be bioassayed. To compensate for the lack of a known buprofezin susceptible strain, susceptible data from from the SUD-S strain (Cahill *et al.*, 1996) has been used for comparison with the Australian data. SUD-S (Table 3.1) is an insecticide susceptible strain of *B. tabaci* that originated from Sudan and is held in culture at IACR-Rothamsted UK. The bioassay methods used by Cahill and in this thesis were identical.

Compared to the SUD-S susceptible strain, the unselected Australian B-biotype *B. tabaci* (F_0) was 11 fold resistant to buprofezin. Subsequent selected generations were 117 (F_2), 348 (F_4) and 2203 (F_6) fold resistant to buprofezin respectively. Bioassay data of unselected and resistant B-biotype *B. tabaci* Australian populations are also shown in comparison with published and unpublished data for other strains of *B. tabaci* in Table 3.1.

Table 3.1 Response of populations of *B. tabaci* nymphs to buprofezin. Biotypes 1, 2 and 3 refer to B-biotype strains successively selected in the laboratory at Tamworth.

*unpublished Oz B data were kindly provided by M. Cahill.
** SUD-S, PAK-1 and NED-3 data obtained from Cahill *et al.* (1996). SUD-S is the reference susceptible laboratory strain, PAK-1 and NED-3 were B-biotypes, and originated from Pakistan and the Netherlands respectively.

Strain	LC_{50} (ppm)	Fiducial Limits	Slope	Resistance Factor
Buprofezin Unselected B-biotype	3.00	(1.8 - 5.0)	0.76	11.00
Buprofezin Selected B-biotype 1	31.60	(15 - 67)	1.40	117.00
Buprofezin Selected B-biotype 2	94.00	(53 - 169)	1.40	348.00
Buprofezin Selected B-biotype 3	595.00	(336 - 1054)	3.70	2203.00
Oz. B*	1.50	(1.3 - 1.9)	1.60	5.50
SUD-S**	0.27	(0.23 - 0.32)	1.70	n.a.
PAK-1**	0.53	(0.46 - 0.62)	1.70	2.00
NED-3**	24.70	(18 - 30)	2.80	217.00

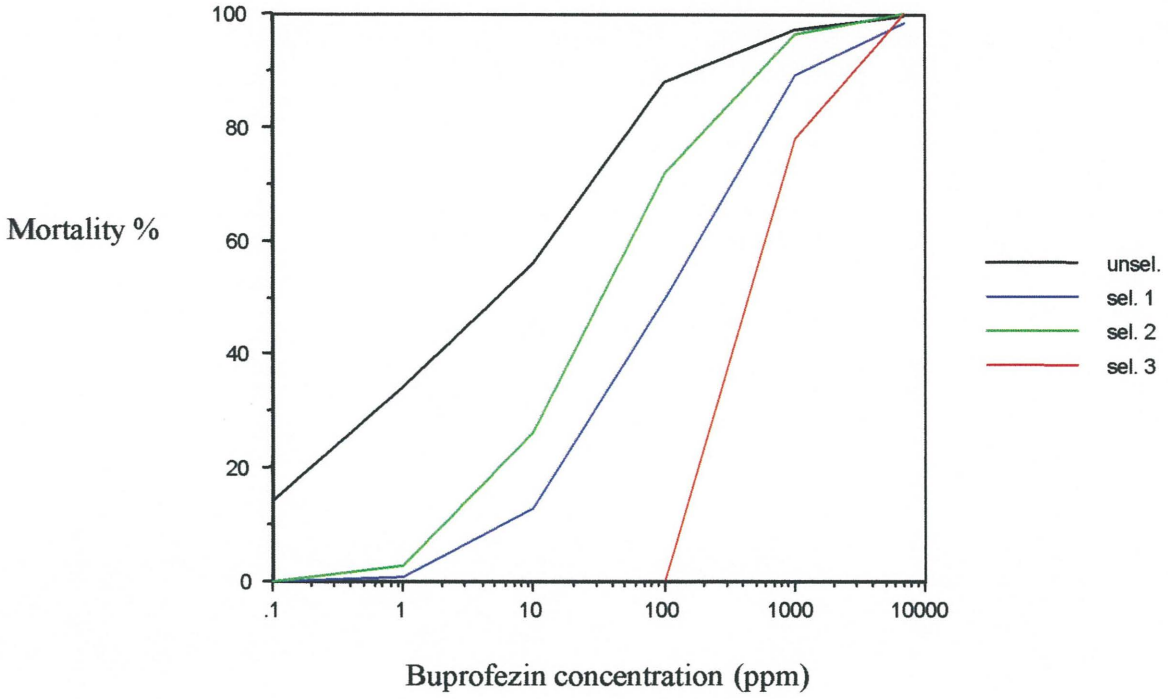


Figure 3.1 Effects of buprofezin on unselected and selected populations over eight generations of B-biotype *B. tabaci.*

B. tabaci strains used are buprofezin non-selected B-biotype (unsel.) (F_0) and buprofezin selected B-biotype selected with buprofezin over time (sel. 1 (F_2), sel. 2 (F_4), sel. 3 (F_6) respectively).

3.4 Discussion and conclusions

Results of buprofezin bioassays in Australian B-biotype *B. tabaci* showed that, compared to insecticide susceptible SUD-S *B. tabaci*, there was pre-existing resistance to buprofezin in the Australian B-biotype population. The unselected population was 11 fold resistant to buprofezin. These data are similar to previously unpublished results from Mathew Cahill (Cahill, unpublished). Cahill bioassayed Australian B-biotype *B. tabaci* (designated the "Oz B" strain) in 1996, and reported a resistance factor of 5.5 (Table 3.1). Cahill's data indicates that when B-biotype *B. tabaci* entered Australia, a low level of resistance was already present.

Overseas, tolerance or resistance to buprofezin has been reported in *B. tabaci* populations from Spain, the Netherlands, Japan, UK, Pakistan and Israel (Horowitz & Ishaaya, 1994; Horowitz *et al.*, 1994; Cahill *et al.*, 1996). Buprofezin resistance levels of 2-fold in Pakistan and up to 217-fold in a glasshouse population from the Netherlands were reported by Cahill *et al.* (1996) (Table 3.1).

Buprofezin selection on Australian B-biotype *B. tabaci* population rapidly produced high levels of buprofezin resistance, in excess of 2000-fold after 4 selection events, or after six generations. This rapid selection for resistance is consistent with a pre-existing low level of resistance to buprofezin in the Australian population. These findings should serve as a warning that overuse of buprofezin in Australia may result in rapid selection of resistance in the field, and that strict resistance management guidelines should be developed and adhered to. The origin of buprofezin resistance was probably from the United States, as

the source of B-biotype *B. tabaci* introduction in Australia was traced to the USA (Australian Quarantine and Inspection Service). B-biotype *B. tabaci* was first detected in Australia in 1994 (Gunning *et al.*, 1995a), entering the country via international trade in poinsettias. Buprofezin played an important role in managing *B. tabaci* resistance in the United States in 1995 (Simmons *et al.*, 1997) and it is highly probable that low frequencies of buprofezin resistant *B. tabaci* formed part of the invading population.

Chapter 4: Buprofezin Resistance Mechanisms - Esterases

4.1 Introduction

Buprofezin bioassay data (Chapter 3) have shown that resistance was easily selected for in Australian B-biotype *B. tabaci* populations. Having selected a buprofezin resistant strain, the next logical step for the research was to investigate the resistance mechanisms. Overseas, virtually nothing is known about mechanisms of resistance to buprofezin or other IGRs (Denholm *et al.*, 1996). This chapter reports metabolic mechanisms of resistance to buprofezin in B-biotype *B. tabaci* in Australia.

In *B. tabaci*, esterases are particularly interesting because they have been found to mediate a number of insecticide resistances. A correlation between high esterase activity, hydrolysis of naphthyl esters and organophosphate and pyrethroid resistance in *B. tabaci*, was recorded by other researchers (Dittrich *et al.*, 1985; Byrne & Devonshire, 1991; Byrne & Devonshire, 1993; Byrne *et al.*, 2000). Esterase activity has also been associated with the detoxification of bifenthrin and other pyrethroids (Riley *et al.*, 1999; Byrne *et al.*, 2000), and profenofos (Byrne & Devonshire, 1991). Gunning & Moores (2000) have also reported that esterases bind to imidacloprid in imidacloprid resistant *B. tabaci*.

The aims of this Chapter include:

a) to determine whether esterases differ in buprofezin resistant and unselected B-biotype *B. tabaci* and

b) to investigate the ability of buprofezin to bind *in vitro* and *in vivo* to esterase iso-enzymes in B-biotype *B. tabaci* strains.

4.2 Comparison of esterase bands and esterase activity in selected and unselected B-biotype *B. tabaci*

4.2.1 Materials and methods

Insects

Insect strains used in this chapter included native non B-biotype, buprofezin unselected and resistant B-biotype *B. tabaci*. Other *B. tabaci* biotypes include a susceptible strain that originated from Sudan (SUD-S susceptible), Egypt (Egypt B-biotype) and the USA (USA B-biotype). Egypt, SUD-S and USA-B are kept in culture at IACR - Rothamsted, UK, and were kindly supplied by Dr Graham Moores and Kevin Gorman. Level of resistance to buprofezin is shown in Table 3.1

Electrophoresis

The polyacrylamide gel electrophoresis method used was that of Byrne and Devonshire (1991). Individual adult whiteflies were homogenised in 20μL of 1.6% Triton X-100 (especially purified for membrane research (Boehringer Mannheim, Appendix 2)) containing 10% sucrose and a few grains of bromocresol purple (Sigma-Aldrich, Appendix 2). Aliquots of 15μL homogenate (0.75 insect equivalent) were pipetted into wells of polyacrylamide gels. Gels were similar to those used by Williams & Reisfeld (1964) containing 7.5% polyacrylamide, but to achieve optimum resolution, the triton

X-100 concentration was 0.20% in the stacking gel and 0.05% in the resolving gel (Byrne & Devonshire, 1991). Specially designed gel combs (Burkard Scientific, Appendix 2) that cast wells with 4.5mm spacing in the stacking gel (ffrench-Constant *et al.*, 1988) were used.

The gels were run at 250V maximum current for 90 minutes at 5°C (Gel Electrophoresis Apparatus, GE-2/4) (Pharmacia Australia Pty Ltd., Appendix 2). Gels were stained for esterase activity, using 0.5mM α-naphthyl butyrate (Sigma-Aldrich) and 0.2% Fast Blue RR (Sigma-Aldrich) in 0.02M phosphate buffer pH 6.0. Gels were fixed in 5% acetic acid. Electrophoretic mobilities (R_m) ratios were measured as the distance that esterase bands travelled down the gel, relative to the buffer interface.

Total esterase assay

The total esterase assay method for the microplate reader used was similar to that described by Grant *et al.* (1989). Samples from each whitefly population containing 50 adult *B. tabaci* were homogenised in 1mL of 0.02M pH 7.0 phosphate buffer containing 0.05% Triton X-100 (20μL/whitefly). Total esterase activity was determined on 10μL aliquots. The reaction was initiated by adding 240μL of 0.2M pH 6.0 phosphate buffer with 0.6% Fast Blue RR Salt and 1.86% 1-naphthyl butyrate (Sigma-Aldrich). Kinetic assays were performed at 25°C, using a Bio-Rad 3550 microplate reader (Bio-Rad Laboratories) and Kinetic Collector 2.0 software (Bio-Rad Laboratories) run on an Apple Macintosh SE computer. The assay was run for 15 minutes, taking absorbance readings (450 nm) at 14 second intervals. Linear regressions were performed by the computer.

The kinetic velocity was calculated by the computer as the slope of the fitted regression line.

4.2.2 Results and discussion

In vitro esterase activity in buprofezin resistant and unselected B-biotype *B. tabaci* strains was examined by polyacrylamide gel electrophoresis and total esterase assay. The buprofezin resistant strain showed additional strongly stained bands in the adult lifestage ($E_{0.14\text{-}0.29}$) (Fig. 4.1), compared to those of unselected B-biotype *B. tabaci*. These additional bands, while present, are not easily seen in the nymphs in Fig. 4.1. These bands appeared at and below the B-band and were not evident in unselected B-biotype *B. tabaci* or native non B-biotype *B. tabaci*. Total esterase activity was approximately 2-fold higher in adults of the buprofezin resistant B-biotype *B. tabaci* strain, than in the unselected B-biotype population (Fig. 4.2). Adults of native non B-biotype *B. tabaci* had esterase activity approximately 15 fold lower than those of the buprofezin resistant B-biotype strain (Fig. 4.2).

The presence of extra esterases could be very useful in rapid diagnosis of buprofezin resistant *B. tabaci*. Extra esterase bands were also detected in field collected *B. tabaci* after buprofezin spray trials (R. Gunning, pers. comm. 2000). The presence of these extra esterase bands in both buprofezin resistant nymphs and adults that had not been directly treated with buprofezin suggests that this trait is genetically inherited, and not induced. Some sub-lethal effects of insect growth regulators can be transmitted from

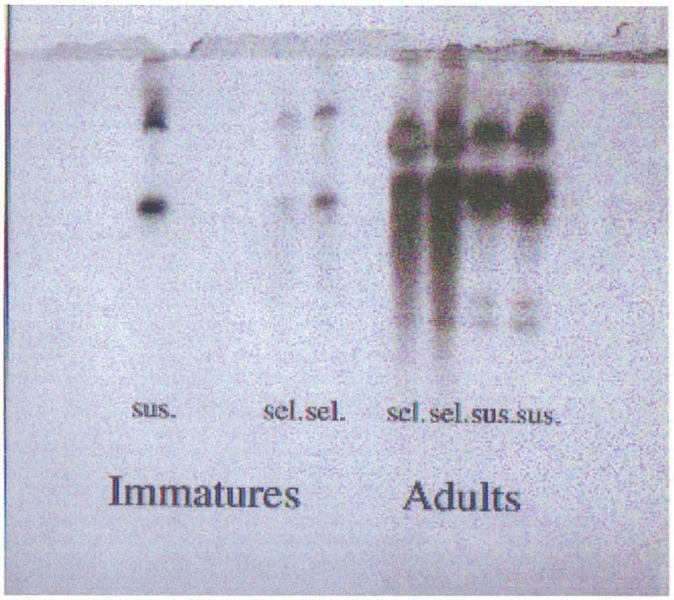


Figure 4.1 Polyacrylamide gel showing esterase iso-enzyme patterns of buprofezin selected and unselected B-biotype *B. tabaci* nymphs and adults.

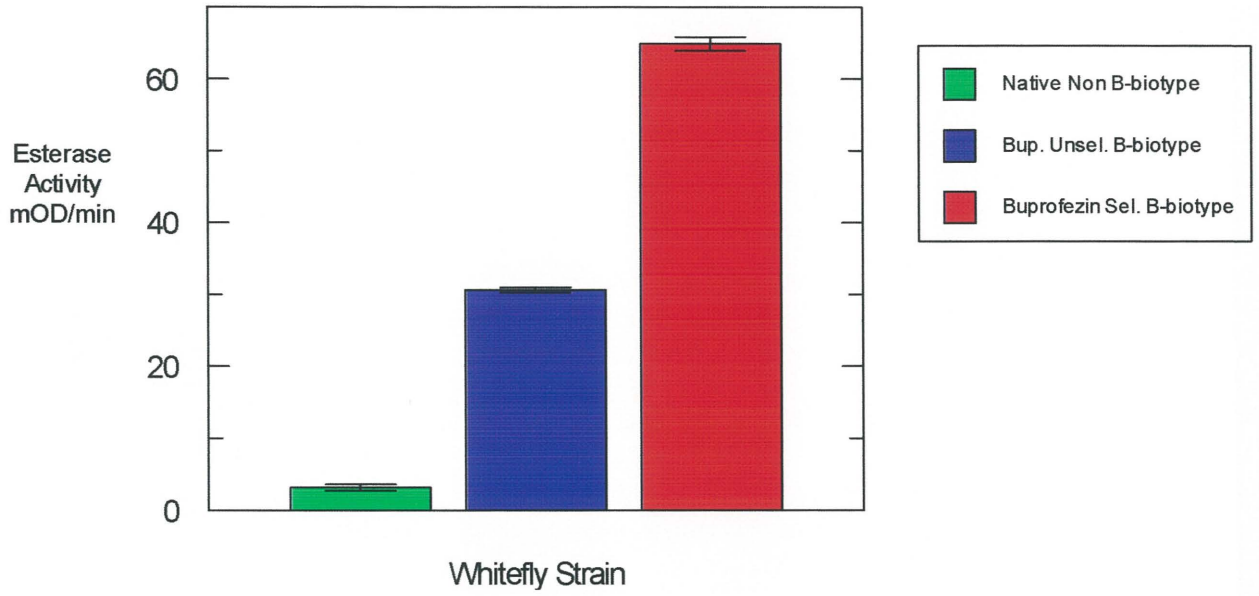


Figure 4.2 Total esterase activity of adult *B. tabaci.*

B. tabaci strains were Native Non B-biotype, buprofezin unselected B-biotype Bup. Unsel. B-biotype) and buprofezin resistant B-biotype (Buprofezin Sel. B-biotype).

female to offspring, however, the extra banding was also observed in resistant individuals that had not been exposed to buprofezin for several generations.

Results also demonstrate that buprofezin resistance in Australian B-biotype *B. tabaci* is positively correlated with increased esterase activity. Increased esterase activity has been linked with insecticide resistance in other insect species. For example, Australian pyrethroid resistant *H. armigera* have additional esterase bands and up to 50-fold increased esterase activity compared to susceptible *H. armigera* (Gunning *et al.,* 1996a). Organophosphate and carbamate resistant *Myzus persicae* have enormously increased E4 esterase activity compared to susceptible *M. persicae* (Devonshire, 1977; Devonshire, 1979; Devonshire & Moores, 1982; Devonshire, 1989). Increased esterase activity in *B. tabaci* is associated with resistance to profenofos and cypermethrin (Ishaaya *et al.,* 1987; Byrne & Devonshire, 1991).

4.3 Esterase inhibition by buprofezin

Model substrates are used extensively in detecting enzyme activity, as they give products which are readily assayed, usually spectrophotometrically (Devonshire, 1990). 1-naphthyl butyrate is the standard substrate used in *B. tabaci* studies (Byrne & Devonshire, 1991; Byrne & Devonshire, 1993; Byrne *et al.*, 2000). *In vitro*, when insect homogenates possessing an esterase mediated resistance mechanism towards an insecticide are incubated with that insecticide and assayed for activity, then the enzyme-insecticide complex prevents substrate binding, thus lowering activity.

4.3.1 Materials and methods

In vitro esterase inhibition by buprofezin

Eighty *B. tabaci* adults each from the buprofezin unselected B-biotype, buprofezin resistant B-biotype and native non B-biotype *B. tabaci* strains were homogenised in 1.6 mL 0.02M pH 7.0 phosphate buffer containing 0.05% Triton X-100. Eight 200µL aliquots were each pipetted in 0.6mL Snap-Cap Microtubes (ICN Biomedicals Inc.).

Methanol (Lomb Scientific, Appendix 2) was added to each aliquot to a total concentration of 5%. Methanol (5%) has been shown to increase insecticide solubility without affecting esterase activity (Devonshire & Moores, 1989). Technical grade buprofezin (in 5µL acetone) (ICN Biomedicals, Appendix 2) and added to homogenate to make final concentrations of 0.1ppm, 0.2ppm, 0.5ppm, 1ppm, 5ppm and 10ppm buprofezin. Samples containing 200µL of whitefly homogenate with methanol and acetone only were used as the uninhibited control. Homogenates were incubated at 25°C for 30, 60 and 90 minutes. After incubation, 10µL aliquots were taken and total esterase activity determined. Each assay was replicated three times. In addition, 15µL aliquots were loaded onto polyacrylamide gels for electrophoresis and stained for esterase activity.

In vivo esterase inhibition by buprofezin

Nymphs

Adult *B. tabaci* were caged on six poinsettia (*Euphorbia pulcherrima*) cuttings and were allowed to oviposit for two days, thus ensuring a similar age of nymphs. Fifteen days

later, poinsettia cuttings were dipped in aqueous solutions of formulated buprofezin (with 0.01% Agral® as a wetting agent) and the leaves were allowed to dry at 25°C. Buprofezin concentrations used were 0.1, 0.5, 1, 5 and 10ppm active ingredient. Control cuttings were dipped in a solution of water with 0.01% Agral® alone. *B. tabaci* nymphs were gently removed with a fine needle from leaves at 0, 12, 24, 36, 48 and 72 hours after insecticide treatment. Three fourth instar nymphs from each insecticide treatment were mass homogenised in 5µL of Triton X-100 with 10% sucrose and a few grains of bromocresol purple, and then made up to 60µl in the homogenising solution. A 15µL sample was pipetted into wells of polyacrylamide gels. At each sampling time, three fourth instar nymphs were also removed from the leaves at each insecticide concentration for total esterase assays. Nymphs were homogenised in 60µL of 0.02M pH 7.0 phosphate Triton X-100 buffer. Total esterase activity was determined on 10µL aliquots, and replicated three times.

Adults

Formulated buprofezin was mixed in a solution of distilled water and 0.01% Agral®, at concentrations of 0.1, 0.5, 1, 5 and 10 ppm active ingredient. Poinsettia cuttings were dipped and allowed to dry. A cutting dipped in a solution of water with 0.01% Agral® was used as a control. Adult *B. tabaci* were placed on each poinsettia cutting in insect cages, as described in Section 2.4.2, and removed for esterase assays at 0, 12, 24, 36, 48 and 72 hours after insecticide exposure. Esterase activity was studied by gel electrophoresis and total esterase assay. Each experiment was replicated three times.

4.3.2 Results and discussion

Native non B-biotype and SUD-S susceptible *B. tabaci* strains showed no inhibition of esterase activity (Fig. 4.3). Buprofezin unselected B-biotype showed approximately 20% inhibition of esterase activity at 1 hour (Fig. 4.3). USA B-biotypes showed a slight inhibition of esterase activity when incubated at higher concentrations of buprofezin (6.7μM) (Fig. 4.3). The Egypt B-biotype strain showed esterase activity losses of up to 30% when incubated with buprofezin (2.0μM) (Fig. 4.3).

In vitro incubation of buprofezin with *B. tabaci* esterase showed a very marked inhibition of esterase activity in the buprofezin resistant B-biotype strain. After an incubation of one hour, approximately 50 - 60% esterase activity was inhibited by buprofezin concentrations as low as 10^{-9}M.

Results from *in vivo* studies with buprofezin treated nymphs (Fig. 4.4a) also showed esterase inhibition in the resistant B-biotype *B. tabaci* strain. Approximately 12 hours after exposure to buprofezin there was 85% enzyme inhibition, however, inhibition was gradual. Esterase activity took some 50 hours to recover to the level of the uninhibited control. A similar pattern of esterase inhibition was observed in non selected B-biotype *B. tabaci,* although levels of inhibition were much reduced. Maximum esterase inhibition occurred at 12 hours, where some 70% of esterase activity was inhibited in this strain.

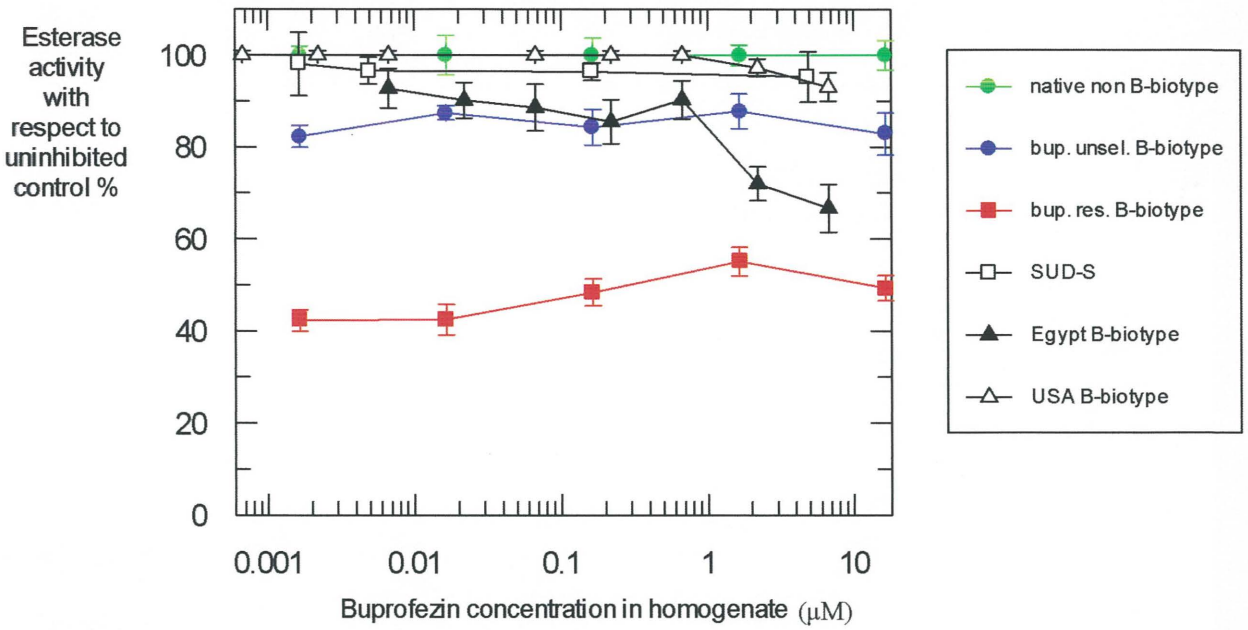
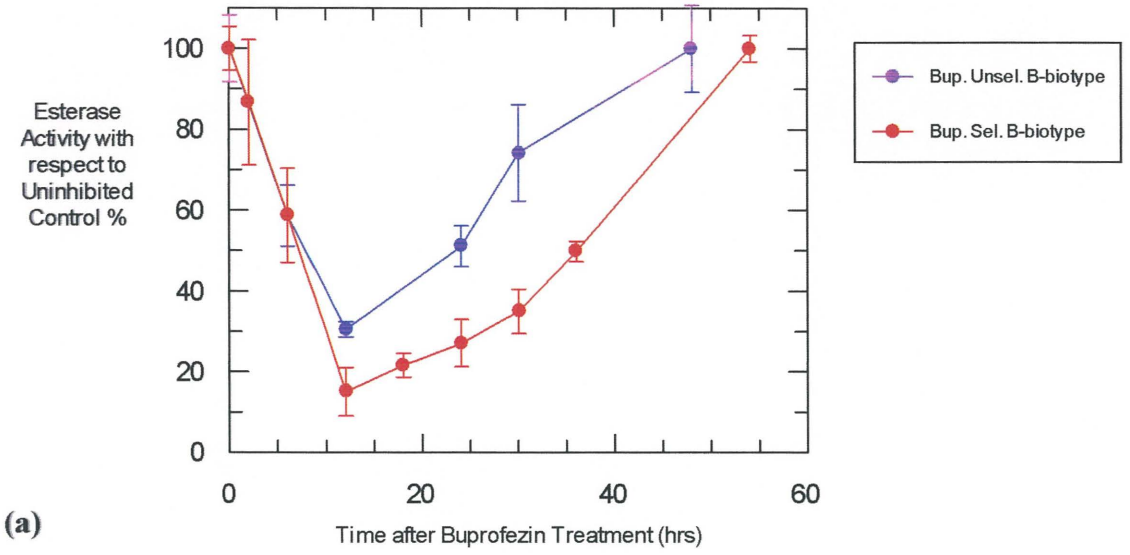
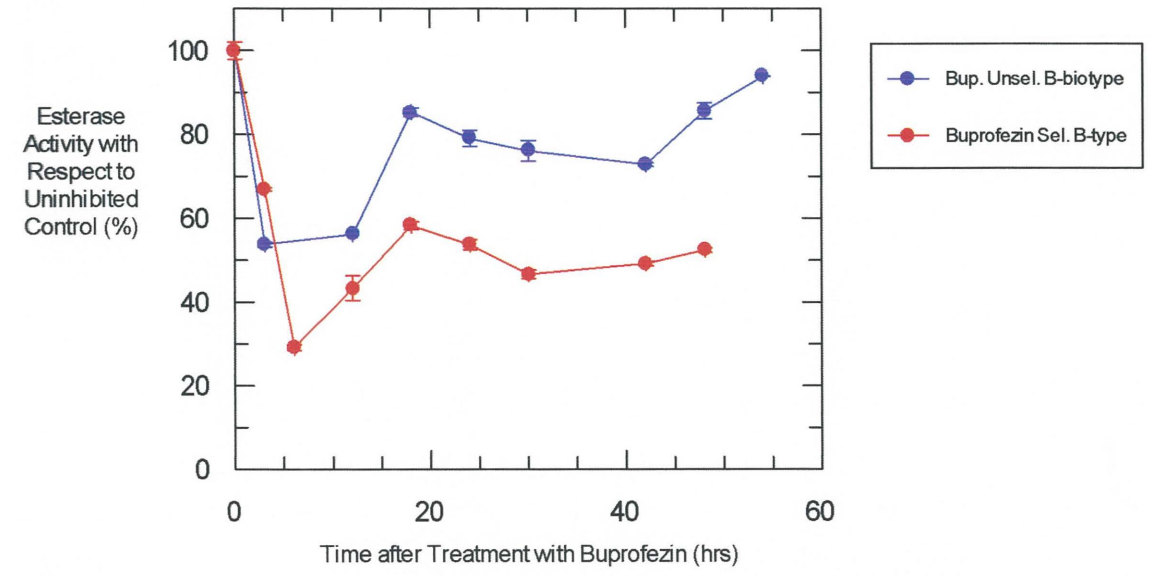


Figure 4.3 *In vitro* total esterase activity (%) of adult *B. tabaci* homogenates incubated with buprofezin for 1 hour.

B. tabaci strains used were native non B-biotype (native non B-biotype), buprofezin unselected B-biotype (bup. res. B-biotype), buprofezin resistant B-biotype (bup. sel. B-biotype), a B-biotype that originated from Egypt (Egypt B-biotype), a buprofezin susceptible strain that originated from Sudan (SUD-S susceptible) and a B-biotype that originated from the USA (USA B-biotype). Egypt, SUD-S and USA-B are kept in culture at IACR- Rothamsted, UK.



(a)



(b)

Figure 4.4 *In vivo* esterase inhibition of buprofezin treated *B. tabaci* nymphs (a) and adults (b). *B. tabaci* strains were buprofezin resistant B-biotype (Bup. sel. B-biotype) and unselected B-biotype (Bup. unsel. B-biotype).

In vivo studies with buprofezin treated adults (Fig. 4.4b) produced similar results to nymphs, with the buprofezin resistant strain showing far greater esterase inhibition than did unselected B-biotype *B. tabaci*. Maximum esterase inhibition occurred at approximately 5 hours after treatment with buprofezin, at 75% and 45% for resistant and non-selected B-biotype *B. tabaci* strains respectively. Esterase activity took approximately 55 hours to recover in the unselected B-biotype strain. Resistant B-biotype *B. tabaci* still had 50% of esterase activity remaining uninhibited at the conclusion of the experiment.

Results (Fig. 4.3) also showed clear and considerable *in vitro* inhibition of esterase by buprofezin in buprofezin resistant B-biotype *B. tabaci*. Inhibition was shown also in buprofezin unselected B-biotype *B. tabaci*, though to a much lesser extent (Fig. 4.3). *In vitro* studies failed to show esterase inhibition in non B-biotype unselected B-biotype *B. tabaci* and SUD-S strains (Fig. 4.3). As the level of esterase inhibition in the buprofezin resistant B-biotype strain (Fig. 4.3) is independent of increasing insecticide concentration, esterase sequestration, rather than through hydrolysis is indicated. Alternatively, it is possible that more inhibitor would lead to greater binding and hence less esterase activity.

In vivo studies showed esterase inhibition by buprofezin in both unselected and buprofezin resistant B-biotype *B. tabaci* strains (Figs. 4.4a & b). As bioassay data for these strains showed (Table 3.1), both strains have resistance to buprofezin, albeit at a low level (11 fold) in the unselected strain. It is therefore not surprising that esterase

inhibition was observed in the unselected strain in *in vitro* studies (Fig. 4.3). However, *B. tabaci* used for the *in vivo* studies were exposed to buprofezin for some considerable time and probably received a much larger dose than the *in vitro* homogenates, giving rise to much higher internal concentrations of buprofezin than were in *in vitro* studies. This could explain the greater level of inhibition measured *in vivo* than *in vitro*. Sufficient native non B-biotype *B. tabaci* were not available for *in vivo* inhibition studies.

Buprofezin is not an ester insecticide, therefore esterase/buprofezin binding cannot involve hydrolysis on the catalytic site of the enzyme. Esterases, however, are adhesive molecules and are well known for the ability to sequester toxins (Devonshire & Moores, 1982; Devonshire, 1991). Therefore, it seems possible that buprofezin resistant B-biotype *B. tabaci* possess esterases which can sequester buprofezin. Sequestration of insecticides by esterases has already been shown to be an important mechanism of pyrethroid resistance in Australian *H. armigera* (Gunning *et al.,* 1996a) and of organophosphate and pyrethroid resistance in *M. persicae* (Devonshire, 1977; Devonshire & Sawicki, 1979; Devonshire *et al.,* 1998). *M. persicae* may contain up to 10 picomoles of E4 esterase per aphid (Devonshire, 1989). These large amounts of E4 esterase can sequester large quantities of insecticide.

Granett and Hejazi (1983), Ishaaya & Degheele (1988) and Ishaaya (1990) and have also implicated esterase involvement in resistance to IGRs. Esterase inhibitors S,S,S-tributylphosphorothioate (DEF) and profenofos increased the toxicity of diflubenzuron in the red flour beetle *Tribolium castaneum* (Herbst), omnivorous leafroller *Platynota*

stultana (Walshingham) and cotton leafworm *Spodoptera littoralis* (Boisduval) larvae. Diflubenzuron is a benzoylphenyl urea chitin inhibitor, with similar action to buprofezin. Over 90% of diflubenzuron hydrolase activity was inhibited when *S. littoralis* larvae were treated with 2.4 x 10^{-4}% profenofos. Addition of a sublethal profenofos dose of 9 x 10^{-5}% to diet containing 9 x 10^{-5}% diflubenzuron resulted in mortality increasing to 48%, as opposed to the diflubenzuron only treatment, which had a mortality of 15% (Ishaaya & Degheele, 1988). Furthermore, the addition of 100ppm dietary DEF increased the toxicity of diflubenzuron to both *T. castaneum* and *S. littoralis* (Gazit *et al.*, 1989; Ishaaya & Klein, 1990), although it was unknown whether these species were resistant to diflubenzuron.

The present finding of esterase involvement in resistance to buprofezin in *B. tabaci* is the first report of a resistance mechanism to buprofezin in *B. tabaci*. In other insect species, esterase mediated resistance can be overcome using esterase inhibitors. The organophosphate profenofos is an excellent esterase inhibitor in *B. tabaci* (Byrne *et al.*, 1991), but esterase inhibition did not take place until 24 hours after exposure to this insecticide. This study with buprofezin showed that esterase inhibition by buprofezin takes place largely in the first 24 hours after treatment, so that organophosphates would be too slow acting to be able to synergise buprofezin when applied as a tank mix to resistant *B. tabaci*. Previous studies (Cottage & Gunning, 1998) showed that other organophosphate insecticides (dimethoate, profenofos and fenthion) are no quicker. Nevertheless, pre-treatment with an organophosphate and then spraying with buprofezin at least 24 hours later could be a valuable field control technique.

4.4 Esterase inhibition by other insecticides

4.4.1 Introduction

Since there are other IGRs with similar modes of action to buprofezin to *B. tabaci*, it is possible that the esterase in buprofezin resistant *B. tabaci* may inhibit other novel insecticides. This may give rise to cross-resistance between buprofezin and these compounds. Insect growth regulators, such as buprofezin and novaluron, have a similar mode of action (both affect chitin activity), and may be prone to cross-resistance problems.

Although cross-resistance is usually associated with insecticides with similar modes of action, in insect growth regulators, cross-resistance cannot be necessarily predicted by chemical structure or mode of action alone. Studies by Devine *et al.* (1999) showed that, while pyriproxyfen and fenoxycarb are similar structurally and have the same mode of action, there was no cross-resistance between pyriproxyfen and fenoxycarb in Israeli *B. tabaci.* It is therefore essential to investigate whether esterase mediated buprofezin resistance conferred cross-resistance to other insecticides with novel modes of action. Consequently, the aim of these experiments was to determine whether buprofezin resistant *B. tabaci* esterase bound to other novel insecticides.

4.4.2 Materials and methods

In vitro incubation of *B. tabaci* homogenate with insecticides

Insecticides used were pyriproxyfen (technical grade 97.4%) (Sumitomo Australia Ltd., Appendix 1), novaluron (technical grade 98.6%) (Nufarm Ltd., Appendix 1), pymetrozine (technical grade 98.9%) (Novartis, Appendix 1), flufenoxuron (EC grade 10% a.i.) (BASF Australia, Appendix 1) and fenoxycarb (EC grade 25% a.i.) (Novartis).

Eighty buprofezin resistant B-biotype *B. tabaci* adult whiteflies were homogenised in 1.6mL 0.02M pH 7.0 phosphate buffer containing 0.05% Triton X-100. This was repeated for buprofezin unselected B-biotype *B. tabaci.* Aliquots (200µL) were pipetted into 0.6mL snap-top microtubes, and made up to 5% MEOH to increase insecticide solubility. Insecticides were added in acetone to make final concentrations of 1, 2, 5, 10, 50 and 100ppm in the homogenates. Homogenates (200µL) from each strain with methanol and acetone only, were used as the uninhibited controls. The homogenates were incubated for up to 3 hours at 25°C, and samples were taken for esterase assays at one and three hours, (with the exception of flufenoxuron, where samples were taken at one hour and 90 minutes). Aliquots of 10µL and 15µL were used in total esterase assays and gel electrophoresis respectively, as previously described (Section 4.2.1).

Pyriproxyfen bioassay

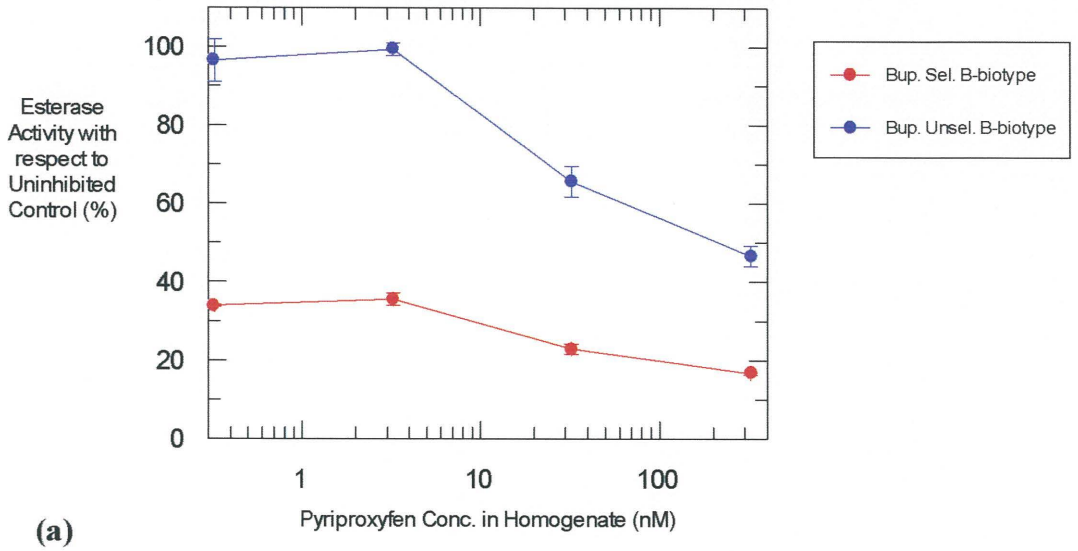
The bioassay method used for IGRs was described in Section 2.3.2. *B. tabaci* were allowed to oviposit on poinsettia cuttings for 48 hours, then removed. Eight days after oviposition, 1st/2nd instar nymphs were counted and cuttings were dipped for 20 seconds

in six pyriproxyfen (10 EC) concentrations, ranging from 1ppm up to 100ppm. Leaves were dipped in Agral® and water only, as controls. Twenty days after oviposition, a final count of live 3rd/4th instar was made and the percentage mortality at each dose calculated. Data were corrected for control mortality and analysed by probit analysis, as described in Section 2.5.

4.4.3 Results and discussion

Experiments with pyriproxyfen showed inhibition of esterase activity in both unselected and buprofezin resistant B-biotype *B. tabaci* at both one and three hours incubation (Fig. 4.5a & b). Inhibition was greater in the resistant strain. Some 70-80% of buprofezin resistant B-biotype *B. tabaci* esterase was inhibited by concentrations between 0.1 and 200nM of pyriproxyfen. Esterases from buprofezin unselected B-biotype *B. tabaci* were also inhibited between concentrations of 1-200nM. This is consistent with bioassay data which showed a low level of cross-resistance between buprofezin and pyriproxyfen (Table 4.1, Fig. 4.6). Buprofezin resistant B-biotype *B. tabaci* were 7.43 times more resistant to pyriproxyfen (Table 4.1) than the buprofezin unselected population.

For novaluron, total esterase assays showed that unselected B-biotype *B. tabaci* esterase activity was uninhibited at concentrations between 0.01-100nM. However, after three hours incubation 10-15% of resistant esterase was inhibited between these concentrations (Fig. 4.7b). Buprofezin resistant and unselected B-biotype strains tested for esterase activity prior to three hours did not show any inhibition (Fig. 4.7a). Esterase binding to



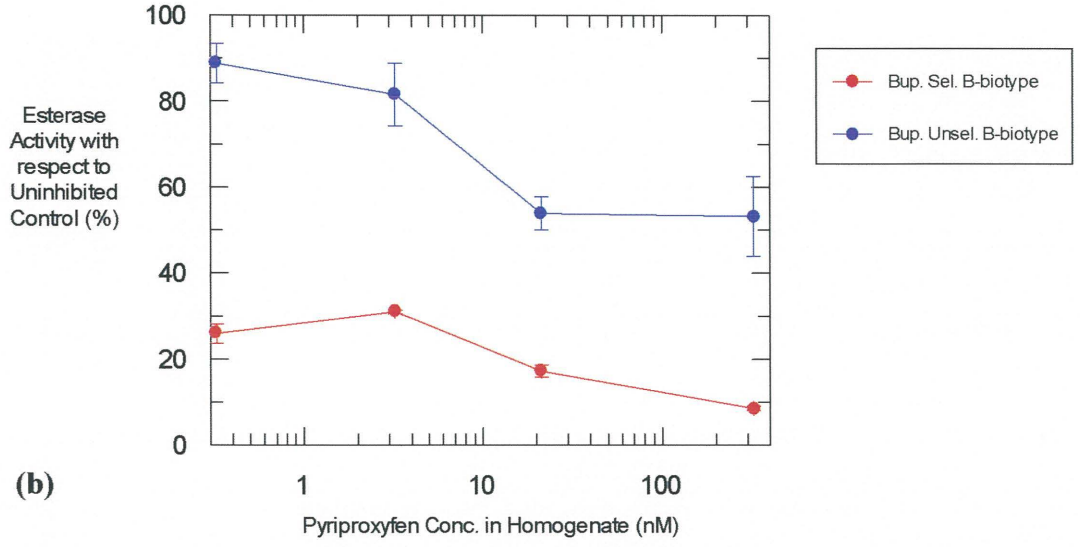


Figure 4.5a & b Esterase activity of adult *B. tabaci* homogenate incubated with pyriproxyfen for one hour (a) and three hours (b). Strains used were buprofezin resistant (bup. sel. B-biotype) and unselected (bup. unsel. B-biotype) B-biotype *B. tabaci*.

Table 4.1: Response of buprofezin non selected and resistant B-biotype *B. tabaci* populations to pyriproxyfen

Strain	LC_{50} (ppm)	Fiducial Limits	Slope	Resistance Factor
Buprofezin Unselected B-biotype	3.23	(1.15 - 9.09)	1.29	n.a.
Buprofezin Selected B-biotype	24	(15.1 - 38.3)	3.03	7.43

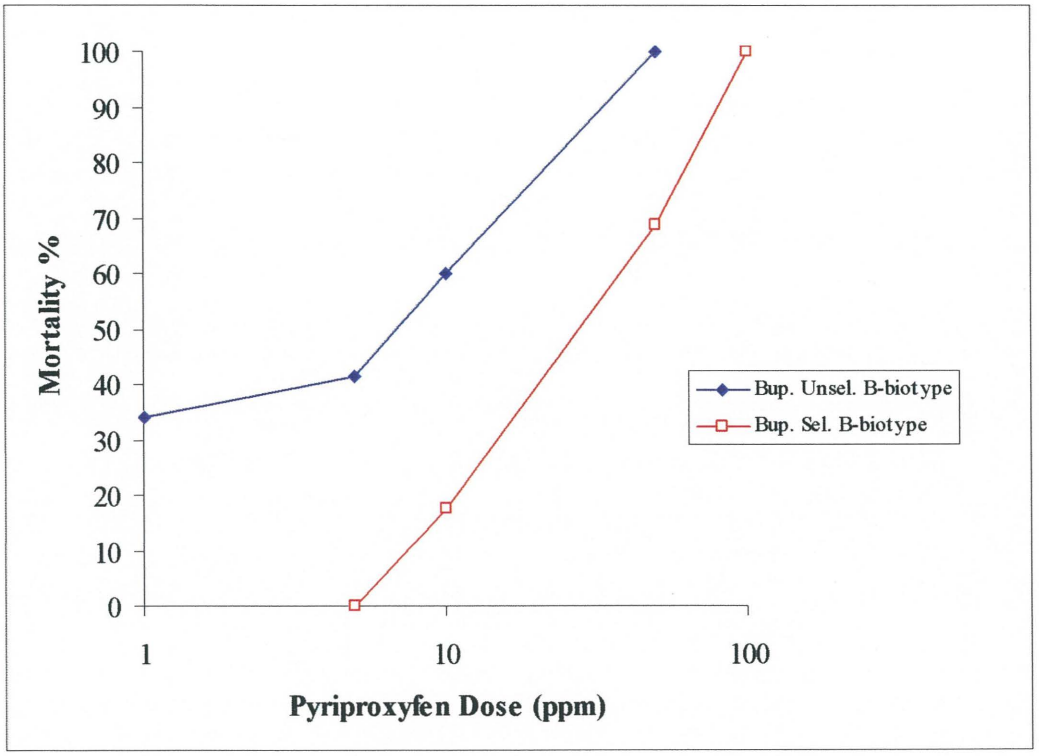
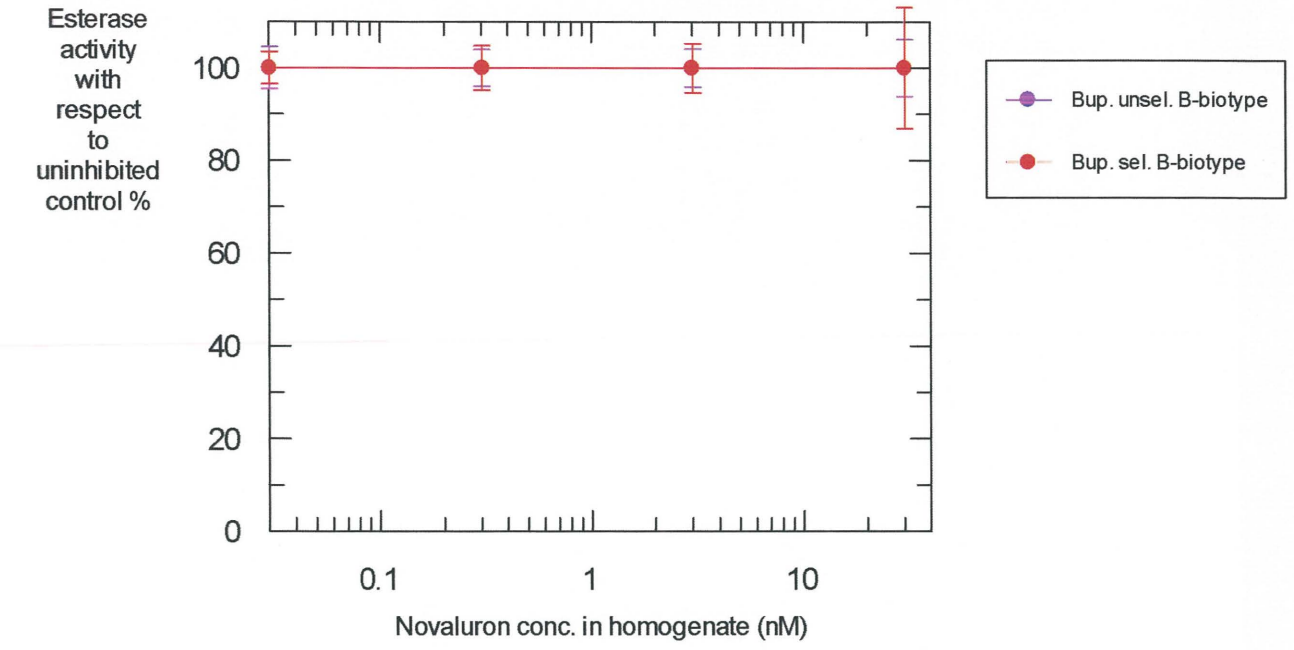
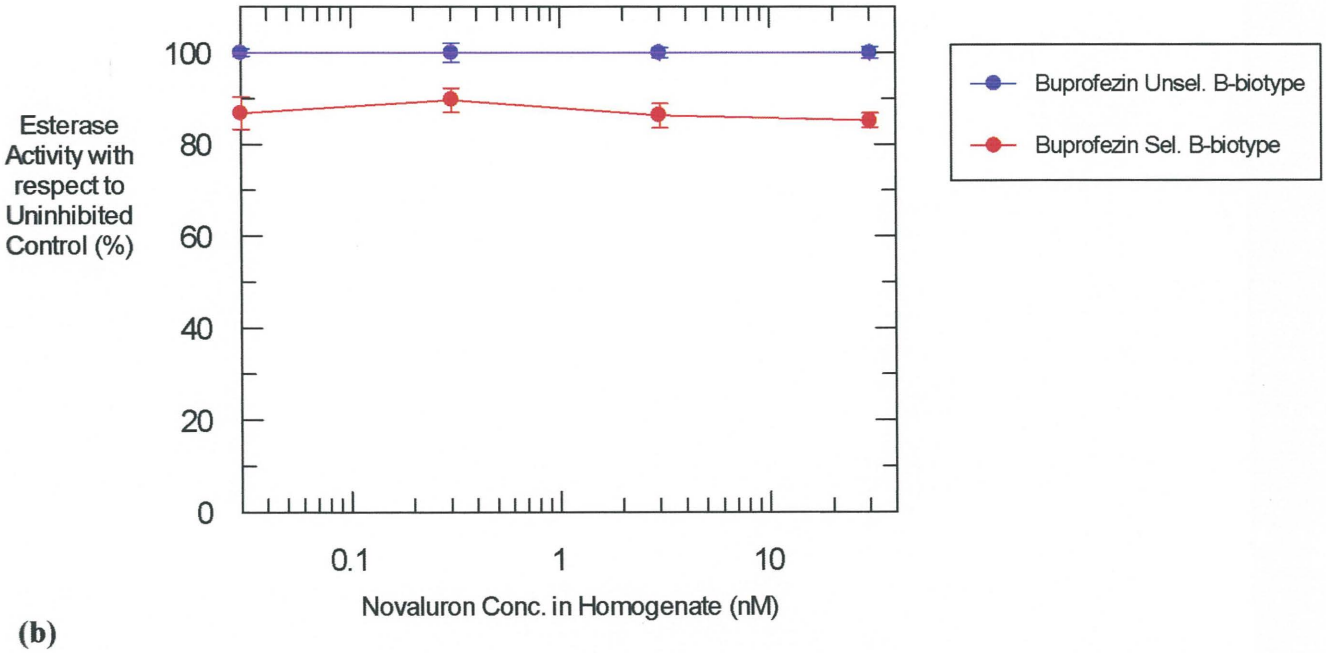


Figure 4.6 Mortality of buprofezin unselected and selected B-biotype *B. tabaci* nymphs treated with pyriproxyfen.



(a)



(b)

Figure 4.7 Esterase activity of B-biotype *B. tabaci* homogenate incubated with novaluron for one (a) and three hours (b). Strains used were buprofezin resistant (Bup. sel. B-biotype) and unselected (Bup. unsel. B-biotype) B-biotype *B. tabaci*.

novaluron was not detectable on the gels (Fig. 4.8) and it is likely that the complex did not survive gel sieving. Buprofezin selected or unselected B-biotype *B. tabaci* did not show any significant inhibition of esterase activity after incubation with fenoxycarb at one (Fig. 4.9a) or three hours (Fig. 4.9b). This lack of esterase inhibition was also shown in gels in the buprofezin resistant B-biotype strain (Fig. 4.10b).

Buprofezin resistant esterase was inhibited by approximately 10% after incubating for an hour with pymetrozine concentrations between 0.5 to 500nM pymetrozine (Fig. 4.11a). Inhibition did not increase with further incubation (Fig. 4.11b). Esterase of unselected B-biotype *B. tabaci* was uninhibited by pymetrozine (Fig. 4.11a &b). Esterase inhibition by pymetrozine was not shown in the gel of buprofezin non-selected B-biotype *B. tabaci* (Fig. 4.12a). Esterase bands produced by insecticide treated buprofezin resistant *B. tabaci*, were more faintly stained than those produced by the untreated control (Fig. 4.12b).

Like buprofezin and novaluron, flufenoxuron is also a chitin inhibiting insecticide, although not used against whiteflies. When both buprofezin resistant and non-selected B-biotype *B. tabaci* homogenates were incubated with flufenoxuron, esterase inhibition was evident in both strains when the insecticide concentration was highest (approximately 1nM) after one hour (Fig. 4.13a) and 90 minutes (Figs. 4.13b).

These experimental data indicated that esterases from buprofezin resistant B-biotype *B. tabaci* were inhibited by two other insect growth regulators, in addition to buprofezin. Some 10-15% of esterase was inhibited by novaluron in buprofezin resistant B-biotype

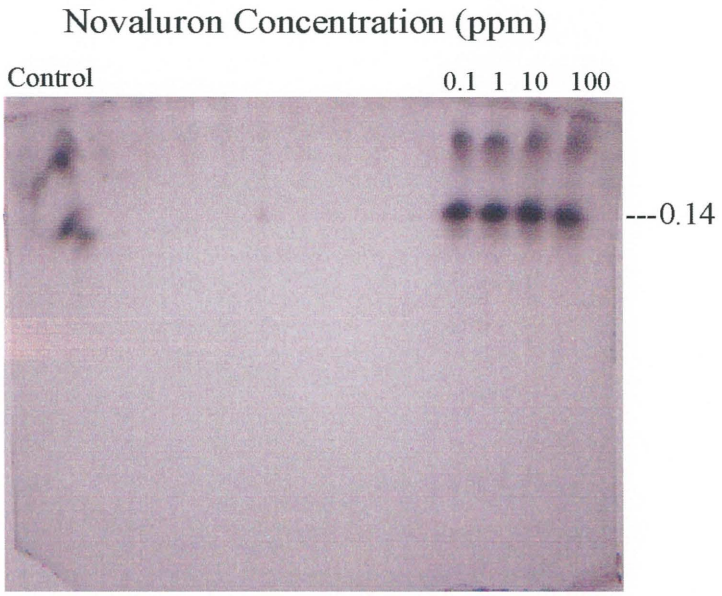
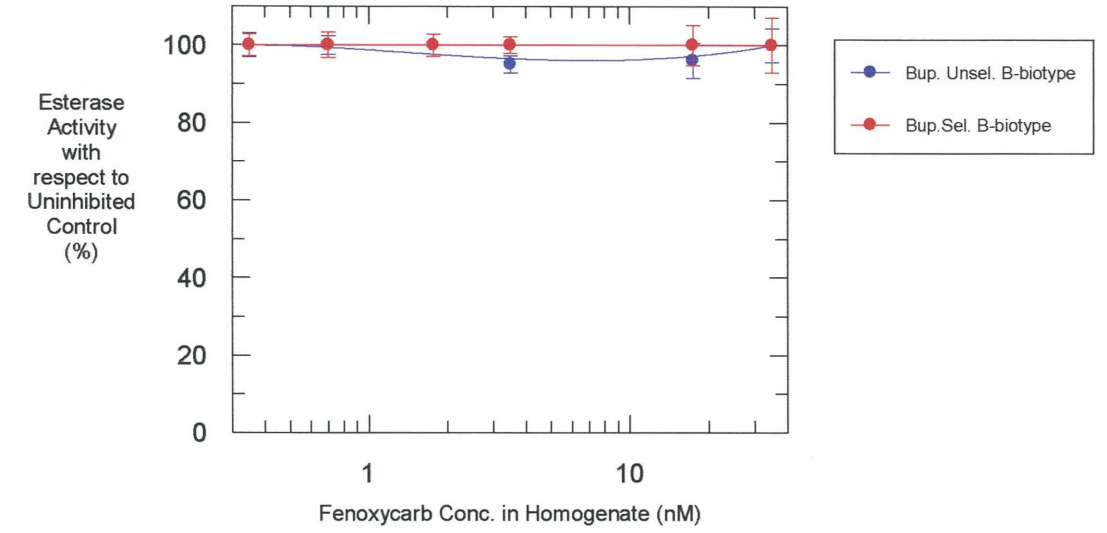
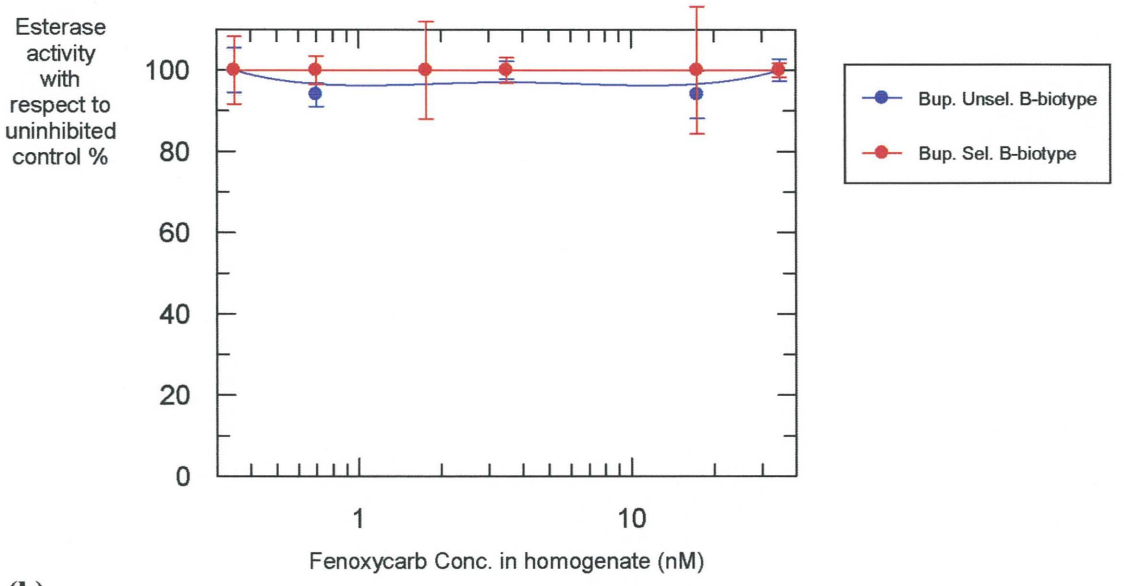


Figure 4.8 Polyacrylamide gel showing isoenzyme patterns of buprofezin unselected B-biotype *B. tabaci* incubated with novaluron for one hour.



(a)



(b)

Figure 4.9 Esterase activity of B-type adult *B. tabaci* homogenate incubated with fenoxycarb for one hour (a) and three hours (b). Strains used were buprofezin resistant (Bup. sel. B-biotype) and unselected (Bup. unsel. B-biotype) B-biotype *B. tabaci*.

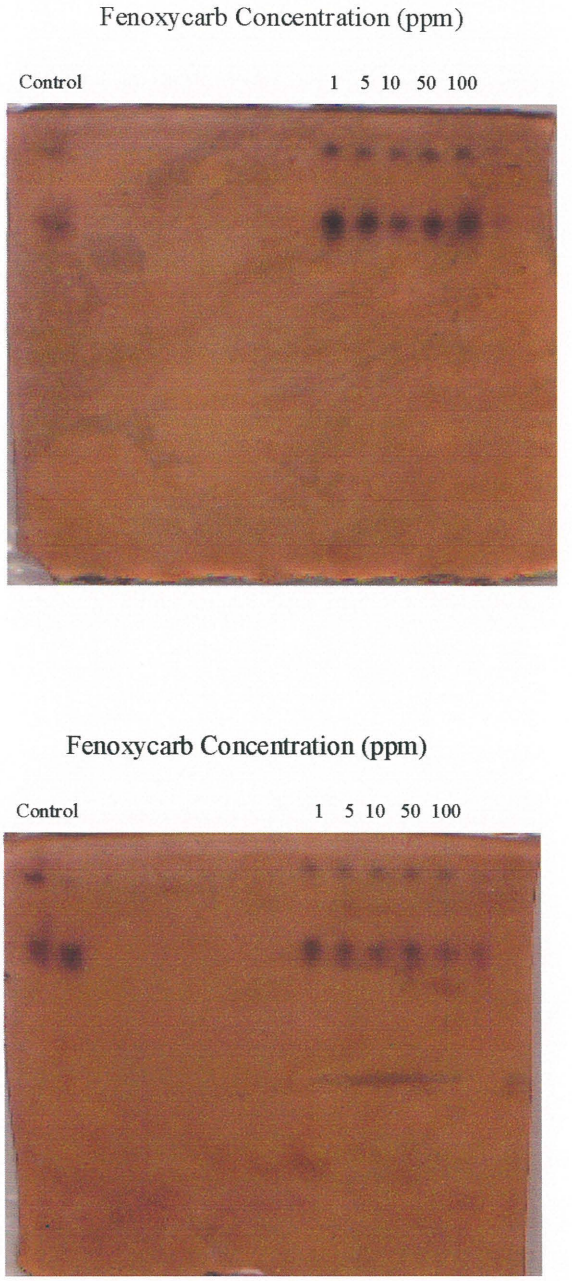
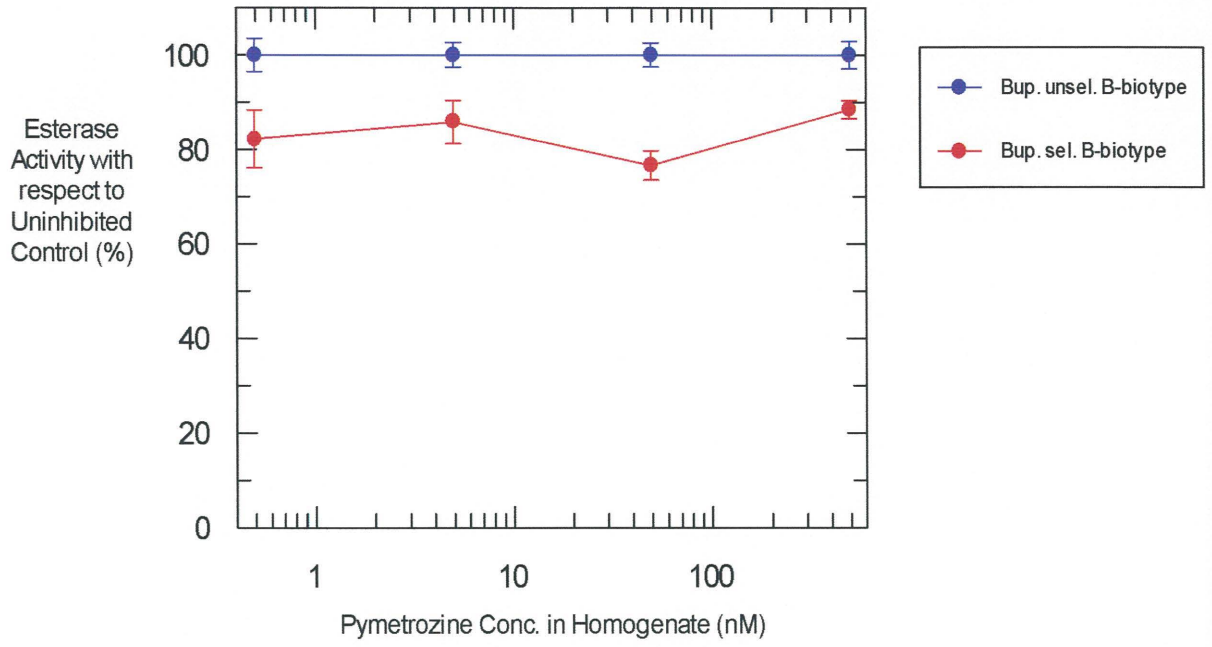
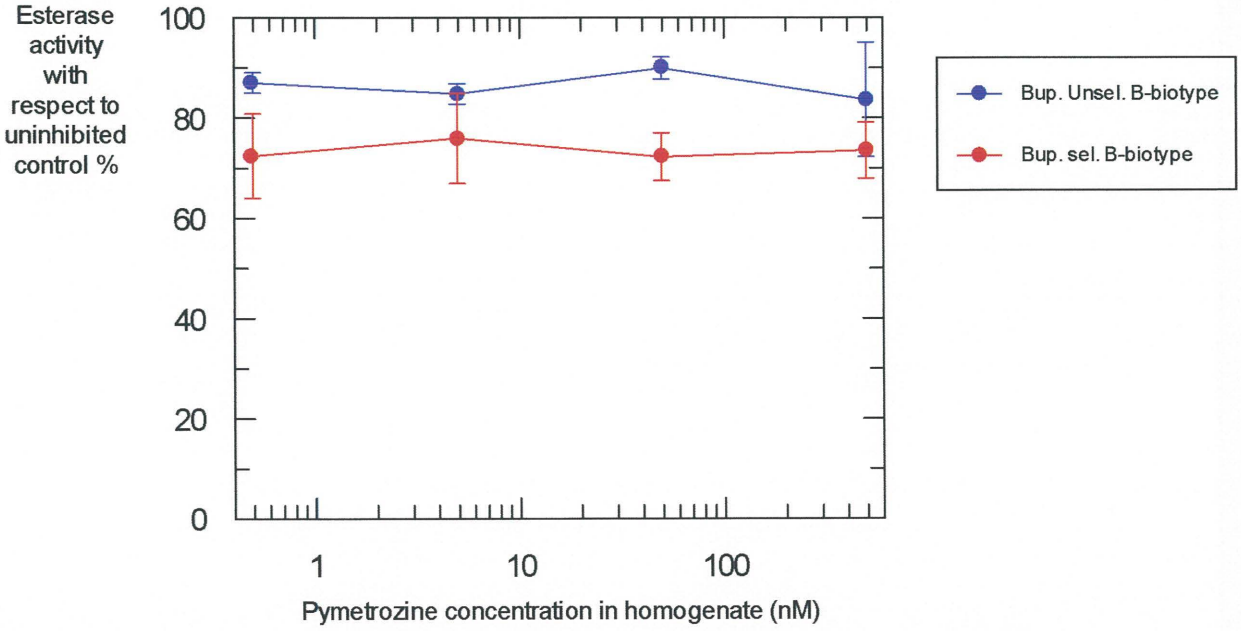


Figure 4.10a & b Esterase isoenzyme patterns of buprofezin unselected (a) and resistant (b) B-biotype *B. tabaci* homogenates incubated with fenoxycarb for one hour.

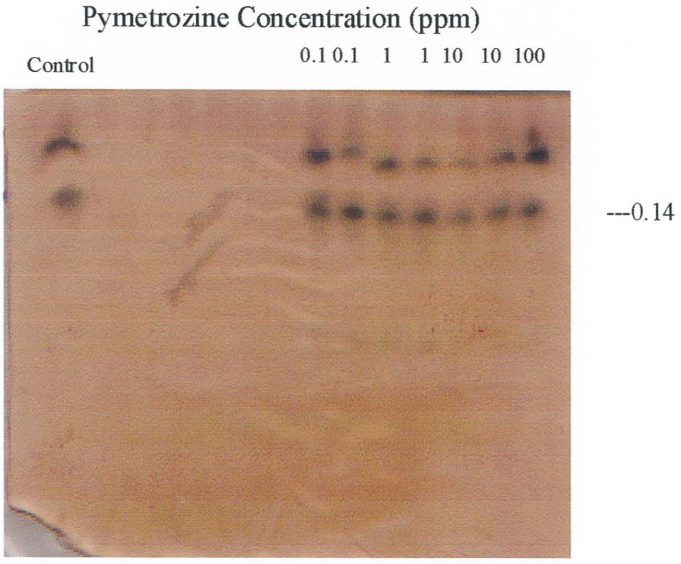


(a)

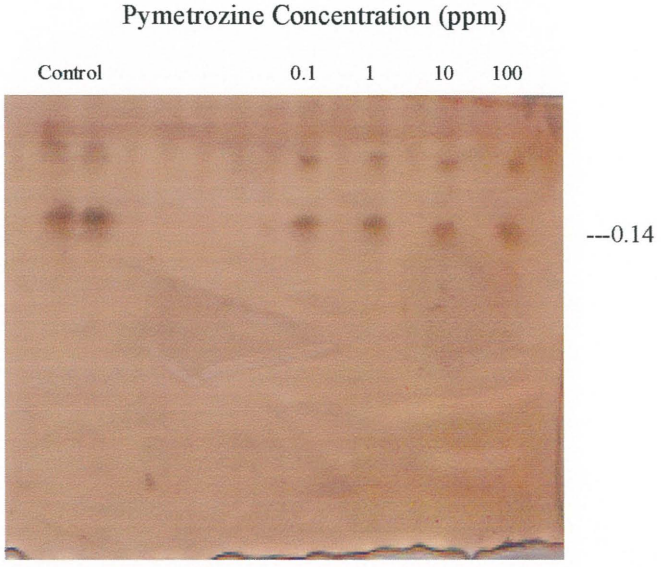


(b)

Figure 4.11a & b Total esterase activity of B-biotype *B. tabaci* homogenates incubated with pymetrozine for one hour (a) and three hours (b). Strains used were buprofezin resistant (bup. sel. B-biotype) and unselected (bup. unsel. B-biotype) B-biotype *B. tabaci*.

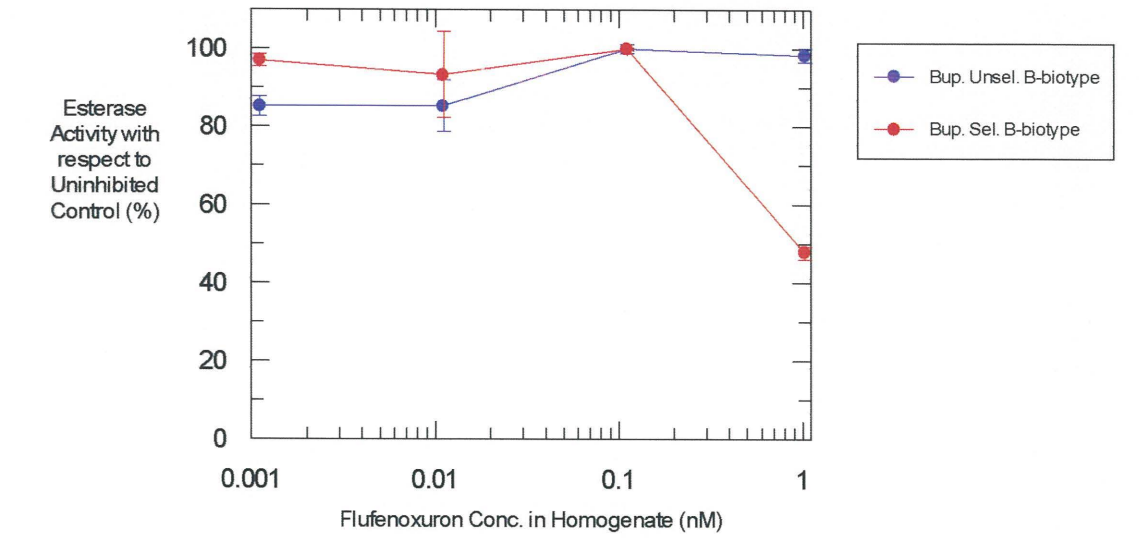


(a)

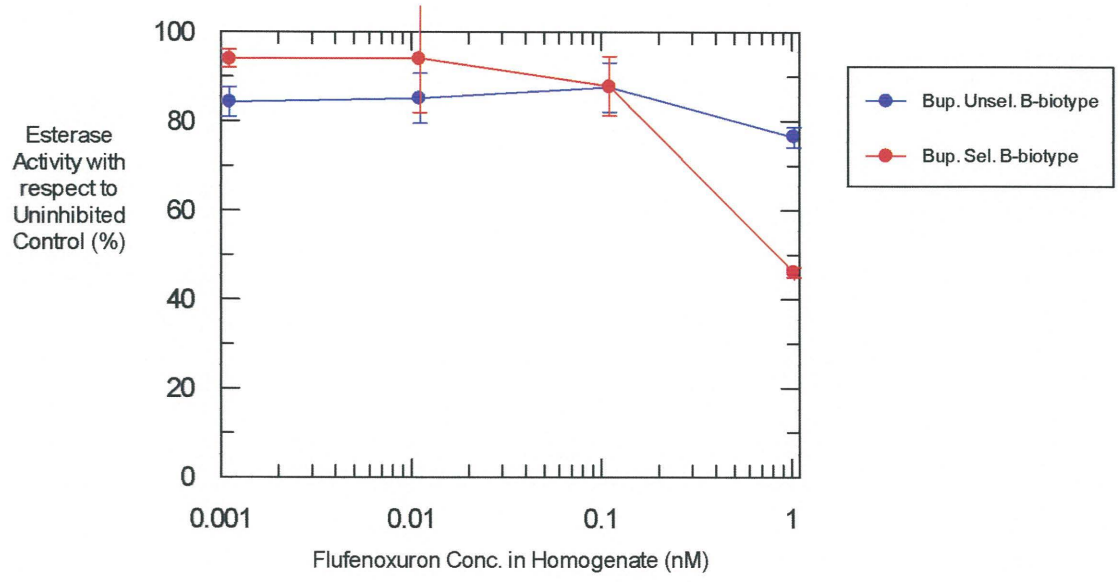


(b)

Figure 4.12 Esterase isoenzyme patterns of buprofezin unselected B-biotype *B. tabaci* (a) and buprofezin resistant (b) incubated with pymetrozine for one hour. Strains used were buprofezin resistant (bup. sel. B-biotype) and unselected (bup. unsel. B-biotype) B-biotype *B. tabaci*.



(a)



(b)

Figure 4.13 Total esterase activity of adult B-biotype *B. tabaci* homogenates incubated with flufenoxuron for one hour (a) and 90 minutes (b).

Strains used were buprofezin resistant (bup. sel. B-biotype) and unselected (bup. unsel. B-biotype) B-biotype *B. tabaci*.

B. tabaci. Novaluron, like buprofezin, is a chitin synthesis inhibitor and its inhibition in the buprofezin resistant strain suggests that there may be cross-resistance between buprofezin and novaluron. If this is proven to be the case in field material, management strategies such as treating IGRs as one group and not allowing consecutive use of IGRs, even if they are different chemicals, should be a part of any use in Australia.

Unlike pyriproxyfen, the juvenile hormone analogue fenoxycarb did not inhibit esterase in either the buprofezin resistant or unselected B-biotype strains. While both buprofezin unselected and resistant strains showed esterase inhibition by pyriproxyfen, inhibition in the buprofezin resistant strain was much greater. Bioassay data (Fig. 4.6) showed buprofezin resistant *B. tabaci* had a higher tolerance (approximately 10 fold) to pyriproxyfen than its unselected counterparts. Cross-resistance between buprofezin and pyriproxyfen was also demonstrated in Emerald field B-biotype *B. tabaci* in 2002 (R. Gunning, unpublished).

In the USA, pyriproxyfen resistant *B. tabaci* also showed greater tolerance to buprofezin (T. Dennehy, pers. comm. 2002). Buprofezin selection in my studies produced a lower level of resistance to pyriproxyfen, the data demonstrating the potential for cross-resistance between these two insecticides. If both buprofezin and pyriproxyfen were used simultaneously, the level of cross-resistance to both would probably be quite high. It is also interesting to note that in the field, buprofezin was the stronger selecting agent for cross-resistance than pyriproxyfen (R. Gunning, unpublished). These data would support

the use of pyriproxyfen over buprofezin if only one IGR was to be used in a resistance management strategy.

Possible cross-resistance between buprofezin and pyriproxyfen is perhaps an unexpected finding, given that these two insecticides have distinctly different modes of action. An explanation for this phenomenon lies in esterases being very non specific enzymes, apparently able to sequester more than one type of toxicant. Since pyriproxyfen and buprofezin are among the promising compounds for *B. tabaci* control in Australia, future work should further explore this possible cross-resistance, and its importance for field control. Reciprocal selection experiments using field material are essential, because the insecticide may have a different impact on the pest under field conditions. Unpredictable, low level cross-resistance relationships are not uncommon and may not accurately reflect field resistance problems caused by major genes.

In the literature, little is known about pyriproxyfen resistance mechanisms in *B. tabaci*. Devine *et al.* (1999) have suggested that low mono-oxygenase levels may prevent activation to a more toxic metabolite. However, these workers used piperonyl butoxide (PBO) synergism as an indicator of mono-oxygenase activity and it is becoming increasingly well known that PBO is an esterase inhibitor in a variety of insects, including B-biotype *B. tabaci* (Gunning *et al.*, 1998; S. Young, R. Gunning & G. Moores, unpublished).

The feeding inhibitor pymetrozine was also found to inhibit approximately 10% esterase activity in buprofezin resistant B-biotype *B. tabaci,* while esterases from unselected B-biotype *B. tabaci* remained unaffected. Therefore, it is also possible that there is cross-resistance between buprofezin and pymetrozine. This could also form the subject of further studies.

4.4.4 Conclusions

It is obvious from these data that much future work concerning cross-resistance and novel insecticides is required. Cross-resistance between novaluron and buprofezin could not be verified by bioassay as there were insufficient buprofezin resistant B-biotype *B. tabaci* available. Such bioassays could form the subject of further studies. Future research could also include use of radiolabelled buprofezin to further investigate buprofezin/esterase binding and other techniques such as surface plasmon resonance to detect ligand formation. Synergism studies with buprofezin and other insect growth regulators using esterase inhibitors should also be undertaken. A complete understanding of buprofezin resistance and cross-resistance is required before any rational resistance management strategy for insect growth regulators can be formulated for B-biotype *B. tabaci* in Australia.

Chapter 5: Metabolism of Buprofezin

5.1 Introduction

Data from experiments described in the previous chapter indicated that buprofezin was probably being sequestered by esterases in resistant B-biotype *B. tabaci*. As sequestration may lead to eventual metabolism, thin layer chromatography experiments were designed to detect any buprofezin metabolites in B-biotype *B. tabaci*.

5.2 Materials and methods

Thin layer chromatography is a separation method involving the use of a chromatographic adsorbent in the form of a thin layer affixed to an inert rigid support (Stock & Rice 1967). The movement of substances in thin layer chromatography is a result of opposing forces. The driving force is the mobile phase (eg solvent) which moves substances from the origin in the direction of the mobile phase flow. The retarding force is the sorbent, which drags the substances out of the mobile phase and back onto the sorbent. The distance (R_f) travelled by the substance is the result of the driving and retarding forces. Thin layer chromatography is commonly used to separate and identify insecticide metabolites. R_f is calculated as follows:-

$$R_f = \frac{\text{distance of the substance from the start}}{\text{distance of the solvent front from the start}}$$ (Randerath, 1966).

To investigate any buprofezin metabolism in resistant B-biotype *B. tabaci* and native non B-biotype *B. tabaci* the following methods were employed. Ten adult whiteflies from each strain were homogenised in 200µL pH 7.0 0.02M phosphate buffer. Tubes were centrifuged at 3000 rpm for 5 minutes. The supernatant was decanted in 90µL aliquots into clean centrifuge tubes and incubated at 25°C with buprofezin (0.001%) for up to 22 hours. After 1, 2, 4 and 22 hours, 5µL samples were taken and the reaction stopped by the addition of 200µL of very cold acetone (-20°C) (Gunning *et al.*, 1995b).

Each sample was spotted onto a TLC plate (Whatman silica gel 60A LK6DF) (Crown Scientific, Appendix 2), dried, placed in a chromatography tank and co-chromatographed alongside buprofezin. The chromatography solvent was toluene (saturated with formic acid) in a ratio of 5:1 by volume with ether. Buprofezin and any metabolic products were located under ultra violet light and R_f values calculated. There were three replications of the experiment. Controls consisting of homogenate only were also run.

5.3 Results

Data from the thin layer chromatography experiment are summarised in Table 5.1. Buprofezin was easily distinguished on the TLC plate under UV light. R_f values of buprofezin ranged from 0.68 to 0.71. The solvent system used was therefore a suitable medium for TLC of buprofezin.

As well as the parent compound buprofezin, homogenate showed the presence of an unknown compound in both *B. tabaci* strains. The quantity of the metabolite did not

change during the course of the experiment in either strain. The R_f of the unknown compound ranged from 0.62 –0.64.

Table 5.1 Compounds isolated from *in vitro* metabolism of buprofezin in homogenates of buprofezin resistant B-biotype *B. tabaci* and native non B-biotype *B. tabaci.*

TLC Isolate	R_f	Res. 1hr	Res. 2hrs	Res. 9hrs	Res. 22hrs	Nat. 1hr	Nat. 2hrs	Nat. 4hrs	Nat. 2hrs
Buprofezin	0.68-0.71	0.68-0.71	0.68-0.71	0.68-0.71	0.68-0.71	0.68-0.71	0.68-0.71	0.68-0.71	0.68-0.71
Unknown Compound	0.62-0.64	0.62-0.64	0.62-0.64	0.62-0.64	0.62-0.64	0.62-0.64	0.62-0.64	0.62-0.64	0.62-0.64

Legend: R_f = distance (R_f) travelled by the substance is the result of the driving and retarding forces.
Res = buprofezin resistant B-biotype *B. tabaci*
Nat. = native non B-biotype *B. tabaci*
hrs = No. hours homogenate incubated with buprofezin

5.4 Discussion and conclusions

Buprofezin metabolites were easily identified by TLC. The unknown TLC isolate (R_f 0.62-0.64) was not further characterised. Nonetheless, the TLC experiments have produced interesting information. The unknown compound had a R_f value similar to buprofezin and therefore possible that it was a buprofezin-like compound. The fact that the unknown compound was detected in both resistant and susceptible strains suggests that it was a normal breakdown product of buprofezin and perhaps unrelated to resistance. It seems, therefore, that sequestration of buprofezin by the resistant strains

does not necessarily result in detectable metabolites. Additional homogenate incubation experiments adding NADPH, which engages the mono-oxygenase system, may help to resolve this issue.

Chapter 6: Buprofezin Resistance Mechanisms - Acetylcholinesterase

6.1 Introduction

The mode of action of insect growth regulators such as buprofezin has never been fully understood. Given the findings detailed in previous chapters that esterases are involved in *Bemisia tabaci* resistance to IGRs, it seems possible that these insecticides may have other effects. The cholinesterase system is a common source of resistance mechanisms in *B. tabaci*, therefore it was decided to investigate the effect of buprofezin on acetylcholinesterase activity of B-biotype *B. tabaci*.

6.2 Materials and Methods

6.2.1 Microplate assay of acetylcholinesterase activity in *B. tabaci*

Acetylcholinesterase activity was measured using the method of Ellman *et al.* (1961). This method involves the hydrolysis of the substrate analogue acetylthiocholine iodide (ATChI) (Sigma-Aldrich), which is measured colourimetrically by the absorbance of 2-nitro-5-thiobenzoate at 405nm, after the reaction of 5-5'-dithio-bis(-2-nitrobenzoate) (DTNB) (Sigma-Aldrich) with the liberated thiocholine. Assays were done in a microplate, as described for houseflies by Moores *et al.* (1988).

Native non B-biotype *B. tabaci*, buprofezin unselected and resistant B-biotype *B. tabaci* were homogenised individually in 20µL 0.1M pH 7.5 phosphate buffer containing 0.1%

Triton X-100. Aliquots (10μL) were pipetted into a 96 well microplate. ATChI and DTNB solutions were dissolved in buffer (0.1M pH 7.5 phosphate buffer containing 0.1% Triton X-100), and 100μL of each added, to give final concentrations of 0.5μM and 0.05mM, respectively. The microplate was placed in a Bio-Rad microplate reader (Bio-Rad Laboratories) and read at 405nm for 30 minutes at 14 second intervals, utilising Kinetic Collector software (Bio-Rad Laboratories) to fit linear regressions to the kinetic plots.

6.2.2 Polyacrylamide gels stained for acetylcholinesterase activity

B. tabaci (native non B-biotype, buprofezin unselected and resistant B-biotypes) were homogenised individually in 20μL of 1.6% Triton X-100 containing 10% sucrose. Aliquots (15μL) were pippetted into wells of 7.5% polyacrylamide gels (Byrne & Devonshire, 1991). Gels were run for 90 minutes at 250V at maximum current (0.55% barbitone buffer system).

The method for gel staining for acetycholinesterase activity was that of Karnovsky and Roots (1964). Acetylthiocholiniodide (25mg) was dissolved in 32.5 mL of AChE buffer (0.1M pH 7.5 phosphate buffer in 0.1% Triton X-100). Then, 2.5mL of 0.1M sodium citrate (Sigma-Aldrich), 5mL of 39mM copper sulfate (Sigma-Aldrich), 5mL of distilled and deionised water and 5mL of 5mM potassium ferricyanide (Sigma-Aldrich) were added in consecutive order. The gel was stained in the solution until bands appeared. Gels were photographed.

6.2.3 Microplate assay of AChE activity in *B. tabaci* homogenates incubated with buprofezin

Fifty buprofezin resistant and non selected B-biotype *B. tabaci* were mass homogenised in 1mL of 0.1M phosphate buffer (pH 7.5 and containing 0.1% Triton X-100). Buprofezin was serially diluted in acetone to the following concentrations: 1, 10, 100, 1000 and 10,000 ppm. Aliquots (100µL) of homogenate were made up to buprofezin concentrations of 0.02, 0.05, 0.2, 0.5, 2, 5, 20, 50, 200 and 500ppm. Each aliquot, including the control, contained a total volume of 5µL acetone. Previous studies have shown that this does not significantly inhibit AChE activity. Solutions were incubated for one hour at 25°C. Aliquots (10µL) were pipetted onto a clean microplate and assayed for AChE activity (Section 6.2.1). AChE activity was plotted as a percentage of the uninhibited control against insecticide concentration. This experiment was replicated four times, data pooled and standard errors calculated.

6.2.4 Electrophoresis of AChE inhibition to buprofezin

Aliquots (15µl) of homogenate used for the AChE insensitivity studies were pippetted into gel wells. The gel was run for 90 minutes at 250V at maximum current. Gels were stained for AChE activity using the method of Karnovsky and Roots (1964). R_m values were determined. The gels were photographed.

6.2.5 Relationship to organophosphate and carbamate resistant acetylcholinesterase

Buprofezin resistant and unselected B-biotype *B. tabaci* with known insensitive AChE to organophosphates and methomyl (R. Gunning, pers. comm. 2000) were mass homogenised (100 whiteflies) in 2mL of 0.01M pH 7.5 phosphate buffer containing 0.1% Triton X-100. Aliquots (10µL) were pipetted in 96-well microplates.

Insecticides used were technical grade methyl paraoxon (99.9%, Bayer Australia, Appendix 1), azamethiphos (Novartis), demeton-*S*-methyl (Bayer Australia) and methomyl (DuPont Australia Ltd., Appendix 1). Insecticides were dissolved in acetone and six concentrations of 0.475, 0.95, 1.9, 4.75, 9.5 and 19 mM were prepared in buffer (0.1M pH 7.5 phosphate buffer with 0.1% Triton X-100) containing ATChI. DTNB (100µL) and insecticide/ATChI mix (100µL) were added to homogenate aliquots. Wells containing 100µL of ATChI/buffer only served as uninhibited controls. The AChE assay was run immediately at 405nm for 80 readings at 14 second intervals utilising Kinetic Collector software (Bio-Rad Laboratories) to fit linear regressions to the kinetic plots. AChE activity in the presence of insecticides was calculated as a percentage of the corresponding uninhibited rate. Curves of enzyme activity versus final insecticide concentration in the wells were plotted. Experiments were repeated three times, and means and standard errors calculated.

6.3 Results and discussion

There were important differences detected between AChE activity of buprofezin resistant and non-selected B-biotype *B. tabaci*. Results (Fig. 6.1) showed that native non B-biotype had higher mean AChE activity (10.61mOD/min) than either buprofezin resistant (2.74 mOD/min) or buprofezin unselected B-biotypes (2.34 mOD/min).

The electrophoretic mobility of AChE bands differed between buprofezin unselected and resistant strains, with relative mobility (Rm) values of 0.15 and 0.16 respectively (Fig. 6.2a). The different Rm values suggest that these are two different forms of AChE in these two strains (Fig. 6.2). There were no differences between the relative mobilities of AChE bands of native non B-biotype and buprofezin unselected B-biotype *B. tabaci*, both having relative mobility values of 0.15 (Fig. 6.2b).

Buprofezin resistant B-biotype *B. tabaci* had higher mean AChE activity (2.74 mOD/min) than the buprofezin unselected strain (2.34 mOD/min) (Fig. 6.1). This finding is interesting because organophosphate insensitive forms of AChE in *B. tabaci* are much less active than AChE from the susceptible strain (Byrne & Devonshire, 1997). However, the AChE form that demonstrates insensitivity to paraoxon but sensitivity to azamethiphos is higher AChE activity than the susceptible strain (G.D. Moores, pers. comm. 2003). Increased AChE activity may reflect either increased production of the enzyme or a modified enzyme with higher catalytic efficiency. Larger amounts of AChE expressed in the insect nervous system may function as a scavenger of insecticide molecules, leading to increased tolerance (Fournier *et al.*, 1992).

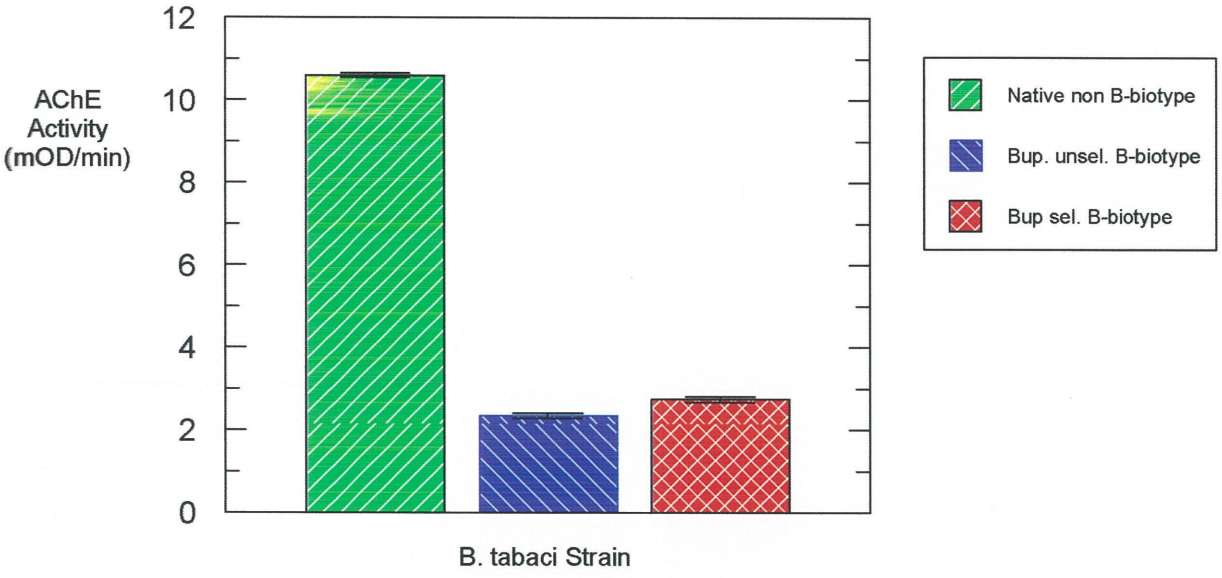
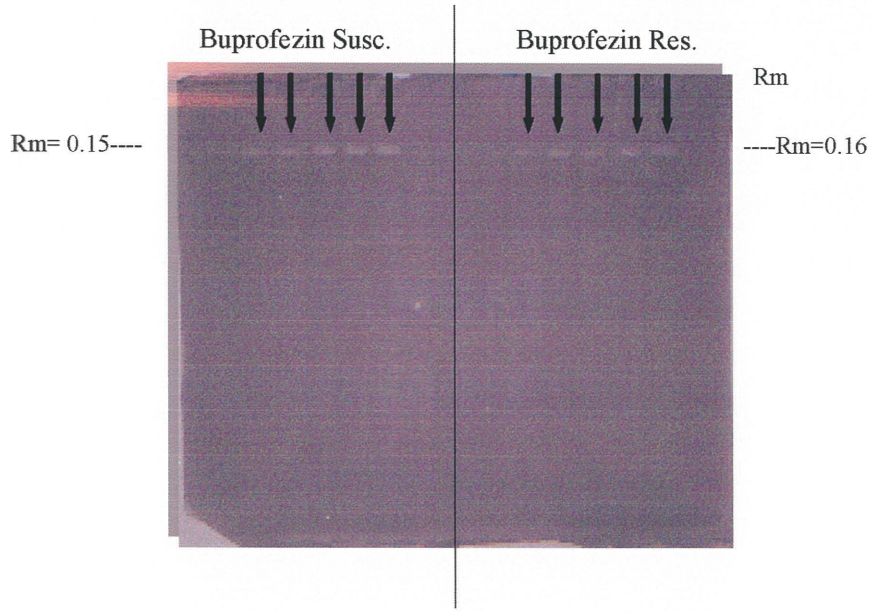
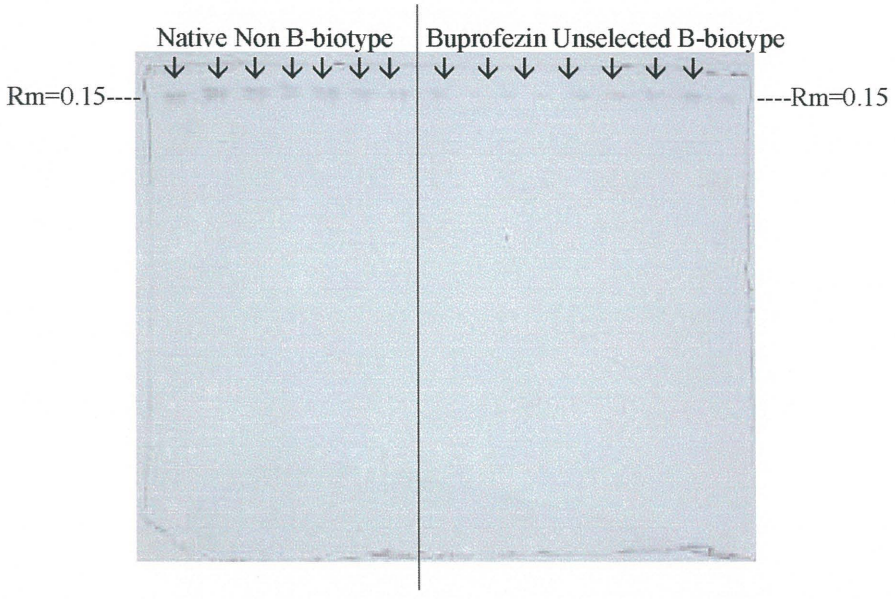


Figure 6.1 Acetylcholinesterase activity of homogenates of adult *B. tabaci.*

Strains used were non B-biotype (native non b-biotype), unselected (Bup. unsel. B-biotype) and buprofezin resistant (Bup. sel. B-biotype) B-biotype *B. tabaci*. Error bars represent standard errors.



(a)



(b)

Figure 6.2a & b Polyacrylamide gels showing acetylcholinesterase bands of buprofezin unselected and resistant B-biotype *B. tabaci* (a) and buprofezin unselected B-biotype and non B-biotype *B. tabaci* (b).

Examples of increased AChE activity associated with resistance include the greenbug *Schizaphis graminum* (Rondani), where three organophosphate resistant strains showed both significantly increased activity of AChE (1.5, 2.2 and 2.0 fold) and reduced sensitivity to inhibition by paraoxon, in comparison to a susceptible strain (Zhu & Gao, 1999). Increased AChE activity has also been linked to OP insensitivity in a strain of organophosphate resistant housefly (*M. domestica*) with a new form of insensitive AChE that had 3.4-fold greater affinity for acetylthiocholine than the wild type enzyme (Devonshire & Moores, 1984). This means that the modification that imposed insensitivity also increased enzyme activity. Fournier *et al.* (1992) also showed that increased AChE activity was correlated with insecticide resistance in *Drosophila melanogaster* (L.).

Gel electrophoresis indicated that the buprofezin insensitive AChE had a different electrophoretic mobility to the susceptible AChE (Fig. 6.2a). It has also been reported that some other altered AChE could be distinguished by different electrophoretic mobility (Morton & Singh, 1982; Bonning *et al.*, 1991).

The change in AChE relative mobility in the buprofezin resistant strain was small (R_m 0.16, compared to R_m 0.15 in the unselected strain) (Fig. 6.2a), perhaps representing a small number of amino acid substitutions. In the Colorado potato beetle (*Leptinotarsa decemlineata*), a serine to glycine point mutation of acetylcholinesterase occurred in an azinphosmethyl resistant strain (Zhu *et al.*, 1996). Zhu *et al.* (1996) suggested that the transition from the turn in the α-helix occurs earlier in the sequence when serine is

replaced by glycine. These conformational changes in the AChE were expected to impinge on binding sites, resulting in binding of organophosphorous insecticides and other ligands to these modified sites.

Results of AChE inhibition experiments with buprofezin are shown in Fig. 6.3. AChE from the resistant B-biotype *B. tabaci* strain showed only a slight response to buprofezin, with inhibition level at approximately 20% at the highest buprofezin concentration tested. On the other hand, AChE of unselected B-biotype *B. tabaci* was markedly inhibited by buprofezin. At 0.5μM concentration some 90% of AChE activity was inhibited (Fig. 6.3).

Results have indicated that buprofezin is an inhibitor of AChE in B-biotype *B. tabaci* and that buprofezin resistant *B. tabaci* AChE is insensitive to buprofezin. These data show a new mode of action for buprofezin, in addition to its more conventional action of moult disruption. This is the first report of a commercial insect growth regulator acting as an AChE inhibitor. However, it is possible, that as the AChE used in these experiments was not purified (whitefly numbers were too low to permit this process) the esterase in the buprofezin selected strain may have been sequestering the insecticide, thus giving the appearance of an insensitive AChE.

Piperonyl butoxide (PBO), a synergist commonly assumed to inhibit mono-oxygenases, has been shown to have insect growth regulator effects by retarding development of immature *B. tabaci* (Devine & Denholm, 1998a; Devine & Denholm, 1998b; Devine *et*

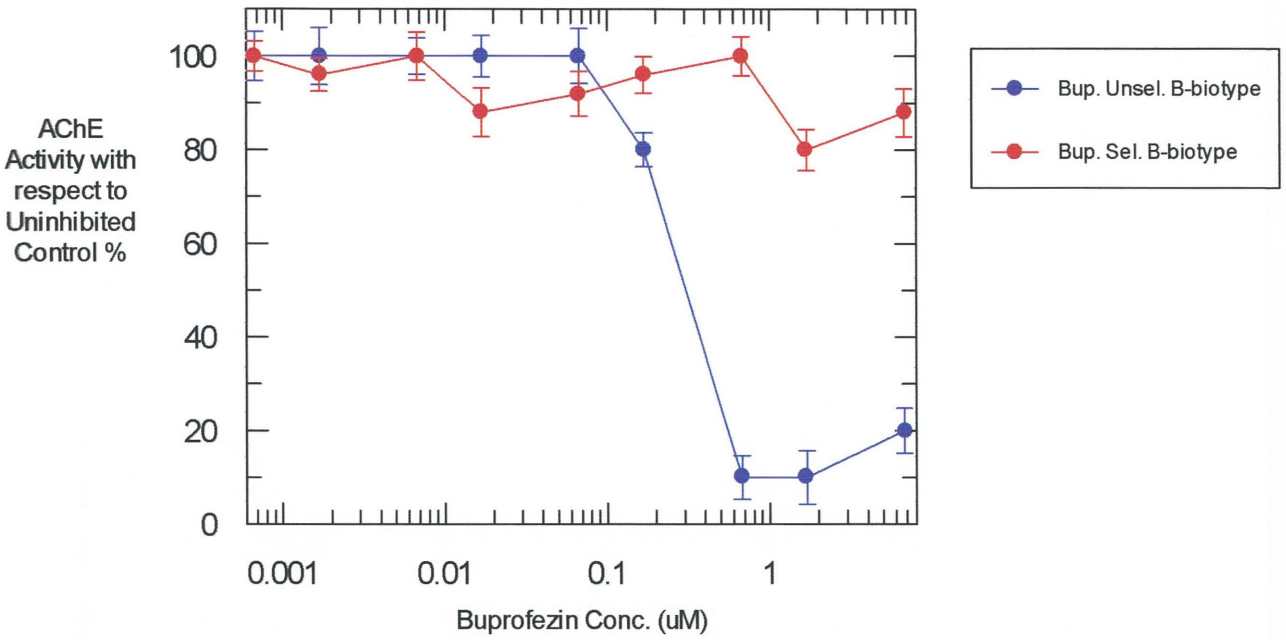


Figure 6.3 Effects of buprofezin on *in vitro* AChE activity in adult B-biotype *B. tabaci* (1hr incubation).

Strains used were buprofezin resistant (buprofezin selected B-biotype) and unselected (B-biotype) B-biotype *B. tabaci*. Error bars represent standard errors.

al., 1998), yellow mealworm beetle *Tenebrio molitor* (L.) and milkweed bug *Oncopeltus fasciatus* (Dallas) (Bowers, 1968; Adams, 1988). Most significantly, PBO has also been shown to act as an inhibitor of acetylcholinesterase of B-biotype *B. tabaci, H. armigera*, the cotton aphid *A. gossypii* and *Helicoverpa puntigera* (Gunning, 2002; G. Devine, pers. comm., 1999). PBO may also be an AChE inhibitor in other invertebrate species such as the aquatic snail *Limnaea acuminata* (Radix) (Singh & Singh, 2000).

Other compounds that inhibit insect growth and development have also been known to inhibit AChE activity. For example, gedunin, a known insect growth regulator from *Cedrela* spp. also caused AChE inhibition in the fall armyworm *Spodoptera frugiperda* (Smith) (Calderón *et al.*, 2001). Limonoids from the Celastraceae botanical family, such as the romasillo tree *Maytenus disticha* (Hook), are being investigated for potential insecticides. Such compounds include two agarofurans which have been recorded to cause both AChE and growth inhibition in *S. frugiperda* (Cespedes *et al.*, 2001).

Most importantly, experimental results showed that buprofezin resistant B-biotype *B. tabaci* have evolved a mutant resistant form of AChE which appears chemically different to wild type AChE in buprofezin unselected B-biotype and native *B. tabaci* (Fig. 6.2b). Differences in electrophoretic mobility indicate some sort of structural modification. This finding that resistant B-biotype *B. tabaci* have a mutant form of AChE (Fig. 6.3) further emphasises the importance of AChE attack as a mode of action of buprofezin.

It is not clear how buprofezin might act as an AChE inhibitor. Normal insecticide inhibitors like organophosphates and carbamates bind at the target site mimicking the action of acetylcholine. Three-dimensional X-ray crystallography studies on AChE from the electric ray *Torpedo californica* (Ayres) revealed that the active site is located near the bottom of a deep and narrow aromatic gorge. This aromatic gorge reaches halfway into the protein and widens out close to the base (Sussman *et al.*, 1991; Sussman & Silman, 1992). The active site of acetylcholinesterase is often represented as containing two subsites; the anionic site and the esteratic site. The acetylcholine molecule spans these two sites. The anionic site binds the choline moiety of acetycholinesterase and is believed to serve as an effective reactivator of organophosphate AChE (Sussman *et al.*, 1991). The esteratic subsite corresponds to the catalytic machinery centred around the serine residue. In addition to these two subsites, there are one or more binding sites for AChE and other quaternary ligands, which composes the periphatic site. The periphatic site is localised at the rim of the active site gorge, while phosphorylation takes place below (Sussman *et al.*, 1991). Some mutations that effect the binding of the insecticide or the phosphorylation rate may also modify the periphatic site (Fournier & Mutero, 1994). It is important to note that, although *T. californica* is an invertebrate, its AChE may not be identical to insect AChE.

It has been suggested that, while PBO is unlikely to be acting as a conventional inhibitor of insensitive AChE, it may be entering a physiochemical reaction with insensitive AChE, thus making it unavailable to the substrate (Gunning, 2002). While it is unknown how AChE structure differs between these insensitive variants, PBO apparently has a

three dimensional structure able to penetrate to the AChE active site (Gunning, 2002). Perhaps buprofezin is such a compound. Buprofezin may disrupt AChE function by methods such as:-

- steric hindrance, as buprofezin may cap the entrance of the gorge, thus blocking its admittance,
- dynamically inhibiting the widening fluctuation of the gorge,
- by changing the opening behaviour of alternative passages, and
- disrupting the active site conformation (Tai *et al.*, 2002; K. Tai, pers. comm., 2002).

Many organophosphorous and carbamate insecticides contain an aromatic ring and it has been postulated that a binding site for the aromatic ring lies between the anionic site and the esteratic site (Baillie & Wright, 1985). Buprofezin also contains an aromatic ring, as does PBO, which may explain the ability of these compounds to inhibit AChE.

The resolution of the three dimensional structure of acetylcholinesterase in *T. californica* allowed further discovery of mutations that were located around the active site (Sussman *et al.*, 1991). There are many mutations that can change the conformation of the active site and/or reactivity giving rise to a resistant enzyme while preserving acetylcholinsterase hydrolytic activity. For example, in most *D. melanogaster* organophosphate resistant strains, several mutations were found, and combination of these mutations gave enzymes highly resistant to malathion (Fournier *et al.*, 1996). In *M. domestica*, mutations introduced amino acid substitutions that are larger than the

corresponding wild type residues, which implied a steric effect that still allowed access for the smaller acetylcholine molecule, but restricted the larger inhibitors (Walsh *et al.*, 2001).

It is not known why such insect growth regulators should inhibit AChE activity. However, the modes of action of these compounds, especially buprofezin, are not well understood. A sub-lethal inhibition of AChE would certainly contribute to the general toxic effects of buprofezin in B-biotype *B. tabaci*. It has also been suggested that AChE inhibition in itself slows insect growth and development (G. Moores, pers. comm., 2002).

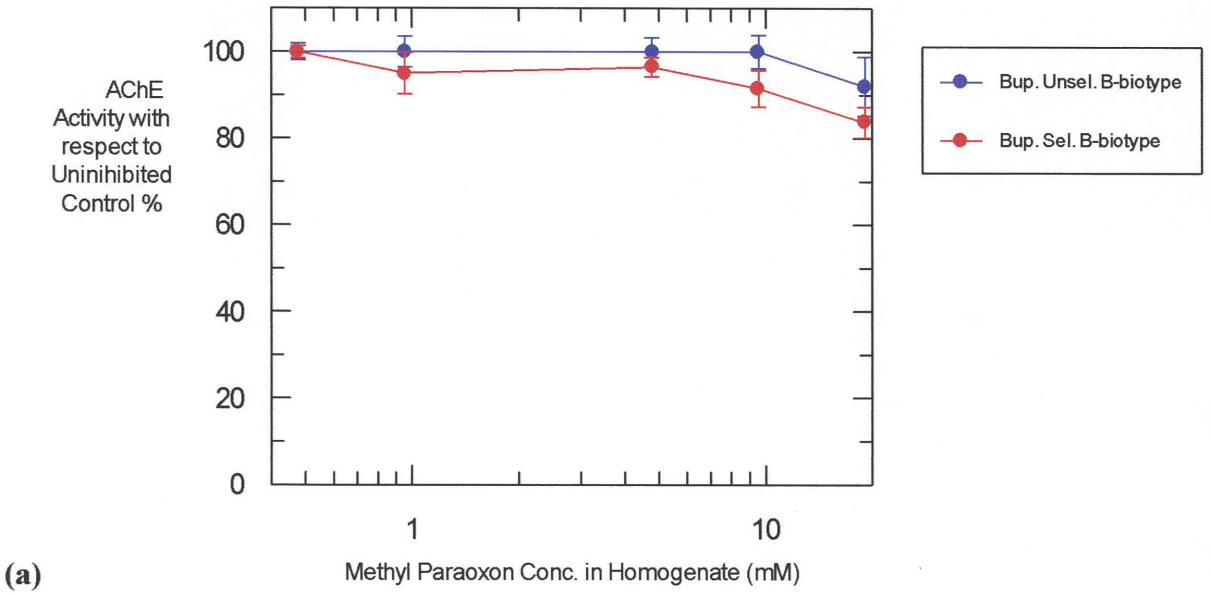
Both an AChE insensitivity to buprofezin and higher intrinsic AChE activity have evolved in the buprofezin resistant strain of *B. tabaci*, which means that AChE attack in IGRs must be an important mode of action. AChE insensitivity in the buprofezin resistant strain may delay these toxic effects of buprofezin to AChE (Chapter 4). However, the possibility that the insensitive AChE is linked to another mechanism should also be considered.

On discovering another form of altered AChE in buprofezin resistant B-biotype *B. tabaci*, it was decided to investigate whether this form of AChE had any relationship to the three forms of organophosphate insensitive AChE already associated with organophosphate resistance (Byrne *et al.*, 1992; Byrne & Devonshire, 1993; Moores *et al.*, 2000). These three insensitive AChE variants are differentiated by their responses to paraoxon and azamethiphos. The AChE forms found in buprofezin resistant and unselected B-biotype

B. tabaci were therefore examined, to ascertain any relationship with organophosphate and carbamate insensitive AChE.

There were no differences in B-biotype *B. tabaci* AChE sensitivity to methyl paraoxon (Fig. 6.4a) or demeton-*S*-methyl (Fig. 6.4b) in the presence or absence of buprofezin selection. However, the AChE of buprofezin resistant B-biotype *B. tabaci* appeared to be slightly more resistant to azamethiphos (Fig. 6.5a) and the carbamate methomyl (Fig. 6.5b). However, the only way to ensure a new AChE variant would be to include variants identified by other authors in the same assay.

The buprofezin resistant AChE in B-biotype *B. tabaci* was therefore not related to AChE which is insensitive to methyl paraoxon (Fig. 6.4a) or demeton-*S*-methyl (Fig. 6.4b). Consequently, these results indicate the existence of a fifth form of *B. tabaci* AChE, in addition to the four forms already documented (Byrne & Devonshire, 1993; Byrne *et al.*, 1994; Moores *et al.*, 2000).



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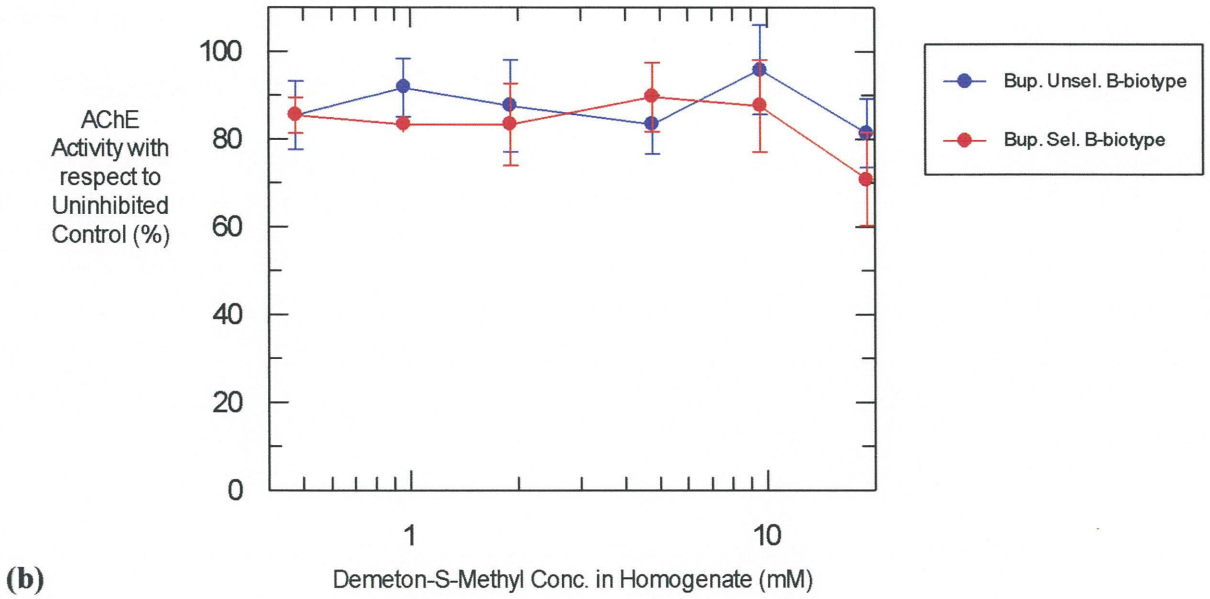
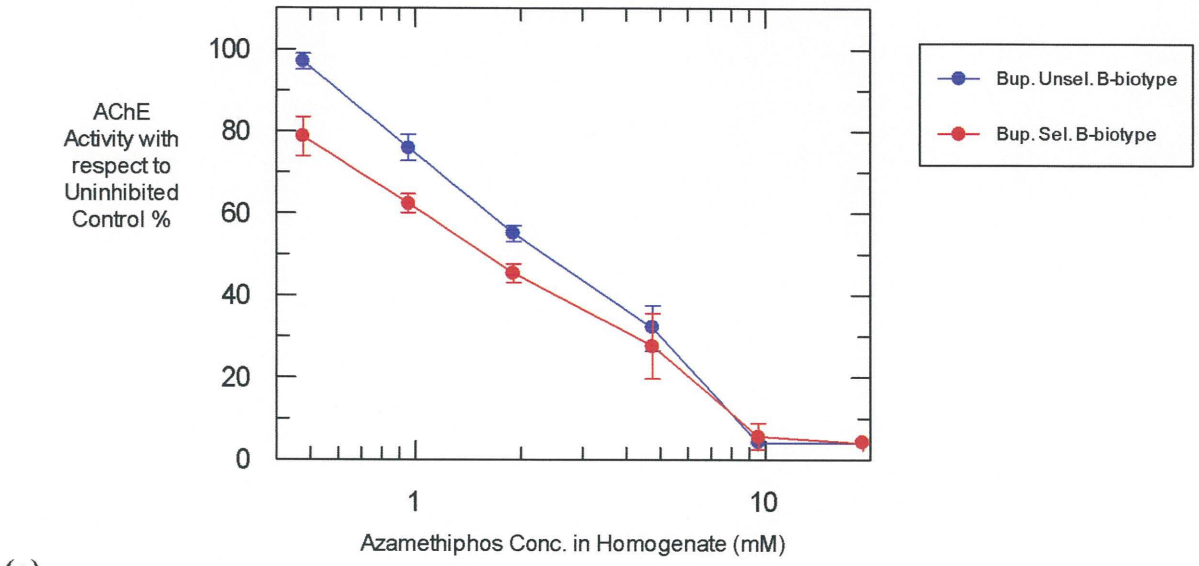


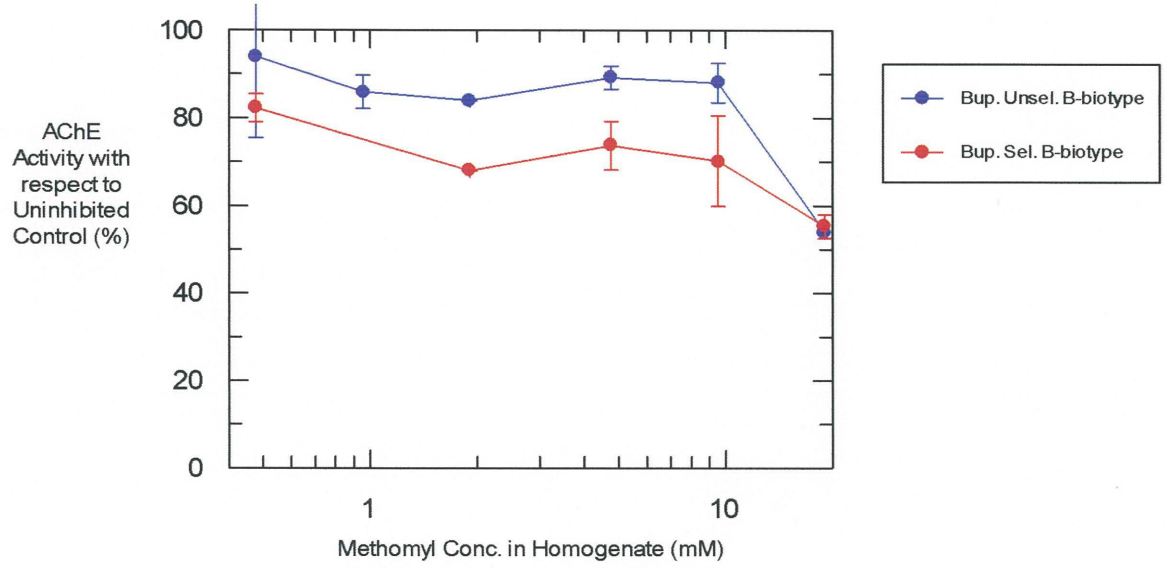
(b)

Figure 6.4 Acetylcholinesterase inhibition in adult B-biotype *B. tabaci* by methyl paraoxon (a) and demeton-S-methyl.

Strains used were buprofezin resistant (buprofezin sel. B-biotype) and unselected (buprofezin unsel. B-biotype) B-biotype *B. tabaci*. Error bars represent standard errors.



(a)



(b)

Figure 6.5 Acetylcholinesterase inhibition in B-biotype *B. tabaci* by azamethiphos (a) and (b) methomyl.

Strains used were buprofezin resistant (bup. sel. B-biotype) and unselected (bup. unsel. B-biotype) B-biotype *B. tabaci*. Error bars represent standard errors.

Effects of other novel insecticides on AChE

6.4 Introduction

The discovery that buprofezin inhibited AChE activity in buprofezin unselected B-biotype *B. tabaci* was further extended into an investigation to determine whether other IGRs and novel insecticides may also have this rather unexpected mode of action and to determine whether the cross-resistance already indicated by bioassay and esterase studies may extend to insensitive AChE.

Insecticides used were novaluron, flufenoxuron, fenoxycarb, pyriproxyfen, pyridaben and pymetrozine. Novaluron and flufenoxuron inhibit chitin synthesis. Pyriproxyfen and fenoxycarb are juvenile hormone analogues. Pyridaben acts on the electron transport system and pymetrozine immobilises the insect's feeding mouthparts, causing death through starvation.

6.5 Materials and methods

Insecticides used were novaluron (technical grade 98.6%) (Nufarm Ltd), flufenoxuron (EC grade 10% a.i.) (BASF Australia Ltd), fenoxycarb (technical grade 96.2%) (Novartis), pyriproxyfen (technical grade 97.4%) (Sumitomo Australasia Ltd), pyridaben (25% a.i.) (BASF Australia Ltd) and pymetrozine (technical grade 98.9%) (Novartis). All insecticides were dissolved in acetone.

Adult whiteflies (50 buprofezin unselected and resistant B-biotype *B. tabaci*) were homogenised in 1mL of 0.1M pH 7.5 phosphate buffer containing 0.1% Triton X-100, then divided into 200µL aliquots. Insecticides were added in acetone, bringing the final concentrations in homogenate to 0.1ppm, 1ppm, 10ppm, 100ppm and 1000ppm. The volume of acetone in each aliquot, including the control, was 5µL. Methanol (10µl) was added to each treatment to increase insecticide solubility to a total concentration of 5% (Devonshire & Moores, 1989). Homogenates were incubated at 25°C, and aliquots were taken for AChE assays (10µL) and for AChE electrophoresis (15µL). AChE assays run after 30, 60 and 90 minutes incubation. Methods for AChE assays and gel electrophoresis are described in Section 6.3.2. The buprofezin resistant B-biotype *B. tabaci* strain was only incubated with novaluron as there were insufficient numbers of the strain available to assess AChE response to other insecticides.

6.6 Results and discussion

Results showed that AChE was inhibited in the unselected strain after 30 minutes incubation with novaluron, but the buprofezin resistant strain remained uninhibited (Fig. 6.6a). It is noted that the AChE inhibition was very gradual and it is possible that the mutant enzyme is inherently unstable. AChE activity in unselected B-biotype *B. tabaci* was inhibited by up to 30% when incubated with 100 ppm novaluron. The buprofezin unselected B-biotype *B. tabaci* homogenate incubated with novaluron were also run on a polyacrylamide gel and stained for AChE activity (Fig. 6.6b). The AChE bands (R_m = 0.15) were quite distinct in the uninhibited control samples and became progressively fainter as the novaluron concentration in the homogenate increased. At concentrations of

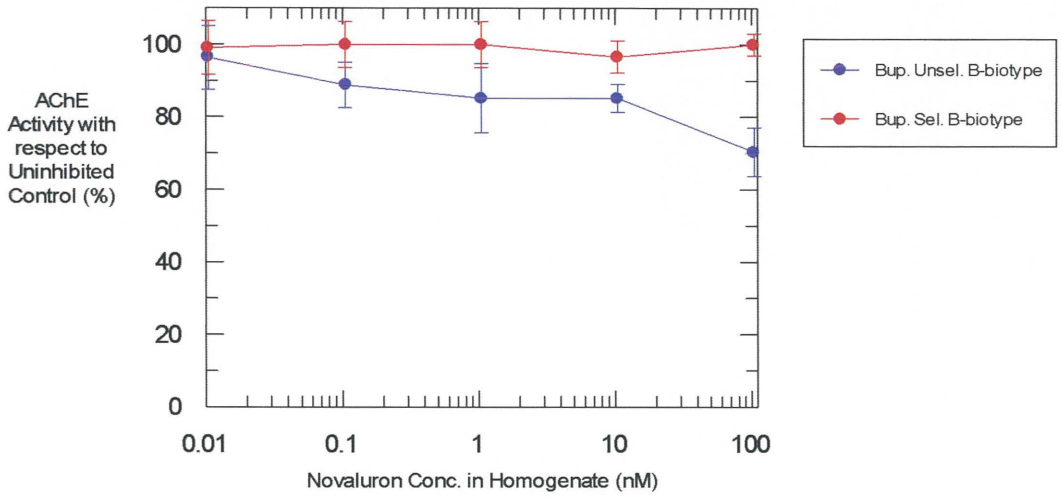


Figure 6.6a Acetylcholinesterase activity of unselected and buprofezin resistant B-biotype *B. tabaci* incubated with novaluron for 30 minutes (Error bars represent standard errors).

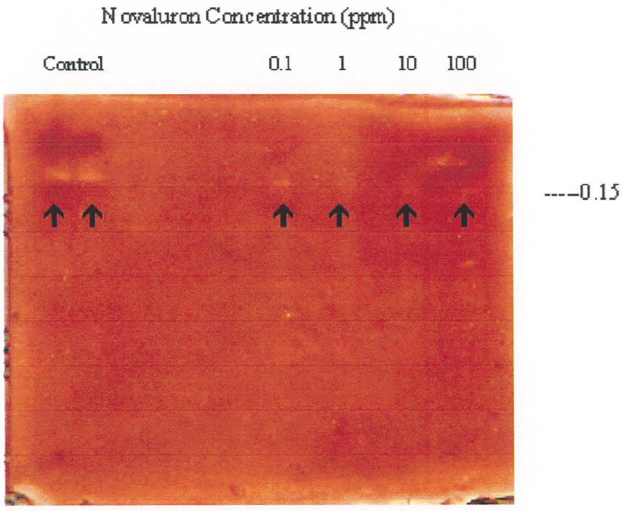


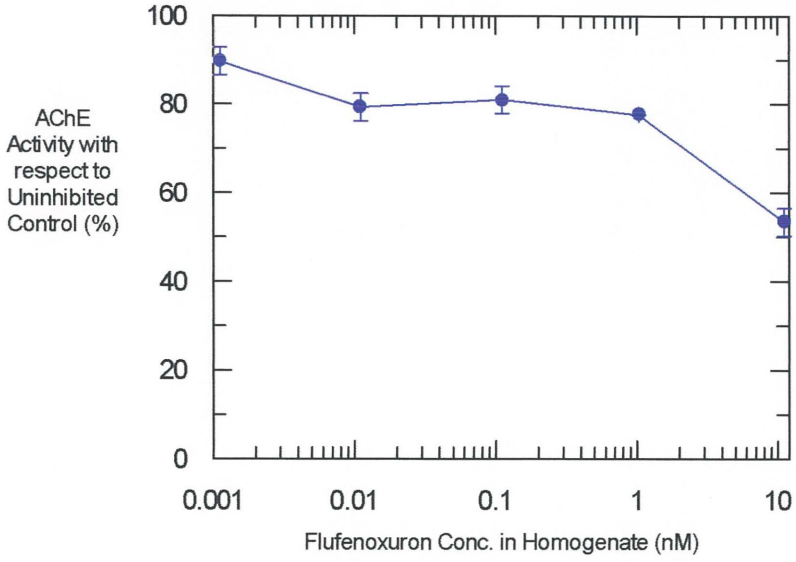
Figure 6.6b Gel stained for acetylcholinesterase of unselected and buprofezin resistant B-biotype *B. tabaci* incubated with novaluron for 30 minutes.

10 and 100ppm novaluron, AChE bands were so faint that they were undetectable. There were insufficent buprofezin resistant B-biotype *B. tabaci* to investigate AChE inhibition by novaluron on a gel.

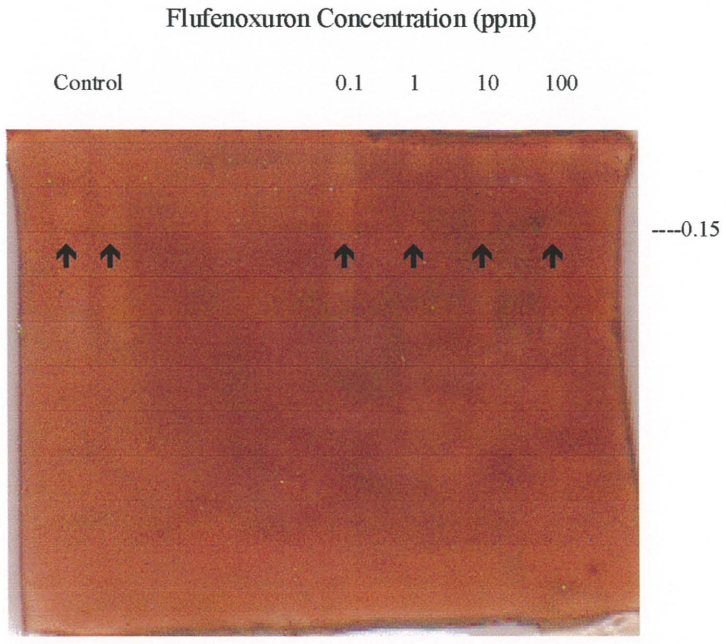
In buprofezin unselected B-biotype *B. tabaci,* nearly 50% of AChE activity was inhibited by a concentration of 10nM flufenoxuron (Fig. 6.7a). Electrophoresis data showed that fainter bands on the gel were also observed in homogenates incubated with flufenoxuron when compared to the non-insecticide control (Fig. 6.7b).

After incubating with approximately 1100ppm pyridaben, up to 40% inhibition of AChE activity was observed in the buprofezin unselected B-biotype strain (Fig. 6.8a). Incubation experiments with homogenates from the unselected B-biotype *B. tabaci* strain indicated that pymetrozine did not inhibit AChE activity to any marked degree (Fig. 6.8a). With pyriproxyfen, approximately 30% of buprofezin unselected B-biotype *B. tabaci* AChE was inhibited when incubated with pyriproxyfen for one hour (Fig. 6.8b). There were insufficient buprofezin resistant whiteflies to examine the effect on AChE activity with pyridaben, pymetrozine, fenoxycarb and pyriproxyfen.

Fenoxycarb, pyriproxyfen and novaluron are all insect growth regulators and it is therefore not entirely unexpected, in view of the finding that buprofezin also inhibits *B. tabaci* AChE (Section 6.3), that these compounds also have inhibitory effects on *B. tabaci* AChE. Inhibition was shown by both AChE assay (Figs. 6.6a, 6.7a, 6.8a & b) and staining for AChE on gels (Figs. 6.6b, 6.7b). Pyridaben, however, is a mitochondrial

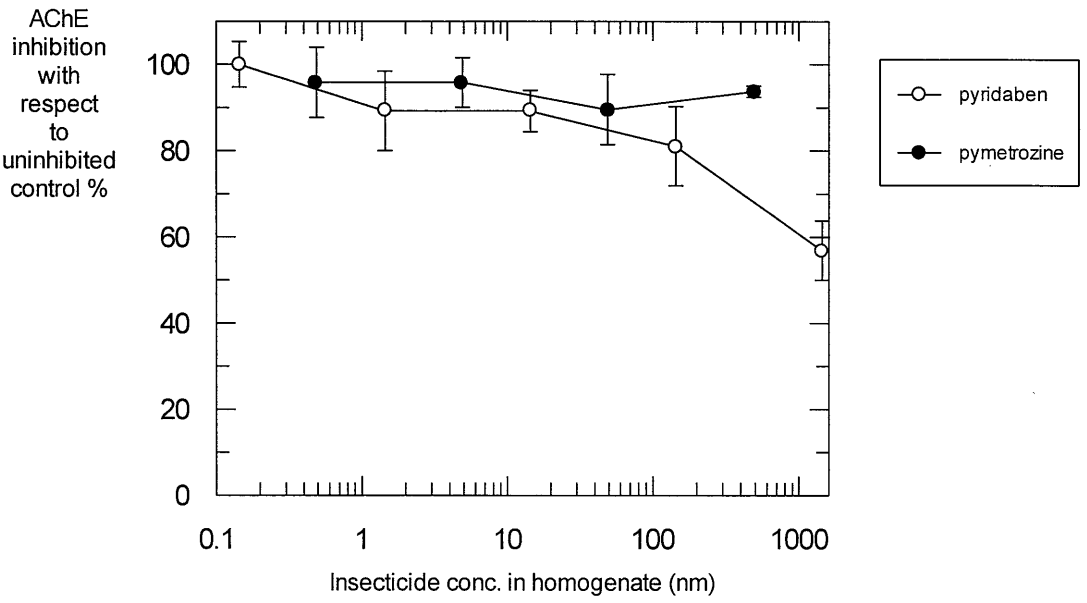


(a)

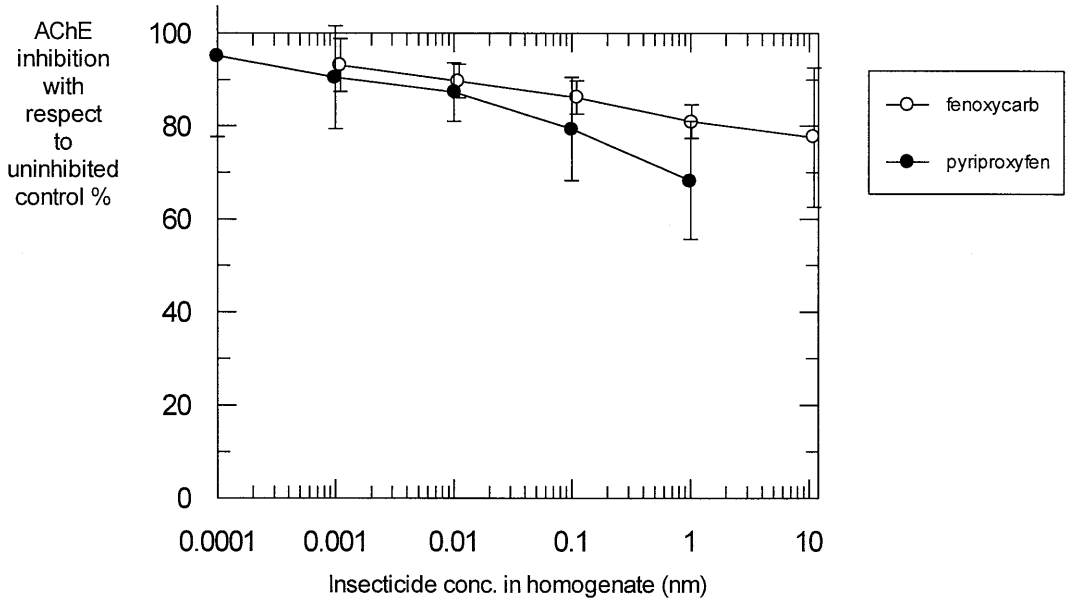


(b)

Figure 6.7 Acetylcholinesterase activity (a) and polyacrylamide gel stained for AChE (b) of buprofezin unselected B-biotype *B. tabaci* homogenates incubated with flufenoxuron for one hour. Error bars represent standard errors.



(a)



(b)

Figure 6.8 Acetylcholinesterase inhibition of buprofezin unselected B-biotype *B. tabaci* homogenates with pyridaben and pymetrozine (30 mins incubation) (a), and fenoxycarb and pyriproxyfen (60 mins incubation) (b). Error bars represent standard errors.

electron transport inhibitor (Devine *et al.*, 2001) with an unrelated mode of action. None the less, pyridaben also caused inhibition of AChE activity, meaning that it is a target site in *B. tabaci* (Fig. 6.8b). AChE inhibition by pyridaben occurred at higher insecticide concentrations (Fig. 6.8a) than was required by the IGRs pyriproxyfen and fenoxycarb (Fig. 6.8b).

Novaluron, like buprofezin, is a chitin synthesis inhibitor and neither compound inhibited AChE of buprofezin resistant B-biotype *B. tabaci*. These data may indicate that there could be cross-resistance between buprofezin and novaluron through an altered acetylcholinesterase as well as by esterase mediated mechanisms (Fig. 4.7). Bioassays need to be done to test this hypothesis. The change in the AChE of the buprofezin resistant B-biotype *B. tabaci* strain that allows this target site to be bypassed has conferred resistance to novaluron as well.

Insensitive AChE in *B. tabaci* has given cross-resistance between insecticides to a number of OP and carbamate insecticides. For example, dimethoate and monocrotophos select for the same insensitive AChE (Dittrich *et al.*, 1990). In Nicaragua and Sudan B-biotype *B. tabaci* populations, insensitive AChE variants conferred resistance to monocrotophos, profenofos and paraoxon, but differed in their response to azamethiphos (Byrne & Devonshire, 1993).

Insensitive AChE commonly confers cross-resistance in other insect species as well. For example, cross-resistance between methomyl and thiodicarb in *H. armigera*, was

attributed to an altered AChE (Gunning *et al.*, 1996b). In the cotton aphid *Aphis gossypii* (Glover), a clone was resistant to pirimicarb and a range of organophosphate insecticides (demeton-s-methyl, monocrotophos, omethoate, pirimiphos-methyl) through an altered AChE resistance mechanism (Moores *et al.*, 1996).

Pymetrozine did not significantly inhibit the AChE activity of buprofezin unselected B-biotype *B. tabaci* (Fig. 6.8a). This insecticide belongs to a new class of chemistry of which it is not known to have any insect growth regulatory effects. Toxic effects are thought to be due to irreversible cessation of feeding (Fuog *et al.*, 1998). This lack of inhibition agrees with studies on the migratory locust *Locusta migratoria* (L.) where pymetrozine was not active in all tests of established target mechanisms of insecticides and was hypothesised to have a mechanism involving a novel neuronal receptor as a binding site (Kaufmann *et al.*, 2000).

It is interesting to note that all of the insecticides tested that induced AChE inhibition (pyridaben, flufenoxuron, pyriproxyfen, fenoxycarb and novaluron) have at least one benzene derivative aromatic ring. Acetylcholine is orientated in the active site by interaction of the quaternary nitrogen of choline with Trp-84, and is thought to be attracted to this site by a strong electrostatic dipole aligned with the gorge, which is lined with 14 aromatic residues (Ripoll *et al.*, 1993). Tryptophan has a benzene ring. It is possible that one of the benzene rings of these insecticides may be attracted to the positive charge of Trp-84 and binding to it, thus 'stacking' the benzene rings upon each other (G. Moores, pers. comm., 2002).

Further investigation of why altered AChE does not allow buprofezin and novaluron to move down the acetylcholinesterase gorge in the buprofezin resistant population could form the subject of further research. Future studies should also consider the possibility that the B esterases possess ATChI hydrolysing ability. Genetic studies on inheritance of mutant AChE would also be fascinating and give some valuable information about how rapidly resistance might spread in field situations. It would have very interesting to compare the response of the buprofezin resistant B-biotype *B. tabaci* with insecticides other than novaluron. Insect unavailability prevented this extension of this research. Further studies of mutant AChE structure using X-ray crystallography would be fascinating and would assist greatly in adding to the information already known about *B. tabaci* acetylcholinesterase.

Chapter 7: Buprofezin Resistance Mechanisms – Mono-oxygenases

7.1 Introduction

While data from this research project have shown strong evidence of esterase involvement in resistance to buprofezin in B-biotype *B. tabaci*, mono-oxygenase involvement in this resistance is a possibility. The apparent lack of substrate specificity of mono-oxygenases allows them to metabolise insecticides of many different groups. Lack of specificity may have caused a broad spectrum of cross-resistance, which has already extended to juvenile hormones and juvenile hormone mimic insecticides in some insects (Oppenoorth & Welling, 1976).

The cyclodiene insecticide aldrin is epoxidised into dieldrin by a reaction involving cytochrome P-450 mono-oxygenase enzyme systems. Aldrin is commonly used as a substrate for the characterisation of mono-oxygenase activity (Kulkarni & Hodgson, 1980; Rose, 1984; Rose, 1985; Rose & Wallbank, 1986). The epoxidation of aldrin to dieldrin is shown in Fig. 7.1.

$$NADPH_2 + O_2$$

$$\text{Aldrin} \;>\!>\!>\!>\!>\!>\!>\!>\!>\!>\!>\!>\; \text{Dieldrin}$$

Microsomes

Figure 7.1 Epoxidation of aldrin to dieldrin

This reaction has been used to monitor mono-oxygenase activity in larval *Spodoptera litura* (Rose, 1984), other larval Lepidopteran species (Rose, 1985) and *Oryzaephilus surinamensis* (Rose & Wallbank, 1986). A known concentration of aldrin and dieldrin can be measured by using gas chromatography and the level of mono-oxygenase activity can be measured by comparing peak heights with those of standard concentrations.

P-450 mono-oxygenase enzymes are involved in endogenous metabolism as well as metabolism of toxins. In insects, these enzymes are essential for the synthesis and degradation of the steroid moulting hormones and juvenile hormones (Hodgson, 1983; Wilkinson, 1985; Bergé *et al.*, 1999). As buprofezin acts by disrupting the moulting cycle, perhaps this is why mono-oxygenases are commonly assumed to be involved in *B. tabaci* resistance to buprofezin.

The purpose of this study was to investigate any involvement of mono-oxygenase enzyme systems in resistance of B-biotype *B. tabaci* to buprofezin.

7.2 Materials and methods

7.2.1 Microassay

The method used to measure dieldrin production was that of Rose and Wallbank (1986). The method was first tested on a strain of *Oryzaephilus surinamensis* (L.) with known mono-oxygenase resistance levels, in order to determine if the assay was working correctly. Twenty buprofezin selected and non-selected adult B-biotype *B. tabaci* were homogenised in 0.01g polyvinyl-pyrrolidone (BDH Chemicals Ltd.) and a small amount

of 0.1 M pH 7.5 potassium phosphate buffer in a Snap-Cap Microtube (ICN Biomedicals Inc.). Reduced glutathione (Sigma-Aldrich) (0.0075g), 0.01g glucose 6-phosphate (Sigma-Aldrich) and 0.001g 9-nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich) were dissolved in 1mL of buffer and added to whitefly homogenates. The volume was made up to 1.5mL with buffer.

A 20µl aliquot of aldrin (274 nmoles/0.1mL methyl cellusolve) (Shell Research Ltd, Appendix 1) was added to each of the homogenate solutions, then incubated in a water bath at 25°C. After ten minutes, the reaction was terminated by adding 0.5mL of HCl, and 100µL of hexane to each solution. Solutions were mixed for 30 seconds, which enabled the hydrophobic dieldrin and aldrin to migrate to the hexane layer.

Dieldrin production was measured with a Varian Model 3700 gas chromatagraph (GC) containing a Ni^{63} detector. The nitrogen gas flow rate was 30 mL/min, with an injector temperature of 210°C, a detector temperature of 320°C and a column temperature of 205°C. Under these conditions the retention time of dieldrin was ten minutes. A standard was run initially, so to be able to compare the dieldrin and aldrin peak heights with those produced by buprofezin selected and unselected B-biotype *B. tabaci*.

7.3 Results and discussion

Both buprofezin unselected and resistant *B. tabaci* showed no detectable conversion of aldrin to dieldrin, which indicated no significant mono-oxygenase activity in either strain. While both *B. tabaci* strains had a level of resistance to buprofezin, if mono-oxygenases

were involved in resistance, then the highly buprofezin resistant (resistance factor 2203) might have been expected to show increased mono-oxygenase activity compared to the unselected strain (resistance factor 11).

While these studies showed no differences in mono-oxygenase activity between these B-biotype *B. tabaci* strains, it is possible that the failure to detect dieldrin may be due to the small amounts of whitefly tissue used and it would be useful to repeat these experiments using a greater quantity of *B. tabaci* tissue.

Chapter 8: Cold Tolerance of *B. tabaci*

8.1 Introduction

Insects avoid cold in three ways, by migrating, overwintering, or by cold tolerance. *B. tabaci* rarely migrates long distances and does not have an overwintering diapause and therefore has to overcome cold temperatures in order to survive winter in cooler climates. Whilst using cold to facilitate handling of whiteflies during the course of the research described in this thesis, it was observed that buprofezin resistant B-biotype *B. tabaci* seemed to have a greater cold tolerance than the non selected strain and experiments were designed to investigate this phenomenom.

The aims of this experiment were to investigate if there were any differences in cold tolerance between native non B-biotype, buprofezin resistant and buprofezin unselected B-biotype *B. tabaci* and to see if increased tolerance was reflected in whitefly protein levels.

8.2 Materials and methods

8.2.1 Cold treatment

Ten whiteflies from each *B. tabaci* strain (native non B-biotype, buprofezin selected and non selected B-biotypes), were placed in a refrigerator at 4°C for 5, 10 or 20 minutes or in the freezer at -4°C for 1, 2 or 3 minutes. After cold treatment, the whiteflies were held at 26°C and observed under a fluorescent, heat free magnifying lamp. The time (s) taken for

each strain to recover and fly again was recorded. Selecting the time taken for 50% of the sample to resume flight rather than 100% represents a median value of the population, which is less likely to be biassed by extreme outlying data points.

8.2.2 Protein assay

Calculation of protein standard curve

Protein concentration in *B. tabaci* homogenates was determined using a Bio-Rad protein kit (Bio-Rad Laboratories). This kit is a colourimetric assay for protein concentration following detergent solubilisation and is based on the Lowry assay (Lowry *et al.*, 1981). A protein standard curve was prepared using bovine serum albumin (Bio-Rad Protein Assay Standard II: Bovine Serum Albumin Lyophilized, supplied by Bio-Rad Laboratories). The protein standard was serially diluted in distilled water containing 0.05 % Triton X-100 to a range of concentrations from 0.2mg/mL to 1.5mg/mL protein. Aliquots (5μL) of each dilution were pipetted into a microplate. Reagent blanks were carried as a control.

BioRad reagent A (25μL) was pippetted into each microplate well, followed by 200μL of BioRad reagent B. The plate was gently agitated to mix the reagents. Blanks and samples were incubated at 25°C for 20 minutes. Absorbance was read at 750nm as an endpoint, using the microplate reader with BioRad Microplate Manager software (Bio-Rad Laboratories). The absorbance versus protein concentration was plotted to form a standard curve.

Protein assay in *B. tabaci* strains

Twenty adult whiteflies from the native non B-biotype, buprofezin unselected and selected B-biotype *B. tabaci* strains were mass homogenised, each in 200μL of 0.02M phosphate buffer pH 7.0 containing 0.05% Triton X-100. Aliquots (5μL) were taken for the protein assay. The protein assay was as described for the standard curve. Protein concentrations in whitefly samples were calculated from absorbances read from the standard curve. Protein content per whitefly (w/w%) was calculated.

8.3 Results

The buprofezin unselected B-biotype strain had the longest cold recovery times, then the native non B-biotype. Buprofezin selected B-biotype *B. tabaci* were the quickest in recovering flight ability. The difference in cold recovery times between strains was similar when held at both 4°C and -4°C. At –4°C (Fig. 8.2), there was little difference in the recovery times of native non B-biotype and buprofezin unselected B-biotype when held for one minute, but when held in cold for longer, a longer recovery time for unselected B-biotype *B. tabaci* became more apparent.

A protein standard curve was plotted (Fig. 8.3), from which protein percentages of *B. tabaci* strains were determined. The highest protein level was found in the buprofezin resistant strain (4.34 ± 0.13%), followed by the native non B-biotype (3.66 ± 0.16%), and finally the buprofezin unselected B-biotype *B. tabaci* (2.29 ± 0.11%) (Fig. 8.4).

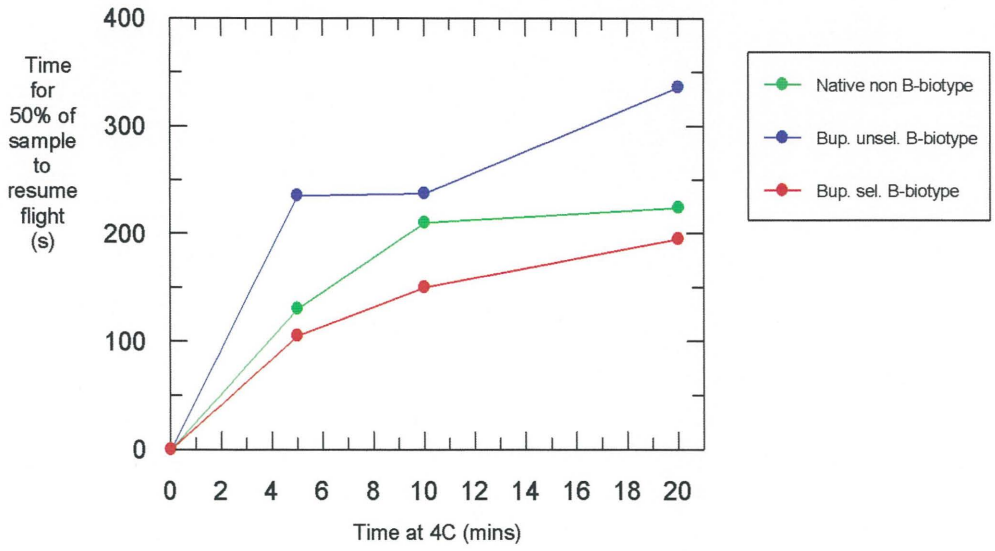


Figure 8.1 Cold recovery time of 50% of *B. tabaci* cooled at 4°C (s).

B. tabaci strains used were native non B-biotype (Native), buprofezin unselected B-biotype (Bup. Unsel. B-biotype) and selected B-biotype (Bup. Sel. B-biotype).

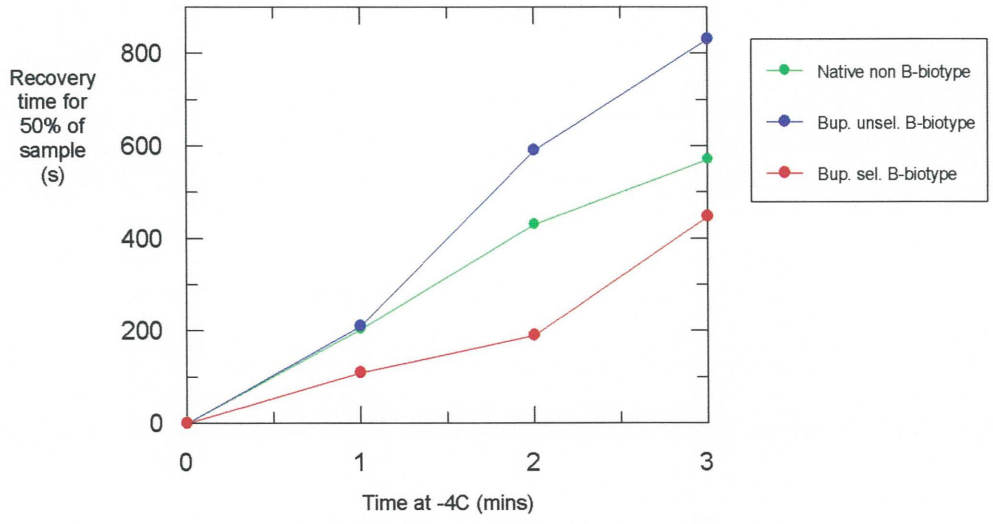


Figure 8.2 Cold recovery time of 50% *B. tabaci* cooled at -4°C (s).

B. tabaci strains used were native non B-biotype (Native), buprofezin unselected B-biotype (Bup. Unsel. B-biotype) and selected B-biotype (Bup. Sel. B-biotype).

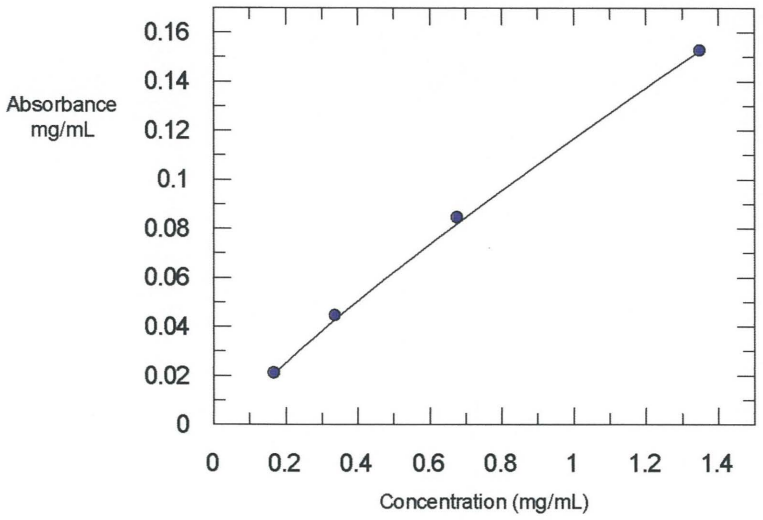


Figure 8.3 Protein standard curve for bovine serum albumin (BSA) using the Bio-Rad protein kit.

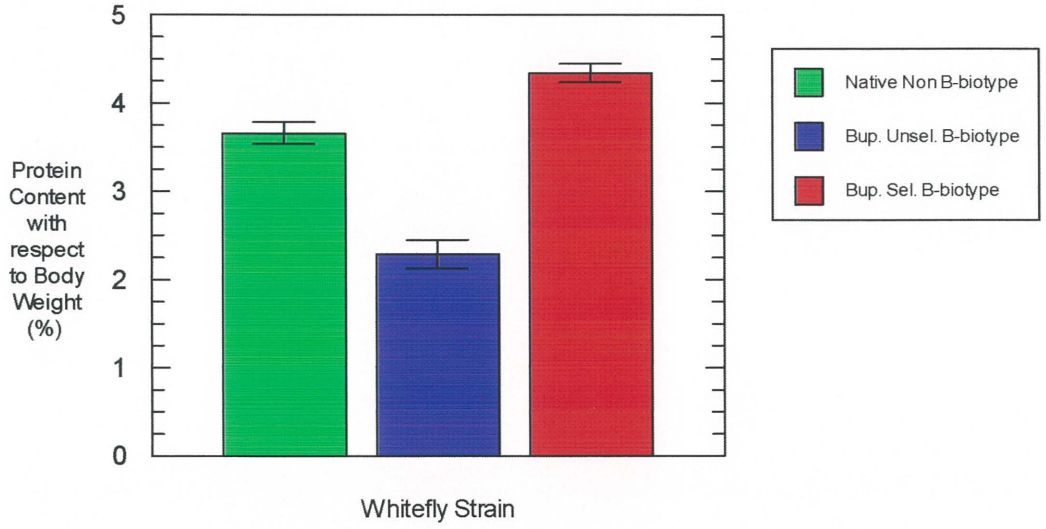


Figure 8.4 Protein content of Australian *B. tabaci* strains (% body weight).

B. tabaci strains were native non B-biotype (Native non B-biotype), buprofezin unselected B-biotype (Bup. unsel. B-biotype) and buprofezin resistant B-biotype (Bup. sel. B-biotype). Error bars are standard errors.

8.4 Discussion and Conclusions

Personal observations about different cold tolerances between different *B. tabaci* populations were confirmed. Experimental data (Figs 8.1-8.2) indicate that in the *B. tabaci* strains tested, cold tolerance is associated with increased protein content (Fig. 8.4). The highest protein content was found in the buprofezin resistant strain, then native non B-biotypes, which had 1.89 and 1.60-fold more protein than the buprofezin unselected B-biotypes respectively. It is possible that higher protein content found in the buprofezin resistant population may be, at least partially, a cause of higher amino acid content in the haemolymph. Numerous proteins are known to be present in the haemolymph and some may well have a role to act as an anti-freeze by binding incipient ice crystals and preventing the addition of more water molecules (Chapman, 1969). In *Sitophilus granarius* (L.) and *Cryptolestes ferrugineus* (Steph.), levels of amino acids proline, asparagine, lysine and glutamic acid were found to be considerably higher in cold adapted populations (Fields *et al.*, 1998).

It is also possible that increased esterase content in the buprofezin resistant B-biotype *B. tabaci* may have resulted in the increased cold tolerance of that strain, or some other factor may have also been independently selected for. Increased esterase activity was unrelated to protein content or cold tolerance, because despite native non B-biotype having the lowest esterase activity of all three strains, it had higher protein content than the unselected B-biotype strain (Fig. 4.2). Having a higher protein content of the native non B-biotype than the buprofezin unselected B-biotype *B. tabaci* strain (Fig 8.4) agreed with past results from R. Gunning & F. Byrne (unpublished).

The superior cold tolerance of buprofezin selected *B. tabaci* may allow it to better overwinter in the field, or survive in cooler climates than other *B. tabaci* strains. This could have far reaching effects on its pest status in Australia. CLIMEX, developed by CSIRO, is an interactive model which predicts the potential geographic distribution and relative abundance of a species using climatic preferences derived from overseas data (De Barro, 1995b). Predictions by CLIMEX show, excluding protective glasshouse environments, that suitable climatic conditions include parts of Queensland, Western Australia (north of Perth), Northern Territory and New South Wales (north of Sydney) (De Barro, 1995a; De Barro, 1995b). Victoria and Tasmania were deemed unsuitable because of low temperatures over winter (De Barro, 1995a). However, increased cold tolerance of this pest, as shown by buprofezin resistant B-biotype *B. tabaci*, could extend this range considerably. In addition, better winter survival would result in earlier insect pressure for consecutive cropping systems.

Immature *B. tabaci* have been shown to have a higher tolerance to cold, sometimes able to complete their development even when low temperatures kill the host plant (Canas *et al.*, 2001). Therefore, the immature stages of the buprofezin selected B-biotype *B. tabaci* may have even greater tolerance to cold than the adults studied. The work described in this chapter can only be regarded as a preliminary study, and it should be further replicated. Such studies could be extended by including more treatments and greater replication, and by measuring levels of various polyols.

Chapter 9: General Conclusions

B-biotype *B. tabaci* is an insect pest of escalating importance in Australia, particularly in the horticultural, grain and cotton industries. Recent population explosions (2001/2002 cotton season) in cotton areas of central Queensland (Emerald and Biloela/Theodore) have highlighted the importance of this pest. High levels of resistance to insecticides with conventional modes of action (nerve poisons) such as organophosphates, carbamates and pyrethroids, have made control very difficult. Insecticides with novel modes of action such as insect growth regulators, including the chitin inhibitor buprofezin, are options for control of B-biotype *B. tabaci* in Australia. This thesis describes new information regarding resistance to buprofezin and *B. tabaci*.

Laboratory selection of a B-biotype *B. tabaci* population with buprofezin rapidly increased levels of buprofezin resistance from 11-fold to in excess of 2000-fold in 4 selections. The rapid selection for resistance is a result of a pre-existing, low level resistance to buprofezin in the Australian population. The speed and extent of resistance development in the laboratory should serve as a warning that buprofezin should not be overused in Australia, and strict resistance management guidelines, such as using IGRs as one group, should be adhered to.

Prior to this research, there was no information about the mechanisms of resistance to buprofezin in *B. tabaci*. This research has produced evidence showing the involvement of an esterase mediated resistance mechanism, probably via sequestration. Compared to

the susceptible and unselected strains, buprofezin resistant *B. tabaci* were shown to have additional esterase bands (Rm 0.14-0.29), a higher total esterase activity and these esterases were inhibited by buprofezin both *in vitro* and *in vivo*.

The presence of extra esterase band(s) could be very useful in a rapid PAGE diagnosis of buprofezin resistant *B. tabaci*. The fact that extra esterase bands were inherent in both resistant nymphs and adults which had not been treated with buprofezin indicates that this trait is genetically inherited and not induced by exposure to buprofezin. Some sub-lethal effects of insect growth regulators are known to be transmitted from female to offspring, however, the extra PAGE banding was observed in resistant *B. tabaci* not exposed to buprofezin for several generations.

Esterase of buprofezin resistant B-biotype *B. tabaci* also showed binding with pyriproxyfen and cross-resistance between buprofezin and pyriproxyfen was confirmed by bioassay. Esterase inhibition by novaluron, pymetrozine and flufenoxuron was also demonstrated in buprofezin resistant B-biotype *B. tabaci* and it is possible that buprofezin confers cross-resistance to these other compounds.

In insects, P-450 mono-oxygenase enzymes are essential for the synthesis and degradation of the steroid moulting hormones and juvenile hormones (Hodgson, 1983; Wilkinson, 1985; Bergé *et al.*, 1999) and it was considered that they might have a role in resistance. However, the present research found no indication of the involvement of monooxygenases in buprofezin resistance in *B. tabaci*.

The findings that firstly, AChE attack is a new undescribed mode of action for buprofezin, and secondly, that resistant individuals have evolved a buprofezin insensitive form of AChE are exciting new discoveries. Given that the buprofezin resistant *B. tabaci* population have a buprofezin insensitive AChE, it would appear that the AChE target site of buprofezin could be quite an important mode of action. Prior research to this, only three novel variants of AChE in *B. tabaci* had been reported (Byrne & Devonshire, 1993; Byrne *et. al.*, 1994; Moores *et al.*, 2000) in addition to the sensitive AChE of the susceptible SUD-S strain. Buprofezin insensitive AChE in buprofezin resistant B-biotype *B. tabaci* was not related to any of the organophosphate insensitive AChE forms of AChE. Therefore, a fourth form of *B. tabaci* insensitive AChE joins the three variants already documented.

The buprofezin resistant form of AChE in B-biotype *B. tabaci* also had increased AChE activity compared to the unselected form. The finding is quite unusual because the organophosphate insensitive forms of AChE in *B. tabaci* are much less active than the susceptible AChE (Byrne & Devonshire, 1997). A greater affinity for a substrate could mean that AChE may be partially protected from inhibitors, even if the enzyme were not also intrinsically insensitive to inhibition (Devonshire & Moores, 1984).

Buprofezin insensitive AChE also had a different electrophoretic mobility compared to the sensitive AChE, which indicates there have been some structural changes in the enzyme which could affect the ability of buprofezin to bind to AChE. Findings of AChE

inhibition by novaluron suggests the insensitive AChE contributes cross-resistance as well as by esterase sequestration to novaluron and buprofezin.

This research also showed that flufenoxuron, pyriproxyfen, fenoxycarb and pyridaben also inhibited AChE of unselected B-biotype *B. tabaci* and indicate a new mode of action for these insecticides.

All of the insecticides that showed AChE inhibition (buprofezin, pyridaben, flufenoxuron, pyriproxyfen, fenoxycarb and novaluron) have at least one benzene derivative aromatic ring. Many organophosphorous and carbamate insecticides contain an aromatic ring, and it has been postulated that a binding site for this ring lies within the active site of the AChE enzyme. The possession of an aromatic ring may explain the ability of these novel insecticides to inhibit AChE.

Pymetrozine differs because both of its aromatic rings have positions where carbon is substituted by nitrogen, thus pymetrozine contains pyridine derivatives. It is possible that the addition of these pyridine derivatives alters pymetrozine's structure to a degree that does not allow the binding in or near the active site gorge of AChE.

There is much further research that could be done with *B. tabaci* in this area, including bioassays to confirm the possibility of cross-resistance between buprofezin, novaluron and pymetrozine. Synergism studies using esterase inhibitors and buprofezin should also be undertaken, as well as genetic studies on the inheritance of resistance mechanisms.

It would have been very interesting to compare the response of AChE from buprofezin resistant B-biotype *B. tabaci* with insecticides other than novaluron. Unfortunately, due to whitefly population (which was beyond my control), this was not possible. Further investigation of the structural change(s) in resistant AChE which do not allow buprofezin binding in or around the active site in buprofezin resistant B-biotype *B. tabaci* could form the subject of some fascinating further research. Studies using X-ray crystallography would be helpful in understanding the structure of mutant AChE.

The rapid rate at which buprofezin resistance was selected for in *B. tabaci*, and potential for cross-resistance between these novel insecticides is a warning that buprofezin should be used sparingly and only in rotation with other insecticides. The results have already provided valuable information in forming an insecticide resistance management strategy for B-biotype *B. tabaci* in central Queensland. With the escalating problem of B-biotype *B. tabaci* in many Australian crop production systems, including cotton, an effective resistance management strategy is vital.

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Appendix 1: Insecticide Suppliers in Australia, for the products used in this thesis

BASF Australia
867 Norwest Business Park,
Maitland Place,
Baulkham Hills, NSW 2153

Bayer Australia Ltd.,
875 Pacific Hwy,
Pymble NSW 2073
Australia

Crop Care Australasia Ltd.
77 Tingira St.,
Pinkenba, Qld 4008

Dow AgroSciences Pty. Ltd.
20 Rodborough Rd, Frenchs Forest,
NSW 2086

DuPont Australia Ltd.
168 Walker St.,
North Sydney NSW 2060

Novartis Australasia Pty Ltd
PO Box 249
Wentworthville, NSW 2145

Nufarm Ltd.
30 Pritchard St,
Lytton, Qld, 4178

Shell Research Ltd,
Sittingbourne Research Centre,
Sittingbourne, Kent, ME9 8AG
England

Sumitomo Australia Ltd.
16 Coombes Drive,
Penrith, NSW 2750

Appendix 2: Suppliers of Chemicals (other than insecticides) and Equipment used in this thesis

Arthur Yates and Co., Ltd.,
244 Horsly Rd.,
Milperra, NSW 2214
Australia

Australian Entomological Supplies,
PO Box 250,
Bangalow, NSW 2479
Australia

BDH Chemicals Ltd.,
Poole, Dorset BH15 ITD
England

Bio-Rad Laboratories
Po Box 210
Regents Park, NSW 2143
Australia

Boehringer Mannheim
PO Box 455
Castle Hill NSW 2154
Australia

Burkard Scientific,
PO Box 55,
Uxbridge, Middlesex,
Kent UB8 2RT
UK.

CERIT,
University of NSW,
Randwick, NSW 2031
Australia

Crown Scientific
Private Mail Bag 4
Moorebank, NSW 2170
Australia

ICN Biomedicals Inc.,
Unit 12, 167 Prospect Hwy,
Seven Hills, NSW 2147
Australia

Leiner Davis Gelatin
PO Box 409
Beaudesert, Qld, 4285
Australia

Lomb Scientific
PO Box 2223
Taren Point, NSW 2229
Australia

Oasis Horticulture Pty Ltd,
PO Box 2,
Winmalee, NSW 2777

Pharmacia Australia Pty Ltd.
59 Kirby St
Rydalmere NSW 2116
Australia

Sigma-Aldrich
Unit 2, 14 Anella Ave,
Castle Hill NSW 2154
Australia