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RESISTANCE AND METABOLISM OF IMIDACLOPRID IN COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* Say (COLEOPTERA: CHRYSOMELIDAE)

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By

David Mota Sánchez

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ABSTRACT

RESISTANCE AND METABOLISM OF IMIDACLOPRID IN COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* Say (COLEOPTERA; CHRYSOMELIDAE)

By

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ABSTRACT: Since 1995, imidacloprid, a neonicotinoid compound and an agonist of nicotinic acetylcholine receptors, has been vital for the control of the Colorado potato beetle in many areas of the United States. High levels of resistance to this compound were detected in beetle populations collected in Long Island, NY after less than three years of use. In 1998, the imidacloprid resistance ratios ranged from 27-fold to 155-fold. Three imidacloprid resistant Long Island populations expressed very low levels of cross resistance to another neonicotinoid insecticide, thiamethoxam (resistance ratios ranged from 1.7-fold to 3.1-fold) and no cross resistance to bensultap, a nereistoxin derived compound, acting as an antagonist of the insect acetylcholine receptor. In 1999, significant survivals (17 to 80 %) were found in populations from Long Island treated with a discriminating doses of 3.16 μg / beetle. High correlation was found between KD_{50} and mortality 10 days after treatment ($r = 0.90$, $p = 0.0007$). The susceptible populations had faster knock down than resistant populations, and this fast knock down was significant correlated with high mortality 10 days after treatment. Mix of imidacloprid + the synergist piperonyl butoxide partially suppressed resistance in the Long Island strains.

Rapid penetration and excretion of ^{14}C-imidacloprid were observed in a susceptible and a resistant strain of Colorado potato beetle. Comparison of the pharmacokinetics of ^{14}C-imidacloprid in the resistant and susceptible strains treated with a low dose (16 ng / beetle) indicated a slightly lower rate of uptake in the resistant strain, but no significant difference was seen in the percentage of the dose excreted and present in the body. The pharmacokinetics in the resistant strain treated with a low dose and a high dose (900 ng / beetle) also indicated a similar pattern for the percent of external radioactivity, excretion and internal radioactivity of ^{14}C-imidacloprid. Thus no significant differences were found between the susceptible strain and the resistant strain in either the metabolism or excretion of imidacloprid that could explain resistance, and the internal levels of imidacloprid were comparable in both strains. Both resistant and susceptible strains showed minimal metabolic conversion. Only a single major radioactive metabolite was formed. This was probably the olefine analog of imidacloprid. The resistant strain was also cross-resistant to the olefine compound which was less toxic than the parent imidacloprid.

The lack of differences in the pharmacokinetics and metabolism of imidacloprid observed in these experiments between resistant and susceptible beetles together with differences in intoxication symptoms suggest that resistance could be due to a modification of the target site, the nicotine AchRs. Further neurophysiology studies using the isolated nervous systems, together with studies of binding site competition of the nAChRs between resistant and susceptible strains of Colorado potato beetle are essential to determine if the target site modification is the mechanism of resistance to imidacloprid in the NY Selected strain.

To the memory of my mother

Sara Sánchez Valverde

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INTRODUCTION

INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* Say, is the principal pest of potato, *Solanum tuberosum* L. in North America and Europe (Weber and Ferro 1994). Foliar feeding by overwintered adults, spring larvae, summer adults and summer larvae cause severe damage, often times total loss of tuber production when the attack occurs before tuber initiation (Hare 1980), and limited potato production in some regions of the United States (Wyman et al. 1994). This insect is a formidable pest because of its high reproductive potential, adaptation to subtropical and colder weather (Harcout 1971), adaptation to wild and cultivated hosts such as tomato, *Lycopersicon esculentum* Mill and eggplant, *Solanum melogena* L., and resistance to many pesticides. In fact, the potato beetle is resistant to more than 37 compounds and is ranked seventh in development frequency of pest resistance worldwide (Mota-Sanchez et al. 2002). Although synthetic insecticides have only been used for the last 50 years, the co-evolution of plants and insects has been occurring for millions of years. Plants have been producing secondary compounds like repellents and toxins to defend themselves. Insects in turn have evolved to detoxify or resist xenobiotics from the hosts, developing an arsenal of genetic defenses to metabolize plant compounds and insecticides (Georghiou 1986). For example, species of the Solanaceae family, including the genera *Solanum* and *Lycopersicon,* hosts of the Colorado potato beetle, are loaded with steroidal glycoalkaloids (Schreiber 1979) that provide chemical protection by repellent, deterrent or toxic effects against herbivorous insects. Adaptation of Colorado potato beetle to these plants probably aids in the beetles resistance to insecticides (Ferro 1993).

The potato and the Colorado potato beetle are native species from the Americas. However, the interaction between the beetle and the potato is likely only about 150 years old (Hare 1990). The potato has its origin in Peru, where native Americans cultivated it as early as 2,500 B.C. After the Spanish conquered South America, potatoes were introduced to Europe in 1570. Potatoes were then introduced to Virginia, North America in 1621 (Harris 1978). Colorado potato beetle is indigenous to Southern Mexico (Hsiao 1985) where it feeds on wild species of the Solanacea family such as the burweed, or buffalo bur, *Solanum rostratum* Mill. and *Solanum angustifolium* Dunal. It is believed that dispersion of the Colorado potato beetle into North America probably occurred as a result of the dispersion of S. *rostratum* burs by cattle movement from Mexico to Texas and from there northward (Lu and Lazell 1996). The Colorado potato beetle was reported present on the eastern slope of the Rocky Mountains in 1824 (Riley 1875) At that time, the insect was reported feeding only on the native host, *S. rostratum*, despite the presence of potato plants. However, in 1859 the potato beetle was reported as a pest of potatoes in eastern Nebraska (Walsh 1865). Fifteen years later the beetle reached the Atlantic Coast. Less than 25 years later, the Colorado potato beetle was reported as a serious pest throughout the Northeastern US. In 1920, evidence of Colorado potato beetle as a pest was reported in France and it then was disseminated into Eastern Europe and other countries in Asia.

As Colorado potato beetle disperses in the eastern and western portions of North America, its adaptation spread to cultivated species of the Solanaceae and wild species of *Solanum*. In the eastern US the beetle adapted to the horse-nettle, *Solanum carolinensis* L. and *Solanum dulcamara* L., the latter species originally from Europe. In the western

US, the Colorado potato beetle adapted to henbane, *Hyoscyamus niger* L., and *Solanum sarrachoides* Sendtn.

This host and weather adaptation of different races have been associated with a pericentric inversion of the chromosome 2 of the Colorado potato beetle (Hsiao 1985). For instance, the Mexican and some southern US races feed only on wild species of *Solanum* and are adapted to hot and dry climates. Cytogenetic analysis of the chromosomes showed that this race carries methacentric chromosomes. However, the race that adapted to potatoes and colder weather has an acrocentric chromosome. Probably the acrocentric race has its origin in the methacentric race. In Europe the only race found is the acrocentric chromosome race. Analysis of mitocondrial DNA suggests that populations from Texas probably have their origin in Mexico (Azeredo-Espin et al. 1991, Azeredo-Espin et al. 1996). Hsiao (1985) pointed out that hybridization of the two races has been occurring in the last decades. Hybrids of these two races could be more vigorous, adapted to a wider range of environments and hosts including tomatoes and eggplant, and have high levels of resistance to various pesticides in the northeastern United States. However, there is currently no convincing proof that hybridization is responsible for resistance to insecticides. Once the Colorado potato beetle expanded its range to cultivated crops, it nevertheless retains its ability to recognize and utilize original hosts despite isolation for more than 100 generations (Harrison 1987). Today, the host range of Colorado potato beetle includes 20 species of the Solanaceae family (Hsiao 1988).

Up to now, there are no known natural enemies that regulate Colorado potato beetle populations efficiently and have the capacity to survive the winter weather

conditions in temperate regions where Colorado potato beetle is located (Hare 1990).

Many methods have been used to control Colorado potato beetle, including hand-picking, bird predation, resistant potato varieties, trapping, border sprays, trench traps, propane flamers, crop vacuums, and crop rotation. None of these methods, including biological control (de Wilde and Hsiao 1981), have been completely effective and farmers depend primarily on the use of chemical insecticides (Casagrande 1987).

Host plant resistance to Colorado potato beetle has also been studied. Solanine and chaconine, are two glycoalkaloids present in the foliage of *Solanum tuberosum* that deters feeding only at very high doses (Gregory et al. 1981). However, there are other effective foliar glycoalkaloids including leptines and specialized glandular trichomes (Sikinyi et al. 1997). Sinden et al. (1986) demonstrated that leptines were a powerful deterrent for adults and larvae of Colorado potato beetles. Wild species of *Solanum berthaultii* Hawkes, a species from Bolivia, produce sticky adhesive substance in glandular thrichomes on the leaves that affects larval growth and the capacity of females to lay eggs (Casagrande 1982, Wright et al. 1985). Traditional plant breeding aimed at the production of Colorado potato beetle resistant varieties with glycoalkaloids has been proactive for decades. However, the presence of glycoalkaloids in tubers has raised concerns for human health (Tingey et al. 1984). Subsequent breeding attention focused on the presence of glandular trichomes on leaves. These compounds affect the growth rate, survival and oviposition of the potato beetle (Dimock and Tingey 1988). However, Colorado potato beetle adapted and overcame the effects of the glandular trichomes in only two generations of selection (Groden and Casagrande 1986). Thus a selected strain

of Colorado potato beetles laid eggs and survived on *S. berthaultii* at rate similar to commercial potatoes (Groden and Casagrande 1986).

Other characteristics such as leptines have been introgressed into commercial varieties (Douches et al. 2001). In addition, insertion of Bt genes has been created, deployed, and yielded effective control of Colorado potato beetle. Strategies of resistance management including pyramiding potatoes with Bt genes, leptines and trichomes have been developed and evaluated (Douches et al. 2001, Coombs et al. 2002). Transgenic potatoes are highly effective against Colorado potato beetle, but the only commercially available line, Newleaf (Monsanto Corp.) was withdrawn from the market in 2002 because of consumer and buyer concerns about genetically modified organisms. In addition, a limiting factor for the deployment of this variety is the competition from insecticides like imidacloprid.

Due to overuse of insecticides in potatoes and the remarkable adaptability of the Colorado potato beetle, whole classes of insecticides have failed through of the development of insecticide resistance. The contribution factors to resistance in Colorado potato beetle are a result of: a) rapid population growth; b) high percentages of the population being treated in each generation in potatoes while populations on untreated alternate hosts are much less abundant; c) selection of all stages, except pupa; d) sometimes cross-resistance to other compounds is due to a single mechanism of resistance; e) use of systemic soil insecticides that perpetuate foliar residues and select beetles for longer periods than conventional sprayed insecticides; and f) foliar applications of the same insecticide perpetuating the selection process still further (Roush and Tingey 1991).

Since the introduction of DDT, Colorado potato beetle resistance to insecticides has followed a familiar pattern: new chemicals provide good to excellent control, but the beetle develops resistance within 1-3 years. The potato beetle has developed resistance to 41 different compounds (Table 1), 39 of these compounds have documented field resistance (Mota-Sanchez et al. 2002, MSU Resistance Database 2002) while two have developed resistance to abamectin (Argentine 1991) and the microbiological insecticide *Bacillus thuringiensis tenebrionis* Berliner (Whalon et al. 1993) under laboratory selection. The insecticides in Table 1 are classified in ten groups of chemicals and eight modes of action including effects on the sodium channel for DDT and pyrethroids, inhibition of acetylcholinesterase for carbamates and organophosphates, blockage of chloride channels for cyclodienes, activation of GABA receptors for avermectin, agonist activity at nicotine acetylcholine receptors for neonicotinoids, antagonism for the same receptors for nereistoxin compounds, and binding of receptors in the midgut cells by the endotoxin of *Bacillus thuringiensis* var. *tenebrionis*.

The cost associated with resistance has not only been in terms of yield reduction, but also in the amount of money spent on insecticides. In New York the cost for seasonal insecticide treatment for Colorado potato beetle was more than $987/ha and in Massachussets it was $568/ha (Roush et al. 1990). Resistance was so severe that it caused the return of the use of insecticides that were applied before DDT, namely rotenone and cryolite (Roush et al. 1990). In Michigan, growers in the county most affected by insecticide resistance, spent $412/ha for insecticide treatment (Grafius 1997). Conversely, in the Upper Peninsula of Michigan where Colorado potato beetle exhibits only one

Table 1. Number of compounds and chemical groups that Colorado potato beetle has developed resistance

a) ORGANOCHLORINES
- 1.DDT
- 2.methoxychlor

b) CYCLODIENES
- 3. aldrin
- 4. dieldrin
- 5. endrin
- 6. chlordane
- 7. endosulfan
- 8. lindane
- 9. BHC
- 10. toxaphene

c) ORGANOPHOSPHATES
- 11. azinphos methyl
- 12. phoxim
- 13. chlorfenviphos
- 14. malathion
- 15. methamidophos
- 16. phorate
- 17. phosmet
- 18. monocrotophos
- 19. methidation
- 39. bensultap
- 20. parathion
- 21. methyl parathion
- 22. tetrachlorvinphos
- 23. quinalphos
- 24. thriclorfon

d) CARBAMATES
- 25. aldicarb
- 26. carbaryl
- 27. oxamyl
- 28. carbofuran
- 29. chloetocarb
- 30. dioaxacarb
- 31. propoxur

e) PYRETHROIDS
- 32. permethrin
- 33. deltamethrin
- 34. fenvalerate
- 35. deltamethrin

f) FUMIGANT
- 36. hydrogen cyanide

g) MICROBIAL
- 37. *B. thuringiensis*

h) AVERMECTINS
- 38. abamectina

i) NEREISTOXINS
- 39. cartap
- 40. bensultap

j) NEONICOTINOIDS
- 41. imidacloprid

Source: MSU Resistance database 2002

generation per season and insecticides are less frequently applied, the cost was $35-74 (Grafius 1997).

The desperate need to control resistant Colorado potato beetle in Michigan has led growers to implement various IPM strategies. Scouting was implemented in 98% of the area and crop rotation was employed in 78% of potato fields (Grafius 1997). Other strategies used to reduce resistant Colorado potato beetle populations included the use of propane flamer (Moyer et al. 1997). The intense heat of the propane flamer kills beetles, but this measure is only effective when the potato plant is less than 15 cm in height. In addition, the heat of the flames is dissipated on a windy day.

Crop rotation is effective in delaying the beetle infestation and reducing the density of overwintered beetles. Beetles in diapause tend to overwinter close to the areas where potatoes were grown the prior year (Voss et al. 1988). The distance between the previous crop and the new crop determines the arrival and the level of infestation of overwintered beetles (Wyman et al. 1994). In addition, after spring emergence, beetles tend to disperse by walking (Ng and Lashomb 1983), and only after temperatures reach more than 18 °C will they start flying (Johnson 1969). Therefore, crop rotation delays the establishment of Colorado potato beetle in rotated potato fields and reduces at least one insecticide treatment early in the season (Wright 1984). One of the inconveniences of crop rotation is that some growers do not have an alternate field for potato production due to soil type, irrigation, among others, and this method may no be possible in some situations.

Known mechanisms of resistance of the Colorado potato beetle to conventional insecticides include target site insensitivity, reduced insecticide penetration, and

enhanced metabolism (Argentine 1991, Argentine et al. 1993, Wierenga and Hollingworth 1993, Argentine et al. 1995, Grafius 1995, Grafius and Bishop 1996, Zhao et al. 2000). Of these mechanisms, penetration is a small factor in resistance while the others are major. Argentine (1993) pointed out a slight, but significant decrease in penetration of azinphosmethyl in a strain resistant to this compound. However, penetration was not an important factor in carbofuran resistance (Wierenga and Hollingworth 1993).

Among change in sensitivity at the site of action an important mechanism is decreased acetylcholinesterase sensitivity first observed in strains of Colorado potato beetle from Michigan (Ioannidis et al. 1992, Wierenga and Hollingworth 1993). Acetylcholinesterase insentivity seems to be specific for some carbamates and organophosphates. Acetylcholinesterase from one strain (Michigan) is insensitive to carbamates and from another strain (Long Island NY) to organophosphates (Wierenga and Hollingworth 1993). This work has been expanded much further by Clark et al. (2001) including sequencing of the enzyme involved. A serine point mutation to glycine in acetylcholinesterase was associated with azinphosmethyl resistance in Colorado potato beetle (Zhu et al. 1996).

The *kdr* factor (knockdown resistance due to altered sodium channels) may also be involved in resistance to pyrethroids in Colorado potato beetle (Ioannidis and Grafius 1988). Further studies by Argentine et al. (1995) pointed out that permethrin resistance was also associated with nerve insensitivity and increased levels of carboxylesterase activity. This carboxylesterase activity was due to sequestration of permethrin by hemolymph carboxylesterases rather than rapid hydrolysis (Lee and Clark 1998).

Enhanced metabolism is one of the principal mechanisms of resistance. Monooxygenases may be involved in Colorado potato beetle resistance to carbofuran and carbaryl (Rose and Brindley 1985). Mixed function oxidase enzymes are an important mechanism of resistance to carbofuran from Long Island NY (Ioannidis et al. 1992).

More than one mechanism may be responsible for resistance. Wierenga and Hollingworth (1993) reported that altered acetylcholinesterase and enhanced metabolism by mixed function oxidase activity were involved in insecticide resistance in Colorado potato beetle. An azinphosmethyl resistant strain was resistant due to multiple mechanisms including slightly reduced penetration, enhanced xenobiotic metabolism by glutathione transferase, and target site insentivity (Argentine et al. 1993). Esterases are also involved in resistance to azinphosmethyl and pyrethroids(Argentine et al. 1989, Ioannidis et al. 1992, Ahammad-Sahib et al. 1994, Anspaugh et al. 1995).

Natural compounds that interact with the insect nicotinic acetylcholine receptor including nicotine (Yamamoto and Casida 1999) and nereistoxin (Okaichi and Hashimoto 1962) have been used for many years to control pests. These compounds mimic acetylcholine that is an important neurotransmitter that mediates the communication between nerve cells. Acetylcholine binds the receptors in the postsynaptic membrane causing a synaptic potential. Once acetylcholine has acted it is broken down by acetylcholinesterase in coline + acetic acid. Coline is recycled to the presynaptic membranes. In nature, as a mechanism of defense, natural compounds produced by plants, invertebrates and annelids interact with the nicotinic acetycholine receptors. Nicotine, present in the tobacco plant, *Nicotiana tabacum* L. and *Nicotiana rustica* L., is one of the oldest compounds still applied in greenhouses to control pests of the order

Homoptera (Nauen et al. 1999). After the Second World War, use of nicotine was about 2,500 ton per year. However, high mammalian toxicity together with the discovery of cheap broad spectrum pesticides reduced the use of nicotine to only 200 tons (Ujváry 1999). Other alkaloids including nornicotine are present in the Australian shrub, *Duboisia* hopwoodii F.Muell. (anabasine) and in the Asian plant, *Anabasis aphylla* L. (Chenopodiaceae) (Ujváry 1999). The Panamanian frog *Dendrobates pumilio* produces pumiliotoxins that interact at the acetycholine receptors and Ca2+-dependent ATPase (Witkop and Gössinger 1983). The marine annelid, *Lumbriconereis heteropoda* Marenzeller, used in Japan as a fish bait, produces the nereistoxin, a substance that paralyzes insects (Okaichi and Hashimoto 1962) by interaction with the insect acetylcholine receptors (Konishi 1972). Synthetic derivates of nereistoxins originated cartap which is metabolized by insects to nereistoxins. Similar situation occurs with thyociclam, and bensultap (Ujváry 1999). In contrast with nicotine, cartap is an antagonist of the nicotine acetylcholine receptors. Paralysis of insects is due to the blocking of the cholinergic transmission by nereistoxin. Throughout the blocking by nereistoxin there is no excitation of the postsynaptic membrane.

The nicotinic acetylcholine receptors (nAChR) in the insect nervous system are the primary target for cartap and its nereistoxin parent (Eldefrawi et al. 1986), nicotinoids (nicotine), and for neonicotinoid insecticides (including imidacloprid) (Schroeder and Flattum 1984), (Bai et al. 1991). Due to their similar structure and mode of action, nicotinoids and neonicotinoids are grouped as "nicotinoid insecticides" (Yamamoto and Casida 1999). Figure 1 presents the chemical structure of the neonicotinoids. Niathizine was the first compound that inspired the discovery and synthesis of neonicotinids

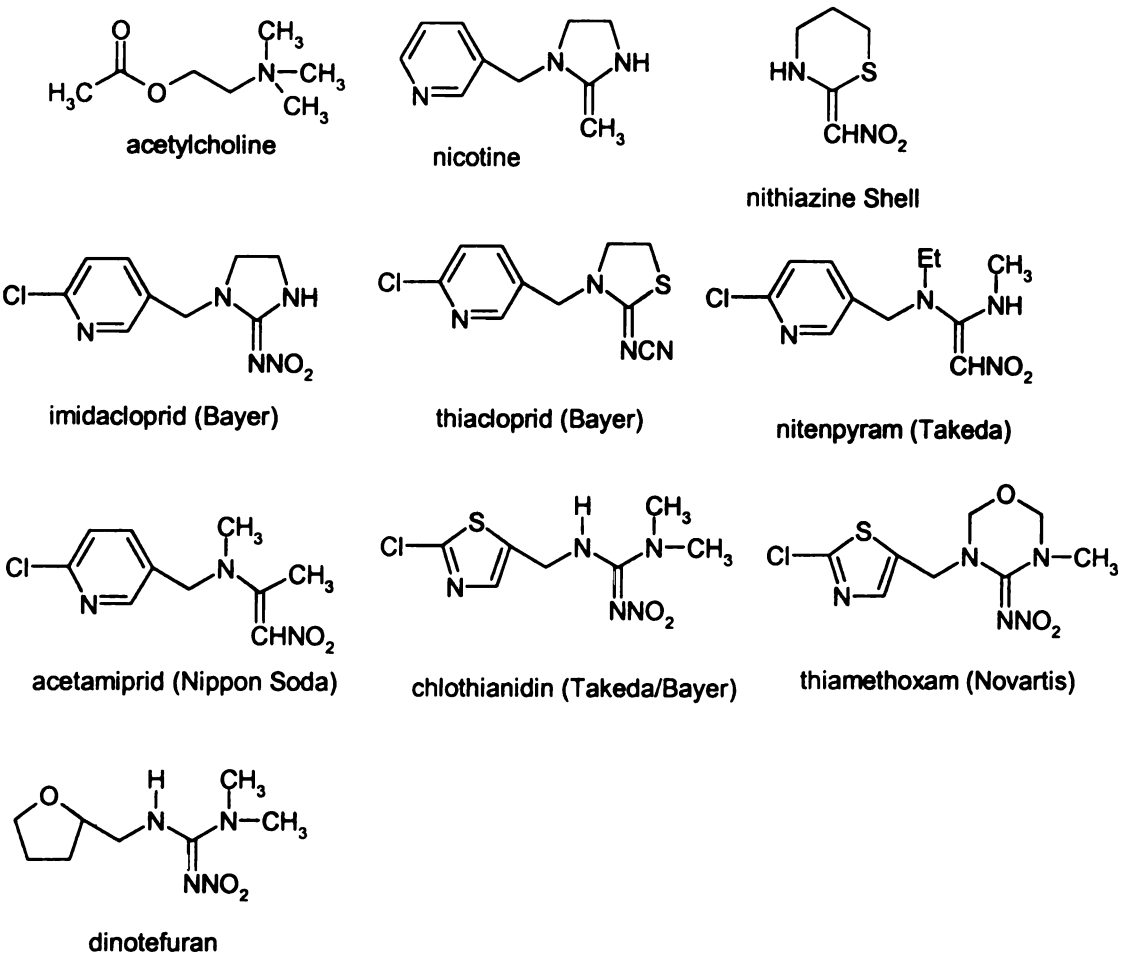

Figure 1. Chemical structure of compounds that interact at the insect acetylcholine receptors (nAChRs).

However, reduced photoestability limited its used in the field (Yamamoto 1999). Later more stable compounds, including imidacloprid, acetamiprid, thiamethoxam, thycloprid, and nytempiram, were very attractive for field deployment. Imidacloprid was the first active ingredient of this chemical class to reach the market (Thyssen and Machemer 1999). Currently, 10 to 15% of the insecticide world market are shared by the neonicotinoid compounds (Wollweber and Tiejten 1999).

Strong differences in binding potency of imidacloprid between insect and mammalian acetylcholine receptors make this product effective for insect control and safe for use (Thyssen and Machemer 1999). Imidacloprid is quickly eliminated from mammals. Twenty-four hours after oral administration in rats, 90% of imidacloprid has been eliminated, and in 48 h the entire compound was excreted without being distributed in the fat tissue, central nervous system or bones (Thyssen and Machemer 1999). Metabolism of imidacloprid in mammals includes two principal pathways: The first is the hydroxylation of the molecule in the imidazolidine ring, followed by water elimination and the production of an unsaturated metabolite (Figure 2) (Thyseen and Machemer 1999). The second pathway is the oxidative cleavage to imidazolidine moiety and 6-chloronicotinic acid. The first is excreted in the urine, and the second is conjugated by glutathione to a relative of mercapturic acid and later on to methyl mercaptonicotinic acid. The metabolism of imidacloprid in cell suspension cultures of potato, wheat and maize followed three patways: the first is the oxidation of the ethylene bridge of the imidazolidine ring to produce the mono- and bis-hydroxy-, the keto and the olefine metabolite; second, oxidation of the pyridinyl-methylene group and then the conjugation with glucose to produce the 6-chloropyridinyl glucopyranoside, and third, reduction of

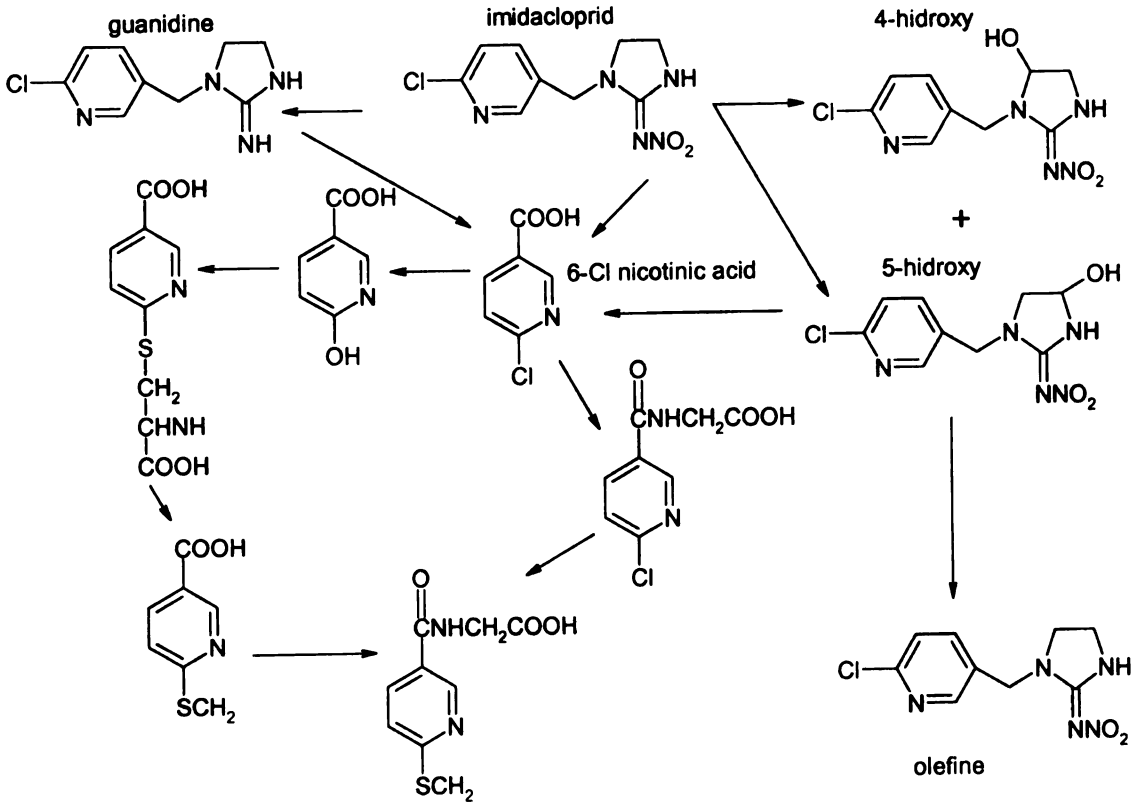

Figure 2. Metabolism of imidacloprid in rats.

the nitro- to the nitroso group (Koester 1992). The major metabolites produced in potato were the 4-hydroxy, and the olefine metabolite. (8 and 4% of transformation), showing the parent compound little transformation (83% of unchanged).

Imidacloprid was registered for potatoes in the US in 1995 and immediately became the primary means to control organophosphate, pyrethroid, and carbamate-resistant Colorado potato beetles in Michigan and other areas in the US (Grafius 1997). Imidacloprid has been very effective against Colorado potato beetle. However, in Poland, Colorado potato beetle has developed resistance to cartap (Georghiou and Lagunes-Tejeda 1991). Therefore, Colorado potato beetle has demonstrated the potential to develop resistance to compounds that have the nicotinic acetylcholine receptors as a molecular target. Imidacloprid and other insecticides with the same mode of action could have a reduced use-life if cross-resistance occurs as with previously deployed and related insecticides classes.

In 1996, a low level of resistance to imidacloprid was detected in a Colorado potato beetle population collected from a commercial potato field in Michigan (Grafius and Bishop 1996). There is no evidence that these levels of resistance in Michigan caused a reduction in crop yield or significant foliar damage. However, in Long Island NY, only 50% control of Colorado potato beetle was achieved in a conventional potato field (Moyer et al. 1997). Topical bioassays of this population with imidacloprid exhibited 100.8 fold levels of resistance (Zhao et al. 2000). If this resistance to imidacloprid continues to develop and become more widespread, growers will be left with few control options as well as reduced potato productivity and increased control costs. If imidacloprid and related compounds is to remain a viable option for growers, it will be necessary to

rapidly develop and implement resistance management strategies for neonicotinoid compounds. Monitoring the resistance and understanding the mechanisms of both resistance and cross-resistance is the cornerstone of these strategies.

The objectives of this research were to: 1) determine if resistance to imidacloprid was widespread in field populations of Colorado potato beetle from Long Island NY; 2) determine the pattern of cross-resistance to other compounds that act at the nicotine acetylcholine receptor including the second generation neonicotinoid, thiamethoxam and a nereistoxin compound, bensultap; and 3) use ^{14}C-imidacloprid to determine the pharmacokinetics and metabolism of imidacloprid by Colorado potato beetle.

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Chapter 2

DETECTION OF RESISTANCE OF COLORADO POTATO BEETLE TO IMIDACLOPRID

INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* Say, is the principal pest of potatoes in North America and Europe (Weber and Ferro 1994). Many methods have been used to control Colorado potato beetle, including hand-picking, bird predation, resistant potato varieties, trapping, border sprays, trench traps, propane flamers, and crop rotation. These methods, as well as control by natural enemies, have not been completely effective and farmers depend primarily on the use of chemical insecticides (Casagrande 1987). Resistant varieties have been developed containing specific glycoalkaloids or glandular trichomes that reduce the growth and survival of this insect, including *Solanum chacoense* Bitt. and *Solanum berthaultii* Hawkes (Tingey et al. 1984). However, Colorado potato beetle has adapted to a glandular trichome variety in only two generations (Groden and Casagrande 1986). Genetically engineered resistant varieties by the insertion of the genes of *Bacillus thuringiensis* are highly effective, but production is limited because of consumers concerns and the limited number of varieties available. Due to intensive use, whole classes of insecticides have failed because of insecticide resistance development. Since the introduction of DDT, the pattern of Colorado potato beetle resistance has followed a familiar pattern: new chemistries provided good to excellent initial control, but Colorado potato beetle developed resistance within 1-3 years. The potato beetle has developed resistance to every insecticide used for its control (Forgash 1985, Georghiou and Lagunes-Tejeda 1991) reported field resistance to 37 insecticides across several classes including organophosphates, carbamates, and pyrethroids. Under laboratory selection, this insect has developed resistance to abamectin

(Argentine et al. 1992) and the microbiological insecticide *Bacillus thuringiensis tenebrionis* Berliner (Whalon et al. 1993) as well.

The nicotinic acetylcholine receptors (nAChR) in the insect nervous system are the primary target for neonicotinoid insecticides, including imidacloprid, and nicotinoids (nicotine) (Schroeder and Flattum 1984, Bai et al. 1991), and cartap and its nereixtoxin parent (Eldefrawi et al. 1986). Due to their similar structure and mode of action, nicotinoids and neonicotinoids are grouped as "nicotinoid insecticides" (Yamamoto and Casida 1999). Imidacloprid is the first active ingredient of its chemical class to reach the market (Thyssen and Machemer 1999). Strong differences in binding potency between insect and mammalian acetylcholine receptors made this product safe for use and effective for insect control (Thyssen and Machemer 1999). Currently, it is the compound that leads the sales with US $ 455 millions in 1999 (Maienfisch et al. 2001). Incorporation of imidacloprid in the soil provided long control of overwintered adults and larvae of Colorado potato beetle (Boiteau et al. 1997). Foliar applications control summer generations at intervals of seven days or more. Imidacloprid was registered for potatoes in 1995 and soon became the primary means to control organophosphate, pyrethroid, and carbamate-resistant Colorado potato beetles in Michigan and other potato areas in the US (Grafius 1997). Imidacloprid has been very effective against Colorado potato beetle. However, in Poland, Colorado potato beetle has developed resistance to cartap (Georghiou and Lagunes-Tejeda 1991). Therefore, Colorado potato beetle has demonstrated the potential to develop resistance to compounds that have the nicotinic acetylcholine receptors as a molecular target.

In 1996, a low level of resistance to imidacloprid was detected in a Colorado potato beetle population collected from an imidacloprid-treated commercial potato field in Michigan (Grafius and Bishop 1996). One year later, 16-fold imidacloprid resistance was found in another Michigan field population (unpublished data). There is no evidence that these levels of resistance in Michigan caused a reduction in crop yield or significant foliar damage. However, in Long Island, NY, only 50% control of Colorado potato beetle was achieved in a potato field (Moyer et al. 1997) which exhibited 100.8-fold levels of resistance (Zhao et al. 2000). Resistance to insecticides in Colorado potato beetle builds up year after year until economic yield reductions occur. If resistance to imidacloprid continues to develop, growers will be left with few control options, and reduced potato productivity and greatly increased control costs could result. Although resistance of Colorado potato beetle to imidacloprid was detected in one site in Long Island, NY (Zhao et al. 2000), it is essential to know the extent of resistance to design strategies of resistant management.

Imidacloprid is a compound that kills insects slowly unless applied at very high doses. One of the major effects of imidacloprid is the knock down of beetles. Results of topical bioassays for detection of resistance in Colorado potato beetle usually takes from seven to 10 days because some beetles recover from the effects of the insecticide in a period of time from three to 10 days. Other beetles die during this period of time. However, some beetles do not recover from the effects of imidacloprid and stay knocked down with slow movements of legs. Knock down beetles that do not recover in 7 or 10 days from the effects of imidacloprid will eventually die. Therefore, mortality is defined as either dead beetles or beetles that were knocked down 10 days after treatment. These results allow

for the estimation of the Lethal Dose fifty (LD_{50}). However, getting faster data about levels of the resistance from the field populations could be compromised due to the long time to evaluate the mortality. An alternative to conventional bioassays is the use of the Knockdown Fifty (KD_{50}), defined as the time that is necessary to knock down 50% of beetles treated with a single dose of imidacloprid. A high correlation between fast KD_{50} and mortality 10 d after treatment (as defined above) would be an indication that this method of bioassay could be used for a fast detection of resistance.

The Colorado potato beetle had also developed resistance to several insecticides due to metabolic resistance (see Chapter 4 of this thesis). In this research, I also explored the effects of the synergist piperonyl butoxide (PBO) in the suppression of resistance in beetles from Long Island resistant to imidacloprid collected in 1999.

The objectives of this study were to evaluate if resistance to imidacloprid is widespread in field populations of Colorado potato beetle from Long Island, to assess if the use of KD_{50} is a reliable and rapid method of resistance detection, and to explore the effect of PBO in the suppression of resistance to imidacloprid in Colorado potato beetle.

MATERIALS AND METHODS

Insecticides

Imidacloprid (98.7%, technical grade) was provided by Bayer Corporation (Kansas City, MO). Piperonyl butoxide (90%, technical grade) was bought to Aldrich Chemical Company, Inc.

Populations.

Long Island 1998. Five field populations of Colorado potato beetle adults were collected from Long Island, NY in August 1998 (Table 2). The Suffolk Long Island population was

Table 2. Number of applications (in parentheses), formulation, and rate of imidacloprid used on Colorado potato beetle From 1995 to 1998 at the five locations where adults were collected in 1998.

	Products (number of applications)				Number of applications		
location	1995	1996	1997	1998	Admire®	Provado®	Total
Cutchogue	Provado[a] (3)	Admire[b] (1) Provado[a] (1)	Admire[b] (1) Provado[a] (1)	Admire[c] (1) Provado[a] (1)	3	6	9
Mattituck	Provado[a] (4)	Provado[a] (4)	Admire[b] (1)	Admire[c] (1)	2	8	10
Calverton	Provado[a] (4)	Provado[a] (4)	Provado[a] (2)	Provado[a] (2)	0	12	12
Janesport	Admire[d] (1)	Admire[d] (1) Provado[a] (1)	Admire[d] (1) Provado[a] (1)	Admire[c] (1) Provado[a] (1)	4	3	7
Riverhead	Admire[e] (1) Provado[a] (2)	Admire[f] (1) Provado[a] (1)	Admire[f] (1) Provado[a] (1)	Admire[g] (1)	4	4	8

[a]Provado 0.273 l/ha (3.75 fl oz / acre, Spot treatment)

[b]Admire 1.007 l/ha (13.8 fl oz / acre)

[c]Admire 1.168 l/ha (16 fl oz / acre)

[d]Admire 0.956 l/ha (13.1 fl oz / acre)

[e]Admire 0.985 l/ha (13.5 fl oz / acre, applied in borders)

[f]Admire 0.985 l/ha (13.5 fl oz / acre)

[g]Admire 1.095 l/ha (15.0 fl oz / acre)

collected in July 1997 and reared on cv. Russet Burbank potato in the laboratory at Michigan State University. The approximate distance between collection sites in 1998 was 8 km except for the Mattituck and Cutchogue sites, which were only 2.5 km apart. In the field, each population collected was exposed to potatoes treated with imidacloprid applied in furrow at planting (Admire® Bayer Corp., Kansas City, MO) and/or to foliar applications of Provado® (Bayer Corp., Kansas City, MO) from 1995 to 1998 (Table 3). The susceptible colony was originally collected from several commercial potato fields in Michigan and has been reared without selection pressure in the laboratory at MSU for 10 years. Beetles were maintained on cv. Superior potato plants grown in the greenhouse.

Long Island, NY collection, 1999. Five adult field populations of the Colorado potato beetle were collected in Long Island, NY. The Suffolk population collected in July 1997 was also included in the study. Two additional populations were collected from Hudson, NY and Midland, MI.

Bioassays

Long Island 1998. Topical bioassays were used to assay adult resistance. Technical grade insecticide was diluted with acetone. Five doses that resulted in more than 0% and less than 100% mortality based in preliminary assays were used. Ten beetles (1 to 2 wk old in the case of the susceptible S64 and unknown age for the field populations) were treated with 1 µl of solution on the ventral area of the abdomen with a 50 µl microsyringe connected to a microapplicator (Hamilton Company, Reno NV). The control beetles were treated with 1 µl of acetone only. Two to three replications per concentration were performed. After treatment, beetles were placed in Petri dishes and fed potato leaves and kept at 28 °C, 50 % relative humidity, photoperiod 16:8 (L:D). Mortality as defined in the

introduction was assessed at 1, 3, 5, 7, and 10 d after treatment. Beetles that were unable to stand on their legs and walk a distance equal to their own body length were counted as knock down. Previous experiments reported that beetles treated with imidacloprid may recover from intoxication from three to 10 days (Zhao et al. 2000) unpublished experiments of this research). Recovery was more common in resistant than in susceptibles beetles. Beetles that did not recover within 10 days after treatment died in the following days. Thus knock down beetles and dead beetles 10 days after treatment were considered dead.

Long Island 1999. To determine resistance in field populations of Colorado potato beetle from Long Island, N.Y. in 1999, a similar topical bioassay procedure described above was followed. However, only one dose of 3.16 µg of a.i./ beetle was tested. This dose was selected because some beetles of the Long Island strains survived doses of more than 10 µg/beetle (Results from 1998). However, this dose would be enough to cause knock down of resistant beetles. Three replications were performed, each consisting of 10 beetles. Knockdown beetles were counted periodically for 24 hours after the treatment. In addition, mortality and knockdown were assessed at 3, 5, 7, and 10 days after treatment.

Bioassays of single dose of imidacloprid + piperonyl butoxide.

The bioassays and the dose were similar to the above procedure for bioassays in Long Island 1999, except that 1 h before the application of imidacloprid, beetles were treated with 5 µg of piperonyl butoxide. Previous experiments had indicated that this dose of synergist did not cause any mortality (unpublished data).

Analysis of results

Data were analyzed by Probit analysis (SAS 1995, SAS 2000). Abbott's formula was used to correct for natural mortality (Abbott 1925). Lethal Dose fifty (LD_{50}), Knockdown fifty (KD_{50}), fiducial limits, slope ± SE, and chi-square goodness of fit values were determined. Resistance ratios were calculated as the LD_{50} value of the resistant colony / LD_{50} value of susceptible colony, and the KD_{50} value of the resistant colony / KD_{50} value of the susceptible colony. To analyze the data for knockdown beetles 10 d after the treatment from Long Island, NY 1999, an arcsine transformation was used. A regression analysis was made between the KD_{50s} for the beetle populations and mortality 10 days after treatment for the same populations.

RESULTS AND DISCUSSION

Resistance to imidacloprid 1998.

Widespread resistance to imidacloprid was found in Long Island. Resistance ratios of the field-collected populations ranged from 27.8 to 155.3 fold (Table 3). The highest resistance levels were found at Cutchogue, Mattituck, and Calverton where populations were heavely treated with imidacloprid. They were treated eight or more times with imidacloprid from 1995 to 1998 (Admire® and/or Provado®) (Table 2). The Janesport population, which was treated seven times from 1995 to 1998, exhibited intermediate levels of resistance. Although the Riverhead site was treated eight times, we did not find a significant difference in the LD_{50} level between this population and the susceptible population. Perhaps field to field crop rotation with small grains helped mitigate resistance in this population. Growers from Long Island have reported reduced control by

Table 3. The mortality response of a laboratory susceptible strain and six field collected strains of Colorado potato beetle from Long Island, NY to topical applications of imidacloprid.

Population	n	slope ± SE	χ^2	[a]LD_{50} (95% fiducial limits) (µg/beetle)	[c]RR
Susceptible	160	5.2 ± 0.8	0.692	0.076 (0.067, 0.089)	1
13Mile, MI	180	2.6 ± 0.4	0.503	0.010 (0.082, 0.132)	1
Riverhead, LI NY	108	0.5 ± 0.8	0.846	0.620 (0.003, 2.160)	8
Jamesport, LI NY	117	1.0 ± 0.2	0.255	2.100 (1.060, 3.890)	28
Suffolk, LI NY	180	1.7 ± 0.2	0.546	2.150 (1.580, 3.090)	28
Calverton, LI NY	114	1.0 ± 0.4	0.077	5.710[b]	75
Mattituck, LI NY	117	1.0 ± 0.2	0.144	10.400(5.470, 28.400)	133
Cutchogue, LI NY	107	0.5 ± 0.2	0.776	11.810 (3.500, 56.200)	155

[a]LD_{50} = Lethal dose fifty

[b]Unable to calculate fiducial values

[c]RR = Ratio of resistance (LD_{50} field population / LD_{50} susceptible population)

Admire® and Provado® in some potato fields which was confirmed in field efficacy trials. The reduced effectiveness of imidacloprid has led Long Island producers to start using avermectins, another class of compound, as an alternative insecticide.

The LD_{50} for the Suffolk population (2.42 μg / beetle 95% CL= 1.58-3.71 μg / beetle) determined by Zhao et al.(2000) was confirmed in our studies. However, due to differences in the susceptible populations used in the two studies, their susceptible LD_{50} was lower (0.021 μg / beetle) than in the susceptible strain used in this study (0.076 μg / beetle). Consequently the resistance ratio reported by Zhao et al.(2000) for the Suffolk population was 100.8 fold while we reported a 28.2 fold ratio. If we use the Zhao et al.(2000) susceptible value the resistance ratio for the Suffolk population would be 100.9 fold and for the most resistant Long Island population, the Cutchogue population, the ratio for resistance would be 562.3 fold. The LD_{50} for the unselected Suffolk population was 2.42 μg/beetle in 1997 and after 14 months of continuous culture without selection, the LD_{50} was 2.15 μg/beetle. These results indicate that imidacloprid resistance may be stable under insectary rearing conditions since there was no significant decrease in susceptibility.

Growers with the highest resistant populations used more foliar applications of Provado® than Admire® in-furrow treatments. The Riverhead and Janesport sites used Admire® from 1995-1998 and four or less applications of Provado®. Beetles at these sites exhibited the lowest resistance populations. These differences probably occur because imidacloprid in-furrow treatment may result in higher dosages and longer persistence in potato plants than for foliar application. The higher, more persistent dosage of Admire® may reduce the survival of resistant heterozygous beetles, slowing resistance

development. In contrast, Provado® (foliar) treatments result in an initially high peak concentration followed by rapid attenuation creating a low dose exposure to beetles that avoid the initial dosage peak.

Colorado potato beetle resistance to imidacloprid is autosomal and is inherited in an incompletely recessive manner (Zhao et al. 2000). F1 offspring of R x S parents are 13.2 fold resistant compared with a 110.8 fold for the resistant parents. A high dose strategy is the use of a dose that kills RS heterozygotes (to make the R gene functionally recessive) to maximize impact of susceptible immigrants (Tabashnik and Croft 1982). Both soil and foliar treatments could be defined as high dose strategies because they kill both susceptible and heterozygote genotypes. However, low persistence of Provado® treatments could allow survival of heterozygotes at some point after application. In addition, foliar treatments of Provado® may result in uneven coverage of the plant and some beetles could be in locations receiving lower doses. Rapid foliage expansion early in the season may also contribute to untreated plant areas in treated fields.

Significantly lower values of the probit regression slopes were observed in most of the Long Island populations compared with the susceptible population (Table 3 and Figure 3). This indicates a high degree of variability in the resistant populations and the potential for even higher resistance if high selection pressure continues.

The higher levels of resistance to imidacloprid found in Long Island have not yet occurred in Michigan or other areas of the country, possibly because growers are not using both Admire® and Provado® in the same season against the overwintering and summer generations. Other factors present on Long Island include the extremely low threshold to control Colorado potato beetle, the presence of more than one generation per

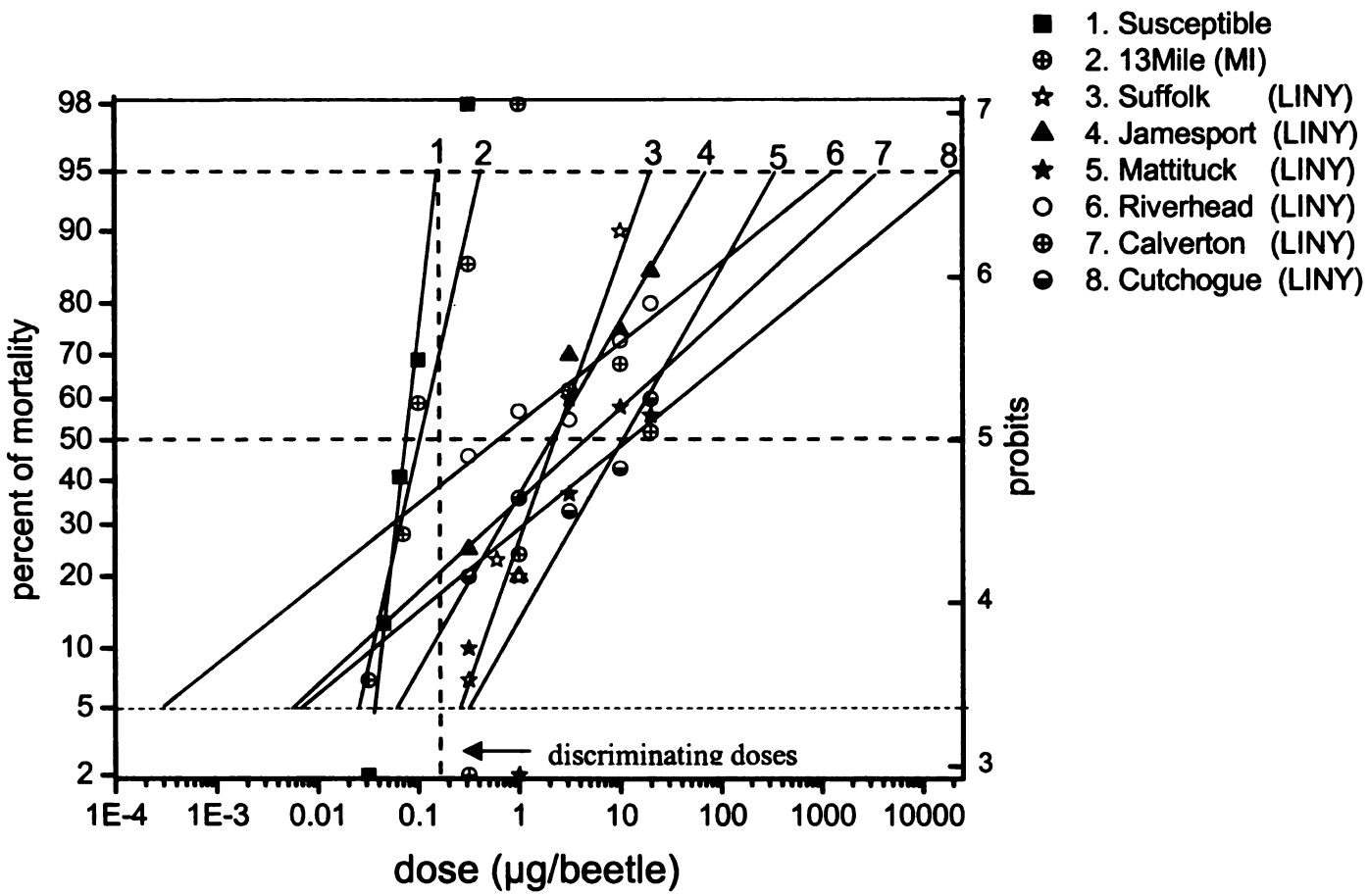

Figure 3. Log doses probit lines of resistant and susceptible populations of Colorado potato beetle to imidacloprid.

year, and the lack of crop rotation and cultural control in conjunction with very reduced alternative non-crop hosts have led to the worst case scenario for the development of resistance (Weber and Ferro 1994). In addition, initial susceptibility to insecticides is important in the development of resistance. Prior to imidacloprid use in the field on Long Island, two Long Island populations were 16-fold less susceptible than the most susceptible populations from Wisconsin (Olson et al. 1996). In 1998, a significant shift in susceptibility was found in Long Island populations (Olson et al. 2000). Olson et al. (2000) used first instar larvae to monitor resistance, but Zhao et al. (2000) reported that resistance in second instar larvae only expressed 13-fold resistance in comparison with a 100 fold of the adults. Thus expression of resistance in early instars can be masked due to lack of full physiological development in first instars to survive insecticide treatment. In addition, symptoms of intoxication by imidacloprid inhibit the feeding of the larvae, resulting in starvation and the death of the larvae. In contrast, adults of Colorado potato beetle treated with imidacloprid also reduced their feeding, but higher amount of reserves allow them to survive after they recovered from the effects of intoxication. Monitoring resistance by using adults is particularly important when growers use foliar sprays of imidacloprid. Immediately after foliar treatment the insecticide can eliminate susceptible adults and larvae. However, some resistant beetles could recover after some days of the exposure and lay eggs when there is too little insecticide to kill early instars larvae. A comparable situation could happen with imidacloprid applied in-furrow treatment. High initial doses in potato plants protect the plants from beetles, but after some period of time residues inside the plant wouldn't be sufficient enough to kill resistant beetles. As a result, the beetles would be able to lay eggs when the amount of imidacloprid is not large

enough to kill the first instar larvae. Another important factor of monitoring resistance in adults involves the fact that the beetles overwinter. Determination of resistance can be done at the end of the season and a year before the next crop season.

Other factors such as selection pressure, pest biology, and migration affect the development of resistance to insecticides. Due to the limited dispersal of Colorado potato beetle, local selection plays an important role in resistance (Grafius 1995). Periodic dispersal caused by limited food availability (e.g. crop rotation) in the spring also contributes. However, gene flow caused by periodical dispersal usually occurs over long periods of time and can be responsible for disseminating individuals that carry resistant alleles (Grafius 1995). Rapid development of resistance to imidacloprid is more likely due to high selection pressure and common mechanisms of resistance already present in Long Island populations. Intense use of insecticides including imidacloprid and a behavior history of high selection pressure by growers has resulted in a legacy of widespread multiple and cross-resistance to many different insecticides. In fact, Colorado potato beetle is the seventh most resistant species to pesticides in the world (Mota-Sanchez et al. 2002), and beetle populations from Long Island have developed resistance to most of the compounds listed for this species.

A similar trend in pesticide use has been recorded in Spain in the Almeria region where intense pesticide use led to the rapid development of imidacloprid resistance of the whitefly, *Bemisia tabaci* Gennadius (Cahill et al. 1996). Resistance to imidacloprid (15-fold) was also reported in a field population of *Bemisia argentifolii* Bellows & Perring collected in the Imperial Valley, California. This level of resistance, did not result in field

failure, yet subsequent selection over 32 generations led to a 78-fold level of resistance in this species (Prabhaker et al. 1997).

Discriminating doses.

A discriminating dose that separates the susceptible from the resistant beetles could be chosen from Figure 3. The LD_{95} corresponds to about 0.11 μg/beetle for the susceptible population. This dose would kill from 40% to 0 % of the Long Island beetles. A steep slope was found in the susceptible population and in the Michigan field populations. This condition generally indicates the degree of homozygosity regarding susceptibility. Lower values of the slope indicated heterozygosity and progress to high levels of resistance. However, (Chilcutt and Tabashnik 1995) pointed out that higher slopes did not mean necessarily low or high resistance or that low values of slope mean a high degree of genetic variation. The slope indicates the phenotypic variation (environment plus genetic variation). In this research, the environmental conditions were similar in all bioassays, except the age of the beetles from the field populations. These beetles were collected at the end of the season (overwintered generation). Therefore, the shallow slope of the Long Island populations may be due to a bigger genetic variation. The Michigan population also was collected in the field at the end of the season (overwintered population). If the age were the cause of bigger phenotypic variation, a low value of the slope would be expected. However, this population has similar values of the slope to that of the susceptible population.

Resistance to imidacloprid in 1999.

Knock down fifty ratios of resistance of Long Island populations ranged from 1.5 to 7.5 (Table 4 and Figure 4). The highest levels of Colorado potato beetle resistance were

Table 4. The knockdown response of a laboratory susceptible strain and seven field collected strains from New York and one from Michigan to topical applications of imidacloprid.

Population	[a]KD_{50} (95% confidential limits) (minutes)	slope ± SE	χ^2	[b]RR	Mortality 10 days after treatment (percent)
Midland, MI	32 (28, 36)	3.0 ± 0.3	0.9133	0.46	100
Susceptible	68 (63, 75)	4.0 ± 0.3	0.8183	1	99
Hudson, NY	77 (69, 85)	3.3 ± 0.3	0.9449	1.1	100
Suffolk, LI	106 (96, 116)	3.9 ± 0.3	0.4441	1.5	83
Jamesport, LI	135 (117, 157)	2.1 ± 0.1	0.4350	2	66
Calverton, LI	142 (125, 160)	3.1 ± 0.2	0.6762	2.1	70
Zilverton, LI	266 (216, 322)	2.8 ± 0.3	0.0049	3.9	80
Rutkoski, LI	318 (293, 344)	3.4 ± 0.2	0.6985	4.6	33
Wells, LI	515 (466, 574)	3.2 ± 0.4	0.9894	7.5	20

[a]Resistance ratios = KD_{50} of field populations / KD_{50} of the susceptible population

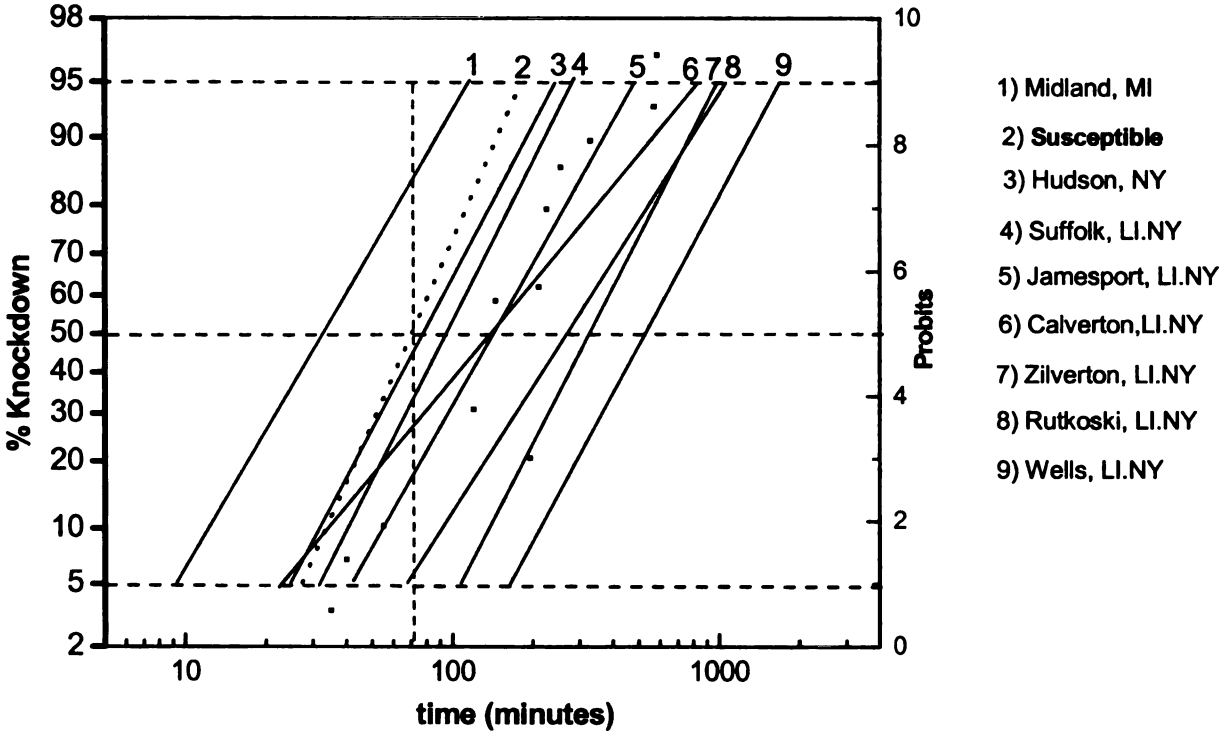

Figure 4. Log time probit lines of resistant and susceptible populations of Colorado potato beetle to imidacloprid (a single dose of 3.16 μg / beetle).

found at the Wells, Rutkoski, and Zilverton locations where these populations also experienced eight or more imidacloprid treatments since 1995 (Admire and/or Provado). The Hudson, NY and Midland, MI populations exhibited lower levels of resistance than the Long Island populations. The levels of resistance using KD_{50} seemed to be lower in comparison with bioassays using an entire range of doses (results of bioassays in 1998 indicated up to 150 fold levels of resistance). However, use of KD_{50} is a quick way of diagnosing resistance because it allows a separation of resistant populations from the susceptible strain at the level of KD_{50}.

Ten days after treatment, a high percent of mortality was found in the susceptible populations from Midland, MI and Hudson, NY. Conversely, significant survival was found in beetles from all Long Island populations (Table 4). Figure 5 shows a high correlation between KD_{50} and mortality ($r= 0.908$, $p=0.0007$). These results confirm that susceptible populations will have faster knock down than resistant populations. In addition, this fast knock down was correlated with high mortality 10 days after treatment. One of the disadvantages of using KD_{50} is that it is very important to observe the beetles for up to 10 h during the experiment. However, bioassays of Colorado potato beetles by using KD_{50} could be a valuable tool for fast detection of resistance. In addition, less effort and time in beetle collection and bioassays are invested because only a maximum of 30 beetles per population are needed to do the bioassays in comparison with the minimum of 100 beetles needed for getting an LD_{50} for a topical bioassay using an entire set of doses.

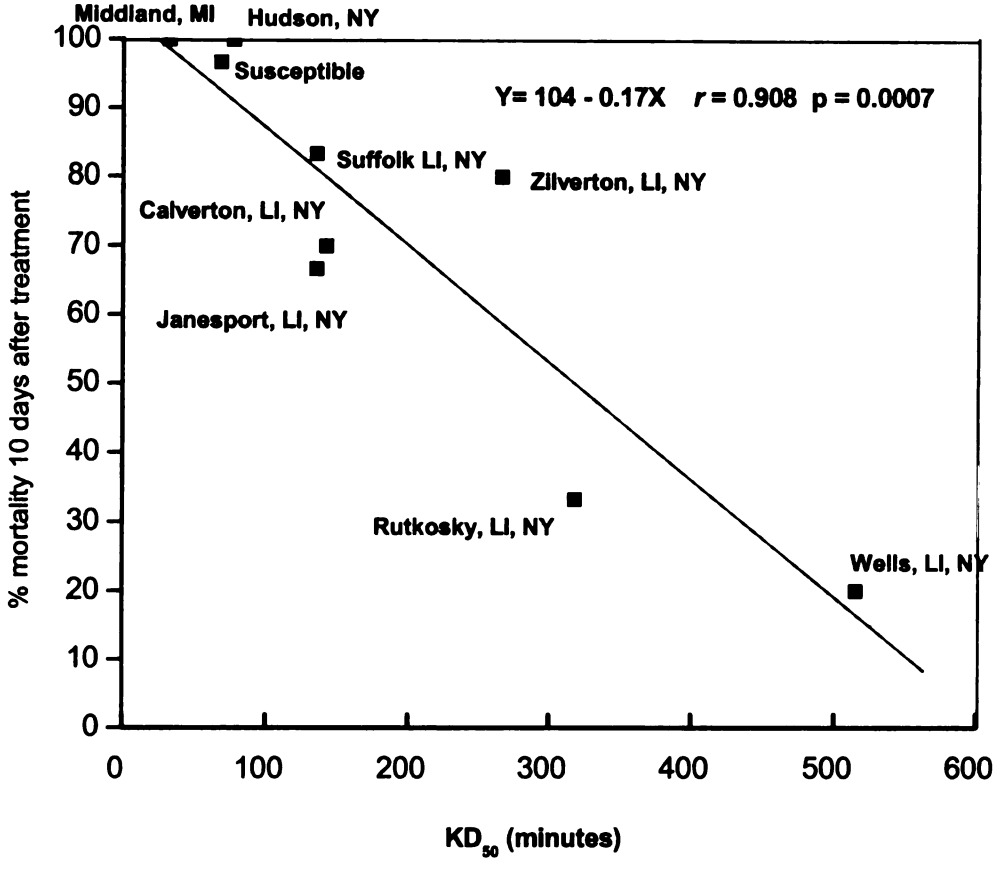

Figure 5. Regression mortality 10 days after treatment versus KD_{50}s of resistant and susceptible populations of Colorado potato beetle.

Bioassays of single doses of imidacloprid + piperonyl butoxide.

Piperonyl butoxide partially increased the mortality of resistant beetles from Long Island, NY (Table 5 and Figure 6). The method of bioassays using only a single dose had some limitations because the toxicity of the compounds is not measured to the full range lethal doses. However, the results would suggest that the P450 mechanism of resistance is a factor in the resistance to imidacloprid from beetles collected in Long Island, NY, but it was a surprise that results from pharmacokinetics and metabolism (Chapter 4) indicated that metabolic resistance is not important in the resistance of Colorado potato beetle to imidacloprid. Probably the use of piperonyl butoxide supressed the cytocrome P450 mechanism that is vital for other physiological functions, resulting in a indirect effect in the interaction of the P450 mechanism and imidacloprid.

CONCLUSIONS

Until 2002 imidacloprid was the only registered insecticide effective for Colorado potato beetle control in many potato growing regions in the US. Abamectin is also registered but is limited to two applications per season. High levels of resistance to imidacloprid detected in many sites on Long Island, NY in 1998 and significant survival in the laboratory of beetles to a high single dose in 1999 should be a warning of potential major problems in the near future. Management strategies that do not rely exclusively on the use of imidacloprid and other neonicotinoid compounds must be developed and implemented. The KD_{50} method could be used as a resistance assay if a rapid diagnostic method for insecticide resistance is needed.

Table 5. The knockdown response of a laboratory susceptible strains and seven field collected strains from New York and Michigan to topical application of imidacloprid +PBO.

Population	**[a]KD_{50} (95% confidential limits) (minutes)**	**slope ± SE**	χ^2	**[b]RR**	**Mortality 10 days after treatment (percent)**	**[c]SR**
Midland, MI	27 (25, 30)	5.0 ± 0.5	0.3606	1	100	1.2
Susceptible (lab)	28 (26, 31)	4.5 ± 0.5	0.3009	1	99	2.4
Hudson, NY	58 (54, 61)	7.1 ± 0.6	0.1888	2	100	1.3
Suffolk, LI NY	91 (84, 98)	6.8 ± 0.9	0.7883	3.2	57	1.2
Jamesport, LI NY	132 (120,146)	4.1 ± 0.3	0.7655	4.6	96	1.0
Calverton, LI NY	89 (81, 99)	3.9 ± 0.3	0.3672	3.1	95	1.6
Zilverton, LI NY	172 (152,193)	2.8 ± 0.2	0.4095	6	95	1.5
Rutkoski, LI NY	164 (141,185)	2.7 ± 0.3	0.5685	5.7	72	1.9
Wells, LI NY	325 (281, 337)	2.7 ± 0.3	0.5467	11	53	1.6

[a]Resistance ratios = KD_{50} of field populations / KD_{50} of the susceptible population

[b]Sinergistic ratio = KD_{50} of population treated with imidacloprid / KD_{50} of the population trated with imidacloprid + PBO

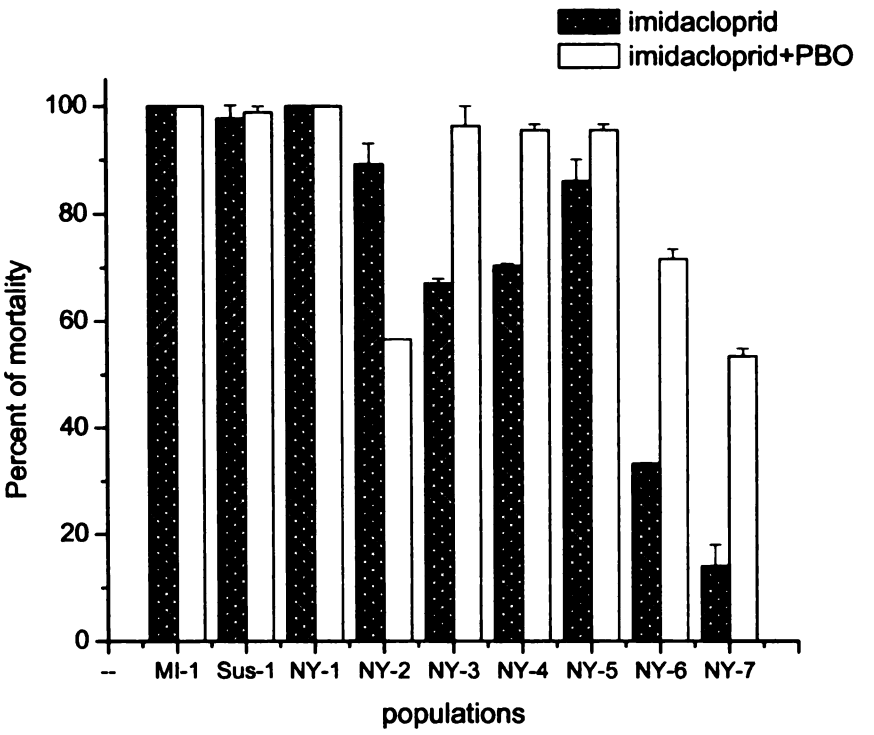

Figure 6. Percent of mortality of Colorado potato beetle in susceptible and field populations from Michigan and New York to topical treatments of imidacloprid and imidacloprid + PBO. MI-1 = Midle, MI, Sus-1 = Laboratory susceptible, NY-1 = Hudson, NY, NY-2 = Suffolk, LI, NY, NY-3 = Janesport, LI, NY, NY-4 = Calverton, LI, NY, NY-5 = Zilverton, LI, NY, NY-6 = Rutkosky, LI, NY, NY-7 = Wells, LI, NY.

Insecticide rotation, crop rotation, propane flamers, and trench traps were widely used in some areas during the early 1990s when no effective insecticides were available. However, except for crop rotation, they were generally abandoned following the introduction of imidacloprid. If re-integrated into the control program for CPB, these strategies and tactics may prolong the useful life of the neonicotinid insecticides for CPB control.

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CHAPTER 3

REDUCED CROSS-RESISTANCE OF COLORADO POTATO BEETLE TO COMPOUNDS THAT INTERACT WITH THE INSECT ACETYLCHOLINE RECEPTORS.

INTRODUCTION

Oppenoorth and Welling (1976) defines cross-resistance as " resistance in a strain of insects to compounds other than the selective agent, due to the same mechanism. In contrast, multiple resistance is the resistance of a single strain to several different compounds but resulting from different mechanisms." Numerous examples of cross-resistance occur in insects, for instance, cross-resistance in cyclodienes led to many problems in western corn rootworm, *Diabrotica virgifera virgifera* LeConte and the malaria mosquito, *Anopheles gambiae* Giles. Examples of multiple resistance are found in the cattle tick, *Boophilus microplus* (Canestrini) that is resistant to organochlorines, organophosphates, and carbamates (Metcalf 1983). Cross-resistance has been observed in Colorado potato beetle between aldicarb and permethrin, carbofuran, endosufan, and azinfosmethyl (Ioannidis et al. 1991).

The nicotinic acetylcholine receptors (nAChR) located solely in the central nervous system of insects are the primary targets for cartap and its nereixtoxin parent (Eldefrawi et al. 1986), nicotine and neonicotinoid insecticides including imidacloprid (Schroeder and Flattum 1984, Bai et al. 1991). Due to their similar structure and mode of action, nicotine and neonicotinoids are grouped as nicotinoid insecticides (Yamamoto 1999). Application of neonicotinoids to the insect nervous system resulted in a increase in the frequency of spontaneous discharge followed by a total block of the impulse propagation (Schroeder and Flattum 1984). However, cartap acts as an antagonist of the nAChRs (Nagata et al. 1997). Imidacloprid was the first active ingredient of this chemical class to reach the market (Thyssen and Machemer 1999). After the discovery and patent of imidacloprid, there was significant research on molecules with similar structure to the 6-

chloro-3-pyridymethyl moiety (Sheets 2001), including, acetamiprid, nitenpyram and thiachloprid (Takahashi et al. 1992, Minamida et al. 1993, Sheets 2001). Recently the changed of the chloropyridinyl moiety for a chlorothiazol group resulted in the subgroup called" the second generation" of neonicotinoid insecticides (Boelle et al. 1998). Compounds in this group are chothinidin and thiamethoxam (Maienfisch et al. 1999). Thiamethoxam was the first compound of this group to be used in the market after its discovery. This compound is used for foliar and seed treatments (Maienfisch et al. 2001). In standard laboratory assays thiamethoxam is slightly more effective than imidacloprid and superior to acetamiprid and nitenpyram. Thiamethoxam was first registered in the U.S. for control of Colorado potato beetle and other pests in potatoes in 2001. Since Colorado potato beetle has developed resistance to imidacloprid (Chapter one), cross-resistance could be expected to other compound with the same mode of action. Colorado potato beetle has developed resistance to bensultap in potato growing areas of Hungary after being exposed to this compound for more than three years (Pap et al. 1997).

The objectives of this research were to determine if cross-resistance to thiamethoxam and bensultap is present in populations of Colorado potato beetle that are resistant to imidacloprid.

MATERIAL AND METHODS

Insecticides.

Three insecticides that act at the insect acetylcholine receptors were used: imidacloprid (98.7%, technical grade) and nytempiran (98.5%, technical grade) (Bayer Corporation, Kansas City, MO), thiamethoxam (99%, technical grade, Novartis Crop Protection, Inc.,

Greensboro, NC), bensultap (96%, technical grade, Takeda Chemical LTD, Tokyo, Japan), and nicotine (98% technical grade, Aldrich Chem. Co. city).

Long Island, NY collection 1998.

Beetles resistant to imidacloprid from Riverhead, Cutchogue, Janesport, Mattituck, and Suffolk were tested for resistance to thiamethoxam. See Chapter 2 for procedures of beetle collection. The susceptible population was the same tested in 1998 (see Chapter 2). Due to the limited number of beetles available only the Suffolk, and susceptible population were assayed for cross-resistance to bensultap.

Bioassays.

The method of bioassay was similar to the detection of resistance to imidacloprid (first chapter), except that the doses for thiamethoxam were 20, 10, 3.2, 1, 0.32, 0.10, 0.03, 0.01, 0.0032, or 0.001 μg/beetle. A similar criterion of mortality defined in Chapter 2 was used in this experiment.

Data analysis. Mortality was evaluated 3, 5, and 10 days after treatment. However, only the 10 day results were submitted to Probit analysis (SAS 2000). Statistical differences were determined based on overlapping or non-overlappping of 95% confidence limits.

RESULTS AND DISCUSSIONS

Cross-resistance to neonicotinoids

Low levels of resistance to the second generation neonicotinoid compound, thiamethoxam, were found in Janesport, Mattituck and Suffolk populations (Table 6).

Table 6. The mortality response of a laboratory susceptible strain and five field collected strains of Colorado potato beetle from Long Island, NY to topical applications of thiamethoxam.

Population	**n**	**slope ± SE**	χ^2	**[a]LD_{50} (95% fiducial limits) (µg/beetle)**	**[b]RR**
Susceptible	180	3.1 ± 0.5	0.576	0.159 (0.123, 0.192)	1
Riverhead, LI NY	112	1.7 ± 0.4	0.116	0.228 (0.140, 0.322)	1.4
Cutchogue, LI NY	104	2.7 ± 0.5	0.913	0.256 (0.189, 0.333)	1.6
Janesport, LI NY	109	2.8 ± 0.5	0.543	0.270 (0.206, 0.348)	1.7
Mattituck, LI NY	118	2.5 ± 0.4	0.143	0.373 (0.287, 0.507)	2.3
Suffolk, LI NY	180	1.4 ± 0.3	0.484	0.497 (0.352, 0.864)	3.1

[a]LD_{50} = Lethal dose fifty

[b]RR = Ratio of resistance (LD_{50} field population / LD_{50} susceptible population)

Resistance ratios for thiamethoxam ranged from 1.7 to 3.1 fold. The Long Island populations have not been exposed to this compound and there is no cross-resistance to bensultap in the Suffolk population (Table 7). A limited number of reports show cross-resistance in insects to compounds that affect the acetylcholine receptors including evidences of cross-resistance between imidacloprid and nicotine in aphids (Cahill et al. 1996). The most significant example is the cross-resistance between imidacloprid and thiamethoxam and acetamiprid in *Bemisia tabaci* in Southern Spain (Elbert and Nauen 2000). The use of a diagnostic concentration of imidacloprid, thiamethoxam and acetamiprid resulted in a mortality of whiteflies of less than 30% in 1998. Conversely, in the laboratory strain the discriminating doses caused more than 80% mortality (Elbert and Nauen 2000). In greenhouses trials applications of imidacloprid and thiamethoxam resulted in reduced control in Almeria (Elbert and Nauen 2000). Less dramatic examples including the diamondback moth, *Plutella xylostella* (L.) in which a population with 9.1-fold resistant to cartap expressed negligible levels of resistance to acetamiprid (2.1-fold) (Akayama and Minamida 1999). However, diamondback moth resistant to cartap was also cross- resistance to bensultap and thyociclan (Hama 1986). All of these compounds are metabolized to nereistoxin, compound that is responsible for the interaction at the nicotine acetylcholine receptors. There may be similar pathways that also confer cross-resistance. Insects resistant to conventional insecticides (such as organophosphates, carbamates, and pyrethroids) have expressed very low levels of cross-resistance to neonicotinoid compounds in some species such as the small brown plant hopper, *Laodelphax striatellus* Fallen, Lygus bug, *Lygus hesperus* Knight (Dennehy and Russel 1996), and German cockroach, *Blatella germanica* L. (Wen and Scott 1997). However,

Table 7. The mortality response of a laboratory susceptible strain and a field collected strain of Colorado potato beetle from Long Island, NY to topical applications of bensultap.

Population	**n**	**slope ± SE**	χ^2	**[a]LD_{50} (95% fiducial limits) (µg/beetle)**	**[b]RR**
Susceptible	180	3.4 ± 0.4	0.804	7.1 (5.5, 8.7)	1
Suffolk, LI NY	180	2.2 ± 0.3	0.634	11.1 (8.2, 14.4)	1.5

[a]LD_{50} = Lethal dose fifty
[b]RR = Ratio of resistance (LD_{50} field population / LD_{50} susceptible population)

neonicotinoids have good efficacy against a wide range of pests resistant to conventional insecticides such as aphids, whiteflies, leafhoppers and planthoppers (Elbert et al. 1991, Elbert et al. 1998).

A history of exposure of Colorado potato beetle to many classes of insecticides on Long Island has selected many mechanisms of resistance in the Colorado potato beetle. It however is unknown the principal mechanism for imidacloprid resistance. (Zhao et al. 2000) pointed out that esterases and oxidative metabolism are involved in the resistance of Colorado potato beetle to imidacloprid. In the results of Chapter 2, it is explained that PBO partially suppressed resistance of Colorado potato beetle to imidacloprid in Long Island populations. However, studies using radiolabeled imidacloprid show no differences in metabolism in resistant and susceptible strains of Colorado potato beetle (see Chapter 4). Different binding sites on the nicotine acetylcholine receptors may also explain the mechanism of resistance. However, in other imidacloprid resistant insects, *Myzus persicae* (Sulzer) and *Myzus nicotianae* (Blackman), no detectable differences in target site binding were demonstrated with binding assays using ($^{+3}$H) imidacloprid in both species (Nauen et al. 1996). Although *M. nicotianae* was 10-fold more resistant to imidacloprid than *M. persicae*, there were no differences in the binding kinetics between the strains, suggesting that target site insensitivity was not involved in the aphids' resistance to imidacloprid (Nauen et al. 1996). Target site modification has also been dismissed in strains of *Bemisia tabaci* from Almeria, Spain (Elbert and Nauen 2000). However, to determine the imidacloprid resistance mechanism in different species, additional target site studies are essential. Bensultap, as well as other nereistoxin analogs, neonicotinoids, and nicotinoids, interact with the nAChR and, due to a similar

molecular target it would be expected cross-resistance. However, ligand experiments in honeybee nAChR defined three types of binding interactions: 1) neonicotinoids (imidacloprid and acetamiprid) that have higher affinity to the (3H)-Bungarotoxin binding site; 2) nereistoxins that have higher affinity for (3H) phecyclizine (3H)PCP binding site; and 3) nicotinoids (nicotine and anabasine) and the neonicotinoid nitempyram, which have a higher affinity for both binding sites (Yamamoto 2000). (Kayser and Lee 2002) reported that in binding studies imidacloprid binds with membranes from *M. persicae* and there is not competitive interaction between imidacloprid and thiamethoxam suggesting that there are differences binding sites for both compounds. However, N-methyl desmethyl derivative of thiamethoxam compete with imidacloprid. In addition, N-methyl imidacloprid behave as a noncompetitive inhibitor of imidacloprid. May be presence of the methyl group in the structure is responsible for differences in interaction at the binding site of nAChRs. Other nenonicotinoids such as acetamiprid, nitempyram, thiacloprid, chlotianidin and nithiazine shared similar site than imidacloprid. Reduce interaction at the nAChRs probably explain low levels of resistance and inconsistence in all populations to thiamethoxam. In the case of resistance of white flies to imidacloprid and cross-resistance to acetamiprid and thiamethoxam in Spain it is particularly interesting that there were not evidences of target site modification to imidacloprid(Nauen et al. 1998). However, the authors pointed out that they are conducting studies in resistant strains collected in 1999. It is interesting to see if in whiteflies also there is different binding sites for thiamethoxam and also if target site modification confer cross-resistance. Strains from these places have developed resistance to pyrethroids, cyclodienes, organophosphates, buprofezin and pymetrozine.

(In chapter 4 is shown that still metabolic transformation produce toxic metabolites to Colorado potato beetle). Therefore, may be target site modification is the mechanism of resistance. If there is modification of the target sites, binding interactions together with other chemical properties of the compounds that interact at the insect acetylcholine receptor would determine the development of resistance.

CONCLUSIONS

Despite that imidacloprid and bensultap act at the same molecular target, there was not cross-resistance between the Colorado potato beetle resistant to imidacloprid and the nereistoxin, bensultap. The strains Mattituck, Janesport and Suffolk from Long Island expressed low levels of resistance to thiamethoxam. Probably these levels are too low to cause field failures. However, should be a warning for conduct further studies on the mechanism of resistance to neonicotinoids compounds and monitoring should also be an important tool to detect resistance to this important group of new insecticides.

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Chapter 4

PHARMACOKINETICS OF ^{14}C-IMIDACLOPRID IN RESISTANT AND SUSCEPTIBLE COLORADO POTATO BEETLES

INTRODUCTION

The Colorado potato beetle is ranked the seventh most resistant species to pesticides in the world (Mota-Sanchez et al. 2002). The potato beetle has developed resistance to 41 different compounds, 39 of which have documented field resistance (MSU Resistance Database 2002). While under laboratory selection, the potato beetle has developed resistance to abamectin (Argentine et al. 1992) and the microbiological insecticide *Bacillus thuringiensis* var. *tenebrionis* Berliner (Whalon et al. 1993). These 41 insecticides are classified in ten groups of chemicals and eight modes of action including effects on the sodium channel for DDT and pyrethroids, inhibition of acetylcholinesterase for carbamates and organophosphates, blockage of chloride channels for cyclodienes, activation of GABA receptors for avermectin, agonist activity at nicotine acetylcholine receptors for neonicotinoids, antagonism for the same receptors for nereistoxin compounds, and binding of receptors in the midgut cells by the endotoxin of *Bacillus thuringiensis* var. *tenebrionis*.

Known mechanisms of resistance of the Colorado potato beetle to conventional insecticides include reduced insecticide penetration, target site insensitivity, and enhanced metabolism (Argentine 1991, Argentine et al. 1993, Wierenga and Hollingworth 1993, Argentine et al. 1995, Grafius 1995, Zhao et al. 2000). Of these mechanisms, penetration is a small factor in resistance while the others are major. Argentine et al. (1993) pointed out a slight, but significant decrease in penetration of azinphosmethyl in a strain resistant to this compound. However, penetration was not an important factor in carbofuran resistance (Wierenga and Hollingworth 1993).

Among the changes in sensitivity at the site of action, an important mechanism is decreased acetylcholinesterase sensitivity. This was first observed in strains of Colorado potato beetle from Michigan (Ioannidis et al. 1992, Wierenga and Hollingworth 1993). The acetylcholinesterase insensitivity seems to be specific for either carbamates or organophosphates. Acetylcholinesterase from one strain (Michigan) is insensitive to carbamates and from another strain (Long Island, NY) is insensitive to organophosphates (Wierenga and Hollingworth 1993). This work has been expanded further by Clark et al. (2001) and included sequencing of the enzyme. A point mutation of serine to glycine of acetylcholinesterase was associated with azinphosmethyl resistance (Zhu et al. 1996).

The *kdr* factor (knockdown resistance due to altered voltage-dependent sodium channels) can be also involved in resistance to pyrethroids (Ioannidis and Grafius 1988). Further studies by Argentine et al. (1995) pointed out that permethrin resistance was associated with nerve insensitivity and increased levels of carboxylesterase activity. This led to resistance through due to sequestration of permethrin by hemolymph carboxylesterases rather than rapid hydrolysis (Lee and Clark 1998).

Enhanced metabolism is one of the principal mechanisms of resistance. Rose and Brindley (1985) pointed out that monooxygenases may be involved in Colorado potato beetle resistance to carbofuran and carbaryl in Canada. Mixed function oxidase enzymes are an important mechanism of resistance to carbofuran from a Long Island population (Ioannidis et al. 1992).

More than one mechanism may be responsible for resistance. Wierenga and Hollingworth (1993) reported that altered acetylcholinesterase and enhanced metabolism by mixed function oxidase activity were involved in insecticide resistance in Colorado

potato beetle. An azinphosmethyl resistant strain combined multiple mechanisms including slightly reduced penetration, enhanced xenobiotic metabolism by glutathione transferase, and target site insensitivity (Argentine et al. 1993). Esterases were also involved in resistance to azinphosmethyl and pyrethroids (Argentine et al. 1989, Ioannidis et al. 1992, Ahammad-Sahib et al. 1994, Anspaugh et al. 1995).

The nicotinic acetylcholine receptors (nAChR) in the insect nervous system are the primary targets for cartap and its nereistoxin parent (Eldefrawi et al. 1986), nicotinoids (e.g. nicotine), and neonicotinoid insecticides including imidacloprid (Schroeder and Flattum 1984, Bai et al. 1991). Due to their similar structure, nicotinoids and neonicotinoids have a common mechanism of action (Yamamoto and Casida 1999). Imidacloprid is the most important ingredient of this chemical class to reach the market (Thyssen and Machemer 1999). Currently, 10-15% of the insecticide world market is shared by neonicotinoid compounds (Wollweber and Tiejten 1999). Strong differences in the binding potency of imidacloprid between insect and mammalian acetylcholine receptors make this product effective for insect control and safe for use (Thyssen and Machemer 1999).

Imidacloprid was registered for use on potatoes in 1995 and soon became the primary means to control Colorado potato beetles resistant to organophosphates, pyrethroids, and carbamates in Michigan and other areas in the US (Grafius 1997). Imidacloprid has been very effective against Colorado potato beetle. However, in Poland, the Colorado potato beetle has developed resistance to cartap, a nicotinic antagonist (Georghiou and Lagunes-Tejeda 1991). Thus, the Colorado potato beetle has demonstrated the potential to develop resistance to compounds that have nicotinic acetylcholine receptors as a molecular target.

This is reinforced, as shown in Chapter 2, by the development of resistance to imidacloprid and low levels of resistance to thiamethoxam (Chapter 3) in beetles from Long Island, NY. Strategies of resistance management must be instituted if the use of neonicotinoid compounds is to be sustained. Determination of the mechanisms of resistance is the cornerstone of these strategies. Therefore, the objective of this research was to compare the pharmacokinetics including metabolism of ^{14}C-imidacloprid in resistant and susceptible strains of Colorado potato beetle to determine whether this can explain resistance.

MATERIAL AND METHODS

Insects. A resistant strain (NY Selected) of Colorado potato beetle was collected from Long Island, NY in 1998 and selected for 10 generations with 5 to10 µg/beetle of imidacloprid applied topically. The susceptible strain (Hughes) was collected from an organic farm in the Upper Peninsula of Michigan in 1999. The LD_{50} of the susceptible population was 0.027 µg/beetle after topical application of imidacloprid. Both strains were maintained in the laboratory and reared on potato plants. The LD_{50} for the NY selected strain was about 10 µg/beetle.

Insecticide. ^{14}C-Imidacloprid (labeled at the methylene bridge, and with a specific activity of 125.5 µCi/mg, 32.1 mCi/mmole) and the olefine metabolite of imidacloprid were generous gifts of Dr. Ralph Nauen from Bayer, Germany. ^{14}C-Imidacloprid was purified on TLC plates (1000 mm thick silica gel plates Whatman PK6F, Maidstone, England) using a mobile phase of methylene chloride + methanol (186 + 14). ^{14}C-imidacloprid was localized by phosphoroimaging (Biorad, Personal Fx) and removed

from the TLC plates by scraping the areas where the compound was localized and then eluting the silica with ethanol. After extraction, the imidacloprid had a radiochemical purity of 98.08%.

Dosing. Insecticide was applied topically to beetles with a calibrated Hamilton microapplicator connected to a 50 µl syringe which delivered 1 µl doses. Two doses of ^{14}C-imidacloprid were used: a low dose (4400 dpm/beetle, ~16 ng) in both the resistant and susceptible strains and a high dose (248600 dpm/beetle, ~900 ng) in the resistant strain only. One microliter of the ^{14}C-imidacloprid dissolved in acetone solution was applied to the third ventral abdominal sclerite of the adult. The low dose only caused sublethal effects to the susceptible strain including hyperexcitation and knock down in some of the beetles, and mild effects to the resistant strain such as minor excitation. In contrast, the high doses caused the knockdown of beetles of the resistant strain, but did not cause mortality. Beetles treated at the high dose started recovering from the effects of intoxication 3 d after treatment. To handle beetles, they were held by the dorsal part of the body using the tip of a pipe connected to a vacuum line. Following insecticide application, beetles were held until the drop of solution dried. They were then transferred to a 20 ml glass scintillation vial. Up to four beetles were held in a vial. Each replication consisted of two sets of four beetles. Nine time intervals were used for the low dose experiments (0.5, 1, 2, 4, 8, 24, 72, 120, and 240 h) and seven times intervals for the high dose experiment (1, 2, 4, 8, 24, 72, and 120 h). Three replications were performed per exposure time. To reduce any contamination by the transfer of material such as the excreta from the vials to the beetles, beetles were transferred to new vials at each evaluation interval, except at 0.5 h.

External rinse. At the end of each time interval, both sets of beetles were transferred to a scintillation vial containing 4 ml of acetone. They were gently swirled for 20 s, and then transferred to a second scintillation vial for a second acetone rinse. To quantify the radioactivity remaining in the cuticle, two aliquots of 400 µl of acetone from the first and second rinse were put in a scintillation vial with 15 ml of cocktail fluid (Safety Solve, Research Product International Corp., Mount Prospect, IL) and measured in a liquid scintillation counter (Mark V Series, Tm Analytic) for 10 min. Background radioactivity was subtracted from the recorded radioactivity.

Excretion. To calculate the amount of excreted ^{14}C from imidacloprid, the two holding vials for each time period were rinsed and swirled for 30 s with 3 ml of methanol, and then the methanol washes from each vial was combined. The radioactivity of an aliquot of 400 µl of methanol was counted in the LSC. To get the total amount of ^{14}C excreted, the results of all fractions were combined.

Internal radioactivity and non-extractable radioactivity. After rinsing with acetone, the beetles were homogenized in a tube with 10 ml of acetonitrile using a high speed mechanical homogenizer (VirTishear) After homogenization, tubes were centrifuged for 5 min at 7000 rpm. The supernatant was decanted into a scintillation vial. The pellet was resuspended with 6 ml of methanol, vortexed, and centrifuged again. An aliquot of 400 µl was taken from each extract to count the radioactivity. To quantify the non-extractable radioactivity, the second pellet was transferred to a scintillation vial, and then 3 ml of tissue solubilizer was added (Protosol®, New England Nuclear, Boston, MA 02118). The vials were heated in a bath of water for 1 h at 45 ^{o}C, and then a few drops of hydrogen peroxide (30%) were added to decolorize the sample, and 70 µl of glacial acetic acid

were added to reduce chemiluminescence. After this procedure, 15 ml of scintillation fluid was added and the samples were held in the dark for 24 h before counting. An internal standard (^{14}C-toluene) was used to evaluate the efficiency of the counting and correct for quenching. The results of penetration, excretion, internal, and non-extractable radioactivity were expressed in terms of the percent of the dose of ^{14}C-imidacloprid applied.

Analysis of results

Statistical analysis was conducted using SAS software (SAS Institute, Cary, NC 2000). The two factors used for the pharmacokinetics of the low dose were the strain (resistant and susceptible), and time of exposure (0.5, 1, 2, 4, 8, 24, 72, and 120 h). The two factors used for the pharmacokinetics of the resistant strain treated with a low and high dose were: dose (low and high doses), and time of exposure (0.5, 1, 2, 4, 8, 24, 72, and 120 h). Pair wise comparisons were performed and t tests were used to determine significant comparisons. The response was considered the percent of radioactivity remaining in the cuticle, excreta, and inside of the insect body.

Metabolism

Extracts from the excreted and internal samples were put in scintillation vials and dried with a gentle stream of nitrogen in a bath of water at 40^{o} C. Samples were reduced to a volume of 100 µl for the low dose and 200 µl for the high dose experiments. Thirty µl of the samples were spotted on TLC plates (250 mm thick silica gel plates Whatman LK5F, Clifton, NJ). The parent imidacloprid was run on one strip as a marker. For the low dose experiment, the plates were developed by using a mobile phase of methylene dichloride + methanol (186 + 14) to a distance of 14 cm from the point of application. After drying,

the plates were covered by a thin mylar film (0.001 mm) and put in a phosphor screen. Three days after exposure, the screen was scanned in a phosphorimager analyzer (BioRad). In addition, the TLC plates were scraped in bands according to the pattern of metabolites and the silica gel was transferred to a scintillation vials for reading in the liquid scintillation counter (LSC). For the high dose experiment, the procedure for drying samples, developing TLC plates and reading was similar to the low doses. However, the non-labeled olefine metabolite was co-chromatographed with samples of internal extract and excreta. Two single dimensions systems and one double dimension systems were used to confirm the identification of the olefine metabolite in these samples. The solvents used for the single dimension systems were methylene chloride + methanol (96 + 4) and ethyl acetate + toluene + methanol + acetic acid (80 + 20 + 20 + 1). For the double dimension, the solvents were: system 1, ethyl acetate + toluene + methanol + acetic acid (80 + 20 + 20 + 1), system 2, ethyl acetate + 2-propanol + water (65 + 23 + 12). The non-labeled olefine on the plates was identified after developing using a UV chamber. The area of the metabolite was marked with a pencil, and then the plates were put with phosphor screens and scanned. The image was printed as a transparency, and matched with the original plate to determine the co-location of the ^{14}C-unknown metabolite and the non-labeled olefine. In addition, before developing the TLC plates a lane was spotted with ^{14}C-imidacloprid to identify the parent compound.

Bioassays with the olefine metabolite. A similar procedure of topical application of ^{14}C-imidacloprid was used to treat beetles with the non-label olefine metabolite. Two doses of (300 ng/insect and 700 ng/insect) were applied. Knockdown was evaluated 24 h after treatment, and the mortality was assessed 10 d after treatment. The assay in the

susceptible strain was replicated two times with 6 beetles at each dose, and in the resistant strain the assay was replicated three times with 8 beetles at each dose.

RESULTS AND DISCUSSION.

Pharmacokinetics: low dose

External rinse. ^{14}C-imidacloprid disappeared rapidly from the surface of the susceptible Hughes strain and the resistant NY Selected strain (Figure 7A). Thirty minutes after treatment more than 25% of the insecticide had penetrated into the body in both strains. A continuous reduction in the amount of ^{14}C-imidacloprid recoverable from the integument was observed in both strains at all times. Eight hours after treatment 81% of the compound in the susceptible strain and 73% in the resistant strain was lost from the surface. After 24 h, there was a reduction in the rate of penetration as compared with the earlier times, due to the low quantity of insecticide that remained in or on of the cuticle. Five days after treatment only 10% of the insecticide in the resistant strain and 3% in the susceptible strain was recoverable by the acetone wash. The results showed that the resistant strain had significantly less extractable radioactivity remaining on the cuticle than the susceptible strain ($p=0.005$). Despite a reduced percent of ^{14}C extractable from the insect cuticle of the resistant strain as compared with susceptible strain, the only significantly lower penetration of ^{14}C-imidacloprid in this strain occurred at 2 h ($p<0.012$) after treatment (Figure 7A).

Excretion and internal radioactivity. Although excretion appeared to be slightly faster in the susceptible strain, the percentage of excreted radioactivity in both strains was not significantly different ($p=0.073$). Eight hours after treatment, 68% of ^{14}C-imidacloprid had been excreted from the susceptible strain and 63% from the resistant strain (Figure

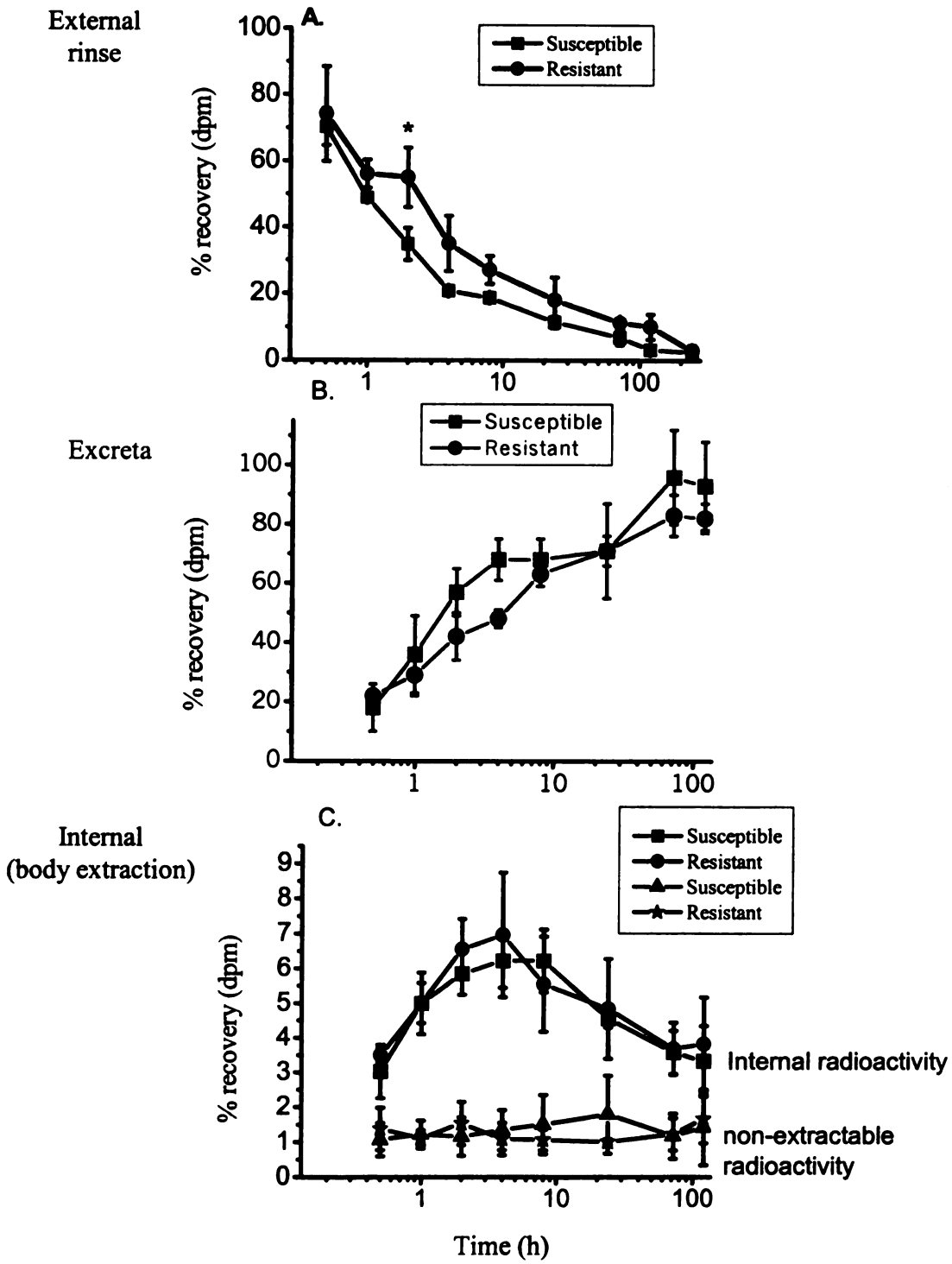


Figure 7. Pharmacokinetics of a low dose of ^{14}C-imidacloprid (16 ng/beetle) in a susceptible and a resistant strain of Colorado potato beetle.

7B). Despite that the susceptible population excreted slightly more insecticide at 4 h after application than the resistant strain (Figure 7B), there were no statistical differences in this individual comparison ($p=0.123$). Five days after treatment, 93% of the ^{14}C-dose in the susceptible strain and 80 % of the dose in the resistant strain had been excreted.

Because of this rapid excretion, only a small amount of radioactivity was found in the insect body. Most of this was solvent-extractable (Figure 7C). Internal radioactivity increased beginning 0.5 h after applied dose with a peak (6-7% of the dose) at 4 h in the resistant strain and 8 h in the susceptible insects. It then declined slowly over the next 3 days. The internal amount of ^{14}C-imidacloprid was not significantly different between the strains ($p=0.461$). The percentage of non-extractable radioactivity was very low (less than 2%) and did not differ between the two strains or with time after dosing.

These pharmacokinetic results may explain the intoxication and recovery of beetles treated with imidacloprid (Zhao et al. 2000, Mota et al. 2000, Chapter 2). Beetles treated with imidacloprid showed symptoms 20 to 30 min after treatment. These symptoms included hyperexcitability, tremors, bending of the antenna and tarsi, vomiting, and defecation. When the beetles were knocked down, they became prostrate with feeble movement of the appendages. These early symptoms could be attributed to the initial penetration of the compound and distribution to the target sites. Once the compound (which is water soluble) enters the body it is easily transported through the hemolymph to the central nervous system, where the nicotinic acetylcholine receptors are located (Yamamoto 1999). The agonist effect of imidacloprid on the nicotinic acetylcholine receptors triggered adverse physiological responses mentioned above due to the initial stimulation of the receptors by depolarization and ultimate block of the nicotinic system

due to receptor desensitization. However, 3 to 10 d after treatment, some beetles, recovered from the insecticide exposure. This situation may be due to the elimination and reduction in the internal radioactivity of most of the radiolabeled compound as it was found in this Chapter. A similar process of intoxication and recovery occurs in susceptible and resistant beetles. However, the susceptible beetles were knocked down at much lower doses than the resistant ones. The severity of symptoms and recovery of beetles in each strain were correlated with the dose used in the bioassays; high doses caused severe symptoms and less recuperation of the beetles.

Although a lower penetration rate in the resistant strain was observed from 1 to 8 h after treatment, the rate in the resistant strain was reduced by only about 1.5-fold compared to the susceptible strain over this period. Reduced penetration has been reported as a minor factor in the insect resistance and it is usually accompanied by other factors including enhanced metabolism (Oppernorth 1985). However because excretion is probably also faster in the susceptible strain, internal levels of imidacloprid were comparable in both strains. Therefore, it is unlikely that this reduced penetration rate is a major factor in the resistance of the NY selected strain.

Pharmacokinetics: high dose.

Symptoms of intoxication in beetles of the NY Selected strain were more severe and long lasting at this dose. However, there was no mortality at any of the exposure times. One hour after treatment, 26% of the ^{14}C-imidacloprid had penetrated the cuticle (Figure 8). At 8 h 58% of the compound had been internalized, and 5 d after treatment only 5% remained outside of the body. Excretion of ^{14}C-imidacloprid also was relatively fast. At 8 h, 58% has been excreted, and 5 d after treatment 80% had been removed from the

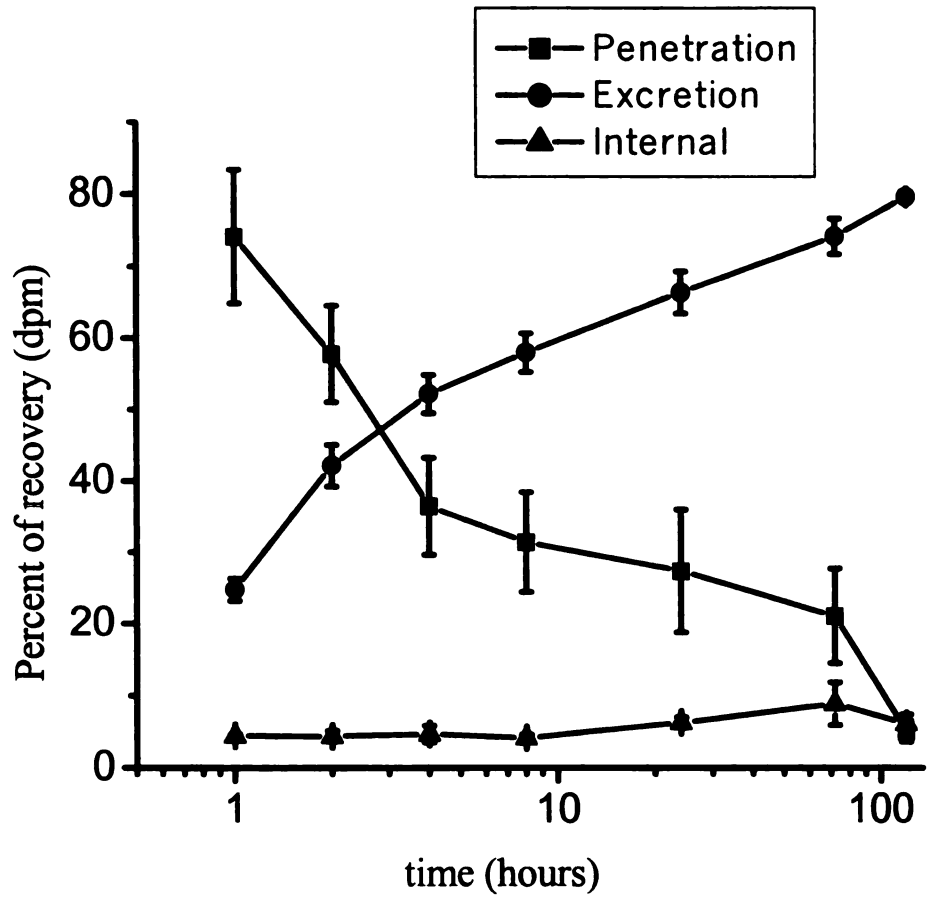


Figure 8. Pharmacokinetics of a high dose of ^{14}C-imidacloprid (900 ng/beetle) in a resistant strain of Colorado potato beetle.

body. Internal levels of radioactivity were 6% of the dose at exposure times from 1 to 24 h, about 9% after 3 d and 6% at 5 d (Figure 8).

Comparison of the pharmacokinetics of the low and high doses indicated a slightly lower rate of uptake and excretion at the high dose, but no significant difference was seen in the percentage of the dose present in the body ($p=0.132$ for the external radioactivity, and a $p=0.227$ for the excreta) (Figure 9). The only difference in external radioactivity for individual pair comparisons were observed 1 h after treatment; the resistant strain treated with a high dose had a significantly higher percent of ^{14}C-imidacloprid on the cuticle ($p=0.0489$) as compared to the same strain treated with the low dose (Figure 9).

The percent of internal ^{14}C-imidacloprid in beetles treated with a low and a high dose was not significantly different ($p=0.754$) and also was not not time dependent ($p=0.7713$). However, at 72 h the beetles treated with the high dose had a significantly higher percent of internal ^{14}C-imidacloprid than the low dose ($p=0.0073$).

The key factor in the process of the intoxication was likely the amount of internal ^{14}C-imidacloprid. Severity of symptoms of the Colorado potato beetle intoxication were dose dependent, but they usually started 0.5 h after treatment, and they are correlated with the amount of internal ^{14}C-imidacloprid that arrive and interact at the nicotinic acetylcholine receptors of the central nervous system. At 1 h after application in the low dose experiment, 0.99 ng/beetle was found inside of the body in the susceptible strain, and 0.97 ng/beetle inside of the body in the resistant strain. In contrast, in the high dose experiment, 39.5 ng was present at the same time. At a dose of 0.99 ng, susceptible beetles started suffering hyperexcitation, tremors, and knock down of some beetles. However, at similar dose (0.97 ng), the resistant beetles suffered only mild symptoms. To

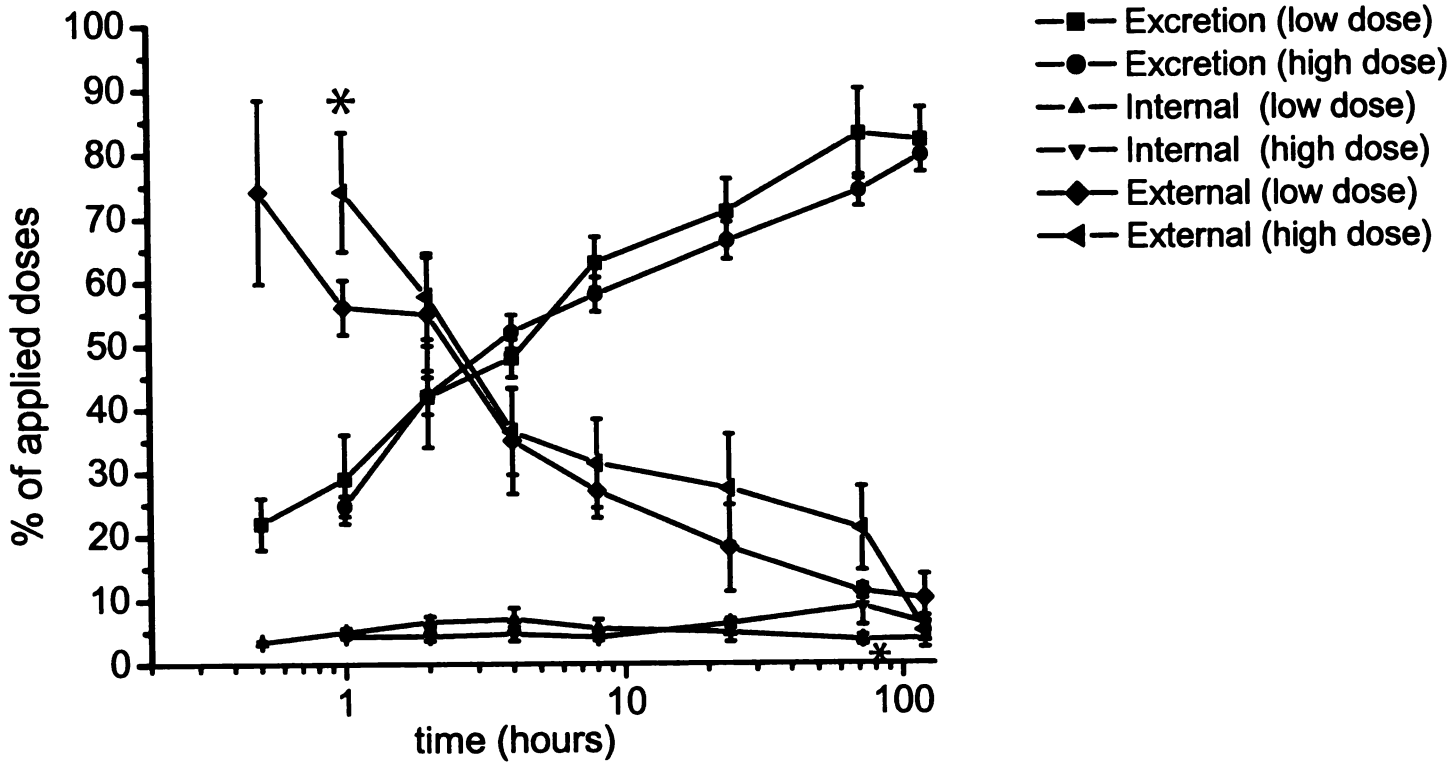


Figure 9. Pharmacokinetics of a low dose (16 ng/beetle) and a high dose (900 ng/beetle) of ^{14}C-imidacloprid in a resistant strain of Colorado potato beetle.

get similar symptoms in the resistant strain as observed in the susceptible strain, it was necessary to increase the internal by 40-fold. Susceptible beetles treated with a high dose of ^{14}C-imidacloprid (900 ng/beetle) showed extremely severe symptoms including hyperexcitation, tremors, and extrusion of part of the hindgut outside of the body 1 h after treatment, and eventually they died. Therefore, severe symptoms are very probably caused by massive stimulation of the nAChRs by imidacloprid. As mentioned above, a similar amount of internal insecticide is present in both strains (at the low doses, 1 h after treatment), but this dose is not enough to cause similar symptoms in the resistant strain as in the susceptible beetles. Possible scenarios for this lack of sensitivity are to: 1) modification in the target site; 2) a pump that that reduces the amount of imidacloprid in the central nervous system, or 3) other morphological or physiological mechanisms that also decrease the penetration of the insecticide into the nervous system, and 4) tolerance for oxidative stress and the ability to withstand general stress.

Metabolism: low doses.

Analysis by TLC indicated that most of the excreted radiolabeled insecticide was the parent compound (Figure 10). Both resistant and susceptible strains showed minimal metabolic conversion. Only a single major radioactive metabolite was formed. This was probably the olefine analog of imidacloprid (see high dose metabolism). This metabolite was first visible in the excreta 24h after treatment in both strains (Figure 10). At five d and 10 d after treatment, a higher percent of the radioactive metabolite was found in the excreta of both resistant and susceptible strains (Figure 10). There was a tendency for

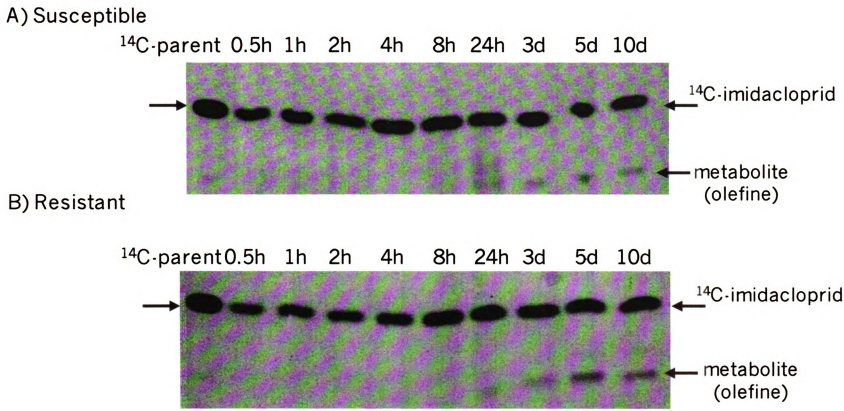


Figure 10. TLC separation of parent compournd and metabolite in the excreta of a susceptible and a resistant strain of Colorado potato beetle after a low dose (16 ng/beetle).

lower conversion to the olefine in the resistant insects in the excreta and internal extracts (Figure 11), but the data are variable due to the low amount of radioactivity present.

A similar pattern was found in the internal fraction (Figures 11 and 12), but the metabolite appeared 4h after treatment and increased in concentration at all times of exposure. The delay in the presence of metabolized ^{14}C radioactivity in the excreta (visible detection at 24 hr) may be due to fact that the metabolite is in the process of being excreted at 4-8 h after treatment and was not visible until the next time of analysis (24 h). At this time most of the radioactive insecticide was eliminated. These results indicated that the excretion of the parent compound is the principal means of insecticide elimination in both strains. The compound was slowly metabolized later in both strains, which may act as detoxification process since the olefine is probably less toxic than imidacloprid (see later). Solanaceous feeders eliminate alkaloids from their diet in various ways including rapid excretion or enzymatic detoxification (Blum 1983). The fact that imidacloprid has a high water solubility probably explains why such a high degree of direct excretion occurs. No significant differences were found between the strains in either the metabolism or excretion of imidacloprid that could explain resistance.

Metabolism in the resistant strain treated at a high dose.

As at the lower dose, metabolic conversion was relatively minor in resistant beetles treated with a high dose (900 ng/beetle) of ^{14}C-imidacloprid, and most of the excreted and internal ^{14}C-radioactivity was the parent compound. Again, only one major metabolite was produced. This metabolite started being visible 2 h after treatment in the internal fractions and 24 h after treatment in the excreta (Figure 12). Co-chromatography of this metabolite with the non-labeled olefine compound in two single dimension

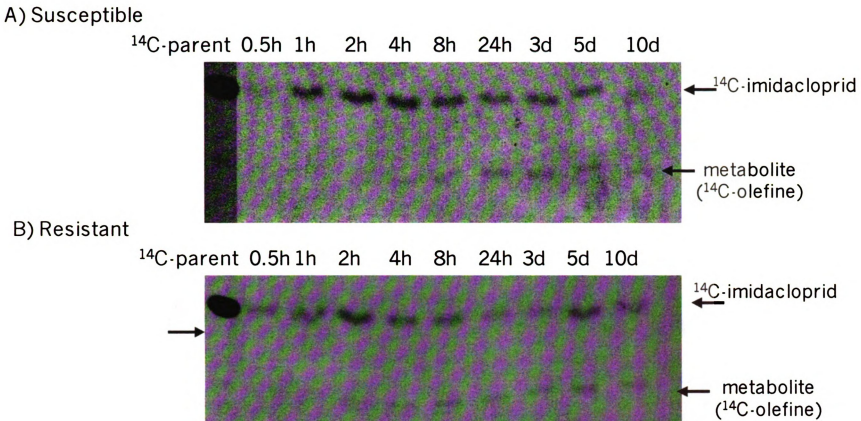


Figure 11. TLC separaration of parent compound and metabolite in an internal extract of a susceptible and resistant strain of Colorado potato beetle after a low dose (16 ng / beetle)

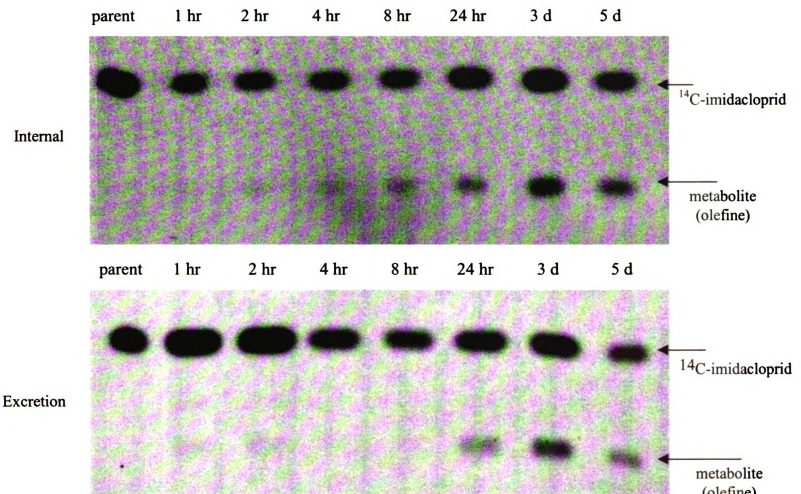
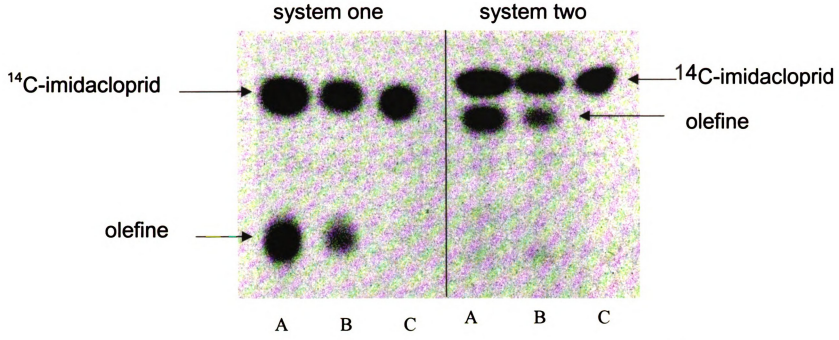


Figure 12. TLC separation of parent compound and metabolite of excreta and internal extracts of a resistant strain of Colorado potato beetle treated with a high dose (900 ng/insect).

systems (Figure 13) and the one double dimension chromatography (Figure 14) was exact and suggested that the metabolite is very likely the olefine analog of imidacloprid. No compound with the chromatographic behavior of 6-chloronicotinic acid was observed. In some cases, another very small metabolite was observed (Figure 14). This metabolite was not identified. The high dose of ^{14}C-imidacloprid in the resistant strain also resulted in a low percent of the metabolite in the excreta (Figure 15B) and in the internal extract (Figure 15D).

Comparison of the pharmacokinetics of the low and high doses in the resistant strain indicates that excretion of the parent compound is similar (Figure 16A). However, there is a tendency for the resistant strain to excrete higher ^{14}C-olefine in the low dose especially at 3 d and longer after treatment (Figure 16B). This situation may be due to the fact that beetles treated at the high dose are physiologically more affected than beetles treated at the low dose. The trend in pharmacokinetics of the parent compound at the low dose is different from the high dose. At the low dose, there is an increase in the accumulation of parent compound from 0.5 to 8 hr, and then there is a decrease in the internal amount of parent compound (Figure 16C). Conversely, at the high dose, the amount of parent compound remained without changes until 24 hr after treatment, and then an increase in the amount of internal ^{14}C-imidacloprid occurs, followed by a decrease 5 d after treatment (Figure 16C). The percentage of ^{14}C-olefine is bigger at 3 d after treatment at the low dose (Figure 16D). However, the differences are less than 2% between the two doses, and overall there is little difference in the pharmacokinetics at the low and high doses.



Rf (^{14}C-imidacloprid A and B) = 0.58
Rf (^{14}C-imidacloprid C) = 0.57
Rf (olefine A) = 0.32
Rf (olefine B) = 0.31

Rf (^{14}C-imidacloprid A, B, and C) = 0.59
Rf (olefine A) = 0.51
Rf (olefine B) = 0.52

Figure 13. Thin layer chromatography of internal and excreta radioactivity samples taken three days after exposure of ^{14}C-imidacloprid. A. internal body. B. excreta, and C. ^{14}C-imidacloprid. System one, methanol (96+4). Systems two, ethyl acetate + toluene + methanol + acetic (80+20+20+1). Note: This figure is an expanded section of the image, not the whole TLC plate.

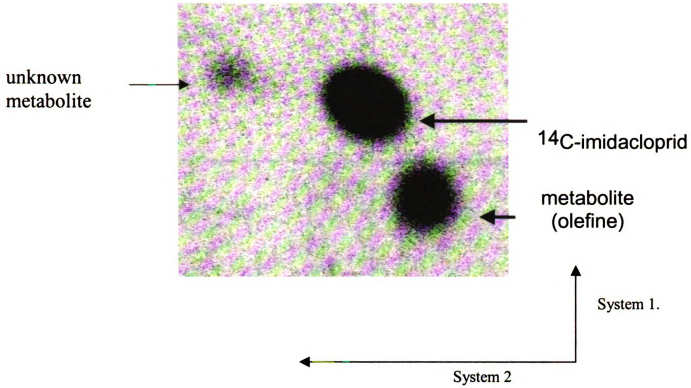


Figure 14. Double dimension chromatography of a 10 µl sample of the internal extract three days after treatment with ^{14}C-imidacloprid at a high dose in a resistant strain of Colorado potato beetle. System 1, ethyl acetate + toluene + methanol + acetic acid (80 + 20 + 20 + 1). System 2, ethyl acetate + 2-propanol + water (65 + 23 + 12). Note: this figure is an expanded section of the image, no the whole TLC plate.

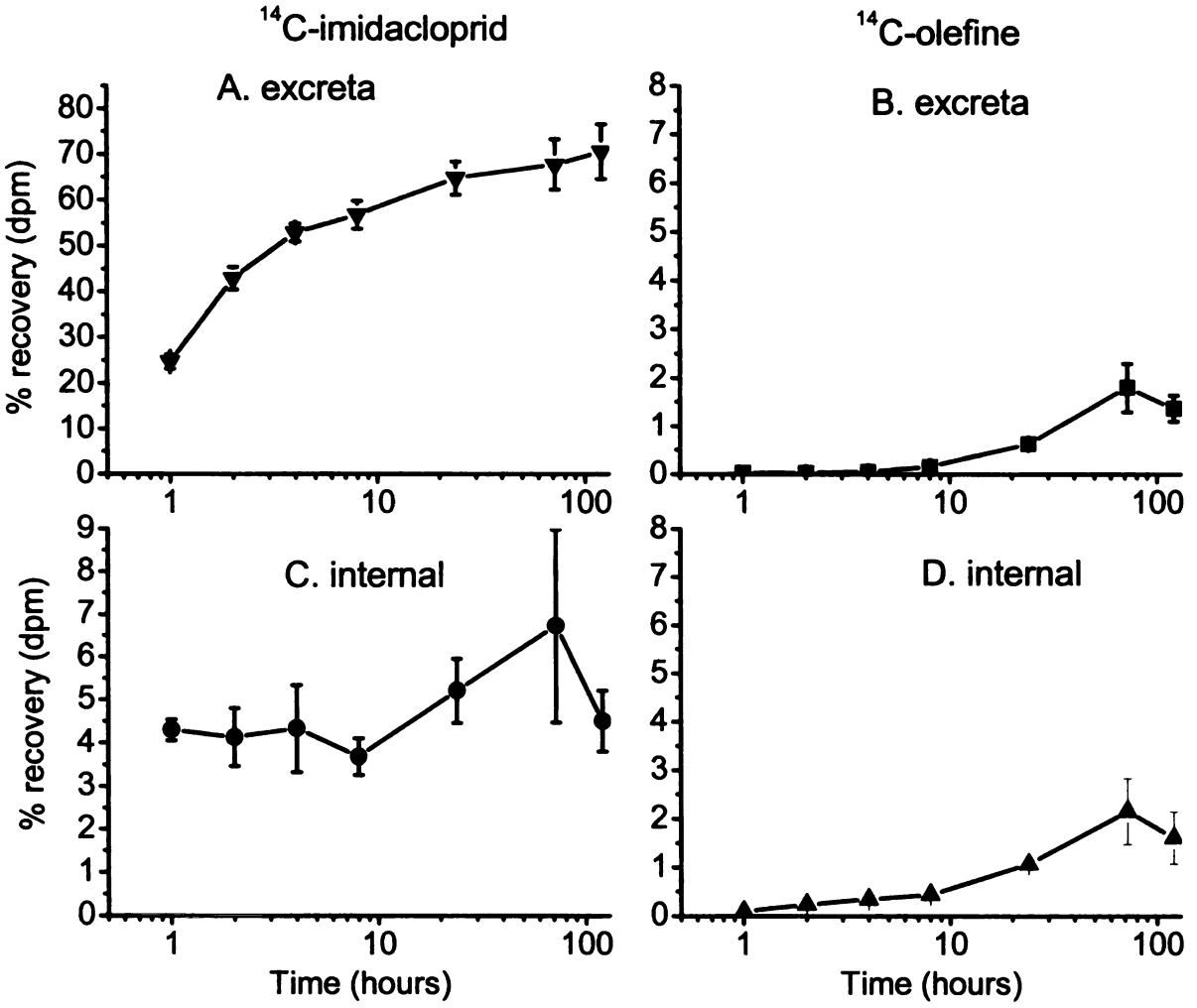


Figure 15. Pharmacokinetics of ^{14}C-imidacloprid and ^{14}C-olefin in a resistant strain of Colorado potato beetle after treatment with a high dose of 900 ng of ^{14}C-imidacloprid. A. Parent compound in the excreta. B. ^{14}C-olefine in the excreta. C. Parent compound inside the beetle. D. ^{14}C-olefine inside the beetle. Note: n=192 beetles/strain.

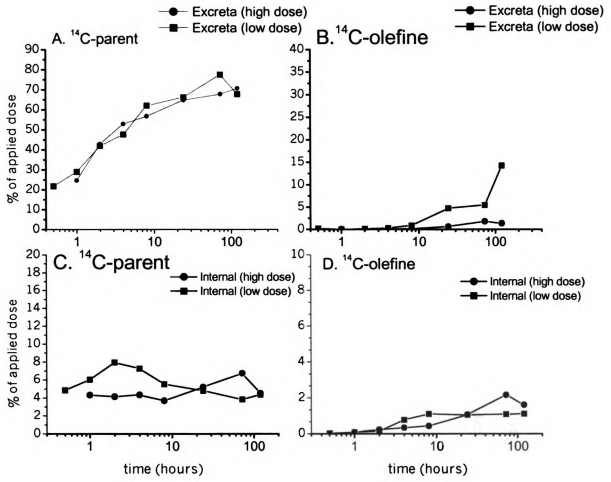


Figure 16. Pharmacokinetics of ^{14}C-imidacloprid and ^{14}C-olefine in a resistant strain of Colorado potato beetle after treatment with a low and a high doses of ^{14}C-imidacloprid. A. Parent compound in the excreta. B. ^{14}C-olefine in the excreta. C. Parent compound inside the beetle. D. ^{14}C-olefine inside the beetle. Note: n=64 beetles/strain in the low dose, n=192 beetles/strain in the high dose.

A possible pathway for ^{14}C-imidacloprid metabolism is by hydroxylation of ^{14}C-imidacloprid in the imidazolidine ring to give 4-(or 5-) hydroxy ^{14}C-imidacloprid (Figure 17). Water is then removed spontaneously from the hydroxy ^{14}C-imidacloprid to get the unsaturated ^{14}C-imidacloprid, the ^{14}C-olefine. The 4–(or 5-) hydroxy ^{14}C-imidacloprid most likely has a very short life inside of the insect body since the only major metabolite in the excreta and the internal extracts was the olefine. The major metabolites produced in house flies are the olefine and 4-(or 5-) hydroxy imidacloprid (Miyagawa et al. 2002). These are also major metabolites of imidacloprid in plant cells (Koester 1992) and mammals (Thyssen and Machemer 1999).

Results of the olefine metabolite

Results of bioassays using two doses of the olefine compound indicated that the New York Selected strain is cross-resistant to the olefine compound (Table 9). A dose of the 300 ng/insect caused 0% knock down in the resistant strain 24 h after treatment. Conversely, 66% knock down was observed in the susceptible strain. Twenty four h after treatment, a dose of 700 ng caused 3% and 83% of knock down in the resistant and susceptible strain, respectively. Recovery of beetles was observed in the susceptible strain, especially at the 300 ng dose, 10 d after treatment (Table 9). In previous experiments, a dose of 300 ng of imidacloprid killed more 100% of susceptible beetles. In contrast, a similar dose of the olefine killed only 8.3% in the same strain (Table 9). One of the best ways to compare the toxicity of two compounds is by the use of the LD_{50} value and its fiducial limits. However, bioassays to get an LD_{50} for the olefine compound were not conducted because lack of material, and so it was not possible to

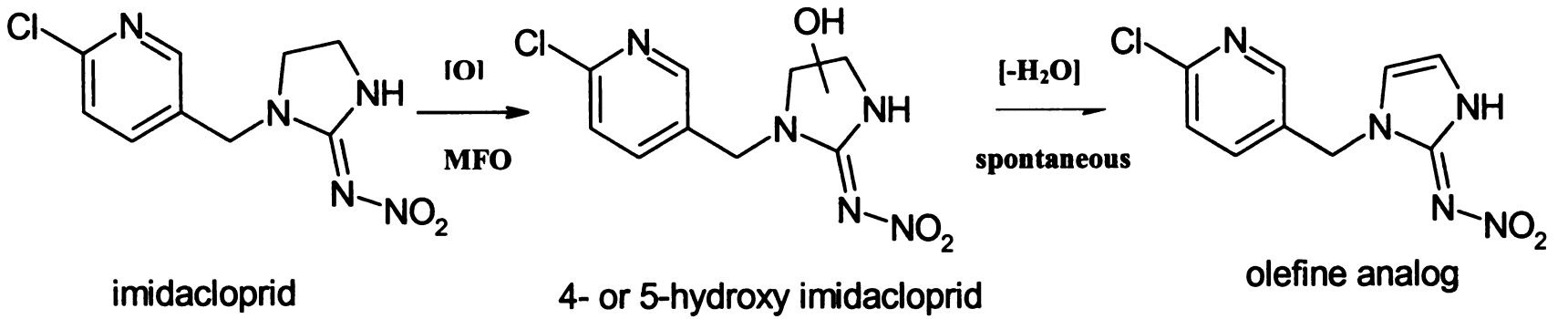


Figure 17. Proposed metabolism of imidacloprid in the Colorado potato beetle.

Table 8. Percent of mortality of a susceptible and resistant strain of Colorado potato beetle to the imidacloprid metabolite, the olefine.

	Strains/time			
Dose (ng/beetle)	Susceptible 24 h Knock down	Resistant 24 h Knock down	Susceptible 10 d Mortality	Resistant 10 d Mortality
300	66%	0%	8.3%	0%
700	83%	3%	60%	3%

compare the LD_{50} of imidacloprid and the olefine for both resistant and susceptible strains. Despite this situation, these results indicate that the olefine is less toxic than imidacloprid to Colorado potato beetle, a situation that is surprising giving the examples of high toxicity of the olefine to imidacloprid in other species of insects.

It might be expected that plant or insect metabolic transformation to the hydroxy imidacloprid and olefine metabolites would reduce the toxicity of imidacloprid in insects. However, it is reported in *Bemisia tabaci* Gennadius that the olefine metabolite (LC_{50} = 0.024 ppm) is 10-fold more toxic than imidacloprid (LC_{50} =0.24 ppm) (Nauen et al. 1999). The 4-hydroxy metabolite has about the same toxicity as imidacloprid (1.6-fold lower), and the 5-hydroxy metabolite is 10 times less toxic than imidacloprid. In *Apis mellifera* L., imidacloprid and the olefine metabolite are similar in toxicity in oral bioassays (41 ng and about 36 ng per bee, respectively). In addition, the oral LD_{50} for 4,5-dihydroxyimidacloprid is about 49 ng and it is 159 ng for 5-OH-imidacloprid (Nauen et al. 2001). The toxicity of imidacloprid and metabolites has been associated with the capacity of binding the nAChRs in membrane preparations of *A. mellifera*. In studies of binding with isolated nAChRs, the value of the IC_{50} for imidacloprid was 2.9 nM compared to 0.45 nM for the olefine metabolite, and 24 nM for the dihydroxyimidacloprid (Nauen et al. 2001). The olefine metabolite expressed higher affinity for nAChRs in ligand competitions than did imidacloprid and its other metabolites. The urea metabolite of imidacloprid and 6-chloronicotinic acid are not toxic to bees and were ineffective in displacing (^3H)-imidacloprid from its binding site in nAChRs in membrane preparations of honey bees (Nauen et al. 2001).

The relationship between metabolism, synergism and excretion in the toxicity of imidacloprid to the Colorado potato beetle are complex and so far, poorly explained by known metabolic reaction in some cases. The use of synergists such as piperonyl butoxide (PBO) suppresses the cytochrome P450-based oxidation mechanism of resistance. (Liu et al. 1993) reported that topical treatments with imidacloprid analogs in house flies in combination with cytochrome P450 inhibitors, including piperonyl butoxide (PBO) and O-propyl O-(2-propynyl) phenylphosphonate (PPP), increased the toxicity of the insecticides, meaning that these insecticides suffer oxidative metabolic detoxification. Liu et al. (1993) attributed the low sensitivity of house flies to imidacloprid in topical application to poor penetration and rapid oxidative detoxification, and not to a low affinity of the target site. Recently, Miyagawa et al. (2002) noted that imidacloprid is rapidly excreted from the body of house flies after injection, with only 10% remaining internally after 6 h. In addition, PPP and piperonyl butoxide significantly reduced the metabolism of imidacloprid and the excretion of ^{14}C-imidacloprid. However, the relationship between metabolism and toxicity is unclear. In the green peach aphid, the use of PBO did not suppress tolerance to imidacloprid, indicating that oxidative metabolism is not responsible for tolerance (Nauen et al. 1998).

Fruit flies bearing a *Rst*(2) DDT resistance alleles including *Rst*(2)DDT$^{Hikone\text{-}R}$, a gene located in a cluster of cytochrome P450 genes, show pre-existing cross-resistance to imidacloprid indicating a key role for oxidative metabolism in resistance in this strain (Daborn et al. 2001). Chemical mutagenesis had been used to select mutants of fruit flies resistant to imidacloprid. Imidacloprid resistant fruit flies also show cross-resistance to DDT (Daborn et al. 2001). This insecticide resistance is associated with the over-

expression of *Cpy6g1*, a gene also mapping at the same location as the *Rst*(2) gene. In Colorado potato beetles, PBO as been reported to decrease resistance to imidacloprid in Colorado potato beetle from Long Island, NY (Mota-Sanchez et al. 2000, Zhao et al. 2000). However, the effect of PBO on the toxicity of imidacloprid is quite small and the results presented here indicate that metabolism is not an important factor responsible for resistance in beetles from Long Island, NY.

The lack of differences in the pharmacokinetics and metabolism of imidacloprid observed in these experiments between resistant and susceptible beetles suggests that resistance could be due to a modification of the target site, the nicotinic AChR. In other imidacloprid-tolerant insects, *Myzus persicae* (Sulzer) and *Myzus nicotianae* (Blackman), no detectable differences in target site binding were demonstrated with binding assays using (^{3}H)imidacloprid in either species (Nauen et al. 1996). Although *M. nicotianae* was 10-fold more tolerant to imidacloprid than *M. persicae*, there were no differences in the binding kinetics between the strains, suggesting that target site insensitivity was not involved in the aphid tolerance to imidacloprid (Nauen et al. 1998). Target site modification has also been ruled out in resistant strains of *B. tabaci* from Almeria, Spain (Elbert and Nauen 2000, Nauen et al. 2002). However, to determine the imidacloprid resistance mechanism in different species, additional target site studies are essential. If a less-sensitive target site is present in the resistant beetles, it is surprising that cross-resistance to a closely related neonicotinoid, thiamethoxam, is low (Chapter 3). However, a recent report by Kayser and Lee (2002) shows that imidacloprid and thiamethoxam do not compete for the same binding site on the nAChR in aphids. Thus a change could occur at the imidacloprid site which had little or no effect on thiamethoxam binding.

CONCLUSIONS

Rapid penetration and excretion of ^{14}C-imidacloprid were observed in the susceptible and resistant strains of Colorado potato beetle. Comparison of the pharmacokinetics of ^{14}C-imidacloprid in the resistant and susceptible strains for the low dose indicated a slightly lower rate of uptake in the resistant strain, but no significant difference was seen in the percentage of the dose excreted and present in the body. The pharmacokinetics in the resistant strain treated at a low dose and a high dose also indicated a similar pattern for the percent of external radioactivity, excretion and internal radioactivity of ^{14}C-imidacloprid. Thus no significant differences were found between the susceptible strain and the resistant strain in either the metabolism or excretion of imidacloprid that could explain resistance, and the internal levels of imidacloprid were comparable in both strains. Differences in symptoms at the low and high doses were observed for both strains. The low dose caused symptoms as hyperexcitation, tremors and knock down of some susceptible beetles at one h after treatment. Conversely, the resistant beetles only expressed very mild symptoms at this dose and time. A 40-fold increase in the internal dose in the resistant strain is necessary to cause similar symptoms as the susceptible strain treated at the low dose. Both resistant and susceptible strains showed minimal metabolic conversion. Only a single major radioactive metabolite was formed. This was probably the olefine analog of imidacloprid. The resistant strain was also cross-resistant to the olefine compound which was less toxic than the parent imidacloprid.

The lack of differences in the pharmacokinetics and metabolism of imidacloprid observed in these experiments between resistant and susceptible beetles together with differences in intoxication symptoms suggest that resistance could be due to a

modification of the target site, the nicotine AchRs. Further neurophysiology studies using the isolated nervous systems, together with studies of binding site competition of the nAChRs between resistant and susceptible strains of Colorado potato beetle are essential to determine if the target site modification is the mechanism of resistance to imidacloprid in the NY Selected strain. An affirmative answer to this question also would be a valid reason to isolate and clone genes that express nicotinic receptor subunits to determine if a mutation exists and to determine its nature.

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GENERAL CONCLUSIONS

Until 2002, imidacloprid was the only registered insecticide effective to control Colorado potato beetle resistant to conventional insecticides in many potato growing regions in the United States. Abamectin is also registered but is limited to two applications per season. High levels of resistance to imidacloprid detected in many sites on Long Island, NY in 1998 and significant survival in the laboratory of beetles to a high single dose in 1999 should be a warning of potential major problems in the near future. Consequences of resistance of Colorado potato beetle to imidacloprid have not yet been as dramatic as occurred in the past in Long Island to conventional insecticides. However, reduction in the effective protection time has been observed. In addition, some farmers have switched to the use of avermectins to control Colorado potato beetle. Two factors may play an important role in the continuous use of imidacloprid. The first one is that virus transmission by the green peach aphid, *Myzus persicae* (Sulzer) is an important problem in the area, and imidacloprid is very effective in controlling this aphid. The second is that despite the reduced effective protection time and reduced control in some places, imidacloprid still effectively controls Colorado potato beetle. However, there is a potential risk for the development of a high degree of homozigosity for resistance that would lead to lost of the compound. Since imidacloprid is essential to control Colorado potato beetle it is very important to delay the development of resistance Other mortality factors must be implemented to manage the resistance in Colorado potato beetle. Management strategies that do not rely exclusively on the use of imidacloprid and other neonicotinoid compounds must be developed. Crop rotation is another important component of IPM of Colorado potato beetle. In the future, deployment of transgenic

crops together with use of neonicotinoid compounds and lactones macrocyclic may serve as important tools to slow the evolution of resistance.

Rapid diagnostic of resistance of Colorado potato beetle is often necessary, especially before spraying. However, imidacloprid is a slow kill compound and it usually takes 10 days to get LD_{50} data. In this research, it is demonstrated that the use of lethal time $_{50}$ may be an important tool as a rapid method of detection of resistance. Fast knock down in susceptible field populations were highly correlated with high mortality 10 days after treatment. In contrast, resistant field populations expressed slow knock down and less percent of mortality 10 days after treatment. The KD_{50} method could be used as a resistance assay if a rapid diagnostic method for insecticide resistance is needed.

Even though imidacloprid and bensultap act at the same molecular target, there was not cross-resistance between the Colorado potato beetle resistant to imidacloprid and the nereistoxin, bensultap. The strains Mattituck, Janesport and Suffolk from Long Island expressed low levels of resistance to thiamethoxam in 1998. Probably these levels are too low to cause field failures. The situation may have changed because in recent studies, other researches have found higher levels of resistance (Grafius, personal communication). In other studies, Nauen et al. (2002) detected white flies with high levels of resistance to imidacloprid and also high levels of cross-resistance to thiamethoxam and acetamiprid. Target site modification has been ruled out as a mechanism of resistance in resistant strains of *B. tabaci* from Almeria, Spain (Elbert and Nauen 2000). Similar binding kinetics between the strains suggested that target site insensitivity was not involved in the aphid tolerance to imidacloprid (Nauen et al. 1998). However, to determine the imidacloprid resistance mechanism in different species,

additional target site studies are essential. If a less-sensitive target site is present in the resistant beetles, it is surprising that cross-resistance to a closely related neonicotinoid, thiamethoxam, is low (Chapter 3). However, a recent report by Kayser and Lee (2002) shows that imidacloprid and thiamethoxam do not compete for the same binding site on the nAChR in aphids. Thus a change could occur at the imidacloprid site which had little or no effect on thiamethoxam binding. Another possibility is that metabolism may be an important factor in thiamethoxam resistance. Further studies on the mechanism of resistance to neonicotinoids compounds and monitoring would also be an important tool to detect resistance to this important group of new insecticides. Insecticide rotation, crop rotation, propane flamers, and trench traps were widely used in some areas during the early 1990s when no effective insecticides were available. However, except for crop rotation, they were generally abandoned following the introduction of imidacloprid. If re-integrated into the control program for CPB, these strategies and tactics may prolong the useful life of the neonicotinid insecticides for CPB control.

Rapid penetration and excretion of ^{14}C-imidacloprid were observed in the susceptible and resistant strains of Colorado potato beetle. Comparison of the pharmacokinetics of ^{14}C-imidacloprid in the resistant and susceptible strains for the low dose indicated slightly lower rate of uptake in the resistant strain, but no significant difference was seen in the percentage of the dose excreted and present in the body. Pharmacokinetics of the resistant strain treated at a low dose and a high dose also indicated a similar pattern for the percent of external radioactivity, excretion and internal radioactivity of ^{14}C-imidacloprid. No significant differences were found between the Hughes susceptible strain and the resistant NY Selected strain in either the metabolism or excretion of

imidacloprid that could explain resistance. In addition, internal levels of imidacloprid were comparable in both strains. Therefore, it is unlikely that this reduced penetration rate is a major factor in the resistance of the NY selected strain. Differences in symptoms at the low and high doses were observed for both strains. The low dose caused symptoms as hyperexcitation, tremors and knock down of some susceptible beetles at one hour after treatment. Conversely, the resistant beetles only expressed very mild symptoms at this dose and time. A 40-fold increase in the internal low dose in the resistant strain is necessary to cause similar symptoms as the susceptible strain treated at low dose. In other experiments (unpublished results), susceptible beetles treated with a high dose of imidacloprid (900 ng / beetle) showed severe hyperexcitation, tremoring, evertion of the gut, and higher excretion of fluids (including imidacloprid) than the resistant strain in less than an hour. This means that the insecticide reached the target site, and the elimination of imidacloprid by susceptible beetles was not sufficient to remove the insecticide and protect the nervous system from effects of the chemical. The resistant strain also had a large amount the insecticide inside the body. However, the symptoms and mortality were less severe than the susceptible strain. This disparity between the susceptible and resistant strain may be due to reduced insensitivity in the target site of the resistant strain. In other insects including *Manduca sexta* it had been demonstrated that a pump is responsible for elimination of nicotine from the body. This mechanism also be a possibility for resistance to imidacloprid in Colorado potato beetle.

Both resistant and susceptible strains showed minimal metabolic conversion. Only a single major radioactive metabolite was formed. This was very probably the olefine analog of imidacloprid. The resistant strain was also cross-resistant to the olefine

compound which was less toxic than the parent imidacloprid. Pyperonil butoxide (PBO) partially suppress resistance in the Long Island strains. This situation is likely caused by suppression of metabolism of imidacloprid to the olefine compound that is less toxic than imidacloprid in Colorado potato beetle. This factor may be is not as important as other mechanisms in the resistant strain (such as target site modification), but use of PBO probably allowed more internal amounts of imidacloprid affecting the target site.

The lack of differences in the pharmacokinetics and metabolism of imidacloprid observed in these experiments between resistant and susceptible beetles together with differences in intoxication symptoms suggest that resistance could be due to a modification of the target site, the nicotine AchRs. Further neurophysiology studies using the isolated nervous systems, together with studies of binding site competition of the nAChRs between resistant and susceptible strains of Colorado potato beetle are essential to determine if the target site modification is the mechanism of resistance to imidacloprid in the NY Selected strain. An affirmative answer to this question also would be a valid reason to isolate and clone genes that express nicotinic receptor subunits to determine if a mutation exists and to determine its nature.

Appendices

Appendix 1

Record of Deposition of Voucher Specimens*

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

Voucher No.: 2002-10

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

RESISTANCE AND METABOLISM OF IMIDACLOPRID IN COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* Say (COLEOPTERA: CHRYSOMELIDAE)

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

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)
David Mota-Sanchez

Date 08/15/2002

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

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

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

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

Appendix 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Leptinotarsa decemlineata Say	MI, Ingham Co., East Lansing, MSU 9 August 2002, E.Grafius coll.					5	5		MSU
Myzus persicae (Sulzer)	WA, Benton Co., Prosser, USDA Lab culture, 2002			10		10			MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)
David Mota-Sanchez

Date 08/14/2002

Voucher No 2002-10
Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date 14 Aug 2002

Appendix 2

RESISTANCE TO METHAMIDOPHOS OF THE GREEN PEACH APHID, *Myzus persicae* (Sulzer) (HOMOPTERA: APHIDIDAE) FROM POTATO SEED PRODUCTION AND COMMERCIAL POTATO AREAS IN THE PACIFIC NORTHWEST

Resistance to methamidophos of the green peach aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae) from potato seed production and commercial potato areas in the Pacific Northwest

Abstract: Green peach aphid, *Myzus persicae* (Sulzer) has developed resistance to more than 68 compounds and is ranked third in worldwide pesticide resistance (MSU resistance database). It causes severe damage by direct feeding and transmission of viral diseases. To determine resistance to methamidophos of the green peach aphid in the Pacific Northwest, 26 field populations were collected from potato seed production fields, commercial potato fields and alternate hosts in the Pacific Northwest and submitted to an aphid dip bioassay. Populations from commercial potato field in Washington in Skagit, County (northwest area), and Adams and Grant Counties (central) showed 3.5, 4.3 and 13-fold resistance, respectively. A discriminating dose of 3,160 ppm of methamidophos, resulted in 25% survival of aphids of the Grant County population. Field failure of methamidophos against *M. persicae* was also observed at Grant County. Aphid populations collected from certified potato seed areas in Kootenai County (south-west) and Jefferson County (central) Oregon showed resistance ratios of 3.9 and 9.4 fold, respectively. Insecticide resistance of aphids in potato seed fields is more critical than in commercial areas, because even low levels of aphid survivals can cause severe damage due to transmission of potato viruses. Implementation of strategies of insecticide management strategies for green peach aphid and methamidophos is important if virus problems are to be managed in potato production.

INTRODUCTION

The green peach aphid, *Myzus persicae* (Sulzer), is one of the key pests of vegetables, fruits and ornamental crops. Evolutionary development of alternation of a sexual phase and a parthenogenetic asexual phase combine with host alternation (heteroecy) enables the green peach aphid to colonize more than 140 different host plants (Blackman and Eastop 1984). The primary hosts of green peach aphid include peach (*Prunus persicae* Miller) and other *Prunus* species. In the fall holocyclic populations of the aphid lay overwintered eggs on *Prunus* spp., in mild winters, the aphid also survives on various perennials and winter annuals, such as tumble mustard, flixweed, shepherds purse, mallow, horseweed, pennycress and redstem filaree (Pike 2000). In the spring the eggs hatch and the fundatrix initiates the first of many clonal generations. Winged parthenogenetic females emigrate from their overwintering hosts to summer hosts including potatoes (Unruh et al. 1996).

Green peach aphid causes damage by direct feeding and transmission of viral diseases (Van Emden and Bashford 1969). Both types of injury, especially the latter, force producers to rely on repeated insecticide treatments to control this pest. The green peach aphid's ability to transmit viruses even at low population densities and its ability to disperse widely make this pest very difficult to control (Lecrone and Smilowitz 1980). Intense use of insecticides selects individuals that carry alleles to resist insecticides. This pressure, together with biological and ecological characteristics of green peach aphid have led to many instances where economically important insecticide resistance has developed. In fact, green peach aphid has developed resistance to about 68 compounds

and is ranked third in worldwide pest resistance development frequency (Mota-Sanchez et al. 2002).

In potato seed production of the Pacific North west, one of the major concerns is the transmission of viruses- principally potato leafroll virus (PLRV) by the green peach aphid. Potato seed producers usually apply methamidophos (Monitor ®) to control the peach aphid. However, some growers have been reported lack of control in the field and increase incidence of the field and increase incidence of virus diseases. If this tendency continues we will see higher frequency of virus damage in the potato fields and the risk of PLVR in seed stocks. Early season detection of methamidophos resistance is very important to avoid virus dissemination resulting from failure to control aphids. If producers know they have resistant populations in their fields, then alternative insecticides or other methods may be chosen in time to control green peach aphid and prevent PRLV spread.

Field resistance monitoring is the cornerstone of resistance management strategies. Therefore, the objectives of this research was to determine the resistance to methamidophos in green peach aphid in potato seed production, commercial potato areas and on alternate hosts in the Pacific Northwest.

MATERIALS AND METHODS

Aphid Sampling. Adult field populations of Green peach aphid were sampled live from potatoes and selected weed hosts during mid summer 2000, and subsequently used to start insectary cultures maintained at Washington State University, Prosser, WA. In total, 26 populations were established for Monitor® (Bayer Corporation, Kansas City, KS)

resistance assessment (Table 10). Collected aphids were transported from the field to the insectary on cut foliage in screen-covered plastic containers (300 ml, 10 cm diameter x 4 cm height) held in a cool ice chest. Subsamples of each population were examined and identified under microscope to insure that the species was green peach aphid.

All cultures were reared on broadleaf mustard, *Brassica juncea* in individual cages. Each culture was started with 5-10 apterous aphids placed on month old mustards (plant stage: 3-4 leaf ca. 15 cm in high). The mustards were grown singly in 15 cm diameter pots using a greenhouse soil mix. The cultures were maintained at 21 C, under 16:8 (L:D) fluorescent lighting. Lights were supersaver cool white, 34 watts/bulbs, 4 bulbs/2 cages mount 10 cm above the cages. Cages were made of nylon fabric (AB Ludvig Svensson, Kinna, Sweden, 38 x 44 mesh with a 0.15 x 0.35 mm hole size). Frame dimensions were 61 cm x 53 cm x 38 cm.

All bioassays were done at Michigan State University. Aphids from cultures at Washington were sent to Michigan by overnight mail on host foliage in petri dishes (ca. 15 cm diameter x 2 cm ht) in insulated boxes. To allow for replicate testing and analysis, multiple mailings of each population were sent.

Bioassays. The method of application to assay adult resistance was the aphid dip bioassay recommended by the FAO (Busvine 1980). Metamidophos (Monitor ®) (Bayer Corporation, Kansas City, KS) was diluted with water and an adjuvant was added. Doses of insecticide 1000, 316, 100, 31.6, 10, 3.16, 1, and 0.316mg of A.I. / L were used. Ten to 20 adult aphids were used per dose. Each aphid was transferred by a fine hair paint brush to a cup with a fine screen bottom. The cup was dipped in the insecticide solution for 10 seconds and then was placed on a filter paper to dry the aphids. The control aphids were

Table 10. Green peach aphid populations sample sites in Northwest USA, and site statistics, 2000-crop year.

Plant host	Setting[v]	Field location State, county (general area)	Insecticide treatment[a] At-plant or layby	aphids/ plant
Potato, Russet Burbank	C	WA, Grant Co. (west-central)	aldicarb, methamidophos, carbofuran, fulfill	<1
Potato, Umatilla Russet	S	OR, Baker Co. (central)	imidacloprid	70-150
Potato, Russet Norkotah	S	OR, Jefferson Co. (central)	aldicarb, imidacloprid, methamidophos	<1
Potato, Russet Norkotah	C	WA, Adams Co. (south-west)	methamidophos	10-15
Potato, Russet Burbank	C	WA, Grant Co. (south-west)	aldicarb, methamidophos, carbofuran	<1
Potato, Russet Burbank	R	WA, Benton Co. (west-central)	No application	2.5
Potato, Russet Norkotah	C	WA, Grant Co. (central)	Thimet, asana, dimethoate, methamidophos	25
Potato, Ranger Russet	S	OR, Jefferson Co. (south-central)	Admire	<1
Weed, Indian mustard	L	ID, Owyhee Co. (north-west)	No application	50+
Potato, Ranger Russet	C	WA, Franklin Co. (north-west)	methamidophos	3
Potato, Russet Burbank	C	WA, Grant Co. (west-central)	Temik, methamidophos	<1
Potato, Russet Burbank	C	WA, Benton Co. (south-west)	carbofuran, methamidophos	<1
Potato, NorValley	S	WA, Kittitas Co. (east-central)	Top-MZ Gaucho	<1

Table 10 (cont'd)

Weed, tumble mustard	B	WA, Walla Walla Co. (west-central)	No application	250
Weed, hairy nightshade	B	WA, Grant Co. (west-central)	methamidophos	15
Potato	C	WA, Skagit (west-central)	No application	<1
Potato, Umatilla Russet	S	ID, Kootenai (south-west)	Genesis	<1
Potato	C	WA, Skagit (west-central)	No application	5
Potato, Russet Burbank	S	WA, Whatcom Co. (north-central)	unknown	5-10
Potato	S	WA, Whatcom Co. (north-central)	unknown	<1
Potato, volunteer	V	MT, Gallatin Co. (west-central)	No application	2-3
Potato, Russet Burbank	S	ID, Fremont Co. (south-central)	Admire, fulfill	5
Weed, broadleaf mustard	L	WA, Benton Co. (west-central)	No application	50+
Potato, Russet Burbank	S	MT, Lake Co. (central)	Thimet	2-3
Potato, Ranger Russet	S	WA, Klickitat Co. (north-west)	imidacloprid	2-4
Potato, Russet Norkotah	S	WA, Spokane Co. (west-central)	Thimet, dimetoato, methamidophos	<1

[c]Setting. B, field border; C, commercial field; L, lab colony; R, research field; S, certified seed field; V, volunteer potato.

treated with water and the adjuvant only. After drying, aphids were placed in Petri dishes and fed on a Nappa disk. Three to five replications per concentration were performed. Petri dishes containing the aphids were placed in a room at 28 °C, 50 % relative humidity and a photoperiod of 16:8 (L:D). Mortality was assessed at 24 h after treatment. Aphids unable to move after probing with a hairbrush were recorded as dead. Data were analyzed by Probit analysis (SAS 2000). Resistance ratios were calculated as the LC_{50} value of the field colony / LC_{50} of the most susceptible population.

RESULTS AND DISCUSSION

Commercial potato fields. Ratios of resistance ranged from 1.2 to 13 fold (Table 11 and Figure 18). Populations from Skagit, Co. (northwest), WA, Adams Co., and Grant Co. (central) were significant different at the LC_{50} level value from the reference population (Klickitat Co., northwest). Population from Grant Co. (central) expressed high levels of resistance (13 fold). However, it was not significant different from the reference population due to overlapping of the fiducial limits at the LC_{50} level. This overlapping of the Grant Co. (central) population with the Klickitat Co. is due to the high variability of the fiducial limits in the Grant Co. population.

Additional bioassays by using a single higher dose of 3,160 ppm in aphids, resulted in 25% of survivals in the population Grant, Co. Farmers from this county have been reported field failure of methamidophos applications in the season. Both results of laboratory bioassays and field applications corroborated that field resistance is present in green peach aphid from this site.

Table 11. LC_{50} and resistance ratio values of methamidophos in Green peach aphids from commercial potato fields.

Population	n	slope	LC_{50} (ppm)	Fiducial limits	RR
Franklin (north-west)	300	0.95	61	*	1.2
Grant (west-central)	324	1.8	79	(63, 98)	1.6
Benton (south-west)	155	3.5	87	(73, 104)	1.7
Grant (south-west)	273	1.1	133	(14, 851)	2.7
Skagit (west-central)	237	3.2	137	(113, 167)	2.7
Grant (west-central)	248	2.0	156	(74, 323)	3.1
Adams (south-west)	433	1.9	177	(63, 755)	3.5
Skagit (west-central)	256	2.42	216	(174, 271)	4.3
Grant Co. (central)	223	1.9	659	(91, 3553)	13

*Unable to calculate fiducial limits

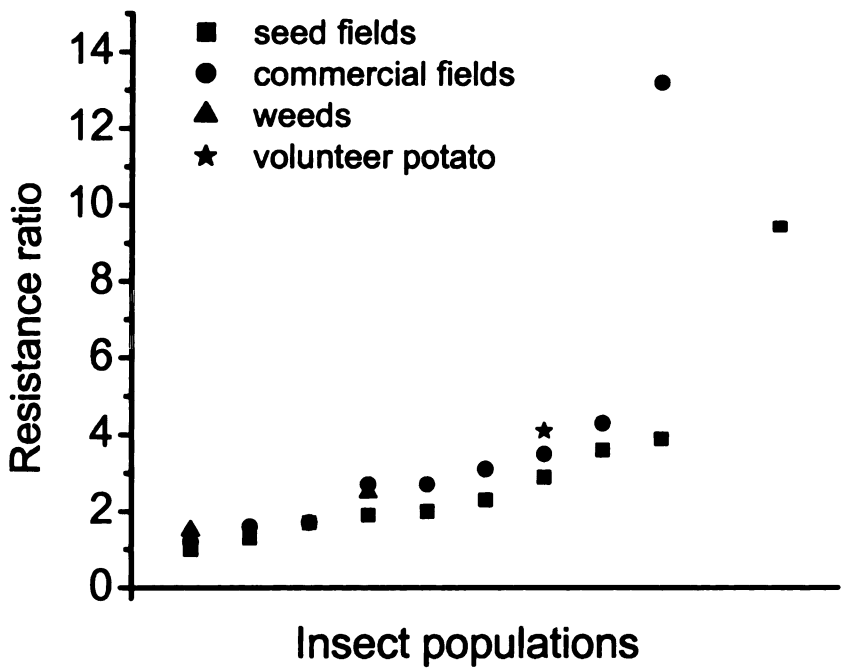

Figure 18. Resistance ratios of Green peach aphid populations from commercial and seed fields, and other hosts to methamidophos.

Aphid population from Skagit Co. (west central) has not been exposed to insecticides in the season. However, the Grant, Co. has been exposed in the season to organophosphates (forate, dimethoate and methamidophos) and pyrethroids (esfenvelarate) (Table 10). This repeated insecticide sprays might screen aphids with resistant alleles to withstand methamidophos.

Mechanism of resistance of green peach aphid to organophosphates and carbamates is insecticide-insensitive acetylcholinesterase; to pyrethroids is knockdown resistance (kdr); and the most common green peach aphid aphid mechanism of resistance to organophosphates, carbamates and pyrethroids: the overexpression of detoxyfing esterases. Esterases (E4 or FE4) can be found in up to 1 percent of the total body protein of the green peach aphid. This enzyme sequestrates and hydrolyses organophosphates, carbamates and pyrethroid insecticides (Devonshire and Moores 1982, Devonshire et al. 1999). Therefore, insecticide treatments of organophosphates might select aphids with overexpression of detoxyfing esterases. However, biochemical tests have to be conducted to confirm this hypothesis.

Not differences in susceptibility to methamidophos was found between the WA, Grant Co. (west central) population collected in commercial potatoes (from Table 11 the LC_{50} = 79 (63, 98) ppm) and the aphid population collected in a weed, hairy nightshade in the field border of the same location (WA, Grant Co., west central) and another aphid population collected in tumble mustard in Walla Walla Co. (west central) (Table 12).

Seed production areas. Ratios of resistance ranged from 1.3 to 9.4 fold. Populations from Kootenai Co. (south-west) and Jefferson Co. (central), were significant different from the reference population (Klickitat) (Table 13). Additional treatment by using a

Table 12. LC_{50} and resistance ratio values of methamidophos in Green peach aphid from weeds and volunteer potato fields.

Population	n	slope	LC_{50} (ppm)	Fiducial limits	RR
Grant, WA (west-central)[1]	461	1.0	77	(14, 426)	1.5
Walla, WA (west-central)[2]	384	1.4	124	(69, 228)	2.5
Gallatin, MT (west-central)[3]	230	2.1	209	(160, 260)	4.1

[1]Weed, hairy nightshade
[2]Weed, tumble mustard
[3]Volunteer potato

Table 13. LC_{50} and resistance ratio values of methamidophos in GPA from seed potato fields.

Population	n	slope	LC_{50} (ppm)	Fiducial limits	RR
Klickitat(northwest) WA	304	1.1	50	(20, 118)	1.0
Kittitas (east-central) WA	327	1.8	67	(14, 187)	1.3
Lake (central) MT	232	3.0	85	(69, 106)	1.7
Whatcom (north-central) WA	175	1.3	96	(64, 145)	1.9
Jefferson (south-central) OR	395	1.7	98	(78, 123)	2.0
Spokane (west-central) WA	215	1.5	117	(85, 159)	2.3
Baker (central) OR	187	1.7	143	(64, 557)	2.9
Fremont (south-central) ID	332	1.8	181	(90, 416)	3.6
Kootenai (south-west) ID	194	2.0	192	(147, 256)	3.9
Whatcom (north-central) WA	292	1.1	231	(90, 105)	4.6
Jefferson (central) OR	365	1.9	469	(194, 1793)	9.4

single dose of 3,160 ppm resulted in 3 % of aphid survivals in the Jefferson population. Treatments of aldicarb, methamidophos and imidacloprid were applied in the field where the Jefferson Co. (central) population was collected. High percent of aphids that survive insecticide treatments in commercial potato fields may not is as important as the aphids that survive in certified seed field; where low percent of survivals would cause a high indirect and severe damage due to viruses

Comparison of results of this research with other results is difficult because despite many reports of resistance green peach aphid to insecticides by using topical application and leaf-dip bioassays, there are not many results of methamidophos by using the method of aphid immersion. In addition, values of LC_{50} of susceptible aphid populations to methamidophos are rear to find. However, I found two reports of determination of the LC_{50} value by using aphid dip bioassays and one of Potter tower bioassay. Using the LC_{50} value of 39.4 ppm determined by Ambrose and Regupathy (1992) the highest ratio of resistance for commercial potato fields increased to a maximum of 17. The ratio of resistance was smaller if the LC_{50} value of 132 ppm determined by Herron and Rophail (1994) was used, having a maximum ratio of resistance of 5.1. Conversely, the highest ratio of resistance increase to 37 if the LC_{50} value of 63 ppm determined in a field population by Potter tower spray by McClanahan and Founk (1983) was used. Ratios of resistance are variable according with the crop and the pest threshold. For instance, smaller ratios of resistance will cause serious damage in certified potato field that in commercial potato fields. Few percent of survival of the aphids in certified potato field would be enough to cause serious viruses transmission in the seeds.

CONCLUSIONS

History of insecticide application in previous years together with factors such as immigration and host alternation would have an impact on the resistance. Results of this research together with the lack of efficacy in the field demonstrated the onset of the resistance on seed certified potato fields and commercial fields. Additional determination of the mechanism of aphid resistance to methamidophos is very important for designing strategies of resistance management. Farmers now are switching to other insecticides options to control GPA with pymetrozine and imidacloprid. History of insecticide treatment pointed out that compounds with novel chemistries provide excellent initial control. However, green peach aphid develops resistance. Wise use of this compounds will guarantee that the efficacy last for longer period of time. Determination of the initial susceptibility (base lines) is a key point of management of resistance. Resistance to methamidophos in some potato fields could teach us that management of resistance before occurs.

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