Quantification of Insecticide Resistance in the Tobacco-Adapted Form of the Green Peach Aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

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Abstract

The tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer), is one of the most important insect pests of tobacco in the United States and around the world. Insecticides play a major role in controlling the aphid on tobacco because natural enemies usually fail to maintain its populations below damaging levels. The aphid has a history of developing resistance to many insecticides. Therefore, baseline information on the aphid's susceptibility to imidacloprid and other insecticides is critical for developing future resistant management programs to minimize losses attributed to the aphid. Studies were conducted on colonies of the tobacco-adapted form of the green peach aphid collected from nine states in the eastern United States in 2004-2007. The susceptibility of 151 colonies to imidacloprid was determined in serial leaf-dip bioassays. When combined over the four years, 18, 14, and 4% of the colonies had 10- to 20-fold, 20- to 30-fold, and 30- to 90-fold resistance ratios, respectively, suggesting that high levels of resistance to imidacloprid are present in field populations of the aphid. A colony collected near Clayton, NC had the highest LC_{50} value (31 ppm) combined over six tests and three years, but the average resistance ratios for the first three runs was over 130-fold (48 ppm). Geographic location had little effect on susceptibility to imidacloprid. Aphid colonies (136) including red, green, and orange color morphs were screened for total esterase activity using microplate assay with 1-Naphthyl acetate as the substrate. The green morphs generally had lower esterase levels than the red and orange morphs. All orange morphs had among the highest esterase activities. Esterase activities of red and green morphs were positively correlated with LC_{50} values as determined by leaf-dip bioassays for acephate and methomyl. All 25 colonies tested for esterase gene amplification had either E4 or FE4 gene amplification. The amplification of both E4 and FE4 seen as an 865-bp band characteristic of the FE4 gene and an additional 381-bp band characteristic of a deleted upstream region of the E4 gene occurred in all (4) orange morphs and one (1 of 9) green morph. Target-site insensitivity of acetylcholinesterase (AChE), as modified AChE resistance (MACE) was assessed in 65 colonies of field-collected tobacco-adapted forms of *M. persicae.* Eight colonies over a range of AChE activity were selected to study inhibition of AChE in the presence of two carbamate insecticides, methomyl and pirimicarb. IC_{50} values for

methomyl ranged from 0.35 to 2.4 μM while six of eight colonies had lower values with a range of 0.16 to 0.30 μM for pirimicarb. Two colonies that were inhibited by methomyl had very high IC_{50} values of 40.4 and 98.6 μM for pirimicarb. Such insensitivity may be due to mutations in the *ace2* gene, but this needs to be confirmed by genetic and molecular analysis. Glutathione *S*-transferases (GSTs), isoenzymes that are involved in the metabolism and detoxification of many xenobiotic compounds were quantified for 100 colonies by CDNB conjugation. There was a wide range of GST activity for the red (8 to 343 pmol/min/mg protein) and green (15.3 to 330 pmol/min mg protein) morphs, but all six orange morphs collected in 2007 had a narrower range (160 to 211 pmol/min/mg protein). About 45% of the red morphs had GST activity from 200-300 pmol/min/mg of protein, while 53% of the green morphs had a range of 100-200 pmol/min/mg protein. The influence of temperature-mediated synergisms on the toxicity of insecticides in red and green color morphs of the tobacco-adapted from of *M. persicae* were evaluated using leaf-dip bioassay procedures in laboratory incubators. Post-exposure temperatures of 15, 20, and 25^{o}C were evaluated for four classes of insecticides, acephate, imidacloprid, lambda-cyhalothrin, and methomyl. The temperature change from 15 to 20^{o}C caused almost a 3-fold increase in toxicity to the red and green color morphs for methomyl, acephate, and imidacloprid. In contrast, the toxicity of lambda-cyhalothrin decreased as the temperature increased, showing a negative temperature coefficient. Bioassay experiments conducted with the red morph for indirect estimates of imidacloprid concentrations in flue-cured tobacco showed that leaf position, imidacloprid rate and time after application affected the concentration of the toxicant in the leaf. The differences in aphid mortality between the lower and upper leaf positions indicate that the concentration of imidacloprid and its metabolites were unevenly distributed with the lowest mortality for aphids feeding on the younger, upper leaves and the highest for those feeding on the older, lower leaves. In field experiments, higher aphid populations occurred on tobacco treated with imidacloprid less than the field recommended rate of 41.4 ml/1,000 plants. The development of aphid populations in the field was consistent with the laboratory bioassays. Field trials were conducted to evaluate the performance of various insecticides currently registered for aphid control on tobacco. Imidacloprid applied as a tray drench treatment and acephate as foliar sprays were the most effective treatments. Moderate declines in control with imidacloprid were observed at 75-87 d after transplanting in 2006 and 2007. Aldicarb gave good to excellent control in one of three years, but only fair to poor control

in the other two years. Methomyl and lambda-cyhalothrin gave good control in all three years except the residual was shorter. The poor performance of aldicarb in the two years may have been related to the presence of E4 or FE4 resistance in the naturally occurring TGPA in the experimental plots.

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Chapter 1
Introduction

Tobacco, *Nicotiana tabacum* (L.), is one of the most important cash crops in Virginia with a value of more than $70 million in farm cash receipts from 8,300 ha in 2007 (USDA/ARS 2007). In the United States, tobacco produced about $1.3 billion in farm income in 2007 (USDA/ARS 2007). The tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer), is one of the most important insect pests of tobacco. The TGPA and other forms of *M. persicae* are important pests of various crops including potatoes, peppers, sugar beets, peaches, bedding plants, and nursery crops. They are also vectors of several important plant viruses (Van Emden et al. 1969).

The green peach aphid, *M. persicae,* is a highly polyphagous species, colonizing over 500 species of host plants from at least 40 different families (Blackman and Eastop 2000). There is considerable genetic variation between clones in host plant adaptation or specialization (Van Emden et al. 1969, Weber 1985, 1986, Edwards 2001). In the United States, economically injurious populations of *M. persicae* were not observed on tobacco until 1946 (Dominick 1949). However, *M. persicae* had been present in low numbers on tobacco for many years and occasionally colonized tobacco in greenhouses as early as 1908 (Kulash 1949). *M. persicae* overwinters on alternate hosts, especially on *Brassicae* and the alates migrate into tobacco fields soon after transplanting in the spring.

In Virginia, the TGPA has an anholocyclic life cycle (Clements et al. 2000a)**,** passing through many generations on tobacco with each generation lasting for about 7 d under typical summer conditions. Since populations can double in as little as 2.2 d, TGPA populations increase rapidly when control fails. High populations of TGPA can reduce tobacco yield by five to 25% depending on the growing conditions and the level of control (Reed and Semtner 1992). Aphids deposit honeydew on the tobacco leaves and a dark, sooty mold, *Fumago vagans* (Pers.) and other species of fungi, often grows on the honeydew. The combination of aphid feeding damage, sooty mold, and honeydew interferes with curing, reduces the leaf quality, and often remains on tobacco even after aphids have been controlled (Mistric and Clark 1979).

Insecticides play a major role in controlling the TGPA. The TGPA is usually an annual problem on untreated tobacco because natural enemies and cultural practices cannot maintain

populations below damaging levels. During 1986-1988, numerous accounts of control failure against the TGPA were reported in most tobacco-growing regions of the United States (Harlow et al. 1991). This problem occurred immediately after the previously predominant green form of the TGPA was replaced by the 'new' red-colored morph in most parts of the world (Blackman 1987, McPherson 1989). Compared with the green morph, the red morph is better able to survive, develop, and reproduce at temperatures of 25°C and higher, partially explaining its predominance in the field (Reed and Semtner 1991). Insecticide resistance arose about the same time that the red morph became established on tobacco (Blackman 1987, Harlow and Lampert 1990, McPherson and Bass 1990). Among the color morphs, the red form has predominated on tobacco since 1985. The color of pigmentation includes yellow and various shades of red and green. The association between the red form and resistance to organophosphorous insecticides is well established for the TGPA in the United States (Harlow et al. 1991, Clements et al. 2000b, Sorenson et al. 2002).

Tobacco farmers rely on several insecticides to control the TGPA and other insect pests on tobacco. The cost of controlling insect pests on tobacco in Virginia ranges from 0.5 to $1 million annually and losses attributed to insect damage often exceed $1 million (Semtner 2003). In 2003, a year with unusually high populations of hornworms and budworms, 30% of the flue-cured tobacco acreage in Virginia was sprayed three or more times for hornworms and budworms compared with over 80% of the crop in Florida, Georgia, and South Carolina (Caldwell and Collins 2004). The amount of insecticide used on tobacco decreased from 11% of the total pesticide active ingredient in 1992 to only 6% in 1998 (USDA 2003).

Imidacloprid was the first commercially available representative of a new chemical class, the chloronicotinyl or neonicotinoid insecticides (Elbert et al. 1990). The molecule exhibits a novel mode of action as it acts as an agonist of the nicotinic acetylcholine receptor (nAChR) leading to paralysis and death of pest organisms (Tomizawa and Casida 2005). Imidacloprid (Admire®) was registered as a tray drench application to greenhouse transplants for the control of TGPA, tobacco flea beetles, *Epitrix hirtipennis* (Melsheimer); wireworms, *Conoderus* spp., and other species on field tobacco in 1996, and as a transplant water treatment in 1997. Systemic insecticides like imidacloprid, generate higher selection pressure for resistance under prolonged residual persistence and higher exposure levels than do foliar sprays (Taylor and Georgiou 1982, Roush 1995) as all stages of the pest receive prolonged exposure. When coupled with the long-

term persistence of imidacloprid in plants, the selection pressure for resistance development in insects also is likely to be higher than for contact insecticides. Tolerance or resistance to Admire has been reported in green peach aphid (Devine et al. 1996, Kerns et al. 1998, Tomizawa and Casida 2002, Margaritopoulos et al. 2007), whiteflies, *Bemisia tabaci* (Gennadius) (Prabhaker et al. 1997), and in Colorado potato beetles, *Leptinotarsa decemlineata* (Say) (Zhao et al. 1998). However, information is lacking on imidacloprid tolerance to the TGPA in the United States. Imidacloprid now is being used on over 80% of the tobacco acreage annually in Virginia, North Carolina, and Georgia. The extensive use of imidacloprid will likely increase the chances of TGPA developing resistance to this insecticide. In the last 7 to 10 yr there were several reports of high TGPA populations developing late in the season on tobacco treated with imidacloprid. This may indicate that a tolerance has developed or just that the residues had dissipated to a point where they no longer provided effective control of TGPA. These sublethal doses of pesticides may lead to increased tolerance due to hormoligosis seen in several insect and acari species (James and Price 2002, Ako et al. 2004).

Acephate (Orthene®) applied as transplant water treatments and foliar sprays and Temik® (aldicarb) applied to the soil are still registered and often effective for TGPA control on tobacco in Virginia. Georghiou (1990) reported that *M. persicae* has developed resistance to more insecticides than any other insect species since many chemicals with different modes of action have been registered and used to control the *M. persicae* complex. The genetic feature relevant to the evolution of resistance in *M. persicae* is their karyotype. It has long been recognized that a heterozygous translocation between autosomes 1 and 3 is associated with esterase-based resistance (Blackman et al. 1978) and was proved to be the same for the development of resistance to the organophosphates in Virginia (Barnes 1990). The translocation appears to reduce the ability of the aphid to reproduce sexually, which slows the spread of resistance genes among the populations (Devonshire et al. 1998).

M. persicae has developed resistance worldwide to organophosphorous, carbamate, and pyrethroid insecticides through the increased production of a carboxylesterase, E4, or its closely related variant FE4. These enzymes inactivate insecticides by sequestration and ester hydrolysis (Devonshire and Moores 1982). In the most highly resistant aphids there is approximately 60 times as much esterase activity as in susceptible aphids, accounting for 1-2% of total body protein (Field and Foster 2002). Molecular genetic studies have shown that this increase in

esterase production is primarily due to gene amplification, i.e. the presence of multiple copies of the esterase gene in resistant aphids (Field et al. 1993). These developments set the stage for scientists to explore both the particulars of the applied problem (insecticide resistance) and the basic biology/life history of the organism.

The number of aphicides registered for use on tobacco has declined over the past 30 years and several others may be lost in the future. Some aphicides have been withdrawn from the market due to poor performance, safety, or environmental concerns. Acephate (OP) and aldicarb (carbamate) could be lost in the near future due to regulatory and business decisions. A 24(c) special local needs label for aldicarb was reviewed by the US Environmental Protection Agency and the final decision has not been made on its use on tobacco (US EPA, 2007). If these chemicals were discontinued, only the neonicotinoids such as imidacloprid and pymetrozine, an aphid antifeedant, would remain for managing the TGPA on tobacco. Imidacloprid is very effective and easy to use and has become the most commonly used insecticide for TGPA and flea beetle control on tobacco in the United States. Despite the introduction of this very effective insecticide, research to assess the level of resistance in *Myzus* to this chemical is limited.

Baseline studies are needed to determine the genetic diversity of the tobacco-feeding forms of *Myzus* in Virginia and adjacent states and to determine the types of resistance being developed. This information would help us develop and evaluate strategies for preserving the effectiveness of insecticides, increasing their performance, and finding alternative cultural and natural controls. It is critical to establish baseline information on the levels of resistance to imidacloprid and other insecticides that are commonly being used to control TGPA. Early detection of resistance would allow the implementation of resistance management plans to delay further development of insecticide resistance, and, thereby, protect the most valuable insecticides.

Objectives:

In view of the potential for the development of insecticide resistance in the tobacco-adapted form of the green peach aphid, *M. persicae*, aphid colonies were collected from different geographic locations in the eastern United States to address the following objectives:

1. Determine the differences in tolerance / resistance levels to imidacloprid, a neonicotinoid insecticide in the aphid populations.

2. Assess esterase-based resistance in populations of the tobacco-adapted form of the green peach aphid to develop baseline information for long-term monitoring.
3. Determine the levels of modified acetylcholinesterase resistance (MACE) in aphid populations.
4. Quantify glutathione *S*- transferase activity in the aphid colonies.
5. Measure the influence of post-exposure temperature on the toxicity of insecticides to the tobacco-adapted form of the green-peach aphid.
6. Determine the distribution and persistence of imidacloprid within the tobacco plant system and its impact on the aphid.
7. Evaluate response of tobacco-adapted form of the green peach aphid to chemicals in four classes of insecticides on flue-cured tobacco in the field.

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Chapter 2

Literature Review of *Myzus persicae*

The green peach aphid, *Myzus persicae* (Sulzer), is an important agricultural pest worldwide. *M. persicae* is a serious pest of several major agricultural crops including peaches, cole crops, potatoes, sugar beets, tobacco, and various ornamental crops grown in landscapes and greenhouses (Mason 1940, Chamberlin 1958, Van Emden et al. 1969, Blackman and Eastop 2000). High populations of *M. persicae* can injure plants by removing the sap and depleting the nutrients sometimes causing wilting or stunting of the affected plants. *M. persicae* also causes indirect injury by producing honeydew that accumulates on the leaves making them susceptible to sun scald. In addition, honeydew provides food for sooty mold, *Fumago vagans* (Pers) and other species, which reduces the value of ornamental plants and tobacco leaf quality (Dominick 1949, Mistric and Clark 1979). *M. persicae* is also one of the most important vectors of several persistent and nonpersistent viruses that cause serious losses in tobacco and other crops (Sylvester 1954, Lucas and Hill 1980, Kanavaki et al. 2006).

2.1 Life history

The tobacco-adapted form of the green peach aphid (TGPA), *M. persicae,* is a hemimetabolous insect belonging to the order Hemiptera, family Aphididae. *M. persicae* originated in southeast Asia (China), the origin of its primary host the peach, *Prunus persicae* (L.) (Blackman 1981), and the source of the two most popular common names, the green peach aphid and the peach-potato aphid. *M. persicae* uses the peach as its primary host in regions where the aphid goes through a sexual phase in life cycle. Many species of *Myzus*, including *M. persicae*, have holocyclic life cycles where the sexual phase is completed on a primary host plant and a parthenogenic (asexual) phase occurs on secondary host species. Anholocyclic forms of aphids have a continuous parthenogenetic life cycle, without a sexual reproductive phase (Ilharco and van Harten 1987). Permanent anholocyclic life cycles associated with geographic and climatic conditions fail to produce one or both sexuparae (the male and oviparous female forms). Intermediate life cycles, such as an androcyclic life cycle, have sexual males, but no sexual females (Ilharco and van Harten 1987).

Secondary hosts of *M. persicae* include species from more than 60 plant families (Patch 1938). Some species of *Myzus* were described in association with their secondary host plants

(Mason 1940). The green peach aphid has many synonyms is because it has a very large number of secondary hosts, including grasses, lilies, legumes, peppers, mustards, geraniums, spurges, dogbanes, nettles, cacti, and nightshades (Patch 1938, Blackman and Eastop 2000).

2.2 Color morphism

M. persicae was first observed as an economic pest of tobacco in southeast Asia (Blackman 1987). In fact, the earliest reports of injurious infestations were from Sumatra (Kuijker 1930). In the United States, high aphid populations were not observed on tobacco until 1946 (Dominick 1949, Kulash 1949, Chamberlin 1958). When *M. persicae* first became a problem in tobacco fields in the United States in the 1940's, a light green color morph was predominant, while dark green and red forms were rare (Dominick 1949, Chamberlin 1958, Blackman 1987). In the 1980s, two changes happened to aphid populations on tobacco at about the same time, significant insecticide resistance in tobacco fields and a red-colored form became the dominant morph (McPherson 1989, Reed and Semtner 1989, Harlow and Lampert 1990). This red morph was also noticeably larger than the light green morph (Blackman 1987). A serious agricultural problem, insecticide resistance, arose at the same time that this striking visual change took place (Blackman 1987, Harlow and Lampert 1990, McPherson and Bass 1990). The red morph became the main form found on tobacco in 1986 (Reed and Semtner 1989, McPherson and Bass 1990). *M. persicae* has several distinctive color morphs and phenotypes, described as some or several variations of green and shades of red (Mason 1940, Blackman and Eastop 2000). It has both alate (winged) and apterous (wing-less) forms.

Studies by Reed and Semtner (1991) demonstrated that the red morph is more tolerant of high temperatures than the green morph. Research in Japan (Ueda and Takada 1977) showed that red anholocyclic populations of *M. persicae* survived the winter better than the green strains. Environmental factors, nutritional conditions of the host, and population density also contribute to changes in color (Takada 1981). Studies in Greece (Margaritopoulos et al. 2002) established that yellow and green clones may produce progeny of two different colors under short day conditions. This color morphism in *M. persicae* results from the presence of a series of glycosides in the aphid hemolymph (Blackman 1974).

2.3 Taxonomic issues

Sulzer first described *M. persicae* in 1776 in a German publication as *Aphis persicae* and Passerini transferred *Aphis persicae* to the genus *Myzus* in 1860 (Mason 1940). The genus *Myzus* has four distinctive morphological characteristics, including: six-segmented antennae; convergent antennal tubercles; long, cylindrical siphunculi; and short, conical cauda without much constriction (Mason 1940, Blackman and Eastop 2000).

Blackman (1987) described the tobacco-adapted form of *M. persicae* as a new species, *M. nicotianae* based on multivariate morphometric analysis of canonical variates to separate the two species. In addition to the morphological and host adaptation differences, Blackman (1987) assumed that *M. nicotianae* was reproductively isolated from the *M. persicae* based on a permanently parthenogenic life cycle. Since the tobacco-adapted form of the green peach aphid was described as a new species, its taxonomic status has been controversial (Clements et al. 2000b). The taxonomic status of the tobacco-feeding forms of *M. persicae* has several implications for managing the spread of insecticide resistance both on tobacco-adapted forms and forms on other crops (Clements et al. 2000a, Clements et al. 2000b).

Margaritopoulos et al. (1998) in Greece using RAPD-PCR (random amplified polymorphic DNA polymerase chain reaction) techniques found no differences between the tobacco-adapted *M. nicotianae* and *M. persicae* from non-tobacco hosts. Clements et al. (2000b) in the United States used RAPD-PCR, mitochondrial cytochromic oxidase II (COII), and EF-1a tests to assess differences between tobacco and non-tobacco associated *M. persicae*, but found no differences. Therefore, they concluded that the tobacco and non-tobacco forms of the aphid were the same species. Margaritopoulos et al. (2002) regarded the tobacco-adapted forms of the aphid as a race of *M. persicae* on tobacco, but not as a separate species. Fuentes-Contreras et al. (2004) found that a single predominant red clone of TGPA of *M. persicae* complex may coexist within the same geographic regions of Chile, since they colonize different host plants and have different life cycles similar to those reported in Greece (Margaritopoulos et al. 2003).

Blackman and Eastop (2007) named the tobacco-adapted form as a subspecies, *M. persicae nicotianae* (Blackman) because some inbreeding would have occurred between the two host-adapted forms. European and South American scientists working with the tobacco-adapted forms now refer to them as *M. persicae nicotianae* (Olivares-Donoso et al. 2007, Margaritopoulos et al. 2007).

I am referring to this aphid as the tobacco-adapted form of the green-peach aphid (TGPA), *Myzus persicae* (Sulzer), because both *M. persicae* and *M. nicotianae* have the same resistance and biochemical mechanisms and taxonomists have had difficulty in separating the two forms using the morphometric methods described by Blackman (1987) (Field et al. 1994, Nauen and Elbert 1997, Clements et al. 2000b). This taxonomic issue is beyond the scope of my research.

2.4 Cultural control of *Myzus* on tobacco

Several cultural practices may help delay aphid infestations or reduce aphid populations on tobacco, but they usually do not keep the rapidly increasing populations under control. However, cultural practices may help improve the effectiveness of foliar insecticides and reduce the need for insecticide applications after topping. Cultural practices such as keeping alternate host crops and weeds away from plant beds or greenhouses, early planting to reduce mid-season aphid infestations (Semtner 1984), using recommended rates of nitrogen (not excessive), topping early, and controlling suckers reduce the impact of aphids on tobacco. Conservation tillage also shows promise for reducing TGPA infestation on tobacco (Semtner et al. 2001).

2.5 Insecticides to control *M. persicae* on tobacco

Insecticides are applied in several ways to control the TGPA on tobacco, as a greenhouse tray drenches to seedlings, transplant water treatments, soil treatments, and foliar treatments for remedial control. Remedial treatments are recommended if the TGPA populations reach economic threshold levels, i.e., when at least five of 50 plants are infested with 50 or more aphids on any one leaf (Semtner 2007). Insecticides from four major chemical classes provide several choices for managing insecticide resistance in the TGPA. The organophosphate, Acephate (Orthene) has provided effective control of TGPA on tobacco since the mid-1970s. The carbamates, methomyl (Lannate) and aldicarb (Temik) are still labeled for use on tobacco. Methomyl applied as a foliar spray provides only marginal control of TGPA due to its short residual activity under field conditions. Aldicarb has been effective under most situations, but control is sometimes inadequate. Pymetrozine, a unique class of insecticide that paralyzes the salivary pump apparatus of the aphids is registered for aphid control on tobacco, and gives good control when used as a foliar treatment (Semtner 2007).

However, two insecticides could be withdrawn from the market soon. Endosulfan (Thiodan), an organochlorine, is in the later stages of reregistration and will no longer be

available for use on tobacco after the process has been completed (US-EPA 2002). Aldicarb is also undergoing reregistration and Bayer CropScience has indicated that they will voluntarily discontinue its 24c label for tobacco in Virginia and North Carolina (US-EPA 2007).

Several neonicotinoid insecticides, imidacloprid (Admire), acetamiprid (Assail), clothianidin (Belay), and thiamethoxam (Platinum) are now registered to control TGPA on tobacco in the United States. However, few alternatives to the neonicotinoids may be available for TGPA control, especially if endosulfan, aldicarb, and acephate are withdrawn from the market. Since the green peach aphid has a history of developing resistance to many insecticides (Foster et al. 2007), the extensive use of imidacloprid and other neonicotinoids on tobacco and the reduced availability of insecticides with other modes of action to rotate with the neonicotinoids could create conditions that favor the development of neonicotinoid resistance in the TGPA.

2.6 Insecticide resistance in *Myzus*

Insecticide resistance is defined as a significant, genetically-based shift in the biochemical, molecular, or behavioral bases of quantal responses in populations of arthropods. It represents one extreme of response, compared with susceptibility, the other extreme (Robertson et al. 2007). Quantal response bioassays are useful to identify shifts in population tolerance and provide information that is used for statistical comparisons of entire regression lines and individual dose levels of interest (Robertson et al. 2007).

In the early 1990's, *M. persicae* was resistant to more insecticides than any other insect species (Georghiou 1990). Insecticide resistance in the TGPA did not appear in the United States until 1981 when resistance to the OP, acephate, was reported in Virginia (carbamates and organochlorines) (Koziol and Semtner 1984). Resistance to acephate was not common, and there was no observed cross-resistance to other commonly used compounds. Despite this early report of resistance, acephate is one of a few insecticides that still provides effective control of TGPA on tobacco (Srigiriraju and Semtner 2006). By the mid-1980s, TGPA outbreaks due to control failures with some insecticides were common in the tobacco-growing regions of southeastern United States. This was attributed to the development of high levels of resistance in the red morph to several organophosphorous insecticides including monocrotophos, malathion, and methyl parathion (Harlow and Lampert 1990, McPherson and Bass 1990).

Insecticide resistance in populations of TGPA from tobacco and other hosts coincided with a shift in predominant body color from green to red, and was positively correlated with an autosome-1,3 chromosomal translocation (Barnes 1990, Harlow et al. 1991, Blackman et al. 1995). At high temperatures (25^{o}C and above), red morphs live longer, develop faster, and are more fecund than green morphs. Therefore, the red morphs have a selective advantage when temperatures are high during the tobacco growing season (Reed and Semtner 1991). However, green morphs have not disappeared and Foster et al (1997) found that insecticide resistant red morphs of *M. persicae* had reduced overwintering ability on winter host plants than the green forms.

Three distinct resistance mechanisms have been identified in the green peach aphid: (1) insecticide sequestration and detoxification by elevated levels of carboxylesterases (E4 and FE4), (2) target-site insensitivity because of altered acetylcholinesterase (MACE), and (3) mutation at the *para II* sodium channel gene mutation conferring knockdown resistance (*kdr)*. So far, the most common mechanism of resistance is the increased production of carboxylesterases (E4 or FE4), which causes enhanced degradation and sequestration of xenobiotic esters (Field and Devonshire 1998, Devonshire et al. 1998). Two forms of target-site resistance involving changes in the acetylcholinesterase (*AChE*) (Moores et al. 1994) and sodium channel (*kdr*) (Martinez-Torres et al. 1997) genes have been described. Several biochemical and DNA diagnostic methods were used to identify all three mechanisms in individual aphids, and prove their spatial distributions and temporal dynamics (Devonshire et al. 1992, Field et al. 1996). Amplified genes, responsible for the increased production of esterases with their expression modulated by DNA methylation were evaluated. Amplification of the *E4* gene is in strong linkage disequilibrium with the *kdr* gene that may initiate strong insecticidal selection favoring aphids with multiple mechanisms, tight chromosomal linkage, and prominence of parthenogenesis (Devonshire et al. 1998).

2.7 Neonicotinoids to control *Myzus* pest complex

Insecticidal properties of heterocyclic nitromethylenes discovered by Nihon Tokushu Noyaku Seizo K.K of Japan led to the synthesis of many active compounds belonging to the same mode of action. The nitromethylenes are effectors of the nicotinic acetylcholine receptor (Schroeder and Flattum 1984, Benson 1989). One of the most encouraging developments in pest management was the discovery of the neonicotinoid, imidacloprid (Elbert et al. 1990).

Imidacloprid represents a milestone in insecticide research over the past three decades because it is an important insecticide belonging to a new class of compounds, the neonicotinoids, which are the fastest-growing class of insecticides since the pyrethroids (Nauen and Bretschneider 2002). Like nicotine, the neonicotinoids act on the insect central nervous system as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) (Bai et al. 1991, Liu and Casida 1993, Chao et al. 1997, Wollweber and Tietjen 1999, Zhang et al. 2000, Nauen et al. 2001). Unlike nicotine, however, they have marked selectivity within the Insecta and have little mammalian toxicity (Tomizawa and Casida 2002). Because of this specific selectivity and mode of action, there is little or no cross-resistance with older insecticide classes such as pyrethroids, chlorinated hydrocarbons, organophosphates, and carbamates (Nauen and Denholm 2005). As a result, today the neonicotinoids group is the most widely used chemical class for insect control on many major crops (Denholm et al. 2002). Neonicotinoids comprise a distinct, single mode of action (MOA) group as defined by the Insecticide Resistance Action Committee (IRAC) for resistance-management purposes (IRAC 2004).

The synthesis and later commercial development of neonicotinoid insecticides has provided agricultural producers with invaluable tools for managing some of the world's most destructive crop pests. Insect groups targeted by neonicotinoids, mainly Hemiptera (aphids, whiteflies, and planthoppers) and Coleoptera (beetles), include species with a long history of resistance to older products (Cahill and Denholm 1999). After its registration in 1996, many tobacco farmers quickly adopted imidacloprid and it is still the commercial leader for managing TGPA, flea beetles, wireworms and thrips to control tomato spotted wilt virus (McDougall 2004). A single application at transplanting offers almost season-long protection against the TGPA and early season control of flea beetles, thrips, and wireworms in the United States (Semtner and Wilkinson 2003). Imidacloprid is used on over 80% of the flue-cured tobacco acreage in Virginia (Dimock et al. 2001, Semtner 2004). The neonicotinoids applied as transplant water or greenhouse tray drench treatments are effective, much safer, and have less impact on the environment than the insecticides that they replaced.

Imidacloprid's distinguishing mode of action compared with most conventional insecticides provides excellent control of present-day multiresistant pests that have evolved many resistance mechanisms to detoxify the existing xenobiotics (Prabhaker et al. 1997). However, the speed and scale with which imidacloprid is used in control strategies around the

world prompted widespread concern over the development of imidacloprid resistance (Cahill and Denholm 1999). However, imidacloprid has proven remarkably resilient to resistance, and cases of resistance that have been reported are still manageable or geographically localized (Zhao et al. 2000, Denholm et al. 2002, Nauen and Bretschneider 2002).

Systemic insecticides, including imidacloprid, create higher selection pressure for resistance under prolonged residual persistence and higher exposure levels than do foliar sprays (Taylor and Georghiou 1982). The tray drench or transplant water applications of imidacloprid persist longer in the crop than foliar sprays and all stages of the pest receive a prolonged exposure to the insecticide (Roush 1995). When coupled with the long-term persistence of imidacloprid in plants, the selection pressure for the development of resistance may be higher than for contact insecticides. The existence of strong resistance in some species such as the silverleaf whitefly, *Bemisia tabaci* (Gennadius) (Prabhaker et al. 1997), brown plant hopper, *Nilaparvata lugens* (Stal) (Zewen et al. 2003), Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Zhao et al. 2000), has displayed the potential of pests to adapt and resist field applications of neonicotinoids.

Imidacloprid provides effective control of aphids because of its excellent systemic nature and its relative selectivity to sucking pests (Elbert et al. 1991, Nauen et al. 1996, Nauen et al. 1998a). Because of its translaminar bioavailability, imidacloprid is suitable for controlling both early- and late-season pests (Anke Buchholz 2002). Affinity studies (Lind et al. 1998) of the binding of imidacloprid to the receptors show a high affinity, in the nano-molar range specifically for the green peach aphid, *M. persicae*, and leaf-hoppers, *Nephotettix cincticeps* (Uhler). The aphicidal potency of plant metabolites of imidacloprid (imidazoline derivative, olefine metabolite) has higher activity towards head nicotinic acetylcholine receptors than the parent compound (Nauen et al. 1998a). Sublethal doses of imidacloprid have a substantial antifeedant effect similar to nicotine (Woodford and Mann 1992, Nauen 1995, Devine et al. 1996). This response is important in controlling plant viruses vectored by aphids and thrips (McPherson et al. 2005).

Use of imidacloprid on tobacco has increased over the years, and it is used as either as transplant water or tray-drench treatments (Semtner 2007, Sorenson and Southern 2007). Since the mode of action of imidacloprid is identical with that of nicotine, the possible resistance of tobacco-associated populations of *M. persicae* to imidacloprid and potential spread of resistance

genes to populations on other crops is a concern. However, no examples of true resistance have been reported for this compound in the TGPA (Nauen and Denholm 2005). It is suggested that since aphids are phloem feeders and nicotine is transported in the xylem there is no selection with nicotine in TGPA populations (Guthrie et al. 1962), and there should be no cross-resistance to imidacloprid (Elbert et al. 1996). However, low levels of tolerance (insensitivity) to imidacloprid, correlated with TGPA tolerance to nicotine were observed on tobacco. This was attributed to the antifeedant activity of the compound and the overall hardiness of the natural sensitivity in the tobacco-adapted populations (Devine et al. 1996, Elbert et al. 1996, Nauen and Elbert 1997, Nauen et al. 1998b). No differences in the binding of titrated imidacloprid to the nicotinic acetylcholine receptor was found between tolerant and susceptible strains, suggesting no target site differences (Nauen et al. 1996). Synergistic experiments with PBO and DEF failed to increase mortality in tobacco-associated strains, suggesting no differences in metabolic enzymes between susceptible and tolerant aphids (Nauen et al. 1998b). Nauen et al. (1998) also found that tolerance to nicotine was higher in the tobacco-associated populations that were morphologically identified as *M. nicotianae.*

Low level differences in imidacloprid susceptibility have also been reported on cabbage in Arizona (Kerns et al. 1998), but those differences were attributed to variability in starvation tolerance resulting from overall hardiness of the aphids. Recent studies in Greece (Margaritopoulos et al. 2007) show higher tolerance of TGPA towards imidacloprid, compared to other host-adapted forms from the same regions. As mentioned above, imidacloprid is now widely used around the world on various crops. In addition, newer compounds acting at the same target site, such as acetamiprid, clothianidin, and thiamethoxam, are being used widely in the United States and in other countries (Nauen and Denholm 2005).

Resistance monitoring studies with imidacloprid have been conducted for several aphid species (Elbert et al. 1996, Foster et al. 2003, Nauen and Elbert 2003, Weichel and Nauen 2003, Margaritopoulos et al. 2007) including strains resistant to other classes of insecticides, such as organophosphates, carbamates, and pyrethroids. Monitoring work done in conjunction with the release of imidacloprid showed low levels of tolerance (3 to 10-fold) to neonicotinoids in European and Japanese samples of *M. persicae* (Devine et al. 1996, Nauen et al. 1996), but they found no link of this tolerance to specific biochemical markers (Nauen et al. 1998b). Lower

susceptibility to imidacloprid and other neonicotinoids has generally been correlated with decreased efficacy of nicotine (Devine et al. 1996, Nauen et al. 1996).

Nauen and Elbert (2003) observed variation due to the greater test survival in field strains of *M. persicae* bioassayed against imidacloprid as soon as they were received compared with laboratory-maintained reference strains. This allowed strains to survive longer than susceptible laboratory populations when exposed to imidacloprid-treated leaves (Nauen and Elbert 1997). Such effects disappeared when the exposure time was extended from 48 to 72 h, and after maintaining such strains under laboratory conditions for at least two weeks (Nauen and Elbert 1997, Nauen and Elbert 2003). In other cases, strains have maintained reduced susceptibility over several years of laboratory culture (Foster et al. 2003b).

M. persicae with up to 18-fold resistance were reported from other regions including Zimbabwe, the United States, and southern and northern Europe (Cox et al. 2001, Foster et al. 2003a). Two colonies that were collected from United States in 1991 had high tolerance to imidacloprid and the green morph was more tolerant than the red one (Nauen et al. 1998b). Resistance to imidacloprid was significantly correlated with resistance to nitenpyram, acetamiprid, and nicotine (Foster et al. 2003a). It is believed that the low-level neonicotinoid resistance seen in red-colored morphs of *M. persicae* arose originally as an adaptation to feeding on tobacco rather than from field exposure (Nauen et al. 1998b). The practical implications of neonicotinoid resistance in *M. persicae* have been researched in the laboratory and the field. Under field conditions, imidacloprid applied at reduced rates did not distinguish between aphids fully susceptible to neonicotinoids and ones with up to 6-fold resistance in bioassays (Haylock et al. 2002). In laboratory cages, however, aphids with higher levels of resistance (up to 15-fold) showed increased survival and reproduction on cabbage and tobacco treated with lower than recommended rates of imidacloprid (Foster et al. 2003). The ability of some aphids to survive better than others under such conditions explains the potential for further selection, leading to a more marked impact on the extent and duration of control efficacy (Nauen and Denholm 2005).

2.8 Esterase-based resistance

Esterases are a large, heterogeneous, and diverse group of enzymes metabolizing various exogenous and endogenous substrates with ester linkages. They are associated with insecticide resistance in over 50 species of insects, ticks and mites (Devorshak and Roe 1998). The roles of esterases or carboxylesterases in pesticide resistance are xenobiotic metabolism and

sequestration (Devorshak and Roe 1998). Esterases have also been used successfully as markers for detecting insecticide resistance (Devorshak and Roe 1998). Esterase-mediated insecticide resistance will have a practical importance to manage resistance in the context of integrated pest management for several compounds currently used for TGPA control on tobacco (Clements et al. 2000).

Green peach aphids, like many insect species, have a strong positive correlation between general esterase activity and resistance to organophosphorus insecticides (Needham and Sawicki 1971). This increased esterase was attributed to overproduction of a single esterase enzyme (E4), resulting from successive tandem duplications of the E4 structural gene (Devonshire and Sawicki 1979). The number of duplications, and, therefore, the amount of detoxification enzyme (E4) produced, determined the degree of resistance an aphid clone expressed. E4 was subsequently purified, characterized, and shown to hydrolyze insecticidal esters from the organophosphorus, carbamate, and pyrethroid chemical classes (Devonshire and Moores 1982).

The large amounts of the enzyme *in vivo* (up to 3 % of the total protein in highly-resistant aphids) indicates that sequestration has a significant role in conferring resistance (Devonshire 1989). It eventually became clear that resistant aphids lacking the autosomal-1,3 translocation have slightly different amplified esterase, which was later named as FE4 (Devonshire et al. 1983). Two closely related variant forms of the enzyme, E4 and FE4, were subsequently distinguished, the latter having a slightly higher catalytic center activity (1.5-fold difference) towards OPs (Devonshire et al. 1983) and a molecular weight of 66 kDa compared to 65 kDa for E4 (Field et al. 1988). The difference was in the primary structure rather than resulting from different post-translational change (Devonshire et al. 1986b).

Studies on the catalytic and substrate specificity of E4 showed its efficiency in catalyzing the hydrolysis of organophosphates, carbamates, and pyrethroids that confers resistance primarily from the binding and sequestration (Devonshire et al. 1983, Soderlund and Bloomquist 1990, Devonshire and Field 1991).

Devonshire et al. (1986b) showed the increase in copy number of E4 and FE4 esterase genes was accompanied by an increase in the corresponding mRNAs, compared to susceptible clones. They found that the nascent, resistance-conferring enzymes underwent post-translational glycosylation. Southern blot hybridization studies using cDNA clones confirmed that elevated esterase content in resistant *M. persicae* is due to amplification of esterase structural gene (Field

et al. 1988). Field et al. (1993) sequenced E4 and FE4 cDNA clones that exhibited extremely high nucleotide identity in the coding regions of the gene. The most significant difference between the two clones was a single nucleotide difference (A in E4 and T in FE4) that results in a stop codon in E4 and an additional 36 bases of open reading frame for FE4.

In the United States, resistant populations of aphids had approximately 2.5 times the carboxylesterase activity as susceptible ones (Harlow and Lampert 1990). Furthermore, the toxicity of organophosphosphate insecticides toward the resistant strains was synergized by the esterase inhibitor DEF, suggesting the mechanism of resistance was due to increased ester hydrolysis caused by higher levels of carboxylesterase. They also found that the aphicidal activity of acephate and endosulfan (a cyclodiene insecticide) was the same for susceptible and resistant populations. Carboxylesterase activity in both tobacco and non-tobacco adapted forms of *Myzus* was determined using 1-Naphtholic esters. Abdel-Aal et al. (1990) found that the carboxylesterase activity of resistant strains was greater for each substrate compared to the susceptible ones and the degree of resistance to malathion was positively correlated with this activity. They also showed that TGPA resistance to malathion was always associated with the autosomal-1,3 translocation first noted in the TGPA by Blackman (1987) and also present in E4 resistant green peach aphids.

Studies conducted in the 1990's on field-collected cultures of the TGPA showed that all red morphs were translocated and resistant, whereas green morphs were either translocated (resistant) or of normal karyotype (susceptible) (Harlow et al. 1991). Results from a later study (Abdel-Aal et al. 1993) concluded that TGPA resistance associated esterase cross-reacted with an antibody raised against esterase E4 from green peach aphids. The esterase associated with resistance in the TGPA was purified and characterized by Wolff et al. (1994). Its molecular weight and isoelectric point (pI) were identical with those previously reported for E4 green peach aphid.

Investigations with cotton aphids, *Aphis gossypii* (Glover) from midsouth of United States found a strong correlation between resistance to chlorpyrifos and the amount of esterase produced using isoelectric polyacrylamide focusing gel techniques (O'Brien et al. 1992). Differences in carboxylesterases were seen as differing banding patterns and expression of comigrating bands.

2.9 Biochemical detection of esterase-based resistance

Single-insect biochemical assays for detection and monitoring of resistance have been most successful in aphids and mosquitoes, in which resistance is conferred by overexpression of one or more carboxylesterases. Resistance in *M. persicae*, as mentioned above was first surveyed in single-insect assays of 1-Naphthyl acetate-hydrolyzing activity either in whole aphid homogenates or after electrophoretic separation of aphid esterases (Sawicki et al. 1977). A further refinement of this approach involved the use of antibodies to the E4 esterase in immunoassays of the esterase content of individual aphids (Devonshire et al. 1986a). Both of these assays are adaptable to high-throughput microplate assays for use in esterase-based resistance monitoring.

A direct comparison of these approaches showed that immunoassays were superior in identifying aphids with differing levels of esterase over-expression but colorimetric total esterase assays provided a simple and robust means of identifying resistant aphids (Devonshire et al. 1992). The detection of "revertant" aphids, which have low esterase levels but retain amplified esterase genes posed a special risk for the reselection of resistance (Hick et al. 1996). Combination of the immunoassay for esterase levels with assessments of E4 esterase gene content in individual aphids in dot blots of individual aphid homogenates provided a means of detecting revertants at the single insect level (Field et al. 1989). The detection of amplified esterases in individual mosquito homogenates following electrophoretic separation has been employed extensively to monitor the development and spread of resistance in mosquito populations (Brogdon 1989, Soderlund 1997). Several studies have used the modified-microplate assay to detect esterase-based resistance in the TGPA in the United States and elsewhere (Ffrench-Constant and Devonshire 1988, Harlow et al. 1991, Abdel-Aal et al. 1992, Foster et al. 2000, Fuentes-Contreras et al. 2004, Margaritopoulos et al. 2007).

2.10 Target-site insensitivity involving AChE

Acetylcholinesterase (AChE) is a key enzyme in the nervous system, terminating neurotransmission by the hydrolysis of the neurotransmitter acetylcholine (Pitman 1971). Insect AChE is the target site of organophosphate (OPs) and carbamate insecticides that were introduced to the market decades ago and still represent the economically largest groups of insecticides (Nauen and Bretschneider 2002). Russell et al (2004) revised two major classes of target-site-insensitivity mutations conferring resistance to OPs and carbamates in many insect

species. AChEs that are less sensitive to inhibition have been reported in *Tetranynchus urticae* (Koch) (Smissaert et al. 1975, Stumpf et al. 2001), *Musca domestica* (Linnaeus) (Devonshire and Sawicki 1974, Steele and Smallman 1976), *Drosophila melanogaster* (Meigen) (Salam and Pinsker 1981, Fournier et al. 1992), *Leptinotarsa decemlineata* (Say) (Zhu et al. 1996), *M. persicae* (Foster and Devonshire 1999, Foster et al. 2002). The rate of reaction of the modified AChE with the inhibitor (insecticide) is reduced, while the catalytic role is either unimpaired or still sufficient (Oppenoorth 1985, Gao et al. 1998).

Kinetic properties of AChE mediated resistance have been well studied over in the past decade (Zhu and Brindley 1992, Gao et al. 1998, Zhu and Gao 1999, Pruett 2002, Byrne et al. 2003, Cheng et al. 2004). Molecular mechanisms coffering resistance to insecticides due to amino acid substitutions and mutations were confirmed in several aphid species (Benting and Nauen 2004, Toda et al. 2004). In *D. melanogaster,* the *Ace* locus is responsible for AChE activity in the central nervous system (CNS) and the regions have been cloned and sequenced (Bender et al. 1983, Hall and Spierer 1986). Hemizygous flies displayed only 20-25% of the wild-type AChE activity and were susceptible to other organophosphate insecticides (Fournier et al. 1992). This conferred resistance to organophosphates and is correlated with the amount of AChE in the CNS. The cDNAs encoding two acetylcholinesterases, MpAChE1 and MpAChE2, were isolated from the peach potato aphid and were ortho and paralogous with the *ace* gene of *D. melanogaster* (Nebeshima et al. 2003). However, a single amino acid substitution of Ser431Phe on MpAchE2 was found in the pirimicarb-resistant strains.

Recently, the modified acetylcholinesterase phenotype (MACE) has been correlated with a mutation in the *ace2* gene in the green peach aphid (Nabeshima et al. 2003). Resistance to OPs and carbamates in the melon (cotton) aphid, *A. gossypii*, is due to insensitive AChE (O'Brien and Graves 1992). Several studies were carried out with emphasis on pirimicarb in *M. persicae* (Silver et al. 1995, Moores et al. 1996, Han et al. 1998, Foster et al. 2003a). Studies conducted in France on the aphid, *Nasonovia ribisnigri* (Mosley), by Rufingier et al. (1999) demonstrated that populations with resistance to pirimicarb and paraoxon, were susceptible to methomyl, suggesting there may be a structure-activity relation. Insensitivity of AChE is linked to mutations at the target protein conferring insecticide resistance to OPs and carbamates in *A. gossypii* (Li and Han 2004). Studies on resistance mechanisms in grain aphid, *Sitobion avenae* (Fabricius), have shown that pirimicarb-resistant populations are susceptible to thiodicarb, which

can be used to screen for resistant populations (Chen et al. 2007). Recently, resistance to pirimicarb among the *M. persicae* populations in Greece were studied using 100 µM diagnostic dose of pirimicarb in microplate assay to confirm the target site insensitivity in AChE seen as MACE resistance (Margaritopoulos et al. 2007).

2.11 Glutathione *S*- transferase activity

Enayati et al. (2005) and Hayes et al. (2005) reviewed glutathione *S*- transferases (GSTs) as secondary plant metabolites and their role in insecticide detoxification. Insects are known to develop a wide variety of adaptations to protect themselves from the xenobiotic compounds. Many defensive enzymatic systems help insects overcome insecticide toxins (Francis et al. 2001). Glutathione *S*- transferases are a diverse family of enzymes found in several aerobic organisms (Wilce and Parker 1994). They play a central role in detoxification of both endogenous and xenobiotic compounds and are involved in intracellular transport, biosynthesis of hormones, and protection against oxidative stress (Enayati et al. 2005). The main focus of insect GSTs has been on their role in insecticide resistance (Salinas and Wong 1999). GSTs catalyze the conjugation of the tripeptide glutathione to electrophilic center of lipophilic compounds, thereby increasing their solubility and aiding excretion from the insect system (Habig et al. 1974). In addition, some GSTs catalyze a dehydrochlorination reaction using reduced glutathione as a cofactor rather than a conjugate (Clark et al. 1986).

GSTs are important in cancer epidemiology and drug resistance (Tew 1994, Hayes and Pulford 1995) and well studied in mammals. Most of the studies involving insect GSTs have focused on their role in detoxifying xenobiotic compounds, in particular insecticides and plant allelochemicals and, more recently, their role in mediating oxidative stress responses (Clark et al. 1986, Francis et al. 2001, Konanz and Nauen 2004, Francis et al. 2005).

2.12 Postexposure temperature effect on insecticide toxicity

Temperature influences the biochemical reactions and resultant arthropod activity by increasing or limiting the effectiveness of various insecticides (Horn 1998). Temperature catalyzes many reactions in the biological systems which lead to a wide array of outcomes as it influences physical and chemical properties of insecticides including stability, vaporization, penetration, activity, degradation, uptake, and translocation (Johnson 1990). Post-treatment temperature effects on the toxicity of fenvalerate, pyrethrins, and DDT were found to be more toxic to *Spodoptera litura* (Fabricius) larvae at 15^{o}C than at 25^{o}C both by topical and foliar

application method (Hirano 1979). Musser and Shelton (2005) studied the influence of postexposure temperature on the toxicity of three classes of insecticides, a pyrethroid, a carbamate, and the nicotinic acetylcholine receptor agonist, spinosad, to *Ostrinia nubilalis* (Hubner). They found that the toxicities of the pyrethroids decreased about 10-fold, spinosad decreased 9-fold and methomyl was not affected when the temperatures were changed from 24 to 35^{o}C. McLeod (1991) investigated the effect of temperature on translaminar and systemic toxicities of carbamate and organophosphorus insecticides for control of *M. persicae* on spinach. He found the mortalities were lower at temperatures < 16^{o}C because it hindered the chemicals' systemic activity.

Studies on the effects of pre- and post-treatments of lambda-cyhalothrin toxicity in susceptible and *kdr*-resistant German cockroaches, *Blattella germanica* (Linnaeus) found a negative temperature coefficient towards the susceptible, but not the resistant strain (Valles et al. 1998). There was no synergistic effect for piperonyl butoxide (PBO) on *kdr*-resistant strains, irrespective of temperature differences. Differences in toxicity of esfenvalerate towards the differential grasshopper *Melanoplus differentialis* (Thomas) had no temperature-dependent activity (10 to 35^{o}C), while spinosad was ineffective at lower temperatures, diflubenzuron was most efficient at higher temperatures (Amarasakare and Edelson 2004). Investigations on the effect of temperature and synergistic action of PBO on imidacloprid toxicity to cat fleas, *Ctenocephalides felis* (Bouche) showed a marked difference between larval and adult mortality rates (Richman et al. 1999). Imidacloprid was highly toxic to adults at higher temperatures (35^{o}C compared to 20^{o}C), but mortality was higher for the larvae at lower temperature. PBO acted as a synergistic doubling the toxicity of imidacloprid only at the higher temperatures.

2.13 Persistence of imidacloprid in the plant system

Imidacloprid is transported mainly in the xylem and its systemic properties allow it to spread evenly within young growing plants of both mono- and dicotyledonous crops (Nauen et al. 1999). In tobacco, imidacloprid is recommended to be used as a single application at transplanting or as foliar spray to control the TGPA (Semtner 2007, Sorenson and Southern 2007). To reduce the potential for resistance to imidacloprid after it is applied as transplant water or greenhouse tray drench treatments, chemicals with other modes of action should be used to help control late-season aphid infestations. These chemicals include the OPs and carbamates to which the TGPA has developed various degrees of resistance (Clements et al. 2000).

Imidacloprid is applied either as a greenhouse tray drench treatment or in the transplant setter water during transplanting. A hand sprayer or a greenhouse boom sprayer is used to make drench applications to the tobacco seedlings that are still in the trays inside or outside the greenhouse from 12 hr to 7 d before transplanting. Immediately after application, water is used to rinse the chemical off the foliage and into the root plug where it moves systemically into the individual transplants (Sur and Stork 2003). Transplant setter water treatments are applied by mixing the insecticide formulation into the setter water that is applied by the transplanter as it transplants each plant. Imidacloprid applied in the transplant setter water or as a greenhouse tray drench treatment gives almost season-long control of TGPA and are superior to foliar applications (LaMondia and Rathier 1999). But, the limited amount of imidacloprid in the root zone becomes exhausted over time due to its systemic uptake as the plant grows and to the chemical's normal degradation in the soil (Cox et al. 1997). LaMondia and Rathier (1999) reported that a band treatment was more effective when adequate soil moisture or irrigation moved the insecticide into the root zone.

When imidacloprid is applied as a transplant setter water treatment, the roots will grow out of the treatment zone over time. This may cause an unequal distribution of imidacloprid within the plant, so younger, upper leaves receive a lower amount of active metabolites (Olson et al. 2004). Westwood et al. (1998) showed the concentration of imidacloprid in sugar beets was six times higher in the bottom leaves than in the top leaves at 49 d after application. A similar study in potato plants found significant differences in the percentage mortality of Colorado potato beetles fed on leaves collected from imidacloprid-treated plants (Olson et al. 2004). Percent mortality from lower canopy leaves decreased from 90 to 20% from wk 8 to wk 12 after transplanting, while mortality for the upper canopy decreased from 30 to 18% during the same period. Studies conducted in Arkansas on imidacloprid distribution in Tabasco peppers, showed minor differences in translocation of the compound from 24 to 96 h after application (Diaz and McLeod 2005). They found consistently lower mortality on the upper leaves (away from the soil) compared with the lower canopy leaves. They also found that the efficacy of imidacloprid against artificial infestations of the green peach aphid decreased from 100% at 35 d after treatment to 85% and 25% at 42 and 56 d after treatment, respectively. Investigations in California citrus plantations on spatial and temporal distribution of the two neonicotinoids,

imidacloprid and thiamethoxam demonstrated no spatial differences in concentration over the study period using commercially available ELISA kits (Castle et al. 2005).

Buchholtz and Nauen (2001) applied imidacloprid in the laboratory to the upper leaf surfaces of cabbage and cotton and tested it against the aphids, *M. persicae* and *A. gossypii*, respectively. They found that cabbage leaves had higher uptake and translocation of imidacloprid than cotton leaves. Buchholtz and Nauen (2001) reported differences in translaminar residual activity between the two species and the host plants, but no differences were observed in oral ingestion bioassays using an artificial double membrane feeding system.

Van Iersel et al. (2001) investigated the effects of imidacloprid application technique and irrigation on the control of silverleaf whiteflies, *Bemisia argentifolii* (Bellows & Perring) on poinsettias in Georgia. They found higher concentrations of imidacloprid in the lower canopy after drench applications, but whitefly control was not improved. A field trial conducted in a commercial vineyard in California showed high correlations of imidacloprid application rate with xylem fluid concentration, and effectively managed populations of the sharpshooter *Homalodisca coagulata* (Say) (Byrne et al. 2005). Up to 95% of the imidacloprid in flue-cured tobacco plants was recovered using HPLC with liquid-liquid partition clean-up in another trial (Liu et al. 2005).

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Chapter 3

Monitoring for imidacloprid resistance in the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) in the eastern United States.

Abstract

The tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer) is a major pest of tobacco in most tobacco-producing regions of the world. Imidacloprid is the primary insecticide used for its control. This study was conducted to establish baseline information on imidacloprid resistance in the TGPA. The susceptibility of the tobacco-adapted form of the green peach aphid to imidacloprid was determined in serial leaf-dip bioassays for 151 field-collected colonies from nine states in the eastern United States from 2004 through 2007. When combined over the 4-yr study, 18, 14, and 4% of the TGPA had resistance ratios of 10-20 fold, 20-30 fold, and 30-90 fold, respectively suggesting the development of high levels of tolerance in some colonies. A colony collected near Clayton, NC had highest LC_{50} value (31 ppm) when combined for six tests over 3 yr, but the resistance ratios for the first three runs conducted in 2005 and 2006 averaged over 130 (48 ppm). Bioassays conducted on the same colony in 2007 indicated a 40-fold resistance ratio. The most susceptible colony and a standard colony that had not been exposed to imidacloprid for over 7 yr had similar LC_{50} values for each test over the same period. Geographic location was not a factor contributing to differences in susceptibility. Overall, the green morphs had higher mean LC_{50} values than the red morphs collected from the same location, but both color morphs had wide ranges in resistance ratios ranging 1 to 90 for the green morphs, 1.6 to 62 for the red morphs and 5 to 23 for orange morphs first observed in 2006. The percentages of colonies with moderate resistance ratios suggest that there is some natural tolerance or the development of resistance to imidacloprid in some populations of the TGPA. The implications of the study for resistance management schemes against *M. persicae* are discussed.

Introduction

The tobacco-feeding form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer), is a persistent annual problem that can reduce the value of flue-cured tobacco by up to 25% (Reed and Semtner 1992). It also transmits several important nonpersistent viruses to tobacco and other crops (Shew and Lucas 1991). Before 1996, Virginia tobacco farmers controlled TGPA with various foliar insecticides or soil applications of aldicarb (Temik®), a toxic insecticide-nematicide (Semtner et al. 1993, Semtner et al. 1999). Unfortunately, some forms of the TGPA developed low to moderate levels of resistance to several of these insecticides (Koziol and Semtner 1984, Harlow and Lampert 1990, McPherson and Bass 1990, Brierley et al. 1994, Clements et al. 1999). In 1996, the neonicotinoid, imidacloprid was registered on tobacco as a greenhouse tray drench treatment to transplants within a week before transplanting for insect control in the field. When imidacloprid is applied to the roots of transplants, it is taken into the plants and acts as a systemic to provide early-season control of flea beetles and wireworms, and almost season-long control of the TGPA (Mullins 1993).

The neonicotinoids act selectively and have a high affinity for the acetylcholine site of the nicotinic acetylcholine receptor in the insect central nervous system. This mode of action is much different from those for the carbamates, organophosphates (OPs), and pyrethroids and is related to a low rate of cross-resistance between the neonicotinoids and most other insecticides (Nauen et al. 1998). After its registration in 1996, tobacco farmers in most states in the United States quickly adopted imidacloprid for managing TGPA, flea beetles, and wireworms. Imidacloprid is currently used on over 80% of the flue-cured tobacco acreage in Virginia (Dimock et al. 2001, Semtner 2004). The neonicotinoids applied as either a greenhouse tray drench or a transplant setter water treatment are effective, much safer, and have less impact on the environment than other conventional insecticides. Since imidacloprid also aids in the control of tomato spotted wilt virus (TSWV), the rates and the proportions of acreage treated will likely increase if the disease becomes an annual problem in Virginia.

Beside the resistance to OPs, carbamates, and pyrethroids, *M. persicae* has developed resistance to several chemical classes, the mechanisms of which are not fully understood. Of these chemical classes, neonicotinoids are among the most important. Resistance (< 20-fold) to imidacloprid has been measured in *M. persicae* clones collected from the UK, mainland Greece,

USA, Zimbabwe, and Japan (Devine et al. 1996, Foster et al. 2002, Foster et al. 2003). Some of these responses was attributed to an antifeedant response to the chemical (Nauen and Elbert 1997). Neonicotinoid resistance has already occurred in the cotton aphid, *Aphis gossypii* Glover (Wang et al. 2001), the tobacco whitefly, *Bemisia tabaci* (Gennadius) (Nauen et al. 2002), and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Zhao et al. 2000). Recently, TGPA populations in Greece have shown considerable tolerance to imidacloprid compared to the strains tested from other host-adapted forms (Margaritopoulos et al. 2007).

The objective of this study is to assess imidacloprid resistance in TGPA populations collected primarily from tobacco in the eastern United States. These data are crucial for establishing baseline information for resistance management strategies and to preserve imidacloprid and other neonicotinoids for future usage. To create baseline information for long-term monitoring, several TGPA colonies were collected from across the tobacco-growing areas in the eastern United States and tested for their susceptibility to imidacloprid.

Materials and Methods

Field sampling. *M. persicae* were collected on leaf samples from tobacco, *Nicotiana tabacum* L. (Family Solanaceae), farms in eight different states (Fig. 3.1) over a 4-yr period (2004 - 2007). Samples were also taken from other host plants in Virginia and New York. Information about their body color under field conditions and host plant was recorded for each locations. Most of the colonies were collected personally with the help of Virginia Cooperative Extension agents. Collaborators in other states (Georgia, South Carolina, North Carolina, Tennessee, Maryland, New York, and Kentucky) made the collections on tobacco leaves. The leaves were placed in Ziplock® plastic bags containing a piece of paper towel to reduce moisture and sent overnight (FedEx®) to the laboratory in Styrofoam® or corrugated containers with ice packs. Whenever possible, both red and green color morphs of the aphid were collected at each location. All aphids were collected from tobacco, except the colonies from NY-Riverhead red, 2006; Semtner (red and green) 2007 and Clay's green, 2007. After collection, all colonies were maintained on insecticide-free excised tobacco leaves (flue-cured tobacco cultivar, 'K-326'). The petioles of the leaves were inserted into agar medium in Styrofoam® cups and kept at 21 ± 1°C, 60% RH and 16:8 (L:D) h photophase in laboratory incubators until the bioassays were

completed. A total of 151 field samples were tested from 2004 through 2007.

Greenhouse plants. As there was a heavy use of tobacco leaves for maintaining colonies in the laboratory and conducting bioassays, a continuous supply of leaves was achieved by maintaining at least 100 to 200 plants in the greenhouse at a single point of time. Plants were rotated among two or three greenhouses to manage natural infestations of aphids and whiteflies. Flue-cured tobacco 'K-326' was seeded in Styrofoam® seeding trays (288 cells) filled with Carolina's Choice Tobacco Mix® (a soil-less greenhouse growing medium). Trays were placed in float bays filled with water and fertilized according to the VA Cooperative Extension recommendations (Reed 1998). Three to four-wk-old seedlings were transplanted from the trays, into 15-cm diameter pots filled with growing medium and placed in the water-filled bays and fertilized with 200 ppm of Peters® 20-20-20 water dispensable fertilizer. Plants were discarded once they reached the button stage (preflowering stage), as they became sticky and less desirable for aphids.

Leaf-dip bioassay. Each test population was increased in numbers by placing 20 to 50 adult apterous aphids on a large tobacco leaf, with its petiole dipped in a 25 ml glass vial filled with agar and placed in clear, vented plastic containers. Five to six-day-old aphids of similar size were used for the bioassay. Each test population consisted of adult apterous aphids of the same color (green or red). Aphids were bioassayed by leaf dip method using water dispersions of imidacloprid (Admire 2F; Bayer CropScience, Research Triangle Park, North Carolina, USA). Leaf disks, 100 mm in diameter, were cut from fresh leaves (mid-stalk position) from greenhouse-grown tobacco plants (flue-cured tobacco cultivar, 'K-326'). Leaf disks were dipped for 5 s in the designated concentrations, air-dried, and placed on slightly moistened filter papers in labeled Petri dishes. The inside lips of the Petri dishes were coated with Insect-a-slip® (Fluon) to restrict the aphids to the leaves and to keep them from escaping. Ten healthy adults from the test colony were placed on each leaf disk with a camel's hair brush. Covers were placed on each Petri dish and secured with Parafilm®. A total of 30 to 40 aphids were used for each dose. Mortality was assessed 72 h after treatment to overcome any antifeedant effect of imidacloprid (Nauen et al. 1998). If an aphid did not move or only had slight twitching after being probed with a camel's hair brush, it was considered dead. At least six doses ranging from 0.1 to 256 ppm with a deionized water check were tested each time with each dose replicated at least three times. All bioassays were conducted in environmental chambers kept at 21 ± 1°C, 60% RH and

16:8 (L: D) h photophase. A greenhouse-maintained colony (NC State Red) of the TGPA collected from tobacco with no history of imidacloprid exposure for almost seven yr was obtained from North Carolina State University (provided by Dr. Clyde Sorenson) and used for comparison.

Resistance ratios (RR) were calculated as:

$$RR = \frac{LC_{50} \text{ of the test colony}}{LC_{50} \text{ of the most susceptible colony}}$$

Statistical analysis: Abbott's formula (Abbott 1925) was used to correct for mortality. LC_{50} values were calculated using POLO PC (LeOra Software, Berkeley, CA). Normality of the data was tested using Kolmogorov-Smirnov test and nonparametric Kruskal-Wallis test was conducted to compare the LC_{50} values between the color morphs using SAS software (SAS 2001). Wilcoxon non-parametric matched paired t-test was used to test the significance of the LC_{50} values between the two color morphs collected from the same location using SAS software (SAS 2001).

Results

The responses (LC_{50} values) of tobacco-adapted forms of *M. persicae* to imidacloprid are summarized by yr in Appendices 1 through 4. Out of the 149 aphid colonies collected from eight tobacco-growing states and screened for possible resistance to imidacloprid, the LC_{50} values ranged from 0.4 ppm for a sample from a natural infestation in a greenhouse (Southern Piedmont AREC, Blackstone, VA collected on tobacco in 2004) to 33.5 ppm for a green morph collected from tobacco at the Central Crops Research Station, Clayton, Johnston County, NC in 2005.

Fig. 3.2 shows the difference in susceptibility of the aphid colonies to imidacloprid by (combined over yr) color morph, with mean LC_{50} values of 5.3 ± 0.72, 5.4 ± 0.93, and 4.4 ± 0.36 ppm for green, orange, and red color morphs, respectively. The LC_{50} values of paired colonies that were collected from the same locations and individually for each color morph over a 4-yr period are presented in Fig. 3.3. Deviation from normality was detected for both red and green

morph dosage-morality data using Kolmogorov-Smirnov test (K-S = 0.225, $P < 0.0001$ and K-S = 0.202, $P = 0.0009$, respectively). Therefore, the mean LC_{50} values were compared using the nonparametric Kruskal-Wallis test. The mean LC_{50} values of the green morphs were significantly higher than those of red morphs collected from the same location, when combined over years (Kruskal-Wallis: $H = 71.06$, df = 3, $P < 0.0001$; paired t test: t = 2.01, df = 34, $P < 0.05$), but not within year.

The LC_{50} value for the reference colony (NC State, red) was 1.9 ppm. However, the most susceptible colony in the study was for a green morph collected in a greenhouse at the Southern Piedmont AREC, Blackstone, VA in 2004. Therefore, the latter, more sensitive colony was used instead of the reference colony for calculating the resistance ratios (RR) as shown in Fig. 3.4. When combined over yr, the RR ranged from 2 to 91 with 15.3% below 5; 39.3% between 5 and 10; 16% between 10 and 15; 12% between 15 and 20; 7.3% between 20 and 25; 6.6% between 25 and 30 and < 4% above 30.

2004 Colonies. The results of the 2004 bioassays are shown in Appendix 1. Eighteen colonies collected from Virginia were tested against imidacloprid in leaf-dip bioassays. The LC_{50} values ranged from 0.4 ppm (the most susceptible colony, collected from the greenhouse infestation, Southern Piedmont AREC, Nottoway County, VA) to 9.3 ppm (red color morph from Hatchet farm in Franklin County, VA). A 25-fold difference was noted in RR values compared with the most susceptible colony.

Both red and green morphs were collected at three locations. In one, the most susceptible green colony was more susceptible to imidacloprid than the red morph collected with it. At the other two locations, the green morphs had higher LC_{50} values than the red ones, but there was no significant difference between the toxicities exhibited by the two color morphs (Wilcoxon non-parametric matched paired t-test; $P>0.05$).

2005 Colonies. In 2005, TGPA colonies were collected from three states, Virginia, North Carolina, and Georgia (Appendix 2). Out of 24 colonies tested, 60% were from various locations in Virginia, 28% were from North Carolina and 12% from Georgia. The LC_{50} values of the colonies ranged from 0.6 (red color morph, Witcher farm, Franklin county, VA) to 33.5 ppm for a green color morph from Clayton, Johnston County, NC. The standard, NC State red colony was tested six times and there was no apparent change in this colony's response to imidacloprid (LC_{50}=2.1 to 1.9) in six tests from 2005 through 2007 (Fig. 3.5).

The Clayton green colony was most resistant with an LC_{50} value of 61 ppm when tested for the first time soon after it was collected from the field (Table 3.1, Fig. 3.5). Subsequent testing of the Clayton green colony over the next three yr of continuous laboratory maintenance showed a 4-fold decline in LC_{50} values. The imidacloprid resistance declined steadily over the next five tests, from LC_{50} = 51.1 ppm in the second test in early 2006 (about 5 mo after the first bioassay) to a 15.5 ppm in LC_{50} value when the colony was tested for the sixth time in the early fall of 2007 (Fig. 3.5).

2006 Colonies. Sixty-two colonies were screened for tolerance to imidacloprid in 2006 (Appendix 3). The LC_{50} values ranged from 0.8 (Walker, Oxford, NC - green color morph) to 22.9 ppm (Mitchell, Franklin County, VA - red color morph). Colonies of both color morphs collected from Georgia had the lower LC_{50} values for the respective morphs. A red color morph collected from Florence, SC had a 10-fold higher LC_{50} value compared to the green color morph collected from the same location (Appendix 3). However, contrasting results were obtained from the colonies collected from Horry County, SC, where the green morph was more tolerant than the red one. The colonies collected from Virginia had a wide range of tolerance, with the LC_{50} values ranging from 0.8 to 22.9 ppm, but only two colonies of red morphs (Clary, Mecklenburg, VA and Mitchell, Franklin) had LC_{50} values higher than 10 ppm.

The mean LC_{50} values did not differ significantly among the color morphs that were collected from the same locations (Wilcoxon non-parametric matched paired t-test; $P>0.05$) (Fig. 3.3).

2007 Colonies. Of 45 colonies screened against imidacloprid in 2007, 63% had LC_{50} values above 3 ppm with a range of 0.7 to 23.6 ppm (Appendix 4). A green colony from Bowen farm, Tift, GA had the highest LC_{50} (23.6 ppm). This was much higher than the green morph (1.6 ppm) collected from the same location in 2006 and moderately higher than the green morph (7.5 ppm) collected there in 2005. The six orange morphs collected had moderate tolerance with LC_{50} values ranging from 2.5 to 8.4 ppm (Fig. 3.2). The green morph from Clayton, Johnston County, NC had notable tolerance again with a LC_{50} of 10.2 ppm, although this was much less than the colony collected from the same location in 2005. Colonies from Tennessee, Kentucky, and Maryland had higher LC_{50} than those from South Carolina. Colonies collected from Virginia had LC_{50} values ranging from 1.2 to 8.4 ppm with an average RR of 10 compared to the susceptible colony. All orange morphs collected from the Southern Piedmont AREC,

Blackstone, VA had LC_{50} values above 6.8 ppm. The mean LC_{50} values of the color morphs collected from the same location were not significantly different from each other (Wilcoxon non-parametric matched paired t-test; P=0.083) (Fig. 3.3). On the whole, we found several colonies with high levels of resistance to imidacloprid in both color morphs, with a higher percentage of resistance in the green morphs.

Discussion

Imidacloprid has been used to control aphids on tobacco since its introduction in 1996, and it is now the most widely used insecticide on tobacco in the U.S. (McDougall 2004). So far, there have been no verified reports of control failures in the field, or of cross-resistance to other neonicotinoids such as thiamethoxam, clothianidin, and acetamiprid. Nevertheless, it is important to learn more about TGPA susceptibility to the neonicotinoids and to assess changing trends of resistance to these compounds. Development of insecticide resistance in field populations is a dynamic phenomenon that requires regular monitoring.

The LC_{50} values of several colonies of the TGPA in this study suggest that there is strong tolerance or resistance to imidacloprid in aphid populations in the southeastern U.S. Forty-five percent of the colonies had imidacloprid RR above 10. However, low-level tolerance to imidacloprid was noted in several colonies from other locations. Localized variation in the populations may indicate a potential for resistance development as seen in the colonies collected from the same location over the 4-yr study. Though we noticed a visible difference between the averages of the color morphs in 2004, 2005, and 2007, the differences were not statistically significant due to the variation within the color morphs.

Imidacloprid has been used to control TGPA on tobacco for more than a decade in United States with some possible control failures reported by Extension agents and farmers. Our results show medium to higher levels of tolerance to imidacloprid in many of the colonies collected in the past four yr. Though direct comparisons cannot be made (different bioassay methods) with earlier reports of tolerance in *M. persicae* populations in England (Nauen and Elbert 2003) and Greece (Margaritopolus et al. 2007), some populations in my study appeared to have higher tolerance to imidacloprid. Seventeen percent of the colonies had RR values > 20 suggesting a development of higher tolerance in many of the colonies. Several other studies conducted on

samples from Greece (Margaritopolus et al. 2007), Japan (Nauen et al. 1996), Europe (Nauen and Elbert 2003), France (Nauen and Elbert 1997), and the U.S. (Foster et al. 2003) over the last decade found low- to medium-level tolerance to imidacloprid and the colonies from tobacco were more tolerant than other host-adapted forms. Studies using the FAO dip method on aphid populations from tobacco in France gave an LC_{50} value of 16 ppm (Nauen and Elbert 2003).

No mechanism of resistance to imidacloprid has been reported in *M. persicae*. Neonicotinoid resistance in the whitefly *Bemisia tabaci* (Gennadius) is due to metabolic-based resistance conferred by overexpression of P450 genes (Rauch and Nauen 2003). In the brown planthopper, *Nilaparvata lugens* (Stahl), resistance to imidacloprid is associated with a mutation of the target-site, nicotinic acetylcholine receptor (Liu et al. 2005). The only cases of reduced susceptibility to imidacloprid were described for red morphs of *M. persicae* associated with tobacco plants (Nauen et al. 1996). However, this was interpreted as natural variation rather than resistance developed directly through selection due to use of less than recommended rates under field conditions (Elbert et al. 1996, Nauen et al. 1998). In previous studies performed on the tobacco-adapted form of *M. persicae*, tolerance to imidacloprid has been correlated with a decreased efficacy towards nicotine (Devine et al. 1996, Nauen et al. 1996). However, binding of the titrated imidacloprid (H^3) was similar in different host-adapted forms, and target-site insensitivity was ruled out (Nauen et al. 1996).

In *M. persicae,* three well-known and widespread resistance mechanisms, esterase overproduction ((Nauen et al. 1996, Devonshire et al. 1998), MACE (Moores et al. 1994), and kdr (Martinez-Torres et al. 1997) do not affect imidacloprid or other neonicotinoids. Interestingly, many colonies with high RR values in my studies had low total esterase activity. All other mechanisms of resistance had no cross-tolerance conferred towards imidacloprid; one possibility is that the tolerance is conferred due to the selection pressure of imidacloprid alone.

Imidacloprid derived plant metabolites are more active against *M. persicae* and *A. gossypii* than the parent compound itself (Nauen et al. 1998). The most active metabolite, the imidazoline derivative (olefine metabolite) had 16-fold higher activity than imidacloprid on both aphid species after oral ingestion. The other factor is 'hardiness' in the tobacco-adapted forms due to natural tolerance to nicotine. An explanation linking imidacloprid tolerance to naturally acquired nicotine tolerance is supported by studies from Greece where Margaritopolus et al. (2007) reported tolerant/resistant populations of *M. persicae* complex to imidacloprid were

mostly from tobacco-adapted forms. *M. persicae* colonies collected from non-tobacco hosts in Virginia (Clay's green morph – *Bougainvillea spectablilis*; Semtner's garden red and green morphs – turnips, *Brassica napus* L. and NY-Riverhead red morph – *Calibrachoa* sp.) had low RR values compared with most of the tobacco-adapted forms.

Future studies should test imidacloprid-tolerant in tobacco-adapted populations on non-tobacco hosts to assess the natural tolerance caused by nicotine adaptation. The decrease in tolerance to imidacloprid in the most tolerant colony (Clayton, Johnston County, NC – green morph from 2005) indicates that selection pressure may be an important factor responsible for the tolerance. The differences in tolerance to imidacloprid among the aphid populations indicate that selection pressure for neonicotinoids is an important factor due to their higher persistence in the plant system (Prabhaker et al. 1997). The variation in tolerance indicates that the resistance genes must have been present in the populations at low frequencies. The stability of the tolerance in the standard (NC State red morph, 2005) and the most susceptible colonies (Southern Piedmont-AREC, Blackstone, VA - Green morph) explains this phenomenon to some extent. The populations with moderate to low RR values may be able to preserve the genes responsible for tolerance when present in lower frequencies.

Since imidacloprid is so widely used on tobacco and with growing concerns about imidacloprid tolerance in many insect species, it is important to develop baseline information for monitoring imidacloprid resistance to the TGPA. The importance of imidacloprid and other insecticides belonging to the neonicotinoids is increasing rapidly, and it is important to find ways to help sustain their effectiveness. This research provides a basis for ascertaining continued susceptibility to and efficacy of neonicotinoids in use for aphid resistance-management. Future studies should assess aphid cross-resistance among the neonicotinoids. This information is vital for developing successful resistance-management plans and to save this important class of chemicals.

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Table 3.1. Difference in susceptibility of a highly resistant colony of the tobacco-adapted form of the green peach aphid, Clayton (green morph), Johnston County, NC, tested over a 3-yr period, 2005-2007.

Test Period	LC_{50}*	N	CF (95%)	Slope	H†
Summer 2005	61.0	210	41.9 - 100.1	1.51 ± 0.23	0.37
Spring 2006	51.1	210	37.0 - 97.8	1.30 ± 0.20	0.31
Fall 2006	47.2	210	36.8 - 60.1	2.26 ± 0.20	0.36
Spring 2007	35.6	210	24.8 - 51.3	1.41 ± 0.18	0.83
Summer 2007	19.7	180	13.2 - 27.7	1.51 ± 0.24	0.64
Fall 2007	15.5	180	12.3 - 19.1	3.01 ± 0.45	0.30

* LC_{50} values expressed as parts per million (mg/L) imidacloprid (Admire 2F formulation)
† Heterogeneity factor = Observed chi-square value / degrees of freedom

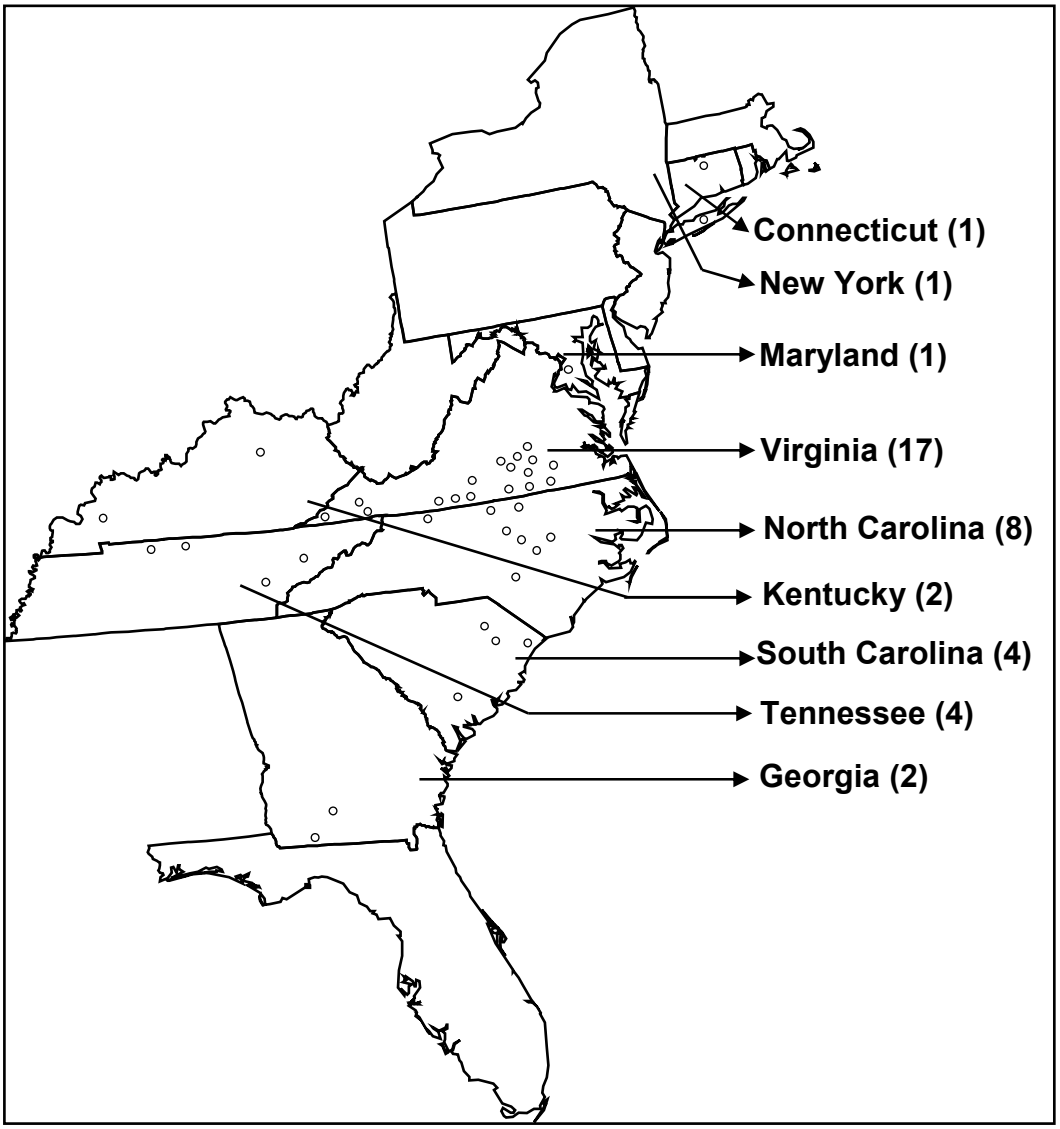

Fig. 3.1. Sampling sites of *Myzus persicae* populations screened for imidacloprid resistance. The numbers of counties from each state tested are presented in the parenthesis.

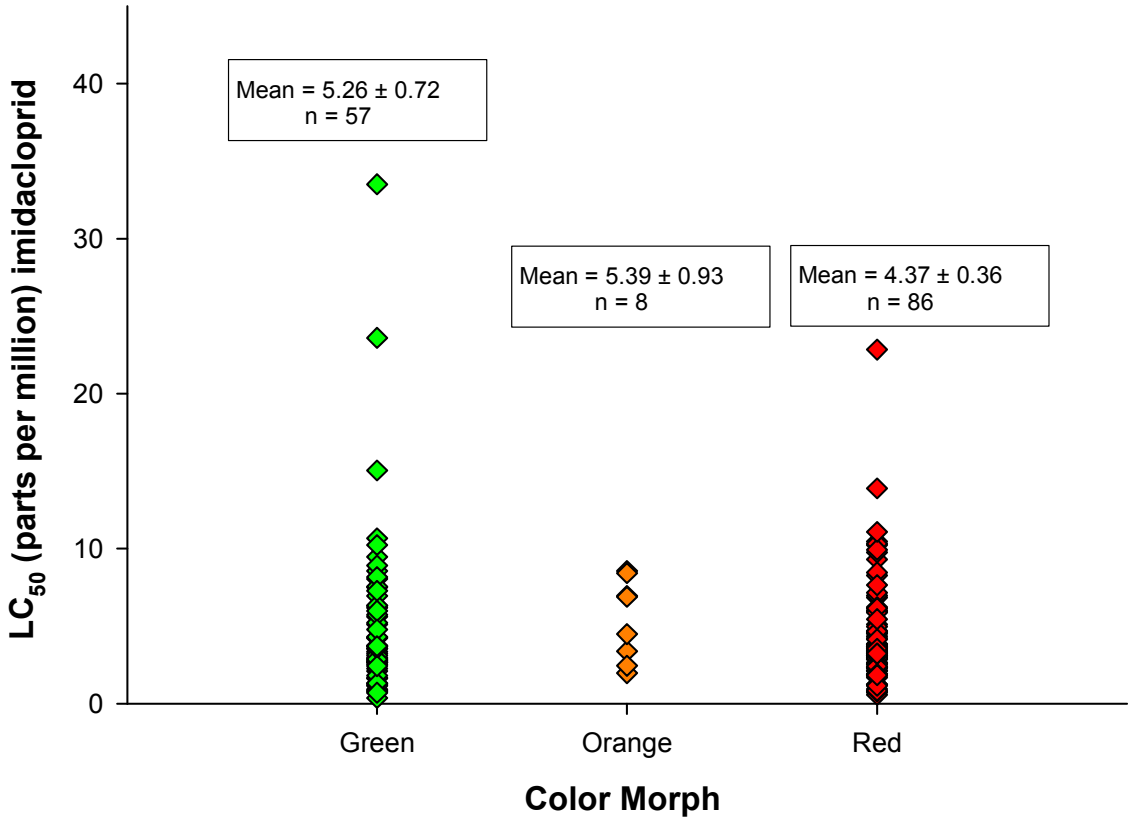

Fig. 3.2. Activity of imidacloprid (LC_{50}, ppm) in leaf-dip test bioassays with various field samples of *Myzus persicae* collected from tobacco in the eastern United States; n = number of field samples examined (2004 - 2007).

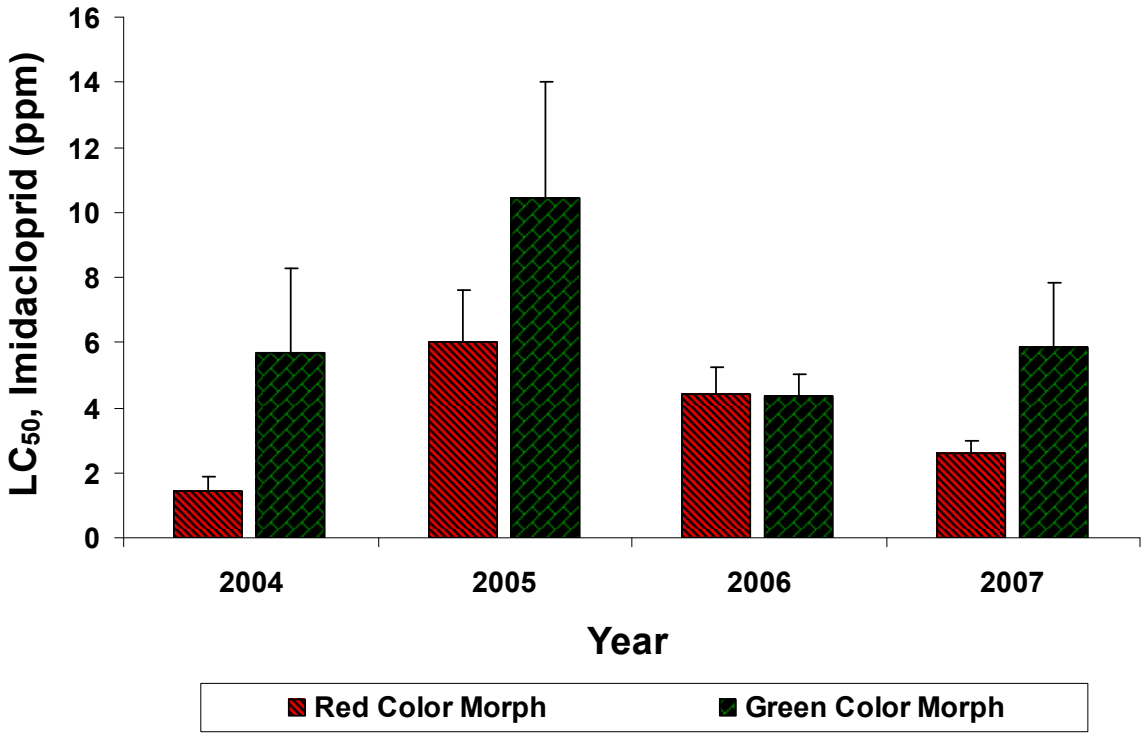

Fig. 3.3. Mean LC_{50} values for imidacloprid against two color morphs of the tobacco-adapted form of the green peach aphid collected from the same locations (3 pairs combined for 2004, 8 pairs combined for 2005, 13 pairs combined for 2006 and 11 pairs combined for 2007). Data are mean values ± SEM.

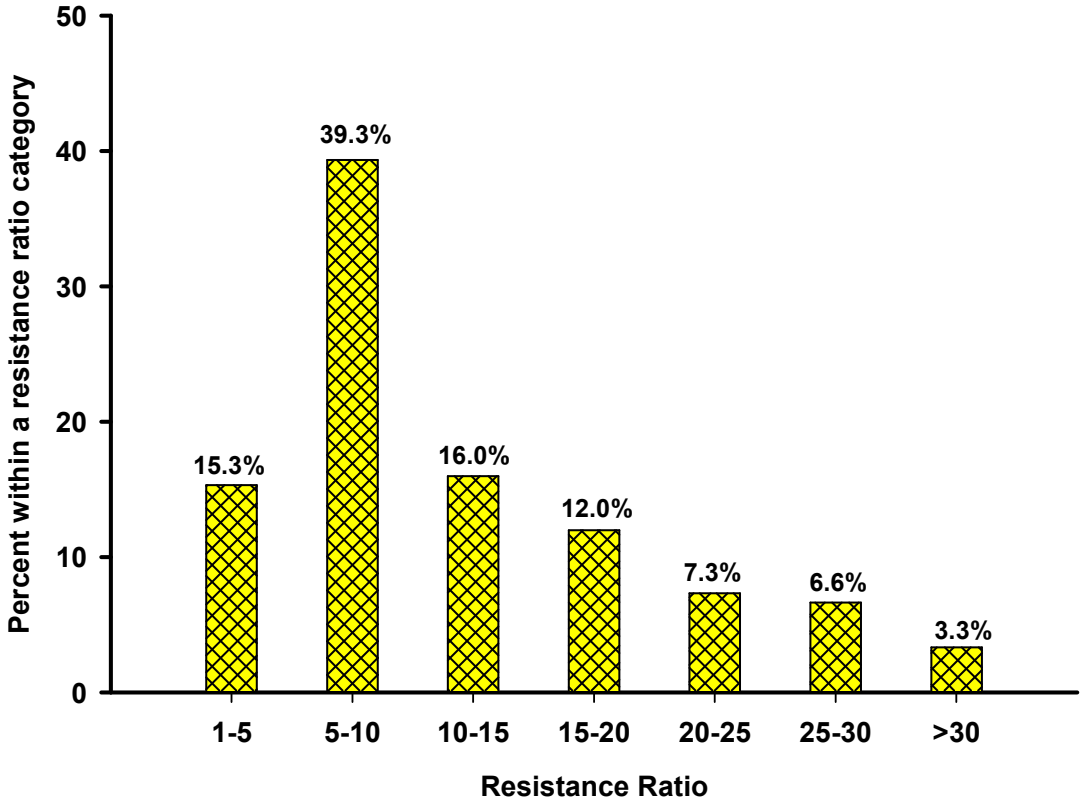

Fig. 3.4. Frequency of imidacloprid resistance ratios of the tobacco-adapted forms of the green peach aphid calculated from the susceptible strain (Southern Piedmont- AREC, Green morph – LC_{50} 0.37 ppm) in the leaf-dip bioassays (151 colonies combined over yr and location).

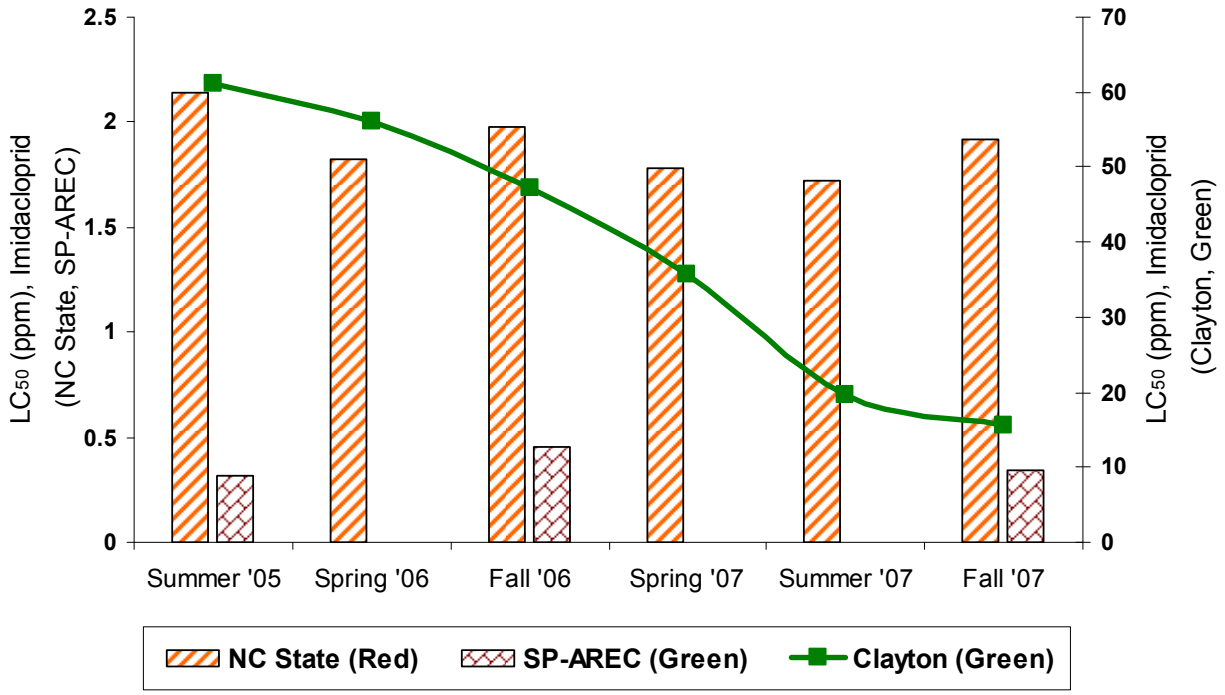

Fig. 3.5. Changes in susceptibility to imidacloprid among three colonies of the tobacco-adapted form of the green peach aphid, (i) most resistant colony, Clayton (green morph) (ii) Standard colony, NC State (red morph) and (iii) most susceptible colony, SP-AREC (green morph) tested over a 3-yr period, 2005-2007.

Chapter 4

Esterase-based resistance in the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae).

Abstract

Organophosphates and carbamates represent alternative insecticides in managing the tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer), a major pest of tobacco in United States and around the world. General esterases that detoxify these insecticides were assessed in three color morphs of field-collected *M. persicae*. A total of 136 aphid colonies collected from 2004 though 2007 were screened for total esterase activity by microplate assay using 1-Naphthyl acetate as the substrate. The green morphs had lower esterase levels with a mean of 73 nmol/min/mg protein than the red (83 nmol/min/mg protein) and orange morphs (172 nmol/min/mg protein). Seventy percent of the green morphs had esterase values below 70 nmol/min/mg protein, over 80% of the red morphs had levels ranging from 60 and 120 nmol/min/mg protein, and all of the orange morphs had esterase activity above 120 nmol/min/mg protein. Overall esterase activities, and those for the red and green morphs were positively correlated with LC_{50} values for acephate (organophosphate) and methomyl (carbamate) assessed in leaf-dip bioassays. Esterase genes responsible for higher esterase activities were diagnosed by gene amplification studies using PCR-RFLP procedure. All colonies tested had either E4 or FE4 gene amplified that confers esterase-based resistance. Fifteen out of the 24 colonies tested had amplified E4 gene and four colonies had FE4 gene amplification. All four orange morphs and one green morph had both E4 and FE4 genes amplified. This unique phenotype had an 865-bp band characteristic of the FE4 gene and an additional 381-bp and characteristic of a deleted upstream region of the E4 gene. Changes that occurred in esterase-based resistance in the TGPA over the past two decades and their implications on insecticide resistance management are discussed.

Introduction

The tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer) is one of the most important insect pests of tobacco in Virginia and the southeastern United States. The combination of aphid feeding damage, sooty mold, and honeydew interferes with curing and reduces the leaf quality (Mistric and Clark 1979). Injury attributed to the TGPA reduces the value of untreated tobacco by 5 to 30% annually (Reed and Semtner 1992). Before 1985, a green morph of the TGPA was the only form reported on tobacco in the southeastern United States (Blackman 1987). A red morph of the TGPA first occurred on tobacco in 1985 and quickly replaced the green morph as the most common form (Blackman 1987, Lampert and Dennis 1987, McPherson 1989, Harlow et al. 1991). Widespread insecticide resistance to traditional organophosphates (OPs), carbamates arose about the same time (Blackman 1987, Harlow and Lampert 1990, McPherson and Bass 1990). The association between the red form and resistance to OP insecticides is well established for the TGPA in the United States (Harlow et al. 1991, Clements et al. 2000). All red morphs and some green morphs have a translocated karyotype that is associated with an amplified gene that produces high levels of carboxylesterases and gives resistance to OPs and carbamate insecticides (Harlow et al. 1991, Field and Devonshire 1998).

The green peach aphid, *M. persicae* has developed resistance worldwide to OP, carbamate, and pyrethroid insecticides through the increased production of a carboxylesterase, E4, or its closely related variant FE4. These enzymes inactivate insecticides by sequestration and ester hydrolysis (Devonshire and Moores 1982). Molecular genetic studies have shown that this increase in esterase production is primarily due to gene amplification, i.e. the presence of multiple copies of the esterase gene in resistant aphids (Field et al. 1993).

Though some studies conducted in Europe involved clones collected from the United States, none were report to be from tobacco. Since the studies conducted by Harlow et al. (1990, 1991), there has been little effort to quantify resistance and the factors involved with the help of modern biochemical and molecular techniques. This was neglected partly due to the species controversy that arose between the tobacco-feeding-form, *M. nicotianae* and the green peach aphid, *M. persicae* (Blackman 1987, Clements et al. 2000a, b). It has been almost 14 yr since insecticide resistance has been documented for the TGPA in the United States.

Acephate (Orthene®) (OP) and aldicarb (Temik®) (carbamate) remain important alternatives in TGPA resistance management program. If these chemicals were cancelled for use on tobacco, only the neonicotinoids and pymetrozine (Fulfill®), an aphid antifeedant, would remain for managing the aphid. The present studies were conducted to create baseline information to determine the biochemical and molecular diversity of esterase-based resistance in TGPA from Virginia and seven other tobacco-producing states in the eastern United States. This information will aid in developing strategies for preserving their effectiveness, increasing their performance, and finding alternative cultural and natural controls.

Materials and Methods

Chemicals

Leaf-dip bioassays. Acephate (Orthene 97®) was obtained from Valent BioSciences Corporation, Libertyville, IL and methomyl (Lannate 90SP®) was obtained from DuPont, Wilmington, DE. Fluon (Insect-a-slip®) was purchased from Bioquip, Rancho Dominguez, CA.
Esterase analysis. Fast Blue RR salt, sodium dodecyl sulphate (SDS), α-Naphthyl acetate, α-Naphthol, bicinchoninic acid and bovine serum albumin (BSA) were obtained from Sigma-Aldrich, St. Louis, MO. Anhydrous mono- and di-basic sodium phosphate and Triton X-100 were obtained from Fisher Scientific, Pittsburg, PA.
Electrophoresis. Tris base, hydrochloric acid, glycerol, bromophenol blue, lysine, and arginine were obtained from Sigma Aldrich, St. Louis, MO. Phosphoric acid and acetone were obtained from Fisher Scientific, Pittsburg, PA.
Esterase gene amplification. DNA purification kit was purchased from QIAGEN, Valencia, CA. *Spe* I, *Hind* III, *Taq* DNA polymerase, magnesium chloride and DNA-primers were purchased from Invitrogen Corporation, Carlsbad, CA.

Field sampling. Tobacco-adapted forms of *M. persicae* were collected on leaf samples from tobacco, *Nicotiana tabacum* L. (Family Solanaceae), fields across nine different states over a 4-yr period (2004 - 2007). Information about their body color under field conditions was recorded for all the locations. Most of the colonies were collected personally with the help of agricultural Extension agents. Collaborators in other states (Georgia, South Carolina, North

Carolina, Tennessee, Maryland, New York and Kentucky) collected aphids on tobacco leaves by removing whole or pieces of infested leaves from the plants. The aphids from New York were shipped on live *Calibrachoa* plants from Riverhead, NY. The infested leaves were placed in Ziplock® plastic bags containing a piece of paper towel to reduce moisture and sent overnight (FedEx®) to the laboratory in Styrofoam® or corrugated containers with ice packs. Where possible, both red and green color morphs of the aphid were collected at each location. All aphids were collected from tobacco, except the colonies from Riverhead, NY AREC red (*Calibrachoa* spp.), 2006; Semtner (red and green) 2007 (*Brassica napa*, 'Purpletop turnip', and Clay's green, 2007 (*Bougainvillea spectabilis* Willd). All colonies were maintained on insecticide-free excised tobacco leaves (Flue-cured variety, K-326). Leaf petioles were inserted into agar medium in Styrofoam® cups and kept at 21 ± 1°C, 60% RH and 16:8 (L:D) photoperiod in laboratory incubators until discarded after the bioassays were conducted.

Greenhouse plants. Plants were grown in at least two different locations to overcome possible natural insect-pest infestation in the greenhouse. Tobacco, flue-cured variety, K-326 was seeded in Styrofoam® seeding trays (288 cells) filled with Carolina Tobacco Mix® (a soilless greenhouse growing medium). The trays were placed in float bays filled with water and fertilized based on fertilizer recommendations (Reed 1998). Three to four-week-old seedlings were transplanted from the trays, in to 15 cm diameter pots filled with growing medium (Carolina Tobacco Mix®) and placed in the float bays filled with water and fertilized with 200 ppm of Peters® 20-20-20 water soluble fertilizer. Plants were discarded once they reached button stage (pre-flowering stage), as they become sticky and undesirable for aphids.

Leaf-dip bioassay. Test populations were increased on excised tobacco leaves having the cut petiole end inserted into water agar plugs contained in glass vials (50 ml). Leaves were placed in individual, ventilated plastic containers. Five to six-day-old aphids of similar size were used for the bioassays. Each test population consisted of adult apterous aphids of the same color (green or red) from the same tobacco field. Aphids were bioassayed by leaf dip method using water dispersions of acephate (Orthene 97®) and methomyl (Lannate SP®). Leaf disks, 100 mm in diameter, were cut from fresh leaves (midstalk position) from greenhouse-grown tobacco plants (Flue-cured tobacco cultivar, 'K-326'). Leaf disks were dipped for 5 s in the designated concentrations, allowed to air-dry, and placed on slightly moistened filter papers in labeled 15 x 100 mm Petri dishes. The inside lips of the Petri dishes were coated with Fluon® (Insect-a-slip®)

to keep the aphids on the leaves and to prevent escape. Ten healthy adults from the test colony were placed on each leaf disk with a camel's hairbrush. Covers were placed on each Petri dish and secured with Parafilm®. Each dose included 30-40 aphids.

Mortality was assessed 24 h after aphicide exposure. The aphids were probed lightly with the camel's-hair brush to elicit a response. If an aphid did not move or only twitched slightly, it was considered dead. At least six doses and a deionized water check were tested each time with each dose replicated at least three times. Treated leaf disks and aphids were kept in environmental chambers at 21 ± 1°C, 60% RH and 16:8 h light: dark photophase. Abbott's formula (Abbott 1925) was used to correct for mortality in the control. Results represent the combined data from at least two separate bioassays. LC_{50} values were calculated using POLO PC (LeOra Software, Berkeley, CA). Differences among means of LC_{50} values between the color morphs were evaluated using one way ANOVA followed by a *post hoc* Tukey's multiple comparison test (SAS Institute, 2001). The level of significance for all statistical analyses was chosen *a priori* to be $p \leq 0.05$.

Resistance ratios were calculated as:

$$RR = \frac{LC_{50} \text{ of the test colony}}{LC_{50} \text{ of the most susceptible colony}}$$

Microplate assay for quantification of total esterase activity. Determination of the levels of enzyme systems that are involved in degradation of organophosphate and carbamate insecticides is a traditional method of estimating the resistance to these compounds. An extremely sensitive colorimetric technique for estimation of total esterases uses the novel-substrate, α-Naphthyl acetate in an end point assay. General esterase activity was measured according to the method of Van Asperen (1962) as modified by Zhu & Gao (1999). This assay is based on the estimation of naphthol produced from the hydrolysis of naphtholic ester. Five apterous (wingless) adult or last (4^{th}) instar aphids were homogenized in 0.5 ml ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.3% Triton X-100 (v/v). The aphid homogenates were centrifuged at 12,600 rpm for 15 min at 4°C and the supernatants were transferred to separate clean microcentrifuge tubes. Three to four aliquots of 15 µl enzyme preparation from each

colony were pipetted into separate wells of the 96-well microplate (NUNC flat bottom, Fisher Scientific, USA). Enzyme preparations were kept on ice at all times. Enzyme reaction started with the addition of 0.3 mM α-Naphthyl acetate [0.1 ml of 30 mM α-Naphthyl acetate in 9.9 ml of 0.1 M phosphate buffer (pH 7.5)], with a final concentration of 0.27 mM per well. Plates were covered with Parafilm and incubated for 30 min at 37^{o}C. Fifty µl (30 mg Fast Blue RR Salt that couples to naphthol and produces a colored conjugate in 5% SDS that solubilizes the naphtholic azo dye conjugate) was added to stop the reaction. The mixture was set aside at room temperature for 15 min to develop color. Production of α-Naphthol as a final product was determined at 595 nm using a microplate reader (TRIAD multimode detector, Dynex Technologies, USA). The amount of α-Naphthol produced was calculated based on the optical density value obtained from α-Naphthol standard curve. Each colony was replicated at least four times for each run and every colony was repeated at least three times. All chemicals were prepared fresh for each run. A total of 136 field samples were tested for the years 2004-2007.

The mean esterase activity was calculated and standardized per mg of protein for each colony. Analysis of variance was used to determine if the activities differed significantly (α=0.05) (SAS Institute, 2001).

Protein determination. Protein contents of the enzyme preparations of each colony were standardized according to Smith et al. (1985) using bovine serum albumin (BSA) as the standard. Measurement was performed using 20 µl of the enzyme preparation (as described above) and incubated with 180 µl of bicinchoninic acid in 4% cupric sulphate solution (Sigma Aldrich, St. Louis, MO). The formation BCA/Cu^{+} complex with the protein after 30 min incubation period was measured at 595 nm using a microplate reader (TRIAD multimode detector, Dynex Technologies, USA). Protein content was calculated based on the optical density value obtained from a BSA (Sigma Aldrich, St. Louis, MO) standard curve.

Electrophoresis. Isoelectric focusing of samples having a range of esterase activity was performed on wide range (pH 3.5 - 9.5) slab gels (Bio-Rad, Hercules, CA). Enzyme preparation of individual colonies was made as described above in 0.1 M phosphate buffer (pH 7.5) containing 3% (v/v) Triton X-100. Gels were focused at 5^{o}C, 100 V for 60 min, 250 V for 60 min and 500 V for 30 min using different electrode buffers with wide range of pI (anode buffer: 7 mM phosphoric acid; cathode buffer: 20 mM lysine + 20 mM arginine) on a Mini-Protein II dual slab cell (Bio-Rad, Hercules, CA). Esterases were visualized by incubating gels in 0.02%

(w/v) α-Naphthyl acetate in sodium phosphate buffer (0.1 M, pH 7.2) for 30 min. Gels were washed in distilled water and incubated for 20 min in 0.13% (w/v) Fast Blue RR salt. After staining, the gels were washed and stored in distilled water at 4°C. The density of the resolved bands was analyzed using densitometric analysis (Kodak, 2000).

Detection of amplified esterase genes (E4 and FE4). Twenty-five colonies that had a range of general esterase activity were chosen to look at the esterase gene amplification responsible for the activity. Clones were established from individual aphids and DNA was extracted from a single aphid from each clone using Qiagen DNA extraction kit. The amount of DNA extracted was quantified using spectrophotometer (NanoDrop® ND-1000, Wilmington, DE). The amplified genes encoding carboxylesterases E4 and FE4 genes in individual aphids were investigated according to a polymerase chain reaction - restriction fragment length polymorphism (PCR–RFLP) method described by Field et al. (1996). New polymerase chain reaction (PCR) primers reported by Guillemaud et al. (2003) based on the E4 and FE4 esterase gene sequences reported by Field et al. (1996) were used with the aim of improving PCR yield. The PCR protocol is presented in Appendix 5. An aliquot of genomic DNA containing at least 100ng DNA was used in a PCR reaction with E4 and FE4 primers. The primers used were:

Est3N 5′-AAATCATATTCCCGGGTTC-3′ and
Est4p 5′-TGAGTAATCTTAGTGAACCTGA-3′

The PCR products were digested overnight at 37°C with *Hind*III (specific for FE4 alleles) or *Spe*I (specific for E4 alleles) giving either a 572-bp fragment for E4 genes or an 865-bp fragment for FE4 genes. The basis of this non-competitive PCR diagnostic is a deletion at the 5' end of the FE4 gene, as described by Field et al. (1999). The outcome is either both bands for aphids without amplified genes (i.e. susceptible, with a single copy of each), or a single band of 572-bp if amplified E4 genes are present or an 865-bp band from amplified FE4. The PCR products were run on 1.5% agarose gels and visualized by staining with ethidium bromide. Individuals were classified as FE4, E4, E4/FE4, or S aphids.

Results

Toxicity of methomyl and acephate in leaf-dip bioassays. Differences in mortality among colonies tested against acephate and methomyl are shown in Appendices 6 and 7. Thirty-eight colonies representing red, green, and orange color morphs are selected for a range of total esterase activity were screened against methomyl. The LC_{50} values for red and green morphs for methomyl (Lannate 90SP) ranged from 49.9 ppm to 682.2 ppm (Appendix 6). Methomyl was more toxic to the green morphs than the red and orange morphs. The toxicity of methomyl to red morphs ranged from 95.6 (Ayres, Patrick, 2006) to 627.7 ppm (TN-AREC, Loudon, TN, 2007). All of the orange morphs had LC_{50} values greater then 400 ppm. An orange morph from Patrick County, VA had the highest toxicity value (LC_{50}=682 ppm). Three green colonies had high LC_{50} values compared to other green morphs (Johnson, Surry, NC, 2006 – 545.7; Semtner, Nottoway, VA, 2006 – 482.2; SC-AREC, Horry, SC, 2006 – 520.5). Two of the red morphs had high toxicity values, Johnson, Darlington, SC, 2007 – 582.6 ppm and Rogers, Henry, VA, 2007 – 443.5 ppm. The means of the LC_{50} values for orange morphs were significantly higher than both green and red ones (Tukey's multiple comparison, $p < 0.05$).

Acephate (Orthene 97®) toxicity for red and green morphs is presented in Appendix 7. Forty-nine colonies with a range of total esterase activity for each color morph were tested against acephate. The toxicity of acephate to red morphs ranged from 122.2 (Ayres, Patrick, 2006) to 423.6 ppm (Mitchell, Franklin County, VA, 2006). The toxicity values for the orange morphs ranged between 389.2 to 523.5 ppm. As seen with the toxicity towards methomyl, an orange morph from SPAREC, Nottoway, VA collected in 2006 had highest LC_{50} value for acephate (523.5 ppm). While five of the 18 green colonies had high LC_{50} values greater than 400 ppm, three out of 23 red morphs had toxicity values greater than 400 ppm (Johnson, Darlington, SC, 2007 – 402.2 ppm; Townsend, Dinwiddie, VA, 2006 – 423.5 ppm and Mitchell, Franklin County, VA, 2006 – 423.6 ppm). The means of the LC_{50} values for orange morphs were significantly higher than both green and red ones (Tukey's multiple comparison, $p < 0.05$).

Total esterase quantification by microplate assay. Results of the total esterase analyses for the TGPA collected from nine different states in the eastern United States are presented by year in Appendices 8 through 11. There were significant differences in esterase activity among the 136 colonies tested ($F = 19.07$; df = 136, 272; $P = 0.0001$). Over four years,

the total esterase activity ranged from 32 to 241 nmol/min/mg protein. The frequency distribution of the total esterase, within color morph is show in Fig. 4.1. Though almost 70% of the green morphs had esterase values below 70 nmol/min/mg protein, 8% of the green morphs had values higher than 120 nmol/min/mg protein. Lowest esterase activities were recorded in the green morphs each yr from 2004 through 2007. Higher activities were also recorded in the green morphs consistently from 2004 through 2006 (Townsend, Dinwiddie, VA - 203.2; Witcher, Franklin, VA - 139.3 and Manning, Mecklenburg, VA - 241 nmol/min/mg protein respectively). In 2007 the orange morph collected from Dellenback, Patrick, VA had the highest total esterase activity for the year (214.2 nmol/min/mg protein). Esterase activity in the red morphs ranged from a low of 40.4 (Jackson, Henry, VA, 2006) to 185.4 nmol/min/mg protein (Greenhouse, Greene, NC, 2007). Just over 80% of the red morphs had activities that ranged from 60 and 120 nmol/min/mg protein. Five percent of the total red morphs had esterase values higher than 120 nmol/min/mg protein. All of the orange morphs had esterase activity values higher than 120 with 75% of them above 150 nmol/min/mg protein. In 2007, the orange morph collected from Dellenback, Patrick, VA had the highest total esterase activity for the yr (214.2 nmol/min/mg protein). The first orange morph that was collected late in September 2006 from SPAREC, Nottoway, VA also had high activity (153.5 nmol/min/mg protein).

Electrophoresis: Isoelectric focusing. Homogenates of 25 colonies with a range of esterase activity were resolved on wide range (pH 3.5 - 9.5) isoelectric focusing (IEF) gels using α-Naphthyl acetate as substrate. The resolved esterase isozymes were visualized using ester-fast blue staining protocol like in the microplate assay. The most anodic resistance associated esterases (RAEs) in the TGPA appear to be a multiple of at least two isozymes (Fig. 4.2). Only one isozyme is apparent in this region in resistant TGPA, seen as a distinct peak in moderately resistant (R1) and highly resistant individuals (R2). This anodic RAE is appreciably missing in the susceptible individuals of the TGPA. It is rather interesting that the cathodic RAEs are seen in all the strains (S, R1 and R2) unlike that reported earlier (Abdel-Aal et al. 1990) though not seen as a distinct peak as reported in their findings. Both the moderately resistant strain (R1) of the TGPA and the highly resistant strain (R2) showed similar band intensity of those isozymes as the susceptible strain (S), the latter strain (R2) showed a orange body coloration while the former (R1) was red. The data presented in Fig. 4.2 also re-confirmed the information obtained from the total esterase activity (Appendix 8) in that the homogenates from resistant aphids exhibited

darker bands than those of the reference susceptible strain. From the densitometric analysis there are at least two esterases (see arrows in Fig. 4.3) that are associated with resistance in the TGPA.

Esterase gene amplification. Of the 136 aphid colonies screened by microplate assay, 24 colonies exhibiting a range of esterase activity were selected to study the molecular mechanisms responsible for the elevated esterases (Table 4.1). A single predominant clone collected on tobacco in Chile, South America provided by Dr. Fuentes-Contreras exhibiting E4 allele was used as a standard.

Twenty-four out of 25 colonies diagnosed for the esterase gene amplification had either E4, FE4, or both in the colonies (Fig. 4.4). Fifteen out of 24 colonies collected between 2004 and 2007 had amplified E4 gene and 4 colonies had FE4 gene amplified. Both E4 and FE4 amplification was observed in five of 25 colonies, four of four orange morphs and one of nine green morphs (Semtner Garden, Nottoway County, VA, 2006) (Fig. 4.5). This unique phenomenon is seen as an 865-bp band characteristic of FE4 but having an additional 381-bb band (Fig. 4.5).

Discussion

Esterases are a large, heterogeneous and diverse group of enzymes metabolizing various exogenous and endogenous substrates with ester linkages. They are associated with insecticide resistance in over 50 species of insects, ticks and mites (Devorshak and Roe 1998). The roles of esterases or carboxylesterases in pesticide resistance are xenobiotic metabolism and sequestration. Esterases have also been used successfully as markers for detecting insecticide resistance. Several studies have used the modified-microplate assay to detect esterase-based resistance in the TGPA in the United States and elsewhere (Ffrench-Constant and Devonshire 1988, Harlow et al. 1991, Abdel-Aal et al. 1992, Foster et al. 2000, Fuentes-Contreras et al. 2004, Margaritopoulos et al. 2007).

Relationship between general esterase activity and the toxicity to acephate and methomyl: The activity of the total esterases in the 136 colonies had up to 5-fold differences. Most green morphs had activities below 70 nmol/min/mg protein while 80% of the red morphs ranged between 60 and 120 nmol/min/mg protein. Interestingly, a low percent of the red and green morphs also had either high or low esterase activities, especially the green morphs, which

had the highest activities in the years 2004, 2005, and 2006. The orange morphs were sampled extensively in 2007, although one orange morph was collected late in the 2006 season at SP-AREC, Blackstone, VA on Oriental tobacco, 'Bamsa' grown in a high tunnel structure. In 2007, colonies of the orange morph were almost ubiquitous in most of the farms visited in Virginia, and this color morph was also received from Georgia and Maryland in the same season. Orange morphs were seen throughout the season at the Southern Piedmont AREC at Blackstone, VA. Though this was not the first occurrence of this unique color morph, (Semtner, Personal communication), the frequency of detection of this colored aphid was greater in 2007. Interestingly all the orange morphs had high esterase activities, greater than 150 nmol/min/mg protein, which is almost three times the amount found in most of the green morphs.

The susceptibility of TGPA colonies towards acephate and methomyl in leaf-dip bioassays were compared with total esterase activities (Table 4.2). Total esterases were highly correlated with LC_{50} values for both methomyl and acephate. The plots of LC_{50} values against insecticides, methomyl (Fig. 4.6) and acephate (Fig. 4.7) showed distinct groupings for color morphs, with esterase values always correlated with the toxicity values. The orange morphs, which had very high esterase activities, also had higher LC_{50} values than most red and green morphs for both methomyl and acephate. Several green and red morphs had unusually high or low esterase activities. However, all the colonies with high esterase activities also had higher LC_{50} values regardless of their body color. The LC_{50} values for red and green morphs of the TGPA collected from Virginia in 1988 and 1989 by Barnes (1990) tested against acephate ranged from 87 ppm to 413 ppm. The range of toxicity was very similar in the present study, with the exception for the orange morphs, which had LC_{50} values ranging from 485 ppm to 682 ppm.

All of the orange morphs had both higher esterases and toxicity values for acephate and methomyl than most green and red morphs. The carboxylesterase activity reported by Harlow (1990) ranged from 17.2 to 77.4 µM 1-Naphthol produced per minute per aphid. With an exception of one colony (Duplin County green morph, NC) all other green morphs had lower esterase activity compared to the red morphs in the 36 clonal cultures collected from North Carolina in his study. His results also indicated that there is a noticeable difference in the esterase activity between the color morphs, though there is an overlap detected due to some susceptible red morphs. Earlier findings by Abdel-Aal et al. (1990) found the highest esterase

activity in highly resistant (R2) aphids to be around 60 nmol/min/mg protein towards α-Naphthyl acetate. Generally, my studies indicate that the esterase activity in many of the colonies was almost two-fold higher.

My findings agree with Harlow and Lampert (1990) and Harlow et al. (1991) that, the body color alone could not be used to separate aphids based on the esterase activities. As the esterase activities were positively correlated with LC_{50} values, presumably, body color would not separate susceptible and resistant populations.

As with many insect species, a strong positive correlation between general esterase activity and resistance to organophosphate, carbamate, and pyrethroid chemical classes has been observed in the green peach aphid (Needham and Sawicki 1971, Devonshire and Moores 1982). Large amounts of the enzyme produced *in vivo* which is up to 3 % of the total protein in highly resistant aphids has been attributed to sequestration by esterase E4 resulting from successive tandem duplications of the E4 structural gene (Devonshire and Sawicki 1979, Devonshire 1989). Later, it became clear that resistant aphids lacking the autosomal-1,3 translocation have slightly different amplified esterase, which was named as FE4 (Devonshire et al. 1983). Two closely related variant forms of the enzyme, E4 and FE4, were subsequently distinguished, the latter having a slightly higher catalytic center activity (1.5-fold difference) towards OPs (Devonshire et al. 1983) and a molecular weight of 66 kDa compared to 65 kDa for E4 (Field et al. 1988). The difference was in the primary structure rather than resulting from different post-translational change (Devonshire et al. 1986).

Electrophoresis: The intensity of the esterase band seen in the orange morphs is very high compared to the other colonies. Though the susceptible strain shown in Fig. 4.3 is a green morph with a low esterase activity, other green morphs that had higher esterase activities also had darker esterase bands. It is quite evident now that higher esterase activities could be found in other color morphs regardless of the body coloration. This study resolved a pending question whether esterase banding patterns are associated with the body coloration. The overall differences in total esterase activities as seen in the densitometric analysis with peaks in the resistant-associated colonies compared to the susceptible ones. This procedure confirms both quantitative (total esterase activities in microplate assay) and qualitative (toxicity towards acephate and methomyl) differences between resistant and susceptible colonies.

Esterase gene amplification: Overproduction of insecticide detoxifying esterases in *M. persicae* is a form of resistance which was first implicated in the late 1960's by the demonstration that all resistant strains showed an increased ability to hydrolyze the model esterase substrate, 1-Naphthyl acetate (Needham and Sawicki 1971). It was subsequently shown to have developed from an increased production of one of two forms of esterase, E4 or FE4, that were distinguishable electrophoretically (Devonshire 1986). These esterases can account for as much as one percent of the aphid's total body protein, and give a broad spectrum of resistance to organophosphate, carbamate, and pyrethroid insecticides as a consequence of both ester hydrolysis and sequestration (Devonshire and Moores 1982). Compared to E4, FE4 hydrolyzes insecticides slightly faster (Devonshire et al. 1983). Field et al. (1994) have shown that both tobacco-adapted and non-tobacco adapted forms of *M. persicae* have the same esterase genes, E4 and FE4. Several studies, thereafter, have used this PCR-based diagnostic tool to separate out the two forms of esterase based resistant genes (Guillemaud et al. 2003, Margaritopolus et al. 2007).

We found 24 of 25 colonies exhibiting esterase gene amplification and had either E4, FE4 or both in the colonies. One colony, a red morph collected from Southern Piedmont - AREC, Nottoway County, VA, in 2006 is considered as susceptible aphid because it had very faint bands that were very difficult to visualize. This colony also exhibited very low level of esterase activity (54.5 nmol/min/mg protein). Fifteen out of 24 colonies collected between 2004 and 2007 had amplified E4 and 4 colonies had amplified FE4 gene.

An interesting aspect of the amplified E4 genes is that expression can be lost in some aphid clones, which is associated with the loss of DNA methylation (5-methylcytocine) present in expressed genes (Field et al. 1989, Field 2000, Field et al. 2004). This phenomenon occurred in 8 out of 15 colonies that had E4 gene amplification that had total esterase values lower than 65 nmol/min/mg protein (Fig. 4.8). Aphid colonies with such unexpressed E4 genes are called 'revertants'. Accordingly, they would be considered as either susceptible of low level resistant phenotypes when classified with the values obtained in biochemical analysis. Such 'revertants' were shown to have an E4 allele that is underexpressed due to no apparent selection pressure. Such colonies can only be detected at the molecular level. PCR-restriction enzyme diagnostic tool developed by Field et al (1996) allows us to identify whether individual resistant aphids had amplified E4 or FE4 genes in them. This tool uses E4 and FE4 primers to amplify the gene in a

PCR-RFLP procedure and digest them with E4 or FE4 specific restriction enzymes to quantify which gene is responsible for resistance.

One important observation was the detection of both E4 and FE4 amplification in 5 of the 25 colonies tested, and four of them being orange morphs. This is seen as an 865-bp band characteristic of FE4 but having an additional 381-bp band. This is not a common phenomenon and occurs less frequently. This phenomenon was first noticed in only two of the 205 colonies collected and screened in England (Field and Foster 2002). Further cloning and sequencing of the PCR products in their study showed that the 865-bp band in those clones was identical to the expected FE4 gene sequence but the 381-bp band was in all cases an E4 sequence with 191-bp deleted (Field and Foster 2002). This deleted region is upstream from the predicted transcription start site of the E4 gene (Field and Devonshire 1997) so it might be the product of the gene that would be a normal E4 enzyme (Field and Foster 2002). This means that clones that had both 865-bp and 381-bp bands will have elevated levels of both E4 and FE4. This is the first time that this phenomenon is reported in the TGPA. It is interesting that four of the five colonies with the deleted E4 gene were orange morphs collected from three different states in 2007 season. As seen in eastern England (Cambridgeshire), there may be a single origin of this version of the E4 gene associated with the orange morph.

The 'deleted' version of the E4 gene in the orange morphs, although will not affect the enzyme production and the subsequent resistance status of the aphid, can be present with amplified FE4, which was never seen for the 'normal' amplified E4 gene in the green peach aphids (Field and Foster 2002). Since some green peach aphids have holocyclic life cycles in England, the individuals with amplified E4 gene may co-exist with amplified FE4. Therefore, the offspring that inherit both were unlikely to survive because of the chromosomal translocation associated with the amplified E4 genes (Blackman et al. 1996, Field and Foster 2002). Crossing experiments by Blackman et al. (1996) showed that it is difficult to produce individuals with both amplifications.

Deletion of amplified E4 genes are not associated with a chromosome translocation and, if this is true, they may be able to spread and associate with FE4 genes by sexual crossing in holocyclic forms more easily than the 'normal' E4 genes (Field and Foster 2002). Since the TGPA in the United States is considered to be anholocyclic, it opens a new avenue of research to determine how the genes are transmitted and their association with the color morphs. As

mentioned earlier, since orange morphs were collected only in the 2006 and 2007, and the origin of the E4+FE4 gene in these morphs is not merely a coincidence. Functional and phylogenetic aspects of evolution of such a phenomenon need to be addressed in future experiments. As this deleted E4 amplified fragment was also seen in one green morph that had a high level of total esterase activity, it is more complicated than a mere association with body coloration.

As mentioned earlier, predominant use of neonicotinoids in managing TGPA would have adverse effects, and in other studies we have shown that some TGPA populations have already developed (low-level) resistance to these compounds. Acephate (OP) aldicarb and methomyl (carbamates) are the only chemicals from the two major classes of insecticides that are still alternatives in a TGPA resistance management program. There is every need to preserve these chemical formulations. If they were lost, only the neonicotinoids and pymetrozine (Fulfill®), an aphid antifeedant, would remain for managing the aphid. Continuous monitoring with the use of these simple techniques would create baseline information for monitoring esterase-based resistance in the TGPA in the United States. This information would not only help develop strategies for preserving these compounds, but also increase their effectiveness and performance.

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Table 4.1. Esterase activity and the corresponding amplified genes responsible for the activity in the tobacco-adapted form of the green peach aphids, *Myzus persicae* (Sulzer).

S. No.	Location	County	Year	Color	State	Esterase[†]	Esterase Gene
1	Ayres	Patrick	2006	Red	VA	48.6 ± 4.9	E4
2	Clay's	Nottoway	2007	Green	VA	110.8 ± 5.7	E4
3	Clayton	Johnston	2005	Green	NC	56.5 ± 4.9	E4
4	Cross Creek	Cumberland	2006	Red	NC	58.7 ± 5.6	E4
5	D Johnson	Russell	2006	Red	VA	59.9 ± 2.3	E4
6	Dellenback	Patrick	2007	Orange	VA	214.1 ± 2.5	E4 & FE4
7	Glasscock	Prince Edward	2006	Green	VA	47.2 ± 8.1	E4
8	Greenhouse	Greene	2007	Red	NC	185.4 ± 2.1	FE4
9	Johnson	Darlington	2007	Red	SC	149.7 ± 3.8	FE4
10	Johnson	Surry	2006	Green	NC	106.9 ± 7.8	FE4
11	La Batalla	Talca	2005	Red	Chile	1.83[*]	E4
12	Manning	Mecklenburg	2006	Green	VA	241 ± 4.3	FE4
13	MD-AREC	Prince Georges	2007	Orange	MD	193.6 ± 3.4	E4 & FE4
14	Melvin Owen	Pittsylvania	2006	Red	VA	72.4 ± 2.3	E4
15	Mitchell	Franklin	2006	Red	VA	107.8 ± 4.3	E4
16	Mitchell	Franklin	2007	Green	VA	39.8 ± 1.2	E4
17	NC-State	Wake	2005	Red	VA	94 ± 8.1	E4
18	NY-AREC	Suffolk	2006	Red	NY	89.6 ± 2.3	E4
19	Pee Dee -AREC	Florence	2006	Green	SC	45.9 ± 7.6	E4
20	Semtner	Nottoway	2006	Green	VA	133 ± 4.5	E4 & FE4
21	SP-AREC	Nottoway	2007	Orange	VA	165.7 ± 14.6	E4 & FE4
22	SP-AREC	Nottoway	2005	Green	VA	54.4 ± 3.6	S ?
23	UGA	Tift	2007	Orange	GA	178.2 ± 8.9	E4 & FE4
24	Wyatt	Pittsylvania	2005	Red	VA	89.6 ± 8.5	E4
25	Yanceyville	Caswell	2006	Green	NC	63.2 ± 1.5	E4

[†] Total esterase in nmol/min/mg protein ± SE

[*] Esterase values expressed as absorbance per µg fresh weight (aphids from Chile, South America)

Table 4.2. Relationship between general esterase activity and toxicity to methomyl and acephate in three color morphs of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) determined by Pearson correlation analysis.

Color Morph	General Esterase Activity* - LC_{50}† Acephate	General Esterase Activity* - LC_{50}† Methomyl
Red	r = 0.692 ; P = 0.0003 (N=23)	r = 0.775 ; P = 0.0238 (N=13)
Green	r = 0.732 ; P = 0.0008 (N=17)	r = 0.889 ; P = 0.0001 (N=12)
Orange	r = 0.196 ; P = 0.2329 (N=9)	r = 0.633 ; P = 0.0202 (N=8)

*General esterase activity measured in microplate assay using α-Naphthyl acetate as substrate (nmol/min/mg protein).

†Toxicity to acephate and methomyl determined in leaf-dip bioassays.

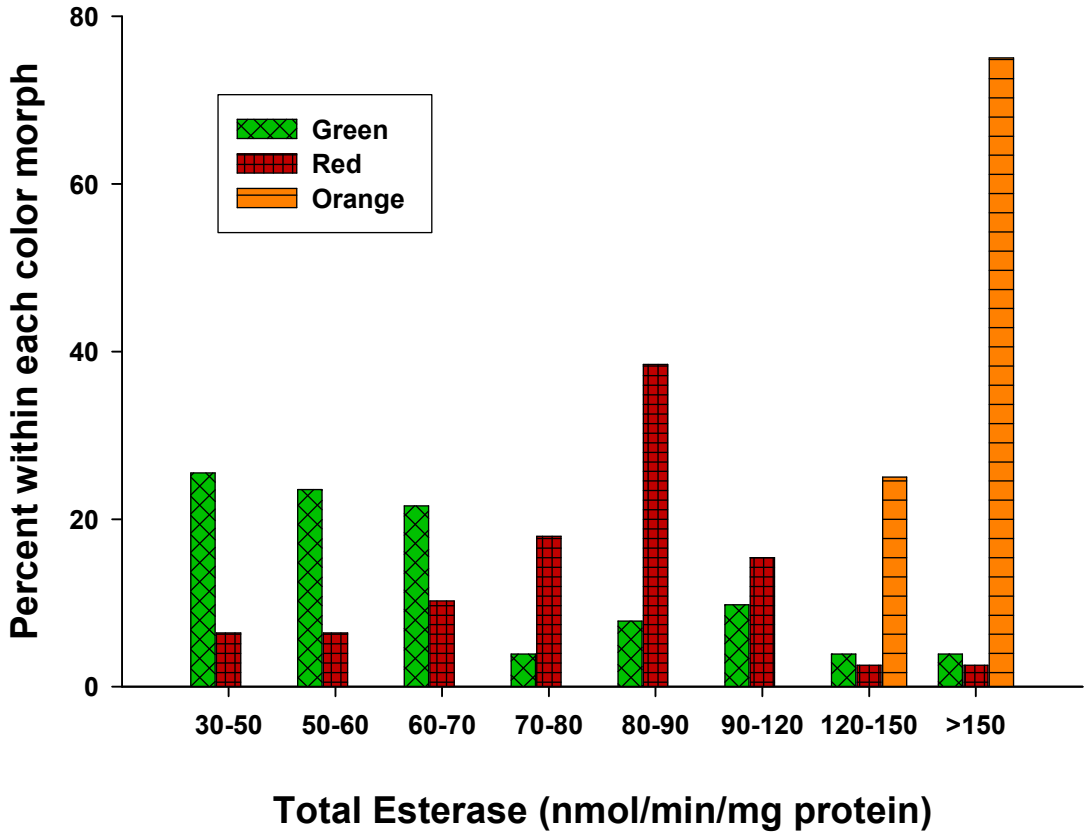

Fig 4.1. Frequency of the tobacco-adapted form of the green peach aphid colonies according to the general esterase activity within each color morph, eastern United States, 2004 through 2007.

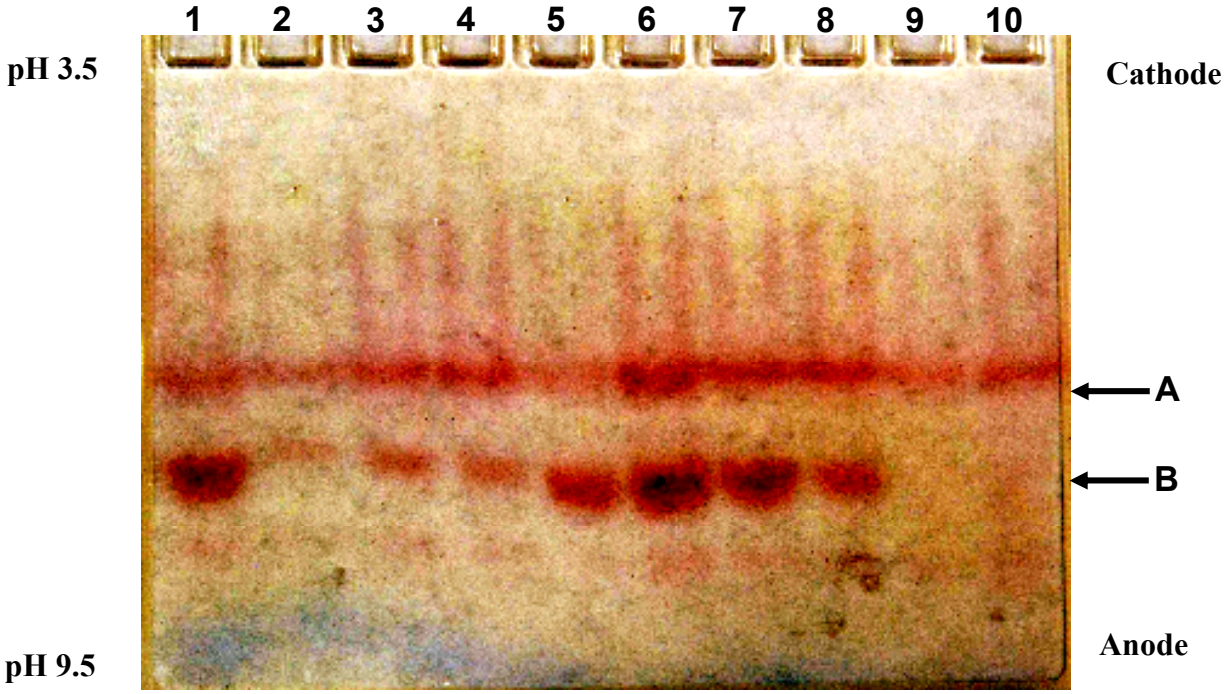

Fig. 4.2. Isoelectric focusing (IEF - pH 3.5 to 9.5) of 1-Naphthyl acetate hydrolyzing isozymes in resistant and susceptible tobacco-adapted form of the green peach aphid colonies from eastern United States.

Lane	Colony	Esterase Activity*
1	UGA, Tift, GA, 2007 - Orange	**178.2 ± 8.9**
2	Wyatt, Pittsylvania, VA, 2005 - Red	**89.6 ± 8.5**
3	Mitchell, Franklin, VA, 2006 - Red	**107.8 ± 4.3**
4	NC-State, Wake, NC, 2007 - Red	**82.9 ± 2.8**
5	AREC, Prince Georges, MD, 2007 - Orange	**193.6 ± 3.4**
6	Dellenback, Patrick, VA, 2007 - Orange	**214.2 ± 2.6**
7	Semtner, Nottoway, VA, 2006 - Green	**133.1 ± 4.6**
8	Clay's, Nottoway, VA, 2007 - Green	**110.8 ± 5.7**
9	Yanceyville, Caswell, NC, 2006 - Green	**63.2 ± 1.5**
10	Glasscock, Prince Edward, VA, 2006 - Green	**47.3 ± 8.2**

* Total esterase activity in nmol/min/mg protein ± SE measured in microplate assay.

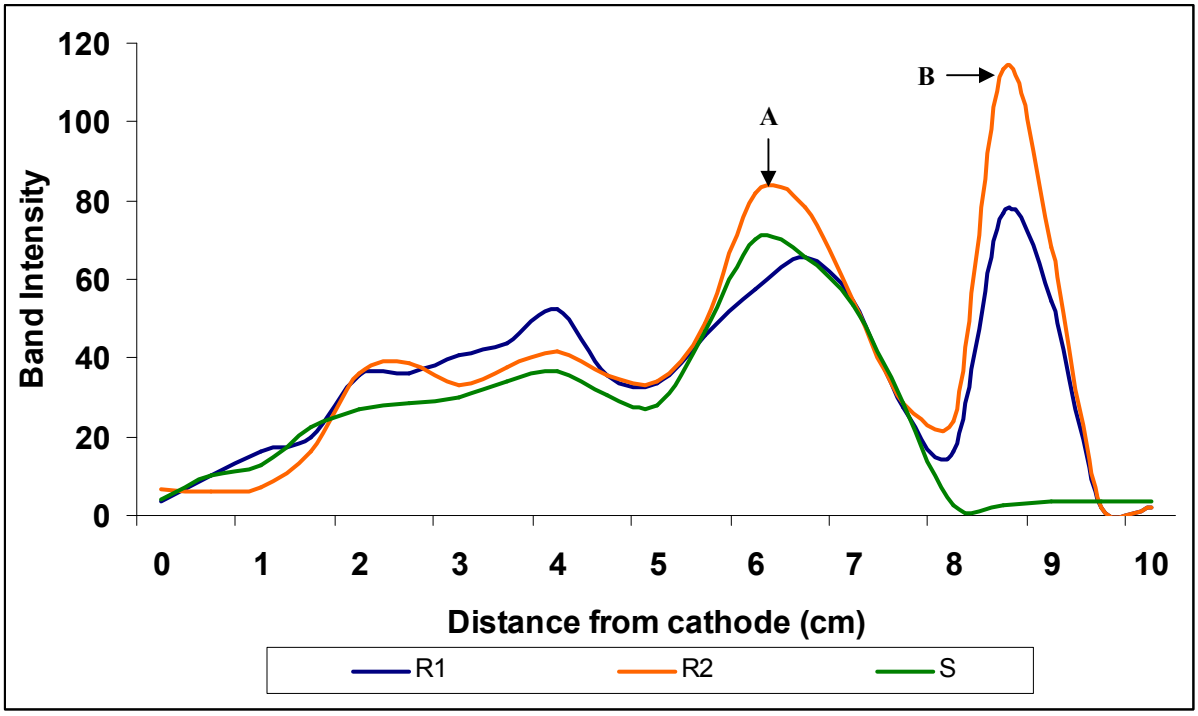

Fig. 4.3. Densitometric analysis of the esterase bands in the IEF gel among the colonies with a range of esterase activity: R1: NC-State, Wake, NC, 2007, red morph (82.9) - Lane 4; R2: Dellenback, Patrick, VA, 2007, orange morph (214.2) - Lane 6; S: Glasscock, Prince Edward, VA, 2006, green morph (47.3) - Lane 10. A and B represent the resistance associated esterases (RAEs).

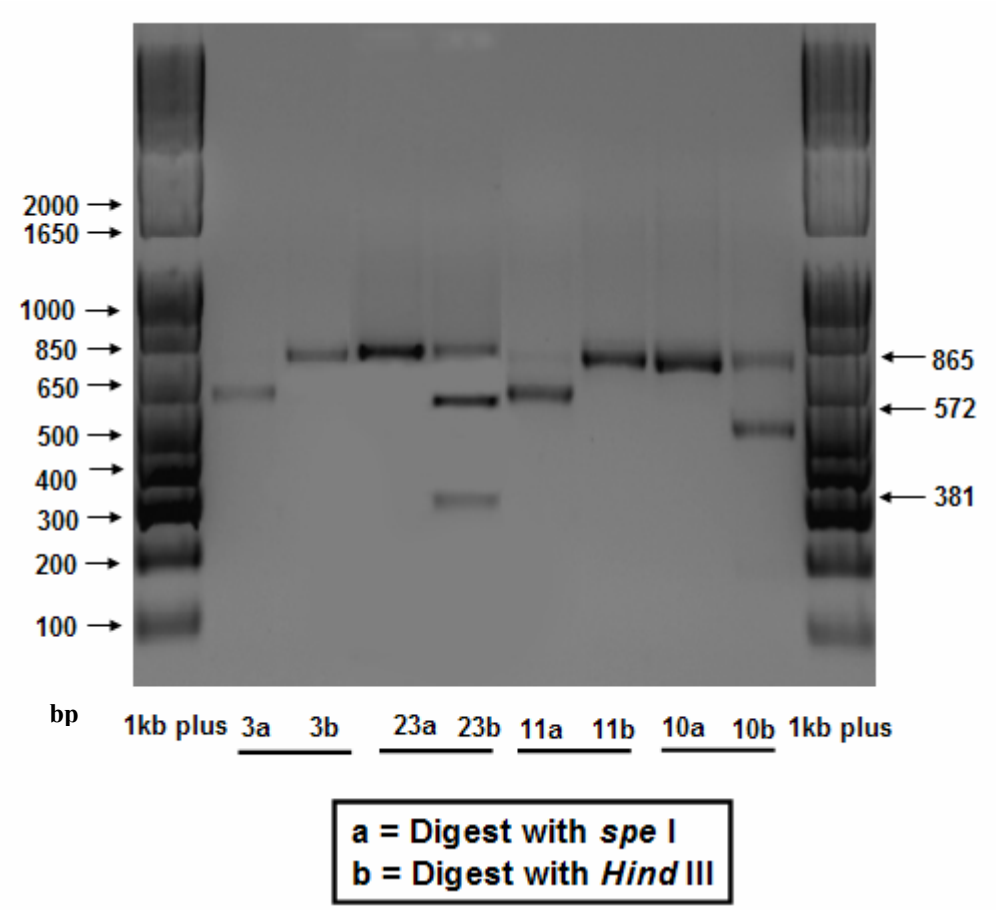

Fig. 4.4. Products of PCR digests, using E4/FE4 specific restriction enzymes (see text) on the tobacco-adapted form of the green peach aphid, *Myzus persicae* DNAs run on 1.5% agarose gel and visualized by staining with ethidium bromide. 1kb plus = 1kb plus DNA ladder (Invitrogen®). Colonies 3, 23, 11 and 10 showing E4, E4+FE4, E4 and FE4 respectively. Colony numbers correspond to their numbers in Table 3.1.

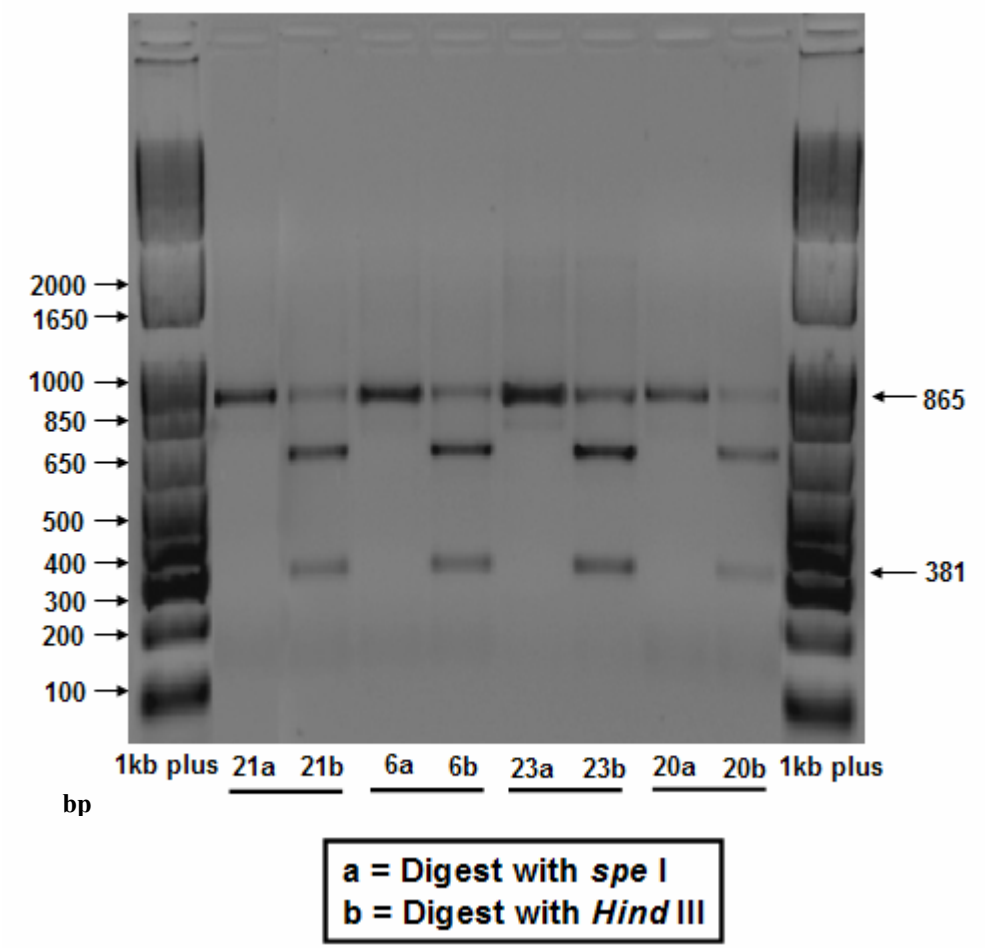

Fig. 4.5. Products of PCR digests, using E4/FE4 specific restriction enzymes (see text) on the tobacco-adapted form of the green peach aphid, *Myzus persicae* DNAs run on 1.5% agarose gel and visualized by staining with ethidium bromide. 1kb plus = 1kb plus DNA ladder (Invitrogen®). Colonies showing E4 & FE4 alleles seen as additional 381-bp fragment. Colony numbers correspond to their numbers in Table 3.1.

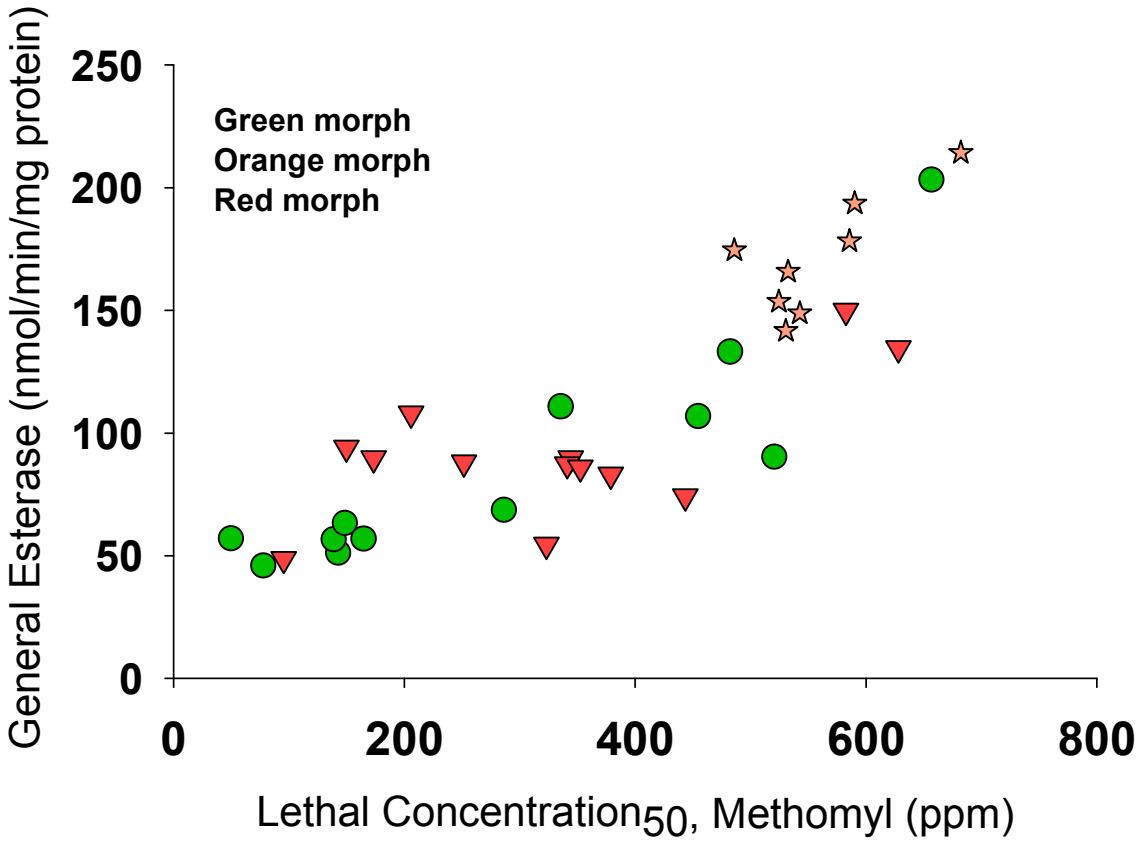

Fig. 4.6. Relationship between the toxicity to methomyl (Lannate SP) in the tobacco-adapted form of the green peach aphid colonies and total esterase activity combined for all the color morphs (Pearson $r = 0.857$, $P<0.0001$).

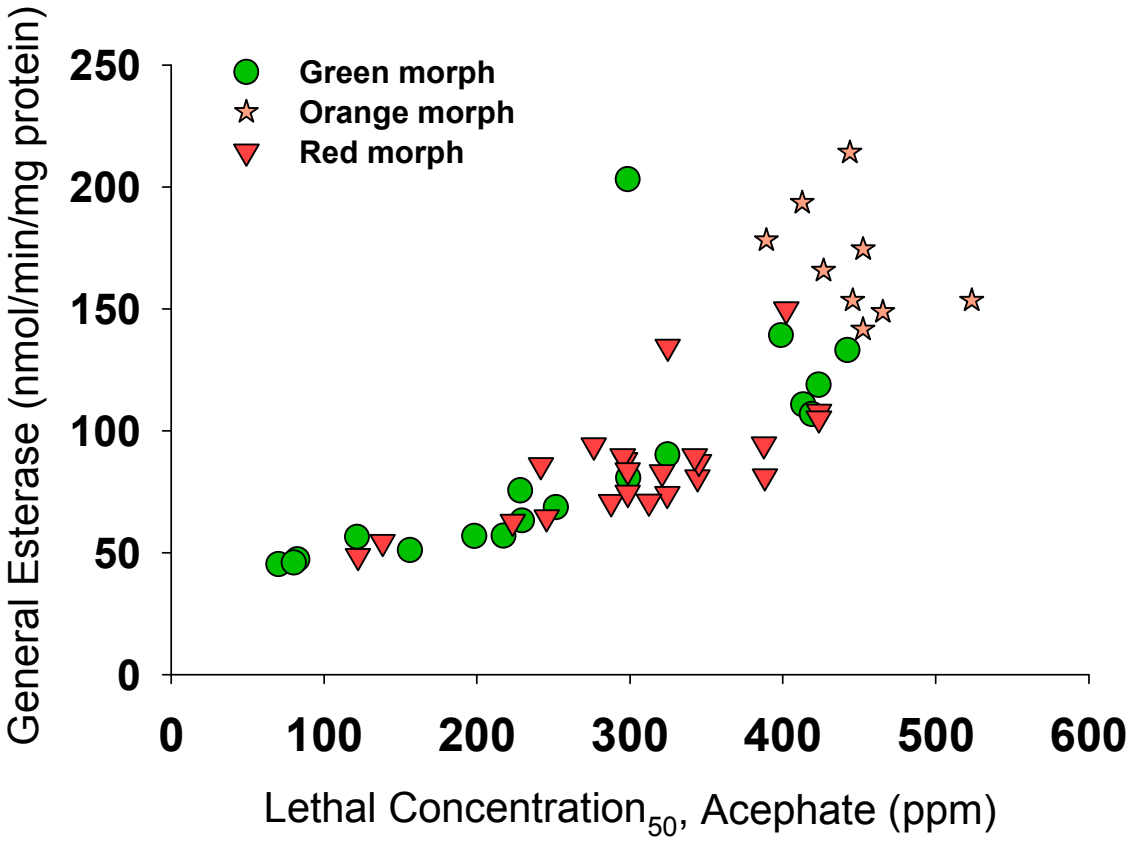

Fig. 4.7. Relationship between the toxicity to acephate (Orthene 97) in the tobacco-adapted form of the green peach aphid colonies and total esterase activity combined for all the color morphs (Pearson $r = 0.762$, $P<0.0001$).

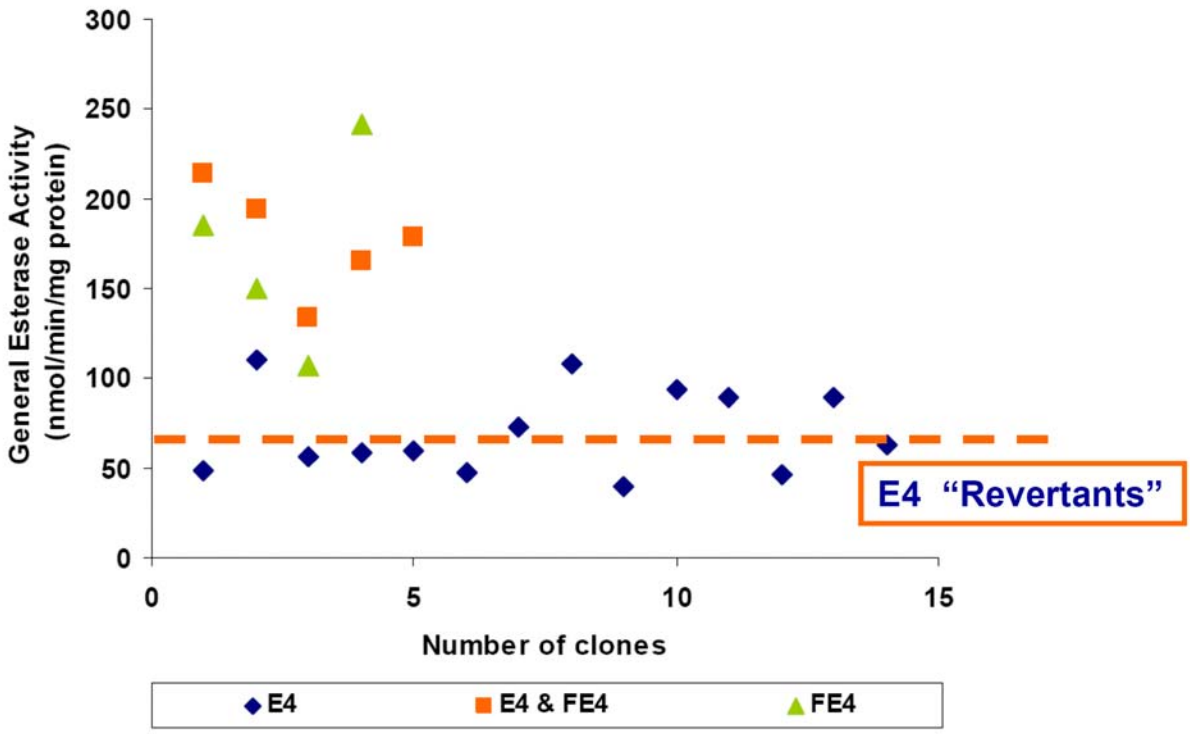

Fig. 4.8. Relationship between general esterase activity and corresponding amplified esterase gene in 24 colonies of the tobacco-adapted form of the green peach aphid.

Chapter 5

Acetylcholinesterase (AChE) activity in the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

Abstract

Organophosphate and carbamate insecticides exert their neurotoxic effects by inhibiting acetylcholinesterase (AChE), thereby, prolonging the contact of acetylcholine at cholinergic synapses, resulting in hyperexcitation. Mutations at the AChE target site confer modified acetylcholinesterase (MACE) phenotypes. Target-site insensitivity of AChE was characterized in field-collected, tobacco-adapted forms of the green peach aphid, *Myzus persicae* (Sulzer), from nine different states in the eastern United States from 2004 through 2007. The specific activity of the AChE among the 65 aphid colonies screened by Ellman's assay ranged from 0.017 to 0.259 U/min/mg protein. Eight colonies, with a wide range of specific activities were chosen to study the inhibition of AChE in the presence of two carbamate insecticides, methomyl and pirimicarb. IC_{50} values for methomyl ranged from 0.35 to 2.4 μM, while six out of eight colonies had lower values that ranged from 0.16 to 0.30 μM for pirimicarb. Two colonies that were inhibited by methomyl had very high IC_{50} values for pirimicarb, 40.4 and 98.6 μM respectively. The target-site insensitivity in these two colonies that are resistant to pirimicarb could be due to *ace2* gene mutation though it needs to be confirmed. The results indicate that the possible insensitivity due to MACE resistance in some colonies may render selected carbamate insecticides ineffective. Concerns of MACE resistance in managing the tobacco-adapted form of the green peach aphid on tobacco in the United States are discussed.

Introduction

Acetylcholinesterase (AChE) is a key enzyme in the nervous system, terminating neurotransmission by the hydrolysis of the neurotransmitter acetylcholine (Pitman 1971). Insect AChE is the target site of organophosphate (OP) and carbamate insecticides, which were introduced to the market decades ago and still represent the economically largest group of insecticides (Nauen and Bretschneider 2002). Russell et al. (2004) revised two major classes of target-site-insensitivity mutations conferring OP and carbamate resistance in many insect

species. Recently, a modified acetylcholinesterase (MACE) phenotype has been correlated with a mutation in the *ace2* gene in the green peach aphid, *Myzus persicae* (Sulzer) (Nabeshima et al. 2003). Resistance to OPs and carbamates in the melon (cotton) aphid, *Aphis gossypii* Glover, is also due to an insensitive AChE, and several studies have emphasized pirimicarb (O'Brian and Graves 1992, Silver et al. 1995, Moores et al. 1996, Han et al. 1998, Foster et al. 2003). Resistance to pirimicarb among thetobacco-adapted form of the green peach aphid, *M. persicae* (TGPA) populations on tobacco in Greece was confirmed as target-site insensitivity in the AChE site (Margaritopoulos et al. 2007). Mutations in acetylcholinesterase associated with insecticide resistance also occur in *A. gossypii* (Li and Han 2004). AChE mediated insensitivity is seen in other aphid species, including greenbug, *Schizaphis graminum* (Rond) (Zhu and Gao 1999), lettuce aphid, *Nasonovia ribisnigri* (Mosley) (Rufinger et al. 1999), and English grain aphid, *Sitobion avenae* F. (Chen et al. 2007).

The, a tobacco-feeding form of the green peach aphid, *M. persicae* (Sulzer) is one of the most important insect pests of tobacco. Aphids build up very high populations that deposit honeydew on the tobacco leaves resulting in the subsequent growth of a black sooty mold *Fumago vegans,* Pers., and other species, The combination of aphid feeding damage, honeydew, and sooty mold interferes with normal leaf ripening and curing, reducing leaf quality (Mistric and Clark 1979). Injury attributed to the TGPA reduces the value of untreated tobacco by five to 30% annually (Reed and Semtner 1992).

Acephate (Orthene®, an OP) and aldicarb (Temik®, a carbamate) remain important alternatives to neonicotinoids in TGPA resistance management program for tobacco. If these chemicals were no longer available, only the neonicotinoids [imidacloprid (Admire®) and others] and pymetrozine (Fulfill®) (an aphid antifeedant) would remain for managing aphids on tobacco. Since these viable insecticide classes still need to be protected, studies were conducted to quantify possible insensitivity of the target enzyme system, seen as MACE resistance. MACE resistance is a major mechanism of resistance for OP and carbamate insecticides in the green peach aphid in Europe. The specific activity of the target enzyme (AChE) was quantified and its inhibition by various carbamate insecticides was studied. This is the first study to look at MACE resistance in TGPA populations from the United States.

Materials and methods

Insects. Sixty-five colonies of TGPA tested in this study were collected from tobacco and other secondary hosts across nine different states over a 4-yr period (2004 - 2007). Colonies were maintained without insecticide selection pressure on excised tobacco leaves (flue-cured variety, K-326) with their petioles inserted into agar medium in Styrofoam® cups and kept at 21 ± 1°C, 60% RH and 16:8 (L:D) h photophase in laboratory incubators. Colonies were transferred to fresh leaves every 7 to 14 d.

Insecticides and chemicals. Acetone (American Chemical Society certified, 99% purity) was from Fisher Scientific (Pittsburg, PA, USA). Acetylthiocholine iodide (ATC), bicinchoninic acid solution, bovine serum albumin, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), triton X-100, were from Sigma (St. Louis, MO, USA). Commercial formulation of methomyl, Lannate®2.4 LV, was from DuPont (Wilmington, DE, USA). The technical insecticides, methomyl, pirimicarb, and thiodicarb with greater than 98% purity were also from Sigma (St. Louis, MO, USA). All other chemicals came from Sigma, (St. Louis, MO, USA) unless otherwise stated. Insecticidal stock solutions were prepared in acetone.

Aphid bioassay. Aphids were bioassayed by leaf dip method. Formulated test chemicals [commercial grades methomyl (Lannate®2.4 LV) and technical grade of pirimicarb] were serially diluted for at least six doses with a de-ionized water check. Each dose was replicated at least three times. Leaf disks, 100 mm in diameter, were cut from fresh leaves from greenhouse-grown tobacco plants (Flue-cured tobacco cultivar, 'K-326'), dipped for 5 s in the designated concentrations, air-dried, and placed on slightly moistened filter papers in labeled Petri dishes, with their lips coated with Fluon®. Ten healthy adults from the test colony were placed on each leaf disk with a camel's hair brush. Covers were placed on each Petri dish and secured with Parafilm®. Mortality was assessed 24 h after treatment. All bioassays were conducted in environmental chambers maintained at 21 ± 1°C, 60% RH, and 16:8 (L:D) h photophase. Abbott's formula (Abbott 1925) was used to correct for mortality. Tests were repeated at least once and the data were pooled for analysis. LC_{50} values were calculated by probit analysis using POLO PC (LeOra Software, Berkeley, CA).

Enzyme assay. AChE activity was measured according to the method developed by Ellman et al. (1961) and later modified by Zhu et al. (1996). Twenty aphids from each test

colony were homogenized in 200 µl of ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.3% (v/v) Triton X-100. Homogenates were centrifuged at 12,600 g for 15 min at 4°C, and the supernatants were transferred to new tubes. Ten µl of the supernatant were used to measure the residual AChE activity using a Dynex kinetic microplate reader (Magellan Biosciences, USA) at 405 nm immediately after 100 µl of the mixture of ATC and DTNB was added. The final concentrations of ATC and DTNB in the reaction mixture were 0.25 and 0.40 mM, respectively. Each colony was replicated at least three times for each run.

The specific activity of the enzyme (U) was calculated as:

$$U = \frac{(\text{mOD/min/1000}) \text{ X } 10^6 \text{ µM/min/ml X Dilution factor}}{\text{Extinction coefficient of DTNB X path length X protein concentration}}$$

Where the extinction coefficient of DTNB = $1.36 \text{ X } 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and the path length = 0.45 (200µl in a 86 X 128 mm well).

The concentration of total protein in each AChE preparation was determined using the method of Smith et al. (1985) with bovine serum albumin as a standard. The measurements were made using a Dynex microplate reader (Magellan Biosciences, USA) at 560 nm.

AChE inhibition experiments. AChE inhibition experiments were carried out on eight colonies with a range of AChE specific activity to determine the inhibitory potential of three carbamates (methomyl, pirimicarb, and thiodicarb) on enzyme activity. Crude aphid homogenates for AChE inhibition studies were prepared as described above. The experiments were conducted in the absence of a substrate (10 min inhibitor pre-incubation with enzyme solution) in 96-well microplates, as described by Stumpf et al. (2001). Five concentrations of each inhibitor were used for each colony tested along with a control and a blank. Each concentration was replicated at least three times, and each experiment was repeated twice. Progression of inhibition was monitored at 30 s intervals for 10 min. The percent residual activity was plotted against the elapsed time, and the line was regressed between the time to 50% inhibition extrapolated from the regression equation. Data were analyzed by non-linear regression analysis using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA) and IC_{50}

values (molar concentration of inhibitor needed to inhibit enzyme activity by 50%) were calculated.

Results and Discussion

The specific activities of AChE in the sample aphids are presented by year in Tables 5.1 through 5.3. The specific activity of the enzyme AChE among the 65 colonies screened ranged from 0.017 to 0.259 U/min/mg protein. The colonies were categorized into three groups based on the enzyme specific activity: (I) 0.017 to 0.06 U/min/mg protein, (II) 0.06 to 0.15 U/min/mg protein and (III) >0.15 U/min/mg of protein. Approximately, 63% of the colonies fell in the second category, and only 6% of the total colonies had activity higher than 0.15 U/min/mg protein.

Eight colonies, two colonies each from groups I and II and four colonies from group III were chosen to study the inhibition of AChE in the presence of two carbamate insecticides, methomyl and pirimicarb. With low values obtained for the activity of the target enzyme (AChE), the diagnostic dose of 100 µM of pirimicarb (Moores et al. 1994) that was recommended for identification of modified AChE-site was considered too high in this study. Therefore, I used the logarithmic inhibitor concentration on the enzyme activity, with concentrations ranging from 0.1 to 1,000 µM giving final concentrations of 0.01 to 100 µM of the inhibitor in their respective wells.

Six of eight colonies, all those from Groups I and II and two of the four colonies from group III tested against methomyl (Table 5.4) and pirimicarb (Table 5.5) had very low IC_{50} values. Lower IC_{50} values were obtained for pirimicarb (0.16 to 0.30 µM) than for methomyl (0.35 to 2.40 µM). The inhibition rates were similar for these six colonies irregardless of the difference in their enzyme specific activity (Table 5.4 and 5.5). The red morph from Peek, Washington County, VA had the lowest IC_{50} value (0.35 µM) for methomyl compared with the other colonies with values above 1 µM.

Two colonies (Windsor - red morph from tobacco and Clay's - green morph from *Bougainvillea*) had very high IC_{50} values for pirimicarb, with 40.5 and 98.6 µM respectively (Fig. 5.1 and 5.2). This marked difference was seen as a plateau at approximately 85% of the enzyme activity remaining. The enzyme activity in the presence of pirimicarb for these two

colonies was 130 and 330-fold higher compared with the highest IC_{50} value among the other six colonies (0.30 µM). Comparatively, methomyl inhibited the enzyme activity in these two colonies, with IC_{50} values of 1.21 and 0.99 µM respectively. This indicates that these two colonies have either a modified target site or higher catalytic center activity.

Elevated carboxylesterases have been described as a primary mechanism of resistance against organophosphates and carbamates in *M. persicae.* Measurement of carboxylesterase levels revealed similar activities among the colonies that were selected for inhibition assays (Table 5.6). Assuming that resistance is not conferred by higher esterase levels, rather than by AChE insensitivity in the two colonies that had higher IC_{50} values for pirimicarb. Methomyl showed stronger inhibition of the insensitive AChE that was not inhibited by pirimicarb. This would suggest possible negative cross-insensitivity within the carbamate group. This may be due to the structure-activity relationship between the target-site and the inhibitor.

Studies conducted to unfold resistance mechanisms in the English grain aphid, *S. avenae* have shown that populations with resistance to pirimicarb were susceptible to thiodicarb, which can be effectively used to screen the resistant populations *in vivo* (Chen et al. 2007). Though thiodicarb is considered a pro-insecticide that is converted to methomyl oxime derivatives by hydrolysis in rats (Huhtanen and Dorough 1976) and several insect and acari (Gayen and Knowles 1981, Gencsoylu et al. 1998), it could strengthen inhibition by carbamate insecticides with structural similarity to methomyl. This diagnostic method has been used *in vivo* analysis to separate colonies of lettuce aphids with differential sensitivity within the carbamate insecticides caused by mutations in the a*ce* genes (Rufingier et al. 1999). This structure-activity relationship could be used to differentiate the pirimicarb-resistant populations through *in vitro* studies. The two colonies, Windsor red and Clay's green, with higher IC_{50} values for pirimicarb were tested with thiodicarb along with two sensitive colonies, Dellenback, dark green and Peek, red morphs. The IC_{50} values of the enzyme inhibition showed a marked decrease in the enzyme activity in all four colonies (Table 5.6).

Generally, the colonies with lower IC_{50} values for methomyl and pirimicarb also had lower LC_{50} values in the leaf-dip bioassays (Table 5.4 and 5.5). Furthermore, there was a significant weak correlation ($r = 0.395$, $P = 0.038$) between AChE specific activity (in the absence of inhibitor) and the toxicity of commercial formulations of methomyl (Lannate®2.4 LV) determined in leaf-dip bioassays (Fig. 5.3). This shows that the difference in the catalytic

efficiency of the target-enzyme plays a role in conferring some resistance to carbamate insecticides.

In other aphid species, such as lettuce aphid, resistance to pirimicarb and propoxur results from decreased sensitivity of the target AChE (Rufingier et al. 1999). However, this decreased sensitivity of the enzyme did not confer resistance to methomyl, paraxon, and acephate, further validating the results in this study. Similarly, in the greenbug, *S. graminum* (Rond), increased activity was associated with reduced sensitivity of AChE in organophosphate-resistant stains (Zhu and Gao 1999).

Recent studies on AChE genes led to the cloning of two AChE genes from aphids (termed *ace1* and *ace2*) that have been identified as single point mutations in the aphid *ace1* genes and correlated with MACE phenotypes in *A. gossypii* (Li and Han 2002, 2004). In *M. persicae*, the MACE phenotype was correlated with an S431F mutation in the *ace2* gene (Nabeshima et al. 2003) which is orthologous to *A. gossypii ace1* gene. The colonies with high IC_{50} values towards pirimicarb in the present study may have an *ace2* gene mutation in the target protein, AChE, but further experimentation is needed for verification.

A recent concern has been the selection and spread of MACE aphids that carry a modified AChE, the target enzyme for OP and carbamate insecticides. This mechanism has been identified in green peach aphids and cotton aphids and confers strong resistance to dimethylcarbamates such as pirimicarb and triazamate (O'Brian et al. 1992, Moores et al. 1996, Silver et al. 1995, Han et al. 1998). These compounds retain good activity against aphids carrying the more common elevated esterase and kdr resistance mechanisms, so continued selection and spread of MACE clearly has serious implications for the control of these pests in many crops (Foster and Devonshire, 1999).

Baseline studies such as these are needed to determine the development and diversity of the insecticide resistance in the tobacco-adapted forms of *Myzus* in Virginia and adjacent states. Such studies will assist in determining the levels and types of resistance being developed. This information will help scientists improve strategies for preserving the effectiveness and increasing the performance of insecticides currently used for TGPA control on tobacco, and finding alternative cultural and natural controls. Early detection of resistance will allow resistance management plans to be implemented, thereby, delaying further development of insecticide

resistance and protecting the most valuable groups of insecticides. Further studies on the molecular mechanisms of the AChE target site protein would help validate these results.

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Table 5.1. AChE specific activity in the tobacco-adapted form of the green peach aphid, *Myzus persicae* collected from various locations in Virginia and North Carolina, 2004 & 2005.

S.No	Colony	County	State	Year	Color	S Activity (U/min/mg protein)[a] ± SEM
1	Bledsoe	Nottoway	VA	2004	Red	0.031 ± 0.01
2	Wyatt	Pittsylvania	VA	2005	Red	0.053 ± 0.04
3	SPAREC	Nottoway	VA	2004	Red	0.056 ± 0.02
4	Hatchet	Franklin	VA	2004	Red	0.064 ± 0.03
5	NC State	Wake	NC	2005	Red	0.066 ± 0.05
6	SPAREC (C-till)	Nottoway	VA	2005	Green	0.066 ± 0.04
7	SWAREC	Washington	VA	2004	Red	0.068 ± 0.03
8	Adkinson	Pittsylvania	VA	2004	Green	0.072 ± 0.02
9	Keates	Franklin	VA	2004	Red	0.091 ± 0.01
10	SPAREC (Sys)	Nottoway	VA	2005	Green	0.102 ± 0.01
11	Howard	Lunenburg	VA	2005	Red	0.102 ± 0.03
12	Witcher	Franklin	VA	2005	Green	0.104 ± 0.04
13	Clayton	Johnston	NC	2005	Green	0.107 ± 0.03
14	Bass	Campbell	VA	2005	Green	0.119 ± 0.02
15	Peek	Washington	VA	2004	Red	0.173 ± 0.01

[a]U = Enzyme unit (Conversion of 1 µM substrate - DTNB in 1 minute)

Table 5.2. AChE specific activity in the tobacco-adapted form of the green peach aphid, *Myzus persicae* collected from various locations in the eastern United States, 2006.

S.No	Colony	County	State	Color	S Activity (U/min/mg protein) [a] ± SEM
1	Dudley	Wayne	NC	Green	0.017 ± 0.02
2	Ayres	Patrick	VA	Red	0.018 ± 0.01
3	Barnard	Amelia	VA	Red	0.033 ± 0.03
4	Wallace	Dinwiddie	VA	Green	0.057 ± 0.01
5	NY-Riverhead	Suffolk	NY	Red	0.061 ± 0.01
6	R Moore	Lunenburg	VA	Green	0.063 ± 0.03
7	Turbeville	Halifax	VA	Red	0.068 ± 0.02
8	Glasscock	Prince Edward	VA	Red	0.068 ± 0.04
9	Pee Dee-AREC	Florence	SC	Green	0.076 ± 0.01
10	Darrel Johnson	Russell	VA	Red	0.078 ± 0.02
11	Pee Dee-AREC	Florence	SC	Red	0.080 ± 0.05
12	Bobby	Russell	VA	Red	0.080 ± 0.01
13	UGA	Grady	GA	Red	0.083 ± 0.01
14	Yanceyville	Caswell	NC	Red	0.095 ± 0.01
15	Green Bay	Prince Edward	VA	Red	0.096 ± 0.02
16	Glasscock	Prince Edward	VA	Green	0.107 ± 0.02
17	Mitchell	Franklin	VA	Red	0.109 ± 0.01
18	SPAREC (Reed)	Nottoway	VA	Green	0.110 ± 0.03
19	Semtner	Nottoway	VA	Green	0.116 ± 0.04
20	Barnard	Amelia	VA	Green	0.120 ± 0.05
21	Washburn	Mecklenburg	VA	Red	0.122 ± 0.03
22	Patrick Henry	Charlotte	VA	Green	0.133 ± 0.02
23	Cross Creek	Cumberland	NC	Green	0.137 ± 0.01
24	D Moore	Pittsylvania	VA	Red	0.141 ± 0.03
25	SC-AREC	Horry	SC	Green	0.143 ± 0.04
26	R Moore	Lunenburg	VA	Red	0.147 ± 0.02
27	Angel	Franklin	VA	Red	0.162 ± 0.01
28	Johnson	Surry	NC	Green	0.186 ± 0.01
29	Windsor	Hartford	CT	Red	0.259 ± 0.04

[a] U = Enzyme unit (Conversion of 1 μM substrate - DTNB in 1 minute)

Table 5.3. AChE specific activity in the tobacco-adapted form of the green peach aphid, *Myzus persicae*, collected from various locations in the eastern United States, 2007.

S.No	Colony	County	State	Color	S Activity (U/min/mg protein) [a] ± SEM
1	Witcher	Franklin	VA	D Green	0.030 ± 0.02
2	NC State	Wake	NC	Red	0.047 ± 0.03
3	Bowen	Tift	GA	Red	0.051 ± 0.02
4	Johnson	Darlington	SC	Red	0.051 ± 0.01
5	UK-AREC	Caldwell	KY	Green	0.062 ± 0.02
6	KY-AREC	Lexington	KY	Red	0.071 ± 0.02
7	TN-AREC	Loudon	TN	Red	0.076 ± 0.01
8	Highland-RIM	Robertson	TN	Red	0.078 ± 0.02
9	Clayton	Johnston	NC	Green	0.085 ± 0.03
10	Hutchinson	Halifax	VA	Green	0.087 ± 0.02
11	UGA	Grady	GA	Orange	0.092 ± 0.01
12	Hale	Macon	TN	Red	0.094 ± 0.02
13	Dellenback	Patrick	VA	D Green	0.099 ± 0.03
14	Johnson	Colleton	SC	Green	0.100 ± 0.04
15	Clayton	Johnston	NC	Red	0.109 ± 0.01
16	Johnson	Colleton	SC	Red	0.114 ± 0.01
17	Hite	Lunenburg	VA	Orange	0.123 ± 0.03
18	NC State	Wake	NC	Green	0.125 ± 0.02
19	Dellenback	Patrick	VA	Orange	0.149 ± 0.01
20	Ayres	Patrick	VA	Red	0.161 ± 0.03
21	Clay's	Nottoway	VA	Green	0.208 ± 0.02

[a]U = Enzyme unit (Conversion of 1 μM substrate - DTNB in 1 minute)

Table 5.4. Toxicity and inhibition of AChE in selected colonies of tobacco-adapted form of the green peach aphid, *Myzus persicae*, with methomyl.

S. No.	Colony	County	State	Year	Color	AChE SA [a †]	IC_{50} [b]	LC_{50} (ppm)	95% FL	Slope
1	Bledsoe	Nottoway	VA	2004	Red	0.031	1.78	298.6	242.8 - 324.6	1.64
2	Peek	Washington	VA	2004	Red	0.173	0.35	244.3	206.4 - 316.8	1.86
3	R Moore	Lunenburg	VA	2006	Green	0.063	1.96	286.2	242.8 - 318.2	1.58
4	Angel	Franklin	VA	2006	Red	0.162	1.95	324.6	286.2 - 341.3	1.42
5	Windsor	Hartford	CT	2006	Red	0.259	1.21	331.5	316.8 - 364.4	1.28
6	Highland-RIM	Robertson	TN	2007	Red	0.078	2.40	341.2	322.8 - 386.4	1.52
7	Dellenback	Patrick	VA	2007	D Green	0.099	1.07	242.6	192.8 - 284.1	1.72
8	Clay's	Nottoway	VA	2007	Green	0.208	0.99	335.5	302.8 - 366.4	1.40

[a] Acetylcholinesterase (AChE) Specific Activity
[b] Inhibitor concentration to reduce the AChE enzyme activity by 50%

Table 5.5. Toxicity and inhibition of AChE in selected colonies of the tobacco-adapted form of the green peach aphid, *Myzus persicae*, with pirimicarb.

S. No.	Colony	County	State	Year	Color	AChE SA[a]	IC_{50}[b]	LC_{50} (ppm)	95% FL	Slope
1	Bledsoe	Nottoway	VA	2004	Red	0.031	0.16	196.2	172.6 - 246.8	1.45
2	Peek	Washington	VA	2004	Red	0.173	0.30	180.4	146.4 - 216.2	1.41
3	R Moore	Lunenburg	VA	2006	Green	0.063	0.24	172.2	144.8 - 202.4	1.72
4	Angel	Franklin	VA	2006	Red	0.162	0.28	168.2	122.6 - 216.8	1.32
5	Windsor	Hartford	CT	2006	Red	0.259	40.45	356.3	313.5 - 394.3	1.24
6	Highland-RIM	Robertson	TN	2007	Red	0.078	0.24	186.4	104.5 - 276.2	1.10
7	Dellenback	Patrick	VA	2007	D Green	0.099	0.13	174.2	142.4 - 224.3	1.34
8	Clay's	Nottoway	VA	2007	Green	0.208	98.64	452.3	302.4 - 755.3	0.89

[a] Acetylcholinesterase (AChE) Specific Activity
[b] Inhibitor concentration to reduce the AChE enzyme activity by 50%

Table 5.6. AChE inhibition with thiodicarb and carboxylesterase activity in selected colonies of the tobacco-adapted form of the green peach aphid, *Myzus persicae.*

S. No.	Colony	County	State	Year	Color	AChE SA[a]	IC_{50}[b]	Esterase[c]
1	Bledsoe	Nottoway	VA	2004	Red	0.031	-	78.1
2	Peek	Washington	VA	2004	Red	0.173	4.67	64.6
3	R Moore	Lunenburg	VA	2006	Green	0.063	-	68.7
4	Angel	Franklin	VA	2006	Red	0.162	-	95.8
5	Windsor	Hartford	CT	2006	Red	0.259	5.80	81.9
6	Highland-RIM	Robertson	TN	2007	Red	0.078	-	87.3
7	Dellenback	Patrick	VA	2007	D Green	0.099	5.10	98.6
8	Clay's	Nottoway	VA	2007	Green	0.208	4.61	110.8

[a] Acetylcholinesterase (AChE) Specific Activity
[b] Inhibitor concentration to reduce the AChE enzyme activity by 50%
[c] Esterase activity in nmol/min/mg protein

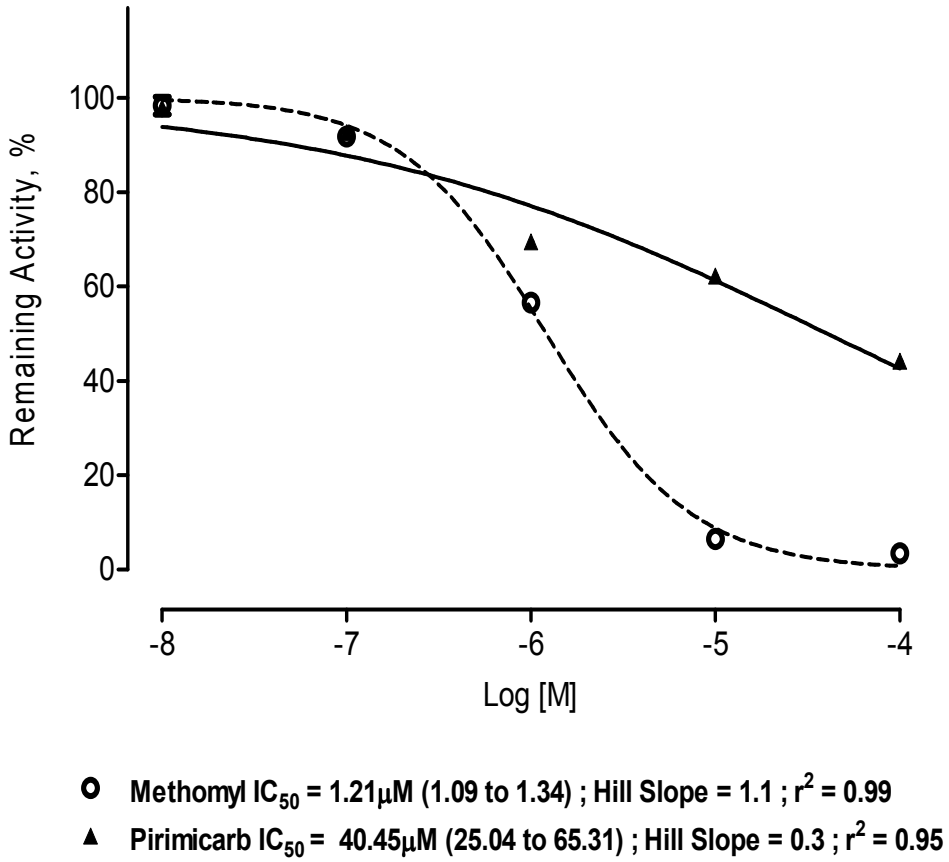

Fig. 5.1. Inhibition of AChE activity by two carbamate insecticides, methomyl and pirimicarb in the red color morph of the tobacco-adapted form of the green peach aphid, *Myzus persicae* from tobacco, Windsor, CT, 2006.

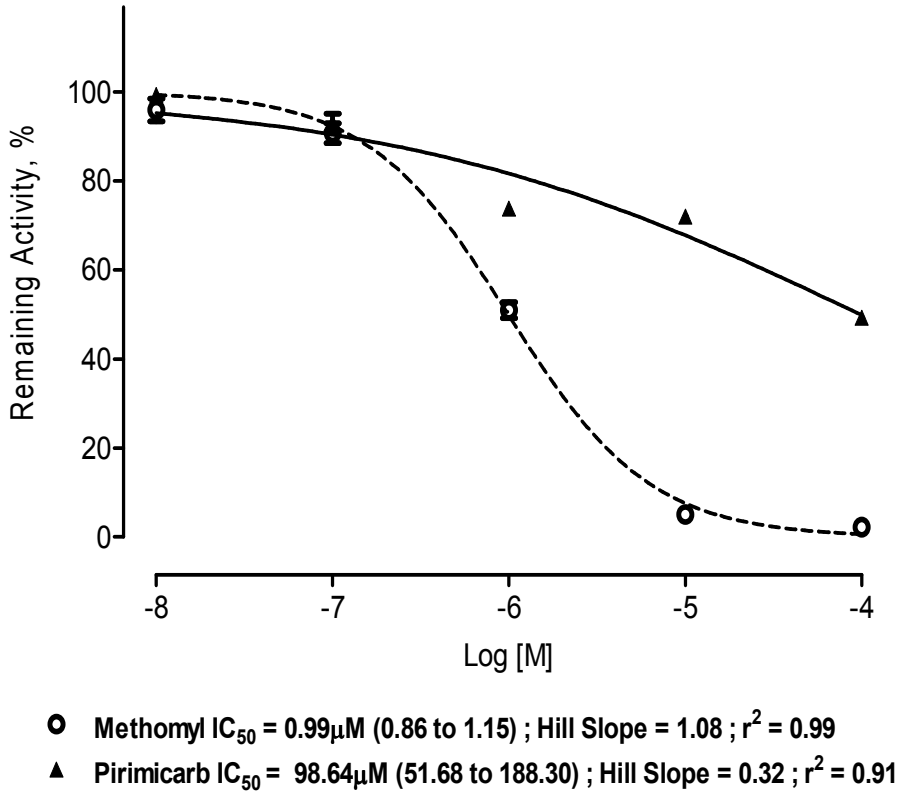

Fig. 5.2. Inhibition of AChE activity by two carbamate insecticides, methomyl and pirimicarb, in green color morph of *Myzus persicae* collected from *Bougainvillea*, Clay's Garden Center, Blackstone, VA, 2007.

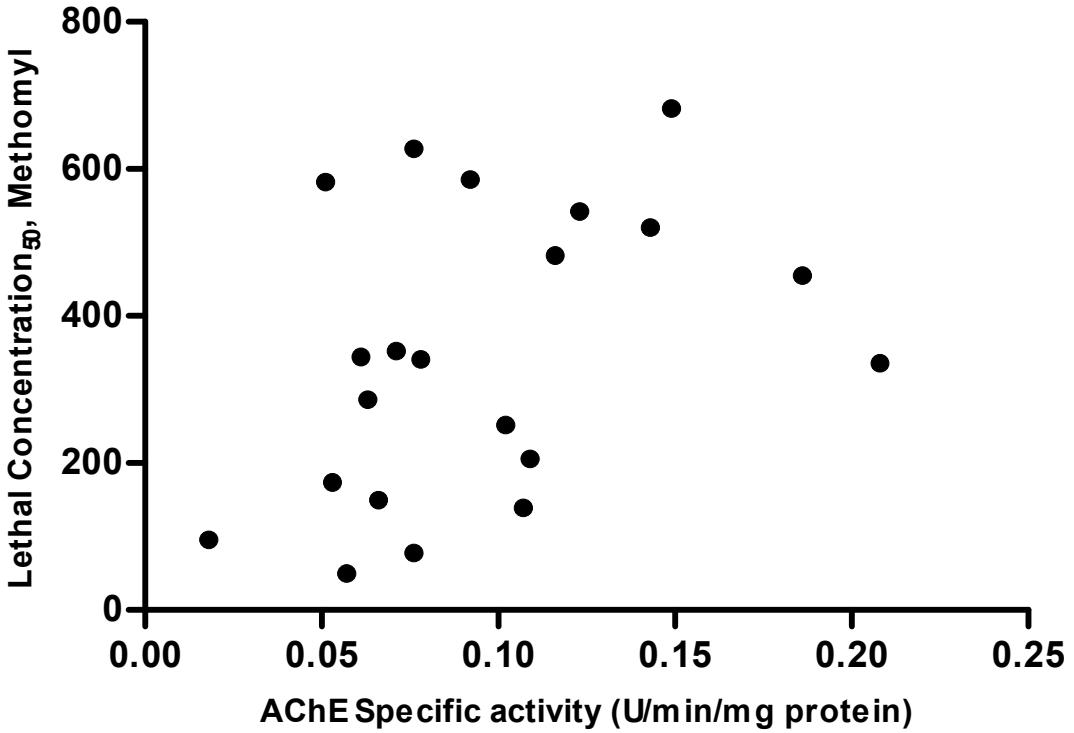

Fig. 5.3. Correlation of methomyl mortality of the tobacco-adapted form of the green peach aphid, *Myzus persicae* colonies in leaf-dip bioassays to AChE specific activity (Combined for all color morphs – 21 colonies; $r = 0.395$, $P = 0.038$).

Chapter 6

Glutathione *S*- transferase activity in the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

Abstract

The tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer), is an important insect pest on tobacco in the United States and around the world. Insecticides play a substantial role in keeping this pest under control. Glutathione *S*- transferases (GSTs), are the isoenzymes that are involved in the metabolism and detoxification of a wide variety of endogenous and xenobiotic compounds. These isozymes, which could potentially confer insecticide resistance, were quantified by CDNB conjugation. GST activity was measured in the three color morphs, in 100 TGPA colonies collected in nine different states in the eastern United States in the yr 2004 through 2007. The results revealed a wide range of activities among red morphs, which ranged from 7.9 to 342.8 pmol/min/mg protein with a mean of 176.7 ± 11.3 pmol/min/mg protein; green morphs, 15.3 to 329.9 pmol/min/mg protein with a mean of 188.4 ± 10.9 pmol/min/mg protein. About 45% of the total red morphs had activity in the range of 200-300 pmol/min/mg of protein, while 53% of the total green morphs were in the range of 100-200 pmol/min/mg protein. The six orange morphs collected on tobacco farms in 2007 had a narrow range of activity falling between 160 to 200 pmol/min/mg protein. Impact of GST activities on the resistance management of the tobacco-adapted form of *M. persicae* is discussed.

Introduction

Glutathione *S*- transferases (GSTs) are a family of isoenzymes involved in the metabolism of a wide variety of electrophilic substrates found in several in aerobic organisms (Hodgson and Levi 1994, Wilce and Parker 1994). They play a central role in detoxification of both endogenous and xenobiotic compounds and are involved in intracellular transport, biosynthesis of hormones, and protection against oxidative stress (Enayati et al. 2005, Hayes et al. 2005, Francis et al. 2005). They catalyze conjugations by facilitating the nucleophilic attack of the sulfhydryl group of endogenous reduced glutathione (GSH) on electrophilic centers of a

vast range of xenobiotic compounds, including insecticides and acaricides (Konanz and Nauen 2004). Thus the xenobiotics have increased solubility and are excreted from the insect system by the formation of mercapturic acid derivatives (Habig et al. 1974).

GSTs play a major role as a detoxification mechanism for insecticides, thus contributing to insecticide resistance in economically important pest species in diverse agronomic cropping systems as well as disease vectors such as mosquitoes (Clark 1989, Huang et al. 1998, Vontas et al. 2001, Yu 2002). GSTs are also involved in the detoxification of several chemical classes of insecticides, i.e., organophosphates, pyrethroids, carbamates, and chlorinated hydrocarbons (Ranson et al. 1997, Sun et al. 2001).

The green peach aphid complex colonizes over 500 species of plants from at least 40 different families (Blackman & Eastop 2000). It is a pest of many crops including potatoes, peppers, sugar beets, peaches, bedding plants, and it transmits several important plant viruses (Van Emden et al. 1969). There are many host-adapted forms of the green peach aphid with genetic variation in host plant adaptation among clones (Weber 1985, Edwards 2001). The tobacco-feeding form of *Myzus persicae* (Sulzer) (TGPA) is one of the most important insect pests of tobacco (Semtner et al. 1993). Aphids deposit honeydew on the tobacco leaves and a dark, sooty mold often grows on the honeydew. The combination of aphid feeding damage, sooty mold and honeydew interferes with curing and reduces the leaf quality. Injury attributed to the TGPA reduces the value of untreated tobacco by five to 30% annually (Reed & Semtner 1992). Our recent research indicates that some TGPA are tolerant to the insecticides most commonly used for aphid control on tobacco.

As GSTs play a major role in metabolizing many insecticidal compounds used for controlling the TGPA. This study was conducted to learn more about isoenzyme activities and to ascertain whether they influence insecticide resistance in the aphid. GST activity was determined in the TGPA collected from nine tobacco-producing states across the eastern United States from 2004 to 2007.

Materials and Methods

Chemicals. Acetone (American Chemical Society certified, 99% purity), anhydrous mono- and di-basic sodium phosphate and Triton X-100 were purchased from Fisher Scientific, Pittsburg, PA. Glutathione (reduced), 1-chloro-2, 4-dinitrobenzene (CDNB), bicinchoninic acid and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, St. Louis, MO. NUNC flat bottom 96-well microplates were purchased from Fisher Scientific, USA.

Field sampling. *M. persicae* were collected on leaf samples from tobacco, *Nicotiana tabacum* L. (Family Solanaceae), fields across nine different states over a 4-yr period (2004 - 2007). Information about their body color under field conditions was recorded for all locations. Most of the colonies were collected personally with the help of agricultural extension agents. Collaborators in other states (Georgia, South Carolina, North Carolina, Tennessee, Maryland, New York and Kentucky) collected aphids on tobacco leaves by removing whole or pieces of infested leaves from the plants. The infested leaves were placed in Ziplock® plastic bags containing a piece of paper towel to reduce moisture and sent overnight (FedEx®) to entomology laboratory in Styrofoam® or corrugated containers with ice packs. Where possible, both red and green color morphs of the aphid were collected at each location. All aphids were collected from tobacco, except the colonies from Riverhead, NY red morph, 2006 (*Calibrachoa parviflora* Juss. - Family Solanaceae), Semtner (red and green) 2007 (*Brassica napa* L. 'Purple Top'- Family Brassicaceae), and Clay's green morph, 2007 (*Bougainvillea spectabilis* Willd - Family Nyctaginaceae). All colonies were maintained on insecticide-free excised tobacco leaves (Flue-cured cultivar, 'K 326'). The petioles were inserted into agar medium in Styrofoam® cups and kept at 21 ± 1°C, 60% RH and 16:8 (L:D) h photoperiod in laboratory incubators until the bioassays were performed. The leaves in the Styrofoam® cups remained healthy for almost 10 d.

Greenhouse plants and aphid colony maintenance. As there was heavy use of tobacco leaves both for maintaining colonies in the laboratory and conducting bioassays, a continuous supply of healthy leaves was achieved by maintaining at least 100 to 200 plants in the greenhouse at a time. Plantings of tobacco were rotated among two or three greenhouses to manage natural infestations of aphids and whiteflies. Tobacco flue-cured cultivar, 'K-326' was seeded in Styrofoam® seeding trays (288 cells) filled with Carolina Tobacco Mix® (a soilless greenhouse growing medium). The trays were placed in float bays filled with water and

fertilized based on recommendations for tobacco transplant production (Reed 1998). Three to four-week-old seedlings were transplanted from the trays, into 15 cm diameter pots filled with growing medium (Carolina Tobacco Mix®), and grown hydroponically in bays of water 6 to 8 cm deep, and fertilized with Peters® 20-20-20 fertilizer. When plants began to flower they were discarded and replaced by a new planting. Plants were discarded at this time to reduce the potential for aphid and whitefly infestations and because the plants become sticky and undesirable for aphids.

Microplate assay for determining GST activity. Batches of 20-25 adult apterous aphids from each colony were homogenized in 200 µl ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.3% (v/v) Triton X-100 using multiple homogenizer (Burkard Scientific, Middx, UK). Homogenates were transferred to a micro-centrifuge tube, and centrifuged at 15,000g for 15 min at 4^{o}C. The supernatants were transferred to a new centrifuge tube, labeled, and used as enzyme sources.

GST activity was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) and reduced GSH as substrates (Habig et al. 1974) with modifications (Zhu et al. 2000, Rauch and Nauen 2004). The total reaction volume per well of a 96-well microplate was 300 µl, consisting of 50 µl enzyme preparation (equivalent to five aphids). CDNB (containing 1% (v/v) ethanol) and GSH in buffer, giving final concentrations of 0.4 mM and 4 mM of CDNB and GSH were added to each well respectively. The non-enzymatic reaction of CDNB with GSH measured without homogenate served as the control. The change in absorbance was measured continuously for 5 min at 340 nm and 25^{o}C in 15 s intervals using a monochromatic kinetic microplate reader (TRIAD multimode detector, Dynex Technologies, USA). Changes in absorbance per minute were converted into pmol CDNB conjugated/min/mg protein using the extinction coefficient of the resulting 2,4-dinitrophenyl-glutatione: e340nm = 8.6 mM^{-1} cm^{-1} (Habig et al. 1974). A total of 100 colonies were screened with each colony replicated internally at least three times and each experiment repeated at least twice.

Protein determination. Protein contents of the enzyme preparations of each colony were standardized according to Smith et al. (1985) using bovine serum albumin (BSA) as the standard. The measurement was performed using 20 µl of the enzyme preparation (as described above) and incubating it with 180 µl of bicinchoninic acid in 4% cupric sulphate solution (Sigma Aldrich, St. Louis, MO). The formation BCA/Cu^{+} complex with the protein after 30 min

incubation period was measured at 595 nm using microplate reader (TRIAD multimode detector, Dynex Technologies, USA). Protein content was calculated based on the optical density value obtained from BSA (Sigma Aldrich, St. Louis, MO) standard curve.

GST specific activity was calculated as:

$$\text{GST activity} = \frac{\Delta \text{ Absorbance / minute}}{8.6 \text{ mM/cm X 1 cm}} \text{ X } 10^6 = \text{pmol/min/ml}$$

$$\text{Specific GST activity} = \frac{\text{pmol/min/ml}}{\text{mg protein/ml}} = \text{pmol/min/mg protein}$$

Statistical Analysis. The results of the GST specific activities in the two color morphs were analyzed using one way ANOVA followed by a *post hoc* Tukey's multiple comparison test. Correlation analyses were performed to look at the relationship between the LC_{50} values obtained for acephate (Orthene 97), methomyl (Lannate 90SP), and imidacloprid (Admire 2F) (SAS Institute, 2001). The level of significance for all statistical analyses was chosen *a priori* to be $p \leq 0.05$.

Results and Discussion

Results of the glutathione S-transferase activity determined by CDNB conjugation in 100 TGPA colonies collected in nine different states across eastern United States are presented by yr in Appendices 12-15. The results revealed a wide range in GST levels among both the red and green morphs (Fig. 6.1). GST activity in the red morphs ranged from 7.9 to 342.8 pmol/min/mg protein and the green morphs ranged from 15.3 to 329.9 pmol/min mg protein. The frequency distribution of the activity by color morph indicates that the majority of both the red and green morphs had activities that ranged between 100 to 300 pmol/min/mg of protein (Fig. 6.2). About 45% of the red morphs had activity in the range of 200-300 pmol/min/mg of protein, while 53% of the green morphs were in the range of 100-200 pmol/min/mg protein (Fig. 6.2).

An orange colored morph was collected on tobacco farms from across south-central Virginia in 2007. Seven orange morphs were collected including one from the Southern Piedmont AREC, Blackstone, VA late in the season (September). Of the seven orange morphs, five were collected on various farms in Virginia and one each from Maryland and Georgia in 2007. All orange morphs had GST activity ranging from 160 to 211 pmol/min/mg protein, with almost 86% of them falling in a very narrow range of 160 to 200 pmol/min/mg protein. Other than the red morphs from 2005 and 2006 (Fig. 6.3), significant differences between the means of GST activity of the colonies were not detected by Tukey's test ($P<0.05$).

The relationship between GST specifica activities and toxicity values (LC_{50}) obtained from the leaf-dip bioassays (data not shown), using a carbamate, methomyl (r = 0.2497; P = 0.2392), an organophosphate, acephate (r = 0.327; P = 0.1984); and the neonicotinoid, imidacloprid (r = 0.1987; P = 0.4321) were not significantly correlated.

GST-based DDT resistance is common in several anopheline mosquito species, reflecting the heavy use of this insecticide for malaria control over several decades (Hemmingway and Ranson 2000). These specific isozymes also have a secondary role in organophosphate resistance (Hemmingway et al. 1985). GST specific activity in the whiteflies, *Bemisia tabaci* (Gennadius) was shown to be around 400 nmol/min/mg protein in the laboratory susceptible colony, which did not differ significantly with the resistant strains (Rauch and Nauen 2004). The GST specific activities in organophosphate resistant and susceptible greenbugs, *Schizaphis graminum* (Rondani), is about 260 nmol/min/mg protein (Zhu et al. 2000). One other substrate commonly used in measuring GST activity, 3,4-dichloronitrobenzene (DCNB) was also tested along with CDNB in preliminary studies had no detectable activity in the TGPA tested, the same response observed in greenbugs (Zhu et al. 2000). This leads to the hypothesis that the colonies with higher GST activities to CDNB conjugation might contain an over expressed genetic mechanism with higher substrate specificity. Such an over-expression could be the consequence of constant selection pressure with insecticides.

The *Drosophila melanogaster* delta class GST (GST-2) has a major antioxidant role due to its conjugating activity towards lipid peroxidation products (Agianian et al. 2003). However, Vontas et al. (2002) recently described that a GST purified from insecticide resistant *Nilaparvata lugens* (Stal) had a role in resistance through the conjugation of lipid peroxidation products induced by pyrethroids (a class of neurotoxic insecticides interfering with voltage-gated sodium

channels). Further analysis of the aphid enzyme with regard to its peroxidase activity is necessary in order to clarify its metabolic role. Further studies with the aid of molecular tools like quantitative PCR could specify if the GST is over-expressed in some aphid colonies resistant to different classes of insecticides. In addition, GST has been shown to be a biochemical marker possibly linked to the resistance -associated degradation of abamectin one of the most important commercial acaricides in a strain of *Tetranychus urticae* (Koch) from the Netherlands (Stunpf and Nauen 2002).

Many of the insect species studied previously involved purification of the GST isozymes. With a wide range of GST specific activity in the TGPA colonies, it would be interesting to look at the kinetic parameters of the purified enzyme and subsequent inhibition by GST inhibitors. The orange morphs that had a narrow range of activity need to be studied more extensively to characterize the underlying molecular mechanisms for the activity.

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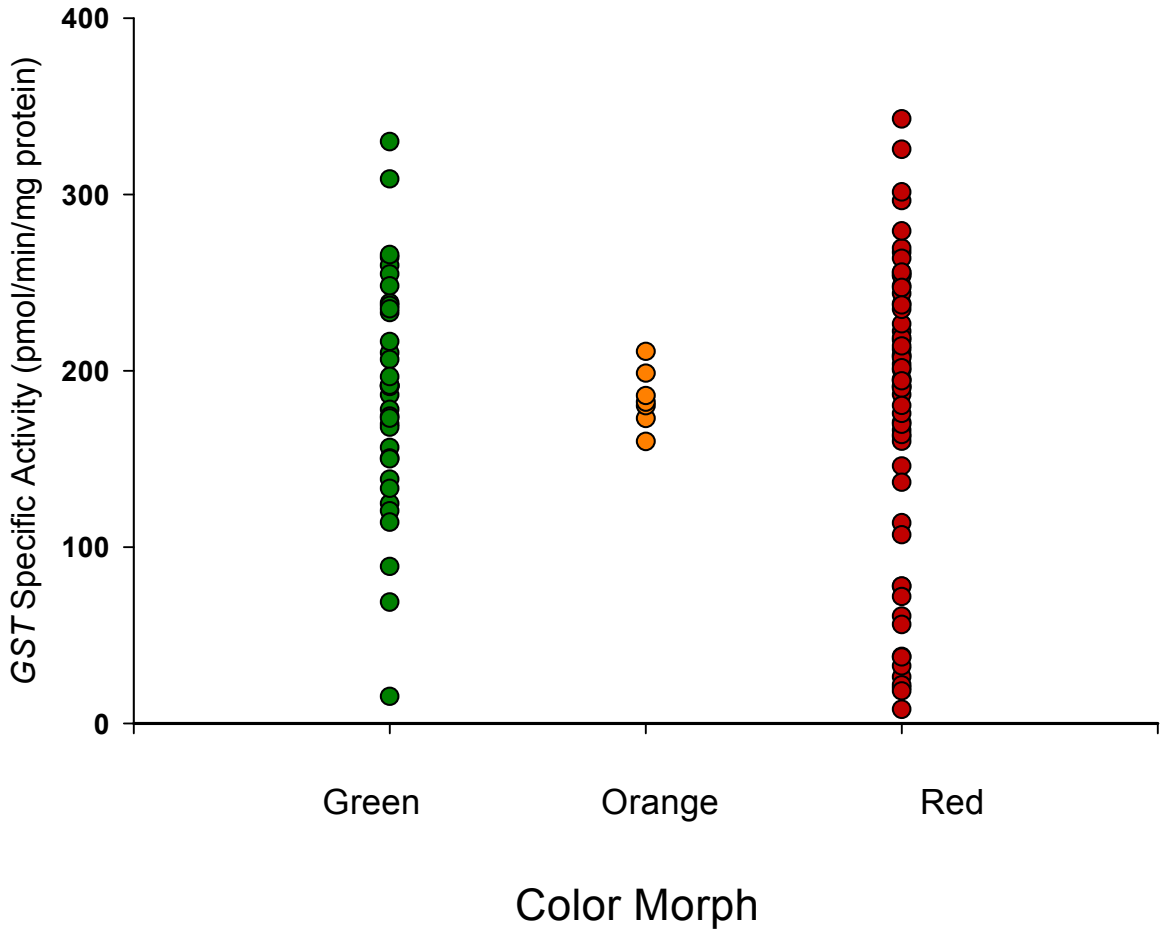

Fig. 6.1. Range of glutathione *S*-transferase activities in the tobacco-adapted form of the green peach aphid colonies collected from eight tobacco growing states across eastern United States (2004 to 2007). The colonies represent those provided in Appendix 8.

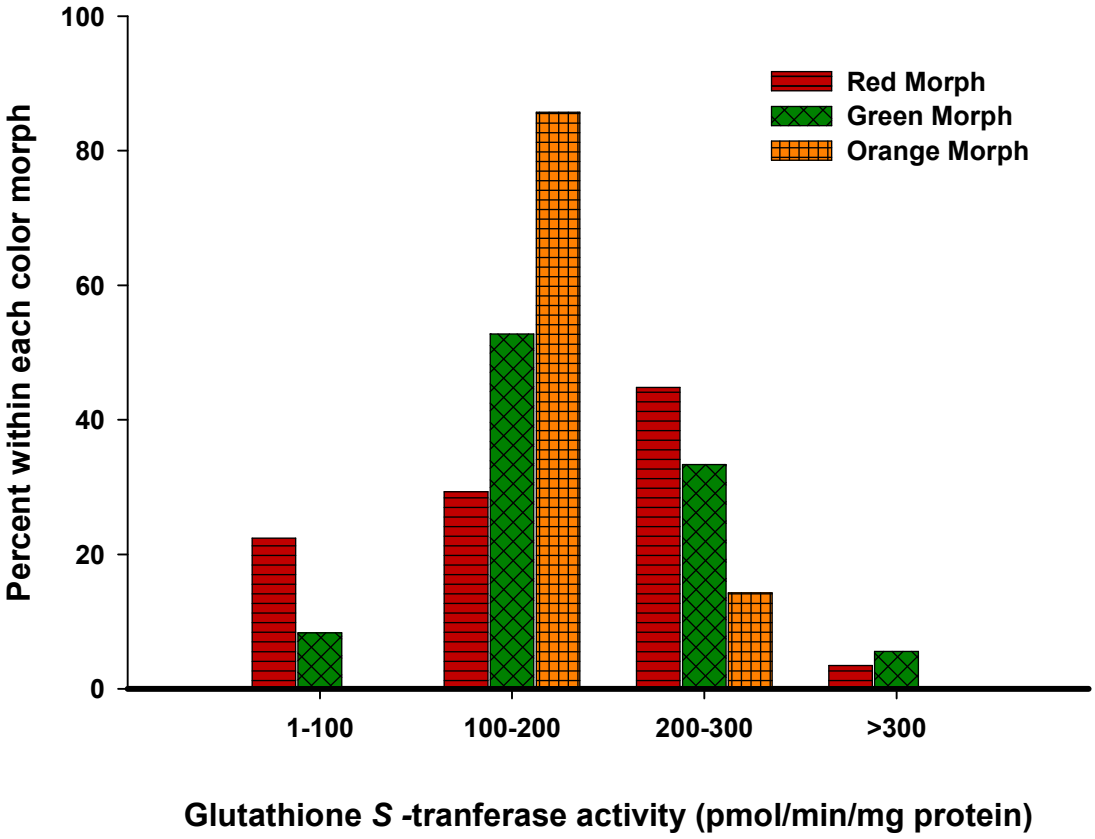

Fig. 6.2. Frequency distribution of glutathione *S*-transferase activity in the three color morphs of the tobacco-adapted form of the green peach aphid as determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Activity is presented according to the color morph in the 100 aphid colonies screened.

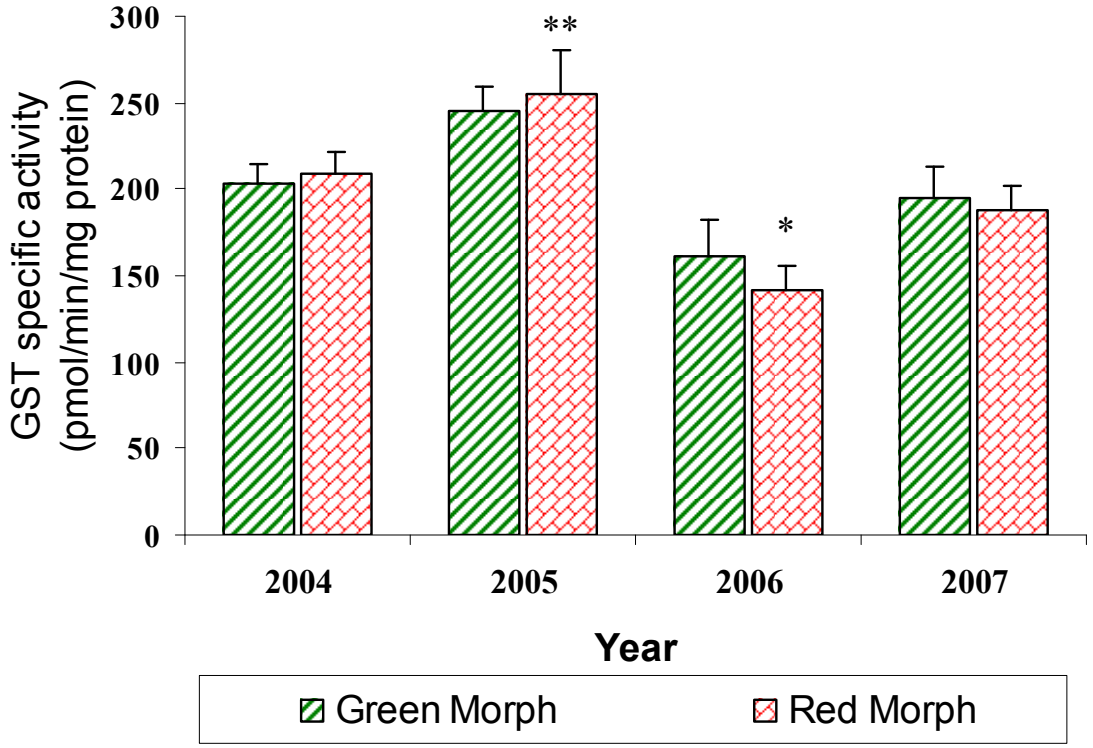

Fig. 6.3. Glutathione *S* – tranferase activity in two color morphs in the tobacco-adapted form of the green peach aphid, *Myzus persicae*. Data presented are means ± SE for the colonies mentioned in Appendix 8. Symbol (*) indicates the means are significantly different from each other [Tukey's multiple comparison (α=0.05)].

Chapter 7

Influence of post-exposure temperature on the toxicity of insecticides to the tobacco- adapted form of the green-peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

Abstract

Abiotic factors such as temperature play important roles in the activity and performance of insecticidal compounds, as they influence biochemical reactions that may either enhance or limit the effectiveness of an insecticide. The influence of these temperature-mediated synergisms on the toxicity of insecticides in red and green color morphs of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer), were evaluated using leaf-dip bioassay procedures in laboratory incubators. Post-exposure temperatures of 15, 20, and 25^{o}C were evaluated for four classes of insecticides, organophosphate (acephate), carbamate (methomyl), pyrethroid (lambda-cyhalothrin), and neonicotinoid (imidacloprid). Except for lambda-cyhalothrin, all the insecticides had positive temperature coefficients for *M. persicae* at both 5- (15-20 and 20-25^{o}C) and 10^{o}C (15-25^{o}C) temperature ranges. The post-exposure temperature had the same effect on insecticide toxicity to both color morphs. The temperature change from 15 to 20^{o}C resulted in almost a 3-fold increase in toxicity for methomyl, acephate, and imidacloprid in both color morphs. In contrast, the toxicity of lambda-cyhalothrin decreased as the temperature increased, showing a negative temperature coefficient. Since insecticide resistance monitoring is typically conducted in the laboratory bioassays, standardized temperatures are necessary for diagnosing problems and making recommendations for resistance management programs.

Introduction

Abiotic factors play important roles in the activity and performance of insecticidal compounds used in integrated pest management programs. Temperature influences the rate of biochemical reactions, the resultant arthropod activity, and may either enhance or limit the effectiveness of an insecticide (Horn 1998). Temperature catalyzes many reactions in biological systems that lead to a wide array of outcomes. It affects both physical and chemical properties of

insecticides such as stability, vaporization, penetration, activity, degradation, uptake, and translocation (Johnson 1990). These temperature-mediated synergisms play an important role, owing to the complexities that each class of insecticides exhibits. Extreme temperatures are common during the growing season when insecticide applications may be necessary to keep insect pest populations below economic threshold levels. Therefore, the application of insecticides at very high or low temperatures can drastically affect their performance under field conditions. Variations in temperature and other abiotic factors can also affect bioassays for monitoring insecticide resistance in the laboratory.

The green peach aphid, *Myzus persicae* (Sulzer) is a highly polyphagous species, colonizing over 500 species of secondary host plants from at least 40 different families (Blackman and Eastop 2000) with several genetically different host-adapted clones (Van Emden et al. 1969, Weber 1985, 1986, Edwards 2001). The tobacco-adapted form of the green peach aphid (TGPA), *M. persicae,* is an important pest of tobacco. High populations of TGPA can reduce tobacco yield by 5-25% depending on the growing conditions and the level of control (Reed and Semtner 1992). Honeydew produced by the TGPA accumulates on the tobacco leaves and a dark, sooty mold often grows on the honeydew (Mistric and Clark 1979). The combination of aphid feeding damage, sooty mold, and honeydew interferes with curing, reduces leaf quality, and often remains on tobacco even after aphids are controlled.

TGPA control depends extensively on insecticide applications and, in Virginia; control expenditures often exceed $500,000 annually (Semtner, personal communication). Presently, there are four important classes (organophosphate, carbamate, pyrethroid, and neonicotinoid) of insecticides that are registered for TGPA control on tobacco. To express the difference in the toxicity of insecticides with variation in temperature, temperature coefficients are often used (Gordon, 2005). Temperature coefficient is the change in rate or activity of a process for a $1^{o}C$ change in temperature (IUPS, 2001). Organophosphate and carbamate insecticides generally have stable toxicities at all temperatures, but some studies have found slight positive or negative temperature coefficients to several insect species (McLeod 1991, Musser and Shelton 2005). Pyrethroid insecticides often have reduced efficacy at high temperatures (Horn 1998, Musser and Shelton 2005), although they have a positive temperature coefficient against some species (Valles et al. 1998). Imidacloprid, a neonicotinoid class of insecticide is extensively used to control TGPA, and the effect of temperature on the toxicity of this group of compounds has not

been studied extensively. As temperature sensitivity varies between insecticide classes and is sometimes pest and product-specific, there is a need for more information to allow those responsible for making pest management decisions to select the best product for the existing environmental conditions.

Limited information is available on the influence of temperature on the toxicity of insecticides to the TGPA (or *M. persicae* complex) even though this insect pest is primarily controlled with insecticides in many important field and vegetable crops. As insecticide resistance monitoring is primarily evaluated in laboratory bioassays, the effect of temperature on the toxicity of insecticides would play a critical role and should be considered in resistance management programs. As insecticides from the same class often possess similar temperature responses, this study examines the effects of post-treatment temperature on the efficacy of four types of insecticides against the TGPA.

Materials and Methods

Insects. Red and green morphs of TGPA were collected by removing infested tobacco leaves from untreated plants grown in the field at the Virginia Tech Southern Piedmont AREC, Blackstone, Virginia on 11 July 2007. The infested leaves were kept on moistened paper towels for 2 d to allow the colonies to adapt to laboratory conditions and to make sure that the colonies were free of disease. Healthy adults and last instar nymphs were then exposed to pesticides as described below.

Formulations and preparation. This experiment evaluated commercial formulations of four insecticides: acephate (Orthene 97, Valent USA), an organophosphate; lambda-cyhalothrin (Warrior, Syngenta, Greensboro, NC, USA), a pyrethroid; methomyl (Lannate LV, DuPont, Wilmington, DE, USA), a carbamate; and imidacloprid (Admire 2F, Bayer CropScience, Kansas City, MO), a neonicotinoid. Aphids were bioassayed by leaf dip method using water dispersions of each formulated insecticide. Leaf disks, 100 mm in diameter, were cut from fresh leaves (mid-stalk position) from greenhouse-grown tobacco plants (flue-cured cultivar, 'K-326'). Leaf disks were dipped for 5 s in the designated concentrations, air-dried, and placed on slightly moistened filter papers in labeled (15 X 100 mm) Petri dishes. The inside lips of the Petri dishes were coated with Fluon® to keep the aphids on the treated leaves and to prevent escape. A

camel's hair brush was used to place 10 healthy adults on each leaf disk. Covers were placed on each Petri dish and secured with Parafilm®. Ten aphids of each color morph were used per Petri dish for each dose and replicated five times.

Mortality was assessed 72 h after treatment with imidacloprid to over come the antifeedant effect (Nauen et al. 1998), and 24 h for the other insecticides. The aphids were touched (or brushed) lightly with the camel's-hair brush for 5 s. If an aphid did not move or only twitched slightly, it was considered dead. Six doses of each chemical were tested to cover the range of expected partial mortality of *M. persicae* at 21°C based on preliminary tests and each dose replicated four times. Leaf disks dipped in distilled water served as the untreated check. All bioassays were conducted in environmental chambers maintained at 15, 20, and 25°C, 60% RH, and 16:8 (L:D) h photophase. Three separate growth chambers were used for each temperature. To ensure that temperature effects were independent of the growth chamber used, separate runs were conducted to check the mortality using aphids on untreated leaf disks. Abbott's formula (Abbott 1925) was used to correct for mortality.

Statistical analysis. LC_{50} values were calculated by probit analysis (POLO PC, LeOra Software, Berkeley, CA). Temperature coefficients were calculated and the likelihood ratios, hypothesis of equality and parallelism (slopes and intercepts) were tested at $P < 0.05$. The difference in toxicity due to temperature with-in and between the color morphs for each insecticide class is discussed.

Results

Except for lambda-cyhalothrin, all the insecticides had positive temperature coefficients for *M. persicae* at both the 5 and 10°C temperature intervals (Table 7.1). Methomyl, with a temperature coefficient of +5.7 and +4.7 for red and green morphs, respectively, required 5.7 and 4.7 times less methomyl at 25°C than was needed to achieve equal control at 15°C. The temperature change from 15 to 20°C resulted in almost a 3-fold increase in toxicity for methomyl in both color morphs (Table 7.1). The difference in the toxicity over the temperature range was tested for the hypothesis of parallelism and equality (slopes and intercepts) was not significant ($P>0.05$) between the color morphs or for the three temperatures. This shows that temperature

had greater effects on toxicity than the color morphs and the responses to temperature were consistent for both color morphs.

A similar trend was observed for acephate, with temperature coefficients of +4.8 and +2.9 for the red and green morphs, respectively (Table 7.1). Except at 20^{o}C, temperatures were not significantly different between the color morphs, as indicated by overlapping 95% confidence intervals. The statistical test performed to check this difference revealed that the slopes are parallel ($p > 0.05$) with different intercepts ($P<0.05$). The differential toxicity was evident with the non-overlapping confidence intervals at 15 and 25^{o}C within and between color morphs at those temperatures respectively.

Imidacloprid on the other hand, had a steady increase in toxicity with the increase in post exposure temperature (Table 7.1). The toxicity is seen as temperature coefficients at the 10^{o}C interval of +3.4 and +6.0 fold for red and green morphs respectively. At 15^{o}C both red and green morphs had higher LC_{50} values, which were significantly different from the toxicity values seen at 20 and 25^{o}C, respectively (likelihood ratio, $P<0.05$). The 95% confidence interval overlap of the toxicity values at 20^{o}C between the color morphs is seen as the difference in their likelihood ratios (hypothesis of parallelism not rejected at $P>0.05$). The variations in toxicities were not different at both 20 and 25^{o}C for either color morphs.

In contrast, the toxicity of the pyrethroid, lambda-cyhalothrin, decreased with increased temperature (Table 7.1). The difference in the toxicity over the 10^{o}C interval was -4.5 and -3.2 for red and green morphs, respectively. The trend in toxicity remained the same at each temperature for both color morphs and was not statistically significant ($P>0.05$). Except at 15^{o}C, differences among color morphs were not significant ($P>0.05$, 95% confidence interval and likelihood ratio test).

Discussion

The biochemical reactions leading to the toxicity of many insecticide molecules proceed more rapidly at higher temperatures. Some insecticides, like the pyrethroids, often have negative temperature coefficients, and are usually more toxic at lower temperatures (Wadleigh et al. 1991, Satpute et al. 2007).

Results indicated that environmental temperature did have an influence on the efficacy of the four insecticides tested for control of the TGPA. As insecticide resistance monitoring in aphids are usually conducted at 21±1^{o}C in laboratory incubators, the changes in the temperature over a range of 5-10^{o}C could result in drastic changes in the toxicity of an insecticide.

Methomyl had a positive temperature coefficient regardless of the color morph. The temperature change from 15 to 20^{o}C resulted in almost a 3-fold increase in toxicity for methomyl in both color morphs (Fig. 7.1). Similar trends were observed against acephate for both the color morphs. The differential toxicity was evident with the non-overlapping confidence intervals at 15 and 25^{o}C within and between color morphs. Interestingly, for both methomyl and acephate the differences in toxicity between the color morphs disappeared at 25^{o}C. As the efficacy of any insecticide molecule is a function of the interrelationship between vaporization and cuticular penetration, the insecticide formulation plays an important role in determining the actual toxicity of the molecule and not the carrier. The lower susceptibility of TGPA to methomyl at lower temperatures could be due to a higher vaporization effect that leads to higher toxicity at higher temperatures (Hill et al. 1996).

Despite intensive studies between temperature and organophosphorous toxicity to arthropods, little is known about the mechanism responsible for this relationship. Studies have shown that altered distribution within the insect body, target site interactions, penetration, and metabolism of OPs and carbamates differ considerably within insect species (Scott, 1995). A positive temperature coefficient, often characteristic of organophosphates could be due to higher penetration at higher temperatures. If this occurs in the field, there will be shorter residual activity along with increased penetration into insects at higher temperatures (Hill et al. 1996) In the present findings, organophosphate, carbamate, and neonicotinoid insecticides were more effective at higher temperatures, showing positive correlations of temperature with toxicity. Pawinska (1994) reported that organophosphorous compounds had increased activity from 20 to 30^{o}C, which also supports the findings in the current study. Morytz et al. (1997) also reported increased activity of an organophosphorous compound (chlorpyriphos) between 15 and 35^{o}C against both *M. domestica* and *Leptinotarsa decemlineata* (Say).

Esterase-based resistance is the primary factor conferring resistance to organophosphate and carbamate insecticides in *M. persicae* (Devonshire and Moores 1982, Field et al. 1994). General esterase activity in aphids used in this study was measured according to the method of

Van Asperen (1962) as modified by Zhu and Gao (1999) using α-Naphthyl acetate as substrate. Both red and green morphs had similar general esterase activity (62 and 54 nmol/min/mg protein, respectively). This further strengthens that the difference in the toxicity seen in this study is mainly due to temperature.

The neurotoxic action of pyrethroids was extensively reviewed by Soderlund and Bloomquist (1989). In general, pyrethroids have negative temperature coefficients due to increased effectiveness at the target site at lower temperatures. Both natural pyrethrins and synthetic pyrethroids have higher toxicity at lower temperatures (Hartzell and Wilcoxon 1932, Harries et al. 1945, Blum and Kearns 1956, Hirano 1979). Electrophysiological studies indicated that pyrethroids have increased activity in the form of repetitive discharges (Gammon 1978, Starkus and Narahashi 1978), increased negative after potential (Narahashi, 1962), and conduction block of the central nervous system (Wang et al. 1972). Pyrethroid-induced membrane depolarization (Bloomquist 1993) and sodium currents (Vijverberg et al. 1983) are greatly prolonged with decreasing temperature, and depend on several kinetic parameters of the voltage dependent sodium channels that change with temperature. Lambda-cyhalothrin belongs to the type II pyrethroid class that prolongs sodium currents for up to several minutes which increases as temperatures decrease. The increase in toxicity with decrease in temperature in my study is consistent with most studies that have examined the impact of temperature on efficacy of pyrethroids on various species of insects (Scott 1995).

Only a few studies have reported that temperature affects the toxicity of imidacloprid. As the neonicotinoids are among the most heavily used insecticides, any information regarding the activity of these newer compounds may play a critical role in insecticide resistance monitoring. Richman et al (1999) demonstrated that the toxicity of imidacloprid to cat fleas was higher at 35^{o}C compared to 20^{o}C. In the current study, imidacloprid toxicity increased steadily with increasing post-exposure temperature (Fig. 7.1). At 15^{o}C, both red and green morphs had significantly higher LC_{50} values than the toxicities at 20 and 25^{o}C.

As temperature may positively interact with the expression of insecticide resistance, its effects should be considered in resistance management. Tolerance of citrus thrips, *Scirtothrips citri*, to acephate and fluvalinate decreased as temperatures increased from 16° to 32°C (Zareh and Morse 1989). A field population of *Drosophila melanogaster* selected for cyclodiene resistance displayed a temperature-related paralysis at high temperature (38°C) in the laboratory,

and such an effect could reduce fitness at high temperatures (ffrench-Constant et al. 1993). Brown (1987) noted that a pyrethroid-tolerant strain of *Heliothis virescens* (Fabricius) displayed resistance to fenvalerate, fluocythrinate, and permethrin at 26 °C but not at 16°C, indicating that resistance in the field could go unnoticed at low temperatures. Foster et al. (1996) reported apparent loss of insecticide resistance in the green peach aphid, *M. persicae*, after exposure to low temperature and theorized that selection for resistance could be negated by cold winter weather. The biochemical mechanisms responsible for these effects have not been thoroughly investigated.

Regardless of other impacts, when insecticides from different classes are available to control a pest, knowledge of a product's temperature coefficient will enable pest managers to select a product that is efficacious under given environmental conditions. Insecticide resistance monitoring by laboratory bioassay methods is affected by variations in the abiotic factors, especially temperature. This study shows that changes in temperature greatly influence insecticide toxicity to the TGPA, but the mechanisms responsible for these effects need to be pursued further.

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Table 7.1. Effect of post-exposure temperature on insecticide toxicity for the tobacco-adapted form of the green peach aphid, *Myzus persicae.*

Insecticide	Color Morph	Temperature (°C)	N[a]	LC_{50} (95% CF) ppm[b]	Slope ± SE	H[c]	Temperature Coefficient[d]	
							5°C	10°C
Methomyl	Red	15	350	203.9 (155.3 - 269.4) a	1.49 ± 0.14	0.50		
		20	350	68.3 (53.1 - 86.7) b	1.98 ± 0.19	0.64	+ 3.0	+ 5.7
		25	350	35.5 (28.4 - 43.9) c	2.33 ± 0.25	0.92	+ 1.9	
	Green	15	350	163.1 (123.2 - 216.4) a	1.43 ± 0.14	0.83		
		20	350	62.4 (48.7 - 79.3) b	2.16 ± 0.21	1.08	+ 2.6	+ 4.7
		25	350	34.6 (26.6 - 44.1) c	1.89 ± 0.21	0.76	+ 1.8	
Acephate	Red	15	350	364.2 (272.7 - 497.1) a	1.41 ± 0.13	0.53		
		20	350	226.8 (171.2 - 303.8) a	1.61 ± 0.14	1.05	+ 1.6	+ 4.8
		25	350	76.1 (59.8 - 97.5) b	1.82 ± 0.18	0.44	+ 3.0	
	Green	15	350	216.6 (159.2 - 299.1) a	1.25 ± 0.12	0.36		
		20	350	97.3 (74.7 - 127.2) b	1.59 ± 0.15	0.53	+ 2.2	+ 2.9
		25	350	73.8 (56.2 - 96.4) b	1.56 ± 0.15	0.52	+ 1.3	
λ-cyhalothrin	Red	15	350	61.1 (38.8 - 89.9) a	0.95 ± 0.11	0.44		
		20	350	181.1 (127.1 - 261.3)b	1.13 ± 0.11	1.04	- 3.0	- 4.5
		25	350	275.7 (192.5 - 409.5)b	1.02 ± 0.11	0.50	- 1.5	
	Green	15	350	87.2 (49.1 - 143.1) a	0.69 ± 0.10	0.29		
		20	350	135.9 (92.8 - 197.8) b	0.98 ± 0.11	0.70	- 1.6	- 3.2
		25	350	276.0 (192.3 - 411.2) b	1.01 ± 0.11	0.64	- 2.0	
Imidacloprid	Red	15	350	6.4 (4.6 - 9.0) a	1.10 ± 0.14	0.18		
		20	350	3.6 (2.3 - 4.9) a	1.54 ± 0.27	0.83	+ 1.8	+ 3.4
		25	350	1.9 (0.4 - 2.7) b	0.64 ± 0.13	0.14	+ 1.9	
	Green	15	350	3.6 (2.4 - 5.2) a	0.96 ± 0.13	0.30		
		20	350	1.2 (0.8 - 1.7) b	1.48 ± 0.18	1.01	+ 3.0	+ 6.0
		25	350	0.6 (0.3 - 0.8) b	1.57 ± 0.21	0.40	+ 2.0	

[a] Total number of *Myzus persicae* apterae tested for each temperature/color morph/insecticide treatment.

[b] LC_{50} values followed by the same letter are not significantly different within each insecticide treatment ($P<0.05$)

[c] Heterogeneity factor = Observed chi-square value / Degrees of freedom

[d] Ratio of LC_{50} value at 5 to 10°C differences in temperature. A negative coefficient indicates a higher LC_{50} at the higher temperature.

[b] LC_{50} values followed by the same letter are not significantly different within each insecticide treatment ($P<0.05$)

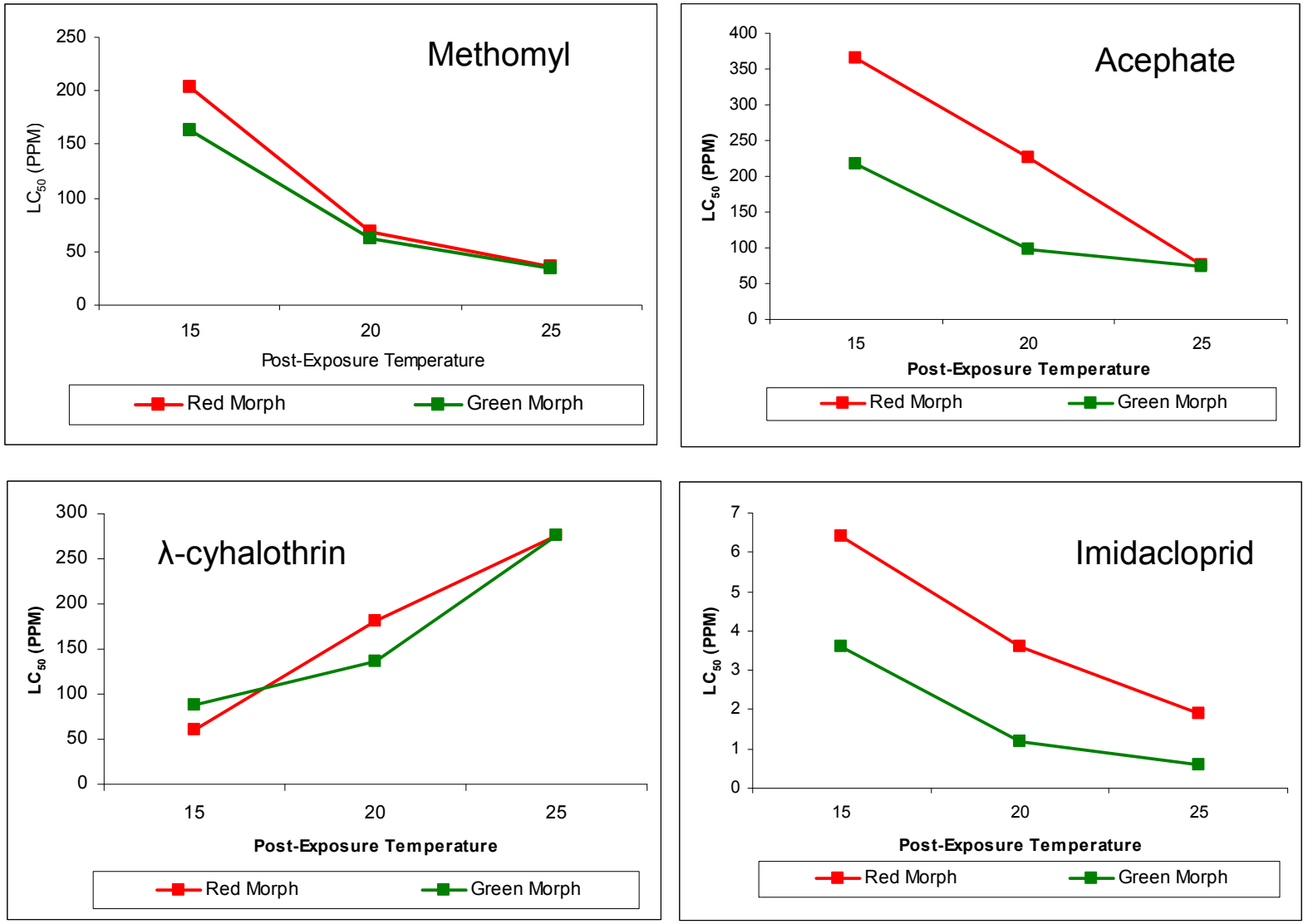

Fig. 7.1. Influence of post-exposure temperature on insecticide toxicity of methomyl, acephate, lambda-cyhalothrin, and imidacloprid on the tobacco-adapted form of the green peach aphid, *Myzus persicae.*

Chapter 8

Indirect Estimation of distribution of imidacloprid within the tobacco plant system and its impact on the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae).

Abstract

Research was conducted to investigate the dynamics of imidacloprid (Admire 2F) distribution, movement, and persistence in tobacco plants and its impact on the tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer), in field and greenhouse experiments. Bioassay experiments were conducted with the red morph of the aphid to obtain indirect estimates of imidacloprid concentrations in flue-cured tobacco plants. Both the greenhouse and field studies showed that imidacloprid concentration, leaf position, and sampling date had significant effects on aphid mortality on leaf disks in laboratory bioassays. Bioassay responses indicated that the concentration of imidacloprid was lowest in the younger, upper leaves and highest in the older, lower leaves. The differences between the lower and upper leaf positions indicate that the concentration of imidacloprid and its metabolites were unevenly distributed in the tobacco plant during the study period between 5-12 wk after treatment. The percent mortality in the aphids declined in all the rates of imidacloprid as the season progressed. In field experiments, higher aphid populations occurred on tobacco treated with imidacloprid at rates less than the field recommended rate of 41.4 ml of Admire 2F/1,000 plants. The development of aphid populations in the field was consistent with the laboratory bioassays. The impact of the sublethal concentrations of imidacloprid on the aphid populations and their influence on the resistance management programs are discussed.

Introduction

Imidacloprid was the first commercially available representative of a new chemical class, the chloronicotinyl or neonicotinoid insecticides (Elbert et al. 1990). The molecule exhibits a novel mode of action as it acts as an agonist of the nicotinic acetylcholine receptor (nAChR)

leading to paralysis and death of pest organisms (Bai et al. 1991, Nauen et al. 1998, Tomizawa and Casida 2005). Systemic insecticides including the neonicotinoid-based imidacloprid have site-specific modes of action that increase the possibility that resistance will develop in insect populations (Roush 1995, Prabhaker et al. 1997).

Systemic insecticides are transported throughout the plant and provide effective control when concentrations are high and well enough distributed within the plant when the insects are feeding. Resistance management has a greater impact with this unequal distribution (Olson et al. 2004). The susceptible individuals that colonize the plant parts with sublethal concentrations may become more tolerant over time. Imidacloprid is transported mainly in the xylem, and its systemic properties allow the active metabolites to become evenly distributed in young growing plants of both mono- and dicotyledonous crops (Nauen et al. 1999). The amount of imidacloprid in the root zone is limited and becomes depleted over time due to declining systemic uptake as the plant grows and the normal degradation processes in the soil (Cox et al. 1997). Since the tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer), may colonize imidacloprid-treated tobacco plants within 6 wk after transplanting, studies were conducted to assess the impact of imidacloprid concentrations within the plant on aphid mortality over time. Understanding the dynamics of imidacloprid distribution, movement, and persistence in tobacco plants is crucial because little is known about these factors. Therefore, research was conducted to investigate the distribution and persistence of imidacloprid within the tobacco plant system and its impact on the tobacco-adapted form of the green peach aphid. This study included a combination of field, greenhouse, and laboratory bioassay experiments to characterize the persistence and distribution of imidacloprid in tobacco plants. Bioassay experiments were conducted with the tobacco-adapted form of the green peach aphids to obtain indirect estimates of imidacloprid concentrations in flue-cured tobacco.

Materials and methods

Study site. Field experiments were conducted at the Virginia Tech Southern Piedmont AREC, Blackstone, Virginia, in 2006 and 2007. The imidacloprid (Admire 2F) treatments for field experiments were applied as tray drenches to greenhouse seedlings at 0.25X (10.4 ml/1,000 plants), 0.5X (20.7 ml/1,000 plants), 1.0X field rate (41.4 ml/ 1000 plants), and 2.0X (82.8 ml/

1000 plants) rates. Only water was applied as an untreated check. The experiment was arranged in a randomized complete block design with four replications. The experimental plots were 2.4 × 12.2 m (2 rows × 22 plants) separated by single untreated border rows, and blocks were separated by 1.5 m vacant alleys. The soil type was a Durham sandy-loam with a pH of 5.8 and less than 2% organic matter. A CO_2-pressurized back-pack sprayer with an 8004LP tip and operated at 213 kPa was used to apply imidacloprid treatments in 400 ml of water to plants in 288-cell float trays 1 d before transplanting. Additional water was used to wash the insecticide residue off the plants into the media around the roots. 'K-326' flue-cured tobacco was transplanted into experimental plots on 10 May 2006 and 2 May 2007.

Greenhouse experiments. Greenhouse experiments were conducted in the summers of 2006 and 2007. Treatments in the greenhouse experiments were applied as drenches to the medium 2 d after transplanting. Seedlings were transplanted into 30 cm pots. Medium was prepared by mixing 40 parts of Miracle-Gro® garden soil mix (composition - peat, compost forest products, sphagnum peat moss, manure, wetting agent and 0.15-0.05-0.10 fertilizer), 40 parts of sand and 20 parts of Carolina tobacco mix. Plants were selected at random from a pool of 40 plants. Leaching of the insecticide was minimized by placing each pot in separate water pans, so that any leached chemical would be taken up by the plant after watering. Imidacloprid rates were the same as those used the field study (0, 10.4, 20.7, 41.4 and 83.8ml/1,000 plants), but treatments were replicated eight times instead of four.

Sampling. In the field study, 20 plants were selected at random in each plot. The first fully expanded (the first leaf above the bedding leaves) after transplanting leaf was marked with a tag at approximately 4 wk after transplanting. The leaves were sampled four times at approximately 10-d intervals beginning 7 wk after transplanting. On each sampling date, two new plants were sampled with the tagged leaf as the bottom most leaf and a top leaf just wide enough to make a 10 cm diameter leaf disk. The top leaf moved up the stalk as the plant grew. Two new plants were sampled in each plot on each sampling date.

Sampling in the greenhouse experiments started at 5 wk after transplanting and continued at approximately 10 d intervals for four sampling dates. On the first sampling date, each plant had a minimum of four leaves between the lowest harvested leaf and the top leaf wide enough to produce a leaf disk. Studies were terminated when the plants began to flower. Unlike in the

field experiments, where new plants were sampled each time, each plant was sampled repeatedly, four times during the entire study period.

Leaf bioassay. Sampled leaves were taken to the laboratory and a scalpel was used to cut 10-cm leaf disks from the center of each leaf. Individual leaf disks were placed into Petri dishes coated with Fluon® on the sides to prevent aphids from crawling off the disks. The moistened filter paper reduced evaporation so the leaf disk remained healthy during the study. A single colony of a red morph of the tobacco-adapted form of the green peach aphid, collected from the field and reared in the laboratory was used for the bioassay to obtain an indirect estimate of imidacloprid concentrations in the leaves. A fine camel's hair brush was used to place ten apterous 6-d-old aphids on the abaxial leaf surface. The Petri dishes were sealed with Parafilm and placed in an incubator that was set at a 16:8 (L: D) h cycle and 21±2°C with 35-40% RH. Mortality was recorded at 72 h after treatment. An aphid was recorded as dead if no movement was observed when probed with a camel's hair brush.

Estimation of total alkaloids. This study was conducted to determine the influence of imidacloprid concentration and leaf position on total alkaloids in the green tobacco leaves. In 2007, leaf samples from the greenhouse [36, 46, and 67 d after treatment (DAT)] and the field studies (52, 61, and 82 DAT) were dried in force-air laboratory oven and ground with using a mortar and pestle. Total alkaloids were determined by the method of Davis et al. (1976) adapted for a Technicon Auto-Analyzer II.

Statistical analysis. Percent mortality was transformed using arcsin square-root transformation (Snedecor and Cochran 1989). Data were checked for normality and homogeneity of variances using Shapiro-Wilk's W test and Kolmogorov-Smirnov D test (Royston 1982). The PROC MIXED repeated measures procedure of ANOVA (SAS Institute 2001) was used to test for main effects and interactions for treatment, leaf position, DAT. For the greenhouse study, the experimental design was split-plot (CRD-RCBD) design, where the treatments are nested. Post-treatment interval and leaf position as crossover factors. In the field study, the experimental design was split-plot (RCBD-RCBD) design with treatment rate as the whole plot factor, nested, while the post-treatment interval and leaf position were crossover factors. If F tests were significant, means were separated using Tukey's multiple comparison adjustment ($P < 0.05$). Difference in total alkaloids was checked individually over treatment rates and DAT using ANOVA and significant means separated by Duncan's multiple range test

($P < 0.05$). Paired comparisons were performed using student paired *t*-test to determine significant differences in total alkaloids for the two leaf positions ($\alpha=0.05$). All tests were performed using SAS software (SAS Institute 2001).

Results

Greenhouse experiment, 2006. Tobacco plants in the greenhouse reached 7-8 leaf stage at about 5 wk after transplanting. Imidacloprid rate ($F = 303.4$; df = 4, 35; $P < 0.0001$), leaf age (upper and lower) ($F = 265.6$; df = 1, 212; $P < 0.0001$), and post-treatment interval (d after treatment - 36, 46, 56 and 67 DAT) ($F = 25.6$; df = 3, 140; $P < 0.0001$) had significant effects on aphid mortality (Fig. 8.1A, B). Mortality increased with increasing rates of imidacloprid, declining with both post-treatment interval and leaf age. The interaction of rate with time interval was significant, indicating that aphid mortality declined more rapidly on leaves treated with the two lowest rates of imidacloprid than the two highest rates ($F = 7.8$; df = 12, 140; $P < 0.0001$). The imidacloprid rate by leaf position (upper and lower) interaction was also significant indicating a difference in the concentration gradient ($F = 9.3$; df = 4, 212; $P < 0.0001$) (Table 8.1).

Greenhouse experiment, 2007. The tobacco plants in the greenhouse reached 7-8 leaf stage at the same time (36 DAT) as in the 2006 trial. Imidacloprid rate ($F = 274.1$; df = 4, 35; $P < 0.0001$), leaf position ($F = 286.2$; df = 1, 231; $P < 0.0001$), and sampling interval (36, 46, 56 and 67 DAT) ($F = 68.1$; df = 3, 134; $P < 0.0001$), had significant effects on aphid mortality (Fig. 8.2. A, B). There were also significant interactions of imidacloprid rate with sampling interval ($F = 27.8$; df = 12, 134; $P < 0.0001$) and imidacloprid rate by leaf position ($F = 8.2$; df = 4, 231; $P < 0.0001$) (Table 8.1).

Field study, 2006. The plants reached the 5-6 leaf stage at 7 wk (49 DAT) after transplanting. The imidacloprid rate ($F = 741.7$; df = 4, 14.6; $P < 0.0001$), leaf position ($F = 341.9$; df = 1, 72.2; $P < 0.0001$), and time interval (d after treatment) (49, 59, 69, 83 DAT) ($F = 136.1$; df = 3, 40.2; $P < 0.0001$) each had significant effects on aphid mortality in the leaf-dip bioassays (Fig. 8.3A, B). There were significant interactions for rate by time interval ($F = 54.3$; df = 12, 40.2; $P < 0.0001$) and rate by leaf position ($F = 16.8$; df = 4, 72.2; $P < 0.0001$) (Table 8.1) for aphid mortality.

Field study, 2007. The plants reached the 5-6 leaf stage at about the same time as in the 2006 field test and the first samples were taken at 52 DAT. The rate of imidacloprid ($F = 218.3$; df = 4, 12; $P < 0.0001$), leaf position ($F = 309.5$; df = 1, 105; $P < 0.0001$), and time interval (52, 61, 72 and 82 DAT) ($F = 8.04$; df = 3, 105; $P < 0.0001$) had significant effects on the aphid mortality in the bioassay tests (Fig. 8.4A, B). The interactions of imidacloprid rate with time interval ($F = 10.9$; df = 12, 105; $P < 0.0001$) and imidacloprid rate with leaf position ($F = 2.93$; df = 4, 105; $P = 0.0241$) were significant once again (Table 8.1).

Field observations, 2006. Aphid populations in the untreated check increased steadily from 19 June, 40 d after transplanting through 21 July, 72 d after transplanting. All rates of imidacloprid were effective on 19 June and provided significant control through 21 July. The recommended 1.0X rate of imidacloprid [146 g/(AI)/ha] was the most effective treatment through 21 July, 72 d after transplanting (Table 8.2). The 2.0X rate of imidacloprid, 291 g (AI)/ha, tended to have higher aphid populations (significantly higher on 11 July) than the 1.0X (AI)/acre rate. The lowest rate of imidacloprid (0.032 lb (AI)/acre) provided control that was not significantly different from the highest rate. However, the two lowest rates gave < 80% control on 21 July, the last observation date. Imidacloprid rate had little effect on early-season aphid control, but control with the two lowest rates declined more dramatically than the two highest rates between 11 and 21 July (Table 8.2).

Field observations, 2007. Populations of the tobacco-adapted form of the green peach aphid in the untreated check increased steadily from 7 June, 36 d after transplanting through 13 July, 72 d after transplanting. All rates of imidacloprid were effective on 7 June and provided significant control through 29 June (Table 8.3). The 1.0X and 2.0X rates of imidacloprid [146 and 291 g (AI)/ha] provided similar control of aphids throughout the test including >97% control on 13 July, 72 d after transplanting. Control with the two lowest rates of imidacloprid was significantly less than the two highest rates after 22 June, providing 68 and 74% control on 13 July (Table 8.3).

Total Alkaloids: 2007. Imidacloprid rate did not affect the percentage of total alkaloids either in the greenhouse or in the field samples on any of the sampling dates (Table 8.4). However, the percentages of total alkaloids on a dry weight basis in the lower leaves were significantly higher on each sampling date than for the upper leaves in both greenhouse ($t=20.82$; df=112; $P < 0.0001$) and field samples ($t=29.58$; df=79; $P < 0.0001$) (Fig. 8.5).

Discussion

Greenhouse and field studies with flue-cured tobacco showed that imidacloprid concentration, leaf position, and the time interval after treatment had significant effects on TGPA mortality in laboratory bioassays. Differences between the lower and upper leaf positions indicate that the concentration of imidacloprid and its metabolites was unevenly distributed in the tobacco plant during the study period between 5-12 wk after treatment. Bioassay responses suggest that the concentration of imidacloprid was lowest in the younger, upper leaves and highest in the older, lower leaves. In field experiments, higher TGPA populations occurred on tobacco treated with imidacloprid at the 0.25X and 0.5X rates than the 1.0X field recommended rate (41.4 ml/1,000 plants). This demonstrates the decrease in the efficacy of imidacloprid in the field experiments. The field observations are consistence with the laboratory bioassays.

These findings show that the reservoir of imidacloprid in the soil (TW treatment-greenhouse) or in the root plug and early foliage (TD treatment-field) appears sufficient to provide a toxic concentration of the chemical throughout the tobacco plant. However, as the plant biomass increases with the concurrent degradation and depletion of imidacloprid and its metabolites within the plant system, the concentration in the newest plant tissues decreases as the season progresses.

The two lower rates (0.25X and 0.5X) of imidacloprid applied either as TW or TD treatments resulted in lower aphid mortality in the bioassays when compared with 1.0X and the 2.0X rate. Given this scenario, the rates lower than the recommended labeled rate of imidacloprid may cause problems by creating a fitness differential among aphid genotypes feeding on certain parts of the plant. These sublethal doses may functionally render the tolerant genotype dominant by discriminating between susceptible and tolerant individuals.

In agreement with previous research (Semtner et al. 1993, LaMondia and Rathier 1999, Semtner et al. 2007) imidacloprid is equally potent regardless of the mode of application and had no effect on the depletion of the concentration gradients within the tobacco plant. This study demonstrated marked differences in TGPA responses to imidacloprid-treated tobacco plants over time. This suggests possible scenarios of how different concentrations of the systemic insecticide that may affect the selective fitness differential between individuals in the aphid populations.

Differences in the TGPA response to the leaf positions were probably only partially due to the uneven distribution of imidacloprid. As the tobacco plant grows, the older, lower canopy leaves, begin to senesce making them less palatable for aphid growth and development. The older leaves are of lower quality and are probably less nutritious than the younger leaves on upper part of the plant. This phenomenon was seen in the field, where all rates of imidacloprid and the untreated check had higher mortality on the lower leaves on the later sampling dates when the plants were more mature. The relocation of imidacloprid from old to new leaves is limited due to minimal translocation through the phloem tubes (Nauen et al. 1999). This phenomenon has a direct impact on phloem feeders such as aphids, because the active metabolites of imidacloprid like olefin derivatives are not translocated to the newer foliage.

In the tobacco plant, nicotine synthesis and breakdown are a continual process. Many biotic and abiotic factors influence the amount of nicotine in the tobacco plants as the leaves begin to senesce. Nicotine is known to evaporate from the mature tobacco leaves (Tso 1990). The pattern of accumulation of nicotine in the tobacco leaves begins soon after transplanting, and increases until maturity. Maximum nicotine content of individual leaves occur at successively higher stalk positions as the plant matures. The rate and amount of nicotine accumulation is dependent upon the type of tobacco grown and the cultural practices used to produce it. The amount of total alkaloids in the cured leaf is a concern and is usually measured in the cured leaves, not the green leaves. But, as imidacloprid is a neonicotinoid, which has structural similarity to nicotine, the major alkaloid present in the tobacco plants, it is a concern that the natural tolerance to nicotine in the tobacco-adapted forms of the green peach aphid could have cross-resistance to the newer generation neonicotinoids such as imidacloprid.

As nicotine is translocated within the plant in the xylem vessels and phloem is the preferred feeding sites for aphids (Guthrie et al. 1962), logically the higher imidacloprid-tolerant individuals should not have any cross tolerance to nicotine (Devine et al. 1996). However, electrical penetration graphs have shown that the TGPA actually encounters the xylem vessels as it probes in search of the phloem and imbibes small amounts of nicotine on regular basis (Tjalingii 1985, Nauen and Elbert 1994). Since imidacloprid and nicotine share a common target site, nicotinic acetylcholine receptor (nAChR) (Tomizawa and Casida 2005), the possibility of aphids becoming cross-resistant to both is a logical concern. My results suggest that the influence of differential exposure of tobacco plants to imidacloprid and its influence on the

amount of nicotine can be ruled out, as the total alkaloid concentrations were not significantly different among the imidacloprid rates. Interestingly, the lower leaves had significantly higher alkaloid levels than the upper leaves, similar to differences in the aphid mortality in response to the rates of imidacloprid. This opens up the question of cross-tolerance in the tobacco adapted-forms of the green peach aphids, due to differential exposure to both imidacloprid and nicotine.

The results of this study are in agreement with the findings of Westwood et al. (1998) on sugar beet and can be explained in terms of a supply and demand relationship. A similar study by Olson et al (2004) in potato plants demonstrated significantly higher Colorado potato beetle mortality on the lower leaves collected from imidacloprid-treated plants. Van Iersel et al. (2001) also found higher accumulations of imidacloprid in the lower canopy leaves than in the upper canopy leaves of poinsettia. Differences in spatial and temporal distribution of imidacloprid to control glassy-winged sharpshooter, *Homalodisca coagulata* (Say) have ineffective uptake resulting in sublethal concentrations of toxicant (Byrne et al. 2005, Castle et al. 2005, Byrne and Toscano 2006).

Monitoring the incidence of imidacloprid resistance in field populations is critical to resistance management of the TGPA. Although recommended field dose rates are in the lethal range, I observed very marked differences in mortality, as affected by leaf position and the time interval after treatment. Such uneven distribution could result in a lower concentration within the plant, which could allow the development of resistant individuals due to discrimination between homozygous susceptible and heterozygous insects (Roush and Daly 1990). This would be a completely different story if the insect species has a continuous parthenogenetic life cycle like the TGPA. In this case, heterozygotes would produce same resistant alleles as offspring that survive a field dose of the insecticide (Olson et al. 2004). Resistance management would have a greater impact with the unequal distribution of the pesticide in the plant. The susceptible individuals that tend to colonize the plant parts with sublethal concentrations may receive sublethal doses and become more tolerant over time.

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Table 8.1. Comparison of differences in the aphid mortality between upper and lower canopy leaves determined in the laboratory bioassays, SPAREC, Blackstone, VA, 2006-2007. Data show the significance determined by Tukey's multiple comparison (α=0.05) in the corrected mortality within each treatment on each sampling interval after treatment.

		Post-Treatment Interval [a]			
	Treatments	**1**	**2**	**3**	**4**
Greenhouse 2006	Control	N	N	N	Y
	10.4 ml/1000 plants	Y	Y	Y	Y
	20.7 ml/1000 plants	Y	Y	Y	Y
	41.4 ml/1000 plants *	Y	Y	Y	Y
	82.8 ml/1000 plants	N	N	Y	Y
Greenhouse 2007	Control	N	N	Y	Y
	10.4 ml/1000 plants	N	Y	Y	Y
	20.7 ml/1000 plants	Y	Y	Y	Y
	41.4 ml/1000 plants *	N	Y	Y	Y
	82.8 ml/1000 plants	N	N	Y	Y
Field 2006	Control	N	N	N	Y
	10.4 ml/1000 plants	N	N	N	N
	20.7 ml/1000 plants	Y	Y	Y	Y
	41.4 ml/1000 plants *	N	Y	Y	Y
	82.8 ml/1000 plants	N	Y	Y	Y
Field 2007	Control	N	N	N	Y
	10.4 ml/1000 plants	Y	N	N	N
	20.7 ml/1000 plants	N	Y	Y	Y
	41.4 ml/1000 plants *	N	Y	Y	Y
	82.8 ml/1000 plants	N	Y	Y	Y

* Field recommended application rate
Y = Mortality significantly different between upper and lower canopy leaves.
N = Mortality not significantly different between upper and lower canopy leaves.

[a] Post treatment intervals:

Greenhouse 2006 : 36, 46, 56 and 67 d after treatment
Greenhouse 2007 : 36, 46, 56 and 67 d after treatment
Field 2006 : 49, 59, 69 and 83 d after treatment
Field 2007 : 52, 61, 72 and 82 d after treatment

Table 8.2. Influence of rate of imidacloprid applied as a tray drench treatment on populations of the tobacco-adapted form of the green peach aphid on flue-cured tobacco the incidence of naturally occurring TGPA populations in the field, SPAREC, Blackstone, VA, 2006.

Treatment/	Rate g	Aphids/ 10 plants[a]			
formulation	(AI)/ha	19-Jun	29-Jun	11-Jul	21-Jul
Imidacloprid 2F	291	6b	74bc	425b	3,934cd
Imidacloprid 2F	146	3b	26c	236c	2,930d
Imidacloprid 2F	73	9b	113b	783b	13,906b
Imidacloprid 2F	36	8b	60bc	394b	8,168bc
Untreated check		445a	3,347a	17,500a	40,455a
(P > F)		0.0034	0.0001	0.0001	0.0001

[a]Means within a column followed by the same letter(s) are not significantly different as indicated by Waller-Duncan k-ratio t-test (k-ratio=100). Data were transformed to $Log_{10}(x+1)$ before analysis.

Table 8.3. Influence of rate of imidacloprid applied as a tray drench treatment on the incidence of naturally occurring populations of the tobacco-adapted form of the green peach aphid on flue-cured tobacco in the field, SPAREC, Blackstone, VA, 2007.

Treatment/	Rate g	Aphids/ 10 plants					
formulation	(AI)/ha	7-Jun	15-Jun	22-Jun	29-Jun	6-Jul	13-Jul
Imidacloprid 2F	291	2b	4c	32c	75c	185d	850c
Imidacloprid 2F	146	5b	9c	26c	84c	296d	1,080c
Imidacloprid 2F	73	8b	16c	142b	456b	590c	10,240b
Imidacloprid 2F	36	9b	24b	254b	824b	1,045b	12,480b
Untreated check		192a	543a	9,183a	8,940a	37,175a	38,875a
(P > F)		0.0012	0.0001	0.0001	0.0001	0.0001	0.0001

Means within a column followed by the same letter(s) are not significantly different as indicated by Waller-Duncan k-ratio t-test (k-ratio=100). Data were transformed to $Log_{10}(x+1)$ before analysis.

Table 8.4. Effect of post-treatment interval on the percentage of total alkaloids in dried green leaves on various sampling dates in greenhouse and field studies conducted at SPAREC, Blackstone, VA, 2007.

Greenhouse Study 2007				
	Mean*	*F*	df	*P*
37 DAT[a]	0.58	0.17	11, 68	0.95
47 DAT[a]	0.55	0.11	11, 68	0.99
71 DAT[a]	0.79	1.99	11, 60	0.10
Field Study 2007				
52 DAT[a]	0.76	0.23	7, 72	0.93
61 DAT[a]	0.81	0.13	7, 72	0.97
82 DAT[a]	1.73	0.88	7, 72	0.82

* Mean % total alkaloids
[a] Days after treatment

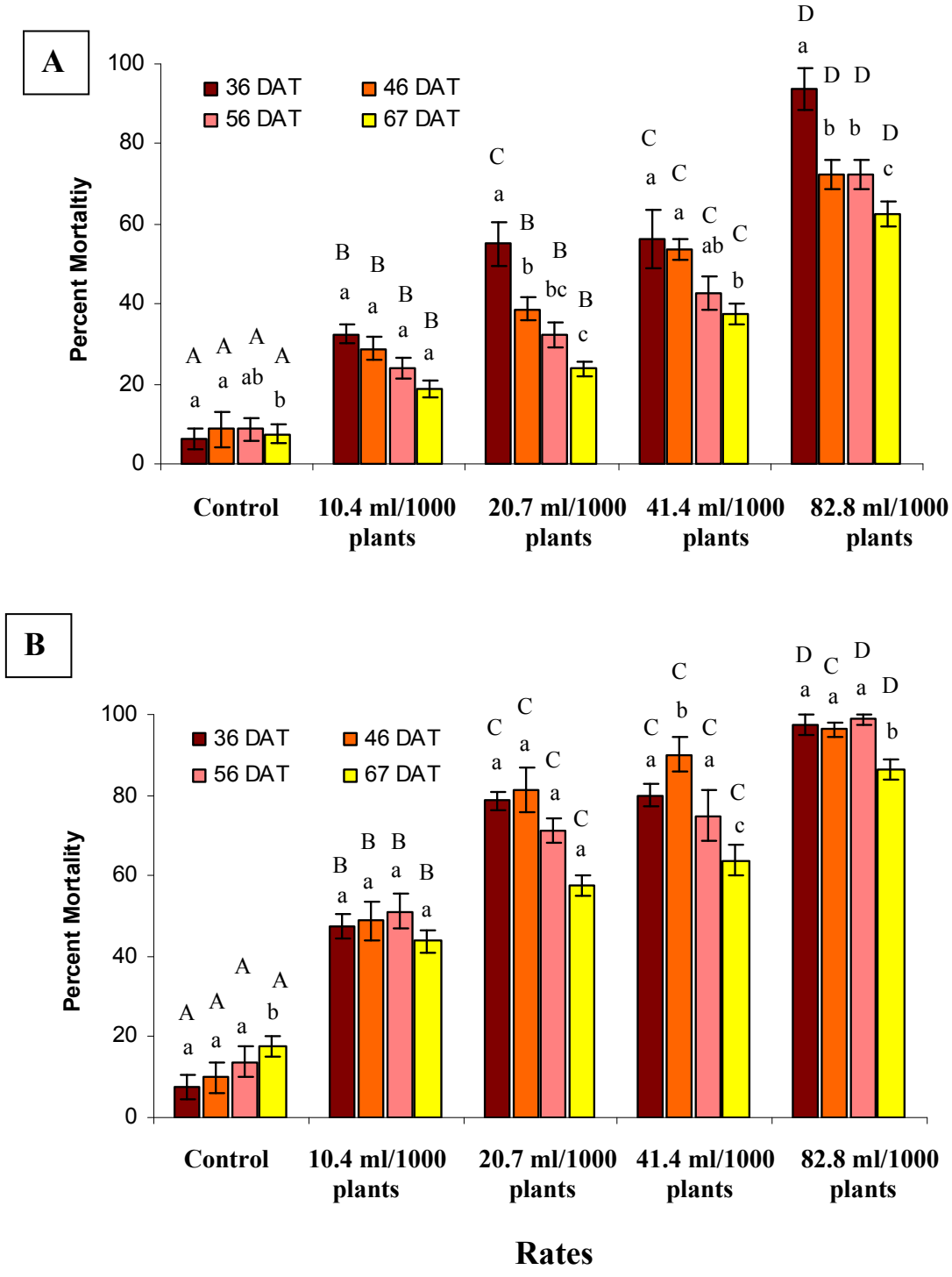

Fig. 8.1. Influence of imidacloprid rate, time interval, and leaf position on mortality of the tobacco-adapted form of the green peach aphid in laboratory bioassays, greenhouse experiments Virginia Tech SPAREC, Blackstone, VA, 2006 (A) percent mortality on upper canopy leaves (B) percent mortality on lower canopy leaves. Data shown are mean percent mortality from eight replications ± SE. Bars within a rate followed by the same lower case letter are not significantly different over time interval. Percent mortalities within a time interval followed by the same upper case letter are not significantly different among treatment rates (Tukey's multiple comparison, α=0.05).

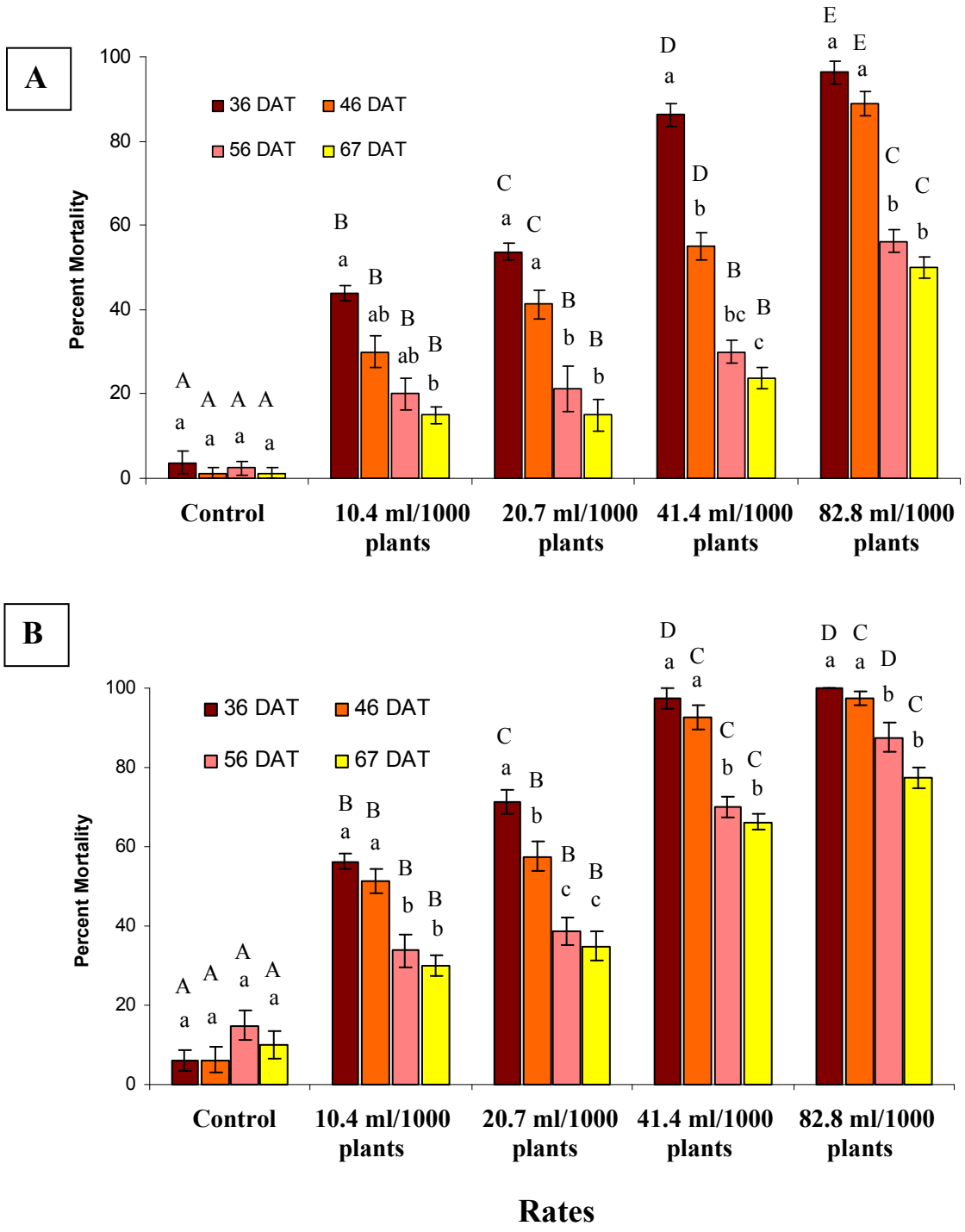

Fig. 8.2. Influence of imidacloprid rate, time interval, and leaf position on mortality of the tobacco-adapted form of the green peach aphid in laboratory bioassays, greenhouse experiments Virginia Tech SPAREC, Blackstone, VA, 2007 (A) percent mortality on upper canopy leaves (B) percent mortality on lower canopy leaves. Data shown are mean percent mortality from eight replications ± SE. Bars within a rate followed by the same lower case letter are not significantly different over time interval. Bar within a time interval followed by the same upper case letter are not significantly different among treatment rates (Tukey's multiple comparison, α=0.05).

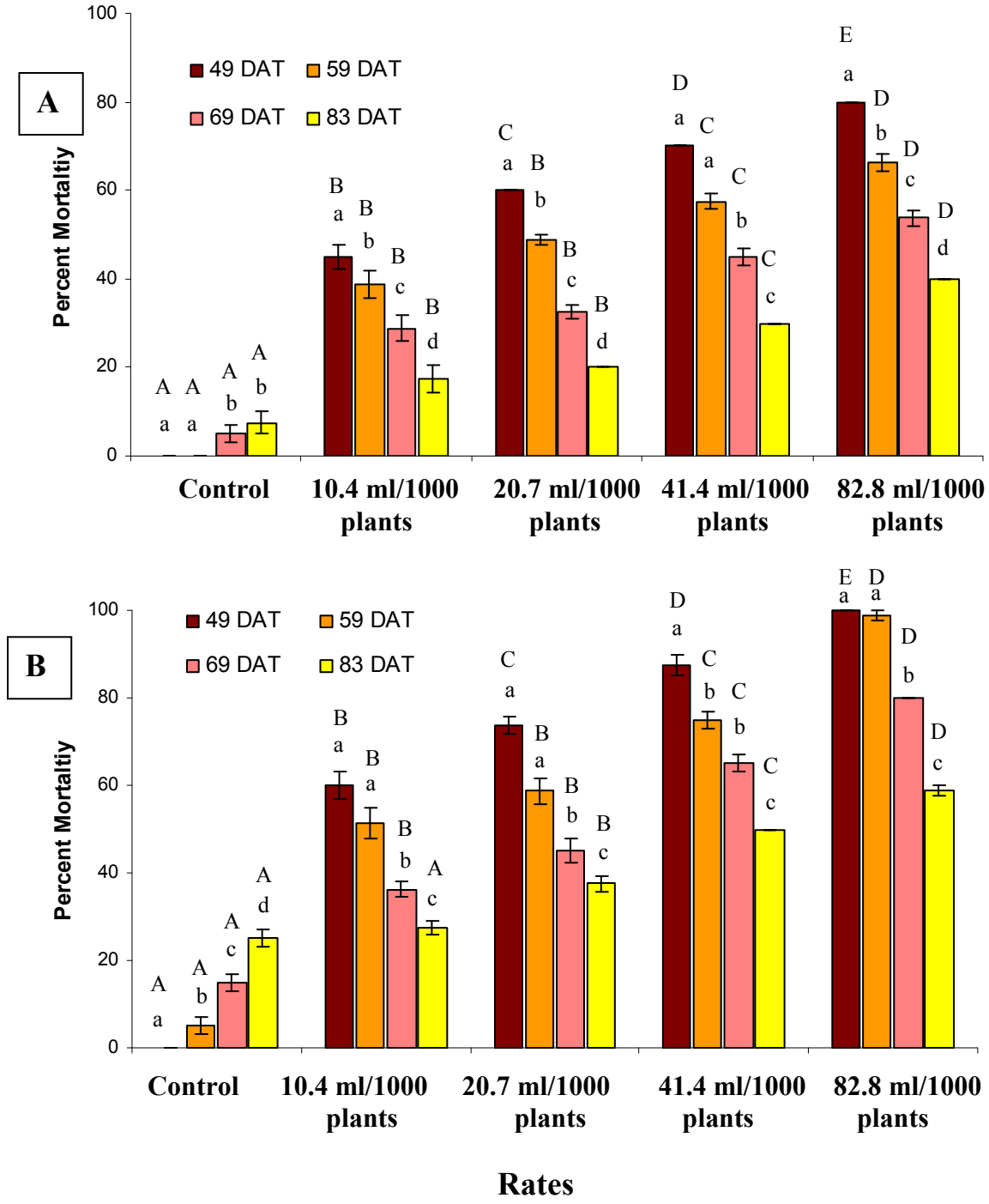

Fig. 8.3. Influence of imidacloprid rate, time interval, and leaf position on mortality of the tobacco-adapted form of the green peach aphid in laboratory bioassays, field experiments Virginia Tech SPAREC, Blackstone, VA, 2006 (A) percent mortality on upper canopy leaves (B) percent mortality on lower canopy leaves. Data shown are mean percent mortality from eight replications ± SE. Bars within a rate followed by the same lower case letter are not significantly different over time interval. Bars within a time interval followed by the same upper case letter are not significantly different among treatment rates (Tukey's multiple comparison, α=0.05).

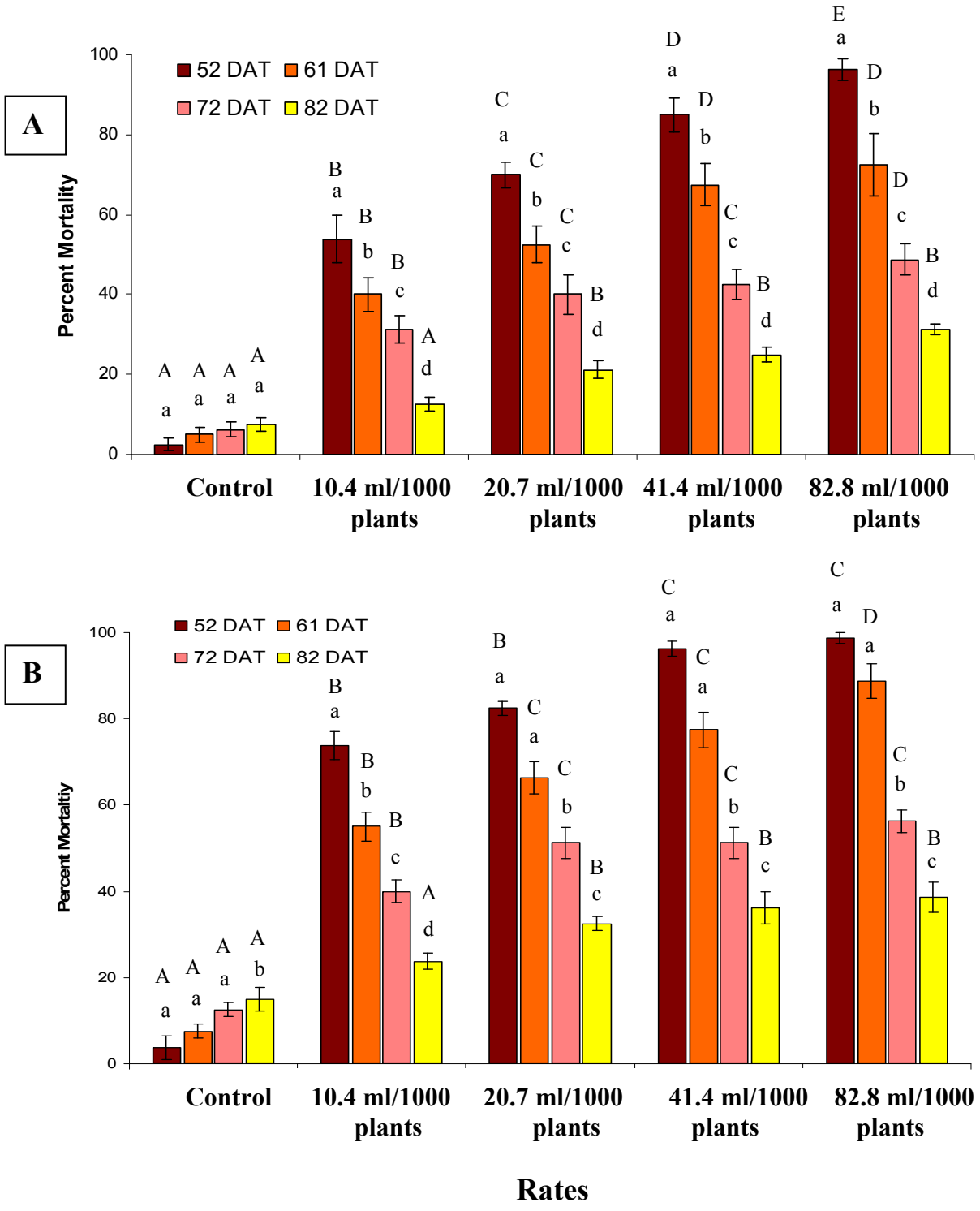

Fig. 8.4. Influence of imidacloprid rate, time interval, and leaf position on mortality of the tobacco-adapted form of the green peach aphid in laboratory bioassays, field experiments Virginia Tech SPAREC, Blackstone, VA, 2007 (A) percent mortality on upper canopy leaves (B) percent mortality on lower canopy leaves. Data shown are mean percent mortality from eight replications ± SE. Bars within a rate followed by the same lower case letter are not significantly different over time interval. Bars within a time interval followed by the same upper case letter are not significantly different among treatment rates (Tukey's multiple comparison, α=0.05).

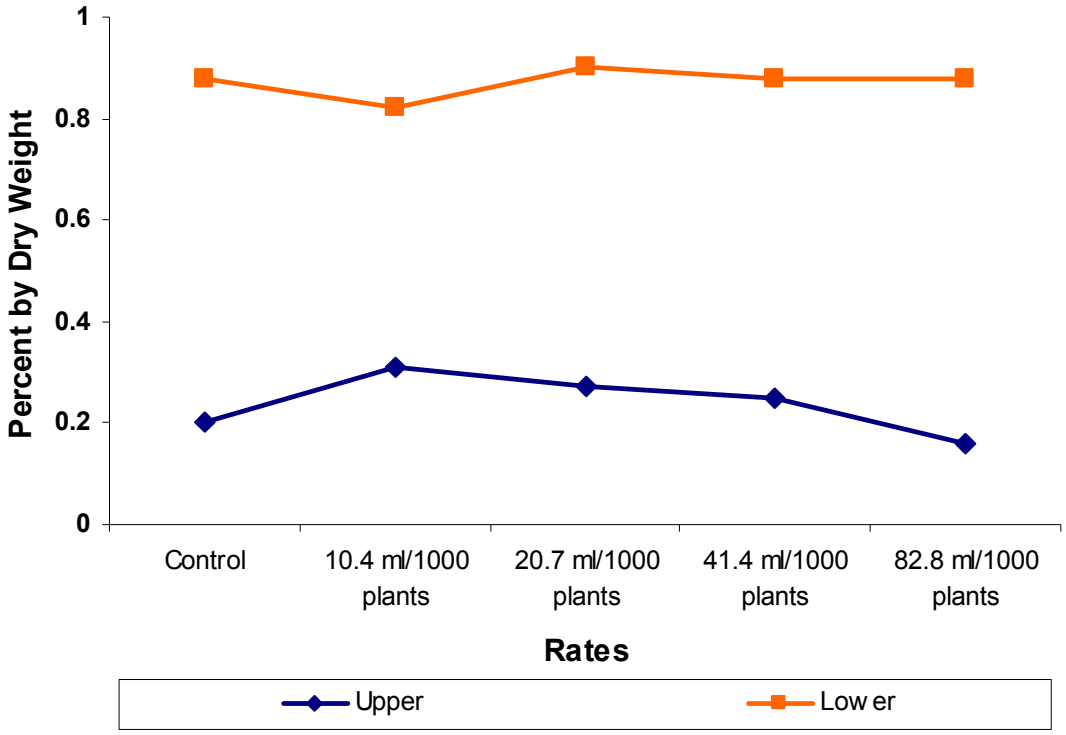

Fig. 8.5. Effect of imidacloprid rate on the total alkaloids in dried green leaf samples of the upper and lower leaf positions of flue-cured tobacco plants in the field studies conducted at Virginia Tech SPAREC, Blackstone, VA, 2007.

Chapter 9

Response of tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) to insecticides in four chemical classes on flue-cured tobacco

Abstract

Field trials were conducted to evaluate the performance of various insecticides currently registered for controlling the tobacco-adapted form of the green peach aphid, (TGPA), *Myzus persicae* (Sulzer) on tobacco. Imidacloprid applied as a tray drench treatment and acephate applied as foliar sprays were the most effective treatments. Moderate declines in control with imidacloprid were observed at 75-76 d after transplanting in 2006 and 2007 and 14% reduction in control at 87 d after treatment. Aldicarb gave good to excellent control in one of three yr and poor to fair control in the other two yr. Methomyl and lambda-cyhalothrin gave fair to good control in all three years. The poor performance of aldicarb in the two yr may have been related to the presence of E4 or FE4 resistance in the naturally occurring TGPA in the experimental plots. None of the insecticides gave 100% control with any of the insecticide treatments. This may indicate that some aphids may have a low level of resistance to the insecticides that I used in my experiments or that they are not receiving enough chemical to kill them.

Introduction

Tobacco, *Nicotiana tabacum* (L.), is one of the major cash crops in Virginia producing $69.7 million in farm cash receipts from 8,340 ha in 2007 (USDA-NASS 2007). In the United States, tobacco produced about $1.3 billion in farm income in 2007 (USDA-NASS 2007). The tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer), is a major insect pest of tobacco worldwide (Chamberlin 1958, Blackman 1987). In North America, it is a major insect pest of the flue-cured tobacco in Virginia (Reed and Semtner 1992, Semtner et al. 1993), North Carolina (Harlow and Lambert 1990, Harlow et al. 1991), Ontario (Cheng 1985) and of burley (Thurston 1965, Sedlacek and Townsend 1990), and Maryland tobaccos (Harrison 1969). Tobacco farmers rely on several insecticides to control this pest.

Many of the insecticides previously used for TGPA control on tobacco have been withdrawn from the market due to poor performance, safety, or environmental concerns. Various federal and state laws, company business decisions, and public concerns may also affect the future availability of several of most important aphicides including acephate, endosulfan, and aldicarb (US EPA 2007). If these chemicals were discontinued, only the neonicotinoids and pymetrozine would remain for managing the TGPA. Imidacloprid, a neonicotinoid that is effective, easy to use, has become the most commonly used insecticide for TGPA and flea beetle control on tobacco in the United States. However, there have been several reports of TGPA resistance to imidacloprid in Europe (Margaritopolus et al. 2007) and some indications of poor control in the United States (Semtner Personal Communication). Therefore, it is critical that baseline information be established on the toxicity of imidacloprid and other insecticides commonly used for TGPA control for future monitoring of insecticide resistance. Early detection of insecticide resistance would allow resistance management plans to be implemented to delay the further development of insecticide resistance.

Studies were conducted to compare the field efficacy against the TGPA for five insecticides in four chemical classes registered for insect control on tobacco in 2004, 2006, and 2007. Effectiveness of different compounds with relation to insecticide resistance monitoring is discussed.

Materials and Methods

Experimental plots were established at the Southern Piedmont Agricultural Experiment Center, Blackstone, VA in 2004, 2006, and 2007. Flue-cured tobacco 'K-326' was transplanted into the plots 1.2 x 12.2 m (1 row x 22 plants) on 14, 10, and 2 May 2004, 2006, and 2007, respectively. Plots were separated by single guard rows. The guard rows to the right of the plots treated with the designated soil, transplant drench (TD), or transplant water (TW) treatments were also treated with that insecticide. The border rows to the right of the foliar treatments were not treated. Five treatments and an untreated check were established in a randomized complete block design with four replications (Tables 1-3).

The treatments included:

1. Aldicarb (Temik 15G) – 3.36 kg (AI) /ha [36-cm band and incorporation at bedding]
2. Imidacloprid (Admire 2F) - 0.146 kg (AI)/ha [Transplant drench]
3. Acephate (Orthene 97WG) - 0.840 kg (AI)/ha [Transplant water & Foliar]
4. Methomyl (Lannate 2.4LV) - 0.504 kg (AI)/ha [Foliar]
5. Lambda-cyhalothrin (Warrior Zeon 1SC) - 0.03 kg (AI)/ha [Foliar]
6. Untreated control

Normal production practices, recommended by Virginia Cooperative Extension were followed for weed and disease control, fertilization, topping and sucker control. The aldicarb treatment was applied in a 36-cm band with a Gandy® granular applicator and covered with 16 cm of soil at bedding 2 d before transplanting in 2004 and 2006. In 2007, the aldicarb treatment was applied as side-dress using shanks to inject the aldicarb 8-12 cm into the soil about 10 cm on each side of the row on 29 May, 27 d after transplanting (DAT). A CO_2-pressurized backpack sprayer with an 8004E nozzle and operated at 241 kPa was used to apply the imidacloprid TD treatment in 360 ml of water to plants in 288-cell float trays 1 d before transplanting each year. Additional water (500 ml) was used to wash the chemical residue off the transplants and into the media. Immediately after transplanting, the acephate transplant water (TW) treatment was applied with a measuring cup that delivered 118 ml (1729 liter/ha) of mixture to the base of each plant in the designated plots. The tests were transplanted on 10 May 2004, 14 May 2006, and 2 May 2007. Foliar treatments were applied once each year with a CO_2-pressurized backpack sprayer that delivered 235 liter/ha at 414 kPa through TX-12 (TeeJet®) hollow cone nozzles (3 per row) on 8 July 2004, 13 July 2006, and 23 July 2007. Temperatures during application ranged from 22 to 25^{o}C on 8 July 2004, 29 to 31^{o}C on 13 July 2006, and 28 to 32^{o}C on 23 July 2007.

The numbers of TGPA were estimated on the upper 4 leaves of 10 plants/plot every 6 to 11 d from 17 June-29 July 2004; 6 June-28 July 2006 and 7 June-27 July in 2007. TGPA data were transformed to Log10(x+1), analyzed by ANOVA, and significantly different means were separated by Waller-Duncan k-ratio t-test (k-ratio=100) (SAS 2001).

Results and Discussion

2004. The imidacloprid tray drench treatment gave the best season-long control of the TGPA, providing >97% control through 29 July (Table 9.1). The aldicarb band treatment at bedding was almost as effective as imidacloprid, through 20 July, but control declined to 72% on 29 July compared with 97% in the imidacloprid treatment, 11 wk after transplanting (Table 9.1). The acephate transplant water treatment provided 66-77% control through 7 July. The foliar application of acephate on 8 July gave excellent control of TGPA through the remainder of the test. The methomyl, lambda-cyhalothrin, and acephate foliar treatments were not applied until the TGPA thresholds were reached on 8 July. However, the TGPA populations in the lambda-cyhalothrin plots were significantly lower than those in the other foliar insecticide plots before the foliar treatments were applied, possibly due to natural variation in the test populations of TGPA. The methomyl and lambda-cyhalothrin were significantly less effective than the acephate treatment on 20 and 29 July, 12, and 21 DAT. The methomyl and lambda-cyhalothrin treatments had severe declines in control at 21 DAT since populations rebounded rapidly after 13 July. The acephate TW treatment provided 66 to 77% control of the TGPA through 7 July, while the aldicarb and imidacloprid treatments provided 98-100% control 23 June- 7 July (Table 9.1). The foliar application of acephate on 8 July drastically reduced TGPA populations, exceeding 99% control on 13 and 20 July, and 93% control on 29 July.

2006. The imidacloprid TD treatment provided excellent control (>99%) of TGPA through 11 July (Table 9.2), 62 DAT. Control declined gradually to 93% on 28 July. Unlike in 2004, aldicarb gave poor control, ranging from 57-73% from 6 June-11 July and 38-55% from 17-28 July. The acephate TW treatment did not control the TGPA and aldicarb was ineffective throughout the study. However, control was better in the aldicarb plots located in two of the four replications, where the plants grew more slowly. On 11 July, TGPA populations exceeded the economic threshold in all plots except those treated with imidacloprid. After the foliar treatments were applied on 13 July, all foliar treatments gave significant control through 28 July, 15 d after the sprays were applied. The foliar applications of acephate and methomyl, and the imidacloprid TD treatment gave similar 96 to 99% controls at 4 and 7 d after the foliar treatments were applied. TGPA populations started to build up in the imidacloprid treatment on 17 July, 11 wk after transplanting, but control was still about 93% on 28 July, 79 d after transplanting. Although the acephate TW treatment was ineffective, the foliar application of

acephate provided excellent control (99%) on 28 July. The lambda-cyhalothrin was the least effective foliar treatment providing 75-90% control. The performance of aldicarb may have been affected by >1,750 mm of rainfall on 4-5 July. A very high incidence of the fungal pathogen, *Pandora neoaphidis* (Humber), contributed to the TGPA population collapse after 28 July so the counts were discontinued.

2007. Imidacloprid TD provided excellent (>97%) control of TGPA through 6 July, 65 d after transplanting (Table 9.3). Control declined to 93% by 16 July, 75 d after transplanting, and 86% on 27 July, 86 d after transplanting. Aldicarb was ineffective in reducing the TGPA populations throughout the study, ranging from 43-71% control from 7-26 June, 40% on 6 and 16 July, and only 11% on 27 July (Table 9.3). This poor control was probably related to the late side-dress application of aldicarb (29 May) and dry weather through June. The acephate TW treatment provided some early control of TGPA, but control had declined to 54% on 22 June, 51 d after transplanting. Since TGPA populations exceeded the economic threshold on 22 June, the foliar treatments were applied on 23 June. The foliar application of acephate provided > 96% control through 16 July, 23 DAT. The foliar applications of methomyl and lambda-cyhalothrin gave significant control through 27 July (Table 9.3). The acephate foliar and imidacloprid TD treatments provided the best season-long control. TGPA populations started to buildup on 6 July and increased steadily through 27 July. The foliar applications of methomyl and lambda-cyhalothrin provided moderate control, but they were significantly less effective than the imidacloprid and acephate treatments. Despite the differences in TGPA infestation levels in this study, there were no significant differences in yield ($F = 2.131$; $P=0.1176$) (Table 9.3). The aldicarb treatment had a numerical 450 to 500 kg/ha higher yield than the untreated check and the lambda-cyhalothrin and methomyl foliar treatments (Table 9.3).

Over the 3-year study, imidacloprid, a neonicotinoids provided good to excellent season-long control of TGPA through 15 wk after transplanting (Tables 9.1-9.3) and its efficacy began to decline steadily after 15 wk. Acephate, the organophosphate, provided good to excellent control when applied as a foliar spray, but gave fair to poor control as a TW treatment (Tables 9.1-9.3). The carbamates (aldicarb, methomyl) and pyrethroid (lambda-cyhalothrin) provided moderate control of the TGPA. Systemic insecticides, especially the newer neonicotinoids formulations need to be monitored more frequently to determine whether TGPA is developing resistance to the neonicotinoids and provide important information on the changes that might

happen in the field populations. To assure the success of any insecticide resistance management program, in addition to reliable resistance testing and monitoring, field efficacy of the available insecticide molecules is critical in order to be in a position to carry out resistance risk analysis in the future.

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Table 9.1. Control of the tobacco-adapted form of the green peach aphid with insecticides belonging to four different classes on flue-cured tobacco, Virginia Tech Southern Piedmont AREC, Blackstone, VA, 2004.

Treatment and formulation	Rate g (AI)/ha	Application method [b]	Aphids/10 plants [a,c] 17-Jun	23-Jun	1-Jul	7-Jul	13-Jul	20-Jul	29-Jul
Aldicarb 15G	3,360	Band	1c	0d	5e	83d	1,005b	134c	606b
Imidacloprid 2F	146	TD	1c	0d	1e	70d	214c	56c	67d
Acephate 97WG + Acephate 97WG	840 840	TW + Foliar	4b	64c	169b	1,597b	34d	4d	152c
Methomyl 2.4LV	504	Foliar	24a	178a	525a	3,854a	1,287b	2,384b	1,780a
Lambda-cyhalothrin 1SC	33	Foliar	33a	121b	135b	595c	904b	2,120b	1,327a
Untreated control			17a	190a	604a	4,941a	17,074a	20,494a	2,219a

[a] Means within a column not followed by the same letter(s) are significantly different as indicated by Waller-Duncan k-ratio t-test (k-ratio = 100).
[b] Application method: TD= tray drench; TW = transplant water; Band=14 inch band at bedding on 8 May, Foliar spray on 8 July.
[c] Data were transformed to Log10(x+1) before analysis. Actual means are presented.

Table 9.2. Control of the tobacco-adapted form of the green peach aphid with insecticides belonging to four different classes on flue-cured tobacco, Virginia Tech Southern Piedmont AREC, Blackstone, VA, 2006.

Treatment and formulation	Rate lb g(AI)/ha	Application method [b]	Aphids/10 plants [a,c] 6-Jun	19-Jun	29-Jun	11-Jul	17-Jul	21-Jul	28-Jul
Aldicarb 15G	3,360	Band	15a	116b	1,171a	2,880b	10,982ab	10,456b	10,660b
Imidacloprid 2F	146	TD	0a	1c	19b	61c	359cd	844d	1,347c
Acephate 97WG +	840	TW +	4a	114ab	1,386a	6,311a	348cd	664cd	388d
Acephate 97WG	840	Foliar							
Methomyl 2.4LV	504	Foliar	17abc	64b	424a	3,194ab	205d	570cd	1,216c
Lambda-cyhalothrin 1SC	33	Foliar	27a	334a	882a	8,831a	1,874bc	2,476bc	5,216b
Untreated control			56a	271ab	3,405a	9,123a	17,760a	23,451a	20,606a

[a] Means within a column not followed by the same letter(s) are significantly different as indicated by Waller-Duncan k-ratio t-test (k-ratio = 100).
[b] Application method: TD= tray drench; TW = transplant water; Band=14-inch band at bedding on 12 May; Foliar spray on 13 July
[c] Data were transformed to Log10(x+1) before analysis. Actual means are presented.

Table 9.3. Control of the tobacco-adapted form of the green peach aphid with insecticides belonging to four different classes on flue-cured tobacco, Virginia Tech Southern Piedmont AREC, Blackstone, VA, 2007.

Treatment and formulation	Rate g g (AI)/ha	Application method [b]	Aphids/10 plants [a,c] 7-Jun	15-Jun	22-Jun	26-Jun	6-Jul	16-Jul	27-Jul	Yield lb/acre
Aldicarb 15G	3,360	Bands-SD	16b	87b	1,564b	3,452b	12,689b	16,780a	12,640a	3,272a
Imidacloprid 2F	146	TD	0c	1c	24c	167d	598d	1,568c	2,134c	3,026a
Acephate 97WG +	840	TW +	8c	48b	1,276b					
Acephate 97WG	840	Foliar				246d	456d	982d	2,345c	2,922a
Methomyl 2.4LV	504	Foliar	42a	240a	3,124a	567c	1,564c	3,124b	8,567b	2,836a
Lambda-cyhalothrin 1SC	33	Foliar	37a	186a	2,451a	789c	1,824c	2,589b	5,672b	2,769a
Untreated control			56a	237a	2,765a	10,454a	21,359a	28,341a	15,532a	2,800a

[a] Means within a column not followed by the same letter(s) are significantly different as indicated by Waller-Duncan k-ratio t-test (k- ratio=100).
[b] Application method: TD= tray drench; TW = transplant water; Bands-SD applied sidedress on 29 May; Foliar spray on 23 June.
[c] Data were transformed to Log10(x+1) before analysis. Actual means are presented.

Chapter 10

Summary, Significance, Unresolved Issues, and Future Work

This research investigates several factors responsible for insecticide resistance in the tobacco-adapted form of the green peach aphid (TGPA), *Myzus persicae* (Sulzer). TGPA is one of the most important insect pests of tobacco in the United States and world-wide. Insecticides play a significant role in managing TGPA, as cultural controls and natural enemies usually fail to maintain TGPA populations below economically damaging levels. It has been over 15 years since the insecticide resistance status of TGPA has been assessed (McPherson 1989, Harlow and Lampert 1990). Several new insecticides, mostly neonicotinoids, have been registered for use on tobacco since 1996. Since *M. persicae* has a history of developing resistance to many different insecticides, it is critical that baseline information be developed on the levels of susceptibility to the TGPA for both the older and the newer insecticides used on tobacco. Studies were conducted on TGPA colonies collected from nine different states in the eastern United States from 2004 through 2007 to assess insecticide resistance and the factors responsible for resistance development. These factors dealt with identifying the levels and causes of insecticide resistance in the TGPA in order to create a robust monitoring system for reporting its status in the United States. In Europe, a well-established resistance-monitoring program for the green peach aphid includes the collaboration of institutions in the European Union combined with support from the pesticide industry (Nauen and Denholm 2005). The Insecticide Resistance Action Committee (IRAC) was set up to monitor the resistance buildup in some of the most notorious pests around the world, including the green peach aphid (http://www.irac-online.org).

My research on TGPA resistance to the neonicotinoid, imidacloprid, is one of the most valuable contributions made by this research. Information on insecticide resistance is important due to the extensive usage of neonicotinoids for managing TGPA, as more than 80% of the tobacco acreage in Virginia is treated with imidacloprid or other neonicotinoids. Imidacloprid susceptibility was assessed in 151 field-collected colonies of TGPA including colonies of green and red colored morphs collected over a 4-yr period and a new orange morph collected in 2006 and 2007. I found that 14% percent of the test colonies tested had resistance ratios of 20 to 30-fold higher than the most susceptible colony and 4% of the colonies had 30 to 90-fold resistance

ratios. This suggests that high levels of resistance are present in some colonies. The most resistant colony in this study had an LC_{50} value for imidacloprid of 31 ppm and a 90-fold resistance ratio when combined for six tests and over 3 yr. However, the first three tests conducted in 2005 and 2006 had an average of 130-fold resistance compared with the most susceptible colony in the study. The last two tests, although relatively high, had lower resistance ratios, 42- and 52-fold, respectively. This suggests that resistance levels in highly resistant field-collected colonies of the TGPA may decline markedly under continuous laboratory maintenance without insecticidal pressure. The same thing could happen under field conditions where resistance may not carry over from one year to the next. TGPA colonies were sampled from the same geographic locations in consecutive years, but no correlations were found among the tolerance factors. There were large variations within the green and red color morphs. Both the most susceptible and the most resistant aphids were green morphs. We found considerable variations in some colonies that were sampled from more than one location within a county in the same year. For the green and red colored morphs collected from the same locations, the green morph generally had higher LC_{50} values than the red morphs, although the red morphs often had the highest LC_{50} values at some locations. With such a variation, it was difficult to reach a conclusion based on the statistical tests. Therefore, geographic location and the color morphism do not seem to be a factor contributing to differences in susceptibility to imidacloprid.

Recent studies from Greece have shown higher tolerance to imidacloprid among colonies collected from tobacco compared with colonies from other host plants (Margaritopoulos et al. 2007). Such differences are termed 'natural hardiness' of the tobacco-adapted forms compared with other host-adapted forms. This natural hardiness is attributed to an acquired tolerance to nicotine in the tobacco-adapted forms (Devine et al. 1996). Nicotine is transported apoplastically through xylem vessels, and the preferred feeding site for the aphids on tobacco is the phloem (Guthrie et al. 1962). Nevertheless, electrical penetration graph recordings in many aphid species including *M. persicae*, have shown that small amounts of nicotine may be imbibed on a regular basis when penetrating mesophyll cells in search of the phloem (Tjalingii 1985, Nauen and Elbert 1994). However, it was shown that this natural tolerance has nothing to do with the receptor affinity towards imidacloprid regardless of the host adaptation of *M. persicae* (Nauen et al. 1998). Therefore, the nicotine tolerance and the 'natural-hardiness' issue is still a pending question that needs to be resolved in the tobacco-adapted forms of *M. persicae.*

We found low to moderate resistance to the neonicotinoids in some TGPA colonies and several recent reports suggest that there is a potential for cross-resistance in the neonicotinoids, which have one mode of action. Therefore, alternative conventional insecticides such as several organophosphates and carbamates must be preserved to avoid over-reliance on the neonicotinoids. Abdel-Aal et al. (1992) described several kinetic parameters for TGPA esterases for different substrates and described their impact on organophosphate-resistant aphids. Even with this information, the monitoring of insecticide resistance in the TGPA has been ignored for the past 15 years. This was partly due to improved TGPA control practices and the introduction of the neonicotinoids for aphids on tobacco. For this reason, the TGPA has caused very few crop failures on tobacco. However, the efficacy of several organophosphate and carbamate insecticides has declined and only a few are still effective aphicides. Some of the organophosphates and carbamates could be removed from the market at any time leaving tobacco farmers with only the neonicotinoids and pymetrozine to manage the aphid.

To investigate changes in the status of TGPA resistance to these cholinesterase inhibitors, we used a microplate assay using 1-Naphthyl acetate as the substrate to screen 137 aphid colonies for total esterase activity. The green morphs had lower esterase levels than the red and orange morphs, while all orange morphs had high esterase activity. Esterase activities of red and green morphs were positively correlated with LC_{50} values for acephate (organophosphate) and methomyl (carbamate) as determined by leaf-dip bioassays. Of 25 colonies tested for esterase gene amplification, each had either E4 or FE4 gene amplified. However, I found that all orange morphs and one green morph had a rare combination of both an 865-bp band characteristic of the FE4 gene and an additional 381-bp band characteristic of a deleted upstream region of the E4 gene. This means that clones that had both 865-bp and 381-bp bands have elevated levels of both E4 and FE4. This is the first report of this phenomenon for TGPA and has only previously reported in two of 205 colonies of *M. persicae* collected and screened in England (Field and Foster 2002). Four of the five colonies with the deleted E4 gene in the current study were orange morphs collected from three different states in 2007, while the two colonies previously reported to have this characteristic were collected from a single location in Cambridgeshire, England. Since this 'deleted' version of the E4 gene in the orange morphs does not affect enzyme production and the subsequent resistance status of the aphid, it can be present with amplified FE4 gene (Field and Foster 2002). Since some green peach aphids have holocyclic life cycles in

England, the individuals with amplified E4 gene may co-exist with amplified FE4 and produce offspring that inherit both. However, they were unlikely to survive, as the chromosomal translocation associated with the amplified E4 genes would not allow it to happen (Blackman et al. 1996, Field and Foster 2002).

Since the TGPA in the United States has an anholocyclic life cycle, it opens a new avenue of research to determine how the genes are transmitted and how they relate to the color morphs. Since orange morphs were collected only in the 2006 and 2007, the origin of the E4+FE4 gene in these morphs cannot be viewed as a mere coincidence. It would be interesting to see if such amplifications are a coincidence by monitoring the occurrence of these genes in the orange morphs over the next few years. Functional and phylogenetic aspects of evolution of such a phenomenon need to be addressed in future experiments. Since this deleted E4 amplified fragment also occurred in one green morph with a high level of total esterase activity, it is more complicated than a mere association with body coloration.

Another way that insects develop resistance to organophosphate and carbamate insecticides is by target-site insensitivity. Mutations at the acetylcholinesterase (AChE) site, seen as modified AChE resistance (MACE) is a predominant mechanism seen in *M. persicae* in Europe. Such mechanism may have direct impact on the TGPA populations just like esterase-based resistance. Therefore, I characterized MACE resistance in field-collected TGPA. Sixty-six aphid colonies were screened for specific activity of the AChE. Of these, eight colonies with a range of specific activities were chosen to study the inhibition of AChE in the presence of two carbamate insecticides, methomyl, and pirimicarb. IC_{50} values for methomyl ranged from 0.35 to 2.4 µM while six out of eight colonies had lower values that ranged from 0.16 to 0.30 µM for pirimicarb. Two colonies that were inhibited by methomyl had very high IC_{50} values of 40.4 and 98.6 µM for pirimicarb showing target site insensitivity. Such insensitivity may be due to mutations in the *ace2* gene, which still needs to be confirmed by genetic and molecular analysis.

Other metabolic-based resistance that may be of prime importance is the glutathione *S*-transferases (GSTs), the isoenzymes that are involved in the metabolism and detoxification of a wide variety of xenobiotic compounds. The GST activity was measured in the three color morphs in 100 field-collected TGPA colonies. The results revealed a wide range of activity among the red and green morphs. About 45% of the red morphs had activity in the range of 200-300 pmol/min/mg of protein, while 53% of the green morphs were in the range of 100-200

pmol/min/mg protein. The six orange morphs collected on tobacco farms in 2007 had a narrow range of activity falling between 160 to 211 pmol/min/mg protein. This is an interesting phenomenon just like the general esterase activity, wherein, all the orange morphs had a narrow range of activities. This is the first study to quantify GST activity in the crude aphid homogenates. Earlier studies have revealed higher GST activities in other insect species (Nauen and Stumpf 2002, Konanz and Nauen 2004) as they dealt with purified enzyme, which would be a tedious and time-consuming process when dealing with the large number of colonies as in this study. My findings have created baseline information of the GST activity in colonies of the TGPA in the United States that will support future research.

Cytochrome P450 monooxygenases mediate the degradation and bioactivation of many xenobiotic compounds in insects. This is the prime mechanism responsible for neonicotinoid resistance in the Q-biotype of whitefly, *Bemisia tabaci* (Gennadius) (Rauch and Nauen 2003). The impact of monooxygenase activity was determined by one of the most sensitive methods available, the fluorometric assay. This assay involves detection of O-deethylation of 7-ethoxycoumarin by monooxygenases to the highly fluorescent 7-hydroxycoumarin (Rauch and Nauen 2003). However, we were able to generate a 7-hydroxycoumarin standard curve, P450 activity for the aphid homogenates were always below the detection limit of the instrument (TRIAD multimode detector, Dynex Technologies, USA). We tried many deviations in the substrate concentrations, incubation timings, and numbers of insects used for enzyme extraction, but were unable to detect and quantify the P450 activity in the TGPA colonies.

The influence of temperature-mediated synergisms on the toxicity of insecticides to colonies of red and green morphs of the TGPA was evaluated using leaf-dip bioassay procedures in laboratory incubators. Post-exposure temperatures of 15, 20, and 25^{o}C were evaluated for insecticides representing four different chemical classes. The temperature change from 15 to 20^{o}C caused almost 3-fold increases in the toxicity for methomyl, acephate, and imidacloprid in both color morphs. In contrast, the toxicity of lambda-cyhalothrin decreased as the temperature increased, showing a negative temperature coefficient. This shows that even minor deviations in temperature during insecticide bioassays can have important impacts on the test results obtained for reporting insecticide resistance in the TGPA. Our findings emphasize the importance of conducting bioassay under controlled environmental conditions. The results indicate the

importance of abiotic factors that may influence the toxicity of insecticides when reporting resistance status.

Imidacloprid continues to be the most commonly used insecticide to control aphids on tobacco. Therefore, studies were conducted to determine the persistence and distribution of imidacloprid in the tobacco plant system under field and greenhouse conditions. Imidacloprid concentrations were determined by indirect bioassay experiments with a field-collected red morph. The results showed that leaf position, imidacloprid rate, and time after imidacloprid application all affected the indirect measurement of the concentration of the toxicant in the leaf. Aphid mortality was higher on the lower, older leaves than the upper, younger leaves indicating that the concentrations of imidacloprid and its metabolites were unevenly distributed. In field experiments, higher tobacco-adapted form of the green peach aphid populations occurred on tobacco treated with imidacloprid at rates below the field recommended rate. The development of TGPA populations in the field was consistent with the laboratory bioassays. This study evaluated the concentrations of imidacloprid in an indirect method, but additional studies need to be conducted to estimate the actual concentrations of imidacloprid and its active metabolites in the leaves. This study indicates that there is a differential concentration of the toxicant that could have sublethal effects on the aphid populations on tobacco. Future studies should be conducted to learn more about the fecundity of the survived aphids to see if hormoligosis increases survival due to sublethal exposure to the insecticide.

Field trials were conducted to evaluate the performance of various insecticides currently registered for TGPA control on tobacco. Imidacloprid applied as a tray drench treatment and acephate applied as foliar sprays were the most effective treatments. Moderate declines in control with imidacloprid were observed at 75-76 d after transplanting in 2006 and 2007 and 14% reduction in control at 87 d after treatment. Aldicarb gave good to excellent control in one of three yr and poor to fair control in the other two yr. Methomyl and lambda-cyhalothrin gave fair to good control in all three years. The poor performance of aldicarb in the two yr may have been related to the presence of E4 or FE4 resistance in the naturally occurring TGPA in the experimental plots. None of the insecticides gave 100% control with any of the insecticide treatments. This may indicate that some aphids may have a low level of resistance to the insecticides that I used in my experiments or that they are not receiving enough chemical to kill them.

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Appendix 1. Susceptibility to imidacloprid of various colonies of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer), collected from tobacco, 2004.

S.No	Colony	County	State	Color	N	LC_{50}*	95% CI	Slope ± SE	H†	RR‡
1	**SP-AREC (GH)**	**Nottoway**	**VA**	**Green**	**720**	**0.4**	**0.1 - 1.0**	**0.77 ± 0.19**	**0.64**	**1**
2	Giles	Campbell	VA	Red	480	0.7	0.1 - 0.8	1.22 ± 0.32	0.57	2
3	Walker	Washington	VA	Green	640	0.9	0.2 - 2.3	2.61 ± .0.85	0.45	2
4	SP-AREC (GH)	Nottoway	VA	Red	640	1.2	0.7 - 3.1	1.12 ± 0.22	0.54	3
5	SW-AREC	Washington	VA	Red	640	2.1	0.7 - 3.6	2.01 ± .0.90	0.32	6
6	Townsend	Dinwiddie	VA	Red	720	2.3	1.1 - 3.8	1.24 ± 0.22	0.40	6
7	Semtner	Nottoway	VA	Green	720	2.5	1.2 - 3.8	2.02 ± 0.97	0.42	7
8	Opie	Mecklenburg	VA	Green	720	2.9	0.3 - 7.9	1.54 ± 0.41	0.42	8
9	Peek	Washington	VA	Red	640	3.0	1.9 - 4.1	1.67 ± 0.23	1.04	8
10	Hutcherson	Campbell	VA	Red	440	3.1	2.2 - 4.9	1.72 ± 0.22	0.43	8
11	SP-AREC (TR)	Nottoway	VA	Red	780	3.5	1.4 - 5.2	1.34 ± 0.23	0.44	9
12	Keates	Franklin	VA	Red	380	4.2	2.9 - 5.9	1.92 ± 0.22	0.64	11
13	SP-AREC	Nottoway	VA	Red	480	5.1	0.2 - 16.8	1.11 ± 0.32	0.44	14
14	Adkinson	Pittsylvania	VA	Green	720	5.6	4.3 - 7.8	2.13 ± 0.37	0.34	15
15	Bledsoe	Nottoway	VA	Red	640	6.9	5.3 - 8.7	2.01 ± 0.35	0.32	19
16	Giles	Campbell	VA	Green	740	8.1	3.3 - 18.4	1.55 ± 0.31	0.48	22
17	Townsend	Dinwiddie	VA	Green	640	8.6	5.4 - 12.7	1.19 ± 0.17	1.80	23
18	Hatchet	Franklin	VA	Red	820	9.3	5.9 - 14.1	1.22 ± 0.18	0.41	25

* LC_{50} values expressed as parts per million (mg/L) imidacloprid (Admire 2F formulation)

† Heterogeneity factor = Observed chi-square value / Degrees of freedom

† Correction factor when the Pearson's chi-square statistic is significant at $P = 0.05$

‡ Resistance ratio (RR): LC_{50} value of field population/LC_{50} value of susceptible strain

Appendix 2. Susceptibility of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer), to imidacloprid in the colonies collected in 2005.

S.No	Colony	County	State	Color	N	LC_{50}*	95% CI	Slope ± SE	H†	RR‡
1	Witcher	Franklin	VA	Red	640	0.6	0.2 - 0.9	1.93 ± 0.49	0.36	2
2	NC State	Wake	NC	Red	1340	1.9	1.1 - 2.7	1.28 ± 0.23	0.33	5
3	Francis	Charlotte	VA	Green	960	2.1	1.1 - 3.0	1.93 ± 0.44	0.33	6
4	SP-AREC	Nottoway	VA	Red	640	2.4	1.3 - 4.1	1.72 ± 0.25	0.54	6
5	Walker	Granville	NC	Green	720	2.8	1.4 - 8.6	0.64 ± 0.21	0.30	7
6	Bowen	Tift	GA	Red	480	3.4	2.6 - 4.7	1.69 ± 0.25	0.49	9
7	Howard	Charlotte	VA	Red	320	3.4	0.2 - 12.6	1.74 ± 0.45	1.12	9
8	Oxford	Granville	NC	Green	640	3.8	2.5 - 6.9	1.07 ± 0.22	0.15	10
9	Farris	Campbell	VA	Red	640	4.4	2.0 - 7.3	2.38 ± 0.31	2.10	12
10	Keates	Franklin	VA	Red	380	4.7	2.8 - 6.9	1.23 ± 0.19	1.64	13
11	Wyatt	Pittsylvania	VA	Red	320	5.0	3.0 - 8.2	1.17 ± 0.25	0.23	13
12	SP-AREC	Nottoway	VA	Green	480	5.1	3.0 - 8.1	1.85 ± 0.23	0.42	14
13	Farris	Campbell	VA	Green	480	6.3	4.6 - 9.2	2.15 ± 0.32	1.21	17
14	Giles	Campbell	VA	Green	720	7.0	3.8 - 11.7	0.87 ± 0.16	0.65	19
15	Bowen	Tift	GA	Green	720	7.5	5.3 - 12.5	1.22 ± 0.23	0.89	20
16	Clayton	Johnston	NC	Red	960	8.3	5.2 - 20.5	1.24 ± 0.25	0.54	22
17	Wyatt	Pittsylvania	VA	Green	640	9.5	4.0 - 12.9	0.66 ± 0.25	0.33	26
18	Anderson	Halifax	VA	Red	480	9.8	6.8 - 14.3	2.46 ± 0.29	1.13	26
19	Mitchell	Franklin	VA	Red	740	10.2	6.9 - 15.2	1.82 ± 0.22	0.52	28
20	Walker	Granville	NC	Red	480	10.5	6.3 - 29.6	1.28 ± 0.27	0.16	28
21	Bass	Campbell	VA	Green	620	10.7	5.4 - 17.6	0.85 ± 0.23	0.10	29
22	Oxford	Granville	NC	Red	720	13.9	7.4 - 46.8	1.02 ± 0.29	0.95	38
23	Witcher	Franklin	VA	Green	720	15.1	6.4 - 30.1	0.75 ± 0.23	0.45	41
24	Clayton	Johnston	NC	Green	1020	33.5	28.0 - 38.8	1.54 ± 0.08	1.07	91

* LC_{50} values expressed as parts per million (mg/L) imidacloprid (Admire 2F formulation)

† Heterogeneity factor = Observed chi-square value / Degrees of freedom

† Correction factor when the Pearson's chi-square statistic is significant at $P = 0.05$

‡ Resistance ratio (RR): LC_{50} value of field population/LC_{50} value of susceptible strain

Appendix 3. Susceptibility of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer), to imidacloprid in the colonies collected in 2006.

S.No	Colony	County	State	Color	N	LC_{50}*	95% CI	Slope ± SE	H†	RR‡
1	Walker	Washington	VA	Green	640	0.8	0.1- 1.5	2.16 ± .0.93	0.42	2
2	Washburn	Mecklenburg	VA	Red	760	0.8	0.1 - 2.4	1.00 ± 0.25	1.33	2
3	NC State	Wake	NC	Red	640	1.0	0.5 - 2.7	1.36 ± 1.17	0.42	3
4	Semtner	Nottoway	VA	Green	720	1.2	0.1 - 1.8	2.48 ± 1.03	0.45	3
5	Pee Dee-AREC	Florence	SC	Green	840	1.2	0.8 - 1.6	2.46 ± 0.52	0.29	3
6	SP-AREC (GH)	Nottoway	VA	Green	640	1.3	0.5 - 2.6	1.80 ± 0.22	0.64	4
7	Bowen	Tift	GA	Green	720	1.6	1.1 - 2.2	2.52 ± 0.41	0.23	4
8	Parker	Pittsylvania	VA	Green	640	1.7	1.0 - 2.6	1.07 ± 0.56	0.45	5
9	Parker	Pittsylvania	VA	Red	720	1.7	0.7 - 2.4	2.15 ± 0.56	0.35	5
10	Cross Creek	Cumberland	NC	Red	720	1.9	1.0 - 2.5	0.90 ± 0.43	0.35	5
11	SP-AREC (RE)	Nottoway	VA	Red	480	2.2	1.9 - 3.6	1.43 ± 0.22	0.39	6
12	Witcher	Franklin	VA	Green	640	2.3	1.4 - 3.3	1.55 ± 0.23	0.51	6
13	Rogers	Brunswick	VA	Red	320	2.3	1.7 - 2.8	3.84 ± 0.85	0.32	6
14	NY-Riverhead	Suffolk	NY	Red	720	2.4	1.6 - 3.3	1.76 ± 0.26	0.29	6
15	SC-AREC	Horry	SC	Red	560	2.6	1.6 - 3.7	1.76 ± 0.27	0.18	7
16	Jackson	Henry	VA	Red	960	2.6	1.7 - 3.3	2.8 ± 0.60	0.28	7
17	Neal	Lee	VA	Red	320	2.6	1.2 - 4.1	1.25 ± 0.25	0.80	7
18	D Moore	Brunswick	VA	Red	640	2.7	1.9 - 3.9	3.04 ± 0.59	0.38	7
19	Johnson	Surry	NC	Green	640	2.7	1.7 - 3.8	1.77 ± 0.26	0.49	7
20	Manning	Mecklenburg	VA	Green	640	2.7	1.9 - 3.6	1.93 ± 0.28	0.37	7

(Continued)

Appendix 3. Susceptibility of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer), to imidacloprid in the colonies collected in 2006 (Continued)

S.No	Colony	County	State	Color	N	LC_{50}*	95% CI	Slope ± SE	H†	RR‡
21	Roberts	Brunswick	VA	Green	480	2.7	1.4 - 4.2	1.35 ± 0.22	0.35	7
22	Darrell Johnson	Russell	VA	Red	720	2.9	2.0 - 3.9	1.92 ± 0.27	0.33	8
23	Lunenburg	Lunenburg	VA	Red	320	2.9	1.7 - 4.0	1.69 ± 0.30	1.50	8
24	Mt. Airy	Pittsylvania	VA	Red	720	2.9	2.1 - 3.9	3.37 ± 0.62	0.63	8
25	Patrick Henry	Charlotte	VA	Green	480	3.0	1.3 - 3.4	1.92 ± 0.47	0.35	8
26	Ayres	Patrick	VA	Red	320	3.0	2.3 - 4.1	2.36 ± 0.35	0.35	8
27	Adams	Lee	VA	Red	640	3.1	2.0 - 5.1	2.23 ± 0.35	0.54	8
28	Bobby	Russell	VA	Red	480	3.1	2.3 - 5.8	1.34 ± 0.22	0.38	8
29	UGA	Grady	GA	Red	720	3.1	1.8 - 4.7	1.25 ± 0.18	0.21	8
30	Sparrow Ln	Halifax	VA	Red	480	3.3	0.1 - 7.5	0.97 ± 0.39	1.01	9
31	Turbeville	Halifax	VA	Red	720	3.3	2.1 - 4.7	1.46 ± 0.20	0.25	9
32	Cross Creek	Cumberland	NC	Green	480	3.4	2.5 - 4.3	2.50 ± 0.47	0.26	9
33	Clayton	Johnston	NC	Red	480	3.5	2.5 - 4.8	1.86 ± 0.26	0.45	9
34	Washburn	Mecklenburg	VA	Green	480	3.5	2.1 - 5.4	2.30 ± 0.22	0.83	10
35	Glasscock	Prince Edward	VA	Red	960	3.6	2.2 - 5.2	1.64 ± 0.28	1.41	10
36	Anderson	Brunswick	VA	Red	720	3.7	2.8 - 4.9	2.25 ± 0.30	0.44	10
37	Barnard	Amelia	VA	Green	640	3.7	2.6 - 5.0	1.88 ± 0.25	0.42	10
38	Semtner	Nottoway	VA	Red	840	3.8	2.0 - 4.8	2.40 ± 0.25	0.56	10
39	Yanceyville	Caswell	NC	Red	480	3.9	2.8 - 5.1	2.06 ± 0.27	0.50	10
40	Manning	Mecklenburg	VA	Red	320	3.9	2.7 - 5.4	1.71 ± 0.23	0.79	10

(Continued)

Appendix 3. Susceptibility of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer), to imidacloprid in the colonies collected in 2006 (Continued)

S.No	Colony	County	State	Color	N	LC_{50}*	95% CI	Slope ± SE	$H^{\dagger}$	$RR^{\ddagger}$
41	R Moore	Lunenburg	VA	Green	480	4.3	3.0 - 5.9	1.73 ± 0.23	0.65	12
42	Yanceyville	Caswell	NC	Green	380	4.3	2.9 - 6.1	1.50 ± 0.20	0.38	12
43	Angel	Franklin	VA	Red	480	4.5	3.0 - 6.5	1.47 ± 0.19	0.32	12
44	Owen	Pittsylvania	VA	Red	640	4.6	2.0 - 6.3	1.64 ± 0.22	0.35	12
45	Windsor	Hartford	CT	Red	640	4.6	3.2 - 6.5	1.56 ± 0.23	1.06	12
46	SP-AREC (A/FB)	Nottoway	VA	Green	720	5.2	3.8 - 9.2	1.23 ± 0.24	0.35	14
47	SP-AREC (RE)	Nottoway	VA	Green	480	5.7	3.9 - 8.0	1.58 ± 0.19	0.41	15
48	Townsend	Dinwiddie	VA	Red	320	5.9	4.3 - 7.9	1.99 ± 0.25	0.39	16
49	Dudley	Wayne	NC	Red	840	6.0	2.0 - 9.5	1.53 ± 0.22	1.20	16
50	Wallace	Dinwiddie	VA	Green	420	6.0	4.4 - 8.0	2.06 ± 0.27	0.66	16
51	Barnard	Amelia	VA	Red	720	6.0	3.6 - 8.7	2.09 ± 0.38	0.38	16
52	Glasscock	Prince Edward	VA	Green	480	6.2	3.2 - 8.2	1.23 ± 0.21	1.12	17
53	Coffee	Lunenburg	VA	Green	480	6.4	1.9 - 13.9	1.31 ± 0.28	1.84	17
54	Green Bay	Prince Edward	VA	Red	480	7.0	3.1 - 10.8	1.93 ± 0.43	0.83	19
55	MD-AREC	Prince Georges	MD	Red	320	7.2	1.0 - 20.0	1.11 ± 0.28	0.65	19
56	SC-AREC	Horry	SC	Green	640	7.6	5.5 - 10.1	1.71 ± 0.19	1.65	20
57	SP-AREC	Nottoway	VA	Orange	960	8.6	6.2 - 11.8	1.78 ± 0.21	0.39	23
58	Townsend	Dinwiddie	VA	Green	640	8.9	5.3 - 12.8	1.27 ± 0.24	0.32	24
59	Coffee	Lunenburg	VA	Red	840	9.9	3.7 - 14.4	1.78 ± 0.35	1.16	27
60	Clary	Mecklenburg	VA	Red	720	10.3	7.2 - 14.8	1.47 ± 0.18	0.61	28
61	Pee Dee-AREC	Florence	SC	Red	720	11.1	8.4 - 14.6	2.29 ± 0.29	0.34	30
62	Mitchell	Franklin	VA	Red	480	22.9	16.8 - 31.4	1.88 ± 0.24	0.69	62

* LC_{50} values expressed as parts per million (mg/L) imidacloprid (Admire 2F formulation)

† Heterogeneity factor = Observed chi-square value / Degrees of freedom

† Correction factor when the Pearson's chi-square statistic is significant at $P = 0.05$

‡ Resistance ratio (RR): LC_{50} value of field population/LC_{50} value of susceptible strain

Appendix 4. Susceptibility of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer), to imidacloprid in the colonies collected in 2007.

S.No	Colony	County	State	Color	N	LC_{50}*	95% CI	Slope ± SE	H†	RR‡
1	Johnson	Colleton	SC	Green	720	0.7	0.2 - 1.1	1.72 ± 0.41	0.28	2
2	Johnson	Colleton	SC	Red	640	0.9	0.2 - 2.0	1.23 ± 0.43	0.32	2
3	Route 360	Pittsylvania	VA	Red	640	1.2	0.5 - 3.0	0.74 ± 0.22	0.32	3
4	Witcher	Franklin	VA	Red	320	1.2	0.8 - 1.6	2.60 ± 0.55	0.36	3
5	Johnson	Darlington	SC	Red	640	1.2	0.2 - 1.9	1.93 ± 0.42	0.25	3
6	Hutchinson	Halifax	VA	Green	480	1.3	0.8 - 1.8	2.21 ± 0.42	0.25	4
7	Mitchell	Franklin	VA	Red	840	1.8	1.2 - 2.5	1.91 ± 0.31	0.59	5
8	Clays	Nottoway	VA	Green	640	1.8	1.1 - 2.6	1.58 ± 0.25	0.28	5
9	SP-AREC	Nottoway	VA	Red	620	1.8	0.9 - 3.2	1.64 ± 0.38	0.67	5
10	Pittard	Mecklenburg	VA	Red	380	1.9	1.2 - 4.8	2.42 ± 0.32	0.25	5
11	Hite	Lunenburg	VA	Red	740	2.1	1.2 - 3.9	2.50 ± 0.25	0.22	6
12	Greenhouse	Greene	NC	Red	640	2.3	1.7 - 3.1	2.27 ± 0.34	0.26	6
13	Hale	Macon	TN	Red	480	2.4	1.7 - 3.3	1.84 ± 0.27	0.24	7
14	Pittard	Mecklenburg	VA	Green	320	2.4	1.8 - 3.2	2.36 ± 0.35	0.22	7
15	MD-AREC	Prince Georges	MD	Orange	840	2.5	1.7 - 3.3	2.01 ± 0.29	0.24	7
16	Hite	Lunenburg	VA	Green	620	2.9	2.0 - 5.7	1.38 ± 0.22	0.22	8
17	Dellenback	Patrick	VA	Green	640	3.1	2.0 - 4.5	1.50 ± 0.21	0.43	8
18	Semtner	Nottoway	VA	Red	720	3.2	2.3 - 4.9	3.13 ± 0.45	0.23	9
19	Ayres	Patrick	VA	Red	480	3.2	2.2 - 5.0	2.01 ± 0.33	0.33	9
20	Elvis	Franklin	VA	D.Green	620	3.2	1.7 - 5.7	2.12 ± 0.34	0.23	9
21	UGA	Tift	GA	Red	640	3.2	2.0 - 4.9	1.52 ± 0.23	0.22	9
22	Hite	Lunenburg	VA	Orange	780	3.4	2.3 - 4.8	1.57 ± 0.21	0.12	9
23	Rogers	Henry	VA	Red	480	3.5	1.5 - 5.6	1.74 ± 0.23	0.23	10
24	Mitchell	Franklin	VA	Green	720	3.6	1.8 - 5.4	1.95 ± 0.34	0.43	10
25	Hutchinson	Halifax	VA	Red	440	3.6	2.6 - 4.9	1.89 ± 0.25	0.55	10
26	Bowen	Tift	GA	Red	480	3.7	2.4 - 5.3	1.48 ± 0.20	0.42	10
27	Clayton	Johnston	NC	Red	840	3.7	2.0 - 5.0	2.32 ± 0.35	0.25	10
28	SP-AREC	Nottoway	VA	Green	640	3.7	2.3 - 5.2	1.43 ± 0.22	0.47	10
29	KY-AREC	Lexington	KY	Red	720	4.2	2.8 - 5.9	1.50 ± 0.20	0.38	11

(Continued)

Appendix 4. Susceptibility of the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) to imidacloprid in the colonies collected in 2007.

S.No	Colony	County	State	Color	N	LC_{50}*	95% CI	Slope ± SE	$H^{\dagger}$	RR‡
30	Dellenback	Patrick	VA	D.Green	720	4.2	2.7 - 6.1	1.85 ± 0.27	0.88	11
31	UGA	Tift	GA	Orange	840	4.5	3.3 - 6.1	2.03 ± 0.26	0.32	12
32	Dellenback	Patrick	VA	Red	740	4.7	3.4 - 6.3	1.99 ± 0.26	0.45	13
33	Semtner	Nottoway	VA	Green	720	4.8	3.4 - 6.9	2.11 ± 0.34	0.22	13
34	MD-AREC	Prince Georges	MD	Red	980	5.5	3.7 - 8.2	1.69 ± 0.22	0.32	15
35	Highland - RIM	Robertson	TN	Red	380	6.2	4.2 - 8.9	1.42 ± 0.18	0.60	17
36	Bledsoe	Nottoway	VA	Red	640	6.2	5.3 - 8.2	2.22 ± 0.35	0.35	17
37	SP-AREC	Nottoway	VA	Orange	840	6.9	5.3 - 9.0	2.43 ± 0.32	0.34	19
38	Dellenback	Patrick	VA	Orange	840	7.0	5.2 - 9.4	2.04 ± 0.25	0.47	19
39	UK-AREC	Caldwell	KY	Green	480	7.3	5.3 - 10.1	1.75 ± 0.21	0.42	20
40	Univ TN	Knoxville	TN	Red	480	7.7	5.8 - 10.1	2.42 ± 0.32	0.25	21
41	Witcher	Franklin	VA	Green	540	8.2	5.7 - 10.2	2.57 ± 0.54	0.22	22
42	SP-AREC (Late)	Nottoway	VA	Orange	840	8.4	6.6 - 10.7	3.11 ± 0.50	0.28	23
43	TN-AREC	Loudon	TN	Red	720	8.5	6.5 - 11.1	2.49 ± 0.34	0.39	23
44	Clayton	Johnston	NC	Green	840	10.2	8.1 - 15.0	2.12 ± 0.30	0.23	28
45	Bowen	Tift	GA	Green	720	23.6	17.6 - 31.8	2.07 ± 0.26	0.60	64

* LC_{50} values expressed as parts per million (mg/L) imidacloprid (Admire 2F formulation)

† Heterogeneity factor = Observed chi-square value / Degrees of freedom

† Correction factor when the Pearson's chi-square statistic is significant at $P = 0.05$

‡ Resistance ratio (RR): LC_{50} value of field population/LC_{50} value of susceptible strain

Appendix 5: Protocol PCR-RFLP on *Myzus persicae*

(Modified from Field et al. 1996 Insect Mol. Biol. 5: 197-202)

PCR

Water	30 µl
Taq 10X buffer	5 µl
$MgCl_2$ (50 mM)	3 µl
dNTP (5 mM of each)	1 µl
Est3N (5 µM)	3 µl
Est4P (5 µM)	3 µl
Taq DNA Polymease (Invitrogen)	1 µl
DNA (atleast 100ng)	4 µl
Total PCR volume =	50 µl

DNA extracted from Qiagen DNA extraction kit and amount of DNA was quantified by spectrophotometer.

PCR cycles:		
95°C	5'	
94°C	1"	
52 °C	1'30"	X 35 cycles
72°C	2'	

Primers:
Est3N: 5'-aaa tca tat tcc cgg gtt c-3'
Est4P: 5'-tga gta atc tta gtg aac ctg-3'

Digestion with *Spe*I (or) *Hind*III

SpeI (actagt) cuts E4 allele
*Hind*III (aagctt) cuts FE4 allele

DNA (=PCR product)	10 µl
Enzyme buffer (10X)	2.5 µl
Enzyme	1 µl
(*Hind*III or *Spe*I = 1 µl)	

All samples were incubated at 37^0C over night for digestion.
Migration on a 1.5% Agarose gel, stained with ethidium bromide.

Appendix 6. Responses of the tobacco-adapted form of the green peach aphid colonies to leaf-dip bioassays for methomyl (Lannate 90SP) and general esterase activity.

S.No.	Colony	County	Year	Color	State	Esterase[a]	LC_{50} (ppm)	95% CI	Slope ± SE	RR[b]
1	Angel	Franklin	2006	Red	VA	95.8	324.6	286.2 - 341.3	1.42 ± 0.024	7
2	Ayres	Patrick	2006	Red	VA	48.6	95.6	42.5 - 135.4	1.12 ± 0.032	2
3	Bledsoe	Nottoway	2004	Red	VA	78.1	298.5	253.6 - 388.5	1.64 ± 0.022	6
4	Bowen	Tift	2007	Green	GA	56.9	164.8	123.6 - 230.9	1.43 ± 0.021	3
5	Clay's	Nottoway	2007	Green	VA	110.8	335.5	302.8 - 366.4	1.40 ± 0.026	7
6	Clayton	Johnston	2005	Green	NC	56.6	138.7	96.7 - 178.7	1.74 ± 0.023	3
7	Dellenback	Patrick	2007	D Green	VA	98.5	242.6	192.8 - 284.1	1.72 ± 0.026	5
8	Dellenback	Patrick	2007	Orange	VA	214.2	682.2	564.5 - 780.6	1.42 ± 0.013	14
9	Highland-RIM	Robertson	2007	Red	TN	87.3	341.2	322.8 - 386.4	1.52 ± 0.021	7
10	Hite	Lunenburg	2007	Orange	VA	174.5	485.9	435.5 - 520.6	1.11 ± 0.050	10
11	Howard	Charlotte	2005	Red	VA	88.0	251.7	192.5 - 320.6	1.31 ± 0.010	5
12	Peek	Washington	2004	Red	VA	64.6	244.3	206.4 - 316.8	1.86 ± 0.023	5
13	Johnson	Darlington	2007	Red	SC	149.8	582.6	516.5 - 648.3	1.16 ± 0.012	12
14	Johnson	Surry	2006	Green	NC	106.9	454.7	360.8 - 580.9	1.22 ± 0.037	9
15	Keates	Franklin	2004	Green	VA	51.1	142.7	98.4 - 212.5	1.74 ± 0.027	3
16	KY-AREC	Lexington	2007	Red	KY	86.0	352.5	321.5 - 394.3	2.74 ± 0.025	7
17	MD-AREC	Prince Georges	2007	Orange	MD	193.6	590.3	520.3 - 670.8	1.45 ± 0.014	12
18	Mitchell	Franklin	2006	Red	VA	107.9	205.9	156.4 - 235.5	1.43 ± 0.032	4
19	NC-State	Wake	2005	Red	VA	94.1	149.9	85.6 - 260.8	1.11 ± 0.034	3

(Continued)

Appendix 6. Responses of the tobacco-adapted form of the green peach aphid colonies to leaf-dip bioassays for methomyl (Lannate 90SP) and general esterase activity (Continued).

S.No.	Colony	County	Year	Color	State	Esterase[a]	LC_{50} (ppm)	95% CI	Slope ± SE	RR[b]
20	NY-Riverhead	Suffolk	2006	Red	NY	89.6	344.4	210.6 - 450.7	1.42 ± 0.032	7
21	Pee Dee-AREC	Florence	2006	Green	SC	46.0	77.9	43.2 - 91.6	1.61 ± 0.045	2
22	R Moore	Lunenburg	2006	Green	VA	68.7	286.2	242.8 - 318.2	1.58 ± 0.034	6
23	Rogers	Henry	2007	Red	VA	74.2	443.6	394.3 - 512.6	1.32 ± 0.033	9
24	SC-AREC	Horry	2006	Green	SC	90.2	520.6	480.2 - 580.1	1.48 ± 0.023	10
25	Semtner	Nottoway	2006	Green	VA	133.1	482.2	394.6 - 520.5	1.21 ± 0.016	10
26	SP-AREC	Nottoway	2006	Orange	VA	153.5	524.5	493.7 - 560.3	1.52 ± 0.024	11
27	SP-AREC(CON)	Nottoway	2005	Red	VA	54.5	323.0	294.5 - 356.7	1.32 ± 0.023	6
28	SP-AREC (OR)	Nottoway	2007	Orange	VA	165.8	532.6	420.4 - 640.8	1.12 ± 0.022	11
29	SP-AREC (OR2)	Nottoway	2007	Orange	VA	141.7	530.6	456.2 - 610.3	1.26 ± 0.015	11
30	TN-AREC	Loudon	2007	Red	TN	134.5	628.0	554.3 - 720.6	1.54 ± 0.020	13
31	Townsend	Dinwiddie	2004	Green	VA	203.2	656.7	584.1 - 712.3	1.36 ± 0.028	13
32	UGA	Tift	2007	Orange	GA	178.2	585.7	430.5 - 720.1	1.13 ± 0.017	12
33	Univ TN	Knoxville	2007	Red	TN	83.1	378.8	310.2 - 420.1	1.54 ± 0.014	8
34	Wallace	Dinwiddie	2006	Green	VA	57.1	49.9	23.6 - 69.4	1.95 ± 0.034	1
35	Windsor	Hartford	2006	Red	CT	81.9	331.5	316.8 - 364.4	1.28 ± 0.025	7
36	Wyatt	Pittsylvania	2005	Red	VA	89.7	173.4	137.8 - 230.4	1.64 ± 0.013	3
37	Yanceyville	Caswell	2006	Green	NC	63.3	148.6	94.8 - 212.6	1.13 ± 0.025	3

[a] Total general esterase activity in nmol/min/mg protein

[b] Resistance ratio (RR): LC_{50} value of field population/LC_{50} value of most susceptible strain

Appendix 7. Responses of the tobacco-adapted form of the green peach aphid colonies to leaf-dip bioassays for acephate (Orthene 97) and general esterase activity.

S.No.	Colony	County	Year	Color	State	Esterase[a]	LC_{50} (ppm)	95% CI	Slope ± SE	RR[b]
1	Adams	Lee	2006	Red	VA	62.7	223.5	184.5 - 280.9	1.74 ± 0.025	3
2	Anderson	Brunswick	2004	Red	VA	70.8	287.6	154.6 - 350.8	1.16 ± 0.012	4
3	Ayres	Patrick	2006	Red	VA	48.6	122.2	87.6 - 160.5	1.43 ± 0.032	2
4	Barnard	Amelia	2006	Green	VA	47.4	82.6	38.2 - 134.5	1.11 ± 0.034	1
5	Bledsoe	Nottoway	2006	Green	VA	118.9	423.5	386.2 - 480.8	1.42 ± 0.032	6
6	Bowen	Tift	2007	Green	GA	56.9	198.2	123.5 - 250.7	1.61 ± 0.045	3
7	Clary	Mecklenburg	2006	Red	VA	81.4	388.2	267.2 - 476.8	1.58 ± 0.034	6
8	Clay's	Nottoway	2007	Green	VA	110.8	413.4	345.6 - 481.3	1.32 ± 0.033	6
9	Clayton	Johnston	2005	Green	NC	56.6	121.6	87.6 - 143.5	1.48 ± 0.023	2
10	Dellenback	Patrick	2007	Orange	VA	214.2	443.8	384.6 - 493.1	1.21 ± 0.016	6
11	Dudley	Wayne	2006	Red	NC	94.5	387.6	310.4 - 420.6	1.52 ± 0.024	6
12	Witcher	Franklin	2007	D. Green	VA	75.6	228.4	182.4 - 278.2	1.23 ± 0.023	3
13	Witcher	Franklin	2005	Green	VA	139.3	398.8	230.7 - 460.3	1.12 ± 0.022	6
14	Green Bay	Prince Edward	2006	Red	VA	81.0	344.2	285.7 - 410.8	1.95 ± 0.034	5
15	Highland-RIM	Robertson	2007	Red	TN	87.3	345.2	294.6 - 392.4	1.28 ± 0.025	5
16	Hite	Lunenburg	2007	Orange	VA	174.5	452.5	384.6 - 498.9	1.64 ± 0.013	6
17	Howard	Charlotte	2005	Red	VA	88.0	296.7	232.5 - 342.1	1.13 ± 0.025	4
18	Peak	Washington	2004	Red	VA	64.6	245.6	202.6 - 289.1	1.11 ± 0.010	3
19	Johnson	Darlington	2007	Red	SC	149.8	402.2	383.6 - 432.1	1.86 ± 0.023	6
20	Johnson	Surry	2006	Green	NC	106.9	419.0	389.4 - 456.7	1.16 ± 0.012	6
21	Keates	Franklin	2004	Green	VA	51.1	156.2	102.5 - 186.6	1.22 ± 0.037	2
22	KY-AREC	Lexington	2007	Red	KY	86.0	241.8	210.4 - 276.8	1.74 ± 0.027	3
23	MD-AREC	Prince Georges	2007	Orange	MD	193.6	412.7	378.5 - 445.7	1.36 ± 0.028	6
24	Mitchell	Franklin	2006	Red	VA	107.9	423.6	365.4 - 487.2	1.13 ± 0.017	6

(Continued)

Appendix 7. Responses of the tobacco-adapted form of the green peach aphid colonies to leaf-dip bioassays for acephate (Orthene 97) and general esterase activity (Continued).

S.No.	Colony	County	Year	Color	State	Esterase[a]	LC_{50} (ppm)	95% CI	Slope ± SE	RR[b]
25	NC-State	Wake	2005	Red	VA	94.1	276.4	234.5 - 324.7	1.54 ± 0.014	4
26	NY-Riverhead	Suffolk	2006	Red	NY	89.6	295.3	267.4 - 321.3	1.95 ± 0.034	4
27	Patrick-Henry	Charlottee	2006	Green	VA	45.3	70.2	54.3 - 86.7	2.28 ± 0.025	1
28	Pee Dee-AREC	Florence	2006	Green	SC	46.0	80.2	54.7 - 112.6	1.64 ± 0.013	1
29	R Moore	Lunenburg	2006	Green	VA	68.7	251.6	213.6 - 287.9	1.13 ± 0.025	4
30	Rogers	Henry	2007	Red	VA	74.2	324.5	298.7 - 345.6	2.11 ± 0.010	5
31	SC-AREC	Horry	2006	Green	SC	90.2	324.6	313.5 - 365.8	2.86 ± 0.023	5
32	Semtner	Nottoway	2006	Green	VA	133.1	442.3	398.2 - 486.5	1.16 ± 0.012	6
33	SP-AREC	Nottoway	2006	Orange	VA	153.5	523.5	501.2 - 550.1	1.45 ± 0.014	7
34	SP-AREC (CON)	Nottoway	2005	Red	VA	54.5	138.2	112.3 - 154.6	1.54 ± 0.014	2
35	SP-AREC (GH)	Nottoway	2004	Green	VA	80.8	299.0	264.5 - 330.9	1.95 ± 0.034	4
36	SP-AREC (OR)	Nottoway	2007	Orange	VA	165.8	426.6	385.7 - 467.1	1.28 ± 0.025	6
37	SP-AREC (OR2)	Nottoway	2007	Orange	VA	141.7	452.5	423.8 - 498.4	1.64 ± 0.013	6
38	TN-AREC	Loudon	2007	Red	TN	134.5	324.7	294.6 - 345.7	1.13 ± 0.025	5
39	Townsend	Dinwiddie	2004	Green	VA	203.2	298.6	243.1 - 330.7	1.43 ± 0.032	4
40	Townsend	Dinwiddie	2006	Red	VA	105.1	423.6	378.5 - 465.9	1.11 ± 0.034	6
41	UGA	Tift	2007	Orange	GA	178.2	389.2	356.7 - 430.8	1.42 ± 0.032	6
42	Univ TN	Knoxville	2007	Red	TN	83.1	321.0	302.1 - 356.8	1.61 ± 0.045	5
43	Walker	Oxford	2005	Red	NC	83.8	298.6	256.8 - 330.6	1.58 ± 0.034	4
44	Wallace	Dinwiddie	2006	Green	VA	57.1	217.5	189.7 - 240.6	1.32 ± 0.033	3
45	Washburn	Mecklenburg	2006	Red	VA	74.6	298.6	276.2 - 324.1	1.48 ± 0.023	4
46	Wyatt	Pittsylvania	2005	Red	VA	89.7	342.3	316.7 - 387.3	1.21 ± 0.016	5
47	Yanceyville	Caswell	2006	Green	NC	63.3	229.5	206.5 - 240.2	2.52 ± 0.024	3
48	Yanceyville	Caswell	2006	Red	NC	71.0	312.5	286.4 - 369.2	1.28 ± 0.025	4

[a] Total general esterase activity in nmol/min/mg protein

[b] Resistance ratio (RR): LC_{50} value of field population/LC_{50} value of most susceptible strain

Appendix 8. Total esterase activity in the tobacco-adapted forms of the green peach aphids collected in 2004 and screened in a microplate assay uisng α-Naphthyl acetate as a substrate.

S.No.	Colony	County	Color	State	Esterase Activity[a]	SE [b]
1	Giles	Campbell	Green	VA	42.9	1.22
2	Semtner	Nottoway	Green	VA	48.9	1.45
3	Keates	Franklin	Green	VA	51.1	1.84
4	Walker	Washington	Green	VA	52.3	1.72
5	SP-AREC (HB)	Nottoway	Red	VA	59.5	2.34
6	Keates	Franklin	Red	VA	59.8	5.16
7	Hutcherson	Campbell	Green	VA	62.9	2.38
8	Peek	Washington	Red	VA	64.6	2.48
9	SP-AREC (RE)	Nottoway	Green	VA	66.2	1.23
10	SW-AREC	Washington	Green	VA	66.5	3.41
11	Wright	Washington	Red	VA	70.1	1.42
12	Giles	Campbell	Red	VA	70.6	3.26
13	Anderson	Brunswick	Red	VA	70.8	1.24
14	SP-AREC (TR)	Nottoway	Red	VA	75.5	2.80
15	SP-AREC (A/FB)	Nottoway	Red	VA	77.0	3.44
16	Beldsoe	Nottoway	Red	VA	78.1	3.76
17	SW-AREC	Washington	Red	VA	79.3	1.37
18	SP-AREC (GH)	Nottoway	Green	VA	80.8	2.12
19	Hatchet	Franklin	Red	VA	84.7	4.67
20	Hutcherson	Campbell	Red	VA	85.0	2.05
21	SP-AREC (GH)	Nottoway	Red	VA	88.2	2.32
22	Townsend	Dinwiddie	Green	VA	203.2	1.34

[a] Total carboxylesterase activity using α-Naphthyl acetate as substrate

[b] SE: Standard error mean of at least three separate runs replicated atleast three times within each run

Appendix 9. Total esterase activity in the tobacco-adapted forms of the green peach aphids collected in 2005 and screened in a microplate assay uisng α-Naphthyl acetate as a substrate.

S.No.	Colony	County	Color	State	Esterase Activity[a]	SE [b]
1	SP-AREC (CON)	Nottoway	Green	VA	54.5	3.69
2	SP-AREC (2/4)	Nottoway	Green	VA	54.7	1.32
3	Mitchell	Franklin	Green	VA	56.5	2.34
4	Clayton	Johnston	Green	NC	56.6	4.94
5	Bass	Campbell	Green	VA	65.7	8.67
6	Mitchell	Franklin	Red	VA	81.9	11.64
7	Walker	Oxford	Red	NC	83.9	8.93
8	SP-AREC(SYS)	Nottoway	Red	VA	85.6	4.72
9	SP-AREC (2/4)	Nottoway	Red	VA	85.9	5.47
10	Howard	Lunenburg	Red	VA	88.0	5.99
11	Wyatt	Pittsylvania	Red	VA	89.7	8.52
12	NC-State	Wake	Red	VA	94.1	8.19
13	SP-AREC (CON)	Nottoway	Red	VA	97.2	1.11
14	Witcher	Franklin	Green	VA	139.3	3.54

[a] Total carboxylesterase activity using α-Naphthyl acetate as substrate

[b] SE: Standard error mean of at least three separate runs replicated atleast three times within each run

Appendix 10. Total esterase activity in the tobacco-adapted forms of the green peach aphids collected in 2006 and screened in a microplate assay uisng α-Naphthyl acetate as a substrate.

S.No.	Colony	County	Color	State	Esterase Activity[a]	SE [b]
1	SP-AREC (RE)	Nottoway	Green	VA	32.0	4.56
2	Townsend	Dinwiddie	Green	VA	39.0	3.62
3	Jackson	Henry	Red	VA	40.4	1.23
4	Mt. Airy	Pittsylvania	Red	VA	44.2	4.56
5	Patrick Henry HWY	Charlotte	Green	VA	45.3	4.16
6	Pee Dee-AREC	Florence	Green	SC	46.0	7.69
7	Bobby	Russell	Red	VA	46.6	4.57
8	Mt. Airy	Pittsylvania	Green	VA	47.1	2.34
9	Lunenburg	Lunenburg	Green	VA	47.2	10.31
10	Glasscock	Prince Edward	Green	VA	47.3	8.15
11	Barnard	Amelia	Green	VA	47.4	10.31
12	Ayres	Patrick	Red	VA	48.6	4.88
13	SP-AREC (RE)	Nottoway	Red	VA	49.6	3.45
14	Wallace	Dinwiddie	Green	VA	57.1	5.62
15	Cross Creek	Cumberland	Red	NC	58.7	5.62
16	Farris	Campbell	Red	VA	59.8	2.34
17	D Johnson	Russell	Red	VA	60.0	2.31
18	Dudley	Wayne	Green	NC	60.8	7.73
19	Johnson	Florence	Red	SC	61.9	6.23
20	SP-AREC (A/FB)	Nottoway	Green	VA	62.3	2.67
21	Adams	Lee	Red	VA	62.7	9.66
22	Manning	Mecklenburg	Red	VA	63.1	2.22
23	Yanceyville	Caswell	Green	NC	63.3	1.52
24	Clayton	Johnston	Red	NC	63.5	8.33
25	SP-AREC (GH)	Nottoway	Green	VA	63.5	4.54
26	R Moore	Lunenburg	Green	VA	68.7	2.81
27	Owen	Pittsylvania	Green	VA	69.9	9.23
28	Yanceyville	Caswell	Red	NC	71.0	3.68
29	Lunenburg	Lunenburg	Green	VA	71.3	3.23
30	Owen	Pittsylvania	Red	VA	72.5	2.34

(Continued)

Appendix 10. Total esterase activity in the tobacco-adapted forms of the green peach aphids collected in 2006 and screened in a microplate assay uisng α-Naphthyl acetate as a substrate (Continued).

S.No.	Colony	County	Color	State	Esterase Activity[a]	SE[b]
31	Neal	Lee	Red	VA	73.5	7.04
32	Washburn	Mecklenburg	Red	VA	74.6	7.30
33	Green Bay	Prince Edward	Red	VA	81.0	1.67
34	J Parker	Pittsylvania	Red	VA	81.2	5.22
35	Clary	Mecklenburg	Red	VA	81.4	3.47
36	Windsor	Hartford	Red	CT	81.9	10.79
37	Wylliesburg	Charlotte	Red	VA	83.1	1.56
38	Owen	Pittsylvania	Red	VA	84.4	2.34
39	Semtner	Nottoway	Red	VA	85.1	3.45
40	Ligun	Mecklenburg	Green	VA	87.3	4.31
41	Cross Creek	Cumberland	Green	NC	87.9	3.52
42	R Moore	Lunenburg	Red	VA	88.0	6.13
43	UGA	Grady	Red	GA	88.6	3.43
44	Turbeville	Halifax	Red	VA	89.0	7.59
45	NY-Riverhead	Suffolk	Red	NY	89.6	2.38
46	SC-AREC	Horry	Green	SC	90.2	6.11
47	Anderson	Brunswick	Red	VA	91.3	3.32
48	Dudley	Wayne	Red	NC	94.5	2.17
49	Prince Edward	Prince Edward	Red	VA	94.9	9.11
50	Angel	Franklin	Red	VA	95.8	4.24
51	Barnard	Amelia	Red	VA	98.5	9.36
52	Pee Dee-AREC	Florence	Red	SC	103.5	2.34
53	Townsend	Dinwiddie	Red	VA	105.1	2.38
54	Johnson	Surry	Green	NC	106.9	7.89
55	Mitchell	Franklin	Red	Va	107.9	4.32
56	Bledsoe	Nottoway	Green	VA	118.9	2.34
57	Semtner	Nottoway	Green	VA	133.1	4.56
58	SP-AREC	Nottoway	Orange	VA	153.5	4.14
59	Manning	Mecklenburg	Green	VA	241.0	4.32

[a] Total carboxylesterase activity using α-Naphthyl acetate as substrate

[b] SE: Standard error mean of at least three separate runs replicated atleast three times within each run

Appendix 11. Total esterase activity in the tobacco-adapted forms of the green peach aphids collected in 2007 and screened in a microplate assay uisng α-Naphthyl acetate as a substrate.

S.No.	Colony	County	Color	State	Esterase Activity[a]	SE[b]
1	Mitchell	Franklin	Green	VA	39.9	1.24
2	Johnson	Colleton	Green	SC	48.6	2.18
3	SP-AREC	Nottoway	Green	VA	50.3	5.38
4	Semtner	Nottoway	Green	VA	50.8	10.89
5	Hite	Lunenburg	Green	VA	55.8	3.42
6	Bowen	Tift	Green	GA	56.9	2.56
7	Witcher	Franklin	Green	VA	59.3	2.17
8	Pittard	Mecklenburg	Green	VA	59.5	2.14
9	Route 360	Pittsylvania	Red	VA	68.0	2.45
10	Dellenback	Patrick	Green	VA	70.0	1.11
11	Hite	Lunenburg	Red	VA	70.3	5.62
12	Rogers	Henry	Red	VA	74.2	1.17
13	Witcher	Franklin	D.Green	VA	75.6	1.28
14	Bledsoe	Nottoway	Red	VA	78.6	8.92
15	Bowen	Tift	Red	GA	78.6	2.56
16	Mitchell	Franklin	Red	VA	79.9	3.41
17	MD-AREC	Prince Georges	Red	MD	81.6	4.21
18	NC-State	Wake	Red	NC	82.9	2.82
19	Pittard	Mecklenburg	Red	VA	83.0	2.34
20	Univ TN	Knoxville	Red	TN	83.0	3.65

(Continued)

Appendix 11. Total esterase activity in the tobacco-adapted forms of the green peach aphids collected in 2007 and screened in a microplate assay uisng α-Naphthyl acetate as a substrate (Continued).

S.No.	Colony	County	Color	State	Esterase Activity[a]	SE [b]
21	Ayres	Patrick	Red	VA	84.5	2.34
22	KY-AREC	Lexington	Red	KY	86.0	2.41
23	Highland-RIM	Robertson	Red	TN	87.3	3.14
24	Witcher	Franklin	Red	VA	87.5	3.41
25	Hutchinson	Halifax	Red	VA	88.7	5.42
26	UGA	Tift	Red	GA	89.7	1.76
27	Dellenback	Patrick	Red	VA	90.5	2.67
28	SP-AREC	Nottoway	Red	VA	91.3	8.23
29	Dellenback	Patrick	D.Green	VA	98.5	1.12
30	NCSU	Wake	Green	NC	103.3	3.42
31	Clay's	Nottoway	Green	VA	110.8	5.74
32	TN-AREC	Loudon	Red	TN	134.5	2.21
33	SP-AREC (OR2)	Nottoway	Orange	VA	141.7	2.34
34	Johnson	Darlington	Red	SC	149.8	3.89
35	Semtner	Nottoway	Red	VA	161.0	3.54
36	SP-AREC (OR)	Nottoway	Orange	VA	165.8	14.63
37	Hite	Lunenburg	Orange	VA	174.5	19.18
38	UGA	Tift	Orange	GA	178.2	8.94
39	Greenhouse	Greene	Red	NC	185.4	2.18
40	MD-AREC	Prince Georges	Orange	MD	193.6	3.41
41	Dellenback	Patrick	Orange	VA	214.2	2.57

[a] Total carboxylesterase activity using α-Naphthyl acetate as substrate

[b] SE: Standard error mean of at least three separate runs replicated atleast three times within each run

Appendix 12. Glutathione *S*-transferase specific activity in the tobacco-adapted form of the green peach aphid colonies *Myzus persicae* (Sulzer), 2004.

S.No	Colony	County	State	Color	Specific Activity[a]	SE[b]
1	Peek	Washington	VA	Red	145.97	13.78
2	Giles	Campbell	VA	Red	166.36	10.34
3	Walker	Washington	VA	Green	177.99	9.84
4	Semtner	Nottoway	VA	Green	186.29	15.67
5	SP-AREC (A/FB)	Nottoway	VA	Red	200.46	12.18
6	Keates	Franklin	VA	Red	209.04	17.64
7	Adkinson	Pittsylvania	VA	Green	210.16	12.83
8	Hatchet	Franklin	VA	Red	212.21	15.84
9	Hutcherson	Campbell	VA	Red	217.40	12.56
10	SW-AREC	Washington	VA	Red	222.48	15.23
11	Wright	Washington	VA	Green	238.47	20.78
12	Bledsoe	Nottoway	VA	Red	248.22	6.23
13	SP-AREC (TR)	Nottoway	VA	Red	254.32	14.52

[a] pmol/min/mg protein

[b] Standard error of mean

Appendix 13. Glutathione *S*-transferase specific activity in the tobacco-adapted form of the green peach aphid colonies *Myzus persicae* (Sulzer), 2005.

S.No	Colony	County	State	Color	Specific Activity[a]	SE[b]
1	Townsend	Dinwiddie	VA	Green	191.69	9.87
2	Mitchell	Franklin	VA	Red	203.66	8.97
3	SP-AREC (sys1)	Nottoway	VA	Green	216.57	21.76
4	R Howard	Charlotte	VA	Red	238.00	14.52
5	SP-AREC (cons)	Nottoway	VA	Red	243.82	20.76
6	Clayton	Johnston	NC	Green	265.86	12.84
7	Wyatt	Pittsylvania	VA	Red	267.26	10.98
8	SP-AREC (sys2)	Nottoway	VA	Red	278.54	22.83
9	NC-State	Wake	NC	Red	296.38	12.98
10	Bass	Campbell	VA	Green	308.78	21.73

[a] pmol/min/mg protein

[b] Standard error of mean

Appendix 14. Glutathione *S*-transferase specific activity in the tobacco-adapted form of the green peach aphid colonies *Myzus persicae* (Sulzer), 2006.

S.No	Colony	County	State	Color	Specific Activity[a]	SE[b]
1	Glasscock	Prince Edward	VA	Red	7.94	13.98
2	Pee Dee-AREC	Florence	SC	Red	18.37	12.67
3	Bobby	Russell	VA	Red	20.24	8.64
4	J Parker	Pittsylvania	VA	Red	21.70	9.86
5	Cross Creek	Cumberland	NC	Red	26.37	10.93
6	D Johnson	Russell	VA	Red	32.56	12.32
7	Clayton	Johnston	NC	Red	56.07	12.37
8	Ayres	Patrick	VA	Red	60.76	15.64
9	Dudley	Wayne	NC	Green	68.80	14.54
10	Owen	Pittsylvania	VA	Red	77.62	11.92
11	Yanceyville	Caswell	NC	Red	77.83	12.52
12	SP-AREC	Nottoway	VA	Green	114.13	11.32
13	SP-AREC (A/FB)	Nottoway	VA	Green	120.48	15.23
14	Pee Dee-AREC	Florence	SC	Green	124.72	18.98
15	Wallace	Dinwiddie	VA	Green	133.21	13.42
16	R Moore	Lunenburg	VA	Green	138.49	14.53
17	Johnson	Surry	NC	Green	156.37	8.74
18	NY-Riverhead	Suffolk	NY	Red	159.99	14.54
19	Barnard	Amelia	VA	Red	162.64	15.67

(Continued)

Appendix 14. Glutathione *S*-transferase specific activity in the tobacco-adapted form of the green peach aphid colonies *Myzus persicae* (Sulzer), 2006 (Continued).

S.No	Colony	County	State	Color	Specific Activity[a]	SE[b]
20	Semtner	Nottoway	VA	Green	168.00	14.52
21	Barnard	Amelia	VA	Green	169.67	19.87
22	Yanceyville	Caswell	NC	Green	174.30	9.87
23	Neal	Lee	VA	Red	186.46	13.56
24	Manning	Mecklenburg	VA	Green	190.95	6.89
25	D Moore	Brunswick	VA	Red	194.59	19.76
26	SC-AREC	Horry	SC	Red	195.21	13.65
27	Cross Creek	Cumberland	NC	Green	206.41	12.34
28	Mitchell	Franklin	VA	Red	207.22	12.87
29	Clary	Mecklenburg	VA	Red	208.10	5.78
30	SP-AREC	Nottoway	VA	Orange	210.85	17.43
31	Adams	Lee	VA	Red	218.73	13.98
32	Turbeville	Halifax	VA	Red	226.54	10.32
33	Glasscock	Prince Edward	VA	Green	232.92	9.87
34	Windsor	Hartford	CT	Red	234.80	10.84
35	Washburn	Mecklenburg	VA	Red	253.76	9.72
36	Townsend	Dinwiddie	VA	Green	254.78	12.89
37	Anderson	Brunswick	VA	Red	269.56	14.56
38	Green Bay	Prince Edward	VA	Red	342.80	11.87

[a] pmol/min/mg protein

[b] Standard error of mean

Appendix 15. Glutathione *S*-transferase specific activity in the tobacco-adapted form of the green peach aphid colonies *Myzus persicae* (Sulzer), 2007.

S.No	Colony	County	State	Color	Specific Activity[a]	SE[b]
1	Pittard	Mecklenburg	VA	Green	15.36	8.93
2	Route 360	Pittsylvania	VA	Red	37.52	10.34
3	Dellenback	Pittsylvania	VA	Red	71.95	16.78
4	Hite	Lunenburg	VA	Green	89.06	10.89
5	Townsend	Dinwiddie	VA	Red	106.96	13.67
6	TN-AREC	Loudon	TN	Red	113.76	10.54
7	Johnson	Darlington	SC	Red	136.70	8.76
8	Mitchell	Franklin	VA	Green	149.88	10.26
9	Clayton	Johnston	NC	Green	150.54	14.43
10	SP-AREC (OR1)	Nottoway	VA	Orange	159.91	15.43
11	Hutchinson	Halifax	VA	Red	163.79	16.76
12	Mitchell	Franklin	VA	Red	169.64	9.56
13	Ayres	Patrick	VA	Red	170.80	13.87
14	Dellenback	Patrick	VA	Orange	172.91	12.45
15	Bowen	Tift	GA	Green	173.11	7.89
16	Hite	Lunenburg	VA	Orange	180.17	15.65
17	SP-AREC (OR2)	Nottoway	VA	Orange	182.17	18.54
18	UGA	Tift	GA	Orange	185.92	14.52
19	NC-State	Wake	NC	Red	190.10	8.64
20	UGA	Tift	GA	Red	190.22	12.08

(Continued)

Appendix 15. Glutathione *S*-transferase specific activity in the tobacco-adapted form of the green peach aphid colonies *Myzus persicae* (Sulzer), 2007 (Continued).

S.No	Colony	County	State	Color	Specific Activity[a]	SE[b]
21	Highland-RIM	Robertson	TN	Red	190.86	11.76
22	Hutchinson	Halifax	VA	Green	191.37	13.45
23	Johnson	Colleton	SC	Green	191.78	9.87
24	Univ TN	Knoxville	TN	Red	194.25	16.57
25	UK-AREC	Caldwell	KY	Green	196.63	10.87
26	MD-AREC	Prince George's	MD	Orange	198.53	14.76
27	Rogers	Henry	VA	Red	201.55	13.87
28	Dellenback	Patrick	VA	Green	235.04	13.65
29	Clay's	Nottoway	VA	Green	237.15	8.96
30	SP-AREC	Nottoway	VA	Red	237.16	8.76
31	KY-AREC	Lexington	KY	Red	247.18	11.32
32	SP-AREC	Nottoway	VA	Green	248.15	14.32
33	Greenhouse	Greene	NC	Red	255.83	10.34
34	Witcher	Franklin	VA	D Green	259.78	9.34
35	Hite	Lunenburg	VA	Red	263.75	9.78
36	Dellenback	Patrick	VA	D Green	264.32	15.54
37	Witcher	Franklin	VA	Red	279.25	8.79
38	MD-AREC	Prince George's	MD	Red	325.57	23.65
39	Witcher	Franklin	VA	Green	329.91	12.43

[a] pmol/min/mg protein

[b] Standard error of mean

Appendix 16. GPS coordinates of locations where aphid colonies were collected.

S. No.	Location	County	State	Latitude *	Longitude *
1	Adams	Lee	VA	36°44'36.57"N	83°13'24.10"W
2	Adkinson	Pittsylvania	VA	36°50'20.92"N	79°24'12.56"W
3	Anderson	Brunswick	VA	36°47'3.65"N	77°51'3.70"W
4	Anderson	Halifax	VA	36°49'.496"N	77°46'.672"W
5	Angel	Franklin	VA	36°55'.172"N	79°40'.827"W
6	Ayres	Patrick	VA	36°33'.407"N	80°12'.835"W
7	Barnard	Amelia	VA	37°14'.606"N	77°47'.929"W
8	Bass	Campbell	VA	37°13'40.98"N	79° 8'0.77"W
9	Bledsoe	Nottoway	VA	37°00'99.50"N	78°02'0.55"W
10	Bobby	Russell	VA	39°12'2.89"N	78° 0'11.51"W
11	Bowen	Tift	GA	31°27'10.39"N	83°25'54.52"W
12	Clary	Mecklenburg	VA	36°42'57.90"N	78°23'2.15"W
13	Clay's	Nottoway	VA	37° 3'48.53"N	78° 0'20.67"W
14	Clayton	Johnston	NC	35°40'.178"N	78°30'.697"W
15	Coffee	Lunenburg	VA	36°56'53.27"N	78°14'54.00"W
16	Cross Creek	Cumberland	NC	35° 4'0.29"N	78°53'57.53"W
17	D Moore	Brunswick	VA	36°47'3.65"N	77°51'3.70"W
18	D. Johnson	Russell	VA	39°12'2.89"N	78° 0'11.51"W
19	Dellenback	Patrick	VA	36°33'.407"N	80°12'.835"W
20	Dudley	Wayne	NC	35°13'.436"N	77°59'.174"W
21	Witcher	Franklin	VA	37° 0'24.76"N	79°54'50.13"W
22	Hite	Lunenburg	VA	36°53'2.85"N	78° 3'4.69"W
23	Farris	Campbell	VA	37°13'40.98"N	79° 8'0.77"W
24	Francis	Charlotte	VA	36°58'24.06"N	78°40'25.92"W
25	Giles	Campbell	VA	37°13'40.98"N	79° 8'0.77"W
26	Glasscock	Prince Edward	VA	37°14'26.39"N	78°15'18.72"W
27	Green Bay	Prince Edward	VA	37° 7'58.61"N	78°18'53.71"W
28	Greenhouse	Greene	NC	35°29'59.93"N	77°39'3.67"W
29	Hale	Macon	TN	36°31'32.95"N	86°0'29.83"W
30	Hatchet	Franklin	VA	37° 0'24.76"N	79°54'50.13"W
31	Highland-RIM	Robertson	TN	36°30'3.69"N	86°51'25.62"W
32	Hite	Lunenburg	VA	36°56'53.27"N	78°14'54.00"W
33	Howard	Charlotte	VA	36°58'24.06"N	78°40'25.92"W
34	Hutcherson	Campbell	VA	37°13'40.98"N	79° 8'0.77"W
35	Hutchinson	Halifax	VA	36°36'29.31"N	79° 2'31.95"W
36	Peek	Washington	VA	36°45'41.81"N	81°58'7.34"W
37	Jackson	Henry	VA	36°41'56.54"N	79°52'4.59"W

(Continued)

Appendix 16. GPS coordinates of locations where aphid colonies were collected (Continued).

S. No.	Location	County	State	Latitude *	Longitude *
38	Johnson	Colleton	SC	32°47'21.82"N	80°39'11.25"W
39	Johnson	Darlington	SC	34°18'37.61"N	79°58'21.94"W
40	Johnson	Surry	NC	36°23'57.71"N	80°42'29.12"W
41	Keates	Franklin	VA	37° 0'24.76"N	79°54'50.13"W
42	KY-AREC	Lexington	KY	38° 2'56.06"N	84°29'59.42"W
43	Manning	Mecklenburg	VA	36°42'57.90"N	78°23'2.15"W
44	MD-AREC	Prince Georges	MD	38°51'32.15"N	76°53'55.69"W
45	Mitchell	Franklin	VA	36°5'0.698"N	79°47'.026"W
46	Mt. Airy	Pittsylvania	VA	36°56'17.39"N	79°11'40.83"W
47	NC State	Wake	NC	35°47'51.97"N	78°37'30.31"W
48	Neal	Lee	VA	36°35'.651"N	83°16'.954"W
49	NY-AREC	Suffolk	NY	40°55'20.37"N	72°38'14.11"W
50	Opie	Mecklenburg	VA	36°42'57.90"N	78°23'2.15"W
51	Owen	Pittsylvania	VA	36°40'.459"N	79°18'.961"W
52	Oxford	Granville	NC	36°18'.617"N	78°37'.426"W
53	Patrick Henry	Charlotte	VA	37° 2'56.78"N	78°33'52.66"W
54	Pee Dee-AREC	Florence	SC	34° 2'29.09"N	79°41'35.86"W
55	Pittard	Mecklenburg	VA	36°42'57.90"N	78°23'2.15"W
56	R Moore	Lunenburg	VA	36°56'53.27"N	78°14'54.00"W
57	Rogers	Brunswick	VA	36°47'3.65"N	77°51'3.70"W
58	Rogers	Henry	VA	36°41'56.54"N	79°52'4.59"W
59	Route 360	Pittsylvania	VA	37° 5'25.19"N	79°17'21.21"W
60	SC-AREC	Horry	SC	33°56'8.94"N	78°55'9.26"W
61	Semtner	Nottoway	VA	37° 0'412.90"N	78°00'08.48"W
62	SPAREC	Nottoway	VA	37° 05'50.70'N	78°4'46.51"W
63	Sparrow Ln	Halifax	VA	36°48'33.69"N	78°51'17.29"W
64	SWAREC	Washington	VA	36°46'54.90"N	81°48'54.50"W
65	TN-AREC	Loudon	TN	35°44'3.72"N	84°21'33.99"W
66	Townsend	Dinwiddie	VA	37°0'1.137"N	77°48'.202"W
67	Turbeville	Halifax	VA	36°36'29.31"N	79° 2'31.95"W
68	UGA	Grady	GA	30°52'38.58"N	84°13'36.54"W
69	UK-AREC	Caldwell	KY	38°48'21.75"N	84°23'27.54"W
70	Univ. TN	Knoxville	TN	35°57'59.70"N	83°55'27.76"W
71	Walker	Granville	NC	36°16'55.70"N	78°37'49.63"W
72	Walker	Washington	VA	36°44'.322"N	81°52'.406"W
73	Washburn	Mecklenburg	VA	36°55'25.50"N	78° 8'55.17"W
74	Windsor	Hartford	CT	41°51'10.01"N	72°38'37.64"W
75	Wyatt	Pittsylvania	VA	36°40'.459"N	79°18'.961"W
76	Yanceyville	Caswell	NC	36°23'.232"N	79°23'.434"W

* Latitude and longitude are shown in degrees, minutes and seconds.

Photographic Plate 1. Pictures of three main aphid color morphs of the tobacco-adapted form of the green peach aphid included in the study.

(A) Green Morphs

Green Morphs Close-up

(B) Orange Morphs

Orange Morphs Close-up

(C) Red Morphs

Red Morphs Close-up