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Behavior of Colorado Potato Beetle (Coleoptera: Chrysomelidae) Larvae: Effect of *Bacillus thuringiensis*-transgenic Potato on Feeding by *B. thuringiensis*-resistant and Susceptible Strains, and Position of Larvae on Individual Plants in the Field

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Jennifer Anne Altre

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BEHAVIOR OF COLORADO POTATO BEETLE (COLEOPTERA: CHRYSOMELIDAE) LARVAE: EFFECT OF *BACILLUS THURINGIENSIS*-TRANSGENIC POTATO ON FEEDING BY *B. THURINGIENSIS*-RESISTANT AND SUSCEPTIBLE STRAINS, AND POSITION OF LARVAE ON INDIVIDUAL PLANTS IN THE FIELD

By

Jennifer Anne Altre

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ABSTRACT

BEHAVIOR OF COLORADO POTATO BEETLE (COLEOPTERA: CHRYSOMELIDAE) LARVAE: EFFECT OF *BACILLUS THURINGIENSIS*-TRANSGENIC POTATO ON FEEDING BY *B. THURINGIENSIS*-RESISTANT AND SUSCEPTIBLE STRAINS, AND POSITION OF LARVAE ON INDIVIDUAL PLANTS IN THE FIELD

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Within 4 h, *Bacillus thuringiensis* -transgenic potato reduced feeding and increased resting by *B. thuringiensis*-resistant and susceptible Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) second instars. In contrast, behavior of *B. thuringiensis*-resistant larvae was unaffected after 4 h on leaves dipped in *B. thuringiensis* at 1.6 times their LC_{50}. Larval development was similar in the two strains. In the field, wild-type egg masses occured throughout the canopy. Small larvae were in lower leaf axils, and large larvae were on upper seven-leaflet leaves. Large larvae fed and rested on the tops of upper leaves in the morning; in the afternoon, larvae fed over the whole plant.

To Mom and Dad and John-
I love you!
Thanks for your loving support, and for many happy times together,
past, present, and future!!

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INTRODUCTION

Potatoes (*Solanum tuberosum* L.) are an important source of food energy; the average American eats over 100 pounds of potatoes each year (Anonymous 1990). Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) is the most damaging insect pest of potatoes in the United States (Olkowski et al. 1992). Large larvae and adults can completely defoliate a potato field, reducing yield by at least 66% (Hare 1980). In 1991, the $70 million potato industry in Michigan lost approximately $15 million due to Colorado potato beetle damage and control costs (Anonymous 1992; Olkowski et al. 1992). Proper management of the Colorado potato beetle is vital to the $3.1 billion U.S. potato industry.

Colorado Potato Beetle Natural History and History of Pest Status

Depending on weather and food supply, Colorado potato beetle can have one to three generations per year (Radcliffe 1982). Adult Colorado potato beetles overwinter approximately 10 cm below the soil surface (Hare 1990). Females lay clusters of approximately 30 eggs on the undersides of leaves of potato or another appropriate host. After four stadia on host foliage, larvae drop to the soil and pupate underground.

Surprisingly little is known about the distribution of Colorado potato beetle eggs and instars on individual host plants in the field. Inaccurate assumptions about the distribution of a specific life stage could lead to inaccurate estimates of pest abundance and inappropriate pest management decisions. Studies of the distribution of Colorado potato beetle eggs and larvae on potato plants in the field are discussed in Chapter Two.

Most host plants of the Colorado potato beetle are in the same genus as potato (Hare 1990). Colorado potato beetle occurs on at least a dozen different host plants in the U.S., including potato, eggplant (*Solanum melongena* L.), and tomato (*Lycopersicon esculentum* Mill) (Hsiao 1985). Potato is the most suitable host for most Colorado potato beetle populations in the United States (Hare 1990). However, adult Colorado potato beetles from different parts of the U.S. sometimes differ in feeding behavior on the same Solanaceous plants (Harrison 1987).

In Southern Mexico, where Colorado potato beetle originated, beetles feed on *Solanum rostratum* Dunal and *Solanum angustifolium* Mill (Hsiao 1985). The original distribution of the Colorado potato beetle also included the following states: Texas, Oklahoma, Kansas, Nebraska, Colorado, New Mexico, and Arizona (Hsiao 1985). Thomas Nuttal collected Colorado potato beetle on *S. rostratum* near the Iowa-Nebraska border in 1811 (Jacques 1988; Casagrande 1987). In 1824, Thomas Say described the species as *Doryphora decemlineata*. Throughout the Civil War period, many farmers brought potatoes westward into the range of the Colorado potato beetle.

The first outbreak of Colorado potato beetle damage to potatoes occurred in Nebraska in 1859 (Jacques 1988). The Colorado potato beetle spread eastward on potatoes at a rate of 80 km per year, reaching the Atlantic coast by 1870 (Hurst 1975). Early control methods for Colorado potato beetle included hand-picking beetles off plants, or letting poultry loose in the field to eat them (Casagrande 1987). In the 1870's, early-emerging, Colorado potato beetle-resistant potato varieties were recommended. Growers were urged to space potato fields far apart and to rotate their fields between potatoes and non-Solanaceous crops in alternate years. C. V. Riley recommended placing tubers in the field before crop emergence as a means of attracting many beetles to a small area where they could be more easily exterminated.

By 1875, the paint pigment Paris Green was commonly used to control Colorado potato beetle (Casagrande 1987). Lead and calcium arsenate became the most common insecticides in the early 1900's, and they were replaced by DDT in 1945. The first DDT-resistant Colorado potato beetles were reported in 1952 (Forgash 1985). As more synthetic organic insecticides were invented, Colorado potato beetle often developed resistance to new compounds more quickly than to previous ones. Today, Colorado potato beetle can be expected to develop resistance to new conventional pesticides within 3.5 years (Olkowski et al. 1992). Long Island Colorado potato beetles are notorious for being resistant to all registered potato pesticides, except for cryolite and the delta-endotoxin of bacterium *Bacillus thuringiensis* (Ferro & Voss 1985). Insecticide resistance can come at a cost of otherwise reduced fitness. For example, one Colorado potato beetle strain resistant to permethrin and azinphosmethyl has a slower developmental rate and reproductive rate than a susceptible strain (Argentine et al. 1989).

Resistance to an insecticide may be delayed by reducing the exposure of Colorado potato beetle to that insecticide. This can be achieved by reducing the frequency of applications, the amount of insecticide sprayed per application, and the amount wasted per application. The frequency of sprays can often be reduced when growers switch from spraying on a pre-determined calendar schedule to spraying only when pest abundance or crop damage warrants a spray. Damage or pest abundance is quantified by "scouting" fields and recording observations. Insecticides should only be applied when the pest damage or density reaches a pre-determined tolerance level, or "action threshold", beyond which there is unacceptable danger of economic loss. For example, one Colorado potato beetle action threshold prescribes control measures for Colorado potato beetle when there are 0.5 adults per vine, or 1.5 large larvae per vine, or 4 small larvae per vine (Nyrop & Wright 1985).

Potato plants can tolerate considerable defoliation without reduced tuber yield, except during tuber formation and growth (Hare 1980). This occurs at approximately the same time that the summer generation Colorado potato beetle adults emerge and lays eggs. Even during tuber formation and growth, 25% defoliation can be tolerated (Hare 1990). Zehnder & Evanylo (1989) studied defoliation tolerance of potato plants infested with different Colorado potato beetle densities at different times, and concluded that currently recommended action thresholds are probably unnecessarily conservative, especially for plants which have already bloomed.

Alternative Tactics for Colorado Potato Beetle Control

Crop Rotation. Using insecticides in combination with other control factors could help reduce the amount of insecticide necessary. For instance, crop rotation reduces the abundance of Colorado potato beetle, so less insecticide is needed (Roush et al. 1990). Rotation means that a crop other than potato, tomato, or eggplant is grown in a field where potatoes were grown the previous year. Ideally, new potato crops are planted as far from the rotated field as possible, because adult beetles emerging in the rotated field will leave in search of host plants. Crop rotation is thought to be one of the most important non-chemical tactics for Colorado potato beetle management.

Wheat is a good rotation crop because it slows the emergence of overwintering adults (Lashomb & Ng 1984). The tall, dense stems physically obstruct beetle emigration from the wheat field to potato fields elsewhere. When potatoes are planted again in a field used for wheat the previous year, overwintering adults invade later and produce 90% fewer egg masses as compared to adults re-colonizing non-rotated potato fields.

Volunteer potatoes left in a corn or carrot rotation crop further reduce the number of adult Colorado potato beetles which leave to attack other fields (Grafius et al. 1992b). Straw mulch (Zehnder & Hough-Goldstein 1990) and no-till crop residue (Zehnder & Linduska 1987) can also enhance the benefits of rotation. However, optimal rotation crops have not been identified yet. Ideally, a rotation crop would be high-value, tolerant of acid soils, and effective in blocking beetle emigration.

Other Cultural and Mechanical Control Measures. Border strips of rye are used by some Michigan growers to block movement of adult Colorado potato beetles into tomato fields. If a potato field has a history of colonization by adult Colorado potato beetles walking in from a predictable direction, a plastic-lined trench can be positioned along that edge of the field to act as a pitfall trap.

Early season Colorado potato beetle control may be necessary to ensure that plants are not eaten to the ground, and that stems are sufficiently refoliated by the time tubers start to form. Late season control may not affect potato yield, but could be useful for reducing the number of overwintering beetles that will attack the next year's potato fields. Non-pesticide control tactics are appropriate for these less critical periods.

One strategy for early and late Colorado potato beetle control is to attract adult Colorado potato beetles to a small planting of potato, and then exterminate them mechanically. This can be done before the rest of the plants in the field emerge, or after they have been harvested (Olkowski et al. 1992). On an early "trap crop" of young plants, a propane flamer can be used to kill Colorado potato beetle. For the late season, a vacuum is preferable, because it can be used repeatedly on mature plants without killing them (Olkowski et al. 1992). Flaming and vacuuming can also be used to treat patches of high Colorado potato beetle density throughout the season.

For growers who choose not to rotate their potato fields, the following strategy was devised in 1870: harvest potatoes early one year, and plant late the following year (Casagrande 1987). Overwintering Colorado potato beetle adults must survive nearly a year without food when this is done. However, it may be more difficult to market early and late potato varieties, and their yield and market value would also need to be competitive for this strategy to be feasible.

Biological Control. Insect predators and parasitoids of the Colorado potato beetle could become effective control tools as conventional insecticides are phased out of IPM programs. In Michigan, adult *Lebia grandis* Hentz (Coleoptera: Carabidae) are indigenous predators of Colorado potato beetle eggs, consuming up to 47 eggs per day (Groden 1989). *L. grandis* larvae are ectoparasitoids of Colorado potato beetle prepupae, infesting up to 50% of the prepupae in the field. *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae) is another significant indigenous predator of Colorado potato beetle eggs (Groden 1989). It thrives on corn pollen, making corn an especially desirable rotation crop. *Edovum puttleri* Grissel (Hymenoptera: Eulophidae) cannot overwinter, but parasitizes 50% of Colorado potato beetle eggs and persists for almost four months when introduced into potato fields (Schroder & Athanas 1989). *Perillus bioculatus* (F.) (Hemiptera: Pentatomidae) can be effective in controlling low populations of Colorado potato beetle and can be reared on an alternate host and released into potato fields (Hough-Goldstein & Keil 1991). Additional species of Hemiptera, Coleoptera, Diptera and Hymenoptera have been identified as biocontrol candidates for Colorado potato beetle, as have Neuroptera (G. E. Heimpel et al., 1991, unpublished paper, University of Delaware). The Colorado potato beetle multiplies faster than these beneficial insects, so beneficial insects must be considered as one component of multiple-strategy IPM programs rather than a sole source of control.

The most successfully exploited pathogen of Colorado potato beetle is the bacterium *Bacillus thuringiensis* Berliner. The interaction between *B. thuringiensis* var. *tenebrionis* and Colorado potato beetle is the focus of Chapter One.

Bacillus thuringiensis Delta-endotoxins

Insecticidal Activity and Pesticide Status. An isolate of the spore-forming bacterium *Bacillus thuringiensis* Berliner (*B.t.*) from Mediterranean flour moth, *Anagasta kuehniella* (Zeller) (Pyralidae), was described by Berliner in 1911. Some isolates of *B. thuringiensis* produce insecticidal crystal protein inclusions called delta-endotoxins. At least 19 different types of delta-endotoxin have been described (McGaughey & Whalon 1992).

Delta-endotoxins are usually specific for one or two insect orders, and are not known to be toxic to humans. Most of the known delta-endotoxins are active against Lepidoptera, and are classified as cryI. CryII toxins are toxic to both Lepidoptera and Diptera. Delta-endotoxins active against Colorado potato beetle and other Coleoptera are classified as cryIII. CryIV delta-endotoxins are toxic to Diptera. Typically, a delta-endotoxin is most toxic to a subset of insect species within a family. For example, cryIIIA is toxic to Colorado potato beetle, but not to southern corn rootworm, *Diabrotica undecimpunctata* howardi Barber (Chrysomelidae) (Donovan et al. 1992).

Lepidopteran-active *B. thuringiensis* was first registered as a pesticide in the U.S. by Nutralite Corporation in 1961. A mix of spores and cryI crystals from *B. thuringiensis* var. *kurstaki* is still the most widely used microbial insecticide today (Tabashnik et al. 1990). *B. thuringiensis* accounts for over 90% of the biopesticide market (Feitelson et al. 1992). In 1990, biopesticides were a $100 million dollar industry, accounting for less than one percent of the world pesticide market (Twombly 1990). Biopesticides are expected to be a billion-dollar industry in the year 2000 (Twombly 1990), and *B. thuringiensis* alone will account for at least $300 million (Feitelson et al. 1992). More than 30 *B. thuringiensis* patents were issued in the U.S. from 1988-1991, and 18 companies had *B. thuringiensis* research programs in 1992 (Feitelson et al. 1992).

Delta-endotoxin Mode of Action and Insect Resistance. The mode of action of *B. thuringiensis* endotoxin is described by Gill et al. (1992). To become active, an endotoxin crystal must first be ingested by an insect with a gut pH which will solubilize the crystal into its component endotoxins. Large protoxins, such as cryI and some cryIV, must then be cleaved at the carboxyl end by insect midgut proteases to become actively toxic. CryIII toxins lack this extra C-terminal sequence, and may not need to be proteolytically activated.

Endotoxin receptors are on the insect midgut columnar epithelial cells. *B. thuringiensis* binds to the anterior midgut in Lepidoptera, and to the posterior midgut in Colorado potato beetle (Bravo et al. 1992). The C-terminal cell-binding domain of the endotoxin binds to the receptor, and then the N-terminal toxic domain inserts into the cell membrane. Multiple toxin molecules penetrate the membrane and bind with each other to form an oligomer. The result is a pore in the membrane through which cations, water, and other small molecules pass (Knowles & Ellar 1987). Electrical, pH, and cation gradients are disrupted by the pore. Water and cations first rush into the epithelial cell, causing it to swell and lyse. If enough cells are lysed, disintegration of the epithelium allows mixing of hemolymph and gut contents.

When cottonwood leaf beetle (*Chrysomela scripta* F.) larvae consume a mixture of *B. thuringiensis* spores and cryIIIA, no damage to midgut epithelial cells is evident until 2 h after ingestion (Bauer & Pankratz 1992). In contrast, *B. thuringiensis* damage to

Lepidopteran midgut cells is evident within a few minutes (Percy & Fast 1983).

After 2 h, the infoldings of the basal plasma membrane of beetle midgut epithelium expand, pushing the nuclei toward the gut lumen (Bauer & Pankratz 1992). Cells swell at their apex and project into the gut lumen. After 3 h, cells start to burst and leak cytoplasm into the gut lumen, and microvilli are broken. If present, *B. thuringiensis* spores germinate, and subsequent generations of the dividing bacteria provide still more endotoxin.

B. thuringiensis endotoxin-binding receptors on midgut epithelial cells may vary in abundance and affinity for toxin molecules. Changes in these parameters can cause resistance or enhance susceptibility to the toxin. Surprisingly, low receptor binding affinity is correlated with high toxicity in gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) (Wolfersberger 1990). In contrast, cryIA receptors of cryIA-resistant Indian meal moth, *Plodia interpunctella* (Huebner) (Lepidoptera: Pyralidae), have a lower binding affinity for cryIA than those of cryIA-susceptible *P. interpunctella* (van Rie et al. 1990). In cryIA-resistant *P. interpunctella*, the concentration of cryIC receptors on the midgut epithelium is increased, as is their susceptibility to cryIC.

Mild resistance to *B. thuringiensis* occurs in *P. interpunctella* in some *B. thuringiensis*-treated grain bins (McGaughey 1985). The resistance increases rapidly during laboratory selection, and stabilizes at 100-fold. Resistance does not diminish after seven unselected generations. Either resistance has no fitness cost in the laboratory setting, or the lab population is fixed for the resistance allele. *B. thuringiensis*-resistance is a recessive trait in *P. interpunctella*.

Some field populations of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), are 30-fold resistant to *B. thuringiensis* (Tabashnik et al. 1990). Such *B. thuringiensis*-resistant diamondback moths from the field become 820-fold resistant to *B. thuringiensis* after nine generations of laboratory selection. *B. thuringiensis* reistance fails to decline in diamondback moth in the absence of selection, even after 15 generations. Altered toxin binding sites have been demonstrated as a mechanism of *B. thuringiensis* resistance in *P. xylostella* (Ferre et al. 1991).

B. thuringiensis resistance in laboratory-selected tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), reaches a plateau of 16-fold after seven generations (Stone et al. 1988). *H. virescens* is far more resistant to toxin in transformed *Pseudomonas* than to toxin alone. Stone & Sims (1993) noted an 8-fold range in cryIA(c) endotoxin LC_{50}'s for populations of *H. virescens* from 14 states. Corn earworm (*Helicoverpa zea* [Lepidoptera: Noctuidae]) from the same states had a 16-fold LC_{50} range. Mild *B. thuringiensis* exists in at least two other Lepidopteran species and two species of mosquitoes (McGaughey & Whalon 1992).

Bacillus thuringiensis and Behavior of Lepidoptera

Behavioral "Resistance" to *B. thuringiensis* in *Cydia pomonella.* Codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) is susceptible to *B. thuringiensis* on artificial diet or apple slices in the laboratory (Andermatt et al. 1988). However, *C. pomonella* spits out the epidermis as it enters a whole apple. Thus, *B. thuringiensis* fails to control these larvae in the field because they never ingest it; this "resistance" is based on normal feeding behavior.

Detection and Avoidance of *B. thuringiensis* by *Heliothis virescens.* *Heliothis virescens* larvae are able to avoid *B. thuringiensis* in some situations, indicating that cthey can detect *B. thuringiensis*. In arenas containing both *B. thuringiensis*-treated and untreated diet, small *H. virescens* larvae avoid all but the two highest *B. thuringiensis* concentrations used by Gould et al. (1991). This suggests that they move away from *B. thuringiensis*-treated areas after consuming small quantities of *B. thuringiensis*, but are immobilized too quickly to escape when *B. thuringiensis* concentrations are high.

Late *H. virescens* instars avoid only growth-inhibiting doses of *B. thuringiensis.* The difference in consumption between *B. thuringiensis* and control diet is amplified in large experimental arenas where only one of the diets shows evidence of feeding after 24 h. In a small arena, both types of diet are tasted within 24 h. In 23 out of 31 cases, the untreated diet was the one chosen in this study. Assuming the larva encounter the *B. thuringiensis* diet first 50% of the time, *B. thuringiensis* is virtually untouched when encountered. Instead, larvae probably wander until they find untreated diet.

Resistant and susceptible individuals may differ in behavioral response to *B. thuringiensis.* At a low concentration of pure endotoxin, neither *B. thuringiensis*-resistant nor *B. thuringiensis*-susceptible *H. virescens* larvae avoid *B. thuringiensis* (Gould & Anderson 1991). At the two lowest concentrations of Dipel (which contains toxin plus spores), only *B. thuringiensis*-susceptible larvae avoid *B. thuringiensis.* At all higher concentrations of Dipel and pure toxin, both strains avoid *B. thuringiensis*. The survival advantage of resistant larvae is much more pronounced on no-choice *B. thuringiensis* diet than in choice situations where untreated diet is also available.

Bacillus thuringiensis **and Colorado Potato Beetle**

CryIII Endotoxins. Several different strains of *B. thuringiensis* produce cryIII proteins, including *B. thuringiensis* var. *tenebrionis* (Krieg et al. 1983; McPherson et al. 1988), which was once called *B. thuringiensis* var. *san diego* (Ferro & Gelernter 1989; Herrnstadt et al. 1986; Herrnstadt et al. 1987), as well as *B. thuringiensis* EG2158 (Donovan et al. 1988), *B. thuringiensis* EG2838 (Rupar et al. 1991), *B. thuringiensis* NCIMB 40152 (Cidaria et al. 1991), *B. thuringiensis* var. *tolworthi* (Sick et al. 1990), *B.*

thuringiensis var. *kurstaki* (Lambert et al. 1992), and *B. thuringiensis* EG4961 (Rupar et al. 1991; Johnson et al. 1993). Several different types of cryIII proteins have been discovered. The known cryIII proteins are about 650 amino acids long and 74 kd in size. Coleoptera from families other than Chrysomelidae that are susceptible to at least one cryIII toxin include: yellow mealworm, *Tenebrio molitor* L. (Tenebrionidae), from which *B. thuringiensis* var. *tenebrionis* was isolated (Krieg et al. 1983); boll weevil, *Anthonomus grandis grandis* Boheman, and black vine weevil, *Otiorhynchus sulcatus* (F.) (Curculionidae) (Herrnstadt et al. 1986); and Japanese beetle, *Popilla japonica* Newman (Scarabaeidae) (M. E. Whalon, Michigan State University, personal communication). Chrysomelidae susceptible to at least one cryIII toxin include: Colorado potato beetle; cottonwood leaf beetle, *Chrysomela scripta* F. (Bauer & Pankratz 1992); imported willow leaf beetle, *Plagiodera versicolora* (Laicharting) (Bauer 1992); southern corn rootworm, *Diabrotica undecimpunctata* howardi Barber (Donovan et al. 1992); *Agelastica alni* L. (Krieg et al. 1983); elm leaf beetle, *Pyrrhalta luteola* (Mueller) (Francardi 1990); and Tasmanian eucalyptus leaf beetle, *Chrysophtharta bimaculata* (Olivier) (Elliott et al. 1993).

CryIII Insecticides for Colorado Potato Beetle Control. *Bacillus thuringiensis* var. *tenebrionis* Berliner is the source of all *B. thuringiensis* products commercially available for Colorado potato beetle control. Products registered for use against Colorado potato beetle include Foil (Ecogen, Langhorne, PA), Novodor (NovoNordisk/Entotec, Davis, CA), Trident I (Sandoz Des Plains, IL), Trident II, and M-Trak (Mycogen, San Diego). M-Trak is the only approved recombinant-DNA *B. thuringiensis* product for Colorado potato beetle control (Feitelson et al. 1992). It was approved by EPA in 1991. The *B. thuringiensis* gene is inserted into the bacterium *Pseudomonas fluorescens,* which expresses the toxin. The *Pseudomonas* are then killed and fixed with iodine, and the cell walls enclose the toxin molecules; this may increase the persistence of the toxin in the field.

B. thuringiensis var. *tenebrionis* provides good field control of Colorado potato beetle. Potato yield is equal for plots sprayed with M-One (the forerunner to M-Trak) and with the pyrethroid Asana (Ferro & Gelernter 1989). Larval mortality peaks 3 to 4 d after first exposure, making this a relatively slow-acting insecticide (Ferro & Gelernter 1989). First and second instars are more susceptible to a given concentration of cryIIIA than larger life stages (Zehnder & Gelernter 1989). Thus, for successful control of Colorado potato beetle, field sprays of M-Trak must be applied when early instars are the predominant life stage (Anonymous 1992). A newer formulation of cryIIIA, Novodor, provides some control of large larvae (Olkowski et al. 1992).

Temperature and duration of larval exposure affect *B. thuringiensis* toxicity. First-instar mortality due to *B. thuringiensis* is reduced as temperature decreases (Ferro & Lyon 1991). The effectiveness of cryIIIA can be hindered if it is washed off by irrigation or rain soon after spraying (Anonymous 1992). Maximum larval mortality is achieved when early instars are exposed to *B.*

thuringiensis for at least 6 to 8 h at temperatures above 24° C (Ferro & Lyon 1991).

Even when it is not washed off plants, endotoxin is not persistant in the field environment. A concentration of M-Trak which initially causes 90% mortality of small larvae exposed for 1 h breaks down within 3 d so that the residue causes only 20% mortality (Ferro et al. 1993). Since small larvae occur throughout the growing season, repeated spraying of cryIIIA is necessary (Anonymous 1992).

High-concentration CryIII-transgenic Potato in the Field. Wash-off and breakdown of cryIIIA in the field can be avoided by a new alternative to spray formulations of delta-endotoxin. The gene for endotoxin has been integrated into the genome of potato plants, which are thus able to produce endotoxin (Monsanto Co., St. Louis). Such plants are referred to as *B. thuringiensis*-transgenic. The first generation of *B. thuringiensis*-transgenic potato plants contains endotoxin at 100 times the LC_{90} of Colorado potato beetle larvae in the field. *B. thuringiensis* toxin comprises approximately 0.1% of the total soluble protein in the plant tissue (J. Wierenga, personal communication). These plants are called constitutive transgenics because they express *B. thuringiensis* in all tissues, at all times. Constitutive transgenic potato plants which produce a high concentration of delta-endotoxin are almost immune to all Colorado potato beetle life stages throughout the season (Grafius et al. 1992; Boylan-Pett et al. 1991). One key problem with this new resource is that transgenic minitubers emerge more slowly than conventional potato seed pieces and thus produced a somewhat lower yield than might be expected from a conventional crop. Yield needs to be improved by 1996-7, when transgenic potato is expected to become commercially available for planting.

Ideally, even the most *B. thuringiensis*-resistant insects will be killed when they attempt to feed on these plants. However, beetles which can tolerate this level of exposure will probably gradually increase in prevalence. Although continuous transgenic monocrops would initially be free of Colorado potato beetle damage, this strong selection for resistance might shorten the useful life of cryIIIA transgenics and sprays against Colorado potato beetle. One proposed solution is to mix some endotoxin-free potato plants, or refugia, in stands of cryIIIA-transgenic potato to ensure survival of some susceptible Colorado potato beetles. The economic feasibility of sacrificing enough unprotected potato plants to maintain sufficient *B. thuringiensis*-susceptible alleles in the Colorado potato beetle gene pool has not been demonstrated.

***Bacillus thuringiensis* Resistance in Colorado Potato Beetle.** Colorado potato beetle has been selected for resistance to *B. thuringiensis* in the laboratory (Whalon et al. 1993). Maintenance selection keeps the laboratory colony's average resistance level at approximately 90-fold. Second instars are selected with a concentration of M-Trak rendering less than 2% survival. After the first 12 generations of selection, the *B. thuringiensis*-resistant strain was 60-fold resistant to *B. thuringiensis* compared to a *B.*

thuringiensis-susceptible laboratory strain, and 30-fold more resistant to *B. thuringiensis* than larvae from other insecticide-resistant strains.

B. thuringiensis-resistant Colorado potato beetles also partially resist the inhibitory effects of endotoxin on oviposition. In caged experiments, the *B. thuringiensis*-susceptible strain is unable to oviposit at all when exposed to a high *B. thuringiensis* concentration (Whalon et al. 1993). Although the resistant beetles oviposit at a reduced rate in the presence of *B. thuringiensis*, oviposition increases with increasing resistance levels.

Colorado potato beetle may have multiple resistance mechanisms against *B. thuringiensis* endotoxin, each of which may have associated fitness costs. Possible physiological mechanisms of resistance include: altered gut pH leading to decreased toxin solubility; altered enzymes leading to increased or more rapid proteolytic inactivation of toxin; increased non-midgut binding of toxin leading to reduced binding at the midgut epithelium (for instance, increased binding and removal of toxin by the peritrophic membrane); faster movement of gut contents through and out of the midgut; alteration of specific midgut binding sites leading to reduced binding of toxin to midgut epithelium; membrane changes leading to reduced pore formation; ability to counteract ion imbalances caused by pores in the epithelial membranes; increased ability to repair epithelial damage; and enhanced resistance to septicemia. As previously mentioned, altered toxin binding sites have been demonstrated as a mechanism of *B. thuringiensis* resistance in some Lepidoptera.

Colorado potato beetle *B. thuringiensis* resistance declines in the absence of selection (Whalon et al. 1993). Rotating use of *B. thuringiensis* sprays or trangenics with years of *B. thuringiensis*-free control measures could therefore slow the buildup of *B. thuringiensis* resistance.

Research on *B. thuringiensis*-resistant diamondback moth in the field and laboratory suggests that *B. thuringiensis* should be used in combination with other biological and cultural control tools (Tabashnik et al. 1991). An IPM program with crop rotation and *B. thuringiensis* as components can be less expensive than conventional Colorado potato beetle management (Olkowski et al. 1992). Releases of arthropod biocontrol agents may someday be a companion to *B. thuringiensis*. For instance, releasing two-spotted stink bugs, *Perillus bioculatus* (F.) (Hemiptera: Pentatomidae), in conjunction with *B. thuringiensis* sprays causes 75% greater mortality than *B. thuringiensis* alone (Hough-Goldstein & Keil 1991).

***Bacillus thuringiensis* and Colorado Potato Beetle Behavior.** At a concentration equivalent to 12.5% of field rate, formulated cryIIIA has little effect on leaf area consumed by susceptible Colorado potato beetle adults and does not cause adult mortality (Zehnder & Gelernter 1989). At 20% of field rate, cryIIIA affects the movement of susceptible adults only (Whalon et al. 1993). At 75% of field rate, both cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle adults move less on treated plants than on untreated plants (Whalon et al. 1993). At 20% and 75% of field rate, cryIIIA causes cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle adults to clip or chew the stems of potato plants more frequently than adults on untreated potato plants (Whalon et al. 1993).

Like adult Colorado potato beetles, third instars are capable of feeding recovery after 24 hours of cryIIIA exposure at field rate (Zehnder & Gelernter 1989). In contrast, second instars that survive cryIIIA exposure do not recover their normal feeding rate within two days on untreated foliage (Zehnder & Gelernter 1989). Feeding stimulants increase Colorado potato beetle consumption of cryIIIA-treated foliage in the field, but the increased feeding does not increase mortality (Hough-Goldstein et al. 1991). In contrast, treatment of foliage with antifeedant citrus limonoids before a *B. thuringiensis* application reduces adult colonization and oviposition and increases larval development time (Murray et al. 1993).

Neonate Colorado potato beetles feed and rest equally on *B. thuringiensis*-treated and untreated foliage in a choice situation (Arpaia & Ricchiuto 1993). Fourth instars with access to both cryIIIA-treated and untreated leaves stay on the first leaflet they encounter and feed as often and as readily on cryIIIA-treated as on untreated foliage (Ferro & Lyon 1991). Adult Colorado potato beetles are equally abundant on treated leaves and untreated leaves in a choice situation; this is true for both cryIIIA-susceptible and cryIIIA-resistant strains. Egg masses are also deposited equally on cryIIIA-susceptible Colorado potato beetle adults move less on treated plants than on untreated plants (Whalon et al. 1993). At 20% and 75% of field rate, cryIIIA causes cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle adults to clip or chew the stems of potato plants more frequently than adults on untreated potato plants (Whalon et al. 1993).

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CHAPTER ONE

Behavior of cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle larvae on cryIIIA-treated and trangenic potato foliage, and comparative development of the two beetle strains

Introduction

Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is the most serious defoliating pest of potato in the United States and Europe (Radcliffe 1982). Potatoes are important both as human food and as feed for livestock (Radcliffe 1982). In Michigan, 1991 potato yield loss due to Colorado potato beetle averaged 12.2% (Anonymous 1992).

The soil bacterium *Bacillus thuringiensis* var.*tenebrionis* Berliner is the most effective microbial weapon against Colorado potato beetle. M-Trak (Mycogen, San Diego) is one of several Colorado potato beetle insecticides derived from the cryIIIA endotoxin of *B. thuringiensis* var. *tenebrionis*. In addition to cryIIIA sprays, the bacterial gene for cryIII endotoxin has been integrated into the genome of potato plants (Monsanto Co., St. Louis). These first-generation cryIII-transgenic potato plants contain endotoxin at 100 times the LC_{90} of Colorado potato beetle larvae in the field; endotoxin comprises 0.1% of the total soluble protein in the plant tissue (L. Bauer & J. Wierenga, Michigan State University, personal communication). These plants are called constitutive transgenics because they constantly express cryIII in all tissues.

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At the currently recommended field rate, *B. thuringiensis* is lethal to susceptible first and second instars, but not to larger larvae. Ingested *B. thuringiensis* causes susceptible first and second instars to stop feeding hours or days before death. Larvae which survive exposure to cryIIIB as first instars weigh significantly less as second instars than unexposed larvae (Arpaia & Ricchiuto 1993).

Colorado potato beetle second and third instars feed significantly less during 24 h on potato foliage treated with M-One at 6.0% of field rate than the same life stages on untreated foliage (Zehnder & Gelernter 1989). Second instars do not recover food intake at all during the next 2 d on untreated foliage. Third instars recover significantly, but still consume significantly less than third instars never exposed to *B. thuringiensis. Bacillus thuringiensis*-susceptible third and fourth instars which survive exposure to *B. thuringiensis*-transgenic foliage lose weight (J. Wierenga, Michigan State University, personal communication).

Colorado potato beetle has not yet developed resistance to *B. thuringiensis* in the field, but one laboratory-selected population is more than 200-fold resistant to *B. thuringiensis* (Whalon et al. 1993). *B. thuringiensis*-resistant second, third, and fourth instars which survive on transgenic foliage gain weight, though at a reduced rate (J. Wierenga, Michigan State University, personal communication).

Colorado potato beetle larvae are apparently unable to detect and avoid *B. thuringiensis.* When both untreated and *B. thuringiensis*-treated foliage is available for 3 d to first instars in a Petri dish, the larvae tend to be equally distributed on both treated and untreated foliage (Arpaia & Ricchiuto 1993). In 30-minute choice tests, fourth instars also show no bias toward untreated or *B. thuringiensis*-treated foliage (Ferro & Lyon 1991).

Objectives. My general objective was to compare larval behavior in the presence and absence of endotoxin within and between cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle strains. More specifically, the primary goals of the behavior study were: 1) to provide a quantitative demonstration of the "dramatic decrease or complete cessation of feeding within a few hours" of cryIIIA ingestion by susceptible Colorado potato beetle larvae, as noted by Zehnder & Gelernter (1989); 2) to compare the effect of cryIIIA on the feeding behavior of cryIIIA-susceptible larvae and cryIIIA-resistant larvae; 3) to characterize the effect of *B. thuringiensis*-transgenic foliage on the feeding behavior of cryIIIA-resistant larvae; and 4) to characterize *B. thuringiensis*-induced changes in other behaviors besides feeding.

My approach was to measure the proportion of time that larvae spent feeding, resting, and walking on potato foliage in non-choice situations. My expectation was that susceptible larvae would spend less time feeding and walking and more time resting on cryIIIA-treated foliage as compared to untreated foliage. (This was my expectation in part because paralysis is a symptom of *B. thuringiensis* ingestion in some Lepidoptera [Heimpel & Angus 1959].) I also measured time spent off the foliage to determine

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whether larvae leave cryIIIA-treated foliage in a no-choice situation.

For the experiment with cryIIIA-dipped foliage, behavior at two intervals of cryIIIA exposure was studied: behavior during a single hour of cryIIIA exposure, and behavior after 4 h of cryIIIA exposure. A second experiment was performed with cryIIIA-transgenic plants and observation after a 4 h exposure interval.

Our impression from working with immature stages of the cryIIIA-resistant strain was that they developed more slowly than the cryIIIA-susceptible strain. The objective of the final experiment in this chapter was to compare durations of stadia, weights, and rates of weight gain between cryIIIA-resistant and susceptible larvae.

Materials and Methods

The following two behavior studies measured the percentage of 1 h that cryIIIA-resistant and cryIIIA-susceptible larvae spent feeding, resting, and walking in the presence and absence of *B. thuringiensis* var. *tenebrionis* cryIIIA endotoxin. The first study included potato foliage dipped in cryIIIA; the second included *B. thuringiensis*-transgenic potato foliage. Feeding inhibition by *B. thuringiensis* occurs prior to mortality (Zehnder & Gelernter 1989), so behavioral assay is a more immediate method than mortality counts for recognizing relative *B. thuringiensis* toxicity of transgenic versus treated foliage.

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Egg masses of cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetles were obtained from Dr. M. E. Whalon. Dr. Whalon's group established both strains in 1988 (Whalon et al. 1993). The cryIIIA-susceptible strain was founded with eight egg masses from fields which had never been sprayed with cryIIIA. The cryIIIA-resistant strain was founded with 2000 adults from fields previously sprayed with cryIIIA. Successive generations of cryIIIA-resistant second instars were selected with M-Trak (Mycogen Corp., San Diego) to yield less than 2% survival per generation.

Behavior Experiment with Leaves Dipped in CryIIIA. The cryIIIA-resistant larvae in this experiment were at least 60-fold more resistant to cryIIIA than the susceptible larvae. This level of resistance has been obtained after 12 generations of continuous selection (Whalon et al. 1993). However, the cryIIIA-resistant larvae in this experiment were the progeny of a culture that was first selected continuously with *B. thuringiensis* for approximately 20 generations to yield 200-fold resistance; the culture was then maintained for a few generations without selection, during which the resistance level dropped to 60-fold.

Four-hour Pre-treatment. Two five-leaflet leaves from greenhouse-grown 'Atlantic' potato were dipped in a *B. thuringiensis* solution containing cryIIIA delta-endotoxin (0.16 mg/ml, M-Trak batch #4505460) plus Silwet 77 (2 µl/ml, for more uniform coating of leaves; Loveland Industries, Inc., Greeley CO). This concentration of delta-endotoxin was approximately 100 times the LC_{50} of the cryIIIA-susceptible second instars, and 1.6 times the LC_{50} of the cry

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cryIIIA-resistant second instars. Two five-leaflet potato leaves were dipped in 2 µl/ml Silwet 77 only (foliage dipped only in Silwet will be referred to as "untreated", because it was not dipped in *B. thuringiensis*). Petioles of all four leaves were placed in 3.5 cm microvials. The leaves were air-dried and placed in four separate 9 cm Petri dishes lined with filter paper. Four to six cryIIIA-resistant early second instars were placed in one Petri dish containing a cryIIIA-treated leaf, and four to six more were place in a dish containing an untreated leaf. Four to six cryIIIA-susceptible early second instars were placed in each of the other two dishes. Larvae were left in the dishes for 4 h.

The total number of larvae used in a single experiment ranged from 20 to 24. The experiment was repeated seven times, so the final sample sizes for the 4 h treatment were: 34 resistant larvae on treated foliage, 40 resistant larvae on untreated foliage, 41 susceptible larvae on treated foliage, and 40 susceptible larvae on untreated foliage.

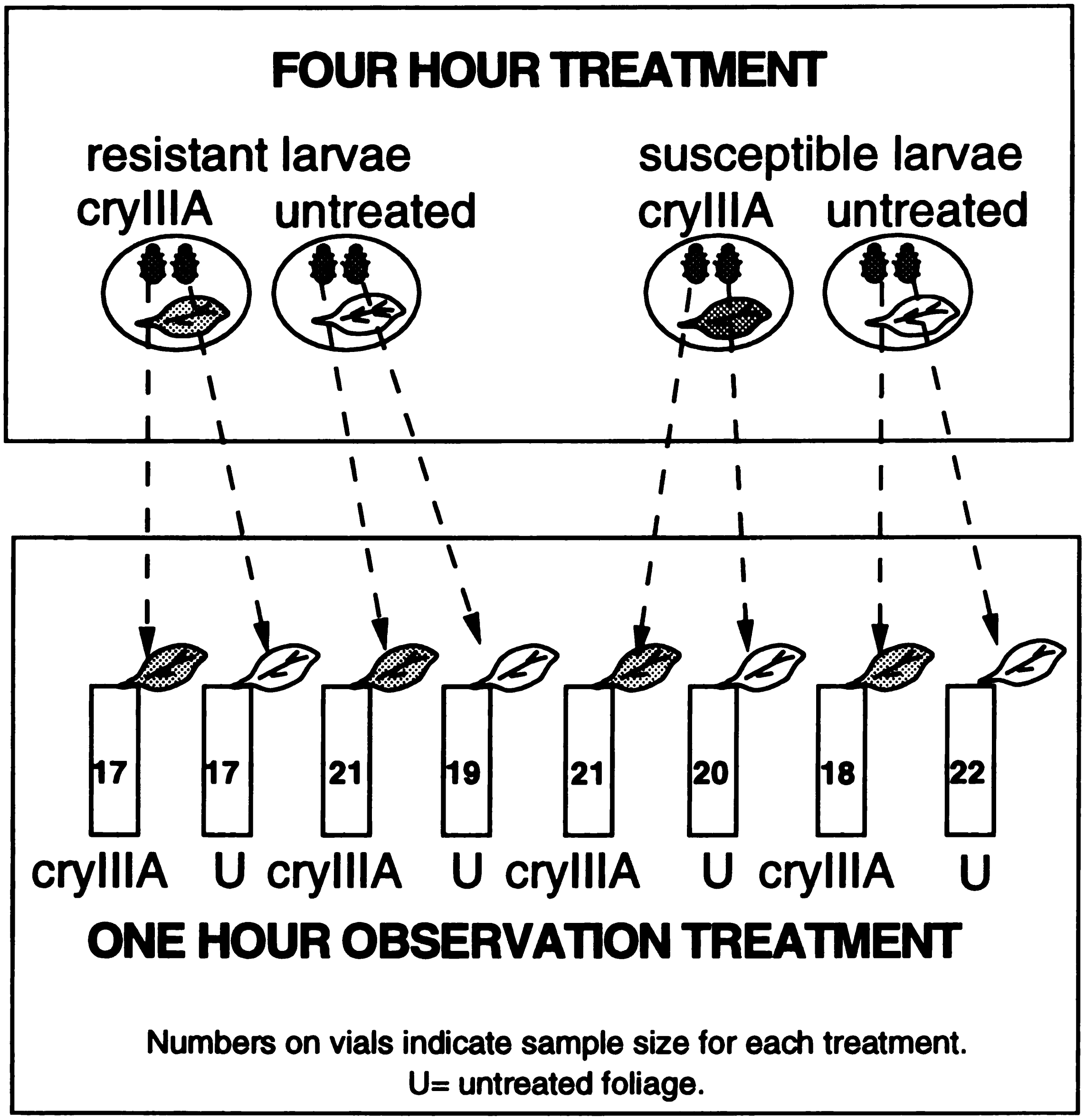
One-hour observation. The observation setup was prepared during the 4 h when larvae were in the Petri dishes. Ten to 12 terminal leaflets from five-leaflet leaves were dipped in the cryIIIA and Silwet solutions, and ten to 12 leaflets were dipped only in the Silwet solution. The petiole of each leaflet was placed in a 7 cm vial filled with tap water, and the vial was plugged with wet cotton. Leaflets were positioned with the adaxial leaf surface facing upward.

After leaflets had air-dried, the vials were labelled with three pieces of information: 1) the solution into which the leaflet had been dipped, 2) the strain of the larva to be placed on the leaflet, and 3) whether the larva had been on a cryIIIA-treated leaf or an untreated leaf for the 4 h exposure period. Vials were labeled so that half of the larvae in each Petri dish were placed on a cryIIIA-treated leaflet, and the other half of the larvae in each dish were placed on an untreated leaflet (Figure 1).

The vials were lined up in one row on a white bench, parallel with a fluorescent light (two GTE Sylvania cool white long bulbs, #F96T12/CW; AC) situated 1 m above. Petroleum jelly was rubbed around the circumference of the vial near the bottom, in order to prevent larvae from wandering onto the bench.

After the 4 h had elapsed, each larva was removed from its Petri dish and placed in the center of a leaflet that was in a vial labeled with the correct strain, 4 h pre-treatment, and 1 h observation treatment. After all larvae were on leaflets, the vial labels were turned away from the observer. The vials were scrambled within the row, in order to ensure that the observer did not know the strain of the larvae or the leaf treatments. The vials were then numbered consecutively with a permanent marker, starting with the vial on the left end of the row of vials. Vials were left undisturbed for 0.5 h before the hour of observation, to allow larvae to recover from handling.

Speaking into a microcassette recorder, the observer noted the time, larval ID number, and new behavior whenever a larva changed



Numbers on vials indicate sample size for each treatment.
U= untreated foliage.

Figure 1. Treatments for the experiment with cryIIIA-treated foliage.

behavior. Behaviors were described as "feeding" or "walking"; larvae that were not feeding or walking were described as "resting". The position of each larva on the upper surface, lower surface, or edge of the leaflet was also noted. Larvae that left leaflets and spent time on the cotton plug or the glass vial were described as "off", with no specific behavioral description.

The observer was able to monitor up to 24 larvae at a time, because changes in behavior were infrequent. This experiment was repeated seven times, and a total of 155 larvae were observed.

Times spent feeding, walking, resting, on upper leaf surface, and off leaf were recorded. Results were analyzed using three-way analysis of variance (SuperANOVA; Abacus Concepts, 1989), followed by Fisher's Protected LSD where ANOVA indicated significant differences (Abacus Concepts, 1989).

Behavior Experiment with *B. thuringiensis*-transgenic Leaves. This experiment was similar to the previous experiment. However, *B. thuringiensis*-transgenic "Russet Burbank" leaves replaced cryIIIA-dipped leaves, and the cryIIIA-resistant larvae provided by Dr. Whalon were at least 90-fold resistant to cryIIIA. Each larva stayed on the same type of foliage (*B. thuringiensis*-transgenic or untransformed) for both the 4 h and 1 h phases of the experiment. Eighty larvae were observed in this experiment (ten cryIIIA-resistant larvae and ten cryIIIA-susceptible larvae in each of four repetitions).

Dr. Whalon's laboratory received *B. thuringiensis*-transgenic minitubers from Hybritech Seed and grew them in the greenhouse.

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The transgenic foliage contained endotoxin at a concentration 30 times greater than that used in the cryIIIA leaf dip experiment. The concentration of endotoxin in *B. thuringiensis*-transgenic foliage was approximately 100 times recommended field rate. This was approximately 100 times the LC_{95} of susceptible second instars and equal to the LC_{95} of resistant second instars.

As in the previous experiment, larvae were exposed to a 4 h pre-treatment of *B. thuringiensis*-transgenic or untransformed foliage in Petri dishes. Each larva was then placed on a leaflet held in a vial; each larva stayed on the same type of foliage (transgenic or untransformed) for both the 4 h and 1 h phases of the experiment. Larvae were allowed to rest 0.5 hours before the hour of behavioral observation.

Times spent feeding, walking, resting, on upper leaf surface, and off leaf were recorded. Results were analyzed using two-way ANOVA (Abacus Concepts, 1989), followed by Fisher's Protected LSD where ANOVA indicated significant differences.

Development of CryIIIA-resistant and CryIIIA-Susceptible Larvae. Resistant larvae used in this experiment were at least 90-fold resistant. Larvae were reared to the second instar from three cryIIIA-susceptible egg masses and two cryIIIA-resistant egg masses. Because so few egg masses were used, the validity of generalizing the results of this study is uncertain. However, eggs within an egg mass can have different fathers (Boiteau 1988), so there may have been considerable variation among eggs within the egg masses used.

On day one of the experiment, new second instars were weighed and placed individually into wells of 24-microwell plates lined with 2% agar. Leaf discs (1 cm diameter) were available to larvae in excess. Two plates (48 larvae) of each strain were maintained for 8 d. Larval stadium was recorded twice daily. The mean durations of the egg stage and the first three stadia were compared between 40 susceptible larvae and 44 resistant larvae. Comparisons were made using 95% confidence intervals (Devore & Peck 1986). Duration of the first through third stadia combined was compared between strains with a Chi-squared test (Devore & Peck 1986).

Larvae were weighed individually on days one, two, three, and eight. Only larvae that molted to the second instar on day one and to the third instar on day two were used in these analyses; 36 susceptible larvae and 22 resistant larvae were used. Significance of differences between mean weights, mean weight gains, and mean relative growth rates of resistant and susceptible larvae was determined with 95% confidence intervals. Mean weights of early second (day one) instars, early third (day two) instars, and fourth instars (day eight) were compared between strains. Mean weight gains during the second stadium and during the eight-day period were also compared between strains. The relative growth rates (percentage body weight gain per day = [100 x weight gain]/[time interval x mean weight of larva during the interval]; Waldbauer 1968) were calculated for larvae of both strains for two time intervals: day one through day three, and day three through day eight.

Results and Discussion

Behavior Experiment with Leaves Dipped in CryIIIA.

One-hour observation. One h treatment did not affect larval feeding behavior (*F* = 2.4; df = 1, 147; *P* = 0.13) or resting or walking behaviors (*F* = 0.004; df = 1, 147; *P* = 0.95). This failure to respond to short-term *B. thuringiensis* exposure suggested that these second instars did not "taste" cryIIIA (and the formulation ingredients) on the foliage. Previous studies have shown that first instars do not avoid cryIIIB and fourth instars do not avoid cryIIIA (Ferro & Lyon 1991; Arpaia & Ricchiuto 1993). The lack of response to 1 h leaf treatment also suggested that larvae did not experience toxic effects within 1 h at the concentration of endotoxin used. This is not surprising, since midgut disruption is not visible in cottonwood leaf beetle until 3 h after ingestion of endotoxin (Bauer & Pankratz 1992).

An experiment with a larger sample size and no pre-treatment might compensate for the variability of behavior and reveal significant behavioral effects of 1 h of feeding on foliage dipped in cryIIIA. Because 1 h treatment did not cause significant behavior differences in this experiment, larvae were lumped into four groups for analysis of the effects of 4 h leaf treatment; they were grouped according to their strain and the 4 h leaf treatment they experienced. The total sample sizes for the seven repetitions of the experiment over time were: 34 cryIIIA-resistant larvae on

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cryIIIA-treated leaves, 40 cryIIIA-resistant larvae on untreated leaves, 41 cryIIIA-susceptible larvae on cryIIIA-treated leaves, and 40 cryIIIA-susceptible larvae on untreated leaves.

Spending 4 h on cryIIIA-treated foliage affected the behavior of cryIIIA-susceptible larvae, but not cryIIIA-resistant larvae (Table 1 and Figure 2). CryIIIA-susceptible larvae that spent 4 h on cryIIIA-treated leaves fed less (Table 1 and Figure 2; $P = 0.0001$, $F = 65.3$) and rested more (Table 1; $P = 0.0001$, $F = 42.0$) than cryIIIA-susceptible larvae on untreated foliage. In contrast, cryIIIA-resistant larvae that spent 4 h on cryIIIA-treated leaves did not differ significantly from those on untreated leaves in percentage of time spent feeding (Table 1 and Figure 2; $P = 0.26$, $F = 0.13$) or resting (Table 1; $P = 0.44$, $F = 0.6$). Both within and between strains, 4 h leaf treatment did not affect time spent walking, so larvae did not become immobilized (Table 1; $P = 0.40$, $F = 1.4$). Four h leaf treatment did not affect time spent on top of the leaf (Figure 3; $P = 0.25$, $F = 1.4$). Thus, cryIIIA did not markedly alter the positioning preference of larvae, and even susceptible larvae on cryIIIA-treated foliage were able to move from the upper leaf surface on which they were placed. Time spent off the leaf was also not significantly different between treatment groups ($P = 0.06$; $F = 3.5$). More than 75% of all larvae remained on the leaf throughout the observation period. The mean time spent off the leaf was 4.4 minutes, or 7.3% of the observation time.

On cryIIIA-treated leaves, cryIIIA-susceptible larvae spent more time resting (Table 1; $P < 0.05$, $F = 5.8$) and less time feeding

Table 1. Behavior of cryIIIA-resistant and cryIIIA-susceptible larvae on cryIIIA-treated and untreated foliage.

		Mean percentage of 1 h spent on behavior ± 95% CI	
	Larval behavior	CryIIIA-resistant	CryIIIA-susceptible
CryIIIA-treated foliage	feed	11.3 ± 3.6	1.8 ± 1.1
	rest	73.0 ± 5.0	87.1 ± 3.6
	walk	11.2 ± 3.8	6.0 ± 1.6
Untreated foliage	feed	15.2 ± 2.9	27.6 ± 3.8
	rest	67.5 ± 4.6	48.9 ± 4.9
	walk	14.4 ± 3.3	12.9 ± 2.8

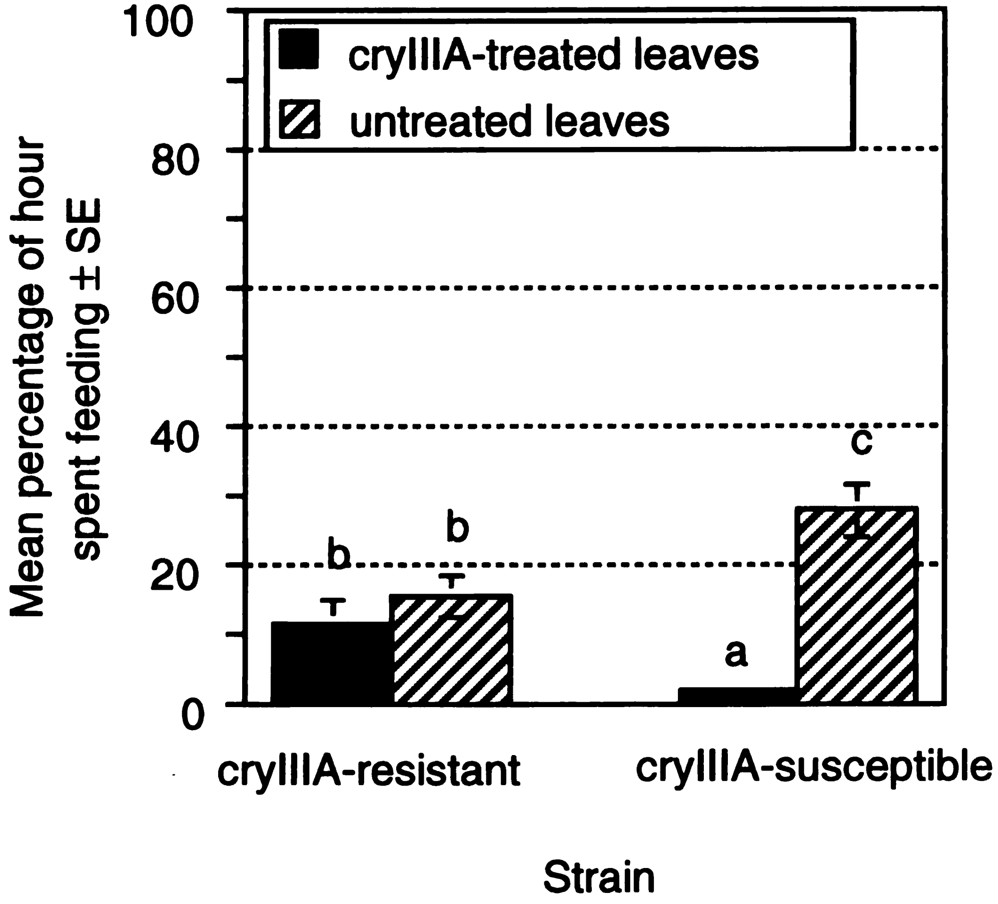
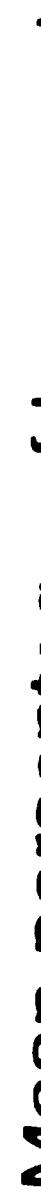


Figure 2. Mean percentage of 1 h that cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle second instars spent feeding on leaves treated with cryIIIA vs. untreated leaves. Means with different letters are significantly different (ANOVA and Fisher's protected LSD, significance at $P < 0.05$).



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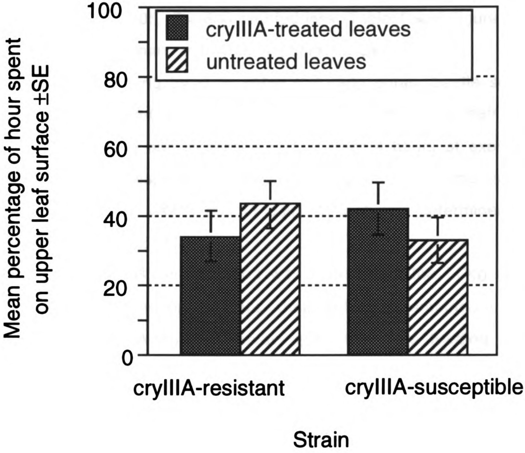

Figure 3. Mean percentage of 1 h that cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle second instars spent on the upper surfaces of leaves treated with cryIIIA vs. untreated leaves. No significant differences (ANOVA).

(Table 1 and Figure 2; *P* < 0.005, *F* = 11.4) than cryIIIA-resistant larvae. On untreated leaves, cryIIIA-resistant larvae rested more (Table 1; *P* < 0.005, *F* = 8.4) and fed less (Table 1 and Figure 2; *P* < 0.05, *F* = 6.9) than cryIIIA-susceptible larvae.

Thus, feeding by susceptible Colorado potato beetle second instars is inhibited within 4 h by cryIIIA at 100 times their LC_{50}. Reduced feeding and increased resting after 4 hours of exposure were probably responses to midgut disruption, since this is longer than the time to visible midgut damage that was determined by Bauer & Pankratz (1992).

Though this concentration was higher than the LC_{50} of the cryIIIA-resistant second instars, their behavior remained unchanged after 4 h. CryIIIA-resistant larvae spent less time feeding on untreated foliage than cryIIIA-susceptible larvae in this study. Since Colorado potato beetle larvae apparently cannot detect cryIII before toxic effects occur, cryIII-resistant larvae might also initially ingest cryIII-treated foliage at a slower rate than susceptible larvae, until the toxic effect of cryIII causes susceptible larvae to decrease feeding. However, this is not likely to enhance the resistant phenotype significantly.

Behavior Experiment with *B. thuringiensis*-transgenic Leaves. Unlike formulated cryIIIA applied to foliage in the previous study, *B. thuringiensis*-transgenic foliage reduced feeding by cryIIIA-resistant larvae as much as feeding by cryIIIA-susceptible larvae (Table 2 and Figure 4; *P* = 0.70, *F* = 0.15). This difference is probably because the *B. thuringiensis*-transgenic foliage contained a

Table 2. Behavior of cryIIIA-resistant and cryIIIA-susceptible larvae on cryIIIA-transgenic and untransformed foliage.

		Mean percentage of 1 h spent on behavior ± 95% CI	
	Larval behavior	CryIIIA-resistant	CryIIIA-susceptible
CryIIIA-transgenic foliage	feed	2.2 ± 1.3	0.0 ± 0.0
	rest	87.8 ± 5.7	90.8 ± 5.3
	walk	11.4 ± 5.4	9.2 ± 5.3
Untransformed foliage	feed	18.1 ± 4.3	20.6 ± 5.1
	rest	71.7 ± 6.4	67.6 ± 6.9
	walk	8.0 ± 3.1	12.0 ± 5.4

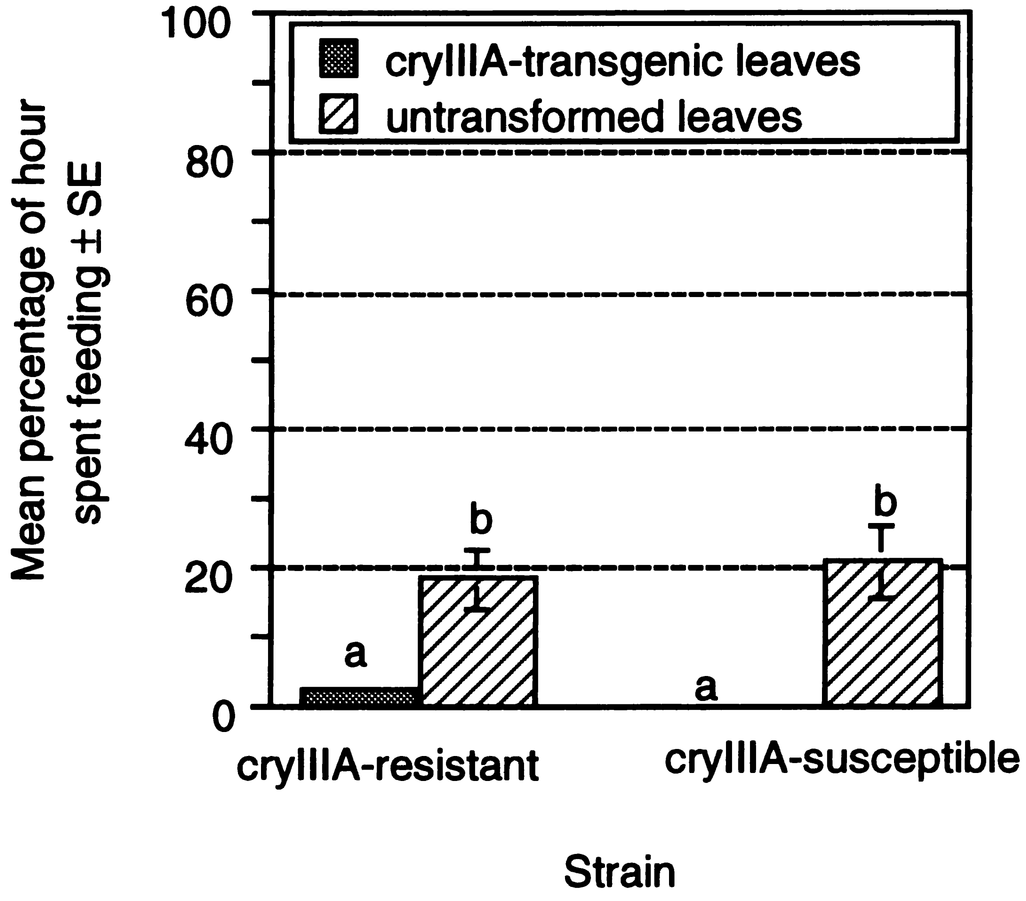

Figure 4. Mean percentage of 1 h that cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle second instars spent feeding on cryIIIA-transgenic leaves vs. untransformed leaves. Means with different letters are significantly different (ANOVA and Fisher's protected LSD, significance at $P < 0.05$).

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higher concentration of *B. thuringiensis* than the cryIIIA-dipped foliage. Within each strain, larvae spent more time feeding on untransformed foliage than on *B. thuringiensis*-transgenic foliage (Table 2 and Figure 4; *P* = 0.0001, *F* = 32.7).

There was no significant difference between larval strains in the amount of time spent resting on *B. thuringiensis*-transgenic and conventional foliage (Table 2; *P* = 0.91, *F* = 0.12). Both strains spent more time resting on the *B. thuringiensis*-transgenic foliage than on conventional foliage (Table 2; *P* = 0.0003, *F* = 14.0).

Larvae spent similar amounts of time walking, regardless of larval strain (Table 2; *P* = 0.97, *F* = 0.001) or foliage type (Table 2 ; *P* = 0.64, *F* = 0.22). Proportion of time spent on the upper surface of the leaf was also independent of larval strain (Figure 5; *P* = 0.84, *F* = 0.04) and foliage type (Figure 5; *P* = 0.50, *F* = 0.45). In contrast, J. Murphy and J. Wyman (University of Wisconsin, unpublished data) found that the frequency with which second instars are found on lower leaf surfaces is significantly greater on cryIIIA transgenic foliage than on untreated potato foliage. Little time was spent off leaflets during the observation period, and time spent off the leaf was independent of strain (*P* = 0.32, *F* = 0.99) and treatment (*P* =0.22, *F* = 1.52).

This study shows that 90-fold *B. thuringiensis*-resistant larvae respond to the high concentration of endotoxin in transgenic plants in the same way that cryIIIA-susceptible larvae respond to a relatively lower concentration of formulated endotoxin. Within 4 h, HybriTech's *B. thuringiensis*-transgenic foliage is capable of

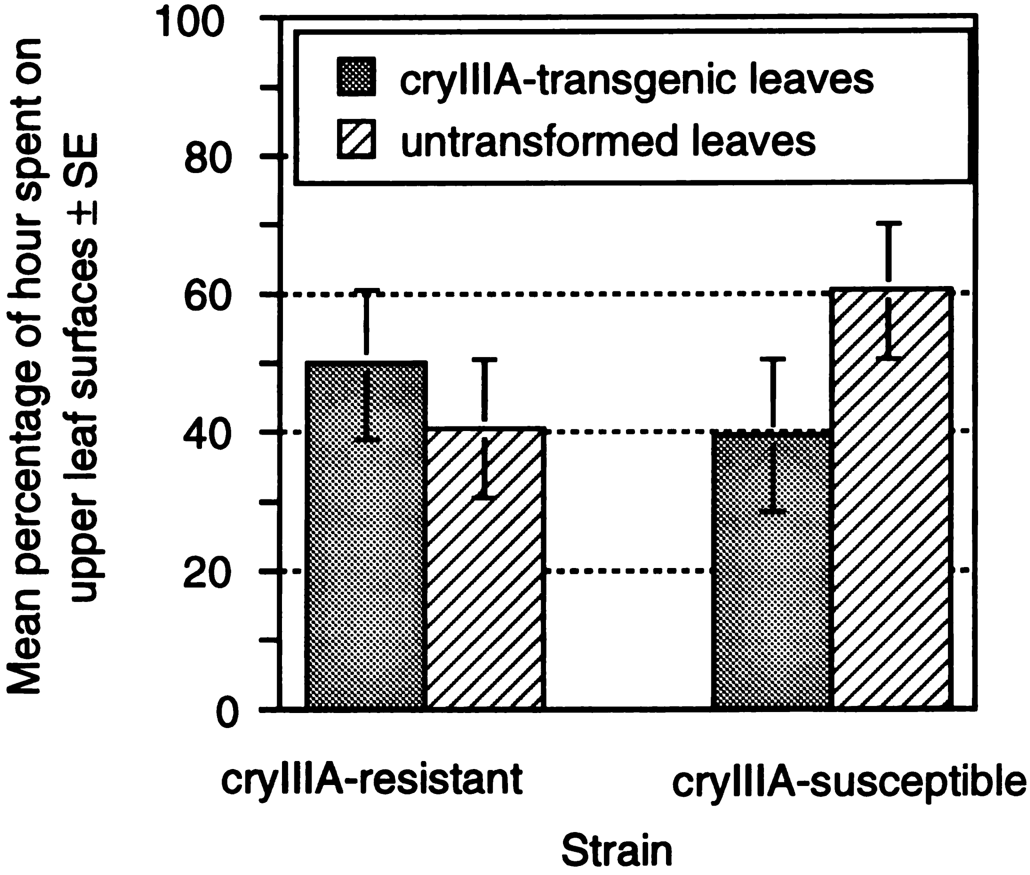

Figure 5. Mean percentage of 1 h that cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle second instars spent on the upper surfaces cryIIIA-transgenic leaves vs. untransformed leaves. No significant differences (ANOVA).

virtually eliminating feeding by both 90-fold cryIIIA-resistant Colorado potato beetle larvae and cryIIIA-susceptible larvae.

Development of CryIIIA-resistant and CryIIIA-Susceptible Larvae. All three cryIIIA-susceptible egg masses hatched in 4 d, whereas the two cryIIIA-resistant egg masses took 4.5 to 5.5 d (Figure 6). This lag is probably too slight to have any practical significance. A larger sample size would be necessary before any conclusions could be drawn about the duration of the egg stage.

Forty cryIIIA-susceptible larvae and 44 cryIIIA-resistant larvae were included in this analysis of instar duration. The first instar lasted 2.5 d for cryIIIA-susceptible larvae and 2 d for cryIIIA-resistant larvae (Figure 6; $P = 0.05$). A shorter first instar could be an advantage to larvae in fields treated with *B. thuringiensis* because the first instar is very susceptible to cryIIIA.

For the majority of larvae in both strains, the second stadium lasted 1.5 d and the third stadium lasted 2.5 d. The mean duration of the second stadium was statistically shorter for susceptible larvae than resistant larvae (Figure 6; duration [d] ± 95% CI: cryIIIA-susceptible, 1.55 ± 0.07; cryIIIA-resistant, 1.76 ± 0.13). In reality, I did not measure stadium duration with this degree of precision; I estimated to the nearest half day. The mean duration of the third stadium was not significantly different for the two strains (Figure 6; duration [d] ± 95% CI: cryIIIA-susceptible, 2.55 ± 0.04; cryIIIA-resistant, 2.41 ± 0.12). There was no significant difference between strains in the amount of time required to complete stadia one

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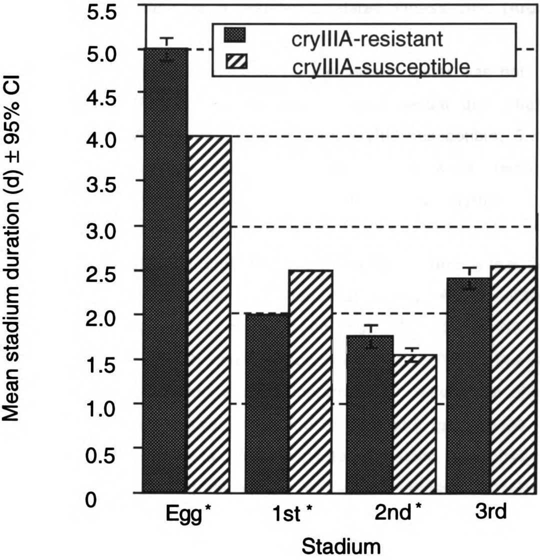

Figure 6. Mean stadium duration for cryIIIA-resistant and cryIIIA-susceptible egg through 3rd instar. *= significantly different between cryIIIA-resistant and cryIIIA-susceptible.

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through three ($P = 0.05$, df = 1, $X^2 = 0.01$). In contrast, azinphosmethyl-resistant Colorado potato beetles have longer developmental time as a resistance-related fitness cost (Argentine et al. 1989).

The mean weight gain during the second instar was not significantly different between strains (mean weight gain [mg] ± 95% CI: cryIIIA-resistant, 5.46 ± 0.49; cryIIIA-susceptible, 5.70 ± 0.43); neither was the mean weight gain over the week (mean weight gain [mg] ± 95% CI: cryIIIA-resistant, 143.2 ± 8.2; cryIIIA-susceptible, 137.9 ± 8.0).

Mean weight of cryIIIA-resistant second instars within 24 h of molting was significantly less than that of cryIIIA-susceptible second instars (Figure 7; mean weight [mg] ± 95% CI: cryIIIA-resistant, 2.65 ± 0.09; cryIIIA-susceptible, 3.43 ± 0.16). The smaller size of cryIIIA-resistant second instars might reflect the shorter first instar, which allowed less time to feed and gain weight before the molt to second instar. A slower rate of feeding might have also contributed to the lighter weight of cryIIIA-resistant second instars compared to cryIIIA-susceptible instars.

Smaller size of second instars might have been a physiological cost of cryIIIA resistance. However, smaller size did not lead to higher mortality; five or six larvae of each strain died. Moreover, the relative growth rate of cryIIIA-resistant larvae was significantly greater than that of cryIIIA-susceptible larvae during the period from early-middle third instar on day three to early-middle fourth

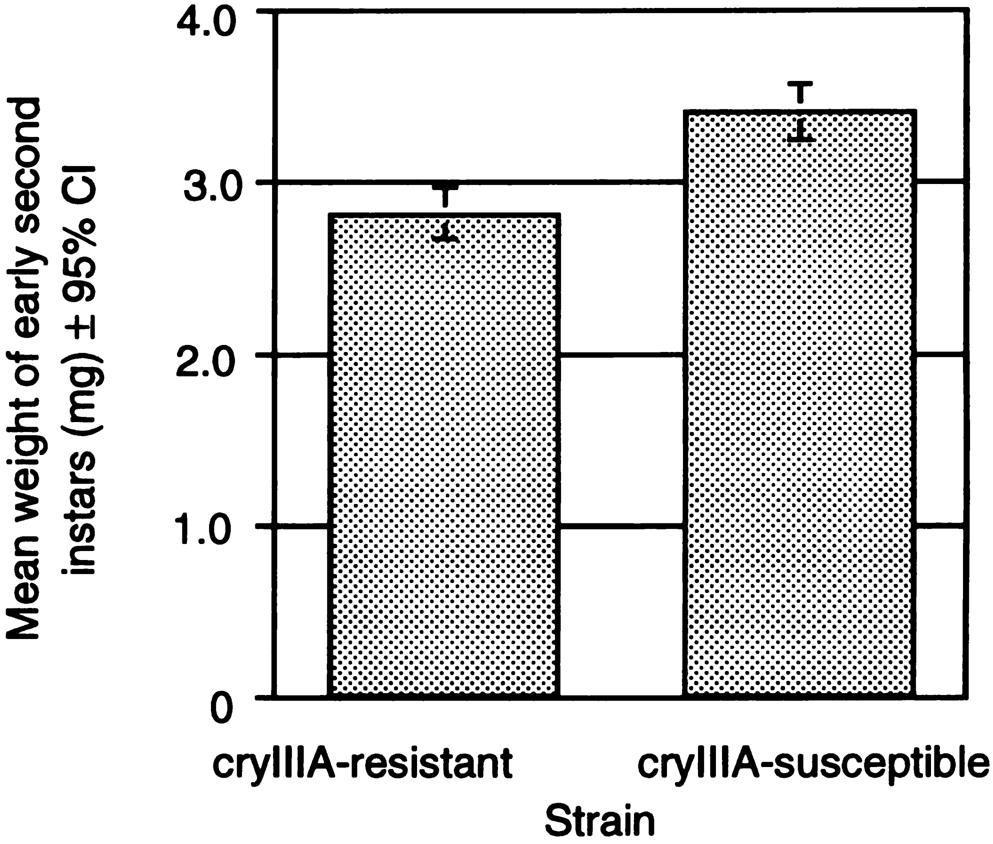

Figure 7. Mean weight of cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle second instars, less than 24 h after molting. Means are significantly different.

instar on day eight (Figure 8; mean rate (%) ± 95% CI: cryIIIA-resistant, 30.6 ± 0.5; cryIIIA-susceptible larvae, 28.6 ± 0.7). As a result, mean weights of cryIIIA-resistant and cryIIIA-susceptible third instars were not significantly different (Figure 9; mean weight [mg] ± 95% CI: cryIIIA-resistant, 8.11 ± 0.54; cryIIIA-susceptible, 9.13 ± 0.50). The mean weights of fourth instars of each strain were also not different on the final day of the experiment (mean weight [mg] ± 95% CI: cryIIIA-resistant, 145.9 ± 8.2; cryIIIA-susceptible, 141.3 ± 8.0).

Thus, cryIIIA-resistant Colorado potato beetles showed a temporary lag in larval size, but ultimately had no developmental disadvantages of practical significance. Under less ideal conditions in the field, the smaller size of cryIIIA-resistant early instars might be a more significant survival disadvantage. If this is the case, then cryIII-resistant larvae in the field might be more susceptible to non-*B. thuringiensis* control tactics than cryIII-susceptible larvae. In that case, cryIII resistance genes would be selectively removed from the Colorado potato beetle population when such tactics were alternated or combined with *B. thuringiensis*. The ability of second and third instars of each strain to survive and grow under stress should be compared.

In addition to removing *B. thuringiensis*-resistance genes from the Colorado potato beetle population, further selection for resistance should be reduced. As previously mentioned, selection for *B. thuringiensis* resistance can be minimized by applying *B. thuringiensis* sprays only when necessary, and providing refugia

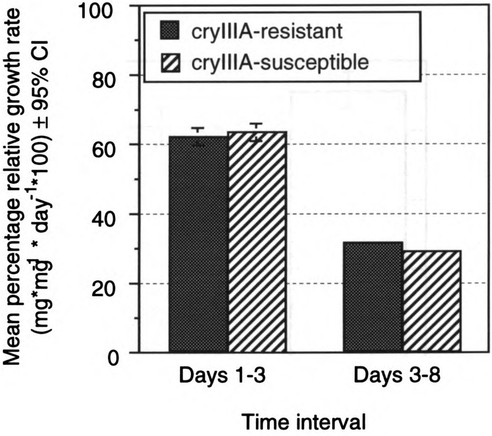

Figure 8. Mean percentage relative growth rate for cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle larvae. Day 1= early second instar, 3= middle third instar, 8= middle fourth instar. Means significantly different in the second interval only.

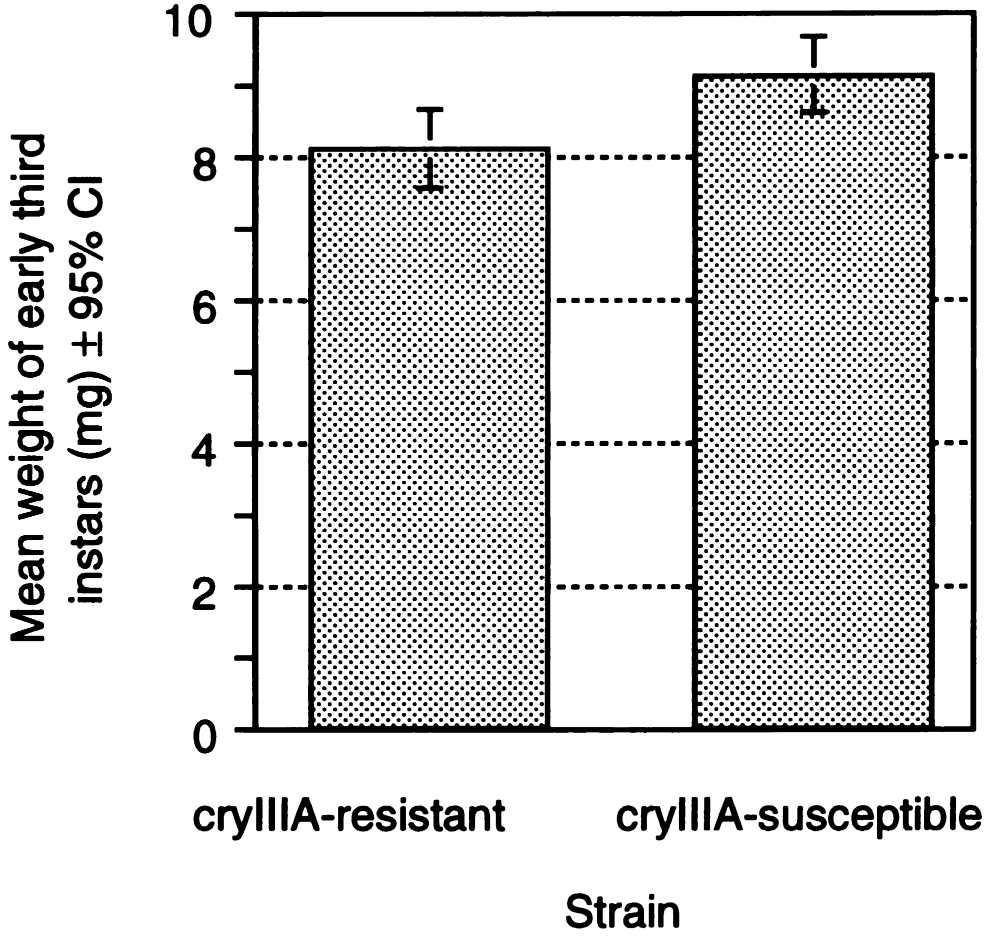

Figure 9. Mean weight of cryIIIA-resistant and cryIIIA-susceptible Colorado potato beetle third instars, less than 24 h after molting. Means are not significantly different.

among *B. thuringiensis*-transgenic plants. The amount of *B. thuringiensis* used could also be reduced by selectively directing *B. thuringiensis* onto portions of the plant where small larvae are most abundant. The next chapter focuses the location of Colorado potato beetle larvae on plants in the field.

CHAPTER TWO

Position of Colorado potato beetle egg masses and larvae on potato plants in the field

Introduction

The following study describes the height at which Colorado potato beetle eggs and larvae were found on potato plants in the field. A few previous studies have calculated the degree of aggregation of different life stages and determined optimal sampling methods and sample sizes for large larvae and adults (Zehnder et al. 1990; Zehnder & Linduska 1988; Martel et al. 1986; Nyrop & Wright 1985).

Position of Egg Masses on Plants. Proper timing of *B. thuringiensis* sprays against Colorado potato beetle is determined by locating a sample of egg masses and determining what percent have hatched. *B. thuringiensis* should be applied within nine days of peak egg mass hatch, when approximately 30 to 60% of the egg masses in the field have hatched (Zehnder et al. 1992). When rainfall occurs, *B. thuringiensis* spray timing must be even more precise to be effective. Knowledge of the distribution of egg masses on plants could improve the accuracy of egg hatch estimates and therefore of *B. thuringiensis* spray timing.

Colorado potato beetle egg masses are consistently laid on the undersides of leaves. Zehnder & Linduska (1988) determined that egg masses are less aggregated than Colorado potato beetle larvae and adults. Colorado potato beetle egg masses are commonly thought to occur mainly on lower leaves.

Position of Larvae on Plants. After eggs hatch, larvae consume the chorion and begin feeding on leaf tissue near the egg mass; larger larvae feed on leaf edges (Hsiao 1988). First and second instars are more aggregated than third and fourth instars (Zehnder & Linduska 1988). Aggregation of small larvae on potato is probably due to the fact that larvae in a clump hatched from the same egg mass and have not yet spread out over the plant. Feeding in a group helps small larvae chew through the tough leaves of some host plant species (Hsiao 1988); however, potato leaf toughness is probably not a major obstacle for early Colorado potato beetle instars.

It is possible that larval developmental rate and survival are affected by position on potato plants. Abiotic environment, natural enemies, and nutritional, chemical, and physical leaf characteristics could all influence larval survival and larval position on the plant. The specific location most favorable for growth and survival could differ between instars. Even if position on the plant has little effect on larval survival, different instars might be found at different, predictable locations on the plant.

For instance, the majority of large larvae (54 to 72%) are found on the upper leaves of potato plants (Zehnder et al. 1990). In contrast, evidence suggests that first and second instars are on the lower plant; *Bacillus thuringiensis* sprays are most effective when sprayed with drop nozzles, which direct spray upward from the base of the plant (Zehnder & Speese 1991). The vertical distribution of different Colorado potato beetle instars on plants has not been quantitatively compared.

Though small and large larvae may generally be found at certain positions on the plant, the actual location of larvae on plants changes in response to temperature (May 1981). Larval growth rate peaks at about 30° C and drops off at higher temperatures (May 1981). At 35° C, the majority of Colorado potato beetle larvae are located in shady interiors of potato plants; below 30° C, fewer than one-third of all larvae are in the shade, and the majority are on upper leaf surfaces. May (1981) also found that the percentage of Colorado potato beetle larvae feeding in the field increases from 20% at 23° C to 60% at 38° C.

Objectives. The first objective of this study was to determine the relative height at which Colorado potato beetle egg masses were laid on individual plants of different sizes. Our hypothesis from informal observations was that egg masses are laid on the undersides of lower leaves of potato plants.

A second goal of this study was to document the relative height at which first through fourth instar Colorado potato beetles were found on potato plants in the field, and the plant parts on which they were found. Another objective was to monitor changes in the position and behavior of third and fourth instars during a single day in the field. Finally, observations were made on the position of late instars during a light rain.

Materials and Methods

All field studies were performed at the Michigan State University Montcalm Research Farm in Entrican, Michigan. "Snowdon" potatoes were planted on 6 May in 3 m border plots on both ends of a 130 m potato field. Four plots of three rows each were planted with 0.3 m between plants and 0.9 m between rows. The four plots were separated by 1.8 m of bare ground.

Height of Egg Masses. Potato plants (56) were flagged for studies of the heights of egg masses above the ground and of larval behavior. On 12 June, 41 plants were flagged, and on 14 June, 15 additional plants were flagged. One egg mass was randomly selected on each flagged plant (a random number table was used). The chosen egg mass was flagged with a piece of red yarn, and all other egg masses were removed from the flagged plant and the two adjacent plants. The heights of the chosen plants and egg masses were recorded. For convenience, the plants flagged on 12 June plus the 15 plants flagged on 14 June are collectively referred to as the 12 June sample.

On 14 June, 20 additional plants were sampled; plant heights and the heights of all egg masses were measured. On 19 June, plant heights and the heights of 51 egg masses on another five plants were recorded.

The correlation between egg mass height and plant height was plotted, Pearson's sample and Spearman's rank correlation coefficients were calculated, and the equation of the least squares line was calculated. The height of each egg mass was expressed as a percentage of the height of its host plant; ANOVA was used to compare plant heights and relative and actual egg mass heights between samples. The frequency of three ranges of egg mass height as a percentage of plant height were compared between dates by Chi-squared analysis: 21- 40%, 41- 60%, and 61- 100%.

Behavior of Different Instars in the Field. Of the 56 flagged egg masses, 31 remained on 19 June. These 31 egg masses provided larvae for this study. Most egg masses hatched on 20 June (cool minimum daily temperatures [12° C] may have prolonged the egg stage). Each flagged plant was examined on 21, 23, 26, and 29 June. Height and instar were recorded for every larva observed on each plant. The plant parts on which larvae were found were also recorded: stem, leaf axil, growing tip, or leaf. The lowest, oldest leaves on the plant have fewer leaflets than the newer, upper leaves. For example, seven-leaflet leaves are younger and higher on the plant than five-leaflet leaves.

ANOVA and Fisher's protected LSD were used to compare the heights of different instars on the plants. Chi-squared tests were used to compare the abundance of instars on different plant parts.

Behavior of Third and Fourth Instars throughout the Day. On 5 July, the behavior of third and fourth instars was recorded in the morning, at midday, and in the late afternoon. Fourth instars were more common than third instars. Morning observations were from 6:30 am to 9:00 am (15 plants , 293 larvae). The temperature was 23.5° C at 6:30 am, with 50% cloud cover and no wind. At 11:00

am, four of the plants were re-examined (n = 66 larvae). At that time, the temperature was 29° C, cloud cover was less than 10%, and the wind was approximately 30 kph. At 4:00 pm, the same four plants were examined again (n = 73 larvae). The wind and temperature were the same, but the cloud cover had increased to 40%.

Larval behavior was recorded as feeding, resting, or walking. Larval position on the upper or lower surfaces of leaves was noted, as well as whether larvae were on the top of the plant or on the middle to lower plant. Chi-squared tests were used to compare location and behavior of larvae between observation times.

Behavior of Third and Fourth Instars on a Rainy Day. On 30 June, six plants were examined during trace rainfall (<0.5 cm/hr) at approximately 10 am. Third and fourth instars were most common on this date. Plant heights and the height of each larva above the plant base was noted, as well as whether the larva was on a leaf axil, upper leaf or lower leaf. Behavior and position of third and fourth instars during rainfall was analyzed with a Chi-squared test.

Results and Discussion

Height of Egg Masses. Plants were significantly taller on each successive sampling date (Figure 10a, *P* = 0.0001, *F* = 202.5). Mean plant heights were as follows: 12 June, 11.7 cm (n = 56); 14 June, 16.7 cm (n = 20); and 19 June, 24.8 cm (n = 5). Egg masses were significantly higher above the soil on 19 June than on 12 June and 14 June (Figure 10b, *P* = 0.0001, *F* = 29.2; n = 51, 56, and 106 egg masses, respectively). Plant height is a weak predictor of egg mass height (Figure 11; n = 213, *r* = 0.44).

On 14 June, egg masses were most abundant on the lower plant, as anticipated (Fig. 12). The percentage of egg masses at 21 to 40% of plant height was significantly greater on 14 June than on 19 June. However, on 12 and 19 June, egg masses were more evenly distributed at all heights on the plant. The percentage of egg masses at 41 to 60% of plant height was greater on 12 June than on 14 June, and the percentage of egg masses at 61 to 100% of plant height was greater on both 12 June and 19 June than on 14 June. Egg masses were at a significantly lower mean percentage of total plant height on 14 June than on 12 June or 19 June (Figure 13; mean ± 95% CI: 29.8 ± 3.8, 51.0 ± 6.1, and 42.8 ± 7.2%, respectively). Mean percentage of plant height for egg masses including all dates was 39%.

Thus, egg masses were generally on the lower leaves of potato plants, particularly on 14 June. Humidity, light, and temperature can affect oviposition (Hurst 1975); these factors may be optimal on lower to middle leaves. However, egg masses were also common on upper and middle potato leaves, especially on 12 and 19 June. Selectivity of females for oviposition sites may decline as egg mass crowding increases; this might explain why egg masses within potato fields sometimes occur on inappropriate substrates such as soil and on non-host weeds.

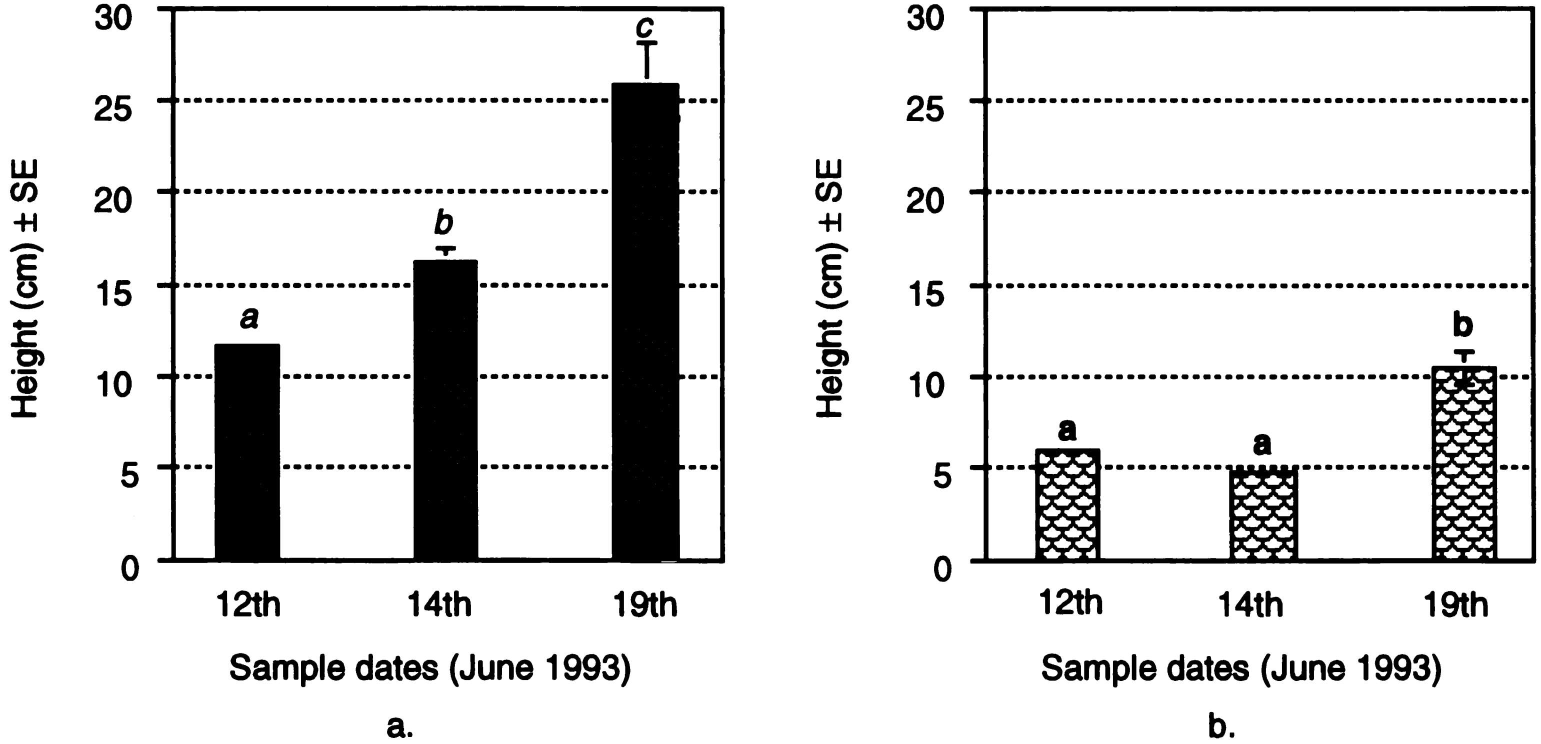

Figure 10. Mean heights (cm) of a. potato plants and b. Colorado potato beetle egg masses on three different sample dates. Within each graph, means with different letters are significantly different (ANOVA and Fisher's protected LSD, $P= 0.0001$).

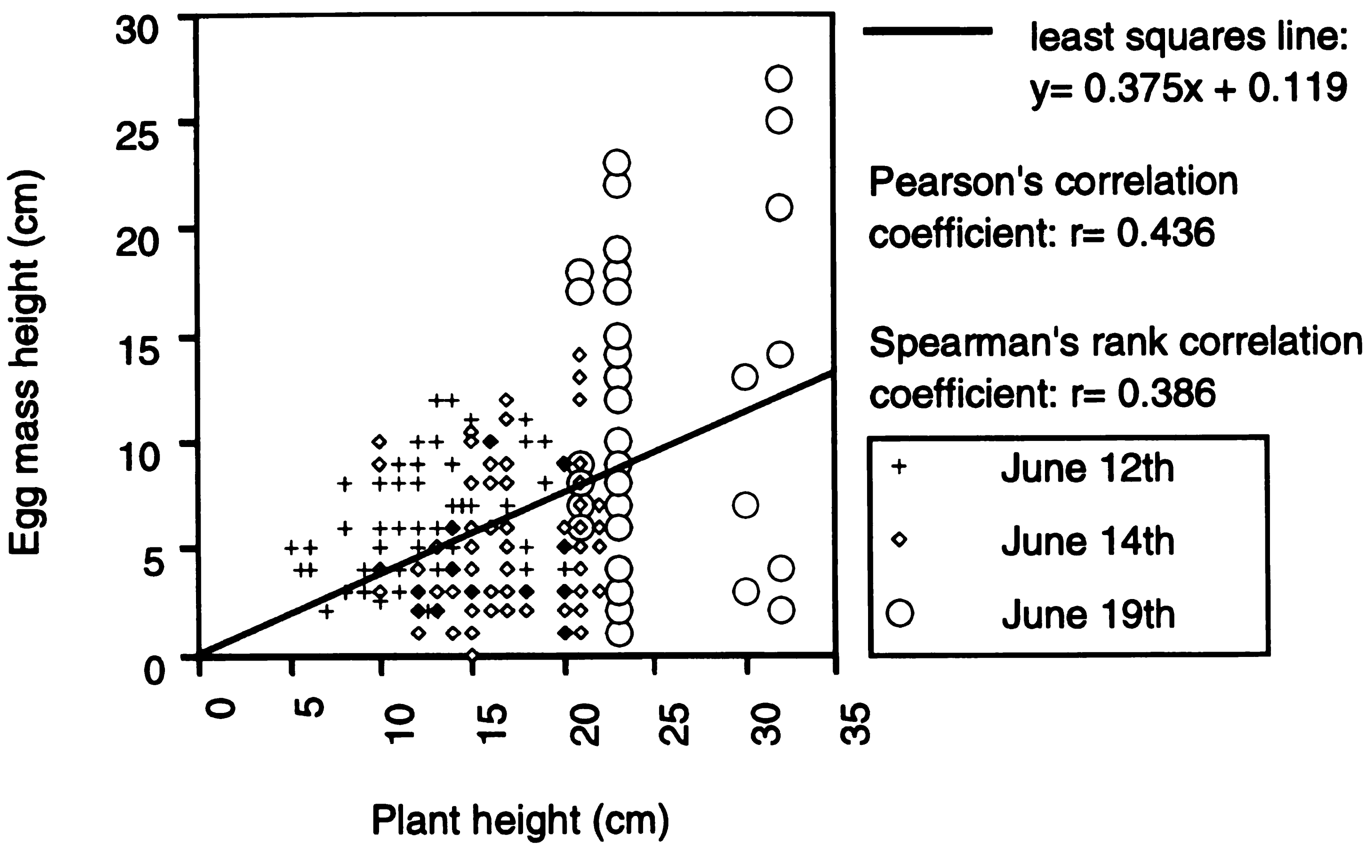

Figure 11. Heights of Colorado potato beetle egg masses and the potato plants on which they were found, on three sample dates.

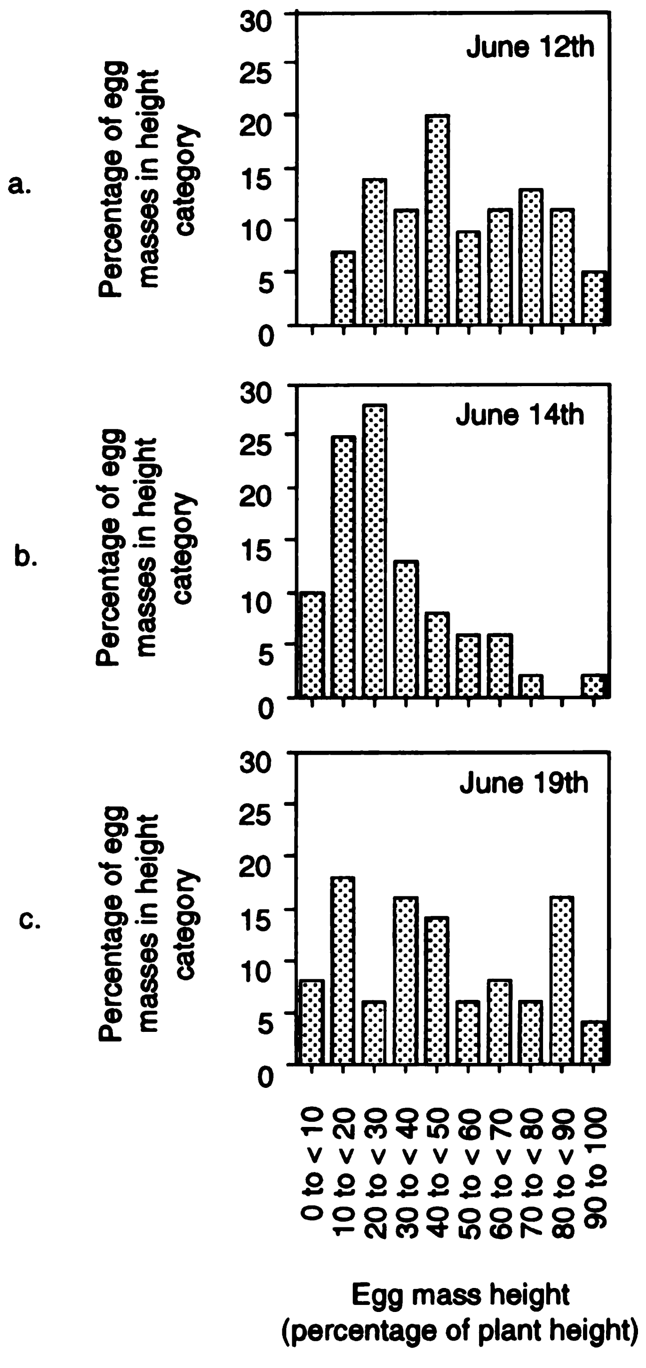

Figure 12. Percentage of Colorado potato beetle egg masses in ten percent plant height categories. a. June 12th (n= 56 egg masses on 56 plants); b. June 14th (n= 106 egg masses on 20 plants); c. June 19th (n= 51 egg masses on 5 plants).

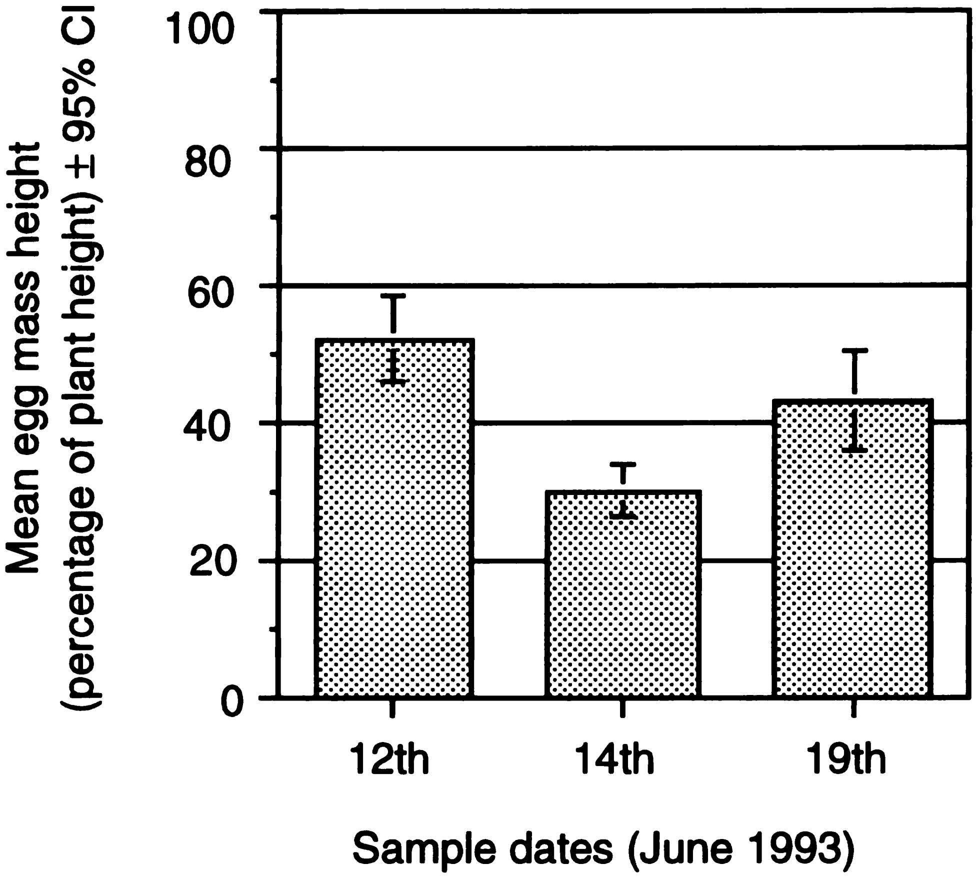

Figure 13. Mean Colorado potato beetle egg mass height expressed as a percent of plant height for three different sample dates. Mean egg mass height for the June 14th sample is significantly lower than the other two means.

Temperature could also indirectly influence egg mass location by affecting the location of adult females. Adult Colorado potato beetles are found almost entirely on the tops of plants prior to daily maximum temperature, most likely because flight muscles are warmed by the sun in this position (Zehnder et al. 1990). As temperatures increase above 25° C, the proportion of adult beetles in the shade increases (May 1981).

Higher temperatures are not likely to have caused the lower mean egg mass height on 14 as compared to 12 and 19 June, because the maximum temperature on 14 June was between the 12 and 19 June maximum temperatures (Figure 14). The 14 June minimum temperature was also intermediate between those of the other two days. The average of the maximum and minimum temperatures was 20.6° C for 14 June versus 18.8° C for both 12 and 19 June, but such a small difference is not likely to have been an appreciable influence.

For future studies of egg mass position, sampling at regular intervals over more of the season would give a more complete picture. Using a standard random method of choosing plants and egg masses, measuring a uniform number (or all) of the egg masses on each plant, and ensuring sufficient sample sizes would yield more reliable results.

Because egg masses can be found at any elevation on the plant, search height does not appear to be critical when scouting egg masses for *B. thuringiensis* application. However, the accuracy of

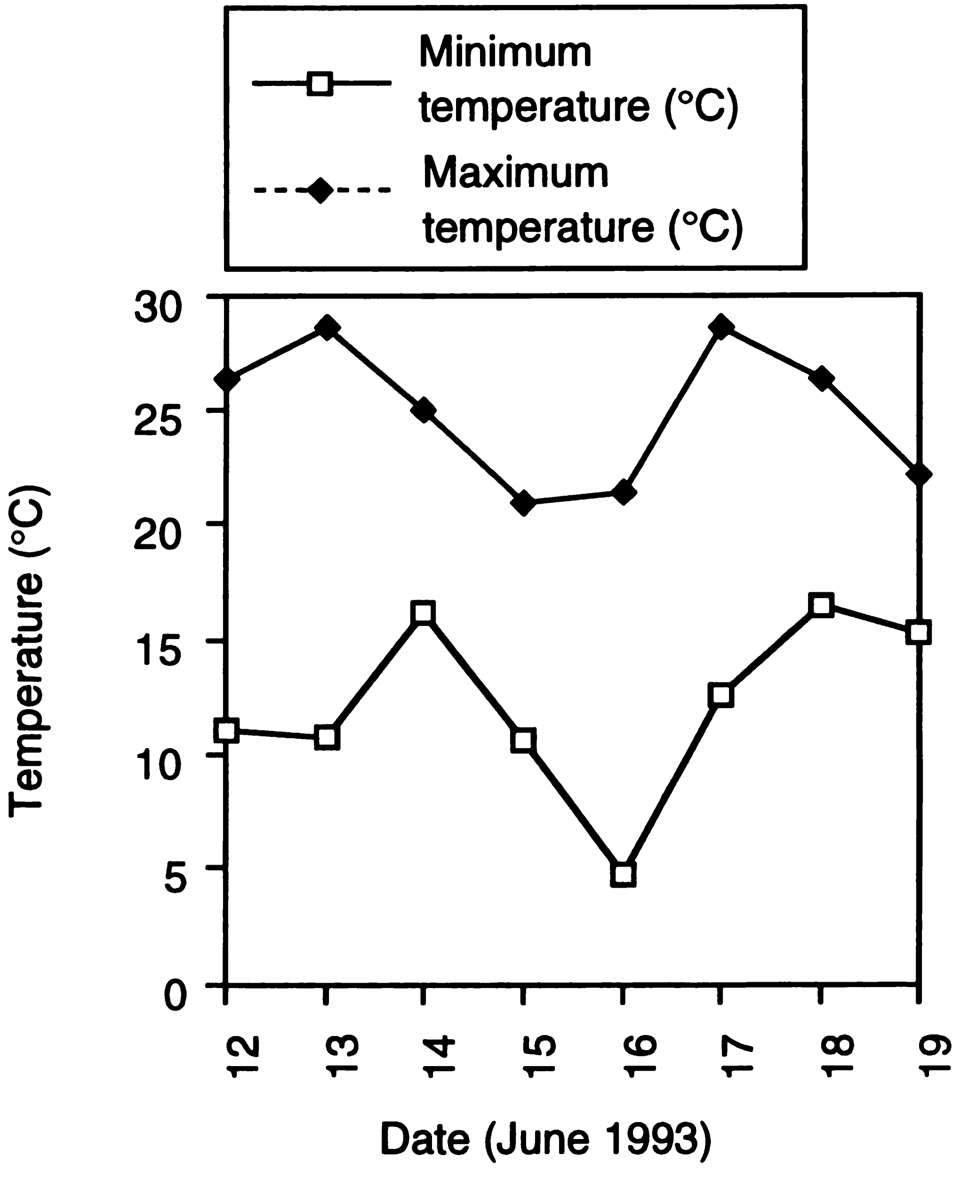

Figure 14. Daily maximum and minimum temperatures for the week during which egg mass heights were sampled. Data are averages from two weather stations, each 15 km from the field site.

percentage hatch estimates could be affected by search height if mean age of egg masses varies with elevation on the plant.

Behavior of Different Instars in the Field. Later instars were higher on the plant. Mean heights of all instars were significantly different (mean heights of 1st through 4th instars ± SE: 8.6 ± 0.3 cm [n = 384], 15.2 ± 0.3 cm [n = 416], 22.9 ± 0.4 cm [n = 478], and 25.1 ± 0.9 cm [n= 61]; $P = 0.0001$, $F = 341$). Larval height expressed as percentage of plant height was different between first, second, and third instars (Figure 15; mean percentage plant height of 1st through 3rd instars ± SE: 36 ± 5 [n = 384], 57 ± 5 [n = 416], and 75 ± 4 [n = 478]; $P = 0.0001$, $F = 268$). The mean percent plant height for first instars matched the overall mean percent plant height of egg masses determined in the above study. Larval height expressed as percentage of plant height was not significantly different between third and fourth instars (Figure 15; mean percentage height of 4th instars ± 95% CI: 79 ± 3 cm [n = 61]; $P = 0.2$, $F = 2.06$).

These results suggest that the previously mentioned effectiveness of drop nozzles for *B. thuringiensis* sprays may be due to low positioning of early instars on potato plants. It is possible that the difference in position between different instars was the result of differences in temperature between the dates and times when they were observed. Temperature should have been carefully tracked during sampling, and sampling of different instars should have been deliberately done at similar temperatures.

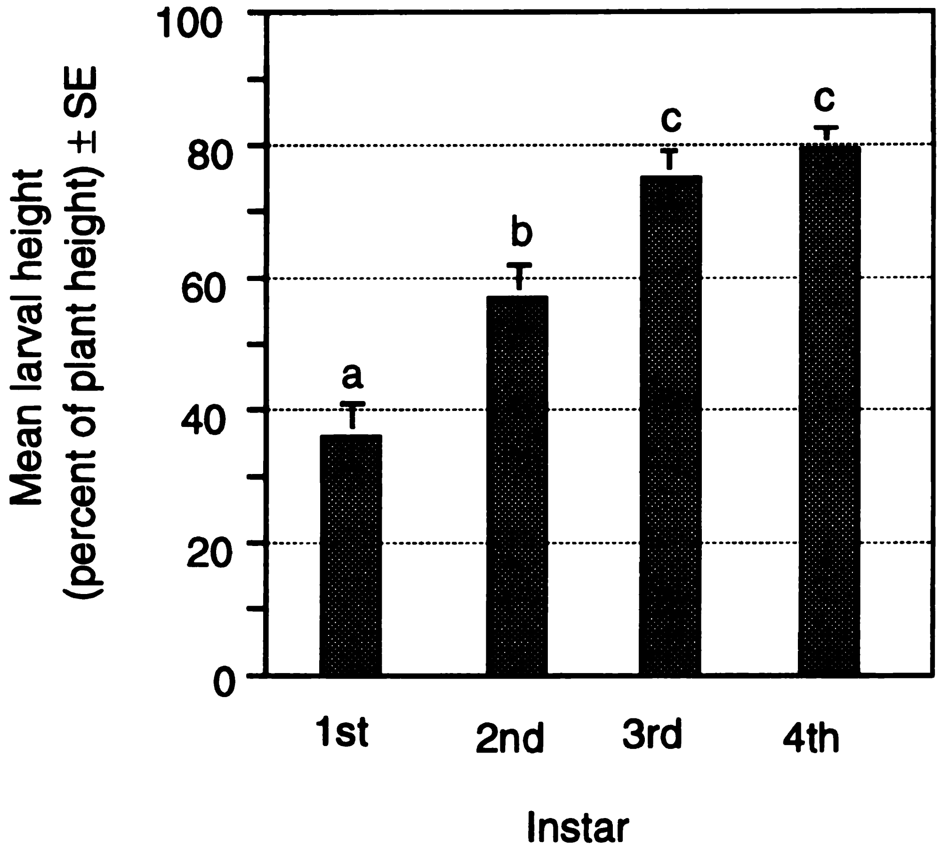

Figure 15. Mean percentage of plant height where 1st, 2nd, 3rd, and 4th instar Colorado potato beetles were found. Means with different letters are significantly different (ANOVA, Fisher's protected LSD, p= 0.0001)

A number of factors could cause small larvae to survive at a higher rate on lower leaves. For instance, the lower plant is sheltered from rain, the major mortality factor for small larvae (Harcourt 1971). Top nozzle pesticide sprayers cover the tops of foliage much more heavily than lower foliage (Grafius et al. 1990). Finally, egg hatch is higher when relative humidity is greater than 80% (Hurst 1975), and small larvae are at a lower risk of desiccation in the more humid lower plant.

In contrast, large larvae are less prone to environmental mortality factors; starvation due to overpopulation is their major source of mortality (Harcourt 1971). Large larvae can maintain higher body temperatures and thus develop more quickly when exposed to the sun on the upper plant (May 1981). This, in turn, is an advantage because it narrows the window of vulnerability to disease, insecticides, starvation, and other mortality agents. Rapid development also allows more generations per season. Light and gravity could be proximate cues used by larvae to position themselves in the appropriate locations.

First instars were found most commonly in leaf axils (Figure 16; n = same as above). Second instars were found in equal abundance in leaf axils and on the growing tips of stems. Hiding in leaf axils may protect small larvae from rain, wind, and natural enemies. The importance of natural enemies as mortality factors is demonstrated by Groden (1989) and Cappaert et al. (1991). Early instars might periodically leave the leaf axils to feed on larger leaflets. Third and fourth instars were found most commonly on leaves with seven or

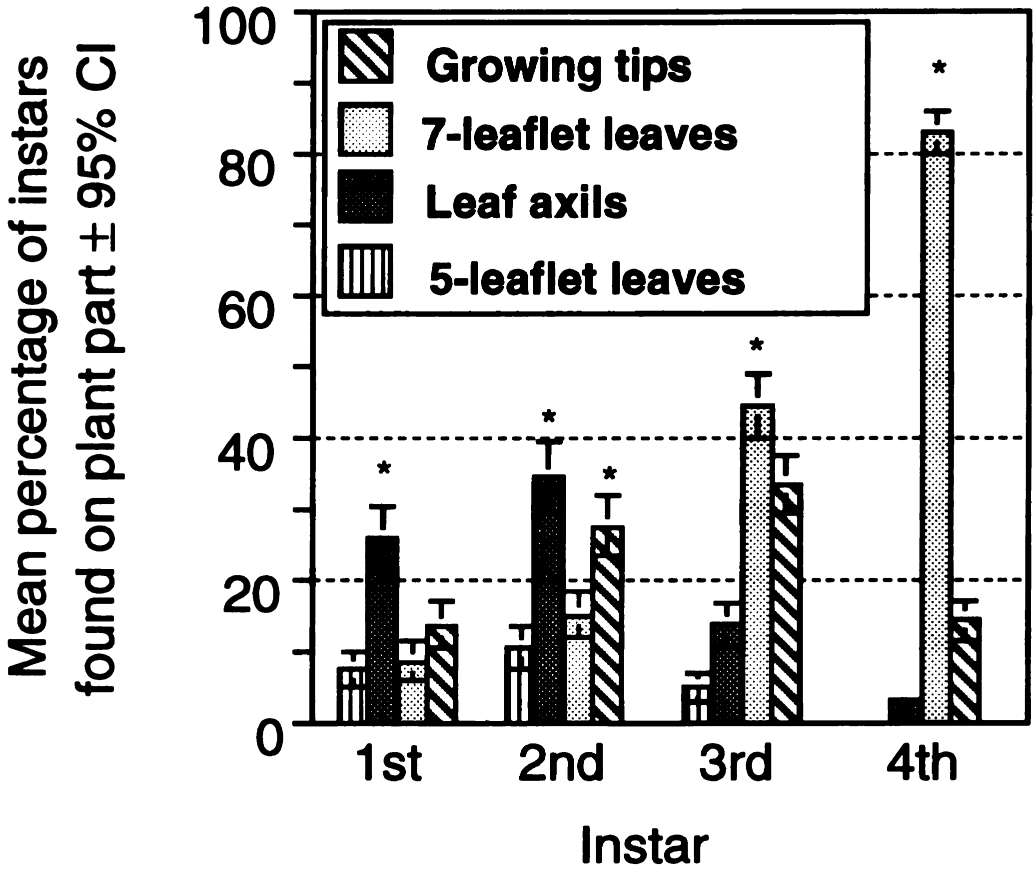

Figure 16. Mean percentage of 1st, 2nd, 3rd, and 4th instar Colorado potato beetles found on potato growing tips, 7-leaflet leaves, leaf axils, and 5-leaflet leaves. Within each instar, the plant part(s) on which the most larvae were found is starred.

more leaflets (Figure 16); this corresponds with their location higher on the plant.

Plant factors that may affect small larvae. Potato leaf toughness factors may affect behavior, development, or location of early Colorado potato beetle instars. The frass of fourth instars contains two such structures: trichomes and lignified spiral fibers from the walls of xylem vessels (Chin 1950). The maximum size of leaf fragments in the frass of third and fourth instars is twice that of first and second instars (Chin 1950). Thus, large larvae might be able to eat whole trichomes or lengths of xylem fiber which pose a feeding obstacle for first and second instars. In addition, larger larvae probably have greater biting force, and therefore probably bite through tough structural tissues more easily than small larvae. My preliminary observations indicate that fourth instars easily break spiral xylem wall fibers, whereas second instars sometimes struggle for five minutes to break off xylem fibers from a feeding site which are tangled in their mouthparts (see appendix). The relative density of xylem vessels with spiral wall fibers in young versus mature potato leaves is not documented. As previously mentioned, trichomes are more dense on young potato leaves than on mature leaves, and this could contribute to the position of egg masses and first instars on lower leaves.

Leaf nutrient content could affect feeding and positioning of any instar. Sucrose, phospholipid, and certain amino acids stimulate feeding by CPB larvae (Hsiao & Fraenkel 1968). If concentrations of these nutrients change with leaf age, they could cause differential arrestment of larvae on leaves of different ages. In general, oligophagous herbivores like CPB prefer younger host plant leaves, which are usually more nutritious (Cates 1980).

Upper potato leaves have a higher total nitrogen and free amino acid content than lower potato leaves, whereas lower leaves have higher concentrations of nitrate nitrogen than upper leaves (Jansson & Smilowitz 1985a). Leaf nitrogen concentration is positively correlated with abundance of Colorado potato beetle on potato (Jansson & Smilowitz 1986), with mass gain of Colorado potato beetle larvae on potato (Zitzman & May 1989), and with survival, development, and pupal mass of CPB on tomato (Hunt et al. 1992).

However, leaf nitrogen may not be an important determinant of larval success or positioning. Under some environmental conditions, leaf nitrogen is not correlated with CPB larval mass gain (Zitzman & May 1989). The developmental rate of CPB larvae in the field is negatively correlated with foliar nitrogen concentrations of 'Katahdin' potatoes, perhaps due to the contribution of glycoalkaloids and protease inhibitors to foliar nitrogen concentration (Jansson & Smilowitz 1985b). For green peach aphid, another potato pest, some significant relationships between leaf nitrogen levels and population parameters exist. However, leaf age appears to be more important (Jansson et al. 1986); green peach aphids are most abundant on older, lower leaves (Jansson & Smilowitz 1985c).

Potato glycoalkaloids are synthesized at the top of the plant, and the concentration of glycoalkaloid in potato leaves decreases with plant age (Osman 1980). Thus, glycoalkaloid content appears to be another factor that could make lower leaves are suitable for small larvae than upper leaves. However, Colorado potato beetle is resistant to potato glycoalkaloids. In laboratory studies, foliar concentrations of the major potato glycoalkaloids solanine and chaconine do not inhibit feeding or survival of Colorado potato beetles in no-choice situations (Harrison & Mitchell 1988; Deahl et al. 1991; Wierenga & Hollingworth 1992). A five-fold increase in the concentration of solanine in potato leaves does not affect feeding by adult Colorado potato beetles (Harrison & Mitchell 1988).

Trichome density is inversely proportional to leaf area (Pelletier 1990), so feeding by small larvae may be less hindered on the broader lower leaflets.

Behavior of Third and Fourth Instars throughout the Day. As visibility increased at dawn, third and fourth instars were seen on the tops of plants, and some were feeding. Adult Colorado potato beetles are known to consume roughly equal amounts of their daily intake at night and during the day, with increased nocturnal feeding at temperatures exceeding 25° C (Hurst 1971).

Throughout the day, many fourth instars were walking on the soil and burrowing into the ground to pupate. Changes in location and feeding frequency in third and fourth instars occurred during an 8.5 h interval with a 5.5 °C temperature increase and an increasingly strong wind.

At 7:30 am and at midday, the numbers of larvae feeding and resting were not significantly different (Figure 17, n = 293). Several

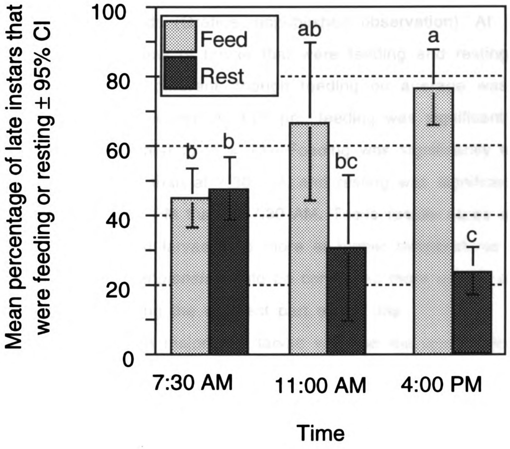

Figure 17. Mean percentage of Colorado potato beetle third and fourth instars that were feeding or resting in the morning, at midday, and in the afternoon. Confidence intervals with matching letters overlap.

larvae were walking and a few were molting. A few were sitting with head and front legs raised; this may have been a reaction to the observer. Similar behavior has been observed in response to attack by tachinid parasitoids (Grafius, unpublished observation). At midday, the proportions of larvae that were feeding and resting were still not significantly different, though feeding on average was twice as common (n = 66). At 4:00 pm, feeding was significantly more common than resting (n = 73). Feeding was significantly more common at 4:00 PM than at 7:30 AM, and resting was significantly less common at 4:00 PM than at 7:30 AM. These results agree with previous findings that larvae feed more at higher temperatures (May 1981). Thus, *B. thuringiensis* would be consumed more quickly on warm days, and during the warmest part of the day.

At 7:30 am, the majority of larvae were on the tops of leaves (Figure 18; n = as above). At 4:00 pm, less than 20% of the larvae were on the tops of leaves; this was a significantly smaller proportion of the larvae than at the other times. This trend of moving to the undersides of leaves as temperatures increase was also was noted by May (1981). Conventional sprayers cover only the upper surfaces of leaves, whereas air-curtain sprayers are able to coat the undersides as well as tops of leaves (Grafius et al. 1990). Therefore, an air curtain sprayer might be more appropriate for applying contact insecticides at warmer temperatures. However, such insecticides normally have long half-lives, so larvae would probably eventually encounter the insecticide on upper surfaces of leaves. Because large larvae consume entire leaves, the leaf surface

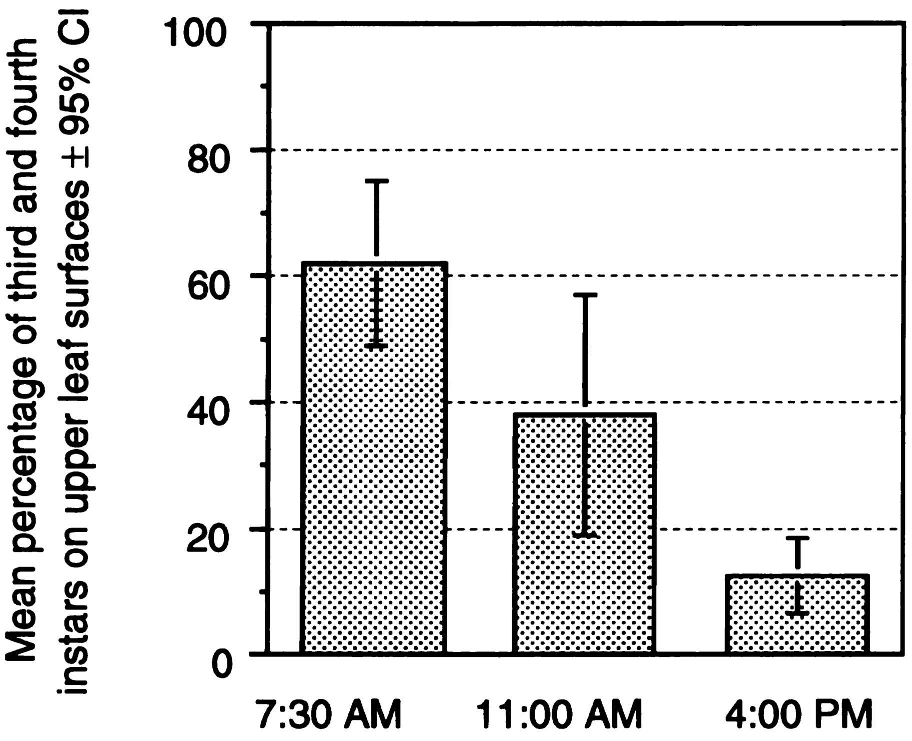

Figure 18. Mean percentage of Colorado potato beetle third and fourth instars that were on upper leaf surfaces in the morning, at midday, and in the afternoon. There were significantly fewer larvae on upper surfaces of leaves at 4:00 pm than at the two earlier times.

they are on will not affect the efficacy of insecticides with oral toxicity.

At 7:30 am, significantly more larvae were on the tops of plants than on the middle to lower plant (Figure 19; n = as above). Equal numbers of larvae were found on the middle to lower plant and on the top of the plant at 11:00 am and 4:00 pm. Insecticide application with a boom sprayer may be most effective in the early morning, when large larvae are exposed. Again, this is not likely to be important with long-lasting chemicals. However, vacuuming or flaming may be significantly more effective if done during cooler periods when larvae are exposed at the tops of plants.

Perhaps the most important IPM opportunity afforded by the position of large larvae on the upper surfaces of leaves at the top of the plant is their increased visibility. A greater proportion of large larvae should be visible from a standing position when sampled during cool weather or in the early morning.

Behavior of Third and Fourth Instars on a Rainy Day. Despite the rain, most large larvae were at the top of the plant rather than in leaf axils (Figure 20; n = 93, $P < 0.05$, df = 1, $X^2 = 36.6$). On average, larvae were located about 70% of the way up the plant from the base. More larvae were on undersides of leaves than on tops, but the difference was not significant (Figure 21; n = 93, $P > 0.05$, df = 1, $X^2 = 3.5$). Rain did not strongly affect the position of large larvae.

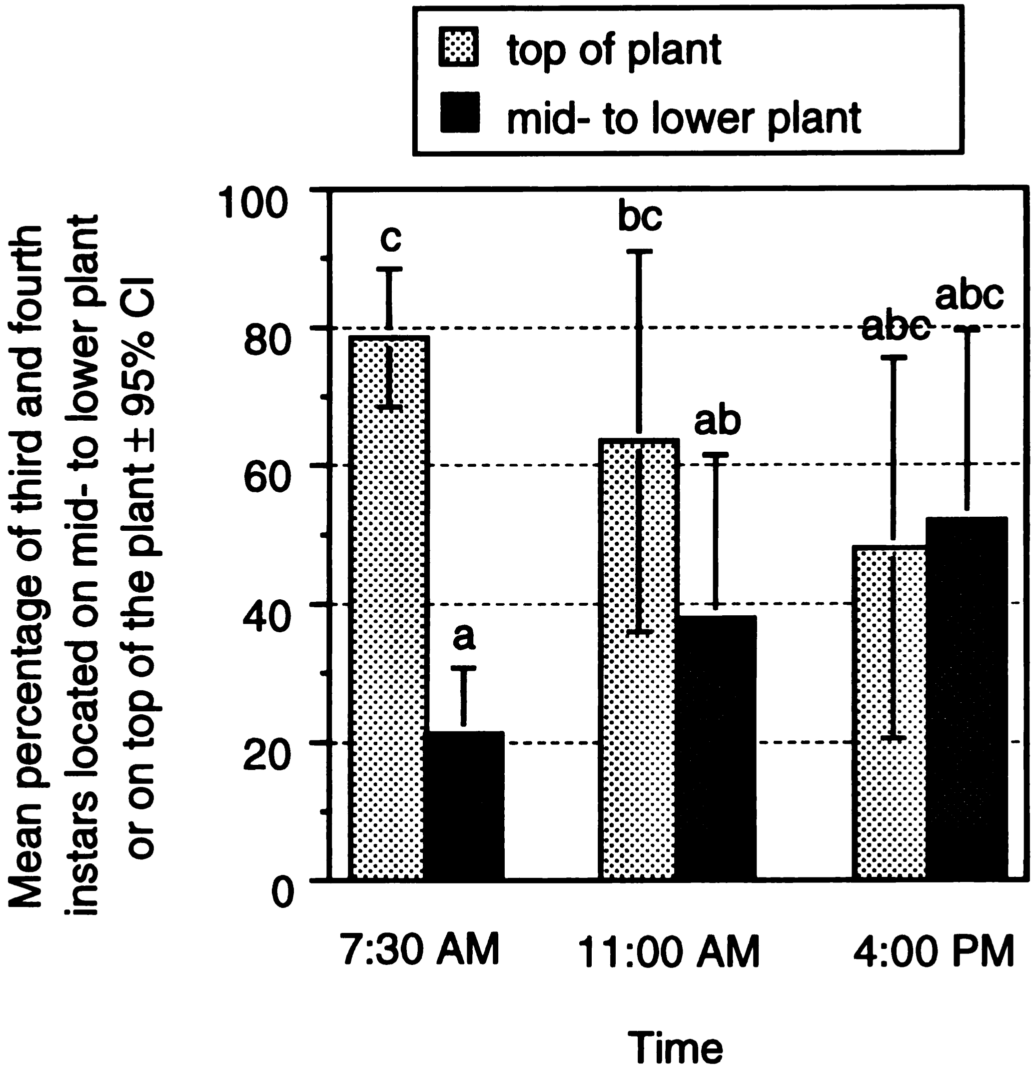

Figure 19. Mean percentage of Colorado potato beetle third and fourth instars on mid- to lower potato leaves or at the top of potato plants in the morning, at midday, and in the afternoon. Means with no matching letters are significantly different.

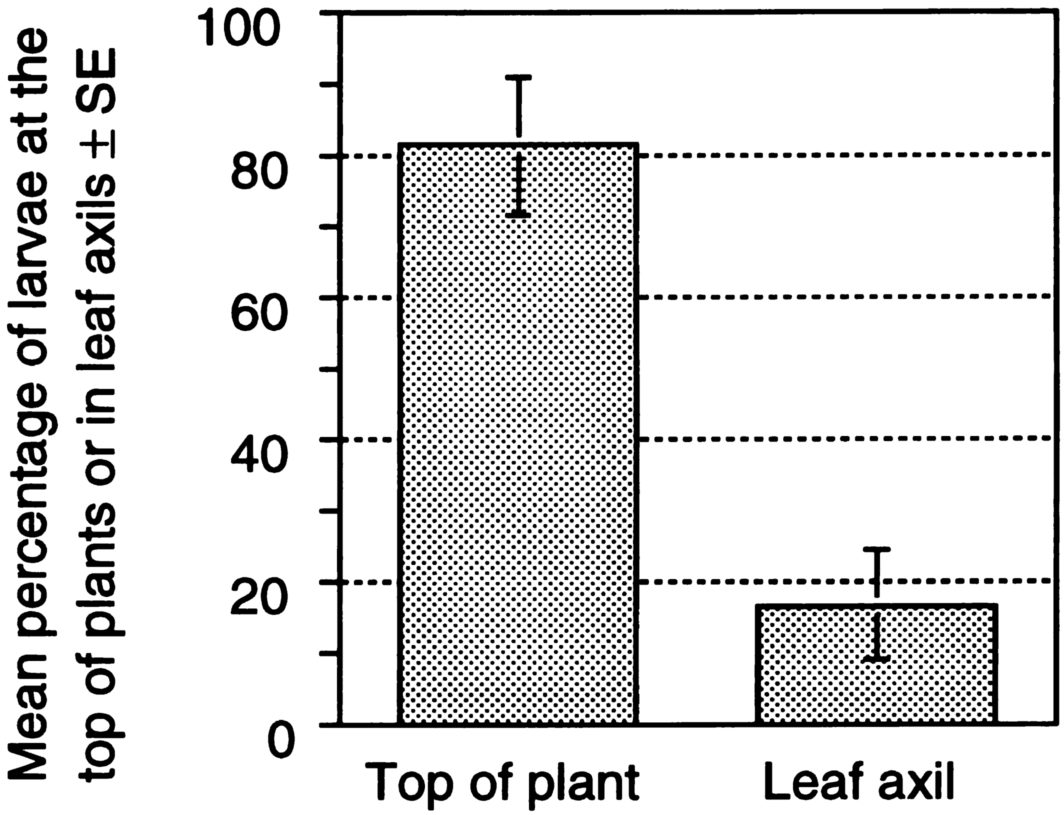

Figure 20. Mean percentage of Colorado potato beetle third and fourth instars at the top of plants and at leaf axils on a rainy morning. Means are significantly different (Chi-squared test, P= 0.05).

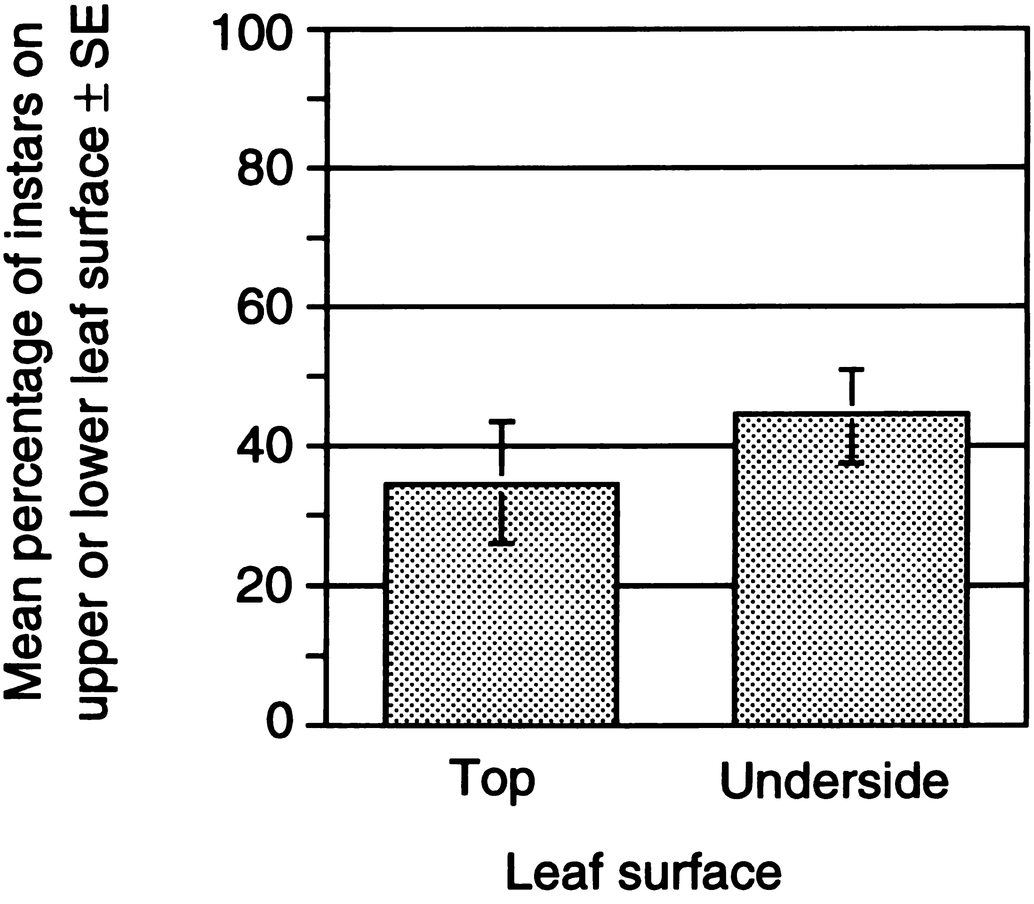

Figure 21. Mean percentage of Colorado potato beetle third and fourth instars on upper and lower leaf surfaces on a rainy morning. Means are not significantly different (Chi-squared test with significance level P= 0.05).

Conclusions. Results of my studies showed that Colorado potato beetle egg masses occured throughout the potato canopy on lower leaf surfaces, small larvae were generally on the lower to middle plant in leaf axils, and large larvae were most common on upper leaves with seven or more leaflets. This information could help those sampling for egg masses or larvae decide what plant parts to examine. However, it is likely that significant effects of various factors (e.g. weather, time, plant characteristics) on positioning of immature Colorado potato beeltes remain to be fully demonstrated. Further study is needed before larval and egg mass positioning can be predicted well enough to develop reliable sampling protocols. Pinpointing the location of larvae on plants will also aid in optimizing the efficacy of control measures such as biorational pesticide sprays, vacuuming and flaming which will comprise sustainable management systems for Colorado potato beetle.

CONCLUSIONS

Action threshold-based spraying could help conserve insecticide resources such as *Bacillus thuringiensis* var. *tenebrionis.* The environment would also benefit from pest-based decision making. Regional efforts should be made to determine appropriate action thresholds for Colorado potato beetle at key stages of potato plant growth. Demonstrating the effectiveness of a threshold-based spray program could encourage growers to use thresholds rather than calendar sprays.

Bacillus thuringiensis var. *tenebrionis* is compatible with Colorado potato beetle natural enemies and with the environment in general. Therefore, growers should be encouraged to use *B. thuringiensis* for Colorado potato beetle control. The improved efficacy of newer formulations should be demonstrated to help convince growers of the value of this insecticide. Growers should be trained to scout for Colorado potato beetle egg masses, to time the sprays at peak egg mass hatch, and to consider factors such as temperature and precipitation when deciding whether to spray. This will help ensure that they have success with *B. thuringiensis.*

If progress is made toward these basic goals, extension workers could begin cooperating with growers to explore other options available with *B. thuringiensis* var. *tenebrionis* sprays, such as use of drop nozzles and the value of integrating *B. thuringiensis* with crop rotation or other alternatives. Growers should be alerted as use of drop nozzles and the value of integrating *B. thuringiensis* with crop rotation or other alternatives. Growers should be alerted to the pros and cons of *B. thuringiensis* var. *tenebrionis* -transgenic plants.

Conclusions from Field Studies. Sampling may be easier and more accurate when the position of the target life stage is considered. For example, upper, middle, and lower potato leaves should all be examined when percent egg hatch is determined for the timing of *B. thuringiensis* sprays. Fairly accurate sampling for large larvae could be done from a standing position on cool mornings or cloudy days, because large larvae are more likely to be visible on the upper surfaces of leaves on the tops of plants. In contrast, sampling for small larvae requires kneeling and close scrutiny, because they are lower on the plant and hidden in leaf axils.

Zehnder & Speese (1989) found that drop nozzles plus top nozzles work better for M-Trak application than top nozzles alone. My finding that early instars are on the lower plant provides an explanation for this. Another option is the air curtain sprayer, which reaches lower as well as upper leaves, and even reaches the undersides of leaves (Grafius et al. 1990). Of course, the easiest option for ensuring optimum coverage will soon be transgenic plants.

Conclusions from Laboratory Studies. By re-confirming the resistance of the cryIIIA-resistant laboratory strain, my laboratory feeding study with cryIIIA-dipped leaves provided further evidence that resistance to cryIIIA in the field is inevitable. Thus, it is desirable to develop strategies to delay resistance to *B. thuringiensis* sprays and transgenic plants.

My laboratory feeding study with *B. thuringiensis*-transgenic potato foliage provided evidence that trangenics would at least be effective in controlling 90-fold resistant Colorado potato beetle. Because transgenic foliage tolerated higher resistance than *B. thuringiensis*-treated foliage, transgenic plants may have the same number of years of potential utility as formulated endotoxin despite their accelerated selection for resistance.

I believe that the relatively higher selection pressure for *B. thuringiensis* resistance by transgenics will not deter growers from using them. Growers will enjoy their superior convenience and efficacy. If growers do choose to spray *B. thuringiensis* rather than planting transgenics, I expect that the reasons will be economic rather than resistance-related. Market value of transgenic potatoes (which will depend on quality and on consumer acceptance of the technology), yield of transgenic potatoes, and price of transgenic minitubers or seed pieces for planting could determine the success of the trangenic potato industry.

CryI was used commercially against Lepidoptera for about 30 years before resistance occurred in the field. This provides hope that cryIII may similarly remain a useful resource for many years.

Beyond *B. thuringiensis*, the potential of pathogens as control agents of the Colorado potato beetle has only begun to be explored and exploited. The insecticide Agri-Mek contains the antibiotic abamectin, which is produced by bacteria. Agri-Mek is as effective as the conventional insecticide Asana for control of Colorado potato beetle (Grafius et al. 1994). Other antibiotics are also known to be effective against Colorado potato beetle (Jizba et al. 1991). Abamectin resistance has been demonstrated and characterized in Colorado potato beetle (Argentine et al. 1992).

The endemic fungal pathogen *Beauveria bassiana* (Balsamo) Vuillemin has been extensively studied. In bioassays (Anderson et al. 1989), foliar applications (Hajek et al. 1987), and soil applications (Cantwell et al. 1986; Gaugler et al. 1989), *B. bassiana* does not consistently cause high mortality of Colorado potato beetle. However, Watt & LeBrun (1984) achieved high pupal mortality with *B. bassiana*. Improving formulation and application techniques and selecting highly virulent *B. bassiana* strains could someday lead to a commercial *B. bassiana* product for Colorado potato beetle control.

Several species of nematodes also seem promising as microbial insecticides against Colorado potato beetle (Nickle & Kaiser 1984; Wright et al. 1987). The microsporidian *Nosema scripta* infects Colorado potato beetle (Bauer & Pankratz 1993). Though microsporidia are generally less likely to be useful microbial insecticides, they cannot be ignored; *Nosema locustae* was registered for use against grasshoppers in 1980.

More pathogens for use as microbial insecticides against Colorado potato beetle may someday be discovered in Colorado potato beetle populations in America or at the species epicenter in Mexico. Pathogens of beetles in the same family (Chrysomelidae) could be screened for activity against Colorado potato beetle. For example, a *Mermis* nematode and a *Cephalosporium* fungus are known to infect *Podontia quatuordecimpunctata* L. in India (Singh & Misra 1989), and these pathogens have probably not been tested for efficacy against Colorado potato beetle. Still farther removed, a relative abundance of pathogens are known for grubs (Scarabaeidae), and to a lesser extent, for weevils (Curculionidae). Such Coleopteran pathogens could also be tested for efficacy against the appropriate Colorado potato beetle life stages. The success of *Bacillus thuringiensis* var. *tenebrionis* may be followed in the future by many more microbial insecticides for Colorado potato beetle management.

APPENDICES

APPENDIX 1

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.: 1993-4

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

Behavior of Colorado potato beetle (Coleoptera: Chrysomelidae) larvae: effect of Bacillus thuringiensis-transgenic potato on feeding by B. thuringiensis-resistant and susceptible strains, and position of larvae on individual plants in the field

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

Entomology Museum, Michigan State University (MSU)

Other Museums: -

Investigator's Name (s) (typed)
Jennifer Altre

Date 4/94

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

Deposit as follows:

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

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

APPENDIX 1.1

Voucher Specimen Data

Page 1 of 1 Pages

	Museum where deposited	MSU
Number of:	Other	5 B.t.-resistant adults, sex undetermined 5 B.t.-susceptible adults, sex undetermined
	Adults ♂	
	Adults ♀	
	Pupae	
	Nymphs	
	Larvae	
	Eggs	
	Label data for specimens collected or used and deposited	[illegible] or Ex: B.t.-susceptible culture
	Species or other taxon	Leptinotarsa decemlineata (Say)

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Jennifer Altre

Date 4/94

Voucher No. 1993-4

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

F. W. Stehr
Curator

12/93
Date

APPENDIX 2

APPENDIX 2

Spiral Xylem Elements of Potato Leaves Entangle Colorado Potato Beetle Larvae

Potato (*Solanum tuberosum*) foliage is generally the preferred food for Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) larvae and adults. Chin (1950) noted that Colorado potato beetle larvae sometimes move their heads up and down while chewing and swallowing leaves of non-preferred hosts. During close-up video taping, I noticed that larvae feeding on potato leaves occasionally rocked their heads back and forth, and clear, shiny strands stretched from their mouths to the leaf surface. Several people viewed the video footage of this behavior, and disagreed on whether the strands were of insect or plant origin. Dr. Walter Pett (Michigan State University, East Lansing MI) generously rendered his services as scanning electron microscopist so that I could more closely view the interfaces of strands with leaf surfaces and with insect mouthparts.

It was clear from the scanning images that the strands arose from the leaf. Either larval mouthparts became entangled in loose ends of strands, or the larvae actually swallowed the strands and were unable to break them off.

The strands extending from the leaves to the larvae appeared to be stretched coils. Coiled continuous strands composed of lignin, cellulose, and hemicellulose form the secondary thickening of walls of early xylem elements in plants

(Jensen & Salisbury 1984; see Plate 63 in Troughton & Donaldson 1972 for SEM of spiral xylem walls in cucumber). The stretchable spiral configuration accommodates expansive growth. Later xylem elements have more rigid, continuous, pitted tubes of secondary wall material.

Xylem fibers can be found in the frass of Colorado potato beetle larvae (Chin 1950). Thus, larvae do encounter the strands while feeding. I believe that the larvae I observed were caught on helical xylem secondary wall fibers which remained intact or unravelled as the larvae tugged to free themselves.

I performed a preliminary assay of the length of time that tangled larvae required to break the strands when I disturbed them with a paintbrush. I placed late second instars and early third instars (age 7 d) on a newly excised terminal potato leaflet and let them feed for 30 minutes. I disturbed 46 feeding larvae by prodding them every 10 s, and observed their response at 250x. For each larva that attempted to escape, the presence or absence of xylem strands in the mouth was noted. If the larva was attached to the leaf by xylem strands, the number of seconds until the strands were broken was noted. I repeated this with second instars less than 2 d old, and I also watched late third instars interact with strands.

The early second instars were stuck on strands in 12 out of 13 instances of feeding. The mean escape time for these larvae was 1 minute. Of the 31 feeding late second instars instars, 13 (42%) were stuck on xylem strands when prodded. Seven did not attempt to leave when prodded. Among the six late second instars that actively struggled to break away, the mean escape time was 4 minutes and 30 s. This weak escape response may have been due to the imminence of molting. Six of 15 early third instars were stuck on strands. Five of these broke away in less than one minute. Late third instars were observed with up to 10 xylem strands attached to their mouths at once, but these large larvae easily broke the strands.

Although entanglement by xylem strands is a relatively common occurrence, second and third instars are able to break the strands within five minutes of struggle. Therefore, entanglement by xylem fibers probably does not have a significant impact on larval survival.

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