Development of diagnostic tools for detecting expression of resistance-associated esterases in the tobacco budworm, Heliothis virescens (F.)

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DEVELOPMENT OF DIAGNOSTIC TOOLS FOR DETECTING EXPRESSION OF RESISTANCE-ASSOCIATED ESTERASES IN THE TOBACCO BUDWORM, *HELIOTHIS VIRESCENS* (F.)

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Entomology

By

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ABSTRACT

Esterase-based metabolic resistance was studied using biochemical and biological assays with organophosphate (OP)- and pyrethroid (PYR)- resistant tobacco budworms, *Heliothis virescens*. In biochemical assays, results suggest that: (1) esterase activities toward all substrates used were enhanced in both resistant strains compared with the susceptible strain, suggesting that esterases were involved in resistance; (2) esterase profiles differed depending on the strain and substrate used, and these differences were visualized by using native polyacrylamide gel electrophoresis; 3) esterase activities toward some pyrethroid substrates were significantly higher (P ≤ 0.01) in the PYR-R strain than those in the OP-R strain. These results suggest that pyrethroid substrates may be useful indicators for detecting esterases associated with pyrethroid resistance. Finally, biochemical assays were modified for use on solid materials, and esterase substrates were tested in filter paper assays. Whereas some differences in color intensity were detected between susceptible and resistant strains, these differences were not dramatic. Thus, utility of these substrates in such assays appears limited at this time, but further research is warranted.

In biological assays, two approaches were taken to improve the precision with which esterases associated with pyrethroid resistance were detected. First, bioassays were used to test effects of pyrethroid substrates and traditional synergists (e.g., piperonyl butoxide) on insecticide toxicity. Non-toxic pyrethroid esters enhanced pyrethroid toxicity to a greater extent than DEF, a compound widely used as an esterase inhibitor. In addition, synergism of profenofos and cypermethrin toxicity in both resistant strains by an oxidase
inhibitor, 1, 2, 4-trichloro-3 (2-propynyl oxy) benzene, suggests that P450 monooxygenases were also involved in resistance. The second method tested was to utilize bioactivated insecticides to detect esterases. Absence of negative cross-resistance to insecticides (i.e., acephate and indoxacarb) that are activated by esterases suggests that detoxication of these compounds in resistant insects proceeds more rapidly than their activation by esterases. Finally, levels of cross-resistance to tefluthrin and trans-fenfluthrin were lower than to permethrin and cypermethrin in both resistant strains, suggesting that resistance to insecticides in which sites for detoxifying enzymes (e.g., oxidases) are blocked develops more slowly than resistance to those in which metabolic sites are present.
CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The tobacco budworm, *Heliothis virescens* (F.), is a devastating cotton pest. Larvae of this polyphagous insect damage a wide range of field, horticultural and ornamental crops (Fitt, 1988). In addition, populations of *H. virescens* and its close relative, *Helicoverpa zea* (Boddie), can increase rapidly to very large size and were reported to cause almost one-third of all insect damage to American cotton from 1981 to 1998 (Suguiyama and Osteen, 1988; Head, 1992, 1993; Williams, 1994-99). During this period, the economic status of this complex consistently ranked as the number one pest in the southern cotton ecosystem. Wide-spread plantings of transgenic cotton have effectively managed *H. virescens* throughout much of midsouth. However, due to relatively low toxicity of currently expressed Bt toxins to *H. zea*, the *H. virescens / H. zea* complex remains the third most damaging of all cotton pests in this country (Williams, 2000, 2001).

1.2 History of Insecticide Resistance in *H. virescens*

Resistance is an inevitable consequence of the repeated applications of any new class of insecticides, and it is one of the major reasons that *H. virescens* is so destructive (Sparks, 1981; Sparks et al., 1993). Resistance has been described as “the ability of an insect population to survive a dose of poison that is lethal to the majority of individuals in a normal population of the same species” (Anonymous, 1957). Over the past 40 years, field populations of *H. virescens* have developed resistance to almost all classes of insecticides. During the 1960’s, organochlorine insecticides (e.g., DDT) could not adequately control the tobacco budworm (Graves, 1967; Lingren and Bryan 1965;
Lowry, 1966), which led to increased reliance on organophosphates (OPs) and carbamates. Shortly thereafter, resistance to many OP insecticides was reported (Graves and Clower, 1971; Harris, 1972), and the pyrethroids became the cornerstone of chemical management strategies in the cotton ecosystem during the late 1970’s (Wolfenbarger et al., 1981). However, reduced efficacy of these highly effective and environmentally-compatible insecticides to manage *H. virescens* spread rapidly throughout the mid-south because of their heavy use and pre-existing cross-resistance to DDT (Plapp and Hoyer, 1968; Nicholson and Miller, 1985; Sparks et al., 1988). At present, populations of this pest are being managed effectively by transgenic cotton that expresses a crystalline toxin (Cry1Ac) from *Bacillus thuringiensis* (Bt). However, three Bt-resistant strains of *H. virescens* have been developed by laboratory selection using Cry1Ac-impregnated artificial diet (Stone, 1989; Gould, 1992, 1995). In addition, Elzen (1997) collected a field strain of *H. virescens* with a significant resistance to a foliar formulation of Bt that was applied in Mississippi from 1993 through 1995.

### 1.3 Insecticide Resistance Mechanisms

A comprehensive knowledge of mechanisms by which insects develop insecticide resistance is a prerequisite for developing resistance management strategies and diagnostic techniques for detecting and monitoring the expression of resistance mechanisms in field populations of insects (Hammock and Soderlund, 1986). Insects develop resistance to insecticides primarily though three mechanisms: decreased penetration, reduced target site sensitivity and enhanced metabolism (Plapp, 1976; Oppenoorth, 1985). Expression of all three mechanisms has been reported in *H. virescens* (Sparks et al, 1993). Moreover, development of resistance to a particular
insecticide may be accompanied by the development of high levels of resistance or hypersensitivity to other insecticides, due to similar (i.e., cross-resistance) or dissimilar (i.e., multiple cross-resistance) resistance mechanisms (Adkisson and Nemec, 1967; Leonard et al., 1988; Pittendrigh, 2000). Cross-resistance can increase or decrease insecticide toxicity. For example, the toxicities of a number of structurally divergent Bt toxins decreased in *H. virescens* following selection with a single Bt toxin (CryIAc) in the laboratory (positive cross-resistance; Gould, 1992). In contrast, the susceptibility of pyrethroid-resistant *H. virescens* to chlorfenapyr (a non-pyrethroid) increased with the development of resistance to pyrethroids (negative cross-resistance; Pimprale et al., 1997).

### 1.3.1 Penetration Resistance

Reduced rate of cuticular penetration of an insecticide delays, but does not prevent the attainment of an effective amount of an insecticide that can kill the insect. Thus, reduced cuticular penetration as a single resistance mechanism confers only low levels of resistance and cross-resistance to an insecticide. However, when combined with other mechanisms, reduced cuticular penetration may enhance resistance in more than an additive fashion (Scott, 1990). In addition, reduced cuticular penetration is thought to be expressed in many cases of insecticide resistance (Sawicki and Lord, 1970; Oppenoorth, 1985) and has been detected in many resistant insects, such as the house fly, *Musca domestica* (Plapp and Hoyer, 1968), American cockroach, *Periplaneta americana* (Soderlund et al., 1983), diamondback moth, *Pultella xylostella* (Noppun et al., 1989), and the cotton bollworm, *H. armigera* (Ahmad and McCaffery, 1988). Despite its prevalence and importance, this mechanism is poorly understood.
Reduced cuticular penetration as a resistance mechanism is also expressed in insecticide-resistant *H. virescens*. For example, the cuticular penetration of endrin into resistant larvae of *H. virescens* was six-fold slower than that into susceptible larvae (Pollens and Vinson, 1972). However, resistance due to a six-fold decrease in the amount of absorbed endrin was amplified by co-expressed resistance mechanisms (e.g., enhanced metabolism, target-site insensitivity) to produce extremely high levels of resistance to endrin in *H. virescens*. In contrast, reduced penetration was a minor resistance factor in profenofos-resistant *H. virescens* (Kanga and Plapp, 1995). Finally, reduced penetration was detected in both larvae and adults of cypermethrin-resistant *H. virescens*, but difference between resistant and susceptible strains were not dramatic (Ottea et al., 2000).

### 1.3.2 Target-Site Resistance

Reduced sensitivity of insecticide target sites is a second major insecticide resistance mechanism. This mechanism results from functional modifications of target sites in resistant insects compared with susceptible ones. Decreased sensitivity of all major insecticide target sites (e.g., acetylcholinesterases (AChEs), the voltage-sensitive sodium channel, and gama-aminobutyric acid (GABA)-gated chloride channel) has been reported in a number of insects. To date, reported modifications have been exclusively point mutations of structural genes encoding the target site protein (Feyereisen, 1995; ffrench-Constant et al., 2000).

Target-site resistance to an insecticide usually confers cross-resistance to other compounds that share the same target site. For example, decreased sensitivity of sodium channel to DDT confers cross-resistance to all pyrethroids, which also act at this target
(Narahashi, 1983). Thus, it is believed that reduced susceptibility of the tobacco budworm to the pyrethroids existed before their widespread use in the field because of previous, extensive applications of DDT in the field (Davis et al., 1975; Brown et al., 1982; Leonard et al., 1988).

1.3.2.1 Reduced Sensitivity of AChEs

The major target site for both OPs and carbamates (O’Brien, 1960; Eldefrawi, 1985) is AChE, which acts by hydrolyzing excess neurotransmitter (acetylcholine) in some synapses of the nervous system. Reduced sensitivity of AChE to these insecticides is well-studied and has been expressed in a number of insects (Oppenoorth, 1985), such as *M. domestica* (Walsh et al., 2001), the aphid, *Nasonovia ribisnigri* (Rufingier et al., 1999), the Colorado potato beetle, *Leptinotarsa decemlineata* (Zhu et al., 1996), and the fruit fly, *Drosophila melanogaster* (Mutero et al., 1992). Reduced sensitivity of AChEs to OPs and carbamates is also expressed in *H. virescens* as a major resistance mechanism. For example, compared with a susceptible strain, AChE activity from a methyl parathion-resistant strain of *H. virescens* was 22-fold less sensitive to inhibition by methyl paraoxon in both larvae and adults. This resistant AChE was also less sensitive to structurally-analogous OPs, and to the N-methyl carbamates (Brown and Bryson, 1992). Similar results were demonstrated in thiodicarb-selected larvae (Zhao et al, 1996) and OP- and carbamate-resistant adults of *H. virescens* (Kanga et al., 1995).

Reduced sensitivity of AChE to OPs and carbamates is conferred by point mutations. Although genetic and biochemical bases of reduced sensitivity of AChE have been examined in a number of insects, they have been most well-studied in *D. melanogaster*, *M. domestica* and *L. decemlineata*. In the nucleotide sequence of AChE in *D.*
mellonogaster, Tyr<sup>109</sup> was first reported as a potential site of a point mutation conferring resistance to insecticides (Mutero et al., 1992). Subsequently, amino acid substitutions at four different sites (Phe<sup>115</sup> to Ser, Ileu<sup>199</sup> to Val, Gly<sup>303</sup> to Ala, and Phe<sup>368</sup> to Tyr) were found in OP-resistant <i>D. melanogaster</i> (Fournier et al., 1993) and at five different sites (Val<sup>180</sup> to Leu, Gly<sup>262</sup> to Ala or Val, Phe<sup>327</sup> to Tyr and Gly<sup>365</sup> to Ala) in <i>M. domestrica</i> (Walsh et al., 2001). The expression of varied combinations of mutations in a single AChE gene resulted in different patterns of resistance among OPs, and levels of resistance increased in an additive way. To date, more mutations in AChEs of <i>D. melanogaster</i> have been reported as being possibly involved in OP and carbamate resistance, but their toxicological significance has not been demonstrated (Villate et al., 2000). Interestingly, reduced sensitivity of AChEs to OPs and carbamates also occurs by replacement of a different amino acid (Ser<sup>238</sup> to Gly) in an azinphosmethyl-resistant strain of the Colorado potato beetle, <i>L. decemlineata</i> (Zhu et al., 1996).

1.3.2.2 Reduced Sensitivity of GABA-gated Chloride Channels

The GABA-gated chloride channel is the major target site for a number of insecticides including cyclodiienes (e.g., α-endosulfan), lindane, fipronil (Casida, 1993; Bloomquist, 2001), the spinosyns (Watson, 2001), and possibly a secondary target site of type II pyrethroids (Gammon and Casida, 1983) and the avermectins (Bloomquist, 1994). To date, reduced sensitivity of GABA-gated chloride channels as a resistance mechanism has been widely studied only in cyclodiene-resistant insects (Bloomquist et al., 1992; ffrench-Constant, 1994). In addition, replacement of Ala<sup>302</sup> in the GABA receptor subunit gene (termed resistance to dieldrin or Rdl) from three different orders is the only mutation that is known to confer reduced target site sensitivity at this site (ffrench-
Constant et al., 1995). There have been no direct studies associating reduced sensitivity
of GABA-gated chloride channel to resistance in *H. virescens* (McCaffery, 1998).

### 1.3.2.3 Reduced Sensitivity of Voltage-gated Sodium Channels

Three classes of insecticides (pyrethroids/DDT analogs, N-alkylamides, and
dihydropyrazoles) can modify sodium channel function, although modifications result
from binding to three distinct target sites on this protein (Soderlund and Knipple, 1995).
Thus, a domain-specific modification of sodium channels as a resistance mechanism
usually does not confer cross-resistance among these three classes, although negative
cross-resistance between pyrethroids and N-alkylamides (Elliott et al., 1987) or
dihydropyrazoles (Khambay et al., 2001) has been reported. Moreover, reduced
sensitivity of the alpha subunit of voltage-gated sodium channel has been widely studied
in DDT and pyrethroid resistance and has been identified in a range of insect species,
such as *D. melanogaster* (Vais et al., 2001) and *M. domestica* (Lee et al., 1999). Reduced
sensitivity of sodium channels to pyrethroids is expressed in pyrethroid-resistant *H.
virescens* (McCaffery, 1991; Ottea et al., 1995; Park and Taylor, 1997).

Substitutions of two different amino acids though point mutations are associated with
pyrethroid/DDT-resistant phenotypes: kdr (knock-down resistance) and super-kdr. First,
substitution of Leu<sup>1014</sup> with Phe results in the kdr phenotype in *D. melanogaster* (Vais et
al., 2001) and *M. domestica* (Lee et al., 1999), which shifts the voltage dependence of
both activation and steady-state inactivation by approximately 5mV towards more
positive potentials and facilitates Na channel inactivation (Vais et al., 2001). In addition,
incorporation of a second mutation (Met<sup>918</sup> to Thr) led to expression of the super-kdr
phenotype in both *D. melanogaster* (Vais et al., 2001) and *M. domestica* (Lee et al.,
1999), which also increased sodium channel inactivation and caused a more than 100-fold reduction in deltamethrin sensitivity in *D. melanogaster* (Vais et al., 2001). Similarly, replacement of Thr<sup>929</sup> with Ile in *D. melanogaster* confers super-kdr-like resistance (Vais, 2001).

### 1.3.3 Metabolic Resistance

Enhanced metabolism of insecticides decreases the attainment of the effective amount of insecticides that can kill insects. Thus, metabolic resistance may significantly decrease the susceptibility of insects to insecticides. Three major detoxifying enzymes are associated with insecticide resistance: cytochrome P450 monooxygenases (CYPs), glutathione S-transferases (GSTs) and esterases (ESTs) (Bull, 1981; Oppenoorth, 1985). Most insecticides can be detoxified by at least one of these enzymes in insects and enhanced detoxication is often involved in resistance. Moreover, the broad substrate spectrum of each of these enzymes and the multiplicity of individual enzymes (ffrench-Constant et al., 1999) is believed to cause cross-resistance among different classes of insecticides, regardless of their target sites.

#### 1.3.3.1 Resistance Associated with CYPs

Detoxifying CYPs are a superfamily of important enzymes that catalyze multiple oxidative reactions and are capable of metabolizing a variety of endogenous and exogenous substrates (Guengerich, 1996). Metabolism by CYPs generally leads to detoxication of substrates, although activation is also possible. For example, the efficacy of phosphorothioate insecticides is based on the activation of P = S to P = O by CYPs, which results in more toxic insecticides than parent compounds (O’Brien, 1960; Oppenoorth, 1972; Prestwich, 1990).
Elevated CYP activities have been detected in a number of resistant insects (Agosin, 1985; Feyereisen, 1999). Increased epoxidation by CYPs was expressed in resistant fall armyworms, *Spodoptera frugiperda* (Yu, 1991). Moreover, enhanced demethylation by CYPs was present in insecticide-resistant diamondback moth larvae (Yu and Nguyen, 1992) and permethrin-resistant soybean loopers, *Pseudoplusia includens* (Thomas et al., 1996). Similarly, elevated deethylation by CYPS was detected in *D. melanogaster* (DeSousa et al., 1995) and heightened levels of hydroxylation by CYPs were measured in *M. domestica* (Li et al., 1989). Finally, enhanced CYP activities including dealkylation (Rose et al., 1995; Zhao et al., 1996) and hydroxylation (Shan and Ottea, 1997) were also expressed in insecticide-resistant *H. virescens*.

Elevated CYP activities are generally thought to occur by over-production of CYP proteins. Rose et al. (1995) demonstrated that elevated CYP activities toward three substrates (p-nitroanisole, benzopyrene and benzphetamine) in multi-resistant *H. virescens* varied from 3- to 33-fold and were associated with enhanced cytochrome P450 content in microsomes from the gut (3.7-fold), fat body (4.4-fold) and carcass (4-fold) of resistant insects. In addition, over-expression of *CYP6* and *CYP9* genes conferred pyrethroid resistance in *H. virescens* (Pimprale, 1998). Similarly, over-expression of a number of CYPs that were associated with insecticide resistance was present in *CYP6A2*, *CYP4E2*, *CYP6A9* genes of *D. melanogaster* (Amichot et al., 1994; Brun et al., 1996; Maitra et al., 1996), *CYP6A1*, *CYP6D1* of *M. domestica* (Cariño et al., 1992; Liu and Scott, 1998), and *CYP6B2* of *H. armigera* (Wang et al., 1995).

Enhanced transcriptional expression is possibly the underlying mechanism of CYP over-expression that confers insecticide resistance. Pimprale (1998) compared the *CYP6*
and CYP9 genes from pyrethroid-resistant and -susceptible H. virescens and showed that structural gene polymorphism and gene amplification were not likely to be responsible for the pyrethroid resistance measured. However, altered transcriptional expression of CYP genes was demonstrated and is believed to underlie resistance in this pest. In studies with M. domestica, an elevated rate of transcription of CYP6D1 from pyrethroid-resistant adults is an underlying cause of insecticide resistance (Liu and Scott, 1998), and enhanced levels of transcription were mapped to factors on autosomes 1 and 2 (Scott et al., 1998).

1.3.3.2 Resistance Associated with GSTs

Detoxifying GSTs are a family of enzymes that catalyze the conjugation of glutathione with electrophilic substrates including insecticides (Soderlund, 1997). The GSTs are involved in O-dealkylation or dearylation of OPs (Hayes and Wolf, 1988). High frequencies of profenofos resistance were moderately correlated with GST activity toward 1-chloro-2, 4-dinitrobenzene in larvae of H. virescens that were collected in Louisiana cotton fields during the 1995 cotton growing season (Harold and Ottea, 1997). Moreover, GSTs are involved in the dehydrochlorination of DDT in M. domestica (Clark and Shamaan, 1984) and are the primary metabolic mechanism of resistance to this insecticide. Finally, a recent study suggests that GSTs act as antioxidant-defense agents and confer pyrethroid resistance in Nilaparvata lugens and possibly in other insects (Vontas et al., 2001).

Enhanced activities of GSTs that confer insecticide resistance result from both quantitative and qualitative alterations in gene expression. First, there is evidence for over-expression of one or more GST isoforms in resistant insects. For example, the high
activity found in an insecticide-resistant strain of *M. domestica* is correlated with high level of *GST1* transcript (Fournier et al., 1992). Similar phenomena were also found in insecticide-resistant *Aedes aegypti* (Grant et al., 1991), *Anopheles gambiae* (Prapanthadara et al., 1993; Ranson et al., 2001), and *P. xylostella* (Ku et al., 1994). Moreover, qualitative differences of GSTs were also present between susceptible and resistant insects. For example, most subcellular fractions from susceptible *M. domestica* had higher conjugation activities toward 1-chloro-2, 4-dinitrobenzene than the fractions from the Cornell-R strain, but all fractions from the susceptible strain had lower conjugation activities toward 1, 2-dichloro-4-nitrobenzene than fractions from the Cornell-R strain (Chien et al., 1995). In addition, quantitative and qualitative alterations can be co-expressed and confer resistance in one resistant strain. For example, GSTs from a DDT-resistant strain of *A. gambiae* had an altered GST profile and one of GSTs was increased 8-fold in a resistant strain compared with the susceptible strain (Prapanthadara et al., 1993).

### 1.3.3.3 Resistance Associated with ESTs

Esterases are a large group of proteins (Benning, 1994; Cousin, 1997; Kim, 1997) that share a highly-conserved catalytic domain characterized by an $\alpha / \beta$ hydrolase fold (Ollis, 1992) in their three-dimensional structures. They use water to hydrolyze endogenous and exogenous esters and produce alcohols and acids as products.

A systematic nomenclature for classifying esterases remains to be established, and multiple classifications are used. For example, based on the nature of inhibition by OPs, esterases can be classified into three types (A, B, C; Aldridge, 1953). Moreover, based on the identity of the functional amino acid (either serine or cysteine) within the catalytic
triad, esterases can be categorized into serine- or cysteine-type esterases (Yan et al., 1994). Finally, esterases can be named according to the substrates they hydrolyze, such as phosphorotriester hydrolase (PTEH, EC 3.1.1.2), AChEs, carboxylesterases (CarbEs; EC 3.1.1.1), or amidases. Although there are multiple classifications, none of them is completely satisfactory for naming individual esterases (La Du, 1992).

1.3.3.3.1 Esterase-based Resistance in Insects

Esterase-mediated metabolic resistance is widespread and has been detected in almost all pests and against all classes of insecticides containing an ester moiety. First, enhanced metabolism by esterases is a major mechanism in OP resistance, and has been detected in a number of dipterans (Holwerda and Morton, 1983; Newcomb et al., 1997; Hemingway, 1981; Whyard et al., 1995), homopterans (Abdel-Aal et al., 1992; Owusu et al., 1996; Chen and Sun, 1994; Chiang and Sun, 1996), coleopterans (Conyers et al., 1998; Mathews, 1980; Argentine et al., 1989), lepidopterans (Beeman and Schmidt, 1982), and hymenopterans (Baker et al., 1998). Similarly, elevated esterase activities are associated with pyrethroid resistance in the sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Prabhaker et al., 1988), *Leptinotarsa decemlineata* (Lee, 1996), and the rice green leafhopper, *Nephotettix cincticeps* (Ulher) (Chiang, 1996). Moreover, esterase-associated resistance to carbamates has also been demonstrated in methomyl-resistant tentiform leaf miners, *Phyllonorycter blancard* (F.) (Pree, 1989), and carbaryl-resistant *S. frugiperda* (Yu, 1992). Finally, an esterase-based resistance mechanism has also been detected in abamectin-resistant tomato leafminers, *Tuta absoluta* (Meyrick) (Siqueira et al., 2001) and *L. decemlineata* (Say) (Argentine, 1991).
Enhanced esterase activities are also expressed in insecticide-resistant *H. virescens*. A field-derived strain of *H. virescens* expressed an enhanced phosphorotriester hydrolase activity as one of three mechanisms conferring resistance to methyl parathion (Konno et al., 1990). More recent studies have shown that increased esterase activities toward model (i.e., non-insecticide) substrates are associated with resistance to carbamate, OP, and pyrethroid insecticides (Goh, 1995; Zhao, 1996), and are correlated with frequencies of profenofos resistance in field-collected and laboratory-selected strains of *H. virescens* (Harold and Ottea, 1997).

### 1.3.3.3.2 Esterases Associated with Insecticide Resistance

There are a large number of cases in which esterases are involved in insecticide resistance. However, there are two relatively specific esterases (PTEH and CarbEs) that are most often associated with metabolic resistance.

Levels of PTEHs (EC 3.1.1.2) are relatively low in arthropods compared with mammals (Dauterman, 1982). However, in resistant insects, these enzymes effectively cleave phosphorus esters. For example, an increase in PTEH activity was observed in all of the malathion-resistant strains of *M. domestica* examined by Kao (1984). Moreover, PTEH activity toward diazoxon was detected in a resistant strain of *M. domestica* and was present in all subcellular fractions examined (Oi et al., 1990). In addition, a field-derived resistant strain of *H. virescens* displayed increased PTEH activities as one of three mechanisms contributing to methyl parathion resistance (Konno et al., 1989, 1990).

The CarbEs can hydrolyze both aromatic and aliphatic carboxylesters and have also been shown repeatedly to be associated with insecticide resistance (Soderlund, 1997). As was seen in GSTs, resistance arises from both quantitative and/or qualitative changes of
CarbE expression. Quantitative increases of CarbEs that confer resistance have been shown to occur by over-expression of the corresponding genes. It is interesting to note that, in many cases, over-expression of CarbEs does not result in a significant change in overall rates of insecticide hydrolysis. Rather, the insecticide is sequestered from its target site, and actual hydrolysis occurs very slowly (Devonshire, 1977; Herath et al., 1987; Karunaratne et al., 1995). This type of resistance has been well studied in the green peach aphid, *Myzus persicae*. A single isozyme (termed E4) from both susceptible and OP-resistant aphid strains exhibited identical catalytic properties toward $\alpha$-naphthyl acetate (1-NA) and paraoxon, but massive amounts of E4 (approximately 3% of the insect’s total proteins) are expressed in resistant aphids (Devonshire, 1977, 1979; 1982). Moreover, further studies showed that E4 is very inefficient in catalyzing the hydrolysis of insecticidal substrates, such as OPs, carbamates and pyrethroids (Devonshire, 1982). These results suggest that the observed resistance to OPs, carbamates and pyrethroids resulted primarily from sequestration of these insecticidal substrates by over-expressed CarbEs rather than from increased efficiency of detoxication of these substrates. Similarly, CarbEs from permethrin-resistant *L. decemlineata* possibly sequester insecticides in a non-specific way, and sequestration is associated with cross-resistance to other hydrophobic insecticides, such as other pyrethroids, DDT, and abamectin (Lee et al., 1998). In addition, resistance conferred by sequestration by over-expressed CarbEs also occurred in culicine mosquitoes and the brown planthopper, *Nilaparvata lugens* (Mouches et al., 1986; Field and Devonshire, 1998; Karunaratne et al., 1998; Small and Hemingway, 2000; Hemingway, 2000). Finally, abundance of an over-expressed CarbE
was quantitatively correlated with pyrethroid resistance in several field-collected strains of *H. armigera* (Gunning et al., 1996).

Overproduction of sequestering esterases may result from either gene amplification (e.g., multiple copies) or gene expression (e.g., methylation; Field et al., 1988; Vaughan and Hemingway, 1995; Field et al., 1996; Rooker et al., 1996). Field et al. (1996) demonstrated that approximately 12 copies of a 24 kb amplicon containing the *E4* gene were responsible for insecticide resistance in the peach-potato aphid *M. persicae* (Sulzer). Similarly, high levels of OP resistance (approximately 800-fold) in *C. pipiens* resulted from over-expressed aliesterase (Ali-E) B1 by *B1* gene amplification, which contained at least 250 copies of a 30 kb amplicon, including a highly conserved "core" of 25 kb (Mouches et al., 1990). However, gene amplification cannot fully account for the levels of resistance expressed. For example, the level of resistance (60-fold) measured in *M. persicae* is substantially higher than the extent of gene amplification (12 copies), suggesting that enhanced *E4* gene expression or additional resistance mechanisms is also responsible for producing resistance measured in these aphids (Field et al., 1996). A similar conclusion may be possible to explain high levels of OP resistance in *C. pipiens*, because the copy number of esterase gene (at least 250) is far below the level of resistance (800-fold; Rooker et al., 1996). In addition, reversion in *M. persicae* from resistant to susceptible phenotypes (Field et al., 1996; Hick et al., 1996; Field et al., 1999) did not arise from the loss of amplified esterase DNA sequence, but rather from decreased gene expression due to methylation at a certain site (CCGG in *M. persicae*) in the amplified DNA sequence, which silences expression of the amplified *E4* gene. A similar phenomenon has been observed for the greenbug aphid, *Schizaphis graminum*.
(Ono, 1999). These results suggest that both gene amplification and transcriptional control affect esterase expression in resistant insects.

Qualitative changes in resistance-associated CarbEs are thought to occur as a result of point mutations in structural genes for these proteins. Qualitative changes in CarbEs are nicely illustrated by a phenomenon known as the “mutant aliesterase hypothesis.” This theory was proposed after the observation that increased degradation of OPs is associated with decreased CarbE activities toward “general” substrates, e.g., 1-NA (Oppenoorth and van Asperen, 1961; Lewis and Sawicki, 1971; Hughes and Raftos, 1985). Recent research demonstrates that OP-hydrolase activities (with a diethyl preference) of a mutated Ali-E increases in parathion-/ diazinon-resistant *L. cuprina* at the expense of activities toward diverse carboxylesters including Ali-E substrates, 1-NA and the carboxylester moiety of malathion. In contrast, OP-hydrolase activities (with a dimethyl, rather than diethyl preference) of a mutated Ali-E increased in malathion-resistant *L. cuprina* at the expense of activities toward Ali-E substrates and 1-NA, but not the carboxylester moiety of malathion (Campbell et al., 1997). These and other studies (Bell and Busvine, 1967; Townsend and Busvine, 1969; Hughes and Raftos, 1985) led to the conclusion that esterase-associated metabolic resistance to OPs in *M. domestica*, *C. putoria*, and *L. cuprina* can be categorized into two related but distinct phenotypes, resulting from different changes in the same major esterase genes (Campbell et al., 1997).

Present evidence from nucleotide sequence of structural genes suggests that point mutations in CarbE genes are responsible for substrate specificity and insecticide resistance. For example, a single substitution (Trp^{51}-Leu) within the E3 CarbE of *L. cuprina* results in resistance to malathion (Campbell et al., 1998). Conversely, a second
substitution (Gly^{137}-Asp) within the same gene confers broad cross-resistance to a range of OPs, but not malathion (Campbell et al., 1998). This same substitution within the E7 CarbE gene confers OP resistance in both *L. cuprina* and *M. domestica* (Claudianos et al., 1999).

As noted earlier, quantitative and qualitative changes of CarbEs can co-occur in resistant insects. Owusu et al. (1996) demonstrated that both changes of CarbEs are responsible for dichlorvivos resistance in the cotton aphid, *Aphis gossypii*. Similarly, both kinds of changes of CarbEs also lead to the high levels of malathion resistance in *C. tarsalis* (Ziegler et al., 1987). In addition, the relative resistance of several field-collected strains of *H. armigera* was quantitatively correlated with the abundance and activities of an over-expressed CarbE, suggesting that quantitative and qualitative changes have occurred in expression of this CarbE (Gunning et al., 1996).

### 1.4 Available Techniques for Detecting Esterases

The ability to detect esterases associated with resistance in a field situation is an important component of insecticide resistance management strategies, because incisive decisions regarding effective implementation of these strategies are primarily based on precise detection of insecticide resistance mechanisms in early stages in the development of resistance. Technology is available to develop a number of different types of diagnostic assays (Brown and Brogdon, 1987) and each has advantages and disadvantages. In general, these methods can be evaluated based on their expense, specificity, the time and equipment required for development/implementation, and ultimately, their field utility.
1.4.1 Chromatographic Techniques

Chromatographic techniques directly detect enhanced metabolism of an insecticide by measuring either the disappearance of insecticide substrates or appearance of products. These techniques include thin-layer chromatography (TLC), gas chromatography (GC) (Abernathy and Casida, 1973) and high-pressure liquid chromatography (HPLC) (Yu, 1988, 1990). The cheapest and most facile one of these techniques is TLC, which can be used to measure qualitative differences in metabolism, but, unless radioactive substrates are used, the quantitative results are not possible. Whereas both GC and HPLC are powerful analytic tools, they are highly dependent on instrumentation, fairly labor-intensive and thus, difficult to use in a field situation.

1.4.2 Immunological Techniques

Immonoassays are analytical methods based on the specific immuno-reaction between an antibody and antigen, and may be used for the quantitation of either product or reactant in a solution. Enzyme Linked Immunosorbant Assays (ELISAs) have been used successfully to detect esterases associated with insecticide resistance (Zhu et al., 1994; Karunaratne et al., 1995, 1998; Siegfried et al., 1997), and are able to detect both low abundance and over–expressed esterases (Devonshire et al., 1986). However, generation of a specific antibody requires identification and purification of the enzyme of interest. Moreover, the specificity of the generated antibody may be suspect (Karunaratne et al., 1995). On the one hand, they may be so specific that their utility is limited to a single esterase that may be unique to a particular insect. On the other hand, resistance-associated esterases that result from a single point mutation may not be readily distinguished from wild type enzymes (Siegfried, 1997).
1.4.3 Molecular Techniques

Monitoring techniques based on molecular biology are used for detecting polymorphisms at the nucleotide level between insect strains. These techniques include the polymerase chain reaction (PCR) and restriction endonucleases, single-stranded conformational polymorphism, and PCR amplification of specific alleles (ffrench-Constant et al., 1995; Zhu et al., 1999). The advantages of these techniques are that a change in one nucleotide may be detected, making them the least ambiguous of presently used diagnostic assays. In addition, this is the only type of method that allows absolute detection of heterozygous individuals (ffrench-Constant et al., 1995). However, the limitation of these techniques may be directly attributed to their unparalleled specificity: the genetic change responsible for resistance must be identified prior to establishing this method (ffrench-Constant, 1994), and this is both time-consuming and expensive. Further, these methods are highly dependent upon instrumentation. Thus, their field utility is limited. Thus, molecular monitoring techniques will probably never replace the use of insecticide bioassays in the initial diagnosis, or even necessarily in the routine monitoring of resistance (ffrench-Constant et al., 1995).

1.4.4 Bioassays with Synergists

Traditional bioassays with synergists are extremely fast and easy to use, but their specificity is often questionable. Synergist assays rely on specific inhibition of resistance-associated enzymes; however, synergists may reduce (or increase) the penetration of insecticides and, thus, decrease (or increase) the accessibility of insecticides to their targets (Martin et al., 1997; Sanchez et al., 2001). Moreover, multiple forms of insecticide detoxifying enzymes may be expressed concurrently, and
the specificity of these enzymes to inhibition by any individual synergist is variable (Jao and Casida, 1976; Khayrandish et al., 1993; Brown et al., 1996; Shan and Ottea, 1997). As a result, the absence of synergism might indicate that: (1) the targeted enzyme is not responsible for resistance, (2) a resistant-associated enzyme is present but not inhibited by the synergist (ffrench-Constant et al., 1990; Brown et al., 1996) or (3) the synergist affects the accessibility of insecticides to their target sites.

1.4.5 Biochemical Assays

Biochemical assays may be based on the hydrolysis of an actual insecticide or surrogate substrate, such as 1-naphthyl acetate (1-NA) by esterases. Most often, hydrolysis results in a color change that can be read by spectrophotometry, and the intensity of this change is directly proportional to the activity of the enzyme of interest. Based on the substrate and modes of measurement, three assays are possible.

1.4.5.1 pH Indicator Assays

A pH indicator assay is a colorimetric, pH responsive method for screening hydrolase libraries, which is based on a pH change that occurs as the reaction proceeds (i.e., as carboxylic acid are generated) (Moris-Varas, 1999). The advantage of this method is that a target insecticide containing an ester bond is hydrolyzed to generate an acid. Thus, it is straightforward to detect whether insecticide hydrolysis is a major mechanism of resistance, and to detect the frequency of resistant individuals in a population. Moreover, it is cheaper and safer compared with using a radiolabeled insecticide. However, these assays are time-intensive compared with other biochemical assays because the change of pH is usually very slow. In addition, it is difficult to use this method to visualize esterases in electrophoretic gels because the color generated is most often transient.
1.4.5.2 Fluorescent Indicator Assays

Fluorescent indicator assays are based on the appearance of a fluorescent product after hydrolysis of a surrogate substrate by an esterase, which can be detected by fluorescent spectrophotometer. They are robust assays and have been used in a number of cases to detect esterases (Homolya et al., 1993; Kitson et al., 2000; Shan et al, 2001); however, the fluorescent product is invisible to the naked eye, and cannot be seen without special lighting, thus their utility is limited for field situations and electrophoresis.

1.4.5.3 Colorimetric Assays

Colorimetric assays increase the appearance of visibly colored metabolites and/or its conjugated complexes after hydrolysis of a surrogate substrate by an esterase. Many of the surrogate substrates for these assays may be used to visualize esterases following separation by polyacrylamide gel electrophoresis (PAGE; Matsumura and Brown, 1961; Hemingway, 1981, 1989; Gopalan et al., 1997; Baker et al., 1998; Harold and Ottea, 2000). In addition, minor adaptations of these techniques for use on solid materials (e.g., filter paper “squash” assays) increase their potential use in field situations (Harold and Ottea, 2000).

Colorimetric assays are widely utilized because they are economical, easy and fast tools for detecting expression of enzymes associated with resistance. Furthermore, compared with other methods, they have potential to be used extensively in the field environment, although, to date, their use has been limited. However, the disadvantages of these assays are similar to those of synergist bioassays: surrogate substrates may or may not be suitable substrates for resistance-associated esterases, or the isozymes that metabolize model substrates may not necessarily be those that detoxify insecticides.
For example, 1-NA is used extensively as a model substrate for esterases, but is a poor indicator of resistance-associated esterases from pyrethroid-resistant *H. virescens* (Dowd, 1987; Ottea et al., 2000). In addition, purified GSTs from rat liver and *M. domestica* also have activity toward 1-NA, suggesting that some of “esterase activity” measured with this substrate may result from the activity of GSTs (Lalah et al., 1995).

### 1.5 Specific Objectives

I) **Synthesize and evaluate substrates for resistance-associated ESTs from the tobacco budworm, *Heliothis virescens* (F.).**

* Synthesize and analyze pyrethroid substrates
* Optimize conditions for biochemical assays with substrates
* Evaluate substrates as indicators of resistance-associated ESTs from *H. virescens*

II) **Investigate the use of existing and novel compounds as tools for detecting esterases associated with resistance.**

* Evaluate whether synthesized pyrethroid substrates can be synergists of cypermethrin toxicity in profenofos- and cypermethrin-resistant strains of *H. virescens*
* Evaluate whether acephate, indoxacarb, tefluthrin and trans-fenfluthrin are diagnostic compounds of esterases associated with insecticide resistance
* Evaluate whether pyrethroid substrates can be used as indicators of esterases associated with resistance in a rapid “squash” assay to detect resistant individuals of *H. virescens*
III) Determine the degree to which resistance to profenofos or cypermethrin confers cross-resistance to other organophosphates or pyrethroids in *H. virescens*.

* Investigate the relationship between pyrethroid structure and cross-resistance

1.6 References


CHAPTER 2
DEVELOPMENT OF PYRETHROID SUBSTRATES FOR ESTERASES ASSOCIATED WITH PYRETHROID RESISTANCE IN HELIOTHIS VIRESCENS (F.)

2.1 Introduction

Esterases are a large group of proteins (Benning, 1994; Cousin, 1996; Kim, 1997) that share a highly-conserved catalytic domain characterized by an $\alpha/\beta$ hydrolase fold (Ollis, 1992) in their three dimensional structures. Esterases play critical roles in insect physiology and detoxify a broad range of xenobiotics including insecticides (Dauterman, 1985). Enhanced esterase activity is a major mechanism of insecticide resistance (Oppenoorth, 1984) and has been detected in a number of insects (Devonshire, 1991; Hemingway, 2000). The role of esterases in insecticide resistance has been actively studied in lepidopteran pests (McCaffery, 1991), where they have been shown to be associated with resistance to organophosphorus (OP; Konno et al., 1990; Harold and Ottea, 1997), carbamate (Goh et al., 1995; Zhao et al., 1996) and pyrethroid insecticides (Konno et al., 1990; Goh et al., 1995; Zhao et al., 1996; Gunning, 1996, 1997).

The ability to detect esterases associated with resistance in a field situation is an important component of insecticide resistance management strategies. Technology is available to develop a number of different types of diagnostic assays (Brown and Brogdon, 1987) and each has advantages and disadvantages. In general, these methods can be evaluated based on their expense, specificity, the time and equipment required for development, and ultimately, their field utility. Molecular techniques detect mutations in specific genes that are associated with resistance. A change in one nucleotide may be detected by this technique, making it the least ambiguous of presently used diagnostic assays (ffrench-Constant et al., 1995). In addition, this is the only method that allows
absolute detection of heterozygous individuals. However, the limitation of this method may be directly attributed to its unparalleled specificity: the genetic changes responsible for resistance must be identified prior to establishing this method, and this is both time-consuming and expensive. Further, this method is highly dependent upon instrumentation; thus, its field utility is limited. Similar constraints limit the field utility of immunoassays, which use antibodies directed against resistance-associated proteins, and chromatographic techniques, which measure directly the enhanced metabolism of an insecticide.

In contrast, insecticide bioassays with synergists and biochemical assays for measuring enzyme activities are extremely fast and easy to use, and have potential field utility. However, the specificity of these techniques is often questionable. These assays rely on specific detection or inhibition of resistance-associated enzymes; however, multiple forms of detoxifying enzymes may be expressed concurrently. For example, the specificity of these enzymes to inhibition by any individual synergist is variable (Jao and Casida, 1976; Brown et al., 1996; Shan and Ottea, 1997), and lack of synergism of insecticide toxicity might indicate either that the targeted enzyme is not responsible for resistance or a resistance-associated enzyme is present but not inhibited by the synergist used (ffrench-Constant and Roush, 1990; Brown et al., 1996). Similarly, biochemical assays have been used to detect changes in esterases expressed in insecticide-resistant insects. In addition, many of the substrates for these assays may be used to visualize esterases following separation by polyacrylamide gel electrophoresis (PAGE; Matsumura & Brown, 1961; Hemingway, 1981, 1989; Gopalan et al., 1997; Baker, 1998; Harold and Ottea, 2000). The disadvantages of these assays are similar to those of synergist bioassays: model (i.e., non-insecticide) substrates for esterases may not be selectively
hydrolyzed by resistance-associated esterases. For example, 1-naphthyl acetate (1-NA), which has been used extensively as a model substrate, is a poor indicator of resistance-associated esterases from pyrethroid-resistant *Heliothis virescens* (Dowd et al., 1987).

Developing specific substrates is vital for enhancing the precision of quantitative biochemical assays for detecting resistance. The hypothesis tested here is that the specificity of detection of resistance-associated esterases can be enhanced by using substrates that are structurally-similar to the insecticide that is resisted. The approach used is to modify the structure of a commonly used pyrethroid insecticide such that the product of hydrolysis is detectable by spectrophotometry. A similar approach has been used to study esterases associated with pyrethroid hydrolysis in the cattle tick *Boophilus microplus* (Riddles, 1983) and in human serum (Butte, 1999).

The major objectives of this Chapter are to: (1) identify whether non-pyrethroid substrates are accurate indicators of esterases associated with pyrethroid resistance in *H. virescens*, and (2) if not, develop and test pyrethroid substrates that are structurally-similar to conventional pyrethroids. Results in this study suggest that esterases associated with pyrethroid resistance in *H. virescens* were more easily detected by pyrethroid substrates than non-pyrethroid substrates.

**2.2 Materials and Methods**

**2.2.1 Chemicals**

Fast blue B salt (90% purity), 1-NA (98% purity), benzenethiol (97% purity), S-methyl thiobutanoate (98% purity), 1-naphthol (99% purity), 2-naphthol (99% purity), acetyl chloride (97% purity), thionyl chloride (99% purity), phosphorus pentoxide (98% purity), 5,5’-dithio (2-nitrobenzoic acid) (DTNB, 99% purity), 4-nitrophenol (99% purity) and benzoyl chloride (99% purity) were obtained from Aldrich Chemical Co.
(Milwaukee, WI). 2-Naphthyl acetate (2-NA) and Silica gel (28-200 mesh, average pore diameter 22 Å) were purchased from Sigma Chemical Co., Inc. (St. Louis, MO). The methyl ester of 3 – (2, 2 –dichlorovinyl) – 2, 2 – dimethylcyclopropane carboxylate (PA; cis/trans = 40/60) was purchased from Fisher Scientific (Pittsburgh, PA). Technical grade profenofos (O-(4-bromo-2-chlorophenyl)–O-ethyl–S-propyl phosphorothioate: 89%) was purchased from NOVARTIS (Greensboro, NC) and cypermethrin (a racemic mixture of trans/cis, 1R/S and α R/S isomers: 96.2%) was from FMC Corporation (Princeton, NJ). All other chemicals were analytical quality and purchased from commercial suppliers.

### 2.2.2 Insects

A susceptible strain of *H. virescens* (LSU-S) was established in 1977 (Leonard et al., 1988) and has been reared in the laboratory since that time without exposure to insecticides. All larvae for resistance selection were fifth instars. Larvae from the LSU-S strain were selected for five consecutive generations with topical applications of profenofos at doses corresponding to the LD<sub>60</sub> for each generation (3.5, 4.6, 8.5, 13.6 and 23.8 μg/individual). After one year without selection, individual larvae were treated with 25μg profenofos (an approximate LD<sub>20</sub>) and the next generation (OP-R strain) was used for biochemical and toxicological assays.

Similarly, larvae from the LSU-S strain were selected for four consecutive generations with topical applications of cypermethrin at doses corresponding to the LD<sub>60</sub> (0.22, 0.4, 0.5 and 0.6 μg/individual). After one generation without selection, larvae were treated with 1 μg of cypermethrin (6% mortality at 72 hours), and the next generation (PYR-R) was used for biochemical and biological assays.
2.2.3 Bioassays

Insecticide susceptibility was measured and compared among strains using a topical bioassay. Fifth instars (day 1), weighing 180±20 mg, were treated on the mid-thoracic dorsum with 1 μl aliquots of profenofos or cypermethrin (in acetone) or with acetone alone (control). The dose-mortality relationship for each compound was assessed from at least five doses with 30 insects treated per dose. After treatment, larvae were maintained at 27°C, and mortality was recorded after 72 hours. The criterion for mortality was the lack of coordinated movement within 30 seconds after being prodded. Results were corrected for control mortality with Abbott’s formula (Abbott, 1925), then analyzed by Finney’s method (Finney, 1971). Resistance ratios (RR) were calculated as the LD$_{50}$ of resistant strain / LD$_{50}$ of susceptible strain.

2.2.4 Synthesis

Preparation of PA followed the procedure of Shan et al. (1997). A mixture of the methyl ester of PA (22.3 g, 0.1 mol), sodium hydroxide (12 g, 0.3 mol) and ethanol-water (1:1, 250 ml) was refluxed for 12 hrs, concentrated under reduced pressure, diluted with 150 ml water, and extracted with ethyl ether (3 x 30 ml). The aqueous phase was acidified with concentrated HCl (37.4%, 30 ml), and the oil precipitate was extracted into ether (3 x 30 ml), washed with water (2 x 25 ml), and then dried (anhydrous Na$_2$SO$_4$) overnight. The solution was filtered and ethyl ether was evaporated under reduced pressure to obtain a solid product, which was re-crystallized from hexane / ether (1:1, v / v). Yield 18.0 g (86%).

The method of Foggassy et al. (1986) was modified and used for separating trans and cis isomers of PA. A mixture of PA (20.9 g, 0.1 mol) and benzene (100 ml) was stirred at 27°C for 5 hrs. The suspension was filtered and the filter residue was recrystallized.
from benzene to give 5.0 g of pure cis – PA (60% recovery). After removal of benzene from the filtrate, the solid product was stirred 5 hrs at 27°C in 100 ml petroleum ether, and then filtered to give 2.5 g solid product, which was recrystallized from hexane to give 1.8 g pure trans – PA (14% recovery).

Preparation of substrates (Scheme 2.1-2.2)

**Scheme 2.1 Pyrethroid Substrates**

![Diagram of Pyrethroid Substrates](image)

Pyrethroids (cypermethrin)

Chromophore (colored)

Substrates

\((X = O, S)\)
Two methods were adopted to prepare esters. First, thionyl chloride (0.1 mole, 8.7 ml) was added dropwise into a mixture of PA (0.025 mole, 5.25 g) and 100 ml benzene (60% in hexane) and then refluxed for 3 hrs at 50-60 °C. The solvent and excess thionyl chloride were removed from the reaction mixture and an oil product (PA-chloride) was obtained (Riddles, 1983). A mixture containing 50 ml benzene (60% in hexane) and p-nitrophenol (3.5 g, 0.025 mol) was prepared and refluxed for 4 hrs at 50-60°C. The reaction mixture was washed with saturated NaHCO₃ (20 ml x 3) and 50% ethanol-water (50 ml x 2), and then purified by column chromatography with hexane: ethyl acetate (70:30) as the developing phase to obtain 5.2 g p-NPPA (62.5% yield). A similar method was used to prepare thiophenyl acetate (TPA) and 1- or 2-naphthyl PA ester (1-NPA & 2-NPA). The yields of TPA, 1-NPA and 2-NPA were 72%, 79.7% and 80.5%, respectively.
The second method used was direct esterification between an acid and an alcohol under dehydrating condition (such as polyphosphate ester; Adams, 1979; Imamoto, 1982). A reaction mixture containing cis/trans-PA (0.01 mol), thiophenol (0.011 mol), polyphosphate ester (20 ml), chloroform (80 ml) and pyridine (10 drops) was stirred 12 hrs at room temperature. The final mixture was washed with saturated NaHCO$_3$ (2 X 50 ml), then extracted with chloroform (2 X 40 ml). The combined organic phase was dried (anhydrous Na$_2$SO$_4$) overnight. After removal of solvent, the solid product was purified by column chromatography with a mixture of hexane: ethyl acetate (70: 30) as the developing phase. The yield of cis-TTPA was 7.30 g (96.5 %).

Identity of products was determined by NMR and GC-MS (Table 2.1). $^1$H NMR spectra were obtained on a Brucker Ac 200 spectrometer using tetramethylsilane as the internal standard and CDCl$_3$ as solvent. Gas chromatography-mass spectrometry (GC-MS) was performed on a Finnigan GCQ (Trace GC 2000 coupled with Polaris MSD). A silica capillary MS column, DB-5MS (30 m $\times$ 0.25 mm $\times$ 0.25 $\mu$m; J &W Scientific, Folsom, CA), was operated at 60$^0$C for 1 min, increased to 150$^0$C (at 5 $^0$C/min increments), where it was held for 15 min at this temperature, then increased to 300$^0$C at 5$^0$C/min, where it was finally held for 10 min. The injector port was operated in splitless mode at 200$^0$C, and helium was used as carrier gas at 1.2 ml/min. The mass spectral detector (MSD) was set on full scan model (m/z 41-400). Purity was calculated by relative peak area.

2.2.5 Enzyme Preparation

Homogenates from individual larvae were used as enzyme source for measuring esterase activities towards all substrates. Individual larvae were dissected and the digestive system was evacuated. The remaining carcass was homogenized using an all-
Table 2.1. Identification and analysis of products by GC-MS and $^1$H NMR

<table>
<thead>
<tr>
<th>Substrates</th>
<th>purity</th>
<th>GC (Retention time, min)</th>
<th>MS m/z (Relative Intensity)</th>
<th>$^1$H NMR (CDCl$_3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-TPPA</td>
<td>98.60% (90.54 98.06% trans-TPPA)</td>
<td>18.19</td>
<td>127.03(100), 191.07 (88.71), 91.05(84.92), 63.02(65.93), 193.05(65.28), 165.01(40.4), 129.04(38.63), 241(10.93), 77(13.3).</td>
<td>δ7.39-7.45 S 5H(aromatic protons); δ6.18-6.27 q 1H (C=C-H); δ 2.21-2.29 q 2H(2 cyclopropane protons); 1.24-1.31 d 6H(2-CH3)</td>
</tr>
<tr>
<td>trans-TPPA</td>
<td>94% (no cis-TPPA)</td>
<td>18.36</td>
<td>127.04(100), 163.01(74.12), 91.06 (74), 191.06(71.85), 165.06(48.37), 193.03(45.06), 129.11 (32.5), 77(11.8)</td>
<td>d7.39-7.45 S 5H(aromatic protons); d5.63-5.77 d 1H (C=C-H); d 2.44-2.49 q 1H (C=C-C-H); d 2.02-2.04 d 1H (H-C-C=O); d1.24-1.27 d 6H(2’-CH3)</td>
</tr>
<tr>
<td>1-NPA</td>
<td>98.67% (cis 51.89%, trans 46.78%)</td>
<td>21.34 (cis) 21.52 (trans)</td>
<td>cis: 191.01(100), 127.04 (88.16), 193.01(68.57), 91.06(53.04), 115.05(41.69), 129.04(29.44), 225 (26.2) trans: 191.02(100), 127.05(89.12), 193.01(69.32), 91.06(58.66), 225(40.4), 115.09(31.06), 129(29.52)</td>
<td>Butte (1999)</td>
</tr>
<tr>
<td>2-NPA</td>
<td>92.21% (cis 40.29%, trans 51.92%)</td>
<td>21.85(cis) 22.03 (trans)</td>
<td>cis: 127.05(100), 191.02 (87.25), 91.05(68.68), 225.02(59.72), 193.03 (58.66), 144.1(57.3), 209 (47.1), 129.11(31.57) trans: 127.06(100), 191.03(89.41), 224.98(87.51), 193.08(77.05), 91.06(65.42), 209.01(65.26), 144.11(60.56), 129.05 (31.83)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table continued
<table>
<thead>
<tr>
<th></th>
<th>p-NPPA</th>
<th>TPA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>96.43%</td>
<td>99.6%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(cis 6.08%, trans 90.35%)</td>
<td>12.89</td>
<td>110.02(100), 43.01(32.66), 109.07(13.5), 66.02(12.37), 65.02(11.05)</td>
</tr>
<tr>
<td></td>
<td>10.07(cis)</td>
<td>127.11</td>
<td>110.02(100), 43.01(32.66), 109.07(13.5), 66.02(12.37), 65.02(11.05)</td>
</tr>
<tr>
<td>cis</td>
<td>187.08(100), 127.11 (53.75), 91.05(49.75), 225.03(39.87), 226.99(35.09), 189.06(33.44)</td>
<td>127.05(58.76), 91.06(40.5), 189.04(34.7), 163.02(25.94)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.19(trans)</td>
<td>226.99(35.09), 189.06(33.44)</td>
<td></td>
</tr>
<tr>
<td>trans</td>
<td>187.07(100), 127.05(58.76), 91.06(40.5), 189.04(34.7), 163.02(25.94)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND: not determined.
glass homogenizer with 500 μl of ice-cold 0.1M sodium phosphate buffer (pH 8.0). Homogenates were centrifuged at 12,600 g for 15 min at 4°C. The resulting supernatants were then held in ice and used in enzyme assays within 20 minutes of preparation.

### 2.2.6 Optimization of Biochemical Assays

Assays with all substrates were optimized (e.g., tissue, pH, temperature, substrate concentrations, and protein dependence) with cytosol from an existing, pyrethroid-resistant strain (CSA) of *H. virescens* (Shan et al., 1997). Individual larvae from the CSA strain were dissected and the digestive system was evacuated. A whole body was rinsed with 1.15% KCl and 0.1 M, pH 7.6 sodium phosphate buffer, then homogenized using an all-glass homogenizer with 0.1M pH 7.6 sodium phosphate buffer containing 1mM dithiothreitol, 1mM EDTA, 1mM phenylthiourea, and 4mM phenylmethylsufonyl fluoride (Rose, 1995). The homogenate was centrifuged at 15,000g for 15 min. The resulting supernatant was filtered through glass wool and further centrifuged at 115,000g for 1 hour. The supernatant was used as the enzyme resource for optimizing the conditions of biochemical assays. Activities of esterases toward all substrates (1-NA, 1-NPA, 2-NPA, *cis*-TTPA, *trans*-TTPA, SMTB, TPA and p-NPPA) were measured using the methods of Gomori (1953) and Ellman (1961) with modifications. The total volumes of each individual well of a microtiter plate were 250 μl for 1-NA, 1-NPA, 2-NPA and 300 μl for SMTB, TPA, *cis*-TTPA, *trans*-TTPA and p-NPPA. The rate of change in absorbency during the initial 10 min (or 1 min for TPA) was measured using a Thermomax Microtiter Reader (Molecular Devices, Palo Alto, CA). Data were corrected for non-enzymatic activity using incubations without protein as the control. Changes in optical density were converted to nmol/min\(^{-1}\cdot\text{mg prot}^{-1}\) using the following extinction
coefficients: 9.25 mM\(^{-1}\)250 µl\(^{-1}\) (Grant, 1989) for 1-NA, 1-NPA, 2-NPA; 8.34 mM\(^{-1}\)300 µl\(^{-1}\) for cis-TTPA, trans-TTPA, TPA and SMTB; and 11.4 mM\(^{-1}\)300 µl\(^{-1}\) for p-NPPA.

Native polyacrylamide gel electrophoresis (PAGE) was used to visualize esterases from three individuals for each strain using a vertical electrophoretic unit (Hoefer Scientific Instruments, San Francisco, CA) and 5% polyacrylamide (Sambrook et al., 1989). Protein concentrations in homogenates were adjusted to contain 75 µg protein in 30 µl of pH 7.0 sodium phosphate buffer containing 0.1% Triton X-100, which was mixed with 10 µl of a 6X tracking dye containing 0.25% bromophenol blue and 40% sucrose in double-distilled water. Electrophoresis occurred in an electrode buffer consisting of 100 mM Tris, 2.4 mM EDTA, and 100 mM boric acid (pH 8.0) at constant voltage (150 V). When the dye marker migrated to within 1 cm of the gel base, each gel was removed and stained in darkness at 25°C with 100 ml of 0.1 M, pH 7.0 sodium phosphate buffer containing 0.5 mM 1-NA and 2-NA, and 0.2% fast blue B for 30 min at room temperature. Gels were de-stained in water and fixed in 5% acetic acid if necessary. Protein concentrations were measured from diluted homogenates by the method of Bradford (1976) using bovine serum albumin as the standard.

### 2.2.7 Statistical Analysis

Data from biochemical assays were analyzed using ANOVA – Tukey’s HSD test (P ≤0.01).

### 2.3 Results

#### 2.3.1 Insecticide Susceptibility

Moderate levels of resistance to profenofos and cypermethrin were expressed in fifth instars from the OP-R and PYR-R strains (Table 2.2). Resistance was greatest against the insecticides to which larvae were selected: RRs of 18.1 to profenofos and 19.6
to cypermethrin were measured in OP-R and PYR-R strains, respectively. Moreover, profenofos selection conferred cross-resistance (12.9-fold) to cypermethrin in the OP-R strain. In contrast, cypermethrin selection conferred relatively low levels (4.53-fold) of cross-resistance to profenofos in the PYR-R strain.

2.3.2 Optimization of Assay Conditions

Optimal conditions for enzymatic assays differed depending on the substrate used. First, differences were measured in optimal pH among the substrates (Table 2.3). Activities with all substrates except p-NPPA were greater in phosphate than in Tris/HCl buffers, and optimal pHs differed. In contrast, highest activities with p-NPPA were measured with Tris/HCl buffer at pH 8.6. Moreover, optimal temperatures for activities also varied with substrates, and optimal amounts of proteins for assays (expressed as the maximal value that was within linear range of activities for reactions) also varied among substrates (Table 2.3).

2.3.3 Esterase Activities toward Non-pyrethroid Substrates

Esterase activities toward non-pyrethroid substrates were generally similar between insecticide-resistant OP-R and PYR-R strains, but were significantly greater (Tukey’s HSD; P \leq 0.01) than that in insecticide-susceptible LSU-S larvae (Table 2.4). Differences were not dramatic, and range from 1.1- and 1.2- fold (for 1-NA) to 1.9- and 2.2- fold (for SMTB) in the OP-R and PYR-R strains, respectively.

Both quantitative and qualitative differences in banding patterns among LSU-S, PYR-R and OP-R larvae were observed in polyacrylamide gels stained with 1- and 2-NA (Fig. 2.1). The staining of some bands (e.g., bands 1 and 6, Rm: 0.25 and 0.83, respectively) in both resistant strains was more intense than those in the susceptible strain. Moreover,
Table 2.2 Susceptibility of larvae to profenofos and cypermethrin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Insecticide</th>
<th>LD$_{50}$ (95% FL)</th>
<th>Slope (± SD)</th>
<th>Chi-square</th>
<th>RR$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU-S</td>
<td>Profenofos</td>
<td>2.90 (2.40-3.56)</td>
<td>2.63 (0.40)</td>
<td>1.38</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Cypermethrin</td>
<td>0.19 (0.16-0.24)</td>
<td>2.81 (0.39)</td>
<td>2.90</td>
<td>1.00</td>
</tr>
<tr>
<td>OP-R</td>
<td>Profenofos</td>
<td>52.3 (46.0-59.2)</td>
<td>3.88 (0.57)</td>
<td>1.76</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>Cypermethrin</td>
<td>2.46 (1.86-3.43)</td>
<td>6.49 (0.88)</td>
<td>1.06</td>
<td>12.9</td>
</tr>
<tr>
<td>PYR-R</td>
<td>Profenofos</td>
<td>13.2 (10.4-16.2)</td>
<td>2.49 (0.39)</td>
<td>2.17</td>
<td>4.53</td>
</tr>
<tr>
<td></td>
<td>Cypermethrin</td>
<td>3.75 (3.16-4.33)</td>
<td>3.34 (0.48)</td>
<td>1.56</td>
<td>19.6</td>
</tr>
</tbody>
</table>

$^a$Strains—LSU-S: insecticide-susceptible; OP-R: profenofos–selected; PYR-R: cypermethrin-selected.

$^b$RR= resistance ratio= LD$_{50}$ (resistant strain/susceptible strain)
Table 2.3 Optimal conditions for continuous spectrophotometric assays

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final conc. (mM)</th>
<th>Protein (µg/assay)</th>
<th>Buffer /pH</th>
<th>Temp. (°C)</th>
<th>Wavelength (nm)</th>
<th>Peak</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-NA</td>
<td>2.15</td>
<td>60</td>
<td>Phosphate / 7.0</td>
<td>25</td>
<td>450</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>1-NPA &amp; 2-NPA</td>
<td>0.67</td>
<td>60</td>
<td>Phosphate / 7.0</td>
<td>25</td>
<td>435</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>SMTB</td>
<td>2.00</td>
<td>30</td>
<td>Phosphate / 7.6</td>
<td>29</td>
<td>435</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>TPA</td>
<td>1.33</td>
<td>30</td>
<td>Phosphate / 8.0</td>
<td>28</td>
<td>414</td>
<td>405</td>
<td>405</td>
</tr>
<tr>
<td>p-NPPA</td>
<td>0.17</td>
<td>360</td>
<td>Tris-HCl / 8.6</td>
<td>27</td>
<td>412</td>
<td>405</td>
<td>405</td>
</tr>
<tr>
<td>cis/trans-TPPA</td>
<td>1.33</td>
<td>120</td>
<td>Phosphate / 7.6</td>
<td>31</td>
<td>412</td>
<td>405</td>
<td>405</td>
</tr>
</tbody>
</table>
some bands (e.g., bands 2 and 3, Rm: 0.37 and 0.50, respectively) were present in the OP-R strain, but not in the LSU-S or PYR-R strains. In contrast, band 4 (Rm: 0.68) was present in both LSU-S and PYR-R strains, but was not apparent in OP-R larvae. Finally, band 5 (Rm: 0.73) was visible in LSU-S and OP-R strains, but not in larvae from the PYR-R strain.

2.3.4 Esterase Activities toward Pyrethroid Substrates

As with non-pyrethroid substrates, esterase activities toward pyrethroid esters were significantly greater (Tukey’s HSD; P ≤ 0.01) in insecticide-resistant strains (OP-R and PYR-R) than those in insecticide-susceptible strain (LSU-S) (Table 2.4). Differences between resistant and susceptible strains were greater than those measured with non-pyrethroid esters, and ranged from 3.1- and 2.8- fold (for 2-NPA) to 8.4- and 2.7- fold (for cis-TTPA) in the PYR-R and OP-R strains, respectively. Moreover, esterase activities toward some pyrethroid substrates (e.g., 1-NPA, cis-TTPA and trans-TTPA) were significantly greater in PYR-R strain than those in OP-R strain (Table 2.4), and differences ranged from 1.3- fold for 1-NPA to 3.4- fold for cis-TTPA in PYR-R compared with OP-R larvae. In contrast, esterase activities toward other pyrethroid substrates (e.g., 2-NPA, p-NPPA) were similar between PYR-R and OP-R strains (Table 2.4).

2.4 Discussion

Enhanced levels of esterase activities are associated with insecticide resistance in the tobacco budworm, *H. virescens* (McCaffery, 1998). In previous studies with non-pyrethroid substrates (especially 1-NA), increased esterase activities were measured in larvae that are resistant to OPs such as methyl parathion, profenofos and azinphosmethyl
Table 2.4 Esterase activities (nmol min\(^{-1}\) mg prot\(^{-1}\)) toward pyrethroid and non-pyrethroid esters \(^a\)

<table>
<thead>
<tr>
<th>Esters</th>
<th>Strain(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSU-S</td>
</tr>
<tr>
<td><strong>Non-pyrethroid</strong></td>
<td></td>
</tr>
<tr>
<td>1-NA</td>
<td>73.5(9.51)(^B)</td>
</tr>
<tr>
<td>TPA</td>
<td>345(79.5)(^B)</td>
</tr>
<tr>
<td>SMTB</td>
<td>98.5(15.5)(^C)</td>
</tr>
<tr>
<td><strong>Pyrethroid</strong></td>
<td></td>
</tr>
<tr>
<td>1-NPA</td>
<td>16.6(2.19)(^C)</td>
</tr>
<tr>
<td>2-NPA</td>
<td>12.6(3.24)(^B)</td>
</tr>
<tr>
<td>cis-TTPA</td>
<td>0.82(1.62)(^C)</td>
</tr>
<tr>
<td>trans-TTPA</td>
<td>1.43(1.64)(^C)</td>
</tr>
<tr>
<td>p-NPPA</td>
<td>0.42(0.36)(^B)</td>
</tr>
</tbody>
</table>

\(^a\) Activities (nmol min\(^{-1}\) mg prot\(^{-1}\)) are expressed as means (± SD) based on assays with 30 larvae from each strain. In tests with LSU-S larvae, no activities were detectable in 5, 4, and 1 individual with cis-TTPA, trans-TTPA and p-NPPA, respectively. Values within rows followed by different letters are significantly different (ANOVA – Tukey’s HSD test, P ≤ 0.01).

\(^b\) Strain: LSU-S: insecticide susceptible; OP-R: profenofos–selected; PYR-R: cypermethrin-selected.
Figure 2.1 Polyacrylamide gels of esterase activities toward 1- and 2-naphthyl acetate in individual larvae from susceptible (LSU-S), profenofos-selected (OP-R) and cypermethrin-selected (PYR-R) strains of *H. virescens*. Each lane contains 75 µg of protein from whole body homogenates of individual fifth stadium (day 1) larvae.
(Goh et al., 1995; Zhao et al., 1996; Harold and Ottea, 2000). Moreover, Goh et al. (1995) concluded that substantially increased esterase activities toward 1-NA may be involved in resistance in a thiodicarb-resistant strain. Enhanced esterase activities also confer pyrethroid resistance to *H. virescens* (Dowd et al., 1987; Graves et al., 1991; Goh et al., 1995; Shan and Ottea, 1997). In the present study, further evidence is presented that esterases are associated, at least in part, with resistance and cross-resistance in both OP-R and PYR-R strains.

Detecting and monitoring insecticide resistance and associated mechanisms in insect populations is possible using biochemical assays with model substrates (Brown and Brogdon, 1987; Devonshire, 1987). Model substrates have been widely used in studies to evaluate whether enhanced esterase activities play a critical role on insecticide resistance. In previous studies, esterase activities toward 1-NA have been associated with insecticide resistance in several insects (Abdel et al., 1993). High frequencies of profenofos resistance were detected in larvae of *H. virescens* from 11 field strains collected in Louisiana during 1995, and there was a strong correlation between profenofos resistance ratios and esterase activities toward 1-NA (Harold and Ottea, 1997). Similarly, activity toward S-methyl thiobutanoate (SMTB) is a good indicator of esterases associated with malathion resistance (Kao et al., 1985), and TPA has been shown to have a similar specificity for esterases as 1-NA (Chambers, 1973).

Staining electrophoretic gels with model substrates (e.g., 1- & 2-NA) can be used to visualize quantitative and qualitative differences in esterases associated with resistance. In a previous study, Harold and Ottea (1997) demonstrated that one esterase was consistently over-expressed and a second band under-expressed in OP-resistant *H. virescens* from both laboratory-selected and field-collected strains. In the present study
using a different OP-resistant strain, under-expression of a band migrating to the same position (Rm = 0.68, Fig. 2.1) was detected and provides further evidence that resistance to profenofos was accompanied by under-expression of this esterase. However, in the present study, two bands (Rm = 0.37 and 0.5, Fig. 2.1) were also over-expressed in the OP-resistant strain. This difference between these two studies may result from the involvement of different esterases in resistance, or from different selection regimes (e.g., insecticide used and selection pressure) used to generate resistant strains.

The pattern of esterase banding was simpler in pyrethroid-resistant (PYR-R) than OP-resistant (OP-R) larvae. Because both strains originated from the same parent colony (i.e., LSU-S), it is likely that these differences in banding patterns are associated with structural differences between profenofos and cypermethrin. Whereas both under-expression (band 5, Rm = 0.73, Fig. 2.1) and over-expression (band 1, Rm = 0.25, Fig. 2.1) of esterases were observed in the PYR-R strain compared with the LSU-S strain, differences between these strains in intensity of staining were not dramatic.

Results from biochemical assays using model substrates to determine causal relationship between enzyme activities and insecticide resistance may be equivocal and must be interpreted carefully. Isozymes that metabolize model substrates may not necessarily be those that detoxify insecticides (McCaffery, 1998). The “mutant ali-esterase” hypothesis originated from the observation that esterase activities toward 1-NA actually decreased (rather than increased) with malathion resistance in some resistant strains of *M. domestica* and other insects (Oppenoorth and van Asperen, 1960; Townsend et al., 1969; Beeman et al., 1982; Hughes et al., 1985; Campbell et al., 1998). Similarly, the model substrate, 1-NA, is a poor indicator of resistance-associated esterases from pyrethroid-resistant *H. virescens* (Dowd et al., 1987; Ottea et al., 2000).
In the present study, esterase activities toward non-pyrethroid substrates including 1-NA, SMTB and TPA were variable. Activities toward non-pyrethroid substrates did not appear to be associated with either OP or pyrethroid resistance because activities were not dramatically different (ranging from 1.1- and 1.2-fold for 1-NA to 1.9- and 2.2-fold for SMTB, Table 2.4) in resistant PYR-R and OP-R strains compared with susceptible LSU-S larvae. Moreover, banding patterns of esterases stained with 1- and 2-NA were very similar between the PYR-R and LSU-S strains. In addition, the trend in esterase activities toward non-pyrethroid substrates was dissimilar to that measured in bioassays with cypermethrin. In contrast, activities toward pyrethroid substrates appeared to be associated with pyrethroid (but not OP) resistance because activities were dramatically different (ranging from 3.1-fold for 2-NPA to 8.4-fold for cis-TTPA, Table 2.4) in the PYR-R strains compared with LSU-S strain. Moreover, the pattern of esterase activities toward these substrates (except for 2-NPA and p-NPPA) was very similar to that measured in bioassays with cypermethrin, but not with profenofos. Thus, esterases associated with pyrethroid resistance may be more easily detected by pyrethroid substrates than non-pyrethroid substrates. However, whether esterases hydrolyzing pyrethroid substrates are those hydrolyzing cypermethrin is still questionable. In previous reports, carboxylesterases from both mammals and insects have been reported to prefer a pyrethroid with a primary alcohol ester (e.g., permethrin) to a pyrethroid with a secondary alcohol ester (e.g., cypermethrin) (Abernathy et al., 1973; Holden, 1979). However, Jersey et al. (1985) found that one esterase in the cattle tick, *Boophilus microplus*, hydrolyzed α-cyano-substituted pyrethroid, trans-cypermethrin, but not trans-permethrin.
Hydrolysis of geometric isomers of pyrethroid-similar esters strongly depends on the sources or three-dimensional structures of esterases. More rapid hydrolysis of trans-isomers compared with cis-isomers may be due to less steric hindrance by the dichlorovinyl group of attack on the carbonyl carbon atom (Riddles, 1983). In previous studies, pyrethroid esterases from pyrethroid-resistant *Amblyseius fallacis* (Chang et al., 1986), *Trichoplusia ni* (Ishaaya and Casida, 1980) and *Spodoptera littoralis* (Ishaaya et al., 1983) hydrolyzed trans-permethrin faster than cis-permethrin. In the present study, trans-TTPA also can be more easily hydrolyzed by esterases than cis-TTPA. However, hydrolysis of pyrethroid substrates cannot be explained only by chemical stability. For example, the ratio of hydrolysis of trans-TTPA over cis-TTPA in the PYR-R strain was 1.54 (trans-TTPA and cis-TTPA: 10.59 and 6.88 nmol.min\(^{-1}\).mg prot\(^{-1}\), respectively, Table 2.4). However, the ratio in the OP-R strain was 3.25 (trans-TTPA and cis-TTPA: 6.57 and 2.02 nmol.min\(^{-1}\).mg prot\(^{-1}\), respectively, Table 2.4). Similarly, isomer specificity of pyrethroid esterases for trans- and cis-permethrin in *Amblyseius fallacies* was observed in pyrethroid-resistant strains, but not in susceptible insects (Chang et al., 1986). Conversely, esterases, from resistant rice green leafhoppers, *Nephotettix cincticeps* (Chang et al., 1996), green lacewings, *Chrysopa carnea* (Ishaaya and Casida, 1981; Ishaaya, 1993) and the rice brown planthopper, *Nilaparvata lugens* (Chen and Sun, 1994), can degrade cis-isomers of permethrin and/or cypermethrin, faster (from 2- to 5-fold) than the corresponding trans-isomers. These studies may imply that the sources of esterases or three-dimensional structures of esterases play a primary role in the hydrolysis of geometric isomers of pyrethroid esters, and the chemical stability of pyrethroid esters may not be the major factor affecting enzymatic hydrolysis.
In summary, enhanced levels of esterase activities may play an important role in both profenofos- and cypermethrin-resistant strains. Moreover, pyrethroid substrates appear to be better indicators of esterases associated with pyrethroid resistance or cross-resistance to pyrethroid insecticides than non-pyrethroid esters. However, whether esterases detoxifying cypermethrin are those hydrolyzing pyrethroid substrates is still not clear.

2.5 References


3.1 Introduction

Insecticide resistance is a major impediment to the effective management of field populations of the tobacco budworm, *Heliothis virescens* (Sparks, 1981; Sparks et al., 1993). Resistance may result from reduced sensitivity of an insecticide’s target site to the action of an insecticide, enhanced enzymatic detoxication of the insecticide or reduced rates of penetration (Plapp, 1976; Oppenoorth, 1985). Expression of all three of these mechanisms has been reported in *H. virescens* (Sparks et al., 1993).

The ability to discriminate among these three mechanisms is essential for development and effective implementation of insecticide resistance management strategies. Such knowledge would allow growers to make timely and rational decisions about what to do once resistance has developed in their fields. For example, when resistance is due to reduced sensitivity of a target site, classes of insecticides that act at different target sites must be used as a countermeasure. Further, if resistance is due to enhanced metabolism (and the enzymes responsible have been identified), synergists may be used. At present, there are a limited number of techniques available to discriminate insecticide resistance mechanisms. However, most methodologies are designed for use in the laboratory and their stringent experimental conditions and need for instrumentation make them difficult to utilize in field situations.

Biological assays have potential to be used as diagnostic assays in field situations. They are cheap and there are no special requirements for equipment. Thus, they are suitable for use by growers and consultants. Bioassays with esterase inhibitors have been
widely used in laboratory settings to detect whether enhanced esterase activities are associated with insecticide resistance. However, the results of such assays are often equivocal. The synergist, $S, S, S$-tributyl phosphorotrithioate (DEF), is extensively used as an inhibitor of esterases to detect involvement of these enzymes in resistance, but DEF also inhibits both glutathione S-transferases (Armes et al., 1997) and NADPH-dependent monooxygenases (Sanchez et al., 2001). Thus, the presence of synergism (i.e., significantly increased toxicity) with esterase inhibitors in bioassays may not be an accurate indication that esterases are associated with insecticide resistance. In addition, a single inhibitor/synergist may not block the activity of all toxicologically-relevant (i.e., resistance-associated) enzymes; thus, the absence of synergism in assays with a single compound might not eliminate involvement of esterases in resistance, because a resistant-associated esterase may be present but not be inhibited by the synergist used (ffrench-Constant and Roush, 1990; Brown et al., 1996). Finally, non-metabolic effects (e.g., reduced penetration) of synergists on insecticide toxicity may be present (Bull and Patterson, 1993; Sanchez et al., 2001).

Improving the precision with which an esterase-based resistance mechanism is detected with bioassays is important and necessary. Bioassays with an insecticide that is bioactivated (i.e., toxicity increased) have potential to detect esterases associated with insecticide resistance and offer advantages over bioassays using an insecticide plus a synergist. For example, the esterase-based metabolites of some insecticides (e.g., acephate and indoxacarb) are more toxic than the parent compounds, and the rate of insecticide bioactivation by esterases may dramatically influence toxicity (Mahajna et al., 1997; Wing et al., 2000). A testable hypothesis is that if these insecticides were activated
faster than they are inactivated, negative cross-resistance to these insecticides would be evident in insects expressing resistance-associated esterases. A second class of chemicals with potential use as diagnostic compounds is insecticides in which metabolically-labile sites for non-hydrolytic enzymes (e.g., oxidases) are blocked. For example, both trans- and cis-isomers of 1 (R)- fenfluthrin suppressed resistance to cypermethrin in a cypermethrin-resistant strain of *H. virescens* because the major target sites for metabolism by cytochrome P450 monoxygenases (i.e., ring hydroxylation) in fenfluthrin were blocked with halogens (Shan et al., 1997). A second, structurally-similar pyrethroid is tefluthrin, which has a phenyl ring substituted with four fluorines and a methyl group. Finally, bioassays with non-toxic pyrethroids acting as inhibitors or competitive substrates of esterases associated with pyrethroid resistance have potential use in diagnostic assays.

In addition to diagnostic bioassays, biochemical assays on solid materials may be used in field situations to detect esterase-mediated resistance in early stages of development of *H. virescens*. Simple colorimetric enzyme assays blotted onto a paper matrix (e.g., filter paper or “squash” assays) have been developed to detect enhanced esterase activities toward 1-naphthyl acetate (1-NA; Ozaki, 1969; Pasteur and Georghiou, 1980, 1981, 1989), and can be used in cases where resistance is positively (or negatively) correlated with esterase activities toward a non-insecticide substrate. Thus, these methods have potential to provide an efficient and rapid means to determine the frequency of resistant individuals in field populations of insects. Using such a filter paper assay, differences in esterase activities toward 1-NA between susceptible and resistant *H. virescens* were observed (Harold and Ottea, 2000). In addition, such assays have been used in other

It is essential to improve the precision and expand the applications of these simple and rapid field assays. To date, these assays have been only used with organophosphate-resistant insects and the surrogate (i.e., non-insecticide) substrate used has been limited to 1-NA. However, 1-NA is a poor indicator of resistance-associated esterases from pyrethroid-resistant *H. virescens* (Dowd et al., 1987; Ottea et al., 2000).

The present study has shown that, compared with non-pyrethroid substrates, pyrethroid esters are better indicators of esterases associated with pyrethroid-resistance in *H. virescens*. The major goals of Chapter 3 are to: 1) develop a filter paper assay (“squash assay”) with pyrethroid substrates to detect esterases associated with pyrethroid resistance; and 2) determine whether bioactivated insecticides (acephate and indoxacarb) and novel pyrethroid esters can be used as diagnostic compounds for esterases associated with insecticide resistance in *H. virescens*.

### 3.2 Materials and Methods

#### 3.2.1 Chemicals

Fast blue B salt (90% purity), 1-naphthyl acetate (98% purity), benzenethiol (97% purity), 1-naphthol (99% purity), 2-naphthol (99% purity), 5,5’-dithio(2-nitrobenzoic acid) (DTNB, 99% purity) and benzenethiol (97% purity) were obtained from Aldrich Chemical Co. (Milwaukee, WI).
Pyrethroid esters tested as synergists included: [1/2-naphthyl, 3–(2, 2–dichlorovinyl)–2, 2 – dimethylcyclopropane carboxylate (1/2-NPA), p-nitrophenyl, 3–(2, 2–dichlorovinyl)–2, 2 – dimethylcyclopropane carboxylate (p-NPPA), cis/trans - thiophenyl, 3–(2, 2–dichlorovinyl)–2, 2 – dimethylcyclopropane carboxylate (cis/trans-TPPA)], and were synthesized as described in Chapter 2. In addition, S, S, S-tributyl phosphorotrichioate (DEF; 98.7% from Bayer Corporation, Kansas City, MO), piperonyl butoxide (PBO; 90-95% from Fluka Chemical Corporation, Milwaukee, WI) and 2, 3, 6-trichloro-3 (2-propyloxy) benzene (TCPB; > 95% purity (synthesized by Shan, 1997) were also tested as synergists.

3.2.2 Insects

A susceptible strain of *H. virescens* (LSU-S) was established in 1977 (Leonard et al., 1988) and has been reared in the laboratory since that time without exposure to insecticides. All larvae for resistance selection were fifth instars. Larvae from the LSU-S strain were selected for five consecutive generations with topical applications of profenofos at doses corresponding to the LD_{60} for each generation (3.5, 4.6, 8.5, 13.6 and 23.8 µg/individual). After one year without selection, individual larvae were treated with 25 µg profenofos (an approximate LD_{20}), and the survivors (OP-R strain) were used for biological assays.

Similarly, larvae from the LSU-S strain were selected for four consecutive generations with topical applications of cypermethrin at doses corresponding to the LD_{60} (0.22, 0.4, 0.5 and 0.6 µg/individual). After one generation without selection, larvae were treated with 1 µg of cypermethrin (6% mortality at 72 hours), and the next generation (PYR-R) was used for biochemical and biological assays.
3.2.3 Bioassays

Fifth instars (day 1), weighing 180±20 mg, were treated on the mid-thoracic dorsum with 1 µl aliquots of either profenofos or cypermethrin (in acetone) or with acetone alone (control). The dose-mortality relationship for each compound was assessed from at least five doses with 30 insects treated per dose. After treatment, larvae were maintained at 27°C, and mortality was recorded after 72 hours. The criterion for mortality was the lack of coordinated movement within 30 seconds after being prodded. Results were corrected for control mortality with Abbott’s formula (Abbott, 1925), then analyzed by Finney’s method (Finney, 1971). Resistance ratios (RR) were calculated as LD50 of resistant strain / LD50 of susceptible strain.

Synergism of cypermethrin or profenofos toxicity was evaluated with LSU-S, OP-R and PYR-R larvae. Compounds were applied to the mid-abdominal dorsum 30 min prior to application of insecticide. Larvae were treated with 1µl acetone or synergist alone as control. Doses of pyrethroid esters (1/2-NPA, cis/trans-TTPA, and p-NPPA) were optimized for maximal mortality with fifth-stadium LSU-S larvae by topical application of different concentrations (0-70 µg/larva) of synergists followed by cypermethrin (0.15 µg/larva). All pyrethroid esters administered were non-toxic to LSU-S larvae at 70 µg/larva. The dose used for tests with PBO, DEF and TCPB was 50 µg/larva (Shan et al., 1997). Synergism ratio (SR) was calculated as: LD50 (without a synergist)/LD50 (with a synergist). Synergism was indicated when SR > 1 and antagonism when SR < 1.

3.2.4 Filter Paper Assays

A “squash assay” was performed on filter paper based on earlier methods (Pasteur and Georgiou, 1981; Harold and Ottea, 2000) with modifications. Second stadium larvae
(approximately 10-25 mg) were individually squashed through a micro-centrifuge tube (cut open at the distal end and washed with double-distilled water) using a spatula tip capped with a 0.5 ml micro-centrifuge tube on a Whatman #3 filter paper moistened with phosphate buffer. The pH values of phosphate buffer used were 7.0 for 1-NA and 1- or 2-NPA, or 7.6 for cis or trans-TTPA. The filter paper containing a homogenized larva was incubated atop a second filter paper that was soaked for 1 minute in a substrate solution that was identical to that used for biochemical assays (see Chapter 2). Incubation time for filters varied: 1 min for 1-NA and cis-TTPA; 30 sec for trans-TTPA and 2 min for 1-/ 2-NPPA. Esterase activities were visualized as color production on the undersurface of the filter paper, which were compared with a color scale prepared with 1- or 2- naphthol (0-0.08 µmoles), or thiophenol (0-0.9 µmoles). Photographs were taken immediately following reactions.

3.3 Results

3.3.1 Dose-response Relationship of Pyrethroid Esters

Generally, synergism of cypermethrin toxicity by pyrethroid esters was quantitatively similar: toxicity increased, then decreased with increasing dose of synergists (Figure 3.1). Moreover, maximal levels of mortality measured (50%-63%) were also similar among compounds. In addition, responses measured between geometric isomers (cis- and trans-TTPA) or stereoisomers (1- and 2-NPA) were also similar. However, optimal doses of synergists were different and ranged from 20 µg/larva (for 1- and 2-NPA) to 50 µg/larva (for p-NPPA).
Figure 3.1 Mortality measured after sequential topical application of various doses of synergists followed by 0.15 µg cypermethrin onto fifth stadium (day 1) larvae of *H. virescens* from the LSU-S strain.
3.3.2 Synergism by Non-pyrethroid Compounds

Effects of non-pyrethroid compounds on insecticide toxicity varied depending on the insect strain and insecticide used for assays (Table 3.1). First, DEF antagonized profenofos toxicity in the LSU-S strain, but had no significant effect on profenofos toxicity in the OP-R strain, or cypermethrin toxicity in the LSU-S or PYR-R strain. Moreover, in tests with LSU-S larvae, PBO synergized cypermethrin toxicity, but was an antagonist of profenofos toxicity. No effect of PBO was observed in either the OP-R or PYR-R strains. In contrast, TCPB was the most potent synergist of profenofos toxicity in the LSU-S and OP-R strains, and significantly increased cypermethrin toxicity in the PYR-R larvae, but no significant effect was observed on cypermethrin toxicity in the LSU-S strain.

3.3.3 Synergism by Pyrethroid Compounds

All pyrethroids esters significantly increased cypermethrin toxicity in both OP-R and PYR-R strains (Table 3.1). In the OP-R strain, SRs were similar among the five pyrethroid esters, and ranged from 2.93 (for 1-NPA) to 3.67 (for cis-TTPA). Similarly, in tests with PYR-R larvae, SRs for the five pyrethroid esters ranged from 1.80 (for 2-NPA) to 3.34 (for p-NPPA).

3.3.4 Insecticides as Diagnostic Compounds

Susceptibility to indoxacarb, acephate, tefluthrin and trans-fenfluthrin was significantly lower in larvae from the OP-R strain than that in the LSU-S larvae (Table 3.2). Resistant was highest to indoxacarb (15.8-fold) but relatively low levels were also expressed toward acephate (2.76-fold), tefluthrin (3.30-fold) and trans-fenfluthrin (2.59-fold). Similarly, susceptibility to these compounds was also lower in the PYR-R larvae.
Table 3.1 Effects of compounds on profenofos and cypermethrin toxicity in fifth-stadium larvae from susceptible (LSU-S) and resistant (OP-R and PYR-R) strains of *H. virescens*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment b(µg)</th>
<th>LD50 (95% fiducial limit) c</th>
<th>SR d</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU-S</td>
<td>cypermethrin</td>
<td>0.19 (0.16-0.24)</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + PBO (50)</td>
<td>0.09 (0.08-0.10)*</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + DEF (50)</td>
<td>0.22 (0.16-0.38)</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + TCPB (50)</td>
<td>0.12 (0.08-0.18)</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>profenofos</td>
<td>2.90 (2.40-3.60)</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>profenofos + PBO (50)</td>
<td>7.24 (5.71-10.4)‡</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>profenofos + DEF (50)</td>
<td>8.02 (6.52-11.2)‡</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>profenofos + TCPB (50)</td>
<td>1.63 (1.23-1.97)*</td>
<td>1.78</td>
</tr>
<tr>
<td>OP-R</td>
<td>cypermethrin</td>
<td>2.46 (1.86-3.43)</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + 1-NPA(20)</td>
<td>0.84 (0.55-1.26)*</td>
<td>2.93</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + 2-NPA(20)</td>
<td>0.68 (0.56-0.81)*</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + <em>cis</em>-TTPA(30)</td>
<td>0.67 (0.56-0.78)*</td>
<td>3.67</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + <em>trans</em>-TTPA (40)</td>
<td>0.70 (0.42-1.05)*</td>
<td>3.51</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + p-NPPA(50)</td>
<td>0.79 (0.53-1.14)*</td>
<td>3.11</td>
</tr>
<tr>
<td></td>
<td>profenofos</td>
<td>52.3 (46.0-59.2)</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>profenofos + PBO (50)</td>
<td>67.7 (58.8-79.8)</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>profenofos + DEF (50)</td>
<td>59.5(42.5-91.2)</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>profenofos + TCPB (50)</td>
<td>8.35 (6.29-10.3)*</td>
<td>6.26</td>
</tr>
<tr>
<td>PYR-R</td>
<td>cypermethrin</td>
<td>3.75 (3.16-4.33)</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + 1-NPA(20)</td>
<td>1.84 (1.27-3.00)*</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + 2-NPA(20)</td>
<td>2.08 (1.80-2.48)*</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + <em>cis</em>-TTPA(30)</td>
<td>1.75 (1.32-2.69)*</td>
<td>2.14</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + <em>trans</em>-TTPA (40)</td>
<td>1.42 (1.17-1.76)*</td>
<td>2.64</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + p-NPPA(50)</td>
<td>1.11 (0.91-1.36)*</td>
<td>3.34</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + PBO (50)</td>
<td>3.28 (2.55-4.20)</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + DEF (50)</td>
<td>4.81 (3.84-6.07)</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>cypermethrin + TCPB (50)</td>
<td>1.74 (1.43-2.13)*</td>
<td>2.16</td>
</tr>
</tbody>
</table>

aStrain-----LSU-S: insecticide-susceptible; OP-R: profenofos–selected; PYR-R: cypermethrin-selected
bTreatment: candidate synergists: **1-NPA**: 1-naphthyl, 3–(2, 2–dichlorovinyl)–2, 2–dimethylcyclo- propane carboxylate; **2-NPA**: 1-naphthyl, 3–(2,2–dichlorovinyl)–2, 2–dimethylcyclopropane carboxylate; **p-NPPA**: p-nitrophenyl, 3–(2, 2–dichloro- vinyl)–2, 2–dimethylcyclopropane carboxylate; **cis/trans-TTPA**: *cis/trans-*thiophenyl, 3–(2, 2–

table continued
dichlorovinyl–2, 2–dimethylcyclo-propane carboxylate; **DEF**: S, S, S-tributyl phosphorotrithioate; **PBO**: piperonyl butoxide; **TCPB**: 2, 3, 6-trichloro-3 (2-propynyloxy) benzene.

<sup>c</sup>(95% fiducial limit): ‡ antagonism, * synergism

<sup>d</sup>SR =LD<sub>50</sub> (without a synergist)/LD<sub>50</sub> (with a synergist)
and resistance was greatest toward indoxacarb (5.57-fold), but was also expressed toward tefluthrin (2.18-fold) and trans-fenfluthrin (2.91-fold). However, the difference in LD_{50}s for acephate between LSU-S and PYR-R larvae was not statistically significant.

### 3.3.5 Filter Paper Assays

Differences in color intensity for each product used in filter paper assays were observed within and between the susceptible (LSU-S) and resistant (PYR-R and OP-R) strains, but differences were not dramatic (Figures 3.2-3.7). For all substrates, color production was more intense in tests with OP-R larvae than with LSU-S larvae. However, no qualitative differences could be observed between PYR-R and LSU-S larvae.

### 3.4 Discussion

Simple and rapid field assays to monitor and detect resistance and resistance mechanisms in early stages of development of *H. virescens* are essential for providing timely and accurate information for effective implementation of resistance countermeasures. Insecticide bioassays have been successfully developed and used for monitoring insecticide resistance in populations of *H. virescens* in cotton (McCutchen et al., 1989; Ernst and Dittrich, 1992; Kanga and Plapp, 1995). For example, a glass vial technique has been developed to monitor pyrethroid resistance in larval stages of *H. virescens* (McCutchen et al., 1989), and this technique was further developed to monitor resistance to biodegradable insecticides (e.g., organophosphates and carbamates) (Daly and Fitt, 1990; Kanga and Plapp, 1995; Kanga et al., 1995). This technique not only provides timely and accurate data for the stage when *H. virescens* is an actual pest, but also provides consultants with a predictive bioassay that is useful in detecting resistance.
Table 3.2 Susceptibility of larvae from the susceptible (LSU-S) and resistant (OP-R and PYR-R) strains of *H. virescens*

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Insecticide</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (95% FL&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>Slope (SD&lt;sup&gt;c&lt;/sup&gt;)</th>
<th>Chi-square</th>
<th>RR&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU-S</td>
<td>indoxacarb</td>
<td>0.27 (0.22-0.33)</td>
<td>4.46 (0.64)</td>
<td>2.05</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>acephate</td>
<td>25.8 (22.3-29.4)</td>
<td>4.26 (0.61)</td>
<td>1.65</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>trans</em>-fenfuthrin</td>
<td>1.64 (1.22-2.12)</td>
<td>2.99 (0.40)</td>
<td>3.73</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>tefluthrin</td>
<td>0.51 (0.42-0.69)</td>
<td>2.65 (0.52)</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>OP-R</td>
<td>indoxacarb</td>
<td>4.29 (3.18-5.87)*</td>
<td>1.62 (0.23)</td>
<td>1.11</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>acephate</td>
<td>69.1 (55.0-86.8)*</td>
<td>3.64 (0.53)</td>
<td>4.76</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td><em>trans</em>-fenfuthrin</td>
<td>4.30 (3.70-4.88)*</td>
<td>4.94 (0.64)</td>
<td>0.16</td>
<td>2.59</td>
</tr>
<tr>
<td></td>
<td>tefluthrin</td>
<td>1.68 (1.38-2.01)*</td>
<td>3.00 (0.419)</td>
<td>2.38</td>
<td>3.30</td>
</tr>
<tr>
<td>PYR-R</td>
<td>indoxacarb</td>
<td>1.51 (1.02-2.06)*</td>
<td>1.68 (0.29)</td>
<td>0.44</td>
<td>5.57</td>
</tr>
<tr>
<td></td>
<td>acephate</td>
<td>39.4 (24.8-62.5)</td>
<td>5.09 (0.61)</td>
<td>9.48</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td><em>trans</em>-fenfuthrin</td>
<td>4.83 (3.91-6.25)*</td>
<td>4.49 (0.65)</td>
<td>3.18</td>
<td>2.91</td>
</tr>
<tr>
<td></td>
<td>tefluthrin</td>
<td>1.11 (0.96-1.31)*</td>
<td>1.77 (0.28)</td>
<td>6.75</td>
<td>2.18</td>
</tr>
</tbody>
</table>

<sup>a</sup>Strain--LSU-S: insecticide-susceptible; OP-R: profenofos–selected; PYR-R: cypermethrin–selected<br>
<sup>b</sup>FL= fiducial limits. *: Significantly different from the corresponding value for the LSU-S strain.<br>
<sup>c</sup>SD=standard deviation.<br>
<sup>d</sup>RR=resistance ratio, defined as LD<sub>50</sub> (resistant strain) / LD<sub>50</sub> (susceptible strain).
Figure 3.2 Filter paper assay with 1-NA and second stadium larvae of susceptible (LSU-S) and resistant (PYR-R and OP-R) strains of *H. virescens*. Color scale developed with known concentrations of 1-naphthol.
Figure 3.3 Filter paper assay with 1-NPA and second stadium larvae of susceptible (LSU-S) and resistant (PYR-R and OP-R) strains of *H. virescens*. Color scale developed with known concentrations of 1-naphthol.
Figure 3.4 Filter paper assay with 2-NPA and second stadium larvae of susceptible (LSU-S) and resistant (PYR-R and OP-R) strains of *H. virescens*. Color scale developed with known concentrations of 2-naphthol.
Figure 3.5 Filter paper assay with cis-TTPA and second stadium larvae of susceptible (LSU-S) and resistant (PYR-R and OP-R) strains of *H. virescens*. Color scale developed with known concentrations of thiophenol.
Figure 3.6 Filter paper assay with trans-TTPA and second stadium larvae of susceptible (LSU-S) and resistant (PYR-R and OP-R) strains of *H. virescens*. Color scale developed with known concentrations of thiophenol.
before making a decision regarding subsequent management strategies. In addition, other bioassays (e.g., topical bioassays, plant residue bioassays) also are used to monitor insecticide resistance of field-collected populations of this pest (Elzen, 1991; Ernst and Dittrich, 1992; Harold and Ottea, 1997).

Currently available bioassays provide no information on the biochemical mechanism underlying resistance. A synergist may be incorporated into these assays to identify the metabolic enzyme associated with resistance; however, synergists may not be specific for the detoxifying enzymes associated with resistance (Armes et al., 1997; Sanchez et al., 2001) or they may affect the accessibility of insecticides to their target sites (Bull and Patterson, 1993; Sanchez et al., 2001). In the present study, three approaches were taken in an attempt to improve the precision with which esterase-based resistance is detected.

The first method was to evaluate and compare effects of novel pyrethroid esters and traditional synergists (PBO and DEF) on the toxicity of profenofos and cypermethrin in resistant strains of *H. virescens*. All pyrethroid esters significantly increased the toxicity of cypermethrin in the susceptible (LSU-S) and resistant (OP-R and PYR-R) strains, further suggesting that esterases were associated with pyrethroid resistance in these strains. However, SRs of each pyrethroid ester did not follow in the same order (i.e., LSU-S < OP-R < PYR-R) of esterase activities toward these substrates. Two explanations are possible for this difference: 1) the optimized dose of each pyrethroid ester with susceptible larvae may not be optimal for synergism in resistant strains because of differential hydrolytic activities of esterases in these three strains; or 2) other, non-hydrolytic, detoxifying enzymes, possibly cytochrome P450 monooxygenases (CYPs)
and glutathione S-transferases, may contribute to resistance (Brattsten et al., 1977; Vontas et al., 2001).

The most widely used esterase synergist (DEF) was not a good indicator of esterases associated with insecticide resistance in *H. virescens*. Antagonism of profenofos toxicity was measured following pretreatment with DEF in both LSU-S and OP-R strains, but the decreased LD$_{50}$ was only statistically significant ($P \leq 0.05$) in the LSU-S strain. Antagonism by DEF of cypermethrin toxicity also was observed in both susceptible and OP-resistant strains, but was not statistically significant ($P \leq 0.05$). A similar result was observed in field populations of cypermethrin-resistant *Helicoverpa armigera*, where esterases were the predominant resistance mechanism and pyrethroid toxicity was antagonized by DEF (Tikar et al., 2001). These studies suggest that DEF may not be a specific inhibitor of esterases associated with cypermethrin resistance in these insects.

Significant synergism by TCPB of profenofos toxicity in the OP-R strain (SR of 6.26) and of cypermethrin toxicity in PYR-R larvae (SR of 2.16) indicated that CYPs are associated with resistance in these strains. In addition, there were only low levels of cross-resistance (less than 3.5-fold) in both resistant strains to fenfluthrin and tefluthrin, which have fluorine atoms or methyl groups blocking sites of attack for oxidases, in both resistant strains.

Effects of the oxidase synergists, PBO and TCPB, varied with the strains tested. For example, in the LSU-S strain, PBO acted as a synergist of cypermethrin toxicity and an antagonist of profenofos toxicity, but did not significantly affect either profenofos toxicity in the OP-R strain, or cypermethrin toxicity in the PYR-R strain. In contrast, TCPB was a synergist of both profenofos toxicity in the OP-R strain and cypermethrin toxicity in the PYR-R strain.
toxicity in the PYR-R strain. These differences may result from multiplicity of CYPs associated with insecticide resistance. Profenofos susceptibility is a function of both bioactivation (Wing et al., 1983) and detoxication by CYPs. In the present study, CYPs associated with profenofos activation may have been inhibited by PBO, which resulted in antagonism of profenofos toxicity. In contrast, CYPs associated with detoxication of profenofos and cypermethrin were inhibited by TCPB (Brown et al., 1996), resulting in synergism of profenofos and cypermethrin toxicity. Moreover, differences in polarity of these two compounds and/or variation of cuticular structure of larvae from these strains may have produced differential effects on accessibility of these insecticides on their targets.

Insecticides that can be activated or inactivated by esterases have potential to detect esterase-mediated resistance. The hypothesis tested here was that negative cross-resistance to acephate or indoxacarb (insecticides that are activated by esterases), or positive cross-resistance to tefluthrin and fenfluthrin (that are insecticides inactivated by esterases) would be present when esterase activity was enhanced with resistance. That is, if resistance-associated esterases also hydrolyze acephate and indoxacarb, these insecticides should be more toxic to resistant than to susceptible insects. However, low levels of resistance to both compounds were measured in resistant strains. These results suggest that either the bioactivation of these insecticides by esterases was much slower than their detoxication, or other mechanisms (e.g., CYPs) were involved in resistance in both resistant strains, which agrees with bioassay data with TCPB. In either case, the utility of acephate and indoxacarb as indicators of esterase-mediated resistance in these strains appears limited. In contrast, only low levels of cross-resistance to tefluthrin and
fenfluthrin (two pyrethroids with metabolically-blocked alcohol moiety) were expressed in the resistant strains. This finding suggests that these compounds are not metabolized by either esterases or oxidases in these strains, and may have potential use in diagnostic bioassays to detect metabolism resistance.

Simple filter paper assays, based on biochemical assays, provide an efficient and rapid means to distinguish resistant phenotypes in field populations of *H. virescens*, and this technique has potential for utilization in field situations. In the present study, differences in color intensity for each substrate used were observed within each strain, suggesting that expression of esterase activities toward these substrates was heterogeneous in these strains. However, differences in color developed using 1-NA, or *cis* and *trans*-TTPA between OP-R and PYR-R strains were not obvious, although color developed using 1 and 2-NPA in the OP-R strain was more intense than that in the PYR-R strain. Patterns of color intensity among strains did not parallel (LSU-S < OP-R < PYR-R) that measured for esterase activities in biochemical assays (Chapter 2). In addition, the intensity of yellow color developed with *cis* and *trans*-TTPA is difficult to discriminate with the naked eye. Thus, potential application of these substrates in filter paper assays needs further evaluation in studies with a greater number of individuals.

In summary, novel pyrethroid substrates for esterases may be used as synergists of pyrethroid toxicity in strains expressing esterase-mediated resistance. In some cases, pyrethroid substrates may be used in filter paper assays in field situations to distinguish resistant phenotypes in populations of *H. virescens*. In addition, the potential utility of bioactivated insecticides (e.g., acephate and indoxacarb) and novel pyrethroid insecticides (e.g., tefluthrin and fenfluthrin) in diagnostic bioassays for esterases
associated with pyrethroid resistance is limited, but they remain candidates for such assays in other resistant insects.

3.5 References


CHAPTER 4

INVESTIGATING THE RELATIONSHIP BETWEEN PYRETHROID STRUCTURE AND RESISTANCE IN THE TOBACCO BUDWORM, HELIOTHIS VIRESCENS (F.)

4.1 Introduction

Resistance is defined as “the ability of an insect population to survive a dose of poison that is lethal to the majority of individuals in a normal population of the same species” (Anonymous, 1957). Resistance of insects to insecticides continues to be a serious barrier to successful management of insect pests (Metcalf, 1994). Field populations of *H. virescens* have developed resistance to almost every class of insecticide over the past 40 years (McCaffery, 1998). Insects can develop resistance through reduced target site sensitivity, enhanced metabolism and decreased penetration (Plapp, 1976; Oppenoorth, 1985). All these mechanisms are expressed in *H. virescens* (Sparks et al., 1993; McCaffery, 1998).

Development of metabolic or target resistance to a particular insecticide may result in hypersensitivity (i.e., negative cross-resistance) to other insecticides that have not been previously applied. Mechanistically, negative cross-resistance may arise in a number of ways. First, enhanced activities of detoxifying enzymes accompanied with resistance to one insecticide may increase the bioactivation of another. For example, increased activities of cytochrome P450 monooxygenases were possibly responsible for observed negative cross-resistance to cypermethrin in a chlorpyrifos-selected strain of the German cockroach, *Blattella germanica* (Scharf et al., 1997), to diazinon in the pyrethroid resistant horn fly, *Haematobia irritans* (Cilek et al., 1995), to chlorpyrifos in dicofol-selected two-spotted spider mites, *Tetranychus urticae* (Hatano et al., 1992) and to
chlorfenapyr in pyrethroid-resistant *H. virescens* (Pimprale et al., 1997) and *Haematobia irritans* (Sheppard and Joyce, 1998). Similarly, enhanced activities of esterases were associated with observed negative cross-resistance to proinsecticides in insecticide-resistant peach-potato aphid, *Myzus persicae* (Hedley et al., 1998). In addition to increased metabolic activation, structural changes in insecticide target sites accompanied with resistance to an insecticide may become more sensitive to other insecticides that share the same target site. For example, the alteration in acetylcholinesterase resulting from selection with dimethoate conferred negative cross-resistance to propoxur in the olive fruit fly, *Bactrocera oleae* (Vontas et al., 2001). Finally, allosteric effects at target sites may result in negative cross-resistance between two types of insecticides that share the same target, but different sites. For example, due to allosteric effects of sodium channels, negative cross-resistance to N-alkylamides (Elliott et al., 1986) or dihydropyrazoles (Khambay et al., 2001) is expressed in pyrethroid resistant *Musca domestica*.

In contrast, development of metabolic or target resistance to a particular insecticide may often result in high levels of resistance (i.e., cross-resistance) to other insecticides that have not been previously applied. For example, DDT and pyrethroids share the same target site on the voltage-sensitive sodium channels. Thus, the wide variation in susceptibility of *H. virescens* to the pyrethroids before their widespread field applications is thought to result from cross-resistance associated with reduced sensitivity of this target site arising from the previous and extensive use of DDT (Davis et al., 1975; Brown et al., 1982; Leonard et al., 1988). Similarly, resistance due to reduced sensitivity of acetylcholinesterases in *H. virescens* conferred cross-resistance among many (but not all)
OPs and carbamates (Brown and Bryson, 1992; Heckel et al., 1998). Similar metabolic mechanisms underlying resistance to OPs, carbamates and pyrethroids in *H. virescens* conferred cross-resistance among these three classes of insecticides (Campanhola and Plapp, 1987; Leonard et al., 1988; Zhao et al., 1996). Finally, cross-resistance to structurally divergent Bt toxins in *H. virescens* was exhibited following selection with a Bt toxin (Cry1Ac) in the laboratory (Gould, 1992) and is not accompanied by significant alterations in toxin binding. Generally, metabolic resistance is believed to confer a broader cross-resistance than target site insensitivity (Casida and Quistad, 1998).

The spectrum of cross-resistance not only depends on the target sites and metabolic pathways of an insecticide, but also on the insect being selected and the chemical structure of the insecticide used for selection. Both natural pyrethrins and synthesized pyrethroids share the same target site (i.e., voltage sensitive sodium channels), but pyrethroid-resistant *M. domestica* had very high levels of resistance to synthesized pyrethroids (e.g., permethrin: 204-fold; phenothrin: 283-fold), but had only 7-fold resistance to natural pyrethrins (Katsuda, 1999). Similarly, permethrin-selected resistant strains of *H. virescens* expressed varying degrees of cross-resistance to other pyrethroids (Brown and Bryson, 1996). Moreover, the pyrethroid, fenfluthrin, was not resisted by a strain of *H. virescens* that was selected for high levels (i.e., 58-fold) of resistance to cypermethrin (Shan et al., 1997). These studies suggest that the chemical structure of an insecticide is an important determinant of metabolism and cross-resistance.

In the present study, the degree to which cross-resistance is conferred between OPs and pyrethroids by selection for enhanced esterase activities was examined. The
objective of this chapter was to investigate the relationship between pyrethroid structure and cross-resistance in *H. virescens*.

### 4.2 Materials and Methods

#### 4.2.1 Chemicals

Permethrin (94.6%), bifluthrin (96%), tefluthrin (97%), cypermethrin (96%) and acephate (97.6%) were kindly provided by FMC Corporation (Princeton, New Jersey). Indoxacarb (100%) and spinosyn A (100%) were kindly offered by Du Pont DE Nemours (Wilmington, DE) and DowElanco Corporation (Indianapolis, IN), respectively. *trans*-Fenfluthrin was originally synthesized by Shan et al. (1997) and re-crystallized before use.

#### 4.2.2 Insects

A susceptible strain of *H. virescens* (LSU-S) was established in 1977 (Leonard et al., 1988) and has been reared in the laboratory since that time without exposure to insecticides. All larvae for resistance selection were fifth instars. Larvae from the LSU-S strain were selected for five consecutive generations with topical applications of profenofos at doses corresponding to the LD$_{60}$ for each generation (3.5, 4.6, 8.5, 13.6 and 23.8 µg/individual). After one year without selection, individual larvae were treated with 25 µg profenofos (an approximate LD$_{20}$), and the survivors (OP-R strain) were used for biochemical and toxicological assays.

Similarly, larvae from the LSU-S strain were selected for four consecutive generations with topical applications of cypermethrin at doses corresponding to the LD$_{60}$ (0.22, 0.4, 0.5 and 0.6 µg/individual). After one generation without selection, larvae were treated
with 1 µg of cypermethrin (6% mortality at 72 hours), and the next generation (PYR-R) was used for biochemical and biological assays.

### 4.2.3 Bioassays

Fifth instars (day 1), weighing 180±20 mg, were treated on the mid-thoracic dorsum with 1 µl aliquots of either profenofos or cypermethrin (in acetone) or with acetone alone (control). The dose-mortality relationship for each compound was assessed from at least five doses with 30 insects treated per dose. After treatment, larvae were maintained at 27°C, and mortality was recorded after 72 hours. The criterion for mortality was the lack of coordinated movement within 30 seconds after being prodded. Results were corrected for control mortality with Abbott’s formula (Abbott, 1925), then analyzed by Finney’s method (Finney, 1971). Resistance ratios (RR) were calculated as LD$_{50}$ of resistant strain / LD$_{50}$ of susceptible strain. Values were considered statistically different if their 95% fiducial limits (FL) did not overlap.

### 4.3 Results

Resistance to all insecticides tested (except for acephate) was present in both OP-R and PYR-R larvae (Table 4.1). Resistance to different insecticides varied depending on the strain used for assays. Resistance was highest to the insecticide used for selection (i.e., to profenofos in the OP-R strain and cypermethrin in the PYR-R strain). In addition, pyrethroid resistance was higher to insecticides with 3-phenoxybenzyl alcohol/aldehyde (e.g., cypermethrin and permethrin) than those with 3-phenyl 2-methylbenzyl alcohol (e.g., bifenthrin), and with fenfluorobenzyl (e.g., fenfluthrin) or 4-methyl tetrafluorobenzyl alcohol (e.g., tefluthrin). Moreover, negative cross-resistance was not measured for either acephate or indoxacarb. In contrast, high levels of cross-
Table 4.1 Susceptibility of larvae from the susceptible (LSU-S) and resistant (OP-R and PYR-R) strains of *H. virescens*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pesticide</th>
<th>LD$_{50}$ (95% FL)$^b$</th>
<th>Slope (SD)$^c$</th>
<th>Chi-square</th>
<th>RR$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU-S</td>
<td>profenofos</td>
<td>2.90 (2.40-3.56)</td>
<td>2.63 (0.40)</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cypermethrin</td>
<td>0.19 (0.16-0.24)</td>
<td>2.81 (0.39)</td>
<td>2.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>permethrin</td>
<td>0.14 (0.11-0.19)</td>
<td>1.91 (0.25)</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tefluthrin</td>
<td>0.51 (0.42-0.69)</td>
<td>2.65 (0.52)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bifenthrin</td>
<td>0.05 (0.04-0.06)</td>
<td>3.12 (0.42)</td>
<td>3.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>trans</em>-fenfuthrin</td>
<td>1.64 (1.22-2.12)</td>
<td>2.99 (0.40)</td>
<td>3.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>spinosyn A</td>
<td>1.84 (1.35-2.66)</td>
<td>1.66 (0.29)</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indoxacarb</td>
<td>0.27 (0.22-0.33)</td>
<td>4.46 (0.64)</td>
<td>2.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>acephate</td>
<td>25.8 (22.3-29.4)</td>
<td>4.26 (0.61)</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>OP-R</td>
<td>profenofos</td>
<td>52.3 (46.0-59.0)$^*$</td>
<td>3.88 (0.57)</td>
<td>1.76</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>indoxacarb</td>
<td>4.29 (3.18-5.87)$^*$</td>
<td>1.62 (0.23)</td>
<td>1.11</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>cypermethrin</td>
<td>2.46 (1.86-3.43)$^*$</td>
<td>6.49 (0.88)</td>
<td>1.06</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>permethrin</td>
<td>1.03 (0.54-1.75)$^*$</td>
<td>2.05 (0.31)</td>
<td>3.99</td>
<td>7.20</td>
</tr>
<tr>
<td></td>
<td>bifenthrin</td>
<td>0.17 (0.13-0.24)$^*$</td>
<td>1.77 (0.34)</td>
<td>2.03</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>tefluthrin</td>
<td>1.68 (1.38-2.01)$^*$</td>
<td>3.00 (0.42)</td>
<td>2.38</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td><em>trans</em>-fenfluthrin</td>
<td>4.30 (3.70-4.88)</td>
<td>4.94 (0.64)</td>
<td>0.16</td>
<td>2.59</td>
</tr>
<tr>
<td></td>
<td>acephate</td>
<td>69.1 (55.0-86.8)$^*$</td>
<td>3.64 (0.53)</td>
<td>4.76</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>spinosyn A</td>
<td>4.03 (2.85-5.49)$^*$</td>
<td>1.52 (0.22)</td>
<td>0.64</td>
<td>2.19</td>
</tr>
</tbody>
</table>

| PYR-R  | cypermethrin    | 3.75 (3.16-4.33)$^*$     | 3.34 (0.48)    | 1.56       | 19.6   |
|        | permethrin      | 5.59 (4.27-7.91)$^*$     | 1.77 (0.28)    | 0.57       | 12.4   |
|        | bifenthrin      | 0.26 (0.18-0.41)$^*$     | 2.97 (0.38)    | 8.40       | 5.10   |
|        | *trans*-fenfluthrin | 4.83 (3.91-6.25)  | 4.49 (0.65)    | 3.18       | 2.91   |
|        | tefluthrin      | 1.11 (0.96-1.31)$^*$     | 1.77 (0.28)    | 6.75       | 2.18   |
|        | profenofos      | 13.2 (10.4-16.2)$^*$     | 2.49 (0.39)    | 2.17       | 4.53   |
|        | spinosyn A      | 5.49 (3.79-7.93)$^*$     | 1.30 (0.21)    | 0.19       | 2.99   |
|        | indoxacarb      | 1.51 (1.02-2.06)$^*$     | 1.68 (0.29)    | 0.44       | 5.57   |
|        | acephate        | 39.4 (24.8-62.5)         | 5.09 (0.61)    | 9.48       | 1.53   |

$^a$Strain--LSU-S: insecticide-susceptible; OP-R: profenofos–selected; PYR-R: cypermethrin-selected

$^b$FL= fiducial limits. $^*$: Significantly different from the corresponding datum in the LSU-S strain.

$^c$SD=standard deviation.

$^d$SR=synergism ratio, defined as LD$_{50}$ (without a synergist) / LD$_{50}$ (with a synergist).

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resistance to indoxacarb existed in both OP-R (RR: 15.8) and PYR-R (RR: 5.57) strains. In addition, a low level of resistance to spinosyn A was measured in OP-R (RR: 2.19) and PYR-R (RR: 2.99) larvae.

4.4 Discussion

Chemical structure, as it relates to metabolic stability, is a major determinant of cross-resistance; however, the influence of structure on cross-resistance spectra is poorly understood. In OP-resistant *Lucilia cuprina*, esterases are primarily responsible for resistance (Campbell et al., 1998). Insects resistant to diazinon (a diethoxy ester) displayed 2-fold more resistance toward other diethoxy OPs than their dimethoxy analogs. Similarly, these strains did not show cross-resistance to either diisopropyl or OPs with chiral phosphorus atoms. In contrast, strains selected with malathion (a dimethoxy ester) displayed 2-5 times more resistance toward the dimethoxy OPs than their diethoxy analogs. Moreover, these strains showed only slight resistance (<3-fold) to either the diisopropyl or optically active OPs, including the diisopropyl analog of malathion.

Structure activity relationships, as they relate to cross-resistance to pyrethroids, have received limited attention. In the present study, a relationship between pyrethroid structure and resistance (Table 4.2) has been examined with profenofos- and cypermethrin-resistant *H. virescens* in which esterases and oxidases were primarily responsible for resistance. Four type-I (non-cyano) pyrethroids (permethrin, bifenthrin, tefluthrin and trans-fenfluthrin) showed much lower resistance ratios than a type-II pyrethroid (cypermethrin) in both OP-R and PYR-R strains. Similar results were
obtained previously with a different, cypermethrin-selected strain of *H. virescens* (Shan et al., 1997). In previous studies, permethrin-selected *H. virescens* expressed greater resistance to type-I (permethrin) than type-II (cypermethrin) pyrethroids (Jensen et al., 1984; Brown et al., 1996). These results suggest that the insecticide used for resistance selection has a major effect on the degree of cross-resistance to other insecticides. In addition, the variability seen in resistance among the pyrethroids tested in this study suggests that target site resistance is not a major mechanism of resistance in the PYR-R strain.

Differences in resistance among four different type-I pyrethroids were present in both OP-R and PYR-R strains. The phenoxybenzyl moiety of cypermethrin and permethrin is a major site for enzymatic detoxication (Little et al., 1989; Lee et al., 1989). Tefluthrin
and trans-fenfluthrin are structurally similar: both have fluorinated phenyl rings, thus, a major site for detoxication by oxidases is no longer present. Resistance to these two insecticides was very similar (from 2.5- to 3.3-fold), but much lower than that to permethrin in both OP-R and PYR-R strains, suggesting that: 1) oxidases were involved in resistance in both resistant strains, which agreed with results from bioassays with TCPB (Chapter 3); 2) a substitution of one chlorine atom in trans-fenfluthrin with a methyl group in tefluthrin did not change the degree of resistance in both OP-R and PYR-R strains; 3) these two insecticides cannot be used as diagnostic compounds of esterases associated with pyrethroid resistance (Chapter 3), but are useful for insects with oxidase-mediated metabolic resistance. Moreover, the alcohol moiety of bifenthrin (i.e., 3-phenyl 2-methyl benzyl alcohol) probably subjects to some metabolisms. This was supported by higher resistance ratios of bifenthrin than those of tefluthrin and trans-fenfluthrin, in the PYR-R strain.

The low level of resistance to non-phenoxybenzyl pyrethroids probably reflects the contribution of a penetration mechanism. Similar levels of resistance to spinosyn A in both OP-R and PYR-R strains (less than 3-fold) further suggest that a penetration resistance may be present in both resistant strains.

In summary, the spectrum of cross-resistance to pyrethroids in both profenofos- and cypermethrin-resistant strains was somewhat specific and was primarily dependent on the insecticide used for resistance selection. However, resistance to tefluthrin and trans-fenfluthrin, in which some metabolic sites for detoxifying enzymes (e.g., oxidases) are blocked, may develop more slowly than those (e.g., permethrin, bifenthrin) in which sites for detoxication are present. These results suggest that simple bioassays with such
compounds may be used to detect metabolic resistance in insects. In addition, similar modification in alcohol moiety of existing pyrethroids may result in insecticides that will be active against tobacco budworms expressing metabolic resistance.

4.5 References


CHAPTER 5
SUMMARY AND CONCLUSIONS

Precise detection of esterase-mediated resistance and cross-resistance is a prerequisite of effective implementation of insecticide resistance management strategies. In this study, non-pyrethroid and novel pyrethroid substrates were evaluated as biochemical indicators of esterases associated with pyrethroid-resistance in laboratory populations of *H. virescens*. Moreover, novel pyrethroid substrates were evaluated as synergists of pyrethroid toxicity in *H. virescens*, and results were compared with traditional synergists of esterases. In addition, insecticides that are either bioactivated or detoxified by esterases were assessed as indicators of esterases associated with pyrethroid resistance in *H. virescens*. Finally, a relationship between pyrethroid structure and the development of cross-resistance was studied with both OP- and pyrethroid-resistant *H. virescens*.

Esterase activities toward non-insecticide substrates were measured in the susceptible (LSU-S) strain, and strains selected with profenofos (OP-R) or cypermethrin (PYR-R). Esterase activities toward non-pyrethroid substrates (e.g., 1-NA, SMTP, TPA) and novel pyrethroid esters (e.g., 1 and 2-NPA, *cis* and *trans*-TTPA and p-NPPA) were significantly (*P* ≤ 0.01) enhanced in both resistant strains compared with those in the susceptible LSU-S strain, suggesting that esterases were involved in resistance in these resistant strains. However, significant differences (*P* ≤ 0.01) in esterase activities between OP-R and PYR-R strains were detected only with SMTP, 1-NPA, *cis* and *trans*-TTPA. In addition, patterns of esterase activities toward pyrethroid esters among three strains matched those measured in that of resistance from bioassays with cypermethrin (i.e., LSU-S < OP-R < PYR-R), but not profenofos. Thus, novel pyrethroid substrates
appear to be better indicators of esterases associated with pyrethroid resistance or cross-resistance to pyrethroid insecticides than non-pyrethroid esters.

Traditional synergists and novel pyrethroid substrates for esterases were evaluated as markers of metabolic resistance in three strains. Novel pyrethroid substrates for esterases significantly increased cypermethrin toxicity in both susceptible and resistant strains, whereas the widely used esterase inhibitor, DEF, antagonized both profenofos and cypermethrin toxicity in these strains. These results suggest that novel pyrethroid esters were better inhibitors of esterases associated with pyrethroid-resistance in *H. virescens* than DEF. Moreover, the oxidase inhibitor, TCPB, synergized both profenofos and cypermethrin toxicity in both resistant strains, suggesting that cytochrome P450 monooxygenases were also involved in resistance in these strains.

Attempts were made to develop simple and rapid filter paper assay with non-pyrethroid (e.g., 1-NA) and novel pyrethroid substrates (e.g., 1 and 2-NPA, *cis* and *trans*-TTPA) using larvae from LSU-S, OP-R and PYR-R strains. Differences in color intensity for each product were observed within and between the susceptible (LSU-S) and resistant (OP-R and PYR-R) strains, but differences were not dramatic. For all substrates, color production was more intense in the tests with OP-R larvae than with LSU-S larvae. However, no qualitative differences could be observed between PYR-R and LSU-S larvae.

Insecticides that are either bioactivated (e.g., acephate and indoxacarb) or detoxified (e.g., tefluthrin and fenfluthrin) by esterases were investigated as indicators of esterases associated with pyrethroid resistance in *H. virescens*. Strong positive cross-resistance to indoxacarb was present in both OP-R and PYR-R strains, no or a low level of cross-
resistance to acephate was present in both OP-R and PYR-R strains. These results suggest that acephate and indoxacarb may not be useful as indicators of esterases in *H. virescens*. In addition, low levels of resistance to tefluthrin and *trans*-fenfluthrin in both OP-R and PYR-R strains were present, which suggests that these insecticides cannot be used as indicators of esterases in *H. virescens*.

The spectrum of cross-resistance to pyrethroids in both profenofos- and cypermethrin-resistant strains was somewhat specific, and was primarily dependent on the insecticide used for resistance selection. Resistance to a type-II pyrethroid (e.g., cypermethrin) developed faster than that to type-I (e.g., permethrin, fenfluthrin, tefluthrin and bifenthrin). However, resistance to the insecticides (e.g., tefluthrin, *trans*-fenfluthrin) in which metabolic sites for detoxifying enzymes (e.g., oxidases) are blocked appears to develop more slowly than to those (e.g., permethrin, bifenthrin) in which sites of metabolism are present. These results suggest that simple bioassays with such compounds may be used to detect metabolic resistance in insects. In addition, similar modification in alcohol moiety of existing pyrethroids may result in insecticides that will be active against tobacco budworms expressing metabolic resistance.
APPENDIX A  SPECTRA OF GC-MS OF CIS -TTPA
APPENDIX B SPECTRA OF GC-MS OF TRANS-TTPA
VITA

Huazhang Huang, the second son of Zhenchu Huang and Qiaoyun Ge, was born on August 27, 1971, in Quxi, Sichuan Province, China. He graduated from Quxi Sanzhong High School, Sichuan Province, China, in 1991. He received his bachelor of science degree in applied chemistry in 1995 from Beijing Agricultural University, Beijing, China. He obtained his master of science in pesticide science under the guidance of Drs. Xuefeng Li and Wenji Zhang in 1997 from China Agricultural University, Beijing, China. Huazhang is presently a candidate for the degree of Doctor of Philosophy in entomology at Louisiana State University.