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IN VITRO AND IN VIVO EVALUATION OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE IN GREEN PEACH APHID (MYZUS PERSICAE (SULZER)).

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ABSTRACT

IN VITRO AND *IN VIVO* EVALUATION OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE IN THE GREEN PEACH APHID, *MYZUS PERSICAE* (SULZER) (HOMOPTERA: APHIDIDAE).

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Green peach aphids (GPA) are effectively controlled by insecticides except in instances of resistance. The only known metabolic mechanism of resistance in GPA is increased carboxyesterase activity (Devonshire and Moores 1982). A rapid, accurate, and simple field test for resistance frequency quantification in GPA is needed. Resistance was diagnosed in this study by two different approaches: an *in vitro* test quantifying esterase levels based on either a colorimetric method or polyacrylamide gel electrophoresis of the enzymes and an *in vivo* dosage mortality Bioassay was used to determine actual insecticide resistance levels. Strong positive correlations exist between the two sets of experiments, resulting in the conclusion that both of these sets of assays provide an effective measure of GPA carboxyesterase-based insecticide resistance. This information was then used to develop and evaluate a colorimetric carboxyesterase-based resistance diagnostic tool for pest management.

This thesis is dedicated to the person who awakened my interest in science and believed I could accomplish a great deal. Thank you, Mom, I will never forget the many sacrifices you made for my future.

O Lord, our Lord, how majestic is your name in all the earth! (The Bible, NIV, Psalm 8:1).

God has made everything beautiful in His time. He has also set eternity in the hearts of men; yet they cannot fathom what God has done from beginning to end. I know that everything God does will endure forever; nothing can be added to it and nothing taken from it. God does it so men will revere him. (The Bible, NIV, Ecclesiastes 3:11, 14).

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LIST OF ABBREVIATIONS

GPA	green peach aphid
PAGE	polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
Bisacrylamide	N,N'-methylene bisacrylamide
SDS	sodium dodecyl sulfate
IPM	Integrated Pest Management
AchE	Acetylcholinesterase
BC	Biological Control
µl	microliter
OP	organophosphate insecticide
MFO	Mixed function oxidase system
GSH	Glutathion S-Transferase

CHAPTER 1

INTRODUCTION

CHAPTER 1. LITERATURE REVIEW AND INTRODUCTION

I. Introduction

The green peach aphid (GPA), *Myzus persicae* (Sulzer), is a principal pest on many important food crops both in the United States and throughout the world (Way 1971, Cancelado and Radcliff 1979, Devonshire 1989). Millions of dollars are spent annually in an attempt to control this insect. On potato, *Solanum tuberosum* (L.), GPA is a major pest because of its vector potential and plant feeding damage (Kennedy *et al.* 1962), and there is a direct correlation between GPA population density (including migration intensity) and the problem of virus transmission (Galecka and Kajak 1971). Until recently, growers were able to control this insect with soil systemic insecticides, such as aldicarb (Temik®). However, the voluntary removal of this compound (and others like it) from agriculture due to suspected ground water contamination has resulted in the use of other systemics followed by less effective foliar aphicides (Preston *et al.* 1990, Reed *et al.* 1990). With the frequency of foliar applications, insects (GPA) can develop high levels of resistance (Tabashnik and Croft 1982). Resistance is the ability of sections of a population to tolerate or avoid potentially lethal or reproductively detrimental factors that would negatively influence a normal population (Pedigo 1989). Resistance results from the strong directional selection caused by repeated insecticide applications or use. The use pattern of foliar aphicides causes high levels of mortality in biological control organism populations (Reed *et al.* 1990). At this time, potato growers are reporting increasing resistance problems. Prior to the period before effective control by aldicarb (1974), control of GPA was difficult to maintain. Currently registered insecticides did not control virus transmission (Adams 1950, Hille Ris Lambers 1953, Powell 1973, Bacon 1976), and the primary source of such viruses as PLRV is infected seed (Flanders *et al.* 1991). Although reduction is possible, complete prevention of viral transmission to potatoes by

insects is not possible via insecticides alone, including aldicarb (Villacarlos 1963, Powell and Mondor 1973, Woodford *et al.* 1983). For this reason, a resistance monitoring system by which growers can detect the incidence of resistance in vectors (including GPA) in their fields could aid agriculture greatly by substantiating whether or not spraying of a specific chemical is the most effective pest elimination technique, or if other management methods need to be employed because the pests are resistant and are likely to survive the chemical application.

II. Integrated Pest Management

A. Definition and Approach

Integrated Pest Management (IPM) is a comprehensive approach to pest management which allows growers to make informed judgments with regards to crop production that are specifically tailored to their own fields. Geier and Clark outlined many of the pest management principles in 1961 (Geier and Clark 1961, Geier 1966), of which a key aim is the conservation of environmental quality while maintaining economically productive crop yields and effective pest control. IPM utilizes a holistic approach for decision making that incorporates multiple tactics with an extensive understanding of the entire agro-ecosystem of which each crop is a part. Included in this need for data is an evaluation of an individual grower's crop situation, economic needs, yield capacity, pest level and pest resistance potential, and many of the other biotic and abiotic factors involved in crop production. In pest management decisions, the more information that is obtained regarding a crop ecosystem, the more effective decisions the grower is able to make.

B. Resistance Management

Inherent in IPM is the concept of pesticide resistance management. This is a method of controlling the development of resistance in pests. As stated previously, insecticidal

compounds exhibit strong directional selection pressures on insect populations: they remove susceptible genotypes from a population. Resistance management involves many factors including selection of the appropriate insecticides for use on a specific pests and timing of chemical applications. Use and timing of insecticides, especially foliar sprays, are important because not only do these compounds vary in overall toxicity and mode of action, they also differ in their effect on non-target organisms (Croft and Brown 1975).

Often when fields are sprayed, populations of biological control organisms die in greater numbers than the targeted resistant pests as a result of both insecticide poisoning and loss of food supply (Croft and Brown 1975). As a result, growers may begin to observe other species exhibiting characteristics detrimental to the crop that were heretofore unnoticed because the population size (and thus amount of damage) was controlled effectively by natural enemies. Once these natural controls are eliminated, the species is able to flourish and cause significant damage to the crop (Peterson 1963, Radcliff 1972, 1973, Mackauer and Way 1979). In addition to the elimination of biological control organisms, a strange phenomenon of resurgence is associated with certain chemicals: they have the ability to cause accelerated growth and increased fecundity (greater numbers of ova produced) in some pest species that are resistant to the chemical (Peterson 1963). In this case, the pest population is not killed off, but increases significantly as a result of the spray. Chemicals targeted at other pests can also significantly accelerate the development of resistance in non-target pests. For this reason, care must be taken to choose the correct insecticide and spray at optimum times for highest target-pest mortality. This may help to eliminate many of the problems associated with sprays and can enable more effective pest control.
One factor in resistance management is the practice of attempting to reduce unwarranted sprays. This is important for maintaining the lowest frequency of resistance possible in pest species while sustaining an economically feasible level of pest population suppression

(Stern *et al.* 1959). In order to determine what is economically feasible pest damage, the concept of a pre-determined economic threshold for spraying crops was established. When pest populations are below a set "threshold", damage caused by pest species results in less financial loss than spraying, and therefore no spray is necessary. However, if pest population numbers are at or above the economic threshold, sprays are necessary to reduce the pest species population because above this threshold level the pests are in significant enough numbers to cause extensive damage to the crop. An example of this is that in Minnesota, there is a 10 GPA/ 100 leaves threshold in seed potato production (Cancelado and Radcliff 1979).

The use of economic thresholds helps to eliminate unnecessary sprays, but it is not as simple to implement as a calendar spray system. This type of pest management involves pest monitoring (sampling and resistance testing) by trained scouts, a detailed knowledge of pest and natural enemy populations present in fields, resistance frequencies of pest species, and similar types of decision criteria. The use of resistance management techniques such as IPM and economic thresholds results in retarded resistance development because pest populations are controlled at the optimum times resulting from monitoring and reduction of unnecessary sprays causing accelerated resistance development (Cancelado and Radcliff 1979).

Another key concept in the elucidation of IPM strategy and resistance management is the extension of pesticide use-life. Pesticide use-life relates closely to avoidance of unnecessary sprays and the use of economic thresholds. Multiple sprays quickly shorten the use life of pesticides and can necessitate the use of synergists or mixtures of several insecticides to increase the toxic action of the compound on the targeted pest insects. Pesticide use-life is the length of time an insecticide (or pesticide) can be used to control

the targeted pest species effectively. In order to lengthen pesticide use-life beyond the initial span of time, some growers resort to insecticide mixtures to promote higher mortality levels in insects, hoping for a multiplicative toxic effect on the insects. Others utilize synergists, such as piperonyl butoxide to promote higher morbidity in pest species. Although synergists and mixtures do increase the toxicity of pesticides, there is much doubt as to the advisability of these methods, especially regarding mixtures of several insecticides. There is some evidence that mixtures of insecticides applied to a crop tend to increase the development of cross resistance in insects exposed to them, and can even accelerate the development of resistance more strongly than a single insecticide alone.

C. The Use of Natural Enemies As IPM Control Strategies

Another IPM strategy involves the promotion of natural enemies, or biological control organisms. This can be challenging in sprayed ecosystems since most commonly biological control organisms are more susceptible to insecticides than pest species. Although some claim that natural enemies are not effective for controlling insect vectors at an economically productively level once they are present on plants (Broadbent 1953) others disagree, claiming that these natural enemies can be relatively effective for control of GPA (Tamaki and Weeks 1972, Tamaki 1973, Powell *et al.* 1974). Among the most important predators of GPA are coccinellids, chrysopids (green lacewings), and syrphid flies (Croft 1989). Some claim that predation has a strong effect on GPA mortality while others disagree. In a population dynamics study conducted in Sicily in 1972, Barbagallo *et al.* observed that few aphids were killed by fungi and parasitoids, while predators were the causative agent in 21% of the total aphid loss. Additionally, 26.4% of the aphid population loss was caused by emigration, and Barbagallo *et al.* claim that this shows that predation is not a reliable means of controlling the pest levels below the economic threshold (Barbagallo *et al.*, 1972). By contrast, Mack and Smilowitz (1978, 1981) claim

that biological control organisms can be effective at controlling GPA populations. They found eighteen species of GPA predators in a field, with coccinellid adults and lacewing adults as the two most important types (Mack and Smilowitz 1978, 1981). Syrphid fly adults, however, were rare. It seems that although natural enemies can be an effective means for GPA control, alone they are not enough to control the spread of viruses to potato by GPA.

D. Additional IPM Techniques

In addition to the pest resistance management methods previously mentioned, there are multitudes of other management tactics. Some of these strategies involve use of the different cultural practices used on crops. Cultural applications can include practices to control pest plants (weeds) and insects, planting (such as monoculture or polycultures and rotation), fertilizing, irrigation, and sanitation practices, selection of tolerant or resistant (to the pest) crop varieties, and any other factors which could influence the crop's quality and resistance or promotion to pest activity. In order to know when to implement these, pest monitoring is a necessary part of both the culture practices and the IPM system as a whole.

III. The Green Peach Aphid

A. Geographic Distribution and Host Plants

Green peach aphids have a very broad distribution and host plant range. They are found throughout most of the world, although most commonly in the northern parts of the temperate zone. This range includes most of Europe, the United States, East Asia, and much of Central America (Blackman 1974, 1984, and Commonwealth Institute of Entomology 1954). GPA are considered polyphagous leaf aphid species with an

extremely large host plant range (Dixon 1985). They are heteroecious and holocyclic[1] between peach, the primary host (and other *Prunus* species), and secondary hosts such as potato (Helle *et al.* 1987). GPA is (able to feed on a variety of hosts) on secondary species where peach is absent, and in temperate climes (Blackman 1974).

The considerable flexibility (climate and host-wise) of this insect is believed by some to contribute to its ability to evade control measures successfully and to rapid adaptation to new habitats. GPA are found on most cultivated plants. Some potential hosts are: plum, cherry, prune, citrus, cabbage, dandelion, endive, mustard greens, parsley, turnip, tobacco, rose, spinach, peppers, beets, celery, lettuce, chard, and potato (Blackman 1984). The sexual generation of GPA oviposit overwintering eggs on peach, plum, and cherry in colder climates (Blackman 1984). In warmer areas of some states, such as Arizona, California, Oregon, and Washington they overwinter as adults, and some believe they do so in colder climes under the snow (Takada 1974).

B. Biology

Green peach aphids are active in the spring and summer, and through the fall. They are found clustered together in an unequal distribution on the plant's rapidly growing or senescent leaves (Bradley 1952, Taylor 1953, 1962, Mack and Smilowitz 1981, Jansson and Smilowitz 1985, Nderitu and Mueke 1989). The reason that they prefer senescent tissue may be that phloem sap concentrations change as the leaves senesce (Thomas and Stoddart 1980) and they contain higher nitrogen concentrations (Kennedy 1958, Jansson and Smilowitz 1985). This in turn induces more rapid growth and reproduction in GPA (Jansson and Smilowitz 1985). Additionally, GPA are found in a clustered distribution

[1]Heterocious (alternating host plants) and holocyclic (having a sexual generation)

because they reproduce parthenogenetically throughout much of the summer, and once an aphid finds a suitable site for feeding, they remain sessile. They also maintain close proximity with other GPA for protection purposes because they do not move very rapidly and their defense mechanisms are limited.

The life cycle of GPA is a sequence of different morphs (forms) triggered by environmental factors (Lees 1966, Hille Ris Lambers 1966). GPA have two principle forms in their life cycle: alate (winged) and apterous (wingless), and they exhibit considerable variation in color. Takada observed the occurrence of both green and red aphids in a sympatric population of GPA with red color controlled by a dominant allele (Takada 1981). The apterous adults are light to medium green with a yellow tint in summer (Blackman 1984). In the fall the common color morph is red. Alate GPA are brown to black with a yellow abdomen. GPA is also known to some as the tobacco aphid (has been defined as a separate solely parthenogenetic species) or the spinach aphid.

The cyclical lifecycle of GPA with several parthenogenetic generations in spring and summer with an annual sexual generation in the fall is called "cyclical parthenogenesis" (Blackman 1974). However, some adults are thought to overwinter under the snows in colder climates (Takada 1974), and other alates could migrate north on wind currents in the spring. Ideally, after several asexual generations, the sexual adults lay eggs in the fall. Those eggs laid the previous spring overwinter and emerge, and the cycle begins anew with parthenogenetic generations the following spring. This alternation of sexual and asexual forms is called "holocycly" by Blackman, while anholocyclic parthenogenetic populations, such as greenhouse populations, are derived from the holocylic populations (Blackman 1974). Any population can exhibit holocycly, anholocycly, or a combination of the two, according to Blackman (1974).

The reproductive category (ovipara or vivipara[2]) is usually determined by environmental stimuli, usually photoperiod (Helle 1987). Generally, the first generation, which is found during springtime with high humidity, are asexual apterous females that are larger than the other stages, and called fundatrices or stem mothers (Helle 1987). Not only is the fundatrix of GPA larger in size and plumper than future progeny, with smaller eyes and head, shorter antennae, legs, cauda, and siphunculi, it is also more fecund due to its having more ovarioles (Blackman 1978, Helle 1987). These characters are called the fundatrix facies (Lees 1961). Later generations are asexual or sexual depending on temperature, photoperiod, plant quality, and possible humidity.

After the fundatrix generation, GPA commonly reproduce parthenogenetically. Some believe that GPA parthenogenetic reproduction is a form of paedogenesis, or reproduction by sexually immature or larval forms of the insect (Uichanco 1924). Others, such as Takahashi disagree, explaining that GPA parthenogenesis is not paedogenesis (1924) but a form of asexual reproduction that is not paedogenesis. Additionally there has been much debate as to whether GPA are apomictic (parthenogenesis which results in progeny genetically identical to the mother) or automictic (allowing for recombination of a sort and thus genetic variation within parthenogenetic lines) (Helle 1987). In line with this, Cognetti coined the term endomeiosis for the form of automicty found in aphids (1961). Endomeiosis involves alleles crossing over, or the exchange of alleles between homologous chromosomes during prophase of meiosis within the nucleus of the oocyte

(Cognetti 1961, Beranek and Berry 1974, Helle 1987). This allows for a segregation of the alleles at the loci for which the mother is heterozygous (Helle 1987). Blackman

[2]Ovipara (Producing eggs which hatch to produce young) and Vivipara (producing live young).

claimed that there was no evidence for endomeoisis in GPA (1978). On a more basic line, Suomalainen *et al.* argued that this was not actual recombination because the alleles are combined within the same nucleus (1980). However, others disagree, claiming that Suomalainen *et al.* (1980) overlook the fact that recombined chromosomes would be lost to the polar body at the maturation division (Helle 1987). Baker claims that endomeiosis could occur, however it must be either rare or require special circumstances. It cannot, according to Baker, account for the large changes of resistance frequencies found in the electrophoresis of biotypes over short time intervals in the field (Baker 1978).

C. Genetic Variation: Recombination vs. Endomeiosis

Because of their ability to reproduce parthenogenetically, aphid lines have the potential to develop a virtually unlimited number of alterations to the ancestral karyotype. Over time these segregated lines can become an all but separate line of progeny (Helle 1987). Aphids are expected to show more variation within species than many other organisms because of the holocentric nature of their chromosomes (no localized centromeres). This allows fragments of their chromosomes to perpetuate themselves through many generations because the entire chromosome has centromeric activity, permitting them to orient correctly at the equatorial plate at mitosis and thus replicate normally and pass into the two daughter cells (Helle 1987). One example of such variation is the genus *Amphorophora*, which shows a range of chromosome numbers form n=2 to n=36. Population differences are due entirely to dissociations and fusions of the autosomes, as the X-chromosomes remain unaffected throughout (Blackman 1980).

In the field there may be a mixture of (GPA) clones (from different mothers) during the summer generations that are genetically isolated and can recombine only in the sexual (fall) generation (Reinink *et al.*, 1989). One example of this type of segregative

adaptation is that although as a species GPA are highly polyphagous[3], clones from one mother can become restricted to certain host plants because of adaptations (Weber 1985). Takada agreed with these findings in a study he conducted in Japan (Takada 1979) This same adaptability seems to apply to insecticide resistance and other similar genetic adaptations, not just to feeding preferences. Thus, a population of GPA may be extremely resistant to one chemical or class of chemicals (such as organophosphates), but susceptible to another chemical from a different (or even the same) class of compounds.

In the sexual generation progeny have genes contributed by both parents, thus yielding classical genetic recombination. This is significant in that the most fit parthenogenetic lines are continued, others will be selected against and eliminated during the sexual generations. The most successful lines will continue to contribute to the population season after season (Blackman 1974). According to Blackman, populations that are continuously parthenogenetic (such as greenhouse populations) do not have this fitness selection mechanism available by genetic recombination (Blackman 1974). He states that parthenogenetic populations appear to have a reduced ability to respond to selection pressures, since adaptive mutations that arise in individuals are not shared with other individuals (do not enter the gene pool) because they cannot recombine by sexual reproduction (Blackman 1974). Blackman claims that although endomeiotic recombination would help to achieve homozygousity, and therefore show the adaptive significance of recessive alleles, more often than not heterozygous individuals are superior

(Blackman 1974). Heterozygousity can be maintained in automictic populations as long as selection favors those individuals (Asher 1970), but maintenance of heterozygousity

[3]Polyphagous means that these insects are capable of feeding on a wide variety of host plants.

does not imply adaptability, since most adaptations involve a loss of some heterozygousity (for example: resistance.). On an opposite tack, Carson (1967) maintains that parthenogenesis maintains heterozygousity. Blackman claims that although endomeiosis could bring about one more adaptive step, its evolutionary significance is dubious and for this reason the sexual generation is very important for maintaining heterozygosity and therefore adaptability within a holocylic aphid population (Blackman 1974). Darlington (1937) and Suomalainen (1950) claimed that parthenogenesis is an evolutionary dead end.

Blackman fails to note that resistance is associated with greenhouse populations of parthenogenetically reproducing aphids, and many resistance researchers feel that field resistance may have originated in greenhouse populations (Mark Whalon, personal communication). He seems to ignore the fact that although heterozygousity increases an organism's ability to adapt, in insecticide resistance situations those individuals homozygous for resistance tend to be better fit to withstand chemical treatment, even without the help of genetic recombination found only in sexual reproduction.

D. Feeding mechanisms

GPA have piercing and sucking mouthparts for feeding. They ingest food by insertion of their mouthparts (styli) into the phloem tissue of the plant, and this enables them to extract fluids. The mechanism of their feeding is such that upon insertion of their styli (the maxillary and mandibular styli) there is a pharyngeal pump which forces the plant fluids into the foregut through the food canal (Ponsen 1987). The sequence is as follows: liquid food is forced through the maxillary and mandibular styli, the pharyngeal duct, and the pharyngeal pump, through the head and over the tentorium. Next it flows into the foregut (where there are spherical symbionts), then to the midgut and on to the hindgut. The hindgut connects to the anus (or rectum), from which honey dew is excreted. The aphid

filter chamber takes the place of the Malpighian tubules which are found in other insects (Ponsen 1987). Wastes, constituted of excess sugars ("honeydew") flows out of the anus. Honeydew is a sticky substance which can be used as a carbohydrate source by other insects (and sooty or other molds) because most of the sugars found in plant phloem are left behind by the aphids' digestive tract (aphids are generally nitrogen-limited rather than carbohydrate-limited). Ants in particular are known for cultivating the aphids that produce honeydew, even to the point of "farming" the aphids, providing shelter in the winter and protection from predators. These ants have developed a "milking" behavior which involves stroking the GPA until they exude some of the honeydew. Thus they have evolved a symbiotic relationship that is mutually beneficial to both parties.

E. Green Peach Aphid Pest Status

GPA are considered secondary pests on many crops, because most of the damage is not direct but a result of viral infection. This is true of the crop of focus for this study, the potato (*Solanum tuberosum* L.). Approximately thirty million dollars is spent annually in attempts to control this pest (Anon. 1991). Damage is caused by both viral infections and direct feeding, although the feeding damage is not economically significant. The primary damage caused by GPA is that of virus transmission to the host plant during feeding. Aphids are the largest group of vectors that transmit disease to plants, transmitting more than 300 known viruses (de Bokx 1987). Barbagallo *et al.* consider GPA to be the most active vectors of potato viruses (Barbagallo *et al.* 1972, Nderitu and Mueke 1989). The most economically important viruses that GPA vectors to potato are Leafroll Virus (PLRV) and Potato Virus Y (PVY) (Beemster 1987, Nderitu and Mueke 1988).

IV. The Virus Problem

A. Virus Definition

Anatomically, viruses are simple particles. Viruses are made up of virions, or virus particles, which consist of nucleic acids in the form of double or single stranded DNA or RNA. Encasing the nucleic acids is a protein coat (envelope), or capsid, which helps protect the genetic material inside (van der Want 1987). These particles require the metabolic capabilities of a cell in order to reproduce. They then lyse the cell and spread to surrounding cells, steadily reproducing and lysing, thereby causing the outward symptoms of dead and necrotic tissue and other forms of disease found in plants (and animals).

There has been much debate over whether or not viruses are living organisms or just organized conglomerations of nucleic acids and proteins. Much of this stems from the fact that although these particles can reproduce, they require the use of another cell's metabolic machinery to accomplish this. Other than the reproductive factor, and the fact that viruses contain nucleic acids, they exhibit no characteristics necessary to define them as living.

B. Mode of transmission

Viruses are transmitted in various ways. The three types of transmission are (1) mechanical, (2) circulative, and (3) propagative. Mechanical transmission of the virus depends on some sort of physical transfer, such as the aphid feeding for a short time on several plants (testing them) before settling on one (Sylvester 1949). The virus can be transmitted by physical contact, such as on the mouthparts (stylets). Some examples of mechanically transmitted viruses are Potato virus M (PVM), and Potato virus S (Rich 1983). If the insect feeds on several plants the virus can be spread to each plant. In this case the virus is often (but not always, as is the case with PVM) stylet-borne, and non-persistent (Sylvester 1969).

Circulative transmission occurs by a persistent means when the plant is colonized. Potato leafroll virus (PLRV) is generally termed a persistent virus, since it is transferred by aphids which colonize the host plant. In this case the virus may be stylet-borne, but may circulate in the insect, but the virus does not infect the insect, it simply "circulates".

The last type of viral transmission is called propagative transmission. This means that the virus multiplies within the aphid as well as within the host plant (Sylvester 1969). PLRV can also be a propagative virus, but only in GPA (Stegman and Ponsen 1958). In this case, not only the host plant, but the aphid are infected with the virus. For this type of transmission, the virus has to have entered the aphid previous to transmission. It requires a period of time for the aphid to obtain a virus while feeding on an infected plant. This period is called the "acquisition period". The virus then enters the aphid's digestive tract, moves through the intestinal wall and into the hemolymph, through the accessory gland and into the salivary canal. In the salivary canal the virus becomes mixed with the saliva and is excreted. Thus it enters the next plant's phloem during feeding (Black 1959).

There is often a latent, or "incubation" period, between acquisition and the next viral transmission. This occurs because the viral nucleic acids (genome) must be liberated and take control of the cell's metabolic protein synthesis mechanisms in order to replicate. The viral genome causes the cell's protein synthesis mechanisms to form complete replicates of the viral genome, down to the protein coat (van der Want 1987). All of this takes time; to travel through the insect's body and to replicate for further transmission. The inoculation period is the time of feeding (by the vector) required for the virus to be transmitted from the aphid to a new host plant.

C. Host Resistance

To differentiate the various abilities of hosts to withstand virus replication, terminology has been adapted to describe hosts that resist virus infection. A virus can infect one of two kinds of hosts: the susceptible host or the resistant host (Swenson 1969). The susceptible host allows a virus to infect it and multiply within its cells. The resistant host may allow a virus to infect it, however the virus cannot replicate within the host's cells (van der Want 1987). In order for a virus to infect a host (to replicate within a host's cells), it must be able to cross a series of barriers. If it is unable to cross any one of these barriers, it cannot infect the organism. There are four main barriers in GPA: (1) the uptake of the virus by the mouthparts during feeding, (2) passage through the gut wall, (3) passage in the body of the aphid via the hemolymph, and (4) the passage into the salivary glands and out into the next plant. The most crucial barrier is the passage through the salivary glands (Rochow 1969). This may be because aphids have no Malpighian tubules, and so the salivary glands may act in part as excretory organs (Rochow 1969). And this is one way the virus can be transmitted (circulatively or propagatively.

D. Viral Infections

The virus can infect the plant either locally or systemically. If the infection is local, the virus is confined to a specific area and it cannot spread throughout the plant. Local infection is to the plant's advantage, because if the part of the plant infected with the virus dies off, so will the virus. In the case of the systemic infection, however, the virus has spread throughout the plant and the plant is infected for its lifetime. Thus, vegetatively reproducing plants (like potatoes) often transmit the virus to their progeny. If the plant is infected as part of the initial inoculation into the field, it is known as a primary infection. If the plant was infected as a result of spreading from the primary infection throughout the field, it is known as a secondary infection (van der Want 1987).

Plants have many means of dealing with viruses and the symptoms they cause. In some cases, the plant will not show any obvious symptoms that it has been infected with a virus. This is called an asymptomatic infection, and could be beneficial because lack of symptoms shows that the host plant is tolerant of the virus (van der Want 1987). The other type of infection is called symptomatic, and this is what is generally recognized as a "viral infection", or disease caused by a virus. Generally, leaf rolling or net necrosis (tuber darkening in potatoes) are examples of symptomatic viral infection. Some species of plants may be more tolerant of certain viruses than others, and although this does not eliminate the virus, it limits the damage done by the virus (economic thresholds).

Another response is hypersensitivity to the virus, which is comparable to an allergic reaction in humans. Hypersensitive individuals have an advantage because they are so sensitive that the material where the virus is located may die off and thus localize the infection. Some plants wall off infected cells, sacrificing those cells and allowing the virus to kill them and then run out of cells to consume in the area and therefore die off. This localizes the infection, and eventually eliminates it. In this way the plant has a kind of field resistance to the virus (van der Want 1987). Other mechanisms used by plants include "pitching out", which involves a release of resins from the plant, or secondary compounds for use both in combating the virus and in keeping the vector away. Some plants have specialized hairs or trichomes in order to keep the vectors away, a few types of these can even release sticky or toxic substances to aid in eliminating vectors. Other plants have a thickened waxy layer (cuticle) to combat pests (and prevent water loss) which can assist the plant by making it more difficult for the vector to transmit the virus. Since most viruses are obtained by plants through some type of injury or another, the waxy layer can assist in keeping such injuries from occurring (de Bokx 1987).

E. Potato Viruses and Problems Associated With Them

Although seldom lethal in potato, viruses reduce both yield and quality (Nderitu and Mueke 1989). One example of a virus transmitted to potato by GPA is potato leafroll virus (PLRV). In a symptomatic infection, PLRV has an outward manifestation of potato phloem necrosis and potato leafroll. The results of infection by PLRV are smaller tubers and net necrosis, with losses up to and even exceeding half of the yield. Transmission of this virus is accomplished in a persistent manner solely by aphids in nature, with GPA as the most efficient vector (Peters 1987, Nderitu and Mueke 1989). There are many other viruses which GPA and other aphids transmit which are major problems. Some of these are shown in Table 1.1 (de Bokx 1987).

One point of interest is that potato viruses, according to Avery Rich (1983) are not transmitted through the true seed, but are tuber-perpetuated through clonal propagation. This means that every new cultivar should be free from viruses (in theory) until infected, which must happen during the testing and vegetative seed increase processes before they are sold to producers. However, the main source of viruses in the field is infected seed (Flanders *et al.* 1991), therefore infection of seed is occurring at some point.

Potato crops are protected from virus infection by three factors: maintaining and planting only virus-free certified seed potatoes, control of aphid virus vectors, and restricting the sources of overwintering viruses (reservoirs) to prevent future re infection the following year (Bishop 1967). With the loss of aldicarb and other soil-systemic insecticides, control of viruses relies largely on maintenance of virus-free certified seed potatoes, cultural practices (sanitation and rotation) and timing of foliar insecticide applications to control aphids at the optimum times (Reed *et al.* 1990). Reed *et al.* (1990) suggest strong insecticide control measures early in the season to eliminate the virus vectors from plants

Table 1.1--Comparison of aphid species which can act as vectors for four economically detrimental potato diseases while colonizing them (de Bokx 1987).

Aphid species	PLRV[a]	PVY[b]	PVA[c]	PVM[d]
Aphis craccivora Koch	.	–	.	.
A. gossypii-frangulae complex	–	±	–	±
A. nasturtii Kaltenbach	+	+	+	+
Aulacorthum solani (Kaltenbach)	+	±	±	+
Macrosiphum euphorbiae (Thomas)	+	+	+	+
Myzus ascalonicus Doncaster	+	–	.	.
Myzus persicae (Sulzer)	+	+	+	+
Rhopalosiphoninus latysiphon (Davidson)	±	+	+	.

[a]Potato Leaf Roll Virus, [b]Potato Virus Y, [c]Potato Virus A, [d]Potato Virus M.
+ aphid is a vector, ± aphid can be a vector, – aphid is not a vector, . not known.

newly emerging form the soil. Such procedures as these can help contain the spread of viruses.

V. Resistance

A. Mechanisms

There are many mechanisms of resistance in insects, they can involve metabolic detoxification or avoidance of mortality factors. There are four main classifications recognized as mechanisms of insecticidal resistance: behavioral, reduced or slowed penetration, altered target site, and metabolic mechanisms.

1. Behavioral

Behavioral resistance is defined by Lockwood *et al.* (1984) as "those actions, evolved in response to the selection pressure exerted by a toxicant". These behaviors can involve the use of refuges during pesticide sprays, avoiding certain types of foods at certain times, and other avoidance types of behaviors. A classic example of behavioral resistance is the avoidance of DDT-treated walls and ceilings of huts in Africa during the malaria eradication programs. Certain Anopheles mosquitoes would not light on the walls and ceilings of huts whereas other mosquitoes would land on the walls and ceilings. After treatment with DDT, those mosquitoes which would not land in the huts were selected for and survived in greater numbers than those which would land, illustrating the advantage of different behaviors for survivability (and the development of resistance).

2. Slowed Penetration

Penetration resistance involves the presence of barriers of some sort (generally physical) which prevent the uptake and concentration of toxicants to lethal levels. This can involve things like thickened cuticles or changes in the chemical structure of the cuticle on insects,

which prevent both absorption of toxins and injuries to the exoskeleton that promote absorption.

3. Altered Target Site

Altered target site types of resistance involve some type of change to the target macromolecule so that it is less sensitive to the action of the toxicant (Wierenga 1992). Altered target site insensitivity has been documented in several species (Hama and Iwata 1978, Devonshire and Moores 1984, Oppenoorth 1985) including the Colorado potato beetle (Wierenga 1992). An example of altered target site resistance is altered acetylcholinesterase (AchE) in the Colorado potato beetle, as an insecticide resistance mechanism against carbofuran (Wierenga 1992).

4. Metabolic

Metabolic resistance involves the prevention of toxicants from reaching lethal levels by enzyme degradation and sequestration or secretion. This type of resistance means that toxins are being attacked by some enzyme found in the body of an insect to produce an excretable metabolite that is not necessarily less toxic to the insect as the parent compound. In some cases production of an excretable metabolite first involves activation, which produces a more toxic substance that is then detoxified. An example of this is malathion detoxification: first malathion is activated to form malaoxon, then this is detoxified.

Metabolic resistance is one of the most important forms of insecticide resistance. There are four main types of metabolic resistance mechanisms. These mechanisms include the glutathion transferases, mixed function oxidases (MFO's), and esterases.

The Glutathion S-Transferases (GSH) are a family of compounds involved in detoxification. Substrates for GSH must be somewhat hydrophobic, must contain an electrophillic carbon atom, and they must react non-enzymatically with glutathion at some rate that is measurable (Klaassen 1986). These compounds can react with insecticides to form excretable metabolites (generally detoxification reactions Williams and Weisberger 1986) and thus enable the insect to be resistant to that compound.

The Cytochrome-P450-containing monooxygenases, mixed function oxidases (MFO's or cytochrome-P450) are part of a coupled enzyme system that contains both cytochrome-P450 and NADPH-cytochrome-P450-reductase (Sipes and Gandolfi 1986). This is one of the most important groups of enzymes involved in the biotransformation of toxins (such as insecticides) (Sipes and Gandolfi 1986). The MFO system has many functions, including catalyzing the oxidation of various compounds. Some examples are desulfuration, oxidative dehalogenation, sulfoxidation, and deamination of toxins (Sipes and Gandolfi 1986). Often compounds are activated by this system, resulting in oxon compounds, which may be more toxic than their parent compound. A common mechanism of resistance in insects, MFO's have not been implicated in GPA insecticide resistance.

Esterases are compounds that hydrolyze ester bonds to form a carboxyl group and an alcohol (Sipes and Gandolfi 1986). Esterases commonly hydrolyze bonds in such substances as organophosphate insecticides. There are several classifications for esterases: (1) arylesterases, which preferentially hydrolyze aromatic esters, (2) carboxylesterases (or carboxyesterases), which hydrolyze aliphatic esters, (3) acetylesterases, which preferentially hydrolyze acetyl esters, and (4) cholinesterases, which hydrolyze esters that have choline as the alcohol moiety (Sipes and Gandolfi 1986). These classifications do allow for some overlap, however, as carboxyesterases also catalyze the hydrolysis of aromatic esters.

Increased metabolism by nonspecific esterases is a common mechanism of resistance to organophosphate insecticides in insects (Oppenoorth 1985), especially in homopterans, such as aphids and whiteflies. The only known mechanism of metabolic resistance in GPA is carboxyesterase hydrolysis of the toxin (Devonshire and Moores 1982). This mechanism is due to amplification of the E4 gene which causes overproduction of esterase-4 (FE4) or E4 (due to an A1,3 translocation) (Devonshire 1977, Devonshire and Sawicki 1979, Devonshire and Moores 1982, Devonshire *et al.* 1986). Resistance due to the increased FE4 is stable and non-inducible (Blackman and Takada 1975, Devonshire *et al.* 1986). Resistance associated with the A1,3 translocated gene (E4 enzyme) is not stable (Blackman *et al.* 1978), due to the fact that the gene can be methylated and thus produce lower levels of carboxyesterase (show a more susceptible phenotype) (Field *et al.* 1989) after several generations in the absence of selection (Bauernfeind and Chapman 1985, Georghiou 1963, Dunn and Kempton 1966). This revertancy and recovery (with selection) is associated only with the A1,3 translocated gene (Ffrench-Constant *et al.* 1988), which is related to extremely high levels of resistance (Devonshire and Sawicki 1979, Sawicki *et al.* 1980, Devonshire *et al.* 1986). Kirknel and Reitzels (1973) and Dunn and Kempton (1966) did not note any revertant tendencies in their populations, however the time of revertancy seems to vary with population, perhaps it is due to genotypes or to external factors other than chemical selection.

B. Green Peach Aphid Resistance Mechanisms

Insecticide resistance was first reported in aphids in 1928 (Boyce 1928). At this time it was artificially induced in *Aphis gossypii* Glov. by selection with hydrocyanic acid. Resistance in the field was reported by Michelbacher *et al.* in 1954 with *Chromaphis juglandicola* (Kalt.) to parathion. Insecticide resistance was first documented in GPA in 1954 by Anthon. Later, Shirk (1960), Georghiou (1963), Gould (1966), Wyatt (1967),

FAO (1967, 1969), Baranyovitz and Ghoush (1969), and Hurkova (1970) confirmed the observation that GPA had begun to exhibit insecticide resistance.

In GPA, resistance is correlated to elevated carboxylesterase level (Needham and Sawicki 1971, Beranek 1974, Devonshire 1975, Devonshire and Needham 1975). However, this is not true in all insect species. In the resistant mosquito, higher carboxylesterase activity was associated with low β–naphthyl benzoate hydrolysis and the opposite was true in the case of the two-spotted spider mite (Motoyama and Dauterman 1974). According to Needham and Devonshire, in all GPA populations tested, resistance was associated with increased carboxylesterase activity (Needham and Devonshire 1974). One interesting thing about this is that it certainly seems to refute the concept of Macro evolution, while supporting Micro evolution. If Macro evolution were the driving force in insecticide resistance, one would expect all insects to have carboxyesterase resistance mechanisms, MFO's, and the like. Additionally, one would expect all insects with carboxyesterases to have similar mechanisms, so that one test would work on all resistant species. But this is not the case, therefore, it looks like evolution on the microscale has more involvement in this case than Macro evolution.

C. The Problem

Green peach aphids are a serious pest in both Michigan and the rest of the United States. In Michigan alone, GPA are estimated to have caused over $0.5 million worth of damage annually, chiefly to the seed potato industry. The conventional and once successful current measures of control were soil-applied granular systemic insecticides united with foliar applications. These chemicals are especially important in seed potato production because other biological control or natural control mechanisms are not sufficient to prevent PLRV and PVY transmission at a high enough level.

Several years ago, GPA were relatively well controlled with aldicarb (Temik®), and other insecticides. It appeared that the agricultural industry had achieved a realistic technique for controlling GPA above the economic threshold. Unfortunately, aldicarb and other soil-applied granular insecticides have been implicated in groundwater contamination. Some of these more than twenty insecticides have been removed by the EPA, and others have been removed voluntarily by their producers. Since aldicarb and other granular insecticides are no longer available most growers have resorted to foliar applied insecticides to control GPA. These foliar insecticides involve a greater frequency of spraying and a relative lack of efficacy (when compared to aldicarb). The more frequent sprayings can lead to a greater opportunity for resistance development thus compounding the problem.

Experience demonstrates that the current measures for controlling GPA lead to resistance development. With the system as it stands now, insecticide-induced resistance is a major problem not only because of selection due to spraying, but because the natural enemies of such pests are lost due to higher susceptibility to sprays. A different strategy must be employed to control the development of resistance in agricultural systems. IPM is one effective system that integrates population ecology and pest management (Smith 1970). The goal is to use pesticides effectively and yet maintain the lowest possible level of resistance in populations, while preserving the environment. (Waterhouse 1969). According to Smith, this can be done by avoiding all but necessary applications and utilizing as many alternative control procedures as possible so that agriculture is not entirely dependent upon insecticides (Smith 1970). Additionally, monitoring is one of the most effective tools at our disposal. Knowledge of when sprays would be effective and when they would not is a necessary component of and integrated management system, and as much knowledge about a pest population as can be obtained would be most beneficial.

VI. Goals and Objectives

A. Overall Goal

The overall goal of this study is to develop and test a field-monitoring tool useful as a resistance diagnostic technique by implementing currently available technology into a simple protocol, by which resistance can be diagnosed quickly and reliably by field workers.

B. Objectives:

1) to develop an *in vitro* carboxyesterase-based insecticide resistance monitoring system for GPA using a modification of the colorimetric system developed by Gomori in 1953.

2) to bioassay (*in vivo*) using standard slide dip techniques and probit analysis to determine the actual resistance level of eight GPA strains using insecticides from each of the three major classes (organophosphates, carbamates, and synthetic pyrethroids).

3) to correlate the carboxyesterase levels in Objective 1 with resistance levels from the probit mortality assays in Objective 2 using polyacrylamide gel electrophoresis and a microplate assay.

C. Hypotheses

Four relevant hypotheses were developed for testing this work:

$\mathbf{H_1}$: Strains of GPA with different LC_{50} values will not have the same carboxyesterase enzyme activity (amount).

$\mathbf{H_2}$: Resistant and susceptible strains of GPA will show quantitative and qualitative differences in polyacrylamide gel electrophoresis of enzymes.

H_3: Resistant and susceptible insects show quantifiable differences in both microplate carboxyesterase assays and portable carboxyesterase assays.

H_4: There is a relationship between carboxyesterase level and resistance in populations of GPA in the United States.

D. Thesis Outline

Figure 1.1 outlines the overall format of this study. First, a literature review is necessary to gain an understanding of the problem. Next, in order to completely evaluate the *in vitro* field resistance monitoring tool, three steps must be evaluated. The first of these steps is to conduct a national resistance survey for an overall concept of resistance frequencies as well as to find suitable strains for further investigations. Second, the portable carboxyesterase tool was developed using the microplate assay as a design guide. Third, the portable tool was evaluated on the basis of the microplate assay, polyacrylamide gel electrophoresis, and an *in vivo* bioassay for resistance. This data next enabled economic, labor, accuracy, and simplicity assessments of each test and then a ranking of each tool as a field diagnostic device for carboxyesterase-based insecticide resistance.

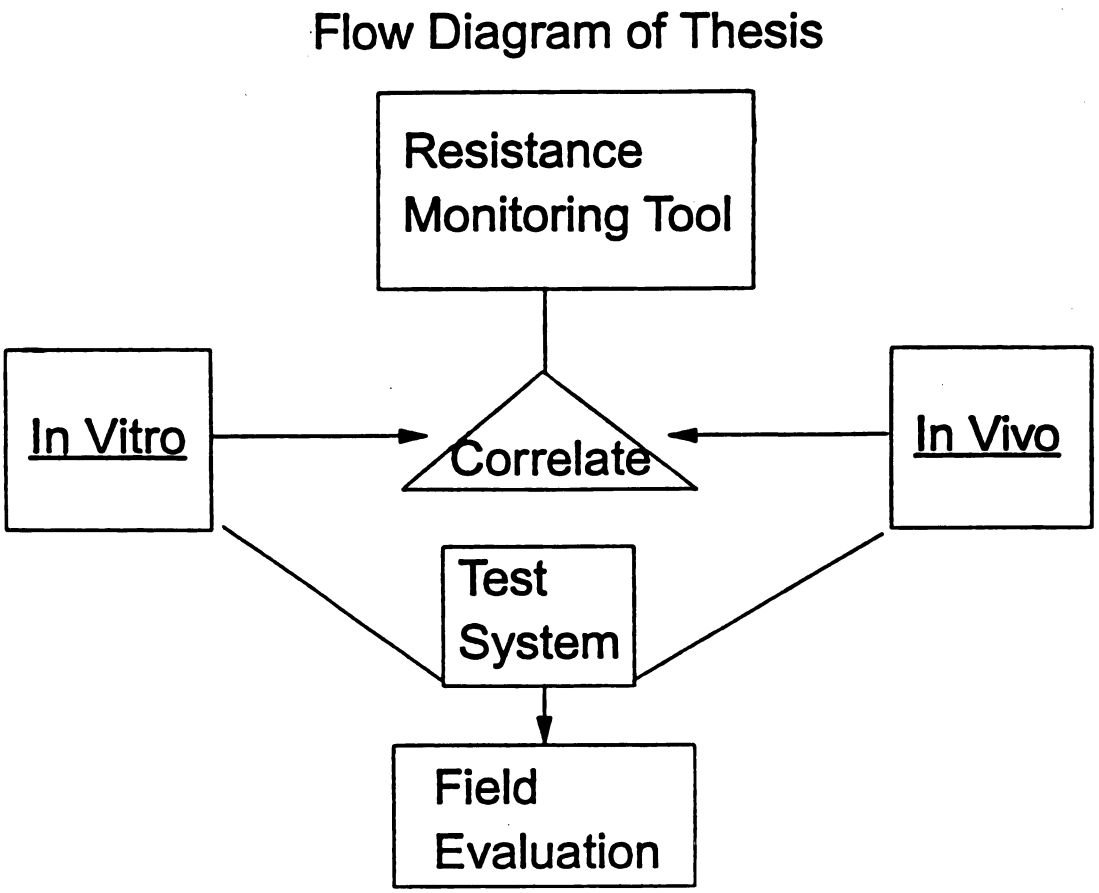


Figure 1.1--Schematic diagram of thesis research project.

CHAPTER 2

IN VITRO EVALUATION OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE

CHAPTER 2. *IN VITRO* EVALUATION OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE.

I. Introduction

A. Green Peach Aphid Identification

Green peach aphids (GPA) have several salient external anatomical characteristics which aid in distinguishing them from other members of the Homopteran Family Aphididae. GPA have a non-pigmented dorsal abdomen (that usually looks yellowish) and long antennae with six or more segments. The terminal antennal segment is longer than the base of the last segment (Blackman and Eastop 1984). Figure 2.1 shows some of the basic external anatomy of aphids, including two important structures helpful for identification purposes: the cauda and the siphunculi. Well-developed, converging tubercles are useful characteristics for differentiation between GPA and other aphids, and some comparisons are depicted in Figure 2.2. Figure 2.2 (e) is a pictorial example of the converging tubercles found on GPA. The shape of the cauda is another character of good taxonomic value, and Figure 2.3 is an illustration of a tongue-shaped cauda as opposed to a rounded, helmet, or knobbed shaped cauda. GPA siphunculi are longer than the cauda and pale (or dark only on distal half), usually tapering or tubular, and about four times as long as the basal diameter (Blackman and Eastop 1984). An example of such siphunculi is found in Figure 2.4 (d) (Blackman and Eastop 1984).

B. Resistance evaluations

Over the past few decades, the amount of crop lost annually to insects has risen to thirteen percent of the total (May and Dobson 1986). Even with increased pesticide usage and better chemistry, insects still manage to take their toll on crops. This is due to the phenomenon of insecticide resistance which is found in over 447 species of insects (Georghiou 1986). In the past thirty years the judicious use of pesticides has been

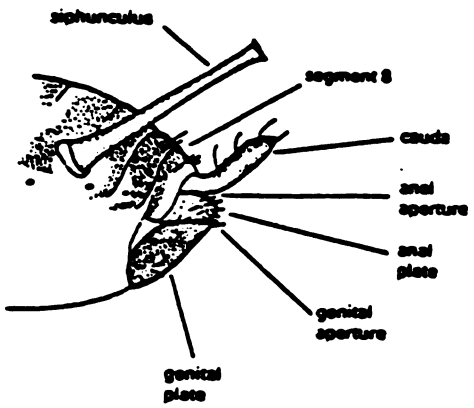


Figure 2.1--Basic external anatomy of the abdomen of an aphid (Aphididae) useful for identifying different Families. Taken from Blackman and Eastop (1984).

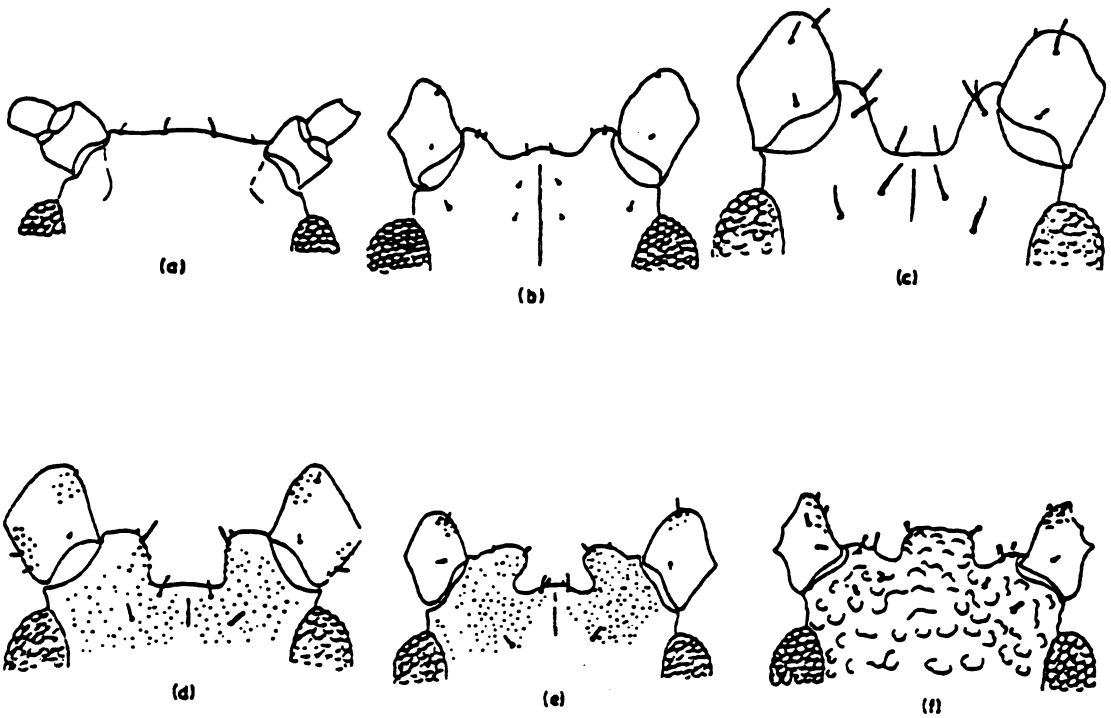


Figure 2.2--Dorsal views of different types of aphid antennal tubercles useful for comparison between Families: (a) undeveloped tubercles, (b) diverging tubercles, (c) well-developed diverging, (d) parallel, (e) converging (*Myzus persicae*), (f) well-developed median frontal projection. Taken from Blackman and Eastop (1984).

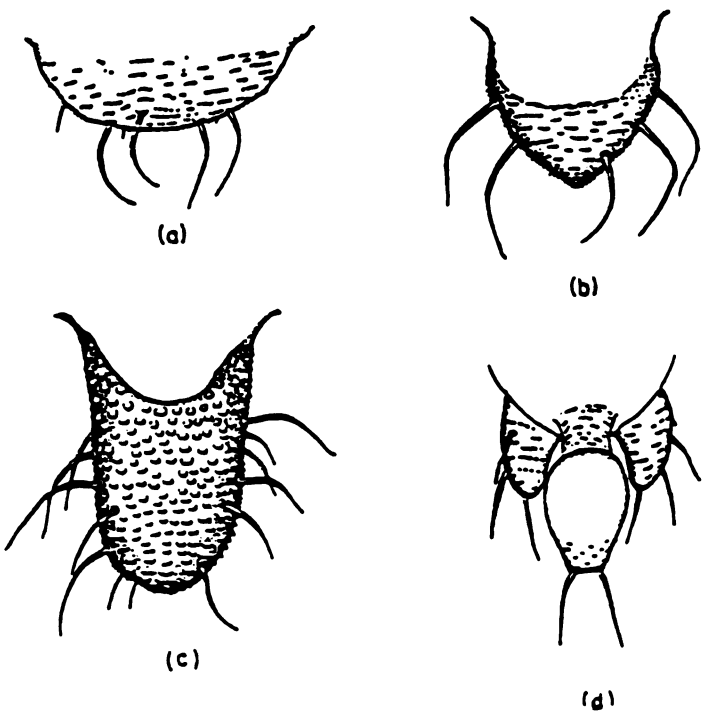


Figure 2.3--Different shapes of cauda found in Aphididae: (a) broadly rounded, (b) helmet shaped, (c) tongue shaped, and (d) knobbed with a bilateral anal plate . Taken from Blackman and Eastop (1984).

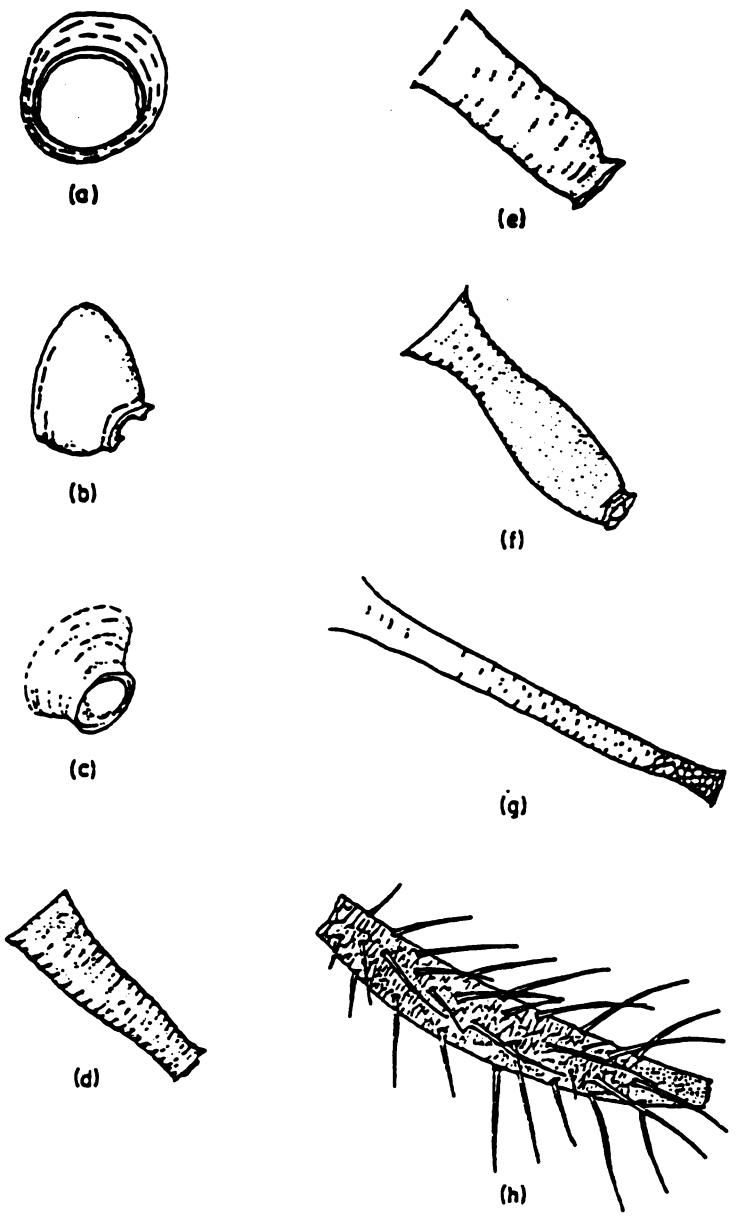


Figure 2.4--Aphid siphunculi of various shapes: (a) pore-like, (b) mammariform, (c) truncate, (d) tapering, (e) swollen proximally, (f) clavate, (g) with a subapical zone of polygonal reticulation, and (h) with sharp spiky hairs. Taken from Blackman and Eastop (1984).

formulated into a mechanism for controlling resistance development called Integrated Pest Management (IPM). One of the key concepts of IPM is the use of pesticides only when damage caused by pest species passes a previously defined limit called the economic threshold (Hammock and Soderlund, 1986). Routine resistance monitoring must be done to quantify pest numbers or damage; it is valuable to have an estimate of the frequency of resistant individuals present in a population (Hammock and Soderlund 1986). Levin (1986) asserts that continuous monitoring of resistance frequencies should be an integral part of all programs to manage resistance. A tool for assessing the insecticide resistance levels of samples is necessary to enable such monitoring.

Most insecticide resistance evaluations are determined by either field failure of insecticides on crops or by bioassays of the insects in a laboratory (Sawicki *et al.* 1977, Brown and Brogden 1987). Both of these methods are extremely time consuming and involve a large number of insects (Devonshire and Needham 1975). According to Sawicki *et al.* (1977), routine biochemical methods of resistance detection are rare and seldom feasible. For this reason, many researchers sought a less labor-intensive and costly method by which to diagnose insecticide resistance both in the field and in the laboratory (Gomori 1953, Brown and Brogden 1987, Brogden 1988, Sawicki *et al.* 1977, van Asperen 1962, Brogden and Dickinson 1983, Pasteur and Georghiou 1981). A rapid biochemical method of resistance detection would be most valuable for such determinations (Sawicki *et al.* 1977, Hammock and Soderlund 1986).

Increased detoxification by nonspecific esterases is a common mechanism of resistance to organophosphate insecticides in insects (Oppenoorth 1985), particularly Homoptera. In GPA, the only known metabolic mechanism of resistance is enzymatic hydrolysis and sequestration of insecticidal esters by carboxyesterases (Needham and Sawicki 1971, Beranek 1974, Sawicki *et al.* 1978, Takada 1979, Devonshire and Moores 1982) also

called carboxylester hydrolases by Brown and Brogden (1987). Mixed function oxidase (MFO) activity may have some impact on resistance, however MFO's have not been implicated in insecticide resistance in strains of GPA (Beranek and Oppenoorth 1977). Devonshire (1973) suggests that this lack of MFO detectability may be due to inhibitors and predicts that these enzymes may still be present. Although MFO's may not be attributed as resistance mechanisms in GPA, they still play a large role in chemical detoxification (oxidation) in other insects and are required in some cases to enable certain carboxyesterases to react more effectively with compounds.

Carboxyesterases hydrolyze carboxyl esters and amides, such as some organophosphate insecticides. This usually results in the formation of a non-toxic (or less toxic) acid, although in some cases enzyme hydrolysis can produce a more toxic secondary compound (Motoyama and Dauterman 1974, Brown and Brogden 1987). The properties of carboxyesterases vary considerably with the species of insect studied (Motoyama and Dauterman 1974). For instance, houseflies show a negative correlation between carboxyesterases and organophosphate insecticide resistance (Van Asperen and Oppenoorth 1959). In GPA, resistance shows a positive correlation with carboxyesterase levels (Needham and Sawicki 1971, Al Khatib 1985, Pasteur and Georghiou 1989). This resistance to organophosphate and carbamate compounds is associated with a quantitative increase in the amount of carboxyesterase, not in an increased affinity of the enzyme for a substrate (Devonshire 1978, Devonshire and Sawicki 1979, Devonshire and Moores 1982). This quantitative increase is due to the amplification on the E-4 gene (Devonshire and Sawicki 1979, Devonshire and Moores 1982), and is associated with the increased ability of aphid homogenates to hydrolyze naphthyl acetate (Needham and Sawicki 1971, Devonshire 1989).

C. Microplate Assay Introduction

The ability of carboxyesterases to hydrolyze ester compounds to acids can be exploited to develop a sensitive test for resistance due to elevated esterase levels. Several of these carboxyesterases are able to degrade naphthyl esters as well as insecticidal esters and this property can be used to identify resistant insects quantitatively by a colorimetric enzyme assay type of test (Gomori 1953, Needham and Sawicki 1971, Devonshire and Needham 1975, Pasteur and Georghiou 1981, Brogden and Dickinson 1983, Raymond *et al.* 1985, Hemmingway *et al.* 1986, Brogden and Barber 1987, Brown and Brogden 1987, Brogden 1988, Moores *et al.* 1988, Pasteur and Georghiou 1989, Dary *et al.* 1991). There is a positive correlation between insecticide resistance in GPA and increased hydrolysis by carboxyesterases, and this property is what can be used to monitor resistance (Needham and Sawicki 1971, Al Khatib 1985).

In 1953, Gomori published a study of a method for the quantitative analysis of esterases based on a colorimetric change resulting when naphthol produced by the reaction of esterases hydrolyzing naphthyl acetate coupled with an azo-dye (Gomori 1953). Gomori's assay has been modified since by Brown and Dickinson (1983) and Dary *et al.* (1990) to detect general esterase activity and by Devonshire *et al.* (1986), on a more specific level. Pasteur and Georghiou describe a filter paper test (1981) and an improved filter paper test for detection and quantification of increased esterase activity (1989). This test is accurate, but difficult to use in field tests and for determination of intermediate levels of resistance (Pasteur and Georghiou 1989). The method involves two reactions: the first is the reaction of the insect homogenate (including carboxyesterases) with α-naphthyl acetate to form α-naphthol and acetic acid. In a second reaction the α-naphthol couples with Fast Garnet GBC (or other azo-dye) to give a colored precipitate (or solution) visible to the naked eye. This color change is then read by a densitometer or microplate reader (Pasteur and Georghiou 1989, Brown and Brogden 1987).

Other researchers have used similar procedures. Van Asperen studied housefly esterases using a very similar methodology (van Asperen 1962, Oppenoorth and van Asperen 1961). To stain the naphthol produced by the esterase-naphthyl acetate reaction, Van Asperen used a diazoblue-sodium laurylsulphate solution. The sodium lauryl sulfate solution denatures the enzyme and causes the color-change reaction to proceed more quickly and the product to be more stable. This is similar to the effects of sodium dodecyl sulfate (SDS) and other detergents.

The reaction sequence of carboxyesterases with naphthyl acetate and then the coupling with *O*-dianisidine for a color change is shown in Figure 2.5. This reaction has been used by many researchers to test for carboxyesterase-based insecticide resistance levels in insects (Gomori 1953, Pasteur and Georghiou 1981, 1983, Brogden *et al.* 1983, 1984). Pasteur and Georghiou (1981) also exploited this reaction to test for resistance in mosquitoes using a squash test. Brogden *et al.* (1983) used a microtitre plate assay for measure esterase activity and protein levels in very small samples several different times (Brogden 1984).

The level of carboxyesterase activity estimated densitometrically appears to be related to the level of insecticide resistance (at LC_{90}) in some insects (Sudderuddin 1973, Beranek 1974, Devonshire and Needham 1975, Devonshire 1975, Pasteur and Georghiou 1989) and is probably responsible for organophosphate degradation (Beranek and Oppenoorth 1977). Although Oppenoorth and Voerman (1975) observed no correlation between esterase activity and insecticide resistance, Pasteur and Georghiou (1989) did observe a significant linear increase in staining intensity as esterase concentration increased, up to optical densities of 0.45. The test appears to be compatible for GPA because these insects have the same organophosphate-detoxifying esterases that hydrolyze naphthyl acetate (Needham and Sawicki 1971, Brookes and Loxdale 1987).

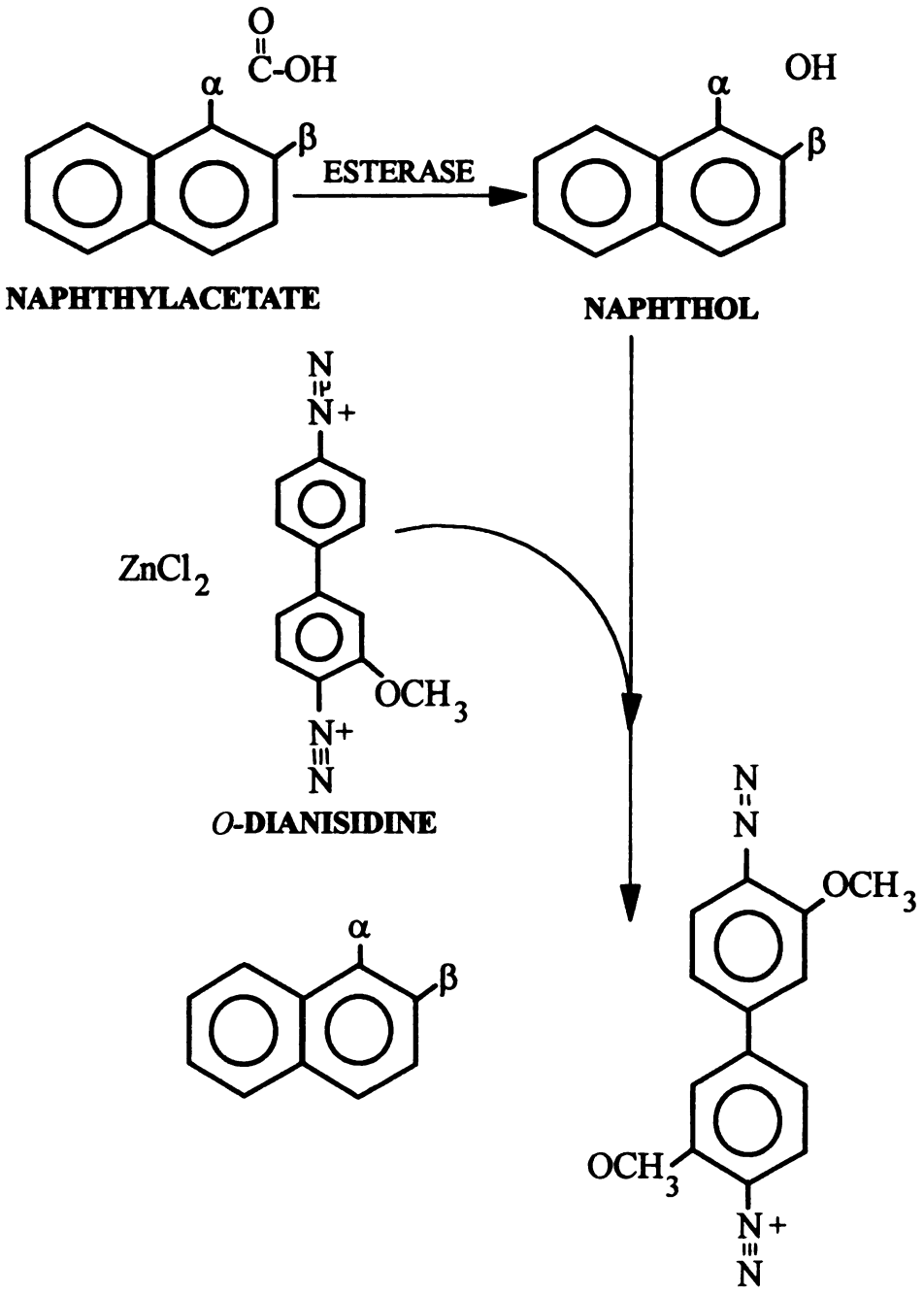


Figure 2.5--Structures and sequence for the reactions of carboxyesterases with naphthylacetate to yield naphthol. Naphthol reacts with *O*-dianisidine (tetrazotized) to yield a colored solution.

The premise upon which this test is based is the fact that insecticide-resistant GPA have greater total esterase levels than susceptible insects (Needham and Sawicki 1971, Devonshire 1975). Total carboxylesterase activity is readily determined in individual GPA, and differences are distinct, provided that activity is corrected for by the weight of individual aphids (Sawicki *et al.* 1977) or total protein concentration. However, in other insects, some scientists have had problems accurately differentiating intermediate esterase activity level strains in field surveys (Pasteur and Georghiou 1989). The increased carboxyesterase levels are associated with insecticide resistance (Devonshire 1975) and have been documented as the only known mechanism for GPA metabolic insecticide resistance (Devonshire and Moores 1982).

C. PAGE Introduction

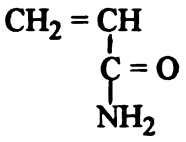
Any charged group or ion will migrate in an electric field (Hames 1981). Since proteins carry a net charge at any pH other than their isoelectric point, they will migrate and the rate of migration depends on the ratio of charge and mass of that protein (Hames 1981). The application of an electric field to a mixture of proteins in solution will result in the different proteins migrating towards one or the other of the electrodes at different rates, depending upon their charge (thus depending on the pH of the buffer, as well) (Hames 1981). This property is the basis of polyacrylamide gel electrophoresis (PAGE).

A supporting medium can be used rather than liquid solution (free electrophoresis) to minimize the disruptive effects of such things as convection (caused by heating) and diffusion (which would prevent effective separation) and to achieve stable, permanent separation of the proteins. Electric current is run through the medium, and proteins migrate along this stable matrix according to charge and density. The resulting banding patterns are stable and permanent and are easily evaluated quantitatively and qualitatively (Hames 1981).

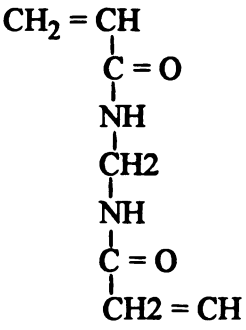
Although various media can be used for electrophoresis, polyacrylamide is an excellent one because it not only prevents diffusion and convection, it interacts physically with the proteins for a molecular sieving effect, separating proteins physically by size, as well as by charge and mass. This sieving effect is can be enhanced because it depends on the pore size of the gel chosen, which in turn depends on the gel concentration used and the type or percentage crosslinker used (Hames 1981).

Polyacrylamide is a polymer (repetitive groups of multiple units of a monomer) made of acrylamide monomer (single acrylamide units) formed into long chains and cross linked with various substances, in this case *N,N'*-methylene bisacrylamide (Bis). The structures of acrylamide and Bis, as well as the structure of polyacrylamide can be found in Figure 2.6. The polymerization reaction occurs as a result of the crosslinkers reacting with functional groups (double bonded areas) at the ends of chains (Hames 1981). The polymerization reaction is catalyzed by either ammonium sulfate or riboflavin. When riboflavin is used, light is necessary to initiate polymerization (by photodecomposition of riboflavin to form free radicals) and *N,N,N',N'*-Tetramethylethylenediamine (TEMED) is usually added to insure polymerization.

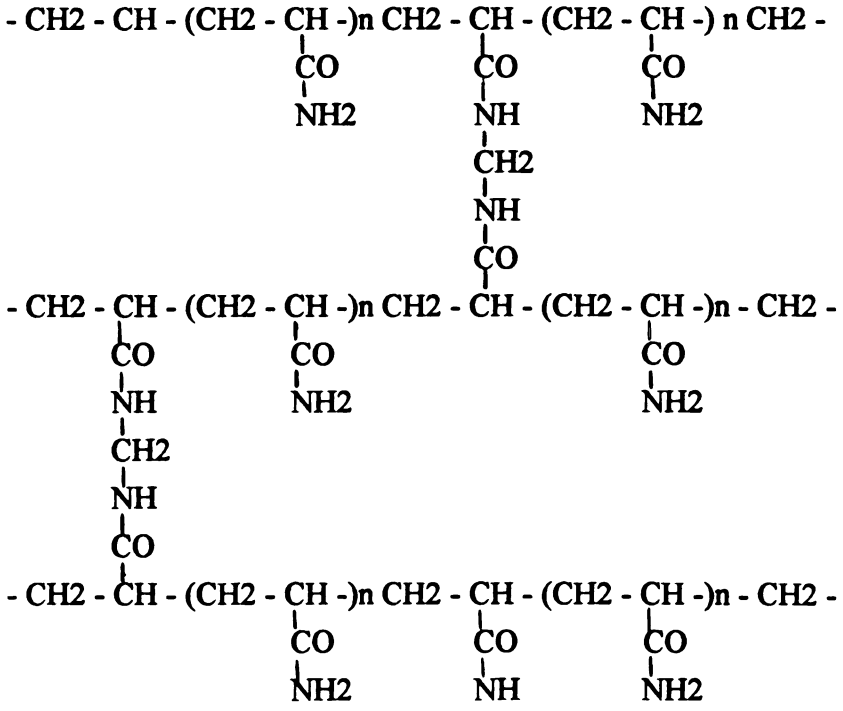
PAGE when used as a native gel is a useful tool for the separation of proteins and active enzymes (Kasciinitz *et al.* 1968, Zacharius *et al.* 1969). Some researchers have used the PAGE-SDS system, which denatures proteins, to study carboxyesterases in GPA. This system does not provide sufficient information about enzymes' activity levels and the effects of insecticides and other inhibitors on the various bands found in the gel. For this reason, native gel PAGE was conducted to determine the banding patterns of several strains of GPA and to elucidate the effect of insecticidal compounds on the enzymes ability to metabolize them. These techniques offer valuable information for strain resistance level characterization and evidence that the enzymes responsible for reaction with the α-



Acrylamide



N,N' - methylene bisacrylamide



Polyacrylamide gel

Figure 2.6--Structures of Acrylamide, *N,N'* - methylene bisacrylamide, and Polyacrylamide gel. (Hames and Rickwood 1984)

naphthyl acetate to form α-naphthol are the same enzymes responsible for breakdown of insecticides, and subsequently responsible for insecticide resistance.

E. Portable Introduction

The ability of carboxyesterases to hydrolyze ester compounds to acids has been used in the past to develop colorimetric tests for resistance diagnosis (Gomori 1953, Needham and Sawicki 1971). Many of these tests must be conducted by specially trained technicians, close to laboratory facilities, and involving a great deal of time and expensive materials or equipment. Some researchers have made efforts to design a simple, field-oriented, inexpensive assay method by which resistance can be diagnosed by non-scientist laborers (Pasteur and Georghiou 1981, Pasteur *et al.* 1984). A monitoring tool of this type would be extremely helpful to growers and would make great strides in assisting IPM decision-makers in resistance monitoring and subsequent pest control on potato.

Simplicity is a key element in developing a portable test. Unfortunately, many field-oriented tests are still too complicated. The development of a novel, extremely simple, highly effective resistance level evaluation tool was still needed. The technique designed here was developed as an extension of the carboxyesterase characterization study and involves the use of a portable colorimetric carboxyesterase assay for detection of field insecticide resistance levels. The microplate assay developed in the first section of this chapter was used to a large extent for the design of the test. The portable assay also needs to be evaluated by several different testing methods for accuracy, precision, economics, labor time, and simplicity.

II. Materials and Methods

A. Strains

For this study a strain is defined as a population of individuals kept in culture that originated from a specific area of the United States. Generally strains were begun from thirty or more GPA. However, some populations were reared from only one or a few aphids. In addition, as with any laboratory-reared colony, there were bottlenecks in which some of the population died off due to an inability to survive under laboratory conditions, parasitism, or plant quality differences. Additionally, each strain has a specific insecticide resistance level, which was measured in the laboratory and used as a means to classify them.

Over one hundred and twenty telephone calls and letters were sent to potential cooperators in targeted areas of potato production in the continental United States. Potato (and selected other crop) growing regions in the following states were solicited: Alabama, California, Colorado, Delaware, Florida, Georgia, Idaho, Indiana, Iowa, Maine, Maryland, Massachusetts, Michigan, Minnesota, Nebraska, New Hampshire, New York, North Carolina, Ohio, Oklahoma, Oregon, Pennsylvania, Rhode Island, South Carolina, Tennessee, Texas, Virginia, Washington, Wisconsin, and Wyoming. Additionally, some strains were hand-collected by persons employed by or associated with our laboratory.

Shipped with the appropriate USDA and Animal Health Inspection Service permits, GPA were sent by cooperators on plant material in plastic (50 ml) centrifuge tubes. Parafilm™ covered the opening, containing the aphids yet allowing for gas exchange. The tubes were contained within brown cardboard shipping containers insulated with paper towels or tissue paper. Upon receipt of shipping containers with GPA from cooperators, the insects were removed from the shipping container, identified positively as GPA using the

diagnostic characters identified previously, and placed on clean potato plants contained in two-liter plastic cages.

Upon arrival, all strains were given a code. The most important factors involved in the code were the crop and the location strains were collected from, including city and state. The code designation can be found in Table 2.1. Peach, the insects' primary host, and potato, the secondary host, were the two crops the study focused on.

Samples of GPA from each of the eight major colonies listed in Table 2.2 were mounted on slides for identification. Initially, GPA were soaked in a 10% potassium hydroxide solution overnight and warmed for three hours to degrade soft tissues. Next, GPA were transferred to a series of four serial increasing concentrations of ethanol (70-100%) to remove water from the insects' tissues. The GPA were subsequently transferred to clove oil for the addition of color, which enabled easier microscope viewing. They were then mounted in Euparol® fixative (ASCO Laboratories, Manchester, England) between a microscope slide and cover, and allowed to dry for a few weeks (Blackman and Eastop 1984, Bob Kriegel personal communication). Finally, they were sent to the USDA/ARS Systematic Entomology Laboratory in Beltsville, Maryland for identification to species (special thanks to Manya Stoetzel and Mary Lacey-Theison for identification services).

GPA were reared on insect and disease-free Superior potato plants (Belding, Michigan). Whalon and Smilowitz (1978) observed that the optimum temperature for rearing GPA with highest survival (87.2%) is 23.9°C. For this reason, GPA were cultured in a greenhouse with a temperature range of approximately 21 to 25°C with a light/dark ratio of 16:8 hours.

Table 2.1--Code designation translations for green peach aphid strains.

	DESIGNATION	FULL NAME
Location:	MOXEE-WA	Moxee, WA
	PRESQUE-ME	Presque Isle, ME
	SALINAS-CA	Salinas, CA
	WOOSTER-OH	Wooster, OH
	STRATHAM-NH	Stratham, NH
	MONTC-MI	Montcalm, MI
	PULLMAN-WA	Pullman, WA
	NEWMAN-CA	Newman, CA
	IMPCO-CA	Imperial County, CA
	GAINESVL-FL	Gainesville, FL
	WILDER-ID	Wilder, ID
	PARMA-ID	Parma, ID
	BINGHAM-ID	Bingham County, ID
	MOSCOW-ID	Moscow, ID
	UNKNOWN-ID	Unknown, ID
	GRDRAPIDS-MI	Grand Rapids, MI
	CARLETON-MI	Carleton, MI
	MONROE-MI	Monroe, MI
	RKINGHM-NH	Rockingham County, NH
	BERRIEN-MI	Berrien County, MI

Table 2.1--Continued.

	DESIGNATION	FULL NAME
Location:	GRATIOT-MI	Gratiot County, MI
	IDAHO	Idaho
	ANSON-MI	Anson, MI
	MUNGER-MI	Munger, MI
	STJOHN'S-MI	St. John's, MI
	AUGRES-MI	Au Gres, MI
	TRAVERSE-MI	Traverse City, MI
	BRDGPORT-NB	Bridgeport, NB
	ALLIANCE-NB	Alliance, NB
	HEMINGFD-NB	Hemmingford, NB
	BEAUFORT-NC	Beaufort, NC
	VANORA-OR	Vanora, OR
	HERMISTON-OR	Hermiston, OR
	CENTER-PA	Center County, PA
	MARYHIL-WA	Maryhill, WA
	QUINCY-WA	Quincy, WA
	YAKIMA-WA	Yakima, WA
	TOPPEN-WA	Toppenish, WA
	RVRSIDE-CA	Riverside, CA
	WALLULA-WA	Wallula Junction, WA
	MADISON-WI	Madison, WI

Table 2.1--Continued.

	DESIGNATION	FULL NAME
Crop:	P1	Peach
	P2	Potato
	T	Tobacco
	O	All other crops

Table 2.2--Strains, cooperators, host plants, and locations of populations on file in the Entomology Museum used for identification of strains as green peach aphid.

Strain	Cooperator	Host	Location
MOXEE1-WA-P1	L. Fox	Peach	Moxee, WA
PRESQUE-ME-P2	G. Sewell	Potato	Presque Isle, ME
SALINAS-CA-O	L. Fox	Other	Salinas, CA
WOOSTER-OH-P2	C. Hoy	Potato	Wooster, OH
STRATHAM-NH-P2	M. Campbell	Potato	Stratham, NH
MONTC1-MI-P2	M. Otto	Potato	Montcalm, MI
PULLMAN-WA-O	T. Mowry	Other	Pullman, WA
WILDER1-ID-P1	L. Fox	Peach	Wilder, ID

B. Microplate Survey

Standard curve data were determined by seven serial dilutions of α– and β–naphthol in potassium phosphate buffer ($KH_2PO_4 + K_2HPO_4$, 0.05 M, pH 7.0) ranging from 1 ng/10 µl to 20 ng/10 µl similar to a procedure used by Brogden *et al.* (1983, Brogden 1984). 10 µl of each naphthol serial dilution were pipetted into a microtitre plate and 190 µl of potassium phosphate buffer were added. No incubation period is necessary, and the 50 µl of freshly made *O*-Dianisidine (0.3% *O*-dianisidine dilute in H_2O), also called Fast Blue B (Aldrich Chemical Co., Milwaukee, Wisconsin) was added. Three replicates were used for each dilution, and the entire assay was conducted in full six times. Microtitre plates were read on an automated Microplate minireader™ MR590 (Dynatech Laboratories Inc., Alexandria, Virginia) at 600 nm (A_{600}) for α-naphthol and 550 (A_{550}) for β-naphthol. These data were then analyzed by regression analysis (Cohen and Cohen 1983) using Systat (Systat Inc., Evanston, Illinois) and the Systat Manual (Wilkinson 1990).

Protein standard curve data were obtained by the serial dilution of 10 mg Bovine Albumin Serum (BSA) per ml potassium phosphate buffer. Controls consisted of 50 µl of potassium phosphate buffer and 200 µl of dilute (1:4) BioRad Protein Reagent, which is a type of Coomassie Brilliant Blue stain (Bradford 1976). Eight serial dilutions ranged from 0.1 µg/10 µl –10 µg/10 µl. 10 µl of each protein dilution was pipetted into a well of a microtitre plate, 40 µl of potassium phosphate buffer was added to each well, and 200 µl of diluted BioRad Protein Reagent was added. Three replicates were taken of each BSA dilution and the microtitre plate was read after five minutes on a Dynatech automated minireader™ MR590 (or Dynatech microplate reader) at 600 nm (A_{600}). The entire assay was replicated in full six times and the data were analyzed by Systat using multiple regression analysis (Cohen and Cohen 1983).

The methods of Gomori (1953) were used to examine each strain of GPA (with modifications similar to Brogden *et al.* 1983) as follows: controls were replicated six times and consisted of 50 µl potassium phosphate buffer and 150 µl either α- or β-naphthyl acetate (consistent with the rest of the plate) added to the first six wells in the A row of a 96-well microtitre plate. After a ten minute incubation (in concert with the rest of the plate) 50 µl *O*-dianisidine (0.3% *O*-dianisidine diluted in H_2O) were added and the plate was incubated again for ten minutes. Controls were read as blanks in the Dynatech minireader at either 600 nm (α) or 550 nm (β) prior to the rest of the plate.

Individual GPA were homogenized in 150 µl of potassium phosphate buffer (0.5M, pH 7.0) within each of 24 wells of a 96 well microtitre bioassay plate (Costar Corp., Cambridge, Massachusetts) with a plastic pestle (Kontes, Vineland, New Jersey). A 50 µl aliquot was pipetted from the original homogenate and diluted with 150 µl of potassium phosphate buffer. Three replicates from each well consisting of 25 µl of diluted homogenate were transferred to a new 96-well microtitre plate, beginning at row B (controls were always pipetted into row A). Each aliquot was diluted further with 25 µl potassium phosphate buffer. 150 µl of either α– or β–naphthyl acetate (diluted to 0.6% in acetone and then 100-fold in buffer) was added to each well in a five second sequence. The plate was incubated for a total of ten minutes, starting with the first well, and then 50 µl of color reagent (0.3% *O*-dianisidine dilute in H_2O) was pipetted into the wells in the same timed 5 second sequence. This was followed by a second ten minute incubation prior to quantitation. Microtitre plates were read on a Dynatech microplate reader at an absorbance density of 600 nm (A_{600}) for α- and 550 nm (A_{550}) for β-naphthyl acetate.

Protein determinations were conducted to correct for differences in protein quantities in GPA bodies. Total protein concentrations of each sample were later to be used as a control for standardizing α- and β-naphthyl acetate sample concentrations. The procedure

of Bradford (1976) was followed, using a type of Coomassie Brilliant Blue stain designed by BioRad, which is stable over time and not influenced by protein molecular weights or PI values (Brogden and Dickinson 1983). Controls were replicated twelve times to eliminate possible variation in the protein reagent and consisted of 50 µl buffer and 200 µl diluted BioRad Protein Reagent™, with a five minute incubation period observed along with the rest of the plate. They were pipetted into the entire A row of a 96-well sterile microtitre plate and were read as blanks in order to standardize the Dynatech microplate reader.

For the actual protein assay, 25 µl of the original homogenate (first dilution) was pipetted in three replicates to each well beginning with row B of a sterile 96-well microtitre plate. Next 25 µl of potassium phosphate buffer was added and then 200 µl of diluted BioRad Protein Reagent™ (diluted 1:4 with H_2O) were pipetted into the wells. After a five minute incubation period, the plate was read on the Dynatech microplate reader at an optical density of 600 nm.

Protein values were first tested using ANOVA (Sheffe 1959) in Systat to obtain mean and standard error values for each individual insect (every three wells originated from one insect). This was done in order to help eliminate some of the variation in protein values due to mispipetting, which is a common error when working with minute volumes. These data were then entered into Systat's Edit spreadsheet and replicated three times (one for each well of the plate), so that each insect would have one protein value and three different α- and β-naphthyl acetate values.

These data were next corrected using the standard curve data that were analyzed by regression analysis. Results of the correction calculations were then transformed to correct for differing protein concentrations and then mathematically computed using

molecular weight equivalents to nmol α– or β–naphthol/ μg protein/ 20 minutes. Next they were analyzed by ANOVA using Systat for the mean and error mean squared values (Carmer and Swanson 1968, Bernhardson 1975, Sheffe 1959). Then they were analyzed using Tukey's Means Separation test in MSTAT-C ($\alpha = .05$, df = 166) (Michigan State University, Departments of Crop and Soil Sciences and Agricultural Economics, East Lansing, Michigan 1990) (Tukey 1977) and plotted in histogram form.

For accuracy comparisons, microplate data were compared with other *in vitro* data using correlation analysis in MSTAT-C and Systat (Systat, Inc., Evanston, Illinois) (both Pearson's linear correlation and Spearman's rho rank correlation test (Cohen and Cohen 1983). Correlation coefficients were determined for each comparison, then tested by using Steel and Torrie's table (A.13) of r values. A χ^2 test for global significance was also conducted (Steel and Torrie 1980).

C. Polyacrylamide Gel Electrophoresis

1. PAGE methodology

For this technique, a 10% solution of acrylamide was used, composed of acrylamide-bisacrylamide (acting as a crosslinker) (30:0.8) in solution with distilled water. This was then added to 12 ml of tris-glycine buffer ([5x], pH 8.64), and 25 ml of double distilled H_2O. After degassing for five minutes under vacuum pressure of 15 psi to remove oxygen, which inhibits polymerization (Hames 1981), 150 μl of 10% ammonium persulfate and 15 μl of TEMED (*N,N,N',N'*-Tetramethylethylenediamine) were added as a catalyst and accelerator, respectively.

The 10% gel was then injected into electrophoresis molds by the use of a syringe, and care was taken to eliminate bubbles from the gel. Combs were then added to the liquid gel, and it was allowed to polymerize into a solid for sixty to ninety minutes. The rapidity of the

reaction depended largely on the concentrations of acrylamide used, the amount of ammonium persulfate or TEMED added, and the temperature and humidity of the laboratory.

Sample solution was pipetted into the gel's sample wells when it was fully polymerized, approximately two hours. The sample solution consisted of tris-glycine buffer ([5x], pH 8.64), glycerol, Bromophenol Blue stain, and double distilled H_2O. Samples were prepared by macerating GPA in 150 µl Tris-glycine buffer ([5x]) over ice using a plastic pestle (Kontes, Vineland, New Jersey) in a microcentrifuge tube. Then the aphid/buffer homogenate was centrifuged at 3000 rpm for five minutes at 4°C in a microcentrifuge (Eppendorf 5415C). After centrifugation, 75 µl of the supernatant (equivalent to one-half of a GPA) was pipetted into a sterile microcentrifuge tube and an equal volume (75 µl) of preparation solution was added. Next, 75 µl of the combined aphid homogenate/buffer and sample solution were added to each well of the gel (one fourth of a GPA), one strain of GPA per well. All of these steps are conducted over ice when possible (but it is not necessary) to retard carboxyesterase enzyme degradation.

Electrophoresis was conducted at 150 volts (constant voltage) for 17 hours with constant mixing of the buffer using a magnetic stir bar. The temperature was maintained at 2°C, in a cold room, to retain a pH of 8.4 in the temperature-dependent Tris-glycine buffer. After electrophoresis, the gels were stained with α-carboxyesterase stain (0.4 M Tris-glycine buffer solution, Fast Blue RR Salt, acetone, α-naphthyl acetate, and double distilled H_2O) for 45 minutes. Then they were washed briefly with double distilled H_2O to remove excess stain solution and preserved by one of several methods (explained later).

2. Insecticide Inhibition in PAGE

For this experiment one chemical was used from each of the three classes of insecticides the *in vivo* bioassays were performed with. The chemicals were a synthetic pyrethroid, a carbamate, and a systemic (oxon) organophosphate. The compounds used for the inhibition study were permethrin, carbaryl, and oxydemetonmethyl.

Using the Basic 10% PAGE protocol, several experiments were conducted. Duplicate gels were poured and samples were loaded as per the general 10% gel technique and run for 17 hours at 150 volts (Constant Voltage) in a cold room (2°C). However, before staining, one of the gels was incubated for 10 minutes in 500 ml of one of three 5.0 mM concentrations of insecticides. Either permethrin, carbaryl, or oxydemetonmethyl was used as the inhibitor. Prior to staining, the inhibited gel was rinsed with double distilled H_2O. The duplicate gel was used as a control and was not inhibited with anything. Both the inhibited gel and the control were placed in α-carboxyesterase stain at the same time in separate glass dishes. Each set of experiments (consisting of the two gels) was repeated three times with the same insecticidal compound.

3. Preservation of Gels and Data Analysis

Data were analyzed by densitometry using the AMBIS Radioanalytical Imaging System (San Diego, California) (with background readings subtracted) and then tested using correlation analysis in MSTAT-C and Systat (both Pearson's correlation analysis and Spearman't rho rank correlation test) for correlations with other *in vitro* data using the r values (Steel and Torrie 1980) significance tests (Cohen and Cohen 1983, Fischer and Yates 1949, Pearson and Hartley 1954). A global χ^2 test was also conducted.

Gels were preserved using BioGel Wrap™ drying apparatus (BioDesign Inc., Carmel, New York), which encases the gel in clear plastic, or a simple sealed Seal-A-Meal bag (Dazey

Corp., Industrial Airport, Kansas) . Pictures were taken of gels as a permanent record of data. Gels were analyzed in one of three ways: by visual methods (pictures), by densitometry analysis using AMBIS, and by statistical analysis of the densitometry data using ANOVA procedures in Systat.

D. Portable Evaluation of Carboxyesterase Levels

Standard curve data were determined by seven serial dilutions of α– or β-naphthol in potassium phosphate buffer (KH_2PO_4 + K_2HPO_4, .05 M, pH 7.0) with a range from 1 ng /10 µl to 20 ng/10 µl. Controls consisted of 2.5 ml of potassium phosphate buffer and were used to set percent transmission was at 100%. This was replicated six times for consistency.

For the standard curve data, 1500 µl α- or β-naphthol solution were pipetted into sterile 16 x 100 mm test tubes (VWR Scientific, San Francisco, California) and 500 µl potassium phosphate buffer was added. Next 500 µl of *O*-Dianisidine/SDS (0.3% tetrazotized *O*-dianisidine dilute in H_2O + 1% sodium dodecyl sulfate) (Aldrich Chemical Co., Milwaukee, Wisconsin), was pipetted into the test tubes. Each dilution was replicated three times and then read on the CHEMetrics USA Portable Photometer (Calverton, Virginia) (for percent transmittance with the correct filter (α=609 nm and β=555 nm). The results were subtracted from 1.00 to obtain percent absorbance values, then the data were analyzed by regression analysis using Systat (Cohen and Cohen 1983).

Bovine Albumin Serum (BSA) was the standard used in serial dilution to obtain values for the protein standard curve. The BSA stock solution consisted of 10 mg BSA/1 ml (10,000 ppm) potassium phosphate buffer. Eight serial dilutions were used with a range in concentrations of 0.156 ng/10 µl to 100 ng/10 µl. Controls consisted of 2.5 ml buffer alone, which was used to set the photometer at 100% transmittance. This was replicated

six times to maintain consistency. For the actual data, 500 ml of the BSA dilutions were pipetted into sterile test tubes in three replicates. Next, 500 ml of potassium phosphate buffer was added and then 2000 ml of diluted BioRad Protein Reagent (diluted 1:4 with H_2O). Results were read after five minutes on the CHEMetrics USA portable photometer with a 609 nm filter. These data were then subtracted from 1.00 to obtain percent absorbance and then analyzed by regression analysis (Cohen and Cohen 1983). To obtain standard curve regression lines, absorbance (Y) was plotted versus protein concentration (μg/100 μl) (X) in Sygraph and Systat.

For this procedure the protocol for Microplate Assay was used with several modifications. Both α- and β-naphthyl acetate assays were conducted but protein assays were not. Sodium dodecyl sulfate (SDS) was introduced into this assay for stability purposes, as well as shortening the total incubation time from twenty minutes prior to reading the absorbance (or transmittance) to ten minutes.

The protocol is as follows: one GPA was homogenized in 500 μl potassium phosphate buffer (pH 7.0, 0.05 M) in a sterile test tube with a glass rod. 0.6% α– or β–naphthyl-acetate was diluted 100-fold and then 1500 μl of it was added to the test tube with homogenate. This was subsequently incubated for ten minutes after which time 500 μl 0.3% tetrazotized O-dianisidine (Fast Blue B) with 1% SDS was added to the test tube and a color change was observed. A CHEMetrics USA portable spectrophotometer was calibrated with a clear buffer solution to 100% transmittance and then percent transmission was read (555 nm for α– and 609 nm for β–naphthyl acetate).

Data were subtracted from 1.00 to adjust percent transmittance to percent absorbance. Next the results were corrected using the standard curve regression values and then calculated with correct conversion factors (inverse of standard curve correlation

coefficients) to obtain nmol naphthol per insect per ten minutes (incubation time) in Systat. Finally Tukey's test for differences between means was conducted on the data in MSTAT-C. (α=.05, df = 16) (Tukey 1977) and the results tabulated in both histogram and table form.

The portable data were compared with the other *in vitro* data using correlation analysis in MSTAT-C and Systat (Pearson's correlation analysis and Spearman's rho rank correlation analysis) (Cohen and Cohen 1983). , and the r values were tested for significance using a table (A.13) from Steel and Torrie (1980). The χ^2 values were also tested as a global test of significance (Fisher and Yates 1949, Pearson and Hartley 1954).

III. Results and Discussion

A. Strain and Microplate Survey Results

Strains were received from thirteen states: California, Florida, Idaho, Maine, Michigan, Nebraska, New Hampshire, North Carolina, Ohio, Oregon, Pennsylvania, Washington, and Wisconsin (Figure 2.7). The high concentration of strains acquired from potato growing regions of the United States, such as Idaho, Washington, Oregon, and parts of Michigan was intentional, as the potato is the major crop of focus for this study. Often multiple samples were taken from these areas of the country. Figure 2.8 shows a detailed map of strain locations in Michigan. Multiple strains were collected from the central Michigan (Montcalm County) area, as this is a strong potato producing area of Michigan, as well as the St. John's and Munger, Michigan areas.

Fifty-five strains were received from cooperators and Figure 2.9 shows the procedure used upon receipt of samples from a cooperator. Insects were received in containers, removed and placed on potato plants using the method shown here. Table 2.3 is a listing of the

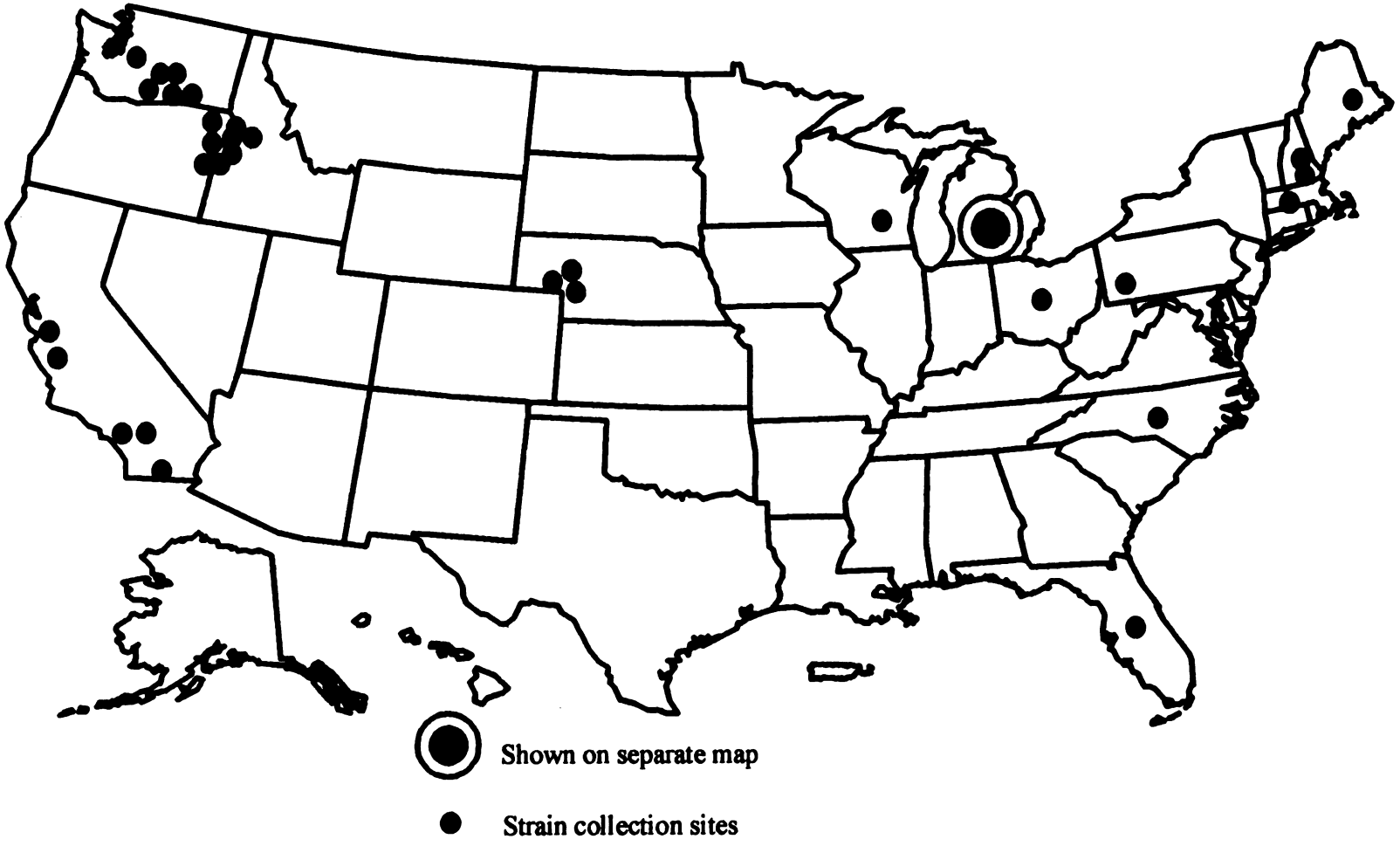


Figure 2.7--Map of locations for green peach aphid strains collected across the United States as part of the Microplate Carboxyesterase Survey.

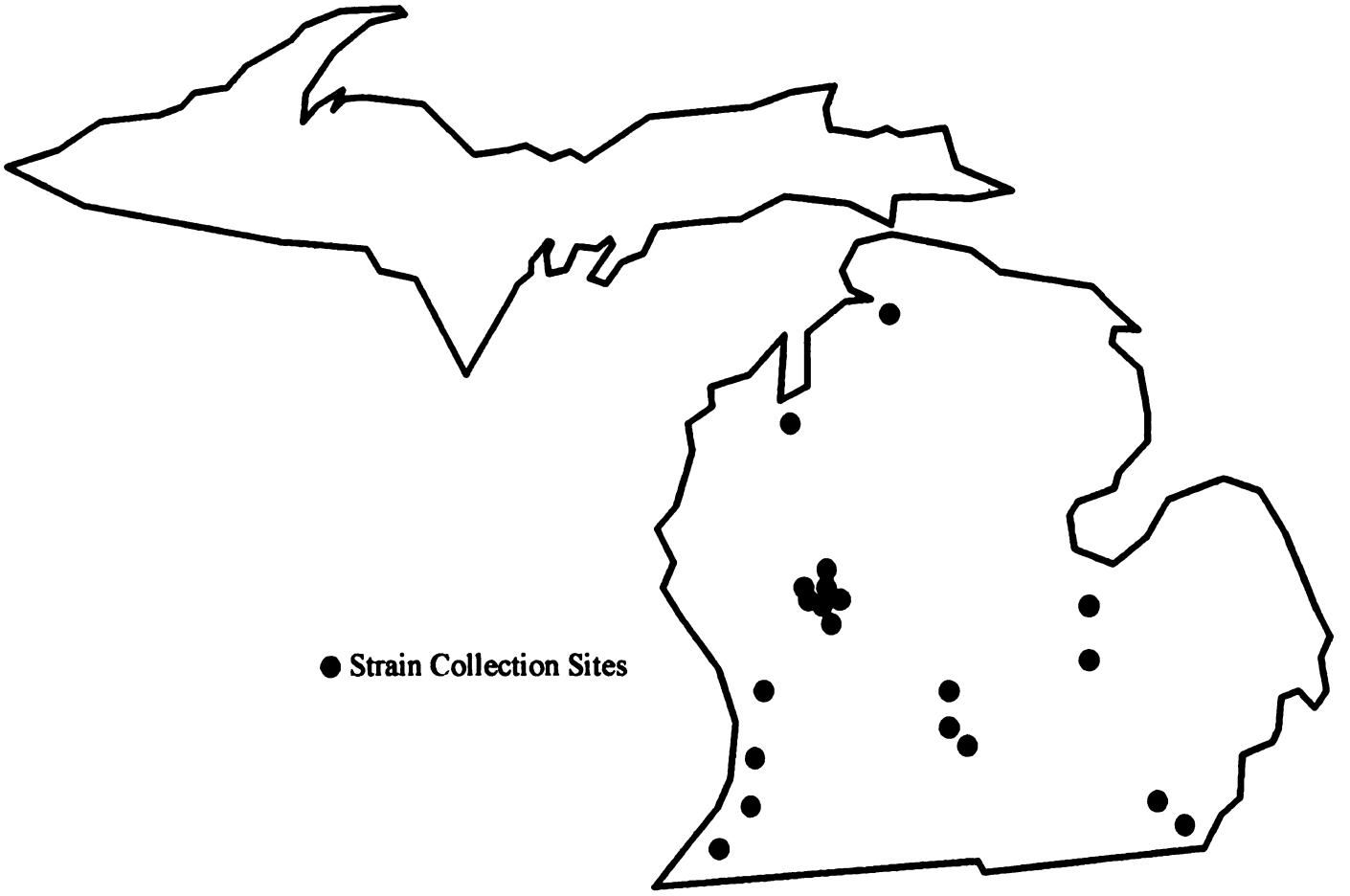


Figure 2.8--Map of green peach aphid strain collection sites in Michigan for the Microplate Carboxyesterase Survey.

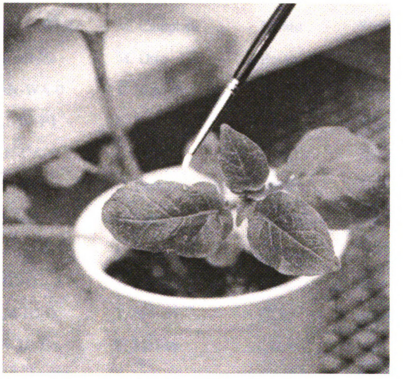


Figure 2.9--A paintbrush was used to transfer green peach aphids to clean potato plants for rearing in the greenhouse at Michigan State University.

Table 2.3--Listing of strains, cooperators, host plants, and locations of populations collected for the national carboxyesterase survey.

Strain	Cooperator	Host	Location
MOXEE1-WA-P1	L. Fox	Peach	Moxee, WA
PRESQUE-ME-P2	G. Sewell	Potato	Presque Isle, ME
SALINAS-CA-O	L. Fox	Other	Salinas, CA
WOOSTER-OH-P2	C. Hoy	Potato	Wooster, OH
STRATHAM-NH-P2	M. Campbell	Potato	Stratham, NH
MONTC1-MI-P2	M. Otto	Potato	Montcalm County, MI
PULLMAN-WA-0	T. Mowry	Other	Pullman, WA
WILDER1-ID-P1	L. Fox	Peach	Wilder, ID
NEWMAN-CA-O	L. Fox	Other	Newman, CA
IMPCO-CA-O	C. Farrar	Other	Imperial County, CA
GAINESVL-FL-T	F. Johnson	Tobacco	Gainesville, FL
WILDER2-ID-P1	L. Fox	Peach	Wilder, ID
PARMA-ID-O	T. Mowry	Other	Parma, ID
BINGHAM-ID-O	T. Mowry	Other	Bingham County, ID
MOSCOW-ID-O	T. Mowry	Other	Moscow, ID
UNKNOWN-ID-P2	T. Mowry	Potato	Unknown, ID
GRDRAPIDS-MI-P1	M. Resch	Peach	Kent County, MI
CARLETON-MI-O	P. Marks	Other	Carleton, MI
MONROE-MI-P2	P. Marks	Potato	Monroe County, MI
BERRIEN-MI-P2	C. Garcia	Potato	Berrien County, MI

Table 2.3--Continued.

Strain	Cooperator	Host	Location
GRATIOT-MI-P2	D. O'Hara	Potato	Gratiot County, MI
MONTC2-MI-P2	D. Miller	Potato	Montcalm County, MI
MONTC3-MI-P2	M. Otto	Potato	Montcalm County, MI
MONTC4-MI-P2	M. Otto	Potato	Montcalm County, MI
MONTC5-MI-P2	M. Otto	Potato	Montcalm County, MI
AUGRES-MI-P2	K. Kernstock	Potato	Au Gres, MI
TRAVERSE-MI-P2	M. Harmon	Potato	Grand Traverse Co., MI
BRDGPORT-NB-P2	M. Whalon	Potato	Bridgeport, NB
ALLIANCE-NB-P2	M. Whalon	Potato	Alliance, NB
HEMINGFD-NB-P2	M. Whalon	Potato	Hemmingford, NB
BEAUFORT-NC-O	K. Sorenson	Other	Beaufort, NC
VANORA-OR-O	L. Fox	Other	Vanora, OR
HERMISTON-OR-P2	G. Reed	Potato	Hermiston. OR
CENTER1-PA-P2	Z. Smilowitz	Potato	Center County, PA
MARYHILL1-WA-O	L. Fox	Other	Maryhill, WA
QUINCY-WA-P1	L. Fox	Peach	Quincy, WA
YAKIMA-WA-P1	L. Fox	Peach	Yakima, WA
TOPPEN1-WA-O	L. Fox	Other	Toppenish, WA
TOPPEN2-WA-O	L. Fox	Other	Toppenish, WA
WALLULA-WA-O	L. Fox	Other	Wallula Junction, WA
MADISON-WI-P2	J. Wyman	Potato	Madison, WI

Table 2.3--Continued.

Strain	Cooperator	Host	Location
IDAHO-P2	T. Mowry	Potato	Idaho
ANSON-MI-P2	C. Garcia	Potato	Anson, MI
MONTC6-MI-P2	M. Otto	Potato	Montcalm County, MI
MONTC7-WI-P2	D. Ragatz	Potato	Montcalm County, MI
MUNGER-MI-P2	U. Rahardja	Potato	Munger, MI
STJOHN'S-MI-P2	D. O'Hara	Potato	St. John's, MI
RVRSIDE-CA-O	T. Unrue	Other	Riverside. CA
MARYHILL2-WA-O	L. Fox	Other	Maryhill, WA
MOXEE2-WA-P1	L. Fox	Peach	Moxee, WA
MONTC8-MI-P2	M. Otto	Potato	Montcalm County, MI
TOPPEN3-WA-O	L. Fox	Other	Toppenish, WA
CENTER2-PA-P2	Z. Smilowitz	Potato	Center County, PA
TOPPEN4-WA-P2	L. Fox	Other	Toppenish, WA
RCKINGHAM-NH-P2	J. Bowman	Potato	Rockingham County, NH

code, crop, location, and cooperator for each strain of GPA. The codes were defined in the Materials and Methods section of this chapter. There were seven strains collected from peach (P1) and thirty from potato (P2), which are the primary and secondary host plant species of GPA, respectively. One strain was collected on tobacco, and eighteen were collected on various other crops.

Slide mounted aphids from each of the eight strains used for the *In vivo* study are preserved as slide mounted specimens in the Michigan State University Department of Entomology Museum Collection, Voucher #1992-05, found in Appendix A. These same eight strains were identified as Myzus persicae (Sulzer) (GPA) by the USDA/ARS Systematic Entomology Laboratory in Beltsville, Maryland (September 28, 1992). Figure 2.10 is a photograph of a slide mounted GPA with identifying characters labeled for easy viewing.

Strains were maintained for two years (some cultures were lost due to poor plant condition and parasitism) in the greenhouse at Michigan State University. Figure 2.11 illustrates the manner in which the strains of GPA were reared and kept free from contamination. Each strain was kept in a separate two-liter plastic cage which was screened with fine gage aphid-proof netting and maintained in water approximately three-quarters of an inch deep. Plants were changed (with clean plants) as needed.

Figure 2.12 shows the standard curves for both the α- and β-naphthol standards. There appears to be a linear progression of increasing optical density with increasing naphthol concentration. Although both absorbance values increase with increasing naphthol concentration at similar same slopes, the β-naphthol curve definitely has a steeper slope than the α-naphthol curve, as is evidenced by the two different Absorbance scales. For example, at 100 ng β-naphthol/ 200 µl, the absorbance value is approximately 0.25,

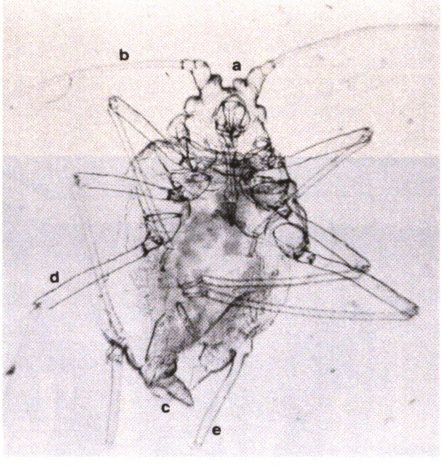


Figure 2.10--Photograph of a slide mounted green peach aphid with the most visible taxonomic characters labeled for easy discrimination: (a) converging tubercles, (b) long antennae (as long as or longer than the body), (c) a tongue shaped cauda, (d) long appendages, and (e) long, tapering or tubular siphunculi.

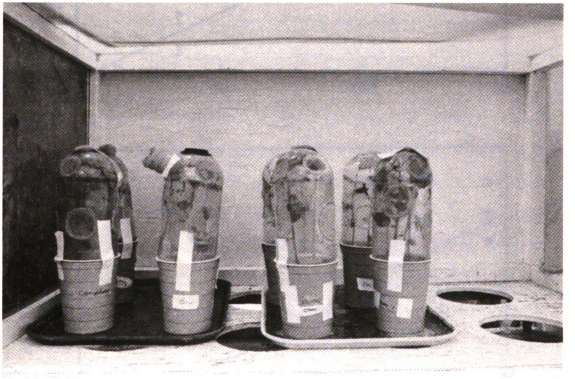


Figure 2.11--Rearing environment for green peach aphids in culture in a greenhouse at Michigan State University.

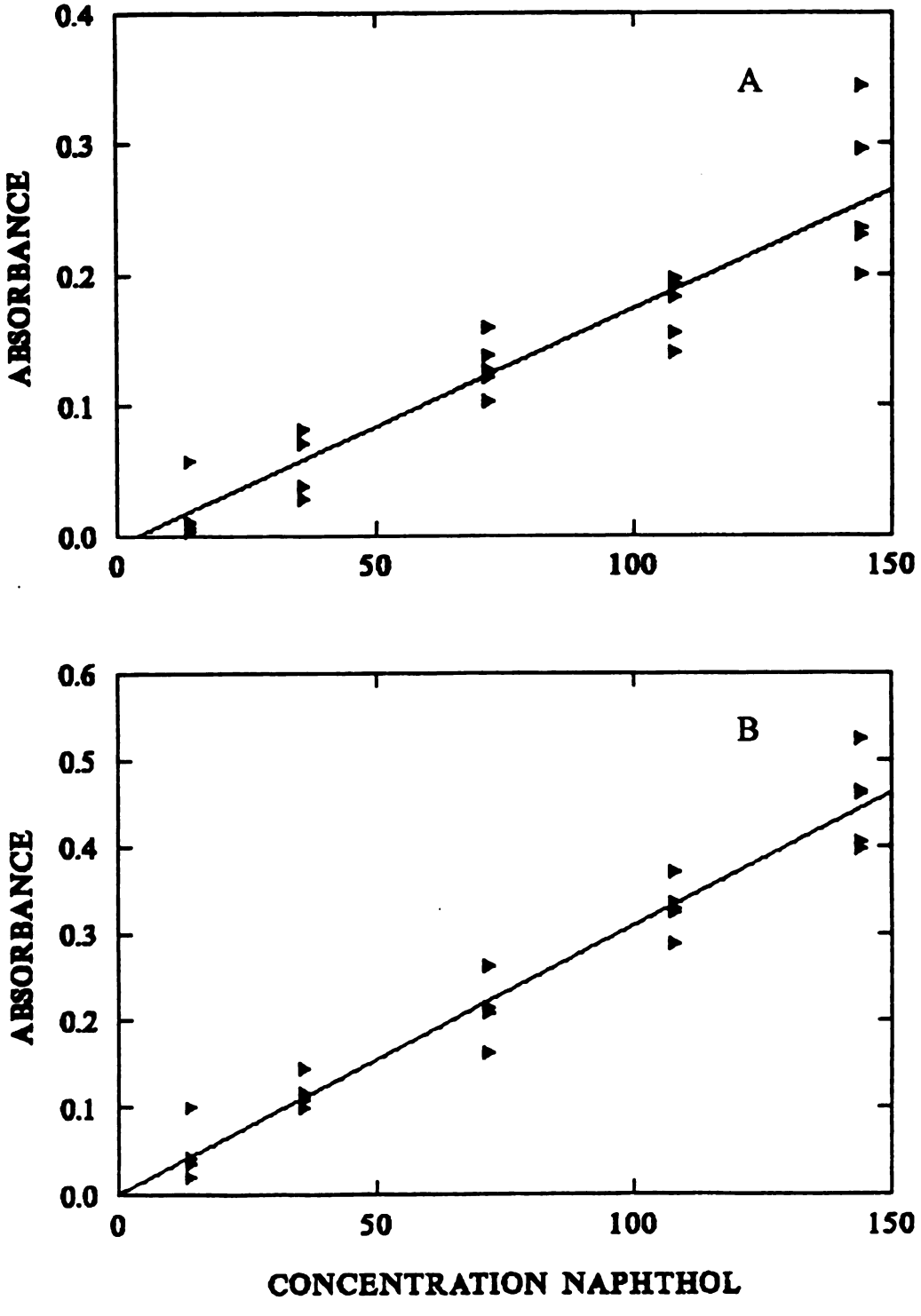


Figure 2.12--Microplate standard curves for α– (A) and β– naphthol (B) standard concentrations (ng)/ 200 µl plotted against absorbance values (600 nm = α, 550 nm = β). Slopes of the lines are found in Appendix B.

whereas for 100 ng α-naphthol/ 200 ml, the absorbance value is approximately 0.16. The β–naphthol absorbance readings increase at a greater rate than the α–naphthol absorbance values do for the same amounts of naphthol. Thus, there is a greater colorimetric change with the β–naphthol than there is for the same amount of α-naphthol. This shows that more α-naphthol is required than β-naphthol is to make the same change in absorbance.

Regression coefficients obtained in the analysis of the standard curve data were used to correct the absorbance values of the microplate assays. The α–regression coefficient was 0.002 and the β was 0.003. The inverse of these, 500 and 333.33, respectively, was multiplied with the data obtained from the microplate assay to standardize the assays.

Figure 2.13 shows the standard curve for the protein standards. The standard solution used was bovine serum albumin (BSA). The protein standard curve does not begin at zero, but above it, at approximately 0.80. This agrees with Bradford (1976) and the BioRad "microtechnique" results, which show a curve that begins slightly above zero on the Y-axis. The protein curve has a large slope value, which can be observed in the figure by the slope of the line. There is a linear relationship between increasing protein concentration and absorbance values in the microplate assay, and this is clearly observable in the Figure. The protein standard curve values were analyzed by regression analysis to obtain the regression coefficient of 0.175. The inverse of this, 5.714, was multiplied with the protein absorbance values to obtain corrected values.

Figure 2.14 shows the results of the α-carboxyesterase microplate assay in histogram form, along with Tukey's Test letters. The susceptible strains are PULLMAN-WA-O and GAINESVILLE-FL-T. The extremely resistant strains are MOXEE2-WA-P1 and HERMISTON-OR-P2. Both of the susceptible and most of the low α-carboxyesterase

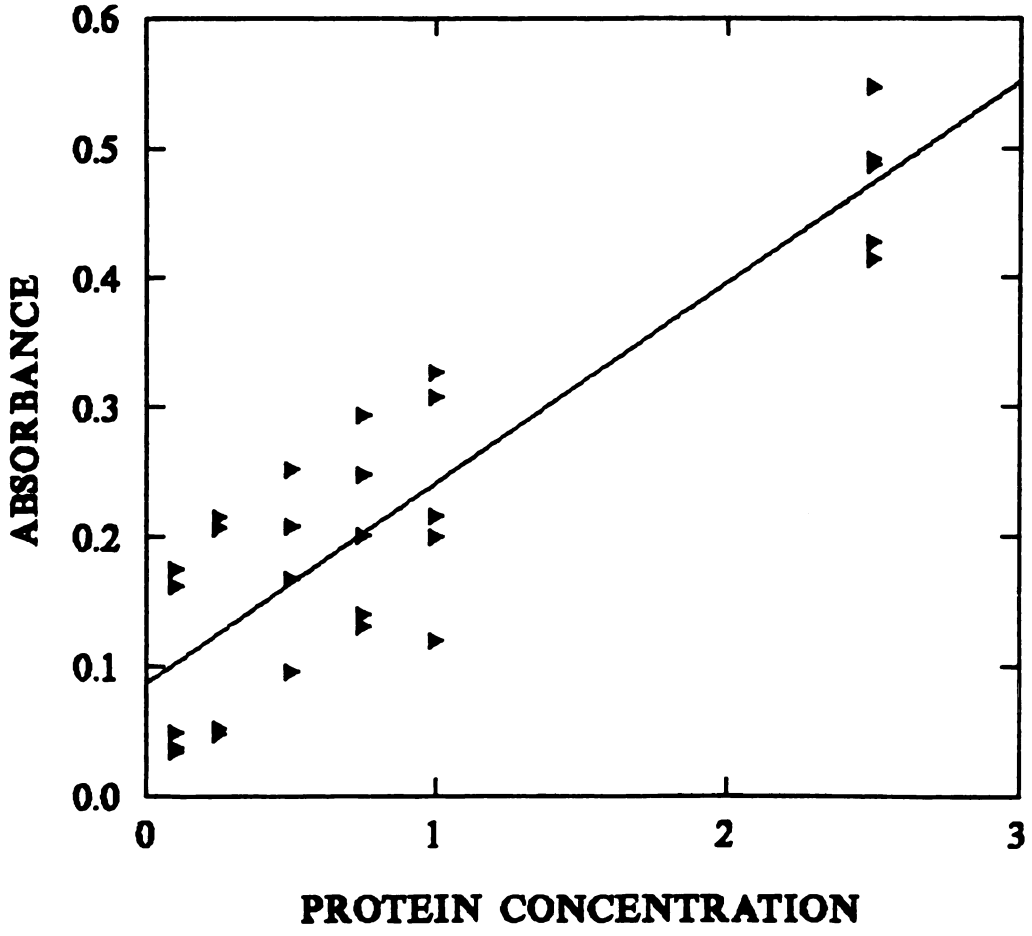
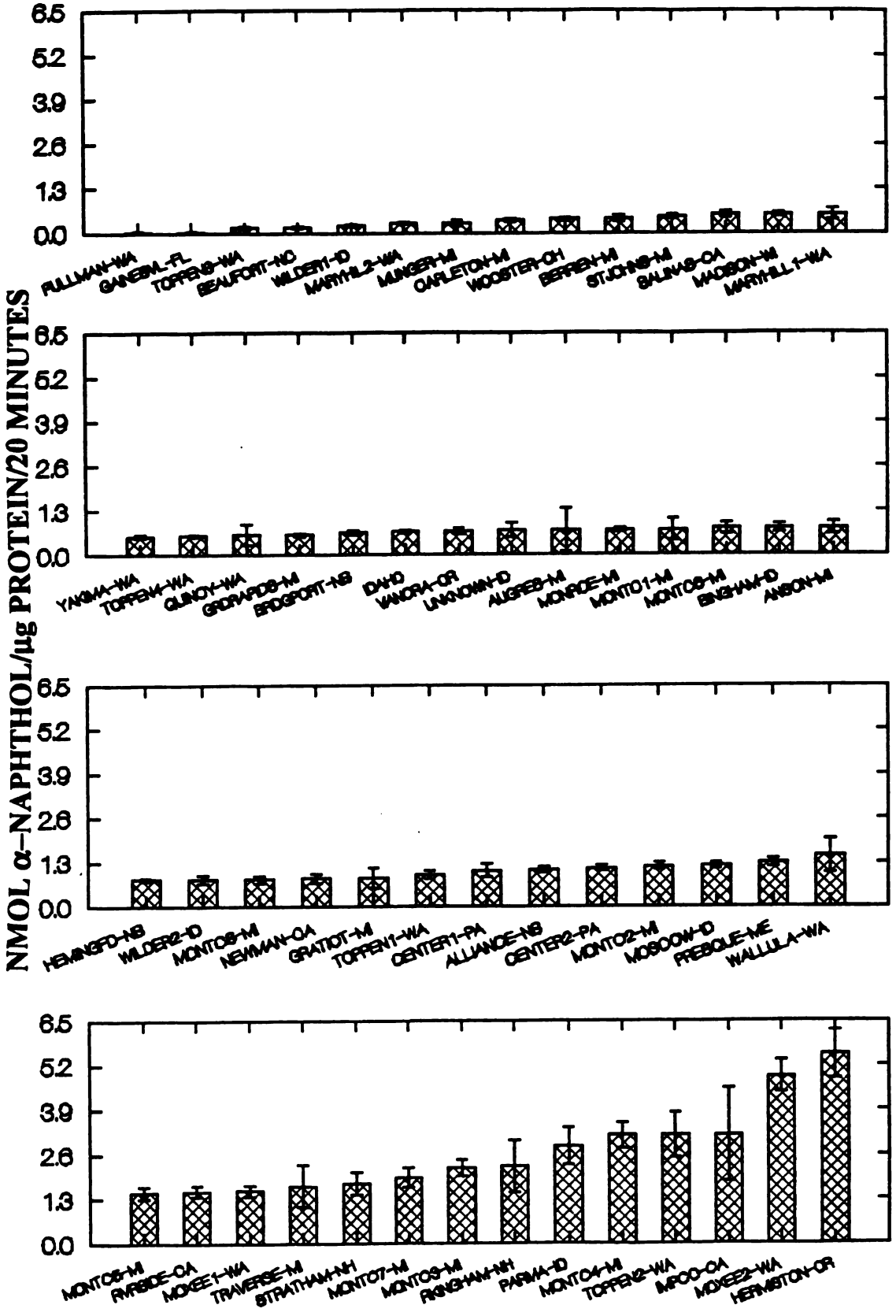


Figure 2.13--Microplate standard curve for total protein (bovine serum albumin) standard concentrations (µg)/ 10 µl plotted against absorbance values (600 nm = protein). The equation of the line is found in Appendix B.

Figure 2.14--Histograms of the α-Naphthylacetate carboxyesterase assay showing total α–carboxyesterase levels, Tukey's test for signifance letters (α = .05, df = 110).


NMOL α–NAPHTHOL/μg PROTEIN/20 MINUTES
6.5
5.2
3.9
2.6
1.3
0.0
PULLMAN-WA
GAINESVL-FL
TOPPER3-WA
BEAUFORT-NC
WILDER1-ID
MARYHL2-WA
MANGER-MI
CARLETON-MI
WOOSTER-OH
BERRIEN-MI
ST.JOHNS-MI
SALINAS-CA
MADISON-WI
MARYHLL1-WA
YAKIMA-WA
TOPPER4-WA
CLINOY-WA
GRDRAPDS-MI
EDGSPORT-NS
IDAHO
VANDRA-OR
UNKNOWN-ID
AUGRES-MI
MONROE-MI
MONTO1-MI
MONTO5-MI
ENGHAM-ID
ANSON-MI
HEMNGFD-NS
WILDER2-ID
MONTO8-MI
NEWMAN-CA
GRATIOT-MI
TOPPER1-WA
CENTER1-PA
ALLIANCE-NS
CENTER2-PA
MONTO2-MI
MOSCOW-ID
PREBOLE-ME
WALLULA-WA
MONTO6-MI
RIVRSIDE-CA
MOXEE1-WA
TRAVERSE-MI
STRATHAM-NH
MONTO7-MI
MONTO3-MI
RNGHAM-NH
PARMA-ID
MONTO4-MI
TOPPER2-WA
IMPCO-CA
MOXEE2-WA
HERMISTON-OR
STRAINS

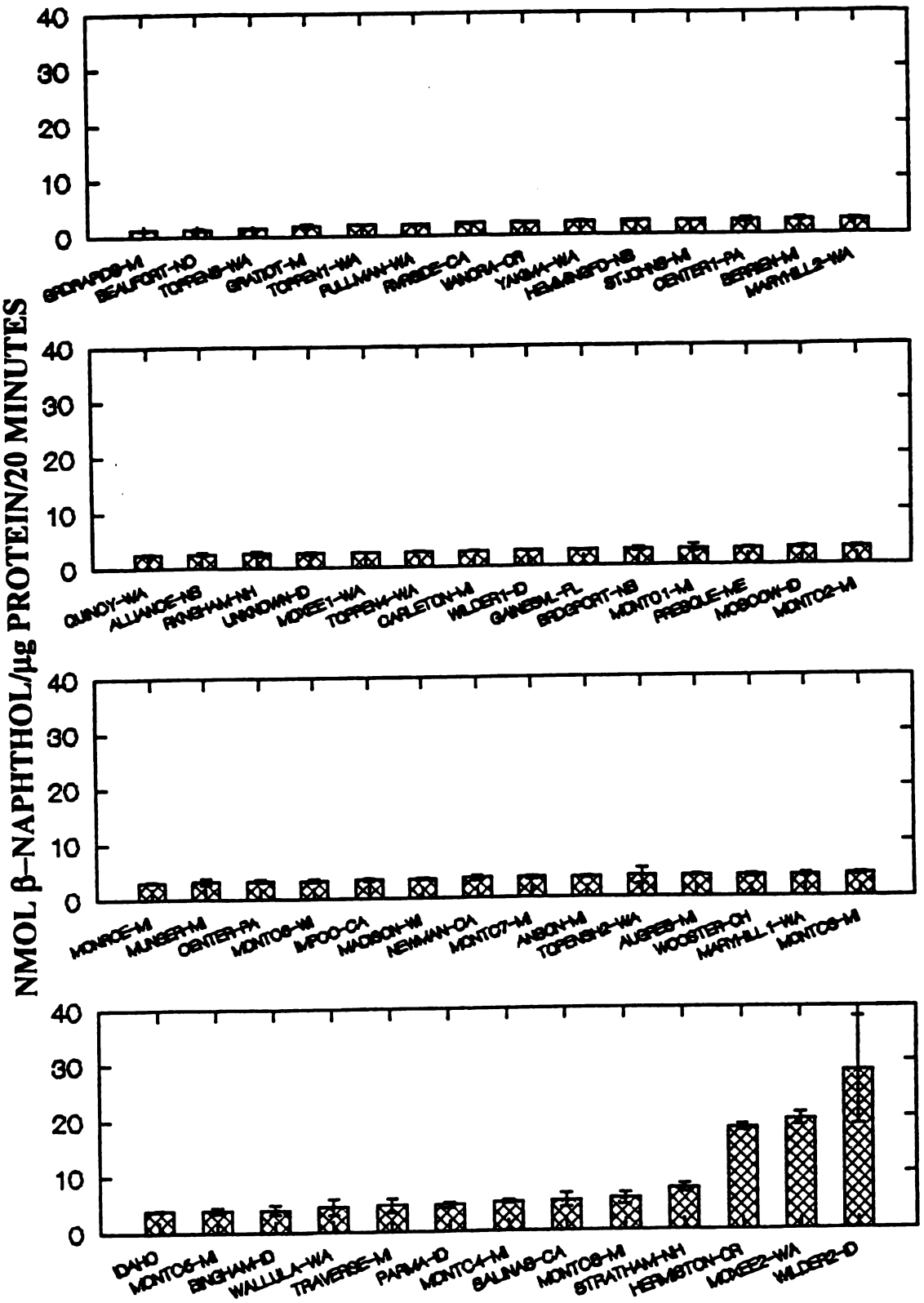
levels originated on host plants other than peach or potato. Most moderate and high α-carboxyesterase levels originated on peach or potato, however, and both of the extremely high α-carboxyesterase levels originated on either the primary host (peach) or the secondary (potato).

Figure 2.15 shows the results of the β-carboxyesterase microplate assay, also in histogram form. The β-assay was used to gain a perspective of the total carboxyesterase level found in each strain. Although some strains have a relatively low α-carboxyesterase level, they may have a rather high β-carboxyesterase level, or general carboxyesterase level. An example of this is the WILDER-2-ID-P1 strain, which was moderate in the α-carboxyesterase assay and extremely high in the β-carboxyesterase assay. Two strains which maintained extremely high levels in both aspects of the assay were the MOXEE2-WA-P1 and HERMISTON-OR-P2 strains.

Table 2.4 elucidates the mean carboxyesterase levels and confidence limits (± standard error of the mean or SEM), resistance levels, and Tukey's test values for each strain (α = .05, df = 110). Strains have been divided into five categories based on the Tukey's tests: susceptible, low, moderate, high, and extreme levels of α-carboxyesterases. The category boundaries, however, are flexible and were designed solely for ease of discussion and understanding, not as absolute measures.

Sawicki *et al.* conducted a similar survey in 1976 of GPA in Great Britain, where 258 populations were collected and assayed for insecticide resistance. Of all the populations collected, only three did not contain demethoate-resistant insects. In 197 of the samples, more than 76% of the GPA were resistant (Sawicki *et al.* 1978). Sawicki *et al.* believe that although bioassays are effective for resistance detection in insects with a large

Figure 2.15--Histograms of the β-Naphthylacetate carboxyesterase assay showing total β–carboxyesterase levels, Tuckey's test for signifance letters ($\alpha = .05$, df = 110).


NMOL β-NAPHTHOL/μg PROTEIN/20 MINUTES
0
10
20
30
40
BROMFIELD-MT
BEALFORT-NO
TOPPENISH-WA
GRATIOT-MI
TOPPENISH-WA
PULLMAN-WA
RARSIDE-CA
WADRA-OR
YAKIMA-WA
HEMMINGFD-NB
ST.JOHNS-MI
CENTER1-PA
BERRIEN-MI
MARYHILL2-WA
QUINDY-WA
ALLIANCE-NB
RINGHAM-NH
UNKNOWN-ID
MOXEE1-WA
TOPPENISH-WA
CARLETON-MI
WILDER1-ID
GAINESVL-FL
BRDGPORT-NB
MONTO1-MI
FREEPORT-ME
MOSCOW-ID
MONTO2-MI
MONROE-MI
MUNGER-MI
CENTER-PA
MONTO9-MI
IMPCO-CA
MADISON-MI
NEWMAN-CA
MONTO7-MI
ANSON-MI
TOPPENISH-WA
AUGRES-MI
WOOSTER-OH
MARYHILL 1-WA
MONTO5-MI
IDAHO
MONTO6-MI
BINGHAM-ID
WALLULA-WA
TRAVERSE-MI
PARMA-ID
MONTO4-MI
SALINAS-CA
MONTO8-MI
STRATHAM-NH
HERMISTON-OR
MOXEE2-WA
WILDER2-ID

STRAINS

Table 2.4--Results of national survey of carboxyesterase levels in green peach aphid using microplate assay ± SEM (Standard Error of the Mean) and Tuckey's test for significance.

Strain	Mean α-carboxyesterase value ± SEM	Mean β-carboxyesterase value ± SEM
MOXEE1-WA-P1	1.53 ± 0.16 gh	2.74 ± 0.01 uvw
PRESQUE-ME-P2	1.29 ± 0.12 hi	2.99 ± 0.15 rst
SALINAS-CA-O	0.53 ± 0.08 qrs	5.30 ± 1.24 f
WOOSTER-OH-P2	0.40 ± 0.04 stuv	3.80 ± 0.30 hijk
STRATHAM-NH-P2	1.75 ± 0.34 fg	7.42 ± 0.80 d
MONTC1-MI-P2	0.74 ± 0.32 mnopq	2.94 ± 0.66 stu
PULLMAN-WA-O	0.03 ± 0.01 x	1.97 ± 0.10 @%
WILDER1-ID-P1	0.23 ± 0.03 uvwx	2.85 ± 0.13 tuv
NEWMAN-CA-O	0.82 ± 0.12 mno	3.56 ± 0.52 lmn
IMPCO-CA-O	3.16 ± 1.36 c	3.37 ± 0.34 nop
GAINESVL-FL-T	0.03 ± 0.004 x	2.85 ± 0.13 tuv
WILDER2-ID-P1	0.80 ± 0.12 mno	28.34 ± 9.67 a
PARMA-ID-O	2.84 ± 0.54 d	4.64 ± 0.48 g
BINGHAM-ID-O	0.79 ± 0.13 mno	4.03 ± 0.90 h
MOSCOW-ID-O	1.20 ± 0.10 ij	3.03 ± 0.24 rst
UNKNOWN-ID-P2	0.73 ± 0.22 mnopq	2.68 ± 0.30 vwx
GRDRAPIDS-MI-P1	0.60 ± 0.05 opqrs	1.19 ± 0.09 #
CARLETON-MI-O	0.37 ± 0.05 stuvw	2.82 ± 0.11 tuv
MONROE-MI-P2	0.73 ± 0.06 mnopq	3.13 ± 0.15 qrs
BERRIEN-MI-P2	0.41 ± 0.08 stu	2.47 ± 0.26 xyz*

Table 2.4--Continued.

Strain	Mean α-carboxyesterase value ± SEM	Mean β-carboxyesterase value ± SEM
GRATIOT-MI-P2	0.85 ± 0.30 lmn	1.90 ± 0.37 %
MONTC2-MI-P2	1.17 ± 0.11 j	3.10 ± 0.21 qrs
MONTC3-MI-P2	2.20 ± 0.23 e	3.92 ± 0.25 hi
MONTC4-MI-P2	3.15 ± 0.38 c	5.09 ± 0.47 f
MONTC5-MI-P3	1.49 ± 0.19 hi	4.01 ± 0.55 h
AUGRES-MI-O	0.73 ± 0.61 mnopq	3.80 ± 0.24 hijk
TRAVERSE-MI-P2	1.67 ± 0.61 gh	4.62 ± 1.20 g
BRDGPORT-NB-P2	0.65 ± 0.07 nopqr	2.86 ± 0.38 tuv
ALLIANCE-NB-P2	1.06 ± 0.09 jk	2.64 ± 0.20 vwxy
HEMINGFD-NB-P2	0.80 ± 0.05 mno	2.35 ± 0.15 z*!
BEAUFORT-NC-O	0.17 ± 0.02 vwx	1.31 ± 0.14 #
VANORA-OR-O	0.70 ± 0.08 mnopqr	2.18 ± 0.12 !@
HERMISTON-OR-P2	5.51 ± 0.70 a	18.21 ± 0.53 c
CENTER1-PA-P2	1.51 ± 0.20 jkl	2.44 ± 0.32 yz*
MARYHILL1-WA-O	0.54 ± 0.17 qrs	3.88 ± 0.37 hij
QUINCY-WA-P1	0.60 ± 0.10 opqrs	2.51 ± 0.25 wxyz
YAKIMA-WA-P1	0.55 ± 0.05 pqrs	2.25 ± 0.09 *!
TOPPEN1-WA-O	0.92 ± 0.12 klm	1.95 ± 0.16 @%
TOPPEN2-WA-O	3.16 ± 0.65 c	3.76 ± 1.39 ijkl
WALLULA-WA-O	1.49 ± 0.49 hi	4.49 ± 1.45 g

Table 2.4--Continued.

Strain	Mean α-carboxyesterase value ± SEM	Mean β-carboxyesterase value ± SEM
MADISON-WI-P2	0.53 ± 0.06 qrs	3.48 ± 0.14 mno
IDAHO-P2	0.69 ± 0.04 mnopqr	3.99 ± 0.23 hi
ANSON-MI-P2	0.79 ± 0.17 mno	3.67 ± 0.23 klmn
MONTC6-MI-P2	0.78 ± 0.17 mnop	3.30 ± 0.25 opq
MONTC7-MI-P2	1.91 ± 0.28 f	3.58 ± 0.29 klmn
MUNGER-MI-P2	0.29 ± 0.09 tuvw	3.22 ± 0.54 pqr
STJOHN'S-MI-P2	0.47 ± 0.06 rst	2.35 ± 0.11 z*!
RVRSIDE-CA-O	1.52 ± 0.16 ghi	2.15 ± 0.10 !@
MARYHILL2-WA-O	0.29 ± 0.02 tuvw	2.48 ± 0.17 xyz*
MONTC8-MI-P1	0.80 ± 0.10 mno	5.64 ± 1.07 e
TOPPEN3-WA-O	0.16 ± 0.04 wx	1.38 ± 0.07 #
CENTER2-PA-P2	1.12 ± 0.08 j	3.29 ± 0.25 opq
TOPPEN4-WA-P2	0.59 ± 0.04 opqrs	2.75 ± 0.09 uv
RCKINGHAM-NH-P2	2.25 ± 0.77 e	2.66 ± 0.44 vwxy
MOXEE2-WA-P1	4.88 ± 0.45 b	19.46 ± 1.19 b

difference between the resistant (R) and susceptible (S) individuals, when phenotypes overlap or have intermediate levels, bioassays are not as effective (1977).

The diazoblue or tetrazotized *O*-dianisidine (Fast Blue B) that van Asperen used is the same that was used in my esterase protocol. However, I did not use sodium laurylsulphate, which van Asperen used to enhance the color of his naphthol solutions as well as to stop the esterase-naphthyl acetate reaction (van Asperen 1962). Also in contrast, van Asperen used β-naphthyl acetate as substrate only occasionally, whereas I used it each time to get an idea of the total carboxyesterase amounts in each strain of insect. We did use the same buffer solutions and pH. van Asperen did not mention protein assays, which are an integral part of my study, as they aid in comparisons of amount of naphthol per insect using total protein variations. Ruud *et al.* (1988) note that in their studies of est-*m*, an esterase found in chrysomelid beetles, that there may be a problem with the BioRad and other total protein staining determinations. This was observed while being unable to stain est-*m* with Coomassie Blue stain during Polyacrylamide Gel Electrophoresis (PAGE). I also observed this in my experiments with GPA carboxyesterases during PAGE, but not in the microplate assays.

I would like to point out that carboxyesterase resistance is associated with increased activity of the enzyme. This is overall activity, not specific activity. It is believed that carboxyesterase resistance in GPA is associated with increased levels of carboxyesterases. This is different than specific activity, which in this case would be associated with a mutant form of the enzyme that has an increased affinity for the substrate or a faster catalytic activity level.

Table 2.5 illustrates the correlation coefficients and their significance between carboxyesterase levels and the other *in vitro* carboxyesterase experiments. These other

Table 2.5--Coefficients for correlation analysis of *in vitro* assays with microplate data across the eight main strains.

	α-Portable Assay	β-Portable Assay	PAGE
α–Microplate Assay	.898**	--	.762*
β–Microplate Assay	--	.405	.568

Significance was tested at $\alpha = .05$ (*) and $\alpha = .01$ (**) levels.
Global χ^2 test = 21.091 * (significant)

techniques involved Basic 10% PAGE banding patterns and a portable carboxyesterase assay tool for field work.

Microplate carboxyesterase assays were compared with the 10% PAGE gels to seek possible correlations between them. The global χ^2 test was significant (21.091). Table 2.5 shows the correlation coefficients for the comparisons. The total densitometry units show a strong correlation coefficient (0.762) with α–carboxyesterase levels (significant at $\alpha = .05$). The β-carboxyesterase microplate data does not show a significant correlation (0.548) with the PAGE densitometry data.

The correlation analysis of the microplate α-carboxyesterase level with portable α–carboxyesterase level was 0.898, significant at $\alpha = 0.01$. The portable β-carboxyesterase level (with the β-microplate assay) showed a positive correlation coefficient of 0.405, which is not significant. However, there is an overall positive correlation which shows that as carboxyesterase level increases with the microplate assay, it also increases with the portable assay. This shows that the portable a-carboxyesterase assay is a valid measure of carboxyesterase level as defined by the microtitre esterase assay, as the correlations are significant at $\alpha = .01$.

B. Gel Results

1. Basic 10% Gel Results

Adult GPA were electrophoresed using PAGE in order to characterize each strain and determine difference in banding patterns or intensity. Mack and Smilowitz found no difference in the protein banding patterns for GPA in PAGE gels (1980). Sudderuddin discusses the use of staining with substrates, such as 1-naphthyl acetate as well as the use of inhibitors like dichlorvos (Sudderuddin 1972). Other inhibitors, such as insecticides could be used as well.

Figure 2.16 is a picture of the two gels used to elucidate the basic banding patterns of each strain. Differences between strains can be observed with the naked eye as well as documented using densitometry techniques. In the figure, susceptible strains (as defined by microplate carboxyesterase data) appear to lack (or it is very faint) one or both of the two bands found in the strains with greater resistance levels. The significant band is the bottom (second band), which is missing in all susceptible strains and present in all resistant ones. Although some resistant strains lack the top band, they still retain the bottom band. Additionally, the top band stains more darkly with β-naphthyl acetate than it does with α, and the bottom more darkly with α-naphthyl acetate. This further illustrates the concept that the second band is the band associated with carboxyesterase resistance due to elevated esterase levels in GPA.

The banding differences are quantified by AMBIS (AMBIS Inc., San Diego, CA), a powerful new image acquisition and analysis system. This data is shown in Table 2.6, which elucidates the various levels of AMBIS analyzed densitometry peaks for each carboxyesterase band and compares them with α–Portable values, α–Microplate values, and resistance ratios (azinphosmethyl LC_{90}). In this way, comparisons can be observed between densitometry units and the other measures. For instance, the two lowest values for the complete densitometry units, PULLMAN-WA and WILDER1-ID are also the two lowest values for the α-Microplate Assay and the α-Portable Assay, and are the two lowest resistance ratio values. The highest densitometry value, SALINAS-CA, is not the highest in any other category, suggesting some error in the gel. However, the highest resistance ratio value, STRATHAM-NH (6.6), also has the highest (tied) α-Portable value and the second highest α-Microplate Value. This strain is also quite close to the densitometry value for SALINAS-CA.

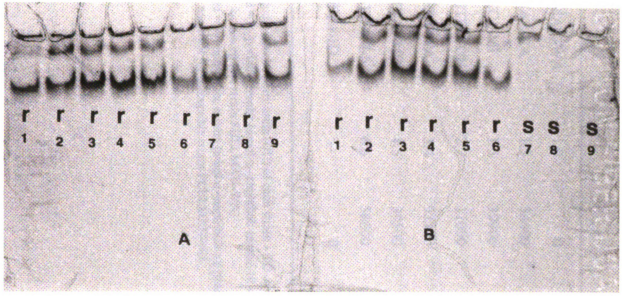


Figure 2.16--Photograph of two native polyacrylamide gels stained for α–carboxyesterase activity. Strains are labeled as either R or S depending on their values in the Microplate assay. The R strains on Gel A are: (1) MONTC1-MI, (2) MOXEE1-WA, (3) SALINAS-CA, (4) PRESQUE-ME, (5) STRATHAM-NH, (6) WOOSTER-OH, (7) CENTER2-PA, (8) MADISON-WI, (9) ROCKINGHAMCO-NH. On Gel B the R strains are the same (1)-(6), with S strains of (7) TOPPENISH3-WA, (8) WILDER1-ID, and (9) PULLMAN-WA.

Table 2.6--Comparison of azinphosmethyl resistance ratios (RR), densitometry unit values, portable values, and microplate values.

STRAIN	RR	DUV[1]	PV[2]	MV[3]
PULLMAN-WA	1.0	0	0.000	0.000
SALINAS-CA	1.3	34600	0.046	0.496
MONTC1-MI	1.5	39900	0.017	0.709
WOOSTER-OH	2.7	11000	0.035	0.370
MOXEE1-WA	3.4	52300	0.052	1.503
PRESQUE-ME	4.5	39500	0.048	1.258
STRATHAM-NH	6.6	58600	0.052	1.720
WILDER1-ID	--	0	0.000	0.204

[1]DU Value = densitometry units (DU) of each strain - DU of susceptible strain (PULLMAN-WA).
[2]P Value = α–portable results (nmol α–naphthol/ insect/ 10 minutes) of each strain – nmol α–naphthol for susceptible strain PULLMAN-WA.
[3]M Value = α– microplate results (nmol α–naphthol/ μg protein/ 20 minutes) of each strain – nmol α–naphthol for susceptible strain PULLMAN-WA.

2. Insecticide-Inhibited Gel Results

Figure 2.17 is a picture of two 10% PAGE gels, one inhibited with an insecticide, one (control) not inhibited. This gel is an example, each insecticide inhibition and control was replicated three times in order to obtain data that was valid for statistical analysis. In the insecticide-inhibited gel there is an absence of bands and in the control gel there is a strong presence of banding, both of which can be observed with the naked eye, as well as in densitometric analysis in the AMBIS Radioanalytic Imaging System.

Table 2.7 shows the percent inhibition for the mean densitometry values (total value minus background - inhibited densitometry units/ uninhibited densitometry units * 100) found for all three replicates of each insecticide. According to the table, the highest percent inhibition was 100%, and the lowest was 66.5% (CENTER2-PA). A visual observance of the gels shows that there were no bands or else very faint ones on the insecticide inhibited gel. The densitometry data confirms this, showing lower values for the inhibited gels than for the controls. This means that the substrate α-naphthol is actually the correct substrate for studying the enzymes that hydrolyze

3. Discussion

Physically, gels can be in many different forms. Vertical slab gels were chosen for these experiments for the ease of preparation and reproducibility. Heat is more easily dissipated in slab gels than in other types (such as rod) and their shape allows procedures such as densitometry to be conducted for quantification purposes. Lastly, many samples can be run under identical purposes on the same gel, which is not true of rod gel type gels (Hames 1981).

Electrophoresis of insect esterases on polyacrylamide gel has been less widely used than starch and agarose gels (Ogita 1963, Cook and Forgash 1965, Salkeld 1965, Benton 1967,

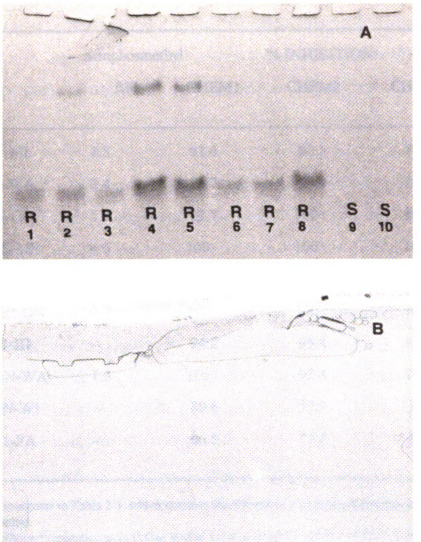


Figure 2.17--Photograph of two native polyacrylamide gels stained for α–carboxyesterase activity. Gel A is the control gel, which was not incubated in any inhibitor (insecticide). Strains are labeled as R or S depending on their classification by Microplate assay. Strains are also numbered: (1) MONTC1-MI, (2) MOXEE1-WA, (3) WOOSTER-OH (4) PRESQUE-ME, (5) STRATHAM-NH, (6) SALINAS-CA, (7) CENTER2-PA, (8) MADISON-WI, (9) PULLMAN-WA, and (10) WILDER1-ID. Gel B is the inhibited gel, which was incubated in one of three insecticides before staining.

Table 2.7--Summary of mean percent inhibition of green peach aphid strains using PAGE gels inhibited by carbaryl, oxydemetonmethyl, and permethrin (three replicates each).

STRAINS[a]	azinphosmethyl	% INHIBITION[c]		
	RR[b]	CHEM1	CHEM2	CHEM3[d]
MONTC1-MI	1.5	82.6	80.1	90.4
MOXEE1-WA	3.4	73.7	98.4	100
SALINAS-CA	1.3	82.7	100	100
PRESQUE-ME	4.5	100	100	100
STRATHAM-NH	6.6	95.5	100	100
WOOSTER-OH	2.7	100	100	100
WILDER1-ID	--	94.2	95.4	100
PULLMAN-WA	1.0	100	97.4	100
MADISON-WI	--	80.6	77.9	96.8
CENTER1-PA	--	66.5	71.2	82.1

[a]STRAINS correspond to Table 2.3, which outlines the alphabetic and numerical codes for each strain of green peach aphid.
[b]RR is the methomyl resistance ratio (LC_{50} strain/ LC_{50} susceptible strain PULLMAN-WA)obtained by *in vivo* assays.
[c]% INHIBITION is calculated by uninhibited total densitometry units (UDU) for each strain – inhibited densitometry units (IDU) for the same strain, divided by UDU and multiplied by 100.
[d]CHEM1=carbaryl, CHEM2=oxydemetonmethyl, and CHEM3=permethrin.

Clements 1967, Matsumura and Sakai 1968, Cook *et al.* 1969, Katzenellenbogen and Kafatos 1971, Sudderuddin 1973). Blackman and Devonshire conducted horizontal starch gel electrophoresis (along with a stain system similar to mine in 1978 (Blackman and Devonshire 1978). However, Sims (1965) studied the esterases of *Drosophila* on PAGE, as did others (Price and Bosman 1966, Arurkar and Knowles 1967, 1968, Ahmad 1968, Ozaki 1969).

Sudderuddin (1972, 1973) observed a higher quantity of one band ("E3") in resistant strains of GPA. The major change was only in activity of est-4 (E4), the enzyme involved in resistance, not in mutation, just amount (Devonshire 1977, Sawicki *et al.* 1980). Devonshire observed in 1977 that the E4 enzyme, responsible for resistance, stains more heavily in PAGE in resistant individuals than susceptibles, even when the insects are only slightly resistant (Devonshire 1977). He showed that the carboxyesterase degrades organophosphate insecticides and α-naphthyl acetate more rapidly in resistant than susceptible insects (Devonshire 1977). Pasteur and Georghiou (1989) used polyacrylamide gel electrophoresis (PAGE) to determine increased or unincreased levels of esterases. They claim that a gap of several optical density units separates susceptible individuals from those with increased levels of esterases (1989). Sawicki *et. al* did note that gel results were semi-quantitative and the levels of resistance could not always be determined by the intensities of the E4 band, and for this reason, they conducted other tests for resistance determinations (1977), as I did. These results were then correlated with the data from dosage mortality assays and carboxyesterase assays.

Sawicki *et al.* (1977) explained that although PAGE is suitable for rapidly estimating the proportion of resistant individuals in a population, the biochemical esterase determination is more reliable for determining resistant types of each individual. I noted excellent correlations with the resistance indicators (α-naphthyl acetate) and rather inconclusive

results with the general esterases, which is to be expected as staining was selective only for α-naphthyl acetate. The results show that PAGE is a valid measure of carboxyesterase differences, although not as quantitatively accurate as is preferable.

Sudderuddin observed that there were (quantitative) differences between resistant and susceptible strains of GPA by their electrophoretic patterns (Sudderuddin 1973). Mack and Smilowitz noted no difference between the number and location of bands, only the banding intensity (Mack and Smilowitz 1980). Total carboxyesterase activity assessed associated with est-4 (Blackman *et al.* 1977).

Sudderuddin notes "interesting" results when gels were first inhibited with dichlorvos then stained for esterases. He notes some slight activity and suggests that this may relate to the fact that they may be arylesterases (Sudderuddin 1972). He also notes that the banding patterns of GPA reveal a relatively constant pattern though quantitative fluctuations can occur over time in laboratory populations (1972). I also noted relatively consistent banding patterns in the PAGE gels tested, and some very slight activity on the insecticide inhibited gels. Mack and Smilowitz noted no differences in banding patterns on two GPA biotypes although there were differences in banding intensity (Mack and Smilowitz 80). I note quantitative differences between strains on my gels, but no qualitative.

For a molecular weight standard, Mack and Smilowitz used Bovine serum albumin (BSA) as a dye marker (mw 68,000) (1980). They do not report difficulties in staining the carboxyesterase as I did and as Ruud *et al.* did (1988). They used SDS gels, however, which could suggest that there is a structural anomaly to the carboxyesterase which is not present when the enzyme is active. However, Field *et al.* report that the molecular weight of esterase E-4 is 65,000 with a polypeptide portion of 57,000. Devonshire and Sawicki report E-4 as 65,000 kDa, and FE-4 as 66,000 kDa (Devonshire *et al.* 1986, Field *et al.*

1989). In the mutant strains of GPA with esterase FE-4, the molecular weight of the polypeptide portion is 58,000 (the whole molecule is a glycoprotein) and the catalytic center of activity is slightly different (Field *et al.* 1988).

In an attempted experiment, I had difficulty with the Coomassie Brilliant Blue dye not stain my carboxyesterases. Ruud *et al.* attributes this fact to the carbohydrate form of some carboxyesterases of other structural peculiarities somehow preventing the Coomassie blue (and in his case silver stain as well) form reacting with enzyme moieties essential to stain uptake. This could also reflect low levels of esterase on the gel (Ruud *et al.* 1988). Ruud *et al.* also suggest that this may also mean that the inability to stain the esterase in the gels may also reflect on the efficacy of staining with BioRad Protein Reagent™ for total protein (Ruud *et al.* 1988).

3. Basic Gel Correlations with other *in vitro* tests

Table 2.8 illustrates the correlation results between the basic gel densitometry data and the carboxyesterase microplate and portable assays. There were positive correlations with the carboxyesterase assay data sets, especially the α-carboxyesterase values. The portable α-values were high 0.831 (Pearson correlation coefficients), and the portable β- values were also high at 0.914 (Pearson correlation), both of which are significant at $\alpha = .01$. The α–carboxyesterase microplate correlation values were significant (0.762) at $\alpha = .05$ (Without WILDER1-ID, the correlation coefficient was 0.919, which is highly significant. This shows that WILDER1-ID is skewing the data to some degree). For the β-microplate assay, the correlation coefficient was not significant (0.548). This is not surprising as the stain system is selective for α-carboxyesterases. There is an overall positive correlation between the α-carboxyesterase assays and PAGE, as the data shows.

Figure 2.8--Coefficients for correlation analysis of *in vitro* assays with PAGE densitometry data across the eight main strains.

	PAGE
α–Microplate Assay	0.919*
β–Microplate Assay	0.548
α-Portable Assay	0.831**
β-Portable Assay	0.914**

Significance was tested at $\alpha = .05$ (*) and $\alpha = .01$ (**) levels.
Global χ^2 test = 21.091 * (significant)

C. Portable Data Results

In Figure 2.18, the regression lines for the standard curves of both the α- and β-naphthol standard curves are shown. Both data sets were replicated three times for statistically correct values for analysis. Standard curve data is useful as a transforming character for standardizing absorbance (or transmittance values of a photometer).

A protein standard curve was attempted but discarded as they are not integral to a functional field assay, but are for a more exact scientific interpretation. All the field workers and pest management decision makers will need is to know the nmol naphthol per insect (GPA), not per μg protein.. Additionally, some difficulties were encountered in calibration of the portable photometer for protein readings, as it quickly reaches the maximum value for accurate readings. According to the graph shown in the appendix, the protein standard curve does not appear to be a linear relationship, but more like a curvilinear one. Additionally, protein values will only be accurately read at protein concentrations above 10 μg/ 100 μl. This curve can be found in Appendix C.

For a graphical representation of the portable assay results, the histograms in Figure 2.19 are one means of elucidation. The amount of naphthol per insect per ten minutes is plotted for each strain. The highest α-carboxyesterase level strain was MOXEE1-WA-P1. The lowest values were WILDER1-ID-P1 and PULLMAN-WA-O, and this is true for the β-carboxyesterase level as well. The highest β-carboxyesterase level strains are SALINAS-CA, PRESQUE-ME-P2, and MOXEE1-WA-P1. For Tukey's tests of differences between means, Table 2.9 was set up with mean nmol α- and β-naphthol per GPA per ten minutes ± standard error of the mean (SEM), and Tukey's test for significant differences between means.

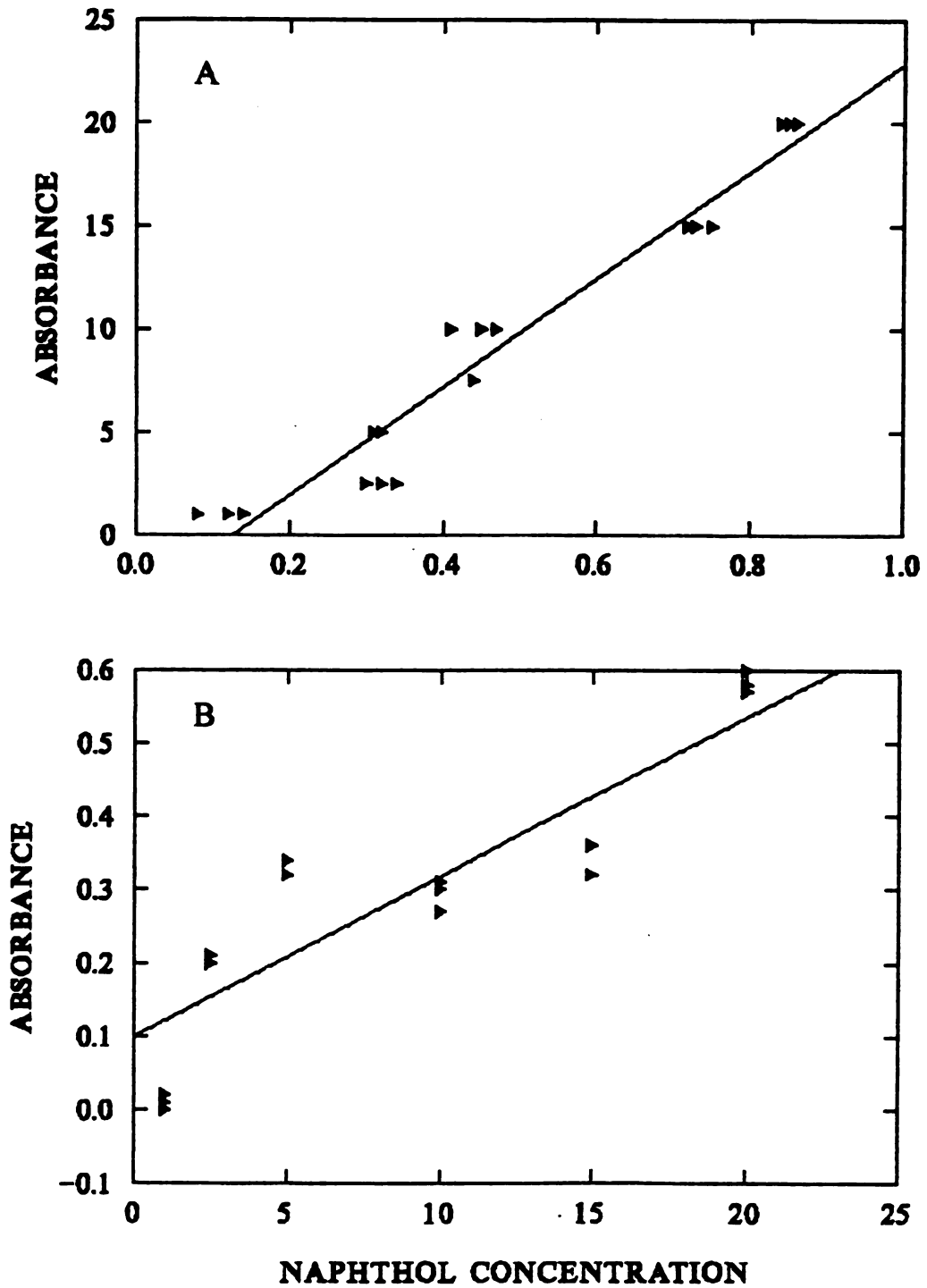
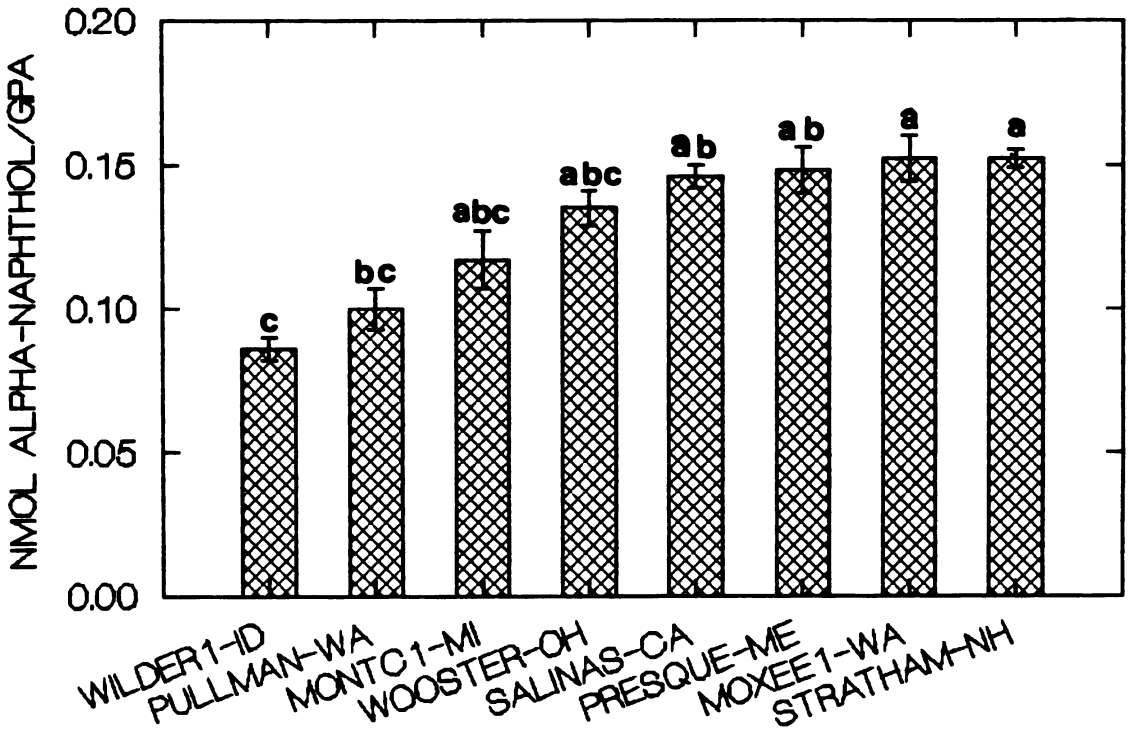


Figure 2.18--Portable *in vitro* assay standard curves for α– (A) and β–naphthol (B) standard concentrations (μg)/ 100 μl plotted against absorbance values (609 nm = α, 555 nm = β). The equations of each line are found in Appendix B.

Figure 2.19--Histograms of portable assay results across eight main strains. Letters signify Tukey's Means Separation Test for significant differences between strains.


NMOL ALPHA-NAPHTHOL/GPA
0.20
0.15
0.10
0.05
0.00
c
bc
abc
abc
ab
ab
a
a
WILDER1-ID
PULLMAN-WA
MONTC1-MI
WOOSTER-OH
SALINAS-CA
PRESQUE-ME
MOXEE1-WA
STRATHAM-NH

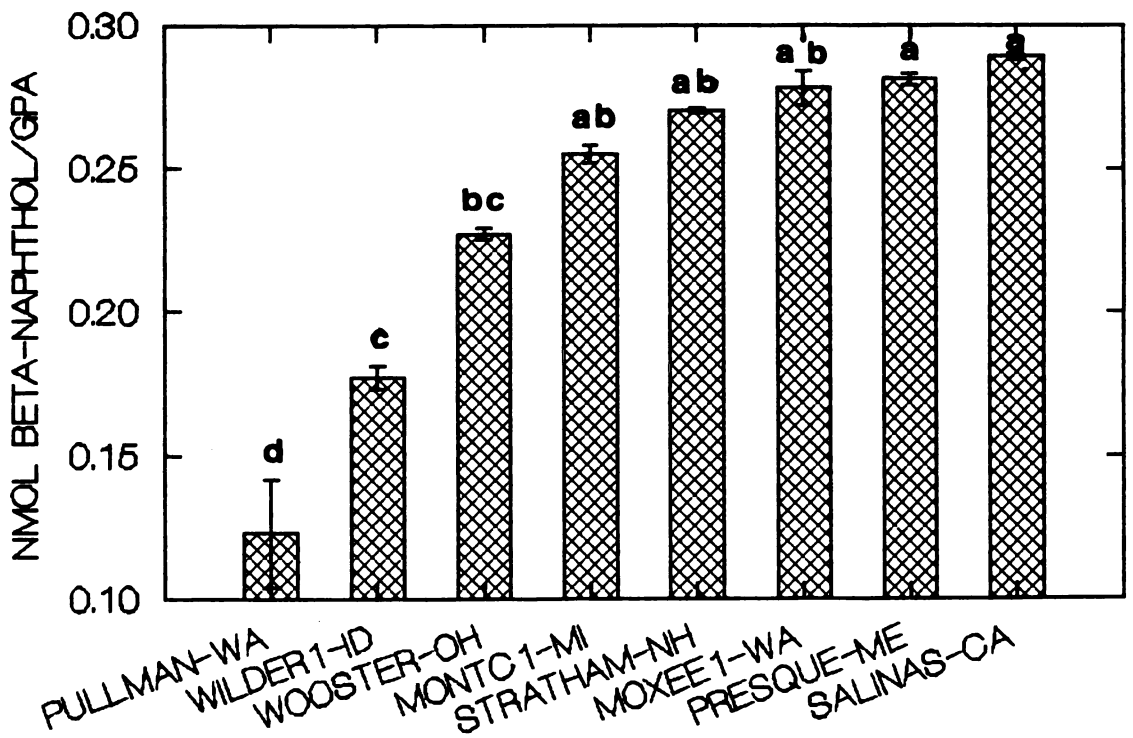

NMOL BETA-NAPHTHOL/GPA
0.30
0.25
0.20
0.15
0.10
d
c
bc
ab
ab
ab
a
a
PULLMAN-WA
WILDER1-ID
WOOSTER-OH
MONTC1-MI
STRATHAM-NH
MOXEE1-WA
PRESQUE-ME
SALINAS-CA

Table 2.9--Portable *in vitro* carboxyesterase assay results for eight main green peach aphid strains including Standard Error of the mean, and Tukey's Test for Pairwise Mean Comparisons with letters signifying statistically significant differences.

Strain	NMOL α–Naphthol[a,c] (±SEM)	NMOL β–Naphthol[b,d] (±SEM)
MOXEE1-WA-P1	.152(±.008)a	.278(±.006)ab
PRESQUE-ME-P2	.148(±.008)a	.281(±.002)ab
SALINAS-CA-O	.146(±.004)a	.289(±.001)a
WOOSTER-OH-P2	.135(±.006)ab	.227(±.002)c
STRATHAM-NH-P2	.152(±.003)a	.270(±.001)ab
MONTCALM1-MI-P2	.117(±.010)bc	.255(±.003)bc
PULLMAN-WA-O	.086(±.004)d	.123(±.019)e
WILDER-ID-P1	.100(± .007)cd	.177(±.004)d

[a]NMOL a-Naphthol/ green peach aphid/10 minutes.
[b]NMOL β-Naphthol/ green peach aphid/10 minutes.
[c]Tukey's Test for pairwise mean comparisons, α=.05, df= 16.
[d]Tukey's Test for pairwise mean comparisons, α=.05, df= 16.

Several other *in vitro* experiments were conducted on the eight strains documented by the portable carboxyesterase assay. The data found by these other tests is correlated in Table 2.10. Positive correlations were found with the microplate assays. The table shows the R values. There is a positive correlation (0.898) between the α-carboxyesterase values for the microplate and the portable assays. This value is significant at $\alpha = 0.01$. The β values, are lower (0.405) and are not significant. The portable α-values correlate with the PAGE densitometry values, 0.802, and are significant (0.05), while the β–portable values correlate 0.914 (0.01 significant), which is strong for both values. These correlations show an overall positive association between α–carboxyesterase portable values and the other *in vitro* assay methods.

D. Conclusions

All three *in vitro* assays were conducted and compared. As a result of the high correlation coefficients, I have shown that the Portable Carboxyesterase Assay is a valid evaluation of Carboxyesterase level when compared with both the Microplate Assay and PAGE. Both resistant and susceptible strains have shown quantifiable differences in carboxyesterase level in both the enzyme assays (Portable Assay and Microplate Assay) and in PAGE. These appears to be a relationship between esterase level and resistance in strains of GPA throughout the United States, with higher levels of resistance found in potato growing regions. The portable assay must now be compared with actual resistance levels as defined by the *in vivo* Dosage Mortality Bioassay.

Figure 2.10--Coefficients for correlation analysis of *in vitro* assays with the portable carboxyesterase data across the eight main strains.

	PAGE	α–Microplate Assay	β–Microplate Assay
α-Portable Assay	0.831**	0.898**	--
β-Portable Assay	0.914**	--	0.405

Significance was tested at $\alpha = .05$ (*) and $\alpha = .01$ (**) levels.
Global χ^2 test = 21.091 * (significant)

CHAPTER 3

IN VIVO EVALUATION OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE

CHAPTER 3. *IN VIVO* EVALUATION OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE

I. Introduction

Field failure of insecticides is the most common means for indicating insecticide resistance in insect pest species. Indeed, field failure is one basis for applied entomologists' definition of insecticide resistance. Although physically spraying a field or other area may be the most common method for discovering resistance, it is certainly not the most efficient means available for such evaluations. For economics reasons and for expediting diagnoses, many researchers utilize biochemical tests for the evaluation of resistance in insects. These tests rely on detection of such anomalies as mutant enzymes with observable differences (ie. increased activity or affinity for a specific substrate) or increased amounts of an enzyme due to gene amplification. These biochemical types of assays have a problem: they do not measure actual resistance levels (survivability) of a population to different insecticides. The only way to accomplish this is by the use of bioassays using different strains of aphids (Needham and Devonshire 1973, 1977).

Evaluations of actual insecticide resistance levels are useful for many purposes. They offer excellent diagnostic information for pest management decision-makers regarding resistance frequencies and chemical specificities within a population. This includes discovering which chemicals and classes of compounds the insects are resistant to and an evaluation of cross resistance or increases in resistance development due to the use of synergists or mixtures. Additionally, actual resistance evaluations offer the benefit of pre-testing compounds on samples taken from field populations in order to observe the effectiveness of control. These evaluations eliminate any experimental conversions and potential conversion errors that can result from inaccurate biochemical-type resistance

evaluations. In other words, actually physically testing field populations of insects with chemicals functions on a true-to-life basis such as what growers would find. Unfortunately, spraying fields with different compounds and then evaluating resistance by the number of survivors is extremely costly, involves a great deal of physical labor and time, and is nearly impossible to truly evaluate. There is no way to count all of the pest insects in a field before spraying, make sure to adequately cover all of them, and then count every last one as dead or alive at the end of the experiment. Sampling methods involve additional cost and time commitments. Furthermore, there are not many farmers who are willing or financially able to spray their fields with many different trials of compounds solely to discover which compounds may or may not offer them reasonable control of pest species. This defeats the purpose of developing pest management techniques and would actively promote pest resistance. Unfortunately, growers often spray on a random chemical choice basis: spraying with one (or more) chemicals until they are no longer effective, then switching to other chemicals or chemicals with synergists after the intial chemicals fail. This system is very costly and is sometimes responsible for the bankruptcy of many small farm operations as well as the high frequencies of insecticide resistance found in many farms at this time.

In vivo insecticide bioassays, however, may offer a viable alternative to trial and error sprays for resistance evaluations. *In vivo* bioassays involve random sampling of field populations of pests, then testing (by one of several means such as dipping or spraying) with different compounds for quantification of resistance proportions. These bioassays also operate on a 1:1 ratio: if ten percent of the insects tested die, the proportion of resistant individuals in the population (if sampling is conducted randomly) is around the 90% range and the utilization of the chemical that the insects were tested with will not serve as an effective means of control. Bioassays of populations may also be conducted to

ascertain the correct chemical to spray pests with at any given time to achieve the necessary mortality levels for control.

The only means by which to measure actual insecticide resistance to different insecticides is by actually physically testing the insects with chemical compounds. Field failure is an extremely costly method to use, and biochemical assays involve highly trained laborers and special equipment along with careful interpretations of data. Bioassays of populations offer the best of both worlds: they can to be conducted in a relatively short amount of time, with minimal cost and data interpretation and yet are a very effective means of determining actual resistance levels of a population.

II. Materials and Methods

A. Strain Selection

Eight strains of GPA were selected in part based on the carboxyesterase values obtained in Chapter 2, utilizing the *in vitro* α-carboxyesterase Microplate Assay results and in part on a random basis. Care was taken to select a proportion of populations from each of the four main resistance levels: susceptible, low, moderate, and high.

B. Chemicals Selection Criterion

Six compounds were chosen to assess the proportion of actual chemical resistance present in each of the eight populations of GPA selected. Chemicals were chosen from each of the three primary chemical classes currently in use against GPA in conventional agricultural systems: organophosphates, carbamates, and synthetic pyrethroids. In addition, the compounds were selected on the basis of their use against GPA by growers in potato fields (Farm Chemicals Handbook 1985, 1992).

C. *In vivo* Assay Methodology

Several different *in vivo* methods for studying actual insecticide resistance in GPA have been used. In the past, topical chemical applications on individuals (Needham and Sawicki 1971, Beranek 1974, Devonshire and Needham 1975, Blackman et al. 1977, Devonshire 1977, Devonshire *et al.* 1977), allowing the GPA to feed on systemical insecticide-treated plants (Devonshire *et al.* 1975), and exposure of aphids to chemical preparations via dips (Devonshire *et al.* 1975, 1977, Sykes 1977, Sawicki *et al.* 1978) are a few of the methods. For this study slide dip bioassays were chosen fore ease of handling, reproducibility, and the ability to keep the control mortality to a minimum.

The dosage mortality study was conducted in two main steps. Several preliminary assays were conducted to determine a relative range for pesticide concentrations. Attempts were made to achieve 90% or above mortality at the highest pesticide concentrations for all strains, while maintaining a maximum control mortality below 10% to 13%.

After observing the results of the Preliminary Assay, eight strains were chosen for intense experimentation. Additionally, five concentrations (and one control) were chosen for each of the six chemicals. Experiments were replicated in full three times, with an n=180 per experiment, 30 GPA per concentration per replicate. Five chemical concentrations and one control solution were used per chemical compound. Fifteen GPA were mounted per slide, with two slides used for each concentration.

Individual GPA were mounted on double sided tape ventral side upwards. After mounting the insects, each slide was dipped in its respective chemical concentration and placed upright in a square glass dish lined with H_2O saturated paper towels. Controls were dipped in either double distilled water or an acetone/double distilled water solution depending on the solubility of the insecticide used. Upon completion of dipping, the glass

dish was sealed with plastic wrap to maintain humidity. Mortality was assessed at 18 hours by brushing their ventral abdoment with a camel's hair paintbrush and evaluating for controlled movement (twitching did not count as controlled movement).

D. Data Analysis

Control mortality was assessed as a percent mortality and computed by MSTAT-C using Abbot's Formula (1925). Each data set was pooled and analyzed by log probit analysis in MSTAT-C (Finney 1952). Probit lines were plotted using log concentration versus probit values for each chemical. These probit lines were graphed in Sygraph (Systat Inc., Evanston, Illinois) for each strain and insecticide and compared for differences in slope. Mean LC_{50} and LC_{90} values and corresponding confidence limits were obtained and compared by overlapping 95% confidence limits for differences between strains for each chemical and for overall trends in chemicals for each strain. These limits were given letters to signify differences. Strains with same letter are the same, different letters signify that two strains are different from each other.

III. Results and Discussion

A. Strains Chosen

Table 3.1 is a listing of the strains chosen for the *in vivo* resistance evaluations. Two strains were collected on peach, the primary host of GPA, and four were collected from potato, the secondary host of GPA. The other two were collected from crops other than the primary or secondary hosts of GPA, such as weed species or peppers. Additionally, the populations were collected from many different areas of the country, most of which are responsible for potato production. There were three strains tested with high carboxyesterase resistance levels (as determined by the *in vitro* carboxyesterase microplate assay), two moderate strains, one low, and two susceptible strains. These strains

Table 3.1--Strains, cooperators, host plants, locations, and resistance levels for populations of green peach aphid used for *in vivo* bioassay.

Strain	Cooperator	Host	Location	Resistance Level
MOXEE1-WA-P1	L. Fox	Peach	Moxee, WA	high
PRESQUE-ME-P2	G. Sewell	Potato	Presque Isle, ME	high
SALINAS-CA-O	L. Fox	Other	Salinas, CA	moderate
WOOSTER-OH-P2	C. Hoy	Potato	Wooster, OH	low
STRATHAM-NH-P2	M. Campbell	Potato	Stratham, NH	high
MONTC1-MI-P2	M. Otto	Potato	Montcalm, MI	moderate
PULLMAN-WA-O	T. Mowry	Other	Pullman, WA	susceptible
WILDER1-ID-P1	L. Fox	Peach	Wilder, ID	susceptible-low

encompass a broad range of resistance levels and yield a wide spectrum of probit lines, slopes, and lethal concentrations.

B. Chemicals Chosen

The chemicals chosen are found in one of three primary classes of compounds: organophosphates, carbamates, or synthetic pyrethroids. Six were chosen in the hope that they would represent a broad spectrum of modes of action for better elucidation of GPA strains of different resistance levels ability to metabolize the compounds. Although the mode of action of organophosphates and carbamates is similar, the mode of toxicity of carbamates is not known for certain. According to Casida (1962), carbamates an exert different mechanisms of action or selective toxicities when hydrolyzed in the bodies of insects. For this reason, the detoxification rates vary highly (Casida 1962).

The chemicals used in the *in vivo* Bioassay are listed found in Table 3.2 along with a schematic of the parent compound structure and a possible metabolite resulting from carboxyesterase hydrolysis of the parent ester bond. Some of these hydrolysis products may not be the primary yields of metabolism, which can also involve oxidation reactions (MFO's) and other types of reactions. However, for this study solely the ester hydrolysis reactions were elucidated because in GPA the only known mechanism of resistance is carboxyesterases (Needham and Sawicki 1971, Devonshire 1975, Devonshire and Needham 1975, Sawicki *et al.* 1980, Baker 1986).

Carbaryl is an aromatic carbamate with two rings. When hydrolyzed by esterases, it forms 1-naphthol, which is also the product of the reaction of 1-naphthylacetate and esterases (Matsumura 1985). This reaction is used for the *in vitro* microplate carboxyesterase assay and for this reason an inhibition reaction with carbaryl would yield activity in an enzyme assay (for 1-naphthol) although the enzyme-1-naphthylacetate reaction is not occurring.

Table 3.2--Listing of insecticides used for the *in vivo* bioassay of insecticide resistance in green peach aphid.

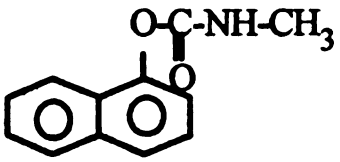
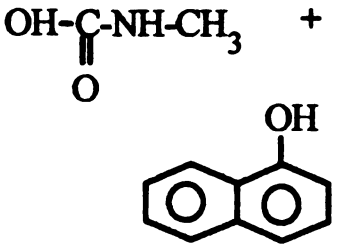
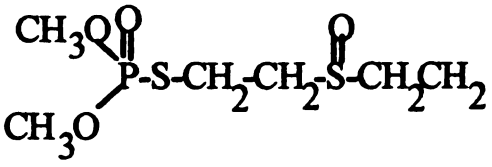
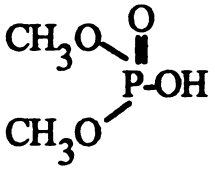
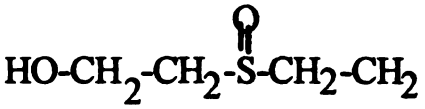
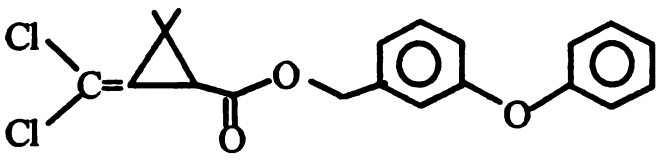
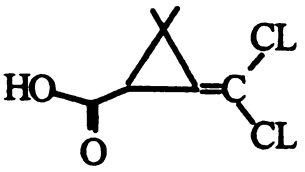
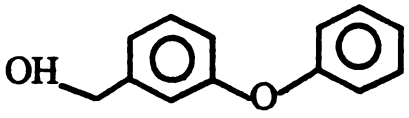
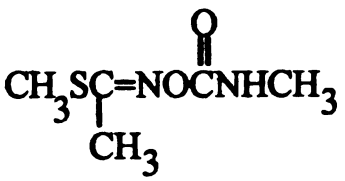
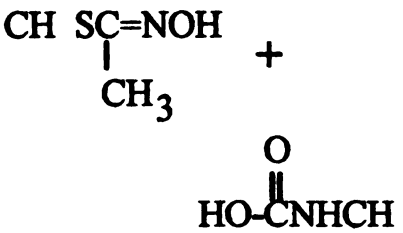
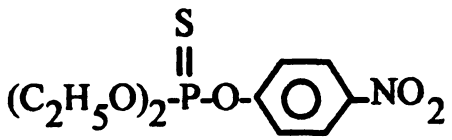
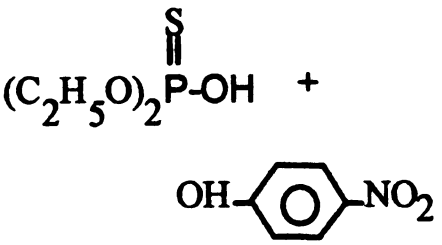
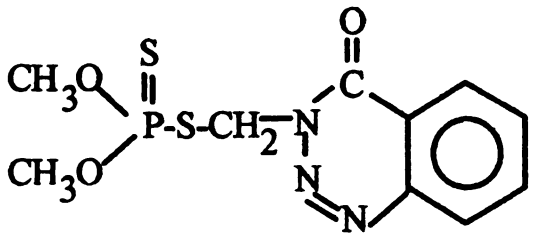
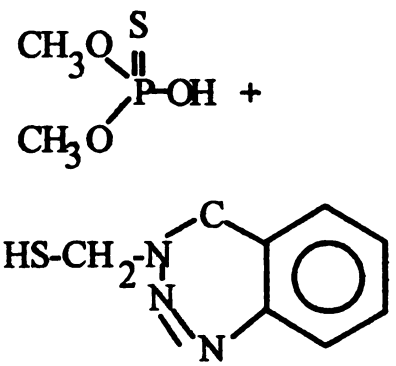
Chemical and Structure	Metabolite
 carbaryl	
 oxydemetonmethyl	 + 
 permethrin	 + 

Table 3.2--Continued.

Chemical and Structure	Metabolite
 methomyl	
 ethyl parathion	
 azinphosmethyl	

Although carbaryl is not a very effective aphicide, it is used on potato for controlling leafhoppers, Tarnished Plant Bug, cutworms, and Flea beetles (MSU Extension Bulletin #312, 1992).

Oxydemetonmethyl is a systemic oxon-organophosphate. For this reason, the compound is very effective once it enters the insect's body. Oxon compounds are generally more toxic than their non-activated parent compounds. Generally, insecticides are activated by the Mixed Function Oxidase (MFO) system, such as when parathion is activated to the more toxic paraoxon before enzymatic degradation. This chemical is commonly applied to potato fields at the rate of 2 pt (SC)/ acre for control of GPA, leafhoppers, and Flea beetles (Epitrix cucumerix (Harris)). It is particularly noted as effective for problem infestations of GPA (MSU Extension Bulletin #312, 1992).

Permethrin is a synthetic pyrethroid containing two aromatic rings. This compound was patterned after the botanical insecticides, such as pyrethrum, but it has the advantage of being more stable in sunlight. Although not a very toxic compound to GPA, it is still used for control of leafhoppers in potato fields (MSU Extension Bulletin #312, 1992).

Methomyl is a carbamate compound. Unlike carbaryl, methomyl has no aromatic rings. Methomyl, or Lannate®, is commonly used for control of GPA, cutworms, flea beetles, and leafhoppers in potato fields at a field rate of 0.5 lb/acre (MSU Extension Bulletin #312, 1992).

Azinphosmethyl is an organophosphate with two rings, one containing nitrogen. Azinphosmethyl is commonly known as Guthion® and although it is generally not used for GPA control on potato, it is used for control of other pests such as Colorado Potato Beetle, flea beetles, and leafhoppers (MSU Extension Bulletin #312, 1992).

The six compounds were not only chosen from several different classes of compounds, but with care taken that they also varied in toxicity level. For this reason, they have different reaction sequences in GPA and perhaps different modes of action. The mammalian toxicities of each compound were also taken into account solely for a general range of toxicity. However, mammalian and insect toxicities are not necessarily related, this was just used as a basis. Care was taken to select compounds not only on the basis of a good diversity of compound classes, but also with respect to a variable range of toxicities.

C. *In vivo* Bioassay

Prelimary assays were conducted solely to gain an understanding of the range of effective concentrations to be used for each compound. The compound concentrations used are elucidated in Table 3.3 They vary extensively from compound to compound depending on toxicity and resistance levels to the compounds found in strains tested. Concentrations were chosen on the basis that they produced approximately 90% mortality or higher at the highest dose, with decreasing mortality percentages with decreasing concentration. The data are presented in ppm in double distilled H_2O.

Figure 3.1 elucidates the probit data for azinphosmethyl and oxydemetonmethyl (two organophosphates) in graphical form. Graph A shows the probit lines for azinphosmethyl (Guthion). Each strain in this graph shows a similar, relatively flattened slope of the line except for MONTC1-MI, which shows a more vertical line. Generally the flatter the slope of the line, more heterogenous the population is. Additionally, there is greater resistance potential for a strain that has a flat slope than a more vertical sloped line that has the same LC_{10} value. Therefore, of the seven strains tested with azinphosmethyl, MONTC1-MI has the most heterogenous population. The strain that has the lowest log concentration values is PULLMAN-WA, at the LC_{10}, LC_{50}, and LC_{90} values consistently. Closely parallel to PULLMAN-WA is SALINAS-CA, also susceptible to azinphosmethyl. The two strains

Table 3.3--Listing of insecticide, compound class, and concentrations used for in vivo bioassays on green peach aphid.

Chemical	Compound Class	Concentration[1]				
		#5	#4	#3	#2	#1
azinphosmethyl	organophosphate	420	168	109	84	29
ethyl parathion	organophosphate	88	57	31	11	7
carbaryl	carbamate	442	178	115	62	22
permethrin	synthetic pyrethroid	1892	1135	757	378	151
oxydemetonmethyl	organophosphate	1288	644	386	129	26
methomyl	carbamate	1750	875	525	175	35

[1]Concentrations measured in ppm.

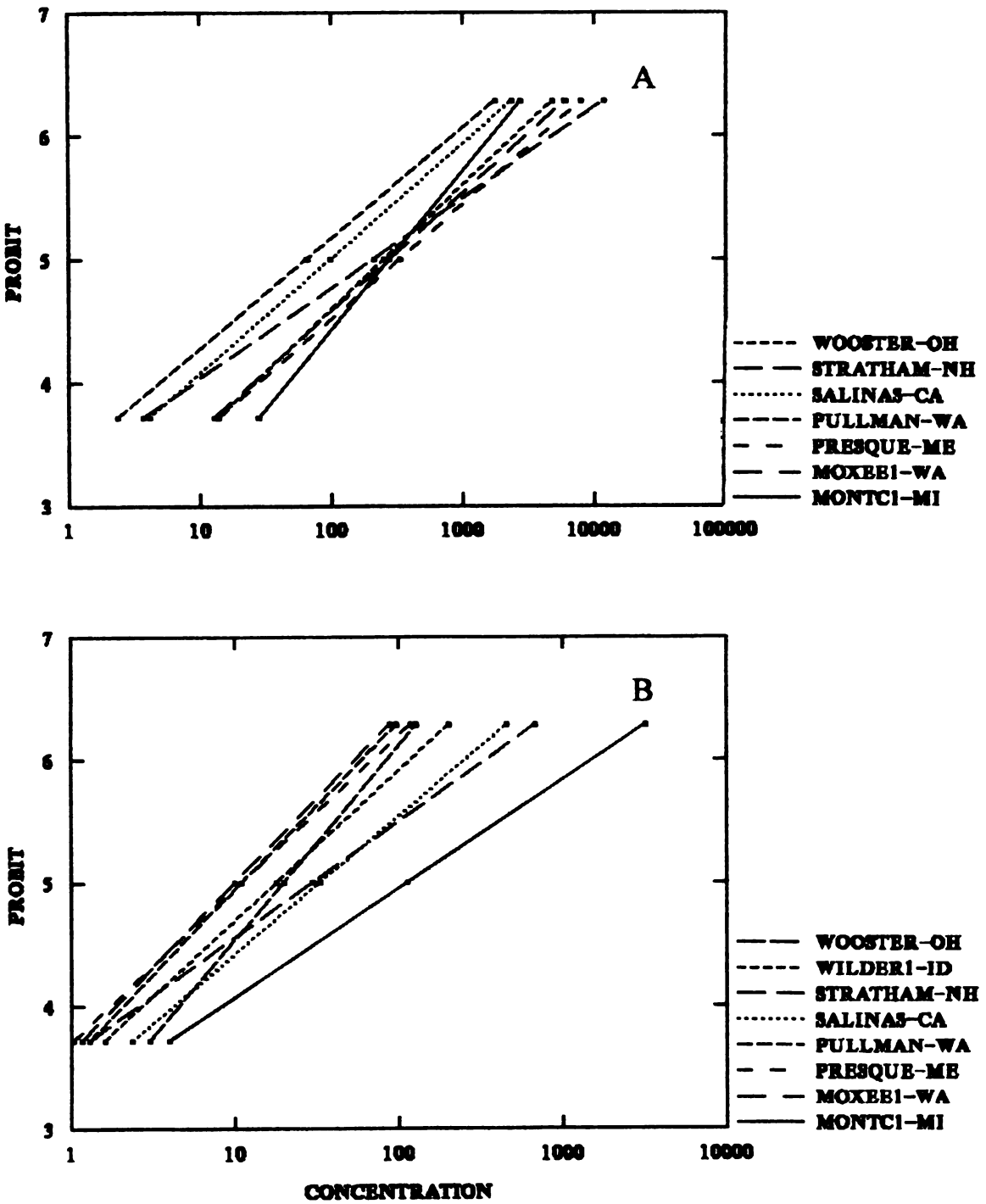


Figure 3.1--Regression lines depicting the relationship between probit values and lethal concentrations for azinphosmethyl (A) and oxydemetonmethyl (B).

have similar slopes, although SALINAS-CA shows a slightly steeper slope than does PULLMAN-WA. The most resistant strain at LC_{10} is MONTC1-MI. However, due to MONTC1-MI's extremely steep slope, by the LC_{90} concentration, MONTC1-MI has teh third lowest LC_{90} value. The other four strains: PRESQUE-ME, MOXEE1-WA, WOOSTER-OH, and STRATHAM-NH are closely grouped after the LC_{50} value and although there is some difference in slope, they remain close after this point. Although there was is a wide spread prior to the LC_{50} point, this rapidly bottlenecks and the strains become grouped hereafter.

Figure 3.1B illustrates the probit line for oxydemetonmethyl, a systemic organophosphate. In this graph there is little crossing over of strains from the LC_{10} to the LC_{50} and LC_{90} values. The strain with the highest resistance potential is MONTC1-MI, due not only to the extremely flat slope of the line but also to the fact that this strain yielded the highest LC_{10}, LC_{50}, and LC_{90} values. When compared with the azinphosmethyl probit line, the opposite was true: MONTC1-MI was the strain with the lowest reistance potential of the seven tested. However, in both graphs, MONTC1-MI began as the most resistant strain at the LC_{10} level, and it was not until the LC_{50} point that the slope-associated difference was elucidated.

In Figure 3.1B (oxydemetonmethyl) the strain with the most vertical slope of the line is WOOSTER-OH. This strain was among the closely grouped strains on the azinphosmethyl probit graph. The first three strains found on the oxydemetonmethyl graph: MOXEE1-WA, PRESQUE-ME, and PULLMAN-WA, are very closely grouped, with similar lethal concentrations and slopes.

Figure 3.2 is a set of two graphs illustrating the probit lines of parathion (A) and permethrin (B). Graph A elucidates the probit lines for ethyl parathion, a very commonly

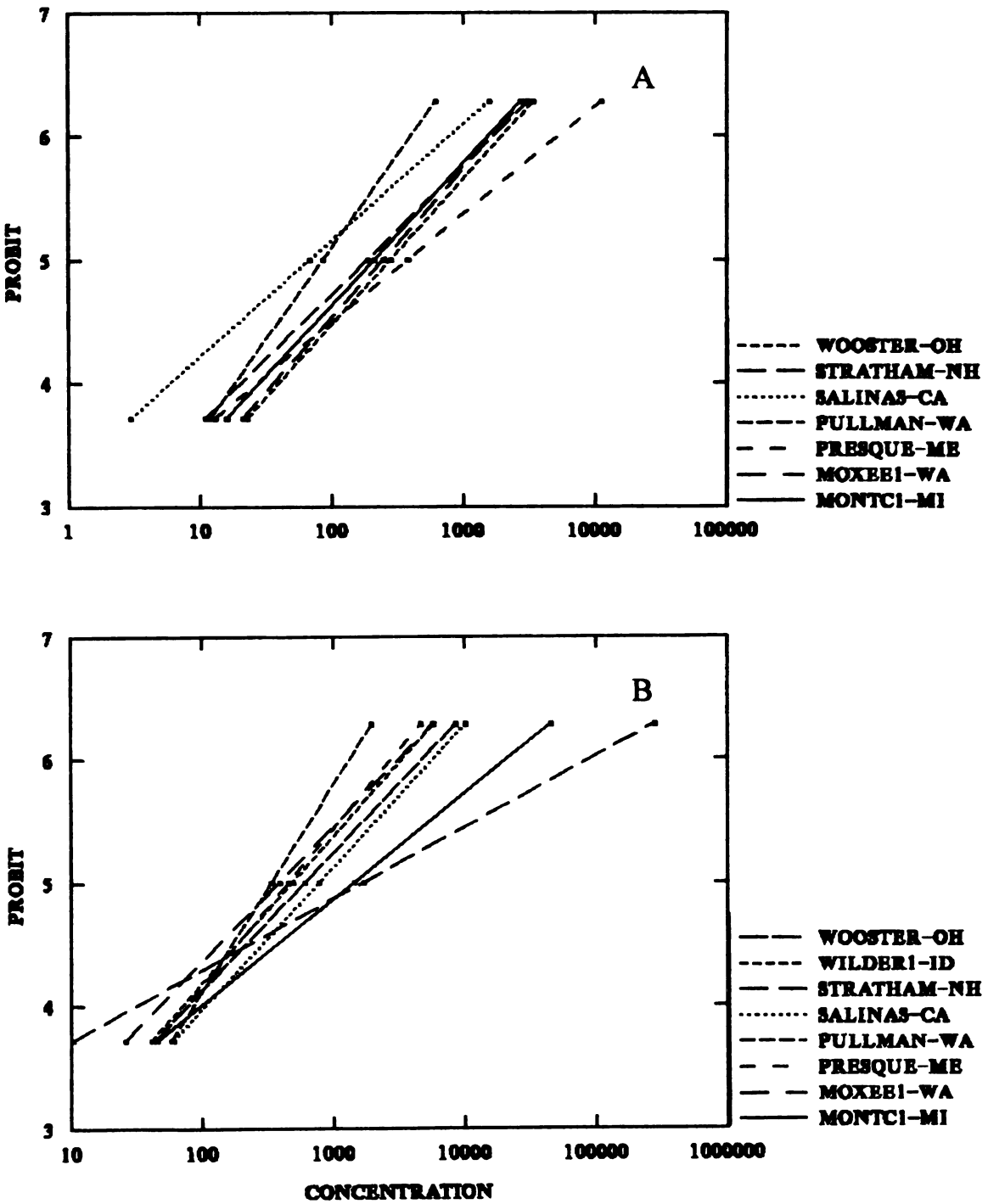


Figure 3.2 -Regression lines depicting the relationship between probit values and lethal concentrations for parathion (A) and permethrin (B).

used organophosphate compound, and the most toxic insecticide used for the *in vivo* Bioassay (Ware 1983). The strain in this graph with greatest vertical slope is PULLMAN-WA, it is also the most susceptible strain at the LC_{90} level, showing that it has the lowest resistance potential of all strains tested. STRATHAM-NH is the most susceptible strain at the LC_{10} level, but it has a more horizontal slope and therefore crosses over the PULLMAN-WA strain at approximately the LC_{50} level and becomes the second most susceptible strain. At LC_{90}, the most resistant strain is PRESQUE-ME, but this is due in part the the flat slope of it's probit line. At the LC_{10} level, it is only a moderately resistant strain, part of a group of six moderate strains. Five of these strains are still grouped at the LC_{50} level, and four remain grouped at the LC_{90} level.

Figure 3.2B illustrates probit lines for the least toxic compound used, permethrin, a synthetic pyrethroid. The strain with the most vertical slope is PULLMAN-WA. The most resistant strain at LC_{90}, STRATHAM-NH, is by far the most resistant strain of all tested. In addition, this strain has the greatest resistance potential, due to the very flat slope of it's probit line. At the LC_{10} level this strain is the most susceptible, as well, showing that this strain is very heterogeneous for resistance to permethrin. The second most resistant strain is MONTC1-MI, which has yielded the highest resistant results to both azinphosmethyl and oxydemetonmethyl, but not to parathion. The other five strains remain closely grouped throughout the graph.

Figure 3.3 is an illustration of the probit lines for methomyl (A) and carbaryl (B), both carbamate insecticides. Graph A shows a very closely grouped association of lines. The highes value at LC_{10} and LC_{50} appears to be SALINAS-CA, however, there do not appear to be any significant differences between lines or slopes of lines in this graph. All eight strains have fairly similar slopes of their probit lines. One exception is

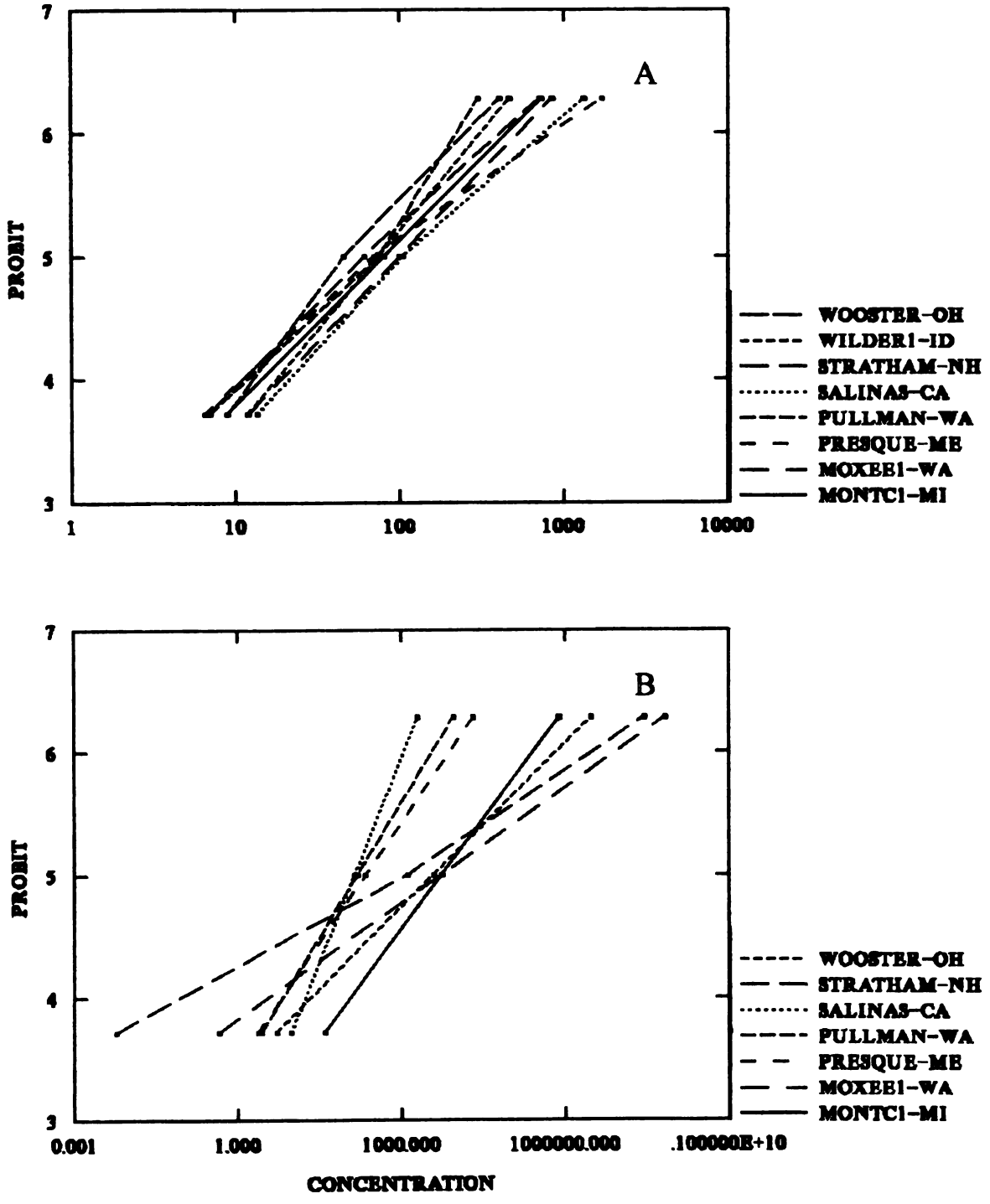


Figure 33--Regression lines depicting the relationship between probit values and lethal concentrations for methomyl (A) and carbaryl (B).

PULLMAN-WA, the strain with the lowest LC_{50} value, and the most susceptible strain across all chemicals. All other strains are very closely grouped.

Figure 3.3B is another carbamate, carbaryl (Sevin®). This graph has the greatest variation and the highest concentrations due to its relatively low toxicity. This chemical yielded broad ranges for concentrations at all levels and a high error level. Difficulties occurred in obtaining a 90% mortality rate across all strains. As a result, some curious probit lines are shown. Strain STRATHAM-NH yielded the flattest slope, however it was paralleled closely by MOXEE1-WA, the strain with the greatest LC_{50} and LC_{90} values. The strain with the steepest slope of the line was SALINAS-CA and this was grouped at the LC_{50} level with PULLMAN-WA (the susceptible for all other chemicals) and PRESQUE-ME. However, the slope of these two strains lines was more horizontal than that of SALINAS-CA, and therefore they did not yield as low LC values.

The calculated LC_{50} and LC_{90} values and 95% confidence limits for each strain and chemical are listed in Table 3.4. In addition, so are the slopes of the lines. Overlapping confidence limits of LC_{50}'s were compared, but a multiple range test was not run because it is not reliable unless weighted. Data for carbaryl is not discussed due to a high error level (very broad confidence limits). This error was due not only to a natural variability in GPA strains but to the low level of toxicity of the compound to GPA. Letters signify differences between strains. Overall, the most susceptible strain was PULLMAN-WA, maintaining the lowest for LC_{50} values. In the case of azinphosmethyl, PULLMAN-WA was significantly lowest of all strains (LC_{50} = 65), with SALINAS-CA the second-most susceptible (LC_{50} = 99). These two strains also had the lowest LC_{50} level for parathion, but not for oxydemetonmethyl (MOXEE1-WA, PRESQUE-ME, and PULLMAN-WA), methomyl, or permethrin. The strain most resistant to azinphosmethyl was PRESQUE-ME (LC_{50} = 334), as well as for parathion (LC_{50} = 381). For methomyl, the most

Table 3.4--LC_{50} values and slopes for each insecticide used in the *in vivo* bioassay.

	Azinphosmethyl		Parathion	
Strain	LC_{50}	Slope (χ^2 value)	LC_{50}	Slope (χ^2value)
MONTC1-MI	279 (253-307)ab	1.28 (18.36)	211 (193-231)cd	1.16 (19.2)
MOXEE1-WA	279 (245-317)abc	0.959 (25.8)	249 (230-270)bc	1.2 (25.8)
SALINAS-CA	99(90-108)e	0.928 (5.91)	68 (59-78)f	0.94 (49.4)
PRESQUE-ME	334(286-389)a	0.928 (12.6)	381 (344-421)a	0.87 (31.3)
STRATHAM-NH	211(183-243)d	0.73 (10.7)	189 (171-209)d	1.05 (49.2)
WOOSTER-OH	252(225-283)bcd	0.998 (12.2)	281 (259-305)b	1.18 (19.9)
PULLMAN-WA	65(58-73)f	0.89 (12.1)	86 (79-94)e	1.49 (18.6)
WILDER1-ID	--	--	--	--

Table 3.4--Continued.

Strain	Permethrin		Carbaryl	
	LC_{50}	Slope (χ^2 value)	LC_{50}	Slope (χ^2value)
MONTC1-MI	1455 (1271-1666)a	0.85 (18.5)	5641 (3555-8953)	0.60 (4.1)
MOXEE1-WA	388 (355-425)e	1.09 (11.5)	5681 (2799-11532)	0.31 (17)
SALINAS-CA	785 (725-849)b	1.15 (16.2)	140 (127-153)	1.11 (1838)
PRESQUE-ME	458 (427-491)d	1.27 (14.7)	225 (197-258)	0.65 (15.7)
STRATHAM-NH	1709 (1369-2132)a	0.58 (29.6)	1338 (768-2331)	0.24 (4.05)
WOOSTER-OH	612 (568-660)c	1.11 (16.6)	3972 (2495-6324)	0.444 (14.3)
PULLMAN-WA	339 (318-362) e	1.68 (21.2)	159 (140-180)	0.73 (8.26)
WILDER1-ID	489 (453-527)d	1.19 (14.3)	--	--

Table 3.4--Continued.

Strain	Oxydemetonmethyl		Methomyl	
	LC_{50}	Slope (χ^2 value)	LC_{50}	Slope (χ^2value)
MONTC1-MI	114 (95-136)a	0.88 (9.57)	81 (76-87)c	1.33 (31.3)
MOXEE1-WA	10 (9-11)d	1.37 (20.48)	101 (95-108)b	1.38 (40.97)
SALINAS-CA	33 (31-36)b	1.12 (16.4)	135 (125-144)a	1.29 (29.1)
PRESQUE-ME	11 (10-12)d	1.24 (12.98)	105 (97-114)b	1.058 (27.5)
STRATHAM-NH	30 (27-33)b	0.94 (4.89)	70 (65-76)cd	1.27 (36.2)
WOOSTER-OH	20 (18.5-21)c	1.57 (6.45)	61 (57-65)d	1.55 (9.30)
PULLMAN-WA	11 (10-12)d	1.37 (15.2)	46 (43.7-50)e	1.58 (10.7)
WILDER1-ID	18 (16.5-19)c	1.22 (8.24)	75 (70-79)c	1.61 (6.74)

resistant strain was SALINAS-CA (LC_{50} = 135), and for permethrin the highest LC_{50} was 1709 for STRATHAM-NH. However, this overlapped confidence limits with MONTC1-MI (1455), so that both strains are considered the most resistant at LC_{50}. MONTC1-MI was also the most resistant strain at LC_{50} for oxydemetonmethyl, a systemic organophosphate.

Overall, data showed excellent differences across strains and chemicals. Carbaryl was the only chemical which showed a high error level and therefore wide 95% confidence limits. Even carbaryl yielded some differences across strains. The most toxic (to mammals) compound tested, parathion, did not show the lowest LC_{50}'s. Oxydemetonmethyl, the systemic oxon-organophophate compound had the lowest LC_{50} values, ranging from 10 to 114, but an average of 31 ppm. Parathion's average LC_{50} was 209 ppm. This is most likely due to two factors: the fact that oxydemetonmethyl is a systemic oxon-organophosphate and therefore probably more toxic to aphids than a regular organophosphate (Ware 1983, Matsumura 1985).

Resistance ratios were computed to aid in comparing between strains. These resistance ratios (RR) are listed in Table 3.5 and are solely indexing tools to observe trends. Some strains maintain approximately the same RR from one chemical to another (PULLMAN-WA, WILDER1-ID, SALINAS-CA), whereas others vary highly (MONTC1-MI, MOXEE1-WA, STRATHAM-NH). Differences also can exist between LC_{50} and LC_{90} RR's, such as STRATHAM-NH shows in carbaryl: a range of 8.4 to 32269. Other strain RR's remain the same for a certain chemical whether it is LC_{50} or LC_{90}, such as MONTC1-MI exemplifies for azinphosmethyl a range of 1.5 to 1.3.

Table 3.5--Summary of resistance ratios for both LC_{50} and LC_{90}'s of six insecticides for eight strains of green peach aphid.

STRAINS	CARBARYL		METHOMYL		PERMETHRIN	
	LC_{50} RR[a]	LC_{90} RR[b]	LC_{50} RR	LC_{90} RR	LC_{50} RR	LC_{90} RR
MONTC1-MI	35.5	86.9	1.7	2.4	4.3	23.5
MOXEE1-WA	35.7	7718.0	2.2	2.8	1.1	2.9
SALINAS-CA	0.9	0.2	2.9	4.4	2.3	5.2
PRESQUE-ME	1.4	2.3	2.3	5.6	1.4	2.4
STRATHAM-NH	8.4	32269.0	1.5	2.3	5.0	143.2
WOOSTER-OH	25.0	337.0	1.3	1.3	1.8	4.4
PULLMAN-WA	1.0	1.0	1.0	1.0	1.0	1.0
WILDER1-ID	--	--	1.6	1.5	1.4	3.0

[a]RR LC_{50} is calculated by LC_{50} Strain/ LC_{50} Susceptible (PULLMAN-WA).
[b]RR LC_{90} is calculated by LC_{90} Strain/ LC_{90} Susceptible (PULLMAN-WA).

Table 3.5--Continued.

STRAINS	PARATHION		AZINPHOSMETHYL		OXYDEMETONMETHYL	
	LC_{50} RR[a]	LC_{90} RR[b]	LC_{50} RR	LC_{90} RR	LC_{50} RR	LC_{90} RR
MONTC1-MI	2.5	4.3	4.3	1.5	10.4	33.3
MOXEE1-WA	2.9	4.6	4.3	3.4	0.9	0.9
SALINAS-CA	0.8	2.5	1.5	1.3	3.0	4.7
PRESQUE-ME	4.4	17.9	5.1	4.5	1.0	1.2
STRATHAM-NH	2.2	5.0	3.2	6.6	2.7	7.1
WOOSTER-OH	3.3	5.5	3.9	2.7	1.8	1.3
PULLMAN-WA	1.0	1.0	1.0	1.0	1.0	1.0
WILDER1-ID	--	--	--	--	1.6	2.1

[a]RR LC_{50} is calculated by LC_{50} Strain/ LC_{50} Susceptible (PULLMAN-WA).
[b]RR LC_{90} is calculated by LC_{90} Strain/ LC_{90} Susceptible (PULLMAN-WA).

B. General Discussion of *in vivo* Resistance Analysis

Many researchers have assayed resistant aphids for toxicology work but only a few have assayed the same strain(s) against a variety of chemicals (Sudderuddin 1973). Bjorling *et al.* (1966) showed a strain of GPA resistant to parathion, demeton, thiometon, phosamidon and thionazin. Multiple resistance has been shown by others (Hurkova 1970). Needham and Sawicki (1971) worked out resistant levels of GPA exposed to several organophosphate and carbamate insecticides (Sudderuddin 1973). Ludvik and Decker (1947 and 1951) determined that an effective insecticide should have the chemical structure $(RO)_2P(O)R'$.

According to Sawicki *et al.*, aphid bioassays are cumbersome and lengthy and seldom give accurate results of the proportion of resistant individuals in a heterogeneous population (Sawicki et al. 1978). As with any single resistance evaluation tool, there are problems as well as benefits associated with it. The bioassay is labor-intensive, time consuming, and involves working with hazardous substances. However, there are several advantages to the technique such as an actual evaluation of resistance and mortality based on the insects' ability to survive chemical applications and the ability to test any compound at any time given the availabilty of insects. No single test can provide all information about resistance for a strain of GPA. For this reason, I conducted several different types of experiments, the dosage (concentration) mortality bioassay among them. The dosage (concentration) mortality assays were conducted in order to determine by actual pesticide concentrations the resistance levels of various populations of GPA. This test will be correlated with data found in the carboxylesterase enzyme microplate assays, the polyacrylamide gels, and a portable carboxyesterase assay.

In some tests, the dose that killed the adults failed to kill nymphs born during the experiment. Sawicki and Rice observed the same phenomenon in leaf-dip bioassays on

GPA (1978). Devonshire and Moores attributed this to increased levels of esterases they found in embryos (1982). I believe it could be because many of the larvae miss being dipped into the insecticide, and so they are only in contact with the chemical through a limited area, the surface of the tape-covered slide they are resting on, and this only after the chemical has had some time in which to degrade. In determining mortality percentages, progeny born during the experiment were disregarded and only adults were quantified.

Results indicate that although resistance is a serious problem, selection of the appropriate insecticide can be of great assistance in achieving satisfactory control. For example, carbaryl would be an ineffective aphicide for all but the most susceptible strains. However, oxydemetonmethyl is a highly effective alternative. Cochran observed similar effects of choice in efficacy of insecticides in the German Cochroach (1989). A tool such as the *in vivo* assay could be invaluable in screening populations for the choice of the proper chemical toxicant.

CHAPTER 4

CORRELATION OF *IN VITRO* AND *IN VIVO* EVALUATIONS OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE

CHAPTER 4. CORRELATION OF *IN VITRO* AND *IN VIVO* EVALUATIONS OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE

I. Introduction

In the past, several different methods for evaluating insecticide resistance have been used. Some of these are: chemical injections (Blackman 1975), systemic insecticides taken up in plants (Devonshire *et al.* 1975). Dip assays (Devonshire *et al.* 1975, 1977, Sawicki *et al.* 1978), topical applications onto individual insects (Needham and Sawicki 1971, Beranek 1974, Devonshire and Needham 1975, Blackman 1977), colorimetric tests (Needham and Sawicki 1971, Sudderuddin 1973, Blackman *et al.* 1977), electrophoresis (Beranek 1974, Blackman 1975, Devonshire 1975, Devonshire and Needham 1975, Oppenoorth and Voerman 1975, Baker 1977, Blackman *et al.* 1977, Devonshire 1977, Devonshire and Moores 1982, Devonshire *et al.* 1982), and placing aphids on treated leaves (Dunn and Kempton 1966, Hurkova 1973).

According to Sawicki *et al.* (1977) resistance to organophosphate insecticides is easy to detect in GPA because there is a positive correlation between level of resistance (as measured by bioassay) and the amount of esterase-4, an α-carboxyesterase (Sawicki *et al.* 1977). They also state that estimating the activity of the enzyme by electrophoresis or carboxyesterase determinations is a rapid and accurate measure of resistance in individuals (1977). For this reason, correlations were sought between both *in vitro* and *in vivo* methodologies for determining resistance levels in GPA.

As stated earlier, there are many positive and many negative aspects associated with different tests for resistance evaluations. No single test eliminates all negative aspects including actual field failures of insecticide chemicals. For this reason, several different evaluations were conducted and then a comparison was made, ranking each test on the

basis of cost, rapidity, accuracy, and overall effectiveness,. Although none of these ranks are absolute measures, they are evaluations to help pest management decision makers decide which test may be the most appropriate one for them to use.

II. Materials and Methods

As described earlier, data were ranked for their performance on the basis of six criteria: (A) Economy (cost), (B) Rapidness (hours of labor), (c) Accuracy, (d) Precision, (e) Sensitivity, and finally (f) Overall Effectiveness. For each examination type there is a separate evaluation procedure.

A. Economic Evaluation

Each data set was evaluated on the basis of economics to determine total cost. From the total cost it is easier to achieve some basis for the determination of a cost-benefit analysis of each experimental set. A basic economic analysis consists of listing expenses in an itemized order, research prices in manufacturers' catalogues, totaling the costs, and ranking each test according to those costs. The analysis was based on personal experience from the experiments conducted in this thesis as well as market research for chemical and equipment pricing, and therefore this evaluation is a valid assessment of costs for each assay procedure. Each experiment was then ranked on the basis of economics.

B. Labor Evaluation

Each technique was evaluated for total time of labor required. For this analysis each experiment is ranked according to the necessary hours of labor per one complete analysis of a data set. These data include sampling time, experiment (including possible incubation times) and experimental analysis and results interpretation time. Once again each technique is ranked according to the labor evaluation.

C. Accuracy Evaluation

Data from the *in vitro* and *in vivo* assays were compared in Systat (1990) using Regression Analysis with a t-test (partial) and an F-test for significance (Cohen and Cohen 1980). Only resistant strains were used for this analysis, as one would only expect a resistance correlation between resistant strains. The strains used in the microplate carboxyesterase assays, portable carboxyesterase assays, PAGE, and the *in vivo* bioassays were observed for the degree of association. During correlation analysis the LC_{90} level values were analyzed as lethal concentrations, using only the resistant strains, as lower correlations would be expected for susceptible and low level resistance populations. Additionally, insecticide-inhibited PAGE gels were tested for degree of association with both the LC_{50} and LC_{90} values found in the *in vivo* study for their corresponding chemical. The correlation coefficients were used for the accuracy rankings.

D. Precision

Each technique was rated on overall precision based on the repeatability of each test while maintaining accuracy. Any extra means by which to reduce errors in replicates was evaluated. This ranking was based on personal experience gained in performing the series of experiments for this thesis.

E. Sensitivity

The sensitivity rankings were based on the amount of insect material necessary to complete an assay. The evaluation was based on data obtained in this study involving the amount of insect material necessary for completion of a statistically valid assay. Additionally, this evaluation was based on the quality of equipment used for each assay.

F. Overall Effectiveness

Overall effectiveness evaluations were calculated based on the results of the economic, accuracy, and labor evaluations. These overall effectiveness rankings are to determine the best tests to be used for field or laboratory diagnosis of insecticide resistance in GPA.

III. Results and Discussion

A. Economic Evaluations

Based on the economic study, Tables 4.1 and 4.2 have been constructed. Table 4.1 is an itemized economic study of the *in vitro* carboxyesterase assays. The total costs for different experiments for resistance evaluations vary. The *in vitro* study costs range from the highest, $374.50 without equipment and $3317.50 with equipment for PAGE, $182.00 per 25 insects without equipment for the microplate assay (complete) ($3785.00 with equipment), and $108.50 for the portable assay (without equipment, $618.50 with equipment). The *in vivo* experiment cost $480.00 without equipment and $1883 with equipment. In addition, when conducting multiple experiments, the *in vivo* procedure is increasingly more expensive, whereas the PAGE procedure does not increase much. It is far easier to assay multiple strains with the PAGE procedure than it is with the *in vivo* procedure because of sample size. This increase is also seen in the microplate and portable assays, which would increase in cost due to increasing time commitments. For this reason, without equipment the cost ranking is ***in vivo* bioassay > PAGE > microplate assay > portable assay**. With equipment, the ranking is **Microplate Assay > PAGE > *in vivo* bioassay > portable assay.**

B. Labor Evaluations

Labor was evaluated on a per population sample basis. Table 4.3 shows an estimate of labor time necessary for experimental, data analysis, and sampling. The *in vivo* bioassay takes approximately 25.5 hours for 190 insects to be assayed (including incubation time of

Table 4.1--Itemized list of costs for *in vitro* assay procedures.

	MICROPLATE	PORTABLE	PAGE
Chemicals	$ 15.00	$ 10.00	$ 20.00
Basic Supplies	$ 9.50	$ 6.00	$ 2.00
Labor			
Solutions	$ 30.00	$ 30.00	$ 60.00
Experiment	$ 52.50	$ 37.50	$ 255.00
Data Analysis	$ 75.00	$ 25.00	$ 37.50
Equipment			
EIA Reader	$3000.00	$ 450.00	
Gel Apparatus			$1095.00
Power Supply			$1095.00
Drying Apparatus			$ 150.00
Portable Reader		$ 450.00	
Portable Filters		$ 60.00	
Pipettes + Tips	$ 603.00		$ 603.00
TOTAL without equipment	$ 182.00	$ 108.50	$ 374.50
TOTAL with equipment	$3785.00	$ 618.50	$3317.50

Figure 4.2--Economics of the *in vivo* bioassay for one chemical.

COMMODITY	BIOASSAY COST
Chemicals	$ 50.00
Basic Supplies	$ 100.00
Labor	
Solutions	$ 30.00
Experiment	$ 250.00
Data Analysis	$ 50.00
Equipment	
Microscope	$ 800.00
Pipettes + Tips	$ 603.00
TOTAL without equipment	$ 480.00
TOTAL with equipment	$1883.00

Table 4.3--Labor evaluation of the four techniques for resistance diagnosis.

	Microplate Assay	Portable Assay	PAGE	*in vivo* Bioassay
Sample	30 min	30 min	15 min	5 hours
Solutions	2 hours	2 hours	4.5 hours	30 min
Experiment	3.5 hours	1 hour	5 hours	5 hours
Data Analysis	--	--	9 hours	18 hours
TOTAL	6 hours	3.5 hours	18.75 hours	28.5 hours

eighteen hours). The portable assay takes only 3.5 hours for 20 insects to be assayed, including sample time. The total hours are ranked as: ***in vivo* bioassay > PAGE > microplate assay > portable assay**.

C. Accuracy Evaluations

Table 4.4 shows the correlation coefficients and an asterisk for significance of the F-test (and t-test), with * indicating significance at α=.05,** α=.02, and *** α=.01 for R^2 values with one degree of freedom in the numerator and six in the denominator. This table shows the relationships between α- and β-carboxyesterase level and LC_{50} for the *in vivo* bioassays (microplate α- and β-). The permethrin LC_{90} correlates strongly (85%***) with the microplate α values. Weaker correlations exist with the Basic PAGE densitometry units (76%*).

There is a strong correlation between methomyl and the α-microplate assay (94%***), the α–portable assay (99%***), and PAGE (95%***). Parathion also showed strong correlations with all three (Microplate = 87%**, Portable 100%***, and PAGE 94%***). Azinphosmethyl showed and extremely strong correlation with the α–portable assay (99%***) and a fairly strong correlation with PAGE (86%**) but none with the α–microplate assay.

These data show that correlations exist between the *in vitro* carboxyesterase evaluations of resistance and the *in vivo* bioassays. An accuracy rating for these tests, taking into account chance for experimental error, would follow a format such as this (from most accurate to least accurate): **microplate assay > PAGE > portable assay > *in vivo* bioassay**. All tests are actually quite close in accuracy, however, the reason the portable assay is next to last is that the handheld spectrophotometer probably does not have as high quality lens as it should for laboratory work. The *in vivo* bioassay is last because there is a

Table 4.4--Correlation coefficients for regression analysis of *in vivo* Bioassays with *in vitro* Assays.

	α-Microplate Assay	α-Portable Assay	PAGE
LC_{90} Permethrin	.85 **	.50	.758*
LC_{90} Methomyl	.94 ***	.99 ***	.95 ***
LC_{90} Parathion	.87 ***	1.00 ***	.94 ***
LC_{90} Oxydemetonmethyl	.82 **	.46	.73 *
LC_{90} Azinphosmethyl	.68	.99 ***	.86 ***

Both a t-test (partial) and an F-test were conducted on the data to test for significance. * signifies significance at $\alpha = .05$, ** signifies significance at $\alpha = .025$, and *** signifies significance at $\alpha = .01$.

great deal of room for experimental error, such as inexact pipetting, difficulty in differentiation between dead and alive insects, and other such errors.

D. Precision Evaluation

The most precise assay type was the Microplate, followed by the Portable Carboxyesterase Assay, followed by PAGE, and finally the *in vivo* Bioassay. This is because although the Microplate and Portable Assays would be easy to make an error such as mispipetting, the repeatability of both assays is excellent. The repeatability of PAGE is quite high as well, however, this technique is so sensitive repeatability can be compromised, and inconsistencies in polymerization of the gel can strongly affect results. The Bioassay is last due to the difficulty repeating this type of assay, and the ease with which errors could occur in multiple replicates.

E. Sensitivity

The technique that is the most precise is the Microplate Assay, then the PAGE evaluation, then the Portable Assay, finally the *in vivo* Bioassay. The Microplate Assay needs approximately 1/6 of an aphid, and the PAGE system utilizes approximately 1/4 of an aphid. The Portable assay uses about 1 aphid, but is sensitive with as little as 1/4 of an aphid. The *in vivo* Bioassay utilizes at least 540 aphids for each insecticide. Thus the ranking is **Microplate Assay > PAGE > Portable > *in vivo* Bioassay**.

F. Overall Effectiveness for Field Diagnostics

Upon overall evaluations of each technique, there are many factors to consider. First economics, time for labor, and accuracy were examined. In this case, the Portable Assay is ranked first, although it is not as accurate as either the Microplate Assay or PAGE. However, when one takes into account that this is a field-diagnostic tool and it is not

expected to be as accurate as the laboratory, the Portable wins the rank of first overall. The rank is: **Portable > Microplate > PAGE > *in vivo* Bioassay**.

IV. Conclusions

The monitoring tool designed in this study has met all of the goals outlined in the Introduction. An *in vitro* carboxyesterase resistance monitoring tool was developed using currently available technology and data found in the national carboxyesterase survey. An *in vivo* dosage mortality bioassay was conducted on eight strains of GPA to determine actual resistance level of each strain. This data was tested for correlations with the diagnostic tool and the other *in vitro* tools used in designing the portable.

The study has proven that the Portable Carboxyesterase Assay is a valid measure of carboxyesterase levels in GPA. The strong correlations with insecticide resistance (*in vivo* Bioassay data) prove that the Portable Carboxyesterase Assay is valid for determining resistance levels due to elevated carboxyesterases. For these reasons, the Portable Carboxyesterase tool has excellent potential as a field resistance diagnostic tool. This tool will aid in distinguishing resistance frequencies in the field and therefore IPM decisions.

V. Future Research Possibilities

In the future, more emphasis should be placed on IPM techniques rather than the quick fix of foliar insecticide sprays. Sprays should be a last ditch effort at control and other tactics such as more effective promotion of biological control organisms and the use of such chemicals as insect growth regulators (IGR) like Kinoprene. According to Bauernfiend and Chapman, Kinoprene can cause high levels of progeny disruption in GPA (1984).

Additionally, if spraying is continued, which I believe it will be, it is important to maintain susceptible individuals in a population, so the use of such things as susceptible population refuges is an excellent idea.

Future research into the enzyme-insecticide system could involve the use of selected substrates for determining actual hydrolysis products of insecticides. This would involved the addition of an insecticide to a homogenized aphid followed by a stain which would stain only for a specific product. Thus if the solution showed a color change, the primary metabolite would be the predicted product. A reaction such as this would further elucidate the mode of action of insecticides and show the primary site of action for the enzyme (such as esterases). This could aid in designing better insecticides with greater effectiveness.

The Portable Carboxyesterase Assay could also use further research. The development of a more accurate photometer would aid in error elimination. The use of other strategies to make the system more portable, such as pre-substrate saturated filter paper (Pasteur and Georghiou 1981, 1989) would be of some use and would help to reduce costs. From the design of the portable system, other portable enzyme assay types of tests could be developed for diagnosis resistance due to altered acetylcholinesterases and Glutathion S-Transferases. All of these tools will be most useful in the future. However, the key to the usefulness of any agricultural tool is that it has to be used. This is the most difficult part of the system: exposure to growers. This tool will be very effective, but only if it is used.

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APPENDICES

APPENDIX A

APPENDIX 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 1992-05

Title of thesis or dissertation (or other research projects):

IN VIVO AND IN VITRO EVALUATION OF CARBOXYESTERASE-BASED INSECTICIDE RESISTANCE IN GREEN PEACH APHID (Myzus persicae (Sulzer) (HOMOPTERA: APHIDIDAE).

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name (s) (typed)
Dorothy O'Hara

Date 20 November 1992

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24:141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.
Copies: Included as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

APPENDIX 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Myzus persicae (Sulzer)	MONTCl-MI...from Montcalm County, MI MOXEEl-WA...from Moxee, WA SALINAS-CA STRATHAM-NH PRESQUE ISLE-ME WOOSTER-OH PULLMAN-WA WILDERl-ID					EIGHT STRAINS (populations), eight samples			MSU DEPARTMENT OF ENTOMOLOGY

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Dorothy O'Hara

Date 20 Nov 1992

Voucher No. 1992-05

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator

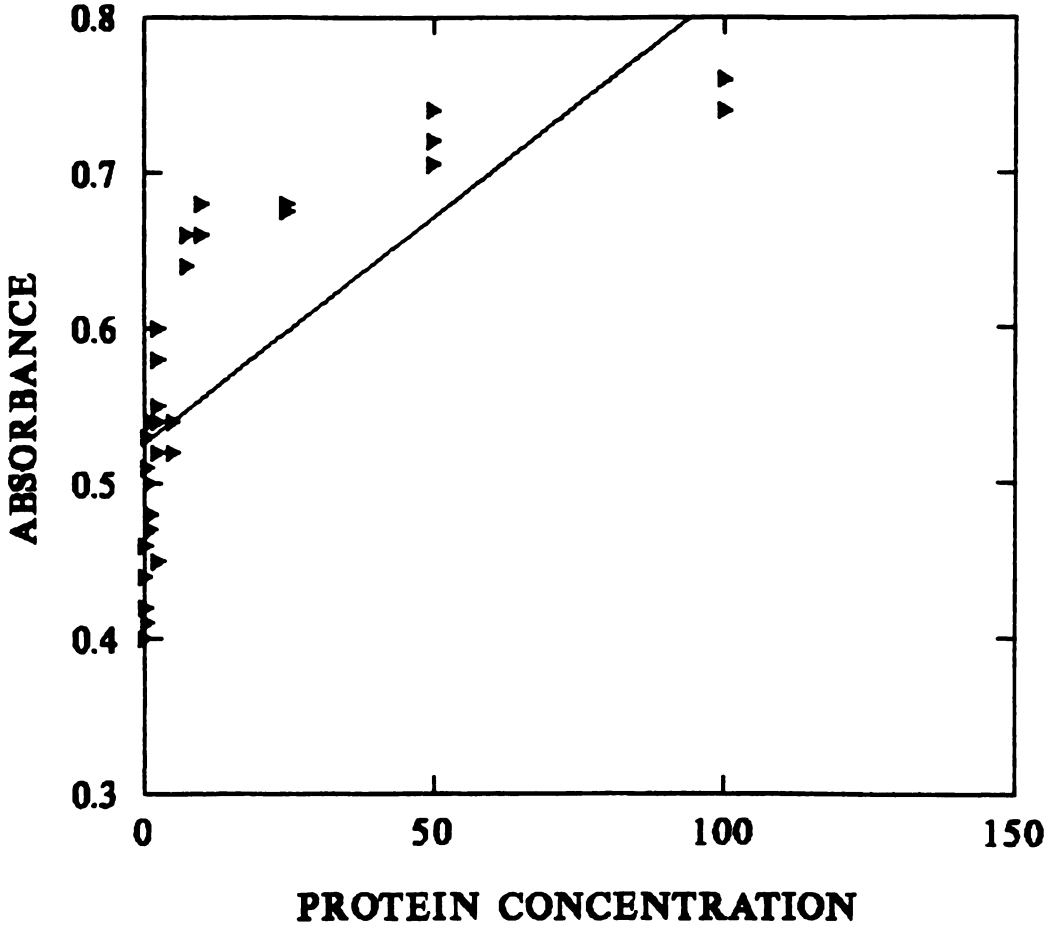
Date

APPENDIX B

APPENDIX B: Equations of regression lines for standard curves found in the text

α–Microplate standard curve	$y = .005 + .002\ x$
β–Microplate standard curve	$y = .001 + .003\ x$
Protein Microplate standard curve	$y = .048 + .175\ x$
α–Portable standard curve	$y = .526 + .003\ x$
β–Portable standard curve	$y = .144 + .036\ x$

APPENDIX C



Appendix C--Portable standard curve of protein concentration versus absorbance (bovine serum albumin) µg/ 100 µl plotted against absorbance (609 nm = protein). This curve is curvilinear and shows that it does not give readings with a linear-type relationship until the protein concentration is at or above 10 µg/ 100 µl.

APPENDIX D

Appendix D--Toxicities of six different insecticides (Ware 1983, Matsumura 1985).

Chemical	Oral rat LD_{50}	Dermal rabbit LD_{50}
azinphosmethyl	5	220
carbaryl	307	2000
ethyl parathion	3	6.8
methomyl	3.6	1000
oxydemetonmethyl	17	100
permethrin	>4000	>4000