Resistance evaluation and management of Colorado potato beetle, *Leptinotarsa decemlineata* (Say), using novel chemistries.

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Abstract

Leptinotarsa decemlineata (Say) is the most important defoliating pest of potato *Solanum tuberosum* L., in North America and Europe. Management of this pest relies heavily on chemical control and insecticide resistance is a persistent problem. This phenomenon has increased the need for developing novel insecticides, resistance evaluation, and the development of alternative control strategies regarding this insect pest. From 2010 to 2013, field and lab experiments were conducted to evaluate the efficacy of a novel insecticide tolfenpyrad on *L. decemlineata*. In leaf-dip assays, tolfenpyrad was highly toxic to *L. decemlineata* with LC_{50} values of 0.013 and 0.164 g ai/L for larvae and adults, respectively. Tolfenpyrad was also toxic to eggs with 0% hatching after being dipped in a field rate concentration. In field efficacy trials, potato plots treated with tolfenpyrad at rates as low as 153 g ai/ha effectively controlled *L. decemlineata*.

In 2012, populations of *L. decemlineata* were collected from the Eastern Shore of VA and subjected to toxicity assays to determine current susceptibility to permethrin and oxamyl. The toxicity assays indicated an increase in toxicity to permethrin in *L. decemlineata* larvae (LC_{50} = 3.931 g ai/L) and an increase in toxicity to oxamyl in adult beetles (LC_{50} = 9.695 g ai/L) compared with LC_{50} values previously reported in 1990. In 2012, populations of *L. decemlineata* from Cheriton, VA, New Church, VA, Painter, VA, and Plymouth, NC were also evaluated for enzyme activity after exposure to sub-lethal concentrations of permethrin, oxamyl, and tolfenpyrad. Adult beetles were subjected to

enzyme assays to measure the activity of cytochrome P450 mono-oxygenase (P450), glutathione-*S*-transferase (GST), general esterases, and protein content. Results from the enzyme assays indicated significantly greater esterase activity in beetles from Painter, VA exposed to permethrin [α-naphthol ($F= 11.66$, $df= 4, 20$, $P<0.0001$) and β-naphthol ($F= 11.86$, $df= 4, 20$, $P<0.0001$)], oxamyl [α- naphthol ($F= 10.64$, $df= 4, 20$, $P<0.0001$) and β-naphthol ($F= 6.94$, $df= 4, 20$, $P=0.0011$)], tolfenpyrad [α- naphthol ($F= 407.62$, $df= 1, 8$, $P<0.0001$) and β- naphthol ($F= 28.15$, $df= 1, 8$, $P= 0.0007$)], and the untreated control [α- naphthol ($F= 28.14$, $df= 3, 16$, $P<0.0001$) and β- naphthol ($F= 28.86$; $df= 3, 16$, $P<0.0001$)] compared to most of the other populations tested. GST activity was significantly greater in tolfenpyrad exposed beetles compared to the non-treated beetles from Painter VA ($F= 17.66$, $df= 5, 24$, $P< 0.0001$).

Through laboratory assays and field experiments in potato, the efficacy of a new bio-pesticide derived from the bacterium *Chromobacterium subtsugae* was evaluated for the control of *L. decemlineata*. Results from the laboratory assays showed *L. decemlineata* feeding was inhibited by the bio-pesticide derived from *C. subtsugae*. However, field efficacy trials in 2010, 2011, and 2012, indicated no control of *L. decemlineata*.

Methyl salicylate is an organic compound produced by potato and other plants in response to insect herbivory. Abundance of predatory arthropods and *L. decemlineata* life stages were measured in plots treated with and without 5 g slow-release packets of methyl salicylate (95% methyl salicylate (Predalure™)). Methyl salicylate treatment had no impact on predator recruitment or cumulative mortality of *L. decemlineata* in potatoes.

This research has provided us with a new tool for *L. decemlineata* management, as well as more information about resistance trends and alternative control strategies from which we can build on to reduce resistance development in *L. decemlineata* and ultimately formulate a stronger integrated pest management strategy for this insect pest.

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The Colorado potato beetle: A literature review

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is one of the most important pests of potato, *Solanum tuberosum* L., in North America and Europe. The severe damage potential of this insect coupled with its ability to develop resistance to insecticides has made it the focus of much research over the years. Useful reviews of its biology, ecology and pest management can be found in Radcliffe 1982, Hare 1990, Capinera 2001, and Alyokhin 2008).

History and Distribution

Colorado potato beetle was first described by Thomas Say, who found the beetle on buffalo bur, *Solanum rostratum* (Dunal), in Missouri and Arkansas; he originally placed the beetle in the genus *Doryphora* (Say 1824). The insect is native to Mexico and probably migrated into North America in the early 1800s (Capinera 2001, Alyokhin 2009). Once the beetle incorporated the cultivated potato into its range of host plants, it spread rapidly and quickly became the most destructive pest of potato in North America (Hare 1990b). Original reports of Colorado potato beetle causing potato damage came from Nebraska in 1859; by 1874, the pest could be found attacking potatoes from the Great Plains region to as far east as Connecticut and Virginia (Capinera 2001). Today, Colorado potato beetle has a wide spread distribution and is a global pest of solanaceous crops, especially potato. It can be found across 8 million km^2 in North America (Hsiao 1985, Alyokhin 2008) and 6 million km^2 in Europe and Asia (Jolivet 1991). Most recently this insect pest has been found in China and Iran (Alyokhin 2008), and has the potential to spread to other parts of the world (Vlasova 1978, Worner 1988, Jolivet 1991, Alyokhin 2008).

Lifecycle and Description

Colorado potato beetle overwinters in the adult stage and emerges from the soil in the spring (Capinera 2001). In Virginia, this typically occurs in late April or early May. Colorado potato beetle is multivoltine and can complete a generation in approximately 30 days. In the U.S., the insect has between one and three generations per year (Capinera 2001). Towards the end of the summer adult beetles will feed and reproduce until the photoperiod shortens causing beetles to begin diapause (Alyokhin 2008). The major factors involved in diapause are day length, temperature, and to a lesser extent, host quality (Capinera 2001). Longer days promote continued reproduction; whereas, short day lengths induce diapause (Capinera 2001). However, there is a lot of variability in diapause behavior and physiology among populations of *L. decemlineata* in the U.S. leading to summer diapause induction, overwintering populations that can include more than one generation, and some overwintering adults that remain in diapause for more than a year (Horton and Capinera 1988, Tauber et al. 1988, Voss et al. 1988, Hare 1990b, Capinera 2001).

Egg

The eggs are oval and elongated between 1.7 and 1.8 mm in length and about 0.8 mm in width (Capinera 2001). Eggs are usually deposited on the underside of leaves in clusters with an average between 20 and 60 eggs per cluster (Hare 1990b, Capinera 2001). The development time for the eggs is variable depending on temperature, 10.7, 6.2, 3.4 and 4.6 days with corresponding temperatures of 15, 20, 24, and 30^{o}C, respectively (Capinera 2001). The eggs within a cluster typically hatch simultaneously and first instars begin feeding on leaf tissue immediately (Hare 1990).

Larva

The larva is reddish in color, and has a plump convex abdomen with two rows of black spots down each side (Capinera 2001, Jacques Jr and Fasulo 2009) (Figure 1). Larvae come equipped with three pairs of thoracic legs as well as a proleg at the tip of the abdomen (Capinera 2001). Colorado potato beetle has four instars distinguishable by the width of their head capsules, 0.65, 1.09, 1.67 and 2.55 mm for instars one through four, respectively. Each instar increases in body length as well as head capsule width as it progress to the next instar (Capinera 2001). Larval development can occur at a minimum temperature of 8^{o}C with an optimal development temperature of 28^{o}C (Capinera 2001). After the larva has completed feeding and development, the prepupa drops from plants to the soil, where it burrows to a depth of 2 to 5 cm where it will pupate (Jacques Jr and Fasulo 2009).

Figure 1. Colorado potato beetle larvae feeding on potato leaves.

Pupa

The pupae measure about 9.2 mm long and 6.4 mm wide and are orange colored and oval in shape (Capinera 2001). The average development time for the pupal stage is about 5.8 days (Capinera 2001). The prepupal, pupal and post pupal stage of development also varies with

temperature and takes an average of 22.3, 14.9, 11.7 and 8.8 days at 15, 20, 24, and 28^{o}C, respectively (Ferro et al. 1985, Capinera 2001).

Adult

After pupation is complete, Colorado potato beetle adults emerge from the soil and seek out host plants on which to feed upon. The adult is approximately 1 cm in length with light yellowish coloration, five black longitudinal stripes down each elytra, and are generally robust and convex oval in shape (Capinera 2001, Wilkerson et al. 2005, Jacques Jr and Fasulo 2009) (Figure 2). The head has a triangular black spot and the thorax is littered with 10 dark markings (Capinera 2001). Colorado potato beetle over winters in the soil as an adult between 7.6 and 12.7 cm until it emerges the following spring (Lashomb et al. 1984, Hare 1990b). As soon as the overwintering adult emerges it walks to the nearest host plant and begins to feed (Hare 1990b). Overwintering females will feed for 5 to 10 days before they mate and oviposit on the undersides of host-plant foliage (Hare 1990b). Beetles will continue to feed and reproduce until environmental conditions are conducive for diapause.

Figure 2. Colorado potato beetle adults.

Host Plants

Colorado potato beetle is an oligophagous insect that feeds on plants in the family Solanaceae (Radcliffe 1982, Hsiao 1988, Hare 1990b). In the early 1800s, the beetle was typically found feeding on buffalo bur (Radcliffe 1982), but upon encountering cultivated potato in North America, it adjusted its host preference to include that plant species by the mid-1800s (Radcliffe 1982). *Leptinotarsa decemlineata* has been observed feeding on the following solanaceous weeds: bittersweet (*Solanum dulcamara* L.), buffalo bur , horsenettle (*S. carolinense* L.), hairy nightshade (*S. sarrachoides* Sendtn.), silverleaf nightshade (*S. elaegnifolium* Cav.), western horsenettle (*S. dimidiatum* Raf.), and henbit (*Hyoscyamus niger* L.) (Hsiao 1988, Weber et al. 1995, Capinera 2001).

Among cultivated crops, *L. decemlineata* feeds on potato, tomato (*Solanum lycopersicum* L.), eggplant (*S. melongena* L.), and rarely on pepper (*Capsicum annuum* L.) (Capinera 2001). Hitchner et al. (2008) conducted field and laboratory-choice tests to better understand host plant choice by *L. decemlineata* in Virginia. In laboratory olfactometer studies, *L. decemlineata* adults oriented to potato over both tomato and eggplant foliage and eggplant over tomato foliage. Field choice tests revealed more *L. decemlineata* adults, larvae, and egg masses on eggplant than on tomato, and virtually none occurring on pepper. However, field studies using counts of live beetles on untreated paired plants and counts of dead beetles on imidacloprid-treated plants found no difference in *L. decemlineata* numbers between potato and eggplant, but there was a significant attraction to eggplant over both tomato and pepper.

Other studies have shown that Colorado potato beetle can adapt its host range to accept the locally abundant *Solanum* species (Horton et al. 1988, Mena-Covarrubias et al. 1996,

Capinera 2001). This ability to adapt to locally abundant hosts increases the survival of beetle populations.

Damage

Leptinotarsa decemlineata is a voracious leaf feeder. The fourth instar is typically responsible for most (~77%) of total leaf consumption (Capinera 2001). Larvae consume approximately 35 to 45 cm^2 of leaf tissue, and adults consume around 7 to 10 cm^2 per day (Ferro et al. 1983, Alyokhin 2009). If larval densities are high enough, they can completely defoliate a potato crop; however, the stage of the plant has a dramatic impact on the amount of actual yield loss that occurs (Cranshaw and Radcliffe 1980, Hare 1980a). Potato is most susceptible to defoliation injury when plants are blooming and shortly after blooming (Shields and Wyman 1984). Defoliation at this stage of plant development could result in significant loss of tuber production and an economic loss for a grower. Based on a decade of insecticide efficacy experiments conducted on the Eastern Shore of Virginia, approximately 40-50% yield loss will occur to potatoes if Colorado potato beetle is not controlled (Kuhar et al. 2006a, 2008, Kuhar and Doughty 2009). In tomato, a Colorado potato beetle density of one beetle every two plants can reduce yields (Schalk and Stoner 1979, Capinera 2001). In eggplant, densities of 8 larvae per plant can reduce yield (Cotty and Lashomb 1982). With any crop, the size and stage of the plant is a major factor affecting yield loss by Colorado potato beetle.

Management

Colorado potato beetle is notoriously difficult to control. There are a variety of control strategies that can be used in an integrated pest management program. Before growers can determine the appropriate control tactic to be utilized, accurate sampling must be conducted to assess the insect pressure within a given season.

Sampling

Populations of Colorado potato beetle within potato fields have traditionally been evaluated visually or through the use of sweep netting (Senanayake and Holliday 1988, Boiteau 2000). Research has concluded that visual estimation was as effective at monitoring Colorado potato beetle populations as whole plant bag sampling and more effective than sweep-net estimates (Senanayake and Holliday 1988). Overwintering adults typically infest fields by walking into them (Hare 1990b). A sampling strategy to accurately monitor early season populations of adult beetles would be vital to population estimates which aid in timely and effective management decisions. Pitfall traps have been used to monitor and estimate walking populations of Colorado potato beetle adults (Voss and Ferro 1990, Boiteau and Osborn 1999, Noronha and Cloutier 1999, Boiteau 2000). Field research has demonstrated that adding a synthetic aggregation pheromone, (S)-3,7-dimethyl-2-oxo-oct-6-ene-1,3-diol ((S)-CPB I), to pitfall traps increased the catch rate of Colorado potato beetle adults by more than five-fold compared with pitfall traps not baited with the aggregation pheromone (Kuhar et al. 2006a). This increased catch rate provides a more sensitive estimate of the adult activity in fields at the beginning of a growing season, which could help growers make a more accurate and effective early season management decision.

Cultural control

Research has shown that cultural practices, such as crop rotation, altering planting date, and the use of straw mulches can significantly reduce Colorado potato beetle pressure (Alyokhin 2009). Crop rotations are most effective when fields are 0.3 and 0.9 km apart (Weisz et al. 1994, Hough-Goldstein and Whalen 1996, Weisz et al. 1996, Sexson and Wyman 2005, Alyokhin 2009). Planting early in the season helps eliminate the impact of the second generation larvae

because as they emerge, the crop is being removed from the field (Weber and Ferro 1994, Alyokhin 2009). The same is true for late planted crops, the summer generation of Colorado potato beetle emerge later in the season and the shorter photoperiod induces a reproductive diapause reducing the impact of second generation larvae (Alyokhin 2009).

Mulches can also have a dramatic impact on Colorado potato beetle populations within potato fields. Research has shown the use of straw mulch can reduce the defoliation in potato fields by two to five fold compared with fields without mulch (Zehnder and Hough-Goldstein 1990, Brust 1994, Alyokhin 2009). Another cultural technique that can be used to take advantage of the behavior of Colorado potato beetle adults is the use of trenches lined with plastic and surrounding fields. Research has demonstrated that trench walls with angles greater than 45^{o} can capture 50% or more of Colorado potato beetle adults that walk into the fields during the early season invasion (Boiteau et al. 1994, Capinera 2001).

Chemical control and insecticide resistance

Since the late 1800s, the use of insecticides has been the most relied-upon method for controlling Colorado potato beetle. Reviews of chemical control in potato can be seen in Gauthier et al. (1981) and Kuhar et al. (2013). The use of insecticides to control Colorado potato beetle dates back to the 1800s with the use of Paris green (Copper[II]-acetoarsenite) (Riley 1871). Other types of arsenical compounds such as lead arsenate and calcium arsenate would continue to be used for its control into the 1940s (Gauthier et al. 1981). However, arsenical insecticides were difficult to mix, difficult to apply effectively, did not have a long residual life on plants, and sometimes caused phytotoxicity (Kuhar et al. 2013). Thus, alternatives to the use of arsenicals in potatoes were sought throughout the early 1900s. These included botanical insecticides such as veratrine alkaloids from Sabadilla, ryania extract, and rotenone (Brown

1951). Although rotenone demonstrated sufficient efficacy against Colorado potato beetle, the focus on botanical insecticides as a replacement for arsenicals would soon be overshadowed with the arrival of DDT in 1939 (Hitchner 1952, Gauthier et al. 1981, Alyokhin 2009). However, by the 1950s, Colorado potato beetle had developed resistance to DDT (Quinton 1955) and was soon resistant to other chlorinated hydrocarbons (Hofmaster et al. 1967, Alyokhin 2009). Since the development of resistance to the chlorinated hydrocarbons, Colorado potato beetle populations have developed resistance to all or some of the compounds classified in the arsenical, organochlorine, carbamate, organophosphate, and pyrethroid classes of chemistry (Alyokhin 2008).

Today, potato and vegetable growers currently rely heavily on the neonicotinoid class of insecticides to control Colorado potato beetle. This group includes imidacloprid, thiamethoxam, clothianidin, acetamiprid and dinotefuran. These compounds are typically applied as in-furrow or seed-piece treatments (insecticide applied directly to the potato seed piece prior to planting); however, foliar applications are also effective. Resistance to imidacloprid in field populations of Colorado potato beetle first appeared in Long Island, NY in the late 1990s (Olson et al. 2004) and has since been documented in other potato-growing regions throughout the U.S. (Mota-Sanchez 2002). More importantly, there appears to be cross-resistance between imidacloprid and thiamethoxam (Mota-Sanchez et al. 2006, Alyokhin et al. 2007).

This seemingly innate ability to develop resistance is greatly influenced by four major factors. First, Colorado potato beetle feeds oligophagously on solanaceous crops (Hare 1990b). These crops have elevated concentrations of toxic glycoalkoloids in their foliage, requiring these insects to develop effective means to detoxify and excrete their diet (Ferro 1993, Bishop and Grafius 1996, Alyokhin et al. 2008). Secondly, the fecundity of Colorado potato beetle results in

large populations in a short period of time (Hare 1990b). A third reason is the host range of this insect is narrow and in agricultural settings this host range comprises heavily managed, high value crops such as potato, eggplant and tomato. This narrow host range reduces the amount of safe refuge Colorado potato beetle can find so beetles that do survive a pesticide application are mating with other survivors, decreasing the number of susceptible insects contributing to the gene pool (Bishop and Grafius 1996, Whalon and Ferro 1998, Alyokhin et al. 2008). The fourth reason is that these insects are continuously exposed to insecticides (Harcourt 1971, Casagrande 1987, Bishop and Grafius 1996, Alyokhin et al. 2008). Colorado potato beetle's propensity for developing resistance has increased efforts by researchers and chemical companies to investigate and develop new compounds with novel modes of action for managing this insect pest.

In addition to the aforementioned broad spectrum insecticides and the neonicotinoids, several other insecticides have demonstrated efficacy on Colorado potato beetle. These include the following: 1) cryolite, an inorganic fluoride insecticide (Noetzel and Holder 1996, Sorensen and Holloway 1997); 2) abamectin (Kuhar et al. 2006b, Marčic´a et al. 2009, Sewell and Alyokhin 2009); 3) azadirachtins found in the seeds of the neem tree (*Azadirachta indica*) (Zehnder and Warthen 1988, Marčic´a et al. 2009); 4) spinosyns such as spinosad and spinetoram (Kuhar and Doughty 2009, Sewell and Alyokhin 2009, 2010); 5) novaluron, an insect growth regulator (Cutler et al. 2007, Kuhar and Doughty 2009, Sewell and Alyokhin 2009, 2010); 6) cyromazine, a triazine chitin synthesis inhibitor (Sirota and Grafius 1994, Linduska et al. 1996); 7) indoxacarb (Linduska et al. 2002, Kuhar and Speese 2005); 8) metaflumizone (Kuhar et al. 2006b, Sewell and Alyokhin 2009, 2010, Hitchner et al. 2012); and most recently, 9) the anthranilic diamides, chlorantraniliprole and cyantraniliprole (Kuhar and Doughty 2009, Sewell and Alyokhin 2009, 2010).

Biological control

There are a number of biological organisms that feed on Colorado potato beetle reducing populations of this pest.

Predators. A number of generalist arthropod predators have been observed to feed on Colorado potato beetle larvae and eggs. Weber (2013) provides an excellent review of the natural enemies of Colorado potato beetle. Three of the most important predators of Colorado potato beetle in North America are the ground beetle *Lebia grandis* Say (Coleoptera: Carabidae) and the predacious stink bugs *Perillus bioculatus* Fabricius (Hemiptera: Pentatomidae) and *Podisus maculiventris* Say (Hemiptera: Pentatomidae), which feed on small and large larvae. There are a number of lady beetles that will feed on the eggs of Colorado potato beetle such as *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae), *Hippodamia convergens* Guerin-Meneville (Coleoptera: Coccinellidae) and the seven spotted lady beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae). All of these insects are commonly observed on potato plants in Virginia (T. P. Kuhar, *unpublished data*).

Parasitoids. There are three major parasitoids that utilize Colorado potato beetle as a host (Weber 2013). These include the egg parasitoid *Edovum puttleri* Grissell (Hymenoptera: Eulophidae), and two dipteran parasitoids, *Myiopharus aberrans* (Townsend) (Diptera: Tachinidae), and *Myiopharus doryphorae* (Riley) (Diptera: Tachinidae) (Weber 2013). Both tachinid parasitoids oviposit in the larvae of Colorado potato beetle. *Myiopharus aberrans* parasitizes larvae in the earlier part of the growing season, and *M. doryphorae* parasitizes larvae later in the season (Weber 2013). *Myiopharus* aberrans has a distinguishing behavior from *M. doryphoare* in that females will larviposit directly into adult Colorado potato beetles late in the season. The parasitoid wasp *E. puttleri*, can parasitize up to 71 to 91% of eggs in an individual

Colorado potato beetle egg mass (Lashomb et al. 1987a, Alyokhin 2009). However, the traditional insecticidal and fungicidal control methods, typical in vegetable production, are not conducive for use with these biological control agents. Thus, integrated pest management for Colorado potato beetle has been a challenge (Alyokhin 2009).

Pathogens. There are also two specific organisms that could potentially be used in a biological control system. First is the entomopathogenic fungi *Beauveria bassiana* (Bals.-Criv.) Vuillemin which is a generalist fungal pathogen. Research has shown this organism can reduce Colorado potato beetle populations by as much as 75% (Cantwell et al. 1986, Alyokhin 2009). Overall the ability of the fungus to control Colorado potato beetle has ranged from poor to excellent (Hajek et al. 1987, Poprawski et al. 1997, Lacey et al. 1999, Wraight and Ramos 2002, Wraight et al. 2007). The second pathogen is a bacterium, *Bacillus thuringiensis* var. tenebrionis, which has shown excellent activity on early instars of Colorado potato beetle (Ghidiu and Zehnder 1993, Weber 2013). However, there are drawbacks to using *B. thuringiensis* var. *tenebrionis*, abiotic factors can have a dramatic impact on the efficacy of the bacterium (Bystrak et al. 1994, Lacey et al. 1999, Wraight et al. 2007, Weber 2013).

Plant resistance.

Pelletier et al. (2013), provides a thorough review of potato plant resistance. There are two main mechanisms of conventional potato resistance, glycoalkaloids, and trichomes (Pelletier et al. 2013). Gylcoalkaloids are compounds present in plants, specifically plants in the family Solanaceae, to include potato (Osman 1983). Research has shown the nature of the glycoalkaloids present in plants is significant to resistance conferral, rather than the amount of glycoalkaloid present (Tingey and Sinden 1982, Sinden et al. 1991, Lyytinen et al. 2007, Pelletier et al. 2013). One such example of an effective group of glycoalkaloid conferring

resistance to Colorado potato beetle is the leptine group (Tingey and Yencho 1994, Pelletier et al. 2013). Crossing *S. tuberosum* and *S. chacoense* Bitt., incorporates leptine into the plant constituents of the resulting hybrid, conferring resistance to the Colorado potato beetle (Lorenzen et al. 2001, Pelletier et al. 2013). The hybrids exhibited antibiotic effects on Colorado potato beetle resulting in slower development, reduction in larval survival, reduction in feeding, and a reduction in oviposition and adult survival (Lorenzen et al. 2001, Pelletier et al. 2013). A commercially-available variety of potato 'Dakota Diamond' has been released with elevated levels of foliar leptine (Thompson et al. 2008, Pelletier et al. 2013).

Trichomes are uni- or multi-cellular structures found on the above ground parts of plants, originating from epidermal cells (Pelletier et al. 2013). Three forms of trichomes have received attention from *Solanum* spp.: 1) tall non-glandular trichomes; 2) the short four lobed type A glandular trichomes; and 3) the hair like type B trichomes, which secrete droplets of sucrose esters of carboxylic acids from their tips (Flanders et al. 1992, Pelletier et al. 2013). There are three tuber bearing species with glandular trichomes, *Solanum ployadenium* Greenm, *S. berthaultii* and *S. neocaardenasii* Hawkes (Gibson 1976, Tingey and Sinden 1982, Dimock et al. 1985, 1986, Lapointe and Tingey 1986, Sanford and Cantelo 1989, Hanzlik et al. 1997, Flanders et al. 1999, Pelletier and Tai 2001, Horgan et al. 2007), that exhibit resistance to Colorado potato beetle, potato tuber moths, aphids, and other insects (Pelletier et al. 2013). However, like most other control options for Colorado potato beetle, there are advantages and disadvantages to trichome-based plant resistance (Pelletier et al. 2013).

There was success on a short term basis with genetically-modified potato cultivars that carried the *Bacillus thuringiensis tenebrionis* Berliner toxin (Wierenga et al. 1996, Capinera 2001). These genetically-modified potato plants provided effective control of the small larvae of

Colorado potato beetle (Capinera 2001). However, these cultivars were discontinued after 5 years of use because of public concerns about genetically engineered food (Alyokhin 2008).

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Research objectives

1. To evaluate the toxicity and field efficacy of tolfenpyrad on *L. decemlineata* larvae and adults.
2. To identify potential detoxification methods and resistance levels of *L. decemlineata* populations in Virginia when exposed to sub-lethal doses of tolfenpyrad, permethrin, and oxamyl.
3. To assess the toxicity and field efficacy of a novel biopesticide derived from *Chromobacterium subtsugae* Martin *et al*., on *L. decemlineata*.
4. To evaluate the effect of methyl salicylate release packets on the population dynamics of *L. decemlineata* in potato.

Chapter 2:

Baseline susceptibility and field efficacy of tolfenpyrad on Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)

Abstract. *Leptinotarsa decemlineata* (Say) is the most important defoliating pest of potato in North America and Europe. Management of this pest relies heavily on chemical control and insecticide resistance is a persistent problem. From 2010 to 2013, we conducted field and lab experiments with the novel insecticide tolfenpyrad for toxicity and field efficacy on *L. decemlineata*. Results from the leaf-dip bioassays revealed that tolfenpyrad was toxic to *L. decemlineata* with LC_{50} values of 0.013 and 0.164 g ai/L for larvae and adults respectively. In addition, *L. decemlineata* eggs masses treated with tolfenpyrad had a 0% hatch rate. In field efficacy trials, potato plots treated with tolfenpyrad, at rates of 153, 186, and 230 g ai/ha, had significantly fewer larvae, less defoliation, and higher tuber yields than the untreated control plots. This research demonstrated tolfenpyrad to be an effective insecticide for managing *L. decemlineata*. Given its novel mode of action, this insecticide should be a useful resistance management tool for regions with neonicotinoid resistant *L. decemlineata* populations.

Key words Toxicity, tolfenpyrad, Colorado potato beetle, LC_{50}, resistance management

Introduction

Leptinotarsa decemlineata (Say) (Coleoptera: Chrysomelidae) is one of the most important insect pests of potato *Solanum tuberosum* L. The beetle is native to Mexico and probably migrated into the Great Plains of North America in the 1800s (Capinera 2001, Alyokhin 2009) and quickly became the most destructive pest of cultivated potato throughout the U.S. and Canada (Hare 1990). Today *L. decemlineata* can be found throughout much of North America, Europe, parts of Asia, and parts of Central America (Capinera 2001). Uncontrolled populations

can completely defoliate potato fields and potentially cause a total loss of tuber production (Hare 1980, Hare 1990). In Virginia, at least 50% loss of yield will typically occur in potato if *L. decemlineata* is not managed (Kuhar et al. 2006, Kuhar et al. 2008, Kuhar and Doughty 2009). In addition to being a voracious defoliator of potato, *L. decemlineata* has demonstrated an innate ability to develop resistance to insecticides.

The seemingly innate ability to develop resistance is greatly influenced by multiple factors. First, *L. decemlineata* feeds oligophagously on solanaceous crops (Hare 1990). These crops have elevated concentrations of toxic glycoalkoloids in their foliage, requiring these insects to develop effective means to detoxify and excrete their diet (Ferro 1993, Bishop and Grafius 1996, Alyokhin et al. 2008). Secondly, the fecundity of *L. decemlineata* results in large populations in a short period of time (Hare 1990). A third reason is the crops this insect feeds on in agricultural settings are traditionally heavily managed with conventional insecticides, high value crops such as potato, eggplant, and tomato. Feeding on heavily managed crops reduces the amount of safe refuge *L. decemlineata* can find so beetles that do survive a pesticide application are mating with other survivors, decreasing the number of susceptible insects contributing to the gene pool (Bishop and Grafius 1996, Whalon and Ferro 1998, Alyokhin et al. 2008). *Leptinotarsa decemlineata*'s propensity for developing resistance has increased efforts by researchers and the agricultural industry to investigate and develop new compounds with novel modes of action for managing this insect pest.

Chemical control of arthropod pests in potato has been the standard pest management practice for more than a century (Kuhar et al. 2013), and will likely be the future of potato pest management (Alyokhin 2009). Tolfenpyrad is a broad spectrum insecticide that was discovered by the Mitsubishi Chemical Corporation (now the Nihon Nohyaku Co. Ltd.) in 1996, and in

Japan was labeled for use in vegetables and ornamentals in 2002, and fruit trees in 2003 (Nonaka 2003). Tolfenpyrad, 4-chloro-3-ethyl-1-methyl-*N*-[4-(*p*-tolyloxy)benzyl]pyrazole-5-carboxamide, is a novel broad spectrum insecticide currently being developed by the Nihon Nohyaku Co. Ltd. It has been classified by the Insecticide Resistance Action Committee (IRAC) in Group 21. Specifically tolfenpyrad inhibits cellular respiration by inhibiting complex I of the mitochondrial electron transport chain. Insect response from exposure to tolfenpyrad is rapid and includes termination of movement and feeding, lack of fecundity, and death of the pest. Tolfenpyrad has a positive mammalian toxicology profile with an acute oral toxicity of 386 mg kg^{-1}, acute dermal toxicity of 2,000 mg kg^{-1}, and an acute inhalation toxicity of 2.21 mg kg^{-1}. Tolfenpyrad was registered as an insecticide in Japan in April of 2002 and until recently, there was little knowledge of or development of the insecticide in the U.S. Nichino America, Inc. (Wilmington, DE) is currently developing tolfenpyrad for use in agricultural markets in the U.S. Tolfenpyrad is a novel insecticide with no reported cross resistance, which is highly attractive for use against *L. decemlineata* (Anonymous 2012). This research reports the LC_{50} of tolfenpyrad on *L. decemlineata* larvae and adults, as well as the ovicidal activity and the field efficacy of tolfenpyrad at different rates for managing this pest.

Materials and Methods

Insecticide. All experiments were conducted with commercially formulated tolfenpyrad 15 EC (15% a.i.; 150 g ai/L) obtained from Nichino America, Inc.

Leaf-dip bioassays. Experiments were conducted from 2010 to 2012 at the Virginia Tech Eastern Shore Agricultural Research and Extension Center (ESAREC) in Painter, VA. *Leptinotarsa decemlineata* adults and small larvae (2^{nd} and 3^{rd} instars) were collected from

insecticide-free potato plots at the ESAREC, and used in leaf-dip bioassays evaluating the toxicity of tolfenpyrad.

Leaf-dip bioassays were conducted separately on small larvae (2^{nd}-3^{rd} instars) and adult *L. decemlineata*. An initial (stock) rate of tolfenpyrad was calculated from a suggested field application rate of 230 g ai/ha. This was equivalent to a concentration of 4.57 mL product/liter (= 0.685 g ai/L). Six rates (serial dilutions) were evaluated in these experiments including a water control. Each rate was replicated four times and each replication consisted of a single dipped potato leaf and ten small larvae. Unblemished potato leaves were completely submerged in each treatment and allowed to air dry. Once the leaves were dry, 10 larvae or 10 adults were placed in either a 9 cm or a 15 cm diameter Petri dish with each treated leaf, respectively. Adult *L. decemlineata* assays included two leaves per Petri dish. Mortality was assessed after 72 hours of exposure to the leaves. Larvae and adults were considered dead or moribund if they did not respond to gentle probing or could not right themselves if turned upside down.

Egg mass bioassays. In 2012 and 2013, *L. decemlineata* egg masses from Virginia and Michigan were collected and exposed to either water or the high field rate of tolfenpyrad, 230 g ai/ha. Egg masses were completely submerged in tolfenpyrad or in a non-treated water control. Once exposed, the egg masses were placed in Petri dishes and observed for 3 to 5 days to determine the number of egg masses that hatched. Egg masses from multiple populations were evaluated to include some lab reared populations resistant to neonicotinoid insceticides (Table 1).

Field efficacy experiments. Experiments were conducted in 2010, 2011, and 2012 at the ESAREC, to evaluate the field efficacy of tolfenpyrad on *L. decemlineata* larvae. Potato seed pieces ‘Superior’ were planted on 25 March, 13 April, and 21 March in 2010, 2011, and 2012,

respectively. Each trial was set up in a randomized complete block design; in 2010 and 2011 each treatment was replicated four times and in 2012 each treatment was replicated 6 times. Individual plots consisted of two rows of potato 0.9 m apart, 6 m long, with plants spaced 30 cm down the row. Three suggested rates of tolfenpyrad were evaluated 153, 186, and 230 g ai/ha. For each experiment, two foliar applications of insecticides were applied one week apart upon the first observation of small larvae in the field. Applications of insecticides were made on 11 and 18 May, 20 and 27 May, and 12 and 21 May in 2010, 2011 and 2012, respectively. In 2010, the tolfenpyrad rates evaluated were compared to a commercial standard, β-cyfluthrin, as well as a non-treated control. In all three years applications were applied using a CO_2 powered backpack sprayer equipped with a four nozzle boom with flat spray tips (110003 VS) spaced 50.8 cm apart at 2.721 atm. Each treatment was evaluated by counting the number of *L. decemlineata* small and large larvae found on 10 randomly chosen potato stems in each plot. Defoliation was measured as a percentage in each plot through visual estimation after larval feeding had ceased on 14 Jun, 10 Jun, and 6 Jun in 2010, 2011, and 2012, respectively. Yield was evaluated by mechanical harvest and tubers were graded by size according to US standards (Grade B, small A, large A, and Chef) (USDA 2011). Potato tubers were harvested on 1 July, 13 July, and 28 Jun in 2010, 2011, and 2012, respectively.

Statistical analysis. Leaf-dip bioassays were analyzed with standard Probit analysis using statistical software, GraphPad Prism, version 5 (Motulsky 2007), to determine the tolfenpyrad LC_{50} and 95% confidence limits for small larvae and adult *L. decemlineata*. Abbott's formula was used to correct for control mortality 15% and higher up to 27% (Abbott 1925).

Data from the field experiments were analyzed using JMP 10 software (SAS 2013). *Leptinotarsa decemlineata* larval counts, percentage defoliation, and marketable yield were analyzed using ANOVA procedures. Insect numbers were square root (x + 0.05) transformed prior to analysis. Defoliation data were arc sine, square root transformed prior to analysis. Mean comparisons were conducted using Fisher's LSD at the $P \leq 0.05$ level of significance. Untransformed data were reported in all tables.

Results

Leaf dip bioassays. Tolfenpyrad was highly toxic to *L. decemlineata* larvae and adults with corresponding LC_{50} values of 13 and 164 ppm, respectively. The 95% confidence intervals for the LC_{50} levels are 10.0 - 16.0 ppm for the larvae and 101.0 - 266.0 ppm for the adults. Thus, tolfenpyrad was approximately 12 times more toxic to larvae than adults. Figure 1 shows the combined concentration-mortality response of *L. decemlineata* populations to tolfenpyrad in Painter VA. The adult and larvae r^2 values were 0.9663 and 0.9584, respectively. Nonetheless, at the proper application rate, this novel insecticide should provide control of both stages of *L. decemlineata* in the field.

Egg mass bioassays. Laboratory bioassays evaluating the ovicidal activity of tolfenpyrad proved to be successful. The high field rate of tolfenpyrad resulted in a 0% hatch rate and the control of water had 100% of the egg masses hatch 5 DAT. It is important to note, of all the treated egg masses only a single egg hatched (Table 1).

Field trials. In the 2010 field season, *L. decemlineata* pressure was moderate with an average of 70 larvae per 10 vines in the untreated control plots. There was a significant treatment effect on numbers of *L. decemlineata* larvae (Table 2). In general, all tolfenpyrad treatments provided effective control of *L. decemlineata*. Three insect counts were conducted

recording the number of small and large larvae on 10 randomly chosen stems per plot. The first count on 17 May indicated significantly less small larvae in the tolfenpyrad treated plots compared to the non-treated plots ($F = 5.69$; df = 5, 15; $P = 0.0039$). On 24 May, there were significantly fewer small larvae ($F = 6.39$; df = 5, 15; $P = 0.0023$) and large larvae ($F = 18.71$; df = 5, 15; $P < 0.0001$) in plots treated with tolfenpyrad compared to the untreated control. There was also a significant treatment effect on defoliation ($F = 9.97$; df = 5, 15; $P = 0.0002$) (Table 2). These data mirrored those of the larvae counts. Plots with the 230 g ai/ha rate of tolfenpyrad and the tolfenpyrad mixed with β-cyfluthrin treatment yielded significantly more marketable potato tubers compared to the control plots ($F = 3.09$; df = 5, 15; $P = 0.0411$).

The 2011 field season had similar results to the 2010 field season. There was a significant treatment effect on numbers of *L. decemlineata* larvae. All treatments of tolfenpyrad provided effective control (Table 3). There was a significant treatment effect for small larvae ($F = 6.06$; df = 3, 9; $P = 0.0152$) and large larvae ($F = 6.08$; df = 3, 9; $P = 0.0153$) on 26 May and on 2 Jun ($F = 31.05$; df = 3, 9; $P < 0.0001$) ($F = 9.90$; df = 3, 9; $P = 0.0033$), respectively. There was also a significant treatment effect on defoliation ($F = 23.01$; df = 3, 9; $P = 0.0001$) (Table 3). Plots treated with tolfenpyrad had significantly less defoliation than the control. There was no significant treatment effect on tuber yield.

The 2012 field season was similar to both the 2010 and 2011 seasons; in general, all treatments of tolfenpyrad provided effective control of *L. decemlineata* larvae (Table 4). Plots treated with tolfenpyrad had a significant treatment effect for small ($F = 19.20$; df = 3, 15; $P < 0.0001$) and large larvae ($F = 53.40$; df = 3, 15; $P < 0.0001$) on 18 May. On 29 May, there was a significant treatment effect on small larvae ($F = 5.11$; df = 3, 15; $P = 0.0124$) and large larvae ($F = 46.28$; df = 3, 15; $P < 0.0001$). There was also a significant treatment effect on defoliation (F

= 70.24; df = 3, 15; $P < 0.0001$), mirroring the larval counts (Table 4). Unlike 2011, there was a significant treatment effect on yield (F = 18.61; df = 3, 15; $P < 0.0001$) and the tolfenpyrad treated plots produced significantly more marketable potato tubers than any of the other treatments.

Discussion

Leptinotarsa decemlineata has demonstrated a high propensity for developing resistance to insecticides. Specifically, *L. decemlineata* is resistant to all or some of the compounds classified in the arsenical, organochlorine, carbamate, organophosphate, pyrethroid, and neonicotinoid classes of chemistry (Mota-Sanchez et al. 2006, Alyokhin 2008, Alyokhin et al. 2008). One strategy for delaying resistance development is to rotate insecticides, particularly with novel modes of action. Our research showed that the pyrazole-5-carboxamide insecticide, tolfenpyrad, is highly toxic to *L. decemlineata* eggs, larvae, and adults. The larval stage of *L. decemlineata* is more susceptible to tolfenpyrad than the adults. Larvae are also more susceptible than adults to many other insecticides including azadirachtin (Trisyono and Whalon 1999, Kowalska 2007, Kuhar et al. 2013), *Bacillus thuringiensis* subsp. *tenebrionis* (Ghidiu and Zehnder 1993, Kuhar et al. 2013), and cyromazine (Sirota and Grafius 1994, Linduska et al. 1996, Kuhar et al. 2013). In all three field trials conducted, *L. decemlineata* was successfully controlled by tolfenpyrad at the lowest rate tested, 153 g ai/ha.

Research has shown tolfenpyrad to be effective on a number of insect pests in a variety of crops. Tolfenpyrad is a broad spectrum insecticide that can control arthropod pests from the orders, Hemiptera, Thysanoptera, Lepidoptera, Coleoptera, Diptera, Orthoptera, and Acari (Anonymous 2012). For example, research by Kuhar et al. (2011a) showed significant fewer green peach aphids *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) on broccoli *Brassica*

oleracea L. treated with tolfenpyrad than the non-treated control. Kuhar et al. (2011b)also reported significantly fewer potato leafhopper nymphs, *Empoasca fabae* (Harris) (Hemiptera: Cicadellidae) in potato plots treated with tolfenpyrad compared to the non-treated control. Researchers in California demonstrated significantly fewer western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) and significantly more marketable heads, in lettuce *Lectuca sativa* var. *longifloria*, Lam. treated with tolfenpyrad than lettuce treated with just water (Natwick 2012). Research from Florida showed significantly fewer Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) adults 58 DAT and significantly fewer ACP nymphs 30 DAT on tolfenpyrad treated orange trees *Citrus sinensis* (L.) compared with non-treated orange trees (Stansly and Kostyk 2012). In a study by Burrack and Chapman (2012), researchers reported significantly fewer thrips in tobacco *Nicotiana tobacum* L. plots treated with tolfenpyrad than untreated tobacco plots resulting in significantly less incidence of tomato spotted wilt virus in the treated plots. Additionally tolfenpyrad has been shown to significantly control the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) in cotton (Peixoto et al. 2009).

Potato pest management, where *L. decemlineata* is present, relies heavily on insecticides. Typically potatoes are treated with a systemic insecticide at planting, usually a neonicotinoid in recent years, with rescue applications of foliar insecticides if needed. Our field trials showed that two foliar applications of tolfenpyrad provided season long protection from *L. decemlineata* pressure. The success of tolfenpyrad in the field and its novel mode of action make it an ideal candidate for resistance management of *L. decemlineata*. Potato producing regions, where *L. decemlineata* has shown resistance to neonicotinoids and other insecticides, could benefit from incorporating tolfenpyrad into pest management strategies.

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Figure caption

Figure 1. Baseline concentration-mortality response of *L. decemlineata* populations to tolfenpyrad. Plotted points are based on cumulative mortality of *L. decemlineata* adults and larvae for each concentration of tolfenpyrad from 2010 to 2012.

Table 1. Number of *L. decemlineata* egg masses that hatched after being exposed to tolfenpyrad (230 g ai/ ha) or a non-treated control (NTC) of water. Egg masses from Virginia were collected from a field population in Blacksburg, VA, in 2012. The remaining egg masses were from laboratory reared colonies established in Michigan and tested in Michigan in 2013.

	Number of egg masses hatching/ten egg masses treated					
Treatment	Evans[z]	Evans[z]	Hadley[y]	New York[z]	New Jersey[x]	Virginia
NTC	10	10	10	10	10	10
Tolfenpyrad	0	0	0[w]	0	0	0

[z] Laboratory populations resistant to imidacloprid.

[y] Laboratory population resistant to thiamethoxam.

[x] Laboratory susceptible population.

[w] A single egg hatched out of the ten egg masses tested.

Table 2. Mean ± SEM of *L. decemlineata* larvae, percent defoliation, and yield in potatoes plots treated with foliar insecticides. All treatments were sprayed on 11 and 18 May in Painter, VA, 2010.

		Mean no. *L. decemlineata* / 10 stems					
		17-May (6 DAT 1)		24-May (6 DAT 2)			
Treatment	Rate (g ai/ha)[z]	Sm. Larv[y]	Lg. Larv[x]	Sm. Larv	Lg. Larv	% Defoliation	Yield (kg / m)
NTC		60.0 ± 16.5 a	12.0 ± 8.7	47.0 ± 21.4 a	30.3 ± 6.3 a	48.8 ± 11.7 a	3.4 ± 0.2 a
Tolfenpyrad	153	10.8 ± 5.6 bc	0.3 ± 2.2	3.5 ± 1.2 b	0.0 ± 2.5 c	7.5 ± 2.7 b	4.2 ± 0.2 bc
Tolfenpyrad	186	16.3 ± 6.2 bc	0.0 ± 2.1	0.0 ± 4.3 b	0.0 ± 2.5 c	7.0 ± 4.2 b	4.3 ± 0.3 bc
Tolfenpyrad	230	12.5 ± 5.1 bc	0.8 ± 1.7	2.8 ± 5.5 b	1.5 ± 1.5 c	7.8 ± 1.8 b	4.4 ± 0.4 bc
Tolfenpyrad +	153 +	5.3 ± 6.5 c	0.8 ± 1.6	0.0 ± 4.3 b	1.3 ± 2.0 c	3.5 ± 4.6 b	4.7 ± 0.3 c
β-cyfluthrin	14						
β-cyfluthrin	14	30.8 ± 8.6 ab	4.3 ± 2.5	6.0 ± 6.6 b	9.3 ± 2.3 b	15.5 ± 2.1 b	3.6 ± 0.3 ab
P-Value from Anova		0.0039	ns	<0.0023	<0.0001	0.0002	0.0411

[z] All treatments received 0.25% v:v non-ionic surfactant.

[y] Values followed by the same letter are not significantly different according to Fisher's LSD, α=0.05.

[x] ns = not significant

Table 3. Mean ± SEM of *L. decemlineata* larvae, percent defoliation, and yield in potatoes plots treated with foliar insecticides. All treatments were sprayed on 20 and 27 May in Painter, VA, 2011.

		Mean no. *L. decemlineata* / 10 stems					
		26-May (8 DAT 1)		2-Jun (8 DAT 2)			
Treatment	Rate (g ai/ha)	Sm. Larv[z]	Lg. Larv	Sm. Larv	Lg. Larv	% Defoliation	Yield (kg / m)[y]
NTC		27.0 ± 8.5 a	44.0 ± 12.2 a	14.0 ± 3.1 a	17.0 ± 4.7 a	23.75 ± 4.4 a	2.0 ± 0.8
Tolfenpyrad	153	2.0 ± 3.4 b	3.0 ± 6.2 b	0.0 ± 1.0 b	0.0 ± 1.1 b	1.25 ± 2.2 b	3.1 ± 1.0
Tolfenpyrad	186	0.0 ± 2.7 b	13.0 ± 8.1 b	0.0 ± 1.0 b	0.0 ± 1.1 b	0.0 ± 1.1 b	2.8 ± 0.4
Tolfenpyrad	230	0.0 ± 2.7 b	0.0 ± 6.1 b	0.0 ± 1.0 b	2.0 ± 2.8 b	0.0 ± 1.1 b	1.8 ± 0.5
P-Value from Anova		0.0153	0.0152	<0.0001	0.0033	0.0001	ns

[z] Values followed by the same letter are not significantly different according to Fisher's LSD, α=0.05.

[y] ns = not significant

Table 4. Mean ± SEM of *L. decemlineata* larvae, percent defoliation, and yield in potatoes plots treated with foliar insecticides. All treatments were sprayed on 11 and 21 May in Painter, VA, 2012.

		Mean no. *L. decemlineata* / 10 stems					
		18-May (7 DAT 1)		29-May (8 DAT 2)			
Treatment	Rate (g ai/ha)[z]	Sm. Larv[y]	Lg. Larv	Sm. Larv	Lg. Larv	% Defoliation	Yield (kg / m)
NTC		127.0 ± 17.4 a	35.5 ± 5.5 a	19.8 ± 5.7 a	25.5 ± 4.2 a	73.33 ± 6.2 a	3.8 ± 0.2 a
Tolfenpyrad	153	20.2 ± 10.6 b	1.2 ± 2.0 b	4.2 ± 3.5 b	1.3 ± 1.5 b	5.8 ± 2.2 b	4.8 ± 0.1 b
Tolfenpyrad	186	8.7 ± 5.3 b	0.2 ± 1.8 b	0.8 ± 2.4 b	1.7 ± 1.5 b	6.7 ± 2.0 b	4.9 ± 0.1 b
Tolfenpyrad	230	12.0 ± 5.0 b	0.2 ± 1.7 b	3.0 ± 1.9 b	1.0 ± 1.3 b	5.8 ± 2.5 b	5.3 ± 0.1 b
P-Value from Anova		<0.0001	<0.0001	0.0124	<0.0001	0.0001	<0.0001

[z] All treatments received 0.25% v:v non-ionic surfactant.

[y] Values followed by the same letter are not significantly different according to Fisher's LSD, α=0.05.

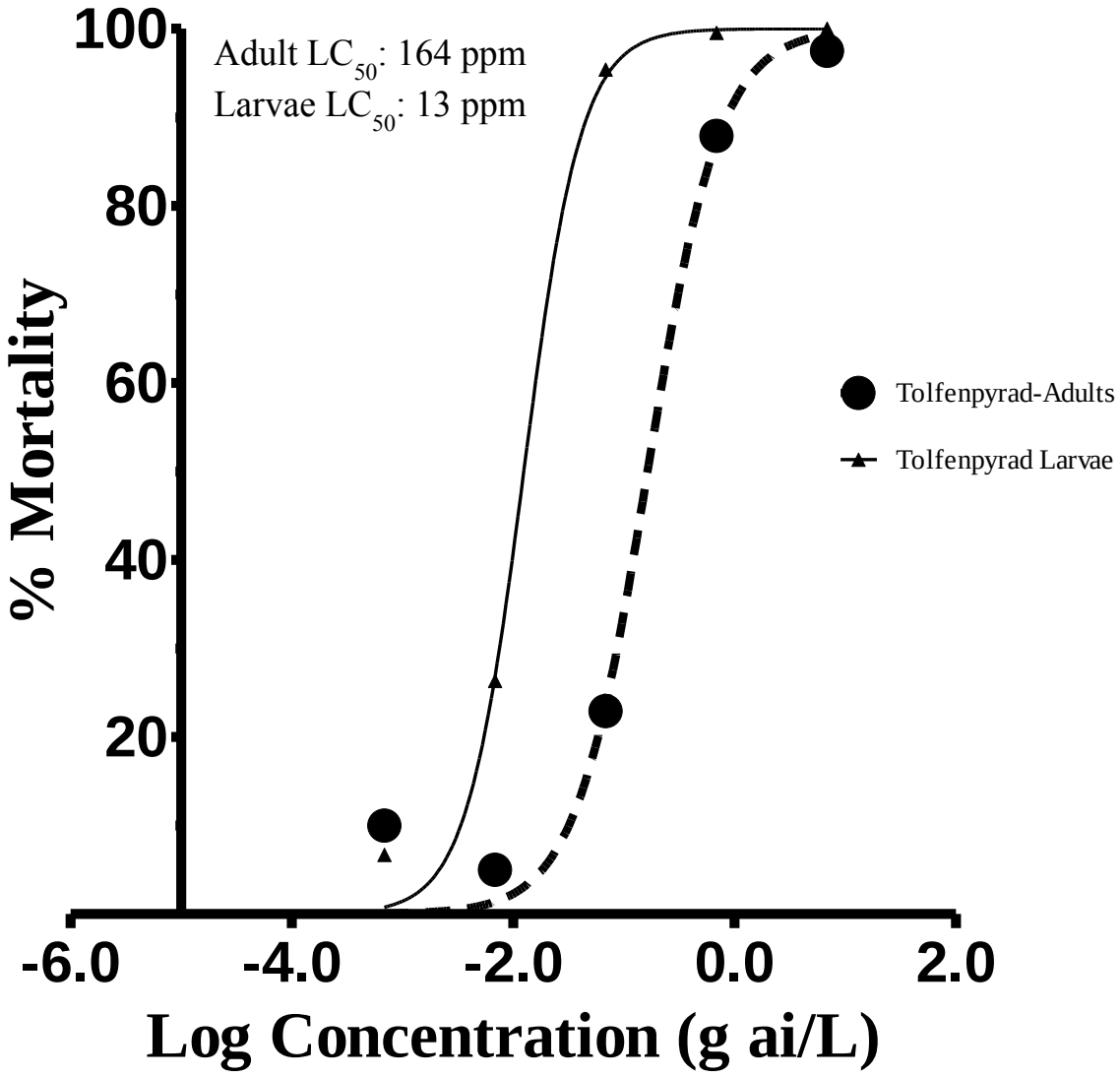

Figure 1. Baseline concentration-mortality response of *L. decemlineata* populations to tolfenpyrad. Plotted points are based on cumulative mortality of *L. decemlineata* adults and larvae for each concentration of tolfenpyrad from 2010 to 2012.

Chapter 3:

Resistance evaluation of *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) populations on the Eastern Shore of Virginia and North Carolina

Abstract. *Leptinotarsa decemlineata* (Say) is the most important insect defoliator of potato in both North America and Europe. Management of this pest relies heavily on the use of insecticides and can be problematic because of resistance development in the pest. In 2012, populations of *L. decemlineata* were collected from the Eastern Shore of VA (Cheriton, New Church, and Painter) and subjected to toxicity assays to determine the present day LC_{50} values of permethrin and oxamyl. These values were compared to those from the same locations >20 years, when the beetle was resistant to the aforementioned insecticides. The 2012 LC_{50} values from the Eastern Shore of VA indicated an increase in toxicity of permethrin in *L. decemlineata* larvae (LC_{50} = 3.931 g ai/L) and an increase in toxicity of oxamyl in adult beetles (LC_{50} = 9.695 g ai/L) compared with LC_{50} values previously reported in 1990. In 2012, multiple populations of *L. decemlineata* were also evaluated for enzyme activity after exposure to sub-lethal concentrations of permethrin, oxamyl, and tolfenpyrad. Adult populations of *L. decemlineata* from Cheriton, VA, New Church, VA, Painter, VA, and Plymouth, NC were subjected to enzyme assays to measure the activity of P450s, GSTs, esterases, and protein content. Results from the enzyme assays indicated significantly greater esterase activity in beetles from Painter, VA exposed to permethrin [α-naphthol ($F= 11.66$, $df= 4, 20$, $P<0.0001$) and β-naphthol ($F= 11.86$, $df= 4, 20$, $P<0.0001$)], oxamyl [α- naphthol ($F= 10.64$, $df= 4, 20$, $P<0.0001$) and β-naphthol ($F= 6.94$, $df= 4, 20$, $P=0.0011$)], tolfenpyrad [α- naphthol ($F= 407.62$, $df= 1, 8$, $P<0.0001$) and β- naphthol ($F= 28.15$, $df= 1, 8$, $P= 0.0007$)], and the untreated control [α-naphthol ($F= 28.14$, $df= 3, 16$, $P<0.0001$) and β- naphthol ($F= 28.86$; $df= 3, 16$, $P<0.0001$)]

compared to most of the other populations tested. GST activity was significantly greater in tolfenpyrad exposed beetles compared to the non-treated beetles from Painter VA ($F= 17.66$, $df= 5, 24$, $P< 0.0001$). Resistance management of *L. decemlineata* needs to be evaluated on an individual farm basis; making management recommendations on a regional scale would be a gross underestimation of the variability that can occur from field to field.

Key words P450, General Esterases, Resistance, *Leptinotarsa decemlineata*, GST

Introduction

Since the 1950s, Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), has developed resistance to over 50 different insecticides (Alyokhin et al. 2008). This major pest of potato, *Solanum tuberosum* L., has been shown to use a number of mechanisms that confer resistance to insecticides including: enhanced metabolism using esterases, carboxylesterases and monooxygenases, target site insensitivity, reduced penetration, increased excretion, and behavioral changes (Rose and Brindley 1985, Argentine et al. 1989, Ioannidis et al. 1991, Ioannidis et al. 1992, Wierenga and Hollingworth 1994, Anspaugh et al. 1995, Hoy and Head 1995, Zhu et al. 1996, Lee and Clark 1998, Alyokhin and Ferro 1999, Clark et al. 2001, Alyokhin et al. 2008). The most common mechanism that *L. decemlineata* utilizes for detoxification of pesticides is the cytochrome P450 monooxygenase (P450) pathway (Alyokhin et al. 2008). One region that has been notorious for resistance problems in *L. decemlineata* is the Eastern Shore of Virginia (Tisler and Zehnder 1990), where most of VA potato production occurs. The insecticide resistance status of Virginia's *L. decemlineata* populations is out of date and needs to be re-evaluated. Providing growers with an updated insecticide resistance profile for *L. decemlineata* populations will help growers to select the

proper management tools and ensure potato production remains profitable for growers in the state.

Metabolic mechanisms that confer insecticide resistance can be classified into two pathways, increased metabolic activity and target site insensitivity (Hemingway et al. 2004). Our research focuses on the metabolic factors that can lead to insecticide resistance, specifically cytochrome P450 mono-oxygenase (P450), general esterase, and glutathione *S*-transferase (GST) activities. Cytochrome P450 mono-oxygenases are important detoxification enzymes in living organisms. They are phase I enzymes that can oxidize both endogenous and exogenous compounds (Li et al. 2007). Detoxification of insecticides via P450s are accomplished via oxidation reactions (Li et al. 2007). Research has shown that insect P450s can detoxify compounds in the pyrethroid, neonicotinoid and carbamate classes of insecticides (Scott 1999). Using a technique developed by Ulrich and Weber (1972), and later modified by Chauret et al. (1999), for use with microassays, the P450 activity of insects can be determined using the substrate 7-ethoxycoumarin (7-EC).

General esterases are hydrolase enzymes capable of hydrolyzing compounds with ester bonds, which in some cases, can reduce the toxicity of a compound as well as prepare it for excretion from the insect (Li et al. 2007). Many insects have demonstrated increased esterase activity in response to pyrethroid, organophosphate, and carbamate insecticides (Hemingway et al. 2004). One method to test the general esterase activity within an insect is to measure the esterase activity on artificial substrates such as α- and β-naphthyl acetate. These substrates are hydrolyzed into α- and β- naphthol and acetic acid, by insect esterases, and when in the presence of fast blue B and SDS a strong blue color is emitted with a maximum absorbance of 600 nm (Jin-Clark et al. 2008). The intensity of the blue color is directly proportional to the amount of

esterase activity which can be quantified using a spectrophotometer (van Asperen 1962). Esterase activity of *L. decemlineata* can be determined by exposing the proteins (enzymes) of the insects to α- and β-naphthyl acetate and using a spectrophotometer to measure the absorbance of the blue color emitted from the remaining concentrations of α- and β- naphthol (Jin-Clark et al. 2008, Srigiriraju et al. 2009).

Glutathione *S*-transferases (GSTs) are phase II metabolic enzymes found in living organisms that allow for the metabolism of multiple electrophilic substrates (Li et al. 2007). In general, the function of GSTs is thought to be the detoxification of endogenous and exogenous compounds either directly or by catalyzing the secondary metabolism of a monooxygenase or esterase-based resistance mechanism (Hemingway 2000, Hemingway et al. 2004). Glutathione *S*-transferases promote conjugation reactions that prepare substrates for elimination from the body. The overall reaction is a glutathione conjugation reaction and the conjugated products are usually more water soluble and easily excreted (Li et al. 2007). Insect resistance via GST activity was first documented for organophosphate insecticides, but GSTs are active on chlorinated hydrocarbons, pyrethroids, and play an important role in detoxifying reactive oxygen species common from oxygen metabolism (Hemingway et al. 2004). These reactions are important for the removal of toxic compounds that are electrophilic.

Research has shown resistance levels in *L. decemlineata* populations can vary within geographic regions (Ioannidis et al. 1991). There is variability in the metabolic processes used to detoxify insecticides with a large amount of overlap between chemical class and metabolic pathway conferring resistance (Hemingway 2000, Li et al. 2007). Herein, we evaluate the current resistance levels of VA populations of *L. decemlineata* to permethrin and oxamyl. In

addition we will profile the metabolic enzyme activity of different *L. decemlineata* populations exposed to sub-lethal doses of these compounds as well as to the novel insecticide tolfenpyrad.

Materials and Methods

Insecticides and *L. decemlineata* populations. Commercial insecticides formulated for agricultural use were used for all experiments and included the following: 1) permethrin (Permethrin 3.2 EC (38.4% ai) Helena Chemical Company, Collierville, TN; 2) oxamyl (Vydate 2L (24% ai) DuPont Crop Protection, Wilmington, DE; and 3) tolfenpyrad (Torac EC (15% ai) Nichino America, Wilmington, DE. The latter insecticide is a mitochondrial electron transport inhibitor that is currently not registered for use in the U.S. (Nichino America, Inc. 2012). The concentrations of each compound used in the bioassays can be seen in Table 1.

Table 2 lists the *L. decemlineata* populations used in the bioassays and the compounds each population was exposed to in 2012.

For each of the enzyme assays, five random adult beetles from each population and each treatment were homogenized. For example, in the Painter VA population, 5 untreated beetles were used, 5 permethrin-exposed beetles via leaf-dip (LD), 5 permethrin-exposed beetles via beetle-dip (BD), 5 tolfenpyrad-exposed beetles, 5 oxamyl-exposed beetles via LD, and 5 oxamyl-exposed beetles via BD. The sub-lethal concentrations for each compound used in the enzyme assays can be seen in Table 1.

Adult toxicity assays. In 2012, multiple *L. decemlineata* populations were exposed to concentrations of oxamyl, permethrin, and tolfenpyrad for toxicity evaluation (Table 2). Bioassays evaluating permethrin and oxamyl toxicity for adult and first instars of *L. decemlineata* were set up following the methods of Tisler and Zehnder (1990). Adult *L.*

decemlineata were exposed by completely submerging groups of ten beetles into various concentrations of permethrin and oxamyl for 30 sec. After 30 sec, the exposed adults were placed onto clean paper towels to absorb excess moisture and then placed into Petri dishes and evaluated for mortality 24 hrs after exposure. Three populations from the Eastern Shore of Virginia (New Church, Painter, and Cheriton) were used to determine the LC_{50} values for permethrin and oxamyl based on adult mortality. Adults pooled from each location (n = 120) were exposed to each concentration of permethrin and oxamyl.

Leaf-dip toxicity assays. Additional adult toxicity assays were conducted using leaf-dip (LD) exposure methods in 2012. Populations of *L. decemlineata* from Painter, VA were exposed to potato leaves treated with each of the following: permethrin, oxamyl, and tolfenpyrad. Unblemished potato leaves were collected from insecticide-free potato plots and completely submerged in different concentrations of oxamyl, permethrin, and tolfenpyrad. Treated leaves were allowed to dry prior to beetle exposure. A non-treated control of water was used in all LD bioassays. The concentrations of permethrin and oxamyl used in the LD bioassays were the same concentrations used in the beetle-dip bioassays (Table 1). Mortality of adult *L. decemlineata* exposed to permethrin or oxamyl was evaluated 24 hrs after exposure. Tolfenpyrad bioassays were only conducted via the LD method. Adults from Painter, VA and Northampton County VA were exposed to potato leaves treated with 5 serial dilutions of tolfenpyrad and a non-treated control of water (Table 1). Adult beetles were assessed for mortality 72 hrs after exposure.

Larval toxicity assays. In 2012, *L. decemelineata* larvae were exposed to permethrin and oxamyl through a contact bioassay via treated filter paper according to the methods described by Tisler and Zehnder (1990). Egg masses were collected from multiple locations and

observed for hatching. After hatching the neonates were isolated for 24 hrs prior to being utilized in a bioassay. Serial concentrations of oxamyl and permethrin were prepared with acetone (Table 1). A 0.5-mL volume of each compound at each concentration was pipetted onto filter paper discs and allowed to dry. A non-treated control of acetone was used in all larval assays. Each treated filter paper was fitted into Petri dishes and 0.5 mL of water was pipetted onto each treated filter paper once and ten *L. decemlineata* neonates were added to each dish. Mortality was assessed 24 hrs after insecticide exposure. For the larval bioassays the same three locations were used as in the adult assays and the LC_{50} values were calculated based on mortality levels of 340 and 330 individuals, pooled from each location, exposed to each concentration of permethrin and oxamyl, respectively.

Protein standard. In 2011, a standard curve was established based on bovine serum albumin (BSA) using the bicinchoninic acid (BCA) assay discussed by Smith et al. (1985). The standard was prepared at a concentration of 1000 µg/mL of BSA in deionized water. Once the standard was prepared, seven protein concentrations were mixed by mixing 0, 5, 10, 15, 25, 35, 45 µL of the standard (BSA at 1000 µg/mL) with 100, 95, 90, 85, 75, 65, and 55 µL of 0.1M sodium phosphate (pH7.8) containing 0.3% Triton X-100, respectively. The mixtures corresponded to seven protein concentrations 0, 50, 100, 150, 250, 350, and 450 µg / mL. Twenty microliters of each protein concentration was pipetted into a 96-well flat bottom microplate along with 180 µL of a protein determination reagent (10 mL of BCA solution and 200 µL of 4% cupric sulfate solution 50:1 v:v). There were three replicates for each of the seven protein concentrations. The microplate was then covered and incubated at 37 ^{o}C for 30 min. After incubation, the microplate was cooled for 5 min at room temperature (~27 ^{o}C) and was then read using a Dynex Triad kinetic microplate reader (Dynex Technologies, Chantilly, VA).

Cytochrome P450 monooxygenase standard. In 2011, a standard curve was made using a stock solution of 5.0 mg of umbelliferone in 5.0 mL of acetone. The stock solution was diluted to a final concentration of 0.4 ng/µL with 0.04% (v:v) acetone. Serial concentrations of umbelliferone were created and added to the wells of a 96 well flat bottom microplate. Each concentration was replicated three times and the concentrations ranged from 0 µL to 16 µL of umbelliferone. Using 0.1 M phosphate buffer, the volume for each microplate well was adjusted to 20 µL for each concentration. The wells received 120 µL of acetonitrile 50% (v:v) with 0.05 M TRIZMA-base buffer and then read using a Dynex Triad kinetic microplate reader.

Esterase standards. In 2011, a stock solution of α-naphthol was made by mixing 10.2 mg of α-naphthol into 5 mL of acetone yielding a concentration of 2 µg / µL. The solution was diluted into 0.1 M sodium phosphate buffer by mixing 0.1 mL of α-naphthol into 9.9 mL of buffer giving a final concentration of 0.02 µg / µL. Serial dilutions were prepared by mixing α-naphthol and 0.1 M sodium phosphate buffer containing 0.3% Triton X-100 and different concentrations of 0.1 M sodium phosphate buffer containing 1% acetone. There were 8 dilutions containing 0, 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, and 2.6 µg of α-naphthol, each dilution was replicated three times. Once mixed, the dilutions of α-naphthol were pipetted into a 96 well flat bottomed microplate and 50 µL of fast blue B-SDS were added to each well. The samples developed for 15 min at room temperature and the optical density was read at 600 and 560 nm using a Dynex Triad Kinetic microplate reader. A standard curve was also made for β-naphthol following the exact procedure for α-naphthol.

Protein. In 2012, the protein content was determined for four insect populations (Cheriton VA, Painter VA, New Church VA, and Plymouth NC) exposed to three insecticides following the methods discussed by Smith et al. (1985). Five insect specimens from each

population exposed to each insecticide were homogenized individually in 750 µL of 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3% v/v Triton X-100. Insect homogenates were centrifuged at 10,000 g for 5 min and 500 µL of the remaining supernatant were collected and stored on ice. Twenty µL of the insect preparations were pipetted into individual wells of a 96-well flat bottom microplate and then 180 µL of BCA-cupric sulfate solution were added to each of the wells. Three replicates of each insect preparation were used in the assay. The microplate was incubated just like the standards and after 5 min at room temperature the plate was read using a microplate reader. The protein concentration of each sample was calculated using the standard linear regression of the BSA standard curve.

P450. Prior to running the experiments, a number of chemical solutions were prepared. The first solution, 50 mM 7-EC, was prepared by dissolving 19.02 mg of 7-EC into 2.0 mL of acetone. Another solution, 62.5 mM β-NADPH, was made by dissolving 5.2 mg of β-NADPH into 100 µL of deionized water (dH_2O). A 100 mM oxidized glutathione solution was made by mixing 61.26 mg of oxidized glutathione in 1.0 mL of dH_2O.

In 2012, experiments followed the general procedures used by Anderson and Zhu (2004). Insect homogenates from the protein assays were used in the P450 assays. Twenty microliters of the insect preparations were added to a 96-well flat bottomed microplate with three replications per insect (60 µL total). A control of 20 µL of 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3% v/v Triton X-100 was replicated 3 times. A solution was prepared by mixing 100 µL of the 62.5 mM β-NADPH, 50 µL of 50 mM 7-EC, and 4.85 mL of 0.1 M phosphate buffer. Each microplate well received 80 µL of the 7-EC and β-NADPH mixture and then the microplate was agitated for 15 min at room temperature on an Eppendorf mixer (400 rpm) (Eppendorf North America, Hauppauge, NY). After incubation, 10 µL of oxidized glutathione

and 10 µL of glutathione reductase were added to each well. The microplate was held at room temperature for another 10 min. After the second incubation period 120 µL of the acetonitrile and TRIZMA-base buffer solution were added to each well and the plate was read using a Dynex Triad kinetic microplate reader. Relative fluorescence units (RFUs) were calculated to determine the P450 activity of each sample using the following equation:

RFU = (fluorescence of each sample) / (protein mg / mL)

Esterase. In 2012, general esterase activity was measured according to the procedures of Srigiriraju et al. (2009), which was modeled after the methods of van Asperen (1962). Esterase activity was determined using the insect preparations from the protein assay. Once supernatant was collected 15 µL of each sample were pipetted into a 96 well flat bottom microplate and replicated 3 times. A control of 15 µL of 0.1M sodium phosphate buffer (pH 7.8) containing 0.3% v/v Triton X-100 was replicated 3 times. A 0.3 mM solution of α-naphthyl acetate was diluted by adding 0.1 mL of 30 mM α-naphthyl acetate into 9.9 mL of sodium phosphate buffer and 135 µL were added to each well containing the insect supernatant. The microplate was incubated at 37^{o}C for 30 min. After incubation, 50 µL of fast blue B-SDS were added to each well and developed for 15 min at room temperature. The methods for β-naphthyl acetate followed the methods described for α-naphthyl acetate. The microplate was read using a Dynex Triad Kinetic microplate reader. The amount of hydrolytic product of each sample was determined using the standard linear regression of the α- and β-naphthol standard curves. The specific activity of general esterase activity was calculated using the following equation:

nmol/min/mg= 15.415 x µg α- or β naphthol / (mg / mL protein)

GSTs. Using the method described by Yu (1982, 1984) GST activity in *L. decemlineata* was determined using a model substrate 3,4-dichloronitrobenzene (DCNB). Two stock solutions

were made, a 10 mM glutathione (GSH) solution and a 150 mM DCNB solution. Insect homogenates prepared in the protein assays were used in the GST assays. Once the supernatant was on ice, 3.7 mL of the 10 mM GSH and 50 µL of the 150 mM DCNB were pipetted into a 15 mL centrifuge tube. Once thoroughly mixed, the 180 µL of the GSH-DCNB solution were pipetted into individual wells of a 96 well flat bottom microplate. Aliquots of 20 µL of the insect preparations (supernatant) were added to the microplate wells containing the GSH-DCNB solution. Three replicates of each insect preparation and three replicates of the control, 20 µL of 0.1 M sodium phosphate buffer containing 0.3% v/v Triton X-100, were read at 340 nm at 10 sec intervals for 10 min. Total GST activity was calculated in order to calculate the specific activity of GST for each sample using the following equations:

Total GST activity = ((Δ absorbanc/min)/ 9.6 mM^{-1} x 1 cm) x 10 (dilution factor) x 1000

Specific GST activity = total GST activity / mg protein / mL

Statistical analysis. Toxicity bioassays were analyzed with standard Probit analysis using statistical software, GraphPad Prism, version 5 (Motulsky 2007), to determine the permethrin and oxamyl LC_{50} values for small larvae and adult *L. decemlineata*. Differences in LC_{50} values between present day populations and those from 1990 were determined based on whether or not there were overlapping standard errors.

Data from the enzyme assays were analyzed using JMP 10 software (SAS 2013). General esterase specific activity, GST specific activity, protein concentration, and P450 RFUs were analyzed using ANOVA procedures. Mean comparisons were conducted using Fisher's LSD at the $P \leq 0.05$ level of significance.

Results

Resistance levels. Results from the bioassays comparing LC_{50} levels of present-day *L. decemlineata* populations from the Eastern Shore of Virginia to the 1990 populations for permethrin and oxamyl are shown in Figure 1. The LC_{50} values of *L. decemlineata* adults and larvae from each population in 2012 were averaged and compared to the average LC_{50} values reported.

Adult LC_{50} values for oxamyl in 2012 were not significantly different from those reported by Tisler and Zehnder (1990). However, there was a significant decrease in the oxamyl LC_{50} value in 2012 larvae compared with the LC_{50} value for the larvae in 1990 (Fig. 1A). Results from the permethrin bioassays showed that *L. decemlineata* adults collected in 2012 had a significantly lower LC_{50} value compared to the adults in 1990. However, there was no significant difference in LC_{50} value for larvae exposed to permethrin in 2012 compared to the larvae LC_{50} value in 1990 (Fig. 1B).

Enzyme activity. In 2012, enzyme assays were conducted on populations of adult *L. decemlineata* from different locations in VA and a location from NC (Table 2). Enzyme activity and protein content were compared in two ways; first, populations from different locations were compared separately by insecticide (Tables 3 to6). Secondly, the activity for the different enzyme pathways (P450, GST, esterases, and protein) were compared across locations based on insecticide exposure (Figs. 2 to5).

The Painter, VA populations of *L. decemlineata* showed significant treatment effects for β-napthol ($F= 2.79$, $df= 5, 24$, $P= 0.0399$), GST activity ($F= 17.66$, $df= 5, 24$, $P< 0.0001$), P450 activity ($F= 2.75$, $df= 5, 24$, $P= 0.0421$), and protein content ($F= 3.13$, $df= 5, 24$, $P= 0.0258$) (Table 3). GST activity was significantly greater in those beetles exposed to permethrin LD,

oxamyl LD and tolfenpyrad compared to the non-treated beetles. Those beetles that were exposed to permethrin BD, and tolfenpyrad had significantly greater β-naphthol than those beetles exposed to oxamyl LD. In those beetles exposed to permethrin LD, the P450 activity was significantly greater than most of the other treatments (Table 3).

The population of *L. decemlineata* collected from New Church, VA had significant treatment effects for α-Naphthol ($F= 5.83$, $df= 2, 12$, $P= 0.0170$), β-Naphthol ($F= 23.81$, $df= 2, 12$, $P< 0.0001$), and GST activity ($F= 14.67$, $df= 2, 12$, $P= 0.0006$) (Table 4). Beetles exposed to oxamyl BD from this location had significantly greater levels of α- and β-naphthol compared to the other treatments. Also, GST activity was significantly greater in the non-treated beetles compared to those beetles exposed to permethrin BD and oxamyl BD.

The *L. decemlineata* populations collected from Cheriton, VA had a significant treatment effect for protein content only ($F= 4.38$, $df= 3, 16$, $P= 0.0197$) (Table 5). The untreated beetles from Cheriton, VA had significantly more protein content than those beetles exposed to permethrin BD and tolfenpyrad.

There was no significant treatment effect for the population of *L. decemlineata* collected from Plymouth, NC (Table 6).

Results from the enzyme assays conducted in 2012, evaluating the different locations where *L. decemlineata* populations were collected, for esterase, GST, and P450 activities are shown in Figures 2 to 5. Results from the non-treated beetles varied according to enzyme assay. Those beetles from Painter, VA showed significantly greater esterase activity compared with the other populations assayed, α- naphthol ($F= 28.14$, $df= 3, 16$, $P<0.0001$) and β- naphthol ($F= 28.86$; $df= 3, 16$, $P<0.0001$) (Fig. 2 A, B). However, GST activity was significantly greater in the population from Plymouth, NC ($F= 16.00$, $df= 3, 16$, $P<0.0001$) compared to the other

populations tested (Fig. 2 C). The populations from Plymouth NC and Painter, VA had significantly greater P450 activity compared with the population from Cheriton, VA ($F= 3.63$, $df= 3, 16$, $P=0.0360$) (Fig. 2 D). There were no significant differences among the untreated populations tested for protein content.

Enzyme activity of the *L. decemlineata* populations exposed to tolfenpyrad showed the Painter, VA population had significantly greater esterase activity compared to the Cheriton, VA population tested, α- naphthol ($F= 407.62$, $df= 1, 8$, $P<0.0001$) and β- naphthol ($F= 28.15$, $df= 1, 8$, $P= 0.0007$) (Fig. 3 A, B). There were no significant differences in GST and P450 activity between the Painter, VA and Cheriton, VA populations exposed to tolfenpyrad (Fig. 3 C, D). However, there was significantly greater protein content in the Painter, VA population compared to the Cheriton, VA population ($F= 8.14$, $df= 1, 8$, $P= 0.0214$).

Results from the enzyme assays evaluating permethrin exposed populations of *L. decemlineata* are shown in Figure 4. In these assays an additional population from Painter, VA was evaluated based on the exposure method to permethrin, a LD population. Results from the esterase assays showed significantly greater esterase activity in the two Painter VA populations compared to the other populations α-naphthol ($F= 11.66$, $df= 4, 20$, $P<0.0001$) and β-naphthol ($F= 11.86$, $df= 4, 20$, $P<0.0001$) (Fig. 3 A, B). The Plymouth, NC and the LD Painter, VA populations had significantly greater GST activity compared to the other populations exposed to permethrin ($F= 12.43$, $df= 4, 20$, $P<0.0001$) (Fig. 4 C). The P450 activity of the populations exposed to permethrin was greatest in the LD Painter VA population and significantly greater than the Cheriton, VA and New Church, VA populations ($F= 3.20$, $df= 4, 20$, $P=0.0349$) (Fig. 4 D). The protein content among the populations exposed to permethrin was greatest in the New Church, VA population ($F= 3.32$, $df= 4, 20$, $P=0.0307$).

Enzyme activity of the *L. decemlineata* populations exposed to oxamyl can be seen in Figure 5. Like the permethrin assays, an additional population from Painter, VA was evaluated based on the exposure method to oxamyl, a LD population. Esterase activity was significantly greater in the LD Painter, VA and the BD Painter, VA populations for α- naphthol ($F= 10.64$, $df= 4, 20$, $P<0.0001$) (Fig. 5 A). However, the β- naphthol activity was greatest in the BD Painter, VA population compared to the other populations tested ($F= 6.94$, $df= 4, 20$, $P=0.0011$) (Fig. 5 B). The GST activity among the populations exposed to oxamyl was significantly greater in the LD Painter, VA and the Plymouth, NC populations exposed to oxamyl ($F= 89.98$, $df= 4, 20$, $P<0.0001$) (Fig. 5 C). The LD Painter, VA and Plymouth, NC populations had significantly more P450 activity compared to the Cheriton, VA and BD Painter, VA populations ($F= 5.83$, $df= 4, 20$, $P=0.0028$) (Fig. 5 D). There was no significant difference in protein content among the populations exposed to oxamyl.

Discussion

Leptinotarsa decemlineata has the ability to overcome insecticides labeled for its control. Current management practices of *L. decemlineata* in potato almost solely rely on the utilization of chemical insecticides. This has led to the development of resistance in this pest to almost every compound labeled for its control (Alyokhin et al. 2008). Without an adequate management program, *L. decemlineata* populations can cause up to 50% loss of yield in potato in VA (Kuhar et al. 2006, 2008, Kuhar and Doughty 2009, 2010)and in China as well (Jiang et al. 2010). A responsible pest management program for *L. decemlineata* should include insecticide resistance management practices to minimize insecticide resistance development. Detection of resistant populations and then understanding the mechanism of resistance are vital for managing *L. decemlineata* when resistant populations are suspected. The purpose of this research was to

evaluate the current resistance levels of *L. decemlineata* populations in the Eastern Shore of VA compared to the resistance levels in 1990 and to determine the specific activities of different enzyme pathways when *L. decemlineata* populations were exposed to sub-lethal doses of permethrin, oxamyl, or tolfenpyrad.

Results from the toxicity assays comparing the LC_{50} values of Eastern Shore *L. decemlineata* populations from 2012 and 1990 varied by compound and beetle life stage. The results indicated a significant decrease of LC_{50} value in adult beetles exposed to permethrin in 2012 compared to 1990. However, the LC_{50} value of the permethrin-exposed larvae in 2012 was not significantly different from the LC_{50} value in 1990; most likely due to the high amount of variability observed within those particular assays (Fig 1 B). In the toxicity assays measuring the LC_{50} value of oxamyl, there was a significant decrease in the larval LC_{50} value in 2012 compared to 1990. The results from the 2012 oxamyl-exposed adults did not show a significant difference in LC_{50} value compared to the 1990 LC_{50} value. One possible explanation of why we saw a decrease in LC_{50} value of oxamyl exposed larvae and not adults is that adults typically are more robust than the larvae and also may have more developed detoxification systems (Silcox et al. (1985). Oxamyl and permethrin are two compounds that *L. decemlineata* populations on the Eastern Shore of VA have historically shown resistance to (Tisler and Zehnder 1990). The resistance to permethrin and oxamyl coupled with the introduction of the neonicotinoid insecticides allowed growers on the Eastern Shore of VA to stop using these compounds. The lack of selection pressure from these compounds should logically result in *L. decemlineata* populations that are more susceptible to oxamyl and permethrin than when these chemicals were frequently applied to potatoes. Alyokhin et al. (2008), suggested that once growers stop using a particular insecticide that a population of *L. decemlineata* is resistant to, there would most likely

be a decline in the alleles that confer that resistance; however, the rate of that decline is unknown. One of the recommendations for managing resistance in this pest is to include an untreated area in a potato field to allow susceptible beetles to survive and contribute to the genetic diversity of the population, preventing the development of homozygously resistant populations (Alyokhin et al. 2008). This resistance management strategy is based on the need for keeping susceptible populations of *L. decemlineata* in the gene pool of a given field population. Research by Whalon and Ferro (1998), suggested 20% of a potato field untreated with insecticides should provide enough survival of a susceptible population to decrease mating between resistant beetles. Although it is unlikely growers on the Eastern Shore of Virginia incorporated untreated portions in their fields, it is likely permethrin and oxamyl were not applied to potato fields because the beetle had become resistant to those compounds and newer more effective insecticides became available. The results from our toxicity assays determined there is a decline in resistance to permethrin and oxamyl in *L. decemlineata* populations from the Eastern Shore of VA after 20 years of non-selection.

Our results from the enzyme assays conducted seem to follow the trend that can be seen in the literature regarding metabolic enzyme activity as potential resistance mechanisms to insecticides. When evaluating the data that examines the enzyme activity for a given population of *L. decemlineata*, regardless of insecticide exposure, we see variability between locations and within populations from a location. A few examples of this variability in enzyme activity can be seen in China (Jiang et al. 2010), Michigan (Ioannidis et al. 1991), and Massachusetts (Argentine et al. 1989). We saw significantly greater esterase activity in the population from Painter, VA compared to most of the other locations examined. This was the case for the untreated beetles and permethrin-treated beetles regardless of exposure method, tolfenpyrad-treated beetles, and

oxamyl treated beetles. Research has shown inhibiting the esterase detoxification pathway (Jiang et al. 2010) and the P450 pathway (Silcox et al. 1985) can increase pyrethroid toxicity in *L. decemlineata*. Increased toxicity of pyrethroids when inhibiting esterases or P450s implicates these detoxification pathways as potential resistance mechanisms to pyrethroid insecticides. The untreated beetles from Painter, VA had significantly greater GST activity than untreated beetles from Plymouth, NC. When comparing beetles exposed to oxamyl we observed significantly greater P450 activity in those populations from Painter VA LD-exposed and Plymouth NC, compared to those populations from Cheriton VA and Painter VA BD exposed.

When we compare enzyme activity of the populations from each location based on insecticide exposure we see another level of variability. For example, when examining the results from the Painter VA location, beetles exposed to permethrin via LD compared to those beetles exposed to permethrin via BD we see significantly greater GST activity in the LD beetles. There appears to be no obvious trend in metabolic activity and insecticide exposure. Painter, VA populations exposed to tolfenpyrad had significantly greater GST activity when compared to the untreated beetles but significantly lower GST activity compared to the LD exposed permethrin and LD oxamyl treated beetles. In those beetles collected from Cheriton, VA, there were no significant differences in esterase, GST, or P450 activities among beetles exposed to permethrin, tolfenpyrad, and oxamyl compared to the untreated beetles. The research shows that resistance mechanisms utilized by *L. decemlineata* cannot be generalized based on insecticide or on location and that resistance to a certain insecticide class does not implicate a specific resistance mechanism is responsible. In other words, insecticide resistant *L. decemlineata* populations from individual farms, rather than regions, need to be evaluated to make conclusions about which resistance mechanism is being employed.

Our research identified GST activity as a potential pathway for resistance development in beetles exposed to tolfenpyrad in Painter, VA. Tolfenpyrad is a novel insecticide class with a novel mode of action. Tolfenpyrad is currently not a registered insecticide in the US; however, it is registered for use on vegetables in Japan (Anonymous 2012). This early realization of a potential mechanism for resistance development should help with developing insecticide resistance management (IRM) approaches.

Our research showed *L. decemlineata* adults exposed to permethrin had significantly greater esterase activity in the Painter, VA location compared to the other locations. When compared to the untreated beetles from Painter, VA, there was no significant difference in esterase activity. These results suggest other factors may be contributing to increased esterase activity. An example of other factors contributing to changes in metabolic activity can be seen in a study by Zhang et al. (2008). Researchers evaluated the effect of diet on the enzyme activity of *L. decemlineata* and reported changes in P450 activity based on changes in diet. Elevated esterase activity seen in the Painter VA populations may be an ominous indication of potential resistance development. Esterase detoxification pathways and subsequent resistance development have been implicated in a number of insect species resistant to organophosphate, carbamate, and pyrethroid classes of insecticides (Li et al. 2007).

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Figure captions

Figure 1. A.) Mean LC_{50} values (g ai/L) of oxamyl for Eastern Shore of VA populations of *L. decemlineata* in 1990 and 2012, (*) indicates significance. B.) Mean LC_{50} values (g ai/L) of permethrin for Eastern Shore of VA populations of *L. decemlineata* in 1990 and 2012, (*) indicates significance.

Figure 2. Enzyme activity for untreated *L. decemlineata* populations in 2012. A.) α-Naphthol specific activity expressed in nmol / min / mg protein. B.) β-Naphthol specific activity expressed in nmol / min / mg protein. C.) Specific activity of GST expressed in nmol / min / mg protein. D.) Relative fluorescent units of P450 activity (fluorescence / (mg protein / mL).

Figure 3. Enzyme activity for tolfenpyrad exposed *L. decemlineata* populations in 2012. A.) α-Naphthol specific activity expressed in nmol / min / mg protein. B.) β-Naphthol specific activity expressed in nmol / min / mg protein. C.) Specific activity of GST expressed in nmol / min / mg protein. D.) Relative fluorescent units of P450 activity (fluorescence / (mg protein / mL).

Figure 4. Enzyme activity for permethrin exposed *L. decemlineata* populations in 2012. A.) α-Naphthol specific activity expressed in nmol / min / mg protein. B.) β-Naphthol specific activity expressed in nmol / min / mg protein. C.) Specific activity of GST expressed in nmol / min / mg protein. D.) Relative fluorescent units of P450 activity (fluorescence / (mg protein / mL).

Figure 5. Enzyme activity for oxamyl exposed *L. decemlineata* populations in 2012. A.) α-Naphthol specific activity expressed in nmol / min / mg protein. B.) β-Naphthol specific activity expressed in nmol / min / mg protein. C.) Specific activity of GST expressed in nmol / min / mg protein. D.) Relative fluorescent units of P450 activity (fluorescence / (mg protein / mL).

Table 1. Insecticide rates evaluated in bioassays conducted on the Eastern Shore of VA investigating *L. decemelineata* resistance and toxicity in 2012.

Permethrin (g ai/L)	Oxamyl (g ai/L)	Tolfenpyrad (g ai/L)
0.0	0.0	0
0.06	0.09	0.000685
0.31	0.47[y]	0.00685
1.53[z]	2.37[z]	0.0685
7.67	11.86	0.685[x]
38.34	59.3	6.85

[z] Survivors frozen at -80 ^{o}C for enzyme assays.

[y] Survivors frozen at -80 ^{o}C for enzyme assays leaf-dip exposed only.

[x] Survivors frozen at -80 ^{o}C for enzyme assays.

Table 2. *Leptinotarsa decemlineata* populations and the insecticides exposed to each population and life stage in 2012.

Population	Adults	Larvae
Painter, VA	Tolfenpyrad, Permethrin, Oxamyl	Permethrin, Oxamyl
New Church, VA	Permethrin, Oxamyl	Permethrin, Oxamyl
Cheriton, VA	Tolfenpyrad, Permethrin, Oxamyl	Permethrin, Oxamyl
Plymouth, NC	Permethrin, Oxamyl	Permethrin, Oxamyl

Table 3. Mean esterase, glutathione-*S*- transferase (nmol / min / mg protein), cytochrome P450 monooxygenase activity (fluorescence units / mg protein) and protein content (mg protein / mL) for the Painter VA population of *L. decemlineata* in 2012 (n = 5).

Insecticide[z]	α-Naphtol[y]	β-Napthol[x]	GST	P450	Protein
Untreated	25.19	13.34 a	0.35 c	75.10 bc	3.13 abc
Permethrin	27.77	14.33 a	4.08 bc	86.16 ab	2.87 bc
Permethrin LD	25.23	11.46 ab	20.90 a	101.79 a	2.64 c
Tolfenpyrad	22.06	12.48 a	8.11 b	72.95 bc	3.57 ab
Oxamyl	20.92	12.02 ab	4.69 bc	57.20 c	3.88 a
Oxamyl LD	21.44	9.29 b	26.81 a	83.40 bc	3.06 bc
P-Value	ns	0.0399	<0.0001	0.0421	0.0258

[z] LD indicates the insecticide was administered via leaf-dip method.

[y] ns = not significant

[x] Means followed by the same letter are not significantly different according to Fisher's LSD, $P \leq 0.05$.

Table 4. Mean esterase, glutathione-*S*- transferase (nmol / min / mg protein), cytochrome P450 monooxygenase activity (fluorescence units / mg protein) and protein content (mg protein / mL) for the New Church, VA population of *L. decemlineata* in 2012 (n = 5).

Insecticide	α-Naphtol[z]	β-Napthol	GST	P450[y]	Protein
Untreated	15.34 b	7.01 b	8.83 a	65.09	3.52
Permethrin	15.52 b	7.14 b	0.47 b	62.44	3.53
Oxamyl	16.16 a	8.46 a	2.50 b	65.90	3.49
P-Value	0.0170	<0.0001	0.0006	ns	ns

[z] Means followed by the same letter are not significantly different according to Fisher's LSD, $P \leq 0.05$.

[y] ns = not significant

Table 5. Mean esterase, glutathione-*S*- transferase (nmol / min / mg protein), cytochrome P450 monooxygenase activity (fluorescence units / mg protein) and protein content (mg protein / mL) for the Cheriton, VA population of *L. decemlineata* in 2012 (n = 5).

Insecticide	α-Naphtol[z]	β-Napthol	GST	P450	Protein[y]
Untreated	13.71	8.42	5.73	54.70	3.59 a
Permethrin	14.37	8.45	5.02	66.39	3.34 b
Tolfenpyrad	14.43	8.10	4.40	65.26	3.35 b
Oxamyl	13.88	7.79	5.48	63.81	3.43 ab
P-Value	ns	ns	ns	ns	0.0197

[z] ns = not significant

[y] Means followed by the same letter are not significantly different according to Fisher's LSD, $P \leq 0.05$.

Table 6. Mean esterase, glutathione-*S*- transferase (nmol / min / mg protein), cytochrome P450 monooxygenase activity (fluorescence units / mg protein) and protein content (mg protein / mL) for the Plymouth, NC population of *L. decemlineata* in 2012 (n = 5).

Insecticide	α-Naphtol[z]	β-Napthol	GST	P450	Protein
Untreated	15.28	8.03	26.32	76.07	3.34
Permethrin	16.60	8.80	24.76	79.61	3.20
Oxamyl	16.24	8.50	25.89	77.57	3.19
P-Value	ns	ns	ns	ns	ns

[z] ns = not significant

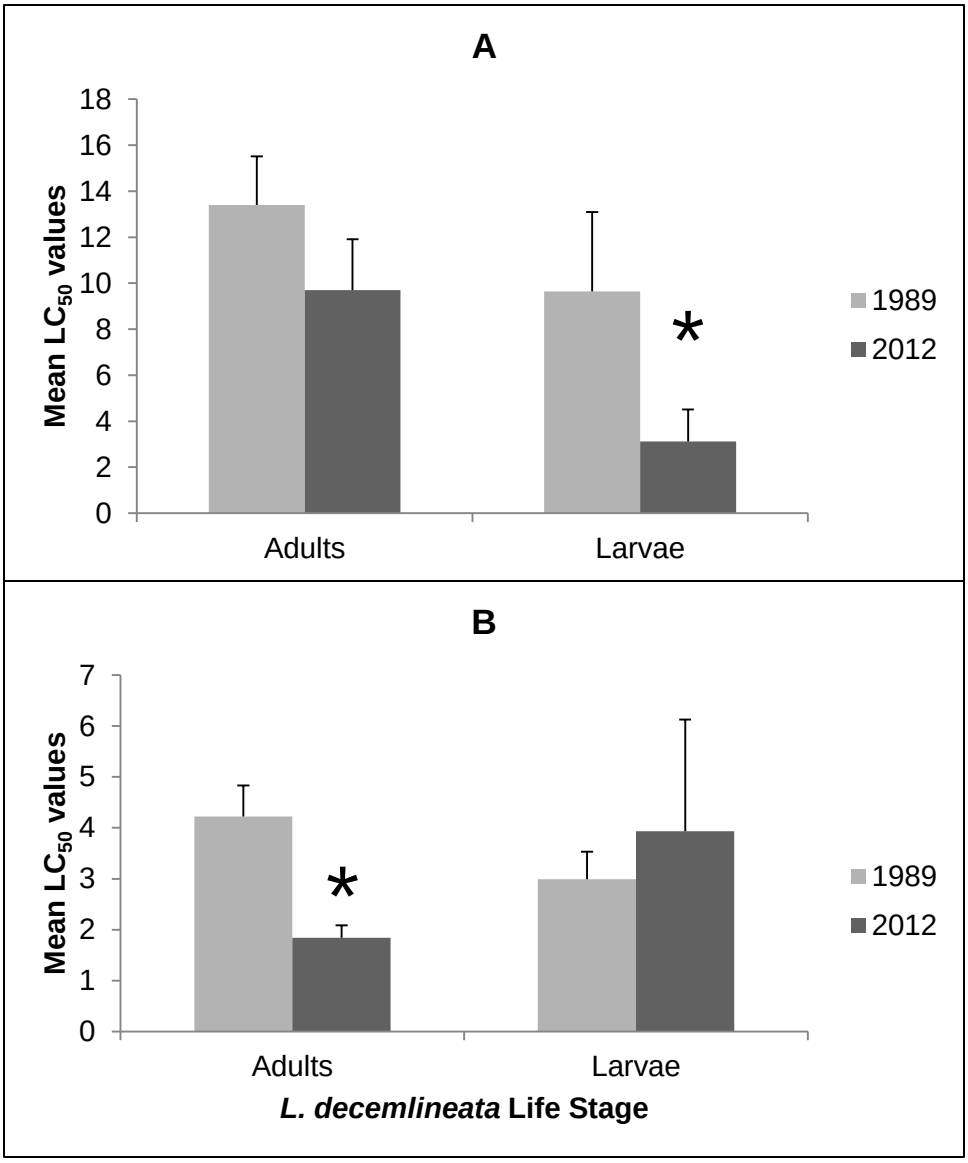

Figure 1. A.) Mean LC_{50} values (g ai/L) of oxamyl for Eastern Shore of VA populations of *L. decemlineata* in 1990 and 2012, (*) indicates significance. B.) Mean LC_{50} values (g ai/L) of permethrin for Eastern Shore of VA populations of *L. decemlineata* in 1990 and 2012, (*) indicates significance.

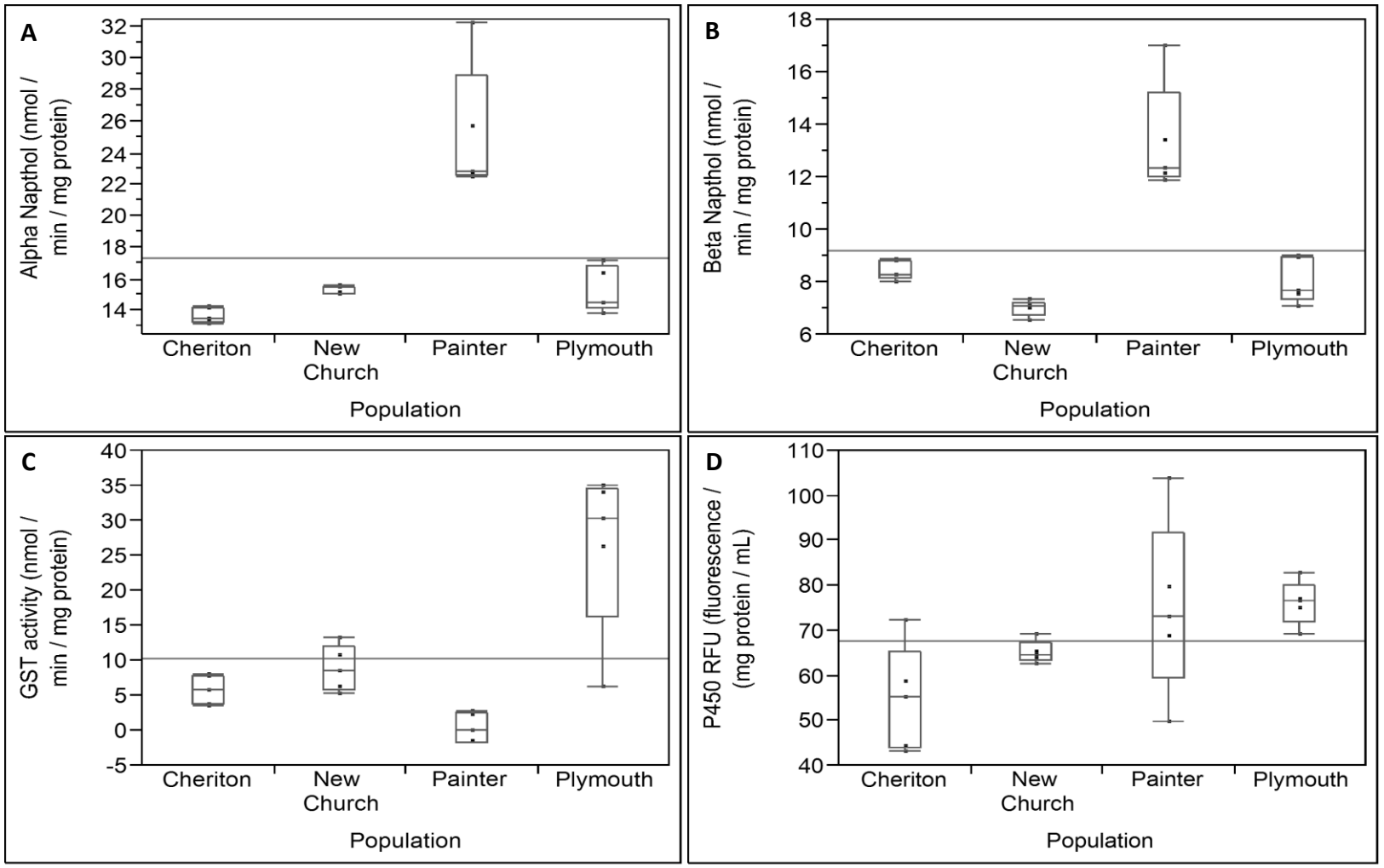

Figure 2. Enzyme activity for untreated *L. decemlineata* populations in 2012. A.) α-Naphthol specific activity expressed in nmol / min / mg protein. B.) β-Naphthol specific activity expressed in nmol / min / mg protein. C.) Specific activity of GST expressed in nmol / min / mg protein. D.) Relative fluorescent units of P450 activity (fluorescence / (mg protein / mL).

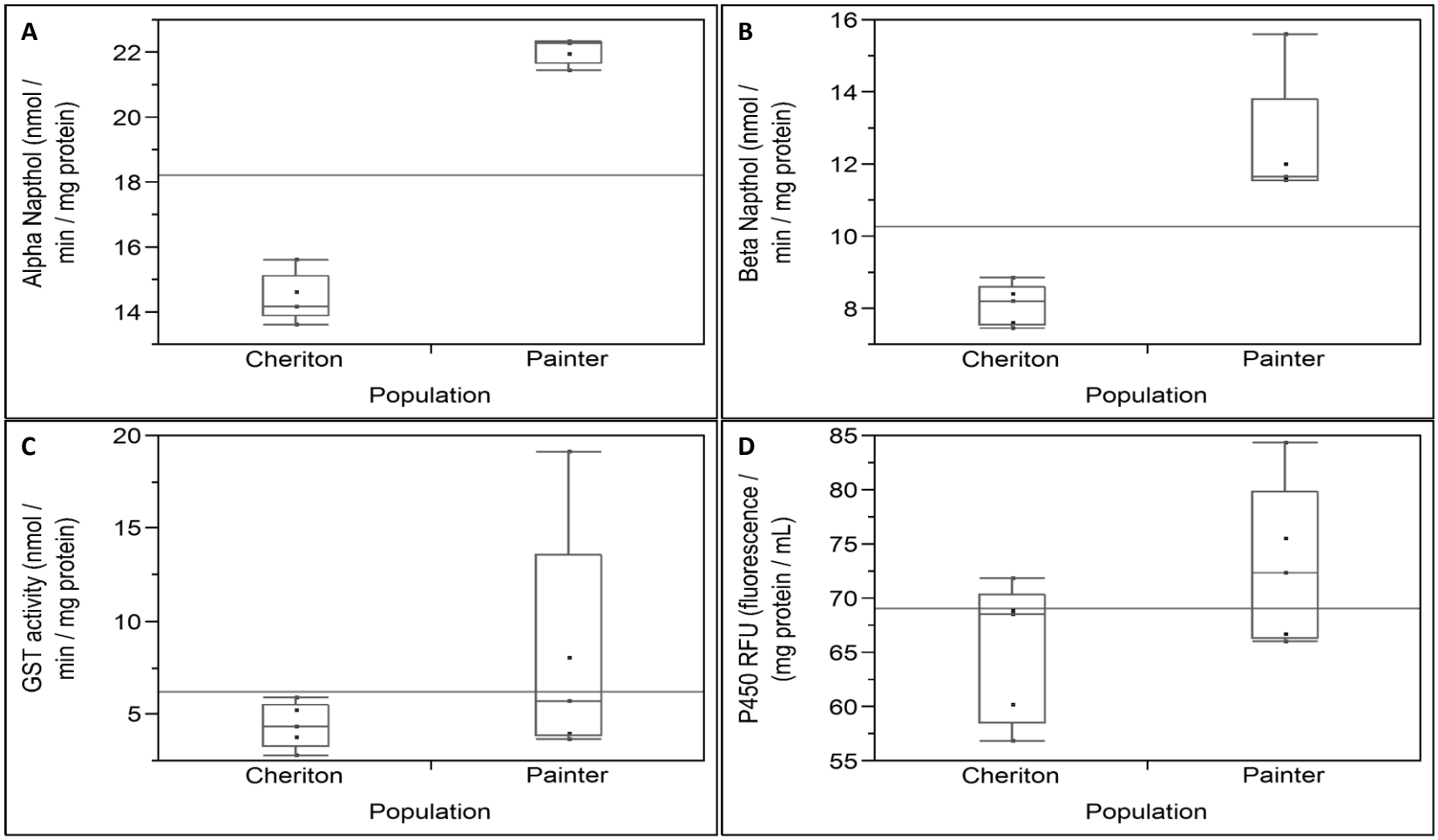

Figure 3. Enzyme activity for tolfenpyrad exposed *L. decemlineata* populations in 2012. A.) α-Naphthol specific activity expressed in nmol / min / mg protein. B.) β-Naphthol specific activity expressed in nmol / min / mg protein. C.) Specific activity of GST expressed in nmol / min / mg protein. D.) Relative fluorescent units of P450 activity (fluorescence / (mg protein / mL).

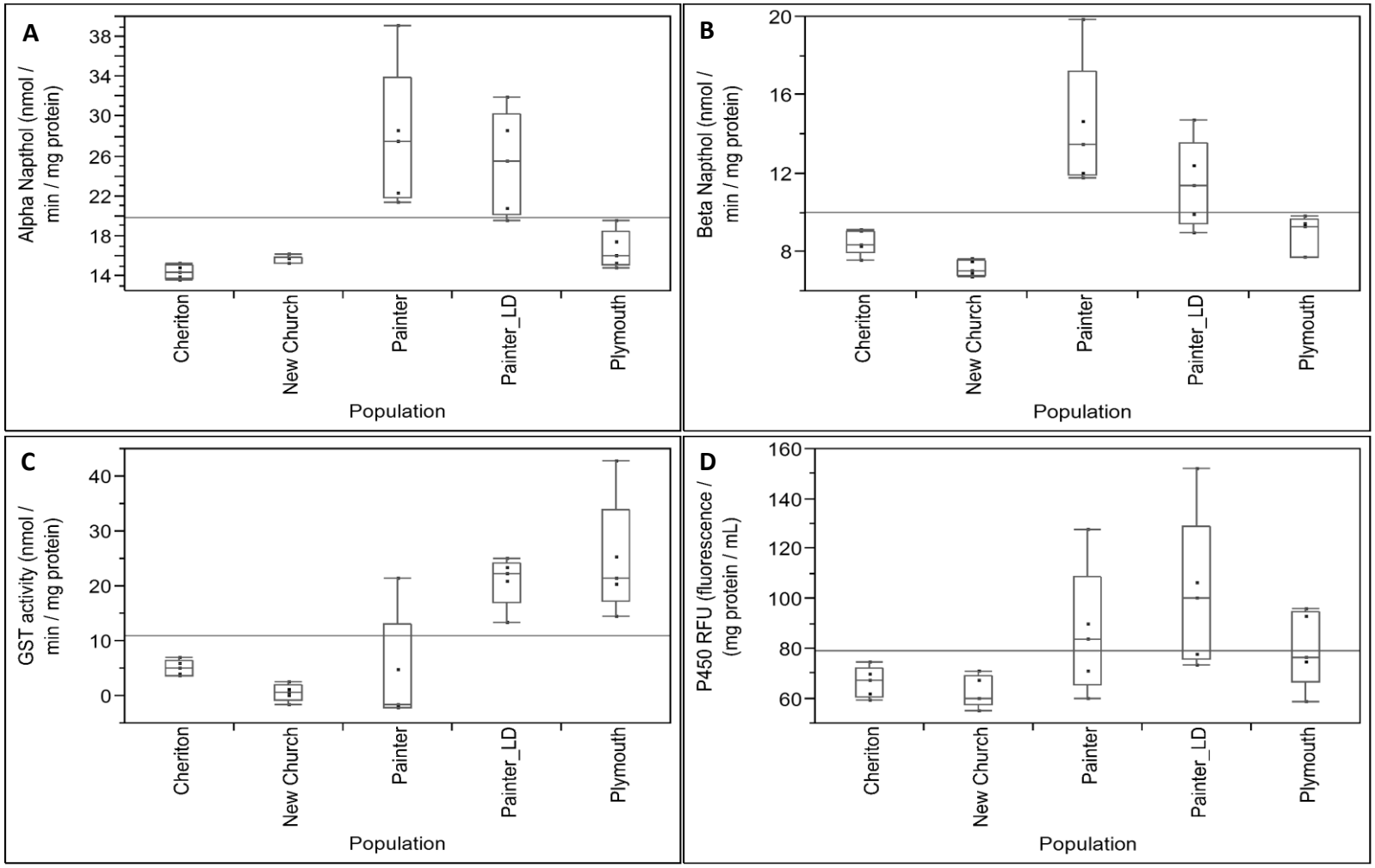

Figure 4. Enzyme activity for permethrin exposed *L. decemlineata* populations in 2012. A.) α-Naphthol specific activity expressed in nmol / min / mg protein. B.) β-Naphthol specific activity expressed in nmol / min / mg protein. C.) Specific activity of GST expressed in nmol / min / mg protein. D.) Relative fluorescent units of P450 activity (fluorescence / (mg protein / mL).

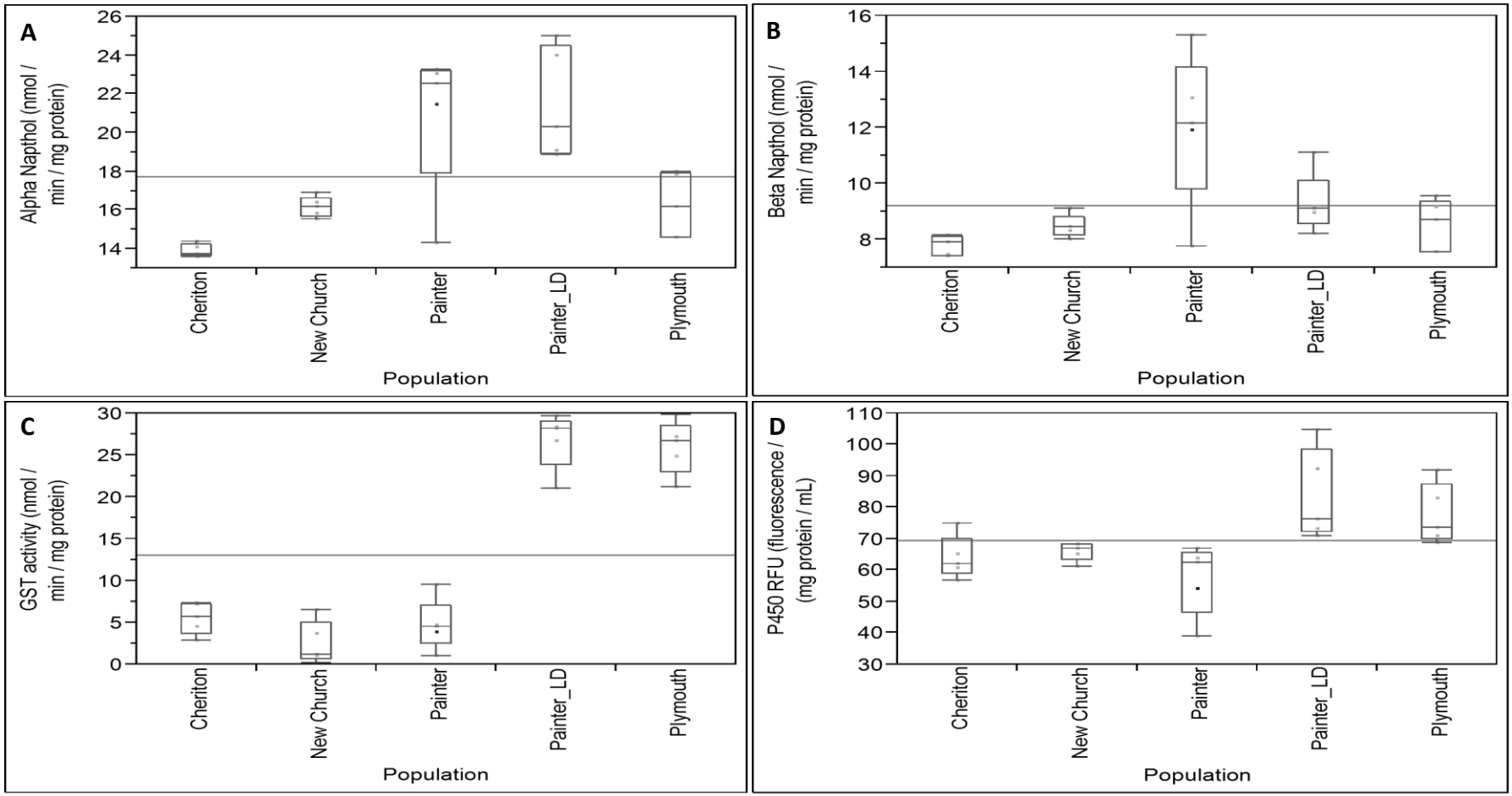

Figure 5. Enzyme activity for oxamyl exposed *L. decemlineata* populations in 2012. A.) α-Naphthol specific activity expressed in nmol / min / mg protein. B.) β-Naphthol specific activity expressed in nmol / min / mg protein. C.) Specific activity of GST expressed in nmol / min / mg protein. D.) Relative fluorescent units of P450 activity (fluorescence / (mg protein / mL).

Chapter 4:

Field efficacy of an experimental bio-pesticide containing metabolites of *Chromobacterium subtsugae* strain PRAA4-1T, for controlling Colorado potato beetle

Abstract. *Leptinotarsa decemlineata* (Say) is an important pest of potato *Solanum tuberosum* L. in many regions of the world, especially the US and Europe. Its ability to develop resistance to insecticides makes management of this pest difficult where resistant populations are present. One alternative is the use of bio-pesticides. Through laboratory assays and field experiments in potato, we evaluated the efficacy of a new bio-pesticide derived from the bacterium *Chromobacterium subtsugae* Martin et al. for the control of *L. decemlineata*. Results from the laboratory assay showed *L. decemlineata* feeding was inhibited by *C. subtsugae*. However, when the compound was taken to the field in 2010, 2011, and 2012, the results indicated *C. subtsugae* treated potato plots were not significantly different in controlling *L. decemlineata* compared to non-treated potato plots.

Key words Bio-pesticide, *Leptinotarsa decemlineata*, *Chromobacterium subtsugae*

Introduction

Chromobacterium subtsugae strain PRAA4-1T is a gram-negative, violet-pigmented bacterium that was isolated from soil under an eastern hemlock tree (*Tsuga canadensis* (L.)) in central Maryland (Environmental Protection Agency 2011). Scientists at the USDA-ARS discovered that this bacterium produces one or more active metabolites that possess insecticidal activity (Martin et al. 2004). In laboratory studies, the supernatant metabolite obtained from *C. subtsugae*, NRRL B-30655, was shown to be orally toxic to a wide range of insects including, *Leptinotarsa decemlineata* (Say), corn rootworm *Diabrotica* spp. (Coleoptera: Chrysomelidae), diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), whiteflies *Bemisia tabaci*

(Gennadius) (Hemiptera: Aleyrodidae), and the southern green stink bug, *Nezara viridula* L. (Hemiptera: Pentatomidae), (Martin et al. 2007). Additional testing showed that *Chromobacterium subtsugae* strain PRAA4-1T-treated diet resulted in reduced feeding in beet armyworm (*Spodoptera exigua*), cabbage looper (*Trichoplusia n*i), tobacco budworm (*Heliothis virescens*), diamondback moth, and southern corn rootworm, suggesting this microbe's insecticidal activity is due to reduction in weight or inhibition of feeding (Environmental Protection Agency 2011). In 2009, the supernatant of *C. substugae* NRRL B-30655 was formulated by Marrone Bio Innovations, Inc. (Davis, CA) into an experimental product called MBI-203. Research trials in Texas showed that the material was highly efficacious against potato psyllid providing 90% control. The objective of this study was to investigate the field efficacy of MBI 203 (*containing metabolites of C. subtsugae strain PRAA4-1T*), for control of *L. decemlineata*.

Materials and Methods

Laboratory bioassays and field efficacy experiments were conducted from 2010 to 2012 at the Virginia Tech Eastern Shore Agricultural Research and Extension Center (ESAREC) in Painter, VA. All specimens of *L. decemlineata* used in the experiments were collected from untreated potatoes (*Solanum tuberosum* L.) var. 'Superior' planted at the ESAREC, or occurred naturally on field plots located at the same location.

Insecticide. All formulations of MBI 203 were obtained from Marrone Bio Innovations, Inc. (Davis, CA). Formulations were changed in each year of the study at the discretion of Marrone Bio Innovations, Inc. The experiments in 2010 were conducted with commercially-formulated MBI 203 supernatant. In 2011, the experiments were conducted with a new formulation of MBI 203 that included an experimental microbial sunblock, MBI 501, in the

formulation. The experiments in 2012 were conducted with Grandevo^{TM}, a commercial granular formulation of MBI 203 (30% a.i.).

Leaf-dip bioassay, 2010. A potato leaf-dip bioassay was conducted on *L. decemlineata* small larvae in the laboratory following the methods described in Hitchner et al. (2012). The bioassay was set up on 17 May and included (dilutions of 1/10 and 1/20 of the formulated of MBI 203 product in water and a non-treated control of water. Four leaves were completely submerged in each treatment and allowed to air dry for > 1hr. Once dry, a single leaf was placed into a 9-cm diameter Petri dish along with 10 small larvae; a total of four dishes (40 insects) were tested per treatment per bioassay. Mortality of the larvae and the amount of feeding that had taken place was assessed at 4 days and 7 days after exposure.

Field-treated leaf bioassay, 2010. The objective of this bioassay was to evaluate the efficacy of MBI-203 after application in the field. Potatoes were planted on 25 March at the ESAREC. Seed pieces were spaced 0.3 m within rows and rows were spaced 0.9 m apart. On 10 Jun, potato plots consisting of two 6-m rows were sprayed with one of the following three treatments: MBI 203 (1/10 dilution), MBI 203 (1/10 dilution) mixed with MBI 501 (at 4% v:v), or an untreated control. All treatments were applied using a CO_2 powered backpack sprayer equipped with a four nozzle boom with flat spray tips (110003 VS) spaced 50.8 cm apart at 2.721 atm. At 0, 4, 9, 24, and 48 hours after treatment, a sample of four random leaves were collected from each plot. Once the leaves were collected, the leaf area was measured using a leaf area meter (LI-COR model no. LI-3100) (LI-COR, Lincoln, NE). Once the area was determined for each leaf, it was placed in a Petri dish along with 5 small *L. decemlineata* larvae. Leaf area was then reassessed after 48 hrs to estimate leaf consumption rate by the larvae.

Field efficacy experiments. Experiments assessing the efficacy of MBI-203 at controlling natural populations of *L. decemlineata* in the field were conducted in 2010, 2011, and 2012. Potato seed pieces 'Superior' were planted on 25 March, 13 April, and 21 March in 2010, 2011, and 2012, respectively. Each trial was set up in a randomized complete block design; in 2010 each treatment was replicated four times, and in 2011 and 2012 each treatment was replicated 6 times. Individual plots consisted of two rows of potato 0.9 m apart, 6 m long, with plants spaced 30 cm down the row. In 2011, the plot size was shortened to 4.6 m in order to include two additional plots per treatment. Two suggested rates of MBI 203 were evaluated in 2010 and 2011, 9.35 L/ha and 18.7 L/ha. In 2011, the MBI 203 formulation included the sunblock MBI 501. It is important to note, in 2011 insecticides applications were made at dusk in order to minimize UV exposure from the sun. In 2012, MBI 203 was formulated into the commercial granular product, Grandevo and was evaluated at 1.008 kg ai/ha. For each experiment, two foliar applications of insecticides were applied one week apart upon the first observation of *L. decemlineata* small larvae in the field. Applications of insecticides were made on 11 and 18 May, 18 and 25 May, and 12 and 21 May in 2010, 2011 and 2012, respectively. Foliar treatments were applied as described in the previous experiment. Beginning at 7 days after treatment, the numbers of live *L. decemlineata* small and large larvae were counted on 10 randomly chosen potato stems in each plot. Defoliation was visually estimated as a percentage after larval feeding had ceased on 14 Jun, 10 Jun, and 6 Jun in 2010, 2011, and 2012, respectively. Yield was evaluated by mechanical harvest and tubers were graded by size according to US standards (Grade B, small A, large A, and Chef) (USDA 2011). Potato tubers were harvested on 1 July, 13 July, and 28 Jun in 2010, 2011, and 2012, respectively.

Statistical analysis. Data from the field treated leaf bioassay and field experiments were analyzed using JMP 10 software (SAS 2013). Leaf-area consumed data was analyzed using ANOVA procedures, and means were separated using Fisher's LSD at the $P \leq 0.05$ level of significance.

Leptinotarsa decemlineata larval counts, percentage defoliation, and marketable yield were analyzed using ANOVA procedures. Larvae data were square root (x + 0.05) transformed prior to analysis. Defoliation data were arc sine, square root transformed prior to analysis. Mean comparisons were conducted using Fisher's LSD at the $P \leq 0.05$ level of significance. Untransformed data were reported in all tables.

Results

Leaf-dip bioassay, 2010. After 96 hours of exposure to treated leaves, mortality of *L. decemlineata* larvae averaged 10 and 23% in the 1/20 and 1/10 dilutions of MBI 203, respectively and 0% in the untreated control (Table 1). By 168 hrs after treatment, virtually all larvae had died in the MBI 203 treatments. Also very little feeding was observed on the potato leaves that were treated with MBI 203 as opposed to >50% of the leaf consumed in the control dishes (Table 1).

Field-treated leaf bioassay, 2010. There was a significant treatment effect on the amount of leaf area consumed by *L. decemlineata* larvae ($F = 29.49$, df = 2, 9, $P = 0.0001$). Potato leaves treated in the field with MBI 203 and MBI 203 + MBI 501 and collected 24 hrs later and exposed to larvae for 48 hrs had significantly less leaf area consumed compared to untreated leaves (Fig. 1).

Field efficacy experiments. In the 2010 field trial, *L. decemlineata* densities averaged 70 to 80 larvae (small and large) per 10 stems in the untreated control plots. There was no

significant treatment effect on numbers of *L. decemlineata* larvae on any sample date or on % defoliation and yield (Table 2). In 2011, as in the previous year, there was no significant treatment effect on numbers of *L. decemlineata* larvae on any sample date or on % defoliation and yield.

Experiments in 2012 testing the granular formulation also revealed no significant treatment effects on any variable.

Discussion

Leptinotarsa decemlineata is of great concern to potato growers where this pest occurs. Current control recommendations rely heavily on chemical insecticides for managing this pest. However, the history of *L. decemlineata* control can be characterized by success followed by failure due to its ability to develop resistance to insecticides. One alternative to synthetic chemical control includes using entomopathogens in an integrated pest management strategy. Benefits of using entomopathogens as control agents for insect pests include lower risk to non-target species, reduction of synthetic chemicals entering the environment, lower pesticide residues on crops, etc. The fungal entomopathogen *Entomophaga maimaiga* is a good example of providing control of gypsy moth *Lymantria dispar* L. (Lepidotera: Erebidae) while not greatly affecting other lepidopterans. Research by Hajek et al. (2000), reported low levels of *E. maimaiga* infection for non-target lepidopteran larvae compared to gypsy moth larvae. *Bacillus thuringiensis* use in agro-ecosystems for control of lepidopteran, coleopteran and dipteran pests allows for the survival of beneficial insects like predators, further increasing the impact on pest insects (Lacey et al. 2001). The use of a broad spectrum insecticide usually not only controls the target pest, but also disrupts naturally enemy populations. This disruption of natural enemies

changes the entire dynamic of the agro-ecosystem in question and can cause increased pest pressure from secondary pests (Lacey et al. 2001).

This research investigated the effectiveness of a bio-pesticide, *C. subtsugae* supernatant, at controlling *L. decemlineata*. Although a reduction in leaf feeding leading to death of larvae was evident in bioassays conducted in the laboratory, the same effectiveness was not realized in any field efficacy test with this material. After MBI 203 neither controlled *L. decemlineata* larvae nor reduced leaf feeding in the field, it was believed that photo-degradation of the material was inhibiting its efficacy. Photo-degradation can be a limiting factor with bio-pesticides. Research by Zhang et al. (2010), reported a greatly reduced half-life of the bio-pesticide pyoluteorin upon exposure to UV-irradiation. In the 2011 field experiment, applications of *C. subtsugae* supernatant were made at dusk to minimize the natural UV exposure from the sun. Also a sun blocking material was added to the formulation. Even with these adjustments, the *C. subtsugae* bio-pesticide still performed poorly in the field. The formulation changed again in 2012, but the *C. subtsugae* bio-pesticide remained ineffective at controlling *L. decemlineata*. The laboratory results indicate there is anti-feeding activity in *L. decemlineata* exposed to this bio-pesticide. Research indicates there is potential for the *C. subtsugae* bio-pesticide; however; further research is needed to understand some of the underlying factors that could be contributing to the lack of efficacy when evaluating this compound in the field. It is possible that the *C. subtsugae* bio-pesticide although effective against potato pysllid and southern green stink bug is not an effective compound for managing *L. decemlineata*, a formidable pest for even conventional pesticides to control.

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Figure caption

Figure 1. Potato leaf consumption by *L. decemlineata* larvae exposed to leaves treated with the *Chromobacterium subtsugae* derived bio-pesticide, MBI 203, with and without the sun blocking agent, MBI 501.

Table 1. Percentage mortality of *L. decemlineata* larvae and leaf feeding in potato leaf-dip bioassays evaluating MBI 203 (*Chromobacterium subtsugae*), ESAREC, Painter, VA, 2010.

	96 hrs		168 hrs	
Treatment	% Mortality	% leaf consumed	% Mortality	% leaf consumed
NTC	0	>50	37.5	>50
MBI 203 1/20 dilution	10.0	<10	90	<10
MBI 203 1/10 dilution	23.1	<10	100	<10

Table 2. Mean ± SEM numbers of *L. decemlineata* larvae, percent defoliation, and tuber yield in potatoes sprayed with different treatments of MBI 203, a bio-pesticide containing metabolites of *Chromobacterium subtsugae* strain PRAA4-1T. All treatments were sprayed on 11 and 18 May in Painter, VA, 2010.

		Mean no. *L. decemlineata* / 10 stems					
		17-May (6 DAT 1)		24-May (6 DAT 2)			
Treatment	Rate (ha)	Sm Larv	Lg Larv	Sm Larv	Lg Larv	% Defoliation	Yield (kg / m)
NTC		60.0 ± 14.5	12.0 ± 7.9	47.0 ± 21.0	30.3 ± 5.7	48.8 ± 7.8	3.4 ± 0.3
MBI 203	9.35 L	55.0 ± 15.3	4.8 ± 5.4	42.3 ± 18.0	33.0 ± 11.0	60.0 ± 6.9	3.6 ± 0.3
MBI 203	18.7 L	72.5 ± 10.1	10.5 ± 2.8	26.0 ± 9.4	18.8 ± 15.0	37.5 ± 7.1	4.0 ± 0.1

Table 3. Mean ± SEM numbers of *L. decemlineata* larvae, percent defoliation, and tuber yield in potatoes sprayed with different treatments of MBI 203 containing a microbial sun block (MBI 501). All treatments were sprayed on 18 and 25 May, 2011 in Painter, VA.

		Mean no. *L. decemlineata* / 10 stems					
		24-May (6 DAT 1)		31-May (6 DAT 2)			
Treatment	Rate (ha)	Sm Larv	Lg Larv	Sm Larv	Lg Larv	% Defoliation	Yield (kg / m)
NTC		22.0 ± 6.9	78.0 ± 10.1	6.0 ± 1.1	28.0 ± 5.5	76.7 ± 3.9	0.8 ± 0.1
MBI 203	9.35 L	28.7 ± 5.3	48.0 ± 9.3	10.0 ± 2.0	51.3 ± 3.0	68.3 ± 3.7	1.0 ± 0.2
MBI 203	18.7 L	22.7 ± 11.2	57.3 ± 8.1	8.0 ± 1.8	35.3 ± 5.8	66.7 ± 2.0	1.1 ± 0.1

Table 4. Mean ± SEM numbers of *L. decemlineata* larvae, percent defoliation, and tuber yield in potatoes sprayed with different treatments of Grandevo™, a commercial granular bio-pesticide containing metabolites of *Chromobacterium subtsugae* strain PRAA4-1T. All treatments were sprayed on 12 and 21 May, 2012 in Painter, VA.

		Mean no. *L. decemlineata* / 10 stems					
		18-May (6 DAT 1)		29-May (8 DAT 2)			
Treatment	Rate (g ai/ha)[z]	Sm Larv	Lg Larv	Sm Larv	Lg Larv	% Defoliation	Yield (kg / m)
NTC		127.0 ± 25.3	35.5 ± 3.7	19.8 ± 5.8	25.5 ± 4.5	73.3 ± 4.7	3.8 ± 0.2
Grandevo	9.35 L	196.8 ± 26.8	23.8 ± 3.7	16.0 ± 5.3	35.7 ± 2.5	78.3 ± 4.6	3.6 ± 0.4

[z] Treatment received 0.25% v:v non-ionic surfactant

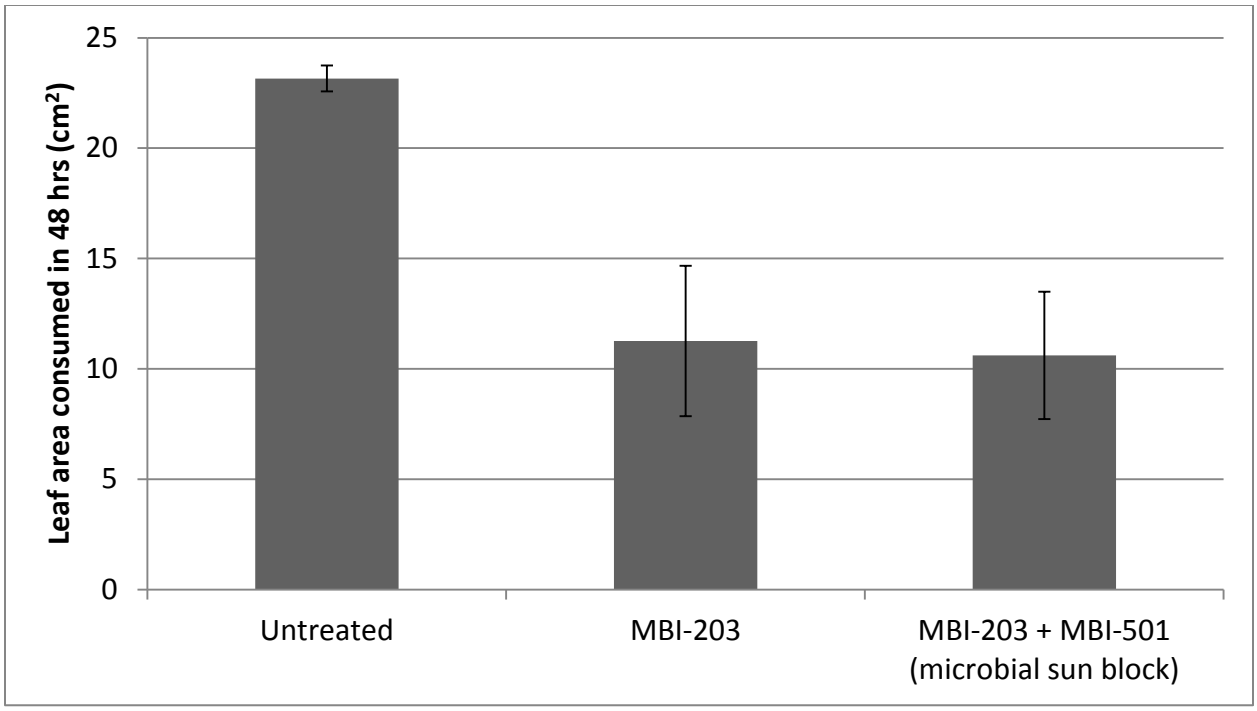

Figure 1. Potato leaf consumption by *L. decemlineata* larvae exposed to leaves treated with the *Chromobacterium subtsugae* derived bio-pesticide, MBI 203, with and without the sun blocking agent, MBI 501.

Chapter 5:

Population dynamics of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae): measuring the effects of methyl salicylate and predator recruitment in potato

Abstract. Methyl salicylate is an organic compound produced by potato and other plants in response to insect herbivory. In other studies the compound has been shown to be attractive to numerous predatory arthropods. Experiments were conducted in Virginia to evaluate methyl salicylate lures for attracting natural enemies in potato plots, as part of a management strategy for populations of *Leptinotarsa decemlineata* (Say). Abundance of predatory arthropods and *L. decemlineata* life stages were recorded in plots treated with and without 90 day, 5 g slow release packets of methyl salicylate (95% methyl salicylate (Predalure™)). Mortality of *L. decemlineata* eggs, small larvae, and large larvae was estimated by calculating the difference in numbers of individuals recruited to subsequent stages using a stage specific life table approach. Methyl salicylate treatment had no impact on predator recruitment or mortality of *L. decemlineata* eggs and small larvae, compared with non-treated plots. Cumulative mortality of *L. decemlineata* ranged from 97.5 to 99.2% in 2010 and 2011. The dominant arthropod predators observed on potatoes included: *Hippodamia convergens* Guerin-Meneville, *Coccinella septempunctata* L., and *Perillus bioculatus* (F.).

Key words *Leptinotarsa decemlineata*, potato, population dynamics, methyl salicylate, natural enemies

Introduction

Methyl salicylate (MeSA), also referred to as oil of wintergreen, is an herbivore induced plant volatile compound. Several studies have shown that MeSA has the potential to attract beneficial insects such as anthocorids (Drukker et al. 2000), geocorids, syrphids, chrysopids, and coccinellids (James 2003, James and Price 2004), as well as predatory mites (Dicke et al. 1990). This has led to the development of a commercially-available synthetic MeSA lure, Predalure™ (AgBio Inc., Westminster, CO), to enhance biological control in gardens and crops by attracting natural enemies. Methyl salicylate has also been shown to have a repellent effect on pest insects such as aphids (Glinwood and Pettersson 2000, Mallinger et al. 2011). Also, Dickens (2000, 2006) found that MeSA is one compound of a blend of potato plant volatiles that is attractive to Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). Thus, it is not known what effect Predalure could have in potato IPM systems.

Leptinotarsa decemlineata is one of the most important insect pests of potato (*Solanum tuberosum* L.) in North America and Europe (Hare 1990, Alyokhin 2009). Uncontrolled populations can completely defoliate potato potentially leading to a total loss of tuber production(Hare 1980). Widespread insecticide resistance problems over the past 50 years have created a strong interest in the development of integrated pest management approaches for this insect (Cassagrande 1987, Alyokhin et al. 2008, Alyokhin 2009).

Leptinotarsa decemlineata has many important natural enemies that can reduce population levels (Weber 2013). These include the predaceous ground beetle *Lebia grandis* Henz (Coleoptera: Carabidae) and several species of Coccinellidae as well as a few parasitoids including *Edovum puttleri* Grissell. Research has shown that mortality of *L. decemlineata* from natural enemies can be quite high. For example, the predaceous lady beetle *Coleomegilla*

maculata De Geer can cause up to 37.8% mortality in the first generation of *L. decemlineata* eggs and an additional 58.1% mortality of eggs in the second generation (Hazzard et al. 1991, Alyokhin 2008). In addition, inundated releases of the predatory stink bugs, *Perillus bioculatus* (Fabricius) (Hemiptera: Pentatomidae) and *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), reduced beetle densities by 62% and decreased defoliation of potato by 86% (Biever and Chauvin 1992, Hough-Goldstein and McPherson 1996, Alyokhin 2008). Moreover, the parasitic wasp *E. puttleri* can parasitize up to 91% of *L. decemlineata* egg masses on eggplant and up to 50% of egg masses on potato (Lashomb et al. 1987, Ruberson et al. 1991, van Driesche et al. 1991, Alyokhin 2008). However, most natural enemies of *L. decemlineata* typically do not sufficiently reduce densities to an acceptable level of control (Hare 1990), forcing growers to rely on insecticides or other means of control.

Using a chemical such as MeSA to attract natural enemies into areas where the *L. decemlineata* is present could contribute to control of the pest. Methyl salicylate has the potential to protect and enhance natural enemies while at the same time reducing the number of insecticide applications needed within a field. Thus, potato plots inundated with MeSA (via Predalure™ (=95% MeSA)) could influence *L. decemlineata* population dynamics by attracting or repelling adults, and by attracting natural arthropod predators that may consume *L. decemlineata* eggs and larvae. Research has shown MeSA is attractive to natural enemies, but may also be attractive to *L. decemlineata*. The purpose of this study, therefore, was to determine the effect of MeSA (Predalure™) on *L. decemlineata* population dynamics.

Materials and Methods

Field plots. The experiment was arranged in a randomized complete block design in two fields at the Virginia Tech Eastern Shore Agricultural Research and Extension Center (ESAREC) located in Painter, VA during the 2010 and 2011 growing seasons. Experimental plots of

'Superior' potatoes were planted on 25 March 2010 and 13 April 2011 in rows spaced 0.9 m apart with plants seeded ≈0.28 m within rows. Potatoes were established and maintained using standard agricultural procedures including fertilizer, herbicide, and fungicide applications according to the commercial vegetable guidelines for Virginia (Wilson et al. 2012); no insecticides were applied to experimental plots. Four replicate blocks were used in each experiment with each block assigned a treated plot containing MeSA release packets and an untreated plot without MeSA release packets. Plots were established as four planted rows of potato ≈3.7 m wide and 7.6 m long and separated by a minimum of 10 m of bare ground.

MeSA application. The experiments were setup on 23 April and 10 May 2010, and on 3 May 2011 when the potato plants were <0.5 m and had not yet bloomed, and *L. decemlineata* adults had just begun to colonize the field. In each of the experiments, treated plots of potato received two 90 d, 5 g slow-release packets of Predalure™ (95% MeSA). For the treated plots, MeSA release packets were fastened with binder clips to wooden stakes 25 cm above the ground and were placed every 2 m in the middle of the plot.

Sampling *L. decemlineata* and arthropod predators. Plots were sampled every 2–5 d after the experiment began. All *L. decemlineata* egg masses, small larvae (instars 1 and 2), large larvae (instars 3 and 4), and adults were counted from 10 randomly-selected plants in each plot. The average number of eggs per egg mass was determined by counting the number of eggs in 10 randomly-selected egg masses. The average number of eggs per egg mass multiplied by the number of egg masses was used to calculate the total number of eggs in each of the treated and untreated plots at each sampling.

Visual counts of all predatory arthropods were recorded every 3–7 d from 10 randomly selected plants in each plot. In 2010, predators were collected and returned to the lab for

identification. In 2011, predators were identified during sampling based on experience from the previous year, but were not removed from plots. Parasitoids were not detected through visual counts and, because *L. decemlineata* egg masses were not collected, the impact of parasitoids on *L. decemlineata* mortality could not be assessed.

Data analysis. The seasonal dynamics of *L. decemlineata* populations in the two treatments was assessed visually by plotting the numbers in each life stage against accumulated degree-day (DD). Degree days were calculated using the Wisconsin model developed by Delahaut (1997), which assumes a minimum developmental temperature threshold of 11°C for *L. decemlineatea*. We then analyzed the data on the numbers of eggs, small larvae, large larvae, and adults collected in each treatment in each year using the Kiritani-Naksuji-Manly (KNM) method for multi-cohort data (Kiritani and Naksuji 1967, Manly 1976, Young and Young 1998). The KNM method provided estimates of the area under the population curve (AUC) for each life stage, which represents the cumulative sum of individuals collected for each stage; the analysis also provided estimates of the numbers entering and dying in each stage.

The data on the mean number of individuals entering and mean number dying in each life stage under each treatment in each year were examined using a χ^2 test of homogeneity of distributions (Ott and Longnecker 2001) with the effect of year as a blocking factor tested using the Cochran-Mantel-Haenszel test. The null hypothesis for the homogeneity test is that of no difference in the distributions with respect to the proportion of individuals in each life stage between treatments (Ott and Longnecker 2001). When the test indicated that the null hypothesis was rejected ($P < 0.05$), we conducted individual tests of the proportions of individuals in each life stage to determine which stage or stages were responsible for the observed difference in the

population distributions between the treatments. All statistical analyses were carried out using JMP 10 (SAS 2013).

The data on predators collected within the treatment plots were pooled across block and year for each treatment. From these data, we calculated the relative abundances of each taxa and the Shannon-Weiner index of diversity as a measure of community structure in each treatment (Krebs 1998, Dively 2005). A significant difference in the estimated Shannon-Weiner indices for the two treatments were determined using a permutation/randomization approach as described in Manly (1997).

Results

***Leptinotarsa decemlineata* populations.** The seasonal dynamics of *L. decemlineata* life stages with respect to DD are shown in Fig. 1. Population levels were higher in 2011 compared with 2010 (Fig. 1; Table 2). In 2010, peak oviposition of *L. decemlineata* occurred between 10 and 19 May and the peak of small larvae occurred between 14 May and 7 June (Fig. 1a and 1b). In 2011, peak oviposition occurred between 10 and 23 May and peak small larvae populations between 21 May and 1 June (Fig. 1c and 1d).

The results of the multi-cohort analysis of the data with the KNM method are presented in Table 2. The homogeneity analysis showed that there was a significant difference in the distribution of the proportions of individuals entering each life stage (χ^2 = 746.58, df = 3, *P* <.0001; Table 2; Fig. 2A). There was also a significant difference between the distributions after blocking for year (χ^2 = 724.65, df = 1, *P* <.0001). Individual analysis of each of the life stages showed that overall the proportion of individuals entering the egg stage was significantly higher in untreated plots compared with the MeSA-treated plots (χ^2 =262.47, df = 1, P<0001). However, a significantly greater proportion of small larvae (χ^2 =4.559, df = 1, P = 0.0327), large

larvae (χ^2 = 162.87, df = 1, P <.0001), and adults (χ^2 = 15.25, df = 1, P<0001) entered the respective stages in the MeSA-treated plots.

The analysis also showed that there was a significant difference between the treatments in the distribution of the proportions of individuals dying within the immature stages (χ^2 = 731.18, df = 2, P <.0001; Fig 2B). A significantly greater proportion of individuals died within the egg (χ^2 = 918.11, df = 1, P <.0001) and small larval (χ^2 = 73.49, df = 1, P <.0001) stages in untreated plots compared with MeSA-treated plots.

Natural enemy abundance. In both years, the three most abundant predatory insects found in potato plots were the convergent lady beetle, *Hippodamia convergens* Guerin-Meneville (Coleoptera: Coccinellidae), seven-spotted lady beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), and the two-spotted stink bug, *P. bioculatus* (Fig. 3). Other arthropod predators observed in low numbers included *P. maculiventris*, *L. grandis*, *C. maculata*, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), chrysopid eggs, various spiders, and predatory mites. The latter three groups were not identified to species. The permutation/randomization analysis showed that there was no significant difference (P = 0.331) in the Shannon-Weiner index for the predatory species collected in untreated (1.567) and MeSA-treated (1.598) potato plots.

Discussion

Our experiments showed the cumulative mortality for *L. decemlineata* based on the egg, small, and large larvae mortalities ranged from 97.6 to 99.2%, which is similar to the cumulative mortality of 99.8% reported by Cappaert et al. (1991b), who observed *L. decemlineata* populations on a native host plant in Mexico. Mena-Covarrubias et al. (1996) also reported a

cumulative mortality of *L. decemlineata* eggs and small larvae ranging from 82 to 99% on horse nettle in Michigan.

Arthropod predators likely play a significant role in early-stage mortality of *L. decemlineata* (Weber 2013). In a study evaluating natural enemies of *L. decemlineata* in Mexico, Cappaert et al. (1991a) observed that over half of the insect natural enemies within their field site consisted of Pentatomidae, Carabidae, and Coccinellidae. We also primarily observed those same three families and some of the same species in Virginia. Heimpel and Hough-Goldstein (1992) observed a similar composition of predators in Delaware potato fields, compared to the predators we observed in VA. Researchers found *C. maculata*, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), *L. grandis*, *P. maculiventris*, and *P. bioculatus* associated with *L. decemlineata*. In our study, the three most abundant natural enemies observed were *H. convergens*, *C. septempunctata* and *P. bioculatus*. As previously mentioned, *P. bioculatus* can reduce *L. decemlineata* populations in potato, specifically when *L. decemlineata* populations are low (Tamaki and Butt 1978). Research has shown that *H. convergens* is associated with *L. decemlineata* populations in Mexico (Cappaert et al. 1991b) and feeds on *L. decemlineata* eggs (Cappaert et al. 1991a, Hough-Goldstein et al. 1993).

Although *C. septempunctata* was one of the most abundant predators detected in our trial, findings from other researchers indicate that this predator may not preferentially feed on *L. decemlineata*. Snyder and Clevenger (2004) examined the survivorship of four Coccinellidae, two native species *H. convergens* and *Coccinella transversoguttata* Brown (Coleoptera: Coccinellidae), and two exotic species *C. septempunctata* and *H. axyridis* when fed diets of the aphid *Myzus persicae* Sulzer, or *L. decemlineata* eggs, or a mixed diet of both. Results indicated that *C. septempunctata* had the highest survivorship compared to the other Coccinellidae species

when fed the mixed diet (Snyder and Clevenger 2004). However, all four coccinellid species in this study had a significantly higher survivorship when fed *M. persicae* versus *L. decemlineata* eggs or a mix (Snyder and Clevenger 2004). Additional research has shown that *C. septempunctata* does not readily feed on *L. decemlineata* larvae or eggs (Heimpel and Hough-Goldstein 1992).

Thus, *L. decemlineata* egg and larval populations incur a tremendous amount of natural mortality and yet *L. decemlineata* remains a major pest of potato year after year. In our plots, over 50% of the potato foliage was consumed by *L. decemlineata*. The resilience of *L. decemlineata* has caused a heavy reliance on chemical control (Kuhar et al. 2013) and has created a cycle of continuously developing new insecticides with novel modes of action that are effective for a number of years before *L. decemlineata* develops resistance and new chemistries are needed for their control (Alyokhin 2009). The high level of natural mortality should be taken into consideration when selecting an insecticide or deciding whether or not a spray is warranted. Methods to further enhance natural mortality could reduce that reliance on chemical control.

Use of chemical attractants to recruit natural enemies is one approach to do just that. Results from our experiments indicated that MeSA release packets (Predalure™) increased the number of *L. decemlineata* adults in treated plots. These results indicate MeSA had attractant properties to *L. decemlineata*. As mentioned earlier, Dickens (2000, 2006) reported MeSA to be a component of plant kairomones attractive to *L. decemlineata*. Our results also showed significantly greater mortality in the large larvae stage in plots treated with MeSA; however, mortality in the egg and small larvae stages was greater in the untreated plots. There was also no effect of treatment on the abundance of arthropod predators. Although MeSA did not significantly impact *L. decemlineata* cumulative mortality or increase the number of natural

enemies within plots in this study, research evaluating the use of volatile compounds should be part of the management of *L. decemlineata*.

Synthetic or naturally-derived volatile compounds with attractant properties to natural enemies can play an important role in biological control of pests. Mallinger et al. (2011) investigated the effects of MeSA lures in organic soybean fields. Researchers found MeSA treated plots had significantly less soybean aphids and significantly greater numbers of syrphid flies (Diptera: Syrphidae) and green lacewings (Neuroptera: Chrysopidae) adjacent to MeSA lures compared to non-treated plots. When MeSA lures were included in exclusion cage studies researchers saw no difference in numbers or population growth rates for soybean aphids (Mallinger et al. 2011). James and Price (2004) showed that slow release MeSA increased the number of natural enemies including *Chrysopa nigricornis* Burmeister (Neuroptera: Chrysopidae), *Hemerobius* sp., *Deraeocoris brevis* (Uhler) (Hemiptera: Miridae), *Stethorus punctum picipes* Casey (Coleoptera: Coccinellidae), and *Orius tristicolor* (White) (Hemiptera: Anthocoridae), and four insect families (Syrphidae, Braconidae, Empididae, and Sarcophagidae) in hop yards and grapes. Both of these crops naturally produce MeSA upon herbivory (James and Price 2004). The interaction between plants and natural enemies is a complex relationship dependent on numerous factors.

Research by McCormick et al. (2012), describe the relationship between herbivore-induced plant volatiles and natural enemy responses as very specific and dependent on dose, blend of the volatiles produced, duration of release, and the type of predator receiving the signal. Research indicates a greater utilization of plant volatiles for specialist predators compared with generalist predators (McCormick et al. 2012). It is important to note that herbivore natural enemies are not exposed to just a single plant volatile compound in nature; but rather a mix of

compounds produced by a plant in response to herbivory. For example, the predatory mite *Phytoseiulus persimilis* Athias-Henriot was attracted to MeSA, one of 5 major herbivore induced compounds of lima bean *Phaseolus lunatus* L., but when presented with a blend of all the major herbivore induced volatiles produced by lima bean there was a greater attraction compared to just MeSA (van Wijk et al. 2008, 2011). Therefore, incorporating a mixture of plant volatiles for attracting herbivore natural enemies may be more successful than just a single compound.

Plants produce a number of volatile compounds with the sole purpose of indirect defense, usually in the form of attracting predaceous insects and arthropods to disrupt the herbivorous insects feeding on these plants. *Leptinotarsa decemlineata* has many natural enemies that feed on its different life stages; manipulating this natural cycle to control *L. decemlineata* populations during oviposition and as eggs hatch could be an effective biological control strategy within an IPM program. However, from this research and the results found in other experiments it appears MeSA alone may not be a useful tool for attracting predatory insects into *L. decemlineata* infested potato fields. Future research should focus on determining the different volatiles, concentrations of those volatiles, timing of when to apply, and ratios of the different volatile blends, that could be used to attract natural enemies of *L. decemlineata* in a potato field. Additional research should also determine which predators primarily feed on *L. decemlineata* and how each volatile as well as mixtures impact predator-prey interactions including type of damage and species specific volatile combinations.

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Table 1. Mean weekly temperatures (°C) recorded in Painter VA, 2010 and 2011.

Week	2010[z]	2011[y]
1	16.1	16.6
2	23.9	17.8
3	17.8	20.9
4	18.1	26.2
5	22.2	23.9
6	25.3	26.7

[z] Trial set up on 23-Apr., weekly temperature recordings start the day after trial set-up.

[y] Trial set up on 3-May, weekly temperature recordings start the day after trial set-up.

Table 2. Results of the multi-cohort analysis with the Kiritani-Nakasuji-Manly method for data on *Leptinotarsa decemlineata* collected in potato plots at Painter, VA.

Year	Treatment[z]	Stage	AUC[y]	Mean Number Entering Stage	Mean Number Dying in Stage
2010	Untreated	Egg	40702.8	7638.2	5580.9
		Small Larva	10887.1	2057.3	1131.4
		Large Larva	6789.6	925.9	753.4
		Adult	1428.6	172.6	
2010	Treated	Egg	42501.6	9627.2	7003.5
		Small Larva	11836.2	2623.8	1582.0
		Large Larva	5536.9	1041.8	806.9
		Adult	2022.1	234.8	
2011	Untreated	Egg	76453.9	18837.5	13000.7
		Small Larva	23457.8	5836.8	4864.9
		Large Larva	13437.1	971.9	821.9
		Adult	2313.6	150.0	
2011	Treated	Egg	74615.5	11832.3	6177.9
		Small Larva	21965.5	5654.4	3511.6
		Large Larva	13329.3	2142.8	1898.7
		Adult	2983.0	244.1	

[z] Treated plots received two 90 d, 5 g slow-release packets of Predalure™ (95% MeSA).

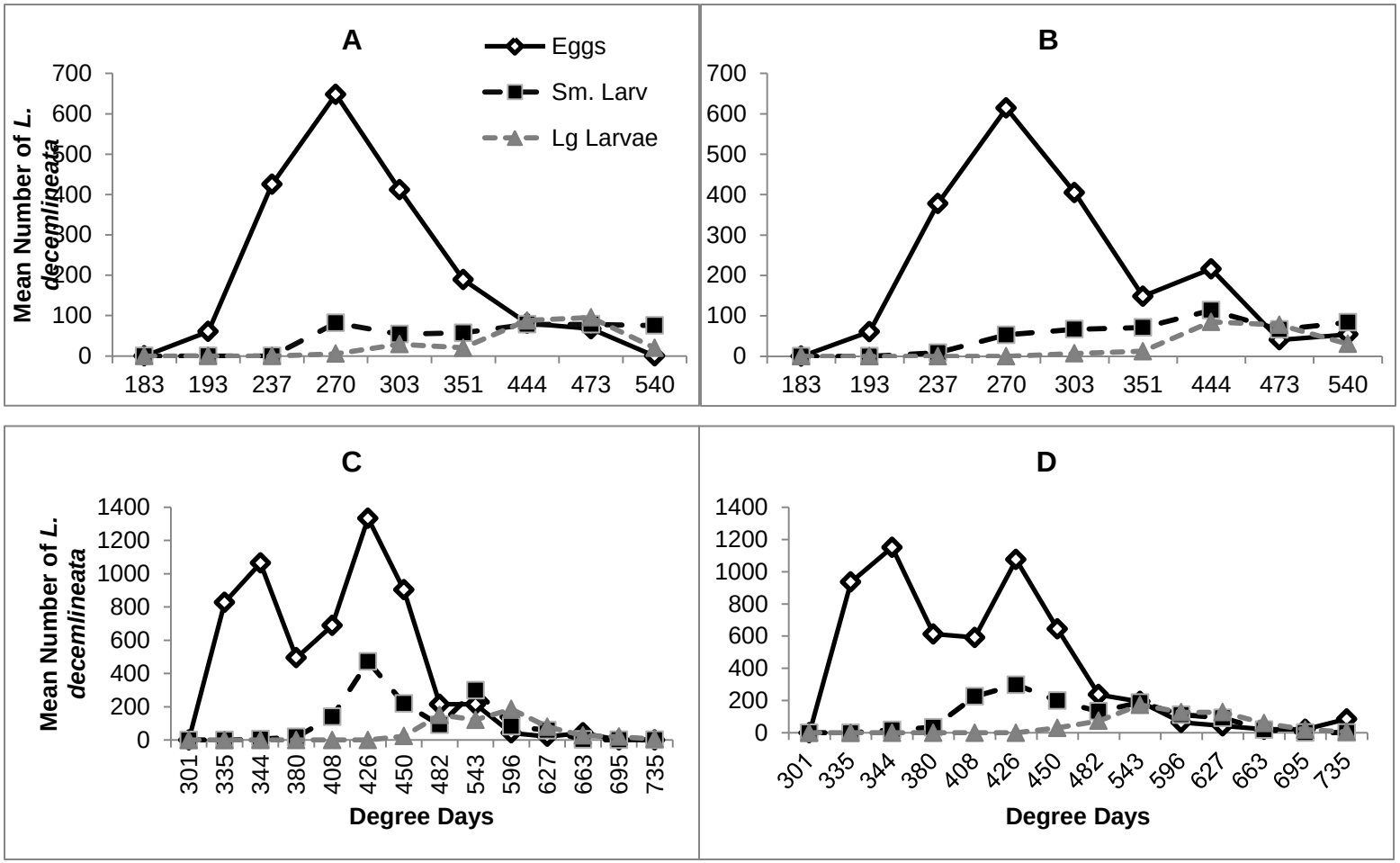
[y] AUC is the mean area under the stage frequency curves for 4 replicates (blocks).

Figure captions

Figure 1. Mean number of *Leptinotarsa decemlineata* eggs, small larvae, and large larvae per ten plants per plot (n=8) in: A.) Untreated potatoes in 2010, B.) Potatoes treated with methyl salicylate in 2010, C.) Untreated potatoes in 2011, and D.) Potatoes treated with methyl salicylate in 2011 in Painter, VA.

Figure 2. Percent of eggs, small larvae, large larvae, and adults in untreated and MeSA-treated plots of potato at Painter, VA; A.) Percent of total number entering each life stage. B.) Percent of total number dying within each life stage.

Figure 3. Arthropod predators collected per 10 plants in potato plots with and without release packets of methyl salicylate in Painter, VA.



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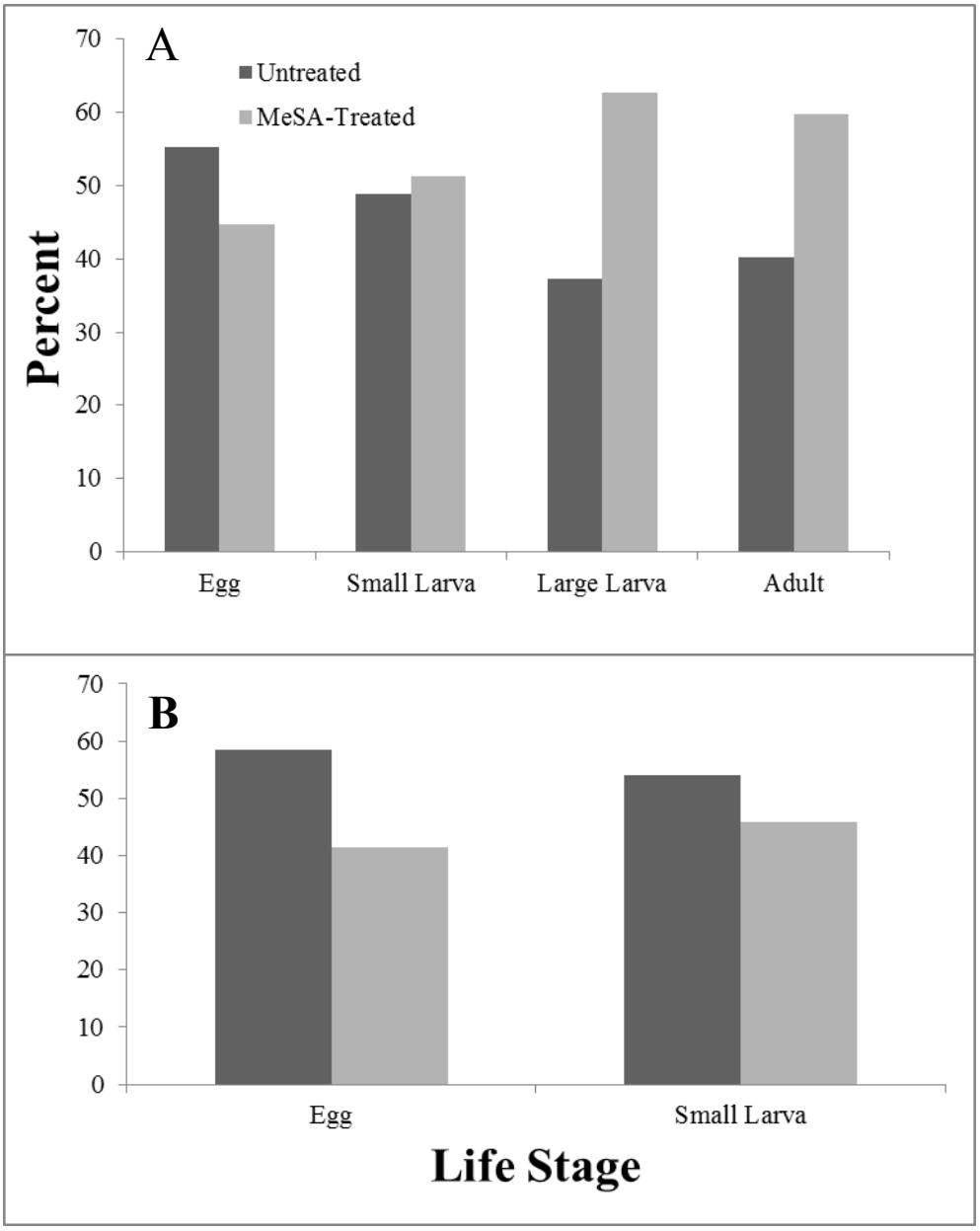

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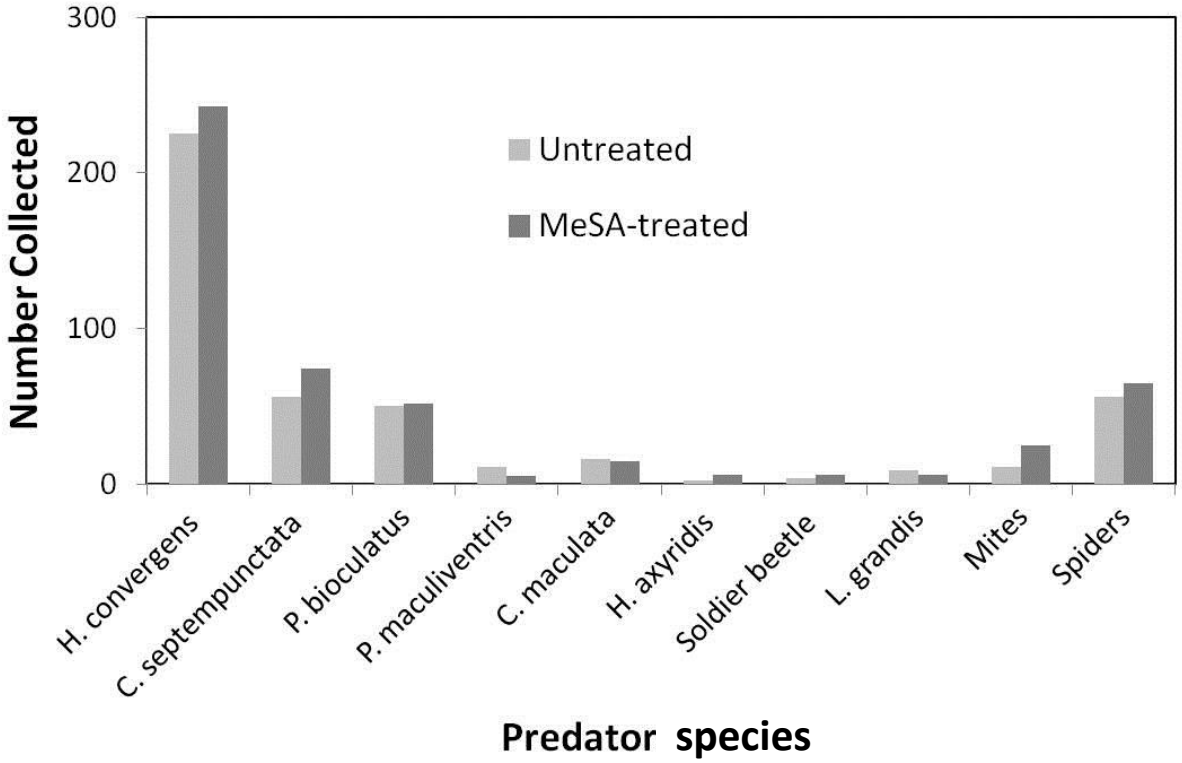

Figure 3. Arthropod predators collected per 10 plants in potato plots with and without release packets of methyl salicylate in Painter, VA.

Overall Conclusion

Leptintotarsa decemlineata is a voracious defoliator of potato and if not properly managed has the potential to devastate potato production where this pest occurs. Management of this pest heavily relies on the use of chemical insecticides. Unfortunately, *L. decemlineata* has developed resistance to most of the insecticides labeled for its control. This ability to develop resistance to insecticides has created an endless cycle of continually developing insecticides with novel modes of action, shortly followed by resistance development by this pest. The current management system is unsustainable and has created the need for alternative management approaches as well as the continuation of novel insecticide development. This research investigated multiple approaches for managing *L. decemlineata*, as well as investigated some of the potential resistance mechanisms employed by this pest.

Two novel insecticides were investigated in this research, tolfenpyrad and a metabolite based bio-pesticides developed from the bacterium *C. subtsugae*. Tolfenpyrad, a novel insecticide being developed by Nichino America, Inc., proved to be an effective insecticide. This compound had excellent activity on *L. decemlineata* in the field and with its novel mode of action, will provide growers with an outstanding resistance management tool. The bio-pesticide developed from the bacterium *C. subtsugae* was also evaluated in the laboratory and field for managing *L. decemlineata*. Unlike tolfenpyrad, this bio-pesticide did not prove to be effective at managing this pest in the field.

Resistant populations of *L. decemlineata* make managing this pest a difficult obstacle. One possible pathway of resistance development in *L. decemlineata* originates from metabolic detoxification of insecticides. Evaluating the specific activity of P450s, general esterases, GSTs, and protein content from resistant beetles can indicate potential detoxification pathways

conferring resistance. From this research it is clear that resistance evaluation and subsequent management of resistant populations should be approached on a farm to farm basis. Even in an area as small as the Eastern Shore of VA, farm to farm variability in resistance and metabolic activity is great among populations of *L. decemlineata*. Managing resistance in this pest is imperative for sustainable potato production.

Alternative management strategies including the use of lures that attract natural enemies of *L. decemlineata* into potato are increasing in popularity because of this innate ability to develop resistance to insecticides. Methyl salicylate is a plant volatile that has been identified as a lure for beneficial insects, especially predators. Field experiments evaluating slow release lures of MeSA were not, however, effective at increasing numbers of predatory arthropods or overall mortality of *L. decemlineata* in potato. In fact MeSA lures attracted greater numbers of adult *L. decemlineata* into treated potato plots compared to untreated plots.